



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 11-150134- -00000-0000

Fecha: 2011-11-04 15:51:49 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 286

Industria y Comercio

S U P E R I N T E N D E N C I A

DELEGATURA PROPIEDAD INDUSTRIAL

SOLICITUD DE PATENTE DE INVENCION - ENTRADA EN FASE NACIONAL -

21. EXPEDIENTE No. _____

54. TITULO SALES DE 6' -SIALIL-LACTOSA Y PROCESOS PARA SU SINTESIS Y PARA LA SINTESIS DE OTROS ALFA-SIALIL-OLIGOSACARIDOS

51. CLASIFICACION INTERNACIONAL CO7H 13/06 (2006.01) - A23L 1/09 (2006.01) - A61K 31/702 (2006.01)

71. SOLICITANTE INALCO S.P.A.

DOMICILIO Via Calabiana 18, I-20139 Milano - ITALIA

74. APODERADO DRA. MAGDA SALZBURG GARROS

22. BOGOTA D.C., NOVIEMBRE 04 DE 2011

PETITORIO

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



Industria y Comercio

SUPERINTENDENCIA



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Act. 411 PRESENTACION Folios: 286

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FORMULARIO ÚNICO DE SOLICITUD DE PATENTE

PCT

ENTRADA EN FASE NACIONAL

SOLICITUD DE:

1

X

Patente de Invención

Patente de Modelo de Utilidad

X

Capítulo I.

Capítulo II.

2

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Número:

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O
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5

PCT (86)

Solicitud Internacional No. **PCT/IB2010/051470**
Publicación Internacional No. **WO 2010/116317 A1**Fecha: **06 ABRIL 2010**
Fecha: **14 OCTUBRE 2010**

6

Título (54)

**SALES DE 6' -SIALIL-LACTOSA Y PROCESOS PARA SU SINTESIS Y
PARA LA SINTESIS DE OTROS ALFA-SIALIL-OLIGOSACARIDOS**

7

Clasificación Internacional (51): **C07H 13/06 (2006.01) - A23L 1/09 (2006.01) - A61K 31/702 (2006.01)**

8

Prioridad

SI

☒

NO

☐

(33) País de Origen

-----ITALIA-----

(32) Fecha

--06/04/2009--

(31) Número de Solicitud

-----FI2009A000071-----

9

Comprobante de pago No. 11 - 107,878

Fecha: **03 NOVIEMBRE 2011**

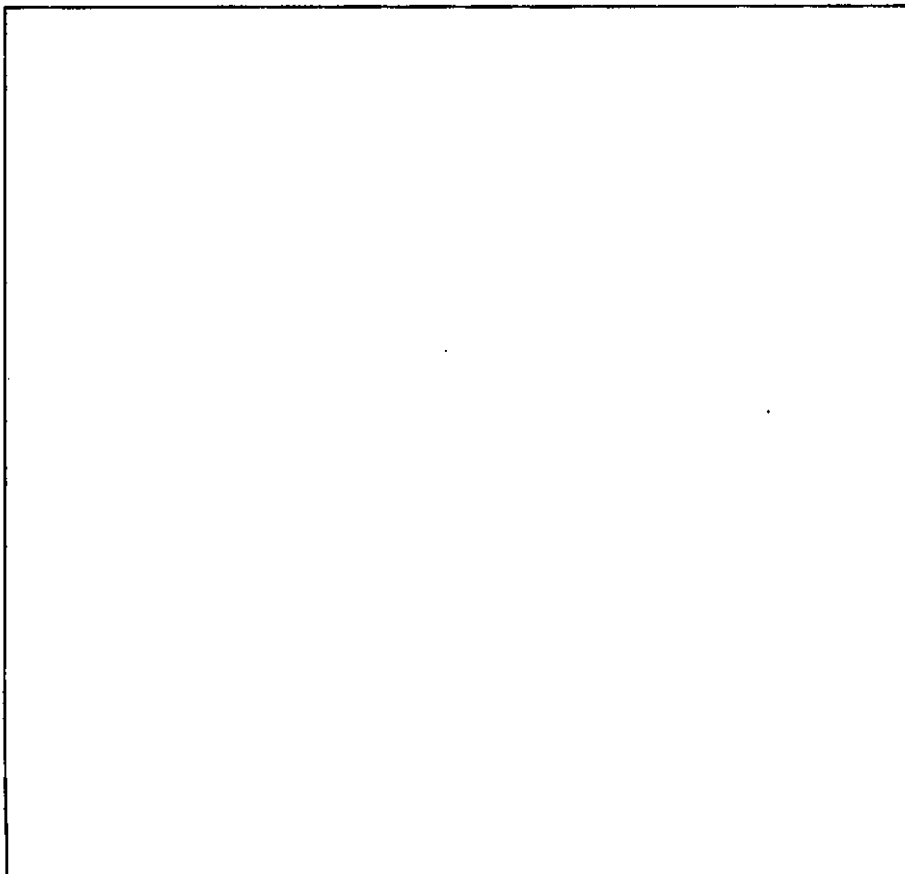
10

ANEXOS

- ☒ Comprobante de pago de la tasa de presentación de la solicitud **\$571.000.00**
- ☐ Comprobante de pago por reivindicación de prioridad
- ☐ Documento que acredita la existencia y representación legal cuando el solicitante sea persona jurídica
- ☐ Poderes, si fuere el caso
- ☐ Documento de cesión del inventor al solicitante o a su causante
- ☐ Declaraciones
- ☒ Copia de la solicitud internacional. (Resumen, descripción, reivindicaciones, dibujos)
- ☒ Traducción de la solicitud internacional al castellano (Resumen, descripción, reivindicaciones, dibujos)
- ☐ Copia del examen preliminar internacional efectuado por la Autoridad Internacional de Examen Preliminar (IPEA)
- ☐ Traducción del examen preliminar internacional efectuado por la IPEA
- ☐ De ser el caso, copia del contrato de acceso
- ☐ De ser el caso, documento que acredite la licencia o autorización de uso de conocimientos tradicionales de las comunidades indígenas
- ☐ De ser el caso, certificado de depósito del material biológico
- ☐ Arte final 12x12
- ☐ De ser el caso, información sobre otras solicitudes de patente o títulos obtenidos en el extranjero por el mismo titular o su causante, relacionadas parcial o totalmente con la invención de esta solicitud
- ☐ De ser el caso, modificaciones efectuadas a la solicitud internacional
- ☐ Otros

11

FIGURA CARACTERÍSTICA:



12

Solicito la concesión de la patente,

NOMBRE: **MAGDA SALZBURG GARROS**

FIRMA:

C.C.: 35.458.610 Usaquén T.P.: 35.337-G.S.J.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 11 - 107,878
FECHA : NOVIEMBRE 3 DE 2011

***** CONSIGNACION *****

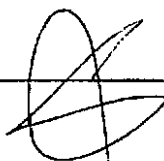
DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	Nº. PAGO	FECHA PGO	Vr. PAGO
MASDA SALZBURG	CONSIGNACION	BANCO DE BOGOTA	062754387	173393920	03/11/2011	571.000.00

***** C O N C E P T O *****

CANT. RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-01 SOLICITUDES	1 TRAMITES DE SOL. DE PATENTE DE IN	571,000.00
	TOTAL :	\$ 571,000.00

SON: QUINIENTOS SETENTA Y UNO MIL PESOS

RESPONSABLE :



SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 11-150134- -00000-0000

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Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 411 PRESENTACION Folios: 286

RECIBO DE CAJA APLICADO AL EXPEDIENTE No.

Banco de Bogotá

NIT: 860.002.894-4

Sistema Nacional de Recaudos Comprobante de Pago Universal Multiple No. 17339392-0

Fecha
Año 2011 Mes 11 Día 03

Código Convenio Cuenta Corriente ☒ Cuenta de Ahorros ☐

Número 062754387

ESPACIO PARA EL TIMBRE

Nombre Convenio o Empresa Recaudadora SUPERINDUSTRIA

Referencia 1

Referencia 2

CÓDIGO FACTURA/No. DOCUMENTO / REFERENCIA		OTROS CONCEPTOS	OTROS DATOS	VALOR
1				\$
2				\$
3				\$
4				\$
5				\$
6				\$
7				\$
8				\$
9				\$
10				\$

Código Banco Número del Cheque Número del Cuenta Ciudad y Plaza

ANEXOS Sin Anexo ☐ Papel ☐ Disquete ☐ Otro ☐

FORMA DE PAGO Efectivo \$ 571.000 Cheque \$ TOTAL A PAGAR \$ 571.000

Nota: Antes de presentar este comprobante, revise diligenciarlo completamente con base en la información que le suministra la empresa. Si paga con cheque, favor anular al momento de la entrega. El número de este comprobante, el nombre y número de la cuenta de la empresa y sus datos personales (Nombre, dirección y teléfono).

Banco de Bogotá 122 Centro Internacional
Srv 2160 BOX P12201 Usq 7955 T533
CC062754387 03/11/11 12:39 H.NO
SUPERINTENDENCIA DE INDUSTRIA
Usq 01
Valor Efectivo: 571,000.00
Valor Cheque: 0.00
Valor TC: 0.00
Valor ND: 0.00
Valor Total: 571,000.00

Nombre del Depositante MAGDA SALZBURG GARZOS Teléfono 2859058

Esta transacción está sujeta a verificación posterior. El cheque (cheques) depositado(s) se reciben "Salvo buen cobro" de manera que la operación solo se efectúa al momento de la liquidación del cheque (cheques) por el banco. El comprobante de pago es un documento de carácter contable y administrativo. No debe ser utilizado como comprobante de pago. En caso de ser utilizado como comprobante de pago, el titular del comprobante de pago debe ser responsable de la verificación de la información contenida en el mismo. En caso de ser utilizado como comprobante de pago, el titular del comprobante de pago debe ser responsable de la verificación de la información contenida en el mismo.

2 COPIA DEPOSITANTE

(12) SOLICITUD INTERNACIONAL PUBLICADA BAJO EL TRATADO DE COOPERACION EN MATERIA DE PATENTES (PCT)		
(19) Organización Mundial de la Propiedad Intelectual Oficina Internacional	PCT	
(43) Fecha de Publicación Internacional 14 octubre 2010 (14.10.2010)		(10) Número de Publicación Internacional WO 2010/116317 A1
(51) Clasificación de patente internacional : C07H 13/06 (2006.01) A23L 1/09 (2006.01) A61K 31/702 (2006.01)		(81) Estados designados (a menos que se indique de otra manera para cada clase de protección nacional disponible): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW. (84) Estados designados (a menos que se indique de otra manera para cada clase de protección regional disponible): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasia (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), Europa (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG). Declaración bajo la Regla 4.17: - en cuanto a la autoridad del solicitante para solicitar y para que le sea otorgada una patente (Regla 4.17(ii)) - de inventoria (Regla 4.17 (iv)) Publicada: - con reporte de búsqueda internacional (Art. 21(3)) - antes de la expiración del límite de tiempo para enmendar las reivindicaciones y para que sea republicada en el caso de la recepción de las enmiendas (Regla 48.2(h))
(21) Número de solicitud internacional: PCT/IB2010/051470		
(22) Fecha de presentación internacional: 6 abril 2010 (06.04.2010)		
(25) Lenguaje de Presentación: Italiano		
(26) Lenguaje de Publicación: Inglés		
(30) Datos de prioridad: FI2009A000071 6 abril 2009 (06.04.2009) IT		
(71) Solicitante (para todos los Estados designados excepto US): INALCO S. P. A. [IT/IT]; Via Calabiana 18, I- 20139 Milano (IT).		
(72) Inventores; y (75) Inventores/Solicitantes (para US solamente): TAMERLANI, Giancarlo [IT/IT]; Via Turati 13, I-40030 Castel Di Casio (IT). LOMBARDI, Ilaria [IT/IT]; Via Sano 17/A, I-51016 Montecatini Terme (IT). BARTALUCCI, Debora [IT/IT]; Via Pietramarina 257, I-50059 Vinci (IT). DANESI, Andrea [IT/IT]; Viale Adua 177, I-51100 Pistoia (IT). SALSINI, Liana [IT/IT]; Via Antonio Gramsci, 238, I-55047 Seravezza (IT). MANONI, Marco [IT/IT]; Via Carmignanesse 103, I-50059 Vinci (IT). CIPOLLETTI, Giovanni [IT/IT]; Via Magolfà 13, I-20143 Milano (IT).		
(74) Mandatario: BORSANO, Corrado et al.; Corso di Porta Vittoria 9, I- Milano 20122 (IT).		
(54) Título: SALES DE 6'-SIALIL-LACTOSA Y PROCESOS PARA SU SINTESIS Y PARA LA SINTESIS DE OTROS ALFA-SIALIL-OLIGOSACARIDOS (57) Resumen: La presente invención se refiere a un proceso para la síntesis de los α -sialil-oligosacáridos y en particular de la 6'-sialil-lactosa y sus sales, que comprende una etapa de unión por la reacción de Koenigs-Knorr bajo condiciones que permiten su uso a una escala industrial.		

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
14 October 2010 (14.10.2010)

(10) International Publication Number
WO 2010/116317 A1

- (51) International Patent Classification:
C07H 13/06 (2006.01) A23L 1/09 (2006.01)
A61K 31/702 (2006.01)
- (21) International Application Number:
PCT/IB2010/051470
- (22) International Filing Date:
6 April 2010 (06.04.2010)
- (25) Filing Language: Italian
- (26) Publication Language: English
- (30) Priority Data:
FI2009A000071 6 April 2009 (06.04.2009) IT
- (71) Applicant (for all designated States except US): INALCO S.P.A. [IT/IT]; Via Calabiana 18, I-20139 Milano (IT).
- (72) Inventors; and
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- (74) Agents: BORSANO, Corrado et al.; Corso di Porta Vittoria 9, I-Milano 20122 (IT).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GI, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- of inventorship (Rule 4.17(iv))

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

WO 2010/116317 A1

(54) Title: 6'-SIALYLLACTOSE SALTS AND PROCESS FOR THEIR SYNTHESIS AND FOR THE SYNTHESIS OF OTHER α -SIALYLOLIGOSACCHARIDES

(57) Abstract: The present invention relates to a process of synthesis of α -sialyl oligosaccharides and in particular of 6'-sialyllactose and its salts comprising a step of coupling by Koenigs-Knorr reaction under conditions that allow its use on an industrial scale.

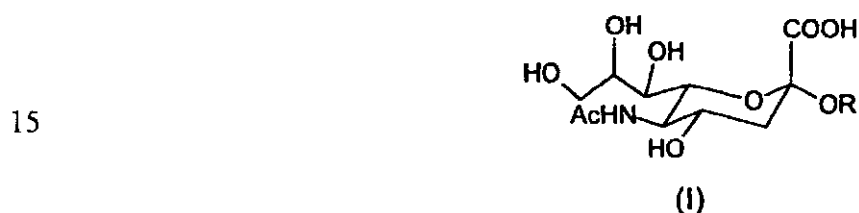
SALES DE 6'-SIALIL-LACTOSA Y PROCESOS PARA SU SINTESIS Y PARA
LA SINTESIS DE OTROS ALFA-SIALIL-OLIGOSACARIDOS

Campo de la Invención

5 La presente invención se refiere al campo de las sales de 6'-sialil-lactosa: la presente invención también se refiere al campo de los procesos para la síntesis de los α -sialil-oligosacáridos y en particular al campo de los procesos para la síntesis de 6'-sialil-lactosa y sus
10 sales.

Antecedentes de la Invención

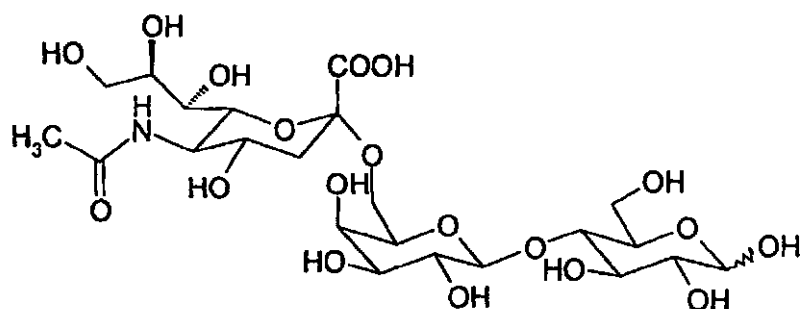
Los α -sialil-oligosacáridos de la fórmula (I)



en donde R es un residuo de mono, di u oligosacárido con grupos hidróxilos libres, están presentes en los tejidos de los mamíferos y las aves y en la forma predominante de los
20 lipooligosacáridos, lipopolisacáridos o glicanos de las glucoproteínas. Los mismos existen en una variedad de enlaces glucosídicos, más típicamente la $\alpha(2-3)$ y $\alpha(2-6)$ galactosa (o lactosa). La función de estos sialósidos varía ampliamente en los animales de acuerdo con la heterogeneidad estructural de
25 la porción del oligosacárido. Los mismos son mediadores de

eventos inter o intra-celulares y en particular desempeñan un papel importante en la fisiología y el crecimiento de muchos agentes patógenos (DK Ress, et al., Current Organic Synthesis, 2004, 1, 31-46).

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(Ia)

Un litro de leche humana contiene aproximadamente 5-10 g de oligosacáridos libres, este contenido es semejante al contenido de las proteínas y excede el contenido de los lípidos. Más de 130 diferentes oligosacáridos fueron identificados en la leche humana (Human Milk Oligosaccharides - HMO), las formulaciones de la leche artificial para bebés se derivan de la leche de bovino y contienen solamente cantidades de traza de estos oligosacáridos que son específicos de la especie humana.

Los bloques de construcción fundamentales de los oligosacáridos de la leche humana son 5 monosacáridos, D-glucosa (Glc), D-galactosa (Gal), N-acetilglucosamina (GlcNAc) L-fucosa (Fuc) y ácido siálico (ácido N-acetilneuramínico, Neu5Ac). El extremo de reducción terminal puede ser formado por la lactosa (Gal β 1-4Glc) o más

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unidades repetitivas (hasta 15 unidades) de N-acetil-lactosamina (Gal β 1-3/4GlcNAc). La lactosa o polilactosamina puede ser tratada con sialilo con los enlaces α 2-3 y/o α 2-6. Los ejemplos de sialósidos de la
5 leche humana son: 3'-sialil-3-fucosil-lactosa (3'S3FL), 6'-sialil-lactosa (6'SL), 3'-sialil-lactosa (3'SL), 3'-sialil-lactosamina (3'SLN), 6'-sialil-lactosamina (6'SLN).

Entre los sialósidos presentes principalmente en
10 los tejidos del mamífero y en la leche humana, el compuesto de la fórmula (Ia) la 6'-sialil-lactosa (N-acetilneuraminil-lactosa, α -NeuNAc-(2 $\rightarrow$ 6)- β - D - Gal-(1 $\rightarrow$ 4)-D-Glc o 6'-LS) es de importancia particular a causa de que es un constituyente importante de las glucoproteínas y los
15 glucolípidos involucrados en varios eventos de las rutas celulares incluyendo el reconocimiento celular y la respuesta inmunológica. La 6'-LS y sus sales son interesantes como suplementos en las formulaciones alimenticias para los infantes. En cuanto a las sales de la 6'-sialil-lactosa en la
20 literatura solamente la sal de sodio (Número de CAS: 157574-76-0; FW: C₂₃H₃₈NO₁₉Na, la sal de sodio de la 6'-sialil-lactosa, sal de sodio de la 6'-N-acetilneuraminil-lactosa) y la sal de amonio, ya son conocidas. Aunque la sal de sodio es aceptable para las sustancias alimenticias y farmacéuticas,
25 la sal de amonio es potencialmente tóxica a causa del ion

amonio. Por esta razón es necesario proporcionar la 6'-LS en formas alternativas de la sal con respecto a unas conocidas que puedan ser aceptables para las sustancias alimenticias y farmacéuticas.

5 En el estado del arte ya se conocen varias estrategias para la síntesis de los sialil-oligosacáridos (incluyendo la 6'-LS) y todas anticipan un método convergente en el cual el fragmento siálico activado (donador) está unido regio y estereo-selectivamente a la porción del oligosacárido
10 (aceptor). Para esta etapa clave de unión en la literatura ya se conocen tres estrategias sintéticas diferentes las cuales anticipan un método exclusivamente enzimático, exclusivamente químico o quimio-enzimático.

 En cuanto a las rutas enzimáticas, se utilizaron
15 las familias de las sialiltransferasas y transialidasas (enzimas que agregan el ácido siálico a los oligosacáridos en una ruta estrictamente específica). Los ejemplos de esta ruta de síntesis son reportados en A. T. Beyer et al *Adv. Enzymol.*, 1981, 52, 23-175, en J. Weinstein et al. *J. Biol.*
20 *Chem.*, 1982, 257, 13845-13853.

Sin embargo, son varias las limitaciones en el uso de estas enzimas:

- 1) la disponibilidad limitada de estas enzimas
- 2) la necesidad de sintetizar el donador del
25 sustrato activado CMP-NeuAc, o PNP-NeuAc

3) la especificidad estricta de las sialiltransferasas que reducen la flexibilidad en el uso para la síntesis de los sialósidos naturales (Ichigawa Y. et al. *Analytical Biochem* 1992, 202, 215-238, S. Sabesan et al. *J. Am. Chem. Soc.*, 1986, 108, 2068-2080, O. Hindsgaul et al. *J. Biol. Chem.*, 1991, 266, 17858-17862, H. J. Gross et al. *Eur. J. Biochem.* 1988, 177, 583-589).

En cuanto a las rutas quimio-enzimáticas, estas incluyen la síntesis química del aceptor y luego la sialilación enzimática como en S. Sabesan et al., *J. Am. Chem. Soc.*, 1986, 108, 2068-2080.

Enfocándose exclusivamente en las rutas químicas se enfatiza que la formación del enlace glucosídico con el ácido siálico es una reacción más bien difícil a causa de que la misma es impedida por el hecho de que el donador está impedido electrónicamente y estéricamente por el grupo carboxilo germinal. Además, la falta de un grupo funcional sobre C-3 regula su asistencia anquimérica para controlar la estereoquímica y conduce a la formación de subproductos por medio de la reacción de eliminación; finalmente la formación de la unión con una configuración α es termodinámicamente desventajosa con relación al efecto anomérico. En un intento por remediar estos defectos en el estado del arte, se han desarrollado varias estrategias para la preparación de donadores y aceptores siálicos activados adecuadamente con

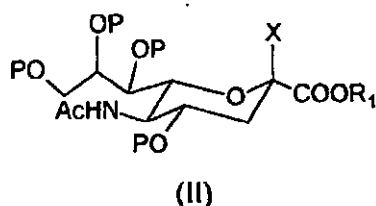
los grupos hidróxilo protegidos adecuadamente que se hacen reaccionar por medio de varios métodos de glucosilación.

Con respecto al donador siálico, se va a enfatizar que la arquitectura molecular complicada imparte un grado
5 substancial de dificultad en su síntesis, protección y activación. La naturaleza multifuncional (3 grupos hidróxilo secundarios) así como el centro anomérico terciario, complican el trabajo del especialista en química sintética. Comúnmente en el estado del arte se sabe bien que el donador
10 siálico puede ser activado como el 2-xantano (A. Marra et al., *Carbohydr. Res.*, 1989, 187, 35), como la 2-aril sulfona (Y. Du et al, 1998, 308, 161), como el 2-fosfito (RR Schmidt et al., *Tetrahedron Lett.*, 1992, 33, 6123 o CH Wong et al., *J. Am. Chem. Soc.*, 1992, 114, 8748) o como el derivado de 2-
15 halo. Entre todos estos grupos los derivados de halógeno son preferibles porque el fosfito y los derivados tio requieren reactivos tóxicos para su síntesis y no son fáciles de manejar en la industria. Entre los halógenos, el derivado del cloro es preferido porque es estable y fácil de sintetizar,
20 en efecto el derivado del bromo es inestable y tiende fácilmente a eliminarse y conduce a mezclas anoméricas durante las reacciones de glucosilación. El derivado de fluoro requiere una síntesis más elaborada que el derivado del cloro y tiende a formar enlaces β -glucosídicos. El
25 derivado del cloro podría ser el donador más fácil de hacer y

utilizar.

Con respecto a la estructura del donador, otras rutas sintéticas, todavía más complejas que algunas previas, requieren la inclusión sobre el C-3 del ácido siálico de un grupo funcional que proporciona asistencia anquimérica en la reacción de glucosilación para prevenir la eliminación competitiva en la posición 2,3. Por esta razón los grupos tales como el feniltio o el fenilselenio han sido introducidos (Y. Ito et al., *Tetrahedron Lett.*, 1988, 29, 3987 o en L. O. Kononov et al., *Tetrahedron Lett.*, 1997, 38, 1599). Estas rutas requieren así etapas múltiples para obtener el donador activo para la reacción de glucosilación partiendo generalmente del 2.3-deshidro NeuAc con rendimientos que varían de acuerdo con la especificidad obtenida y la facilidad de purificación obtenidas de los compuestos intermedios.

Por esta razón, para la síntesis de los sialiloligosacáridos de la fórmula (I) y en particular la 6'SL, podría ser preferible emplear eficientemente un derivado de sialilo simple como el donador de 2-cloro de la fórmula (II)



en donde P es un grupo protector adecuado, R1 es un grupo

alquilo y X es cloro; (que se puede obtener por los métodos reportados en R. Kuhn et al., *Chem. Ber.*, 1966, 99, 611, A. Marra et al., *Carb. Res.*, 1989, 190, 317-322 y NF Byramova et al., *Carb. Res.*, 1992, 237, 161-175) a causa de que también
5 es fácil sintetizarlo a escala industrial sin el uso de reactivos muy tóxicos, y conduce a la formación específica de los enlaces α en las reacciones de glucosilación. El uso de este donador siálico, sin embargo, se redujo significativamente después de sus primeras aplicaciones y el
10 estado del arte se dirige hacia donadores mucho más complejos.

Por lo que respecta a la activación del aceptor para la síntesis de 6'SL, en la literatura existen aceptores substituidos con grupos protectores de éter, por ejemplo
15 grupos bencilo cuya remoción, que requiere hidrogenación, no es manejable fácilmente (G. Pazynina et al. *Tetrahedron Lett.*, 2002, 43, 8011-8013) y por lo tanto difícil de aplicación industrial.

Los α -glucósidos del ácido siálico fueron
20 preparados por la reacción de Koenigs-Knorr involucrando el uso de Ag (I) como un promotor [Koenigs, W., Knorr, E. *Chem. Ber.*, 1901, 34, 957] o utilizando la modificación de Helferich que utiliza el Hg(II) como el promotor [Helferich, B.; Zirner, J. *Chem. Ber.*, 1962, 95, 2604].
25 Los substratos elegidos en estas reacciones son los

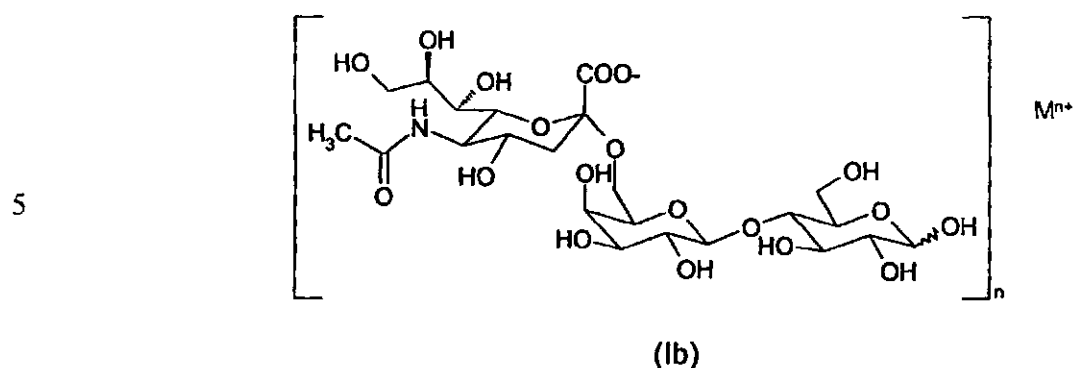
haluros de β -glucosilo. Ya se conocen muchas variaciones de estos métodos clásicos que fueron diseñados para mejorar las oportunidades de empleo y los rendimientos. Las diferencias principales entre estas variaciones están
5 relacionadas con la elección del contra anión del promotor metálico. Los promotores utilizados más comúnmente son AgOTf, Ag₂CO₃, HgX₂ (X = haluro), y Hg(CN)₂. En general ya se sabe que los promotores de Ag(I) son más activos y estereoselectivos (Pazynina G. et al. *Tetrahedron Lett*,
10 2002, 43, 8011-8013) pero estos deben ser utilizados en grandes cantidades (6-7 eq. con relación al aceptor) incrementando así el costo de la síntesis (incluyendo el desecho de la producción residual), mientras que los promotores de Hg(II) proporcionan rendimientos más
15 elevados (H. Paulsen et al. *Angew. Chem. Int. Ed. Engl.* 1982, 927-928) pero puede presentar dificultades en el manejo debido a su toxicidad.

Por lo tanto es evidente la necesidad de un proceso para la síntesis de los compuestos de la fórmula
20 (I) que sea simple y económico, aplicable a escala industrial, y por consiguiente que permita superar los problemas técnicos mencionados anteriormente relacionados con los procesos conocidos en la literatura.

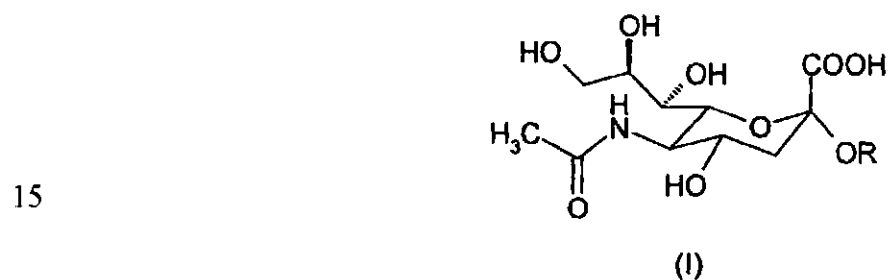
Breve Descripción de la Invención

25 La presente invención resuelve los problemas

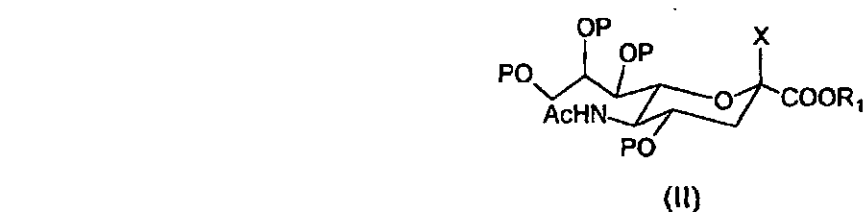
anteriores por los compuestos de la fórmula (Ib)



en donde M^{n+} es elegido del grupo que consiste de K^+ Ca^{2+} Mg^{2+} Sr^{2+} Fe^{2+} Al^{3+} . La materia objeto adicional de la presente
 10 invención es un proceso para la síntesis de los compuestos de α -sialil-oligosacáridos (I) de la fórmula (I)



en donde R es un residuo de mono, di u oligosacárido con los grupos hidróxilo libres; el proceso comprende al menos una etapa:
 a) la unión por medio de la reacción de Koenigs-Knorr del
 20 donador siálico de la fórmula (II)

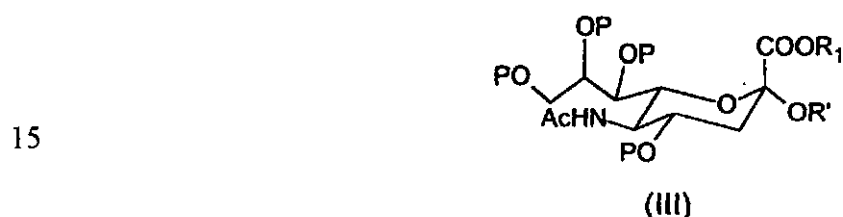


en donde P es un grupo protector adecuado, R1 es un grupo

alquilo, y X es un halógeno; con un aceptor de la fórmula $R'OH$ en donde R' es un residuo de mono, di u oligosacárido protegido adecuadamente con grupos protectores P' y que contienen cero, uno o más grupos
 5 hidróxilo libres; estos grupos protectores P' pueden ser los mismos o diferentes entre sí y de aquellos presentes en el donador;

tal reacción de Koenigs-Knorr caracterizada por el hecho de que el promotor metálico basado en $Ag(I)$ es utilizado en
 10 cantidades molares entre 0.5 y 2.0 eq., con respecto a las moles del aceptor;

para obtener un compuesto intermedio de la fórmula (III)



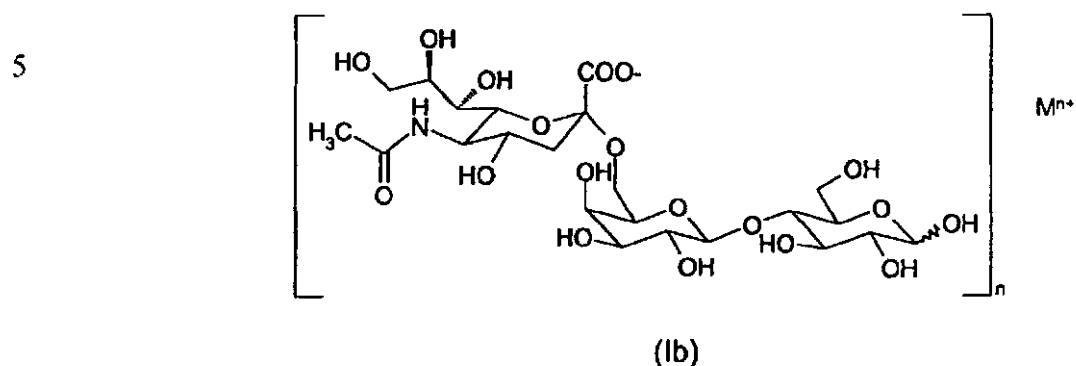
en donde P, R_1 y R' son como se definieron anteriormente.

En particular, el proceso mencionado anteriormente proporciona una ruta conveniente de
 20 síntesis para la 6'SL, y luego para sus sales de la fórmula (Ib) en las cuales M^{n+} es Na^+ , K^+ , NH_4^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} , Al^{3+} .

Otras ventajas de la presente invención son descritas posteriormente en la descripción
 25 detallada.

Descripción Detallada de la Invención

La materia objeto de la presente invención son los compuestos de la fórmula (Ib)



10 en donde M^{n+} es elegido del grupo que consiste de K^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} , Al^{3+} en donde $n = 1, 2, 3$ en correspondencia con el estado de oxidación de M. Preferentemente M^{n+} es Ca^{2+} , Mg^{2+} o K^+ .

Los compuestos de la fórmula (Ib) son todos
15 aceptables alimenticia y farmacéuticamente y son útiles como ingredientes activos potenciales o como suplementos alimenticios (tales como suplementos en formulaciones de leche artificial para infantes).

En particular 6'SL como:

20 - la sal de calcio es potencialmente útil para promover el crecimiento de los huesos;

- las sales de calcio y potasio son potencialmente útiles en el mantenimiento, la promoción o restablecimiento del transporte apropiado a través de las membranas biológicas
25 y la diferencia fisiológica del potencial de la

transmembrana;

- la sal de hierro es potencialmente útil para cualesquiera situaciones patológicas que requieren la integración del Fe.

5 La sal de calcio en particular tiene mejores propiedades físico-químicas que la sal de sodio bien conocida y su cristalización es más fácil. En efecto, durante la fase de cristalización, existe la formación de un sólido cristalino, fácil de ser manejado y por consiguiente más
10 fácil de ser manipulado aún a escala industrial y su filtración no tiene problemas a causa de que es rápida y permite un lavado eficiente del sólido.

 En el caso de la sal de sodio, en lugar de esto, durante la fase de cristalización se obtiene inicialmente
15 un sólido gomoso, difícil de ser agitado, que tiene que ser molido, y su filtración resulta lenta y laboriosa. La estabilidad de las dos sales parece que va a ser semejante.

 Otro aspecto positivo de la sal de calcio es el hecho de que partiendo de una misma matriz de 6'SL en la
20 forma ácida, la sal de calcio es una obtenida con una pureza más elevada: por ejemplo la misma matriz de la 6'SL ha suministrado un cristal de la sal de sodio a una pureza de 87 % de HPLC y de un cristal de la sal de calcio a una pureza de 93 % de HPLC.

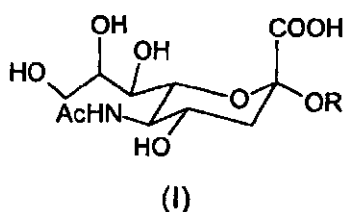
25 Las sales tanto de potasio como de magnesio muestran

características físico-químicas semejantes a la sal de sodio así como su cristalización tiene una tendencia semejante.

Los compuestos de la fórmula (Ib) anteriormente descritos pueden ser preparados por 6'SL siguiendo los métodos conocidos en el estado del arte para la preparación de las sales a partir de los ácidos carboxílicos correspondientes, por ejemplo los mismos pueden ser preparados preferentemente a partir de una solución de 6'SL agregando una base que contiene M^{2+} tales como los hidróxidos, carbonatos o bicarbonatos (es decir, KOH, $Ca(OH)_2$, $Mg(OH)_2$, etc., K_2CO_3 , $CaCO_3$, $MgCO_3$, etc.; $KHCO_3$, etc.) hasta pH 8-10. Después de la remoción del solvente la sal obtenida se purificó por cristalización a partir de los alcoholes o las mezclas de agua/alcohol; preferentemente metanol, etanol y sus mezclas con agua.

Opcionalmente, antes de la remoción del solvente es posible remover el exceso de la base no disuelta por filtración, si está presente.

Para otro aspecto, la presente invención se relaciona con un proceso para la síntesis de los α -sialil-oligosacáridos de la fórmula (I)



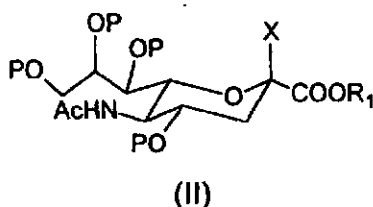
en donde R es un residuo mono, di u oligosacárido con los

grupos hidroxí libres, preferentemente R se selecciona entre la galactosa, glucosa, glucosamina, lactosa, lactosamina, fucosil-lactosa; más preferentemente R se selecciona entre la 6'-galactosa, 3'-glucosa, 3'-glucosamina, 6'-lactosa, 3'-
 5 lactosa, 6'-lactosamina, 3'-lactosamina, 3'-3-fucosil-lactosa;

tal proceso involucra al menos una etapa:

a) la unión por la reacción de Koenigs-Knorr de un donador siálico de la fórmula (II)

10



en donde:

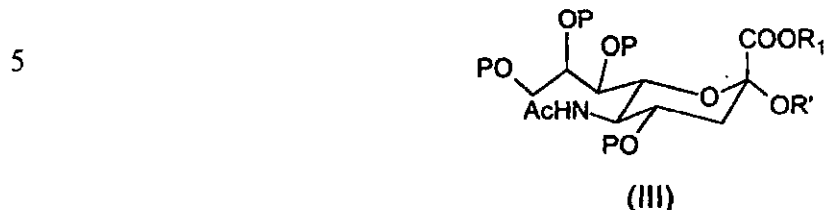
15 P es un grupo protector adecuado;

R1 es un alquilo, preferentemente Me, Et, o Pr;

X es un halógeno, preferentemente cloro;

con un aceptor protegido adecuadamente de la fórmula R'OH en la cual R' es un residuo de mono, di u oligosacárido protegido adecuadamente con los grupos protectores P' y que
 20 contiene cero, uno o más grupos hidróxilo libres: estos grupos protectores P' pueden ser iguales o diferentes entre sí y de aquellos presentes en el donador; la reacción de Koenigs-Knorr está caracterizada por el hecho de que el
 25 promotor metálico basado en Ag(I) es utilizado en cantidades

molares entre 0.5 y 2.0 eq., comparado con las moles del aceptor; para obtener un compuesto intermedio de la fórmula (III)



en donde P, R1 y R' son como se definieron anteriormente.

Para un aspecto preferido, este proceso también incluye después de la etapa (a), la siguientes etapa:

b) la remoción de los grupos protectores P, P' y R1 para obtener los compuestos de la fórmula (I) como se describieron anteriormente.

15 La remoción del grupo protector b) es efectuada de acuerdo con los métodos conocidos en el estado del arte (TW Green y PGM WUTS. Green's Protective Groups in Organic Synthesis. Ed Wiley ed 4. 2006).

Para un aspecto preferido este promotor metálico es utilizado en una cantidad molar entre 0.75 y 0.85 eq. molares comparado con las moles del aceptor.

Para un aspecto preferido el promotor metálico se selecciona entre las sales de Ag(I) tales como AgOTf, Ag₂CO₃, y más preferentemente es Ag₂CO₃.

25 Para un aspecto preferido la unión de a) se lleva a

cabo en un solvente polar aprótico, preferentemente se lleva a cabo en diclorometano.

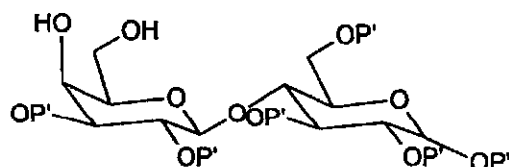
Para un aspecto preferido la unión a) se logra agitando la mezcla a una temperatura entre 20 y 40 °C durante un tiempo entre 5 y 10 días; preferentemente la mezcla se agita a 30 °C durante 7 días.

Para un aspecto preferido P y P' son seleccionados independientemente entre bencilo y acilo, preferentemente P y P' son seleccionados independientemente de acetilo, benzoilo o benzoilo mono o di-substituido con grupos alcoxi, halógeno, nitro.

Para un aspecto preferido P y P' son acilos. Para un aspecto más preferido P y P' son iguales y son acetilo. En donde R es 6'-lactosa en el proceso de la presente invención con $P = P' = \text{Ac}$ existe una distinción adicional de los procesos conocidos para la síntesis de 6'SL porque en este caso el aceptor utilizado en la presente invención no muestra grupos bencilo y en consecuencia tiene la ventaja de evitar la hidrogenación catalítica para la remoción de los grupos protectores.

Para un aspecto preferido, este aceptor R'OH protegido adecuadamente tiene un grupo hidróxilo libre y reactivo en C-6 de la porción galactosídica, en un aspecto preferido particularmente el aceptor es un

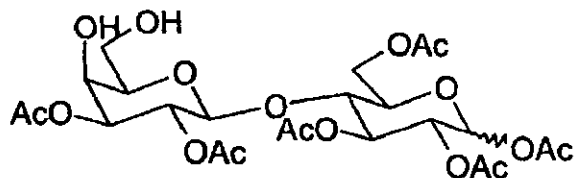
derivado de lactosa de la fórmula (IV):



(IV)

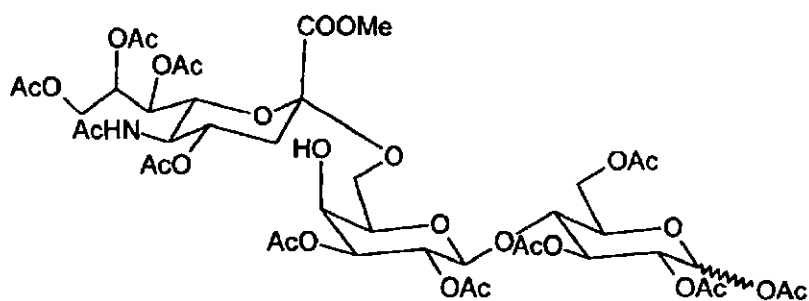
en donde P' es un grupo protector adecuado; preferentemente P' es acilo, más preferentemente acetilo.

Por lo tanto, para un aspecto preferido particularmente, la presente invención se refiere a la síntesis de 6'SL de la fórmula (Ia) en donde R = 6'-lactosa por el proceso descrito anteriormente en el cual P y P' son Ac, X es cloro y R1 es metilo; en esta combinación particular, la reacción de unión en la cual el aceptor es un compuesto de la fórmula (IVa)



(IVa)

proporciona el compuesto de la fórmula (IIIa)



(IIIa)

que sorprendentemente puede ser utilizado como el mismo ha

sido obtenido desde la mezcla sin refinar hasta la reacción subsiguiente de la desprotección consecutiva de los grupos de hidróxilo y de la función de carboxilo hasta el carbón anomérico del ácido siálico y la producción del compuesto 6'SL de la fórmula (Ia). Preferentemente se ha procedido primero a la remoción de los grupos acetilo y luego hasta la hidrólisis del éster metílico.

Esta desprotección consecutiva se lleva a cabo como se sabe en el estado del arte. Preferentemente la remoción de los grupos Ac es efectuada utilizando una base tal como metóxido de sodio, etóxido de sodio o hidróxido de sodio, más preferentemente metóxido de sodio, utilizando un alcohol primario como un solvente, semejante al metanol o etanol, aún más preferentemente metanol. Preferentemente, la hidrólisis del éster metílico en el carbono anomérico del ácido siálico se lleva a cabo en las condiciones básicas con NaOH 1 M.

Preferentemente, cuando se complementa la hidrólisis del éster metílico, la acidificación final de la mezcla de reacción se efectúa por resinas de intercambio iónico, en particular una resina catiónica fuerte y una resina aniónica débil, para obtener un material eluido que contiene 6'SL.

El donador siálico utilizado en el proceso descrito anteriormente, en particular cuando X es cloro y R' es lactosa protegida por la fórmula (IV) con $P = P' = \text{Ac}$, es

simple o fácil de preparar y fácil de utilizar. Esta elección ha revelado sorprendentemente que resuelve adecuadamente los problemas de los procesos conocidos, a pesar de que el estado del arte se ha dirigido hacia donadores mucho más complejos.

5 La cantidad del metal promotor utilizado en la etapa a) es reducida sorprendentemente comparado con el estado del arte conocido, 0.5-2.0 equivalentes contra 6-7 equivalentes, y por lo tanto se reduce el costo de la síntesis y los costos del desecho de la producción de
10 residuos.

 El producto de la reacción de acoplamiento se logra sorprendentemente con suficiente pureza para ser utilizado como está en la reacción subsiguiente de desprotección para obtener los sialil-oligosacáridos desprotegidos con buenos
15 rendimientos y pureza. Además, se debe señalar que el proceso para la reacción de unión de la presente invención es estereoselectiva a causa de que el isómero α es el único obtenido.

 El proceso de la presente invención por lo tanto es
20 factible a escala industrial.

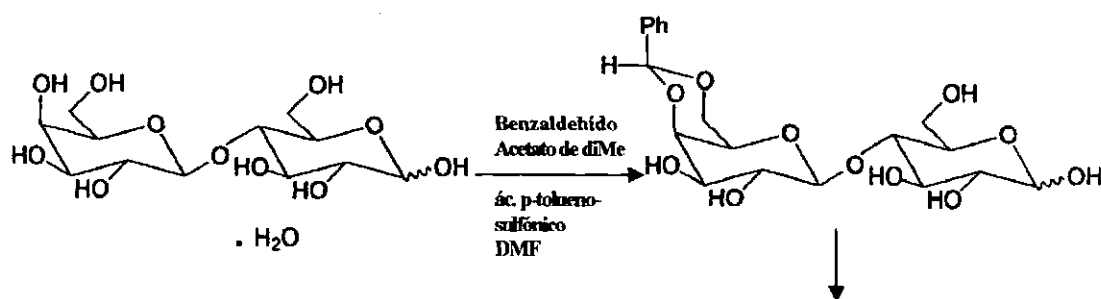
 La presente invención también se refiere a un proceso para la síntesis de los compuestos de la fórmula (Ib) en el cual M^{n+} es Na^+ , K^+ , NH_4^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} , Al^{3+} , tal proceso incluye la preparación de 6'SL por medio
25 del proceso descrito anteriormente; preferentemente las

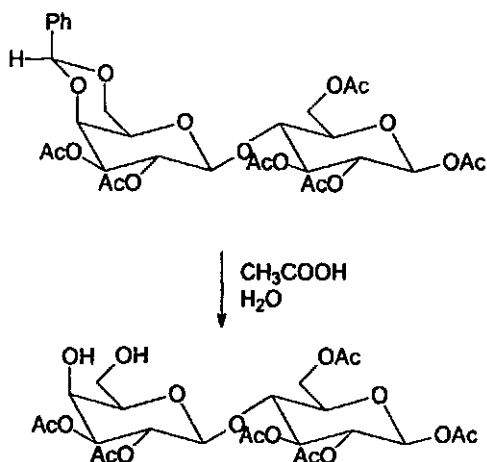
sales de la fórmula (Ib) pueden ser preparadas directamente por el material eluido que contiene la 6'SL, obtenido después del tratamiento con las resinas de intercambio iónico después de la hidrólisis del éster metílico, por la
 5 adición de una base que contiene $M^{(n+)}$ tales como por ejemplo los hidróxidos, carbonatos o bicarbonatos (es decir, KOH, $Ca(OH)_2$, $Mg(OH)_2$, etc., K_2CO_3 , $CaCO_3$, $MgCO_3$, etc.; $KHCO_3$, etc.) hasta pH 8-10.

Los compuestos de la fórmula (II) pueden ser
 10 obtenidos por las técnicas conocidas para el estado del arte tales como los reportados en (R. Kuhn et al., *Chem. Ber.*, 1966, 99, 611, A. Marra et al., *Carb. Res.*, 1989, 190, 317-322 y NF Byramova et al., *Carb. Res.*, 1992, 237, 161-175).

La síntesis del aceptor protegido adecuadamente puede ser llevada a cabo siguiendo el conocimiento del experto en el arte, en particular la síntesis del aceptor de la fórmula (IIIa) en donde P es Ac se lleva a cabo de acuerdo con los métodos conocidos de acuerdo con el Esquema de
 20 Reacción 1.

25





(IVa) Esquema de Reacción 1)

De acuerdo con la invención, alcoxi significa, por ejemplo -OMe, -OEt, -OnPr, -OiPr, -OnBu, -OiBu, -OtBu.

De acuerdo con la invención halógeno significa flúor, cloro, bromo, yodo.

De acuerdo con la invención alquilo es una cadena de alquilo lineal o ramificado que contiene 1 a 6 átomos de carbono posiblemente substituidos por uno o más grupos seleccionados entre halógeno, hidroxilo, alcoxi, nitro.

De acuerdo con la invención arilo es benceno eventualmente reemplazado por uno o más grupos seleccionados de halógeno, alcoxi, nitro.

De acuerdo con la invención acilo significa un grupo -OCO-alquilo, o -OCO-arilo en el cual alquilo y arilo son como se definieron anteriormente.

De acuerdo con la invención, monosacárido significa un polioxialdehído (aldosa) o una polioxiketona (cetosa) o un

azúcar simple de la fórmula $(\text{CH}_2\text{O})_n$, $\text{C}_n\text{H}_{2n}\text{O}_{n-1}$, $\text{C}_n\text{H}_{2n}\text{O}_{n-1}\text{NH}_2$ o $\text{C}_n\text{H}_{2n}\text{O}_{n-1}\text{NHAc}$ con $n = 3, 4, 5, 6, 7$; el mismo significa dentro de la definición la totalidad de los estereoisómeros posibles y todas las formas abiertas o cíclicas o semiacetales o
5 semicetales intramoleculares como un ejemplo de las formas de piranosilo o furanosilo; por ejemplo, gliceraldehído, alosa, altrosa, arabinosa, eritrosa, fucosa, galactosa, glucosa, glucosamina, N-acetil-glucosamina, idosa, lixosa, manosa, psicosa, ribosa, desoxirribosa, sorbosa, tagatosa, treosa,
10 xilosa y las cetosas correspondientes están incluidas en la definición.

De acuerdo con la invención, disacárido significa un compuesto polioxidrilado que consiste de dos monosacáridos enlazados a través de un enlace acetálico o
15 glucosídico tanto O-glucosídico o N-glucosídico; dentro de la definición están incluidos la totalidad de los posibles estereoisómeros y todas las formas abiertas o cíclicas; por ejemplo están incluidas la lactosa, lactosamina, N-acetil-lactosamina, maltosa, celobiosa, sucrosa,
20 trehalosa, turanosa.

De acuerdo con la invención, oligosacárido significa un polímero formado de tres a 6 monosacáridos unidos conjuntamente por enlaces glucosídicos para formar cadenas de sacáridos lineales o ramificadas, por ejemplo
25 están incluidas la rafinosa, melezitosa, maltotriosa,

acarbosa, estaquiosa.

Parte Experimental

Ejemplo 1

Preparación de la di 4',6'-O-bencilidenolactosa

5 Se agregan 200 g (0.555 mol) del monohidrato de lactosa bajo agitación a 1.4 l de N,N-dimetilformamida y luego se agregan 209 ml (1.39 mol) de benzaldehído dimetil acetal y 5.28 g (0.028 mol) del ácido p-toluenosulfónico monohidratado. La suspensión resultante se calienta a 55 °C y
10 la temperatura se mantiene hasta que la TLC fue exitosa (16-18 horas) (Farmacopea). Después de enfriamiento a temperatura ambiente se agregan 4.7 ml de trietilamina hasta pH 7-8. La mezcla se concentra para obtener 700 ml de la solución, la cual se drena en 3 litros de acetona caliente (50-55 °C),
15 manteniendo una agitación vigorosa. La precipitación se complementa por enfriamiento de la mezcla a 0+5 °C. El precipitado se filtra, se lava con 0.7 litros de acetona fría y se seca, obteniéndose 208 g de 4',6'-O-bencilideno lactosa (mezcla de anómeros α/β) como un producto blanco (ensayo de
20 HPLC 66%, 0.319 mol, rendimiento: 57%).

Por cristalización doble, primero por MeOH y luego por MeOH/H₂O 4/1 v/v, se obtiene una muestra analítica enriquecida en un anómero α , la caracterización de RMN es reportada:

25 ¹H RMN (DMSO-d₆, 300 MHz): δ ppm 7.51-7.34 (5H, m, Ph); 6.36

(d, $J_{\text{OH-1}} = 4.8$ Hz, 1H, C1-OH); 5.58 (s, 1H, PhCH); 5.28 (d, $J = 4.2$ Hz, 1H, OH); 5.01 (d, $J = 5.7$ Hz, OH); 4.92 (pseudo t, $J_{\text{OH-1}} = J_{1-2} = 4.0$ Hz, 1H, H-1); 4.68 (d, $J = 6.9$ Hz, 1H, OH); 4.45 (m, 2H, 2xOH); 4.37 (d, $J_{1'-2'} = 7.5$ Hz, 1H, H-1'); 4.16-
 5 3.95 (m, 3H); 3.84-3.11 (m, 9H) (H-2, H-3, H-4, H-5, CH₂-6, H-2', H-3', H-4', H-5', CH₂-6').

¹³C RMN (DMSO-d₆, 75 MHz): 8 ppm 138.5, 128.6, 127.9, 126.2 (Ph); 103.1 (C-1'); 99.8 (PhCH); 92.1 (C-1); 79.6, 75.8, 72.2, 71.6, 71.3, 69.9, 69.8, 68.5 (C2, C2', C3, C3', C4, C4', C5, C5'); 66.2 (C6'); 60.3 (C6).

R_f (farmacopea, UV-vis y naftoresorcina) = 0.7.

Ejemplo 2

Preparación de 4',6'-O-p-metoxibencilinlactosa

Se agregan 200 g (0.555 mol) de monohidrato de
 15 lactosa bajo agitación a 1.4 l de N,N-dimetilformamida y luego se agregan 237 ml (1.39 mol) de p-metoxibenzaldehído dimetil acetal y 5.28 g (0.028 mol) de ácido p-toluenosulfónico monohidratado. La suspensión resultante se calienta a 55 °C y la temperatura se mantiene hasta que
 20 resultó una TLC positiva (16-18 horas) (Farmacopea). Después de enfriamiento a temperatura ambiente se agregan 5.0 ml de trietilamina hasta pH 7-8. La mezcla se concentra y el residuo se cristaliza en 3 litros de acetona caliente (50-55 °C). La precipitación se complementó por el enfriamiento de
 25 la mezcla a 0±5 °C. El precipitado se filtra, se lava con

2x200 ml de acetona fría, obteniéndose 219 g de 4',6'-O-p-metoxibencilideno lactosa (mezcla de anómeros α/β) como un sólido amarillo tenue (ensayo de HPLC 76%, 0361 mol, rendimiento: 65%). La cristalización a partir de acetona
 5 caliente/H₂O 4/1 v/v dio una muestra analítica como una mezcla anomérica α/β (1/1 mol/mol), la caracterización de RMN es reportada:

¹H RMN (DMSO-d₆, 300 MHz): δ ppm 7.38 (d, J = 8.7 Hz, 2H), 6.93 (d, J = 8.7 Hz, 2H) (Ph); 6.70 (d, J_{OH-1} = 6.6 Hz, 1H, C1-OH
 10 β); 6.36 (d, J_{OH-1} = 4.1 Hz, 1H, C1-OH α); 5.52 (s, 1H, PhCH $\alpha+\beta$); 5.27 (m, 1H, OH $\alpha+\beta$); 5.04-4.95 (m, 1H, OH $\alpha+\beta$); 4.92 (pseudo t, J = 4.1 Hz, H-1 α); 4.72-4.60 (m, 1H, OH $\alpha+\beta$); 4.56-4.28 (m, H-1' $\alpha+\beta$ + H-1 β + 2xOH $\alpha+\beta$); 4.12-3.92 (m, 3H); 3.76 (s, 3H, OMe); 3.80-3.11 (m, 9H); 2.98 (m, 1H β).

15 ¹³C RMN (DMSO-d₆, 75 MHz): δ ppm 159.4, 130.9, 127.6, 113.2 (Ph); 103.0 (C-1' $\alpha+\beta$); 99.7 (PhCH $\alpha+\beta$); 96.7 (C-1 β); 92.1 (C-1 α); 79.6, 79.2, 75.7, 74.9, 74.8, 74.6, 72.2, 71.6, 71.3, 69.9, 69.8, 68.4 (C2, C2', C3, C3', C4, C4', C5, C5' $\alpha+\beta$); 66.2 (C6' $\alpha+\beta$); 60.4, 60.3 (C6 $\alpha+\beta$); 55.1 (OMe $\alpha+\beta$).

20 R_f (farmacopea, UV-vis y naftoresorcina) = 0.8.

Ejemplo 3

Preparación de 1,2,3,6,2',3'-hexa-O-acetil-4',6'-O-benciliden- β -D-lactosa

Se agregan 100 g (0.153 moles a partir de HPLC) de
 25 la 4',6'-O-bencilidenolactosa obtenida de acuerdo con el

ejemplo 1 y 256 ml (1.84 mol) de trietilamina a 600 ml de metil etil cetona. La mezcla de la reacción se calienta a 60 °C y se agregan por goteo 174 ml (1.84 mol) de anhídrido acético manteniendo la temperatura interna abajo de 70 °C.

5 La mezcla de la reacción se agita a 70 °C hasta que la TLC fue exitosa (10-12 horas) (AcOEt). El solvente se evapora y el residuo se disuelve en 270 ml de diclorometano y 200 ml de agua. Se agrega NaOH al 30 % bajo agitación hasta pH 9-9.5, luego se separan las capas; la capa acuosa se extrae

10 nuevamente con 75 ml de diclorometano. Las capas orgánicas colectadas se lavan con 200 ml de agua, y luego se agrega una solución de HCl al 32 % bajo agitación hasta pH 1-1.5. La capa acuosa ácida se extrae con 75 ml de diclorometano. Las capas orgánicas colectadas se lavan entonces con 370 ml

15 de NaCl al 20 %, se seca sobre Na₂SO₄ anhidro y se decolora con carbón vegetal y bentonita. El solvente se concentra y el residuo se utiliza como tal en la siguiente reacción. La dosificación de HPLC dio 103 g (0.151 mol) de 1,2,3,6,2',3'-hexa-O-acetil-4',6'-O-bencilideno lactosa

20 esencialmente como el anómero β (anómero α < 10 % mol) (rendimiento: 99 %). Una muestra analítica que contiene 9 % en mol de un anómero α fue obtenida por cristalización a partir de MeOH caliente; la siguiente es la caracterización de RMN (anómero β).

25 ¹H RMN (CDCl₃, 300 MHz): δ ppm 7.54-7.34 (m, 5H, Ph); 5.68 (d,

$J_{1-2} = 8.4$ Hz, 1H, H-1), 5.47 (pseudo s, 1H, CHPh); 5.32-5.21 (m, 2H, H-3+H-2'), 5.07 (dd, $J_{2-3} = 9.6$ Hz e $J_{2-1} = 8.4$ Hz, 1H, H-2), 4.87 (dd, $J_{3'-2'} = 10.4$ Hz e $J_{3'-4'} = 3.8$ Hz, 1H, H-3'), 4.54-4.43 (m, 2H, H-1'+H-6a), 4.38-4.25 (m, 2H, H-4'+H-6'a),
 5 4.14 (dd, $J_{6b-6a} = 12.2$ e $J_{6b-5} = 4.6$ Hz, 1H, H-6b), 4.04 (d, $J_{6'b-6'a} = 12.3$ Hz, 1H, H-6'b), 3.90-3.70 (m, 2H, H4+H5); 3.46 (pseudo s, 1H, H-5'), 2.14-2.00 (6xCOCH₃).

¹³C RMN (CDCl₃, 75 MHz): δ ppm 170.8, 170.4, 170.1, 169.7, 169.0, 168.9 (6xCOCH₃); 137.5, 129.3, 128.3, 126.6 (Ph);
 10 101.4 (CHPh); 101.1 (C-1'); 91.8 (C-1); 75.5, 73.8, 73.2, 72.4, 72.2, 70.5, 69.0, 68.5 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'); 66.6 (C-6'); 61.8 (C-6), 20.9-20.7 (6xCOCH₃).

Rf (AcOEt:hexano = 1:1, UV-vis y H₂SO₄/MeOH) = 0.3.

Ejemplo 4

15 Preparación de 1,2,3,6,2',3'-hexa-O-acetil-4',6'-O-p-metoxibenciliden- β -D-lactosa

Se agregan 100 g (0.165 moles de dosificación por HPLC) de la 4',6'-O-p-bencilidenolactosa obtenida de acuerdo con el ejemplo 2 y 242 ml (1.74 mol) de trietilamina a 600 ml
 20 de metil etil cetona. La suspensión se calienta a 60 °C y se agregan por goteo 164 ml (1.74 mol) de anhídrido acético manteniendo la temperatura interna abajo de 70 °C. La mezcla de la reacción se agita a 70 °C hasta que la TLC positiva fue exitosa (10-12 horas) (AcOEt:hexano = 1:1). El solvente se
 25 evapora y el residuo se disuelve en 270 ml de diclorometano y

200 ml de agua. Se agrega NaOH al 30 % bajo agitación hasta pH 9-9.5, luego se separan las capas; la capa acuosa se extrae nuevamente con 75 ml de diclorometano. Las capas orgánicas colectadas se lavan con 200 ml de agua, y luego se
5 agrega una solución de HCl al 32 % bajo agitación hasta pH 1-1.5. La capa acuosa ácida se extrae con 75 ml de diclorometano. Las capas orgánicas colectadas se lavan entonces con 400 ml de NaHCO₃ saturado, con 400 ml de NaCl al 20 %, se secan sobre Na₂SO₄ anhidro y se blanquea con carbón
10 vegetal y bentonita. El solvente se concentra y el residuo se utiliza como tal en la siguiente reacción. La dosificación de HPLC dio 110 g (0.155 mol) de 1,2,3,6,2',3'-hexa-O-acetil-4',6'-O-p-metoxibenciliden-lactosa esencialmente como el anómero β (rendimiento: 94 %). Una muestra analítica fue
15 obtenida por cristalización a partir de MeOH caliente; la siguiente es la caracterización de RMN (anómero β).

¹H RMN (CDCl₃, 300 MHz): δ ppm 7.36 (d, J = 8.7 Hz, 2H), 6.88 (d, J = 8.7 Hz, 2H) (Ph); 5.66 (d, J₁₋₂ = 8.4 Hz, 1H, H-1); 5.40 (pseudo s, 1H, CHPh); 5.24 (pseudo t, J₃₋₂ = J₃₋₄ = 9.6 Hz, 1
20 H, H-3); 5.23 (dd, J_{2'-3'} = 10.2 Hz e J_{2'-1'} = 7.8 Hz, 1H, H-2'); 5.04 (dd, J₂₋₃ = 9.6 Hz e J₂₋₁ = 8.4 Hz, 1H, H-2); 4.84 (dd, J_{3'-2'} = 10.2 Hz e J_{3'-4'} = 3.6 Hz, 1H, H-3'), 4.46 (dd, J_{6a-6b} = 12.0 Hz e J_{6a-5} = 1.5 Hz, 1H, H-6a); 4.44 (d, J_{1'-2'} = 7.8 Hz, 1H, H-1'); 4.28 (d, J_{4'-3'} = 3.6 Hz, 1H, H-4'); 4.25
25 (d, J_{6'a-6'b} = 12.6 Hz, 1H, H-6'a); 4.12 (dd, J_{6b-6a} = 12.0 e J_{6b-5}

= 4.5 Hz, 1H, H-6b), 4.00 (dd, $J_{6'b-6'a} = 12.6$ Hz e $J_{6'a-5'} = 1.5$ Hz, 1H, H-6'b); 3.87-3.69 (m, 2H, H4+H5); 3.79 (s, 3H, OMe); 3.42 (pseudo s, 1H, H-5'); 2.09, 2.07, 2.03, 2.02, 2.00 (6xCOCH₃).

5 ¹³C RMN (CDCl₃, 75 MHz): δ ppm 170.8, 170.4, 170.1, 169.6, 168.93, 168.89 (6xCOCH₃); 160.3, 130.1, 127.9, 113.7 (Ph); 101.3 (CHPh); 101.1 (C-1'); 91.8 (C-1); 75.5, 73.8, 73.2, 72.4, 72.1, 70.5, 69.0, 68.4 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'); 66.5 (C-6'); 61.8 (C-6), 55.4 (OMe); 20.9-20.6
10 (6xCOCH₃).

Rf (AcOEt:hexano = 1:1, UV-vis y H₂SO₄/MeOH) = 0.2.

Ejemplo 5

Preparación de 1,2,3,6,2',3'-hexa-O-acetil-β-D-lactosa

El jarabe que contiene 100 g (0.15 moles) de la
15 1,2,3,6,2',3'-hexa-O-acetil-4',6'-O-bencilidenolactosa
obtenida de acuerdo con el ejemplo 2 se disuelve en 400 ml de ácido acético glacial. La mezcla de reacción se calienta a 80 °C y luego se agregan 100 ml de agua (precalentada a 80 °C), agitando la mezcla a esta temperatura durante 1.5 horas. La
20 mezcla de reacción se enfría entonces rápidamente a temperatura ambiente y se agregan 500 ml de tolueno y 350 ml de agua y se extrae. La capa acuosa se extrae con 150 ml de tolueno. Las capas de tolueno combinadas contuvieron 1,2,3,6,2',3'-hexa-O-acetil-4',6'-O-bencilidenolactosa que no
25 reaccionó, que puede ser utilizada en otra reacción. La capa

acuosa, que contiene la 1,2,3,6,2',3'-hexa-O-acetil- β -D-lactosa, se extrae sucesivamente con 500 ml y 150 ml de cloruro de metileno; los extractos orgánicos se lavan con 3x150 ml de agua, se secan sobre sulfato de sodio anhidro y se concentran. El residuo se cristaliza a partir de 580 ml de acetato de isopropilo caliente (50-55 °C), obteniéndose después del secado 30.8 g de la 1,2,3,6,2',3'-hexa-O-acetil- β -D-lactosa (0.05 mol) como un sólido blanco yesoso. Considerando que en las capas de tolueno combinadas se recuperaron 20.2 g de la 1,2,3,6,2',3'-hexa-O-acetil-4',6'-O-bencilidenolactosa que no reaccionó (0.03 mol), el rendimiento es del 42 %.

P.f. 188-190 °C.

^1H RMN (CDCl_3 , 300 MHz): δ ppm 5.68 (d, $J_{1-2} = 8.1$ Hz, 1H, H-1), 5.24 (pseudo t, $J_{3-2} = J_{3-4} = 9.3$ Hz, 1H, H-3), 5.19 (dd, $J_{2'-3'} = 10.2$ Hz e $J_{2'-1'} = 7.8$ Hz, 1H, H-2'), 5.05 (dd, $J_{2-3} = 9.3$ Hz e $J_{2-1} = 8.1$ Hz, 1H, H-2), 4.88 (dd, $J_{3'-2'} = 10.2$ Hz e $J_{3'-4'} = 3.3$ Hz, 1H, H-3'), 4.49 (d, $J_{1'-2'} = 7.8$ Hz, 1H, H-1'), 4.49 (dd, $J_{6a-6b} = 11.1$ Hz e $J_{6a-5} = 1.8$ Hz, 1H, H-6a), 4.15-4.05 (m, 2H, H-4'+H-6b), 4.00-3.70 (m, 4H, H-4+H-5+H-6'a+H-6'b), 3.56 (pseudo t, $J = 5.4$ Hz, 1H, H-5'), 2.96 (d, $J_{\text{OH}-4'} = 4.2$ Hz, C4'-OH), 2.59 (dd, $J_{\text{OH}-6'} = 7.5$ e 4.8 Hz, C6'-OH), 2.11, 2.09, 2.08, 2.07, 2.04, 2.03 (6xCOCH₃).

^{13}C RMN (CDCl_3 , 75 MHz): δ ppm 170.6, 170.4, 170.3, 169.7, 169.6, 169.0 (6xCOCH₃); 101.2 (C-1'), 91.7 (C-1), 75.9 (C-4),

74.6 (C-5'), 73.7 (C-3'), 73.6 (C-5), 73.1 (C-3), 70.7 (C-2), 69.7 (C-2'), 67.8 (C-4'), 62.1, 62.0 (C-6, C-6'), 20.9, 20.8, 20.7 (6xCOCH₃).

R_f (AcOEt, UV-vis y H₂SO₄/MeOH) = 0.4.

5 Ejemplo 6

Preparación de di 1,2,3,6,2',3'-hexa-O-acetil-β-D-lactosa

El jarabe que contiene 100 g (0.14 mol) de la 1,2,3,6,2',3'-hexa-O-acetil-4',6'-O-p-metoxibencilidenolactosa obtenido de acuerdo con el ejemplo 4 se disuelve en 400 ml de ácido acético glacial, y la mezcla se agita a temperatura ambiente durante 4.5 horas. Se agregan 500 ml de tolueno y 350 ml de agua, luego se extrae. La capa acuosa se extrae con 150 ml de tolueno. Las capas de tolueno combinadas contuvieron la 1,2,3,6,2',3'-hexa-O-acetil-4',6'-O-p-metoxibencilidenolactosa que no reaccionó, que pueden ser utilizadas en otra reacción. La capa acuosa, que contiene la 1,2,3,6,2',3'-hexa-O-acetil-β-D-lactosa, se extrae sucesivamente con 500 ml y 150 ml de cloruro de metileno; los extractos orgánicos se lavan con 3x150 ml de agua, se secan sobre sulfato de sodio anhidro y se concentran. El residuo se cristaliza a partir de 580 ml de acetato de isopropilo caliente (50-55 °C) obteniéndose, después del secado, 40.6 g de la 1,2,3,6,2',3'-hexa-O-acetil-β-D-lactosa (0.07 mol, rendimiento del 50 %) como un sólido blanco pulvurento cuyas características son semejantes al sólido obtenido del ejemplo 5.

Ejemplo 7

Preparación de la (5-acetamido-4,7,8,9-tetra-O-acetil-3,5-didesoxi-D-glicero- α -D-galacto-non-2-ulopiranosilonato de metilo)-(2 $\rightarrow$ 6)-2,3-di-O-acetil- β -D-galactopiranosil-(1 $\rightarrow$ 4)-

5 1,2,3,6-tetra-O-acetil- β -D-glucopiranososa

100 g (0.168 mol) de la 1,2,3,6,2',3'-hexa-O-acetil- β -D-lactosa, obtenida de acuerdo con el ejemplo 3, se disuelven en 600 ml de diclorometano, luego se agregan 250 g de los tamices moleculares de 3A. La solución se agita durante
10 5-10 minutos y luego se agregan 38.0 g del carbonato de plata (0.14 mol). Se agrega una solución de 128.5 g (0.252 mol) (1.5 eq.) del derivado de cloro de la fórmula (II) en donde P es acetilo, X es cloro y R1 es metilo, en 500 ml de diclorometano y la solución se mantiene en agitación vigorosa a 30 °C
15 durante 7 días hasta que desapareció el derivado de cloro (TLC, CH₂Cl₂: MeOH = 10:1), luego la mezcla de reacción se filtra sobre dicalite y se removi6 el solvente, obteniéndose un residuo sólido quebradizo (aproximadamente 230 g), que contiene una mezcla del producto de la condensación, la
20 1,2,3,6,2',3'-hexa-O-acetil- β -D-lactosa y el producto de la eliminación 2.3 de (II), así como una traza de la 4,7,8,9-tetra-O-acetil-NANA. Por ¹³C RMN se evaluó una conversión de aproximadamente 90 %. Se obtuvo una muestra analítica del producto condensado por cristalización a partir de etanol:éter
25 isopropílico = 1:3 v/v como un sólido amorfo blanco:

^1H RMN (CDCl_3 , 300 MHz): δ ppm 5.66 (d, $J_{1-2} = 8.4$ Hz, 1H, H-1); 5.42-5.10 (m, 5H, H-3+H-2'+H-7''+H-8''+NH); 5.01 (pseudo t, $J_{2-1} = 8.4$ Hz, 1H, H-2); 4.94-4.78 (m, 2H, H-3'+H-4''); 4.53-4.27 (m, 3H, H-1'+H-6a+H-9''); 4.24-3.92 (m, 5H, H-6b+H-4'+H-5''+H-6''+H-9''b); 3.92-3.50 (m, 5H, H-4+H-5+H-5'+H-6'a+H-6'b); 3.80 (s, 3H, COOCH_3), 2.93 (s amp., 1H, OH); 2.55 (dd, $J_{3''\text{eq}-3''\text{ax}} = 12.6$ e $J_{3''\text{eq}-4''} = 4.5$ Hz, 1H, H-3''eq); 2.17-1.97 (31H, 10x CH_3CO e H-3''ax), 1.86 (s, 3H, NHCOCH_3).

^{13}C RMN (CDCl_3 , 75 MHz): δ ppm 171.05, 170.98, 170.5, 170.4, 170.29, 170.28, 170.24, 170.0, 169.6, 169.4, 169.0, 168.0 (OAc, NHAc, COOMe); 100.8 (C-1'); 99.1 (C-2''); 91.7 (C-1); 75.7, 73.7, 73.6, 72.94, 72.90, 72.4, 70.7, 69.7, 68.95, 68.87, 67.4, 66.3 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''); 62.6, 62.4, 62.1 (C-6, C-6', C-9''); 53.2 (OCH_3); 49.4 (C-5''); 37.4 (C-3''); 23.2 (NHCOCH_3), 21.1, 20.9, 20.8, 20.74, 20.67 (10x CH_3CO).

Ejemplo 8

Preparación de la (5-acetamido-3,5-didesoxi-D-glicero- α -D-galacto-non-2-ulopiranosilonato de metilo)-(2 $\rightarrow$ 6)- β -D-galactopiranosil-(1 $\rightarrow$ 4)-(α/β)-D-glucopiranososa

230 g del producto sin refinar, obtenido de acuerdo con el ejemplo 7, se disuelven en 1.4 l de MeOH, luego se agregan 29.6 ml de metóxido de sodio en metanol al 25 % en peso. La solución se mantiene en agitación a temperatura ambiente durante 12 h; en el control de TLC positivo

(Farmacopea) la misma fue neutralizada con 39 g de IR120 (H^+) seca. La resina se filtra y el solvente se remueve por medio de un rotoevaporador, obteniéndose 143 g del residuo, utilizado en siguiente reacción. El rendimiento fue cuantitativo.

- 5 ^1H RMN (D_2O , 300 MHz): δ ppm 5.21 (d, $J_{1-2} = 3.6$ Hz, 1H, H-1 α), 4.66 (d, $J_{1-2} = 7.8$ Hz, 1H, H-1 β), 4.42 (d, $J_{1'-2'} = 7.5$ Hz, 1H, H-1'), 4.15-3.45 (m, 19H), 3.89 (s, 3H, $COOCH_3$), 3.29 (m, 1H), 2.70 (dd, $J_{3''eq-3''ax} = 12.9$ e $J_{3''eq-4''} = 4.8$ Hz, H-3''eq), 2.03 (s, 3H, $NHCOCH_3$), 1.88 (pseudo t, $J_{3''ax-3''eq} = J_{3''ax-4''} =$
 10 12.9 Hz, H-3''ax).
- ^{13}C RMN (D_2O , 75 MHz): δ ppm 175.5 ($NHCOCH_3$), 170.4 ($COOMe$), 103.9 (C-1'), 99.6 (C-2''), 96.3 (C-1 β), 92.5 (C-1 α), 80.5, 80.4, 75.2 (2C), 74.4, 74.0, 73.5, 73.0, 72.2, 71.7, 71.3, 71.2, 70.5, 69.0 (2C), 67.9 (C-2, C-3, C-4, C-5, C-2', C-3',
 15 C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.0 (C-6'), 63.8 (C-9''), 60.8 e 60.7 (C-6 $\alpha+\beta$), 54.1 ($COOCH_3$), 52.3 (C-5''), 39.6 (C-3''), 22.8 ($NHCOCH_3$).

Ejemplo 9

- Preparación de la 5-acetamido-3,5-didesoxi-D-glicero- α -D-galacto-non-2-ulopiranosilonato de sodio-(2 $\rightarrow$ 6)- β -D-galactopiranosil-(1 $\rightarrow$ 4)-(α/β)-D-glucopiranososa

- 143 g del producto de la desacetilación sin refinar, obtenido de acuerdo con el ejemplo 8, se disuelven en 715 ml de agua. El pH se lleva a la neutralidad con NaOH
 25 acuoso y luego se agregan 23 ml de NaOH al 30 %, manteniendo

la temperatura de la solución abajo de 10 °C. La solución se agita a temperatura ambiente durante 24 h. En el control de TLC positivo (Farmacopea) la solución se hace pasar a través de IR120(H⁺)/IRA96(OH⁻). El material eluido se ajusta a pH 9
 5 con NaOH, se concentra hasta un jarabe, y se somete a separación varias veces con EtOH absoluto hasta que se obtiene un sólido blanco quebradizo que es recrystalizado a partir de EtOH al 96 %. Se obtuvieron 77.3 g.

Los datos de ¹H y ¹³C RMN para este compuesto estuvieron de
 10 acuerdo con aquellos reportados en la literatura (L. Dorland et al., *Eur. J. Biochem.* 1978, 87, 323; J.P. Kamerling et al., *Carbohydr. Res.* 1982, 100, 331).

¹H RMN (D₂O, 300 MHz): δ ppm 5.22 (d, J₁₋₂ = 3.8 Hz, 1H, H-1α), 4.66 (d, J₁₋₂ = 7.8 Hz, 1H, H-1β), 4.43 (d, J_{1'-2'} : 7.6 Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, J_{3''eq-3''ax} = 12.5 e J_{3''eq-4''} = 4.7 Hz, H-3''eq), 2.03 (s, 3H, NHCOCH₃), 1.74 (pseudo t, J_{3''ax-3''eq} = J_{3''ax-4''} = 12.5 Hz, H-3''ax).

¹³C RMN (D₂O, 75 MHz, referencia externa de acetona): δ ppm 175.6 (NHCOCH₃), 174.1 (COO⁻), 103.9 (C-1'), 100.9 (C-2''), 96.3 (C-1β), 92.5 (C-1α), 80.4, 80.3, 75.30, 75.26, 74.4, 74.3, 73.2, 73.0, 72.4, 72.3, 71.7, 71.4, 70.6, 69.2, 69.04, 69.01 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.2 (C-6'), 63.3 (C-9''), 60.9 e 60.8 (C-6α+β), 52.4 (C-5''), 40.7 (C-3''), 22.7 (NHCOCH₃).

25 [α]_D^{20°C}: +9.3° (C:1%, H₂O).

P.f.: 191.4+194.2 °C (T descomposición)

Ejemplo 10

Preparación de la 5-acetamido-3,5-didesoxi-D-glicero- α -D-galacto-non-2-ulopiranosilonato de calcio-(2 $\rightarrow$ 6)- β -D-galactopiranosil-(1 $\rightarrow$ 4)-(α/β)-D-glucopiranososa

100 g del producto de la desacetilación sin refinar, obtenido de acuerdo con el ejemplo 8, se disuelven en 500 ml de agua y la solución se enfría a 4 °C. El pH se lleva a la neutralidad con NaOH acuoso y luego se agregan 23 ml de NaOH al 30 %, manteniendo la temperatura de la solución abajo de 10 °C. La solución resultante se mantiene en agitación a temperatura ambiente durante 24 h. En el control de TLC positivo (Farmacopea) la solución se hace pasar a través de IR120(H⁺)/IRA96(OH⁻). El material eluido se ajusta a pH 8.7 con Ca(OH)₂, se filtra y luego se concentra hasta un jarabe a 65° Brix. Esta jarabe se hace gotear en 540 ml de metanol a 50 °C. La suspensión resultante se mantiene en agitación vigorosa a 50 °C durante 1 hora, luego a temperatura ambiente durante 1 hora, y luego se filtra bajo vacío. El sólido se lava con 160 ml de metanol y se seca bajo vacío a 50±55 °C. Se obtuvieron 46.2 g.

¹H RMN (D₂O, 300 MHz): δ ppm 5.22 (d, J₁₋₂ 3.8 Hz, 1H, H-1 α), 4.66 (d, J₁₋₂ 7.8 Hz, 1H, H-1 β), 4.43 (d, J_{1'-2'}: 7.8 Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, J_{3''eq-3''ax} 12.0 e J_{3''eq-4''} 4.5 Hz, H-3''eq), 2.03 (s, 3H, NHCOCH₃), 1.74

(pseudo t, $J_{3''ax-3''eq} = J_{3''ax-4''} = 12.0$ Hz, H-3"ax).

^{13}C RMN (D_2O , 75 MHz, referencia externa de acetonitrilo): 8 ppm 175.5 (NHCOCH_3), 174.1 (COO^-), 103.8 (C-1'), 100.9 (C-2''), 96.2 (C-1 β), 92.4 (C-1 α), 80.3, 80.3, 75.26, 75.22, 5 74.4, 74.3, 73.1, 73.0, 72.4, 72.2, 71.7, 71.4, 70.5, 69.1, 69.13, 69.00 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.2 (C-6'), 63.3 (C-9"), 60.9 e 60.7 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3"), 22.7 (NHCOCH_3).

Ensayo como Ca^{2+} : 98.3 %.

10 $[\alpha]_D^{20^\circ\text{C}}$: $+10^\circ$ (c:1%, H_2O).

P.f.: 204.5-206.6 $^\circ\text{C}$ (T descomposición)

IR $\nu^{\text{KBr}}_{\text{máx}}$: 3400, 1612, 1380, 1033 cm^{-1} .

Ejemplo 11

Preparación de la 5-acetamido-3,5-didesoxi-D-glicero- α -D-galacto-non-2-ulopiranosilonato de potasio-(2 $\rightarrow$ 6)- β -D-galactopiranosil-(1 $\rightarrow$ 4)-(α/β)-D-glucopiranososa

100 g del producto de la desacetilación sin refinar, obtenido de acuerdo con el ejemplo 8, se disuelven en 500 ml de agua y la solución se enfría a 4 $^\circ\text{C}$. El pH se 20 lleva a la neutralidad con NaOH acuoso y luego se agregan 23 ml de NaOH al 30 %, manteniendo la temperatura de la solución abajo de 10 $^\circ\text{C}$. La solución se agita a temperatura ambiente durante 24 h. En el control de TLC positivo (Farmacopea) la solución se hace pasar a través de IR120(H^+)/IRA96(OH^-). El 25 material eluido se ajusta a pH 10 con KOH, se concentra hasta

un jarabe, y se somete a separación varias veces con EtOH absoluto hasta que se obtiene un sólido quebradizo blanco que es recristalizado a partir de EtOH absoluto. El sólido se seca bajo vacío a 50÷55 °C. Se obtuvieron 35.7 g.

5 ^1H RMN (D_2O , 200 MHz): δ ppm 5.22 (d, J_{1-2} 3.8 Hz, 1H, H-1 α), 4.66 (d, J_{1-2} 7.8 Hz, 1H, H-1 β), 4.43 (d, $J_{1'-2'}$ 7.6 Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, $J_{3''\text{eq}-3''\text{ax}}$ 12.0 e $J_{3''\text{eq}-4''}$ 4.4 Hz, H-3''eq), 2.03 (s, 3H, NHCOCH_3), 1.74 (pseudo t, $J_{3''\text{ax}-3''\text{eq}} = J_{3''\text{ax}-4''}$ 12.0 Hz, H-3''ax).

10 ^{13}C RMN (D_2O , 75 MHz, referencia externa de acetona): δ ppm 175.5 (NHCOCH_3), 174.0 (COO^-), 103.8 (C-1'), 100.9 (C-2''), 96.2 (C-1 β), 92.4 (C-1 α), 80.3, 80.2, 75.22, 75.19, 74.4, 74.3, 73.1, 72.9, 72.4, 72.2, 71.7, 71.4, 70.5, 69.1, 69.04, 69.01 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.1 (C-6'), 63.2 (C-9''), 60.8 e 60.7 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3''), 22.6 (NHCOCH_3).

$[\alpha]_{\text{D}}^{20^\circ\text{C}}$: +9.8° (c: 1%, H_2O).

P.f.: 179.3÷182.8 °C (T descomposición)

IR $\nu_{\text{máx}}^{\text{KBr}}$: 3391, 1612, 1379, 1034 cm^{-1} .

20 Ejemplo 12

Preparación de la 5-acetamido-3,5-didesoxi-D-glicero- α -D-galacto-non-2-ulopiranosilonato de magnesio-(2→6)- β -D-galactopiranosil-(1→4)-(α/β)-D-glucopiranososa

25 100 g del producto de la desacetilación sin refinar, obtenido de acuerdo con el ejemplo 8, se disuelven

en 500 ml de agua y la solución se enfría a 4 °C. El pH se lleva a la neutralidad con NaOH acuoso y luego se agregan 23 ml de NaOH al 30 %, manteniendo la temperatura de la solución abajo de 10 °C. La solución se agita a temperatura ambiente durante 24 h. En el control de TLC positivo (Farmacopea) la solución se hace pasar a través de IR120(H⁺)/IRA96(OH⁻). El material eluido se ajusta a pH 9.8 con MgO, se concentra hasta un residuo sometido a separación varias veces con EtOH absoluto hasta que se obtiene un sólido quebradizo blanco que es recristalizado a partir de EtOH absoluto. Se obtuvieron 50.8 g.

¹H RMN (D₂O, 300 MHz): δ ppm 5.22 (d, J₁₋₂ 3.6 Hz, 1H, H-1α), 4.66 (d, J₁₋₂ 8.1 Hz, 1H, H-1β), 4.43 (d, J_{1'-2'}: 7.5 Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, J_{3''eq-3''ax} 12.3 e J_{3''eq-4''} 4.5 Hz, H-3''eq), 2.03 (s, 3H, NHCOCH₃), 1.74 (pseudo t, J_{3''ax-3''eq} = J_{3''ax-4''} 12.3 Hz, H-3''ax).

¹³C RMN (D₂O, 75 MHz, referencia externa de acetonitrilo): δ ppm 175.5 (NHCOCH₃), 174.1 (COO⁻), 103.8 (C-1'), 100.9 (C-2''), 96.3 (C-1β), 92.4 (C-1α), 80.3, 80.2, 75.26, 75.23, 74.4, 74.3, 73.1, 73.0, 72.4, 72.2, 71.7, 71.4, 70.6, 69.1, 69.00, 68.96 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.2 (C-6'), 63.3 (C-9''), 60.9 e 60.7 (C-6α+β), 52.4 (C-5''), 40.7 (C-3''), 22.7 (NHCOCH₃).

Ensayo como Mg^{2+} : 97.5 %

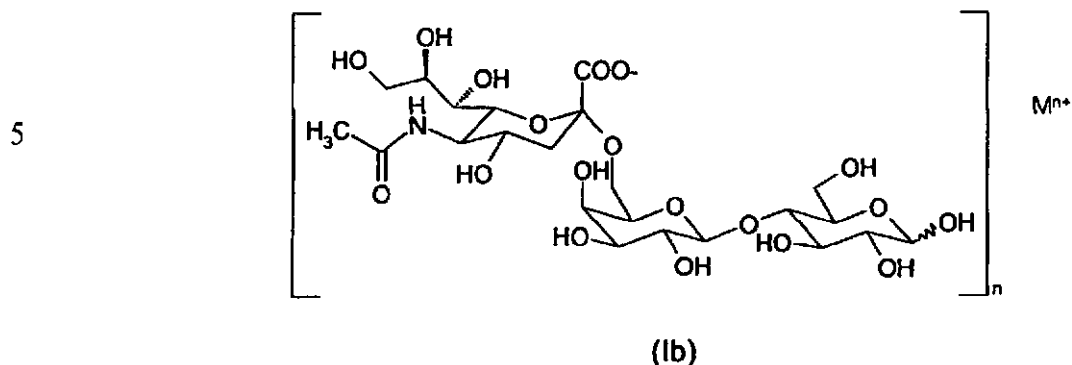
$[\alpha]_{\text{D}}^{20^\circ\text{C}}$: $+9.8^\circ$ (c: 1%, H_2O).

P.f.: $183.1\div 185.1^\circ\text{C}$ (T descomposición)

IR $\nu_{\text{máx}}^{\text{KBr}}$: 3391, 1634, 1379, 1035 cm^{-1} .

REIVINDICACIONES

1. Compuestos de la fórmula (Ib)

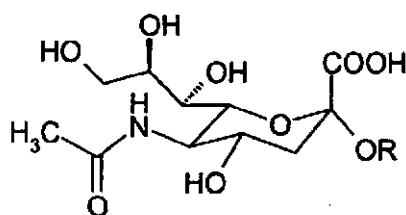


caracterizados porque M^{n+} es elegido del grupo que consiste de K^+ ,
 10 Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} y Al^{3+} .

2. Los compuestos de conformidad con la reivindicación 1, caracterizados porque M^{n+} es elegido del grupo que consiste de K^+ , Ca^{2+} , Mg^{2+} .

3. Un proceso para la preparación de los compuestos
 15 de la fórmula (Ib) de conformidad con cualquiera de las reivindicaciones 1-2 partiendo de la 6'-sialil-lactosa, caracterizado porque comprende agregar a una solución de 6'SL una base que contiene $M^{(n+)}$ para obtener un valor de pH de 8-10.

4. Un proceso para la preparación de los
 20 compuestos de α -sialil-oligosacáridos de la fórmula (I)



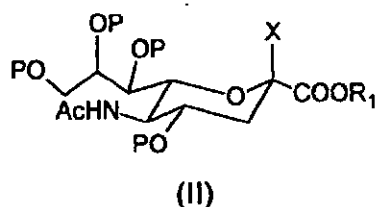
(I)

25 en donde R es un residuo de mono, di u oligosacárido con los

grupos hidroxilo libres; caracterizado porque comprende al menos una etapa de:

a) unir por medio de la reacción de Koenigs-Knorr un donador siálico de la fórmula (II)

5



en donde P es un grupo protector adecuado, R₁ es alquilo, y X es halógeno;

10

con un aceptor de la fórmula R'OH en donde R' es un residuo de mono, di u oligosacárido protegido adecuadamente con grupos protectores P' y que contiene cero, uno o más grupos hidroxilo libres; en donde los grupos protectores P' pueden ser

15

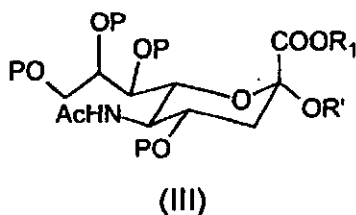
los mismos o diferentes entre sí y de aquellos presentes en el donador;

la reacción de Koenigs-Knorr tiene como particularidad el hecho de que el promotor metálico basado en Ag(I) es utilizado en cantidades molares entre 0.5 y 2.0 eq., con

20

respecto a las moles del aceptor;

para obtener un compuesto intermedio de la fórmula (III)



25

en donde P, R₁ y R' son como se definieron anteriormente.

5. El proceso de conformidad con la reivindicación 4, caracterizado porque además comprende, después de la etapa (a), la siguiente etapa:

5 b) remover los grupos protectores P, P' y R₁ para obtener los compuestos de la fórmula (I) de conformidad con la reivindicación 4.

6. El proceso para la preparación de 6'SL de conformidad con cualquiera de las reivindicaciones 4-5, caracterizado porque R es la 6'-lactosa.

7. Un proceso para la preparación de los compuestos de la fórmula (Ib), en donde Mⁿ⁺ es Na⁺, K⁺, NH₄⁺, Ca²⁺, Mg²⁺, Sr²⁺, Fe²⁺ o Al³⁺, caracterizado porque comprende la preparación de 6'SL por medio del proceso de conformidad con la reivindicación 6.

8. Los compuestos de la fórmula (Ib) de conformidad con cualquiera de las reivindicaciones 1-2, caracterizados porque se utilizan como medicamentos y agentes de integración de los alimentos.

9. Composiciones farmacéuticas y alimenticias, caracterizadas porque contienen los compuestos de la fórmula (Ib) de conformidad con cualquiera de las reivindicaciones 1-2 y al menos otro ingrediente aceptable alimenticia o farmacéuticamente.

RESUMEN DE LA INVENCION

La presente invención se refiere a un proceso para la síntesis de los α -sialil-oligosacáridos y en particular de la 6'-sialil-lactosa y sus sales, que comprende una etapa de
5 unión por la reacción de Koenigs-Knorr bajo condiciones que permiten su uso a una escala industrial.

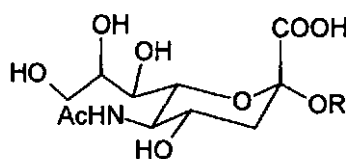
6'-SIALYLLACTOSE SALTS AND PROCESS FOR THEIR SYNTHESIS AND FOR THE SYNTHESIS OF OTHER α -SIALYLOLIGOSACCHARIDES

FIELD OF INVENTION

The present invention relates to the field of salts of 6'-sialyllactose: the present invention also relates to the field of processes for the synthesis of α -sialyl-oligosaccharides and in particular to the field of processes for the synthesis of 6'-sialyllactose and its salts.

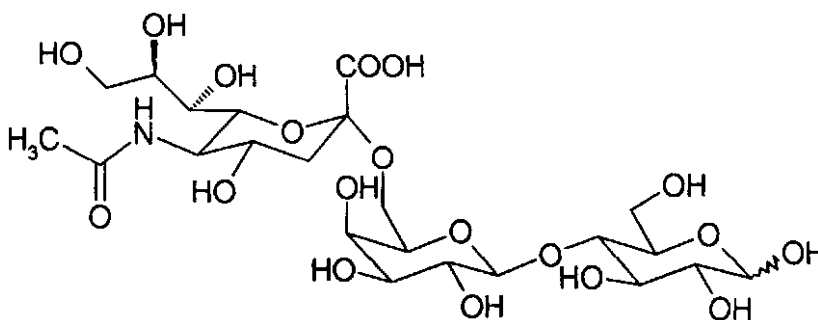
BACKGROUND

α -Sialyl-oligosaccharides of formula (I)



(I)

wherein R is a mono- di- or oligosaccharide residue with free hydroxyl groups are present in mammals and birds tissues and in predominant form of lipooligosaccharides, lipopolysaccharides or glycans of glycoproteins. They exist in a variety of glycosidic bonds, more typically $\alpha(2-3)$ and $\alpha(2-6)$ galactose (or lactose). The function of these sialosides varies greatly in animals according to the structural heterogeneity of the oligosaccharide portion. They are mediators of inter and intra-cells events in particular play an important role in the physiology and growth of many pathogen agents (DK Ress, et al., Current Organic Synthesis, 2004, 1, 31-46).



(Ia)

One litre of human milk contains about 5-10 g of free oligosaccharides, this content is similar to the content of proteins and exceeds the lipid content. More than 130 different oligosaccharides were identified in human milk (Human Milk

Oligosaccharides - HMO), formulations of artificial milk for babies derive from bovine milk and contain only trace amounts of these oligosaccharides that are specific of the human species. The fundamental building blocks of oligosaccharides of human milk are the 5 monosaccharides D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc) and sialic acid (N-acetyl-neuraminic acid, Neu5Ac). The terminal reducing end can be formed by lactose (Gal β 1-4Glc) or more repetitive units (up to 15 unit) of N-acetylglucosamine (Gal β 1-3/4GlcNAc). Lactose or polyglucosamine may be sialylated with α 2-3 and / or α 2-6 bonds. Examples of sialosides of human milk are: 3'-sialyl-3-fucosyllactose (3'S3FL), 6'-sialyllactose (6'SL), 3'-sialyllactose (3'SL), 3'-sialyllactosamine (3'SLN), 6'-sialyllactosamine (6'SLN).

Among sialosides mainly present in mammalian tissues and in human milk the compound of formula (Ia) 6'-sialyllactose (N-acetylneuraminyl-lactose, α -NeuNAc-(2 $\rightarrow$ 6)- β - D-Gal-(1 $\rightarrow$ 4)-D-Glc or 6'-SL) is of particular importance because it is an important constituent of glycoproteins and glycolipids involved in various cell pathway events including cell recognition and immune response. The 6'-SL and their salts are interesting as supplements in food formulations for infants. As for the salts of the 6'-sialyllactose in literature only the sodium salt (CAS Number: 157574-76-0; FW: C₂₃H₃₈NO₁₉Na, 6'-sialyllactose sodium salt, 6'-N-Acetylneuraminyl-lactose sodium salt) and the ammonium salt are known. While the sodium salt is acceptable for food and pharmaceuticals, the ammonium salt is potentially toxic because of the ammonium ion. For this reason it is necessary to get 6'-SL in alternative salt forms to the known ones that may be acceptable for food and pharmaceuticals.

At the state of the art various strategies are known for synthesis of sialyl-oligosaccharides (including 6'SL) and all foresee a convergent approach in which the sialic activated fragment (donor) is regio-and stereo-selectively bound to the oligosaccharide portion (acceptor). For this key step of coupling in literature three different synthetic strategies are known which foresee an exclusively enzymatic approach, exclusively chemical or chemo-enzymatic.

As for the enzymatic pathways families of sialyltransferases and transialidases (enzymes that add the sialic acid to oligosaccharides in a strictly specific way)

were used. Examples of this route of synthesis are reported in A.T.Beyer et al *Adv. Enzymol.*, 1981, 52, 23-175, in J. Weinstein et al. *J.Biol.Chem.*, 1982, 257, 13845-13853.

However several are the limitations in the use of these enzymes:

- 5 1) the limited availability of these enzymes
- 2) the need to synthesize the donor of activated substrate CMP- NeuAc. or PNP-NeuAc
- 3) the strict specificity of the sialyltransferases which reduces the flexibility in use for the synthesis of natural sialosides (Ichigawa Y. et al. *Analytical Biochem* 1992, 202, 215-238, S. Sabesan et al. *J.Am.Chem.Soc.*, 1986, 108, 2068-2080, O. Hindsgaul et al *J.Biol.Chem.*, 1991, 266, 17858-17862, H.J.Gross et al. *Eur.J.Biochem.* 1988, 177, 583-589).

As for the chemo-enzymatic pathways these include chemical synthesis of the acceptor and then enzymatic sialylation as in S. Sabesan et al., *J.Am.Chem.Soc.*,
15 1986, 108, 2068-2080.

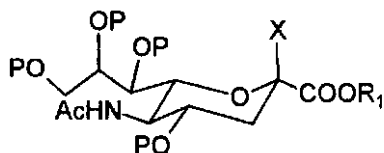
Focusing exclusively on chemical ways it is emphasized that the formation of the glycosidic bond with sialic acid is a reaction rather difficult because it is hampered by the fact that the donor is electronically and sterically hindered by the geminal carboxyl group. Moreover, the lack of functional group on C-3 rules out its
20 anchimeric assistance for controlling stereochemistry and leads to the formation of by-products through the reaction of elimination; finally the formation of the binding with α configuration is thermodynamically disadvantaged in relation to anomeric effect. In an attempt to remedy these defects in the state of the art various strategies have been developed for the preparation of suitably activated sialic
25 donors and acceptor with adequately protected hydroxyl groups that are reacted through various glycosylation methods.

Regarding the sialic donor it is to be emphasized that the complicated molecular architecture imparts a substantial degree of difficulty in its synthesis, protection and activation. The multifunctional nature (3 secondary hydroxyl groups) as well
30 as the tertiary anomeric center, complicate the work of synthetic chemist. Currently to the state of the art is well known that the sialic donor can be activated as 2-xanthate (A. Marra et al., *Carbohydr.Res.*, 1989, 187, 35), as 2-aryl sulphone (Y.

Du et al, *Carbohydr. Res.*, 1998, 308, 161), as 2-phosphite (RR Schmidt et al., *Tetrahedron Lett.*, 1992, 33, 6123 or CH Wong et al., *J.Am.Chem.Soc.*, 1992, 114, 8748) or as 2-halo derivative. Among all these groups the halogen derivatives are preferable as the phosphite and the thio derivatives require toxic reagents for their synthesis and not easy to handle in the industry. Among the halogens the chloro derivative is preferred as it is stable and easy to synthesize, in fact the bromo derivative is unstable and tends easily to eliminate and lead to anomeric mixtures during glycosilation reactions. The fluoro derivative requires a more elaborate synthesis than the chloro derivative and tends to form β glycosidic bonds. The chloro derivative would be the easiest donor to make and use.

Regarding to the structure of the donor, other synthetic routes, still more complex than previous ones, require the inclusion on the C-3 of sialic acid of a functional group that gives anchimeric assistance in the glycosilation reaction to prevent competitive elimination in 2,3 position. For this reason groups such as phenylthio or phenylselenium have been introduced (Y. Ito et al., *Tetrahedron Lett.*, 1988, 29, 3987 or in L.O. Kononov et al., *Tetrahedron Lett.*, 1997, 38, 1599). These pathways thus require multiple steps to obtain the active donor for the glycosylation reaction generally starting from 2,3-dehydro NeuAc with yields that vary according to the obtained specificity and the ease of purification of the intermediates.

For this reason for the synthesis of sialyloligosaccharides of formula (I) and in particular the 6'SL it would be preferable to employ efficiently a simple sialylderivative as the 2-chloro-donor of formula (II)



(II)

where P is a suitable protecting group, R1 is alkyl group and X is chlorine; (obtainable by methods reported in R. Kuhn et al., *Chem.Ber.*, 1966, 99, 611, A. Marra et al., *Carb.Res.*, 1989, 190, 317-322 and NF Byramova et al., *Carb. Res.*, 1992, 237, 161-175) because it is also easily to synthesize on an industrial scale without the use of very toxic reactivities, and it leads to the specific formation of α

bonds in the glycosilation reactions. The use of this sialic donor, however, decreased significantly after its first applications and the state of the art addresses towards much more complex donors.

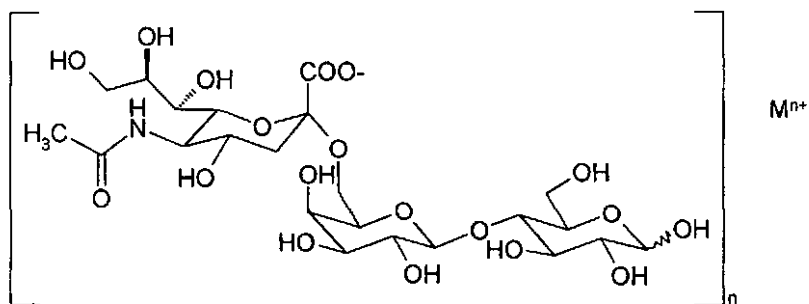
As for the acceptor activation for the synthesis of 6'SL, in the literature there are acceptors substituted with ether protective groups, for example benzyl groups whose removal, requiring an hydrogenation, is not easilymanageable (G . Pazynina et al. *Tetrahedron Lett*, 2002, 43, 8011-8013) and therefore difficult for industrial application.

α -glycosides of sialic acid were prepared by the Koenigs-Knorr reaction involving the use of Ag (I) as a promoter [Koenigs, W., Knorr, E. *Chem. Ber.*, 1901, 34, 957] or by using the Helferich modification that uses Hg (II) as promoter [Helferich, B.; Zirner, J. *Chem. Ber.*, 1962, 95, 2604]. The substrates chosen in these reactions are β -glycosyl-halides. Many variations of these classical methods are known which were designed to improve employment opportunities and yields. The main differences between these variations are related to the choice of counter anion of the metallic promoter. The most commonly used promoters are AgOTf, Ag₂CO₃, HgX₂ (X = halide), and Hg(CN)₂. In general it is known that Ag(I) promoters are more active and stereoselective (Pazynina G. et al. *Tetrahedron Lett*, 2002, 43, 8011-8013) but these should be used in large quantities (6-7 eq in relation to the acceptor) thus increasing the cost of synthesis (including the disposal of waste production), while Hg(II) promoters provide higher yields (H. Paulsen et al. *Angew.Chem.Int.Ed.Engl*, 1982, 927-928) but can present difficulties in handling due to their toxicity.

It is therefore evident the need for a process for the synthesis of compounds of formula (I) which is simple and economical, applicable on industrial scale, and hence allowing to overcome the technical problems above mentioned related to processes known in the literature.

SUMMARY OF THE INVENTION

The present invention solves the above problems by compounds of formula (Ib)

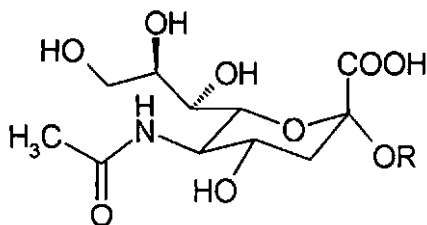


(Ib)

where M^{n+} is chosen from the group consisting of K^+ Ca^{2+} Mg^{2+} Sr^{2+} Fe^{2+} Al^{3+} ,

Further subject-matter of the present invention is a process for the synthesis of α -

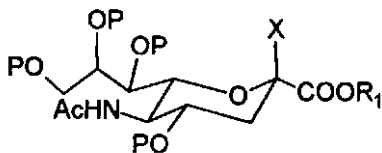
- 5 sialyl-oligosaccharides (I) compounds of formula (I)



(I)

wherein R is a mono-, di-or oligosaccharide residue with free hydroxyl groups;
said process comprising at least one step:

- 10 a) coupling by means of Koenigs - Knorr reaction of a sialic donor of formula (II)

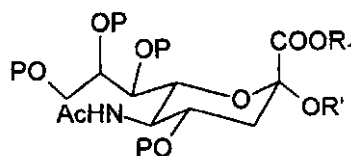


(II)

wherein P is a suitable protecting group, R1 is an alkyl group, and X is a halogen;
with an acceptor of formula $R'OH$ where R' is a mono-, di-or oligosaccharide
15 residue suitably protected with protecting groups P' and containing zero, one or
more free hydroxyl groups; these protecting groups P' may be the same or
different from each other and from those present in the donor;

such Koenigs-Knorr reaction characterized by the fact that the metallic promoter
based on $Ag(I)$ is used in molar quantities between 0.5 and 2.0 eq respect to the
20 moles of acceptor;

to obtain an intermediate of formula (III)



(III)

where P, R₁ and R' are as defined above.

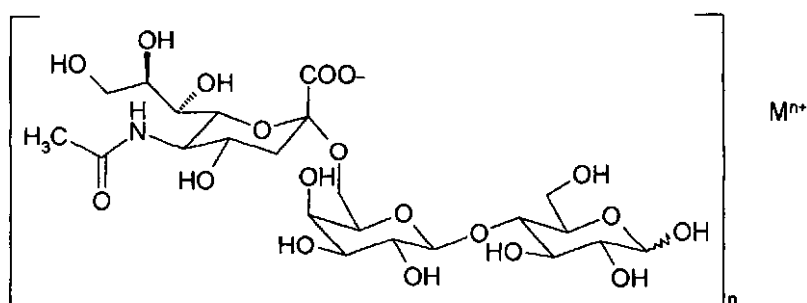
In particular, the process above mentioned provides a convenient route of

- 5 synthesis for the 6'SL and then for its salts of formula (Ib) in which Mⁿ⁺ is Na⁺, K⁺, NH₄⁺, Ca²⁺, Mg²⁺, Sr²⁺, Fe²⁺, Al³⁺.

Other advantages of the present invention are discussed later in the detailed description.

DETAILED DESCRIPTION

- 10 Subject matter of the present invention are compounds of formula (Ib)



(Ib)

wherein Mⁿ⁺ is chosen from the group consisting of K⁺, Ca²⁺, Mg²⁺, Sr²⁺, Fe²⁺, Al³⁺ where n = 1, 2, 3 in correspondence to the oxidation state of M. Preferably Mⁿ⁺ is

- 15 Ca²⁺, Mg²⁺ or K⁺.

Compounds of formula (Ib) are all alimentary and pharmaceutically acceptable and are useful as potential active ingredients or as food supplements (such as supplements in formulations of artificial milk for infants).

In particular 6'SL as:

- 20
- calcium salt is potentially useful to promote bone growth;
 - potassium and magnesium salts are potentially useful in maintaining, promote or restore proper transport through biological membranes and the physiological difference of transmembrane potential;

- iron salt is potentially useful for any pathological situations that require an integration of Fe;

The calcium salt in particular has better chemical-physical properties than the well-known sodium salt and its crystallization is easier. In fact during the crystallization phase, there is the formation of a crystalline solid, easy to be handled and thus easier to be managed even at industrial scale and its filtration has no problem because it is fast and allows an efficient washing of the solid.

In the case of sodium salt, instead, during the crystallization phase a rubbery solid is initially obtained, difficult to be stirred, which has to be grinded, and its filtration results slow and laborious. The stability of the two salts appears to be similar.

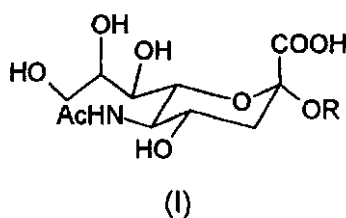
Another positive aspect of the calcium salt is the fact that starting from a same matrix of 6'SL in acid form the calcium salt is the one obtained with higher purity: for example the same matrix of 6'SL has supplied a crystal of sodium salt at 87% HPLC purity and a crystal of calcium salt at 93% HPLC purity.

Both potassium and magnesium salts show chemical-physical characteristics similar to the sodium salt as well as their crystallization has a similar trend.

Compounds of formula (Ib) above described can be prepared by 6'SL following known methods at state of the art for the preparation of salts from the corresponding carboxylic acids; for example they can be preferably prepared from a solution of 6'SL by adding a base containing M^{n+} such as hydroxides, carbonates or bicarbonates (ie, KOH, Ca (OH)₂, Mg (OH)₂, etc., K₂CO₃, CaCO₃, MgCO₃ etc.; KHCO₃, etc..) until pH 8-10. After removal of the solvent the obtained salt are purified by crystallization from alcohols or mixtures of water / alcohol; preferably methanol, ethanol and their mixtures with water.

Optionally before the removal of the solvent it is possible to remove the excess of undissolved base by filtration, if present.

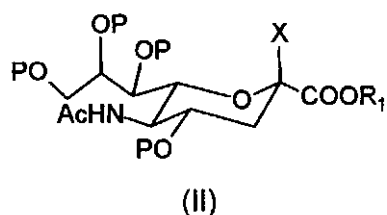
For another aspect the present invention relates to a process for the synthesis of α -sialyl-oligosaccharides of formula (I)



wherein R is a mono-, di- or oligosaccharide residue with free hydroxy groups, preferably R is selected between galactose, glucose, glucosamine, lactose, lactosamine, fucosyllactose; more preferably R is selected between 6'-galactose, 3'-glucose, 3'-glucosamine, 6'-lactose, 3'-lactose, 6'-lactosamine, 3'-lactosamine, 3'-3-fucosyllactose;

such process involving at least one step:

a) coupling by Koenigs-Knorr reaction of a sialic donor of formula (II)



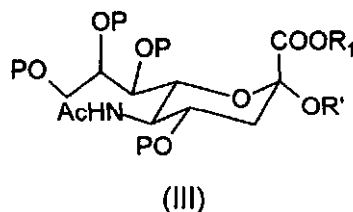
wherein:

P is a suitable protecting group;

R1 is an alkyl, preferably Me, Et, or Pr;

X is a halogen, preferably chlorine;

with a suitably protected acceptor of formula R'OH in which R' is a mono-, di- or oligosaccharide residue suitably protected with protecting groups P' and containing zero, one or more free hydroxyl groups: these protecting groups P' can be equal or different from each other and from those present in the donor; said Koenigs-Knorr reaction is characterized by the fact that the metallic promoter based on Ag (I) is used in molar quantities between 0.5 and 2.0 eq compared to moles of acceptor, to obtain an intermediate of formula (III)



where P, R1 and R' are defined as above.

For a preferred aspect this process also includes after step (a), the following step:

b) removal of protective groups P, P' and R1 to obtain compounds of formula (I) as described above.

The removal of the protective group b) is performed according to methods known to the state of the art (TW Green and PGM WUTS. Green's Protective Groups in Organic Synthesis. Ed Wiley ed 4. 2006).

For a preferred aspect this metallic promoter is used in molar amount between
5 0.75 and 0.85 molar eq compared to moles of acceptor.

For a preferred aspect said metallic promoter is selected between Ag(I) salts such as AgOTf, Ag₂CO₃, and more preferably is Ag₂CO₃.

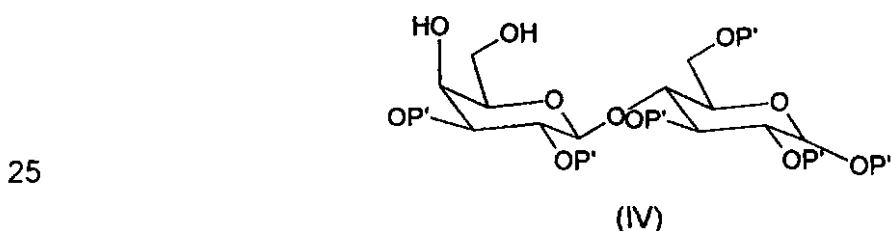
For a preferred aspect the coupling a) is conducted in aprotic polar solvent, preferably is conducted in dichloromethane.

10 For a preferred aspect said coupling a) is achieved by stirring the mixture at a temperature between 20 and 40° C for a time between 5 and 10 days; preferably the mixture is stirred at 30 ° C for 7 days.

For a preferred aspect P and P' are independently selected between benzyl and acyl, preferably P and P' are independently selected from acetyl, benzoyl or
15 benzoyl mono-or di-substituted with alkoxy, halogen, nitro groups.

For a preferred aspect P and P' are acyls. For a more preferred aspect P and P' are equal and are acetyl. Where R is 6'-lactose in the process of the present invention with P = P' = Ac there is an additional distinction from known processes for the synthesis of 6'SL as in this case the acceptor used in the present invention
20 do not show benzyl groups and consequently has the advantage of avoiding catalytic hydrogenation for the removal of protective groups.

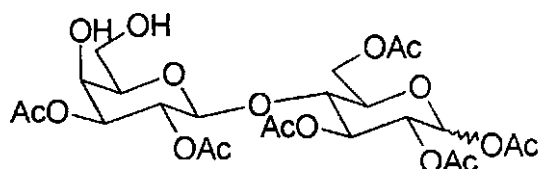
For a preferred aspect this acceptor R'OH adequately protected has a free and reactive hydroxyl group at C-6 of the galactosidic moiety, in a particularly preferred aspect the acceptor is a lactose derivative of formula (IV):



where P' is a suitable protecting group; preferably P' is acyl, more preferably acetyl.

For a particularly preferred aspect therefore the present invention relates to the
30 synthesis of 6'SL of formula (Ia) where R = 6'-lactose by the process above

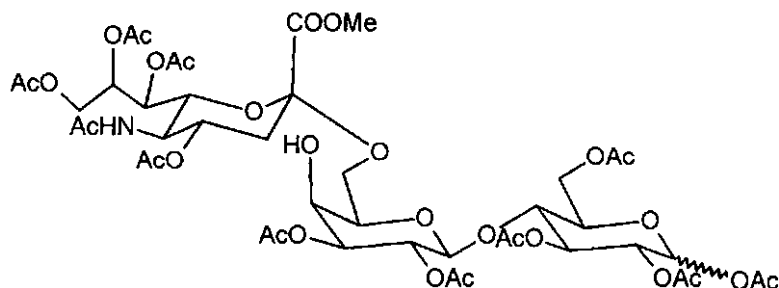
described in which P and P' are Ac, X is chlorine and R1 is methyl; in this particular combination, the coupling reaction in which the acceptor is a compound of formula (IVa)



5

(IVa)

provides the compound of formula (IIIa)



(IIIa)

that surprisingly can be used as it has been obtained from the crude mixture to the subsequent reaction of sequential deprotection of the hydroxyl groups and of the carboxyl function to the anomeric carbon of the sialic acid and production of the compound 6'SL formula (Ia). Preferably it has to proceed first to the removal of acetyl groups and then to the hydrolysis of methyl ester.

This sequential deprotection is carried out as known to the state of the art. Preferably the removal of the Ac groups is performed using a base such as sodium methoxide, sodium ethoxide or sodium hydroxide, more preferably sodium methoxide, using a primary alcohol as a solvent, like methanol or ethanol, most preferably methanol. Preferably the hydrolysis of methyl ester at anomeric carbon of the sialic acid is carried out in basic conditions with NaOH 1M.

Preferably when the methyl ester hydrolysis is completed, final acidification of the reaction mixture is performed by ion exchange resins, in particular a strong cationic resin and a weak anionic resin, to obtain an eluate containing 6'SL.

The sialic donor used in the process above described, in particular when X is chlorine and R' is lactose protected by formula (IV) with P = P' = Ac, is simple or easy to prepare and easy to use. This choice has revealed surprisingly adequate

to solve the problems of known processes, despite the state of the art had directed towards much more complex donors.

The amount of promoter metal used in step a) is surprisingly reduced compared to the known state of the art, 0.5-2.0 eq versus 6-7 eq, and therefore reduces the
5 cost of synthesis and the costs of disposing of waste production.

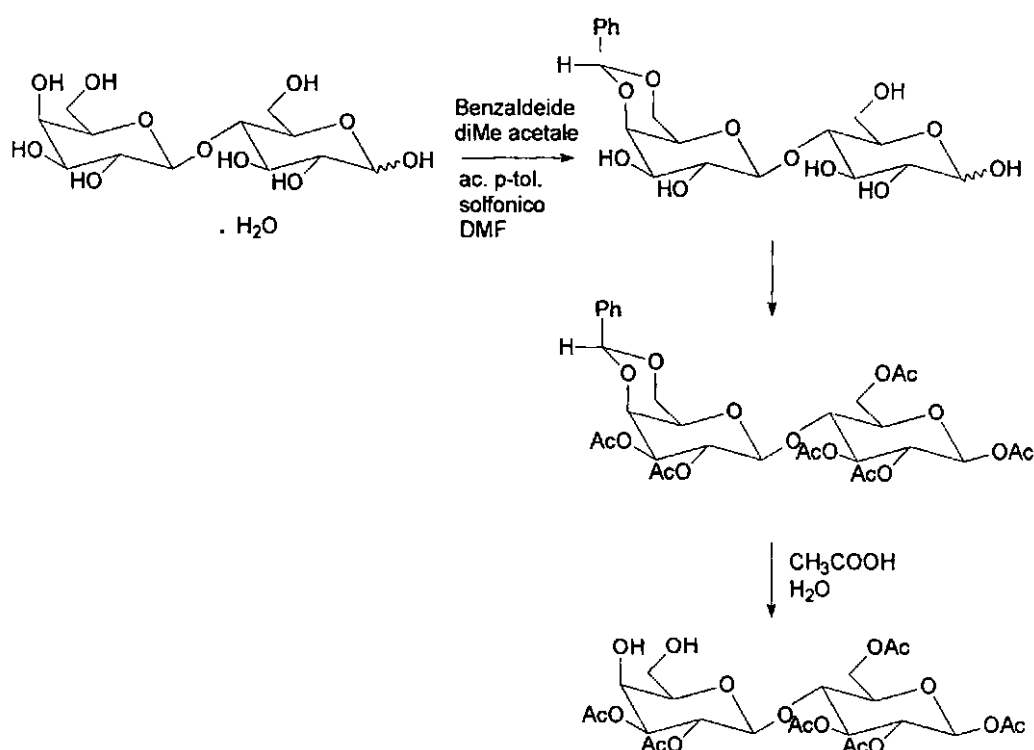
The product of coupling reaction is achieved surprisingly with enough purity to be used as it is in the subsequent reaction of deprotection to obtain the deprotected sialyl-oligosaccharides with good yields and purity. Furthermore it should be noted that the process for the coupling reaction of the present invention is
10 stereoselective because the α isomer is the only one obtained.

The process of the present invention is therefore feasible on industrial scale.

The present invention also refers to a process for the synthesis of compounds of formula (Ib) in which M^{n+} is Na^+ , K^+ , NH_4^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} , Al^{3+} such process including the preparation of 6'SL through the process above described;
15 preferably the salts of formula (Ib) may be prepared directly by the eluate containing 6'SL, obtained after ion exchange resins treatment after hydrolysis of methyl ester, by addition of a base containing $M^{(n+)}$ such eg hydroxides, carbonates or bicarbonates (ie, KOH, $Ca(OH)_2$, $Mg(OH)_2$, etc., K_2CO_3 , $CaCO_3$, $MgCO_3$ etc.; $KHCO_3$, etc.) until pH 8-10.

20 Compounds of formula (II) can be obtained by techniques known to the state of the art such as reported in (R. Kuhn et al., *Chem.Ber.*, 1966, 99, 611, A. Marra et al., *Carb. Res*, 1989, 190, 317-322 and NF Byramova et al., *Carb. Res*, 1992, 237, 161-175).

The synthesis of the suitably protected acceptor can be carried out following the
25 knowledge of the man of the art, in particular the synthesis of the acceptor of formula (IIIa) where P is Ac was carried out according to methods known in agreement with the Scheme 1.



(IVa) Schema 1

According to the invention alkoxy means, for example -OMe, -OEt, -OnPr, -OiPr, -

5 OnBu, -OiBu, -OfBu.

According to the invention halogen means fluorine, chlorine, bromine, iodine.

According to the invention alkyl is a linear alkyl chain or branched containing 1 to 6 atoms of carbon possibly substituted by one or more groups selected between halogen, hydroxy, alkoxy, nitro.

10 According to the invention aryl is benzene eventually replaced by one or more groups selected from halogen, alkoxy, nitro.

According to the invention acyl means a group -OCO-alkyl, or -OCO-aryl in which alkyl and aryl are defined as above.

According to the invention monosaccharide means a polyoxyaldehyde (aldose) or a
15 polyoxyketone (ketose) or a simple sugar of formula $(\text{CH}_2\text{O})_n$, $\text{C}_n\text{H}_{2n}\text{O}_{n-1}$, $\text{C}_n\text{H}_{2n}\text{O}_n$, $\text{C}_n\text{H}_{2n}\text{O}_{n-1}\text{NH}_2$ or $\text{C}_n\text{H}_{2n}\text{O}_{n-1}\text{NHAc}$ with $n = 3, 4, 5, 6, 7$; it means within the definition all the possible stereoisomers and all open or cyclic forms or intramolecular semi

acetals and semiketals as an example the pyranosyl and furanosyl forms; for example, glyceraldehyde, allose, altrose, arabinose, erythrose, fucose, galactose, glucose, glucosamine, N-acetyl-glucosamine, idose, lixose, mannose, psychose, ribose, deoxyribose, sorbose, tagatose, treose, xylose and corresponding ketoses are included in the definition.

5 According to the invention disaccharide means a compound polyoxydrilated consisting of two monosaccharides linked through an acetalic or glycosidic both O-glycosidic or N-glycosidic bond; within the definition are included all of the possible stereoisomers and all forms of open or cyclic; for example lactose, lactosamine, N-
10 acetyl-lactosamine, maltose, cellobiose, sucrose, threulose, turanose are included.

According to the invention oligosaccharide means a polymer formed of three to 6 monosaccharides joined together by glycosidic bonds to form linear saccharide chains or branched, for example raffinose, melezitose, maltotriose, acarbose,
15 stachyose are included.

EXPERIMENTAL PART

EXAMPLE 1

Preparation of di 4',6'-O-benzylidenlactose

200 g (0.555 mol) of lactose monohydrate were added under stirring to 1.4 l of
 5 *N,N*-dimethylformamide and then 209 ml (1.39 mol) of benzaldehyde dimethyl
 acetal and 5.28 g (0.028 mol) of *p*-toluenesulfonic acid monohydrate were added.
 The resulting suspension was heated at 55°C and the temperature was
 maintained until TLC was successful (16-18 hours) (Pharmacopoeia). After cooling
 at room temperature 4.7 ml of triethylamine were added until pH 7-8. The mixture
 10 was concentrated to obtain 700 ml of solution, which is drained in 3 liters of hot
 acetone (50-55°C), maintaining a vigorous stirring. The precipitation was
 completed by cooling the mixture to 0±5°C. The precipitate was filtered, washed
 with 0.7 liters of cold acetone and dried, obtaining 208 g of 4',6'-O-benzylidene
 lactose (mixture of α / β anomers) as white product (assay HPLC 66%, 0319 mol,
 15 yield: 57%).

By double crystallization, first by MeOH and then by MeOH/H₂O 4 / 1 v / v, an
 analytical sample enriched in α anomer was obtained, the NMR characterization is
 reported:

¹H NMR (DMSO-d₆, 300 MHz): δ ppm 7.51-7.34 (5H, m, Ph); 6.36 (d, J_{OH-1}= 4.8
 20 Hz, 1H, C1-OH); 5.58 (s, 1H, PhCH); 5.28 (d, J= 4.2 Hz, 1H, OH); 5.01 (d, J= 5.7
 Hz, OH); 4.92 (pseudo t, J_{1-OH}= J₁₋₂= 4.0 Hz, 1H, H-1); 4.68 (d, J= 6.9 Hz, 1H, OH);
 4.45 (m, 2H, 2xOH); 4.37 (d, J₁₋₂= 7.5 Hz, 1H, H-1'); 4.16-3.95 (m, 3H); 3.84-3.11
 (m, 9H) (H-2, H-3, H-4, H-5, CH₂-6, H-2', H-3', H-4', H-5', CH₂-6').

¹³C NMR (DMSO-d₆, 75 MHz): δ ppm 138.5, 128.6, 127.9, 126.2 (Ph); 103.1 (C-1');
 25 99.8 (PhCH); 92.1 (C-1); 79.6, 75.8, 72.2, 71.6, 71.3, 69.9, 69.8, 68.5 (C2, C2',
 C3, C3', C4, C4', C5, C5'); 66.2 (C6'); 60.3 (C6).

R_f (pharmacopoeia, UV-vis and naftoresorcine)= 0.7

EXAMPLE 2

Preparation of 4',6'-O-p-methoxybenzylidenlactose

- 200 g (0,555 mol) of lactose monohydrate were added under stirring to 1.4 l of N, N-dimethylformamide and then 237 ml (1.39 mol) of p-methoxybenzaldehyde
- 5 dimethyl acetal and 5,28 g (0,028 mol) of p-toluenesulfonic acid monohydrate were added. The resulting suspension was heated at 55°C and the temperature was maintained until positive TLC resulted (16-18 hours) (Pharmacopoeia). After cooling at room temperature 5.0 ml of triethylamine were added until pH 7-8. The mixture was concentrated and the residue was crystallized in 3 liters of hot
- 10 acetone (50-55°C). The precipitation was completed by cooling the mixture at 0÷5°C. The precipitate was filtered, washed with 2x200 ml of cold acetone, obtaining 219 g of 4',6'-O-p-metossibenzyliden lactose (mixture of α/β anomers) as a pale yellow solid (assay HPLC 76%, 0361 mol, yield: 65%) Crystallization from hot acetone/H₂O 4/1 v/v gave an analytical sample as a α/β
- 15 anomeric mixture (1/1 mol/mol), the NMR characterization is reported:.
- ¹H NMR (DMSO-d₆, 300 MHz): δ ppm 7.38 (d, J= 8.7 Hz, 2H), 6.93 (d, J= 8.7 Hz, 2H) (Ph); 6.70 (d, J_{OH-1}= 6.6 Hz, 1H, C1-OH β); 6.36 (d, J_{OH-1}= 4.1 Hz, 1H, C1-OH α); 5.52 (s, 1H, PhCH $\alpha+\beta$); 5.27 (m, 1H, OH $\alpha+\beta$); 5.04-4.95 (m, 1H, OH $\alpha+\beta$); 4.92 (pseudo t, J= 4.1 Hz, H-1 α); 4.72-4.60 (m, 1H, OH $\alpha+\beta$); 4.56-4.28 (m, H-1' $\alpha+\beta$ + H-1 β + 2xOH $\alpha+\beta$); 4.12-3.92 (m, 3H); 3.76 (s, 3H, OMe); 3.80-3.11 (m, 9H); 2.98 (m, 1H β).
- 20 ¹³C NMR (DMSO-d₆, 75 MHz): δ ppm 159.4, 130.9, 127.6, 113.2 (Ph); 103.0 (C-1' $\alpha+\beta$); 99.7 (PhCH $\alpha+\beta$); 96.7 (C-1 β); 92.1 (C-1 α); 79.6, 79.2, 75.7, 74.9, 74.8, 74.6, 72.2, 71.6, 71.3, 69.9, 69.8, 68.4 (C2, C2', C3, C3', C4, C4', C5, C5' $\alpha+\beta$); 66.2 (C6' $\alpha+\beta$); 60.4, 60.3 (C6 $\alpha+\beta$); 55.1 (OMe $\alpha+\beta$).
- 25 R_f (pharmacopoeia, UV-vis and naftoresorcine)= 0.8

EXAMPLE 3

Preparation of 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-benzyliden- β -D-lactose

100 g (0.153 moles from HPLC) of 4',6'-O-benzylidenlactose obtained according to
 5 example 1 and 256 ml (1.84 mol) of triethylamine were added to 600 ml of methyl
 ethyl ketone. The reaction mixture was heated at 60 °C and 174 ml (1.84 mol) of
 acetic anhydride were dropped maintaining the internal temperature below 70°C.
 The reaction mixture was stirred at 70°C until TLC was successful (10-12 hours)
 (AcOEt). The solvent was evaporated and the residue dissolved in 270 ml of
 10 dichloromethane and 200 ml of water. NaOH 30% was added under stirring up to
 pH 9-9.5, then the layers were separated; the aqueous layer was extracted again
 with 75 ml of dichloromethane. The collected organic layers were washed with 200
 ml of water, and then HCl solution 32% was added under stirring up to pH 1-1.5.
 The acid aqueous layer was extracted with 75 ml of dichloromethane. The
 15 collected organic layers were then washed with 370 ml of NaCl 20%, dried over
 anhydrous Na₂SO₄ and decolorized with charcoal and bentonite. The solvent was
 concentrated and the residue used as such in the next reaction. HPLC dosage
 gave 103 g (0,151 mol) of 1,2,3,6,2',3'-hexa-O-acetyl-4', 6'-O-benzylidene lactose
 essentially as β anomer (α anomer <10 mol%) (yield: 99%). An analytical sample
 20 containing 9 mol% of α anomer was obtained by crystallization from hot MeOH;
 following is the NMR characterization (β anomer).

¹H NMR (CDCl₃, 300 MHz): δ ppm 7.54-7.34 (m, 5H, Ph); 5.68 (d, J₁₋₂= 8.4 Hz,
 1H, H-1), 5.47 (pseudo s, 1H, CHPh); 5.32-5.21 (m, 2H, H-3+H-2'), 5.07 (dd, J₂₋₃=
 9.6 Hz e J₂₋₁= 8.4 Hz, 1H, H-2), 4.87 (dd, J_{3'-2}= 10.4 Hz e J_{3'-4}= 3.8 Hz, 1H, H-3'),
 25 4.54-4.43 (m, 2H, H-1'+H-6a), 4.38-4.25 (m, 2H, H-4'+H-6'a), 4.14 (dd, J_{6b-6a}= 12.2
 e J_{6b-5}= 4.6 Hz, 1H, H-6b), 4.04 (d, J_{6'b-6'a}= 12.3 Hz, 1H, H-6'b), 3.90-3.70 (m, 2H,
 H4+H5); 3.46 (pseudo s, 1H, H-5'), 2.14-2.00 (6xCOCH₃).

¹³C NMR (CDCl₃, 75 MHz): δ ppm 170.8, 170.4, 170.1, 169.7, 169.0, 168.9
 (6xCOCH₃); 137.5, 129.3, 128.3, 126.6 (Ph); 101.4 (CHPh); 101.1 (C-1'); 91.8 (C-
 30 1); 75.5, 73.8, 73.2, 72.4, 72.2, 70.5, 69.0, 68.5 (C-2, C-3, C-4, C-5, C-2',C-3', C-
 4', C-5'); 66.6 (C-6'); 61.8 (C-6), 20.9-20.7 (6xCOCH₃).

Rf (AcOEt:hexan=1:1, UV-vis and H₂SO₄/MeOH)= 0.3

EXAMPLE 4

Preparation of 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-*p*-

5 methoxybenzyliden-β-D-lactose

100 g (HPLC dosage 0.165 mol) of 4',6'-O-*p*-methoxybenzylidenlactose obtained according to example 2 and 242 ml (1.74 mol) of triethylamine were added at 600 ml of methyl ethyl ketone. The suspension was heated at 60°C and 164 ml (1.74 mol) of acetic anhydride were dropped maintaining the internal temperature below
 10 70°C. The reaction mixture was stirred at 70°C until positive TLC was successful (10-12 hours) (AcOEt: hexane = 1:1). The solvent was evaporated and the residue dissolved in 270 ml of dichloromethane and 200 ml of water. NaOH 30% was added under stirring up to pH 9-9.5, then the layers were separated; the aqueous layer was extracted again with 75 ml of dichloromethane. The collected organic
 15 layers were washed with 200 ml of water, and then HCl solution 32% was added under stirring up to pH 1-1.5. The acid aqueous layer was extracted with 75 ml of dichloromethane. The collected organic layers were then washed with 400 ml of saturated NaHCO₃, with 400 ml of NaCl 20%, dried on anhydrous Na₂SO₄ and bleached with charcoal and bentonite. The solvent was concentrated and the
 20 residue used as such in next reaction. HPLC dosage gave 110 g (0,155 mol) of 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-*p*-methoxybenzylidenlactose essentially as β anomer (yield: 94%). An analytical sample was obtained by crystallization from hot MeOH; following is the NMR characterization (β anomer).

¹H NMR (CDCl₃, 300 MHz): δ ppm 7.36 (d, J= 8.7 Hz, 2H), 6.88 (d, J= 8.7 Hz, 2H)
 25 (Ph); 5.66 (d, J_{1,2}= 8.4 Hz, 1H, H-1); 5.40 (pseudo s, 1H, CHPh); 5.24 (pseudo t, J_{3,2}= J_{3,4}= 9.6 Hz, 1H, H-3); 5.23 (dd, J_{2,3}= 10.2 Hz e J_{2,1}= 7.8 Hz, 1H, H-2'); 5.04 (dd, J_{2,3}= 9.6 Hz e J_{2,1}= 8.4 Hz, 1H, H-2); 4.84 (dd, J_{3,2}= 10.2 Hz e J_{3,4}= 3.6 Hz, 1H, H-3'), 4.46 (dd, J_{6a-6b} = 12.0 Hz e J_{6a-5}= 1.5 Hz, 1H, H-6a); 4.44 (d, J_{1,2}= 7.8 Hz, 1H, H-1'); 4.28 (d, J_{4,3}= 3.6 Hz, 1H, H-4'); 4.25 (d, J_{6'a-6'b}= 12.6 Hz, 1H, H-6'a);
 30 4.12 (dd, J_{6b-6a}= 12.0 e J_{6b-5}= 4.5 Hz, 1H, H-6b), 4.00 (dd, J_{6'b-6'a} = 12.6 Hz e J_{6'b-5'}

= 1.5 Hz, 1H, H-6'b); 3.87-3.69 (m, 2H, H4+H5); 3.79 (s, 3H, OMe); 3.42 (pseudo s, 1H, H-5'); 2.09, 2.07, 2.03, 2.02, 2.00 (6xCOCH₃).

¹³C NMR (CDCl₃, 75 MHz): δ ppm 170.8, 170.4, 170.1, 169.6, 168.93, 168.89 (6xCOCH₃); 160.3, 130.1, 127.9, 113.7 (Ph); 101.3 (CHPh); 101.1 (C-1'); 91.8 (C-1); 75.5, 73.8, 73.2, 72.4, 72.1, 70.5, 69.0, 68.4 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'); 66.5 (C-6'); 61.8 (C-6), 55.4 (OMe); 20.9-20.6 (6xCOCH₃).

R_f (AcOEt:hexan=1:1, UV-vis and H₂SO₄/MeOH)= 0.2

EXAMPLE 5

10 Preparation of 1,2,3,6,2',3'-hexa-O-acetyl-β-D-lactose

The syrup containing 100 g (0.15 moles) of 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-benzylidenlactose obtained according to example 2 was dissolved in 400 ml of glacial acetic acid. The reaction mixture was heated at 80°C and then 100 ml of water (preheated at 80°C) were added, stirring the mixture at this temperature for 15 1.5 hours. The reaction mixture was then rapidly cooled to room temperature and 500 ml of toluene and 350 ml of water were added and extracted. The aqueous layer was extracted with 150 ml of toluene. The combined toluene layer contained not reacted 1,2,3,6,2', 3'-hexa-O-acetyl-4',6'-O-benzylidenlactose, which can be used in another reaction. The aqueous layer, containing 1,2,3,6,2',3'-hexa-O-20 acetyl-β-D-lactose, was extracted successively with 500 ml and 150 ml of methylene chloride; the organic extracts were washed with 3x150 ml of water, dried over anhydrous sodium sulphate and concentrated. The residue was crystallized from 580 ml of hot isopropyl acetate (50-55°C), obtaining after drying 30.8 g of 1,2,3,6,2',3'-hexa-O-acetyl-β-D-lactose (0.05 mol) as a chalky white solid.

25 Considering that in the combined toluene layers 20.2 g of unreacted 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-benzylidenlactose (0.03 mol) were recovered, the yield is 42%.

Mp 188-190°C.

¹H NMR (CDCl₃, 300 MHz): δ ppm 5.68 (d, J₁₋₂= 8.1 Hz, 1H, H-1), 5.24 (pseudo t, J₃₋₂=J₃₋₄= 9.3 Hz, 1H, H-3), 5.19 (dd, J_{2'-3}= 10.2 Hz e J_{2'-1}=7.8 Hz, 1H, H-2'), 5.05 (dd, J₂₋₃= 9.3 Hz e J₂₋₁= 8.1 Hz, 1H, H-2), 4.88 (dd, J_{3'-2}= 10.2 Hz e J_{3'-4}= 3.3 Hz,

1H, H-3'), 4.49 (d, $J_{1',2'} = 7.8$ Hz, 1H, H-1'), 4.49 (dd, $J_{6a-6b} = 11.1$ Hz e $J_{6a-5} = 1.8$ Hz, 1H, H-6a), 4.15-4.05 (m, 2H, H-4'+H-6b), 4.00-3.70 (m, 4H, H-4+H-5+H-6'a+H-6'b), 3.56 (pseudo t, $J = 5.4$ Hz, 1H, H-5'), 2.96 (d, $J_{OH-4} = 4.2$ Hz, C4'-OH), 2.59 (dd, $J_{OH-6} = 7.5$ e 4.8 Hz, C6'-OH), 2.11, 2.09, 2.08, 2.07 2.04, 2.03 (6xCOCH₃).

- 5 ¹³C NMR (CDCl₃, 75 MHz): δ ppm 170.6, 170.4, 170.3, 169.7, 169.6, 169.0 (6xCOCH₃); 101.2 (C-1'), 91.7 (C-1), 75.9 (C-4), 74.6 (C-5'), 73.7 (C-3'), 73.6 (C-5), 73.1 (C-3), 70.7 (C-2), 69.7 (C-2'), 67.8 (C-4'), 62.1, 62.0 (C-6, C-6'), 20.9, 20.8, 20.7 (6xCOCH₃).

Rf (AcOEt, UV-vis and H₂SO₄/MeOH) = 0.4

10

EXAMPLE 6

Preparation of di 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose

- The syrup containing 100 g (0.14 mol) of 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-*p*-methoxybenzylidenlactose obtained according to example 4 was dissolved in 400
15 ml of glacial acetic acid, then 100 ml of water were added and the mixture was stirred at room temperature for 4.5 hours. 500 ml of toluene and 350 ml of water were added then extracted. The aqueous layer was extracted with 150 ml of toluene. The collected toluene layer contained 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-*p*-methoxybenzylidenlactose not reacted, which can be used in another reaction.
20 The aqueous layer, containing 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose, was extracted successively with 500 ml and 150 ml of methylene chloride; the organic extracts were washed with 3x150 ml of water, dried on anhydrous sodium sulphate and concentrated. The residue was crystallized from 580 ml of hot isopropyl acetate (50-55°C) obtaining, after drying, 40.6 g of 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose (0.07 mol, yield 50%) as white powdery solid whose characteristics are similar to the solid obtained from Example 5.
25

EXAMPLE 7

Preparation of (methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonate)-(2 $\rightarrow$ 6)-2,3-di-O-acetyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-1,2,3,6-tetra-O-acetyl- β -D-glucopyranose

100 g (0,168 mol) of 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose, obtained according
 5 to example 3, were dissolved in 600 ml of dichloromethane, then 250 g of molecular sieves 3A were added. The solution was stirred for 5-10 min and then 38.0 g of silver carbonate (0.14 mol) were added. A solution of 128.5 g (0,252 mol) (1.5 eq) of chlorine derivative of formula (II) wherein P is acetyl, X is chloro and R1 is methyl, in 500 ml of dichloromethane was added and the suspension was
 10 maintained in vigorous stirring at 30°C for 7 days until chlorine derivative disappeared (TLC, CH₂Cl₂: MeOH = 10:1), then the reaction mixture was filtered on dicalite and the solvent removed, obtaining a brittle solid residue (about 230 g), containing a mixture of condensation product, 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose and product of 2.3 elimination of (II), as well as a trace of 4,7,8,9-tetra-O-acetyl-NANA. By ¹³C NMR a conversion of about 90% mol was evaluated. An analytical sample of the condensate product was obtained by crystallization from ethanol: isopropyl ether = 1:3 v/v as a white amorphous solid:

¹H NMR (CDCl₃, 300 MHz): δ ppm 5.66 (d, J_{1,2}= 8.4 Hz, 1H, H-1); 5.42-5.10 (m, 5H, H-3+H-2'+H-7''+H-8''+NH); 5.01 (pseudo t, J_{2,1}= 8.4 Hz, 1H, H-2); 4.94-4.78
 20 (m, 2H, H-3'+H-4''); 4.53-4.27 (m, 3H, H-1'+H-6a+H-9''); 4.24-3.92 (m, 5H, H-6b+H-4'+H-5''+H-6''+H-9''b); 3.92-3.50 (m, 5H, H-4+H-5+H-5'+H-6'a+H-6'b); 3.80 (s, 3H, COOCH₃), 2.93 (broad s, 1H, OH); 2.55 (dd, J_{3''eq-3''ax}= 12.6 e J_{3''eq-4''}= 4.5 Hz, 1H, H-3''eq); 2.17-1.97 (31H, 10x CH₃CO e H-3''ax), 1.86 (s, 3H, NHCOCH₃).
¹³C NMR (CDCl₃, 75 MHz): δ ppm 171.05, 170.98, 170.5, 170.4, 170.29, 170.28,
 25 170.24, 170.0, 169.6, 169.4, 169.0, 168.0 (OAc, NHAc, COOMe); 100.8 (C-1'); 99.1 (C-2''); 91.7 (C-1); 75.7, 73.7, 73.6, 72.94, 72.90, 72.4, 70.7, 69.7, 68.95, 68.87, 67.4, 66.3 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''); 62.6, 62.4, 62.1 (C-6, C-6', C-9''); 53.2 (OCH₃); 49.4 (C-5''); 37.4 (C-3''); 23.2 (NHCOCH₃), 21.1, 20.9, 20.8, 20.74, 20.67 (10xCH₃CO).

30

EXAMPLE 8

Preparation of (methyl 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonate)-(2 $\rightarrow$ 6)- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-(α/β)-D-glucopyranose

230 g of the crude product, obtained according to example 7, were dissolved in 1.4 l of MeOH, then 29.6 ml of sodium methoxide in methanol 25% by weight was added. The solution was kept stirring at room temperature for 12h; at positive TLC control (Pharmacopoeia) it was neutralized with 39 g of dry IR120 (H^+). The resin was filtered and the solvent removed by rotavapor, obtaining 143 g of residue, used in next reaction. The yield was quantitative.

^1H NMR (D_2O , 300 MHz): δ ppm 5.21 (d, J_{1-2} 3.6Hz, 1H, H-1 α), 4.66 (d, J_{1-2} 7.8Hz, 1H, H-1 β), 4.42 (d, $J_{1'-2'}$ 7.5Hz, 1H, H-1'), 4.15-3.45 (m, 19H), 3.89 (s, 3H, $COOCH_3$), 3.29 (m, 1H), 2.70 (dd, $J_{3''eq-3''ax}$ 12.9 e $J_{3''eq-4''}$ 4.8 Hz, H-3''eq), 2.03 (s, 3H, $NHCOCH_3$), 1.88 (pseudo t, $J_{3''ax-3''eq} = J_{3''ax-4''}$ 12.9 Hz, H-3''ax).

^{13}C NMR (D_2O , 75 MHz): δ ppm 175.5 ($NHCOCH_3$), 170.4 ($COOMe$), 103.9 (C-1'), 99.6 (C-2''), 96.3 (C-1 β), 92.5 (C-1 α), 80.5, 80.4, 75.2 (2C), 74.4, 74.0, 73.5, 73.0, 72.2, 71.7, 71.3, 71.2, 70.5, 69.0 (2C), 67.9 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.0 (C-6'), 63.8 (C-9''), 60.8 e 60.7 (C-6 $\alpha+\beta$), 54.1 ($COOCH_3$), 52.3 (C-5''), 39.6 (C-3''), 22.8 ($NHCOCH_3$).

EXAMPLE 9

Preparation of sodium 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonate-(2 $\rightarrow$ 6)- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-(α/β)-D-glucopyranose

143 g of the crude deacetylationproduct, obtained according to example 8, were dissolved in 715 ml of water and the solution was cooled at 4°C. The pH was brought to neutrality with aqueous NaOH and then 23 ml of NaOH 30% were added, maintaining the temperature of the solution below 10°C. The solution was stirred at room temperature for 24h. At positive TLC control (Pharmacopoeia) the solution was passed through IR120(H^+)/IRA96(OH^-). The eluate was adjusted to pH 9 with NaOH, concentrated to syrup, and submitted to stripping several times with absolute EtOH until obtaining a brittle white solid that is re-crystallized from EtOH 96%. Obtained 77.3 gr.

^1H and ^{13}C NMR data for this compound were in agreement with those reported in the literature (L. Dorland et al., *Eur. J. Biochem.* 1978, 87, 323; J.P. Kamerling et al., *Carbohydr. Res.* 1982, 100, 331).

^1H NMR (D_2O , 300 MHz): δ ppm 5.22 (d, J_{1-2} 3.8Hz, 1H, H-1 α), 4.66 (d, J_{1-2} 7.8Hz, 1H, H-1 β), 4.43 (d, $J_{1'-2'}$ 7.6Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, $J_{3''\text{eq}-3''\text{ax}}$ 12.5 e $J_{3''\text{eq}-4''}$ 4.7 Hz, H-3''eq), 2.03 (s, 3H, NHCOCH_3), 1.74 (pseudo t, $J_{3''\text{ax}-3''\text{eq}} = J_{3''\text{ax}-4''}$ 12.5 Hz, H-3''ax).

^{13}C NMR (D_2O , 75 MHz, external reference acetone): δ ppm 175.6 (NHCOCH_3), 174.1 (COO^-), 103.9 (C-1'), 100.9 (C-2''), 96.3 (C-1 β), 92.5 (C-1 α), 80.4, 80.3, 75.30, 75.26, 74.4, 74.3, 73.2, 73.0, 72.4, 72.3, 71.7, 71.4, 70.6, 69.2, 69.04, 69.01 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.2 (C-6'), 63.3 (C-9''), 60.9 e 60.8 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3''), 22.7 (NHCOCH_3).

$[\alpha]_{\text{D}}^{20^\circ\text{C}}$: +9,3° (c:1%, H_2O)

Mp: 191,4÷194,2 °C (T decomposition)

15

EXAMPLE 10

Preparation calcium 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonate-(2→6)- β -D-galactopyranosyl-(1→4)-(α/β)-D-glucopyranose

20 100 g of crude deacetylation, obtained according to example 8, were dissolved in 500 ml of water and the solution was cooled at 4°C. The pH was brought to neutrality with aqueous NaOH and then 23 ml of NaOH 30% were added, maintaining the temperature of the solution below 10°C. The resulting solution was kept stirring at room temperature for 24h. At positive TLC control (Pharmacopoeia)

25 the solution was passed through IR120 (H^+) / IRA96(OH^-). The eluate was adjusted at pH 8.7 with $\text{Ca}(\text{OH})_2$, filtered and then concentrated to syrup at 65 ° Brix. This syrup was dropped in 540 ml of methanol at 50°C. The resulting suspension was maintained in vigorous stirring at 50°C for 1 hour, then at room temperature for 1 hour and then filtered under vacuum. The solid was washed with

30 160 ml of methanol and dried under vacuum at 50÷55°C. Obtained: 46.2 g

^1H NMR (D_2O , 300 MHz): δ ppm 5.22 (d, J_{1-2} 3.8Hz, 1H, H-1 α), 4.66 (d, J_{1-2} 7.8Hz,

^1H , H-1 β), 4.43 (d, J_{1-2} : 7.8Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, $J_{3''\text{eq}-3''\text{ax}}$ 12.0 e $J_{3''\text{eq}-4''}$ 4.5 Hz, H-3''eq), 2.03 (s, 3H, NHCOCH_3), 1.74 (pseudo t, $J_{3''\text{ax}-3''\text{eq}} = J_{3''\text{ax}-4''}$ 12.0 Hz, H-3''ax).

^{13}C NMR (D_2O , 75 MHz, internal reference acetonitrile): δ ppm 175.5 (NHCOCH_3), 174.1 (COO^-), 103.8 (C-1'), 100.9 (C-2''), 96.2 (C-1 β), 92.4 (C-1 α), 80.3, 80.3, 75.26, 75.22, 74.4, 74.3, 73.1, 73.0, 72.4, 72.2, 71.7, 71.4, 70.5, 69.1, 69.13, 69.00 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.2 (C-6'), 63.2 (C-9''), 60.9 e 60.7 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3''), 22.7 (NHCOCH_3).

Assay as Ca^{2+} : 98,3%

10 $[\alpha]_{\text{D}}^{20^\circ\text{C}}$: +10° (c:1%, H_2O)

Mp: 204,5÷206,6 °C (T decomposition)

IR $\nu_{\text{KBr max}}$: 3400, 1612, 1380, 1033 cm^{-1} .

EXAMPLE 11

15 Preparation of potassium 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonate-(2→6)- β -D-galactopyranosyl-(1→4)-(α/β)-D-glucopyranose

100 g of the crude deacetylation product, obtained according to example 8, were dissolved in 500 ml of water and the solution was cooled at 4°C. The pH was brought to neutrality with aqueous NaOH and then 23 ml of NaOH 30% were added, maintaining the temperature of the solution below 10°C. The solution was stirred at room temperature for 24h. At positive TLC control (Pharmacopoeia) the solution was passed through IR120(H^+)/IRA96(OH^-). The eluate was adjusted to pH 10 with KOH, concentrated to syrup, and submitted to stripping several times with absolute EtOH until obtaining a white brittle solid that is re-crystallized from absolute EtOH. The solid was dried under vacuum at 50÷55°C.

Obtained 35.7 gr

^1H NMR (D_2O , 200 MHz): δ ppm 5.22 (d, J_{1-2} 3.8Hz, 1H, H-1 α), 4.66 (d, J_{1-2} 7.8Hz, 1H, H-1 β), 4.43 (d, J_{1-2} : 7.6Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, $J_{3''\text{eq}-3''\text{ax}}$ 12.0 e $J_{3''\text{eq}-4''}$ 4.4 Hz, H-3''eq), 2.03 (s, 3H, NHCOCH_3), 1.74 (pseudo t, $J_{3''\text{ax}-3''\text{eq}} = J_{3''\text{ax}-4''}$ 12.0 Hz, H-3''ax).

^{13}C NMR (D_2O , 75 MHz, internal reference acetone): δ ppm 175.5 (NHCOCH_3), 174.0 (COO^-), 103.8 (C-1'), 100.9 (C-2''), 96.2 (C-1 β), 92.4 (C-1 α), 80.3, 80.2, 75.22, 75.19, 74.4, 74.3, 73.1, 72.9, 72.4, 72.2, 71.7, 71.4, 70.5, 69.1, 69.04, 69.01 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.1 (C-6'), 63.2 (C-9''), 60.8 e 60.7 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3''), 22.6 (NHCOCH_3).

5 $[\alpha]_{\text{D}}^{20^\circ\text{C}}$: +9,8° (c: 1%, H_2O)

Mp: 179,3÷182,8°C (T decomposition)

IR $\nu_{\text{max}}^{\text{KBr}}$: 3391, 1612, 1379, 1034 cm^{-1} .

10 EXAMPLE 12

Preparation of magnesium 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonate-(2→6)- β -D-galactopyranosyl-(1→4)-(α / β)-D-glucopyranose

100 g of the crude deacetylation product, obtained according to example 8, were dissolved in 500 ml of water and the solution was cooled at 4°C. The pH was brought to neutrality with aqueous NaOH and then 23 ml of NaOH 30% were added, maintaining the temperature of the solution below 10°C. The solution was stirred at room temperature for 24h. At positive TLC control (Pharmacopoeia) the solution was passed through IR120(H^+)/IRA96(OH^-). The eluate was adjusted to pH 9.8 with MgO, concentrated to residue submitted to stripping several times with absolute EtOH until obtaining a white friable solid that is re-crystallized from absolute EtOH.

Obtained: 50.8 g

^1H NMR (D_2O , 300 MHz): δ ppm 5.22 (d, J_{1-2} 3.6Hz, 1H, H-1 α), 4.66 (d, J_{1-2} 8.1Hz, 1H, H-1 β), 4.43 (d, $J_{1'-2'}$ 7.5Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, $J_{3''\text{eq}-3''\text{ax}}$ 12.3 e $J_{3''\text{eq}-4''}$ 4.5 Hz, H-3''eq), 2.03 (s, 3H, NHCOCH_3), 1.74 (pseudo t, $J_{3''\text{ax}-3''\text{eq}} = J_{3''\text{ax}-4''}$ 12.3 Hz, H-3''ax).

^{13}C NMR (D_2O , 75 MHz, internal reference acetonitrile): δ ppm 175.5 (NHCOCH_3), 174.1 (COO^-), 103.8 (C-1'), 100.9 (C-2''), 96.3 (C-1 β), 92.4 (C-1 α), 80.3, 80.2, 75.26, 75.23, 74.4, 74.3, 73.1, 73.0, 72.4, 72.2, 71.7, 71.4, 70.6, 69.1, 69.00,

68.96 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.2 (C-6'), 63.3 (C-9''), 60.9 e 60.7 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3''), 22.7 (NHCOCH₃).

Assay as Mg²⁺: 97,5%

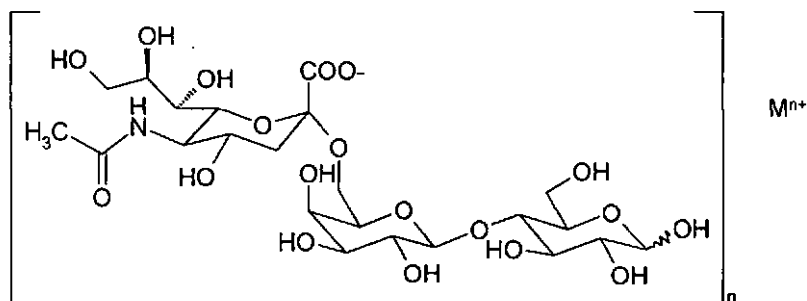
[α]_D^{20°C}: +9,8° (c: 1%, H₂O)

5 Mp: 183.1÷185.1°C (T decomposition)

IR ν ^{KBr}_{max}: 3391, 1634, 1379, 1035 cm⁻¹.

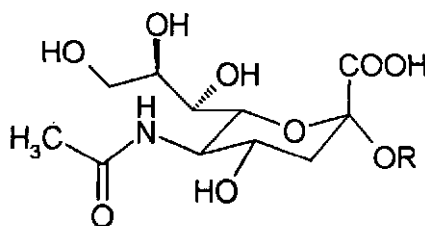
CLAIMS

1. Compounds of formula (Ib)



(Ib)

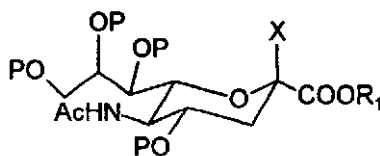
- 5 wherein M^{n+} is chosen in the group consisting of K^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} and Al^{3+} .
2. Compounds according to claim 1 wherein M^{n+} is chosen in the group consisting of K^+ , Ca^{2+} , Mg^{2+} .
3. A process the preparation of compounds of formula (Ib) as defined in any of
 10 claims 1-2 starting from 6'-sialyllactose (6'SL), said process comprising adding to a solution of 6'SL a base containing $M^{(n+)}$ to obtain a pH value of 8-10.
4. Process for the preparation of α -sialyl-oligosaccharides compounds of formula (I)



(I)

wherein R is a mono-, di- or oligosaccharide residue with the hydroxy groups free; said process comprising at least a step of:

- a) coupling by mean of Koenigs-Knorr reaction of a sialyc donor of formula (II)

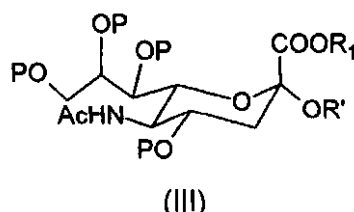


(II)

wherein P is a suitable protecting group; R₁ is alkyl, and X is halogen;
 with an acceptor of formula R'OH wherein R' is a residue mono-, di- or
 oligosaccharide suitably protected with protecting groups P' and containing
 zero, one or more free hydroxy groups; wherein said protecting groups P'
 5 can be the same or different among each others and from those present on
 the donor;

said reaction of Koenigs-Knorr characterised by the fact that the Ag(I) based
 metallic promoter is employed in molar amounts 0.5 - 2.0 eq respect with the
 moles of acceptor;

10 to obtain an intermediate of formula (III)



wherein P, R₁ and R' are as defined above.

5. Process according to claim 4 further comprising, after step (a), the following
 15 step:
 - b) removing protecting groups P, P' and R₁ to obtain compounds of formula (I)
 as defined in claim 4.
6. Process for the preparation of 6'SL according to any of claims 4-5 wherein R
 is 6'-lactose.
- 20 7. A process the preparation of compounds of formula (Ib) wherein Mⁿ⁺ is Na⁺,
 K⁺, NH₄⁺, Ca²⁺, Mg²⁺, Sr²⁺, Fe²⁺ o Al³⁺ said process comprising the
 preparation of 6'SL by mean of the process according to claim 6.
8. Compounds of formula (Ib) according to any of claims 1-2 for use as
 medicaments and food integrators.
- 25 9. Pharmaceutical and foodstuff compositions containing compounds of
 formula (Ib) according to any of claims 1-2 and at least an other ingredient
 pharmaceutically or alimentary acceptable.

**6'-SIALYLLACTOSE SALTS AND PROCESS FOR THEIR SYNTHESIS AND
FOR THE SYNTHESIS OF OTHER A-SIALYLOLIGOSACCHARIDES
ABSTRACT**

The present invention relates to a process of synthesis of α -sialyl oligosaccharides
5 and in particular of 6'-sialyllactose and its salts comprising a step of coupling by
Koenigs-Knorr reaction under conditions that allow its use on an industrial scale.

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PETITORIO PCT

Reimpresión (Original en Forma Electrónica)

0	Para Uso de la Oficina de Recepción solamente	
0-1	Solicitud Internacional No.	PCT/EP2010/051470
0-2	Fecha de Presentación Internacional	06 abril 2010 (06.04.2010)
0-3	Nombre de la Oficina receptora y "Solicitud Internacional PCT"	RO/IB
0-4	Forma - PCT/RO/101 Petitorio PCT	
0-4-1	Preparada utilizando	PCT - SAFE Versión 3.51.044.220 MT/FOP 20100101/0.20.5.18
0-5	Petición Los abajo firmantes solicitan que la presente solicitud internacional sea procesada de acuerdo con el Tratado de Cooperación en Materia de Patentes	
0-6	Oficina Receptora (Especificada por el Solicitante)	Oficina Internacional de la Organización Mundial de Propiedad Intelectual (RO/IB)
0-7	Referencia de registro del Solicitante o del Mandatario	8717PTWO-Dz
I	Título de la Invención	SALES DE 6'-SIALIL-LACTOSA Y PROCESOS PARA SU SINTESIS Y PARA LA SINTESIS DE OTROS ALFA-SIALIL-OLIGOSACARIDOS
II	Solicitante	
II-1	Esta persona es	solicitante solamente
II-2	Solicitante para	Todos los estados designados excepto US
II-4	Nombre	INALCO S. P. A.
II-5	Dirección	Via Calabiana 18 20139 MILANO Italia
II-6	Estado de Nacionalidad	IT
II-7	Estado de residencia	IT
III-1	Solicitante y/o inventor	
III-1-1	Esta persona es	solicitante e inventor
III-1-2	Solicitante para	US solamente
III-1-4	Nombre (APELLIDO, Nombre)	TAMERLANI, Giancarlo
III-1-5	Dirección	Via Turati 13 40030 CASTEL DI CASIO Italia
III-1-6	Estado de Nacionalidad	IT
III-1-7	Estado de residencia	IT

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PETITORIO PCT

Reimpresión (Original en Forma Electrónica)

III-2	Solicitante y/o inventor	
III-2-1	Esta persona es	solicitante e inventor
III-2-2	Solicitante para	US solamente
III-2-4	Nombre (APELLIDO, Nombre)	LOMBARDI, Ilaria
III-2-5	Dirección	Via Sano 17/A 51016 MONTECATINI TERME Italia
III-2-6	Estado de Nacionalidad	IT
III-2-7	Estado de residencia	IT
III-3	Solicitante y/o inventor	
III-3-1	Esta persona es	solicitante e inventor
III-3-2	Solicitante para	US solamente
III-3-4	Nombre (APELLIDO, Nombre)	BARTALUCCI, Debora
III-3-5	Dirección	Via Pietramarina 257 50059 VINCI Italia
III-3-6	Estado de Nacionalidad	IT
III-3-7	Estado de residencia	IT
III-4	Solicitante y/o inventor	
III-4-1	Esta persona es	solicitante e inventor
III-4-2	Solicitante para	US solamente
III-4-4	Nombre (APELLIDO, Nombre)	DANESI, Andrea
III-4-5	Dirección	Via Adua 2177 51100 PISTOIA Italia
III-4-6	Estado de Nacionalidad	IT
III-4-7	Estado de residencia	IT
III-5	Solicitante y/o inventor	
III-5-1	Esta persona es	solicitante e inventor
III-5-2	Solicitante para	US solamente
III-5-4	Nombre (APELLIDO, Nombre)	SALSINI, Liana
III-5-5	Dirección	Via Antonio Gramsci, 238 55047 SERAVEZZA Italia
III-5-6	Estado de Nacionalidad	IT
III-5-7	Estado de residencia	IT

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PETITORIO PCT

Reimpresión (Original en Forma Electrónica)

III-6	Solicitante y/o inventor	
III-6-1	Esta persona es	solicitante e inventor
III-6-2	Solicitante para	US solamente
III-6-4	Nombre (APELLIDO, Nombre)	MANONI, Marco
III-6-5	Dirección	Via Carmignanese 103 50059 VINCI Italia
III-6-6	Estado de Nacionalidad	IT
III-6-7	Estado de residencia	IT
III-7	Solicitante y/o inventor	
III-7-1	Esta persona es	solicitante e inventor
III-7-2	Solicitante para	US solamente
III-7-4	Nombre (APELLIDO, Nombre)	CIPOLLETTI, Giovanni
III-7-5	Dirección	Via Magolfa 13 20143 MILANO Italia
III-7-6	Estado de Nacionalidad	IT
III-7-7	Estado de residencia	IT
IV-1	Mandatario o representante común; o dirección para la correspondencia la persona identificada posteriormente es el que ha sido designado para actuar a favor del solicitante ante las Autoridades Internacional competentes como mandatario	
IV-1-1	Nombre (Apellido, Nombre)	BORSANO, Corrado
IV-1-2	Dirección:	Corso di Porta Vittoria 9 MILANO 20122 Italia
IV-1-3	Teléfono No.	+ 39 2 362161
IV-1-4	Facsímile No.	+ 39 2 36216366
IV-1-6	Dirección de correo electrónico	NOTAGE@NGPATENT.IT
IV-2	Mandatarios Adicionales	los mandatario(s) adicional(es) con la misma dirección que el agente nombrado primero
IV-2-1	Nombre(s)	GERVASI, Gemma; APPOLONI, Romano; CINQUANTINI, Bruno; CATTANEO, Elisabetta; PALLADINO, Massimo Saverio; DE ROS, Alberto

V Designaciones

- V-1 La presentación de este petitorio constituye bajo la Regla 4.9(a), la designación de todos los Estados contratantes afiliados al PCT en la fecha de presentación internacional, para el otorgamiento de cualquier clase de protección disponible y, en donde sea aplicable, para el otorgamiento de las

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PETITORIO PCT

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patentes tanto regional como nacional		
VI-1	Reivindicación de Prioridad de la solicitud nacional más cercana	
VI-1-1	Fecha de presentación	06 abril 2009 (06.04.2009)
VI-1-2	Número	FI2009A000071
VI-1-3	País	IT
VI-2	Incorporación para referencia: en donde un elemento de la solicitud internacional referido en el Artículo 11(1)(iii)(d) o (e) o una parte de la descripción, reivindicaciones o figuras referidas en la Regla 20.5(a) no está contenida de otra manera en esta solicitud internacional pero está contenida completamente en la solicitud previa cuya prioridad es reivindicada en la fecha en la cual uno o más elementos referidos en el Artículo 11(1)(iii) fueron recibidos primero por la Oficina receptora, este elemento o una parte, está sujeto a la confirmación bajo la Regla 20.6 incorporada para la referencia en esta solicitud internacional para los propósitos de la Regla 20.6.	
VII-1	Autoridad de Búsqueda Internacional Elegida	Oficina Europea de Patentes (EPO) (ISA/EP)
VII-2	Petición de uso de los resultados de una búsqueda anterior, referencia a esta búsqueda	
VII-2-1	Fecha de presentación	16 octubre 2009 (16.10.2009)
VII-2-2	Número de solicitud	IO5003 IT FI 20090071
VII-2-3	País (u Oficina regional)	IT
VII-2-5	Los documentos están disponibles para el uso en una forma y manera aceptables para el mismo y por lo tanto no es necesario que sea presentada por el solicitante ante el ISA (Regla 12bis.18f):	Una copia de los resultados de la búsqueda previa
VIII	Declaraciones	Número de declaraciones
VIII-1	Declaración en cuanto a la Identidad del inventor	-
VIII-2	Declaración en cuanto al cargo del solicitante, en cuanto a la fecha de presentación internacional, para la aplicación y para que sea otorgada una patente	1
VIII-3	Declaración en cuanto al cargo del solicitante, en cuanto a la fecha de presentación internacional, para reivindicar la prioridad de la solicitud previa	-
VIII-4	Declaración de la inventoría (solamente para los propósitos de la designación de los Estados Unidos de América)	-
VIII-5	Declaración en cuanto a las descripciones no perjudiciales o excepciones por la falta de novedad	-

VIII-2-1 Declaración: Autorización para Solicitar y para que sea otorgada una patente	
Declaración en cuanto al cargo del solicitante, en cuanto a la fecha de presentación internacional, para solicitar y para que sea otorgada una patente (Reglas 4.17(ii) y 51bis.1(a)(ii)), en un caso en donde la declaración bajo la Regla 4.17(iv) no es apropiada:	con relación a esta solicitud internacional
Nombre:	INALCO S. P. A. está autorizado para solicitar y para que le sea otorgada una patente en virtud de lo siguiente:
VIII-2-1 (ii)	INALCO S. P. A. está autorizado en calidad de empleador del inventor, TAMERLANI, GIANCARLO
VIII-2-1 (ii)	INALCO S. P. A. está autorizado en calidad de empleador del inventor, LOMBARDI, Ilaria
VIII-2-1 (ii)	INALCO S. P. A. está autorizado en calidad de empleador del inventor, BARTALUCCI, Debora
VIII-2-1 (ii)	INALCO S. P. A. está autorizado en calidad de empleador del inventor, DANESI, Andrea
VIII-2-1 (ii)	INALCO S. P. A. está autorizado en calidad de empleador del inventor, SALSINI, Liana
VIII-2-1 -(ii)	un acuerdo entre INALCO S.P.A. y CIPOLLETTI, Giovanni, fechado el 29 de septiembre de 1971 (29.09.1971)
VIII-2-1 (ii)	INALCO S. P. A. está autorizado en calidad de empleador del inventor, MANONI, Marco

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PETITORIO PCT**Reimpresión (Original en Forma Electrónica)**

IX	Lista de verificación	número de hojas	carpetas electrónicas anexas
IX-1	Petitorio	6	si
IX-2	Descripción	26	si
IX-3	Reivindicaciones	2	si
IX-4	Resumen	1	si
IX-5	Figuras	0	no
IX-7	TOTAL	35	
	Elementos anexos	documentos anexos	carpetas electrónicas anexas
IX-8	Hoja de cálculo de derechos	-	si
IX-18	medio físico PCT-SAFE	-	-
IX-20	Figura de los dibujos la cual debe acompañar al resumen		
IX-21	Lenguaje de la solicitud Internacional	Italiano	
X-1	Firma del solicitante o mandatario o representante común	(PKCS7 Firma Digital)	
X-1-1	Nombre	BORSANO, Corrado	
X-1-2	Nombre del que firma		
X-1-3	Cargo		

PARA USO DE LA OFICINA RECEPTORA SOLAMENTE

10-1	Fecha de recepción real de la solicitud internacional pretendida	06 abril 2010 (06.04.2010)
10-2	Figuras:	
10-2-1	Recibidas	
10-2-2	No recibidas	
10-3	Fecha corregida de la recepción real debido a que se recibieron posteriormente pero a tiempo los papeles o dibujos que complementan la solicitud internacional pretendida	
10-4	Fecha de la recepción a tiempo de las correcciones requeridas bajo el Artículo 11(2) del PCT	
10-5	Autoridad de la Búsqueda Internacional	ISA/EP
10-6	Transmisión de la copia de búsqueda retardada hasta que se paguen los derechos de la búsqueda	

RESERVADO PARA LA OFICINA INTERNACIONAL

11-1	Fecha de recepción del ejemplar original por la Oficina Internacional	
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PCT REQUEST

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0	For receiving Office use only	
0-1	International Application No.	
0-2	International Filing Date	
0-3	Name of receiving Office and "PCT International Application"	
0-4	Form PCT/RO/101 PCT Request	
0-4-1	Prepared Using	PCT-SAFE Version 3.51.044.220 MT/FOP 20100101/0.20.5.18
0-5	Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	Receiving Office (specified by the applicant)	International Bureau of the World Intellectual Property Organization (RO/IB)
0-7	Applicant's or agent's file reference	8717PTWO-Dz
I	Title of Invention	SALI DI 6'-SIALILLATTOSIO E PROCESSO PER LA LORO SINTESI E PER LA SINTESI DI ALTRI ALFA-SIALILOLIGOSACCARIDI
II	Applicant	
II-1	This person is	Applicant only
II-2	Applicant for	All designated States except US
II-4	Name	INALCO S.P.A.
II-5	Address	Via Calabiana 18 20139 MILANO Italy
II-6	State of nationality	IT
II-7	State of residence	IT
III-1	Applicant and/or inventor	
III-1-1	This person is	Applicant and inventor
III-1-2	Applicant for	US only
III-1-4	Name (LAST, First)	TAMERLANI, Giancarlo
III-1-5	Address	Via Turati 13 40030 CASTEL DI CASIO Italy
III-1-6	State of nationality	IT
III-1-7	State of residence	IT

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III-2	Applicant and/or inventor	
III-2-1	This person is	Applicant and inventor
III-2-2	Applicant for	US only
III-2-4	Name (LAST, First)	LOMBARDI, Ilaria
III-2-5	Address	Via Sano 17/A 51016 MONTECATINI TERME Italy
III-2-6	State of nationality	IT
III-2-7	State of residence	IT
III-3	Applicant and/or inventor	
III-3-1	This person is	Applicant and inventor
III-3-2	Applicant for	US only
III-3-4	Name (LAST, First)	BARTALUCCI, Debora
III-3-5	Address	Via Pietramarina 257 50059 VINCI Italy
III-3-6	State of nationality	IT
III-3-7	State of residence	IT
III-4	Applicant and/or inventor	
III-4-1	This person is	Applicant and inventor
III-4-2	Applicant for	US only
III-4-4	Name (LAST, First)	DANESI, Andrea
III-4-5	Address	Viale Adua 177 51100 PISTOIA Italy
III-4-6	State of nationality	IT
III-4-7	State of residence	IT
III-5	Applicant and/or inventor	
III-5-1	This person is	Applicant and inventor
III-5-2	Applicant for	US only
III-5-4	Name (LAST, First)	SALSINI, Liana
III-5-5	Address	Via Antonio Gramsci, 238 55047 SERAVEZZA Italy
III-5-6	State of nationality	IT
III-5-7	State of residence	IT

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III-6	Applicant and/or Inventor	
III-6-1	This person is	Applicant and inventor
III-6-2	Applicant for	US only
III-6-4	Name (LAST, First)	MANONI, Marco
III-6-5	Address	Via Carmignanesse 103 50059 VINCI Italy
III-6-6	State of nationality	IT
III-6-7	State of residence	IT
III-7	Applicant and/or Inventor	
III-7-1	This person is	Applicant and inventor
III-7-2	Applicant for	US only
III-7-4	Name (LAST, First)	CIPOLLETTI, Giovanni
III-7-5	Address	Via Magolfa 13 20143 MILANO Italy
III-7-6	State of nationality	IT
III-7-7	State of residence	IT
IV-1	Agent or common representative; or address for correspondence	Agent
	The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	
IV-1-1	Name (LAST, First)	BORSANO, Corrado
IV-1-2	Address	Corso di Porta Vittoria 9 MILANO 20122 Italy
IV-1-3	Telephone No.	+39 2 362161
IV-1-4	Facsimile No.	+39 2 36216366
IV-1-5	e-mail	NOTAGE@NGPATENT.IT
IV-2	Additional agent(s)	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	GERVASI, Gemma; APPOLONI, Romano; CINQUANTINI, Bruno; CATTANEO, Elisabetta; PALLADINO, Massimo Saverio; DE ROS, Alberto
V	DESIGNATIONS	
V-1	The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.	

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VI-1	Priority claim of earlier national application	
VI-1-1	Filing date	06 April 2009 (06.04.2009)
VI-1-2	Number	FI2009A000071
VI-1-3	Country	IT
VI-2	Incorporation by reference : where an element of the international application referred to in Article 11(1)(ii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(ii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.	
VII-1	International Searching Authority Chosen	European Patent Office (EPO) (ISA/EP)
VII-2	Request to use results of earlier search; reference to that search	
VII-2-1	Filing date	16 October 2009 (16.10.2009)
VII-2-2	Application Number	IO5003 IT FI 20090071
VII-2-3	Country (or regional Office)	IT
VII-2-5	Documents are available to the ISA in a form and manner acceptable to it and therefore do not need to be submitted by the applicant to the ISA (Rule 12bis.1(f)):	A copy of the results of the earlier search
VIII	Declarations	Number of declarations
VIII-1	Declaration as to the identity of the inventor	-
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	1
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	-
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	-
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	-

PCT REQUEST

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VIII-2-1	Declaration: Entitlement to apply for and be granted a patent Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent (Rules 4.17(ii) and 51bis.1(a)(ii)), in a case where the declaration under Rule 4.17(iv) is not appropriate: Name (LAST, First)	In relation to this international application INALCO S.P.A. is entitled to apply for and be granted a patent by virtue of the following:
VIII-2-1(i)		INALCO S.P.A. is entitled as employer of the inventor, TAMERLANI, Giancarlo
VIII-2-1(i)		INALCO S.P.A. is entitled as employer of the inventor, LOMBARDI, Ilaria
VIII-2-1(i)		INALCO S.P.A. is entitled as employer of the inventor, BARTALUCCI, Debora
VIII-2-1(i)		INALCO S.P.A. is entitled as employer of the inventor, DANESI, Andrea
VIII-2-1(i)		INALCO S.P.A. is entitled as employer of the inventor, SALSINI, Liana
VIII-2-1(ii)		an agreement between INALCO S.P.A. and CIPOLLETTI, Giovanni, dated 29 September 1971 (29.09.1971)
VIII-2-1(i)		INALCO S.P.A. is entitled as employer of the inventor, MANONI, Marco

PCT REQUEST

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IX	Check list	Number of sheets	Electronic file(s) attached
IX-1	Request (including declaration sheets)	6	✓
IX-2	Description	26	✓
IX-3	Claims	2	✓
IX-4	Abstract	1	✓
IX-5	Drawings	0	-
IX-7	TOTAL	35	
	Accompanying items	Paper document(s) attached	Electronic file(s) attached
IX-8	Fee calculation sheet	-	✓
IX-18	PCT-SAFE physical media	-	-
IX-20	Figure of the drawings which should accompany the abstract		
IX-21	Language of filing of the international application	Italian	
X-1	Signature of applicant, agent or common representative		
X-1-1	Name (LAST, First)		
X-1-2	Name of signatory		
X-1-3	Capacity		

FOR RECEIVING OFFICE USE ONLY

10-1	Date of actual receipt of the purported international application	
10-2	Drawings:	
10-2-1	Received	
10-2-2	Not received	
10-3	Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application	
10-4	Date of timely receipt of the required corrections under PCT Article 11(2)	
10-5	International Searching Authority	ISA/EP
10-6	Transmittal of search copy delayed until search fee is paid	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	Date of receipt of the record copy by the International Bureau	
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TRATADO DE COOPERACION EN MATERIA DE PATENTES

PCT

REPORTE DE BUSQUEDA INTERNACIONAL

(Artículo 18 y Reglas 43 y 44 del PCT)

Referencia de archivo del solicitante o apoderado: 8717PTWO-Dz.	PARA UNA ACCION ADICIONAL	Véase Forma PCT/ISA/220) así como, en donde sea aplicable, el punto 5 posterior.
No. de solicitud internacional: PCT/IB2010/051470	Fecha de presentación internacional (día/mes/año): 06/04/2010	Fecha de prioridad (día/ mes/ año): 06/04/2009
Solicitante: INALCO S. P. A.		

Este reporte de búsqueda internacional ha sido preparado por esta Autoridad de Búsqueda Internacional y se transmite al solicitante de acuerdo al Artículo 18. Una copia se transmite a la Oficina Internacional.

Este reporte de búsqueda internacional consiste de un total de 6 hojas.

☒ También está acompañado de una copia de cada documento de la técnica previa citado en este reporte.

1. Bases del reporte

a. Con respecto al **lenguaje**, la búsqueda internacional se llevó a cabo con base en

- ☒ la solicitud internacional en el lenguaje en el cual se presentó la misma,
☐ en una traducción de la solicitud internacional en _____, el cual es el lenguaje de una traducción
 provista para los propósitos de la búsqueda internacional (Regla 12.3(a) y 23.1(b)).

b. ☐ Este reporte de búsqueda internacional ha sido establecido tomando en cuenta la **rectificación de un error obvio** autorizado por o notificado a esta Autoridad bajo la Regla 91 (Regla 43bis.1(a))

c. ☐ Con respecto a cualquier **secuencia de nucleótidos y/o aminoácidos** descrita en la solicitud internacional, véase Bloque No. I.

2. ☐ Ciertas reivindicaciones se encontró que no son investigables (Véase el Recuadro II).

3. ☒ Está faltando la unidad de la invención (véase el Recuadro III).

4. Con respecto al **título**

- ☒ el texto está aprobado como se presentó por el solicitante.
☐ el texto ha sido establecido por esta Autoridad para leerse como sigue:

5. Con respecto al **RESUMEN**,

- ☒ el texto está aprobado como se presentó por el solicitante.
☐ el texto ha sido establecido, de acuerdo a la Regla 38.2(b), por esta Autoridad como aparece en el bloque IV. El solicitante puede, en el término de un mes a partir de la fecha de envío de este reporte de búsqueda internacional, presentar comentarios a esta Autoridad.

6. Con respecto a las figuras

a. la figura de las figuras que va ser publicada con el resumen es: Figura No.

- ☐ Como se sugiere por el solicitante.
☐ debido a que el solicitante fracaso en sugerir una Figura.
☐ debido a que esta figura caracteriza mejor la invención.

b. ☐ ninguna de las figuras va a ser publicada con el resumen.

REPORTE DE BUSQUEDA INTERNACIONAL		No. de solicitud internacional PCT/IB2010/051470
A. CLASIFICACION DEL TEMA INV. C07H13/06 A61K31/702 A23L1/09 ADD.		
De acuerdo a la Clasificación de Patentes Internacional (IPC) o a la clasificación nacional y a la IPC.		
B FIELDS SEARCHED		
Documentación mínima buscada (sistema de clasificación seguido por los símbolos de clasificación): C07H A23L A61K		
Documentación buscada diferente a la documentación mínima, a la extensión que tales documentos están incluidos en los campos buscados:		
Base de datos electrónicos consultados durante la búsqueda internacional (nombre de la base de datos y, en donde sea práctico, términos de búsqueda utilizados): EPO-Internal, CHEM ABS Data		
C. DOCUMENTOS QUE SE CONSIDERA QUE SON RELEVANTES		
	Cita del documento, con indicación, en donde sea apropiado, de los pasajes relevantes	Relevante para la Reiv. No.
A	RENCUROS I A ET AL: "Human milk Oligosaccharides: an enzymatic protection step simplifies the synthesis of 3'- and 6'-O-sialyllactose and their analogues" CARBOHIDRATE RESEARCH, ELSEVIER SCIENTIFIC PUBLISHING COMPANY, AMSTERDAM, NL, vol. 337, no. 6, 15 marzo 2002 (15-03-2002), páginas 473-483, XP004343543 ISSN: 0008-6215 compuesto (11); página 478 ----- - / - -	1-9
☒ Documentos adicionales se enlistan en la continuación del bloque C ☒ Véase familia de patentes anexa.		
* Categorías especiales de los documentos citados "A" documento que define el estado general de la técnica el cual no se considera ser de particular relevancia. "E" documento anterior publicado en o después de la fecha de presentación internacional. "L" documento el cual puede arrojar dudas sobre la(s) reivindicación(es) de prioridad o el cual se cita para establecer la fecha de publicación de otra cita u otra razón especial (como se especifica) "O" documento que se refiere a una descripción oral, uso, exhibición u otro medio "P" documento publicado antes de la fecha de presentación internacional pero después de la fecha de prioridad reivindicada. "T" documento posterior publicado después de la fecha de presentación internacional o fecha de prioridad y no en conflicto con la solicitud, pero citado para entender el principio o teoría fundamental de la invención. "X" documento de relevancia particular; la invención reivindicada no puede ser considerada nueva o no se puede considerar que involucre una etapa inventiva cuando el documento es considerado de manera aislada. "Y" documento de particular relevancia; no se puede considerar que la invención involucre una etapa inventiva cuando el documento es combinado con uno o más de tales documentos, tal combinación sería obvia para una persona experimentada en la técnica. "&" documento miembro de la misma familia de patentes.		
Fecha de la terminación real de la búsqueda internacional 17 septiembre 2010		Fecha de envío del reporte de búsqueda internacional 23/09/2010
Nombre y dirección de correspondencia de la ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Funcionario Autorizado Bardili, Burkhardt

REPORTE DE BUSQUEDA INTERNACIONAL		No. de solicitud internacional
		PCT/IB2010/051470
C (Continuación) DOCUMENTOS CONSIDERADOS RELEVANTES		
Categoría*	Cita del documento, con indicación, en donde sea apropiado, de los pasajes relevantes	Relevante para la reivindicación No.
A	MULLER H E ET AL: "Occurrence and some properties of neuraminidases in Haemophilus avium and Haemophilus paragallinarum" VETERINARY MICROBIOLOGY, ELSEVIER BV, NL, vol. 2, no. 4, 1 diciembre 1977 (01-12-1977), páginas 303-312, XP023915296 ISSN: 0378-1135 [recuperado el 01-12-1977] tabla 1 -----	1-9
Y	WO 2007/090894 A (NESTEC SA [CH]; SPRENGER NORBERT [CH]; MORGAN FRANCOIS [FR]; BERROCAL) 16 agosto 2007 (16-08-2007) reivindicaciones 1,9 -----	1-3,8,9
Y	WO 92/11017 A (MONSANTO CO [US]) 9 julio 1992 (09-07-1992) reivindicación 4, tabla 1 -----	1-3,8,9
Y	WO 94/03184 A (NEOSE PHARM INC [US]) 17 febrero 1994 (17-02-1994) página 11; reivindicación 1 -----	1-3,8,9
X	PAZYNINA G ET AL: "Simple stereoselective synthesis of alpha2-6 sialooligosaccharides" TETRAHEDRON LETTERS, ELSEVIER, AMSTERDAM, NL LNKD- DOI:10.1016/S0040-4039(02)01983-4, vol. 43, no. 45, 4 noviembre 2002 (04-11-2002), páginas 8011-8013, XP004387177 ISSN: 0040-4039 tabla 1 -----	4-7 4-7

REPORTE DE BUSQUEDA INTERNACIONAL		No. de solicitud internacional
		PCT/IB2010/051470
Bloque II Observaciones en donde ciertas reivindicaciones se encontraron no pudieron ser investigadas (Continuación del punto 2 de la primera hoja)		
Este reporte internacional no ha sido establecido con respecto a ciertas reivindicaciones bajo el Artículo 17(2) del PCT por las siguientes razones:		
1.	☐	Reivindicaciones Nos.: debido a que se relacionan con temas que no requieren ser buscados por esta Autoridad, especialmente:
2.	☐	Reivindicaciones Nos.: debido a que se relacionan con partes de la solicitud internacional que no cumplen con los requerimientos prescritos a tal extensión que ninguna búsqueda internacional significativa se puede llevar a cabo, específicamente:
3.	☐	Reivindicaciones Nos.: debido a que son reivindicaciones dependientes y no están redactadas de acuerdo con las segundas y terceras oraciones de la Regla 6.4(a).
Bloque III Observaciones en donde se carece de unidad de la invención (Continuación del ítem 3 de la primera hoja)		
La Autoridad de Búsqueda Internacional encontró múltiples invenciones en esta solicitud internacional, como sigue: véase hoja adicional		
1.	☒	Ya que todas las cuotas de búsqueda adicionales fueron pagadas a tiempo por la solicitante, este reporte de búsqueda cubre todas las reivindicaciones que se pudieron investigar.
2.	☐	Ya que todas las reivindicaciones que se pudieron investigar pudieron ser buscadas sin esfuerzo que justifique una cuota adicional, esta Autoridad no invita al pago de ninguna cuota adicional.
3.	☐	Ya que solamente algunas de las cuotas de búsqueda adicionales requeridas fueron pagadas oportunamente por la solicitante, este reporte de búsqueda internacional cubre solamente aquellas reivindicaciones para las cuales se pagaron cuotas, específicamente las reivindicaciones Nos.:
4.	☐	Ninguna cuota de búsqueda adicional requerida fue pagada oportunamente por la solicitante. Consecuentemente, este reporte de búsqueda internacional está restringido a la invención primero mencionada en las reivindicaciones; está cubierta por las reivindicaciones Nos.:
Observación en protesta	☐	Las cuotas de búsqueda adicionales estuvieron acompañadas por la protesta de la solicitante, y donde sea aplicable, el pago de una cuota de protesta
	☐	Las cuotas de búsqueda adicionales estuvieron acompañadas por la protesta de la solicitante pero la cuota de protesta aplicable no fue pagada dentro del tiempo límite especificado en la invitación
	☒	Ninguna protesta acompañó el pago de las cuotas de búsqueda adicional.

No. de solicitud internacional

PCT/IB2010/051470

INFORMACION ADICIONAL CONTINUA DE PCT/ISA/ 210

Esta Autoridad de la Búsqueda Internacional encontró (grupos de) invenciones múltiples en esta solicitud internacional, como sigue:

1. reivindicaciones: 1-3, 8,9 (completamente); 7 (parcialmente)

sales de K, Ca, Mg, Sr, Fe y Al de 6'-sialil lactosa y su preparación;
composiciones que comprenden estas sales y su uso como aditivos para las composiciones alimenticias y farmacéuticas.

- - -

2. reivindicaciones: 4-6 (completamente); 7 (parcialmente)

(2a) un método para la preparación de los alfa-sialil oligosacáridos protegidos de la fórmula (I)

(2b) un método para la preparación de los alfa-sialil oligosacáridos desprotegidos correspondientes

(2c) la preparación de la sal de sodio y amonio de la 6'-sialil lactosa que involucra el proceso de la invención (2a)

- - -

REPORTE DE BUSQUEDA INTERNACIONAL Información sobre los miembros de la familia de patentes					No. de solicitud internacional
					PCT/IB2010/051470
Documento de patente citado en el Reporte de Búsqueda	Fecha de publicación	Miembro(s) de la familia de patente(s)			Fecha de publicación
WO 2007090894 A	16-08-2007	AT	468027 T		15-06-2010
		AU	2007213677 A1		16-08-2007
		CA	2641842 A1		16-08-2007
		CN	101400267 A		01-04-2009
		EP	1988786 A1		12-11-2008
		ES	2342625 T3		09-07-2010
		PT	1988786 E		22-06-2010
		US	2009092729 A1		09-04-2009
		ZA	200807763 A		25-11-2009
WO 9211017 A	09-07-1992	US	5164374 A		17-11-1992
WO 9403184 A	17-02-1994	AU	4993893 A		03-03-1994

Forma PCT/ISA/210 (anexo de la familia de patentes) (abril del 2005)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 8717PTWO-DZ	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/IB2010/051470	International filing date (day/month/year) 06/04/2010	(Earliest) Priority Date (day/month/year) 06/04/2009
Applicant INALCO S.P.A.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 6 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed
☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☐ This international search report has been established taking into account the rectification of an obvious mistake authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2. ☐ Certain claims were found unsearchable (See Box No. II)

3. ☒ Unity of invention is lacking (see Box No. III)

4. With regard to the title,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the drawings,

- a. the figure of the drawings to be published with the abstract is Figure No. _____
☐ as suggested by the applicant
☐ as selected by this Authority, because the applicant failed to suggest a figure
☐ as selected by this Authority, because this figure better characterizes the invention
- b. ☐ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2010/051470

Box No. II Observations where certain claims were found unsearchable (Continuation of Item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)

This international Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-3, 8, 9(completely); 7(partially)

K, Ca, Mg, Sr, Fe and Al salts of 6'-sialyl lactose and their preparation; compositions comprising these salts and and their use as additives for food and pharmaceutical compositions

2. claims: 4-6(completely); 7(partially)

(2a) a method for the preparation of protected alpha-sialyl oligosaccharides of formula (I)

(2b) a method for the preparation of the corresponding deprotected alpha-sialyl oligosaccharides

(2c) the preparation of the sodium and ammonium salt of 6'-sialyl lactose involving the process of invention (2a)

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2010/051470

A. CLASSIFICATION OF SUBJECT MATTER INV. C07H13/06 A61K31/702 A23L1/09 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07H A23L A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the International search (name of data base and, where practical, search terms used) EPO-Internal, CHEM ABS Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RENCUROS I A ET AL: "Human milk oligosaccharides: an enzymatic protection step simplifies the synthesis of 3'- and 6'-O-sialyllactose and their analogues" CARBOHYDRATE RESEARCH, ELSEVIER SCIENTIFIC PUBLISHING COMPANY, AMSTERDAM, NL, vol. 337, no. 6, 15 March 2002 (2002-03-15), pages 473-483, XP004343543 ISSN: 0008-6215 compound (11); page 478 ----- -/--	1-9
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art *B* document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
17 September 2010		23/09/2010
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Bardili, Burkhardt

Form PCT/ISA210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/051470

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MULLER H E ET AL: "Occurrence and some properties of neuraminidases in Haemophilus avium and Haemophilus paragallinarum" VETERINARY MICROBIOLOGY, ELSEVIER BV, NL, vol. 2, no. 4, 1 December 1977 (1977-12-01), pages 303-312, XP023915296 ISSN: 0378-1135 [retrieved on 1977-12-01] table I	1-9
Y	WO 2007/090894 A (NESTEC SA [CH]; SPRENGER NORBERT [CH]; MORGAN FRANCOIS [FR]; BERROCAL) 16 August 2007 (2007-08-16) claims 1,9	1-3,8,9
Y	WO 92/11017 A (MONSANTO CO [US]) 9 July 1992 (1992-07-09) claim 4; table I	1-3,8,9
Y	WO 94/03184 A (NEOSE PHARM INC [US]) 17 February 1994 (1994-02-17) page 11; claim 1	1-3,8,9
X	PAZYNINA G ET AL: "Simple stereoselective synthesis of alpha2-6 sialooligosaccharides" TETRAHEDRON LETTERS, ELSEVIER, AMSTERDAM, NL LNKD- DOI:10.1016/S0040-4039(02)01983-4, vol. 43, no. 45, 4 November 2002 (2002-11-04), pages 8011-8013, XP004387177 ISSN: 0040-4039 table 1	4-7

INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/IB2010/051470

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-3, 8, 9(completely); 7(partially)

K, Ca, Mg, Sr, Fe and Al salts of 6'-sialyl lactose and their preparation; compositions comprising these salts and and their use as additives for food and pharmaceutical compositions

2. claims: 4-6(completely); 7(partially)

(2a) a method for the preparation of protected alpha-sialyl oligosaccharides of formula (I)

(2b) a method for the preparation of the corresponding deprotected alpha-sialyl oligosaccharides

(2c) the preparation of the sodium and ammonium salt of 6'-sialyl lactose involving the process of invention (2a)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2010/051470

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007090894 A	16-08-2007	AT 468027 T	15-06-2010
		AU 2007213677 A1	16-08-2007
		CA 2641842 A1	16-08-2007
		CN 101400267 A	01-04-2009
		EP 1988786 A1	12-11-2008
		ES 2342625 T3	09-07-2010
		PT 1988786 E	22-06-2010
		US 2009092729 A1	09-04-2009
		ZA 200807763 A	25-11-2009
WO 9211017 A	09-07-1992	US 5164374 A	17-11-1992
WO 9403184 A	17-02-1994	AU 4993893 A	03-03-1994

TRATADO DE COOPERACION EN MATERIA DE PATENTES

De la
AUTORIDAD DE LA BUSQUEDA INTERNACIONAL

Para: véase la forma PCT/ISA/220		PCT OPINION ESCRITA DE LA AUTORIDAD DE LA BÚSQUEDA INTERNACIONAL (Regla 43bis.1 del PCT)	
		Fecha de envío véase la forma PCT/ISA/210 (día/mes/año): (segunda hoja)	
Referencia de archivo del solicitante o apoderado: véase la forma PCT/ISA/220		PARA ACCION véase el párrafo 2 posterior ADICIONAL	
No. de solicitud internacional: PCT/IB2010/051470	Fecha de presentación internacional (día/mes/año): 06.04.2010	Fecha de prioridad (día/mes/año): 06.04.2009	
Clasificación de Patente Internacional (IPC) o clasificación nacional e IPC: INV: C07H13/06 A61K31/702 A23L1/09			
Solicitante: INALCO S.P.A.			
1. Esta opinión contiene indicaciones que se refieren a los siguientes puntos I ☒ Base de la opinión II ☐ Prioridad III ☐ No se establece opinión con respecto a novedad, paso o etapa inventiva y aplicabilidad industrial IV ☒ Falta de unidad de la invención V ☒ Declaración justificada bajo la Regla 43bis.1(a)(i) con respecto a novedad, etapa o paso inventivo o aplicabilidad industrial; citas y explicaciones que apoyan tal declaración. VI ☐ Ciertos documentos citados VII ☐ Ciertos defectos en la solicitud internacional VIII ☐ Ciertas observaciones en la solicitud internacional			
2. ACCION ADICIONAL Si se hace una solicitud de examen preliminar internacional, esta opinión será considerada usualmente que va a ser una opinión escrita de la Autoridad del Examen Preliminar Internacional ("IPEA"). Sin embargo, esto no aplica en donde el solicitante elija una Autoridad diferente de aquella que la primera que va a ser el IPEA y la IPEA elegida ha notificado a la Oficina Internacional bajo la Regla 66.1bis(c) que las opiniones escritas de esta Autoridad de la Búsqueda Internacional no serán consideradas así. Si esta opinión, como se proporcionó anteriormente, se considera que va a ser una opinión escrita de la IPEA, el solicitante es invitado a presentar ante la IPEA una contestación escrita conjuntamente, en donde sea apropiada, con enmiendas, antes de la expiración de tres meses desde la fecha de envío por correo de la Forma PCT/ISA/220 o antes de la expiración de 22 meses desde la fecha de prioridad, cualquiera que expire al último Para opciones adicionales, véase la Forma PCT/ISA/220.			
3. Para detalles adicionales, véanse las notas de la Forma PCT/ISA/220.			
Nombre y dirección de correspondencia de la ISA: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4465		Fecha de complemento de esta opinión véase la forma PCT/ISA/210	Funcionario Autorizado Bardili, Burkhardt Teléfono No. +49 89 2399-2132

OPINIÓN ESCRITA DE LA		No. de solicitud internacional
AUTORIDAD DE BÚSQUEDA INTERNACIONAL		PCT/IB2010/051470
Recuadro No. I. Base de la opinión		
1. Con respecto al lenguaje, esta opinión ha sido establecida con base en: ☒ la solicitud internacional en el lenguaje en el cual la misma fue presentada, ☐ una traducción del lenguaje original en el siguiente lenguaje , el cual es el lenguaje de una traducción provista para los propósitos de la búsqueda internacional (bajo las Reglas 12.3(a) y 23.1(b)). 2. ☐ Esta opinión ha sido establecida tomando en cuenta la rectificación de un error obvio autorizado por o notificado a esta Autoridad bajo la Regla 91 (Regla 43bis.1(a)) 3. Con respecto a cualquier secuencia de nucleótidos y/o aminoácidos descrita en la solicitud internacional y necesaria para la invención reivindicada, esta opinión ha sido establecida con base en: a. (medios) ☐ en el formato escrito ☐ en la forma electrónica b. (tiempo) ☐ en la solicitud internacional como se presentó ☐ junto con la solicitud internacional en la forma electrónica ☐ subsiguientemente a esta Autoridad para el propósito de búsqueda 4. ☐ además, en el caso de que más de una versión o copia de un listado de secuencias y/o tabla que se relaciona con el mismo, haya sido presentado o provisto, las declaraciones requeridas de que la información en las copias subsiguientes o adicionales es idénticas a aquella en la solicitud como se presentó o que no va más allá de la solicitud como se presentó, cuando sea apropiado, fueron proporcionadas. 4. ☐ además, en el caso de que más de una versión o copia de un listado de secuencias y/o tabla que se relaciona con el mismo, haya sido presentado o provisto, las declaraciones requeridas de que la información en las copias subsiguientes o adicionales es idénticas a aquella en la solicitud como se presentó o no va más allá de la solicitud como se presentó, cuando sea apropiado, en donde sea provisto. 5. Comentarios adicionales:		

OPINION ESCRITA DE LA AUTORIDAD DE LA		No. de solicitud internacional		
BUSQUEDA INTERNACIONAL		PCT/IB2010/051470		
Recuadro IV. Falta de unidad de la invención				
1. ☒ En respuesta a la invitación (Forma PCT/IPEA/206) a restringir o pagar las cuotas adicionales la solicitante ha, dentro del límite de tiempo establecido:				
☒ Pagado derechos adicionales				
☐ pagado las cuotas adicionales bajo protesta, y en donde sea aplicable, los derechos de la protesta				
☐ pagado las cuotas adicionales bajo protesta pero no ha pagado los derechos aplicables de la protesta				
☐ no ha pagado las cuotas adicionales.				
2. ☐ Esta autoridad considera que no se cumple con el requerimiento de unidad de la invención por las siguientes razones y decidió no invitar a la solicitante a restringir o a pagar las cuotas adicionales:				
3. Esta Autoridad considera que el requerimiento de unidad de la invención de acuerdo con la Regla 13.1, 13.2 y 13.3 es				
☐ Satisfecha				
☒ No satisfecha por las siguientes razones <u>véase la hoja separada</u>				
4. En consecuencia, este reporte ha sido restablecido con respecto a las siguientes partes de la solicitud internacional:				
☒ todas las partes de la solicitud				
☐ las partes que se refieren a las reivindicaciones Nos.				
V. Declaración justificada bajo la Regla 43bis.1(a)(i) con respecto a la novedad, etapa inventiva o aplicabilidad industrial; citas y explicaciones que apoyan tal declaración.				
1. DECLARACION				
Novedad (N)		Si:	Reivindicaciones	1-9
		No:	Reivindicaciones	
Etapa Inventiva (IS)		Si:	Reivindicaciones	
		No:	Reivindicaciones	1-9
Campo de aplicación industrial (IA)		Si:	Reivindicaciones	1-9
		No:	Reivindicaciones	
2. Citas y explicaciones				
<u>Véase la hoja separada</u>				

Con respecto al punto IV
Falta de unidad de la invención

La solicitud comprende dos invenciones:

Invención (1):

(1) Sales de K, Ca, Mg, Sr, Fe y Al de la 6'-sialil lactosa y su preparación; composiciones que comprenden estas sales y su uso como aditivos para composiciones alimenticias y farmacéuticas; (reivindicaciones 1-3, 8, 9 (completamente); 7 (parcialmente));

Invención (2):

(2a) un método para la preparación de alfa-sialil oligosacáridos protegidos de la fórmula (I) (reivindicación 4);

(2b) un método para la preparación de alfa-sialil oligosacáridos desprotegidos correspondientes (reivindicaciones 5, 6);

(2c) la preparación de la sal de sodio y amonio de la 6'-sialil lactosa que involucra el proceso de la invención (2a) (reivindicación 7 (parcialmente)).

De acuerdo con la Regla 13.1 y 13.2 del PCT, el requerimiento de unidad de la invención solamente puede ser satisfecho si un grupo de invenciones está relacionado así para formar un solo concepto inventivo general, es decir si existe una relación técnica entre las invenciones que involucra una o más de las mismas características o de características técnicas correspondientes, consideradas como un todo, que se haya heho sobre el arte previo.

Cuando se evalúa la unidad de la invención, la autoridad de la búsqueda internacional está obligada a seguir las Reglas de la Búsqueda y el Examen del PCT. De acuerdo con la Parte III, Capítulo 10, párrafo 10.12 de estas Reglas, una reivindicación independiente para un producto dado, una reivindicación independiente para un proceso especialmente adaptado para la fabricación del producto, y una reivindicación independiente para un uso del producto satisfacen los requerimientos de unidad de la invención a priori. La invención (1) como se definió anteriormente corresponde a esta situación.

Tres invenciones adicionales, es decir las invenciones (2a), (2b) y (2c) como se definieron anteriormente, son reivindicadas. La invención (2a) especifica un proceso para la preparación de una clase de oligosacáridos protegidos que incluyen la 6'-sialil lactosa protegida. De acuerdo con las invenciones (2b) y (2c), se hace uso de este proceso para

preparar los oligosacáridos desprotegidos correspondientes y la sal de sodio y amonio de la 6'-sialil lactosa. En consecuencia, las invenciones (2a), (2b) y (2c) están relacionadas entre sí por un solo concepto inventivo general.

Está pendiente que si las invenciones examinadas (1) y (2) están relacionadas por un solo concepto inventivo general.

De acuerdo con el párrafo 10.15 de las Reglas de la Búsqueda y el Examen del PCT referidas anteriormente la unidad de la invención debe ser considerada cuidadosamente si varias reivindicaciones de la misma categoría están presentes. En el presente caso, varias reivindicaciones de proceso han sido presentadas. Es evidente que los procesos de la invención (2) no están especialmente adaptados para la manufactura del producto de la reivindicación 1 puesto que el proceso de las invenciones (2a) y (2b) está diseñado para la preparación de una amplia clase de oligosacáridos y no para la preparación de la 6'-sialil lactosa y el proceso de la invención (2c) conduce a compuestos que no forman parte de la invención (1). Además, se reconoce en la descripción que los oligosacáridos de acuerdo con la fórmula (I) y en particular la 6'-sialil lactosa y los procesos para su preparación están comprendidos en el estado del arte.

Por consiguiente, estos compuestos no pueden ser considerados que sean características técnicas de las invenciones identificadas anteriormente dentro del significado de la Regla 13.2 del PCT.

Por consiguiente, no existe un solo concepto inventivo general que enlace las invenciones (1) y (2) como es requerido por la Regla 13.1 del PCT.

Con respecto al punto V

Declaración justificada con respecto a la novedad, etapa inventiva o aplicabilidad industrial; citas y explicaciones que apoyan tal declaración

Invención (1):

1. Las sales de sodio y amonio de la 6'-sialil lactosa son descritas en D1/Carbohydr. Res. 337, 473 (2002) y D2/Veterinary Microbiology 2, 303 (1978), respectivamente. D3/WO 2007/090894 y D4/WO 92/11017 se refieren a formulaciones nutritivas y farmacéuticas que comprenden la

6'-sialil lactosa. Puesto que las sales descritas en la reivindicación 1 no son descritas en estos documentos, la materia objeto de la invención (1) es novedosa.

2. El problema técnico que va a ser resuelto por la materia objeto de la invención (1) frente a D3 (o D4) es la provisión de formulaciones de la 6'-sialil lactosa adecuadas para uso farmacéutico o nutritivo. Se sabe bien en el arte que los compuestos activos farmacéuticamente pueden ser utilizados en la forma de sales farmacéuticamente aceptables. D5/WO 94/03184 describe sales farmacéuticamente aceptables de los compuestos semejantes como se reivindicó, es decir las sales de potasio, calcio y magnesio de los oligosacáridos que comprenden una porción de sialil lactosa. Parece que una combinación de los documentos D3 (o D4) y D5 conduce directamente a una persona experta a la materia objeto de las reivindicaciones 1-3, 8 y 9. Por consiguiente, la invención reivindicada no involucra una etapa inventiva (Artículo 33(3) del PCT).

Invención (2):

1. D6/Tetrahedr. Lett. 43, 8011 (2002) describe la síntesis de varios alfa-2-6 sialooligosacáridos en donde un donador de cloruro de sialilo de la fórmula (1) es unido con un aceptor de mono o disacárido en la presencia de carbonato de plata. El carbonato de plata es utilizado en una cantidad de 7 mol por mol del aceptor. La síntesis del alfa-2-6 sialooligosacárido de acuerdo con la reivindicación 4 es novedosa tomando en cuenta el hecho de que significativamente menos promotor es utilizado.

2. La síntesis reivindicada es ilustrada en el ejemplo 7. Sin embargo, la pureza y el rendimiento del producto obtenido no han sido proporcionados. Por lo tanto, no existe evidencia en el registro de que el mismo rendimiento que en D6 sea obtenido por el uso de menos promotor de plata. Por consiguiente, el problema técnico subyacente en la solicitud puede ser observado solamente en la provisión de un método adicional para preparar un alfa-2-6 sialooligosacárido. Una persona experta podría saber que este problema podría ser resuelto por la reducción de la cantidad del promotor sugerido en el arte previo relacionado. Las cantidades reducidas del promotor que todavía hacen posible una reacción de unión entre el donador y el aceptor podría ser identificada por experimentos de rutina. Por consiguiente, la materia objeto de las reivindicaciones 4-7 no involucran una etapa inventiva.

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43bis.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No. PCT/IB2010/051470	International filing date (day/month/year) 06.04.2010	Priority date (day/month/year) 06.04.2009
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International Patent Classification (IPC) or both national classification and IPC
INV. C07H13/06 A61K31/702 A23L1/09

Applicant
INALCO S.P.A.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Fax: +49 89 2399 - 4465	Date of completion of this opinion see form PCT/ISA/210	Authorized Officer Bardili, Burkhardt Telephone No. +49 89 2399-2132 
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**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/B2010/051470

Box No. 1 Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed
 - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))
3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:
 - a. (means)
 - ☐ on paper
 - ☐ in electronic form
 - b. (time)
 - ☐ in the international application as filed
 - ☐ together with the international application in electronic form
 - ☐ subsequently to this Authority for the purposes of search
4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5. Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/B2010/051470

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees, the applicant has, within the applicable time limit:
- ☒ paid additional fees
 - ☐ paid additional fees under protest and, where applicable, the protest fee
 - ☐ paid additional fees under protest but the applicable protest fee was not paid
 - ☐ not paid additional fees
2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is
- ☐ complied with
 - ☒ not complied with for the following reasons:
see separate sheet
4. Consequently, this report has been established in respect of the following parts of the international application:
- ☒ all parts.
 - ☐ the parts relating to claims Nos.

Box No. V Reasoned statement under Rule 43b/s.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	<u>1-9</u>
	No: Claims	
Inventive step (IS)	Yes: Claims	
	No: Claims	<u>1-9</u>
Industrial applicability (IA)	Yes: Claims	<u>1-9</u>
	No: Claims	

2. Citations and explanations

see separate sheet

Re Item IV

Lack of unity of invention

The application comprises two inventions:

Invention (1):

(1) K, Ca, Mg, Sr, Fe and Al salts of 6'-sialyl lactose and their preparation; compositions comprising these salts and their use as additives for food and pharmaceutical compositions; (claims 1-3, 8, 9 (completely); 7 (partially));

Invention (2):

(2a) a method for the preparation of protected alpha-sialyl oligosaccharides of formula (I) (claim 4);

(2b) a method for the preparation of the corresponding deprotected alpha-sialyl oligosaccharides (claims 5, 6);

(2c) the preparation of the sodium and ammonium salt of 6'-sialyl lactose involving the process of invention (2a) (claim 7 (partially))

According to Rule 13.1 and 13.2 PCT the requirement of unity of invention may only be fulfilled if a group of inventions is so linked as to form a single general inventive concept, i.e. if there is a technical relationship among the inventions involving one or more of the same or corresponding technical features that define contribution with each of the claimed inventions, considered as a whole, makes over the prior art. When assessing unity of invention the international searching authority is obliged to follow the PCT Search and Examination Guidelines. According to Part III, Chapter 10, paragraph 10.12 of these Guidelines an independent claim for a given product, an independent claim for a process specially adapted for the manufacture of the said product, and an independent claim for a use of the said product fulfills the requirements of unity of invention *a priori*. Invention (1) as defined above corresponds to this situation.

Three further inventions, i.e. invention (2a), (2b) and (2c) as defined above, are claimed. Invention (2a) specifies a process for the preparation of a class of protected oligosaccharides including protected 6'-sialyl lactose. According to inventions (2b)

and (2c) use is made of this process to prepare the corresponding deprotected oligosaccharides and the sodium and ammonium salt of 6'-sialyl lactose. Consequently, inventions (2a), (2b) and (2c) are linked with each other by a single general inventive concept.

It remains to be examined whether inventions (1) and (2) are linked by a single general inventive concept.

According to paragraph 10.15 of the PCT Search and Examination Guidelines referred to above unity of invention should be looked at carefully if several claims of the same category are present. In the present case, several process claims have been filed. It is apparent that the processes of invention (2) are not **specially adapted** for the manufacture of the product of claim 1 since the process of inventions (2a) and (2b) are designed for the preparation of a broad class of oligosaccharides and not for the preparation of 6'-sialyl lactose and the process of invention (2c) results in compounds which do not form part of invention (1). Furthermore, it is acknowledged in the description that oligosaccharides according to formula (I) and in particular 6'-sialyl lactose and processes for their preparation are comprised in the state of the art. Hence, these compounds cannot be considered to be special technical features of the above-identified inventions within the meaning of Rule 13.2 PCT.

Thus, a single general inventive concept linking inventions (1) and (2) as required by Rule 13.1 PCT does not exist.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Invention (1):

1. The sodium and ammonium salts of 6'-sialyl lactose are described in D1/ Carbohydr. Res. 337, 473 (2002) and D2/ Veterinary Microbiology 2, 303 (1978), respectively. D3/ WO 2007/090894 and D4/ WO 92/11017 relate to nutritional and

pharmaceutical formulations comprising 6'-sialyl lactose. Since the salts recited in claim 1 are not disclosed in these documents the subject-matter of invention (1) is novel.

2. The technical problem to be solved by the subject-matter of invention (1) vis-à-vis D3 (or D4) is the provision of formulations of 6'-sialyl lactose suitable for pharmaceutical or nutritional use. It is well-known in the art that pharmaceutically active compounds may be used in form of pharmaceutically acceptable salts. D5/ WO94/03184 discloses pharmaceutically acceptable salts of similar compounds as claimed, i.e. the potassium, calcium and magnesium salt of oligosaccharides comprising a sialyl lactose moiety. It appears that a combination of documents D3 (or D4) and D5 directly leads a skilled person to the subject-matter of claims 1-3, 8 and 9. Hence, the claimed invention does not involve an inventive step (Article 33(3) PCT).

Invention (2):

1. D6/ Tetrahedr. Lett. 43, 8011 (2002) describes the synthesis of several alpha-2-6 sialooligosaccharides wherein a sialyl chloride donor of formula (1) is coupled with a mono or disaccharide acceptor in the presence of silver carbonate. The silver carbonate is used in an amount of 7 mol per mol of acceptor. The synthesis of the alpha-2-6 sialooligosaccharide according to claim 4 is novel on account of the fact that significantly less promoter is used.

2. The claimed synthesis is illustrated in example 7. However, the yield and purity of the obtained product has not been given. Therefore, there is no evidence on file that the same yield as in D6 is obtained by use of less silver promoter. Hence, the technical problem underlying the application may only be seen in the provision of a further method to prepare an alpha- 2-6 sialooligosaccharide. A skilled person would know that this problem could be solved by reducing the amount of promoter suggested in the related prior art. The reduced amounts of promoter still enabling a coupling reaction between donor and acceptor could be identified by routine experiments. Thus, the subject-matter of claims 4-7 does not involve an inventive step.

Possible steps after receipt of the international search report (ISR) and written opinion of the International Searching Authority (WO-ISA)

General information	For all international applications filed on or after 01/01/2004 the competent ISA will establish an ISR. It is accompanied by the WO-ISA. Unlike the former written opinion of the IPEA (Rule 66.2 PCT), the WO-ISA is not meant to be responded to, but to be taken into consideration for further procedural steps. This document explains about the possibilities.
Amending claims under Art. 19 PCT	Within 2 months after the date of mailing of the ISR and the WO-ISA the applicant may file amended claims under Art. 19 PCT directly with the International Bureau of WIPO. The PCT reform of 2004 did not change this procedure. For further information please see Rule 46 PCT as well as form PCT/ISA/220 and the corresponding Notes to form PCT/ISA/220.
Filing a demand for international preliminary examination	In principle, the WO-ISA will be considered as the written opinion of the IPEA. This should, in many cases, make it unnecessary to file a demand for international preliminary examination. If the applicant nevertheless wishes to file a demand this must be done before expiry of 3 months after the date of mailing of the ISR/ WO-ISA or 22 months after priority date, whichever expires later (Rule 54bis PCT). Amendments under Art. 34 PCT can be filed with the IPEA as before, normally at the same time as filing the demand (Rule 66.1 (b) PCT). If a demand for international preliminary examination is filed and no comments/amendments have been received the WO-ISA will be transformed by the IPEA into an IPRP (International Preliminary Report on Patentability) which would merely reflect the content of the WO-ISA. The demand can still be withdrawn (Art. 37 PCT).
Filing informal comments	After receipt of the ISR/WO-ISA the applicant may file informal comments on the WO-ISA directly with the International Bureau of WIPO. These will be communicated to the designated Offices together with the IPRP (International Preliminary Report on Patentability) at 30 months from the priority date. Please also refer to the next box.
End of the international phase	At the end of the international phase the International Bureau of WIPO will transform the WO-ISA or, if a demand was filed, the written opinion of the IPEA into the IPRP, which will then be transmitted together with possible informal comments to the designated Offices. The IPRP replaces the former IPER (international preliminary examination report).
Relevant PCT Rules and more information	Rule 43 PCT, Rule 43bis PCT, Rule 44 PCT, Rule 44bis PCT, PCT Newsletter 12/2003, OJ 11/2003, OJ 12/2003

Bitte beachten Sie, dass angeführte Nichtpatentliteratur (wie z. B. wissenschaftliche oder technische Dokumente) je nach geltendem Recht dem Urheberrechtsschutz und/oder anderen Schutzarten für schriftliche Werke unterliegen könnte. Die Vervielfältigung urheberrechtlich geschützter Texte, ihre Verwendung in anderen elektronischen oder gedruckten Publikationen und ihre Weitergabe an Dritte ist ohne ausdrückliche Zustimmung des Rechtsinhabers nicht gestattet.

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XS CPRTENFRDE

From the INTERNATIONAL BUREAU

PCT

SECOND AND SUPPLEMENTARY NOTICE
INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION (TO DESIGNATED OFFICES
WHICH APPLY THE 30 MONTH TIME
LIMIT UNDER ARTICLE 22(1))

(PCT Rule 47.1(c))

Date of mailing (day/month/year) 11 August 2011 (11.08.2011)		To: BORSANO, Corrado Corso di Porta Vittoria 9 I-Milano 20122 ITALIE		NOTAR BARTOLO & GERVASI MILANO RECEIVED 22 AGO 2011 AG CA
Applicant's or agent's file reference 8717PTWO-Dz				
International application No. PCT/IB2010/051470		International filing date (day/month/year) 06 April 2010 (06.04.2010)		Priority date (day/month/year) 06 April 2009 (06.04.2009)
Applicant INALCO S.P.A. et al				

IMPORTANT NOTICE

1. **ATTENTION:** For any designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002 (30 months from the priority date), does not apply, please see Form PCT/IB/308(First Notice) issued previously.

2. Notice is hereby given that the following designated Office(s), for which the time limit under Article 22(1), as in force from 1 April 2002, does apply, has/have requested that the communication of the international application, as provided for in Article 20, be effected under Rule 93bis.1. The International Bureau has effected that communication on the date indicated below:
14 October 2010 (14.10.2010)

AU, AZ, BY, CN, CO, DZ, EP, HU, KG, KP, KR, MD, MK, MY, MZ, NA, NG, PG, RU, SY, TM, US

In accordance with Rule 47.1(c-bis)(i), those Offices will accept the present notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

3. The following designated Offices, for which the time limit under Article 22(1), as in force from 1 April 2002, does apply, have not requested, as at the time of mailing of the present notice, that the communication of the international application be effected under Rule 93bis.1:

AE, AG, AL, AM, AO, AP, AT, BA, BB, BG, BH, BR, BW, BZ, CA, CH, CL, CR, CU, CZ, DE, DK, DM, DO, EA, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, ID, IL, IN, IS, JP, KE, KM, KN, KZ, LA, LC, LK, LR, LS, LT, LY, MA, ME, MG, MN, MW, MX, NI, NO, NZ, OA, OM, PE, PH, PL, PT, RO, RS, SC, SD, SE, SG, SK, SL, SM, ST, SV, TH, TJ, TN, TR, TT, UA, UZ, VC, VN, ZA, ZM, ZW

In accordance with Rule 47.1(c-bis)(ii), those Offices accept the present notice as conclusive evidence that the Contracting State for which that Office acts as a designated Office does not require the furnishing, under Article 22, by the applicant of a copy of the international application.

4. **TIME LIMITS for entry into the national phase**

For the designated or elected Office(s) listed above, the applicable time limit for entering the national phase will, subject to what is said in the following paragraph, be 30 MONTHS from the priority date.

In practice, time limits other than the 30-month time limit will continue to apply, for various periods of time, in respect of certain of the designated or elected Office(s) listed above. For regular updates on the applicable time limits (30 or 31 months, or other time limit), Office by Office, refer to the *PCT Gazette*, the *PCT Newsletter* and the *PCT Applicant's Guide*, Volume II, National Chapters, all available from WIPO's Internet site, at <http://www.wipo.int/pct/en/index.html>.

It is the applicant's sole responsibility to monitor all these time limits.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Cecile Chatel
Facsimile No. +41 22 338 82 70	e-mail: mail@wipo.int

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

Date of mailing (day/month/year) 29 April 2010 (29.04.2010)	To: BORSANO, ~~Gerrado~~ Corso di Porta Vittoria 9 I-Milano 20122 ITALIE NOTAR BARTOLO & GERVAZI MILANO RECEIVED 18 MAG 2010
Applicant's or agent's file reference 8717PTWO-Dz	
International application No. PCT/IB2010/051470	International filing date (day/month/year) 06 April 2010 (06.04.2010)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 06 April 2009 (06.04.2009)
Applicant INALCO S.P.A. et al	

- By means of this Form, which replaces any previously issued notification concerning submission or transmittal of priority documents, the applicant is hereby notified of the date of receipt by the International Bureau of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to a date of receipt, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
- (If applicable)* The letters "NR" appearing in the right-hand column denote a priority document which, on the date of mailing of this Form, had not yet been received by the International Bureau under Rule 17.1(a) or (b). Where, under Rule 17.1(a), the priority document must be submitted by the applicant to the receiving Office or the International Bureau, but the applicant fails to submit the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
- (If applicable)* An asterisk (*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b) (the priority document was received after the time limit prescribed in Rule 17.1(a) or the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b)). Even though the priority document was not furnished in compliance with Rule 17.1(a) or (b), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
06 April 2009 (06.04.2009)	FI2009A000071	IT	22 April 2010 (22.04.2010)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Cecile Chatel e-mail RO.IB@WIPO.INT Telephone No. +41 22 338 92 22
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Facsimile No. +41 22 338 82 70

Form PCT/IB/304 (October 2005)

I/EFY1HIEH0

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

To:

BORSANO, Corrado
Borsano, Corrado
Corso di Porta Vittoria 9
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ITALIE

(PCT Rule 44.1)

Date of mailing
(day/month/year)

23 September 2010 (23-09-2010)

Applicant's or agent's
8717PTWO-DL

FOR FURTHER ACTION

See paragraphs 1 and 4 below

International application No.
PCT/IB2010/051470International filing date
(day/month/year)

6 April 2010 (06-04-2010)

Applicant

INALCO S.P.A.

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the International Search Report.**Where?** Directly to the International Bureau of WIPO, 34 chemin des Colombettes
1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
3. ☐ With regard to any protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
- ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminders

Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.

Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.

In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.

See the Annex to Form PCT/ISA/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, National Chapters.

Name and mailing address of the International Searching Authority


 European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040
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Authorized officer

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NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the *PCT Applicant's Guide*.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report and the written opinion of the International Searching Authority, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the letter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only (see *PCT Applicant's Guide*, Annex B).

The attention of the applicant is drawn to the fact that amendments to the claims under Article 19 are not allowed where the International Searching Authority has declared, under Article 17(2), that no international search report would be established (see *PCT Applicant's Guide*, International Phase, paragraph 296).

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 18 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet or sheets containing a complete set of claims in replacement of all the claims previously filed must be submitted.

Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively in Arabic numerals (Section 205(a)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

NOTES TO FORM PCT/ISA/220 (continued)

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments and any accompanying statement, under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the time of filing the amendments (and any statement) with the International Bureau, also file with the International Preliminary Examining Authority a copy of such amendments (and of any statement) and, where required, a translation of such amendments for the procedure before that Authority (see Rules 55.3(a) and 62.2, first sentence). For further information, see the Notes to the demand form (PCT/IPEA/401).

If a demand for international preliminary examination is made, the written opinion of the International Searching Authority will, except in certain cases where the International Preliminary Examining Authority did not act as International Searching Authority and where it has notified the International Bureau under Rule 66.1 b/s(b), be considered to be a written opinion of the International Preliminary Examining Authority. If a demand is made, the applicant may submit to the International Preliminary Examining Authority a reply to the written opinion together, where appropriate, with amendments before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later (Rule 43bis.1(c)).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see the *PCT Applicant's Guide*, National Chapters.

INTERNATIONAL SEARCH REPORT

126

International application No
PCT/IB2010/051470A. CLASSIFICATION OF SUBJECT MATTER
INV. C07H13/06 A61K31/702 A23L1/09
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07H A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RENCUROS I A ET AL: "Human milk oligosaccharides: an enzymatic protection step simplifies the synthesis of 3'- and 6'-O-sialyllactose and their analogues" CARBOHYDRATE RESEARCH, ELSEVIER SCIENTIFIC PUBLISHING COMPANY, AMSTERDAM, NL, vol. 337, no. 6, 15 March 2002 (2002-03-15), pages 473-483, XP004343543 ISSN: 0008-6215 compound (11); page 478 ----- -/-	1-9

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"B" document member of the same patent family

Date of the actual completion of the international search

17 September 2010

Date of mailing of the international search report

23/09/2010

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax: (+31-70) 340-3016

Authorized officer

Bardili, Burkhardt

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/051470

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MULLER H E ET AL: "Occurrence and some properties of neuraminidases in Haemophilus avium and Haemophilus paragallinarum" VETERINARY MICROBIOLOGY, ELSEVIER BV, NL, vol. 2, no. 4, 1 December 1977 (1977-12-01), pages 303-312, XP023915296 ISSN: 0378-1135 [retrieved on 1977-12-01] table I	1-9
Y	WO 2007/090894 A (NESTEC SA [CH]; SPRENGER NORBERT [CH]; MORGAN FRANCOIS [FR]; BERROCAL) 16 August 2007 (2007-08-16) claims 1,9	1-3,8,9
Y	WO 92/11017 A (MONSANTO CO [US]) 9 July 1992 (1992-07-09) claim 4; table I	1-3,8,9
Y	WO 94/03184 A (NEOSE PHARM INC [US]) 17 February 1994 (1994-02-17) page 11; claim 1	1-3,8,9
X	PAZYNINA G ET AL: "Simple stereoselective synthesis of alpha2-6 sialooligosaccharides" TETRAHEDRON LETTERS, ELSEVIER, AMSTERDAM, NL LNKD- DOI:10.1016/S0040-4039(02)01983-4, vol. 43, no. 45, 4 November 2002 (2002-11-04), pages 8011-8013, XP004387177 ISSN: 0040-4039 table 1	4-7

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2010/051470

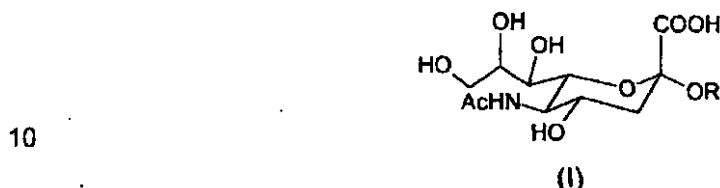
Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 2007090894	A	16-08-2007	AT 468027 T	15-06-2010
			AU 2007213677 A1	16-08-2007
			CA 2641842 A1	16-08-2007
			CN 101400267 A	01-04-2009
			EP 1988786 A1	12-11-2008
			ES 2342625 T3	09-07-2010
			PT 1988786 E	22-06-2010
			US 2009092729 A1	09-04-2009
			ZA 200807763 A	25-11-2009
WO 9211017	A	09-07-1992	US 5164374 A	17-11-1992
WO 9403184	A	17-02-1994	AU 4993893 A	03-03-1994

**6'-SIALYLLACTOSE SALTS AND PROCESS FOR THEIR SYNTHESIS AND
FOR THE SYNTHESIS OF OTHER A-SIALYLOLIGOSACCHARIDES
FIELD OF INVENTION**

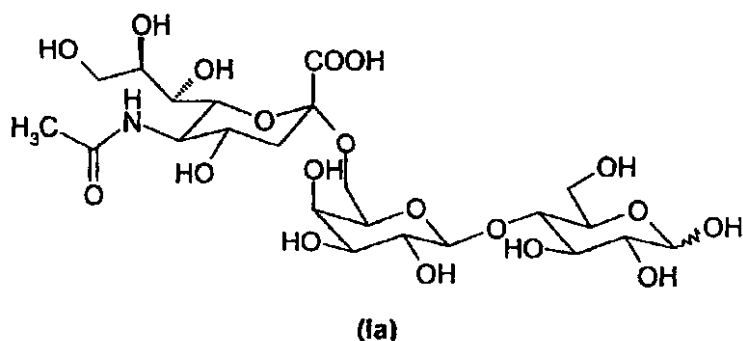
The present invention relates to the field of salts of 6'-sialyllactose: the present
5 invention also relates to the field of processes for the synthesis of α -sialyl-
oligosaccharides and in particular to the field of processes for the synthesis of 6'-
sialyllactose and its salts.

BACKGROUND

α -Sialyl-oligosaccharides of formula (I)



wherein R is a mono- di- or oligosaccharide residue with free hydroxyl groups are
present in mammals and birds tissues and in predominant form of
lipooligosaccharides, lipopolysaccharides or glycans of glycoproteins. They exist in
15 a variety of glycosidic bonds, more typically $\alpha(2-3)$ and $\alpha(2-6)$ galactose (or
lactose). The function of these sialosides varies greatly in animals according to the
structural heterogeneity of the oligosaccharide portion. They are mediators of inter
and intra-cells events in particular play an important role in the physiology and
growth of many pathogen agents (DK Ress, et al., Current Organic Synthesis,
20 2004, 1, 31-46).



One litre of human milk contains about 5-10 g of free oligosaccharides, this
content is similar to the content of proteins and exceeds the lipid content. More
25 than 130 different oligosaccharides were identified in human milk (Human Milk

- Oligosaccharides - HMO), formulations of artificial milk for babies derive from bovine milk and contain only trace amounts of these oligosaccharides that are specific of the human species. The fundamental building blocks of oligosaccharides of human milk are the 5 monosaccharides D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-fucose (Fuc) and sialic acid (N-acetyl-neuraminic acid, Neu5Ac). The terminal reducing end can be formed by lactose (Gal β 1-4Glc) or more repetitive units (up to 15 unit) of N-acetyllactosamine (Gal β 1-3/4GlcNAc). Lactose or polyactosamine may be sialylated with α 2-3 and / or α 2-6 bonds. Examples of sialosides of human milk are: 3'-sialyl-3-fucosylactose (3'S3FL), 6'-sialyllactose (6'SL), 3'-sialyllactose (3'SL), 3'-sialyllattosamine (3'SLN), 6'-sialyllactosamine (6'SLN).
- Among sialosides mainly present in mammalian tissues and in human milk the compound of formula (Ia) 6'-sialyllactose (N-acetylneuraminyllactose, α -NeuNAc-(2 $\rightarrow$ 6)- β - D-Gal-(1 $\rightarrow$ 4)-D-Glc or 6'-SL) is of particular importance because it is an important constituent of glycoproteins and glycolipids involved in various cell pathway events including cell recognition and immune response. The 6'-SL and their salts are interesting as supplements in food formulations for infants. As for the salts of the 6'-sialyllactose in literature only the sodium salt (CAS Number: 157574-76-0; FW: C₂₃H₃₈NO₁₉Na, 6'-sialyllactose sodium salt, 6'-N-Acetylneuraminyllactose sodium salt) and the ammonium salt are known. While the sodium salt is acceptable for food and pharmaceuticals, the ammonium salt is potentially toxic because of the ammonium ion. For this reason it is necessary to get 6'-SL in alternative salt forms to the known ones that may be acceptable for food and pharmaceuticals.
- At the state of the art various strategies are known for synthesis of sialyl-oligosaccharides (including 6'SL) and all foresee a convergent approach in which the sialic activated fragment (donor) is regio-and stereo-selectively bound to the oligosaccharide portion (acceptor). For this key step of coupling in literature three different synthetic strategies are known which foresee an exclusively enzymatic approach, exclusively chemical or chemo-enzymatic.
- As for the enzymatic pathways families of sialyltransferases and transialidases (enzymes that add the sialic acid to oligosaccharides in a strictly specific way)

were used. Examples of this route of synthesis are reported in A.T.Beyer et al *Adv. Enzymol.*, 1981, 52, 23-175, in J. Weinstein et al. *J.Biol.Chem.*, 1982, 257, 13845-13853.

However several are the limitations in the use of these enzymes:

- 5 1) the limited availability of these enzymes
- 2) the need to synthesize the donor of activated substrate CMP- NeuAc. or PNP-NeuAc
- 3) the strict specificity of the sialyltransferases which reduces the flexibility in use for the synthesis of natural sialosides (Ichigawa Y. et al. *Analytical Biochem* 1992,
- 10 202, 215-238, S. Sabesan et al. *J.Am.Chem.Soc.*, 1986, 108, 2068-2080, O. Hindsgaul et al *J.Biol.Chem.*, 1991, 266, 17858-17862, H.J.Gross et al. *Eur.J.Biochem.* 1988, 177, 583-589).

As for the chemo-enzymatic pathways these include chemical synthesis of the acceptor and then enzymatic sialylation as in S. Sabesan et al., *J.Am.Chem.Soc.*,

15 1986, 108, 2068-2080.

Focusing exclusively on chemical ways it is emphasized that the formation of the glycosidic bond with sialic acid is a reaction rather difficult because it is hampered by the fact that the donor is electronically and sterically hindered by the geminal carboxyl group. Moreover, the lack of functional group on C-3 rules out its

20 anchimeric assistance for controlling stereochemistry and leads to the formation of by-products through the reaction of elimination; finally the formation of the binding with α configuration is thermodynamically disadvantaged in relation to anomeric effect. In an attempt to remedy these defects in the state of the art various strategies have been developed for the preparation of suitably activated sialic

25 donors and acceptor with adequately protected hydroxyl groups that are reacted through various glycosylation methods.

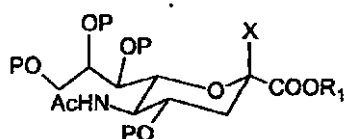
Regarding the sialic donor it is to be emphasized that the complicated molecular architecture imparts a substantial degree of difficulty in its synthesis, protection and activation. The multifunctional nature (3 secondary hydroxyl groups) as well

30 as the tertiary anomeric center, complicate the work of synthetic chemist. Currently to the state of the art is well known that the sialic donor can be activated as 2-xanthate (A. Marra et al., *Carbohydr.Res.*, 1989, 187, 35), as 2-aryl sulphone (Y.

Du et al, *Carbohydr. Res.*, 1998, 308, 161), as 2-phosphite (RR Schmidt et al., *Tetrahedron Lett.*, 1992, 33, 6123 or CH Wong et al., *J.Am.Chem.Soc.*, 1992, 114, 8748) or as 2-halo derivative. Among all these groups the halogen derivatives are preferable as the phosphite and the thio derivatives require toxic reagents for their synthesis and not easy to handle in the industry. Among the halogens the chloro derivative is preferred as it is stable and easy to synthesize, in fact the bromo derivative is unstable and tends easily to eliminate and lead to anomeric mixtures during glycosilation reactions. The fluoro derivative requires a more elaborate synthesis than the chloro derivative and tends to form β glycosidic bonds. The chloro derivative would be the easiest donor to make and use.

Regarding to the structure of the donor, other synthetic routes, still more complex than previous ones, require the inclusion on the C-3 of sialic acid of a functional group that gives anchimeric assistance in the glycosilation reaction to prevent competitive elimination in 2,3 position. For this reason groups such as phenylthio or phenylselenium have been introduced (Y. Ito et al., *Tetrahedron Lett.*, 1988, 29, 3987 or in L.O. Kononov et al., *Tetrahedron Lett.*, 1997, 38, 1599). These pathways thus require multiple steps to obtain the active donor for the glycosylation reaction generally starting from 2,3-dehydro NeuAc with yields that vary according to the obtained specificity and the ease of purification of the intermediates.

For this reason for the synthesis of sialyloligosaccharides of formula (I) and in particular the 6'SL it would be preferable to employ efficiently a simple sialylderivative as the 2-chloro-donor of formula (II)



(II)

where P is a suitable protecting group, R1 is alkyl group and X is chlorine; (obtainable by methods reported in R. Kuhn et al., *Chem.Ber.*, 1966, 99, 611, A. Marra et al., *Carb.Res.*, 1989, 190, 317-322 and NF Byramova et al., *Carb. Res.*, 1992, 237, 161-175) because it is also easily to synthesize on an industrial scale without the use of very toxic reactivities, and it leads to the specific formation of α

bonds in the glycosilation reactions. The use of this sialic donor, however, decreased significantly after its first applications and the state of the art addresses towards much more complex donors.

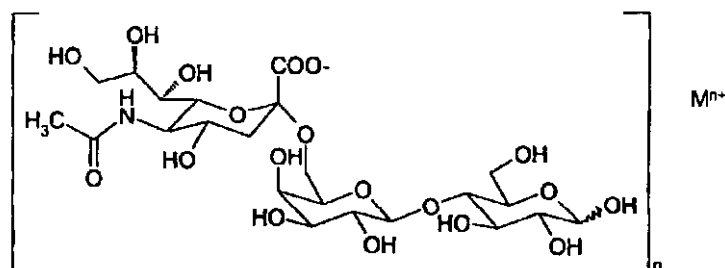
As for the acceptor activation for the synthesis of 6'SL, in the literature there are acceptors substituted with ether protective groups, for example benzyl groups whose removal, requiring an hydrogenation, is not easily manageable (G. Pazynina et al. *Tetrahedron Lett*, 2002, 43, 8011-8013) and therefore difficult for industrial application.

α -glycosides of sialic acid were prepared by the Koenigs-Knorr reaction involving the use of Ag (I) as a promoter [Koenigs, W., Knorr, E. *Chem. Ber.*, 1901, 34, 957] or by using the Helferich modification that uses Hg (II) as promoter [Helferich, B.; Zirner, J. *Chem. Ber.*, 1962, 95, 2604]. The substrates chosen in these reactions are β -glycosyl-halides. Many variations of these classical methods are known which were designed to improve employment opportunities and yields. The main differences between these variations are related to the choice of counter anion of the metallic promoter. The most commonly used promoters are AgOTf, Ag₂CO₃, HgX₂ (X = halide), and Hg(CN)₂. In general it is known that Ag(I) promoters are more active and stereoselective (Pazynina G. et al. *Tetrahedron Lett*, 2002, 43, 8011-8013) but these should be used in large quantities (6-7 eq in relation to the acceptor) thus increasing the cost of synthesis (including the disposal of waste production), while Hg(II) promoters provide higher yields (H. Paulsen et al. *Angew.Chem.Int.Ed.Engl*, 1982, 927-928) but can present difficulties in handling due to their toxicity.

It is therefore evident the need for a process for the synthesis of compounds of formula (I) which is simple and economical, applicable on industrial scale, and hence allowing to overcome the technical problems above mentioned related to processes known in the literature.

SUMMARY OF THE INVENTION

The present invention solves the above problems by compounds of formula (Ib)

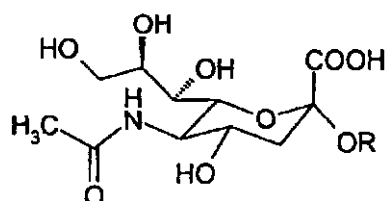


(Ib)

where M^{n+} is chosen from the group consisting of K^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} , Al^{3+} ,

Further subject-matter of the present invention is a process for the synthesis of α -

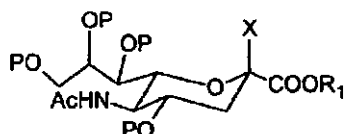
- 5 sialyl-oligosaccharides (I) compounds of formula (I)



(I)

wherein R is a mono-, di- or oligosaccharide residue with free hydroxyl groups;
said process comprising at least one step:

- 10 a) coupling by means of Koenigs - Knorr reaction of a sialic donor of formula (II)



(II)

wherein P is a suitable protecting group, R1 is an alkyl group, and X is a halogen;

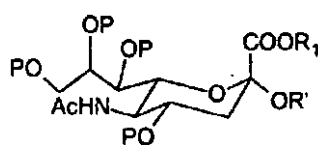
with an acceptor of formula $R'OH$ where R' is a mono-, di- or oligosaccharide

- 15 residue suitably protected with protecting groups P' and containing zero, one or more free hydroxyl groups; these protecting groups P' may be the same or different from each other and from those present in the donor;

such Koenigs-Knorr reaction characterized by the fact that the metallic promoter based on $Ag(I)$ is used in molar quantities between 0.5 and 2.0 eq respect to the

- 20 moles of acceptor;

to obtain an intermediate of formula (III)



(III)

where P, R1 and R' are as defined above.

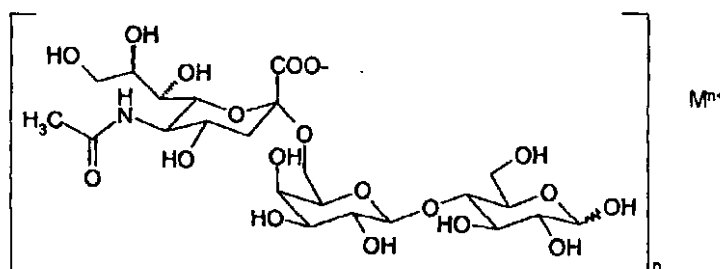
In particular, the process above mentioned provides a convenient route of

- 5 synthesis for the 6'SL and then for its salts of formula (Ib) in which M^{n+} is Na^+ , K^+ , NH_4^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} , Al^{3+} .

Other advantages of the present invention are discussed later in the detailed description.

DETAILED DESCRIPTION

- 10 Subject matter of the present invention are compounds of formula (Ib)



(Ib)

wherein M^{n+} is chosen from the group consisting of K^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} , Al^{3+} where $n = 1, 2, 3$ in correspondence to the oxidation state of M. Preferably M^{n+} is

- 15 Ca^{2+} , Mg^{2+} or K^+ .

Compounds of formula (Ib) are all alimentary and pharmaceutically acceptable and are useful as potential active ingredients or as food supplements (such as supplements in formulations of artificial milk for infants).

In particular 6'SL as:

- 20
- calcium salt is potentially useful to promote bone growth;
 - potassium and magnesium salts are potentially useful in maintaining, promote or restore proper transport through biological membranes and the physiological difference of transmembrane potential;

- iron salt is potentially useful for any pathological situations that require an integration of Fe;

The calcium salt in particular has better chemical-physical properties than the well-known sodium salt and its crystallization is easier. In fact during the crystallization phase, there is the formation of a crystalline solid, easy to be handled and thus easier to be managed even at industrial scale and its filtration has no problem because it is fast and allows an efficient washing of the solid.

In the case of sodium salt, instead, during the crystallization phase a rubbery solid is initially obtained, difficult to be stirred, which has to be grinded, and its filtration results slow and laborious. The stability of the two salts appears to be similar.

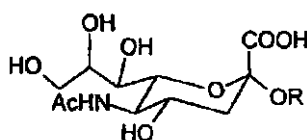
Another positive aspect of the calcium salt is the fact that starting from a same matrix of 6'SL in acid form the calcium salt is the one obtained with higher purity: for example the same matrix of 6'SL has supplied a crystal of sodium salt at 87% HPLC purity and a crystal of calcium salt at 93% HPLC purity.

Both potassium and magnesium salts show chemical-physical characteristics similar to the sodium salt as well as their crystallization has a similar trend.

Compounds of formula (Ib) above described can be prepared by 6'SL following known methods at state of the art for the preparation of salts from the corresponding carboxylic acids; for example they can be preferably prepared from a solution of 6'SL by adding a base containing M^{n+} such as hydroxides, carbonates or bicarbonates (ie, KOH, $Ca(OH)_2$, $Mg(OH)_2$, etc., K_2CO_3 , $CaCO_3$, $MgCO_3$ etc.; $KHCO_3$, etc.) until pH 8-10. After removal of the solvent the obtained salt are purified by crystallization from alcohols or mixtures of water / alcohol; preferably methanol, ethanol and their mixtures with water.

Optionally before the removal of the solvent it is possible to remove the excess of undissolved base by filtration, if present.

For another aspect the present invention relates to a process for the synthesis of α -sialyl-oligosaccharides of formula (I)

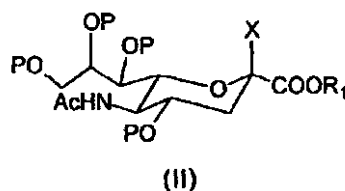


(I)

wherein R is a mono-, di- or oligosaccharide residue with free hydroxy groups, preferably R is selected between galactose, glucose, glucosamine, lactose, lactosamine, fucosyllactose; more preferably R is selected between 6'-galactose, 3'-glucose, 3'-glucosamine, 6'-lactose, 3'-lactose, 6'-lactosamine, 3'-lactosamine, 3'-3-fucosyllactose;

such process involving at least one step:

a) coupling by Koenigs-Knorr reaction of a sialic donor of formula (II)



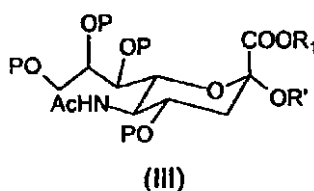
wherein:

P is a suitable protecting group;

R1 is an alkyl, preferably Me, Et, or Pr;

X is a halogen, preferably chlorine;

with a suitably protected acceptor of formula R'OH in which R' is a mono-, di- or oligosaccharide residue suitably protected with protecting groups P' and containing zero, one or more free hydroxyl groups: these protecting groups P' can be equal or different from each other and from those present in the donor; said Koenigs-Knorr reaction is characterized by the fact that the metallic promoter based on Ag (I) is used in molar quantities between 0.5 and 2.0 eq compared to moles of acceptor; to obtain an intermediate of formula (III)



where P, R1 and R' are defined as above.

For a preferred aspect this process also includes after step (a), the following step:

b) removal of protective groups P, P' and R1 to obtain compounds of formula (I) as described above.

The removal of the protective group b) is performed according to methods known to the state of the art (TW Green and PGM WUTS. Green's Protective Groups in Organic Synthesis. Ed Wiley ed 4. 2006).

For a preferred aspect this metallic promoter is used in molar amount between
5 0.75 and 0.85 molar eq compared to moles of acceptor.

For a preferred aspect said metallic promoter is selected between Ag(I) salts such as AgOTf, Ag₂CO₃, and more preferably is Ag₂CO₃.

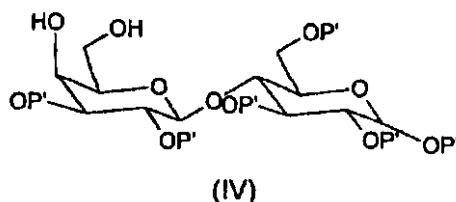
For a preferred aspect the coupling a) is conducted in aprotic polar solvent, preferably is conducted in dichloromethane.

10 For a preferred aspect said coupling a) is achieved by stirring the mixture at a temperature between 20 and 40° C for a time between 5 and 10 days; preferably the mixture is stirred at 30 ° C for 7 days.

For a preferred aspect P and P' are independently selected between benzyl and acyl, preferably P and P' are independently selected from acetyl, benzoyl or
15 benzoyl mono-or di-substituted with alkoxy, halogen, nitro groups.

For a preferred aspect P and P' are acyls. For a more preferred aspect P and P' are equal and are acetyl. Where R is 6'-lactose in the process of the present invention with P = P' = Ac there is an additional distinction from known processes for the synthesis of 6'SL as in this case the acceptor used in the present invention
20 do not show benzyl groups and consequently has the advantage of avoiding catalytic hydrogenation for the removal of protective groups.

For a preferred aspect this acceptor R'OH adequately protected has a free and reactive hydroxyl group at C-6 of the galactosidic moiety, in a particularly preferred aspect the acceptor is a lactose derivative of formula (IV):

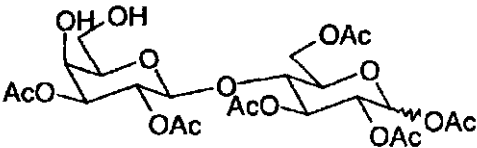


25

where P' is a suitable protecting group; preferably P' is acyl, more preferably acetyl.

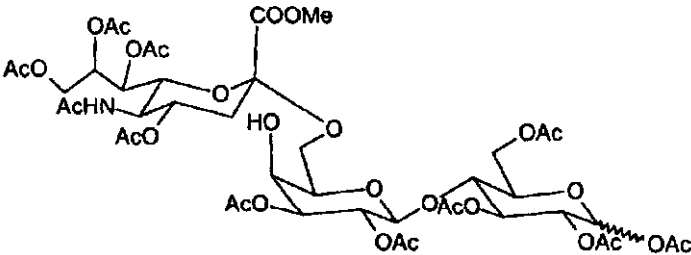
For a particularly preferred aspect therefore the present invention relates to the
30 synthesis of 6'SL of formula (Ia) where R = 6'-lactose by the process above

described in which P and P' are Ac, X is chlorine and R1 is methyl; in this particular combination, the coupling reaction in which the acceptor is a compound of formula (IVa)



(IVa)

provides the compound of formula (IIIa)



(IIIa)

that surprisingly can be used as it has been obtained from the crude mixture to the subsequent reaction of sequential deprotection of the hydroxyl groups and of the carboxyl function to the anomeric carbon of the sialic acid and production of the compound 6'SL formula (Ia). Preferably it has to proceed first to the removal of acetyl groups and then to the hydrolysis of methyl ester.

This sequential deprotection is carried out as known to the state of the art.

Preferably the removal of the Ac groups is performed using a base such as sodium methoxide, sodium ethoxide or sodium hydroxide, more preferably sodium methoxide, using a primary alcohol as a solvent, like methanol or ethanol, most preferably methanol. Preferably the hydrolysis of methyl ester at anomeric carbon of the sialic acid is carried out in basic conditions with NaOH 1M.

Preferably when the methyl ester hydrolysis is completed, final acidification of the reaction mixture is performed by ion exchange resins, in particular a strong cationic resin and a weak anionic resin, to obtain an eluate containing 6'SL.

The sialic donor used in the process above described, in particular when X is chlorine and R' is lactose protected by formula (IV) with P = P' = Ac, is simple or

easy to prepare and easy to use. This choice has revealed surprisingly adequate

to solve the problems of known processes, despite the state of the art had directed towards much more complex donors.

The amount of promoter metal used in step a) is surprisingly reduced compared to the known state of the art, 0.5-2.0 eq versus 6-7 eq, and therefore reduces the
5 cost of synthesis and the costs of disposing of waste production.

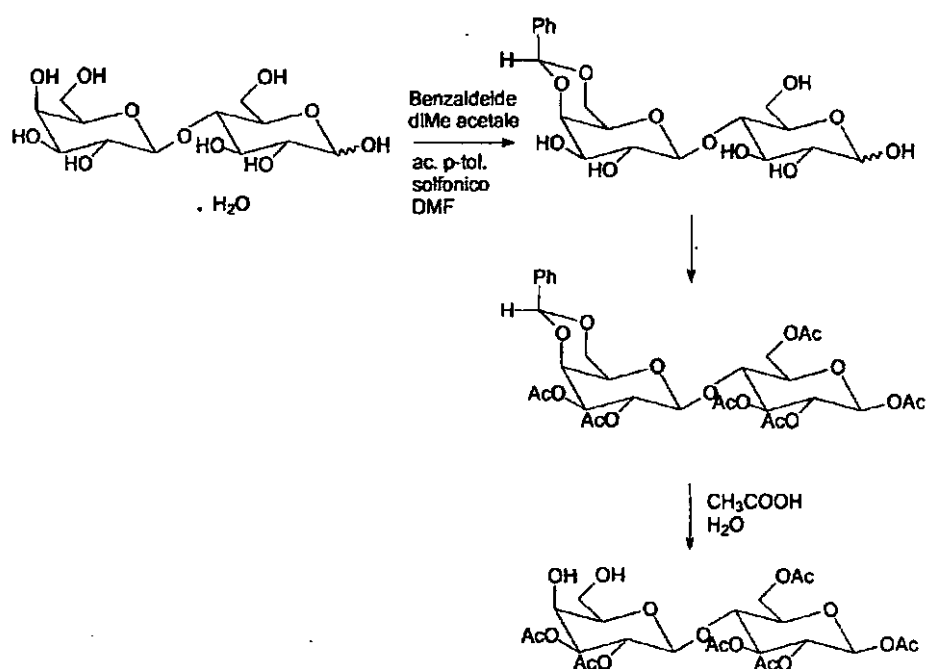
The product of coupling reaction is achieved surprisingly with enough purity to be used as it is in the subsequent reaction of deprotection to obtain the deprotected sialyl-oligosaccharides with good yields and purity. Furthermore it should be noted that the process for the coupling reaction of the present invention is
10 stereoselective because the α isomer is the only one obtained.

The process of the present invention is therefore feasible on industrial scale.

The present invention also refers to a process for the synthesis of compounds of formula (Ib) in which M^{n+} is Na^+ , K^+ , NH_4^+ , Ca^{2+} , Mg^{2+} , Sr^{2+} , Fe^{2+} , Al^{3+} such process including the preparation of 6'SL through the process above described;
15 preferably the salts of formula (Ib) may be prepared directly by the eluate containing 6'SL, obtained after ion exchange resins treatment after hydrolysis of methyl ester, by addition of a base containing $M^{(n+)}$ such eg hydroxides, carbonates or bicarbonates (ie, KOH, $Ca(OH)_2$, $Mg(OH)_2$, etc., K_2CO_3 , $CaCO_3$, $MgCO_3$ etc.; $KHCO_3$, etc.) until pH 8-10.

20 Compounds of formula (II) can be obtained by techniques known to the state of the art such as reported in (R. Kuhn et al., *Chem.Ber.*, 1966, 99, 611, A. Marra et al., *Carb. Res*, 1989, 190, 317-322 and NF Byramova et al., *Carb. Res*, 1992, 237, 161-175).

The synthesis of the suitably protected acceptor can be carried out following the
25 knowledge of the man of the art, in particular the synthesis of the acceptor of formula (IIIa) where P is Ac was carried out according to methods known in agreement with the Scheme 1.



(IVa) Schema 1

According to the invention alkoxy means, for example -OMe, -OEt, -OnPr, -OiPr, -

5 OnBu, -OiBu, -OtBu.

According to the invention halogen means fluorine, chlorine, bromine, iodine.

According to the invention alkyl is a linear alkyl chain or branched containing 1 to 6 atoms of carbon possibly substituted by one or more groups selected between halogen, hydroxy, alkoxy, nitro.

10 According to the invention aryl is benzene eventually replaced by one or more groups selected from halogen, alkoxy, nitro.

According to the invention acyl means a group -OCO-alkyl, or -OCO-aryl in which alkyl and aryl are defined as above.

According to the invention monosaccharide means a polyoxyaldehyde (aldose) or a
15 polyoxyketone (ketose) or a simple sugar of formula $(\text{CH}_2\text{O})_n$, $\text{C}_n\text{H}_{2n}\text{O}_{n-1}$, $\text{C}_n\text{H}_{2n}\text{O}_n$, $\text{C}_n\text{H}_{2n}\text{O}_{n-1}\text{NH}_2$ or $\text{C}_n\text{H}_{2n}\text{O}_{n-1}\text{NHAc}$ with $n = 3, 4, 5, 6, 7$; it means within the definition all the possible stereoisomers and all open or cyclic forms or intramolecular semi

acetals and semiketals as an example the pyranosyl and furanosyl forms; for example, glyceraldehyde, allose, altrose, arabinose, erythrose, fucose, galactose, glucose, glucosamine, N-acetyl-glucosamine, idose, lixose, mannose, psychose, ribose, deoxyribose, sorbose, tagatose, treose, xylose and corresponding ketoses

5 are included in the definition.

According to the invention disaccharide means a compound polyoxydrilated consisting of two monosaccharides linked through an acetalic or glycosidic both O-glycosidic or N-glycosidic bond; within the definition are included all of the possible stereoisomers and all forms of open or cyclic; for example lactose, lactosamine, N-

10 acetyl-lactosamine, maltose, cellobiose, sucrose, threulose, turanose are included.

According to the invention oligosaccharide means a polymer formed of three to 6 monosaccharides joined together by glycosidic bonds to form linear saccharide chains or branched, for example raffinose, melezitose, maltotriose, acarbose,

15 stachyose are included.

EXPERIMENTAL PART

EXAMPLE 1

Preparation of di 4',6'-O-benzylidenelactose

200 g (0.555 mol) of lactose monohydrate were added under stirring to 1.4 l of
 5 *N,N*-dimethylformamide and then 209 ml (1.39 mol) of benzaldehyde dimethyl
 acetal and 5.28 g (0.028 mol) of *p*-toluenesulfonic acid monohydrate were added.
 The resulting suspension was heated at 55°C and the temperature was
 maintained until TLC was successful (16-18 hours) (Pharmacopoeia). After cooling
 at room temperature 4.7 ml of triethylamine were added until pH 7-8. The mixture
 10 was concentrated to obtain 700 ml of solution, which is drained in 3 liters of hot
 acetone (50-55°C), maintaining a vigorous stirring. The precipitation was
 completed by cooling the mixture to 0±5°C. The precipitate was filtered, washed
 with 0.7 liters of cold acetone and dried, obtaining 208 g of 4',6'-O-benzylidene
 lactose (mixture of α / β anomers) as white product (assay HPLC 66%, 0.319 mol,
 15 yield: 57%).

By double crystallization, first by MeOH and then by MeOH/H₂O 4 / 1 v / v, an
 analytical sample enriched in α anomer was obtained, the NMR characterization is
 reported:

¹H NMR (DMSO-d₆, 300 MHz): δ ppm 7.51-7.34 (5H, m, Ph); 6.36 (d, J_{OH-1}= 4.8
 20 Hz, 1H, C1-OH); 5.58 (s, 1H, PhCH); 5.28 (d, J= 4.2 Hz, 1H, OH); 5.01 (d, J= 5.7
 Hz, OH); 4.92 (pseudo t, J_{1-OH}= J₁₋₂= 4.0 Hz, 1H, H-1); 4.68 (d, J= 6.9 Hz, 1H, OH);
 4.45 (m, 2H, 2xOH); 4.37 (d, J₁₋₂= 7.5 Hz, 1H, H-1'); 4.16-3.95 (m, 3H); 3.84-3.11
 (m, 9H) (H-2, H-3, H-4, H-5, CH₂-6, H-2', H-3', H-4', H-5', CH₂-6').

¹³C NMR (DMSO-d₆, 75 MHz): δ ppm 138.5, 128.6, 127.9, 126.2 (Ph); 103.1 (C-1');
 25 99.8 (PhCH); 92.1 (C-1); 79.6, 75.8, 72.2, 71.6, 71.3, 69.9, 69.8, 68.5 (C2, C2',
 C3, C3', C4, C4', C5, C5'); 66.2 (C6'); 60.3 (C6).

R_f (pharmacopoeia, UV-vis and naftoresorcine)= 0.7

EXAMPLE 2

Preparation of 4',6'-O-p-methoxybenzylidenlactose

- 200 g (0,555 mol) of lactose monohydrate were added under stirring to 1.4 l of N, N-dimethylformamide and then 237 ml (1.39 mol) of p-methoxybenzaldehyde
- 5 dimethyl acetal and 5,28 g (0,028 mol) of p-toluenesulfonic acid monohydrate were added. The resulting suspension was heated at 55°C and the temperature was maintained until positive TLC resulted (16-18 hours) (Pharmacopoeia). After cooling at room temperature 5.0 ml of triethylamine were added until pH 7-8. The mixture was concentrated and the residue was crystallized in 3 liters of hot
- 10 acetone (50-55°C). The precipitation was completed by cooling the mixture at 0+5°C. The precipitate was filtered, washed with 2x200 ml of cold acetone, obtaining 219 g of 4',6'-O-p-metossibenzyliden lactose (mixture of α/β anomers) as a pale yellow solid (assay HPLC 76%, 0361 mol, yield: 65%)
- Crystallization from hot acetone/H₂O 4/1 v/v gave an analytical sample as a α/β
- 15 anomeric mixture (1/1 mol/mol), the NMR characterization is reported:.
- ¹H NMR (DMSO-d₆, 300 MHz): δ ppm 7.38 (d, J= 8.7 Hz, 2H), 6.93 (d, J= 8.7 Hz, 2H) (Ph); 6.70 (d, J_{OH+1}= 6.6 Hz, 1H, C1-OH β); 6.36 (d, J_{OH+1}= 4.1 Hz, 1H, C1-OH α); 5.52 (s, 1H, PhCH $\alpha+\beta$); 5.27 (m, 1H, OH $\alpha+\beta$); 5.04-4.95 (m, 1H, OH $\alpha+\beta$); 4.92 (pseudo t, J= 4.1 Hz, H-1 α); 4.72-4.60 (m, 1H, OH $\alpha+\beta$); 4.56-4.28 (m, H-1' $\alpha+\beta$ + H-1 β + 2xOH $\alpha+\beta$); 4.12-3.92 (m, 3H); 3.76 (s, 3H, OMe); 3.80-3.11 (m, 9H); 2.98 (m, 1H β).
- ¹³C NMR (DMSO-d₆, 75 MHz): δ ppm 159.4, 130.9, 127.6, 113.2 (Ph); 103.0 (C-1' $\alpha+\beta$); 99.7 (PhCH $\alpha+\beta$); 96.7 (C-1 β); 92.1 (C-1 α); 79.6, 79.2, 75.7, 74.9, 74.8, 74.6, 72.2, 71.6, 71.3, 69.9, 69.8, 68.4 (C2, C2', C3, C3', C4, C4', C5, C5' $\alpha+\beta$);
- 25 66.2 (C6' $\alpha+\beta$); 60.4, 60.3 (C6 $\alpha+\beta$); 55.1 (OMe $\alpha+\beta$).
- Rf (pharmacopoeia, UV-vis and naftoresorcine)= 0.8

EXAMPLE 3

- Preparation of 1,2,3,6,2',3'-hexa-*O*-acetyl-4',6'-*O*-benzyliden- β -D-lactose
- 100 g (0.153 moles from HPLC) of 4',6'-*O*-benzylidenlactose obtained according to example 1 and 256 ml (1.84 mol) of triethylamine were added to 600 ml of methyl
- 5 ethyl ketone. The reaction mixture was heated at 60 °C and 174 ml (1.84 mol) of acetic anhydride were dropped maintaining the internal temperature below 70°C. The reaction mixture was stirred at 70°C until TLC was successful (10-12 hours) (AcOEt). The solvent was evaporated and the residue dissolved in 270 ml of dichloromethane and 200 ml of water. NaOH 30% was added under stirring up to
- 10 pH 9-9.5, then the layers were separated; the aqueous layer was extracted again with 75 ml of dichloromethane. The collected organic layers were washed with 200 ml of water, and then HCl solution 32% was added under stirring up to pH 1-1.5. The acid aqueous layer was extracted with 75 ml of dichloromethane. The collected organic layers were then washed with 370 ml of NaCl 20%, dried over
- 15 anhydrous Na₂SO₄ and decolorized with charcoal and bentonite. The solvent was concentrated and the residue used as such in the next reaction. HPLC dosage gave 103 g (0.151 mol) of 1,2,3,6,2',3'-hexa-*O*-acetyl-4', 6'-*O*-benzylidene lactose essentially as β anomer (α anomer <10 mol%) (yield: 99%). An analytical sample containing 9 mol% of α anomer was obtained by crystallization from hot MeOH;
- 20 following is the NMR characterization (β anomer).
- ¹H NMR (CDCl₃, 300 MHz): δ ppm 7.54-7.34 (m, 5H, Ph); 5.68 (d, $J_{1,2}$ = 8.4 Hz, 1H, H-1), 5.47 (pseudo s, 1H, CHPh); 5.32-5.21 (m, 2H, H-3+H-2'), 5.07 (dd, $J_{2,3}$ = 9.6 Hz e $J_{2,1}$ = 8.4 Hz, 1H, H-2), 4.87 (dd, $J_{3,2}$ = 10.4 Hz e $J_{3,4}$ = 3.8 Hz, 1H, H-3'), 4.54-4.43 (m, 2H, H-1'+H-6a), 4.38-4.25 (m, 2H, H-4'+H-6'a), 4.14 (dd, $J_{6b,6a}$ = 12.2 e $J_{6b,5}$ = 4.6 Hz, 1H, H-6b), 4.04 (d, $J_{6'b,6'a}$ = 12.3 Hz, 1H, H-6'b), 3.90-3.70 (m, 2H, H4+H5); 3.46 (pseudo s, 1H, H-5'), 2.14-2.00 (6xCOCH₃).
- ¹³C NMR (CDCl₃, 75 MHz): δ ppm 170.8, 170.4, 170.1, 169.7, 169.0, 168.9 (6xCOCH₃); 137.5, 129.3, 128.3, 126.6 (Ph); 101.4 (CHPh); 101.1 (C-1'); 91.8 (C-1); 75.5, 73.8, 73.2, 72.4, 72.2, 70.5, 69.0, 68.5 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'); 66.6 (C-6'); 61.8 (C-6), 20.9-20.7 (6xCOCH₃).
- 30 Rf (AcOEt:hexan=1:1, UV-vis and H₂SO₄/MeOH)= 0.3

EXAMPLE 4

Preparation of 1,2,3,6,2',3'-hexa-*O*-acetyl-4',6'-*O*-*p*-methoxybenzyliden- β -D-lactose

- 100 g (HPLC dosage 0.165 mol) of 4',6'-*O*-*p*-methoxybenzylidenlactose obtained
 5 according to example 2 and 242 ml (1.74 mol) of triethylamine were added at 600
 ml of methyl ethyl ketone. The suspension was heated at 60°C and 164 ml (1.74
 mol) of acetic anhydride were dropped maintaining the internal temperature below
 70°C. The reaction mixture was stirred at 70°C until positive TLC was successful
 (10-12 hours) (AcOEt: hexane = 1:1). The solvent was evaporated and the residue
 10 dissolved in 270 ml of dichloromethane and 200 ml of water. NaOH 30% was
 added under stirring up to pH 9-9.5, then the layers were separated; the aqueous
 layer was extracted again with 75 ml of dichloromethane. The collected organic
 layers were washed with 200 ml of water, and then HCl solution 32% was added
 under stirring up to pH 1-1.5. The acid aqueous layer was extracted with 75 ml of
 15 dichloromethane. The collected organic layers were then washed with 400 ml of
 saturated NaHCO₃, with 400 ml of NaCl 20%, dried on anhydrous Na₂SO₄ and
 bleached with charcoal and bentonite. The solvent was concentrated and the
 residue used as such in next reaction. HPLC dosage gave 110 g (0,155 mol) of
 1,2,3,6,2',3'-hexa-*O*-acetyl-4',6'-*O*-*p*-methoxybenzylidenlactose essentially as β
 20 anomer (yield: 94%). An analytical sample was obtained by crystallization from
 hot MeOH; following is the NMR characterization (β anomer).
¹H NMR (CDCl₃, 300 MHz): δ ppm 7.36 (d, J= 8.7 Hz, 2H), 6.88 (d, J= 8.7 Hz, 2H)
 (Ph); 5.66 (d, J₁₋₂= 8.4 Hz, 1H, H-1); 5.40 (pseudo s, 1H, CHPh); 5.24 (pseudo t,
 J₃₋₂= J₃₋₄= 9.6 Hz, 1H, H-3); 5.23 (dd, J₂₋₃= 10.2 Hz e J₂₋₁= 7.8 Hz, 1H, H-2'); 5.04
 25 (dd, J₂₋₃= 9.6 Hz e J₂₋₁= 8.4 Hz, 1H, H-2); 4.84 (dd, J₃₋₂= 10.2 Hz e J₃₋₄= 3.6 Hz,
 1H, H-3'), 4.46 (dd, J_{6a-6b} = 12.0 Hz e J_{6a-5}= 1.5 Hz, 1H, H-6a); 4.44 (d, J_{1'-2'}= 7.8
 Hz, 1H, H-1'); 4.28 (d, J_{4'-3'}= 3.6 Hz, 1H, H-4'); 4.25 (d, J_{6'a-6'b}= 12.6 Hz, 1H, H-6'a);
 4.12 (dd, J_{6b-6a}= 12.0 e J_{6b-5}= 4.5 Hz, 1H, H-6b), 4.00 (dd, J_{6'b-6'a} = 12.6 Hz e J_{6'b-5'}
 = 1.5 Hz, 1H, H-6'b); 3.87-3.69 (m, 2H, H4+H5); 3.79 (s, 3H, OMe); 3.42 (pseudo
 30 s, 1H, H-5'); 2.09, 2.07, 2.03, 2.02, 2.00 (6xCOCH₃).

^{13}C NMR (CDCl_3 , 75 MHz): δ ppm 170.8, 170.4, 170.1, 169.6, 168.93, 168.89 ($6\times\text{COCH}_3$); 160.3, 130.1, 127.9, 113.7 (Ph); 101.3 (CHPh); 101.1 (C-1'); 91.8 (C-1); 75.5, 73.8, 73.2, 72.4, 72.1, 70.5, 69.0, 68.4 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'); 66.5 (C-6'); 61.8 (C-6), 55.4 (OMe); 20.9-20.6 ($6\times\text{COCH}_3$).

5 Rf (AcOEt:hexan=1:1, UV-vis and $\text{H}_2\text{SO}_4/\text{MeOH}$)= 0.2

EXAMPLE 5

Preparation of 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose

The syrup containing 100 g (0.15 moles) of 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-benzylidenlactose obtained according to example 2 was dissolved in 400 ml of
10 glacial acetic acid. The reaction mixture was heated at 80°C and then 100 ml of water (preheated at 80°C) were added, stirring the mixture at this temperature for 1.5 hours. The reaction mixture was then rapidly cooled to room temperature and 500 ml of toluene and 350 ml of water were added and extracted. The aqueous
15 layer was extracted with 150 ml of toluene. The combined toluene layer contained not reacted 1,2,3,6,2', 3'-hexa-O-acetyl-4',6'-O-benzylidenlactose, which can be used in another reaction. The aqueous layer, containing 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose, was extracted successively with 500 ml and 150 ml of methylene chloride; the organic extracts were washed with 3×150 ml of water,
20 dried over anhydrous sodium sulphate and concentrated. The residue was crystallized from 580 ml of hot isopropyl acetate (50 - 55°C), obtaining after drying 30.8 g of 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose (0.05 mol) as a chalky white solid. Considering that in the combined toluene layers 20.2 g of unreacted 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-benzylidenlactose (0.03 mol) were recovered, the yield is
25 42%.

Mp 188 - 190°C .

^1H NMR (CDCl_3 , 300 MHz): δ ppm 5.68 (d, $J_{1-2}=8.1$ Hz, 1H, H-1), 5.24 (pseudo t, $J_{3-2}=J_{3-4}=9.3$ Hz, 1H, H-3), 5.19 (dd, $J_{2-3}=10.2$ Hz e $J_{2-1}=7.8$ Hz, 1H, H-2'), 5.05 (dd, $J_{2-3}=9.3$ Hz e $J_{2-1}=8.1$ Hz, 1H, H-2), 4.88 (dd, $J_{3-2}=10.2$ Hz e $J_{3-4}=3.3$ Hz, 1H, H-3'), 4.49 (d, $J_{1-2}=7.8$ Hz, 1H, H-1'), 4.49 (dd, $J_{6a-6b}=11.1$ Hz e $J_{6a-5}=1.8$ Hz, 1H, H-6a), 4.15-4.05 (m, 2H, H-4'+H-6b), 4.00-3.70 (m, 4H, H-4+H-5+H-6'a+H-
30

6'b), 3.56 (pseudo t, $J = 5.4$ Hz, 1H, H-5'), 2.96 (d, $J_{OH-4} = 4.2$ Hz, C4'-OH), 2.59 (dd, $J_{OH-6} = 7.5$ e 4.8 Hz, C6'-OH), 2.11, 2.09, 2.08, 2.07, 2.04, 2.03 (6xCOCH₃).

¹³C NMR (CDCl₃, 75 MHz): δ ppm 170.6, 170.4, 170.3, 169.7, 169.6, 169.0 (6xCOCH₃); 101.2 (C-1'), 91.7 (C-1), 75.9 (C-4), 74.6 (C-5'), 73.7 (C-3'), 73.6 (C-5), 73.1 (C-3), 70.7 (C-2), 69.7 (C-2'), 67.8 (C-4'), 62.1, 62.0 (C-6, C-6'), 20.9, 20.8, 20.7 (6xCOCH₃).

R_f (AcOEt, UV-vis and H₂SO₄/MeOH) = 0.4

10 EXAMPLE 6

Preparation of di 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose

The syrup containing 100 g (0.14 mol) of 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-*p*-methoxybenzylidenlactose obtained according to example 4 was dissolved in 400 ml of glacial acetic acid, then 100 ml of water were added and the mixture was stirred at room temperature for 4.5 hours. 500 ml of toluene and 350 ml of water were added then extracted. The aqueous layer was extracted with 150 ml of toluene. The collected toluene layer contained 1,2,3,6,2',3'-hexa-O-acetyl-4',6'-O-*p*-methoxybenzylidenlactose not reacted, which can be used in another reaction. The aqueous layer, containing 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose, was extracted successively with 500 ml and 150 ml of methylene chloride; the organic extracts were washed with 3x150 ml of water, dried on anhydrous sodium sulphate and concentrated. The residue was crystallized from 580 ml of hot isopropyl acetate (50-55°C) obtaining, after drying, 40.6 g of 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose (0.07 mol, yield 50%) as white powdery solid whose characteristics are similar to the solid obtained from Example 5.

EXAMPLE 7

Preparation of (methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonate)-(2 $\rightarrow$ 6)-2,3-di-O-acetyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-1,2,3,6-tetra-O-acetyl- β -D-glucopyranose

- 100 g (0,168 mol) of 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose, obtained according to example 3, were dissolved in 600 ml of dichloromethane, then 250 g of molecular sieves 3A were added. The solution was stirred for 5-10 min and then 38.0 g of silver carbonate (0.14 mol) were added. A solution of 128.5 g (0,252 mol) (1.5 eq) of chlorine derivative of formula (II) wherein P is acetyl, X is chloro and R1 is methyl, in 500 ml of dichloromethane was added and the suspension was maintained in vigorous stirring at 30°C for 7 days until chlorine derivative disappeared (TLC, CH₂Cl₂: MeOH = 10:1), then the reaction mixture was filtered on dicalite and the solvent removed, obtaining a brittle solid residue (about 230 g), containing a mixture of condensation product, 1,2,3,6,2',3'-hexa-O-acetyl- β -D-lactose and product of 2.3 elimination of (II), as well as a trace of 4,7,8,9-tetra-O-acetyl-NANA. By ¹³C NMR a conversion of about 90% mol was evaluated. An analytical sample of the condensate product was obtained by crystallization from ethanol: isopropyl ether = 1:3 v/v as a white amorphous solid:
- ¹H NMR (CDCl₃, 300 MHz): δ ppm 5.66 (d, J_{1,2}= 8.4 Hz, 1H, H-1); 5.42-5.10 (m, 5H, H-3+H-2'+H-7''+H-8''+NH); 5.01 (pseudo t, J_{2,1}= 8.4 Hz, 1H, H-2); 4.94-4.78 (m, 2H, H-3'+H-4''); 4.53-4.27 (m, 3H, H-1'+H-6a+H-9''); 4.24-3.92 (m, 5H, H-6b+H-4'+H-5''+H-6''+H-9'b); 3.92-3.50 (m, 5H, H-4+H-5+H-5'+H-6'a+H-6'b); 3.80 (s, 3H, COOCH₃), 2.93 (broad s, 1H, OH); 2.55 (dd, J_{3''eq-3''ax}= 12.6 e J_{3''eq-4'}= 4.5 Hz, 1H, H-3''eq); 2.17-1.97 (31H, 10x CH₃CO e H-3''ax), 1.86 (s, 3H, NHCOCH₃).
- ¹³C NMR (CDCl₃, 75 MHz): δ ppm 171.05, 170.98, 170.5, 170.4, 170.29, 170.28, 170.24, 170.0, 169.6, 169.4, 169.0, 168.0 (OAc, NHAc, COOMe); 100.8 (C-1''); 99.1 (C-2''); 91.7 (C-1); 75.7, 73.7, 73.6, 72.94, 72.90, 72.4, 70.7, 69.7, 68.95, 68.87, 67.4, 66.3 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''); 62.6, 62.4, 62.1 (C-6, C-6', C-9''); 53.2 (OCH₃); 49.4 (C-5''); 37.4 (C-3''); 23.2 (NHCOCH₃), 21.1, 20.9, 20.8, 20.74, 20.67 (10xCH₃CO).

EXAMPLE 8

- Preparation of (methyl 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylate)-(2 $\rightarrow$ 6)- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-(α/β)-D-glucopyranose
- 230 g of the crude product, obtained according to example 7, were dissolved in 1.4 l of MeOH, then 29.6 ml of sodium methoxide in methanol 25% by weight was

added. The solution was kept stirring at room temperature for 12h; at positive TLC control (Pharmacopoeia) it was neutralized with 39 g of dry IR120 (H^+). The resin was filtered and the solvent removed by rotavapor, obtaining 143 g of residue, used in next reaction. The yield was quantitative.

- 5 ^1H NMR (D_2O , 300 MHz): δ ppm 5.21 (d, $J_{1,2}$ 3.6Hz, 1H, H-1 α), 4.66 (d, $J_{1,2}$ 7.8Hz, 1H, H-1 β), 4.42 (d, $J_{1,2}$ 7.5Hz, 1H, H-1'), 4.15-3.45 (m, 19H), 3.89 (s, 3H, $COOCH_3$), 3.29 (m, 1H), 2.70 (dd, $J_{3^{eq}3^{ax}}$ 12.9 e $J_{3^{eq}4}$ 4.8 Hz, H-3 ^{eq}), 2.03 (s, 3H, $NHCOCH_3$), 1.88 (pseudo t, $J_{3^{ax}3^{eq}} = J_{3^{ax}4}$ 12.9 Hz, H-3 ^{ax}).
- ^{13}C NMR (D_2O , 75 MHz): δ ppm 175.5 ($NHCOCH_3$), 170.4 ($COOMe$), 103.9 (C-1'),
- 10 99.6 (C-2"), 96.3 (C-1 β), 92.5 (C-1 α), 80.5, 80.4, 75.2 (2C), 74.4, 74.0, 73.5, 73.0, 72.2, 71.7, 71.3, 71.2, 70.5, 69.0 (2C), 67.9 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4", C-6", C-7", C-8"), 64.0 (C-6'), 63.8 (C-9"), 60.8 e 60.7 (C-6 α + β), 54.1 ($COOCH_3$), 52.3 (C-5"), 39.6 (C-3"), 22.8 ($NHCOCH_3$).

15 EXAMPLE 9

Preparation of sodium 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonate-(2 $\rightarrow$ 6)- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-(α/β)-D-glucopyranose

- 143 g of the crude deacetylationproduct, obtained according to example 8, were dissolved in 715 ml of water and the solution was cooled at 4°C. The pH was
- 20 brought to neutrality with aqueous NaOH and then 23 ml of NaOH 30% were added, maintaining the temperature of the solution below 10°C. The solution was stirred at room temperature for 24h. At positive TLC control (Pharmacopoeia) the solution was passed through IR120(H^+)/IRA96(OH^-). The eluate was adjusted to pH 9 with NaOH, concentrated to syrup, and submitted to stripping several times
- 25 with absolute EtOH until obtaining a brittle white solid that is re-crystallized from EtOH 96%. Obtained 77.3 gr.

^1H and ^{13}C NMR data for this compound were in agreement with those reported in the literature (L. Dorland et al., *Eur. J. Biochem.* 1978, 87, 323; J.P. Kamerling et al., *Carbohydr. Res.* 1982, 100, 331).

- 30 ^1H NMR (D_2O , 300 MHz): δ ppm 5.22 (d, $J_{1,2}$ 3.8Hz, 1H, H-1 α), 4.66 (d, $J_{1,2}$ 7.8Hz,

1H, H-1 β), 4.43 (d, J_{1-2} : 7.6Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, $J_{3^{eq}-3^{ax}}$ 12.5 e $J_{3^{eq}-4^{ax}}$ 4.7 Hz, H-3 ^{eq}), 2.03 (s, 3H, NHCOCH₃), 1.74 (pseudo t, $J_{3^{ax}-3^{eq}} = J_{3^{ax}-4^{ax}}$ 12.5 Hz, H-3 ^{ax}).

¹³C NMR (D₂O, 75 MHz, external reference acetone): δ ppm 175.6 (NHCOCH₃), 174.1 (COO⁻), 103.9 (C-1'), 100.9 (C-2'), 96.3 (C-1 β), 92.5 (C-1 α), 80.4, 80.3, 75.30, 75.26, 74.4, 74.3, 73.2, 73.0, 72.4, 72.3, 71.7, 71.4, 70.6, 69.2, 69.04, 69.01 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.2 (C-6'), 63.3 (C-9''), 60.9 e 60.8 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3''), 22.7 (NHCOCH₃).
[α]_D^{20°C}: +9,3° (c:1%, H₂O)

10 Mp: 191,4÷194,2 °C (T decomposition)

EXAMPLE 10

Preparation calcium 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylate-(2→6)- β -D-galactopyranosyl-(1→4)-(α / β)-D-glucopyranose

15

100 g of crude deacetylation, obtained according to example 8, were dissolved in 500 ml of water and the solution was cooled at 4°C. The pH was brought to neutrality with aqueous NaOH and then 23 ml of NaOH 30% were added, maintaining the temperature of the solution below 10°C. The resulting solution was kept stirring at room temperature for 24h. At positive TLC control (Pharmacopoeia) the solution was passed through IR120 (H⁺) / IRA96(OH⁻). The eluate was adjusted at pH 8.7 with Ca(OH)₂, filtered and then concentrated to syrup at 65 ° Brix. This syrup was dropped in 540 ml of methanol at 50°C. The resulting suspension was maintained in vigorous stirring at 50°C for 1 hour, then at room temperature for 1 hour and then filtered under vacuum. The solid was washed with 160 ml of methanol and dried under vacuum at 50÷55°C. Obtained: 46.2 g

20

25

¹H NMR (D₂O, 300 MHz): δ ppm 5.22 (d, J_{1-2} 3.8Hz, 1H, H-1 α), 4.66 (d, J_{1-2} 7.8Hz, 1H, H-1 β), 4.43 (d, J_{1-2} : 7.8Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, $J_{3^{eq}-3^{ax}}$ 12.0 e $J_{3^{eq}-4^{ax}}$ 4.5 Hz, H-3 ^{eq}), 2.03 (s, 3H, NHCOCH₃), 1.74 (pseudo t, $J_{3^{ax}-3^{eq}} = J_{3^{ax}-4^{ax}}$ 12.0 Hz, H-3 ^{ax}).

30

¹³C NMR (D₂O, 75 MHz, internal reference acetonitrile): δ ppm 175.5 (NHCOCH₃), 174.1 (COO⁻), 103.8 (C-1'), 100.9 (C-2'), 96.2 (C-1 β), 92.4 (C-1 α), 80.3, 80.3,

75.26, 75.22, 74.4, 74.3, 73.1, 73.0, 72.4, 72.2, 71.7, 71.4, 70.5, 69.1, 69.13, 69.00 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.2 (C-6'), 63.2 (C-9''), 60.9 e 60.7 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3''), 22.7 (NHCOCH₃).

Assay as Ca²⁺: 98,3%

5 $[\alpha]_D^{20^\circ}$: +10° (c:1%, H₂O)

Mp: 204,5÷206,6 °C (T decomposition)

IR ν^{KBr}_{max} : 3400, 1612, 1380, 1033 cm⁻¹.

EXAMPLE 11

10 Preparation of potassium 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylonate-(2→6)- β -D-galactopyranosyl-(1→4)-(α/β)-D-glucopyranose

100 g of the crude deacetylation product, obtained according to example 8, were dissolved in 500 ml of water and the solution was cooled at 4°C. The pH was brought to neutrality with aqueous NaOH and then 23 ml of NaOH 30% were added, maintaining the temperature of the solution below 10°C. The solution was stirred at room temperature for 24h. At positive TLC control (Pharmacopoeia) the solution was passed through IR120(H⁺)/IRA96(OH⁻). The eluate was adjusted to pH 10 with KOH, concentrated to syrup, and submitted to stripping several times with absolute EtOH until obtaining a white brittle solid that is re-crystallized from absolute EtOH. The solid was dried under vacuum at 50÷55°C.

Obtained 35.7 gr

¹H NMR (D₂O, 200 MHz): δ ppm 5.22 (d, J₁₋₂ 3.8Hz, 1H, H-1 α), 4.66 (d, J₁₋₂ 7.8Hz, 1H, H-1 β), 4.43 (d, J₁₋₂: 7.6Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, J_{3'eq-3'ax} 12.0 e J_{3'eq-4'} 4.4 Hz, H-3'eq), 2.03 (s, 3H, NHCOCH₃), 1.74 (pseudo t, J_{3'ax-3'eq} = J_{3'ax-4'} 12.0 Hz, H-3'ax).

¹³C NMR (D₂O, 75 MHz, internal reference acetone): δ ppm 175.5 (NHCOCH₃), 174.0 (COO⁻), 103.8 (C-1'), 100.9 (C-2''), 96.2 (C-1 β), 92.4 (C-1 α), 80.3, 80.2, 75.22, 75.19, 74.4, 74.3, 73.1, 72.9, 72.4, 72.2, 71.7, 71.4, 70.5, 69.1, 69.04, 69.01 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.1 (C-6'), 63.2 (C-9''), 60.8 e 60.7 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3''), 22.6 (NHCOCH₃).

$[\alpha]_D^{20^\circ}$: +9,8° (c: 1%, H₂O)

Mp: 179,3÷182,8°C (T decomposition)

IR $\nu_{\text{max}}^{\text{KBr}}$: 3391, 1612, 1379, 1034 cm^{-1} .

EXAMPLE 12

5 Preparation of magnesium 5-acetamido-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosylate-(2→6)- β -D-galactopyranosyl-(1→4)-(α/β)-D-glucopyranose

100 g of the crude deacetylation product, obtained according to example 8, were dissolved in 500 ml of water and the solution was cooled at 4°C. The pH was brought to neutrality with aqueous NaOH and then 23 ml of NaOH 30% were added, maintaining the temperature of the solution below 10°C. The solution was stirred at room temperature for 24h. At positive TLC control (Pharmacopoeia) the solution was passed through IR120(H⁺)/IRA96(OH⁻). The eluate was adjusted to pH 9.8 with MgO, concentrated to residue submitted to stripping several times with absolute EtOH until obtaining a white friable solid that is re-crystallized from absolute EtOH.

Obtained: 50.8 g

¹H NMR (D₂O, 300 MHz): δ ppm 5.22 (d, J₁₋₂ 3.6Hz, 1H, H-1 α), 4.66 (d, J₁₋₂ 8.1Hz, 1H, H-1 β), 4.43 (d, J_{1-2'} 7.5Hz, 1H, H-1'), 4.02-3.48 (m, 19H), 3.31 (m, 1H), 2.71 (dd, J_{3''eq-3''ax} 12.3 e J_{3''eq-4'} 4.5 Hz, H-3''eq), 2.03 (s, 3H, NHCOCH₃), 1.74 (pseudo t, J_{3''ax-3''eq} = J_{3''ax-4'} 12.3 Hz, H-3''ax).

¹³C NMR (D₂O, 75 MHz, internal reference acetonitrile): δ ppm 175.5 (NHCOCH₃), 174.1 (COO⁻), 103.8 (C-1'), 100.9 (C-2''), 96.3 (C-1 β), 92.4 (C-1 α), 80.3, 80.2, 75.26, 75.23, 74.4, 74.3, 73.1, 73.0, 72.4, 72.2, 71.7, 71.4, 70.6, 69.1, 69.00, 68.96 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 64.2 (C-6'), 63.3 (C-9''), 60.9 e 60.7 (C-6 α + β), 52.4 (C-5''), 40.7 (C-3''), 22.7 (NHCOCH₃).

Assay as Mg²⁺: 97,5%

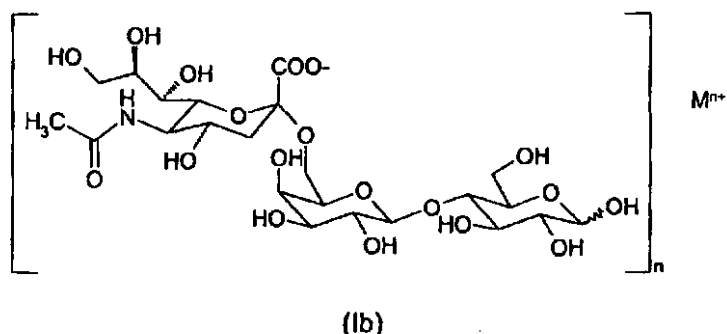
$[\alpha]_{\text{D}}^{20^\circ\text{C}}$: +9,8° (c: 1%, H₂O)

Mp: 183.1÷185.1°C (T decomposition)

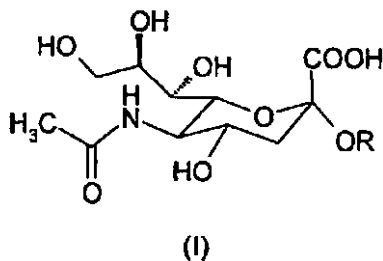
30 IR $\nu_{\text{max}}^{\text{KBr}}$: 3391, 1634, 1379, 1035 cm^{-1} .

CLAIMS

1. Compounds of formula (Ib)

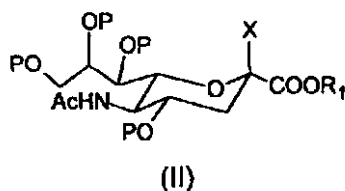


- 5 wherein M^{n+} is chosen in the group consisting of K^+ , Ca^{2+} , Mg^{2+} , Si^{2+} , Fe^{2+} and Al^{3+} .
2. Compounds according to claim 1 wherein M^{n+} is chosen in the group consisting of K^+ , Ca^{2+} , Mg^{2+} .
3. A process the preparation of compounds of formula (Ib) as defined in any of claims 1-2 starting from 6'-sialyllactose (6'SL), said process comprising
- 10 adding to a solution of 6'SL a base containing $M^{(n+)}$ to obtain a pH value of 8-10.
4. Process for the preparation of α -sialyl-oligosaccharides compounds of formula (I)



wherein R is a mono-, di- or oligosaccharide residue with the hydroxy groups free; said process comprising at least a step of:

- a) coupling by mean of Koenigs-Knorr reaction of a sialyc donor of formula (II)



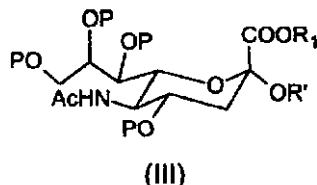
wherein P is a suitable protecting group; R₁ is alkyl, and X is halogen;

with an acceptor of formula R'OH wherein R' is a residue mono-, di- or oligosaccharide suitably protected with protecting groups P' and containing zero, one or more free hydroxy groups; wherein said protecting groups P'

5 can be the same or different among each others and from those present on the donor;

said reaction of Koenigs-Knorr characterised by the fact that the Ag(I) based metallic promoter is employed in molar amounts 0.5 - 2.0 eq respect with the moles of acceptor;

10 to obtain an intermediate of formula (III)



wherein P, R₁ and R' are as defined above.

5. Process according to claim 4 further comprising, after step (a), the following step:

15 b) removing protecting groups P, P' and R₁ to obtain compounds of formula (I) as defined in claim 4.

6. Process for the preparation of 6'SL according to any of claims 4-5 wherein R is 6'-lactose.

20 7. A process the preparation of compounds of formula (Ib) wherein M^{nt} is Na⁺, K⁺, NH₄⁺, Ca²⁺, Mg²⁺, Sr²⁺, Fe²⁺ o Al³⁺ said process comprising the preparation of 6'SL by mean of the process according to claim 6.

8. Compounds of formula (Ib) according to any of claims 1-2 for use as medicaments and food integrators.

25 9. Pharmaceutical and foodstuff compositions containing compounds of formula (Ib) according to any of claims 1-2 and at least an other ingredient pharmaceutically or alimentary acceptable.

Simple stereoselective synthesis of α 2-6 sialooligosaccharides

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Received 4 September 2002; accepted 13 September 2002

Abstract—The sialylation approach reported by A. Khorlin et al. 30 years ago has been modified into a highly practical procedure for stereoselective α 2-6 sialylation of galactopyranose 4,6-diols. © 2002 Elsevier Science Ltd. All rights reserved.

Glycoprotein and glycolipid carbohydrate chains terminated by sialic acids play an exceptional role in cell recognition and cross-talk, cell-pathogen interactions, immune response and in other cellular events. For example, they might act as 'self' ligands that interact with siglecs on myeloid cells, thereby preventing inappropriate self-reactivity.¹ An approach to the systematic understanding of structure-function relationships of sialooligosaccharides requires efficient synthetic methods for regio- and stereoselective sialylation. However, the presence of an electron-withdrawing carboxy group as well as the lack of a co-participating substituent adjacent to the anomeric centre of the sialic acid, often results in low stereoselectivity of sialylation (especially when a primary hydroxyl group is used as the glycosyl acceptor),² and in a high rate of concurrent 2,3-elimination in the sialyl donor.

The first synthesis of oligosaccharides containing the α -N-acetylneuraminic (Neu5Ac) residue was described by Khorlin et al. about three decades ago. In the cited work, chloride **1** was used as the glycosyl donor, silver carbonate was chosen as the promoter-catalyst; the yields of α 2-6-sialodisaccharides reached 18%.³ Later, the search for effective sialylation methods was focused on the modification of the promoter-catalyst, the glycosyl donor, and the reaction conditions. So, the donor **1** was used with silver silicate, silver salicylate and silver carbonate/triflate; thioglycoside donors **2** in combination with various electrophilic promoters were also studied.⁴ An additional group (X = Cl, Br or SeR, see 2, Fig. 1) was introduced at C-3 for co-participation in glycosylation, which gave an improvement of the yield and selectivity, but such groups had to be removed

after the reaction.⁵ Extra acylation of the 5-N-acetyl group or its substitution by N₃ (see 3, Fig. 1) increased the efficiency of glycosylation.^{2,6,7} A significant rise in α -stereoselectivity was observed for donor **4** with the acetoacetoxy groups at C-4 and C-9.⁷ Although these studies improved the yield and/or α -stereoselectivity of sialylation, these advantages were nullified by the multi-step synthesis of the complicated Neu5Ac-donors and by the need for elimination of additional substituents.

We reinvestigated the synthesis of α 2-6-sialooligosaccharides under the classical Koenigs-Knorr conditions used by Khorlin et al., e.g. Ag₂CO₃-promoted glycosylation with the readily prepared donor **1**. In contrast to the cited work, where acceptors with one free hydroxyl group at C-6 were used, we glycosylated less hindered 4,6-diols, namely, the mono- and disaccharide acceptors **5–9** with two free hydroxyl groups (Scheme 1). Monosaccharide **10** with three unprotected hydroxyls was also sialylated (Scheme 2).⁸ A typical procedure for

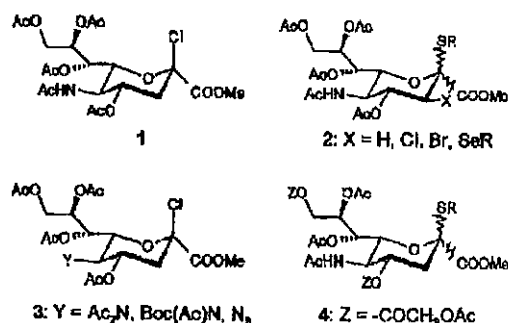


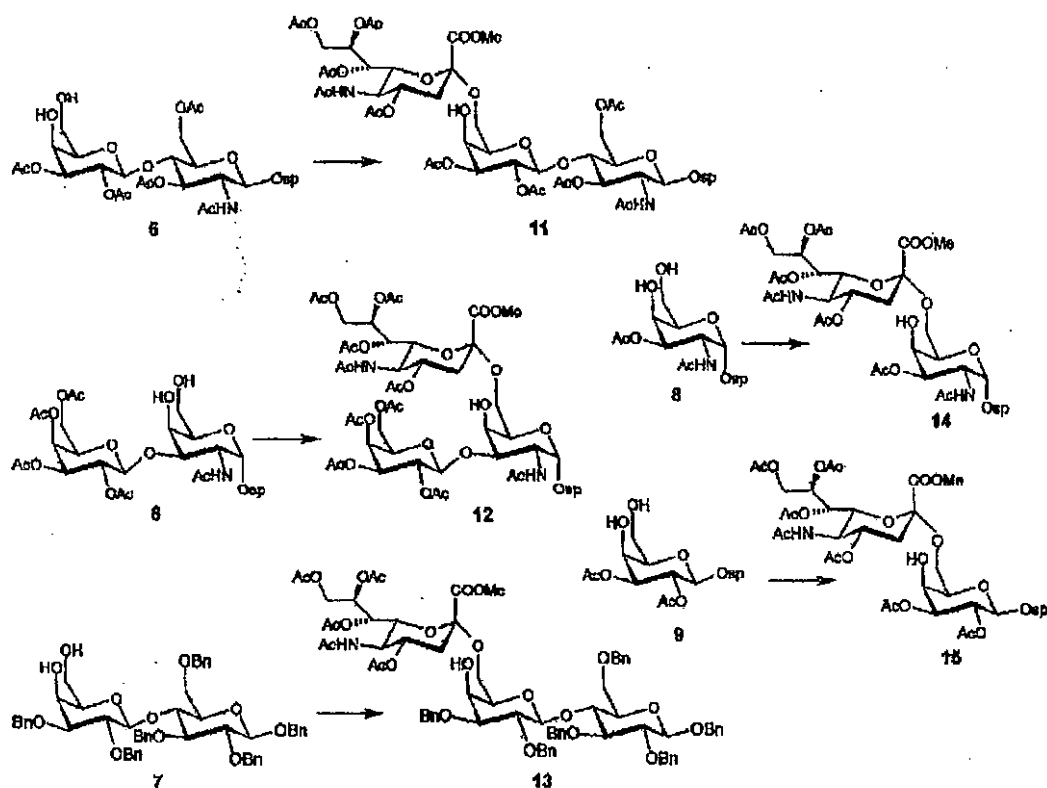
Figure 1. Sialyl donors.^{2–7}

[†] Syntheses of acceptors **5–10** were described earlier;^{7,11,12} synthesis of the glycosyl chloride **1** was performed according to the simple one-pot procedure.¹³

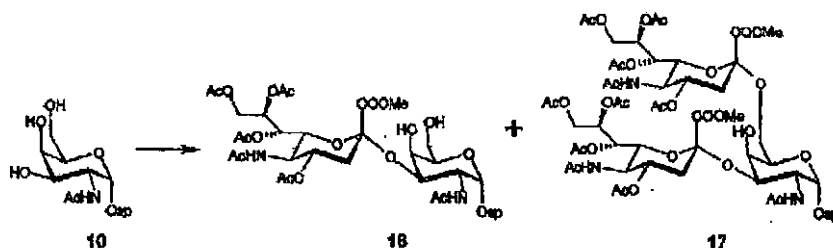
Keywords: oligosaccharides; sialic acids; sialylation.

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PII: S0040-4039(02)01983-4



Scheme 1. Sialylation of mono- and disaccharide diols with 1 under the Koenigs-Knorr conditions, $sp = (CH_2)_3NHCOCF_3$.



Scheme 2. Unusual sialylation of triol 10 with 1 under the Koenigs-Knorr conditions, $sp = (CH_2)_3NHCOCF_3$.

sialylation was as follows. To a mixture of acceptor 5–10 (0.1 mmol), Ag_2CO_3 (0.7 mmol), and molecular sieves 4 Å (1 g) in CH_2Cl_2 or $ClCH_2CH_2Cl$ (5 mL) was

added a solution of the glycosyl chloride 1 (0.3 mmol) in the same solvent (5 mL) (for details see Table 1). The reaction mixture was stirred at room temperature with

Table 1. Sialylation of saccharides under the Koenigs-Knorr conditions (Schemes 1–2)

Glycosyl acceptor	Product	Solvent	Time (days)	Yield of the α -anomer (%) ^a	α/β anomer ratio	Recovery of the acceptor (%)
5	11	CH_2Cl_2	3	95	β in trace	10
6	12	CH_2Cl_2	7	84	6/1	50
7	13	CH_2Cl_2	7	90	β in trace	25
8	14	$ClCH_2CH_2Cl$	5	88	2.5/1	20
9	15	CH_2Cl_2	3	82	β in trace	10
10	16	$ClCH_2CH_2Cl$	7	48	β in trace	10
	17			41		

^a Yields of α -sialooligosaccharides 11–17 are given based on the glycosyl acceptor reacted.

TLC monitoring, then filtered, the solids were washed with $\text{CHCl}_3/\text{MeOH}$ 10/1, and the combined filtrate was concentrated in vacuo. The residue was subjected to chromatography on silica gel. The ^1H NMR spectra of the sialooligosaccharides 11, 12, 14 obtained are identical to those described earlier.^{8–10} The structures of 13, 15, 16 and 17 were confirmed by ^1H NMR; the spectra are available as supplementary material.

Glycosylation of acceptors 5–9 with an excess of donor 1 in the presence of silver carbonate in CH_2Cl_2 or $\text{ClCH}_2\text{CH}_2\text{Cl}$ gave rise to α 2–6 sialooligosaccharides in 85–95% yields. A significant prevalence of the α -anomer was observed; in some cases the β -anomer was detected in only trace amounts. The results obtained are summarised in Table 1.

The stereoselectivity of sialylation and the degree of acceptor conversion depend on the nature of the solvent (CH_2Cl_2 or $\text{ClCH}_2\text{CH}_2\text{Cl}$), which should be chosen so as to provide acceptor solubility. The combination of silver carbonate with silver triflate as a co-promoter accelerates the sialylation, but, at the same time, increases the yields of the β -anomer and the glycal.

The results of sialylation of triol 10 appeared to be rather unexpected (Scheme 2). Earlier, upon glycosylation of this compound by the glycosyl bromides of Gal, GlcNAc, and Gal β 1–4GlcNAc,¹¹ the conventional reactivity of the galactopyranose hydroxyl groups was observed, i.e. the substitution occurred at the primary 6-OH group. However, glycosylation with donor 1 resulted in two products, 3-O-sialylated 16 and 3,6-di-O-sialylated 17, while none of the expected 6-O-sialylated product was isolated. The observed elevated reactivity of the 3-OH group, might be explained by participation of the acetamido group at C-2 of the galactose moiety. Attempts to sialylate the galactose derivatives with a OAc or OH group at C-2 instead of NHAc did not give rise to the 3-O-sialoside. The six saccharides tested as glycosyl acceptors, either with one hydroxyl group at C-3, or with two OH groups at C-2 and C-3, or C-3 and C-4, or with three at C-2, C-3 and C-4 of the galactose yielded no 3-O-sialylated product. Thus, except in one case, the silver carbonate-promoted sialylation of saccharides with the donor 1 occurs at the primary 6-OH group.

A comparison of the results presented here with the data on α 2–6 sialylation^{2–10} with more complex glycosyl donors demonstrates that, being more convenient, Koenigs–Knorr glycosylation is also rather effective with respect to the yield and stereoselectivity. Another advantage is that Ag_2CO_3 -promoted sialylation does

not destroy the acetamido group in the glycosyl acceptor as documented for glycosylation with Neu5Ac thioglycosides.¹² As to generality of this method, it should be mentioned that the synthesis of an oligosaccharide containing *N*-glycolylneuraminic acid has been successfully performed.⁴ The only drawback of the heterogeneous reaction described is that in some cases a part of a glycosyl acceptor remains unreacted; however, the unreacted substance is recovered quantitatively.

Being highly practical, the approach presented gives hope that the synthesis of 6-sialooligosaccharides indispensable for glycobiological investigations, will become routine.

Acknowledgements

The work was supported by RFBR grant #01-04-49300 and NIH grant #1U54GM62116.

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[†] Glycosylation of 8 with *N*-glycolyl analog of chloride 1 gave rise to 76% yield of α -disaccharide, α/β =6:1.



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21/10/2009

RAPPORTO DI RICERCA

Numero della domanda
IO 5003
IT FI20090071

DOCUMENTI CONSIDERATI DI RILIEVO			
Categoria	Citazione del documento con indicazione, se appropriata, delle parti rilevanti	Rivendicazioni rilevanti	CLASSIFICAZIONE DELLA DOMANDA (IPC)
A	RENCUROSÌ A ET AL: "Human milk oligosaccharides: an enzymatic protection step simplifies the synthesis of 3'- and 6'-O-sialyllactose and their analogues" CARBOHYDRATE RESEARCH, ELSEVIER SCIENTIFIC PUBLISHING COMPANY, AMSTERDAM, NL, vol. 337, no. 6, 15 March 2002 (2002-03-15), pages 473-483, XP004343543 ISSN: 0008-6215 -----	1-3,8,9	INV. C07H13/06 A61K31/702 A23L1/09
A	MULLER H E ET AL: "Occurrence and some properties of neuraminidases in Haemophilus avium and Haemophilus paragallinarum" VETERINARY MICROBIOLOGY, ELSEVIER BV, NL, vol. 2, no. 4, 1 December 1977 (1977-12-01), pages 303-312, XP023915296 ISSN: 0378-1135 [retrieved on 1977-12-01] -----	1-3,8,9	
Y	WO 2007/090894 A (NESTEC SA [CH]; SPRENGER NORBERT [CH]; MORGAN FRANCOIS [FR]; BERROCAL) 16 August 2007 (2007-08-16) * claims 1,9 *	1-3,8,9	CAMPI TECNICI RICERCATI (IPC) C07H A23L A61K
Y	WO 92/11017 A (MONSANTO CO [US]) 9 July 1992 (1992-07-09) * claim 4; table I *	1-3,8,9	
Y	WO 94/03184 A (NEOSE PHARM INC [US]) 17 February 1994 (1994-02-17) * page 11; claim 1 *	1-3,8,9	
Questo rapporto di ricerca è stato redatto sulla base di tutte le rivendicazioni			
Munich		Data di completamento della ricerca 16 October 2009	Esaminatore Bardili, Burkhart
CATEGORIA DEI DOCUMENTI CITATI			
X : di particolare rilevanza se considerato singolarmente Y : di particolare rilevanza se combinato con un altro documento della stessa categoria A : informazione generica O : divulgazione orale P : documento intermedio T : teoria o principio alla base dell'invenzione E : documento brevettuale antecedente, ma pubblicato dopo o alla data di deposito D : documento citato nella domanda L : documento citato per altre ragioni & : membro della stessa famiglia di brevetti, documento corrispondente			

2
EPO FORM 1503 07.08 (P04C74)

ALLEGATO AL RAPPORTO DI RICERCA
SULLA DOMANDA DI BREVETTO ITALIANO N. IO 5003
IT FI20090071

Questo allegato enumera i membri della famiglia di brevetti relativi a documenti brevettuali citati nel summenzionato rapporto di ricerca.
I membri sono indicati come da database dell'Ufficio Europeo dei Brevetti al
L'Ufficio Europeo dei Brevetti non si assume alcuna responsabilit  per queste indicazioni, che vengono fornite a solo scopo informativo.

16-10-2009

Documenti brevettuali citati nel rapporto di ricerca	Data di pubblicazione	Membri della famiglia di brevetti	Data di pubblicazione
WO 2007090894 A	16-08-2007	AU 2007213677 A1	16-08-2007
		CA 2641842 A1	16-08-2007
		CN 101400267 A	01-04-2009
		EP 1988786 A1	12-11-2008
		US 2009092729 A1	09-04-2009
WO 9211017 A	09-07-1992	US 5164374 A	17-11-1992
WO 9403184 A	17-02-1994	AU 4993893 A	03-03-1994

EPO FORM P04A3

Per ulteriori dettagli riguardanti questo allegato, si veda la Gazzetta Ufficiale (OJ) dell'Ufficio Europeo dei Brevetti, N. 12/82



Ministero dello Sviluppo
Economico

DIREZIONE GENERALE SVILUPPO PRODUTTIVO E COMPETITIVITA' -
UFFICIO ITALIANO BREVETTI E MARCHI

OPINIONE SCRITTA

N. dossier IO5003	Data di deposito (gg/mm/aa) 06.04.2009	Data di priorit� (gg/mm/aa)	N. domanda ITFI20090071
Classificazione Internazionale dei Brevetti (IPC) INV. C07H13/06 A61K31/702 A23L1/09			
Richiedente INALCO S.P.A.			

Questa opinione fornisce indicazioni riguardanti i seguenti elementi:

- ☒ Riquadro N. I Base dell'opinione
- ☐ Riquadro N. II Priorit 
- ☒ Riquadro N. III Non-redazione di un'opinione a riguardo di novit , attivit  inventiva e applicazione industriale
- ☒ Riquadro N. IV Violazione del requisito d'unit  dell'invenzione
- ☒ Riquadro N. V Dichiarazione motivata a riguardo di novit , attivit  inventiva o applicazione industriale; citazioni e spiegazioni giustificative della dichiarazione
- ☐ Riquadro N. VI Particolari documenti citati
- ☐ Riquadro N. VII Difetti particolari nella domanda
- ☒ Riquadro N. VIII Osservazioni particolari a riguardo della domanda

	Esaminatore Bardili, Burkhart
--	----------------------------------

OPINIONE SCRITTA

N. domanda

ITFI20090071

Riquadro N. 1 Base dell'opinione

1. Questa opinione è stata redatta sulla base delle ultime rivendicazioni depositate prima dell'inizio della ricerca nella tecnica anteriore.
2. Per quanto concerne eventuali sequenze di nucleotidi e/o amminoacidi descritte nella domanda e necessarie per l'invenzione di cui oggetto nelle rivendicazioni, questa opinione è stata redatta sulla base di:
 - a. tipo di materiale:
 - ☐ una sequenza di DNA
 - ☐ una o più tabelle relative alla sequenza di DNA
 - b. formato del materiale:
 - ☐ cartaceo
 - ☐ elettronico
 - c. momento di deposito o presentazione:
 - ☐ depositato insieme alla domanda al momento del deposito della medesima
 - ☐ depositato insieme alla domanda in formato elettronico
 - ☐ presentato successivamente al fine della ricerca d'anteriorità
3. ☐ Inoltre, ove sia stata depositata o presentata più di una versione o copia di una sequenza di DNA e/o tabella ad essa relativa, è stata presentata anche la dichiarazione obbligatoria che le informazioni contenute nelle copie successive o addizionali sono identiche a quelle nella domanda come depositata o che, in ogni caso, non vanno oltre il contenuto della domanda depositata originariamente.
4. Note aggiuntive:

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Riquadro N. III Non-redazione di un'opinione a riguardo di novità, attività inventiva e applicazione Industriale

L'analisi concernente la novità, l'attività inventiva e l'applicazione industriale dell'invenzione rivendicata non è stata condotta per quanto riguarda

- ☐ la domanda in toto
☒ rivendicazioni N. 4-7

poiché:

- ☐ la suddetta domanda, o le suddette rivendicazioni N. hanno il seguente oggetto, per il quale non si necessita di fare una ricerca d'antiorità (*specificare*):
- ☐ la descrizione, le rivendicazioni o i disegni (*specificarne i dettagli di seguito*) o le suddette rivendicazioni N. , mancano a tal punto di chiarezza che non è possibile redigere un'opinione significativa (*specificare*):
- ☐ le rivendicazioni, o suddette rivendicazioni N. sono insufficientemente fondate sulla descrizione a tal punto che risulta impossibile redigere un'opinione significativa (*specificare*):
- ☒ un rapporto di ricerca non è stato redatto sulla domanda in toto o sulle suddette rivendicazioni N. 4-7
- ☐ non è risultato possibile redigere un'opinione significativa in quanto la sequenza di DNA non era presente, oppure non è stata presentata nel formato internazionale (WIPO ST.25).
- ☐ non è risultato possibile redigere un'opinione significativa senza le tabelle relative alle sequenze di DNA; oppure tali tabelle non era presenti nel formato elettronico.
- ☐ Si veda il Riquadro Supplementare per ulteriori dettagli.

Riquadro N. IV Violazione del requisito d'unità dell'invenzione

1. Il requisito di unità dell'invenzione non è soddisfatto per le seguenti ragioni:

si veda l'allegato

2. Questo rapporto è stato redatto per le seguenti parti della domanda:

- ☐ tutte le parti
☒ le parti riguardanti le rivendicazioni N. (si veda il Rapporto di Ricerca)

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N. domanda
ITFI20090071

Riquadro N. V Dichiarazione motivata a riguardo di novità, attività inventiva o applicazione industriale; citazioni e spiegazioni giustificative della dichiarazione

1. 1. Dichiarazione

Novità (N)	Si:	Rivendicazioni	1-3,8,9
	No:	Rivendicazioni	
Attività inventiva (IS)	Si:	Rivendicazioni	
	No:	Rivendicazioni	1-3,8,9
Applicazione industriale (IA)	Si:	Rivendicazioni	1-3,8,9
	No:	Rivendicazioni	

2. 2. Citazioni e spiegazioni

si veda l'allegato

Riquadro N. VIII Osservazioni particolari a riguardo della domanda

si veda l'allegato

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The subject-matter of claims 4-7 has not been searched and is not considered in this Written Opinion.

Re Item IV

Lack of unity of invention

The application comprises two independent inventions:

Invention (1) as defined in claims 1-3, 8, 9 relates to new salts of 6'-sialyl lactose and compositions comprising these salts. Invention (2) as defined in the remaining claims relates to a process to prepare a group of sialyl oligosaccharide acids of formula (I) and new and known 6'-sialyl lactose salts as defined in claim 7 involving a Koenigs-Knorr reaction of a sialyl donor (II) with a suitable saccharide acceptor.

It is acknowledged in the description that sodium and ammonium salts of 6'-sialyl lactose are known. Consequently, as explained at page 3 of the description the technical problem to be solved by the subject-matter of invention (1) is the provision of new formulations of 6'-sialyl lactose. The solution of the problem consists in the salts recited in claim 1. Invention (2) relates to a different problem which may be seen in the provision of a new method to synthesis sialyl oligosaccharides and the use of this method in the preparation of a group of 6'-sialyl lactose salts which is different compared with the group recited in claim 1 and includes the known ammonium and sodium salt of 6'-sialyl lactose. Hence, there is no relationship between these inventions involving a common or corresponding special technical feature making a contribution over the prior art. Thus, a single general inventive concept is not present.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The sodium and ammonium salts of 6'-sialyl lactose are described in D1/ Carbohydr. Res. 337, 473 (2002) and D2/ Veterinary Microbiology 2, 303 (1978), respectively. D3/ WO 2007/090894 and D4/WO 92/11017 relate to nutritional and pharmaceutical formulations comprising 6'-sialyl lactose. The salts recited in claim 1 are not disclosed

**OPINIONE SCRITTA
(RIQUADRO SUPPLEMENTARE)**

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ITFI20090071

in the above-mentioned documents. Thus, the subject-matter of claims 1-3 and 8 and 9 is novel.

The technical problem to be solved by the subject-matter of invention (1) vis-à-vis D3 (or D4) is the provision of formulations of 6'-sialyl lactose suitable for a pharmaceutical or nutritional use. D5 discloses oligosaccharides comprising a sialyl lactose moiety and their pharmaceutically acceptable salts. These salts include the potassium, calcium and magnesium salt. Thus, claims 1-3 and 8-9 lack an inventive step.

Re Item VIII

Certain observations on the international application

The integer "n" in claim is not defined.

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CPR TEN

Human milk oligosaccharides: an enzymatic protection step simplifies the synthesis of 3'- and 6'-O-sialyllactose and their analogues

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Received 6 November 2001; accepted 7 January 2002

Abstract

We describe a chemo-enzymatic synthesis of 3'- and 6'-O-sialyllactose, two trisaccharides occurring in the 'acidic fraction' of the human milk oligosaccharides and endowed with potential antiadhesive activity. The key step is the highly regioselective 6'-O-acylation of benzylactoside, which gave access to suitably protected lactose building blocks to be used as acceptors in the sialylation reaction. Moreover, the synthesis of the carboxymethyl and sulfo analogues of the title compounds is reported. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Human milk oligosaccharides; Enzymatic 6'-O-acylation; Benzylactoside

1. Introduction

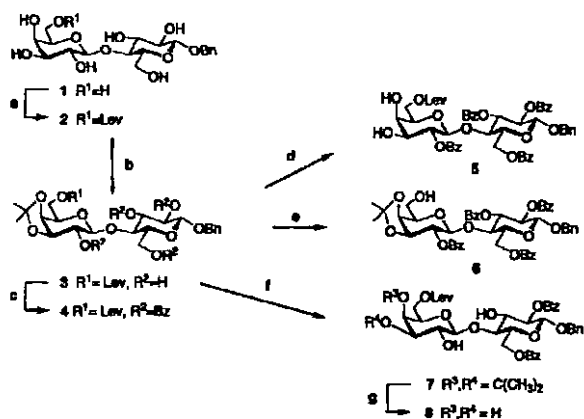
The oligosaccharide fraction is the third most abundant solid constituent (between 12 and 20 g/L) of human milk and consists of more than one hundred different structures. Despite many investigations^{1–3} that have been reported on the subject, the physiological role of these compounds is not completely understood. The fact that human milk oligosaccharides bear structural homology to cell-surface glycoconjugates used as receptors by pathogens may suggest their implication in protecting breast-fed infants from infections. Concerning this issue, evidence has emerged that these oligosaccharides are able to inhibit the adhesion of *Streptococcus pneumoniae* and *Haemophilus influenzae* to human pharyngeal or buccal epithelial cells.² Moreover, the fucosylated fraction of small human milk oligosaccharides inhibits the *Escherichia coli* adhesion to uroepithelial cells.³

In this study, we turned our attention to sialylated human milk oligosaccharides. It has been recently demonstrated⁴ that whey glycoproteins, containing *N*-acetylneuraminic acid, exert a potential inhibitory effect on S-fimbriae mediated adhesion of *E. coli*.

In order to assess the antiadhesive properties of selected oligosaccharides present in human milk, we describe herein the synthesis of 3'- and 6'-O-sialyllactoses and their corresponding simplified analogues where the sialic acid unit is replaced by a negatively charged group such as a sulfate and a carboxymethyl group. Sulfate groups may serve as an effective substituent for sialic acid⁵ in some biological systems and 3'-O-sulfo-lactose has recently been found as an oligosaccharide present in dog milk.⁶ Moreover the carboxymethyl group in analogues of sialyl Lewis X was shown to be useful for mimicking the negative charge of sialic acid.⁷ In previous papers, we reported on the use of lipase catalysed acylation as a tool to prepare useful building blocks for oligosaccharide synthesis.^{8–11} In the present paper, the enzymatically catalysed 6'-O-acylation of lactose has been exploited for designing a versatile chemo-enzymatically protected lactose building block useful for the synthesis of both 3'- and 6'-O-sialyllactoses.

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Scheme 1. Reagents and conditions: (a) trifluoroethyl levulinate, *C. antarctica* lipase, THF, 40 °C, 4 days, 83%; (b) acetone, CSA, Sikkon, refl, 4 h, 78%; (c) BzCl, Py, DMAP, CH₂Cl₂, 0 °C → rt, 48 h, 80%; (d) TFA (60%), CH₂Cl₂, 0 °C → rt, 1 h, 85%; (e) AcONH₂NH₃, EtOH–Et₂O, rt, 20 min, 97%; (f) BzCN, TEA, THF, –25 °C, 17 h, 50%; (g) TFA (60%), CH₂Cl₂, 0 °C → rt, 1.5 h, 92%.

2. Results and discussion

The selective protection of the 6'-OH position of benzyl lactoside 1¹² with a levulinoyl group was achieved in high yield through an enzymatic acylation¹¹ catalysed by *Candida antarctica* lipase in THF (Scheme 1). The subsequent protection of 3',4'-positions of 2 as isopropylidene acetal afforded compound 3. Full benzylation of 3 gave the versatile building block 4 useful for the synthesis of the target compounds. In fact, orthogonal deprotection of isopropylidene or levulinoyl groups afforded acceptors 5 and 6, respectively (Scheme 1), which were separately submitted to sialylation using sialyl phosphite 9¹³ as a donor (Scheme 2).

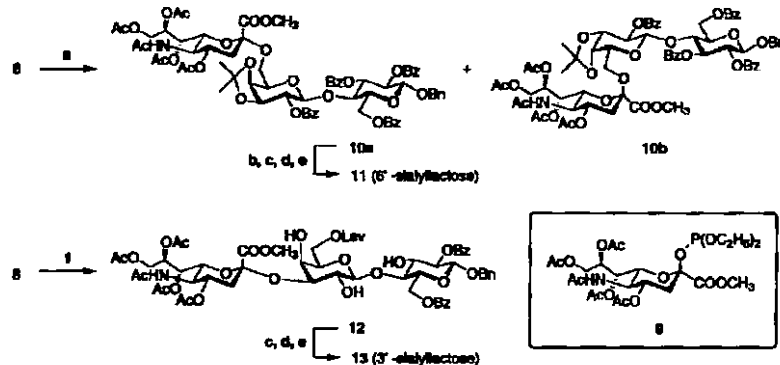
Glycosylation of acceptor 6 in acetonitrile at –40 °C with trimethylsilyltrifluoromethanesulfonate (TMSOTf) as a promoter, afforded the 6'-O-sialyllactoside 10a/b

as a 2:1 α/β mixture in 58% overall yield (Scheme 2). Even though selectivity was not high, the two isomers were completely separated by MP chromatography. Anomeric configuration of the newly formed glycosidic linkage for 10a and 10b was determined by comparison of NMR data of the two compounds. In accordance with the known ¹H NMR empirical rules,^{14–16} δ H-3''eq (α) = 2.73 > δ H-3''eq (β) = 2.61, δ H-4'' (α) = 4.52 < δ H-4'' (β) = 5.49, $J_{7'',8''}$ (α) = 7.9 Hz > $J_{7'',8''}$ (β) = 3.1 Hz.

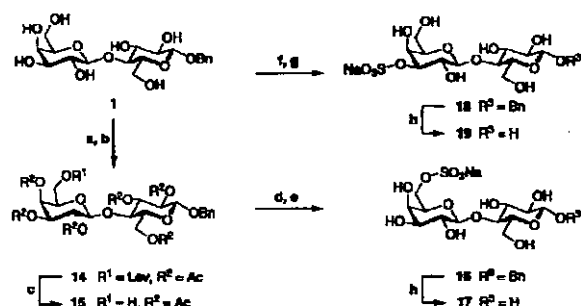
Disappointingly, sialylation of acceptor 5 resulted in a complex mixture of products from which most of the acceptor was recovered unreacted and the supposed 3'-O-sialyllactose derivative was isolated in a very poor yield. These poor results might be due to the steric hindrance and to the presence of electron withdrawing groups close to the reactive centre. Therefore, a new and more reactive acceptor 8 was synthesised through selective benzylation¹⁷ of 3 followed by acidic hydrolysis of the isopropylidene acetal (Scheme 1).

Sialylation of this new acceptor in acetonitrile–THF at –44 °C in the presence of TMSOTf as a promoter, afforded the 3'-O-sialyllactoside 12 in 51% yield (Scheme 2). This result is in line with that reported by Lönn et al.,¹⁸ who used a very similar acceptor employing a glycosyl xanthate as the donor. The (2''→3') glycosylation regiochemistry was ascertained from the observation of COSY cross-peaks between OH-2' (δ 3.17) and H-2' (δ 3.75) and OH-4' (δ 2.38) and H-4' (δ 3.63). Moreover, the HMBC¹⁹ experiment showed the presence of the expected cross-peak between H-3' (δ 4.09) and the quaternary carbon C-2'' (δ 97.6). The α configuration of the newly formed glycosidic linkage was determined on the basis of the occurrence of the Neu5Ac H-4 signal at δ 4.97, as well as the $J_{7'',8''}$ = 8.4 Hz; these data are in accordance with the reported trend for similar compounds.^{18,20}

The synthesis of 6'-O- and 3'-O-sulfated analogues of the corresponding sialyllactoses was based on two different strategies. Lactoside 15 was obtained in three



Scheme 2. Reagents and conditions: (a) 9 (1.7 equiv), TMSOTf, CH₃CN, –40 °C, 1 h, 58%, α/β 2:1; (b) TFA (60%), CH₂Cl₂; (c) MeOH, MeONa; (d) NaOH, water; (e) H₂, Pd/C, H₂O; (f) 9 (2 equiv), TMSOTf, CH₃CN–THF, –44 °C, 2 h, 51%.



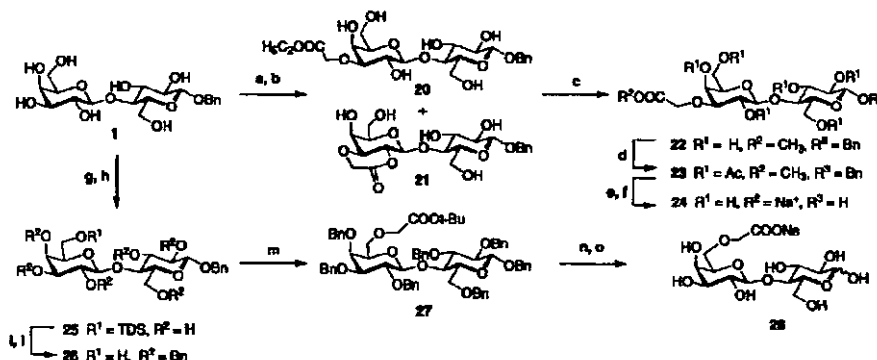
Scheme 3. Reagents and conditions: (a) trifluoroethyl levulinate, *C. antarctica* lipase, THF, 40 °C, 4 days, 83%; (b) Ac₂O, Py, 80%; (c) AcONH₂NH₃, EtOH–Et₂O, rt, 2 h, 98%; (d) SO₃NMe₃, 1.5 equiv, DMF, 60 °C, 2 days; (e) MeONa, MeOH, rt, 81%; (f) Bu₂SnO (1.1 equiv), MeOH, refl, 16 h; (g) SO₃NMe₃ (2 equiv), DMF, rt, 2 days, 75%; (h) H₂, Pd/C, MeOH–water, rt.

steps from benzyl lactoside **1** through enzymatic 6'-*O*-levulinoylation, followed by acetylation (→ **14**) and removal of the levulinoyl group with hydrazinium acetate (Scheme 3). The sulfation of **15** (SO₃NMe₃ complex), followed by deacetylation, provided the 6'-*O*-sulfated lactoside **16**. The 3'-*O*-sulfated lactoside **18** was obtained in a one pot procedure,²¹ by treatment of benzyl lactoside **1** with Bu₂SnO and SO₃NMe₃ complex (Scheme 3).

The same approach was applied to the synthesis of 3'-*O*-(alkoxycarbonyl) methyl lactoside (Scheme 4). In this case, the alkylation of the stannylene acetal obtained from benzyl lactoside **1** produced compound **20** and the corresponding lactone **21**. This mixture was treated without separation with sodium methoxide (→ **22**), followed by acetylation to afford pure compound **23** in good yield. The acetylation step was required in order to better purify the compound. The introduction

of the (alkoxycarbonyl) methyl group at 6'-position was first attempted by treatment of **15** with ethyl bromoacetate in the presence of Ag₂O and TBAI. Disappointingly, a complex mixture of products was obtained, probably derived from acyl migration and/or hydrolysis. Much better results were achieved on the benzylated compound **26**,²² which was synthesised in three steps from benzyl lactoside **1** through regioselective 6'-*O*-silylation,²³ which afforded compound **25**, followed by full benzylation and final 6'-*O*-desilylation. Thus, alkylation of **26** with *tert*-butyl-bromoacetate²⁴ afforded compound **27** in almost quantitative yield (Scheme 4).

Finally, compounds **10a**, **12**, **16**, **18**, **23** and **27** were conventionally deprotected to afford the title compounds **11**, **13**, **17**, **19**, **24**, and **28**, respectively. Full deprotection of compound **10a** was achieved by acidic hydrolysis of the isopropylidene group, followed by Zemplén deacylation, saponification of the methylester with NaOH, and hydrogenolysis of the anomeric benzyl group, affording the 6'-*O*-sialyllactose **11** (Scheme 2). NMR data for **11** are in agreement with those previously reported for the natural compound.²⁵ Compound **12** was converted into the known compound **13** by Zemplén deacylation, saponification with NaOH, and hydrogenolysis of the anomeric benzyl ether. NMR data for **13** are consistent with those reported for the natural compound²⁵ and by Ogawa.²⁶ Sulfated lactose derivatives **17** and **19** were quantitatively obtained from **16** and **18** by hydrogenolysis of the anomeric benzyl ether. Zemplén deacylation of **23**, followed by one-pot saponification with NaOH and hydrogenolysis, afforded compound **24**, whereas **28** was obtained from **27** by acidic hydrolysis of the *tert*-butyl ester followed by hydrogenolysis of the remaining benzyl groups. Final desalting of the deprotected compounds was performed by gel permeation chromatography through a Sephadex G10 column, followed by freeze-drying.



Scheme 4. Reagents and conditions: (a) Bu₂SnO 1.1 equiv, MeOH, refl, 16 h; (b) BrCH₂COOEt 5 equiv, TBAI, DMF, 40 °C, 48 h; (c) MeONa, MeOH, rt, 24 h; (d) Ac₂O, Py, rt, 24 h, 56% from **1**; (e) MeONa, MeOH, rt, 16 h, then water, 20 h; (f) H₂, Pd/C, 91% from **23**; (g) Bu₂SnO 1.1 equiv, MeOH, refl, 16 h; (h) TDSCl 1.1 equiv, THF, rt, 18 h, 76%; (i) BnBr, NaH, DMF, rt, 16 h; (j) TBAI, THF, 0 °C to rt, 6 h, 66% from **25**; (m) BrCH₂COOtBu, NaOH, (Bu)₄NHSO₄, CH₂Cl₂, 4 h, 96%; (n) TFA (10%), CH₂Cl₂, 30 min; (o) H₂, Pd(OH)₂/C, 72 h, 95%.

Results of antiadhesion tests and BIACORE analysis will be reported elsewhere.

3. Experimental

General methods.— ^1H and ^{13}C NMR spectra were recorded on Varian Gemini 200, Bruker AC 300, Bruker Avance 400, Bruker Am 500 and Bruker 600 DRX spectrometers. The HMBC spectra were acquired using 128 scans per series in $1\text{K} \times 256\text{W}$ data points and optimised for the $^3J_{\text{C-H}}$ of 8 and 4 Hz. Melting points were determined with a Büchi apparatus and are not corrected. Optical rotations were measured at rt with a Perkin–Elmer 241 polarimeter. TLC was carried out on E. Merck Silica-Gel 60 F₂₅₄ plates (0.25 mm thickness), and spots were visualised by spraying with a solution containing H_2SO_4 (31 mL), ammonium molybdate (21 g) and $\text{Ce}(\text{SO}_4)_2$ (1 g) in 500 mL water, followed by heating at 110°C for 5 min. Column chromatography was performed by the flash procedure using E. Merck Silica-Gel 60 (230–400 mesh). Elemental analyses were performed using the Carlo Erba elemental analyser 1108. In the description of the ^{13}C spectra, signals corresponding to aromatic carbons were omitted. Lipase from *C. antarctica* was purchased from Roche Diagnostic (Chirazyme® L-2, c.f. C2 lyo). Solvents were dried by standard procedures.

Benzyl 6-O-levulinoyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranoside (2).—Benzyl β -D-lactoside (1)¹² (700 mg, 1.62 mmol) was suspended in dry THF (70 mL). Trifluoroethyl levulinate⁸ (22 g, 113 mmol) and *C. antarctica* lipase (2.1 g) were added, the suspension was shaken for 4 days at 40°C and monitored by TLC (8:1.5:0.5 EtOAc–MeOH–water). The enzyme was filtered, and the solvent was removed under diminished pressure. Purification by flash chromatography (10:1 EtOAc–MeOH) afforded compound 2 as a white foam (713 mg, 83%). Optical rotation value and NMR data are in agreement with those reported by us in a previous publication.⁸

Benzyl 3,4-O-isopropylidene-6-O-levulinoyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranoside (3). Compound 2 (53 mg, 0.1 mmol) was dissolved in acetone (3 mL) in an inert atmosphere, sikkon (180 mg) was added and the mixture was stirred for 30 min. Then a catalytic amount of camphorsulfonic acid (CSA) was added and the suspension was refluxed for 4 h while monitoring the reaction by TLC (9.5:0.5 EtOAc–MeOH). After cooling to rt, the mixture was neutralised with TEA, sikkon was filtered off and the solvent was removed under diminished pressure. Purification by flash chromatography (9:1 EtOAc–MeOH) afforded compound 3 as a white foam (44 mg, 78%); $[\alpha]_{\text{D}}^{20} + 9.8^\circ$ (c 1.7, MeOH); ^1H NMR (CDCl_3 , 300 MHz): δ 7.35–7.20 (m, 5 H, H_{Ar}), 4.87 (d, 1 H, J 11.8

Hz, CHHPh), 4.64 (d, 1 H, CHHPh), 4.42 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1), 4.37 (dd, 1 H, $J_{6a,6b}$ 12.0, $J_{6a,5}$ 3.2 Hz, H-6'a), 4.33 (d, 1 H, $J_{1',2'}$ 8.1 Hz, H-1'), 4.26 (dd, 1 H, $J_{6b,5}$ 8.6 Hz, H-6'b), 4.12–4.05 (m, 3 H, H-4', H-3', H-3), 3.85 (m, 2 H, H-6a, H-6b), 3.64–3.38 (m, 5 H, H-2, H-4, H-5, H-2', H-5'), 2.77–2.73 (m, 2 H, CH_2 lev.), 2.62–2.58 (m, 2 H, CH_2 lev.), 2.20 (s, 3 H, CH_3 lev.), 1.49 (s, 3 H, CH_3 isoprop.), 1.30 (s, 3 H, CH_3 isoprop.); ^{13}C NMR (CDCl_3 , 75.44 MHz): δ 207.0 (CO lev.), 172.6 (COO lev.), 110.6 (Cq isoprop.), 103.0, 101.8 (C-1, C-1'), 81.7, 79.3, 74.9, 74.3, 73.7, 73.2, 73.1, 71.5 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 71.3 (CH_2Ph), 63.4, 62.2 (C-6, C-6'), 37.8 (CH_2COCH_3 lev.), 28.0 (CH_3 lev.), 27.9 (CH_2COO lev.), 26.2 (2 CH_3 isoprop.). Anal. Calcd for $\text{C}_{27}\text{H}_{38}\text{O}_{13}$ (570.583): C, 56.83, H, 6.71. Found: C, 56.95, H, 6.67.

Benzyl 2-O-benzoyl-3,4-O-isopropylidene-6-O-levulinoyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3,6-tri-O-benzoyl- β -D-glucopyranoside (4).—Compound 3 (272 mg, 0.48 mmol) was dissolved in dry CH_2Cl_2 (5 mL) under N_2 atmosphere, then pyridine (410 μL , 5.1 mmol), DMAP (cat) and benzoyl chloride (360 μL , 3.1 mmol) were added at 0°C and the reaction mixture was stirred 48 h at rt. The reaction was quenched with MeOH and the solution was concentrated under diminished pressure. Purification by flash chromatography (6.5:3.5 petroleum ether–EtOAc) afforded 4 as white foam (378 mg, 80%); $[\alpha]_{\text{D}}^{20} + 24.4^\circ$ (c 1.3, CHCl_3); ^1H NMR (CDCl_3 , 300 MHz): δ 8.10–7.08 (m, 25 H, H_{Ar}), 5.63 (t, 1 H, $J_{3,2} = J_{3,4}$ 9.3 Hz, H-3), 5.44 (t, 1 H, H-2), 5.09 (t, 1 H, $J_{1',2'} = J_{2',3'}$ 7.4 Hz, H-2'), 4.80 (d, 1 H, J 12.6 Hz, CHHPh), 4.68 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.64 (dd, 1 H, $J_{6a,5}$ 2.8 Hz, H-6a), 4.62 (d, 1 H, $J_{1',2'}$ 7.6 Hz, H-1'), 4.57 (d, 1 H, CHHPh), 4.50 (dd, 1 H, $J_{6a,6b}$ 11.8, $J_{6b,5}$ 4.8 Hz, H-6b), 4.24–4.18 (m, 2 H, H-4, H-3'), 4.05 (dd, 1 H, $J_{4',5'}$ 1.7, $J_{4',3'}$ 5.8 Hz, H-4'), 3.95 (dd, 1 H, $J_{6a,6b}$ 11.5, $J_{6a,5}$ 4.9 Hz, H-6'a), 3.82–3.73 (m, 2 H, H-5, H-5'), 3.64 (dd, 1 H, $J_{6b,5}$ 7.0 Hz, H-6'b), 2.77–2.73 (m, 2 H, CH_2 lev.), 2.56–2.51 (m, 2 H, CH_2 lev.), 2.20 (s, 3 H, CH_3 lev.), 1.48 (s, 3 H, CH_3 isoprop.), 1.22 (s, 3 H, CH_3 isoprop.); ^{13}C NMR (CDCl_3 , 50.29 MHz): δ 206.3 (CO lev.), 172.2 (COO lev.), 165.9, 165.4, 165.1, 164.8 (COOBz), 110.7 (Cq isoprop.), 100.2, 99.1 (C-1, C-1'), 76.9, 75.5, 73.6, 73.2, 73.0, 72.7, 72.0, 70.9 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 70.3 (CH_2Ph), 62.7 (C-6, C-6'), 37.9 (CH_2COCH_3 lev.), 29.7 (CH_2CO lev.), 27.9 (CH_2COO lev.), 27.3, 26.0 (2 CH_3 isoprop.); Anal. Calcd for $\text{C}_{55}\text{H}_{54}\text{O}_{17}$ (987.007): C, 66.93; H, 5.51. Found: C, 66.99; H, 5.55.

Benzyl 2-O-benzoyl-6-O-levulinoyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3,6-tri-O-benzoyl- β -D-glucopyranoside (5).—Compound 4 (400 mg, 0.41 mmol), was dissolved in CH_2Cl_2 (20 mL) and cooled to 0°C . A 60% aq CF_3COOH (4 mL) was added under vigorous stirring (TLC 1:1 petroleum ether–EtOAc). After 30 min at 0°C and 30 min at rt, the reaction mixture was diluted

with CH_2Cl_2 , neutralised with NaHCO_3 and partitioned between water and CH_2Cl_2 . The organic phase was dried over Na_2SO_4 , filtered and concentrated under diminished pressure. Purification by flash chromatography (1:4 petroleum ether–EtOAc), afforded **6** (325 mg, 85%) as an amorphous white solid: $[\alpha]_{\text{D}}^{20} + 19.7^\circ$ (c 1.0, CHCl_3); ^1H NMR (CDCl_3 , 200 MHz): δ 8.09–7.08 (m, 25 H, H_{Ar}), 5.56 (t, 1 H, $J_{2,3} = J_{3,4}$ 9.5 Hz, H-3), 5.46 (dd, 1 H, $J_{1,2}$ 7.4 Hz, H-2), 5.31 (t, 1 H, $J_{1',2'} = J_{2',3'}$ 8.5 Hz, H-2'), 4.80 (d, 1 H, J 12.5 Hz, CHHPH), 4.65 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1), 4.60–4.47 (m, 4 H, H-6a, H-6b, H-1', CHHPH), 4.13 (t, 1 H, $J_{3,4} = J_{4,5}$ 9.4 Hz, H-4), 3.82–3.35 (2 m, 8 H, H-5, H-5', H-4', H-5', H-6'a, H-6'b, 2 OH), 2.74–2.67 (m, 2 H, CH_2 lev.), 2.51–2.43 (m, 2 H, CH_2 lev.), 2.16 (s, 3 H, CH_3 lev.); ^{13}C NMR (CDCl_3 , 300 MHz): δ 206.7 (CO lev.), 172.4 (COO lev.), 166.1, 166.0, 165.3 (COOBz), 101.1, 98.8 (C-1, C-1'), 76.4, 75.5, 73.5, 73.1, 72.4, 72.3, 71.7, 68.6 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 70.3 (CH_2Ph), 62.8, 61.8 (C-6, C-6'), 37.9 (CH_2COCH_3 lev.), 29.8 (CH_3CO lev.), 27.7 (CH_2COO lev.). Anal. Calcd for $\text{C}_{52}\text{H}_{50}\text{O}_{17}$ (946.943): C, 65.96; H, 5.32. Found: C, 65.92; H, 5.34.

Benzyl 2-O-benzoyl-3,4-O-isopropylidene- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3,6-tri-O-benzoyl- β -D-glucopyranoside (6).—Compound **4** (356 mg, 0.36 mmol) was dissolved in 1:1 EtOH–Et₂O (8 mL), then a 1 M solution of $\text{AcONH}_3\text{NH}_2$ in EtOH (0.4 mL) was dropped at rt. After 20 min, the solvents were removed under diminished pressure and purification by flash chromatography (3:1 toluene–EtOAc) afforded compound **5** as a white solid (309 mg, 97%); mp 202–203 °C; $[\alpha]_{\text{D}}^{20} + 26.7^\circ$ (c 1.0, CHCl_3); ^1H NMR (CDCl_3 , 200 MHz): δ 8.05–7.15 (m, 25 H, H_{Ar}), 5.62 (t, 1 H, $J_{3,4} = J_{2,3}$ 8.8 Hz, H-3), 5.49 (dd, 1 H, $J_{1,2}$ 7.3 Hz, H-2), 5.14 (t, 1 H, $J_{2',3'}$ 7.3 Hz, H-2'), 4.81 (d, 1 H, J 12.5 Hz, CHHPH), 4.71 (d, 1 H, H-1), 4.64 (dd, 1 H, $J_{6a,6b}$ 12.2, $J_{6a,5}$ 2.2 Hz, H-6a), 4.61 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 4.58 (d, 1 H, CHHPH), 4.47 (dd, 1 H, $J_{6b,5}$ 4.7 Hz, H-6b), 4.25–4.16 (m, 2 H, H-4, H-3'), 4.00 (dd, 1 H, $J_{4',3'}$ 5.65, $J_{4',5'}$ 2.01 Hz, H-4'), 3.8 (ddd, 1 H, H-5), 3.56 (m, 1 H, H-5'), 3.43–3.17 (m, 2 H, H-6'a, H-6'b), 1.51 (s, 3 H, CH_3 isoprop.), 1.28 (s, 3 H, CH_3 isoprop.); ^{13}C NMR (CDCl_3 , 50.29 MHz): δ 165.9, 165.5, 165.2, 164.9 (COOBz), 110.7 (Cq isoprop.), 100.5, 99.0 (C-1, C-1'), 77.0, 75.8, 73.9, 73.4, 73.3, 73.0, 71.8 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 70.3 (CH_2Ph), 62.8, 61.8 (C-6, C-6'), 27.4, 26.1 (2 CH_3 isoprop.). Anal. Calcd for $\text{C}_{50}\text{H}_{48}\text{O}_{15}$ (888.907): C, 67.56; H, 5.44. Found: C, 67.62; H, 5.51.

Benzyl 3,4-O-isopropylidene-6-O-levulinoyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,6-di-O-benzoyl- β -D-glucopyranoside (7).—To a solution of **3** (300 mg, 0.53 mmol) in dry THF (7.5 mL), TEA (0.37 mL, 2.65 mmol) was added under Ar atmosphere. The reaction mixture was cooled to -25°C , and a solution of

BzCN (204 mg, 1.56 mmol) in THF (2.5 mL) was added dropwise. The reaction mixture was stirred for 17 h (TLC 1:9 MeOH–EtOAc), diluted with EtOAc, quenched with MeOH (0.3 mL) and allowed to warm at rt. After concentration under diminished pressure, sequential purification by flash and MP chromatography (8:1.5 toluene–acetone) afforded **7** (203 mg, 50%), as a white foam. $[\alpha]_{\text{D}}^{20} - 15.4^\circ$ (c 1.0, CHCl_3); ^1H NMR (CDCl_3 , 600 MHz): δ 8.11–7.15 (m, 15 H, H_{Ar}), 5.21 (dd, 1 H, $J_{2,3}$ 9.4 Hz, H-2), 4.87 (2d overlapped, 2 H, H-6a, CHHPH), 4.70 (d, 1 H, J 12.6 Hz, CHHPH), 4.60 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 4.55 (dd, 1 H, $J_{6a,6b}$ 12.0, $J_{6b,5}$ 5.2 Hz, H-6b), 4.39 (dd, 1 H, $J_{6a,6b}$ 12.1, $J_{6a,5}$ 2.8 Hz, H-6'a), 4.30 (d, 1 H, $J_{1',2'}$ 8.3 Hz, H-1'), 4.25 (dd, 1 H, $J_{6b,5}$ 9.4 Hz, H-6'b), 4.10–4.06 (m, 3 H, H-3', H-4', H-5'), 3.87 (t, 1 H, $J_{3,4}$ 9.2 Hz, H-3), 3.69 (br dd, 1 H, $J_{4,5}$ 10.0 Hz, H-5), 3.63 (br dd, 1 H, $J_{2,3}$ 6.3 Hz, H-2'), 3.61 (t, 1 H, H-4), 2.54–2.35 (m, 4 H, 2 CH_2 lev.), 2.02 (s, 3 H, CH_3 lev.), 1.51 (s, 3 H, CH_3 isoprop.), 1.32 (s, 3 H, CH_3 isoprop.); ^{13}C NMR (CDCl_3 , 150.86 MHz): δ 206.8 (CO lev.), 171.6, 166.8, 165.3 (CO lev., Bz), 110.7 (Cq isoprop.), 103.4 (C-1'), 98.9 (C-1), 82.2 (C-4), 79.1 (C-3' or C-4'), 73.7 (C-3), 73.6 (C-5), 73.4 (C-2'), 73.1 (C-2), 73.0 (C-3' or C-4'), 71.5 (C-5'), 70.3 (CH_2Ph), 63.9 (C-6), 63.2 (C-6'), 37.6 (CH_3 lev.), 29.6, 28.0 (2 CH_2 lev.), 27.8, 26.2 (2 CH_3 isoprop.). Anal. Calcd for $\text{C}_{41}\text{H}_{40}\text{O}_{15}$ (778.795): C, 63.23; H, 5.95. Found: C, 63.20; H, 5.89.

Benzyl 6-O-levulinoyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,6-di-O-benzoyl- β -D-glucopyranoside (8).—Compound **7** (564 mg, 0.72 mmol) was submitted to the same conditions described for the preparation of **5**. Purification by flash chromatography (EtOAc) afforded **8** (490 mg, 92%) as an amorphous glassy solid: $[\alpha]_{\text{D}}^{20} - 37.0^\circ$ (c 1.1, CHCl_3); ^1H NMR (CDCl_3 , 300 MHz): δ 8.15–7.11 (m, 15 H, H_{Ar}), 5.19 (t, 1 H, $J_{2,3}$ 8.7 Hz, H-2), 4.85 (d, 1 H, H-6a), 4.81 (d, 1 H, CHHPH), 4.62 (d, 1 H, $J_{1,2}$ 7.2 Hz, H-1), 4.59 (d, 1 H, J 11.3 Hz, CHHPH), 4.54 (dd, 1 H, $J_{6b,5}$ 5.6, $J_{6a,6b}$ 11.9 Hz, H-6b), 4.44 (br s, 1 H, OH), 4.34 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1'), 4.28 (dd, 1 H, $J_{6a,5'}$ 3.3 Hz, H-6'a), 4.24 (dd, 1 H, $J_{6b,5'}$ 4.4, $J_{6a,6b}$ 11.8 Hz, H-6'b), 4.01 (br s, 1 H, OH), 3.90–3.63 (m, 7 H, H-3, H-4, H-2', H-3', H-4', H-5', OH), 3.54 (m, 1 H, H-5), 3.31 (br s, 1 H, OH), 2.57–2.40 (m, 4 H, 2 CH_2 lev.), 2.03 (s, 3 H, CH_3 lev.); ^{13}C NMR (CDCl_3 , 50.29 MHz, 50°C): δ 207.2 (CO lev.), 173.0, 167.1, 165.7 (COO lev., Bz), 104.3 (C-1'), 99.5 (C-1), 81.8, 73.8, 73.3, 71.3, 68.7 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 70.6 (CH_2Ph), 64.2, 63.6 (C-6, C-6'), 37.9 (CH_2COCH_3 lev.), 29.2 (CH_3 lev.), 28.1 (CH_2COO lev.). Anal. Calcd for $\text{C}_{38}\text{H}_{42}\text{O}_{15}$ (738.731): C, 61.68; H, 5.73. Found: C, 61.63; H, 5.77.

Benzyl (methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero- α -D-galacto-non-2-ulopyranosyl-onate)-(2 $\rightarrow$ 6)-2-O-benzoyl-3,4-O-isopropylidene- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3,6-tri-O-benzoyl- β -D-glucopyranoside (9).—To a solution of **3** (300 mg, 0.53 mmol) in dry THF (7.5 mL), TEA (0.37 mL, 2.65 mmol) was added under Ar atmosphere. The reaction mixture was cooled to -25°C , and a solution of

pyranoside (10a) and its isomer (10b).—Compound 5 (390 mg, 0.44 mmol) and phosphite donor 9¹³ (468 mg, 0.76 mmol) were dissolved in dry CH₃CN (4 mL), and cooled to –40 °C. A 0.5 M solution of trimethylsilyltrifluoromethanesulfonate (TMSOTf) in CH₃CN (150 µL, 0.075 mmol), was dropped under vigorous stirring. After 1 h, the reaction mixture was neutralised with TEA and concentrated. Flash chromatography purification (95:5 toluene–EtOH) of the crude afforded a mixture of 10a, 10b and glycal²⁶ which was further purified by MP chromatography to give 10a (236 mg, 39%) and 10b (115 mg, 19%) as white amorphous solids: 10a, $[\alpha]_D^{25} + 6.8^\circ$ (c 1.0, CHCl₃); ¹H NMR (C₆D₆, 500 MHz): δ 8.30–6.91 (2 m, 25 H, H_A), 5.92 (ddd, 1 H, $J_{6''',7''}$ 7.7 Hz, H-8''), 5.87 (m, 2 H, H-2, H-3), 5.60 (t, 1 H, $J_{2,3}$ 7.2 Hz, H-2'), 5.44 (dd, 1 H, $J_{6'',7''}$ 2.3, $J_{7'',8''}$ 7.9 Hz, H-7'), 4.89 (dd, 1 H, $J_{6a,6b}$ 11.8, $J_{6a,5}$ 6.2 Hz, H-6a), 4.80 (m, 3 H, H 6b, H-4'', H 1'), 4.70 (dd, 1 H, $J_{8'',9''}$ 2.9, $J_{9'',9''b}$ 12.2 Hz, H-9'a), 4.63 (d, 1 H, J 12.7 Hz, CHHPh), 4.52 (m, 1 H, H-4), 4.44 (d, 1 H, CHHPh), 4.41 (m, 2 H, H-1, H-5'), 4.29 (dd, 1 H, $J_{8'',9''}$ 7.7 Hz, H-9'b), 4.10 (dd, 1 H, $J_{6'',7''}$ 2.3, $J_{5'',6''}$ 10.8 Hz, H-6''), 4.01 (dd, 1 H, $J_{4',5'}$ 2.0, $J_{3',4'}$ 5.4 Hz, H-4'), 3.96–3.87 (m, 3 H, H-3', H-6'a, NH), 3.86 (br t, 1 H, H-5'), 3.59 (t, 1 H, $J_{6'a,6'b}$ 9.4, $J_{6'b,5'}$ 8.3 Hz, H-6'b), 3.42 (s, 3 H, COOCH₃), 3.34 (br t, 1 H, H-5), 2.73, (dd, 1 H, $J_{3''eq,3''ax}$ 12.8, $J_{3''eq,4''}$ 4.7 Hz, H-3''eq), 2.20 (s, 3 H, CH₃Ac), 2.07 (s, 3 H, CH₃Ac), 1.96 (t, 1 H, $J_{3''ax,4''}$ 12.0 Hz, H-3''ax), 1.92 (s, 3 H, CH₃Ac), 1.58 (s, 3 H, CH₃Ac), 1.56 (s, 3 H, NHCOCH₃), 1.54 (s, 3 H, CH₃ isoprop.), 1.40 (s, 3 H, CH₃ isoprop.); ¹³C NMR (CDCl₃, 100.62 MHz): δ 171.3, 171.1, 170.7, 170.4, 170.3, 168.3, 166.4, 165.8, 165.6, 165.4 (COOAc, NHAc, Bz, COOCH₃), 110.7 (Cq isoprop.), 100.2, 99.4, 99.4 (C-1, C-1', C-2''), 77.4, 77.6, 74.3, 73.7, 73.2, 72.6, 71.7, 69.3, 69.0, 68.0 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5', C-4'', C-6'', C-7'', C-8''), 70.7 (CH₂Ph), 63.5, 63.1, 62.9 (C-6, C-6', C-9''), 53.2 (COOCH₃), 49.9 (C-5'), 38.1 (C-3''), 27.9, 26.6 (2 CH₃, isoprop.), 23.6 (NHCOCH₃), 21.4–21.2 (4 CH₃Ac). Anal. Calcd for C₇₀H₇₅NO₂₇ (1362.335): C, 61.71; H, 5.55; N, 1.03. Found: C, 61.66; H, 5.52; N, 0.97. 10b, $[\alpha]_D^{25} + 23.5^\circ$ (c 1.0, CHCl₃); ¹H NMR (C₆D₆, 500 MHz): δ 8.19–6.98 (2 m, 25 H, H), 5.98 (t, 1 H, H-3), 5.88 (dd, 1 H, $J_{6'',7''}$ 2.0, $J_{7'',8''}$ 3.1 Hz, H-7''), 5.83 (t, 1 H, $J_{2,3}$ 8.8 Hz, H-2), 5.63 (t, 1 H, H-2'), 5.60 (ddd, 1 H, H-8''), 5.49 (ddd, 1 H, $J_{4'',5''}$ 10.1 Hz, H-4''), 5.26 (dd, 1 H, $J_{9'',9''b}$ 12.4, $J_{9'a,8''}$ 2.5 Hz, H-9'a), 4.96 (d, 1 H, $J_{5'',3''H}$ 9.5 Hz, NH), 4.79 (d, 1 H, $J_{1',2'}$ 7.9 Hz, H-1'), 4.77 (dd, 1 H, $J_{6a,5}$ 2.5, $J_{6a,6b}$ 11.8 Hz, H-6a), 4.74 (dd, 1 H, $J_{6b,5}$ 5.1 Hz, H-6b), 4.69 (d, 1 H, J 12.7 Hz, CHHPh), 4.63 (d, 1 H, $J_{1,2}$ 7.2 Hz, H-1), 4.51 (dd, 1 H, $J_{9''b,8''}$ 8.1 Hz, H-9'b), 4.47–4.43 (m, 3 H, CHHPh, H-6'', H-5''), 4.28 (dd, 1 H, $J_{3,4}$ 8.4, $J_{4,5}$ 9.4 Hz, H-4), 4.17 (dd, 1 H, $J_{3',4'}$ 5.5, $J_{4',5'}$ 1.7 Hz, H-4'), 4.07 (m, 1 H, H-6'a), 3.97 (dd, 1 H, $J_{2,3}$ 7.3 Hz, H-3'), 3.88 (m, 2 H, H-5', H-6'b), 3.61 (ddd, 1 H,

H-5), 3.40 (s, 3 H, COOCH₃), 2.61 (dd, 1 H, $J_{3''eq,3''ax}$ 12.9, $J_{3''eq,4''}$ 4.9 Hz, H-3''eq), 1.92 (s, 3 H, CH₃Ac), 1.85 (s, 3 H, CH₃Ac), 1.82 (t, 1 H, $J_{3''ax,4''}$ 11.5 Hz, H-3''ax), 1.71 (s, 3 H, CH₃Ac), 1.65 (s, 3 H, CH₃ isoprop.), 1.59 (s, 3 H, CH₃Ac), 1.55 (s, 3 H, NHCOCH₃), 1.38 (s, 3 H, CH₃ isoprop.); ¹³C NMR (C₆D₆, 125.72 MHz): δ 170.8, 170.4, 170.3, 169.6, 167.3, 166.1, 165.5, 165.2 (COOAc, NHAc, Bz, COOCH₃), 110.9 (Cq isoprop.), 100.7 (C-1'), 99.8 (C-1), 99.19 (C-2'), 77.1 (C-3'), 76.5 (C-4), 73.9 (C-2), 73.6 (C-3), 73.5 (C-8'), 73.4 (C-4''), 73.3 (C-2), 73.1 (C-5), 72.9 (C-6''), 71.9 (C-5'), 70.4 (CH₂Ph), 69.0 (C-7''), 69.0 (C-4''), 63.6 (C-6), 63.2 (C-9'), 62.5 (C-6'), 52.4 (COOCH₃), 49.9 (C-5'), 38.0 (C-3''), 27.7 (CH₃ isoprop.), 26.7 (CH₃ isoprop.), 22.9 (NHCOCH₃), 20.8, 20.6, 20.48, 20.4 (4 CH₃Ac). Anal. Calcd for C₇₀H₇₅NO₂₇ (1362.335): C, 61.71; H, 5.55; N, 1.03. Found: C, 61.78; H, 5.54; N, 0.96.

Sodium 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-β-D-galactopyranosyl-(1→4)-D-glucopyranose (11). To a solution of 10a (235 mg, 0.17 mmol) in CH₂Cl₂, a 60% aq soln of CF₃COOH was added at 0 °C, and the reaction mixture was stirred at this temperature for 3 h (TLC 9:1 toluene–EtOH). After dilution with water, neutralisation with Na₂CO₃ and extraction with CH₂Cl₂, the organic phase was separated, dried over Na₂SO₄ and concentrated to dryness. The obtained residue was then dissolved in MeOH (4 mL) and treated with a 0.5 M solution of MeONa in MeOH (110 µL) for 48 h at rt (TLC 3:1:0.25 CHCl₃–MeOH–water). The reaction mixture was neutralised with Amberlite IR-120 resin (H⁺ form), concentrated under diminished pressure, dissolved in water (3 mL) and freeze-dried. The obtained compound was dissolved in water (3 mL), treated with 1 M aq NaOH (180 µL) for 24 h and freeze-dried. The residue was dissolved in 1:4 MeOH–water (6 mL) and submitted to hydrogenolysis in the presence of 10% Pd/C for 24 h (TLC 6:3:1 acetone–BuOH–water); the mixture was filtered through Celite and freeze-dried. The residue was loaded onto a Sephadex G 10 column (V_0 = 150 mL, V_t = 300 mL) and eluted with 1:9 EtOH–water. The fractions containing the product were collected and freeze-dried giving 11 (82 mg, 74%) as a white foam. ¹H and ¹³C NMR data for this compound are in agreement with those reported in the literature.²⁵

Benzyl (methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2→3)-6-levulinoyl-β-D-galactopyranosyl-(1→4)-2,6-di-O-benzoyl-β-D-glucopyranoside (12).—Compound 8 (100 mg, 0.13 mmol) and phosphite donor 9¹³ (160 mg, 0.26 mmol) were dissolved in 2:1 CH₃CN–THF (1.5 mL), and cooled to –44 °C. A 0.5 M TMSOTf solution in CH₃CN (50 µL, 0.025 mmol) was dropped under vigorous stirring. After 2 h, the reaction mixture was neutralised with TEA and concentrated.

Flash chromatography purification (95:5 CHCl₃–MeOH) of the crude mixture afforded unreacted **8** (22 mg, 22%) and a mixture of **12**, glycal²⁶ and other unidentified compounds (presumably isomers of **12**), which was further purified by MP chromatography (3:2 toluene acetone) to give **12** (81 mg, 51%) as a white amorphous solid: $[\alpha]_D^{20}$ –14.6° (c 0.7, CHCl₃); ¹H NMR (CDCl₃, 600 MHz): δ 8.09–7.10 (m, 15 H, H_A), 5.31 (td, 1 H, $J_{7,8} = J_{8,9} = 8.4$, $J_{8,9} = 2.5$ Hz, H-8'), 5.27 (br d, 1 H, H-7'), 5.25 (t, 1 H, $J_{1,2} = J_{2,3} = 9.5$ Hz, H-2), 5.22 (d, 1 H, $J_{5,6} = 9.6$ Hz, NH), 4.97 (td, 1 H, $J_{3,4} = J_{4,5} = 10.5$, $J_{3,4} = 4.6$ Hz, H-4'), 4.93 (br d, 1 H, $J_{6,7} = 10.7$ Hz, H-6a), 4.82 (d, 1 H, $J = 12.6$ Hz, CHHPH), 4.61 (d, 1 H, CHHPH), 4.60 (d, 1 H, $J_{1,2} = 7.8$ Hz, H-1), 4.53 (dd, 1 H, $J_{6,5} = 5.6$ Hz, H-6b), 4.52 (d, 1 H, $J_{1,2} = 7.9$ Hz, H-1'), 4.42 (s, 1 H, OH), 4.30 (dd, 1 H, $J_{9,8} = 12.4$ Hz, H-9'a), 4.27 (m, 2 H, H-6'a, H-6'b), 4.10 (br d, 1 H, $J_{6,5} = 9.2$ Hz, H-6'), 4.08 (1 H, dd, $J_{2,3} = 9.1$, $J_{3,4} = 3.4$ Hz, H-3'), 3.93 (m, 2 H, H-5', H-9'b), 3.84 (br t, 1 H, $J_{2,3} = J_{3,4} = 8.8$ Hz, H-3), 3.82–3.68 (m, 4 H, H-4, H-5, H-2', H-5'), 3.80 (s, 3 H, COOCH₃), 3.63 (br s, 1 H, H-4'), 3.17 (d, 1 H, $J = 2.0$ Hz, OH), 2.66 (dd, 1 H, $J_{3',4'} = 13.0$ Hz, H-3'eq), 2.63–2.43 (m, 4 H, 2 CH₂ lev.), 2.38 (d, 1 H, $J_{4',OH} = 3.0$ Hz, OH), 2.13 (s, 3 H, CH₃ lev.), 2.05 (s, 3 H, CH₃Ac), 2.02 (s, 3 H, CH₃Ac), 2.01 (s, 3 H, CH₃Ac), 2.00 (t, 1 H, H-3'ax), 1.96 (s, 3 H, CH₃Ac), 1.88 (s, 3 H, HCOCH₃); ¹³C NMR (CDCl₃, 150.86 MHz): δ 206.9 (CO lev.), 172.6, 170.8, 170.5, 170.2, 170.1, 168.1, 166.2, 165.4 (9 COOAc, NHAc, Bz, COOCH₃ lev.), 104.2 (C-1'), 99.0 (C-1), 97.6 (C-2'), 82.3 (C-5), 76.5 (C-3'), 73.6 (C-3), 73.2 (C-7'), 73.0 (C-6'), 72.9 (C-4), 72.4 (C-5'), 70.1 (CH₂Ph), 69.0 (C-2), 68.8 (C-8'), 68.2 (C-4'), 67.9 (C-4'), 67.0 (C-2), 63.6 (C-6), 63.2 (C-6), 62.4 (C-9'), 53.3 (COOCH₃), 49.8 (C-5'), 37.7, 37.6 (C-3', CH₂COCH₃ lev.), 30.9 (CH₃ lev.), 27.9 (CH₂COO lev.), 23.1 (HCOCH₃), 21.1–20.6 (4 CH₃Ac). Anal. Calcd for C₅₈H₆₉NO₂₇ (1212.159): C, 57.47; H, 5.74; N, 1.16. Found: C, 57.54; H, 6.64; N, 1.19.

Sodium 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylate-(2→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose (13).—To a solution of **12** (35 mg, 0.029 mmol) in MeOH (2 mL), a 0.5 M solution of MeONa in MeOH (12 μL) was added and the reaction mixture was stirred for 48 h at rt (TLC 3:1:0.35 CHCl₃–MeOH–water). The solution was neutralised by Amberlite IR 120 resin (H⁺ form), filtered, concentrated under diminished pressure and, after dilution in water (1 mL), treated with 1 M aq NaOH (80 μL) for 24 h and freeze-dried. The residue was dissolved in 1:4 MeOH–water (3 mL) and hydrogenolysed in the presence of 10% Pd/C for 24 h (TLC 6:3:1 acetone BuOH water). The mixture was filtered through Celite and freeze-dried. Finally the residue was loaded onto a Sephadex G 10 column

(V₀ = 17 mL, V_t = 23 mL) and eluted with 1:9 EtOH–water. The fractions containing the product were collected and freeze-dried giving **13** (16 mg, 84%) as a white powder. ¹H and ¹³C NMR data for this compound are in agreement with those reported in the literature.^{20,25}

Benzyl 2,3,4-tri-O-acetyl-6-O-levulinoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (14).—To compound **2** (110 mg, 0.21 mmol) in pyridine (4 mL), Ac₂O (2 mL) was added and the mixture was stirred at rt for 24 h. The reaction mixture was quenched with MeOH and concentrated. The residue was diluted with EtOAc and washed with 5% HCl, then with water. The organic layer was dried over Na₂SO₄, concentrated and purified by flash chromatography (4:1 EtOAc–petroleum ether) affording **14** (130 mg, 80%) as a white foam: $[\alpha]_D^{20}$ –28.7° (c 1.1, CHCl₃); ¹H NMR (CDCl₃, 200 MHz): δ 7.42–7.23 (m, 5 H, H_A), 5.33 (br d, 1 H, $J_{3,4} = 3.0$ Hz, H-4'), 5.17 (t, 1 H, $J_{2,3} = J_{3,4} = 9.2$ Hz, H-3), 5.10 (dd, 1 H, $J_{1,2} = 7.6$ Hz, H-2), 4.95 (m, 2 H, H-3', H-4'), 4.85 (d, 1 H, $J = 12.3$ Hz, CHHPH), 4.60 (d, 1 H, CHHPH), 4.49 (m, 3 H, H-1, H-1', H-6a), 4.20–4.03 (m, 3 H, H-6b, H-6'a, H-6'b), 3.84 (m, 2 H, H-4, H-5'), 3.60 (ddd, 1 H, $J_{5,6} = 2.0$, $J_{5,6} = 4.9$, $J_{4,5} = 9.8$ Hz, H-5), 2.74 (m, 2 H, CH₂ lev.), 2.53 (m, 2 H, CH₂ lev.), 2.21, 2.16, 2.05, 2.00, 1.94 (5 s, 21 H, CH₃Ac, lev.); ¹³C NMR (CDCl₃, 50.29 MHz): δ 206.1 (CO lev.), 172.1, 170.2, 170.0, 169.7, 169.4, 168.9 (6 COOAc), 100.9, 99.0 (C-1, C-1'), 76.1, 72.7, 71.8, 70.9, 70.5, 69.6, 66.7 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 70.6 (CH₂Ph), 62.0, 60.9 (C-6, C-6'), 37.7 (CH₂COCH₃ lev.), 29.5 (CH₃ lev.), 27.7 (CH₂COO lev.), 20.7–20.5 (6 CH₃Ac). Anal. Calcd for C₃₆H₄₆O₁₉ (782.739): C, 55.24; H, 5.92. Found: C, 55.29; H, 5.96.

Benzyl 2,3,4-tri-O-acetyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-acetyl-β-D-glucopyranoside (15).—Compound **14** (95 mg, 0.12 mmol) was dissolved in 1:1 EtOH–Et₂O (2 mL) and AcONH₃NH₂ (13 mg, 0.14 mmol) was added at rt. After 2 h, the mixture was concentrated and purified by flash chromatography (9:1 toluene–acetone) affording **15** (81 mg, 98%) as a white foam: $[\alpha]_D^{20}$ –13.3° (c 0.8, CHCl₃); ¹H NMR (CDCl₃, 200 MHz): δ 7.35–7.13 (m, 5 H, H_A), 5.34 (d, 1 H, $J_{3,4} = 3.3$ Hz, H-4'), 5.17 (t, 1 H, $J_{2,3} = J_{3,4} = 9.2$ Hz, H-3), 5.14 (dd, 1 H, $J_{1,2} = 7.6$, $J_{2,3} = 10.4$ Hz, H-2'), 4.98 (dd, 1 H, H-3'), 4.96 (dd, 1 H, $J_{1,2} = 7.4$ Hz, H-2), 4.86 (d, 1 H, $J = 12.2$ Hz, CHHPH), 4.62–4.51 (m, 4 H, H-1, H-1', H-6a, CHHPH), 4.09 (dd, 1 H, $J_{6,7} = 11.9$, $J_{6,5} = 5.2$ Hz, H-6b), 3.86 (br t, 1 H, $J_{4,5} = 9.6$ Hz, H-4), 3.70–3.48 (m, 4 H, H-5, H-5', H-6'a, H-6'b), 2.17–1.95 (6 s, 18 H, CH₃Ac); ¹³C NMR (CDCl₃, 75.44 MHz): δ 170.9, 170.4, 170.1, 169.9, 169.6, 169.1 (6 COOAc), 101.1, 99.0 (C-1, C-1'), 76.2, 74.1, 73.5, 72.6, 71.7, 71.0, 69.5, 67.7 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 70.0 (CH₂Ph), 62.1, 60.8 (C-6, C-6'), 20.9–20.7 (6 CH₃Ac). Anal. Calcd for C₃₁H₄₀O₁₇ (684.639): C, 54.38; H, 5.89. Found: C, 54.36; H, 5.82.

Benzyl 6-O-sulfo- β -D-galactopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranoside sodium salt (16).—Compound 15 (186 mg, 0.27 mmol), was dissolved in dry DMF (3 mL) in Ar atmosphere, the SO_3NMe_3 complex (56 mg, 0.40 mmol) was added and the reaction mixture was stirred at 60 °C (TLC 4:1 CH_2Cl_2 –MeOH). After 48 h, the reaction was quenched with a few drops of MeOH and concentrated. Flash chromatography purification (CHCl_3 , then 10:1 CHCl_3 –MeOH, and 5:1 CHCl_3 –MeOH) afforded a white amorphous solid (203 mg). The resulting product was dissolved in dry MeOH (8 mL) under nitrogen; after cooling to 0 °C a 1 M solution of MeONa in MeOH (400 μL) was added and the reaction mixture was stirred at rt for 7 h. The solution was percolated on a column (1 $\times$ 10 cm) filled with Dowex 50 WX8 (H^+ form) resin and the eluate was concentrated and purified by flash chromatography (5:8:0.7 MeOH– CHCl_3 –water). The fractions containing the product were concentrated to a volume of 3 mL and percolated on a Dowex 50 WX8 (Na^+ form) column (1 $\times$ 10 cm). The resulting eluate was finally concentrated to dryness affording 16 (117 mg, 81%), as a white amorphous solid: $[\alpha]_{\text{D}}^{20} -11.5^\circ$ (c 0.6, water); ^1H NMR (D_2O , 500 MHz): δ 7.51–7.42 (m, 5 H, H_{Ar}), 4.94 (d, 1 H, J 11.7 Hz, CHHPb), 4.77 (d, 1 H, CHHPb), 4.57 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 4.48 (d, 1 H, $J_{1,2}$ 7.4 Hz, H-1'), 4.21 (appearing as a d, 2 H, J 6.2 Hz, H-6'a, H-6'b), 4.00 (dd, 1 H, $J_{4,5} < 2$ Hz, H-4'), 3.99–3.98 (m, 2 H, H-6a, H-5'), 3.81 (dd, 1 H, $J_{5,6b}$ 5.1, $J_{6a,6b}$ 12.4 Hz, H-6b), 3.69 (dd, 1 H, $J_{2,3}$ 10.0, $J_{3,4}$ 3.5 Hz, H-3'), 3.64 (m, 3 H, H-3, H-4, H-5), 3.56 (dd, 1 H, H-2'), 3.38 (t, 1 H, $J_{2,3}$ 9.4 Hz, H-2); ^{13}C NMR (D_2O , 125.72 MHz): δ 105.9 (C-1'), 103.9 (C-1), 82.3 (C-4), 77.5 (C-5), 77.3 (C-3), 75.6 (C-2), 75.7 (C-5'), 75.2 (C-3'), 74.3 (CH_2Ph), 73.5 (C-2'), 71.1 (C-4'), 70.0 (C-6'), 63.1 (C-6). Anal. Calcd for $\text{C}_{19}\text{H}_{27}\text{NaO}_{14}\text{S}$ (534.465): C, 42.70; H, 5.09. Found: C, 42.75; H, 5.17.

6-O-Sulfo- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose sodium salt (17).—A solution of 16 (25 mg, 0.046 mmol) in 3:1 MeOH water (4 mL) was hydrogenolysed in the presence of 10% Pd/C for 16 h, (TLC 6:3:1 acetone–BuOH–water). The mixture was filtered through Celite and freeze-dried. The residue was loaded onto a Sephadex G 10 column (V_0 = 17 mL, V_i = 23 mL) and eluted with 1:9 EtOH–water. The fraction containing the product were collected and freeze-dried affording 17 (18 mg, 88%) as a white powder: $[\alpha]_{\text{D}}^{20} +29.4^\circ$ (c 1.0, water); ^1H NMR (D_2O , 200 MHz): δ 5.28 (d, 0.28 H, $J_{1,2a}$ 3.4 Hz, H-1 α), 4.72 (d, 0.7 H, $J_{1,2e}$ 7.9 Hz, H-1 β), 4.57 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1'), 4.27 (appearing as a d, 2 H, H-6'a, H-6'b), 4.06–3.56 (m, 9.3 H, H-2 α , H-3, H-4, H-5, H-6a, H-6b, H-2', H-3', H-4', H-5'), 3.36 (br t, 0.7 H, $J_{2,3}$ 9.1 Hz, H-2 β); ^{13}C NMR (CDCl_3 , 75.44 MHz): 103.9 (C-1'), 96.5 (C-1 β), 92.6 (C-1 α), 80.4, 80.2, 75.5, 75.2, 74.6, 73.7, 73.2, 72.3, 72.0, 71.6, 70.9, 69.1, 68.2 (C-6'), 61.1

(C-6 α), 61.0 (C-6 β). Anal. Calcd for $\text{C}_{12}\text{H}_{21}\text{NaO}_{14}\text{S}$ (444.342): C, 32.44; H, 4.76. Found: C, 32.49; H, 4.72.

Benzyl (3-O-sulfo- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)- β -D-glucopyranoside sodium salt (18).—Compound 1 (218 mg, 0.50 mmol) and Bu_2SnO (135 mg, 0.54 mmol) were stirred in refluxing dry MeOH (4 mL) for 2 h under nitrogen. The reaction mixture was concentrated to dryness and the dry dibutylstannylene intermediate was treated with SO_3NMe_3 complex (144 mg, 1.0 mmol) in dry dioxane (4 mL) at rt. After 48 h, the reaction was diluted with MeOH (3 mL), filtered and concentrated. The residue was then purified by flash chromatography (5:8:1 MeOH– CHCl_3 –water); the fractions containing the product were concentrated, dissolved in MeOH and percolated into a cation exchange resin column (Dowex 50 WX8, H^+ form, 1 $\times$ 10 cm). The eluate was concentrated and percolated on a Dowex 50 column (Na^+ form, 1 $\times$ 10 cm) affording, after removal of the solvents, 18 (200 mg, 75%) as a white amorphous solid: $[\alpha]_{\text{D}}^{20} -17.2^\circ$ (c 1.0, MeOH); ^1H NMR (D_2O , 500 MHz): δ 4.95 (d, 1 H, J 11.4 Hz, CHHPb), 4.78 (d, 1 H, CHHPb), 4.58 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1'), 4.57 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.34 (dd, 1 H, $J_{2,3}$ 9.8, $J_{3,4}$ 3.3 Hz, H-3'), 4.30 (d, 1 H, $J_{4,5} < 1$ Hz, H-4'), 4.01 (dd, 1 H, $J_{5,6a}$ 2.3, $J_{6a,6b}$ 12.4 Hz, H-6a), 3.84 (dd, 1 H, $J_{5,6b}$ 5.2 Hz, H-6b), 3.78 (m, 1 H, H-5'), 3.75 (m, 2 H, H-6'a, H-6'b), 3.70 (t, 1 H, $J_{4,5}$ 9.7 Hz, H-4), 3.70 (t, 1 H, H-2'), 3.64 (t, 1 H, $J_{3,4}$ 8.9 Hz, H-3), 3.59 (ddd, 1 H, H-5), 3.37 (t, 1 H, $J_{2,3}$ 9.2 Hz, H-2); ^{13}C NMR (D_2O , 125.72 MHz): δ 105.4 (C-1'), 103.9 (C-1), 82.9 (C-3'), 81.3 (C-4), 77.7 (C-5'), 77.6 (C-5), 77.2 (C-3), 75.8 (C-2), 74.3 (CH_2Ph), 71.9 (C-2'), 69.6 (C-4'), 63.7 (C-6'), 62.9 (C-6). Anal. Calcd for $\text{C}_{19}\text{H}_{27}\text{NaO}_{14}\text{S}$ (534.465): C, 42.70; H, 5.09. Found: C, 42.77; H, 5.12.

3-O-Sulfo- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose sodium salt (19).—Compound 18 (40 mg, 0.075 mmol) was submitted to the same procedure described for 17, affording 19 (32 mg, 96%) as a white powder: $[\alpha]_{\text{D}}^{20} +40.7^\circ$ (c 1.1, water). ^1H and ^{13}C NMR data are in agreement with those reported in the literature for the natural compound.⁶

Benzyl 2,4,6-O-tri-O-acetyl-3-O-methoxycarbonyl-methyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3,6-tri-O-acetyl- β -D-glucopyranoside (23).—Compound 1 (200 mg, 0.46 mmol) and Bu_2SnO (125 mg, 0.50 mmol) were stirred in refluxing dry MeOH (4 mL) under N_2 for 16 h. The reaction mixture was concentrated to dryness and the dibutylstannylene intermediate, after coevaporation with toluene, was dissolved in dry DMF (4 mL) under Ar and treated with ethyl bromoacetate (255 μL , 2.3 mmol) and TBAI (cat.) at 40 °C for 48 h (TLC 8:1.5 CH_2Cl_2 –MeOH). The reaction mixture was concentrated under diminished pressure and chromatographed to give a pale yellow syrup (181 mg) containing two compounds, namely the expected product 20 and the corresponding lactone derivative 21. The mixture of

these compounds was dissolved in dry MeOH (4 mL) and treated with a 1 M solution of MeONa (30 μ L) in MeOH. After the complete conversion of the faster moving compound into the second (TLC 4:1 CH₂Cl₂–MeOH), the mixture was neutralised with Amberlite IR 120 (H⁺ form) and concentrated leading to crude compound **22**. The residue was dissolved in pyridine (4 mL) and treated with Ac₂O (2 mL). After one night, the reaction mixture was concentrated, diluted with CH₂Cl₂ and washed with water. The organic layer was separated, dried over Na₂SO₄ and concentrated. Purification by flash chromatography (1:1 EtOAc–petroleum ether) afforded **23** as a white foam (193 mg, 56% from **1**): $[\alpha]_D^{20}$ –9.6° (c 1.1, CHCl₃); ¹H NMR (CDCl₃, 200 MHz): δ 7.40–7.21 (m, 5 H, H_{Ar}), 5.39 (br d, 1 H, $J_{3,4}$ 3.1 Hz, H-4'), 5.15 (t, 1 H, $J_{3,4} = J_{2,3}$ 9.0 Hz, H-3), 5.00 (t, 1 H, $J_{2,3}$ 7.9 Hz, H-2'), 4.95 (t, 1 H, $J_{2,3}$ 7.8 Hz, H-2), 4.86 (d, 1 H, J 12.3 Hz, CHHPb), 4.59 (d, 1 H, CHHPb), 4.54 (dd overlapping signal, 1 H, H-6a), 4.52 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.46 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1'), 4.19–4.08 (m, 5 H, H-6b, H-6a', H-6b', OCH₂COO), 3.80 (t, 1 H, $J_{3,4} = J_{4,5}$ 9.8 Hz, H-4), 3.75 (br t, 1 H, $J_{4,5} = J_{5,6}$ 7.0 Hz, H-5'), 3.72 (s, 3 H, COOCH₃), 3.63–3.58 (m, 2 H, H-5, H-3'), 2.13 (s, 9 H, 3 CH₃Ac), 2.08, 2.04, 1.98 (3 s, 9 H, 3 CH₃Ac); ¹³C NMR (CDCl₃, 50.29 MHz): δ 170.3, 170.2, 170.0, 169.6 (6 COOAc, 2 overlapping signals), 101.0, 98.9 (C-1, C-1'), 78.3, 76.2, 72.9, 72.7, 71.7, 70.6, 70.5, 65.2 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 70.6 (CH₂Ph), 66.0, 62.1, 61.2 (C-6, C-6', OCH₂COO), 51.7 (COOCH₃), 20.70–20.59 (CH₃Ac). Anal. Calcd for C₃₄H₄₄O₁₉ (756.702): C, 53.97; H, 5.86; Found: C, 54.01; H, 5.81.

3-O-Carboxymethyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose sodium salt (24**).—To a solution of compound **23** (20 mg, 0.026 mmol) in MeOH (3 mL), a 0.5 M solution of MeONa in MeOH (30 μ L) was added and the reaction mixture was stirred at rt for 16 h. Then water (2 mL) and 0.5 M solution of MeONa in MeOH (30 μ L) were added, the mixture was stirred for additional 20 h at rt, then concentrated under diminished pressure. The residue was dissolved in 1:1 MeOH–water and hydrogenolysed in the presence of 10% Pd/C for 16 h, (TLC 6:3:1 acetone BuOH water). The mixture was filtered through Celite and freeze-dried. The residue was dissolved in 0.1 M NaOH (0.5 mL), loaded onto a Sephadex G 10 column (V_0 = 17 mL, V_i = 23 mL) and eluted with 1:9 EtOH–water. The fraction containing the product were collected and freeze-dried giving **24** (9 mg, 91%) as a white powder: $[\alpha]_D^{20}$ +59.1° (c 0.5, water); ¹H NMR (D₂O, 300 MHz): δ 5.26 (d, H 0.3, $J_{1,2}$ 3.8 Hz, H-1 α), 4.70 (d, H 0.7, $J_{1,2}$ 7.9 Hz, H-1 β), 4.52 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1'), 4.11 (br s, 3 H), 4.06–3.56 (m, 10.3 H), 3.32 (br, 0.70 H, J 8.3 Hz, H-2 β); ¹³C NMR (D₂O, 75.44 MHz): 179.2 (COO[–]), 103.6 (C-1'), 96.6 (C-1 β), 92.6 (C-1 α), 82.7, 79.1, 79.0, 75.9, 75.6, 75.1, 74.6, 73.5, 72.2, 72.0, 70.9, 70.7, 66.2**

(C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5' α and β), 69.3 (OCH₂COO[–]), 61.1 (C-6'), 60.9 (C-6 β), 60.8 (C-6 α). Anal. Calcd for C₁₄H₂₃NaO₁₃ (422.314): C, 39.82; H, 5.49. Found: C, 39.80; H, 5.57.

Benzyl 6-O-thexyldimethylsilyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)- β -D-glucopyranoside (25**).—Compound **1** (200 mg, 0.46 mmol) and Bu₂SnO (125 mg, 0.50 mmol) were stirred in refluxing dry MeOH (4 mL) for 16 h. The reaction mixture was concentrated to dryness and the dibutylstannylene intermediate was dissolved in dry THF (3 mL) and treated with TDSCl (102 μ L, 0.52 mmol) at rt for 18 h. After adding some drops of TEA, the solution was concentrated under diminished pressure. Purification by flash chromatography (95:5 then 9:1 CH₂Cl₂ MeOH) afforded **25** (201 mg, 76%). In order to allow an unambiguous identification of the product, a portion of **25** (50 mg) was submitted to standard acetylation (Ac₂O, Py) and characterised. $[\alpha]_D^{20}$ –29.4° (c 1.3, CHCl₃); ¹H NMR (CDCl₃, 200 MHz): δ 7.38–7.22 (m, 5 H, H_{Ar}), 5.39 (br d, 1 H, $J_{3,4}$ 3.1 Hz, H-4'), 5.14 (t, 1 H, $J_{3,4} = J_{2,3}$ 9.3 Hz, H-3), 5.08–4.91 (m, 3 H, H-2, H-2', H-3'), 4.85 (d, 1 H, J 12.3 Hz, CHHPb), 4.59 (d, 1 H, CHHPb), 4.53–4.45 (m, 3 H, H-1, H-1', H-6a), 4.10 (dd, 1 H, $J_{6a,6b}$ 11.8, $J_{5,6b}$ 4.8 Hz, H-6b), 3.82 (t, 1 H, $J_{3,4} = J_{4,5}$ 9.5 Hz, H-4), 3.70–3.50 (m, 4 H, H-5, H-5', H-6'a, H-6'b), 2.12, 2.11, 2.03, 2.02, 1.99, 1.95 (6 s, 18 H, 6 CH₃Ac), 1.60 (dq, 1 H, H TDS), 0.91–0.79 (m, 12 H, 4 CH₃, TDS), 0.07, 0.04 (2 s, 6 H, 2 CH₃Si TDS).**

Benzyl 2,3,4-tri-O-benzyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3,6-tri-O-benzyl- β -D-glucopyranoside (26**).—To a solution of **25** (165 mg, 0.29 mmol) in dry DMF (2 mL), BnBr (415 μ L, 3.49 mmol) and NaH (60% suspension in mineral oil 104 mg, 2.60 mmol) were added, and the reaction mixture was stirred at rt overnight (TLC 1:9 EtOAc–petroleum ether). After quenching the excess of NaH with MeOH, the reaction mixture was concentrated, the residue was diluted with CH₂Cl₂ and the organic phase was washed with water. The organic layer was separated, dried over Na₂SO₄ and concentrated. The residue was purified by flash chromatography (0.6:10 EtOAc–petroleum ether) affording a colourless syrup (300 mg). The resulting compound was dissolved in THF (10 mL) and, after cooling the solution to –12 °C, treated with a 1 M TBAF solution (540 μ L, 0.54 mmol). The reaction mixture was stirred at rt for 6 h (TLC 2:3 EtOAc–petroleum ether), then the solution was diluted with EtOAc, and washed with satd NH₄Cl. The organic layer was separated, dried over Na₂SO₄, filtered and concentrated. The crude compound was purified by flash chromatography (3:7 then 2:3 EtOAc–petroleum ether) affording **26** (185 mg, 66%) as a white foam: $[\alpha]_D^{20}$ –12.6° (c 1, CHCl₃), lit.²² –14° (c 1.29, CHCl₃). NMR data for this compound are in agreement with those reported in the literature.²²**

Benzyl 2,3,4-tri-O-benzyl-6-O-tert-butoxycarboxymethyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-2,3,6-tri-O-benzyl- β -D-glucopyranoside (27).—To a solution of compound **26** (120 mg, 0.12 mmol) in CH_2Cl_2 (0.2 mL), *tert*-butyl bromoacetate (377 μL , 1.47 mmol), Bu_4NHSO_4 (51 mg, 0.16 mmol) and 33% aq NaOH (0.8 mL) were added and the reaction mixture was stirred at rt for 4 h (TLC 2:3 EtOAc–petroleum ether). The reaction mixture was diluted with water and extracted with CH_2Cl_2 , the organic layer was separated, dried over Na_2SO_4 , filtered and concentrated. The crude compound was purified by flash chromatography (1:9 then 1:4 EtOAc–petroleum ether) affording **27** (129 mg, 96%) as a colourless syrup: $[\alpha]_{\text{D}}^{20} + 3.2^\circ$ (*c* 1.1, CHCl_3); ^1H NMR (CDCl_3 , 300 MHz): δ 5.00 (d, 1 H, *J* 10.7 Hz, CHHPh), 4.98 (d, 1 H, *J* 11.8 Hz, CHHPh), 4.94 (d, 1 H, *J* 12.1 Hz, CHHPh), 4.90 (d, 1 H, *J* 10.9 Hz, CHHPh), 4.80 (d, 1 H, *J* 11.2 Hz, CHHPh), 4.75 (d, 1 H, CHHPh), 4.74 (d, 1 H, CHHPh), 4.73 (d, 1 H, *J* 11.8 Hz, CHHPh), 4.69 (d, 1 H, CHHPh), 4.65 (d, 1 H, CHHPh), 4.63 (d, 1 H, CHHPh), 4.55 (d, 1 H, *J* 12.1 Hz, CHHPh), 4.48 (d, *J* 7.3 Hz, H-1 or H-1'), 4.42 (d, *J* 6.9 Hz, H-1 or H-1'), 4.41 (d, 1 H, CHHPh), 4.15 (m, 2 H), 3.83–3.70 (m, 4 H), 3.65 (d, 1 H, *J* 16.4 Hz, OCHHCOO), 3.57–3.29 (m, 8 H), 1.44 (s, 9 H, 3 CH_3 -*t*-Bu); ^{13}C NMR (CDCl_3 , 75.44 MHz): δ 169.4 ($\text{COO}-t\text{-Bu}$), 102.8, 102.5 (C-1, C-1'), 83.0, 82.5, 81.9, 80.0, 76.7, 75.2, 73.5, 73.0 (C-2, C-3, C-4, C-5, C-2', C-3', C-4', C-5'), 75.3, 75.2, 75.0, 64.7, 73.2, 72.6, 70.9, 69.3, 69.0, 68.3 (7 CH_2Ph , C-6, C-6', $\text{OCH}_2\text{COO}-t\text{-Bu}$), 60.3 (Cq *t*-Bu), 28.1 (CH_3 -*t*-Bu). Anal. Calcd for $\text{C}_{67}\text{H}_{74}\text{O}_{13}$ (1087.297): C, 74.01; H, 6.86. Found: C, 74.09; H, 6.89.

6-O-Carboxymethyl- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose sodium salt (28).—To a solution of **27** (49 mg, 0.045 mmol) in CH_2Cl_2 , 10% CF_3COOH in CH_2Cl_2 (mL) was added. The reaction mixture was stirred at rt for 30 min (TLC 3:7 EtOAc–petroleum ether) and concentrated to dryness. The obtained compound was dissolved in 1:1 EtOAc–MeOH and submitted to hydrogenolysis in the presence of 10% $\text{Pd}(\text{OH})_2/\text{C}$ for 72 h (TLC 6:3:1 acetone–BuOH–water). The mixture was filtered through Celite and freeze-dried. The residue was dissolved in 0.1 M NaOH (0.5 mL), loaded onto a Sephadex G 10 column (V_0 = 17 mL, V_t = 23 mL) and eluted with 1:9 EtOH–water. The fraction containing the product were collected and freeze-dried giving **28** (18 mg, 95%) as a white powder: $[\alpha]_{\text{D}}^{20} + 24.2^\circ$ (*c* 0.6, water); ^1H NMR (D_2O , 300 MHz): δ 5.35 (d, 0.3 H, $J_{1,2}$ 3.8 Hz, H-1 α), 4.81 (H-1 β), 4.58 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1'), 4.17–3.65 (m, 13.3 H), 3.42 (t, 0.7 H, *J* 8.4 Hz, H-2 β); ^{13}C NMR (D_2O , 75.44 MHz): δ 178.7 (COO^-), 103.8 (C-1'), 96.6 (C-1 β), 92.7 (C-1 α), 79.8, 79.7, 75.7, 75.3, 74.7, 74.2, 72.3, 72.1, 71.8, 69.6 (C-2, C-3, C-4, C-5, C-1', C-2', C-3', C-4', C-5' α and β), 70.9, 70.6 (CH_2COO^- , C-6'), 61.1 (C-6 β), 61.0 (C-6 α).

Anal. Calcd for $\text{C}_{14}\text{H}_{23}\text{NaO}_{13}$ (422.314): C, 39.82; H, 5.49. Found: C, 39.88; H, 5.56.

Acknowledgements

A.R. is grateful to Professor R.R. Schmidt for helpful discussion on the sialyllactosides synthesis. This work was supported by EU-NOFA project (grant FAIR CT973142), MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica) and CNR (Centro di Studio sulle Sostanze Organiche Naturali).

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OCCURRENCE AND SOME PROPERTIES OF NEURAMINIDASES IN *HAEMOPHILUS AVIUM* AND *HAEMOPHILUS PARAGALLINARUM*

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(Received 4 May 1977; revised 20 February 1978)

ABSTRACT

Müller, H.E. and Hinz, K.-H., 1978. Occurrence and some properties of neuraminidases in
Haemophilus avium and *Haemophilus paragallinarum*. *Vet. Microbiol.* 2: 303–312.

The enzyme neuraminidase (= sialidase, N-acetylneuraminic acid glycohydrolase) occurs in *Haemophilus avium* and *H. paragallinarum* which are both pathogens of poultry. The neuraminidases of these organisms have different properties, i.e., Michaelis constants (K_m) and substrate specificity. The substrates investigated were N-acetylneuraminylactose (NL), colominic acid sodium salt (Col), and bovine submaxillary mucin (BSM), which bear α -ketosidic 2→3, 2→8, and 2→6 linkages of N-acetylneuraminic acid, respectively. The neuraminidase of *H. paragallinarum* had a narrow substrate spectrum, splitting only the α -ketosidic 2→6 linkage of N-acetylneuraminic acid of BSM. In contrast, the neuraminidase of *H. avium* had a broad substrate spectrum and split all linkages investigated in the different substrates, i.e., the 2→3, 2→6, and 2→8 linkage, with slight preference for the 2→8 linkage. The temperature of optimal activity of the neuraminidase of *H. avium* was between 44 and 52°C. Heat inactivation was found to occur between 52 and 60°C with some reactivation of the neuraminidase at 68°C. Finally, there was an inactivation by EDTA.

INTRODUCTION

The agglutination of red cells by bacteria is a useful screening test for detection of neuraminidase. It has been well known for many years that bacteria of the *Haemophilus* group agglutinate erythrocytes (Kraus and Ludwig, 1902), but for a long time the search for neuraminidase was unsuccessful (Müller, 1971a). Recently, however, weak activity of neuraminidase was described in five *Haemophilus* species affecting human beings by Tuyau and Sims (1974, 1975) and Müller and Hinz (1977).

Therefore, we investigated two *Haemophilus* species occurring in poultry (Page, 1962; Yamamota and Somerset, 1964; Hinz, 1973a, b, 1975, 1976). As a working hypothesis, it was proposed that neuraminidase is a common enzyme of bacteria well adapted to their life in animals or man, because N-acetylneuraminic acid-containing substances as substrates of neuraminidase occur in animals but not in plants. On the other hand, neuraminidase seems to be

involved in the pathogenicity of some organisms for man and animals (Müller, 1974a, b). Thus, the properties of neuraminidases in two different species of a well defined family are interesting, especially since *H. paragallinarum* and also *H. avium* are pathogenic bacteria for some poultry (Page, 1962; Yamamoto and Somersett, 1964; Hinz, 1976; Hinz and Kunjara, 1977a, b; Hinz and Müller, 1977). *H. paragallinarum* is a well defined pathogen causing infectious coryza in fowls (Hinz, 1973a, b, 1975, 1976). *H. avium* is a newly established species which requires the V-factor but not the X-factor or serum for growth. X-factor independence is confirmed by the ability to carry out biosynthesis of porphyrin from δ -aminolevulinic acid. *H. avium* produces nitratase, but gives negative reactions to the urease, indole, oxidase and α -fuconidase tests.

In contrast to *H. paragallinarum*, strains of *H. avium* always produce catalase, alkaline phosphatase and α -glucosidase, produce acid from galactose and trehalose, and do not require serum for optimal growth. Most of the *H. avium* strains are esterase negative, produce yellow pigment and do not require CO₂ for optimal growth. In further contrast, *H. avium* differs from *H. paragallinarum* in its inability to cause infectious coryza of chickens after experimental application on the mucous membrane of the respiratory tract. The type strain of *H. avium* is IPDH 2654. It has been deposited in the American Type Culture Collection (ATCC) under the number 29546.

MATERIALS AND METHODS

Strains

The following strains were investigated:

H. avium 0002 (isolated from the heart of a sick fowl);

H. avium 0003 (isolated from the laryngeal lobe of a sick fowl);

H. avium 64, 94, 1254, and 2654 (isolated from the respiratory tract of sick fowls).

H. paragallinarum 0083 and 0222 (isolated by Page, 1962);

H. paragallinarum 733, 1516, 1596, 1598, 1645, 1655, 2213, 2403, and 2600 (isolated from the respiratory tract of fowls suffering from infectious coryza);

H. paragallinarum 17756 (isolated by Yamamoto and Somersett, 1964).

Growth medium

The strains were cultivated on Difco Brain Heart Infusion Agar with the addition of sterile β -nicotinamide adenine dinucleotide (β -NAD) (100 μ g/ml), L-cysteine hydrochloride (100 μ g/ml) and 1% fowl serum.

Preparation

The 18-h cultures were harvested and washed with a 0.15 M NaCl solution

TABLE I
Properties of substrates used in the investigation of *Haemophilus avium* and *Haemophilus paragallinarum* neuraminidases

No.	Substrate	Origin of the substrates	Purveyor	Molecular weight	Form of linkage of the bound NAN	References
1	N-Acetylneurami-nyllactose, NH ₄ salt (NL)	Cow's milk	Fa. Boehringer, Mannheim, Germany	650	85% α,2→3 15% α,2→6	Von Nicolai (1971), Von Nicolai and Zilliken (1974)
2	Colominic acid, sodium salt (Col.)	<i>Escherichia coli</i>	Dr. Y. Uchida, Uji, Kyoto, Japan	3000	α,2→8	McGuire and Binkley (1964), Uchida et al. (1973)
3	Bovine sub-maxillary mucin	Submaxillary gland of cattle	Fa. Boehringer, Mannheim, Germany	10 ⁶	α,2→6	Muller et al. (1975), Pignan and Gottschalk (1966)

cooled to 4° C. The bacterial suspensions were standardized conventionally in 100 mg protein/ml, as determined by the biuret method, and homogenized for 2 min by an Ultraturrax (Janke & Kunkel KG) with cooling.

Substrates of neuraminidase assay

The substrates used for the studies on neuraminidase activities are listed in Table I.

Neuraminidase assay

The enzyme assay was carried out by mixing 0.1 ml of the homogenized bacterial suspension and 0.1 ml of 1 mg/ml of the different substrates, dissolved in a 0.05 M sodium pyruvate buffer, pH 5.5, in a Britton-Robinson buffer system ranging from pH 3.0 to 9.0, respectively. The reaction mixture was incubated for 60 min at 37° C. The enzymatically liberated N-acetylneuraminate (NAN) was determined using Warren's colorimetric thiobarbituric acid assay (Warren, 1959) as is described in previous papers (Müller, 1971 b).

Cholera neuraminidase

The investigated *Haemophilus* neuraminidases were compared with that of *Vibrio cholerae* neuraminidase, 500 U/ml, established as 100%, obtained from Behringwerke, Marburg, West Germany.

RESULTS

Activities and substrate specificities of the neuraminidases of *H. avium* and *H. paragallinarum*, respectively, are listed in Table II.

Generally, *H. avium* neuraminidase shows a stronger activity than that of *H. paragallinarum*. Furthermore, one can see some differences of the substrate specificity of both enzymes in respect of NL and Col as substrates, but no significant differences were found in respect of BSM. The liberation of NAN from NL and Col was determined as functions of the quantities of substrates (Fig. 1). There are important differences of both enzymes and, furthermore, of both investigated substrates. The Michaelis constants (K_m) are obtained by transformation and graphic extrapolation according to Lineweaver-Burk (Table III). There are stronger affinities of *H. avium* neuraminidase to the studied substrates than of *H. paragallinarum* neuraminidase to these substrates.

Due to the weak activity of *H. paragallinarum* neuraminidase, some other investigations were carried out using the more active neuraminidase of *H. avium*.

The activity of this enzyme, plotted as NAN liberated for the three substrates (NL, Col, and BSM) as a function of pH in a Britton-Robinson buffer system ranging from pH 3.0 to 9.0 (Rauen, 1964, pp. 90–104), is shown in

TABLE II
Activity of neuraminidases using different substrates, expressed as percentage of a *Vibrio cholerae* neuraminidase standard (500 U/ml) = 100%

No.	Strain	Species	% NAN liberation from		
			NL	Col	BSM
1	0002	<i>Haemophilus avium</i>	61	100	11
2	0003	<i>Haemophilus avium</i>	88	82	28
3	64	<i>Haemophilus avium</i>	86	95	30
4	94	<i>Haemophilus avium</i>	47	100	14
5	1254	<i>Haemophilus avium</i>	79	100	35
6	2654	<i>Haemophilus avium</i>	59	100	30
7	0083	<i>Haemophilus paragallinarum</i>	12	1	20
8	0222	<i>Haemophilus paragallinarum</i>	3	1	10
9	733	<i>Haemophilus paragallinarum</i>	2	7	3
10	1516	<i>Haemophilus paragallinarum</i>	11	1	2
11	1596	<i>Haemophilus paragallinarum</i>	13	5	3
12	1598	<i>Haemophilus paragallinarum</i>	2	1	11
13	1645	<i>Haemophilus paragallinarum</i>	5	4	17
14	1655	<i>Haemophilus paragallinarum</i>	4	1	25
15	2213	<i>Haemophilus paragallinarum</i>	3	1	8
16	2403	<i>Haemophilus paragallinarum</i>	9	1	17
17	2600	<i>Haemophilus paragallinarum</i>	7	1	11
18	17756	<i>Haemophilus paragallinarum</i>	3	1	23

TABLE III
The Michaelis constants (K_m) of *Haemophilus avium* and *Haemophilus paragallinarum* neuraminidase, respectively, using different substrates at pH 5.5

Neuraminidase of:	Substrate	K_m (M)
<i>Haemophilus avium</i> (strain 0002 and 64)	NL	8×10^{-3}
	Col	5×10^{-4}
	BSM	10^{-4}
<i>Haemophilus paragallinarum</i> (Strain 1596 and 2403)	NL	3×10^{-3}
	Col	3×10^{-3}

Fig.2. Col as substrate is hydrolysed the best within a broad pH range between 5.5 and 7.0. BSM is cleaved at two distinct optima at pH 5.5 and 8.0, possibly due to the presence of N-acetylneuraminate and O-acetylneuraminate groups in BSM. NL occupies a middle position, probably due to the nonhomogeneity of this substrate, which consists of 85 and 15% N-acetylneuraminy (2-3) lactose and N-acetylneuraminy (2-6) lactose, respectively. Regarding

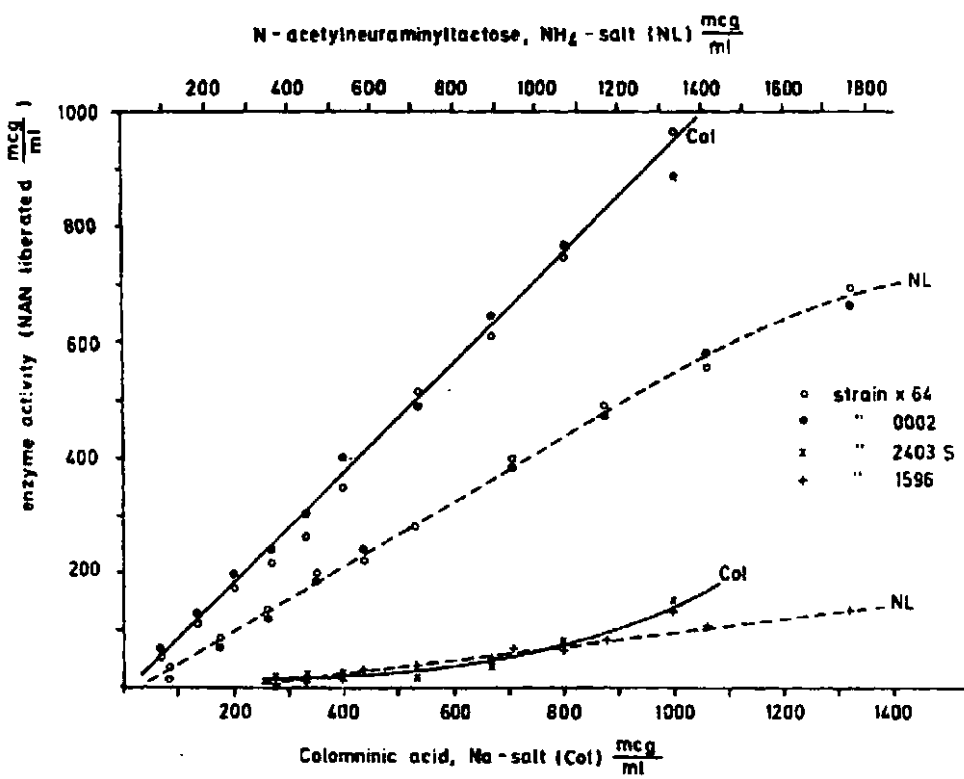


Fig. 1. The liberation of NAN from NL and Col by neuraminidase of *Haemophilus avium* and *Haemophilus paragallinarum*, respectively, in a 0.05 sodium pyruvate buffer, pH 5.5.

the pH optimum at 5.5, there are similar observations with other microbial neuraminidases (Müller, 1974a). The activity of the *H. avium* neuraminidase as a function of temperature is plotted in Fig.3. One can see a considerable temperature gradient and a maximum of enzyme activity within the range between 44 and 52°C. There are some differences in the temperature for optimal enzyme activity for the various substrates.

The thermal stability of neuraminidase from *H. avium* is shown in Fig.4. All other known microbial neuraminidases possess similar properties. They are also, without exception, heat labile.

Regarding the ability of activation by divalent cations, especially by calcium ions, and inactivation by EDTA, respectively, there are some differences between bacterial and viral neuraminidases (Müller, 1974a). In respect of the rough enzyme preparation, containing traces of calcium from the medium, that we investigated, the activity of the enzyme is a function of EDTA concentration, as is shown in Fig. 5. The pH was maintained at a constant level, and one can see evident inactivation of the enzyme by 10⁻³ M EDTA. Therefore, it is suggested that there is also an activation of *H. avium* neuraminidase by divalent cations.

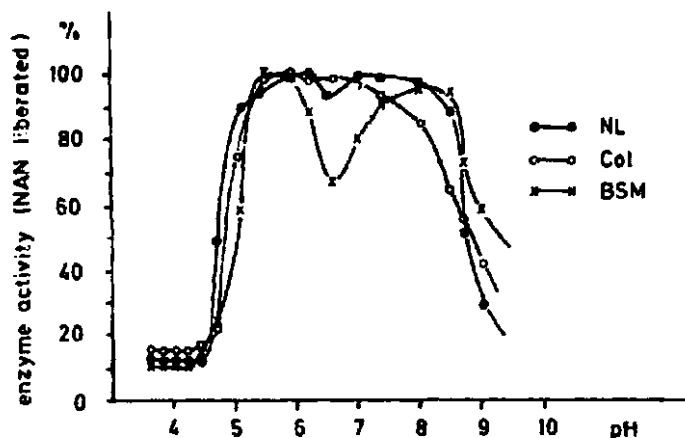


Fig. 2. The activity of neuraminidase of *Haemophilus avium* using NL, Col and BSM as substrates as function of pH in a Britton-Robinson buffer system.

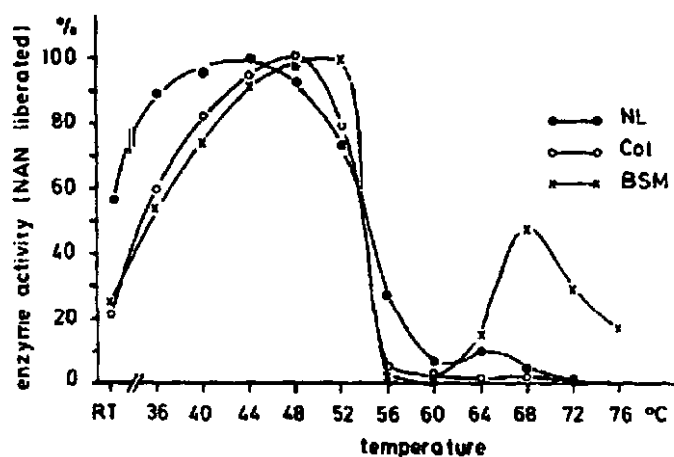


Fig. 3. The activity of neuraminidase of *Haemophilus avium* as function of temperature. NL, Col and BSM were incubated 30 min. The liberated NAN is plotted as a percentage.

DISCUSSION

Two aspects of our results should be discussed: the properties of the two neuraminidases, and, as a conclusion of that, the possible function or pathogenicity of the enzymes. The optimal enzyme temperature, plotted in Fig. 3, can be discussed as a function of the molecular weight of the substrates, because the strongest activity with the low molecular weight NL is at 44°C, whereas the BSM is cleaved best at 52°C. It cannot be excluded that there is a linkage function. Consideration of the enzyme kinetics shows evidence that the molecular weight of the substrates is more important than other factors. Similarly, the neuraminidase activity dependence on the pH is also a function of the molecular weight (Müller, 1974a).

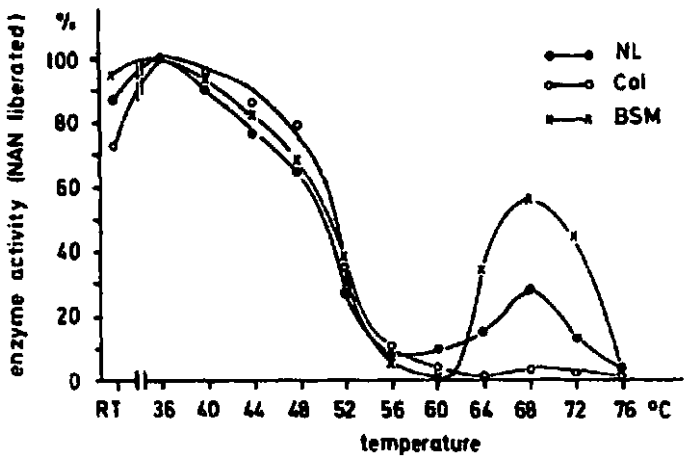


Fig.4. The thermal stability of neuraminidase of *Haemophilus avium*. The enzyme was incubated at different temperatures, after which the enzyme reaction was carried out at 37°C.

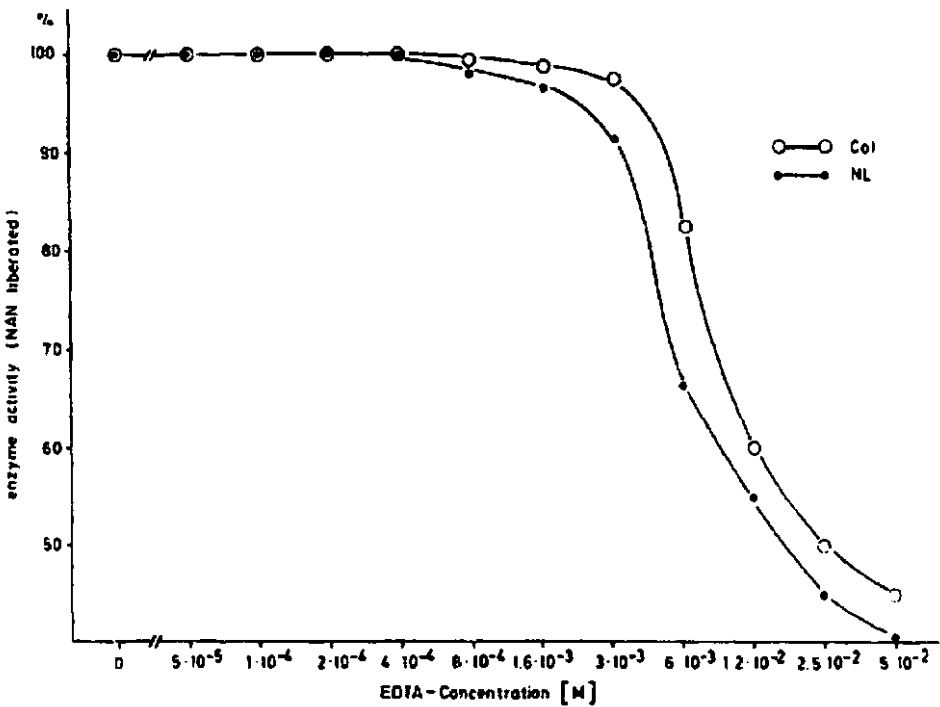


Fig.5. The activity of neuraminidase of *Haemophilus avium* as function of the EDTA concentration.

Regarding the different linkages of the terminal NAN to carbohydrates in the three substrates used (Table I), the results in Table II suggest that the neuraminidase of *H. paragallinarum* splits specifically only the α -ketosidic

2→6 linkage of NAN on BSM, but not the α ,2→3 and the α ,2→8 linkages of NL and Col, respectively. As can be seen in Table II, the neuraminidase activity against NL is somewhat higher than that against Col. This fact might be related to the impurity of the NL because it contains about 15% α ,2→6 bound NAN.

Compared to the *H. paragallinarum* enzyme, the neuraminidase of *H. avium* is generally stronger, has a broader substrate spectrum and less substrate specificity. In other words, the *H. avium* neuraminidase splits all investigated NAN-containing substances more or less in the same manner. Therefore, it resembles the neuraminidase from *Vibrio cholerae*. In relative terms, the α ,2→8 linkage in Col is hydrolysed the best.

The pathogenicity of bacterial neuraminidase can be seen as a function of its activity and breadth of the substrate spectrum, respectively (Müller, 1974a,b; Müller et al., 1975). Therefore, the weak *H. paragallinarum* neuraminidase seems not to be an important pathogenetic factor by splitting effects. The physiological function may be seen more in the reversible attachment on cell surfaces and in the nutrition of the organisms, because N-acetylneuraminate pyruvate lyase, as the following enzyme in the chain of reactions, splits the free NAN into the two metabolisable substances pyruvate and N-acetylmannosamine (Hinz and Müller, 1977).

In contrast, the neuraminidase of *H. avium* shows a strong activity and a broad substrate spectrum, hydrolysing all linkages investigated in this study. Therefore, the enzyme may be involved in the pathogenicity of *H. avium*, as it has been shown to be also in other bacteria with neuraminidases possessing similar broad substrate spectra. This hypothesis is supported by the fact that *H. avium* does not cause a specific clinical picture such as *H. paragallinarum* does.

ACKNOWLEDGEMENTS

This investigation was supported by the Deutsche Forschungsgemeinschaft under Research Project SFB 54, C11.

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(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
16 August 2007 (16.08.2007)

PCT

(10) International Publication Number
WO 2007/090894 A1

- (51) International Patent Classification:
A23L 1/09 (2006.01) A61K 31/702 (2006.01)
A23L 1/29 (2006.01)
- (21) International Application Number:
PCT/EP2007/051288
- (22) International Filing Date: 9 February 2007 (09.02.2007)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
06101556.6 10 February 2006 (10.02.2006) EP
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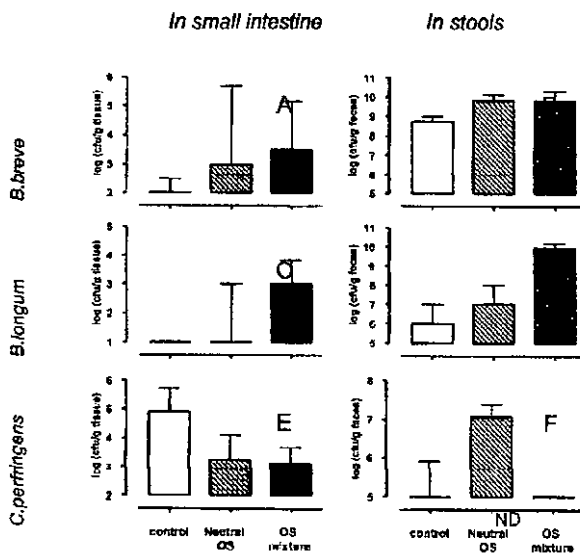
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))
- of inventorship (Rule 4.17(iv))

[Continued on next page]

(54) Title: OLIGOSACCHARIDE MIXTURE



(57) Abstract: An oligosaccharide mixture comprising 5-70 wt% of at least one N-acetylated oligosaccharide selected from the group comprising GalNAc α 1,3Gal β 1,4Glc and Gal β 1,6GalNAc α 1,3Gal β 1,4Glc, 20-90 wt% of at least one neutral oligosaccharide selected from the group comprising Gal β 1,6Gal, Gal β 1,6Gal β 1,4Glc Gal β 1,6Gal β 1,6Glc, Gal β 1,3Gal β 1,3Glc, Gal β 1,3Gal β 1,4Glc, Gal β 1,6Gal β 1,6Gal β 1,4Glc, Gal β 1,6Gal β 1,3Gal β 1,4Glc Gal β 1,3Gal β 1,6Gal β 1,4Glc and Gal β 1,3Gal β 1,3Gal β 1,4Glc and 5-50 wt% of at least one sialylated oligosaccharide selected from the group comprising NeuAc α 2,3Gal β 1,4Glc and NeuAc α 2,6Gal β 1,4Glc; food products comprising said oligosaccharide mixture.

WO 2007/090894 A1

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

OLIGOSACCHARIDE MIXTURE

Field of the invention

- 5 The invention relates to an oligosaccharide mixture, food products comprising said oligosaccharide mixture and processes for producing said oligosaccharide mixture.

Background of the invention

- 10 The human colon is colonised with a wide range of bacteria that have both positive and negative effects on gut physiology as well as having other systemic influences. Predominant groups of bacteria found in the colon include bacteroides, bifidobacteria, eubacteria, clostridia and lactobacilli. The bacteria present have fluctuating activities in response to substrate availability, redox potential, pH, O₂ tension and distribution in the
- 15 colon. In general intestinal bacteria can be divided into species that exert either potentially harmful or beneficial effects on the host. Pathogenic effects (which may be caused by clostridia or bacteroides, for example) include diarrhoea, infections, liver damage, carcinogenesis and intestinal putrefaction. Health-promoting effects may be caused by the inhibition of growth of harmful bacteria, stimulation of immune
- 20 functions, improving digestion and absorption of essential nutrients and synthesis of vitamins. An increase in numbers and/or activities of bacterial groups (such as *Bifidobacterium* and *Lactobacillus*) that may have health promoting properties is desirable.
- 25 As far as infants specifically are concerned, immediately before birth, the gastro-intestinal tract of a baby is thought to be sterile. During the process of birth, it encounters bacteria from the digestive tract and skin of the mother and starts to become colonised. Large differences exist with respect to the composition of the gut microbiota in response to the infant's feeding. The faecal flora of breast-fed infants
- 30 includes appreciable populations of Bifidobacteria with some Lactobacillus species, whereas formula-fed infants have more complex microbiota, with Bifidobacteria, Bacteroides, Clostridia and Streptococci all usually present. After weaning, a pattern of gut microbiota that resembles the adult pattern becomes established.

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Mother's milk is recommended for all infants. However, in some cases breast feeding is inadequate or unsuccessful for medical reasons or the mother chooses not to breast feed. Infant formulae have been developed for these situations.

- 5 One approach to promote the numbers and/or activities of beneficial bacteria in the colon is the addition of prebiotics to foodstuffs. A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health. Such ingredients are non-digestible in the sense that they are not broken down and absorbed in the stomach or small intestine and thus pass intact to the colon where they are selectively fermented by the beneficial bacteria. Examples of prebiotics include certain oligosaccharides, such as fructooligosaccharides (FOS) and galactooligosaccharides (GOS).
- 10
- 15 Human milk is known to contain a larger amount of indigestible oligosaccharides than most other animal milks. In fact, indigestible oligosaccharides represent the third largest solid component (after lactose and lipids) in breast milk, occurring at a concentration of 12-15 g/l in colostrum and 5-8 g/l in mature milk. Human milk oligosaccharides are very resistant to enzymatic hydrolysis, indicating that these
- 20 oligosaccharides may display essential functions not directly related to their calorific value.

- As the composition of human milk becomes better understood, it has also been proposed to add prebiotics to infant formula. Various infant formulas supplemented
- 25 with prebiotics such as mixtures of fructooligosaccharides and galactooligosaccharides for example are commercially available. However, such mixtures approximate only roughly the mixture of oligosaccharides in human milk. Over 100 different oligosaccharide components have been detected in human milk some of which have not been so far detected in animal milks such as bovine milk at all or have been detected
- 30 only in small quantities. Examples of classes of human milk oligosaccharide that are present in bovine milk and colostrum only in very small quantities or not at all are sialylated and fucosylated oligosaccharides.

US Patent Application No. 2003/0129278 describes an oligosaccharide mixture based on oligosaccharides produced from one or several animal milks which is characterized in that it comprises at least two oligosaccharide fractions which are each composed of at least two different oligosaccharides, with free lactose not pertaining thereto. The total spectrum of the oligosaccharides present in the oligosaccharide mixture differs from those present in the animal milk or animal milks from which the oligosaccharide fractions were extracted. Further a) if said oligosaccharides are extracted from only one animal milk, the proportion of neutral oligosaccharides to acidic (sialylated) oligosaccharides is 90-60: 10-40 weight %, or b) if said oligosaccharides are extracted from at least two animal milks, the oligosaccharides extracted from two different animal milks each make up 10 weight % of the total amount of oligosaccharides present in the oligosaccharide mixture.

An object of the invention is to provide an oligosaccharide mixture which is effective as a prebiotic, particularly in the human gut.

Summary of the invention

In one aspect the invention relates to an oligosaccharide mixture which comprises 5-70 wt% of at least one N-acetylated oligosaccharide selected from the group comprising GalNAc α 1,3Gal β 1,4Glc and Gal β 1,6GalNAc α 1,3Gal β 1,4Glc, 20-90 wt% of at least one neutral oligosaccharide selected from the group comprising Gal β 1,6Gal, Gal β 1,6Gal β 1,4Glc Gal β 1,6Gal β 1,6Glc, Gal β 1,3Gal β 1,3Glc, Gal β 1,3Gal β 1,4Glc, Gal β 1,6Gal β 1,6Gal β 1,4Glc, Gal β 1,6Gal β 1,3Gal β 1,4Glc Gal β 1,3Gal β 1,6Gal β 1,4Glc and Gal β 1,3Gal β 1,3Gal β 1,4Glc and 5-50 wt% of at least one sialylated oligosaccharide selected from the group comprising NeuAc α 2,3Gal β 1,4Glc and NeuAc α 2,6Gal β 1,4Glc.

This ingredient is a new protective and immuno-modulating ingredient that is particularly effective as a prebiotic. The mixture is structurally closer to human breast milk oligosaccharides than commercially available prebiotic ingredients, such as FOS and GOS, for example in that it includes a mixture of acidic and neutral oligosaccharides.

In an embodiment the oligosaccharide mixture may be derived from animal milk, such as one or more of cows' milk, goats' milk or buffalo milk.

In another aspect the invention relates to a food product comprising an oligosaccharide mixture as described above. Optionally the food product is an infant food or formula, but the product may be any food or drink consumed by babies, infants or adults. Consumption of a food product containing such an oligosaccharide mixture as a prebiotic will selectively promote the growth and/or activity of one or a limited number of beneficial bacteria in the colon, and thus improve host health.

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Brief Description of the Drawings

Figure 1 shows *Bifidobacterium breve*, (A, B) *B. longum* (C, D) and *C. perfringens* (E, F) counts in the small intestine (jejunum) and stool after 2 weeks of the intervention described in Example 3. Median bacterial counts expressed as log values with robust standard deviation are represented for the control group and group with the OS mixture according to the invention. Probabilities for an effect are indicated based on robust ANOVA. (N = 9 to 10). (NA, not applicable due to values below limit of detection.

Figure 2 shows relative overall metabolic activity of *C. perfringens* over the time of feeding of the intervention described in Example 3. Data are represented as box and whisker plot with median and interquartile range of the control group (grey boxes) and the group with the OS mixture (white boxes). (N=9 to 10). Effect of the prebiotic was significant for day 0 and day 14 as evaluated by median test involving Fisher-Exact test ($p < 0.005$).

Figure 3 shows *Bifidobacterium breve*, *B. longum* and *C. perfringens* counts in small intestine (jejunum)(A,C,E) and stool (B,D,F) after 2 weeks of intervention. Median of bacterial counts expressed as log values with interquartile ranges are represented for the control group, the group with neutral galacto-oligosaccharides only and the group with the oligosaccharide mixture according to the invention. ND, not detected.

Detailed description of the invention

In the present specification, the following words are given a definition that must be taken into account when reading and interpreting the description, examples and claims.

- 5 "Infant formula": foodstuff intended for the complete nutrition of infants during the first four to six months of life. (Article 1.2 of the European Commission Directive 91/321/EEC of 14 May 1991 on infant formulae and follow-on formulae)

10 It has to be understood that infants can be fed solely with infant formulae, or that an infant formula can be fed by the mother or other care-giver as a complement to human milk. The term "infant formula" as used herein is synonymous with the widely used expression "starter formula".

N-acetylated oligosaccharides: oligosaccharides having an N-acetyl residue.

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Neutral oligosaccharides: those oligosaccharides which have no charge and no N-acetyl residue.

20 "Prebiotic": a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health. (Gibson and Roberfroid "Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics" J. Nutr 125:1401 – 1412)

25 "Oligosaccharide": carbohydrate having a degree of polymerisation (DP) ranging from 2 to 20 inclusive but not including lactose.

Sialylated oligosaccharides: oligosaccharides having a sialic acid residue with associated charge

30

The invention provides an oligosaccharide mixture which comprises 5-70 wt% of at least one N-acetylated oligosaccharide selected from the group comprising GalNAc α 1,3Gal β 1,4Glc and Gal β 1,6GalNAc α 1,3Gal β 1,4Glc, 20-90 wt% of at least

- one neutral oligosaccharide selected from the group comprising Gal β 1,6Gal, Gal β 1,6Gal β 1,4Glc Gal β 1,6Gal β 1,6Glc, Gal β 1,3Gal β 1,3Glc, Gal β 1,3Gal β 1,4Glc, Gal β 1,6Gal β 1,6Gal β 1,4Glc, Gal β 1,6Gal β 1,3Gal β 1,4Glc Gal β 1,3Gal β 1,6Gal β 1,4Glc and Gal β 1,3Gal β 1,3Gal β 1,4Glc and 5-50 wt% of at least one sialylated oligosaccharide
- 5 selected from the group comprising NeuAc α 2,3Gal β 1,4Glc and NeuAc α 2,6Gal β 1,4Glc and infant or adult food products comprising such an oligosaccharide mixture.

- Preferably the mixture comprises 10-70 wt% of the specified N-acetylated oligosaccharide(s), 20-80 wt% of the specified neutral oligosaccharide(s) and 10-50
- 10 wt% of the specified sialylated oligosaccharide(s). More preferably the mixture comprises 15-40 wt% of the N-acetylated oligosaccharide(s), 40-60 wt% of the other neutral oligosaccharide(s) and 15-30 wt% of the sialylated oligosaccharide(s). A particularly preferred mixture is 30 wt% of the N-acetylated oligosaccharide(s), 50 wt% of the neutral oligosaccharide(s) and 20 wt% of the sialylated oligosaccharide(s).

- 15 Alternatively, the mixture may conveniently comprise 5-20 wt% of the specified N-acetylated oligosaccharide(s), 60-90 wt% of the specified neutral oligosaccharide(s) and 5-30 wt% of the specified sialylated oligosaccharide(s)

- 20 The oligosaccharide mixture of the invention may be prepared from one or more animal milks. The milk may be obtained from any mammal, in particular from cows, goats, buffalos, horses, elephants, camels or sheep.

- Alternatively the oligosaccharide mixture may be prepared by purchasing and mixing
- 25 the individual components. For example, synthesised galacto-oligosaccharides such as Gal β 1,6Gal β 1,4Glc Gal β 1,6Gal β 1,6Glc, Gal β 1,3Gal β 1,4Glc, Gal β 1,6Gal β 1,6Gal β 1,4Glc, Gal β 1,6Gal β 1,3Gal β 1,4Glc and Gal β 1,3Gal β 1,6Gal β 1,4Glc and mixtures thereof are commercially available under the trade marks Vivinal ® and Elix'or ®. Other suppliers of oligosaccharides are Dextra
- 30 Laboratories, Sigma-Aldrich Chemic GmbH and Kyowa Hakko Kogyo Co., Ltd. Alternatively, specific glycosyltransferases, such as galactosyltransferases may be used to produce neutral oligosaccharides.

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The N-acetylated oligosaccharides may be prepared by the action of glucosaminidase and/or galactosaminidase on N-acetyl-glucose and/or N-acetyl galactose. Equally, N-acetyl-galactosyl transferases and/or N-acetyl-glycosyl transferases may be used for this purpose. The N-acetylated oligosaccharides may also be produced by fermentation technology using respective enzymes (recombinant or natural) and/or microbial fermentation. In the latter case the microbes may either express their natural enzymes and substrates or may be engineered to produce respective substrates and enzymes. Single microbial cultures or mixed cultures may be used. N-acetylated oligosaccharide formation can be initiated by acceptor substrates starting from any degree of polymerisation (DP) from DP=1 onwards. Another option is the chemical conversion of keto-hexoses (e.g. fructose) either free or bound to an oligosaccharide (e.g. lactulose) into N-acetylhexosamine or an N-acetylhexosamine containing oligosaccharide as described in Wrodnigg, T.M.; Stutz, A.E. (1999) Angew. Chem. Int. Ed. 38:827-828.

15 The sialylated oligosaccharides 3'-sialyl-lactose and 6'-sialyl-lactose may be isolated by chromatographic or filtration technology from a natural source such as animal milks. Alternatively, they may also be produced by biotechnology using specific sialyltransferases either by enzyme based fermentation technology (recombinant or natural enzymes) or by microbial fermentation technology. In the latter case microbes may either express their natural enzymes and substrates or may be engineered to produce respective substrates and enzymes. Single microbial cultures or mixed cultures may be used. Sialyl-oligosaccharide formation can be initiated by acceptor substrates starting from any degree of polymerisation (DP) from DP=1 onwards.

25 In a preferred aspect of the invention, the oligosaccharide mixtures described above are incorporated into a food product. In the context of the present invention, the term "food product" is intended to encompass any consumable matter. Hence, it may be a product intended for consumption by humans, in particular infant formula, follow-up formula, baby food such as infant cereals and the like. In particular, the oligosaccharide mixtures of the invention can be incorporated into infant formulas, dehydrated milk or cereal mixtures.

30

The food product may be prepared in any suitable manner known in the art according to the type of product and the oligosaccharide mixture of the invention may be added to the product at an appropriate stage in the manufacturing process. For example, an infant formula may be prepared by blending together the protein source, any
5 carbohydrates other than lactose and the fat source in appropriate proportions. Emulsifiers may be added if desired. Vitamins and minerals may be added at this point but are usually added later to avoid thermal degradation. Any lipophilic vitamins, emulsifiers and the like may be dissolved into the fat source prior to blending. Water, preferably water which has been subjected to reverse osmosis, may then be mixed in to
10 form a liquid mixture.

The liquid mixture may then be thermally treated to reduce bacterial loads. For example, the liquid mixture may be rapidly heated to a temperature in the range of about 80 °C to about 110 °C for about 5 seconds to about 5 minutes. This may be
15 carried out by steam injection or by heat exchanger, e.g. a plate heat exchanger.

The liquid mixture may then be cooled to about 60 °C to about 85 °C, for example by flash cooling. The liquid mixture may then be homogenised, for example in two stages at about 7 MPa to about 40 MPa in the first stage and about 2 MPa to about 14 MPa in
20 the second stage. The homogenised mixture may then be further cooled to add any heat sensitive components such as vitamins and minerals. The pH and solids content of the homogenised mixture is conveniently standardised at this point.

The homogenised mixture is transferred to a suitable drying apparatus, such as a spray
25 drier or freeze drier, and converted to powder. The powder should have a moisture content of less than about 5 % by weight.

The oligosaccharide mixture of the invention is preferably added directly to infant formula by dry mixing. However, if it has been prepared from an animal milk, for
30 example as described below, it may be convenient to add the oligosaccharide mixture without first removing all the lactose. As infant formula contains a carbohydrate component which is often wholly or partially constituted by lactose, it will be apparent to the person skilled in the art that the amount of carbohydrate in the infant formula

will need to be adjusted to take into account the additional carbohydrate that will be provided by the oligosaccharide mixture. The final concentration of the oligosaccharide mixture in the baby or infant food product or formula is preferably from 0.3 to 4%, preferably 0.75 to 1.54% by weight of dry matter. This corresponds to
5 a concentration of from 0.2 to 5 grams per litre of reconstituted formula, preferably 1 to 2 g/l. However, these amounts should not be considered as limitative and should be adapted to the target population, for example based on the weight and age or health of the baby or infant. Preferably, the formula or feed containing the oligosaccharide mixture of the invention is fed to the baby at every feed.

10

Alternatively, the oligosaccharide mixtures may be added to wet infant or adult food products by wet mixing. The mixture may be added to baby or infant formula at concentrations of from about 0.2 to 5 grams of oligosaccharides per litre of product. However, these amounts should not be considered as limitative and should be adapted
15 to the target population, for example based on the weight and age of the baby or infant, or the health of the specific population.

Although it is preferred to supplement food products specifically targeted towards infant or baby nutrition, it may be beneficial to supplement food products not
20 specifically targeted, or targeted to the adult population. For example, the oligosaccharide mixtures of the invention can be incorporated into healthcare nutrition products and nutritional products for the elderly. Such food products may include milk, yoghurt, curd, cheese, fermented milks, milk-based fermented products, ice-creams, fermented cereal based products, or milk-based products, among others.

25

In addition to the oligosaccharide mixture of the invention, a food product such as an infant formula may comprise one or more further oligosaccharides which are added separately.

30 The invention will now be illustrated by reference to the following examples.

Example 1

One method of preparing an oligosaccharide mixture according to the invention will now be described by way of example only.

200,000 litres of a whey ultrafiltration permeate are pre-concentrated to 22% (w/w)
5 total solids (TS), pasteurised at about 75°C for about 30 seconds and then concentrated
by evaporation at 60°C to reach a TS of 59% (w/w). The liquid is cooled in a
crystalliser at a rate of 2°C per hour for a period of 24 hours to crystallise the lactose.
Crystallised lactose is washed then removed by a wringer. The remaining liquid
(mother liquor) is clarified through a decanter. The 77000 litres at 17.7% TS obtained
10 from the clarifier are re-concentrated by evaporation at 60°C to reach a TS of 55%
(w/w) and subject to a second lactose crystallisation step under the same conditions as
before. The 29000 litres at 20.55 TS of the mother liquor thereby obtained are
demineralised by a combination of electrodialysis and ion exchange in a manner known
per se yielding 28500 litres of a 90% demineralised liquor at 17.3 % TS. This liquor,
15 which contains approximately 1.5 grams per litre of a mixture of about 30wt%
GalNAc α 1,3Gal β 1,4Glc and Gal β 1,6GalNAc α 1,3Gal β 1,4Glc, 50 wt% of
Gal β 1,6Gal β 1,6Glc, Gal β 1,6Gal β 1,4Glc and Gal β 1,3Gal β 1,4Glc and 20 wt% of
NeuAc α 2,3Gal β 1,4Glc and NeuAc α 2,6Gal β 1,4Glc, depending upon the starting
material, may either be added directly to a food product such as an infant formula or
20 may be further concentrated in a manner known per se to those skilled in the art.

For example, the lactose remaining in the liquor may be hydrolysed into glucose and
galactose and these monosaccharides may be either be removed by nanofiltration or, if
desired, the galactose may be at least partially polymerised for example by the action of
25 β -galactosidase to produce galacto-oligosaccharides which will also be retained by the
nanofiltration membrane.

Example 2

30 An example of the composition of an infant formula containing a preparation according
to the present invention is given below.

Nutrient	per 100kcal	per litre
Energy (kcal)	100	670

Protein (g)	1.83	12.3
Fat (g)	5.3	35.7
Linoleic acid (g)	0.79	5.3
α -Linolenic acid (mg)	101	675
Lactose (g)	11.2	74.7
OS mixture from Example 1 (g)	0.15	1.0
Minerals (g)	0.37	2.5
Na (mg)	23	150
K (mg)	89	590
Cl (mg)	64	430
Ca (mg)	62	410
P (mg)	31	210
Mg (mg)	7	50
Mn (μ g)	8	50
Se (μ g)	2	13
Vitamin A (μ g RE)	105	700
Vitamin D (μ g)	1.5	10
Vitamin E (mg TE)	0.8	5.4
Vitamin K1 (μ g)	8	54
Vitamin C (mg)	10	67
Vitamin B1 (mg)	0.07	0.47
Vitamin B2 (mg)	0.15	1.0
Niacin (mg)	1	6.7
Vitamin B6 (mg)	0.075	0.50
Folic acid (μ g)	9	60
Pantothenic acid (mg)	0.45	3
Vitamin B12 (μ g)	0.3	2
Biotin (μ g)	2.2	15
Choline (mg)	10	67
Fe (mg)	1.2	8
I (μ g)	15	100
Cu (mg)	0.06	0.4
Zn (mg)	0.75	5

Example 3

The effect of an oligosaccharide mixture according to the invention on the establishment and composition of the intestinal microbiota was investigated in germ free mice.

C3H mice were kept germfree until the age of 8 weeks and fed on a semi synthetic AIN diet. At Day -1 of the intervention, a single dose of a human baby microbiota cocktail was given to each mouse by gavage. The mice were divided into 2 groups and their diet was changed to an AIN diet containing for one group 1.2 % (w/w) lactose as

additional carbohydrate and for the other group 1.2 % (w/w) lactose and 2.5 % (w/w) of an oligosaccharide mixture according to the invention composed of 5 % (w/w) sialyl-oligosaccharides, 5 % (w/w) N-acetylated oligosaccharides and 90% (w/w) neutral oligosaccharides.

5

The microbiota establishment was evaluated in small intestine and stool after 14 days of intervention by plate counting *Bifidobacterium breve*, *B. longum*, and *Clostridium perfringens*. From Figure 1 it may be seen that both resident Bifidobacteria showed increased counts in small intestine and especially in stool. On the other hand *C. perfringens* counts were reduced in small intestine and especially in stool.

10

Over time of feeding the relative metabolic activity of *C. perfringens* was monitored by measuring levels of 16S RNA. Briefly, RNA was extracted from freshly collected faecal samples and RNA was subjected to a RT-PCR reaction to specifically amplify 16S rRNA. PCR products were separated by denaturing gradient gel electrophoresis and *C. perfringens* 16S rRNA was quantified and normalized to the *E. coli* 16S rRNA signal that remained constant during the time of feeding. As may be seen from Figure 2 only one day after the intervention (Day 0 in Figure 2) the metabolic activity of *C. perfringens* was considerably and significantly reduced by the OS mixture according to the invention as compared to the control group and remained lower for the duration of the intervention.

15

20

Example 4

The effect of an oligosaccharide mixture according to the invention on the establishment and composition of the intestinal microbiota was compared with the effect of neutral oligosaccharides alone in gnotobiotic mice.

25

C3H mice were kept germfree until the age of 6 weeks and fed on a semi synthetic AIN diet. A single dose of a human baby microbiota cocktail was given to each mouse by gavage and the microbiota was allowed to establish itself for two weeks. The mice were divided into 3 groups and their diet was changed to an AIN diet containing for the first group 1.2 % (w/w) lactose as additional carbohydrate, for the second group 1.2 %

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(w/w) lactose and 2.5 % (w/w) galacto-oligosaccharides and for the third group 1.2 % (w/w) lactose and 2.5 % (w/w) of an oligosaccharide mixture according to the invention composed of 5 % (w/w) sialyl-oligosaccharides, 5 % (w/w) N-acetylated oligosaccharides and 90% (w/w) neutral oligosaccharides.

5

The microbiota establishment was evaluated in small intestine and stool after 14 days of intervention by plate counting *Bifidobacterium breve*, *B. longum*, and *Clostridium perfringens*. From Figure 3 it may be seen that both resident Bifidobacteria showed increased counts in small intestine and especially in stool in presence of the OS mixture in the diet. In presence of only the neutral galacto-oligosaccharides, the resident *B. longum* did not show increased counts in either small intestine or stool. *C. perfringens* counts were reduced in small intestine and also in stool in the presence of the OS mixture according to the invention. However, in presence of only the neutral galacto-oligosaccharides, increased levels of *C. perfringens* were found in stool. Together, these findings strongly suggest that the oligosaccharide mixture according to the invention has effects on microbiota balance that are superior to neutral oligosaccharides alone.

10

15

Claims

1. An oligosaccharide mixture which comprises 5-70 wt% of at least one N-acetylated oligosaccharide selected from the group comprising GalNAc α 1,3Gal β 1,4Glc and
5 Gal β 1,6GalNAc α 1,3Gal β 1,4Glc, 20-90 wt% of at least one neutral oligosaccharide selected from the group comprising Gal β 1,6Gal, Gal β 1,6Gal β 1,4Glc Gal β 1,6Gal β 1,6Glc, Gal β 1,3Gal β 1,3Glc, Gal β 1,3Gal β 1,4Glc, Gal β 1,6Gal β 1,6Gal β 1,4Glc, Gal β 1,6Gal β 1,3Gal β 1,4Glc Gal β 1,3Gal β 1,6Gal β 1,4Glc and Gal β 1,3Gal β 1,3Gal β 1,4Glc and 5-50 wt% of at least one sialylated oligosaccharide
10 selected from the group comprising NeuAc α 2,3Gal β 1,4Glc and NeuAc α 2,6Gal β 1,4Glc.
2. An oligosaccharide mixture as claimed in Claim 1 which comprises 10-70 wt% of the N-acetylated oligosaccharides, 20-80 wt% of the neutral oligosaccharides and 10-50 wt% of the sialylated oligosaccharides.
15
3. An oligosaccharide mixture as claimed in claim 1 or 2 which comprises 15-40 wt% of the N-acetylated oligosaccharides, 40-60 wt% of the neutral oligosaccharides and 15-30 wt% of the sialylated oligosaccharides.
- 20 4. An oligosaccharide mixture as claimed in any preceding claim which comprises 30 wt% of the N-acetylated oligosaccharides, 50 wt% of the neutral oligosaccharides and 20 wt% of the sialylated oligosaccharides.
5. An oligosaccharide mixture as claimed in Claim 1 which comprises 5-20 wt% of
25 the N-acetylated oligosaccharides, 60-90 wt% of the neutral oligosaccharides and 5-30 wt% of the sialylated oligosaccharides
6. An oligosaccharide mixture as claimed in any preceding claim which is derived from animal milk.
30
7. An oligosaccharide mixture as claimed in claim 6 which is derived from one or more of cows' milk, goats' milk or buffalo milk.

8. An oligosaccharide mixture as claimed in any of claims 1 to 6 which is made synthetically.
9. A food product comprising an oligosaccharide mixture as claimed in any preceding
5 claim.
10. A food product as claimed in Claim 9 which is an infant formula.
11. A food product as claimed in Claim 9 or 10 which comprises from 0.3 to 4% by
10 weight based on dry matter of the oligosaccharide mixture.

Figure 1.

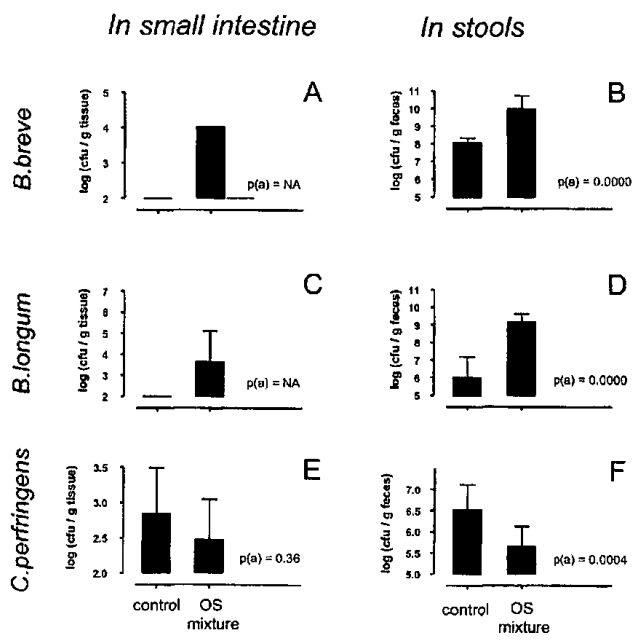


Figure 1 is a box plot showing the relative metabolic activity (% of reference) of control and OS mixture cultures at day 0, day 1, and day 14. The y-axis ranges from 0 to 100. The x-axis is divided into three time points: day 0, day 1, and day 14. For each time point, there are two groups: control and OS mixture. The control group shows a decrease in metabolic activity over time, while the OS mixture group shows a significant decrease in metabolic activity at day 1 and day 14 compared to day 0.

Time Point	Group	Median (%)	Q1 (%)	Q3 (%)	Min (%)	Max (%)
day 0	control	38	23	52	8	63
	OS mixture	10	3	18	1	19
day 1	control	7	4	9	4	11
	OS mixture	1	0	1	0	5
day 14	control	18	11	22	10	23
	OS mixture	1	0	1	0	1

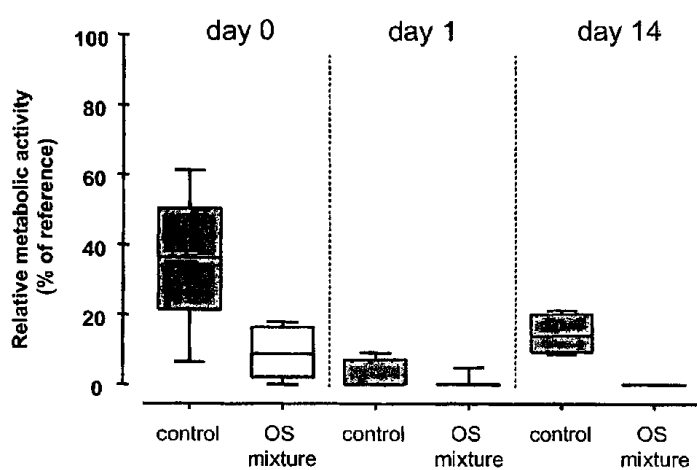
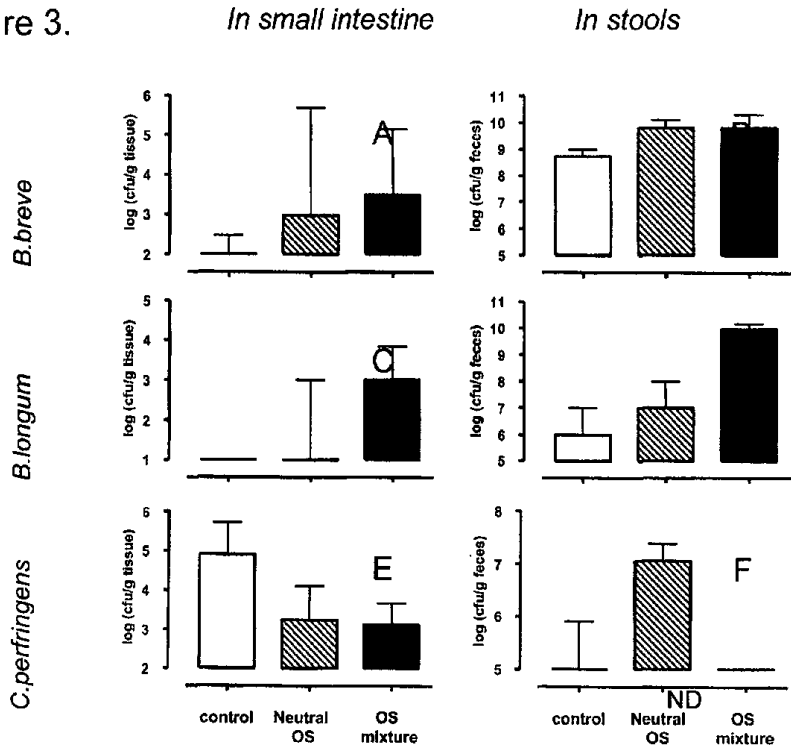


Figure 3.



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2007/051288

A. CLASSIFICATION OF SUBJECT MATTER

INV. A23L1/09 A23L1/29 A61K31/702

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A23L A61K A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, FSTA, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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Date of the actual completion of the international search

5 July 2007

Date of mailing of the international search report

16/07/2007

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INTERNATIONAL SEARCH REPORT

211

International application No

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BNSDOCID: <WO_2007090894A1_1_>



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 31/70	A2	(11) International Publication Number: WO 92/11017 (43) International Publication Date: 9 July 1992 (09.07.92)
(21) International Application Number: PCT/US91/09258 (22) International Filing Date: 9 December 1991 (09.12.91) (30) Priority data: 629,600 17 December 1990 (17.12.90) US (71) Applicant: MONSANTO COMPANY [US/US]; 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US). (72) Inventors: DWEK, Raymond, Allen ; RADEMACHER, Thomas, William ; Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU (GB). (74) Agent: BOLDING, James, Clifton; Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US).		(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: USE OF SIALYLACTOSE FOR THE TREATMENT OF ARTHRITIS (57) Abstract A method for the treatment of arthritis or a related autoimmune disease that exhibits immune complexes in serum or synovial fluid is provided which comprises exposure of the serum or synovial fluid to an oligosaccharide containing a structural component that inhibits or disrupts the degree of occupancy of the Fc carbohydrate binding site on IgG, e.g., sialyllactose.		

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+ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

USE OF SIALYLLACTOSE FOR THE TREATMENT OF ARTHRITIS

5

Background of the Invention

This invention relates to compositions and method
for the treatment of arthritis and related autoimmune
10 diseases.

A known feature of autoimmune diseases is the
presence of immune complexes. Immune complexes help
eliminate foreign bodies such as microorganisms. In
patients with diseases associated with immune complexes,
15 the formation of the immune complex can be a malfunction
of the normal system whereby in some cases the antibody
response is directed against native material (self-
antigen). In other cases the immune response is
directed against the foreign substance repeatedly
20 introduced into the system.

In rheumatoid arthritis, for example, the chronic
inflammation of the synovial membrane of affected
joints, as well as many of the extra-articular
manifestations of the disease, have been ascribed to
25 immune complexes (either localized in the joint or
circulating). The immune complexes in patients with
rheumatoid arthritis consist of IgG homopolymer
complexes [i.e. IgG-(IgG)_n, self-associated] or
heteropolymers [i.e. IgM-(IgG)_n], thereby suggesting
30 that the immunoglobulins are both the antigen and the
antibody. In addition, complement components are
frequently bound to these complexes. The IgG molecules
found in the complexes are referred to as IgG-rheumatoid
factor (IgG-RF) while the IgM molecules in the complexes
35 are called IgM-rheumatoid factors (IgM-RF).

In rheumatoid arthritis it has long been
postulated that auto-sensitization to IgG may play a
pivotal role in the pathogenesis of the disease, and

immune complexes are formed through the binding of the IgM, IgG, or IgA rheumatoid factors to the constant region domains of IgG molecules. The immunogenic site on IgG has been localized to the Fc moiety, but there is
5 no evidence for amino acid changes in the Fc of this IgG. Data on the carbohydrate composition of IgG present in the intermediate complexes isolated from the serum of patients with rheumatoid arthritis invariably show an increased level of sialic acid (usually Fab-associated) as compared to normal serum IgG [Hymes and Mullinax, J. Biol. Chem. 254, 3148 (1979); and Hansson et al., Scand. J. Immunol. 13, 57 (1981)], and decreased
10 content of Fc-associated galactose [Hymes and Mullinax, supra]. In one case, this sialic acid was shown to occur on light chains, and was crucial for IgG self-association, since its removal by neuraminidase treatment abolished complex formation [Hymes and Mullinax, supra]. In a recent study [Pekelharing et al., Ann. Rheum. Dis. 47, 91 (1988)] it was found that
20 serum IgG from patients with rheumatoid arthritis had a lower carbohydrate content and therefore presumably lower Fab N-glycosylation, consistent with the sequestration into complexes of IgG enriched in Fab N-glycosylation. Together, these observations suggest
25 that immune-complex formation in rheumatoid arthritis could involve both Fab N-glycosylation and agalactosyl structures in the Fc. The molecular mechanism whereby these two factors might contribute to IgG auto-antigenicity or self-association are not known, but a
30 study of the crystal structure of Fc provides some insight into this. The crystal structure [Sutton and Phillips, Biochem. Soc. Trans. 11, 130 (1982)] clearly indicates that each N-linked oligosaccharide in the Fc can interact with the protein surface of the CH2 domain,
35 principally via the (NeuNAc α 2-6)Gal β 1-4 segment of the Man α -6 arm. This is the principal non-covalent protein-oligosaccharide interaction in Fc, and serves to restrain the Fc oligosaccharides, and also to mask

certain underlying Fc polypeptide determinants. It is therefore suggested that the change in the degree of occupancy of the Fc carbohydrate-binding site, secondary to decreased outer-arm galactosylation of Fc oligosaccharides, could lead to IgG self-association through one of the following mechanisms. First, through the insertion into this vacant site of an appropriate Fab-linked oligosaccharide from another IgG molecule. Second, through the interaction of the affected IgG with either naturally occurring or induced anti-GlcNAc antibodies. Third, through interaction of the affected IgG with antibodies induced against the peptide (or peptide-oligosaccharide) epitopes previously largely masked by the native oligosaccharide. Self-association of IgG could occur by any of these mechanisms. Further, occupation of the vacant oligosaccharide-binding site by other serum or synovial fluid glycoprotein or cartilage components can also be envisaged.

Additional background information concerning glycosylation patterns in serum IgG of rheumatoid arthritis patients can be had by reference to the following four recent publications:

Parekah et al., "Association of Rheumatoid Arthritis and Primary Osteoarthritis with Changes in the Glycosylation Pattern of Total Serum IgG," Nature **316**, 452-457 (1985).

Parekh et al., "Galactosylation of IgG Associated Oligosaccharides: Reduction in Patients with Adult and Juvenile Onset Rheumatoid Arthritis and Relation to Disease Activity," Lancet **i**, 966-969 (1988).

Parekh et al., "A Comparative Analysis of Disease-Associated Changes in the Galactosylation of Serum IgG," J. of Autoimmunity **2**, 101-114 (1989).

Rademacher et al., "The Role of IgG Glycoforms in the Pathogenesis of Rheumatoid Arthritis," Springer Seminars in Immunopathology **10**, 231-249 (1988).

A recent publication by Pearson and Jamnejad, ICSU Short Rep. **2** (Adv. Gene Technol.), 269-270 (1985),

reported that when rheumatoid factor IgM is incubated with sialyl-N-acetyl-lactose as an additional step in a conventional ELISA test, there was less subsequent interaction with the normal antigen IgG. However, no data are given and the naming of the sugar compound is indefinite. The reported conclusions were that rheumatoid factor from some patients may be specifically blocked by the sialyl-N-acetyl-lactose and that the enzyme galactosyl transferase thus may be involved in the etiology of rheumatoid arthritis.

The known relationships between sialyloligosaccharides and rheumatoid arthritis can be summarized as follows: N-acetylneuraminosyl oligosaccharides of great structural diversity are the major constituents of human milk. That these structures may be important comes from the studies of Witt et al. [Nutr. Metab. 23, 51-61, (1979)] who suggested that these oligosaccharides were not just storage forms of sialic acid but were absorbed and distributed to the tissues intact. Since these studies, it has also become evident that sialyloligosaccharides are important as bacterial anti-adhesions, preventing infection in the newborn.

The urinary excretion of sialyloligosaccharides is known to increase during pregnancy. Interestingly, there is a pregnancy dependence for the excretion of sialyl N-acetyl-lactosamine isomers in urine in contrast to sialyl lactose containing isomers in human milk. The blood serum levels have not been measured. Urinary sialyloligosaccharide excretion can be used as an indicator of disease activity in patients with rheumatoid arthritis [Rheumatol. Int. 1, 7-10, (1981); Eur. J. Clin. Invest. 8, 405-409 (1978)]. Both the urinary excretion of sialyl-lactose and sialyl-N-acetyllactosamine isomers are increased in the disease.

More recent studies have shown that sialyl lactose and sialyl-N-acetyllactosamine may be low-molecular weight acute phase reactants [Ann. of Rheum. Dis. 41, 268-271 (1982)]. In this comparative study rheumatoid

arthritis (RA) patients, with active, aggressive RA had a significantly higher urinary sialyl lactose content, serum CRP and SAA levels than patients with mild disease. In SLE, while sialyl lactose excretion was elevated relative to controls, the excretion correlated neither with serum CRP nor with SAA. This suggests the specific control mechanisms operate for the synthesis of sialyloligosaccharides in RA and pregnancy.

Still more recently, sialyloligosaccharides containing inositol have been found in pregnancy urine [Carb. Res. 115, 221-229 (1983)].

NeuNAc α 2-3Gal β 1-1L-myo-inositol

Their excretion by non-pregnant individuals has not yet been reported.

Research into 'factors' from the urine of pregnant women was initiated in the 1940's by Hensch in his search for the pregnancy factor which causes the remission of arthritis in pregnant women [Mayo Clin. Proc. 24, 167-178, (1949)]. His fractionation of urine led to the eventual discovery of cortisone. Subsequent studies, however, showed that cortisone was not the elusive pregnancy 'factor', which still remains chemically undefined today.

Brief Description of the Invention

In accordance with the present invention, compositions and method for the treatment of arthritis and related autoimmune diseases are provided. The method comprises treating the immune complexes in a body fluid such as serum or synovial fluid by exposure to an oligosaccharide containing a structural component that inhibits or disrupts the degree of occupancy of the Fc carbohydrate binding site on IgG.

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In accordance with conventional nomenclature, the Fc is the crystallizable fragment of the antibody molecule obtained by papain digestion as distinguished from the Fab or antigen-binding fragment.

The preferred oligosaccharides for use in the method of invention are trisaccharides which contain either a reducing terminal glucose or N-acetylglucosamine (GlcNAc) residue substituted with the disaccharide N-acetylneuraminy α -3(6) galactopyranose (i.e. NeuNAc α 3(6)Gal β -). These oligosaccharides are illustrated by the trisaccharide N-acetylneuraminy α -3(6) galactose β -4 glucose, also referred to as sialyllactose, where the galactose and glucose residues are in the D-pyranose configuration.

Sialyllactose is commercially available and also occurs naturally in human milk and bovine colostrum in mixtures of the (2-3) and (2-6)- β -D-galactopyranosyl isomers. In bovine colostrum the mixture is about 83% (2-3) or 3' isomer and 9% (2-6) or 6' isomer; whereas in human milk these isomers exist substantially in the reverse proportions. These commercially available mixtures generally also contain a small amount of 2,6(3)-sialyllactosamine. See, e.g., Table I, below. These oligosaccharide compounds are also found in the urine [Derappe et al., Carbohydr. Res. 145, 341-347 (1985)] and serum [Example 4 hereinafter] of pregnant women.

The effectiveness of the sialyllactose in disrupting or disaggregating immune complexes in the serum and synovial fluid of rheumatoid arthritis patients is demonstrated by comparison with other sugars such as the monosaccharides, glucose, N-acetylglucosamine, N-acetylneuraminic acid (sialic acid) and galactose, and the disaccharide, N-acetyllactosamine, which were ineffective.

Detailed Description of the Invention

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following description taken in conjunction with the accompanying drawings in which, briefly:

FIG. 1 shows the ultracentrifuge analysis of (a) serum from a normal person (Control); (b) serum from a patient with rheumatoid arthritis (RA-); and (c) serum from the same rheumatoid arthritis patient treated with bovine sialyllactose (Sigma mixture, see Table I, below) (RA+) in an embodiment of the invention.

FIG. 2 shows the ultracentrifuge analysis of the serum from a second patient with rheumatoid arthritis (a) without (RA-) and (b) with (RA+) treatment by bovine sialyllactose as in FIG. 1.

FIG. 3 shows the ultracentrifuge analysis of the serum from a third patient with rheumatoid arthritis (a) without (RA-) and (b) with (RA+) treatment by bovine sialyllactose as in FIG. 1.

FIG. 4 is a graphical representation which shows precipitin curves that demonstrate the effectiveness of bovine sialyllactose compared to a control (no sialyllactose) on the binding of rheumatoid factor complexes to heat aggregated IgG in another embodiment of the invention. FIG. 4 also should be compared with FIGS. 5-6.

FIG. 5 is a graphical representation which shows precipitin curves that demonstrate the ineffectiveness of glucose compared to a control (no glucose) on the binding of rheumatoid factor complexes to heat aggregated IgG.

FIG. 6 is a graphical representation which shows the precipitin curves that demonstrate that galactose, N-acetyllactosamine, N-acetylglucosamine and sialic acid all cause an increase in the binding of rheumatoid factor complexes to heat-aggregated IgG when compared to control (no added sugar or +glucose).

FIG. 7 is a graphical representation that shows the effect of sialyllactose (+SL) on rheumatoid factor in human serum compared to similar serum fluid samples without sialyllactose (-SL), both at pH 7, when analyzed by a commercial ELISA (Melisa Rheumatoid HAI Immunoassay), in which absorbance at 495 nm is plotted against serum dilution.

FIG. 8 is a graphical representation that shows the elution profile resulting from high performance anion exchange chromatography (HPEA) of human pregnancy serum using an eluant of 150 mM sodium hydroxide/100 mM sodium acetate, a flow rate of 1 mL/min and triple pulsed amperometric detection (Dionex BioLC System). A, B and C indicate the elution positions of compounds shown in Table I, below.

As illustrated by the results shown in these figures, the effectiveness of oligosaccharides to inhibit or disrupt the formation of immune complexes can be determined by several methods such as, e.g.,

- a) ultracentrifuge analysis;
- b) quantitative precipitin test; and
- c) enzyme-linked immunosorbent assay.

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In the ultracentrifuge analysis, the oligosaccharide-treated serum or synovial fluid of a rheumatoid arthritis patient is compared to rheumatoid arthritis control samples which have not been thus treated and with samples from a normal person. The γ -globulin in a normal person will exhibit a sedimentation rate or velocity 7S which is indicative of monomeric IgG. The sample from a rheumatoid arthritis patient will have a fast-sedimenting material. According to Stanworth and Henney, Immunology 12, 267-274 (1967), the IgG-containing complexes in patients with rheumatoid arthritis can range from 11S to >100S. In the oligosaccharide-treated samples from rheumatoid arthritis patients, this fast-sedimenting material will disappear and be converted essentially to 7S.

The quantitative precipitin test for rheumatoid arthritis is based essentially on serologic reactions. According to this test, the patient's serum is reacted with a variety of serological systems, all of which contain γ -globulin in some form or a component of Cohn Fraction II obtained from plasma or serum, which is largely γ -globulin [Cohn et al., J. Clin. Invest. 23, 417-432 (1944); Cohen et al., J. Amer. Chem. Soc. 68, 459-475 (1946)]. If the patient's serum is positive for rheumatoid arthritis, the presence of the so-called rheumatoid factor will cause the agglutination or immunoprecipitation reaction which can be compared with normal control samples.

In the quantitative precipitin test, the rheumatoid factor preferably reacts with the γ -globulin which has been heat aggregated to form an equivalence precipitate. This test is further described, for example, by Jaffe, J. Lab. Clin. Med. 60 (3), 409-421 (1962).

In the quantitative precipitin test as used herein, the oligosaccharide-treated serum or synovial fluid of a rheumatoid arthritis patient is compared to

the rheumatoid arthritis sample without such treatment and/or control samples from a normal patient.

In a conventional enzyme-linked immunosorbent assay (ELISA), an antigen or antibody is linked to an insoluble carrier surface. The thus sensitized carrier surface captures the corresponding antibody or antigen from the test solution (e.g., serum). An enzyme-labelled antiglobulin attaches to the antigen or antibody complex, which is detected by the enzyme label changing the color of an added substrate. The optical density of the final color is directly proportional to the amount of unknown antibody or antigen in the original test solution. See, e.g., Engvall and Perlmann, Immunochemistry 8, 871-874 (1971); J. Immunol. 109, 129-135 (1972).

In a typical ELISA procedure, horseradish peroxidase is used as the labeling enzyme and o-phenylenediamine is used as the peroxidase substrate. The reactions are conveniently carried out in plastic microplate wells in which the colorimetric end-point is determined by a spectrophotometric reading at, e.g., 492 nm. The optical density can be plotted against concentration of the known antibody linked to the carrier and the concentration of the unknown read from a standard curve.

An ELISA test for rheumatoid arthritis is illustrated hereinafter (Example 3) by use of the commercially-available Melisa Rheumatoid HAI (human anti-immunoglobulin immunoglobulins) Immunoassay Kit from Walker Laboratories Ltd., Cambridgeshire, England. The reagents in this kit react equally with all immunoglobulin classes, whereby it measures IgG plus IgM rheumatoid factors. See, e.g., Gioud-Paquet et al., Annals of the Rheumatic Diseases 46, 65-71 (1987), and PCT Inter. Appln. WO 88/09933, published December 15, 1988. This kit uses IgG coated onto a micropin solid phase and peroxidase labelled IgG as the enzyme blank. Serum samples are mixed with the labelled IgG and

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allowed to interact with the micropins. Rheumatoid factors cross link the labelled IgG with the solid phase IgG. In the absence of rheumatoid factors, label cannot bind, thereby giving very low assay blanks. The enzyme substrate in this test is o-phenylenediamine-HCl in citrate buffer and horseradish peroxidase is used as the enzyme.

The following detailed examples on the use of sialyllactose for the treatment of serum and synovial fluid from rheumatoid arthritis patients will further illustrate the invention although it will be understood that the invention is not limited to these specific examples.

15 Example 1

Human blood serum (about 400 μ l) was dialysed at 4°C for 3 hours against a buffer composed of 20 mM sodium phosphate/0.1 M NaCl, pH 7.4, containing 0.005% sodium azide, using a BRL continuous-flow dialysis apparatus (Bethesda Research Laboratories). Serum was then diluted with an equal volume of this buffer and centrifuged rapidly (about 10 minutes) at room temperature through a 0.5 μ m Teflon® filter. Half of the diluted serum was raised to 10 mM in sialyllactose/lactosamine, pH 7.4, (mixed isomers from bovine colostrum, Sigma Chemical Company), and an equal volume of buffer was added to the other half of the diluted serum. Both samples were incubated at 37°C for 12 hours and then subjected to ultracentrifuge analysis (Beckman Ultracentrifuge, Model E, fitted with a schlieren optical system). Runs were performed at 20°C and 59,200 rpm. Photographs were taken at 6 minute intervals. A control sample from a normal healthy person with very little high molecular weight was also run.

The results from the sera of 3 patients with rheumatoid arthritis are shown in Figures 1 to 3. Three

time frames are shown for each sample (at 6 minutes, 12 minutes and 18 minutes, respectively, left to right). The small peak moving away from the meniscus is of sedimentation velocity 7S, and therefore monomeric IgG.

5 There is very little faster sedimenting material in the control sample (Figure 1a), but a considerable amount of fast sedimenting material in the serum samples from patients with rheumatoid arthritis (Figures 1b, 2a, 3a). Some of this larger material is discrete, and some is

10 very large. Most of this fast sedimenting material disappears upon incubation with sialyl-lactose and the serum has been converted to normal (Figure 1c, 2b, 3b). Of particular interest is the observation that the 7S peak generally has a greater area after incubation with

15 sialyllactose than before, implying that the fast sedimenting material contains IgG which can be dissociated from the complexes with sialyllactose.

It is thus seen that in the ultracentrifuge analysis, IgG has been dissociated from rheumatoid

20 factor complexes by sialyllactose at physiological pH.

Example 2

Heat aggregated IgG was prepared by heating Cohn

25 Fraction II to 63° for 15 minutes. Increasing amounts of heat aggregated IgG were added to a constant volume of the sample being tested. The reaction mixture was incubated for 4 hours at 37°C and retained for 16 hours at room temperature to allow the equivalence precipitate

30 to form. The supernatants were removed after centrifugation; the precipitates were washed twice with phosphate buffered saline and dissolved in 1M NaOH. The optical density (OD) or absorbance at 280 nm of the

35 milligrams of heat aggregated IgG from which they were derived.

The following samples were tested:

(a) Human blood serum containing rheumatoid factor complexes was dialysed against 0.1 M sodium acetate, pH 3.5, to dissociate the complexes. Half of the sample was made 10 mM in sialyllactose (pH 3.5) and then returned to pH 7 with sodium hydroxide. The other half had an equivalent amount of phosphate buffered saline (PBS) added instead of sialyllactose and was also returned to pH 7 with sodium hydroxide.

The results are shown in Figure 4. The precipitin curve obtained from the sample containing sialyllactose was significantly lower than the curve for the control which contained no sialyllactose or other sugar. The amount of precipitate formed at equivalence was decreased by 17% in the sample containing sialyllactose. In a similar assay comparing glucose with control, Figure 5, little or no effect upon the precipitin curve was observed for glucose

(b) Identical assays were run as in (a) except that 10 mM of other sugars were used instead of 10 mM sialyllactose. The other sugars used for comparison with the oligosaccharide sialyllactose were the monosaccharides, glucose, N-acetylglucosamine, N-acetylneuraminic acid (sialic acid) and galactose, and the disaccharide, N-acetyllactosamine.

The results are shown in Figure 6. the precipitin curves obtained from the samples containing these other sugars resulted in an increase in precipitate when compared to the curve for the control which contained no sugar.

It is thus seen that re-association of rheumatoid factors to form complexes was inhibited by the presence of sialyllactose but not by the other sugars.

Example 3

The commercially-available Melisa Rheumatoid HAI Immunoassay Kit (Walker Laboratories Ltd.,
5 Cambridgeshire, England) was utilized to test directly whether or not siallactose/lactosamine (+SL) isomers can prevent the binding of anti-immunoglobulins to rheumatoid factors, i.e. prevent complex formation. Figure 7 shows that in the presence of
10 sialyllactose/lactosamine (+SL), less rheumatoid factor could be detected in the serum of a patient with rheumatoid arthritis than in similar serum in the absence of sialyllactose/lactosamine (-SL). Since immune-complex formation is concentration dependent, the
15 effect of sialyllactose at the higher serum dilution shows this as a specific inhibitory effect. Results of the serum samples were read directly from a standard curve supplied by the manufacturer since the dilution factor of 1:100 is already included in the value stated
20 on the standard labels.

Example 4

This example illustrates the presence and
25 availability of sialyllactose/lactosamine isomers in pregnancy sera from human sources. Pregnancy sera from human third trimester sources were screened by procedures which consisted of preparation of deproteinised serum samples and high performance anion
30 exchange chromatography (HPEA) with pulsed amperometric detection (Dionex BioLC System). The general methodology of HPAE with PAD for the separation of positional isomers of oligosaccharides is described by Hardy and Townsend, Proc. Natl. Acad. Sci. USA 85, 3289-
35 3293 (1988); Townsend et al, Nature 35, 379-380 (1988). The following conditions were used in screening the pregnancy sera of this example:

Serum (100 μ l) is diluted with 500 μ l distilled water and 200 μ l 0.3M barium hydroxide, vortexed and allowed to stand for 10 min at room temperature. After the addition of 200 μ l of 0.18M zinc sulphate, the mixture was vortexed for 2 min and centrifuged at 1500 g. The resulting supernatant of 150 μ l (equivalent to 15 μ l serum) was chromatographed by HPAE (Dionex BioLC) using an eluant of 150 mM sodium hydroxide/100 mM sodium acetate, flow rate of 1 mL/min and utilizing triple pulsed amperometric detection.

The results of this screening are shown in Figure 8 and compared to results obtained in the analysis of sialyllactose/lactosamine isomers in colostrum as set forth in Table I. The results are expressed in percent of isomer and assume an equal response factor for the different isomers.

Table I

Sera Source (Colostrum)	Peak A	Peak B	Peak C
	6' sialyllactosamine	6' sialyllactose or 3' sialyllactosamine	3' sialyllactose
Sigma Bovine	8%	9%*	83%
Sigma Human	trace	77%*	23%
Monsanto Bovine	14%	10%*	76%

*Predominantly 6' sialyllactose

The amount of the sialyllactose or other active oligosaccharide which would normally be administered therapeutically is primarily dependent upon the physical characteristics of the recipient and the severity of the arthritic conditions. The amount to be administered must be an effective amount, that is, an amount which is

medically beneficial but does not present toxic effects which overweigh the advantages which accompany its use. Administration of the active oligosaccharide in physiologically acceptable solutions such as, for example, physiologic saline, is illustrative of a suitable parenteral solution of the oligosaccharide. According to Witt et al., Nutr. Metab. 23, 51-61 (1979), these oligosaccharides are orally absorbed intact. Other suitable formulations of the active oligosaccharide in pharmaceutically acceptable diluents or carriers in therapeutic dosage form can be prepared by reference to general texts in the pharmaceutical field such as, for example Remington's Pharmaceutical Sciences, Ed. Arthur Osol, 16th ed., 1980, Mack Publishing Co., Easton, Pennsylvania. An illustrative method of administration is by direct injection into the synovial fluid contained in the joint cavities, bursae and tendon sheaths of the rheumatoid arthritis patients. An illustrative effective amount of sialyllactose to dissociate immune complexes in the serum or synovial fluid of a rheumatoid arthritic patient and thereby release a relatively small protein having rheumatoid factor activity and a molecular weight of about 14 kDa as determined by SDS-PAGE under reducing conditions is about 10 μ M. This protein contains the sequence (SEQ ID NO:1)

Phe Thr Ile Ser Arg Asn Asp Ser Lys Asn Thr Leu Tyr Leu
5 10

in which the asparagine in the Asn Asp Ser portion of the sequence is optionally glycosylated. Various other examples will be apparent to the person skilled in the art after reading the foregoing disclosure without departing from the spirit and scope of the invention. It is intended that all such other examples be included within the scope of the appended claims.

-17-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rademacher, Thomas W.
Dwek, Raymond A.

(ii) TITLE OF INVENTION: Use of Oligosaccacharides for Treatment
of Arthritis

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Scott J. Meyer
(B) STREET: 800 North Lindbergh Blvd.
(C) CITY: St. Louis,
(D) STATE: Missouri
(E) COUNTRY: U.S.A.
(F) ZIP: 63043

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

-18-

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Meyer, Scott J.
- (B) REGISTRATION NUMBER: 25,275
- (C) REFERENCE/DOCKET NUMBER: 07-27(496)A

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (314) 694-3117

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Phe Thr Ile Ser Arg Asn Asp Ser Lys Asn Thr Leu Tyr Leu
1 5 10

WHAT IS CLAIMED:

- 1. A method for treatment of arthritis or a related autoimmune disease that exhibits immune complexes in a body fluid such as serum or synovial fluid comprising exposing said body fluid to an oligosaccharide containing a structural component that inhibits or disrupts the degree of occupancy of the Fc carbohydrate binding site on IgG.
- 2. The method of Claim 1 in which the body fluid is serum.
- 3. The method of Claim 1 in which the body fluid is that of a rheumatoid arthritis patient.
- 4. The method of Claim 1 in which the oligosaccharide is sialyllactose.
- 5. The method of Claim 2 in which the oligosaccharide is sialyllactose.
- 6. The method of Claim 3 in which the oligosaccharide is sialyllactose.
- 7. The method of Claim 1 in which the body fluid is the serum of a rheumatoid arthritis patient and the oligosaccharide is sialyllactose.
- 8. The method of Claim 1 in which the oligosaccharide is administered in pharmaceutically acceptable aqueous solution.
- 9. The method of Claim 7 in which the oligosaccharide is administered in pharmaceutically acceptable aqueous solution.
- 10. A method for dissociating immune complexes in the serum or synovial fluid of a rheumatoid arthritis patient by treatment of said serum or synovial fluid with a small but effective amount of sialyllactose to thereby release a protein having rheumatoid factor activity and a molecular weight of about 14 kDa as determined by SDS-PAGE under reducing conditions and contains the sequence (SEQ ID NO:1)

Phe Thr Ile Ser Arg Asn Asp Ser Lys Asn Thr Leu Tyr Leu

5

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in which the asparagine in the Asn Asp Ser portion of the sequence is optionally glycosylated.

11. The method of Claim 10 in which the amount of sialyllactose is 10 μ M.

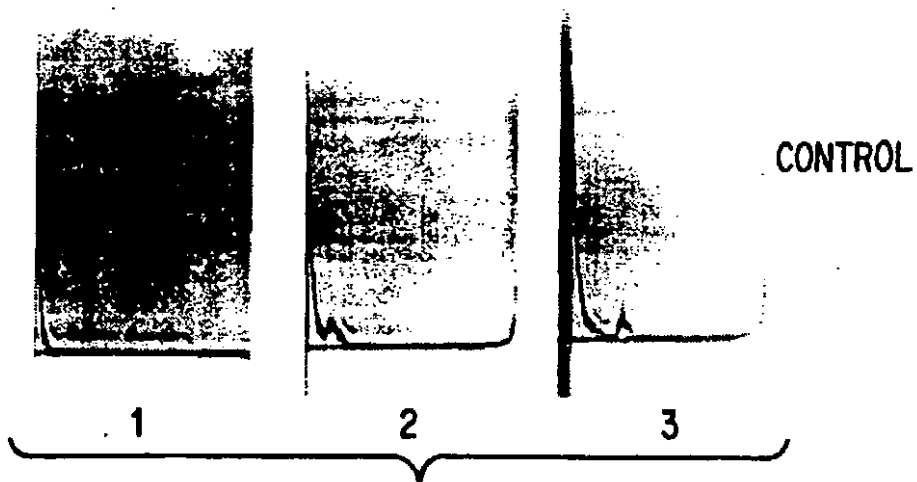


FIG. 1a

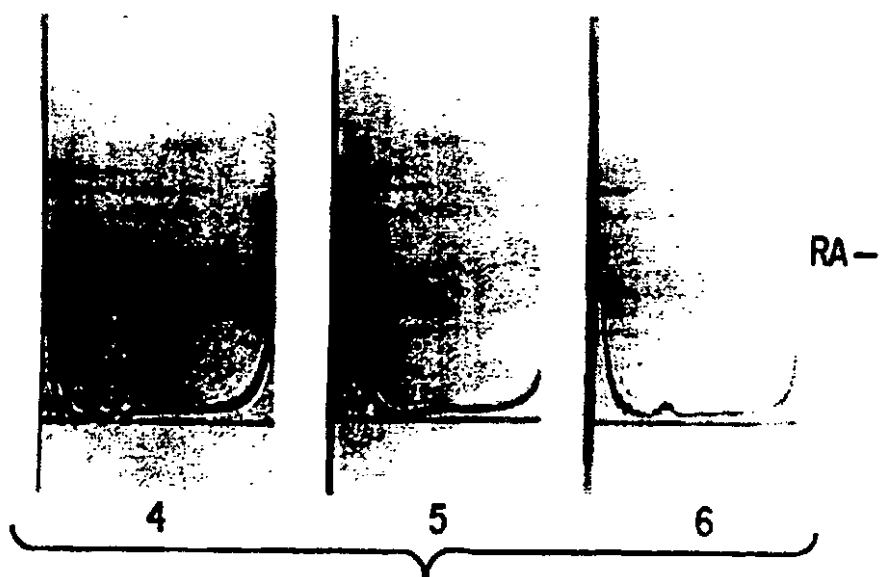


FIG. 1b

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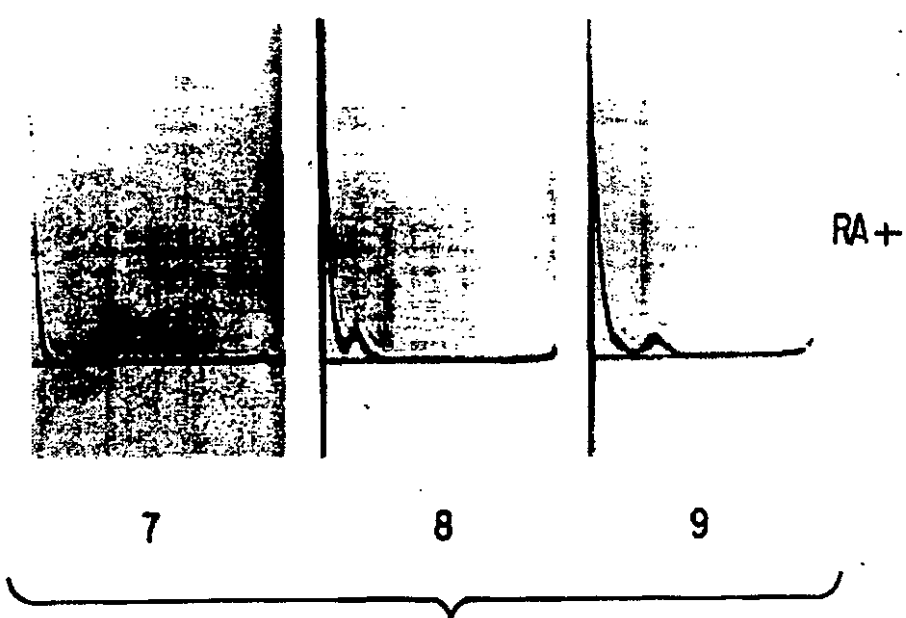


FIG. 1C

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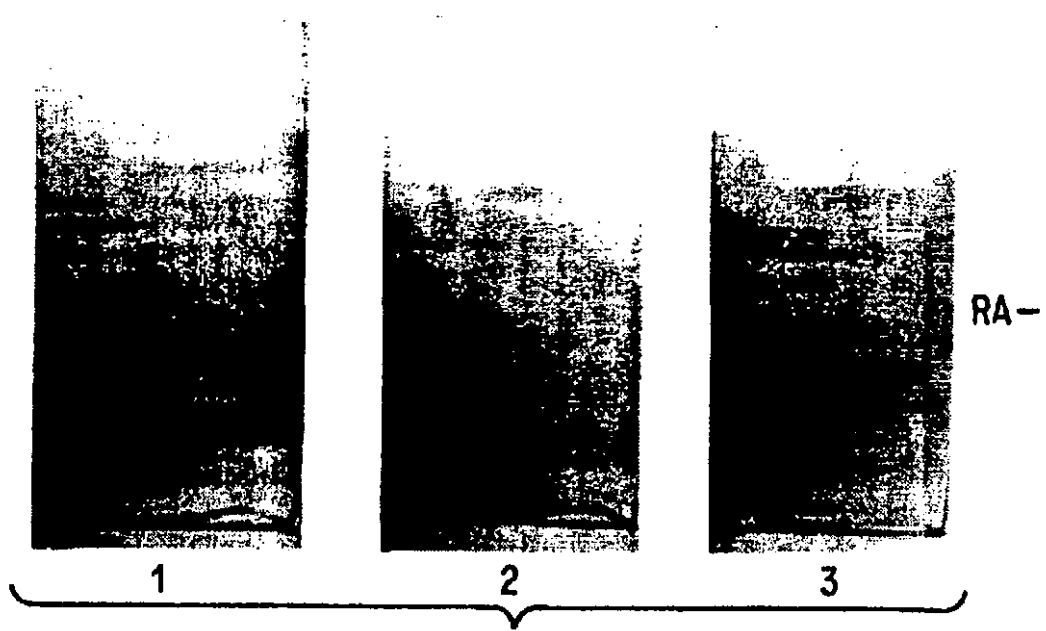


FIG. 2a

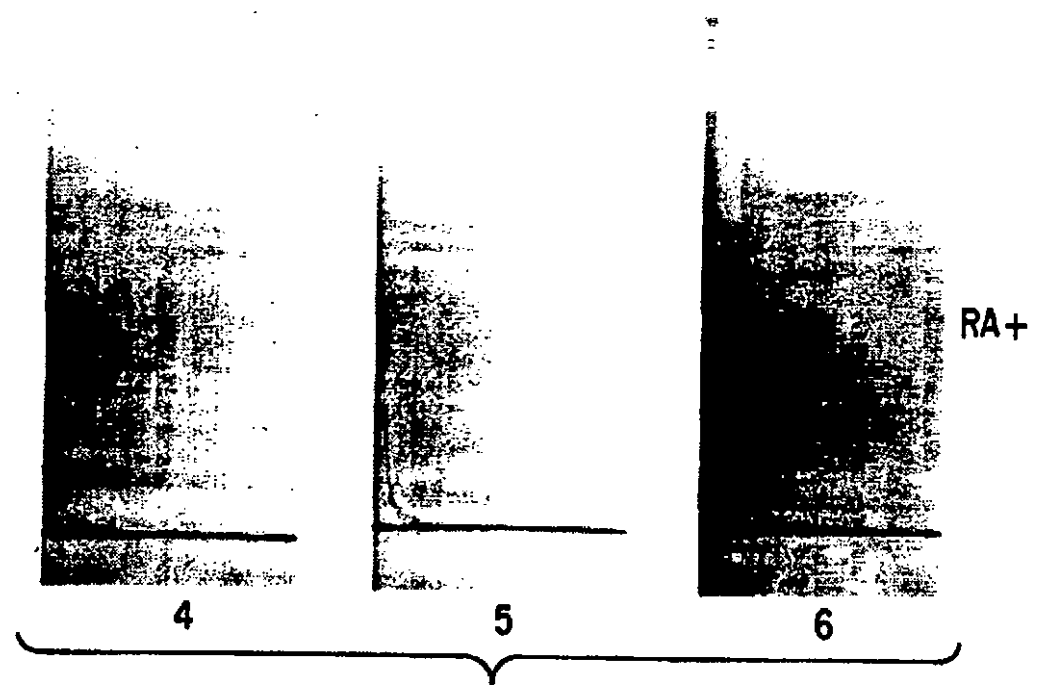


FIG. 2b

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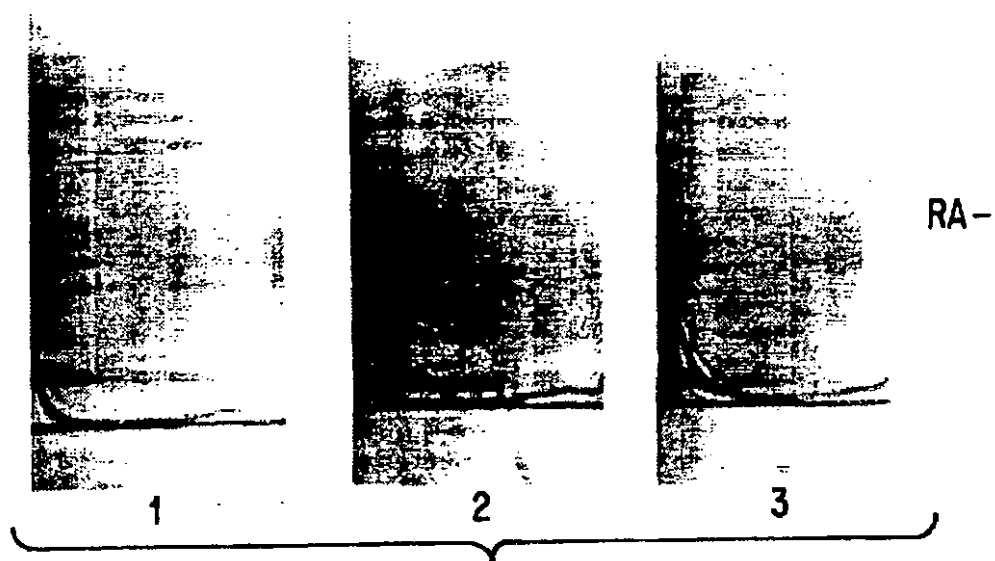


FIG. 3a

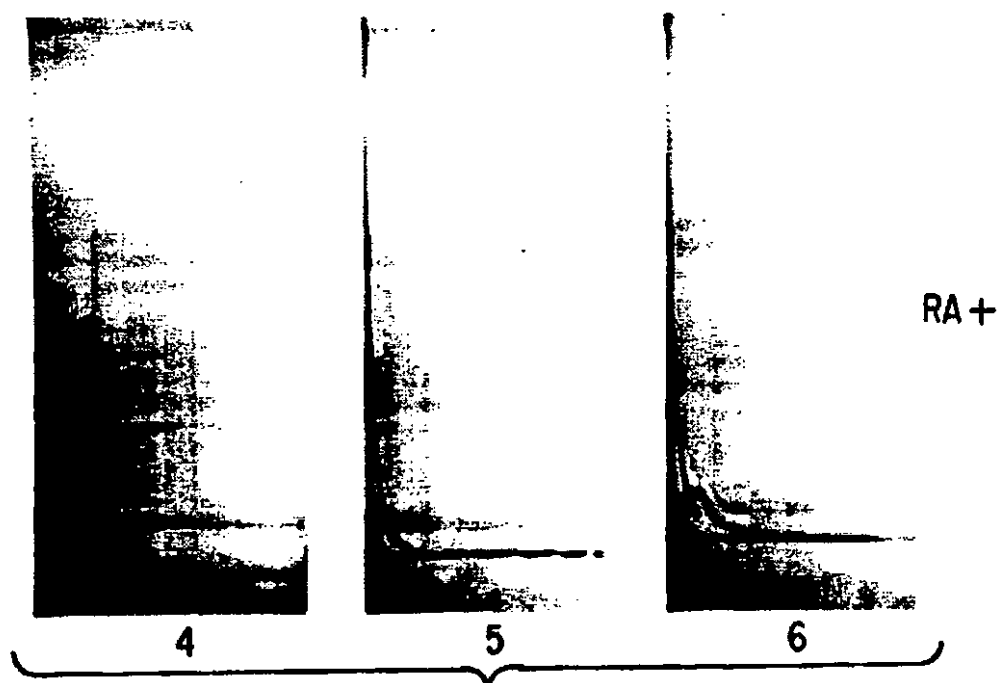


FIG. 3b

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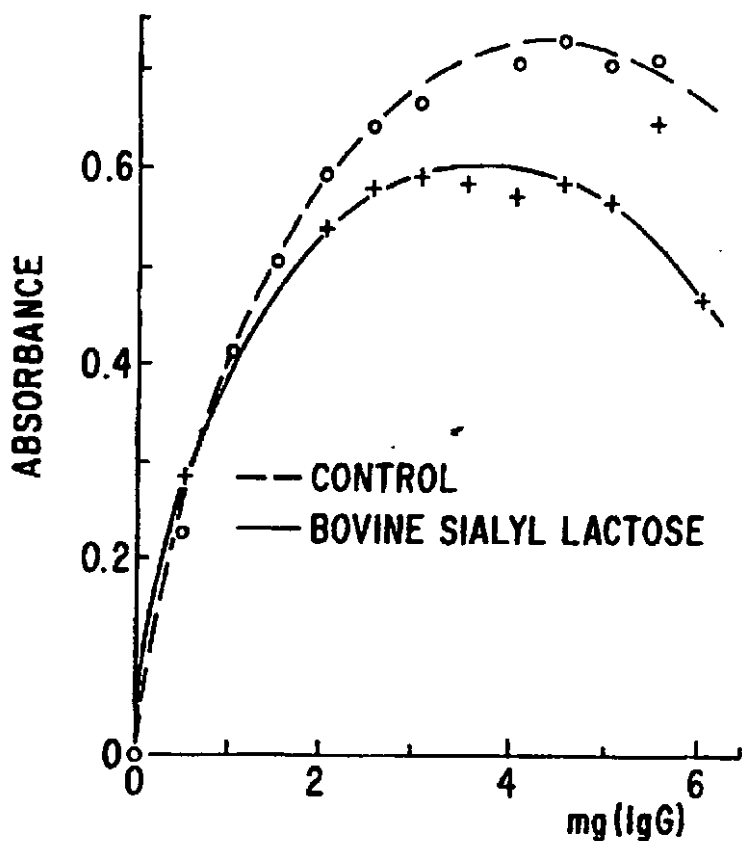


FIG. 4

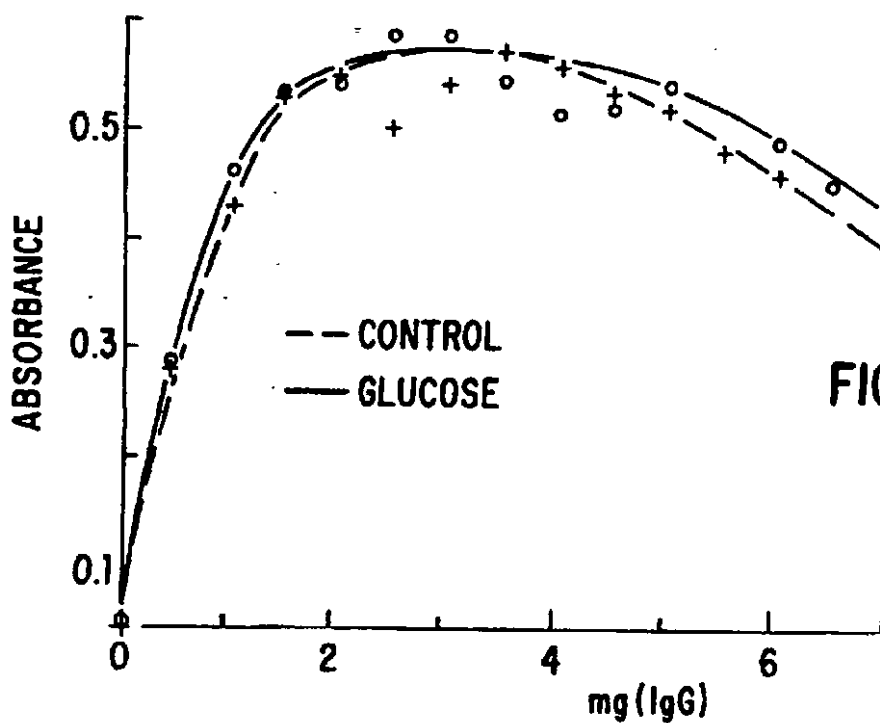


FIG. 5

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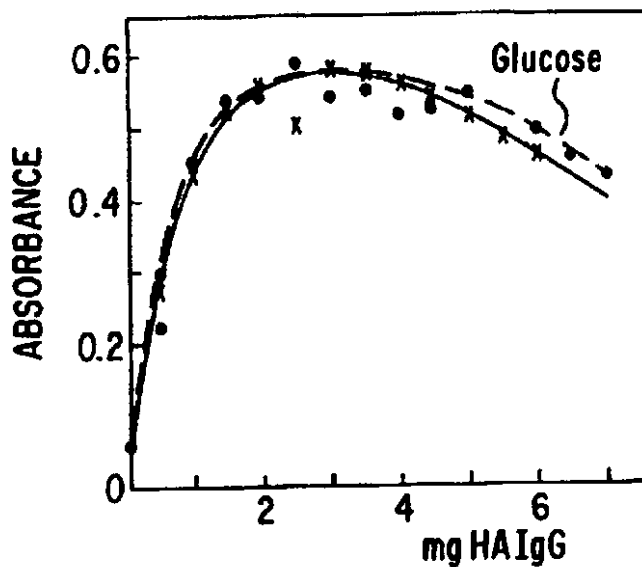


FIG. 6A

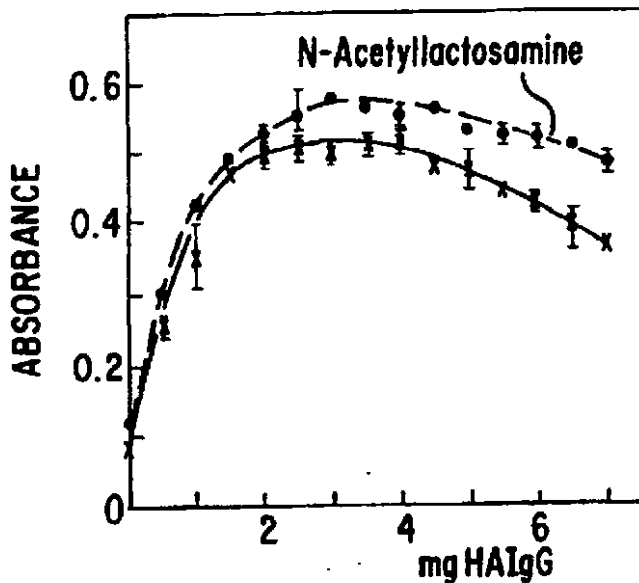


FIG. 6B

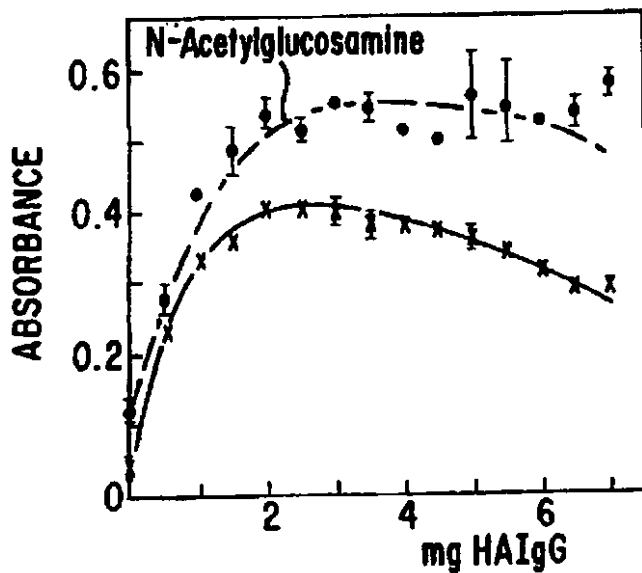


FIG. 6C

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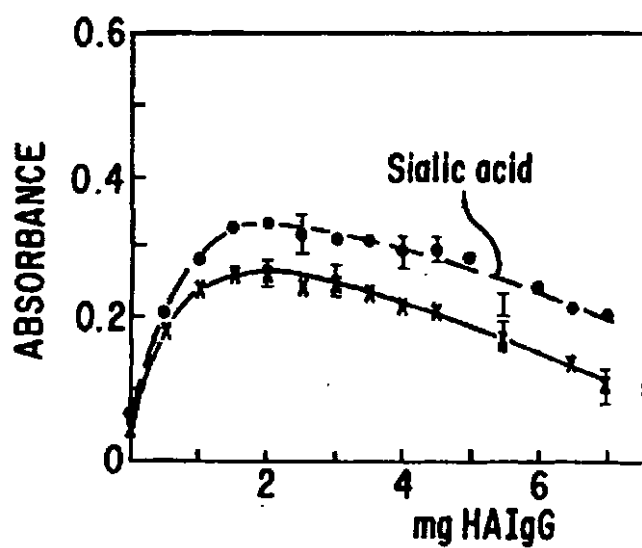


FIG. 6D

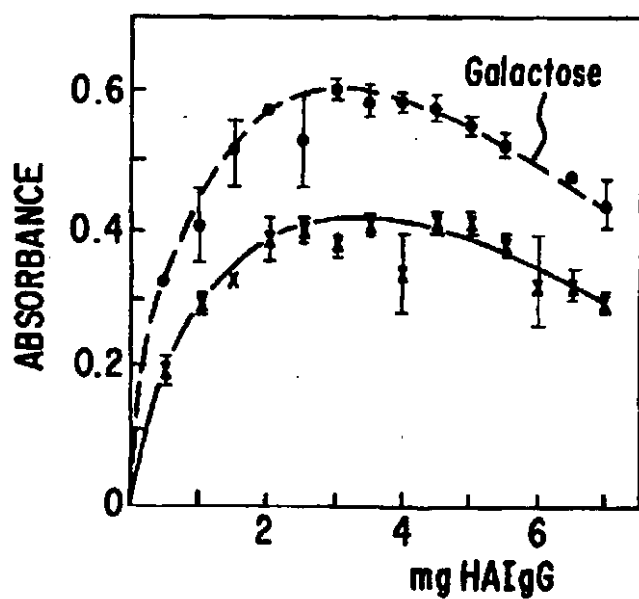


FIG. 6E

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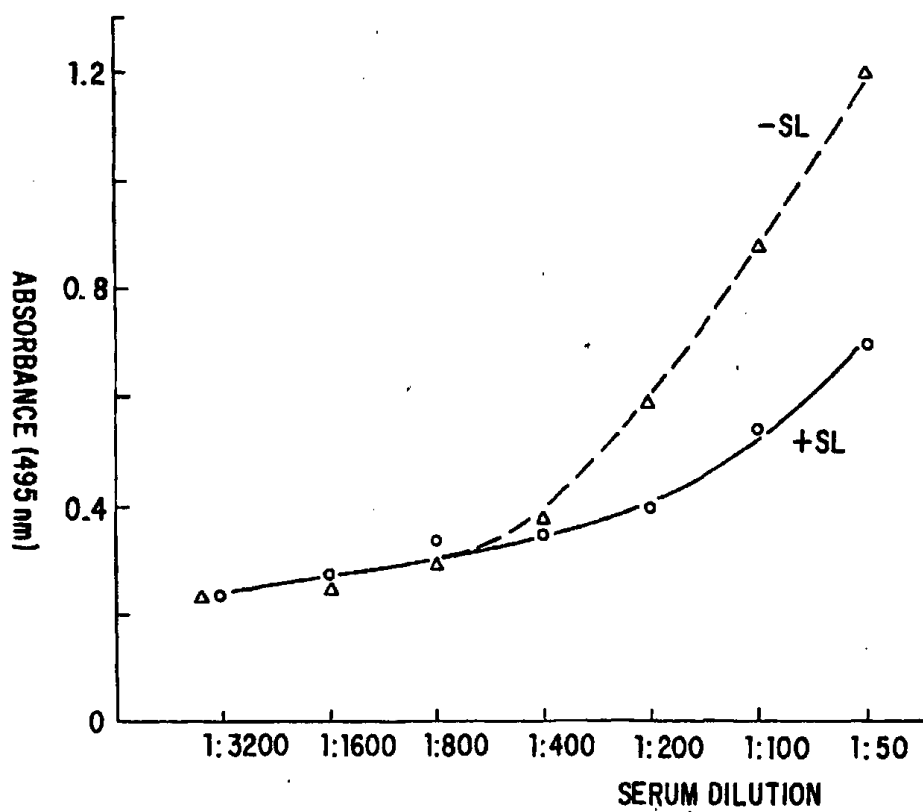


FIG. 7

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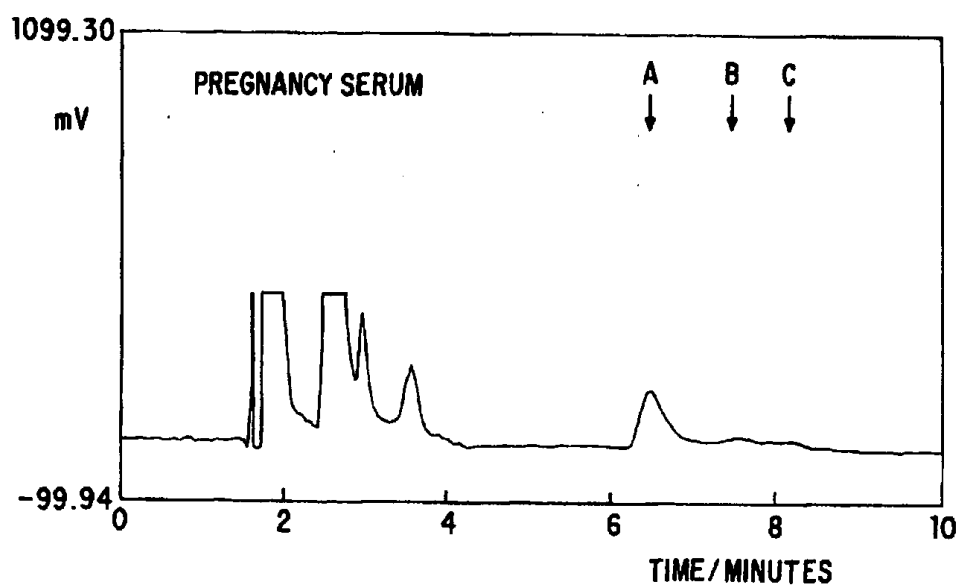


FIG. 8

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 31/70	A3	(11) International Publication Number: WO 92/11017 (43) International Publication Date: 9 July 1992 (09.07.92)
(21) International Application Number: PCT/US91/09258 (22) International Filing Date: 9 December 1991 (09.12.91) (30) Priority data: 629,600 17 December 1990 (17.12.90) US (71) Applicant: MONSANTO COMPANY [US/US]; 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US). (72) Inventors: DWEK, Raymond, Allen ; RADEMACHER, Thomas, William ; Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU (GB). (74) Agent: BOLDING, James, Clifton; Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US).		(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 20 August 1992 (20.08.92)
(54) Title: USE OF SIALYLACTOSE FOR THE TREATMENT OF ARTHRITIS (57) Abstract A method for the treatment of arthritis or a related autoimmune disease that exhibits immune complexes in serums or synovial fluid is provided which comprises exposure of the serum or synovial fluid to an oligosaccharide containing a structural component that inhibits or disrupts the degree of occupancy of the Fc carbohydrate binding site on IgG, e.g., sialylactose.		

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DK	Denmark				
ES	Spain				

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/09258

I. CLASSIFICATION OF SUBJECT MATTER (If several classificative symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.C1.5 A 61 K 31/70		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.C1.5	A 61 K	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	ISCU Short Reports (Advances in Gene Technology: Molecular Biology of the Immune System, Proceedings of the Seventeenth Miami Winter Symposium, Miami, Florida, 11-15 February 1985), vol. 2, D.D. PEARSON et al.: "Interaction of rheumatoid factor and IgG--A blocking effect by the trisaccharide NANA-lactose", pages 269-270, see the whole article ---	1-11
Y	European Journal of Clinical Investigation, vol. 8, no. 6, 1978, Blackwell Scientific Publications, P. MAURY: "Increased excretion of two sialic acid-containing trisaccharides in the urine of patients with rheumatoid arthritis", pages 405-409, see the whole article --- -/-	1-11
* Special categories of cited documents : ¹⁰ "A" documents defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to underlain the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
08-04-1992	10. 07. 92	
International Searching Authority	Signature of ~~Authorized Officer~~ Natalie Weinberg	
EUROPEAN PATENT OFFICE		

Form PCT/ISA/210 (second sheet) (January 1987)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Character of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	The Journal of Biological Chemistry, vol. 247, no. 10, 25 May 1972, (US), P. MAURY; "Increased excretion of N-acetylneuramin-(2+3)-lactose in the urine of pregnant and lactating rats", pages 3153-3158, see the whole article -----	1-11
Y	The American Journal of Medicine, vol. 74, no. 6, 1983, R.M. POPE et al.: "Effect of pregnancy on immune complexes and rheumatoid factors in patients with rheumatoid arthritis", pages 973-979, see the whole article -----	1-11

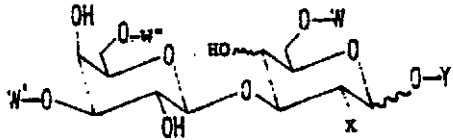
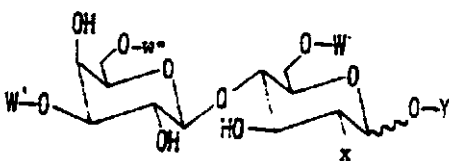
Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹		
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:		
1. ☒ Claim numbers	1-3,8	because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-9 are directed to a method of treatment of the human or animal body the search has been carried out and based on the alleged effects of the compound. Claims 10-11 have been searched in so far as they refer to a method in vitro.		
2. ☐ Claim numbers	...	because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
... Since the oligosaccharide of claim 1 is not sufficiently characterized from the chemical point of view, the search was limited to sialyllactose (see Art.6 of the PCT)		
3. ☐ Claim numbers	...	because they are dissonant claims and are not drafted in accordance with the second and third sentences of PCT Rule 4.4(a).
VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²		
This international Searching Authority found multiple inventions in this international application as follows:		
1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application		
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only these claims of the international application for which fees were paid, specifically claims:		
2. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:		
4. ☐ As all searchable claims could be searched without effort (satisfying an additional fee, the international Searching Authority did not invite payment of any additional fee.		
Remark on Protest		
☐ The additional search fees were accompanied by applicant's protest.		
☐ No protest accompanied the payment of additional search fees.		

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 31/70, 31/715	A1	(11) International Publication Number: WO 94/03184 (43) International Publication Date: 17 February 1994 (17.02.94)
(21) International Application Number: PCT/US93/07010 (22) International Filing Date: 29 July 1993 (29.07.93) (30) Priority data: 922,519 31 July 1992 (31.07.92) US (71) Applicant: NEOSE PHARMACEUTICALS, INC. (US/ US); 102 Witmer Road, Horsham, PA 19044 (US). (72) Inventors: ROTH, Stephen ; 1105 Rose Glen Road, Glad- wyne, PA 19035 (US). McGUIRE, Edward, J. ; 751 Ringgold Street, Philadelphia, PA 19130 (US). LANG- ER, Dennis, H. ; 12 Cleveland Lane, Princeton, NJ 08540 (US).		(74) Agents: GNUSE, Robert, F. et al.; Oblon, Spivak, McClel- land, Maier & Neustadt, Fourth Floor, 1755 South Jef- ferson David Highway, Arlington, VA 22202 (US). (81) Designated States: AU, BB, BG, BR, BY, CA, CZ, FI, HU, JP, KP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.
(54) Title: COMPOSITIONS FOR TREATING AND INHIBITING GASTRIC AND DUODENAL ULCERS  (I)  (II) (57) Abstract A composition for treating and/or inhibiting gastric and duodenal ulcers, comprising an oligosaccharide selected from Formula I, Formula II or mixtures thereof. Furthermore the compound of Formula II is not NAN alpha(2->3) Gal Beta 1-4 Glu or NAN alpha(2->6) Gal Beta 1-4 Glu. Compounds of the Formula I or Formula II detect the presence of <i>Helicobacter pylori</i> which is a microorganism that adheres to mammalian tissue. Also the compounds act to eliminate <i>Helicobacter pylori</i> from the stomach and duodenum.		

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BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LJ	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

Description

Compositions For Treating and Inhibiting Gastric and Duodenal Ulcers

Related Applications:

This application is a Continuation-in-Part Application of U.S. Serial No. 07/922,519, filed on July 31, 1992, the entire contents of which are hereby incorporated by reference.

Technical Field

The present invention relates to compounds and compositions for treating and inhibiting gastric and duodenal ulcers, and to methods of treating and inhibiting gastric and duodenal ulcers.

Background Art

Infection by the gram-negative, spiral, microaerophilic bacterium *Helicobacter pylori* (*H. pylori*), formerly known as *Campylobacter pylori* (*C. pylori*), is a primary cause of non-autoimmune gastritis, is a factor in peptic ulcer disease and is more common in patients with gastric carcinoma. First isolated by Warren (Lancet (1983) 1:1273) and Marshal (Lancet (1983) 1:1273-5), *H. pylori* has been isolated in gastric tissue biopsies in patients throughout the world. While the precise mechanism of inflammation is not well understood, *H. pylori* is found in association with the apical surfaces of gastric mucous-secreting cells.

Due to the site specificity of attachment, it has been suggested that there are specific attachment sites for *H. pylori* which exist on gastric and duodenal mucous-secreting cells. Numerous studies have been undertaken to attempt to identify the specific binding site of *H. pylori*.

Evans et al (Infection and Immunity (1988) 56:2896-2906) reported that *H. pylori* binding to an erythrocyte receptor is preferentially inhibited by N-acetylneuraminyl- α (2 $\rightarrow$ 3)-Gal β 1 $\rightarrow$ 4 Glc [NeuAc(2 $\rightarrow$ 3)-lactose] as compared with NeuAc(2 $\rightarrow$ 6)-lactose. Sialoproteins which contain the NeuAc(2 $\rightarrow$ 3)Gal isomer of NeuAc-lactose, i.e., human erythrocyte glycophorin A, fetuin, and human α_2 -macroglobulin, also inhibited *H. pylori* binding, but at much higher concentrations (mg/ml) than that observed for NeuAc(2 $\rightarrow$ 3)-lactose, while no inhibition was observed for the corresponding asialoproteins.

These researchers further observed that NeuAc-lactose (also called sialyllactose) containing primarily the NeuAc(2 $\rightarrow$ 6)Gal isomer showed no inhibition of binding, leading the researchers to conclude that the receptor on the erythrocytes is a sialoprotein containing NeuAc(2 $\rightarrow$ 3)Gal. Although the NeuAc(2 $\rightarrow$ 3)Gal moiety, which Evans et al believed to be the specific site of binding for *H. pylori*, is a structure which occurs widely in nature, they rationalized the selective binding of *H. pylori* to be due to the unique gastrointestinal environment. This, in their view, accounts for the lack of further colonization by *H. pylori*.

Evans et al (Infection and Immunity (1989) 57:2272-2278) have also observed that *H. pylori* binds to monolayers of Y-1 mouse adrenal cells. But, this adherence can be prevented by pretreating the Y-1 cells with neuraminidase and is blocked by sialyllactose-containing fetuin.

Lingwood et al (Lancet (1989) 2:238-241) have reported the isolation of a gastric glycerolipid material which they observed to behave as a receptor for *H. pylori*. The material was isolated from red blood cells, and mucosal scrapings of pig stomach and human stomach. The investigators postulated that the material was a sulphated alkylacylglycero-lipid, but the actual structure of this material was not been reported. Subsequent investigations (Lingwood et al., Infection and

Immunity (1992) 60:2470-2474) showed that this receptor is phosphatidylethanolamine.

Tzouvelekis et al (Infection and Immunity (1991) 59:4252-4253) reported binding inhibition of *H. pylori* to HEp-2 cells by gastric mucin. The investigators observed that purified mucin showed the greatest inhibition of *H. pylori* binding while asialomucin exhibits somewhat diminished inhibition and periodate-oxidized mucin exhibited the lowest level of binding. On these observations, the researchers concluded that sialic acids are at least partially responsible for the binding interaction between *H. pylori* and human gastric mucin.

Thus the binding inhibition studies all point to a *H. pylori* binding specific receptor which possesses an N-acetylneuraminic acid (sialic acid) (Tzouvelekis et al and Evans et al) bound in a 2-3 manner to a lactose (Evans et al).

In addition to the numerous binding inhibition studies, methods have been pursued to treat gastric and duodenal ulcer patients. Colloidal bismuth subcitrate (CBS) has been used successfully in treating both gastric and duodenal ulcer diseases (for a review, see Lambert in Reviews of Infectious Diseases (1991) 13 (Suppl. 8):S691-5. CBS has proven effective as a histamine H2 antagonist and has been associated with lower relapse rates after cessation of therapy attributed to CBS's ability to eradicate *H. pylori*. Bismuth subsalicylate (BSS) has also been observed to inhibit *H. pylori*.

Additional studies in eliminating *H. pylori* have been conducted using the proton pump inhibitor, omeprazole.

Coleman et al (U.S. Patent No. 4,935,406) reported a method for relieving gastrointestinal disorder, resulting from *H. pylori* population, through the administration of bismuth (phosph/sulf)ated saccharide compositions. The saccharide

compositions according to this method are simple phosphates and sulfates of aldose and ketose monosaccharides.

Clinical trials have been reported (Evans et al, Ann. Internal Med. (1991) August 15, 115(4):266-9) in treating *H. pylori* using ranitidine in conjunction with a "triple therapy" of amoxicillin or tetracycline, metronidazole (an antiprotozoal), and BSS. The clinical studies suggested that ulcer healing was more rapid in patients receiving ranitidine plus the "triple therapy" than in patients receiving ranitidine alone.

However, long-term eradication of this organism has been difficult with these therapies. The antibiotic approach runs the risk of the development of new antibiotic resistant strains. Thus, a method of treating *H. pylori* with good long-term eradication has not yet been developed.

Disclosure of the Invention

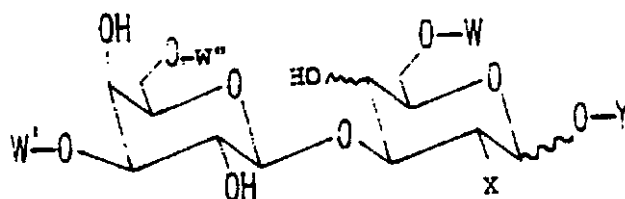
Accordingly, one object of the present invention is to provide novel compositions and methods for inhibiting and/or treating gastric and/or duodenal ulcers.

Another object of the present invention is to provide a method for inhibiting the adhesion of *Helicobacter pylori* to mammalian tissue, including eliminating *Helicobacter pylori* from the stomach and/or duodenum of a in need thereof patient.

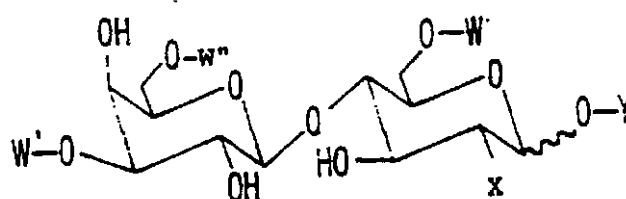
Another object of the present invention is to provide a kit for detecting the presence of *Helicobacter pylori* in a sample.

All of the above objects of the present invention and other objects which are apparent from the description of the invention given herein below have been discovered by the inventors to be satisfied by a composition comprising an

oligosaccharide selected from the group consisting of Formula I



; Formula II



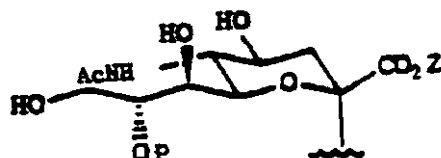
or a mixture thereof;

wherein:

X is independently OH or NHAc;

Y is independently H, or an amino acid or a peptide of 2-100, preferably 2-20, amino acids; and

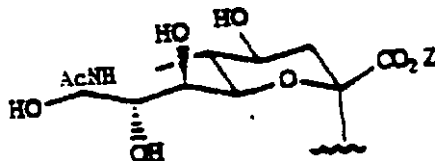
W, W', and W'' are each independently H or



; where Z is independently H or a pharmaceutically acceptable

cation; and

P is independently H or



where Z is defined as above;

wherein at least one of W, W' or W'' is an α -N-acetylneuraminic acid moiety, and

wherein W' and W'' are not simultaneously an α -N-acetylneuraminic acid moiety

with the proviso that the compound of Formula II is not NAN $\alpha(2\rightarrow3)$ Gal $\beta 1-4$ Glu or NAN $\alpha(2\rightarrow6)$ Gal $\beta 1-4$ Glu.

A composition containing at least one oligosaccharide of Formula I or Formula II alone, or in combination with an H₂ blocker, an antibiotic and/or an antiulcerative compound, has been found by the inventors to be effective at inhibiting the binding of *Helicobacter pylori* to the gastric and duodenal mucosa and relieving the effects of gastric and duodenal ulcers.

Best Mode for Carrying Out the Invention

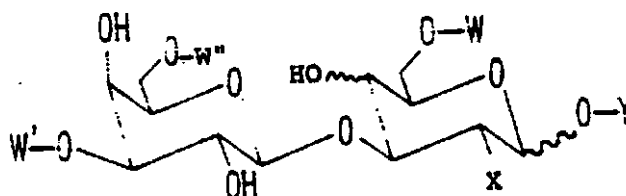
The following abbreviations are used throughout the text: "GalNAc" for N-Acetylgalactosamine; "Gal" for galactose; "Glc" for glucose; "GlcNAc" for N-Acetylglucosamine; "NAN" or "NeuAc" for N-Acetylneuraminic acid; and "ser" for serine.

In the oligosaccharide of Formula I, the OH group at the

4-position of the sugar on the right side of the molecule represents both the axial and equatorial epimeres. When the OH at the 4-position is in the axial position, then the sugar on the right side of the molecule is a Gal or GalNAc moiety. When the OH at the 4-position is in the equatorial position, then the sugar on the right side of the molecule is a Glc or GlcNAc moiety. Thus the core structure of Formula I represents either a Gal β 1-3 Gal, Gal β 1-3 GalNAc, Gal β 1-3 Glc or Gal β 1-3 GlcNAc.

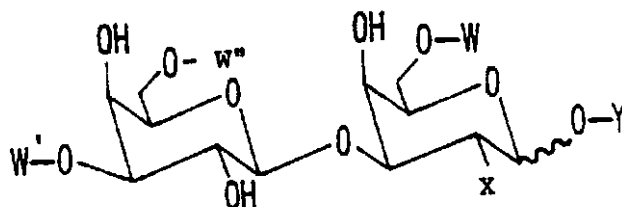
The oligosaccharides according to Formula I of the present invention comprise a core made up of an α -N-acetylneuraminic acid moiety bound via its 2-position to either the 3-position or 6-position of a β -galactose moiety, which, in turn, is bound via its 1-position to the 3-position of a galactose, N-acetylgalactosamine, glucose or N-acetylglucosamine moiety, which is bound via its 6-position to the 2-position of an α -N-acetylneuraminic acid. Alternatively, any of the α -N-acetylneuraminic acid groups, but not all simultaneously, may be replaced with H.

In a preferred embodiment, this core structure further comprises an amino acid bound to the oxygen at the 1-position of the galactose, N-acetylgalactosamine, glucose or N-acetylglucosamine moiety, to provide as shown below an oligosaccharide of Formula I:

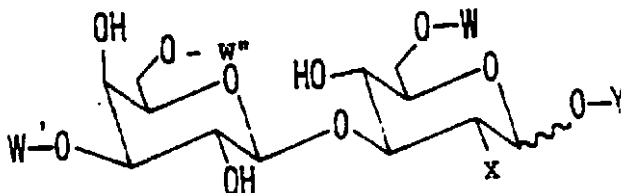


wherein X is OH or NHAc, Y is serine or threonine, W and W' are each an α -N-acetylneuraminic acid moiety, W'' is H and Z is independently H or a known pharmaceutically acceptable cation.

Specifically, the oligosaccharide of Formula I may be Formula Ia



or Formula Ib



where X, Y, W, W', W'', Z and P are as defined above for Formula I.

In other preferred embodiments, the oligosaccharide of Formula I is selected from the group NAN α 2-3Gal β 1-3(NAN α 2-6)GalNAc, NAN α 2-3Gal β 1-3(NAN α 2-6)GalNAc α 1-ser, NAN α 2-3Gal β 1-3GalNAc,

NAN α 2-6Gal β 1-3(NAN α 2-6)GalNAc, NAN α 2-6Gal β 1-3(NAN α 2-6)GalNAc α 1-ser, NAN α 2-6Gal β 1-3GalNAc, Gal β 1-3(NAN α 2-6)GalNAc,

NAN α 2-3Gal β 1-3(NAN α 2-6)Gal, NAN α 2-3Gal β 1-3(NAN α 2-6)Gal α 1-ser, NAN α 2-3Gal β 1-3Gal,

NAN α 2-6Gal β 1-3(NAN α 2-6)Gal, NAN α 2-6Gal β 1-3(NAN α 2-6)Gal α 1-ser, NAN α 2-6Gal β 1-3Gal, Gal β 1-3(NAN α 2-6)Gal,

$\text{NAN}\alpha 2\text{-3Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{GlcNAc}$, $\text{NAN}\alpha 2\text{-3Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{GlcNAc}\alpha 1\text{-ser}$, $\text{NAN}\alpha 2\text{-3Gal}\beta 1\text{-3GlcNAc}$,

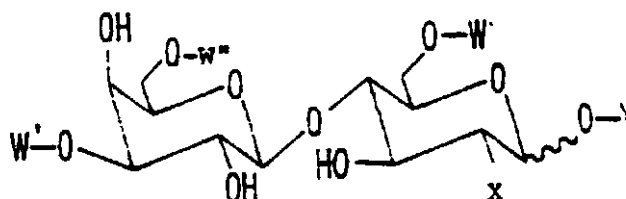
$\text{NAN}\alpha 2\text{-6Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{GlcNAc}$, $\text{NAN}\alpha 2\text{-6Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{GlcNAc}\alpha 1\text{-ser}$, $\text{NAN}\alpha 2\text{-6Gal}\beta 1\text{-3GlcNAc}$, $\text{Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{GlcNAc}$,

$\text{NAN}\alpha 2\text{-3Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{Glc}$, $\text{NAN}\alpha 2\text{-3Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{Glc}\alpha 1\text{-ser}$, $\text{NAN}\alpha 2\text{-3Gal}\beta 1\text{-3Glc}$,

$\text{NAN}\alpha 2\text{-6Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{Glc}$, $\text{NAN}\alpha 2\text{-6Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{Glc}\alpha 1\text{-ser}$, $\text{NAN}\alpha 2\text{-6Gal}\beta 1\text{-3Glc}$, $\text{Gal}\beta 1\text{-3}(\text{NAN}\alpha 2\text{-6})\text{Glc}$ and a mixture thereof.

The oligosaccharides according to Formula II of the present invention comprise a core made up of an α -N-acetylneuraminic acid moiety bound via its 2-position to either the 3-position or 6-position of a β -galactose moiety, which, in turn, is bound via its 1-position to the 4-position of a glucose or N-acetylglucosamine moiety, which is bound via its 6-position to the 2-position of an α -N-acetylneuraminic acid. Alternatively, any of the α -N-acetylneuraminic acid groups, but not all simultaneously, may be replaced with H. However, the compound of Formula II does not include $\text{NAN } \alpha(2\rightarrow 3)\text{Gal } \beta 1\text{-4 Glu}$ or $\text{NAN } \alpha(2\rightarrow 6)\text{Gal } \beta 1\text{-4 Glu}$.

In a preferred embodiment, this core structure further comprises an amino acid bound to the oxygen at the 1-position of the glucose or N-acetylglucosamine moiety, to provide as shown below an oligosaccharide of Formula II:



wherein X is OH or NHAc, Y is serine or threonine, W and W' are each an α -N-acetylneuraminic acid moiety and Z is independently H or a known pharmaceutically acceptable cation.

In other preferred embodiments, the oligosaccharide of Formula I is selected from the group NAN α 2-3Gal β 1-4(NAN α 2-6)GlcNAc, NAN α 2-3Gal β 1-4(NAN α 2-6)GlcNAc α 1-ser, NAN α 2-3Gal β 1-4GlcNAc,

NAN α 2-6Gal β 1-4(NAN α 2-6)GlcNAc, NAN α 2-6Gal β 1-4(NAN α 2-6)GlcNAc α 1-ser, NAN α 2-6Gal β 1-4GlcNAc, Gal β 1-4(NAN α 2-6)GlcNAc,

NAN α 2-3Gal β 1-4(NAN α 2-6)Glc, NAN α 2-3Gal β 1-4(NAN α 2-6)Glc α 1-ser,

NAN α 2-6Gal β 1-4(NAN α 2-6)Glc, NAN α 2-6Gal β 1-4(NAN α 2-6)Glc α 1-ser, Gal β 1-4(NAN α 2-6)Glc and a mixture thereof.

In addition, for the oligosaccharide of Formula I or Formula II the group Y may represent an amino acid or peptide of from 2-100 amino acids, preferably 2-20 amino acids. It is noted that the glycoprotein fetuin, contains a peptide of at least 500 amino acids.

In addition, any one of or all of the free hydroxyl groups on the oligosaccharide of Formula I or Formula II may be acylated with a C₁₋₆ acyl group by treatment with a suitable acylating agent such as acetyl chloride, propionyl chloride, butyryl chloride or acetic anhydride.

The α -N-acetylneuraminic acid moieties as they appear in Formula I or II may further be substituted at the 8-position with an α -N-acetylneuraminic acid moiety. Accordingly for all of the above-identified compounds of Formula I and Formula II any of the α -N-acetylneuraminic acid moieties may be replaced by NAN α 2-8NAN α 2-. This may be done by treating a mono α -N-acetylneuraminic acid compound of Formula I or II with CMP-NAN

and an α -N-acetylneuraminic acid transferase specific for transfer to an α -N-acetylneuraminic acid.

Of the bis sialylated oligosaccharides of the present invention, are for example the bis sialylated compounds of Formula II, $\text{NAN}\alpha 2\text{-8NAN}\alpha 2\text{-3Gal}\beta 1\text{-4Glc}$ and $\text{NAN}\alpha 2\text{-8NAN}\alpha 2\text{-6Gal}\beta 1\text{-4Glc}$.

Any known suitable pharmaceutically acceptable cations may be used with the oligosaccharides of Formula I or Formula II, including the cations of conventional non-toxic salts including a metal salt such as an alkali metal salt (e.g. sodium salt, potassium salt, etc.) or an alkaline earth metal salt (e.g. calcium salt, magnesium salt, etc.), an ammonium salt, an organic base salt (e.g. trimethylamine salt, triethylamine salt, pyridine salt, picoline salt, dicyclohexylamine salt, N, N'-dibenzylethylenediamine salt, etc.), an organic acid salt (e.g. formate, acetate, trifluoroacetate, maleate, tartrate, methanesulfonate, benzenesulfonate, toluenesulfonate, etc.), an inorganic acid salt (e.g. hydrochloride, hydrobromide, sulfate, phosphate, etc.), a salt with an amino acid (e.g. arginine salt, aspartic acid salt, glutamic acid salt, etc.), and the like.

The structure $\text{NAN}\alpha 2\text{-3Gal}\beta 1\text{-3(NAN}\alpha 2\text{-6)GalNAC}$ has been identified as an O-linked carbohydrate found in bovine Factor X (Mizvuchi *et al*, J. Biol. Chem. (1977) 255:3526), bovine high-molecular weight ininogen (Endo *et al*, J. Biochem. Tokyo (1977) 82:545), human plasminogen, (Hayes *et al*, J. Biol. Chem. (1979) 254:8777), immunoglobulin (Chandraskaren *et al*, J. Biol. Chem., (1981) 256:1549), the β -subunit of human chorionic gonadotropin, (M. Kessler *et al*, J. Biol. Chem. (1979) 254:7909), bovine fetuin (R. Spiro *et al*, J. Biol. Chem. (1974) 249:5704) and human apolipoprotein C-III (P. Yaith, Biochem. Biophys. Acta (1978) 541:234).

The oligosaccharide of the present invention may be

obtained using any known method, including (1) enzymatically, using one of the inventor's method described in published international application WO 91/16449, (2) synthetically, using classical organic chemistry, or (3) by degradation of a natural occurring oligosaccharide, glycolipid, or glycopeptide.

The compound $\text{NAN}\alpha 2\text{-3Gal}\beta 1\text{-3(NAN}\alpha 2\text{-6)GalNAc-itol}$, wherein the acetal of the terminal GalNAc has been reduced to the primary alcohol, may be obtained by alkaline hydrolysis and NaBH_4 reduction of fetuin. Illustrative hydrolysis conditions consist of reacting fetuin in 0.05 M NaOH and 1M NaBH_4 at 50°C for 16h in water.

The compound $\text{NAN}\alpha 2\text{-3Gal}\beta 1\text{-3(NAN}\alpha 2\text{-6)GalNAc-peptide}$ may be obtained by protease mediated degradation of fetuin in aqueous solution at about 50°C.

However, as a pharmaceutically acceptable source of this compound, the hydrolysis product of fetuin may be disfavored due to the possible presence of mad cow's disease. While purification and treatment techniques can rule out transfer of this virus to the composition, enzymatic synthesis is still preferred.

The present oligosaccharide may be administered in conjunction with a known proton pump inhibitor or a known H_2 receptor antagonist. A representative proton pump inhibitor is omeprazole, and representative H_2 antagonists include cimetidine, ranitidine, nizatidine and famotidine. The amount of proton pump inhibitor and H_2 antagonist administered in conjunction with the present oligosaccharide is about the same amount administered for their known therapy. Accordingly, effective dosages of the proton pump inhibitor and H_2 can be determined by routine experimentation.

Alternatively a known antiulcerative compound may be used

in conjunction with or as a replacement for the H₂ receptor antagonist. Suitable antiulceratives include aceglutamide aluminum complex, ϵ -acetamidocaproic acid zinc salt, acetoxolone, arbaprostil, benexate hydrochloride, bismuth subcitrate sol, carbenoxolone, cetraxate, cimetidine, enprostil, esaprazole, famotidine, ftaxidide, gefarnate, guaiazulene, irsogladine, misoprostol, nazatidine, ornoprostil, γ -oryzanol, pifarnine, pirenzepine, plaunotol, ranitidine, rioprostil, rosaprostol, rotraxate, roxatidine acetate, sofalcone, spizofurone, sucralfate, teprenone, trimoprostil, trithiozine, troxipide, and zolimidine. The amount of antiulcerative administered in conjunction with the present oligosaccharide is about the same amount administered for its known therapy. Accordingly, effective dosage of the antiulcerative can be determined by routine experimentation.

Alternatively, the present oligosaccharide may be administered in conjunction with an antibiotic with activity against *H. pylori*. Suitable antibiotics include metronidazole, tetracycline, bismuth, erythromycin, macrolide, quinoline and amoxicillin. The amount of antibiotic administered in conjunction with the present oligosaccharide is about the same amount administered for its known therapy. Accordingly, effective dosage of the antibiotic can be determined by routine experimentation.

The anti-*H. pylori* compositions of the present invention contains one or a plurality of oligosaccharides of Formula I or Formula II in association with any suitable liquid or solid, pharmaceutically acceptable carrier or excipient, preferable in a form suitable for oral or enteral administration. In addition, the anti-*H. pylori* compositions of the present invention are preferably pyrogen free.

The anti-*H. pylori* compositions are usually administered as a mixture with a carrier suitably selected depending upon the route for administration using standard formulations. For

example, the compound of the present invention may be administered in the form of tablets which may be prepared using known techniques by adding to a powder of the active ingredient of the present invention an excipient such as starch, lactose, sucrose, glucose, crystalline cellulose, calcium carbonate or kaolin, a hydroxypropylcellulose, a glucose solution, a sucrose solution, water or ethanol, a disintegrator such as starch, agar, gelatin powder, carboxymethylcellulose calcium (CMC-Ca), carboxymethylcellulose sodium (CMC-Na), crystalline cellulose, calcium carbonate or sodium hydrogencarbonate, or a lubricant such as magnesium stearate, calcium stearate, talc, macrogoal 4,000, macrogoal 6,000 or stearic acid.

The mixture is then subjected to compression molding by a conventional tableting method, and if necessary, applying a sugar coating by means of a concentrated sugar solution containing e.g. gum arabic, talc, polyvinylpyrrolidone, polyethyleneglycol and/or titanium oxide, applying a film coating by means of a film-forming agent composed of e.g. polyvinyl acetal diethylaminoacetate, hydroxypropylmethylcellulose, hydroxypropylcellulose, ethylcellulose or polyvinylpyrrolidone or applying an enteric coating by means of a film-forming agent composed of e.g. ethylcellulose phthalate, cellulose acetate phthalate or hydroxypropylmethylcellulose phthalate.

These pharmaceutical compositions may be in the form of granules or fine granules which may be prepared by adding to the active ingredient of the present invention a binder such as starch, gelatin, gum arabic, methylcellulose, sodium carboxymethylcellulose, heavy silicic anhydride or light silicic anhydride, followed by kneading and granulation by usual methods; or as a powder of the active ingredient of the present invention by itself; or as capsules which may be prepared by adding to the active ingredient of the present invention an excipient such as lactose, starch or crystalline

cellulose and/or a lubricant such as magnesium stearate, calcium stearate or talc, and filling the mixture into capsules.

A solution or suspension may be prepared by adding any diluent customarily, used in the art. For example, suitable diluents include water, ethyl alcohol, propylene glycol, polyoxyethylene sorbitol, and sorbitan esters. Sodium chloride, glucose or glycerol may be incorporated into such a liquid preparation in an amount sufficient to prepare an isotonic solution. The therapeutic composition may also further contain ordinary dissolving aids, buffers, pain-alleviating agents, art preservatives, and optionally coloring agents, fragrances, flavors, sweeteners and other pharmacologically active agents such are well known in the art.

Suitable compositions may take the form of a solution, suspension, tablet, coated tablet or any pharmaceutically acceptable form suitable for delivery to the stomach or duodenum.

According to a preferred embodiment of the present invention, the oligosaccharide or anti-*H. pylori* compositions are administered enterally to a patient in need thereof to inhibit *H. pylori* binding or eliminate *H. pylori* colonies from the patient's stomach and/or duodenum.

Typically, suitable patients are humans. However the present method is also applicable to treatment of animals, including but not limited to mammals such as cows, horses, sheep, goats, dogs, cats, rodents and non-human primates, fowl such a chickens, turkeys and ducks, and fish.

Suitable amounts of the composition to be administered include those which produce an effective stomach concentration of oligosaccharide of from 1 μ g to 10,000 mg/ml per dose,

preferably 100 to 1000 µg/ml. When a proton pump inhibitor, H2 antagonist, or antiulcerative is coadministered, the composition is formulated to provide between 10-500 mg, preferably 100-300 mg of the proton pump inhibitor, H2 antagonist, or antiulcerative daily. Dosage forms include such unit dosage forms such as tablets, capsules, solutions or suspensions.

In another embodiment of this invention the oligosaccharide of Formula I or Formula II is provided as a multivalent molecule. In this embodiment, at least one type of the oligosaccharide of Formula I or Formula II is bound to a polymer using known techniques so as to produce a polymer to which more than one individual molecules of the oligosaccharide of Formula I or Formula II are covalently attached. The polymer backbone is sufficiently long to provide a multivalent molecule leaving from between 2-1,000, preferably 10-100, more preferably 20-30 molecules of the compound of Formula I or Formula II bound to the polymer.

The oligosaccharide of Formula I or Formula II is preferably bound to the polymer via the free anomeric carbon of the galactose, N-acetylgalactosamine, glucose or N-acetylglucosamine moiety of Formula I or the glucose or N-acetylglucosamine moiety of Formula II when Y = H, or the peptide when Y = peptide. Suitable polymers are any polymer material which may be reacted with the anomeric carbon of a saccharide, such as a polylysine, a polyacrylamide or a cyclodextrin.

For example, the oligosaccharide of Formula I or Formula II may be bound to a support to form a bead wherein the surface of the bead is bound with the compound of Formula I or Formula II.

Another embodiment of this invention, which relates to one of the inventor's application serial no. 07/241,012, filed September 2, 1988, provides a kit for detecting *H. pylori*. The kit is analogous to a kit for performing ELISAs, but uses a compound of Formula I or Formula II which is bound to a solid support, instead of the antigens or antibodies bound to solid supports normally found with ELISA kits. The kit comprises a container, and contained therein, the compound of Formula I or Formula II bound to a solid support. The compound of Formula I or Formula II is bound to a polymer support through the anomeric carbon of the galactose, N-acetylgalactosamine, glucose or N-acetylglucosamine moiety of Formula I or the glucose or N-acetylglucosamine moiety of Formula II in the case where Y = H and through the amino acid or the peptide when Y = amino acid or peptide. A sample to be tested for *H. pylori* is contacted with the bound compound of Formula I or Formula II. Bound *H. pylori* may be detected by standard means such as labeled antibodies.

* * *

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

* * *

Example 1. Synthesis of tetrasaccharide NAN α 2-3 Gal β 1-3(NAN α 2-6)GalNAc

An aqueous solution of equimolar equivalents of galNAc and UDP-gal is stirred at 40°C in the presence of a β 1-3 galactosyltransferase for a sufficient time to effect coupling of the two moieties. The β 1-3 galactosyltransferase is isolated from porcine submaxillary glands. Next, three molar equivalents of CMP-NAN are introduced along with an α 2-6 sialyltransferase, to form the monosialo compound. Finally an

α 2-3 sialyltransferase is introduced in the presence of three more equivalents of CMP-NAN to form the tetrasaccharide.

Example 2. Synthesis of glycopeptidesaccharide NAN α 2-3 Gal β 1-3(NAN α 2-6) GalNAc β 1-Serine

An aqueous solution of equimolar equivalents of galNAc O-linked to serine and UDP-gal is stirred at 40°C in the presence of a β 1-3 galactosyltransferase for a sufficient time to effect coupling of the two moieties. The β 1-3 galactosyltransferase is isolated from porcine submaxillary glands. Next three molar equivalents of CMP-NAN are introduced along with an α 2-6 sialyltransferase to form the monosialo compound. Finally an α 2-3 sialyltransferase is introduced in the presence of three more equivalents of CMP-NAN to form the glycopeptidesaccharide.

Example 3

An anti-*Helicobacter* composition is prepared by mixing 100 mg of the tetrasaccharide of Example 1 with 250 mg of the H₂ receptor antagonist ranitidine. The mixture is then suspended in a mixture of water and propylene glycol.

Example 4

An anti-*Helicobacter* composition is prepared by mixing 100 mg of the tetrasaccharide of Example 1 with 250 mg of the proton pump inhibitor omeprazole. The mixture is then suspended in a mixture of water and propylene glycol.

Example 5

An anti-*Helicobacter* composition is prepared by mixing 100 mg of the tetrasaccharide of Example 1 with 250 mg of a combination of metronidazole, tetracycline, and amoxicillin. The mixture is then suspended in a mixture of water and

propylene glycol.

Example 6

As a therapeutic treatment, a patient infected with *H. pylori* is treated with the composition of Example 3. The patient is treated orally four times daily with each dosage providing an effective stomach concentration of 100 µg/ml. Therapy is continued for two weeks, after which examination showed eradication of the *H. pylori* bacteria. After eradication, maintenance therapy with the composition of the present invention is continued to prevent recurrence.

Example 7. Synthesis of tetrasaccharide NAN α 2-3 Gal β 1-4 (NAN α 2-6)GlcNAc

An aqueous solution of equimolar equivalents of glcNAc and UDP-gal is stirred at 40°C in the presence of a β 1-4 galactosyltransferase for a sufficient time to effect coupling of the two moieties. The β 1-4 galactosyltransferase is isolated from porcine submaxillary glands. Next three molar equivalents of CMP-NAN are introduced along with an α 2-6 sialyltransferase, to form the monosialo compound. Finally an α 2-3 sialyltransferase is introduced in the presence of three more equivalents of CMP-NAN to form the tetrasaccharide.

Example 8. Synthesis of tetrasaccharide NAN α 2-3 Gal β 1-3 (NAN α 2-6)GlcNAc

An aqueous solution of equimolar equivalents of glcNAc and UDP-gal is stirred at 40°C in the presence of a β 1-3 galactosyltransferase for a sufficient time to effect coupling of the two moieties. The β 1-3 galactosyltransferase is isolated from porcine submaxillary glands. Next three molar equivalents of CMP-NAN are introduced along with an α 2-6 sialyltransferase, to form the monosialo compound. Finally an α 2-3 sialyltransferase is introduced in the presence of three

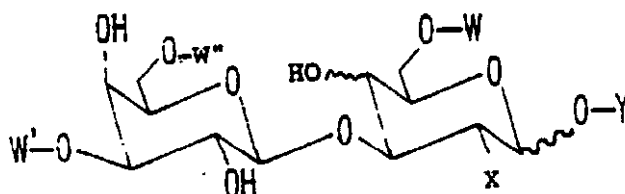
more equivalents of CMP-NAN to form the tetrasaccharide.

* * * *

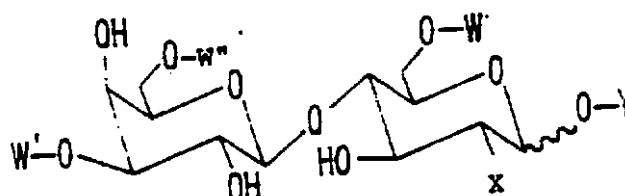
Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

Claims

1. A method of treating or inhibiting an ulcer in the stomach or duodenum of a patient in need thereof, comprising administering to the stomach or duodenum of said patient, an effective amount of a composition comprising an oligosaccharide selected from the group consisting of Formula I



; Formula II



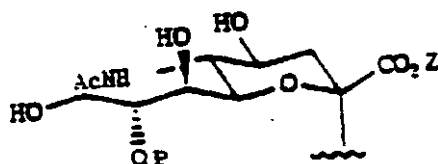
or a mixture thereof;

wherein:

X is independently OH or NHAc;

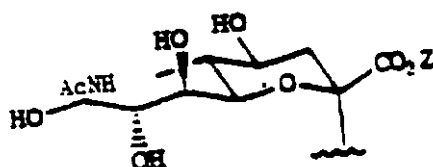
Y is independently H, or an amino acid or a peptide of 2-100, preferably 2-20, amino acids; and

W, W', and W'' are each independently H or



where Z is independently H or a pharmaceutically acceptable cation; and

P is independently H or



where Z is defined as above;

wherein at least one of W, W' or W'' is an α -N-acetylneuraminic acid moiety, and

wherein W' and W'' are not simultaneously an α -N-acetylneuraminic acid moiety

with the proviso that the compound of Formula II is not NAN α (2-3)Gal β 1-4 Glu or NAN α (2-6)Gal β 1-4 Glu.

2. The method of Claim 1, wherein X is OH.
3. The method of Claim 1, wherein X is NHAc.
4. The method of Claim 1, wherein Y is H.
5. The method of Claim 1, wherein Y is serine or threonine.
6. The method of Claim 1, wherein Y is an amino acid or 2 to 20 amino acids long peptide.
7. The method of Claim 1, wherein Y is a peptide of from 2 to 20 amino acids.

8. The method of Claim 1, wherein at least one of Z is H.
9. The method of Claim 1, wherein at least one of Z is a pharmaceutically acceptable cation.
10. The method of Claim 1, comprising inhibiting a gastric ulcer in said patient.
11. The method of Claim 1, comprising inhibiting a duodenal ulcer in said patient.
12. The method of Claim 1, comprising treating a gastric ulcer in said patient.
13. The method of Claim 1, comprising treating a duodenal ulcer in said patient.
14. The method of Claim 1, comprising coadministering an H2 blocker to said patient.
15. The method of Claim 1, comprising coadministering an antiulcerative compound to said patient.
16. The method of Claim 1, comprising coadministering a proton pump inhibitor to said patient.
17. The method of Claim 1, comprising coadministering an antibiotic compound to said patient.
18. The method of Claim 1, wherein a plurality of molecules of said oligosaccharide are covalently bonded to a pharmaceutically acceptable polymer via the free anomeric carbon of the galactose, N-acetylgalactosamine, glucose or N-acetylglucosamine moiety of Formula I or the glucose or N-acetylglucosamine moiety of Formula II when Y is H and via the amino acid or the 2 to 100 amino acids-long peptide moiety

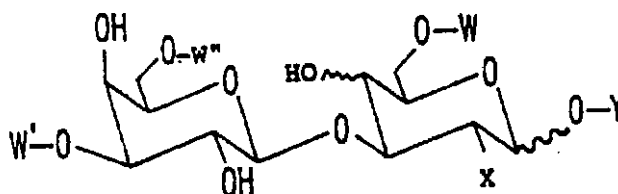
when Y is an amino acid or a 2 to 100 amino acids-long peptide.

19. The method of Claim 18, wherein said pharmaceutically acceptable polymer is a polylysine, a polyacrylamide or a cyclodextrin.

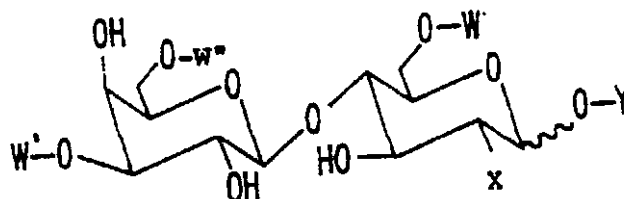
20. The method of Claim 10, wherein a plurality of molecules of said oligosaccharide are covalently bonded to a pharmaceutically acceptable polymer, via the free anomeric carbon of the galactose, N-acetylgalactosamine, glucose or N-acetylglucosamine moiety of Formula I or the glucose or N-acetylglucosamine moiety of Formula II when Y is H and via the amino acid or the 2 to 100 amino acids-long peptide moiety when Y is an amino acid or a 2 to 100 amino acids-long peptide.

21. The method of Claim 20, wherein said pharmaceutically acceptable polymer is a polylysine, a polyacrylamide or a cyclodextrin.

22. A composition comprising, in association with a carrier or excipient suitable for enteral administration, an amount effective for *Helicobacter pylori* binding inhibition, of a composition comprising an oligosaccharide selected from the group consisting of Formula I



; Formula II



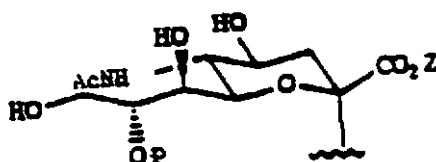
or a mixture thereof;

wherein:

X is independently OH or NHAc;

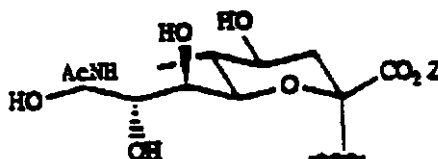
Y is independently H, or an amino acid or a peptide of 2-100, preferably 2-20, amino acids; and

W, W', and W'' are each independently H or



where Z is independently H or a pharmaceutically acceptable cation; and

P is independently H or



where Z is defined as above;

wherein at least one of W, W' or W'' is an α -N-acetylneuraminic acid moiety, and

wherein W' and W'' are not simultaneously an α -N-

acetylneuraminic acid moiety

with the proviso that the compound of Formula II is not NAN $\alpha(2\rightarrow3)\text{Gal } \beta 1-4 \text{ Glu}$ or NAN $\alpha(2\rightarrow6)\text{Gal } \beta 1-4 \text{ Glu}$.

23. The composition of Claim 22, further comprising an amount effective for H2 blocking of an H2 blocker.

24. The composition of Claim 23, wherein said H2 blocker is selected from the group consisting of cimetidine, octreotide, enterogastrone, ranitidine, nizatidine and famotidine.

25. The composition of Claim 22, further comprising an antiulcerative amount of an antiulcerative compound.

26. The composition of Claim 22, further comprising a proton pump inhibiting amount of a proton pump inhibitor.

27. The composition of Claim 26, wherein said proton pump inhibitor is omeprazole.

28. The composition of Claim 22, further comprising an antibiotic effective amount of an antibiotic effective against *H. pylori*.

29. The composition of Claim 28, wherein said antibiotic is selected from the group consisting of metronidazole, tetracycline, bismuth, erythromycin, macrolide, quinoline, amoxicillin and a mixture thereof.

30. A solid composition according to Claim 22.

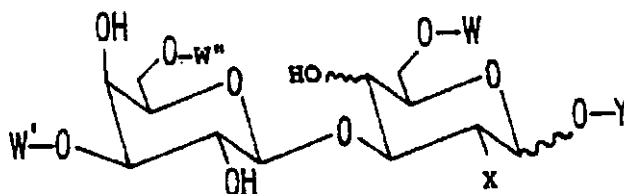
31. A liquid composition according to Claim 22.

32. The composition of Claim 22, wherein X is OH.

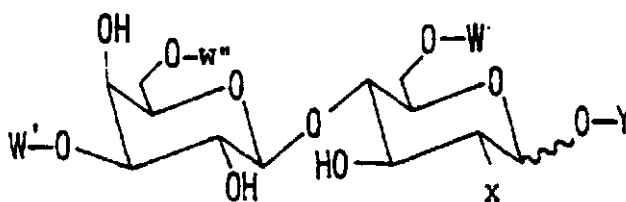
33. The composition of Claim 22, wherein X is NHAc.
34. The composition of Claim 22, wherein Y is H.
35. The composition of Claim 22, wherein Y is an amino acid or 2 to 100 amino acids-long peptide.
36. The composition of Claim 35 wherein Y is a peptide of from 2 to 20 amino acids.
37. The composition of Claim 22, wherein at least one of Z is H.
38. The composition of Claim 22, wherein at least one of Z is a pharmaceutically acceptable cation.
39. A kit for determining the presence of *Helicobacter pylori* in a sample, comprising:

(i) a container and

(ii) an oligosaccharide selected from the group consisting of Formula I



; Formula II



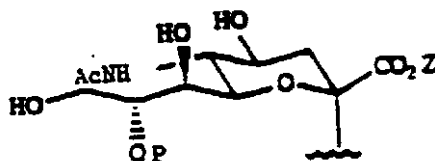
or a mixture thereof;

wherein:

X is independently OH or NHAc;

Y is independently H, or an amino acid or a peptide of 2-100, preferably 2-20, amino acids; and

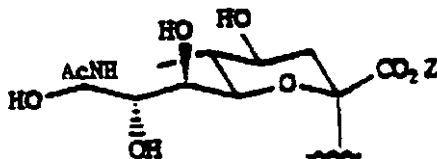
W, W', and W'' are each independently H or



;

where Z is independently H or a pharmaceutically acceptable cation; and

P is independently H or



where Z is defined as above;

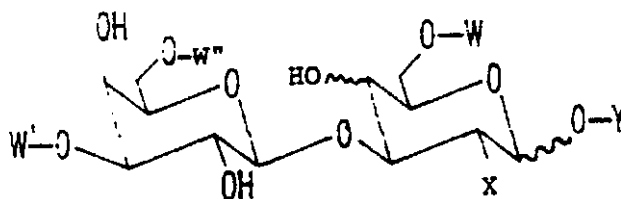
wherein at least one of W, W' or W'' is an α -N-acetylneuraminic acid moiety, and

wherein W' and W'' are not simultaneously an α -N-acetylneuraminic acid moiety

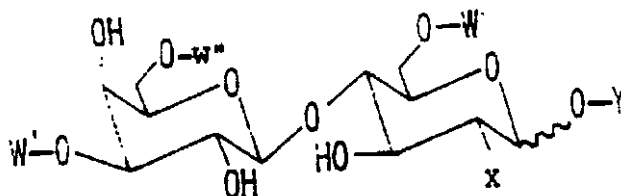
with the proviso that the compound of Formula II is not NAN $\alpha(2\rightarrow3)\text{Gal}\beta1-4\text{Glu}$ or NAN $\alpha(2\rightarrow6)\text{Gal}\beta1-4\text{Glu}$.

40. The kit of claim 39 wherein X is OH.
41. The kit of claim 39 wherein X is NHAc.
42. The kit of claim 39 wherein Y is H.
43. The kit of claim 39 wherein Y is an amino acid or a 2 to 100 amino acids-long peptide.
44. The kit of claim 43, wherein Y is a peptide of from 2 to 20 amino acids.
45. The kit of claim 39 wherein at least one of Z is H.
46. The kit of claim 39 wherein at least one of Z is a pharmaceutically acceptable cation.
47. The kit of Claim 39, wherein a plurality of said oligosaccharide, covalently bonded to a pharmaceutically acceptable polymer via the free anomeric carbon of the galactose, N-acetylgalactosamine, glucose or N-acetylglucosamine moiety of Formula I or the glucose or N-acetylglucosamine moiety of Formula II when Y is H and via the amino acid or the 2 to 100 amino acids-long peptide moiety when Y is an amino acid or a 2 to 100 amino acids-long peptide, are bound to said solid support.
48. The kit of Claim 47, wherein said pharmaceutically acceptable polymer is a polylysine, a polyacrylamide or a cyclodextrin.
49. A method of eliminating *Helicobacter pylori* from the stomach or duodenum of a patient in need thereof, comprising administering to said patient an *Helicobacter pylori*

eliminating effective amount of composition comprising an oligosaccharide selected from the group consisting of Formula I



; Formula II



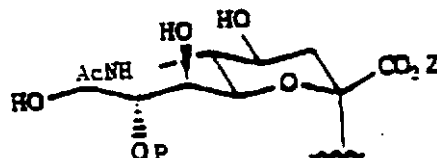
or a mixture thereof;

wherein:

X is independently OH or NHAc;

Y is independently H, or an amino acid or a peptide of 2-100, preferably 2-20, amino acids; and

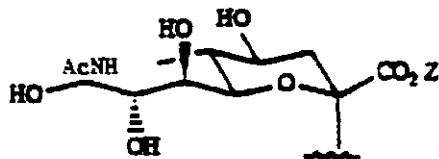
W, W', and W'' are each independently H or



;

where Z is independently H or a pharmaceutically acceptable cation; and

P is independently H or



where Z is defined as above;

wherein at least one of W, W' or W'' is an α -N-acetylneuraminic acid moiety, and

wherein W' and W'' are not simultaneously an α -N-acetylneuraminic acid moiety

with the proviso that the compound of Formula II is not NAN α (2 $\rightarrow$ 3)Gal β 1-4 Glu or NAN α (2 $\rightarrow$ 6)Gal β 1-4 Glu.

50. The method of Claim 49, wherein X is OH.

51. The method of Claim 49, wherein X is NHAc.

52. The method of Claim 49, wherein Y is serine or threonine.

53. The method of Claim 49, wherein Y is an amino acid or a 2 to 20 amino acids long peptide.

54. The method of Claim 49, wherein at least one of Z is H.

55. The method of Claim 49, wherein at least one of Z is a pharmaceutically acceptable cation.

56. The method of Claim 49, wherein said patient has been diagnosed as having a duodenal ulcer.

57. The method of Claim 49, wherein said patient has been diagnosed as having a gastric ulcer.

58. The method of Claim 49, wherein said administration is oral.

59. The method of Claim 49, wherein said administration is enteral.

60. The method of Claim 49, wherein a plurality of said oligosaccharide are covalently bonded to a pharmaceutically acceptable polymer, via the free anomeric carbon of the galactose, N-acetylgalactosamine, glucose or N-acetylglucosamine moiety of Formula I or the glucose or N-acetylglucosamine moiety of Formula II when Y is H and via the amino acid or the 2 to 100 amino acids-long peptide moiety when Y is an amino acid or a 2 to 100 amino acids-long peptide.

61. The method of Claim 60, wherein said pharmaceutically acceptable polymer is a polylysine, a polyacrylamide or a cyclodextrin.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07010

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 31/70, 31/715

US CL :514/42, 53, 58, 61

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/42, 53, 58, 61

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,935,406 (COLEMAN ET AL.) 19 JUNE 1990, SEE ENTIRE DOCUMENT.	1-21, 49-61
Y	US, A, 4,938,967 (NEWTON ET AL.) 03 JULY 1990, SEE ENTIRE DOCUMENT.	22-48
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, VOLUME 260, NO. 14, ISSUED 15 JULY 1985, D. K. PODOLSKY, "OLIGOSACCHARIDE STRUCTURES OF HUMAN COLONIC MUCIN", PAGES 8262-8271, ESPECIALLY SEE TABLE XIV.	1-61

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"g" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 November 1993

Date of mailing of the international search report

NOV 15 1993

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07010

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	INFECTION AND IMMUNITY, VOLUME 56, NO. 11, ISSUED NOVEMBER 1988, D. G. EVANS ET AL., "N-ACETYLNEURAMINYLLACTOSE-BINDING FIBRILLAR HEMAGGLUTININ OF <u>CAMPYLOBACTER PYLORI</u> : A PUTATIVE COLONIZATION FACTOR ANTIGEN", PAGES 2896-2906, SEE ENTIRE REFERENCE.	1-61
Y	INFECTION AND IMMUNITY, VOLUME 57, NO. 8, ISSUED AUGUST 1989, D. G. EVANS ET AL., "RECEPTOR-MEDIATED ADHERENCE OF <u>CAMPYLOBACTER PYLORI</u> TO MOUSE Y-1 ADRENAL CELL MONOLAYERS", PAGES 2272-2278, SEE ENTIRE REFERENCE.	1-61
Y	THE LANCET, ISSUED 29 JULY 1989, LINGWOOD ET AL., "GASTRIC GLYCEROLIPID AS RECEPTOR FOR <u>CAMPYLOBACTER PYLORI</u> ", PAGES 238-241, ENTIRE DOCUMENT.	1-61
Y	INFECTION AND IMMUNITY, VOLUME 59, NO. 11, ISSUED NOVEMBER 1991, L. S. TZOUVELEKIS ET AL., "IN VITRO BINDING OF <u>HELICOBACTER PYLORI</u> TO HUMAN GASTRIC MUCIN", PAGES 4252-4254, ENTIRE DOCUMENT.	1-61
Y	REVIEWS OF INFECTIOUS DISEASES, VOLUME 13 (SUPPL. 8), ISSUED 1991, LAMBERT, "PHARMACOLOGY OF BISMUTH-CONTAINING COMPOUNDS", PAGES S691-S695, ENTIRE DOCUMENT.	1-61
Y	REVIEWS OF INFECTIOUS DISEASES, VOLUME 13 (SUPPL. 8), ISSUED 1991, DUNN ET AL., "ADHERENCE OF <u>HELICOBACTER PYLORI</u> TO GASTRIC CARCINOMA CELLS: ANALYSIS BY FLOW CYTOMETRY", PAGES S657-S664, ENTIRE DOCUMENT.	1-61

Form PCT/ISA/210 (continuation of second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/07010

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

- I. Claims 1-21 and 30-38, drawn to a first method of treating or inhibiting an ulcer in the stomach or duodenum of a patient and first product used thereby, classified 514/62.
- II. Claims 23-24, drawn to second product comprising a H2 blocker, classified 514/646.
- III. Claim 25, drawn to third product comprising an antilulcerative compound, classified 514/54.
- IV. Claims 26-27, drawn to fourth product comprising a proton pump inhibitor, classified 514/438.
- V. Claims 28-29, drawn to fifth product comprising an antibiotic, classified 514/412.
- VI. Claims 39-48, drawn to sixth product comprising Formula I and II, classified 435/174.
- VII. Claims 49-61, drawn to second method of using first product, classified 424/93R.

Note that Claim 22 will be examined with the elected Group II, III, IV or V.

Form PCT/ISA/210 (extra sheet)(July 1992)*



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Tra. 11 PATENTEIYII
Act. 411 PRESENTACION

Dep. 2020 DIR.NUEVASCR
Eve: 378 FASENACIONALI
Folios: 286

PATENTE DE INVENCION ☐

MODELO DE UTILIDAD ☐

Art 33 Decisión 486/00

☐	Indicación que se solicita una patente.
☐	Datos de identificación del solicitante o de la persona que presenta la solicitud
☐	Descripción de la invención
☐	Dibujos de ser estos pertinentes
☐	Comprobante de pago de las tasas establecidas (De ser el caso formato de descuento)
Completa ☐	Incompleta ☐

PATENTE DE INVENCION PCT ☒ MODELO DE UTILIDAD PCT ☐ Art.33 Decisión 486/00, Circular Única

☒	Indicación que se solicita una PCT
☒	Copia de la solicitud en español, tal como fue presentada inicialmente (capítulo descriptivo, reivindicatorio, resumen)
☒	Dibujos de ser estos pertinentes
☒	Comprobante de pago de las tasas establecidas (de ser el caso formato de descuento)
Completa ☒	Incompleta ☐

DISEÑO INDUSTRIAL ☐

(Art. 119 Decisión 486/00)

☐	Indicación que se solicita Diseño industrial
☐	Datos de identificación del solicitante o de la persona que presenta la solicitud
☐	Representación gráfica y fotográfica del Diseño industrial o muestra del material que incorpora el diseño
☐	Comprobante de pago de las tasas establecidas
Completa ☐	Incompleta ☐

ESQUEMA DE TRAZADO ☐

(Art. 92 Decisión 486/00)

☐	Indicación que se solicita un esquema de trazado
☐	Datos de identificación del solicitante o de la persona que presenta la solicitud
☐	Representación gráfica de un esquema de trazado
☐	Comprobante de pago de las tasas establecidas
Completa ☐	Incompleta ☐