64
57

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMI

DIVISION DE NUEVAS CREACIONES

E. S. D.

SUPERINDUSTRIA Y COMERCIO
Radicacion : 00025248 00000001 Folios: 85
Fecha (AMD): 2000-06-20 16:09:11
Tramite : 002 PATENTES D 1 REGISTRO/ 444 REPUESTA
Dependencia: 2020 DIVISION DE NUEVAS CREACIONES

Re: P1.-**ABBOTT LABORATORIES.**-Solicitud de Patente de Invención para **"RESONANCIA MAGNETICA NUCLEAR ^{13}C PARA DETECTAR EL LIGAMIENTO DE UNA MOLECULA OBJETIVO Y UN LIGANDO PUTATIVO".**-Clase A.61.-Expediente número 00-025.248.-

1. Me refiero a la solicitud arriba nombrada de 6 de Abril de 2000 y al auto dictado por este despacho notificado por Estado de 11 de Mayo del mismo año.

2. Por medio del presente atentamente manifiesto a usted que, debidamente autorizado por mi poderdante, modifico el título de la solicitud con el objeto de dar cumplimiento a las exigencias del señor Examinador, para que quede como sigue:

"RESONANCIA MAGNETICA NUCLEAR ^{13}C PARA DETECTAR EL LIGAMIENTO DE UNA MOLECULA OBJETIVO Y UN LIGANDO PUTATIVO"

3. Adicionalmente, declaro a usted que, debidamente autorizado por mi poderdante **excluyo** de la presente solicitud la reivindicación 24.

4. Anexas me permito presentar a usted:

(a) Nuevo Resumen, Objeto y Finalidad de la Invención, **que reemplaza el originalmente presentado,** en el cual ha sido modificado el título de la solicitud;

(b) Nuevas páginas 1 y 36 de la descripción y reivindicaciones, **que reemplazan las originalmente presentadas,** en las cuales (a) se ha modificado el título de la solicitud, (b) se ha cancelado la reivindicación 24 y (c) se ha renumerado la reivindicación 25 como 24;

(c) Nuevo extracto en sus dos formas, **que reemplaza el originalmente presentado**, el cual incluye el nuevo título de la solicitud;

(d) Copia auténtica, debidamente certificada de la solicitud de patente arriba nombrada, **respecto de la cual reclamo prioridad,** a saber la solicitud de patente estadinense número 09/288,924 de 9 de Abril de 1999, junto con la traducción oficial número 5.705 de 9 de Marzo de 2000, que incluye las legalizaciones y reivindicaciones de la solicitud.

5. Anexa también acompaño copia debidamente autenticada por el Notario Sexto de los documentos que acreditan la calidad de Traductor Oficial de la señora María Luisa de Brigard, quien elaboró la traducción de la solicitud estadinense número 09/288,924 respecto de la cual se reivindica prioridad en el presente caso.

66
59

6. Ruego a usted tomar atenta nota de lo anterior y proceder de conformidad.

Señor Superintendente,

JOSE ANTONIO LLOREDA

T.P. 10.784

Código 231

NOTARIA SEXTA DE BOGOTA
RECONOCIMIENTO Y PRESENTACION PERSONAL

Santafé de Bogotá D. C. 20 JUN. 2000

Ante Mí JUAN MANUEL BOTERO MEDINA

NOTARIO SEXTO DEL CIRCULO DE BOGOTA

Compareció(eron) personalmente

quien(es) exhibió(eron) la(s) C. C. 17159681

y la(s) Libreta(s) Militar(es):

domiciliado(s) en Bogotá, y declaró(aron) que la(s) firma(s) que aparece(n) en el presente documento es(son) la(s) suya(s) y que el contenido del mismo es cierto.

En constancia se firma esta diligencia.

NOTARIA SEXTA SANTAFE DE BOGOTA D.C.

JOSE ANTONIO LLOREDA

C.C. 17.159.681 de Bogotá

REPUBLICA DE COLOMBIA
Juan Manuel Botero Medina
Notario Sexto
SANTAFE DE BOGOTA, D.C.

RESUMEN -OBJETO Y FINALIDAD DE LA INVENCION

"RESONANCIA MAGNETICA NUCLEAR ^{13}C PARA DETECTAR EL LIGAMIENTO DE UNA MOLECULA OBJETIVO Y UN LIGANDO PUTATIVO"

La presente invención proporciona métodos para detectar el ligamiento de un ligando putativo a una molécula objetivo enriquecida con ^{13}C, métodos de investigación de la presencia de compuestos que se liguen a una molécula objetivo enriquecida con ^{13}C, métodos para calcular la constante de disociación de un compuesto ligando que se ligue a una molécula objetivo enriquecida con ^{13}C, y métodos empleados para la determinación de los aminoácidos específicos en una molécula objetivo enriquecida con ^{13}C afectada por el ligamiento de un ligando. La presente invención proporciona también compuestos identificados por los métodos de investigación de la presencia de compuestos arriba mencionados.

"RESONANCIA MAGNETICA NUCLEAR ^{13}C PARA DETECTAR EL LIGAMIENTO DE UNA MOLECULA OBJETIVO Y UN LIGANDO PUTATIVO"

Campo de la Invención

La presente invención se refiere al uso de la resonancia magnética nuclear para detectar el ligamiento entre compuestos y moléculas objetivo enriquecidas con el isótopo ^{13}C.

Referencia Cruzada con Solicitudes Relacionadas

La presente solicitud de patente es una continuación, en parte, de la solicitud No. de Serie 09 241 194, radicada en Febrero 1, 1999, la cual es a su vez una continuación de la solicitud No. de Serie. 08/744 701, radicada en Octubre 31, 1996, ahora Patente de los Estados Unidos No. 5, --- ---, la cual es una continuación en parte de la solicitud No. de Serie 08/678 903, radicada en Julio 12, 1996, ahora abandonada, la cual es una continuación en parte de la solicitud No. de Serie 08/558 633, radicada en Noviembre 14, 1995, en la actualidad, Patente de los Estados Unidos No. 5 --- ---.

Antecedentes de la Invención

Una de las herramientas más poderosas en el descubrimiento de nuevos prospectos de drogas es el tamizare (investigación) aleatorio de librerías de productos naturales y sintéticos a fin de descubrir compuestos que se liguen a una molécula objetivo en particular (por ejemplo, la identificación de ligandos de esa molécula objetivo). Usando este método, los ligandos pueden identificarse por su capacidad para formar una asociación física con una molécula objetivo o por su capacidad para alterar una función de una molécula objetivo.

21. El método acorde con la Reivindicación 20, en donde dicha molécula objetivo se selecciona del grupo conformado por proteínas, fragmentos de proteína, y polipéptidos.

22. El método acorde con la Reivindicación 20, en donde dicha molécula objetivo es uniformemente enriquecida con ^{13}C.

23. El método acorde con la Reivindicación 20, en donde dicha molécula objetivo es uniformemente enriquecida con ^{13}C.

24. Un método para la determinación de residuos aminoácido específicos en una molécula objetivo pre-seleccionada enriquecida con ^{13}C afectada por el ligamiento de un ligando a dicha molécula objetivo, método que comprende:

a) generación de un primer espectro de correlación RMN $^{13}C/^{1}H$ bidimensional de dicha molécula objetivo, en donde dichos valores de corrimiento químico de las señales de $^{13}C/^{1}H$ en dicho espectro de correlación bidimensional corresponden a por lo menos una ubicación específica conocida de agrupamientos atómicos presentes en dicha molécula objetivo;

b) formación de una mezcla de dicha molécula objetivo con un compuesto ligando conocido ;

c) generación de un segundo espectro de correlación RMN $^{13}C/^{1}H$ bidimensional de la mezcla del paso (b); y

d) comparación entre el primero y segundo espectros.

74
63

REPUBLICA DE COLOMBIA
MINSTERIO DE RELACIONES EXTERIORES

EL SUSCRITO JEFE DEL AREA DE LEGALIZACIONES DEL MINISTERIO DE RELACIONES EXTERIORES

C E R T I F I C A

Que revisados los registros que se llevan en la oficina de Legalizaciones aparece la firma de la señora **MARIA LUISA DE BRIGARD DE RESTREPO,** como traductora e intérprete oficial para los idiomas **INGLES - ESPAÑOL, ESPAÑOL-INGLES,** según resolución 0793 del 21 de marzo de 1958

La anterior certificación se expide a solicitud de la interesada quien se identificó con cédula de ciudadanía No. 20.024.691 expedida en Santa Fe de Bogotá.

Dada en Santa Fe de Bogotá, D.C. a los veinticinco (25) días de marzo de 1999

CARLOS GUILLERMO DIAZ BUITRAGO
Jefe Area de Legalizaciones

COMO NOTARIO SEXTO ENCARGADO DEL CIRCULO DE SANTAFE DE BOGOTA
HAGO CONSTAR
QUE LA FOTOCOPIA COINCIDE CON LA FOTOCOPIA QUE HE TENIDO A LA VISTA.
SANTAFE DE BOGOTA, 25 ABR. 2000
PABLO MENDEZ BARAJAS

COMO NOTARIO SEXTO ENCARGADO DEL CIRCULO DE SANTAFE DE BOGOTA, HAGO CONSTAR QUE ESTA COPIA FOTOSTATICA COINCIDE CON EL ORIGINAL QUE HE TENIDO A LA VISTA
13 ABR 1999
PABLO MENDEZ BARAJAS

Abbott
5816cc02
72
64

TRADUCCION OFICIAL No. 5.705

de un documento escrito en INGLES, el cual para su identificación se sella con el sello del Traductor al mismo tiempo que la presente. -

---

ESTADOS UNIDOS DE AMERICA

A CUANTOS LLEGAREN LAS PRESENTES:

DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS

Oficina de Patentes y Marcas de Fábrica de los Estados Unidos

23 de febrero del 2000

TRADUCCION OFICIAL No. 5.705

POR LA PRESENTE SE CERTIFICA QUE ANEXA AQUI SE ENCUENTRA UNA COPIA FIEL , TOMADA DE LOS REGISTROS DE LA OFICINA DE PATENTES Y MARCAS DE FABRICA DE LOS ESTADOS UNIDOS, DE LOS DOCUMENTOS DE LA SOLICITUD DE PATENTE IDENTIFICADA ABAJO QUE SATISFACEN LOS REQUISITOS PARA QUE SEA OTORGADA UNA FECHA DE REGISTRO SEGUN 35 USC 111.

NUMERO DE SOLICITUD: 09/288,924

FECHA DE REGISTRO: 9 de abril de 1999.

Por Autorización del

COMISIONADO DE PATENTES Y MARCAS DE FABRICA

(Firma)

P. SWAIN

Funcionario Certificador

Sello: OFICINA DE PATENTES Y MARCAS DE FABRICA - ESTADOS UNIDOS DE AMERICA -

---

73
65

ANEXO :

TRANSMISION DE SOLICITUD DE PATENTE	Expediente del Apoderado	5816.US.P3	
	Primer Inventor o Identificador de Solicitud		Stephen W. Fesik
	Título	Ver 1 en Adición	
	No. de Rótulo de Correo Expreso .		EE835516454US

ELEMENTOS DE LA SOLICITUD	DIRIGIR A: Comisionado Auxiliar para Patentes Apartado de Solicitudes de Patentes Washington, D.C. 20231
1. ☒ *Forma de Derechos de Transmisión 2. ☒ Descripción [Total de Páginas 48] - Título descriptivo de la invención - Referencias cruzadas a Solicitudes Relacionadas - Declaración Con Respecto a R&D respaldada por Fed - Referencia a Apéndice de Microficha - Antecedentes de la Invención - Breve Resumen de la Invención - Breve Descripción de los Dibujos - Descripción Detallada - Reivindicaciones - Extracto de la Exposición 3. ☒ Dibujo(s) (35 U.S.C. 113) [Total de Hojas 5] 4. Juramento o Declaración [Total de Páginas 4] a. ☒ Recién otorgado (original o copia) b. ☐ Copia de una solicitud anterior (37 C.F.R §1.63(d)) i. ELIMINACION DE INVENTOR(ES) Declaración firmada anexa eliminando Inventor(es) nombrado(s) en la solicitud anterior, véase 37 C.F.R §§ 1.63(d)(2) y 1.33(b).	5. ☐ Programa de Computador de Microficha (Apéndice) 6. Presentación de Secuencia de Nucleótido y/o Aminoácido (si corresponde, todo lo necesario) a. ☐ Copia Legible en Computador b. ☐ Copia en papel (idéntica a la copia en computador) c. ☐ Declaración comprobando identidad de tales copias **PARTES DE SOLICITUD ADJUNTAS** 7. ☒ Documentos de Cesión (hoja de cubierta y documento(s)) 8. ☐ Declaración 37 C.F.R. §3.73(b) ☐ Poder (Cuando hay un cesionario) 9. ☐ Documento de Traducción al Inglés (si corresponde 10.☐ Declaración de Exposición de Información (IDS)/PTO-1449 ☐ Copias de IDS Citaciones 11.☐ Reforma Preliminar 12.☒ Tarjeta Postal de Acuse de Recibo (MPEP 503) (Debe detallarse especificamente) 13. ☐ *Declaración de Entidad Pequeña (PTO/SB/09/12) ☐ Declaración presentada en solicitud anterior Situación todavía adecuada y deseada 14. ☐ Copia Certificada de Documento(s) de Prioridad (si se reivindica prioridad extranjera) 15. ☒ Otros: Véase en Adición

5,705

74
66

16. **SI ES UNA SOLICITUD DE CONTINUACION**

☐ Continuación ☐ División ☒ Continuación en Parte (CIP) de solicitud anterior No. 09/241,184
Información de solicitud anterior: Examinador ---------- Grupo/Unidad de Arte ----------

Solamente para SOLICITUDES DE CONTINUACION O DIVISION: La descripción completa de la solicitud anterior, de la cual se suministra un juramento o una declaración en la Casilla 4b, se considera parte de la descripción de la solicitud de continuación o división adjunta y se incluye aquí por referencia. Solo puede confiarse en la inclusión cuando una porción ha sido omitida inadvertidamente de las partes de la solicitud presentadas.

17. DIRECCION DE CORRESPONDENCIA

☐ Número del Cliente o Rótulo
de Código de Barras ---------- o ☒ Dirección de correspondencia indicada abajo

Nombre	Steven F. Weinstock				
	Laboratorios Abbott				
Dirección	100 Abbott Park Road				
	D-377 AP6D				
Ciudad	Abbott Park	Estado	IL	Código Zip	60064-6050
Condado		Teléfono	(847)937-4395	Fax	(847)938-2623

Nombre	Janelle D. Strode	No. de Registro (Apoderado/Agente)	34,738
Firma	(Firma)	Fecha	9 de abril de 1999

5.705

ANEXO :

REIVINDICAMOS :

1. Un método para detectar el enlace entre una molécula determinada ^{13}C-enriquecida ligante putativa y una preseleccionada, que comprende:

a) generar un primer espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de dicha molécula determinada;

b) formar una mezcla de dicha molécula determinada al menos con un compuesto ligante putativo;

c) generar un segundo espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de la mezcla del paso (b); y

5.7.05

Traductor. Oficial
María Luisa Brizard de Restrepo
Intérprete Oficial
Res. 0783 del Min. de Justicia

d) comparar los espectros primero y segundo.

2. El método de la Reivindicación 1 en el que dicha molécula determinada es escogida entre el grupo constituído por lipoproteínas, fragmentos de lipoproteína, glicoproteínas, fragmentos de glicoproteínas, proteínas, fragmentos de proteína, polipéptidos, ADN y ARN.

3. El método de la Reivindicación 1 en el que dicha molécula determinada es escogida entre el grupo constituído por proteínas, fragmentos de proteína y polipéptidos.

4. El método de la Reivindicación 3 en el que dicha molécula determinada se prepara mediante cultivo de una linea de células transformada que contiene un vector de expresión que contiene un polinucleótido que codifica dicha molécula determinada en un medio que contiene fuentes asimilables de ^{13}C.

5. El método de la Reivindicación 4 en el que dichas fuentes asimilables de ^{13}C son uniformemente enriquecidas con ^{13}C.

6. El método de la Reivindicación 5 en el que se emplea glucosa rotulada con ^{13}C (U-^{13}C-glucosa) para producir dichas fuentes asimilables de ^{13}C.

7. El método de la Reivindicación 4 en el que dichas fuentes asimilables de ^{13}C son especificamente enriquecidas con ^{13}C.

PUBLICACION OFICIAL No. 5.705

8. El método de la Reivindicación 7 en el que se emplea yoduro de metilo enriquecido con ^{13}C para producir dichas fuentes asimilables de ^{13}C.

9. El método de la Reivindicación 7 en el que dichas fuentes asimilables de ^{13}C son aminoácidos y sales de estos.

10. El método de la Reivindicación 9 en el que dicho aminoácido es escogido entre el grupo constituido por alanina, leucina, isoleucina y valina.

11. El método de la Reivindicación 8 en el que dichas fuentes asimilables de ^{13}C son precursores biosintéticos de aminoácidos y sales de estos.

12. El método de la Reivindicación 11 en el que dichos precursores biosintéticos de aminoácidos son escogidos entre el grupo constituido por ácido 4(^{13}C)-butírico y ácido 4-(^{13}C)-3-(^{13}C)-metilbutírico.

13. Un método para seleccionar una mezcla de compuestos para enlazarla a una molécula determinada enriquecida con ^{13}C preseleccionada, que comprende:

a) generar un primer espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de dicha molécula determinada;

b) poner dicha molécula determinada en contacto con dicha mezcla de compuestos;

c) generar un segundo espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de la mezcla del paso (b);

d) comparar los espectros primero y segundo.

14. El método de la Reivindicación 13 que comprende además:

e) exponer dicha molécula determinada individualmente a cada uno de los compuestos de dicha mezcla cuando el paso d) revele diferencias entre el primero y el segundo espectro;

f) generar espectros de correlación $^{13}C/^{1}H$ NMR bidimensionales de dicha molécula determinada que ha sido expuesta a cada compuesto;

g) comparar cada uno de los espectros generados en el paso f) con el primer

espectro generado de la molécula determinada sola.

15. El método de la Reivindicación 14 en el que dicha molécula determinada es escogida entre el grupo constituído por lipoproteínas, fragmentos de lipoproteína, glicoproteínas, fragmentos de glicoproteínas, proteínas, fragmentos de proteína, polipéptidos, ADN y ARN.

16. El método de la Reivindicación 15 en el que dicha molécula determinada es escogida entre el grupo constituído por proteínas, fragmentos de proteína y polipéptidos.

OFICIAL No. 5.705

17. El método de la Reivindicación 13 en el que dicha molécula determinada es uniformemente enriquecida con ^{13}C.

18. El método de la Reivindicación 13 en el que dicha molécula determinada es especificamente enriquecida con ^{13}C.

19. Un método para determinar la constante de disociación de un ligante que se liga a una molécula determinada enriquecida con ^{13}C preseleccionada, que comprende:

a) generar un primer espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de dicha molécula determinada;

b) exponer dicha molécula determinada a varias concentraciones de dicho ligante;

c) generar un espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de cada una de las concentraciones de ligante del paso b);

d) comparar cada uno de los espectros del paso c) con dicho espectro del paso (a); y.

e) calcular la constante de disociación.

PUBLICACION OFICIAL No. 5.705

20. El método de la Reivindicación 19 en el que dicha molécula determinada es escogida entre el grupo constituido por lipoproteínas, fragmentos de lipoproteína, glicoproteínas, fragmentos de glicoproteínas, proteínas, fragmentos de proteína, polipéptidos, ADN y ARN.

21. El método de la Reivindicación 20 en el que dicha molécula determinada es escogida entre el grupo constituído por proteínas, fragmentos de proteína y polipéptidos.

22. El método de la Reivindicación 20 en el que dicha molécula determinada es uniformemente enriquecida con ^{13}C.

23. El método de la Reivindicación 20 en el que dicha molécula determinada es especificamente enriquecida con ^{13}C.

24. Un compuesto identificado por el método de selección de la Reivindicación 13.

80
72

25. Un método para determinar los residuos específicos de aminoácidos en una molécula determinada enriquecida con ^{13}C afectada por el enlace de un ligante a ella, que comprende:

a) generar un primer espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de dicha molécula determinada, en donde dichos valores de desviación química de las señales $^{13}C/^{1}H$ en dicho espectro de correlación bidimensional corresponden al menos a un sitio específico conocido de la agrupación atómica de dicha molécula determinada;

b) formar una mezcla de dicha molécula determinada con un compuesto ligante conocido;

c) generar un segundo espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de la mezcla del paso b); y

c) comparar los espectros primero y segundo.

ES TRADUCCION FIEL Y COMPLETA, de la cual queda copia en los archivos de esta oficina de traducciones, para su confrontación y a la que me remito.

Derechos de Traducción: $81.000,oo

BOGOTA, 9 DE MARZO DEL 2000.

TRADUCCION OFICIAL No. 5.705

María Luisa Brigard de Restrepo
Intérprete Oficial
Res. No. 0793 del Min. de Justicia

84
73

25. Un método para determinar los residuos específicos de aminoácidos en una molécula determinada enriquecida con ^{13}C afectada por el enlace de un ligante a ella, que comprende:

a) generar un primer espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de dicha molécula determinada, en donde dichos valores de desviación química de las señales $^{13}C/^{1}H$ en dicho espectro de correlación bidimensional corresponden al menos a un sitio específico conocido de la agrupación atómica de dicha molécula determinada;

b) formar una mezcla de dicha molécula determinada con un compuesto ligante conocido;

5.705
Maria Luisa Briganti de Restrepo
Intérprete Oficial
Orden del Mérito de Justicia

c) generar un segundo espectro de correlación $^{13}C/^{1}H$ NMR bidimensional de la mezcla del paso b); y

c) comparar los espectros primero y segundo.

ANEXO:

PATENTE

EN LA OFICINA DE PATENTES Y MARCAS DE FABRICA

DE LOS ESTADOS UNIDOS

82
74

Solicitantes: S.W. Fesik, et al.	No. de Rótulo de Correo Expreso: EE835516454US
No. de Serie: Todavía no ha sido asignado	Por el presente certifico que este documento (junto con cualquier documento al que se haga referencia como anexo o incluído) se está depositando en el Servicio Postal de los Estados Unidos en la fecha indicada abajo con porte pagado suficiente como Correo Expreso en un sobre dirigido a: Casilla de Solicitud de Patente - Comisionado Auxiliar para Patentes Washington, D.C. 20231, el día: Fecha de Depósito: 9 de abril de 1999
Registrada:	
Título: Uso de Resonancia Magnética Nuclear 13C para Detectar Enlace a Biomoléculas Determinadas	
No. de Caso: 5816.US.P3	
No. de Grupo de Arte: Todavía no ha sido asignado	(Fdo) Kathleen T.L. 9/4/99 Kathleen T. Litz Fecha
Examinador: Todavía no ha sido asignado	

5705

CORRESPONDENCIA OFICIAL No.

Declaración y Poder

para una Solicitud de Patente de los Estados Unidos

Como un inventor mencionado abajo, declaro por la presente que creo que soy el inventor original, primero y conjunto del asunto que se reivindica y para el cual se busca una patente sobre la invención titulada Uso de Resonancia Magnética Nuclear 13C para

Detectar Enlace a Biomoléculas Determinadas, cuya descripción está anexa a la presente.

Mi residencia, dirección postal y ciudadanía son las especificadas abajo a continuación de mi nombre.

Declaro por la presente que he revisado y comprendido el contenido de la descripción mencionada arriba, incluyendo las reivindicaciones.

Reconozco una obligación de revelar a la Oficina de Patentes y Marcas de Fábrica toda la información de la que sé que es importante para poder patentar según lo definido en el Título 37, Código de Reglamentaciones Federales, §1.56. 5.705

TRADUCTOR OFICIAL
María Luisa Briceño de Restrepo
Intérprete Oficial
de otras del año de Justicia

Se reivindica beneficio de solicitud(es) extranjera(s) como sigue: —

Por la presente reivindico beneficios de prioridad extranjera según 35 U.S.C. §119 para las siguientes solicitudes extranjeras de patente o certificado de inventor:

NINGUNA

Las siguientes solicitudes extranjeras de patente o certificado de inventor tienen una fecha de registro anterior a la fecha de registro de la solicitud identificada arriba.

NINGUNA

Se reivindica beneficio de solicitud(es) de los EE.UU anterior(es) como sigue:

Por la presente reivindico el beneficio en virtud de 35 U.S.C. §120 de las siguientes solicitudes de patente de los Estados Unidos registradas anteriormente. Esta solicitud es una continuación en parte de la solicitud No. de Serie 09/241,194, registrada el 1° de febrero de 1999, que es una continuación de la solicitud No. de Serie 08/744,701, registrada el 31 de octubre de 1996, ahora Patente de los Estados Unidos No. 5, ---, ---, que es una continuación en parte de la solicitud No. de Serie 08/678,903, registrada el 12 de julio de 1996, ahora abandonada, que es una continuación en parte de la solicitud No. de Serie 08/558,633, registrada el 14 de noviembre de 1995, ahora Patente de los Estados Unidos 5, ---, ---. Hasta donde el asunto del que es objeto cada una de las reivindicaciones de esta solicitud no está expuesto en las solicitudes de los EE.UU. anteriores en la forma descrita por 35 U.S.C, §112, primer parágrafo, yo reconozco una obligación de revelar a la Oficina de Patentes y Marcas de Fábrica toda la información de la que sé que es importante para poder patentar según lo definido en el Título 37, Código de Reglamentaciones Federales, §1.56 que haya llegado a existir entre la(s) fecha(s) de las solicitudes anteriores y la fecha de registro nacional o de PTC de esta solicitud.

NINGUNA

Yo reivindico por la presente el beneficio según el Título 35, Código de los Estados Unidos § 119(e) de cual(es)quier(a) solicitud(es) provisional(es) de los Estados Unidos enumerada(s) a continuación:

NINGUNA

Por la presente nombro a los siguientes Apoderados y/o agentes para que adelanten esta

solicitud y cualesquiera solicitudes de continuación o división basadas en ella y para que tramiten en la Oficina de Patentes y Marcas de Fábrica todos los negocios relacionados con la misma.

Mona Anand, Reg. No. 34,537
Regina M. Anderson, Reg. No. 35,820
Mark C. Bach, Reg. No. 34,766
Cheryl L. Becker, Reg. No. 35,441
Thomas D. Brainard, Reg. No. 32,459
Dianne Casuto, Reg. No. 40,943
Portia Chen, Reg. No. P44,075
Steven R. Crowley, Reg. No. 31,604
Andreas M. Danckers, Reg. No. 32,652
J. Michael Dixon, Reg. No. 32,410
Mimi C. Goller, Reg. No. 39,046
Matthew R. Hooper, Reg. No. 31,108
Daniel J. Hulseberg, Reg. No. 36,554
Neal D. Marcus, Reg. No.35,267
James D. McNeil, Reg. No. 26,204
Lawrence S. Pope, Reg. No. 26,791
Nicholas A. Poulos, Reg. No. 30,209
Dugal S. Sickert, Reg. No. 33,784
Gregory W. Steele, Reg. No. 33,796
Janelle D. Strode, Reg. No. 34,788
Michael J. Ward, Reg. No. 37,960
David L. Weinstein, Reg. No. 28,128
Steven F. Weinstock, Reg. No. 30,117
Brian R. Woodworth, Reg. No. 33,137
Paul D. Yasger, Reg. No. 37,477

OFICIAL No.
Traducción Oficial
Oficina de Patentes y Marcas

Enviar correspondencia a:

Steven F. Weinstock
Abbott Laboratories
D-388/AP6D
100 Abbott Park Road
Abbott Park, Illinois 60064-3500

Dirigir llamadas telefónicas a:

Janelle D. Strode
(847) 937-4395

Eb
78

INVENTORES

Nombre:	Stephen W. Fesik
Dirección Postal:	1099 Portsmouth Circle
	Gurnee, IL 60031
Residencia:	1099 Portsmouth Circle
	Gurnee, IL 60031
Ciudadanía:	Estados Unidos de América
Nombre:	Philip J. Hajduk
Dirección Postal:	258 Lenox Lane
	Mundelein, IL 60060
Residencia:	258 Lenox Lane
	Mundelein, IL 60060
Ciudadanía:	Estados Unidos de América

TRADUCCION OFICIAL No. 5705
María Luisa Brigard de Restrepo
Intérprete Oficial

Declaro además que todas las declaraciones hechas aquí de mi propio conocimiento son ciertas y que todas las declaraciones hechas con base en información y creencia se consideran ciertas; y además que todas las declaraciones hechas aquí se hicieron con el conocimiento de que las declaraciones voluntariamente falsas y cosas semejantes pueden ser castigadas mediante multa o prisión, o ambas penas, según la Sección 1001 del Título 18 del Código de los Estados Unidos, y que tales declaraciones falsas pueden comprometer la validez de la solicitud o de cualquier patente expedida con base en ella.

(Firma) 18/3/99

Stephen W. Fesik Fecha

~~87~~
79

(Firma) 8/3/99

Philip J. Hajduk Fecha

ES TRADUCCION FIEL Y COMPLETA, de la cual queda copia en los archivos de esta oficina de traducciones, para su confrontación y a la que me remito.

Derechos de Traducción: $130.500

BOGOTA, 9 DE MARZO DEL 2000.

TRADUCTOR OFICIAL No. 5.705

María Luisa Brigard de Restrepo

Intérprete Oficial

Res. No. 0783 del Min. de Justicia

Abbott
C-5816
86
30

PA 211364

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

TRANSLATION OFFICIAL NO. 55

María Luisa Briseño de Rodríguez
Intérprete Oficial
0793 del Min. de Justicia

February 23, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *09/288,924*
FILING DATE: *April 09, 1999*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

P. Swain

P. SWAIN
Certifying Officer

jc649 U.S. PTO 04/09/99

89
81

Please type a plus sign (+) inside this box → [+]

PTO/SB/05 (4/98)
Approved for use through 09/30/2000. OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. § 1.53(b))

Attorney Docket No.	5816.US.P3
First Inventor or Application Identifier	Stephen W. Fesik
Title	See 1 in Addendum
Express Mail Label No.	EE835516454US

jc135 U.S. PTO 09/288924 04/09/99

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231

1. [X] * Fee Transmittal Form *(e.g., PTO/SB/17)* *(Submit an original and a duplicate for fee processing)*
2. [X] Specification [Total Pages 48] *(preferred arrangement set forth below)*
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings *(if filed)*
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. [X] Drawing(s) *(35 U.S.C. 113)* [Total Sheets 5]
4. Oath or Declaration [Total Pages 4]
   a. [X] Newly executed (original or copy)
   b. [ ] Copy from a prior application (37 C.F.R. § 1.63(d)) *(for continuation/divisional with Box 16 completed)*
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §§ 1.63(d)(2) and 1.33(b).

NOTE FOR ITEMS 1 & 13 IN ORDER TO BE ENTITLED TO PAY SMALL ENTITY FEES, A SMALL ENTITY STATEMENT IS REQUIRED (37 C.F.R. § 1.27), EXCEPT IF ONE FILED IN A PRIOR APPLICATION IS RELIED UPON (37 C.F.R. § 1.28).

5. [ ] Microfiche Computer Program *(Appendix)*
6. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
   a. [ ] Computer Readable Copy
   b. [ ] Paper Copy (identical to computer copy)
   c. [ ] Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. [X] Assignment Papers (cover sheet & document(s))
8. [ ] 37 C.F.R.§3.73(b) Statement *(when there is an assignee)* [ ] Power of Attorney
9. [ ] English Translation Document *(if applicable)*
10. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
11. [ ] Preliminary Amendment
12. [X] Return Receipt Postcard (MPEP 503) *(Should be specifically itemized)*
13. [ ] * Small Entity Statement(s) *(PTO/SB/09-12)* [ ] Statement filed in prior application Status still proper and desired
14. [ ] Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
15. [X] Other. See 2 in Addendum

5.705

Maria Luisa Briard de Rodrigo
Interprete Oficial
Traductora Publica de Inglés

16. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in a preliminary amendment:
    [ ] Continuation [ ] Divisional [X] Continuation-in-part (CIP) of prior application No 09/241,184
    Prior application information: Examiner _____ Group / Art Unit: _____
    For CONTINUATION or DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 4b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

17. CORRESPONDENCE ADDRESS

[ ] Customer Number or Bar Code Label (Insert Customer No. or Attach bar code label here) or [X] Correspondence address below

Name	Steven F. Weinstock Abbott Laboratories				
Address	100 Abbott Park Road D-377 AP6D				
City	Abbott Park	State	IL	Zip Code	60064-6050
Country		Telephone	(847) 937-4395	Fax	(847) 938-2623

Name (Print/Type)	Janelle D. Strode	Registration No (Attorney/Agent)	34,738
Signature	Janelle D. Strode	Date	April 9, 1999

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

The Use of ^{13}C Nuclear Magnetic Resonance to Detect Binding to Target Molecules

Field of the Invention

The present invention relates to the use of nuclear magnetic resonance to detect binding between compounds and ^{13}C-enriched target molecules.

Cross-Reference to Related Applications

This application is a continuation-in-part of application Serial No. 09,241,194, filed February 1, 1999, which is a continuation of application Serial No. 08/744,701, filed October 31, 1996, now United States Patent No. 5,---,---, which is a continuation-in part of application Serial No. 08/678,903, filed July 12, 1996, now abandoned, which is a continuation-in-part of application Serial No. 08/558,633, filed November 14, 1995, now United States Patent 5,---,---.

Background of the Invention

One of the most powerful tools for discovering new drug leads is random screening of synthetic and natural product libraries to discover compounds that bind to a

particular target molecule (for example, the identification of ligands to that target). Using this method, ligands may be identified by their ability to form a physical association with a target molecule or by their ability to alter a function of a target molecule.

When physical binding is sought, a target molecule is typically exposed to one or more compounds suspected of being ligands and assays are performed to determine if complexes between the target molecule and one or more of those compounds are formed. Such assays, as is well known in the art, test for gross changes in the target molecule (for example, changes in size, charge, mobility) that indicate complex formation.

Where functional changes are measured, assay conditions are established that allow for measurement of a biological or chemical event related to the target molecule (for example, an enzyme-catalyzed reaction, receptor-mediated enzyme activation, and the like). To identify an alteration, the function of the target molecule is determined before and after exposure to the test compounds.

Existing physical and functional assays have been used successfully to identify new drug leads for use in designing therapeutic compounds. There are, however, limitations inherent to those assays that compromise their accuracy, reliability and efficiency. A major problem with existing assays, for example, relates to the generation of "false positives". In a typical functional assay, a "false positive" result is generated for a

compound that triggers the assay but which compound is not effective in eliciting the desired physiological response. In a typical physical assay, a "false positive" is a compound that, for example, attaches itself to the target but in a nonspecific manner (for example, non-specific binding). False positives are particularly prevalent and problematic when screening higher concentrations of putative ligands because many compounds have non-specific effects at those concentrations.

In a similar fashion, existing assays are frequently plagued by the problem of "false negatives", which result when a compound gives a negative response in the assay but, as found subsequently by some other method, is actually a ligand for the target. False negative results typically occur in assays that use concentrations of test compounds that are either too high (resulting in toxicity) or too low, relative to the binding or dissociation constant of the compound to the target.

Another problem with existing assays is the limited amount of information that is provided by the assay itself. While the assay may correctly identify compounds that attach to or elicit a response from the target molecule, those assays typically do not provide any information about either specific binding sites on the target molecule or structure activity relationships between the compound being tested and the target molecule. The inability to provide any such information

is particularly problematic where the screening assay is being used to identify leads for further study.

It has recently been suggested that X-ray crystallography can be used to identify the binding sites of organic solvents on macromolecules. However, this method cannot determine the relative binding affinities at different sites on the target. It is only applicable to very stable target proteins that do not denature in the presence of high concentrations of organic solvents. In addition, due to the long time needed to determine the individual crystal structures, this approach is not a suitable method for rapidly testing a large number of compounds that are chemically diverse, but is limited to mapping the binding sites of only a few organic solvents.

Rapid, efficient, and reliable methods of determining ligand/target binding, and mapping binding sites on the target substance are disclosed in United States Patent Nos. 5,698,401 and 5,804,390, to Fesik, *et al*. These patents disclose methods of detecting binding of a ligand compound to a target biomolecule by generating first and second nuclear magnetic resonance correlation spectra from target biomolecules which have been isotopically enriched with the NMR-detectable ^{15}N nucleus. The first spectrum is generated from data collected on the target substance in the absence of ligands, and the second in the presence of one or more ligands. A comparison of the two spectra permits determination of which compounds in the mixture of putative ligands bind(s) to the target biomolecule, as

well as specific information about the site of binding. Since the methods elicit information about the binding sites on the target molecule, the methods can be used for optimizing the design of ligands to a pre-selected target.

Because the NMR methods of Fesik, *et al.*, *supra*, require nitrogen-containing target substances due to the dependence of those methods on isotopic-enrichment of the target molecule with ^{15}N, it would be a valuable contribution to the art to provide an alternative method which employs a different type of isotopically-enriched target molecule.

Summary of the Invention

The instant invention provides a method of detecting binding between one or more putative ligands to a pre-selected, isotopically-enriched target molecule.

The instant invention further provides a method of screening a mixture of compounds for binding to a pre-selected, isotopically-enriched target molecule.

Also provided by the instant invention is a method of determining the dissociation constant for a ligand compound that binds to a pre-selected, isotopically-enriched target biomolecule.

Additionally provided by the instant invention are methods employed in the determination of the specific amino acids in a ^{13}C-enriched target molecule affected by the binding of a ligand.

Still further provided by the instant invention is a compound identified by a method of screening a mixture of compounds for binding to a pre-selected, isotopically-enriched target molecule.

Brief Description of the Drawings

Figure 1 illustrates a $^{13}C/^{1}H$ correlation spectrum of uniformly ^{13}C-labeled FKBP.

Figure 2 illustrates the methyl regions of $^{13}C/^{1}H$ correlation spectra of uniformly ^{13}C-labeled FKBP before

(thin multiple contours) and after (thick single contours) addition of 2- phenylimidazole (0.12 mM).

Figure 3 illustrates the methyl regions of $^{13}C/^{1}H$ correlation spectra of FKBP selectively $^{13}C/^{15}N/^{2}H$-labeled at valinyl and leucyl residues before (thin multiple contours) and after (thick single contours) addition of 2-phenylimidazole (0.25 mM).

Figure 4 illustrates the methyl regions of $^{13}C/^{1}H$ correlation spectra of FKBP selectively ^{13}C-labeled at alanyl residues before (thin multiple contours) and after (thick single contours) addition of 2-phenylimidazole (0.25 mM).

Figure 5 illustrates a "stick model" depiction of the three-dimensional structure of FKBP.

Detailed Description of the Invention

Terms used throughout this specification have their usually accepted meanings. The following specific terms have the ascribed meanings.

"DTT" means dithiothreitol.

"FKBP" refers to FK-binding protein.

"HEPES" denotes N-2-hydroxyethylpiperazine-N'-2-ethylsulfonic acid.

"IPTG" means isopropyl-β-D-thiogalactopyranoside.

"PMSF" refers to α-toluenesulfonyl fluoride.

"SCD" refers to the catalytic domain (residues 81-256) of stromelysin.

Any target biomolecule which gives a high resolution NMR spectrum and which can be uniformly or selectively enriched with ^{13}C can be used in the methods of the present invention. These methods are thus applicable to any desired ^{13}C-enriched target biomolecule, including lipoproteins, lipoprotein fragments, glycoproteins, glycoprotein fragments, proteins, protein fragments, polypeptides, DNA and RNA. The natural isotopic abundance of ^{13}C is 1.11%. Thus, the probability that any given carbon atom within an organic molecule is ^{13}C is normally about 0.0111.

In order to increase the strength of a NMR signal evincing data related to spin coupling between the nucleus of a ^{13}C carbon atom and any adjacent hydrogen atoms, it is desirable to increase the natural ^{13}C content of the target molecule being studied. This is accomplished by either uniformly or selectively enriching the target molecule with ^{13}C. As used throughout this specification and the appended claims, the terms "uniform enrichment," "uniformly enriching," "uniformly enriched," uniform labeling" and "uniformly labeled" mean increasing to a value greater than 0.0111, by synthetic means, the probability that a carbon atom randomly selected throughout the target molecule will be ^{13}C. On the other hand, the terms "specific enrichment," "specifically enriching," "specifically enriched," "specifically labeling" and "specifically labeled" mean increasing to a value greater than 0.0111, by synthetic means, the probability that carbon atoms at one or more

specific pre-selected site(s) within the target molecule will be ^{13}C.

For example, biomolecules expressed by genetically-modified microorganisms grown in a nutrient medium containing uniformly ^{13}C-enriched glucose will be uniformly ^{13}C-enriched. On the other hand, a protein expressed by a genetically modified microorganism in a nutrient medium containing alanine that is ^{13}C-enriched only on the methyl side chain will be specifically enriched by ^{13}C at those alanyl residues contained within the expressed protein.

The method of the present invention, which employs isotopic enrichment with ^{13}C, is applicable to any organic target molecule, including those containing nitrogen. The method also permits the analysis of target molecules in which specific carbon atom sites have been enriched (for example, methyl groups of alanyl, leucyl, isoleucyl, and valinyl residues). Methyl groups have favorable relaxation properties compared to amide groups, which is advantageous when applied to larger target biomolecules (MW > 30 kDa).

Polypeptides and proteins perform many pivotal roles in living organisms. The examples provided below employ polypeptides to illustrate the instant method. Polypeptides and protein fragments comprise preferred classes of target substances for the method of the present invention. However, it is to be understood that the method of the present invention is applicable to other target substances which can be ^{13}C-enriched.

The preparation of uniformly and specifically ^{13}C-enriched exemplary polypeptide target molecules is set forth below. One means of preparing adequate quantities of either uniformly or specifically ^{13}C-enriched polypeptide-containing target molecules involves the transformation of a host cell with an expression vector containing a polynucleotide encoding the desired polypeptide. The protein or polypeptide protein fragment is expressed by culturing the transformed cell line in a medium containing assimilable sources of ^{13}C well known in the art. A preferred assimilable source for uniform ^{13}C labeling is uniformly ^{13}C-labeled glucose or U-^{13}C-glucose, available from Cambridge Isotope Laboratories. For site-specific labeling, assimilable sources for ^{13}C-labeling of a target polypeptide include commercially available specifically ^{13}C-labeled amino acids. In an alternative embodiment for specific enrichment, the assimilable sources of ^{13}C contained in the nutrient medium are ^{13}C-labeled biosynthetic precursors of amino acids. For example, α-keto-butyrate is the biosynthetic precursor of isoleucine and α-keto-isovalerate is the biosynthetic precursor of both valine and leucine. Scheme I below shows how the specifically ^{13}C-enriched biosynthetic precursors of leucine, isoleucine, and valine, can be synthesized. The comparatively inexpensive ^{13}C-enriched methyl iodide ($H_3{}^{13}CI$) may be employed as the source for isotopic enrichment to produce C-terminally-labeled α-keto-butyric acid and α-keto-isovaleric acid.

The use of a uniformly ^{13}C-enriched nutrient such as ^{13}C-enriched glucose is a convenient means of introducing ^{13}C-enrichment into a target substance; however, it is very expensive. Furthermore, a vast majority of the carbon sites in uniformly ^{13}C-labeled targets will have a covalently bonded neighbor which is also ^{13}C-labeled, introducing ^{13}C-^{13}C coupling which can negatively impact both the signal-to-noise and relaxation properties of ^{13}C-labeled sites in the target biomolecule. A particular advantage is achieved by site-specifically labeling the target polypeptide with ^{13}C. As stated above, this can be accomplished by including commercially available ^{13}C-labeled amino acids in the nutrient medium. This too, however, is a costly alternative, but may be desirable in some circumstances when labeling of certain types of aminoacyl residues in the target polypeptide is required. However, the preferred method of ^{13}C-labeling a polypeptide target molecule comprises growing the genetically modified cell line in a nutrient medium containing ^{13}C-labeled biosynthetic precursors of amino acids. In particular, preferred amino acid precursors that are labeled include α-keto-butyric acid and α-keto-isovaleric acid. The biosynthetic products of these precursors are leucine, isoleucine, and valine in which particular side-chain methyl groups are ^{13}C-enriched. Because the methyl groups each have three hydrogen atoms connected to a ^{13}C-labeled carbon atom, the corresponding NMR signals are particularly strong and distinctive.

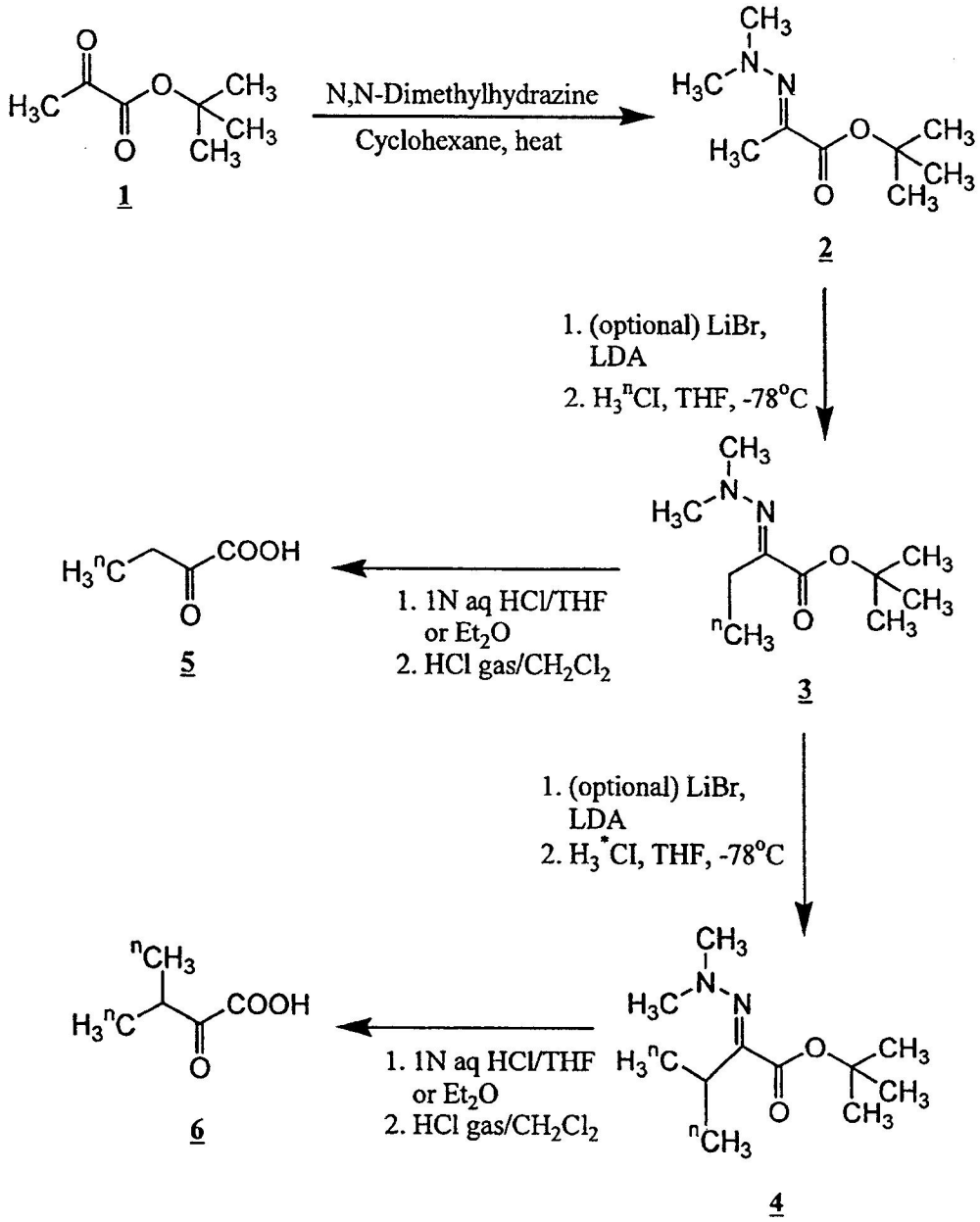
The synthetic sequence for labeled α-keto-butyric acid and α-keto-isovaleric acid involves the methylation of the terminal carbon atom in pyruvic acid with ^{13}C-enriched methyl iodide. Normally, the alkylation of α-keto acids, such as pyruvate, is inherently difficult and is accompanied by decomposition of the enolate intermediate with the formation of numerous side products. However, T. Spencer, *et al.*, *Tetrahedron Letters*, 1975, 3889, and D. R. Williams, *et al.*, *ibid*, 1990, 5881, have shown that alkylation of the corresponding oxime can be easily accomplished, and D. Enders, *et al.*, *Angew. Chem. Int. Eng. Ed.*, 1992, 618 and D. Enders, *et al.*, *Synlett*, 1992, 901 have demonstrated that the hydrazone is also readily alkylated without decomposition.

In Scheme I, *tert*-butyl pyruvate, **1**, is converted to the corresponding N,N-dimethylhydrazone, **2**, by reaction with N,N-dimethylhydrazine in diethyl ether at room temperature. The resulting hydrazone, **2**, is cooled in tetrahydrofuran solution to -78°C, and treated with lithium bromide, followed by lithium diisopropylamide to form the intermediate aza-allyl enolate. The enolate is alkylated with ^{13}C-labeled methyl iodide to produce hydrazone **3**. A second course of alkylation of **3** produces the labeled dimethylated hydrazone, **4**. Treatment of **3** and **4** with trifluoroacetic acid in methylene chloride at 0°C cleaves both the hydrazine and ester groups to produce the corresponding ^{13}C-terminally labeled α-ketoacids, **5**

and 6 (4(^{13}C)-butyric acid and 4-(^{13}C)-3-(^{13}C)-methylbutyric acid, respectively).

Scheme I

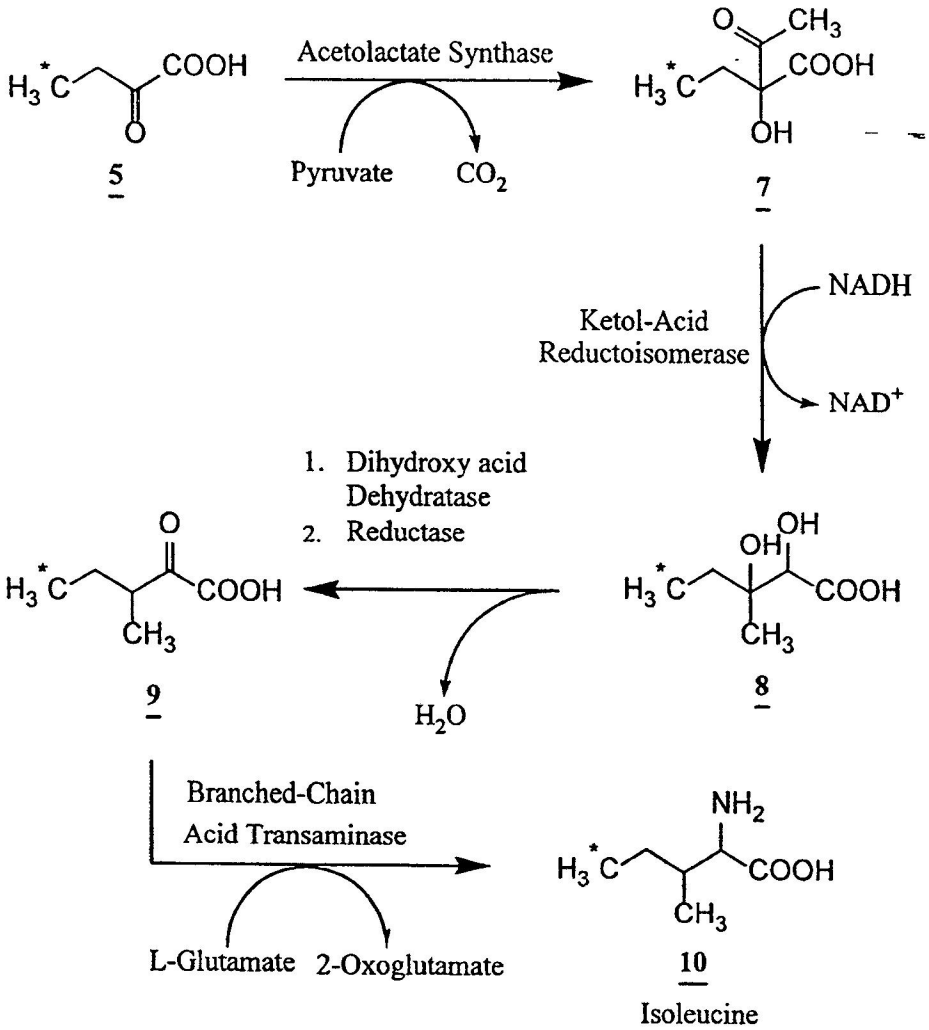
Chemical Synthesis of Labeled Precursors



Reaction Schemes II, III, and IV illustrate, respectively, how these α-ketoacids are biosynthetically converted into ^{13}C-leucine, ^{13}C-isoleucine, and ^{13}C-valine. In all of the Schemes, the sites of isotopic enrichment are indicated by asterisks.

Scheme II

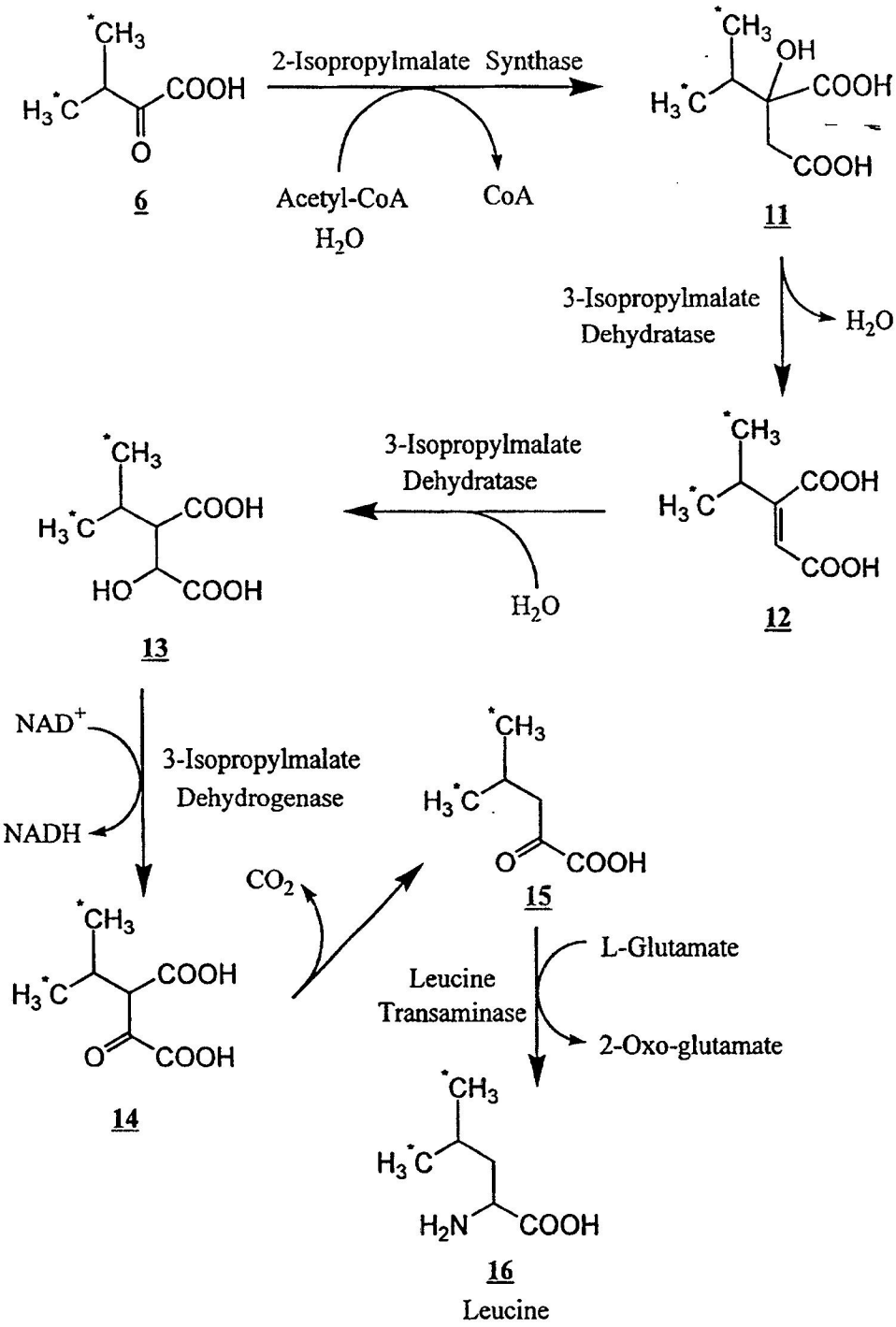
Biochemical Synthesis of Labeled Isoleucine


Acetolactate Synthase
Pyruvate
CO_2
Ketol-Acid Reductoisomerase
NADH
NAD^+
1. Dihydroxy acid Dehydratase
2. Reductase
H_2O
Branched-Chain Acid Transaminase
L-Glutamate
2-Oxoglutamate
5
7
8
9
10
Isoleucine

106
98

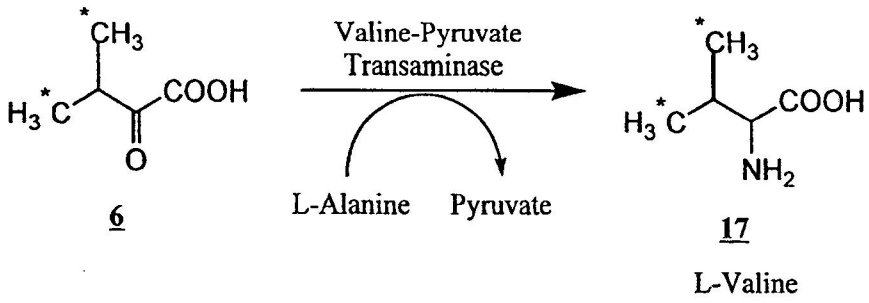
Scheme III

Biochemical Synthesis of Labeled Leucine


2-Isopropylmalate Synthase
Acetyl-CoA
CoA
H_2O
6
11
3-Isopropylmalate Dehydratase
H_2O
12
3-Isopropylmalate Dehydratase
H_2O
13
NAD^+
NADH
3-Isopropylmalate Dehydrogenase
14
CO_2
15
L-Glutamate
Leucine Transaminase
2-Oxo-glutamate
16
Leucine

Scheme IV

Biochemical Synthesis of Valine



6

17

L-Valine

Means for preparing expression vectors that contain polynucleotide sequences coding specific polypeptides and for transforming host cells with those vectors are well known in the art. (*See*, for example, R. W. Old, *et al.*, "Techniques of Gene Manipulation," Blackwell Science, London, 1994, and similar treatises in the field.) Likewise, methods for culturing the transformed cells to express the coded polypeptide and for isolating, purifying and re-folding the polypeptide are also well known in the art. Examples presented below describe the production from modified *E. coli* of ^{13}C-enriched samples of the 81-256 amino acid catalytic region of human stromelysin and FK binding protein (FKBP), and the use of these isotopically-enriched polypeptides in the instant methods.

Where the method is employed to screen more than one compound for binding to the target molecule, for example a mixture or a library of compounds, and where a difference arises between the first spectrum generated from the target molecule alone and that generated from the target molecule in the presence of compound(s),

additional steps are performed to identify which specific compound or compounds contained in the mixture is actually binding to the target molecule. Those additional steps include exposing the ^{13}C-enriched target molecule individually to each compound of the mixture; generating a two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum of the labeled target molecule that has been individually exposed to each compound; and comparing each spectrum to the first spectrum generated from the target molecule alone to determine differences in any of those compared spectra. The differences in the spectra facilitate the identification of a compound that is a ligand.

The chemical shift values of the particular $^{13}C/^{1}H$ signals in the two dimensional correlation spectrum correspond to known specific locations of atomic groupings in the target molecule (for example, the carbon atoms of a particular amino acid residue in the target molecule or, in the case of a polypeptide specifically labeled at the methyl groups of alanyl, leucyl, isoleucyl, and valinyl residues). The screening process of this invention thus allows not only for the identification of which compound(s) bind to a particular target molecule, but also permits the determination of the particular amino acids that are affected by the binding of the compound to the target molecule. The chemical shift values may reflect a change in the conformation of the target molecule, or may reflect the

binding of the ligand compound at the site that corresponds to that particular signal.

In addition, the dissociation constant, K_D, for a given ligand and its target molecule can be determined by this process, if desired, by generating a first two-dimensional ^{13}C /^{1}H NMR correlation spectrum of a ^{13}C-labeled target molecule; exposing the labeled target molecule to various concentrations of a ligand; generating a two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum at each concentration of ligand employed; comparing the spectra generated to the first spectrum of the target molecule; and calculating the dissociation constant between the target molecule and the ligand from those differences using Equation 1:

$$K_D = \frac{([P_0] - x)([L_0] - x)}{x}$$

where $[P_0]$ is the total molar concentration of the target molecule, $[L_0]$ is the total molar concentration of the ligand, and x is the molar concentration of the bound species. The value of x is determined from the NMR chemical shift data according to Equation 2:

$$x = \frac{\delta_{observed} - \delta_{free}}{\Delta}$$

where $\delta_{observed}$ is the observed chemical shift value, δ_{free} is the chemical shift value for the free species, and Δ is

the difference between the limiting chemical shift value for saturation ($\delta_{saturation}$) and the chemical shift value of the target molecule free of bound ligand (δ_{free}).

The dissociation constant is then determined by varying its value until a best fit is obtained with the observed data using standard curve-fitting statistical methods. In those situations where the value of $\delta_{saturation}$ is not directly known, K_D and $\delta_{saturation}$ are varied and the resulting data subjected to the same curve-fitting statistical method.

An advantageous capability of the screening method is its ability to determine the dissociation constant of one ligand of the target molecule in the presence of a second molecule already bound to the ligand. This is generally not possible with other methods which employ "wet chemical" analytical methods of determining binding of a ligand to a target molecule substrate.

The process of determining the dissociation constant of a ligand can be performed in the presence of a second bound ligand. Accordingly, the ^{13}C-labeled target molecule is bound to that second ligand before exposing that target to the test compounds. The screening method is additionally able to provide information regarding the binding of a second or subsequent ligand to the target molecule. This second ligand may be chemically linked to the first ligand bound to the target molecule, thus providing a new composite molecule for use in affecting the target molecule.

The screening method of the present invention begins with the generation or acquisition of a two-dimensional $^{13}C/^{1}H$ correlation spectrum of the isotopically enriched target molecule. As stated above, the target molecule can be either uniformly enriched with ^{13}C, or it can be specifically enriched by the incorporation of ^{13}C-methyl groups in alanyl, leucyl, valinyl, and isoleucyl residues. Means for generating two-dimensional $^{13}C/^{1}H$ correlation spectra are well known in the art. The NMR spectra that are typically recorded are two-dimensional $^{13}C/^{1}H$ heteronuclear single quantum correlation (HSQC) spectra, although other techniques known to those skilled in the art can be used. Because the $^{13}C/^{1}H$ signals corresponding to the protein are usually well resolved, the chemical shift changes for individual $^{13}C/^{1}H$ pairs can be readily monitored. A representative two-dimensional $^{13}C/^{1}H$ correlation spectrum of a ^{13}C-labeled target polypeptide (FKBP) is shown in Figure 1. A representative two-dimensional $^{13}C/^{1}H$ correlation spectrum of a specifically ^{13}C-enriched FKBP is shown in Figure 3.

Following exposure of the ^{13}C-labeled target molecule to one or more test compounds, a second two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum is generated. That spectrum is generated in the same manner as set forth above. The first and second spectra are then compared to determine whether there are any differences between the two spectra. Differences in the two-dimensional $^{13}C/^{1}H$ NMR correlation spectra that indicate the presence of a ligand correspond to ^{13}C-labeled sites in the target

molecule. Those differences are determined using standard procedures well known in the art. Figure 2 shows the methyl regions of uniformly ^{13}C-labeled FKBP before (thin multiple contours) and after (thick single contours) addition of 2-phenylimidazole (0.12 mM).

Particular signals in a two-dimensional $^{13}C/^{1}H$ correlation spectrum correspond to specific carbon and proton atoms in the target molecule (for example, particular methyl groups of the amino acid residues in the protein). By way of example, it can be seen from Figure 3 that chemical shifts observed in two-dimensional $^{13}C/^{1}H$ correlation spectra of FKBP exposed to a test compound occurred at residue positions 97 (leucine) and 55 (valine). The region of the protein that is responsible for binding to the individual compounds is identified from the particular carbon and proton atom pairs that change upon the addition of compound.

EXAMPLES

Preparation 1

Preparation of Uniformly ^{13}C-Enriched Catalytic Domain of Human Stromelysin (SCD)

The 81-256 fragment (SEQ ID NO: 1) of stromelysin (SCD) is prepared by inserting a plasmid which codes for the production of the protein fragment into an *E. coli* strain and growing the genetically-modified bacterial strain in a suitable culture medium. The protein fragment is isolated from the culture medium, purified, and subsequently used in the two-dimensional NMR analysis of its affinity with test compounds in accordance with the method of this invention. The procedures for the preparation processes are described below.

Human skin fibroblasts (ATCC No. CRL 1507) are grown and induced using the procedure described by Clark *et al., Archiv. Biochem. and Biophys.* 241: 36 (1985). Total RNA is isolated from 1 g of cells using a RNAgents® Total RNA Isolation System Kit (Promega Corp.) following the manufacturer's instructions. A 1 µg portion of the RNA is denatured by heating at 80°C for five minutes and then subjected to reverse transcriptase PCR using a GenAmp® RNA PCR kit (Applied Biosystems/Perkin-Elmer) following the manufacturer's instructions.

Nested PCR is performed using first primers (a) GAAATGAAGAGTCTTCAA (SEQ ID NO: 2) and (b)

GCGTCCCAGGTTCTGGAG (SEQ ID NO. 3) and thirty-five cycles of 94°C, two minutes; 45°C, two minutes; and 72°C, three minutes. This is followed by re-amplification with internal primers (c) TACCATGGCCTATCCATTGGATGGAGC (SEQ ID NO: 4) and (d) ATAGGATCCTTAGGTCTCAGGGGA GTCAGG (SEQ ID NO: 5) using thirty cycles under the same conditions described immediately above to generate a DNA sequence coding for amino acid residues 1-256 of human stromelysin.

The PCR fragment is then cloned into PCR cloning vector pT7BIue® (Novagen, Inc.) according to the manufacturer's instructions. The resulting plasmid is cut with NcoI and BamHI and the stromelysin fragment is sub-cloned into the expression vector pET3d (Novagen, Inc.).

A mature stromelysin expression construct coding for amino acid residues 81-256 plus an initiating methionine aminoacyl residue is generated from the 1-256 expression construct by PCR amplification. The resulting PCR fragment is first cloned into the pT7BIue® vector (Novagen, Inc.) and then sub-cloned into the pET3d vector (Novagen, Inc.), using the manufacturer's instructions in the manner described above, to produce plasmid pETST-83-256. This final plasmid is identical to that described by Qi-Zhuang *et al.*, *Biochemistry*, 31: 11231 (1992) with the exception that the present plasmid codes for a peptide sequence beginning two amino acids earlier, at position 81, in the sequence of human stromelysin.

Plasmid pETST-83-256 is transformed into *E. coli* strain BL21(DE3)/pLysS (Novagen, Inc.) in accordance with the manufacturer's instructions to generate an expression strain, BL21(DE3)/pLysS/pETST-255-1.

A pre-culture medium is prepared by dissolving 1.698 g of $NaH_2PO_4 \bullet 7H_2O$, 0.45 g of KH_2PO_4, 0.075 g NaCl, 0.150 g NH_4Cl, 0.3 g U-^{13}C-glucose, 300 µl of 1M aqueous $MgSO_4$ solution, and 15 mL of aqueous $CaCl_2$ solution in 150 ml of deionized water. The resulting solution of pre-culture medium is sterilized and transferred to a sterile 500 ml baffle flask. Immediately prior to inoculation of the pre-culture medium with the bacterial strain, 150 ml of a solution containing 34 mg/ml, of chloramphenicol in 100% ethanol and 1.5 ml of a solution containing 20 mg/ml of ampicillin is added to the flask contents. The flask contents are then inoculated with 1 ml of glycerol stock of genetically modified *E. coli* strain BL21(DE3)/pLysS/pETST-255-1. The flask contents are shaken (225 rpm) at 37°C until an optical density of 0.65 is observed.

A fermentation nutrient medium is prepared by dissolving 113.28 g of $Na_2HPO_4 \bullet 7H2O$, 30 g of KH_2PO_4, 5 g NaCl and 10 mL of 1% DF-60 antifoam agent in 9604 mL of deionized water. This solution is placed in a New Brunswick Scientific Micros Fermenter and sterilized at 121 °C for 40 minutes. Immediately prior to inoculation of the fermentation medium, the following pre-sterilized components are added to the fermentation vessel contents: 100 ml of a 10% aqueous solution of NH_4Cl, 15 g of

uniformly ^{13}C-enriched glucose, 20 ml of an aqueous 1M solution of $MgSO_4$, 1 ml of an aqueous 1M $CaCl_2$ solution, 5 ml of an aqueous solution of thiamin hydrochloride (10 mg/ml), 10 ml of a solution containing 34 mg/ml of chloramphenicol in 100% ethanol, and 1.9 g of ampicillin dissolved in the chloramphenicol solution. The pH of the resulting solution is adjusted to pH 7.00 by the addition of an aqueous solution of 4N H_2SO_4.

The pre-culture of *E. coli* strain BL21(DE3)/pLysS/pETST-255-1 from the shake flask scale procedure described above is added to the fermenter contents and cell growth is allowed to proceed until an optical density of 0.48 is achieved. During this process, the fermenter contents are automatically maintained at pH 7.0 by the addition of 4N H_2SO_4 or 4N KOH as needed. The dissolved oxygen content of the fermenter contents is maintained above 55% air saturation through a cascaded loop which increased agitation speed when the dissolved oxygen content dropped below 55%. Air is fed to the fermenter contents at 7 standard liters per minute (SLPM) and the culture temperature is maintained at 37°C throughout the process.

The cells are harvested by centrifugation at 17,000 x g for 10 minutes at 4°C and the resulting cell pellets are collected and stored at -85°C. The wet cell yield is 3.5 g/L. Analysis of the soluble and insoluble fractions of cell lysates by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) reveals that approximately 50% of the stromelysin is found in the soluble phase.

The stromelysin fragment prepared as described above is purified employing a modification of the technique described by Ye, *et al.*, *Biochemistry*, 31: 11231 (1992). The harvested cells are suspended in 20 mM Tris-HCl buffer (pH 8.0), sodium azide solution containing 1mM $MgCl_2$, 0.5 mM $ZnCl_2$, 25 units/ml of Benzonase® enzyme (Benzon Pharma), and an inhibitor mixture made up of 4-(2-aminoethyl)benzenesulfonyl fluoride ("AEBSF") Leupeptin®, Aprotinin® and Pepstatin® (all at concentrations of 1 mg/ml). AEBSF, Leupeptin®, Aprotinin®, and Pepstatin® are available from American International Chemical. The resulting mixture is gently stirred for one hour and then cooled to 4°C. The cells are then sonically disrupted using a 50% duty cycle. The resulting lysate is centrifuged at 14,000 rpm for 30 minutes and the pellet of insoluble fraction frozen at -80°C for subsequent processing.

Solid ammonium sulfate is added to the supernatant to the point of 20% of saturation and the resulting solution loaded onto a 700 ml phenyl Sepharose fast flow ("Q-Sepharose FF) column (Pharmacia Biotech.). Prior to loading, the Sepharose column is equilibrated with 50 mM Tris-HCl buffer (pH 7.6 at 4°C), 5 mM CaC12, and 1M $(NH_4)_2SO_4$. The loaded column is eluted with a linear gradient of decreasing concentrations of aqueous $(NH_4)_2SO_4$ (from 1M down to 0M) and increasing concentrations of aqueous $CaCl_2$ (from 5 mM to 20 mM) in Tris-HCl buffer at pH 7.6. The active fractions of eluate are collected and concentrated in an Amicon stirred cell (Amicon, Inc.).

The concentrated sample is dialyzed overnight in the starting buffer used with the Q-Sepharose FF column, 50 mM Tris-HCl (pH 8.2 at 4°C) with 10 mM $CaCl_2$.

The dialyzed sample is then loaded on the Q-Sepharose FF column and eluted with a linear gradient comprising the starting buffer and 200 nM NaCl. The purified soluble fraction of the stromelysin fragment is concentrated and stored at 4°C. The pellet is solubilizcd in 8M guanidine-HCl. The solution is centrifuged for 20 minutes at 20,000 rpm and the supernatant added dropwise to a folding buffer comprising 50 mM Tris-HCl (pH 7.6), 10 mM $CaCl_2$, 0.5 mM $ZnCl_2$ and the inhibitor cocktail of AEBSF, Leupeptin®, Aprotinin®, and Pepstatin® (all at concentrations of 1 µg/ml). The volume of folding buffer is ten times that of the supernatant. The mixture of supernatant and folding buffer are centrifuged at 20,000 rpm for 30 minutes. The supernatant from this centrifugation is stored at 4°C and the pellet subjected twice to the steps described above of solubilization in guanidine-HCl, refolding in buffer, and centrifugation. The final supernatants from each of the three centrifugations are combined and solid ammonium sulfate was added to the point of 20% saturation. The resulting solution thus derived from the insoluble fraction is subjected to purification on phenyl Sepharose and Q-Sepharose as described above for the soluble fraction. The purified soluble and insoluble fractions are combined to produce about 1.8 mg of purified

stromelysin 81-256 fragment (SCD) per gram of original cell paste, uniformly enriched with ^{13}C.

Preparation 2

Preparation of Specifically ^{13}C-Enriched Catalytic Domain of Human Stromelysin (SCD)

SCD is expressed by culturing the BL21(DE3)/pLysS/pETST-255-1 modified *E. coli* strain in a medium comprising ^{13}C-enriched α-ketobutyric acid and α-keto-isovaleric acid. The methods used for preparation of the genetically-engineered strain of *E. coli*, and for expressing, isolating, and purifying the protein fragment are as described above, except for the use of U-^{12}C-glucose, instead of U-^{13}C-glucose.

Preparation 3

Preparation of Uniformly and Specifically ^{13}C-Enriched FKBP

A. Preparation of Specifically ^{13}C-Enriched Val/Leu FKBP

Cells transformed with a plasmid encoding for human FKBP (as described in Egan, *et al.*, *Biochemistry* 32: 1920-1927 (1993)) were grown in a 100% D_2O culture medium containing $^{15}NH_4Cl$ (Cambridge Isotopes) as the sole nitrogen source (1.0 g/L) and perdeuterated glucose (1.5 g/L) as the carbon source (Cambridge Isotopes). Cells were grown at 37°C and the flask contents were shaken until an optical density of 1.0 was obtained. One hour prior to induction, 80 mg of L-valine-U-$^{13}C_5$-^{15}N-2,3-d_2 (Cambridge Isotopes) was added to the culture medium. The culture was induced with 1 mM IPTG for 12 hours.

The cells were harvested by centrifugation at 17,000 x g for 10 minutes at 4 °C and the resulting cell pellets were suspended in 50 mM phosphate buffer (pH 7.4) containing 5 mM DTT and 1 mM PMSF and mechanically lysed using a French press. The resulting lysate was centrifuged at 25,000 rpm for 30 minutes. Solid ammoniuum sulfate was added to the supernatant to the point of 40% of saturation and the centrifuged at 18,000 rpm for 5 minutes. The supernatant was dialyzed into 10 mM HEPES (pH 8.0) for 12 hours at 4 °C. The resulting solution was then loaded onto a 10 mL Q-Sepharose fast

flow column (Sigma) pre-equilibrated in the dialysis buffer. Fractions were collected, pooled, and concentrated using an Amicon flow cell. The solution was then dialyzed into 20 mM phosphate buffer (pH 6.5) containing 10 mM DTT and 0.01% sodium azide.

B. Preparation of Specifically ^{13}C-Enriched Val/Leu/Ile FKBP

Cells transformed with a plasmid encoding for human FKBP (as described in Egan, *et al.*, *Biochemistry* 32: 1920-1927 (1993)) are grown in a culture medium containing NH_4Cl as the sole nitrogen source (1.0 g/L) and glucose (1.5 g/L) as the carbon source. Cells are grown at 37°C and the flask contents are shaken until an optical density of 1.0 is obtained. One hour prior to induction, 100 mg of α-keto-butyric acid and α-keto-isovaleric acid are added to the culture medium. The culture is induced with 1 mM IPTG for 12 hours. Expression, isolation, and purification of the expressed protein are as described above.

C. Preparation of Uniformly ^{13}C-Enriched FKBP

Cells transformed with a plasmid encoding for human FKBP (as described in Egan, *et al.*, *Biochemistry* 32: 1920-1927 (1993)) were grown in a culture medium containing $^{15}NH_4Cl$ (Cambridge Isotopes) as the sole nitrogen source (1.0 g/L) and uniformly ^{13}C-enriched

glucose (1.5 g/L) as the carbon source (Cambridge Isotopes). Expression, isolation, and purification of the expressed protein are as described above.

5

Example 1

Screening for Ligands Using $^{13}C/^{1}H$ NMR Correlation Spectra Using Uniformly ^{13}C-Enriched FKBP

Uniformly ^{13}C-enriched FKBP was prepared in accordance with the procedures detailed above. The protein solutions used in the screening assay contained the uniformly ^{13}C-enriched FKBP (0.2 mM) and sodium azide (0.05%) in an H_2O/D_2O (9/1) phosphate buffered solution (20 mM, pH 6.5).

Two-dimensional $^{13}C/^{1}H$ NMR spectra were generated at 30°C on a Bruker DRX500 NMR spectrometer equipped with a triple resonance probe and Bruker sample changer. The $^{13}C/^{1}H$ HSQC spectra were acquired as 64 x 1024 complex points using sweep widths of 3771 Hz (^{13}C, t_1) and 8333 Hz (^{1}H, t_2). A delay of 1 second between scans and 8 scans per free induction decay (fid) were employed in the data collection. All NMR spectra were processed and analyzed on Silicon Graphics computers.

A first two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum was acquired for the ^{13}C-labeled FKBP target molecule as described above. The FKBP target was then exposed to a library mixture of test compounds. Stock solutions of the compounds were made at 100 mM and 1 M. In addition, a combination library was prepared which contained 8-10 compounds per sample at a concentration of 100 mM for each compound.

The pH of the 1 M stock solution was adjusted with acetic acid and ethanolamine so that no pH change was

observed upon a 1/10 dilution with a 100 mM phosphate buffered solution (pH 7.0). It is important to adjust the pH, because small changes in pH can alter the chemical shifts of the biomolecules and complicate the interpretation of the NMR data.

The compounds in the library were selected on the basis of size (molecular weight = 100-300) and molecular diversity. The molecules in the collection had different shapes (for example, flat aromatic rings(s), puckered aliphatic rings(s), straight and branched chain aliphatics with single, double, or triple bonds) and diverse functional groups (for example, carboxylic acids, esters, ethers, amines, aldehydes, ketones, and various heterocyclic rings) to maximize the possibility of discovering compounds that interact with widely diverse binding sites.

The NMR samples were prepared by adding 1.25 μl of the dimethyl sulfoxide stock solution of the compound mixtures that contained each compound at a concentration of 100 mM to 0.5 ml H_2O/D_2O (9/1) buffered solution of the uniformly ^{13}C-labeled protein. The final concentration of each of the compounds in the NMR sample was about 0.25 mM.

In the screening experiment, one compound, 2-phenylimidazole, was found to bind to FKBP. Figure 1 shows the $^{13}C/^{1}H$ correlation spectrum of uniformly ^{13}C-labeled FKBP. The spectrum (128 complex points, 4 scans/fid) was acquired on a 0.1 mM sample of FKBP in 20

mM phosphate (pH 6.5), 0.01% sodium azide and 10% deuterium oxide (D_2O).

Figure 1 shows a $^{13}C/^{1}H$ correlation spectrum of uniformly ^{13}C-labeled FKBP. The spectrum (128 complex points, 4 scans/fid) was acquired on a 0.1 mM sample of FKBP in 20 mM phosphate (pH 6.5), 0.01% sodium azide and 10% deuterium oxide (D_2O). Figure 2 shows the methyl regions of $^{13}C/^{1}H$ correlation spectra (64 complex points, 8 scans/fid) of uniformly ^{13}C-labeled FKBP (0.2 mM) before (thin multiple contours) and after (thick single contours) the addition of 2-phenylimidazole (0.25 mM). The changes in chemical shifts at aminoacyl residues Leu^{97}, Val^{55}, Ile^{56} and Ile^{90} are indicated.

Figure 3 shows $^{13}C/^{1}H$ correlation spectra (48 complex points, 8 scans/fid) of FKBP selectively $^{13}C/^{15}N/^{2}H$ labeled at valinyl and leucyl residues before (thin multiple contours) and after (thick single contours) addition of 2-phenylimidazole (0.25 mM). All other conditions are the same as those employed in generating the spectra illustrated in Figure 1. Selected residues that show significant changes upon binding are indicated. Again, changes in the chemical shift values indicate that binding is occurring at or near the Leu^{97} and Val^{55} residues.

Figure 4 shows $^{13}C/^{1}H$ correlation spectra of FKBP selectively ^{13}C labeled at alanyl residues before (thin multiple contours) and after (thick single contours) addition of 2-phenylimidazole (0.25 mM). The super-position of the chemical shift values before (light

multiple contours) and after (heavy single contour) addition of the ligand indicate that none of the alanyl residues are involved in the binding.

Figure 5 shows a "stick model" depiction of the three-dimensional structure of FKBP. Selected residues are numbered for aid in visualization. The aminoacyl residues involved in the binding site in the protein have been shown in bold.

WE CLAIM:

1. A method of detecting binding between a putative ligand and a pre-selected, ^{13}C-enriched target molecule which comprises:

a) generating a first two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum of said target molecule;

b) forming a mixture of said target molecule with at least one putative ligand compound;

c) generating a second two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum of the mixture of step (b); and

d) comparing the first and second spectra.

2. The method of Claim 1 wherein said target molecule is selected from the group consisting of lipoproteins, lipoprotein fragments, glycoproteins, glycoprotein fragments, proteins, protein fragments, polypeptides, DNA, and RNA.

3. The method of Claim 1 wherein said target molecule is selected from the group consisting of proteins, protein fragments, and polypeptides.

4. The method of Claim 3 wherein said target molecule is prepared by culturing a transformed cell line

which contains an expression vector containing a polynucleotide encoding said target molecule in a medium containing assimilable sources of ^{13}C.

5. The method of Claim 4 wherein said assimilable sources of ^{13}C are uniformly ^{13}C-enriched.

6. The method of Claim 5 wherein uniformly ^{13}C-labeled glucose (U-^{13}C-glucose) is employed to produce said assimilable sources of ^{13}C.

FOLIO OFICIAL No. 5.705
FOLIO OFICIAL
María Luisa Brizuela de Restrepo
Intérprete Oficial

7. The method of Claim 4 wherein said assimilable sources of ^{13}C are specifically ^{13}C-enriched.

8. The method of Claim 7 wherein ^{13}C-enriched methyl iodide is employed to produce said assimilable sources of ^{13}C.

9. The method of Claim 7 wherein said assimilable sources of ^{13}C are amino acids, and salts thereof.

10. The method of Claim 9 wherein said amino acid is selected from the group consisting of alanine, leucine, isoleucine, and valine.

11. The method of Claim 8 wherein said assimilable sources of ^{13}C are biosynthetic precursors of amino acids, and salts thereof.

12. The method of Claim 11 wherein said biosynthetic precursors of amino acids are selected from the group consisting of 4(^{13}C)-butyric acid and 4-(^{13}C)-3-(^{13}C)-methylbutyric acid.

13. A method of screening a mixture of compounds for binding to a pre-selected, ^{13}C-enriched target molecule which comprises:

a) generating a first two-dimensional ^{13}C/^{1}H NMR correlation spectrum of said target molecule;

b) contacting said target molecule with said mixture of compounds;

c) generating a second two-dimensional ^{13}C/^{1}H NMR correlation spectrum of the mixture of step (b);

d) comparing the first and second spectra.

14. The method of Claim 13, wherein said method additionally comprises:

e) exposing said target molecule individually to each compound in said mixture when step d) reveals differences in the first and second spectra;

f) generating two-dimensional $^{13}C/^{1}H$ NMR correlation spectra of said target molecule that has been exposed to each compound; and

g) comparing each spectra generated in step f) to the first spectrum generated from the target molecule alone.

15. The method of Claim 14 wherein said target molecule is selected from the group consisting of lipoproteins, lipoprotein fragments, glycoproteins, glycoprotein fragments, proteins, protein fragments, polypeptides, DNA, and RNA.

16. The method of Claim 15 wherein said target molecule is selected from proteins, protein fragments, and polypeptides.

17. The method of Claim 13 wherein said target molecule is uniformly ^{13}C-enriched.

18. The method of Claim 13 wherein said target molecule is specifically ^{13}C-enriched.

19. A method of determining the dissociation constant for a ligand which binds to a pre-selected, ^{13}C-enriched target molecule which comprises:

a) generating a first two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum of said target molecule;

b) exposing said target molecule to various concentrations of said ligand;

c) generating a two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum at each concentration of ligand in step b);

d) comparing each spectrum from step (c) to said first spectrum from step (a); and

e) calculating the dissociation constant.

20. The method of Claim 19 wherein said target molecule is selected from the group consisting of lipoproteins, lipoprotein fragments, glycoproteins, glycoprotein fragments, proteins, protein fragments, polypeptides, DNA, and RNA.

21. The method of Claim 20 wherein said target molecule is selected from proteins, protein fragments, and polypeptides.

22. The method of Claim 20 wherein said target molecule is uniformly ^{13}C-enriched.

23. The method of Claim 20 wherein said target molecule is uniformly ^{13}C-enriched.

24. A compound identified by the screening method of Claim 13.

25. A method of determining the specific amino acid residues in a pre-selected, ^{13}C-enriched target molecule affected by the binding of a ligand to said target molecule which comprises:

a) generating a first two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum of said target molecule, wherein said chemical shift values of the $^{13}C/^{1}H$ signals in said two dimensional correlation spectrum correspond to at least one known specific location of atomic groupings in said target molecule;

b) forming a mixture of said target molecule with a known ligand compound;

c) generating a second two-dimensional $^{13}C/^{1}H$ NMR correlation spectrum of the mixture of step (b); and

d) comparing the first and second spectra.

Abstract of the Disclosure

Methods of detecting binding of a putative ligand to a ^{13}C-enriched target molecule, methods of screening for compounds which bind to a ^{13}C-enriched target molecule, methods for calculating the dissociation constant of a ligand compound which binds to a ^{13}C-enriched target molecule, and methods employed in the determination of the specific amino acids in a ^{13}C-enriched target molecule affected by the binding of a ligand, as well as compounds identified by these screening methods, are provided herewith.

05050.0049.P3

SEQUENCE LISTING

<110> Fesik, Stephen W.
Hajduk, Philip J.

<120> The Use of ^{13}C Nuclear Magnetic Resonance to Detect Binding to Target Biomolecules

<130> 5816.US.P3

<160> 5

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 174
<212> PRT
<213> Artificial Sequence

<220>
<223> 81-256 Catalytic region of human stromelysin.

<400> 1

Phe Arg Thr Phe Pro Gly Ile Pro Lys Trp Arg Lys Thr His Leu Thr
1 5 10 15
Tyr Arg Ile Val Asn Tyr Thr Pro Asp Leu Pro Lys Asp Ala Val Asp
20 25 30
Ser Ala Val Glu Lys Ala Leu Lys Val Trp Glu Glu Val Thr Pro Leu
35 40 45
Thr Phe Ser Arg Leu Tyr Glu Gly Glu Ala Asp Ile Met Ile Ser Phe
50 55 60
Ala Val Arg Glu His Gly Asp Phe Tyr Pro Phe Asp Gly Pro Gly Asn
65 70 75 80
Val Leu Ala His Ala Tyr Ala Pro Gly Pro Gly Ile Asn Gly Asp Ala
85 90 95

His Phe Asp Asp Asp Glu Gln Trp Thr Lys Asp Thr Thr Gly Thr Asn
100 105 110
Leu Phe Leu Val Ala Ala His Glu Ile Gly His Ser Leu Gly Leu Phe
115 120 125
His Ser Ala Asn Thr Glu Ala Leu Met Tyr Pro Leu Tyr His Ser Leu
130 135 140
Thr Asp Leu Thr Arg Phe Arg Leu Ser Gln Asp Asp Ile Asn Gly Ile
145 150 155 160
Gln Ser Leu Tyr Gly Pro Pro Pro Asp Ser Pro Glu Thr Pro
165 170

<210> 2
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer sequence

<400> 2
gaaatgaaga gtcttcaa 18

<210> 3
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer sequence

<400> 3
gcgtcccagg ttctggag 18

<210> 4
<211> 27
<212> DNA
<213> Artificial Sequence

Substitute Sheet (Rule 26)

137
129

<220>

<223> Primer sequence

<400> 4

taccatggcc tatccattgg atggagc 27

<210> 5

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer sequence

<400> 5

ataggatcct taggtctcag gggagtcagg 30

Doc Number: 1 Version: 1 Type: Amendment Pages: 48

138

130

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: S.W. Fesik, et al.

Serial No.: Not assigned yet

Filed:

Title:Use of 13C Nuclear Magnetic Resonance to Detect Binding to Target Biomolecules

Case No.: 5816.US.P3

Group Art No.: Not assigned yet

Examiner: Not assigned yet

Express Mail Label No.: EE835516454US

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the United States Postal Service on the date shown below with sufficient postage as Express Mail in an envelope addressed to the: Box Patent Application Assistant Commissioner for Patents Washington, D.C. 20231, on: Date of Deposit: April 9, 1999

Kathleen T. Litz 4/9/99
Kathleen T. Litz Date

5,705

Declaration and Power of Attorney for a United States Patent Application

As a below-named inventor, I hereby declare that I believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled Use of 13C Nuclear Magnetic Resonance to Detect Binding to Target Biomolecules, the specification of which is enclosed herewith.

My residence, post office address and citizenship are as stated below next to my name.

I hereby state that I have reviewed and understand the contents of the above-mentioned specification, including the claims.

I acknowledge a duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

Claim to benefit of foreign application(s) as follows:

I hereby claim foreign priority benefits under 35 U.S.C. §119 for the following foreign applications for patent or

inventor's certificate.

NONE

The following foreign applications for patent or inventor's certificate have a filing date earlier than the filing date of the applications identified above.

NONE

Claim to benefit of earlier U.S. application(s) as follows:

OFFICIAL No. 5705
Unidad Bristrd de Restrepo
Interesado Oficial

I hereby claim the benefit under 35 U.S.C. §120 of the following earlier-filed United States patent applications. This application is a continuation-in-part of application Serial No. 09/241,194, filed February 1, 1999, which is a continuation of application Serial No. 08/744,701, filed October 31, 1996, now United States Patent No. 5, ---, ---, which is a continuation-in-part of application Serial No. 08/678,903, filed July 12, 1996, now abandoned, which is a continuation-in-part of application Serial No. 08/558,633, filed November 14, 1995, now United States Patent 5,---,---. Insofar as the subject matter of each of the claims of this application is not disclosed in the prior U.S. applications in the manner required by 35 U.S.C. §112, first paragraph, I acknowledge a duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56 which came into existence between the filing date(s) of the prior applications and the national or PCT filing date of this application.

NONE

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

NONE

I hereby appoint the following Attorneys and/or agents to prosecute this application and any continuation or divisional applications based hereon, and to transact all business in the Patent and Trademark Office connected therewith:

440
132

Mona Anand, Reg. No. 34,537
Regina M. Anderson, Reg. No. 35,820
Mark C. Bach, Reg. No. 34,766
Cheryl L. Becker, Reg. No. 35,441
Thomas D. Brainard, Reg. No. 32,459
Dianne Casuto, Reg. No. 40,943
Portia Chen, Reg. No. P44,075
Steven R. Crowley, Reg. No. 31,604
Andreas M. Danckers, Reg. No. 32,652
J. Michael Dixon, Reg. No. 32,410
Mimi C. Goller, Reg. No. 39,046
Matthew R. Hooper, Reg. No. 31,108
Daniel J. Hulseberg, Reg. No. 36,554
Neal D. Marcus, Reg. No. 35,267
James D. McNeil, Reg. No. 26,204
Lawrence S. Pope, Reg. No. 26,791
Nicholas A. Poulos, Reg. No. 30,209
Dugal S. Sickert, Reg. No. 33,784
Gregory W. Steele, Reg. No. 33,796
Janelle D. Strode, Reg. No. 34,738
Michael J. Ward, Reg. No. 37,960
David L. Weinstein, Reg. No. 28,128
Steven F. Weinstock, Reg. No. 30,117
Brian R. Woodworth, Reg. No. 33,137
Paul D. Yasger, Reg. No. 37,477

Send correspondence to: Steven F. Weinstock
Abbott Laboratories
D-377/AP6D
100 Abbott Park Road
Abbott Park, Illinois 60064-3500

TRADUCTION OFFICIAL No. 5705
Marie Luise Brifford de Restrepo
Traductrice Officiel

Direct telephone calls to: Janelle D. Strode
(847) 937-4395

INVENTORS

Name: (first, middle, last): Stephen W. Fesik
Post Office Address: 1099 Portsmouth Circle
Gurnee, IL 60031
Residence: 1099 Portsmouth Circle
Gurnee, IL 60031
Citizenship: United States of America

Name: (first, middle, last): Philip J. Hajduk
Post Office Address: 258 Lenox Lane
Mundelein, IL 60060
Residence: 258 Lenox Lane
Mundelein, IL 60060
Citizenship: United States of America

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that all statements made herein were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title

18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Stephen W. Fesik 3/18/99
Stephen W. Fesik Date

Philip J. Hajduk 3/18/99
Philip J. Hajduk Date

TRADUCCION OFICIAL No 5.705
Gloria Luisa Espinosa de Rodrigo
Intérprete Oficial
[illegible]

09/03/2004 #0000 09

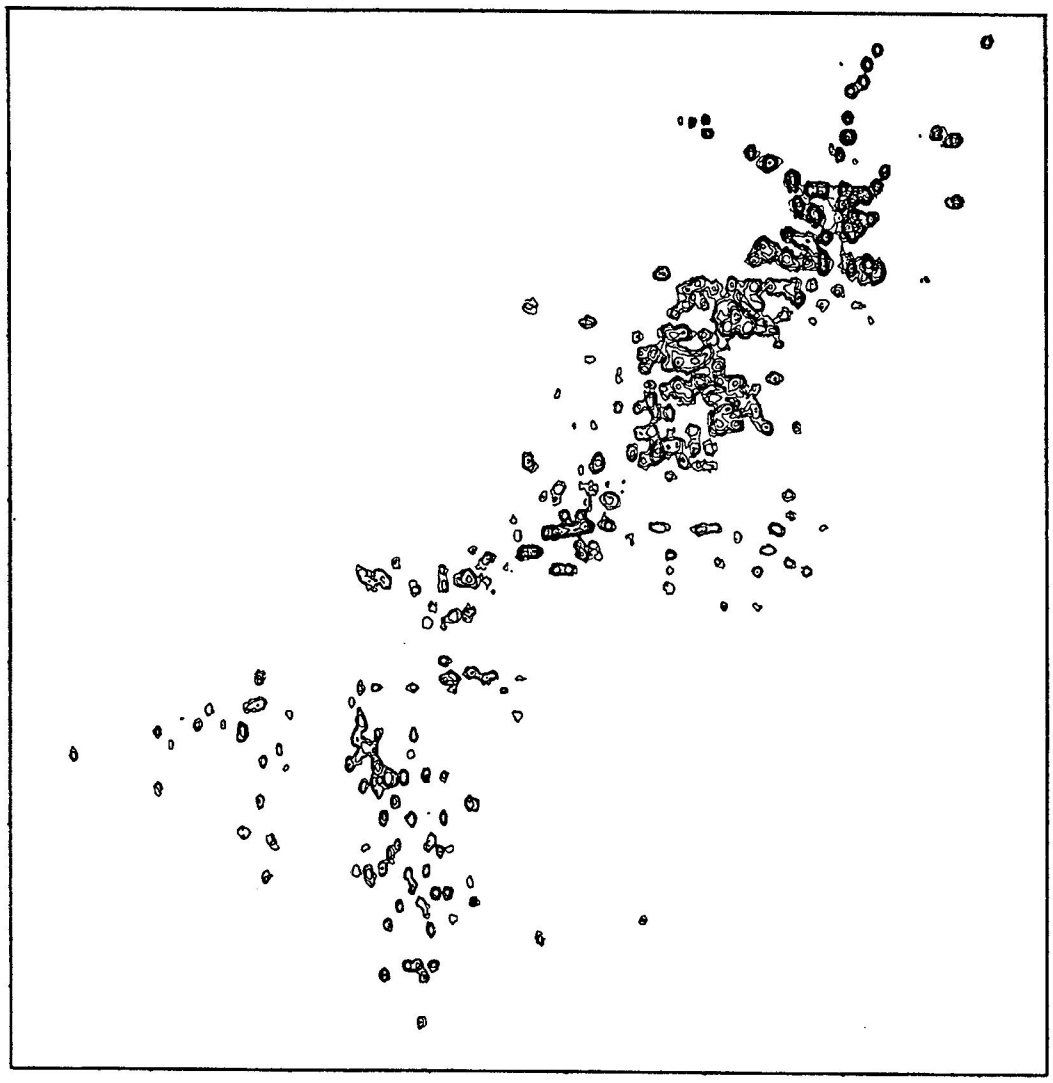


Figure 1

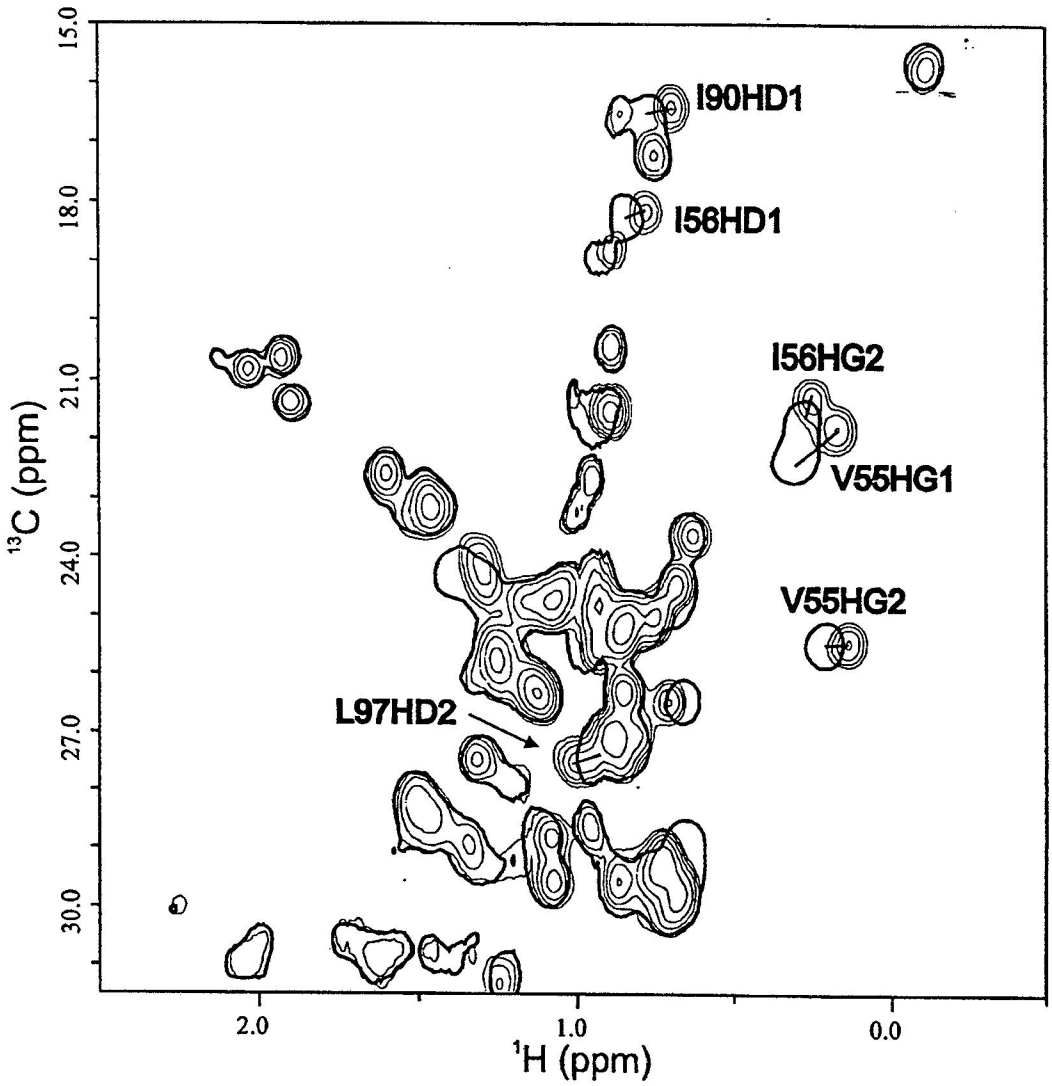

I90HD1
I56HD1
I56HG2
V55HG1
V55HG2
L97HD2
15.0
18.0
21.0
24.0
27.0
30.0
^{13}C (ppm)
2.0
1.0
0.0
^{1}H (ppm)

Figure 2

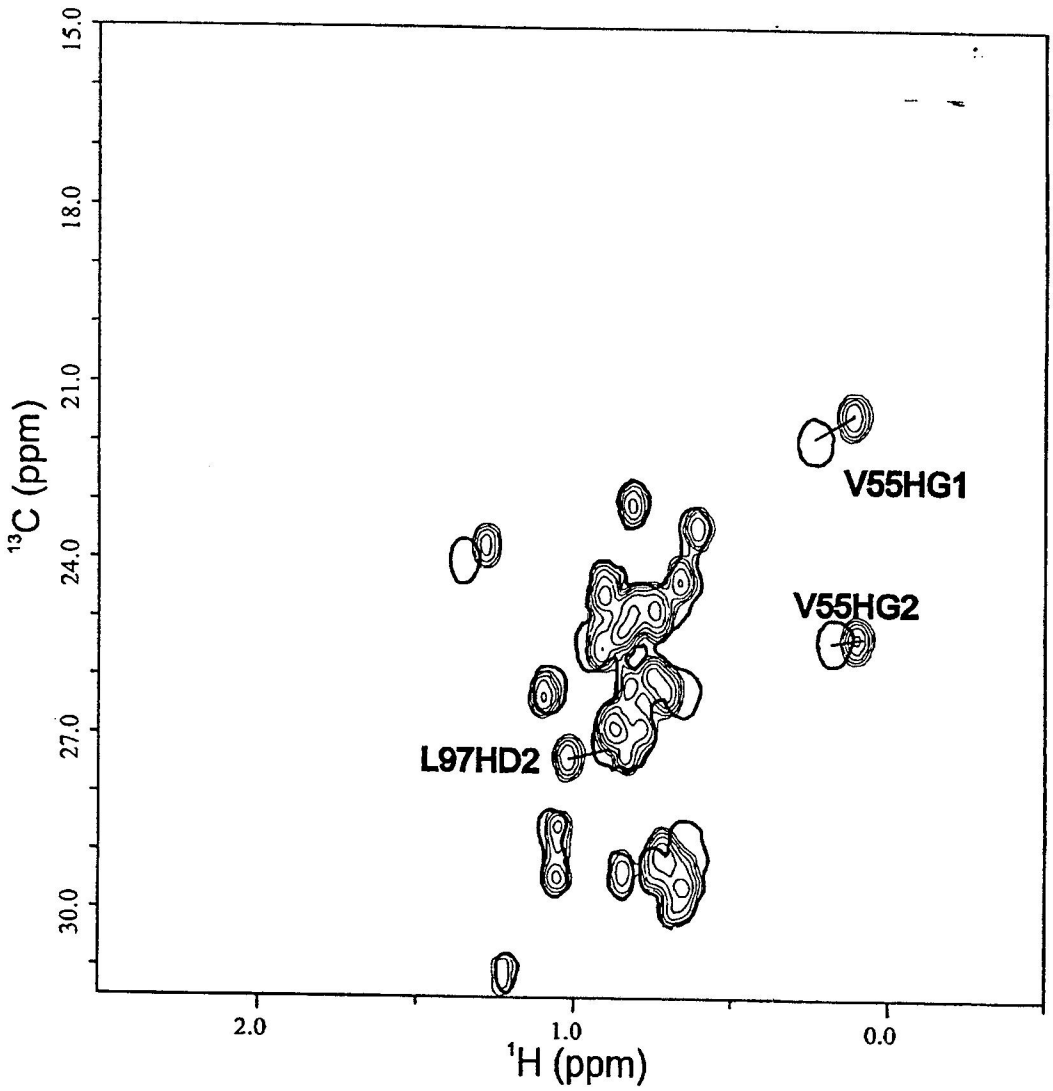

15.0
18.0
21.0
24.0
27.0
30.0
^{13}C (ppm)
V55HG1
V55HG2
L97HD2
2.0
1.0
0.0
^{1}H (ppm)

Figure 3

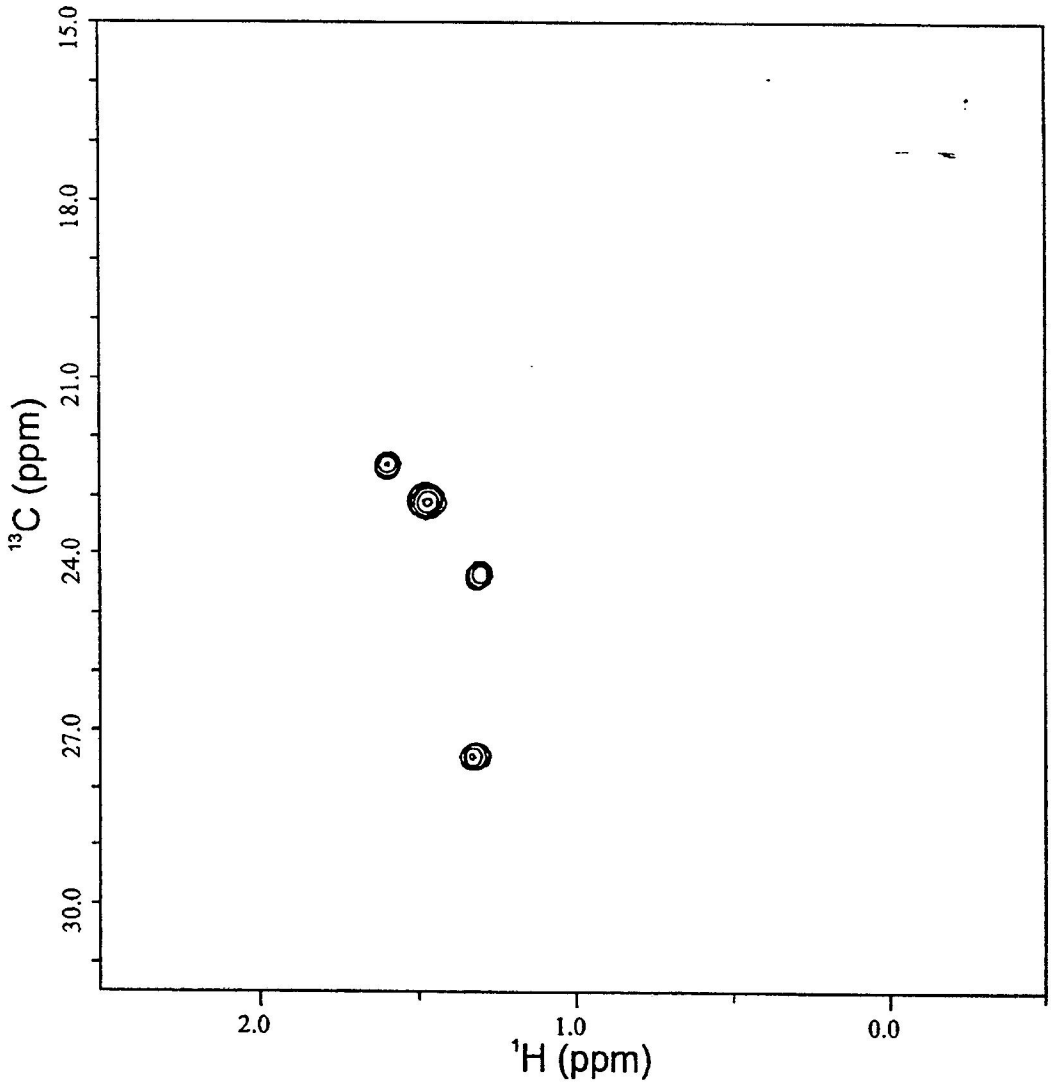


Figure 4

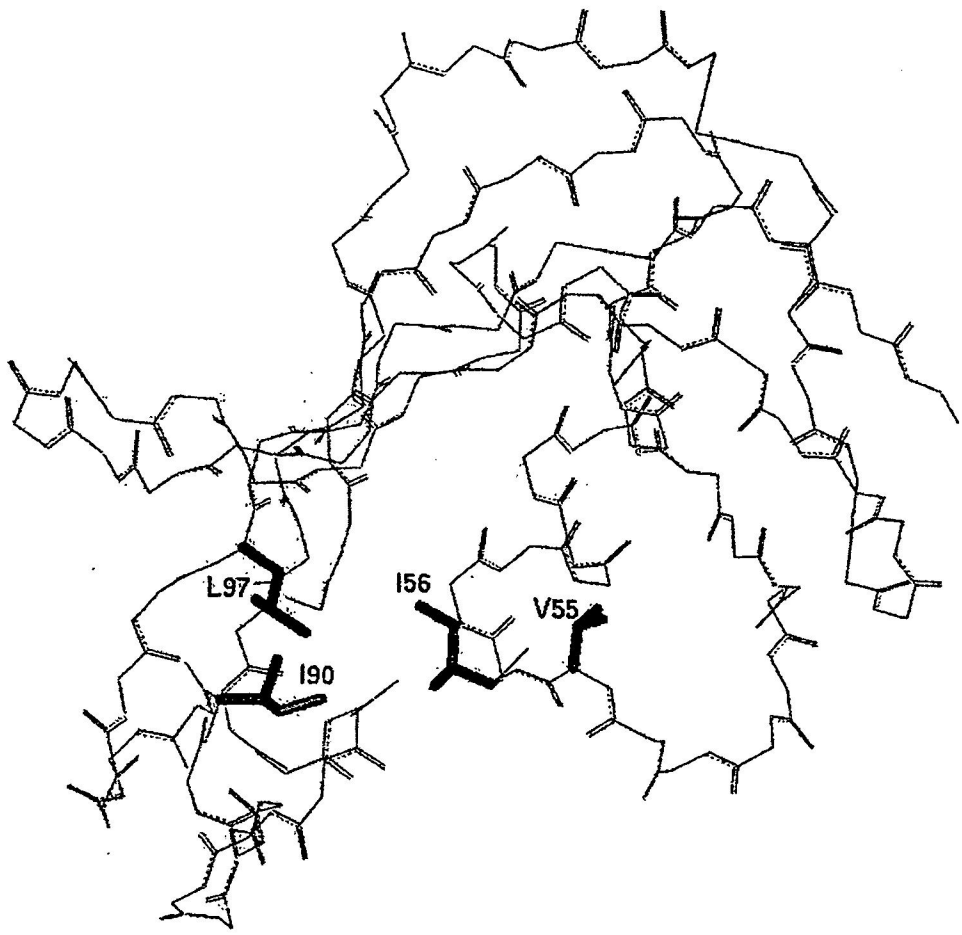


Figure 5

2do. EXAMEN DE FORMA PATENTE DE INVENCION

Folio 148

Radicación no 00-25248

Concepto Técnico no 1451
Patentes G01N 24/08.

Clasificación Internacional de

Practicado el examen de acuerdo a lo establecido en el Art. 38 de la Decisión 486 se determinó que esta solicitud de **Patente de Invención:**

SI [✔] NO [ ] Contiene un resumen de la invención (Art.26-e).

SI [✔] NO [ ] Contiene el título o nombre de la invención (Art.27-d).

SI [✔] NO [ ] Contiene la descripción de la invención (Art.26-b).

SI [✔] NO [ ] NO ES APLICABLE [ ] Contiene los dibujos necesarios para comprender la invención (Art.26-d)

SI [✔] NO [ ] Contiene una o más reivindicaciones. (Art.26-c).

SI [ ] NO [ ] NO ES APLICABLE [✔] Han sido desarrollados los productos y/o procedimientos a partir de recursos genéticos o de sus productos derivados de los que cualquiera de los Países Miembros es país de origen. (Art. 26- h).

SI [ ] NO [ ] NO ES APLICABLE [✔]Los productos y/o procedimientos han sido obtenidos o desarrollados a partir de los conocimientos tradicionales de las comunidades indígenas, afroamericanas, o locales de los países miembros, de acuerdo a lo establecido en la Decisión 391 y sus modificaciones y reglamentaciones vigentes.(Art.26-i).

SI [ ] NO [✔] La invención se refiere a un producto relativo a un material biológico. (Art.26-J).

SI [✔] NO [ ] La prioridad coincide con la materia reivindicada.

SI [✔] NO [ ] CUMPLE LOS REQUISITOS TÉCNICOS

OBSERVACIONES Y OTROS: SI [ ] NO [✔] ver hoja(s) anexa(s)

Según el folio 64, el solicitante desistió de la reivindicación 24

EXAMINADOR TÉCNICO
202016

Bogotá D. C., 2002-09-23

Carrera 13 No. 27-00 Piso 5 y 10
Conmutador: 334 12 21-2-3-4
Fax: 281 31 25 e-mail: info@sic.gov.co
Santa Fe de Bogotá D.C. - Colombia

Industria y Comercio
SUPERINTENDENCIA

Folio 149 2003-01-31 216.

2020
Bogotá DC

Doctor(a)
JOSÉ ANTONIO LLOREDA
Apoderado(a)

REFERENCIA:	Radicación No.	00-25248
	Trámite	002
	Evento	001
	Actuación	416
	Oficio No.	1941
	Folios	001

Teniendo en cuenta que la solicitud de privilegio de patente que se tramita bajo el expediente indicado en la referencia, reúne los requisitos de forma establecidos en los artículos 26 y 27 de la Decisión 486 de la Comisión de la Comunidad Andina, y en las demás disposiciones vigentes sobre la materia, se envía el extracto de la solicitud a la Oficina de Comunicaciones para efecto de su publicación en la Gaceta de Propiedad Industrial, conforme lo establece el artículo 40 de la misma Decisión.

Cúmplase
Dado en Bogotá DC, a los 26 SET. 2002

ALIX CARMENZA CÉSPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones

Bogotá, DC, El extracto de esta solicitud fue publicado en el número 524 de la Gaceta de Propiedad Industrial, de fecha 31-01-03 .El término hábil señalado por la ley expiró el 30-04-03 y SI ___ NO X se presentaron oposiciones por parte de terceros.

202016

Carrera 13 No. 27-00 Piso 5 y 10
Conmutador: 334 12 21-2-3-4
Fax: 281 31 25 e-mail: info@sic.gov.co
Santa Fe de Bogotá D.C. - Colombia