SUPERINTENDENCIA Y COMERCIO
Radicación : 00025848 00000122 Folios: 3
Fecha (AMD): 2003-01-28 17:56:45
Tramite : 382 PATENTES I 1 REGISTRO/ 538 PETICION
Generadores: 2000 DIVISION DE NUEVAS CREACIONES

141

150

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

DIVISION DE NUEVAS CREACIONES

E. S. D.

Re: P1.-**ABBOTT LABORATORIES**.-Solicitud de Patente de Invención para **"RESONANCIA MAGNETICA NUCLEAR ^{13}C PARA DETECTAR EL LIGAMIENTO DE UNA MOLECULA OBJETIVO Y LIGANDO PUTATIVO"**.-Clase **G01N 24/08**.- Expediente número **00-025.248**.-

1. Me refiero a mi memorial de 20 de Junio de 2000.

2. De conformidad con el precepto del Artículo 44 de la Decisión 486 y estando dentro del plazo de seis meses contados desde la fecha de la publicación de la solicitud en la Gaceta de la Propiedad Industrial número 524 de 31 de Enero de 2003, solicito a ese Despacho proceder a realizar el examen de patentabilidad de la presente invención.

3. Anexo me permito acompañar recibos números 02–86.426 y 03-26.378, expedidos por la Superintendencia de Industria y Comercio, que acreditan el pago de la tasa que dicho examen causa.

Señor Superintendente,

JOSE ANTONIO LLOREDA
T.P. 10.784
Código 231

142

Industria y Comercio

SUPERINTENDENCIA

NIT : 860171907 2

SUPERINDUSTRIA Y COMERCIO
Radicacion : 03025243 00000002 Folios: 3
Fecha (A:M:D): 2003-04-28 17:56:45
Tramite : 322 PATENTES D 1 REGISTRO/ 538 PETICION
Modalidad: 2000 DIVISION DE MARCAS PERSONAS

RECIBO OFICIAL DE CAJA : 02 - 86,426
FECHA : DICIEMBRE 24 DE 2002

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	VR. PAGO
LLOREDA [illegible] CIA S. A	CONSIGNACION	BANCO POPULAR	050-00110-6	2139231	24/12/2002	149,623,713.00

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

CANT.	DENOMINACION	CONCEPTO		TOTAL CONCEPTO
1	50005 01-01 SOLICITUDES	85 EXAMEN DE PATENTABILIDAD DE INVEN		310,000.00
		TOTAL :	$	310,000.00

SON: TRESCIENTOS DIEZ Y OCHO MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

Carrera 13 No. 27-00 Pisos 5, 7 y 10
Conmutador 382 0840
Fax 350 5220
E-mail: info@sic.gov.co
www.sic.gov.co
Bogotá, D.C., Colombia

143

SUPERINTENDENCIA Y COMERCIO

Radicacion : 00025848 00000002 Folios: 3

Fecha (AMD): 2003-04-28 17:56:45

Tramite : 000 PATENTES D 1 REGISTRO/ 538 PETICION

Remesa No: 2803 DIVISION DE NUEVAS CREACIONES

NIT : 800176089-2

CERTIFICACION DE EXCEDENTE : 03 - 26,378
FECHA : ABRIL 14 DE 2003

***** C O N S I G N A C I O N *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
LLOREDA Y CIA S. A	CONSIGNACION	BANCO POPULAR	050-00110-6	8699056	14/04/2003	5,675,000.00

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

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-09 OTROS SERVICIOS DE PROPIEDAD I	99 OTROS SERVCIOS ADMINISTRATIVOS	17,000.00
		TOTAL : $	17,000.00

SON: DIEZ Y SIETE MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No._____

ESTA CERTIFICACION DE EXCEDENTE DE PAGO SOLO PODRA SER UTILIZADA COMO PARTE DE LA CANCELACION DE UNA TARIFA VIGENTE Y NO PODRA SER SUSTITUIDA POR NINGUN RECIBO.

2003-10-16.
375
144

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES

2020/

Bogotá, D. C.

Doctor
José Antonio Lloreda
(apoderado o representante sociedad solicitante)

ASUNTO:	Radicación No.	00-25248
	Trámite	002
	Evento	001
	Actuación	430
	Oficio No	8692
	Folios	006

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. DOCUMENTOS ESTUDIADOS:

Para el presente estudio se tendrá en cuenta:

- Capítulo descriptivo comprendido en los folios 6 a 36.
- Las figuras 1 a 11 de los folios 144 a 154
- Las reivindicaciones 1 a 29 del capítulo reivindicatorio encontrado en los folios 37 a 40, con la modificación comprendida en el folio 70.
- De acuerdo con la legislación vigente se acepta la prioridad reivindicada.

2. OBJETO DE LA INVENCIÓN

La solicitud reclama un método para detectar el ligamiento entre un ligando putativo y una molécula objetivo preseleccionada y enriquecida con ^{13}C, que comprende la generación de un primer espectro de correlación RMN $^{13}C/^{1}H$ bidimensional de dicha molécula objetivo, formación de una mezcla de dicha molécula objetivo con al menos un compuesto ligando putativo, generación de un segundo espectro de correlación y comparación del primero y el segundo.

La solicitud también cubre el método en que dicha molécula objetivo se prepara mediante el cultivo de una línea celular transformada con un vector de expresión que contiene un polinucleótido codificador de dicha molécula objetivo en un medio que contienen fuentes asimilables de ^{13}C, como son glucosa, aminoácidos y sus sales, precursores biosintéticos de aminoácidos. Además, es parte de la invención un método para investigar una mezcla de compuestos que se ligan a una molécula objetivo preseleccionada y enriquecida con ^{13}C, que comprende la generación de un primer espectro de correlación RMN $^{13}C/^{1}H$ bidimensional de dicha molécula objetivo, formación de una mezcla de dicha molécula objetivo con al menos un compuesto ligando putativo, generación de un segundo espectro de correlación y comparación del primero y el segundo, opcionalmente, el método comprende la exposición de la molécula marcada individualmente a cada uno de los compuestos de la mezcla.

El objetivo de la invención es el desarrollo de un método rápido, eficiente y confiable que permita determinar el ligamiento de un ligando o molécula objetivo a un compuesto predeterminado, permitiendo la posibilidad de establecer el mapeo de sitos de ligamiento ubicados en la

sustancio objetivo, aun cuando dicha sustancia no contenga nitrógeno.

El objeto de la invención se clasificó, de acuerdo con el IPC, así: G01N 24/08

3. DETERMINACIÓN DEL ESTADO DE LA TÉCNICA

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina que establece que *"El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida.*

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40."

Consultadas las bases de datos y los archivos con que cuenta la entidad se encontró que antes del 9 de abril de 1999, fecha presentación de la solicitud prioritaria, habían sido publicados los documentos FEJZO J. ET AL. Protein Science volumen. 5, no. 9, 1996, paginas 1917 – 1921 y WO97/18471 los cuales se relacionan con pruebas que utilizan NMR para el estudio de las interacciones ligando/receptor.

De acuerdo con lo establecido en el artículo 16 se determinó el estado de la técnica y se encontraron los siguientes antecedentes:

No	Documento	Título	Fecha de Publicación
1	Protein Science vol. 5, no. 9, 1996, paginas 1917 – 1921 Todo el documento.	"Dynamic NMR studies of ligand—receptor interactions: Design and analysis of a rapidly exchanging complex of FKBP-12/ FK506 with a 24kDa calcineurin"	1996
2	WO97/18471 Pág 1623 a 1631.	"Use of nuclear magnetic resonance to identify ligands to target biomolecules"	1997-12-16

*Si se trata de una solicitud colombiana esta fecha corresponde también a la fecha de presentación de la solicitud colombiana aún sin haber sido publicada o sin habérsele cumplido el plazo previsto en el art.40.

4. EVALUACIÓN DE LOS REQUISITOS DE PATENTABILIDAD

El artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina establece que: *"Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial."*

En cumplimiento del artículo 14 de la Decisión 486, se procedió a evaluar los requisitos novedad, nivel inventivo y aplicación industrial con el fin de establecer la patentabilidad de la invención reivindicada en la solicitud en estudio.

4.1. EVALUACIÓN DE LA NOVEDAD

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina dispone que *"Una invención se considerará nueva cuando no está comprendida en el estado de la técnica.*

"El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida.
"Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40."

Las reivindicaciones 1 a 3 de la solicitud reclaman un método para detectar el ligamiento entre un ligando putativo y una molécula objetivo preseleccionada y enriquecida con ^{13}C, que comprende la generación de un primer espectro de correlación RMN $^{13}C/^{1}H$ bidimensional de dicha molécula objetivo, formación de una mezcla de dicha molécula objetivo con al menos un compuesto ligando putativo, generación de un segundo espectro de correlación y comparación del primero y el segundo.

El documento "Dynamic NMR studies of ligand—receptor interactions: Design and analysis of a rapidly exchanging complex of FKBP-12/ FK506 with a 24kDa calcineurin" (ahora en adelante como D1) evidencia que es conocido en el estado de la técnica un método para detectar uniones entre un ligando putativo, como es la subunidad B de calcineurina (CnB) y una molécula objetivo preseleccionada FKBP-$^{12/13}$C-FK506, la cual había sido previamente enriquecida con ^{13}C. Según advierte esta anterioridad, el método comprende la generación de un primer espectro de correlación RMN $^{13}C/^{1}H$ bidimensional (HSQC) de dicha molécula objetivo, la formación de una mezcla de dicha molécula blanco con al menos un compuesto ligando putativo, la generación de un segundo espectro de correlación RMN $^{13}C/^{1}H$ bidimensional (HSQC) de dicha mezcla y la comparación de los espectros.

Teniendo en cuenta que el objeto reclamado en las reivindicaciones 1 a 3 de la solicitud en estudio es el método que comprende un espectro de la molécula objetivo, la mezcla de dicha molécula, un segundo espectro de dicha mezcla y la comparación entre el primer y el segundo espectro, y la anterioridad reporta un métodos con los mismos pasos, esta oficina encuentra que el método reclamado en las reivindicaciones 1 a 3 de la invención en estudio y la formulación reportada en el documento D1 son exactamente iguales, ambos estudian la interacción o unión entre un ligando y una molécula objetivo, los dos se basan en la marcación de la molécula objetivo con ^{13}C, la realización de dos espectros, uno de ellos previo a la mezcla con el ligando y uno posterior a dicha mezcla y finalmente, una comparación los espectros. Siendo así, se establece que es conocido en el estado de la técnica el método que tiene los mismos componentes que la invención definida en las reivindicaciones 1 a 3 de la solicitud colombiana. Más aun cuando la molécula objetivo es una proteína tanto en la anterioridad como en las reivindicaciones en cuestión de la solicitud en estudio. Por consiguiente se establece que la anterioridad D1 afectaría la novedad de las reivindicaciones 1 a 3 y por ende se considera que dichas reivindicaciones no cumplirían con el requisito de novedad exigido por el artículo 14 y definido en el artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina.

4.2. EVALUACIÓN DEL NIVEL INVENTIVO

El artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina dispone que: "Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica."

Las reivindicaciones 4 a 24 reclaman el método definido en el numeral anterior, donde la molécula objetivo se prepara mediante el cultivo de una línea de células transformadas con un

vector a partir del cual se expresa dicha molécula, en un medio que contiene fuentes asimilables de ^{13}C, tales como U-^{13}C-glucosa, yoduro de metilo enriquecido, aminoácidos o sus sales, precursores biosintéticos de aminoácidos o sus sales, tales como el ácido 4(^{13}C)-butírico y el ácido 4-(^{13}C)-3(^{13}C) – metilbutírico.

También es parte de la invención el método para investigar de una mezcla de compuestos cual o cuales se ligan a una molécula objetivo preseleccionada enriquecida con ^{13}C que comprende los mismos pasos que el método definido en el numeral anterior, complementado con la exposición de dicha molécula objetivo individualmente a cada compuesto y la comparación entre el espectro originado por la molécula objetivo sola y el realizado a la molécula luego de enfrentarla con el compuesto individual.

Adicionalmente, la solicitud se refiere al método para determinara la constante de disociación para un ligando que se liga a una molécula objetivo pre-seleccionada que comprende los pasos del método explicado en el numeral anterior, seguido por el cálculo de la constante de disociación, y al método para la determinación de residuos aminoácidos específicos en una molécula objetivo pre-seleccionada enriquecida con ^{13}C y afectada por el ligamiento de un ligando a dicha molécula objetivo, el cual comprende la generación de un primer espectro de correlación RMN 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, y seguido los pasos son los mismos que los del método definido en el numeral anterior.

El documento WO9718471 revela el método para detectar la unión entre un ligando putativo y una molécula blanco enriquecida con el isótopo ^{15}N. El método para identificar compuestos que son ligandos específicos de una molécula objetivo preseleccionada y enriquecida con ^{15}N, que comprende la generación de un primer espectro de correlación RMN $^{15}N/^{1}H$ bidimensional de dicha molécula objetivo, formación de una mezcla de dicha molécula objetivo con al menos un compuesto ligando putativo, generación de un segundo espectro de correlación y comparación del primero y el segundo cualquier diferencia entre ellos implica la presencia de un compuesto que es un ligado. Según esta anterioridad el método comprende adicionalmente el paso de exposición de la molécula objetivo individualmente a cada compuesto y la comparación entre el espectro originado por la molécula objetivo sola y el realizado a la molécula luego de enfrentarla con el compuesto individual. Ahora bien, la anterioridad también se refiere a un proceso para determinar la constante de disociación entre la molécula blanco y el ligando, el cual consiste en el método principal explicado anteriormente, en el cual adicionalmente se calcula la constante de disociación.

Complementando la información anterior, el ejemplo 1 de D2 (líneas 29 a 34) señala que para preparar la molécula objetivo enriquecida con ^{15}N, se insertó en un plásmido una secuencia polinucleótida que codifica para la producción de dicho fragmento de proteína, luego se introdujo dicho plásmido en una cepa de E.coli, se cultivó la célula transformada en un medio de cultivo enriquecido con $^{15}NH_4Cl$ y ^{13}C-glucosa y se aisló el fragmento de proteína enriquecido isotípicamente y con el se realizó el método definido anteriormente. Esta anterioridad también plantea que la proteína sea obtenida a partir de una bacteria recombínate cultivada en medio con ^{15}N-proteína o ^{15}N-cloruro de amonio, entre las proteínas recombinantes marcadas se encuentra la proteína FKBP.

Teniendo en cuenta que el documento D2 evidencia que es conocido en el estado de la técnica un método que comprende los mismos pasos que el método reclamado en la invención en estudio, es decir, el proceso que comprende la generación de un primer espectro de correlación RMN $^{13}C/^{1}H$ bidimensional de dicha molécula objetivo, formación de una mezcla de dicha molécula objetivo con al menos un compuesto ligando putativo, generación de un segundo espectro de correlación y comparación del primero y el segundo, se encuentra que la única

diferencia entre la anterioridad D2 y la solicitud en estudio es que en la anterioridad se utiliza una molécula marcada con un isótopo ^{13}C mientras que la anterioridad D2 emplea ^{15}N.

Siendo que el problema a ser solucionado es la provisión de un método basado en la NMR para detectar la unión entre un ligando putativo y una molécula objetivo enriquecida con un isotopo, resulta evidentemente derivado de la técnica el reemplazar el isótopo ^{15}N por otro isótopo ya conocido en el estado de la técnica y según D1 ya empleado en la determinación de la unión ligando molécula objetivo isotópicamente marcada, como es el isótopo ^{13}C. Efectuar los mismos pasos con un isótopo ya diferente es lógico para cualquier persona versada en la materia, y es obvio que si la molécula objetivo no contienen nitrógeno, no es factible utilizar como isótopo de marcaje ^{15}N. Siendo que la anterioridad D1 había indicado que el isótopo ^{13}C era adecuado para el estudio mediante NMR de la unión de moléculas marcadas isotópicamente y ligados putativos, resulta evidentemente derivado del estado de la técnica, utilizar dicho isótopo en variaciones del método D1 como son los métodos revelados en D2.

Adicionalmente, se advierte que D2 demuestra que era conocido el método de producir moléculas marcadas mediante su expresión en células transformadas y cultivadas en medios que comprenden una fuente asimilable del isótopo ^{15}N y del isótopo ^{13}C, tal como es la ^{13}C-glucosa. Siendo así, se encuentra que es derivado de la técnica el concepto de detectar él ligamiento entre un ligando putativo y una molécula objetivo marcada isotópicamente. Partiendo de este hecho, reemplazar un isótopo por otro resulta evidente y producir dicha molécula marcada de forma recombinante a través del cultivo de la célula transformada en un medio con una fuente asimilable marcada, resulta evidente, más cuando la anterioridad D2 había dejado en claro que se podían producir moléculas isotópicamente marcadas empleando como fuente asimilable la ^{13}C-glucosa. Incluso usar dicho isótopo en forma de otra fuente asimilable, como ^{13}C-proteína, ^{13}C-aminoácido sería absolutamente obvio para una persona versada en la materia.

Por lo expuesto anteriormente se afirma que los documentos D1 y D2 afectarían el requisito de nivel inventivo del método cuya característica esencial es comprender los mismos pasos que revelan las anterioridad D2 y precisa que el isótopo empleado en el marcaje es el que había reportado la anterioridad D1 como isótopo para la detección mediante RMN de la unión de una molécula objetivo marcada y un ligando putativo. En consecuencia, se consideraría que la invención de las reivindicaciones 4 a 24 de la solicitud en estudio no cumpliría con el requisito de nivel inventivo definido en el artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina.

Anexo copia de los documentos D1 y D2 citado como anterioridad.

Nº	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Protein Science vol. 5, no. 9, 1996, paginas 1917 – 1921 Todo el documento.	1 a 3. 4 a 24	Novedad Nivel Inventivo
D2	WO97/18471 Pág 1623 a 1631.	4 a 24	Nivel inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta días contados a partir de la fecha de la notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

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Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e del capítulo quinto (5), del título primero de la circular única número 10 de 2001.

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

El anterior requerimiento fue notificado por fijación en lista, de fecha 16 OCT. 2003

CONCEPTO TÉCNICO Número: 1527	EXAMINADOR TÉCNICO Código # 202016

DI 1-29 150

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FOR THE RECORD

Dynamic NMR studies of ligand--receptor interactions: Design and analysis of a rapidly exchanging complex of FKBP-12/FK506 with a 24 kDa calcineurin fragment

J. FEJZO, C. A. LEPRE, J. W. PENG, MSS. SU, J. A. THOMSON and J. M. MOORE
Vertex Pharmaceuticals Incorporated, 130 Waverly Street, Cambridge, Massachusetts 02139-4242

Dynamic NMR methods, such as differential line broadening and transferred NOE spectroscopy, are normally reserved for the study of small molecule ligand interactions with large protein receptors. Using a combination of isotope labeling and isotope edited NMR, we have extended these techniques to characterize interactions of a much larger protein/drug complex, FKBP-12/FK506 with its receptor protein, calcineurin. In order to examine this multicomponent system by dynamic NMR methods, the 93 kDa, tightly bound FKBP-12/FK506/Cn complex was replaced with a lower affinity, rapidly exchanging system consisting of FKBP-12/FK506 (13 kDa), recombinant calcineurin subunit B (CnB) (20 kDa), and a synthetic peptide (4 kDa) corresponding to the B binding domain (BBD) of calcineurin catalytic subunit A (CnA). Analysis of (1)H-(13)C HSQC data acquired for the FKBP-12/(13)C-FK506 and FKBP-12/(13)C-FK506/CnB/BBD complexes indicates that FKBP-12/FK506 and CnB/BBD are in fast exchange in the quaternary complex. Comparison of proton line widths shows significant broadening of resonances along the macrocycle backbone at 13-CH, 13-OMe, 15-OMe, 18-CH(2), 20-CH, 21-CH, and 25-Me, as well as moderate broadening on the macrocycle backbone at 17-Me, 24-CH, and the pyranose 12-CH(2) protons. The tri-substituted olefin and cyclohexyl groups also show moderate broadening at the 27-Me, 28-CH, and 30-CH(2) positions, respectively. Unexpectedly, little line broadening was observed for the allyl resonances of FK506 in the quaternary complex, although (13)C longitudinal relaxation measurements suggest this group also makes contacts with calcineurin. In addition, intermolecular transfer NOE peaks were observed for the allyl 37-CH(2), 21-CH, 30-CH(2), 13-OMe, 15-OMe, 17-Me, 25-Me, and 27-Me groups, indicating that

these are potential sites on the FK506 molecule that interact with calcineurin.

This article has been cited by other articles:



PROTEIN ENGINEERING ▸HOME

C. McInnes, S. Grothe, M. O'Connor-McCourt, and B. D. Sykes
NMR study of the differential contributions of residues of transforming growth factor alpha to association with its receptor
Protein Eng., March 1, 2000; 13(3): 143 - 147.
[Abstract] [Full Text] [PDF]

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/50, 33/542	A1	(11) International Publication Number: WO 97/18471
		(43) International Publication Date: 22 May 1997 (22.05.97)

(21) International Application Number: PCT/US96/18270

(22) International Filing Date: 13 November 1996 (13.11.96)

(30) Priority Data:
08/555,691 14 November 1995 (14.11.95) US

(71) Applicant: ABBOTT LABORATORIES [US/US]; CHAD 0377/AP6D-2, 100 Abbott Park Road, Abbott Park, IL 60064-3500 (US).

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(57) Abstract

The present invention provides a process for identifying compounds which bind to a specific target molecule. The process includes the steps of: a) generating a first two-dimensional $^{15}N/^{1}H$ NMR correlation spectrum of a ^{15}N-labeled target molecule; b) exposing the labeled target molecule to one or a mixture of chemical compounds; c) generating a second two-dimensional $^{15}N/^{1}H$ NMR correlation spectrum of the labeled target molecule that has been exposed to one or a mixture of compounds in step (b); and d) comparing said first and second two-dimensional $^{15}N/^{1}H$ NMR correlation spectra to determine differences between said first and said second spectra, the differences indentifying the presence of one or more compounds that are ligands which have bound to the target molecule.

Once a suitable linker group is identified, the ligands are linked with that linker. Means for linking ligands are well known in the art and depend upon the chemical structure of the ligand and the linking group itself. Ligands are linked to one another using those portions of the ligand not directly involved in binding to the target molecule.

A detailed description of the design of a drug that inhibits the proteolytic activity of stromelysin, which drug was designed using a process of the present invention is set forth hereinafter in Example 4.

The following Examples illustrate preferred embodiments of the present invention and are not limiting of the specification and claims in any way.

Example 1

Preparation Of Uniformly ^{15}N-Labeled Target Molecules

A. Stromelysin

Human stromelysin is a 447-amino acid protein believed to be involved in proteolytic degradation of cartilage. Cartilage proteolysis is believed to result in degradative loss of joint cartilage and the resulting impairment of joint function observed in both osteoarthritis and rheumatoid arthritis. The protein possesses a series of domains including N-terminal latent and propetide domains, a C-terminal domain homologous with homopexin, and an internal catalytic domain.

Studies have shown that removal of the N-terminal prosequence of approximately eighty amino acids occurs to convert the proenzyme to the 45 kDa mature enzyme. Furthermore, studies have shown that the C-terminal homopexin homologous domain is not required for proper folding of the catalytic domain or for interaction with an inhibitor. (See, e.g., A. I. Marcy, Biochemistry, **30**: 6476-6483 (1991). Thus, the 81-256 amino acid residue internal segment of stromelysin was selected as the protein fragment for use in identifying compounds which bind to and have the potential as acting as inhibitors of stromelysin.

To employ the method of the present invention, it was necessary to prepare the 81-256 fragment (SEQ ID NO:1) of stromelysin in which the peptide backbone was isotopically enriched with and ^{15}N. This was done by inserting a plasmid which coded for the production of the protein fragment into an *E. coli* strain and growing the genetically-modified bacterial strain in a limiting culture medium enriched with $^{15}NH_4Cl$ and ^{13}C-glucose.

The isotopically enriched protein fragment was isolated from the culture medium, purified, and subsequently used as the basis for evaluating the binding of test compounds. The procedures for these processes are described below.

Human skin fibroblasts (ATCC No. CRL 1507) were grown and induced using the procedure described by Clark et al., Archiv. Biochem. and Biophys., **241**: 36-45 (1985). Total RNA was isolated from 1 g of cells using a Promega RNAgents® Total RNA Isolation System Kit (Cat.# Z5110, Promega Corp., 2800 Woods Hollow Road, Madison, WI 53711-5399) following the manufacturer's instructions. A 1 μg portion of the RNA was heat-denatured at 80°C for five minutes and then subjected to reverse transcriptase PCR using a GeneAmp® RNA PCR kit (Cat.# N808-0017, Applied Biosystems/Perkin-Elmer, 761 Main Avenue, Norwalk, CT 06859-0156) following the manufacturer's instructions.

Nested PCR was performed using first primers (A) GAAATGAAGAGTC TTCAA (SEQ ID NO:3) and (B) GCGTCCCAGGTTCTGGAG (SEQ ID NO:4) and thirty-five cycles of 94°C, two minutes; 45°C, two minutes; and 72°C three minutes. This was followed by reamplification with internal primers (C) ATACCATGGCCTATCCAT TGGATGGAGC (SEQ ID NO:5) and (D) ATAGGATCCTTAGGTCTCAGGGGA GTCAGG (SEQ ID NO:6) using thirty cycles under the same conditions described immediately above to generate a DNA coding for amino acid residues 1-256 of human stromelysin.

The PCR fragment was then cloned into PCR cloning vector pT7Blue(R) (Novagen, Inc., 597 Science Drive, Madison, WI 53711) according to the manufacturer's instructions. The resulting plasmid was cut with NcoI and BamHI and the stromelysin fragment was subcloned into the Novagen expression vector pET3d (Novagen, Inc., 597 Science Drive, Madison, WI 53711), again using the manufacturer's instructions.

A mature stromelysin expression construct coding for amino acid residues 81-256 plus an initiating methionine was generated from the 1-256 expression construct by PCR amplification. The resulting PCR fragment was first cloned into the Novagen pT7Blue(R) vector and then subcloned into the Novagen pET3d vector, 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**: 11231-11235 (1992) with the exception that the present codes for a peptide sequence beginning two amino acids earlier, at position 81 in the sequence of human stromelysin.

Plasmid pETST-83-256 was transformed into *E. coli* strain BL21(DE3)/pLysS (Novagen, Inc., 597 Science Drive, Madison, WI 53711) in accordance with the manufacturer's instructions to generate an expression strain, BL21(DE3)/pLysS/pETST-255-1.

A preculture medium was prepared by dissolving 1.698 g of $Na_2HP_4 \cdot 7H_2O$, 0.45 g of KH_2PO_4, 0.075 g NaCl, 0.150 g $^{15}NH_4Cl$, 0.300 13C-glucose, 300 μL of 1M aqueous $MgSO_4$ solution and 15 μL of aqueous $CaCl_2$ solution in 150 mL of deionized water.

The resulting solution of preculture medium was sterilized and transferred to a sterile 500 mL baffle flask. Immediately prior to inoculation of the preculture medium with the bacterial strain, 150 μL 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 were added to the flask contents.

The flask contents were then inoculated with 1 mL of glycerol stock of genetically-modified *E. Coli* , strain BL21(DE3)/pLysS/pETST-255-1. The flask contents were shaken (225 rpm) at 37°C until an optical density of 0.65 was observed.

A fermentation nutrient medium was prepared by dissolving 113.28 g of $Na_2HP_4 \cdot 7H_2O$, 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 was placed in a New Brunswick Scientific Micros Fermenter (Edison, NJ) and sterilized at 121°C for 40 minutes.

Immediately prior to inoculation of the fermentation medium, the following pre-sterilized components were added to the fermentation vessel contents: 100 mL of a 10% aqueous solution of $^{15}NH_4Cl$, 100 mL of a 10% aqueous solution of ^{13}C-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 was adjusted to pH 7.00 by the addition of an aqueous solution of 4N H_2SO_4.

The preculture of *E. Coli* , strain BL21(DE3)/pLysS/pETST-255-1, from the shake-flask scale procedure described above was added to the fermentor contents and cell growth was allowed to proceed until an optical density of 0.48 was achieved. During this process, the fermenter contents were 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 was maintained above 55% air saturation through a cascaded loop which increased agitation speed when the dissolved oxygen content dropped below 55%. Air was fed to the fermenter contents at 7 standard liters per minute (SLPM) and the culture temperature was maintained at 37°C throughout the process.

The cells were harvested by centrifugation at 17,000 x g for 10 minutes at 4°C and the resulting cell pellets were collected and stored at -85°C. The wet cell yield was 3.5 g/L. Analysis of the soluble and insoluble fractions of cell lysates by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed that approximately 50% of the ^{15}N-stromelysin was found in the soluble phase.

The isotopically-labeled stromelysin fragment prepared as described above was purified employing a modification of the technique described by Ye et al., Biochemistry, 31: 11231-11235 (1992).

The harvested cells were suspended in 20 mM Tris-HCl buffer (pH 8.0) sodium azide solution containing 1 mM $MgCl_2$, 0.5 mM $ZnCl_2$, 25 units/mL of Benzonase® enzyme, and an inhibitor mixture made up of 4-(2-aminoethyl)-benzenesulfonyl fluoride ("AEBSF"), Leupeptin®, Aprotinin®, and Pepstatin® (all at concentrations of 1 μg/mL. AEBSF, Leupeptin®, Aprotinin®, and Pepstatin® are available from American International Chemical, 17 Strathmore Road, Natick, MA 01760.)

The resulting mixture was gently stirred for one hour and then cooled to 4°C. The cells were then sonically disrupted using a 50% duty cycle. The resulting lysate was centrifuged at 14,000 rpm for 30 minutes and the pellet of insoluble fraction frozen at -80°C for subsequent processing (see below).

Solid ammonium sulfate was 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., 800 Centennial Ave., P. O. Box 1327, Piscataway, NJ 08855). Prior to loading, the sepharose column was equilibrated with 50 mM Tris-HCl buffer (pH 7.6 at 4°C), 5 mM $CaCl_2$, and 1 M $(NH_4)_2SO_4$. The loaded column was eluted with a linear gradient of decreasing concentrations of aqueous $(NH_4)_2SO_4$ (from 1 down to 0 M) and increasing concentrations of aqueous $CaCl_2$ (from 5 to 20 mM) in Tris-HCl buffer at pH 7.6.

The active fractions of eluate were collected and concentrated in an Amicon stirred cell (Amicon, Inc., 72 Cherry Hill Drive, Beverly, MA 01915). The concentrated sample was 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 was then loaded on the Q-Sepharose FF column and eluted with a linear gradient comprising the starting buffer and 200 mM NaCl. The purified soluble fraction of the isotopically-labeled stromelysin fragment was concentrated and stored at 4°C.

The pellet was solubilized in 8M guanidine-HCl. The solution was centrifuged for 20 minutes at 20,000 rpm and the supernatant was 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 was ten times that of the supernatant. The mixture of supernatant and folding buffer was centrifuged at 20,000 rpm for 30 minutes.

The supernatant from this centrifugation was stored at 4°C and the pellet was 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 were combined and solid ammonium sulfate was added to the point of 20% saturation. The resulting solution thus derived from the insoluble fraction was subjected to purification on phenyl Sepharose and Q-Sepharose as described above for the soluble fraction.

The purified soluble and insoluble fractions were combined to produce about 1.8 mg of purified isotopically-labeled stromelysin 81-256 fragment per gram of original cell paste.

B. Human papillomavirus (HPV) E2 Inhibitors

The papillomaviruses are a family of small DNA viruses that cause genital warts and cervical carcinomas. The E2 protein of HPV regulates viral transcription and is required for viral replication. Thus, molecules that block the binding of E2 to DNA may be useful therapeutic agents against HPV. The protein rather than the DNA was chosen as a target, because it is expected that agents with greater selectivity would be found that bind to the protein rather than the DNA.

The DNA-binding domain of human papillomavirus E2 was cloned from the full length DNA that codes for E2 using PCR and overexpressed in bacteria using the T7 expression system. Uniformly ^{15}N-labeled protein was isolated from

bacteria grown on a minimal medium containing ^{15}N-labeled protein was isolated from bacteria grown on a minimal medium containing ^{15}N-labeled ammonium chloride. The protein was purified from the bacterial cell lysate using an S-sepharose FastFlow column pre-equilibrated with buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, pH = 8.3).

The protein was eluted with a linear gradient of 100-500 mM NaCl in buffer, pooled, and applied to a Mono-S column at a pH = 7.0. The protein was eluted with a salt gradient (100-500 mM), concentrated to 0.3 mM, and exchanged into a TRIS (50 mM, pH = 7.0 buffered H_2O/D_2O (9/1) solution containing sodium azide (0.5%).

C. RAF

Uniformly ^{15}N-labeled Ras-binding domain of the RAF protein was prepared as described in Emerson et al., Biochemistry, **34** (21): 6911-6918 (1995).

D. FKBP

Uniformly ^{15}N-labeled recombinant human FK binding protein (FKBP) was prepared as described in Logan et al., J. Mol. Biol., **236**: 637-648 (1994).

Example 2

Screening Compounds Using Two-Dimensional $^{15}N/^{1}H$ NMR Correlation Spectral Analysis

The catalytic domain of stromelysin was prepared in accordance with the procedures of Example 1. The protein solutions used in the screening assay contained the uniformly ^{15}N-labeled catalytic domain of stromelysin (0.3 mM), acetohydroxamic acid (500 mM), $CaCl_2$ (20 mM), and sodium azide (0.5%) in a H_2O/D_2O (9/1) TRIS buffered solution (50 mM, pH=7.0).

Two-dimensional $^{15}N/^{1}H$ NMR spectra were generated at 29°C on a Bruker AMX500 NMR spectrometer equipped with a triple resonance probe and Bruker sample changer. The $^{15}N/^{1}H$ HSQC spectra were acquired as 80 x 1024 complex points using sweep widths of 2000 Hz (^{15}N, t^1) and 8333 Hz (^{1}H, t2). 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 using in-house-written software.

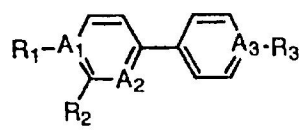
A first two-dimensional $^{15}N/^{1}H$ NMR correlation spectrum was acquired for the ^{15}N-labeled stromelysin target molecule as described above. The stromelysin target was then exposed to a database of test compounds. Stock solutions of the compounds were made at 100 mM and 1 M. In addition, a combination library was prepared that 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 database were selected on the basis of size (molecular weight = 100-300) and molecular diversity. The molecules in the collection had different shapes (e.g., flat aromatic rings(s), puckered aliphatic rings(s), straight and branched chain aliphatics with single, double, or triple bonds) and diverse functional groups (e.g., carboxylic acids, esters, ethers, amines, aldehydes, ketones, and various heterocyclic rings) for maximizing the possibility of discovering compound that interact with widely diverse binding sites.

The NMR samples were prepared by adding 4 μl of the DMSO stock solution of the compound mixtures that contained each compound at a concentration of 100 mM to 0.4 ml H_2O/D_2O (9/1) buffered solution of the uniformly ^{15}N-labeled protein. The final concentration of each of the compounds in the NMR sample was about 1 mM.

In an initial screen, two compounds were found that bind to the catalytic domain of stromelysin. Both of these compounds contain a biaryl moiety. Based on these initial hits, structurally similar compounds were tested against stromelysin. The structure of those biaryl compounds is represented by the structure I, below. (See Table 1 for definitions of R_1-R_3 and A_1-A_3).



In the second round of screening, binding was assayed both in the absence and in the presence of saturating amounts of acetohydroxamic acid (500 mM).

Many of the biaryl compounds were found to bind the catalytic domain of stromelysin. FIG. 4 shows a representative two-dimensional $^{15}N/^{1}H$ NMR correlation spectrum before and after exposure of stromelysin to a biaryl test compound. It can be seen from FIG. 4 that the compound caused chemical shifts of ^{15}N-sites such as those designated W124, T187, A199 and G204.

These sites correspond to a tryptophan (Trp) residue at position 124, a threonine (Thr) at position 187, an alanine (Ala) at position199, and a glycine (Gly) at position 204 of SEQ ID NO. 1. FIG. 9 shows the correlation between the NMR binding data and a view of the NMR-derived three-dimensional structure of the catalytic domain of stromelysin. The ability to locate the specific binding site of a particular ligand is an advantage of the present invention.

Some compounds only bound to stromelysin in the presence of hydroxamic acid. Thus, the binding affinity of some compounds was enhanced in the presence of the hydroxamic acid (i. e. cooperative). These results exemplify another important capability of the present screening assay: the ability to identify compounds that bind to the protein in the presence of other molecules.

Various biaryl compounds of structure I were tested for binding to stromelysin at differing concentrations. The $^{15}N/^{1}H$ spectra generated at each concentration were evaluated to quantify differences in the spectra as a function of compound concentration. A binding or dissociation constant (K_D)was calculated, using standard procedures well known in the art, from those differences. The results of this study are shown in Table 1. The values for R1-R3 and A1-A3 in Table 1 refer to the corresponding positions in the structure I, above.

Table 1

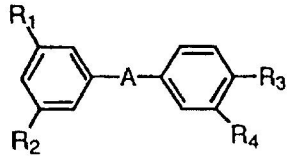
Compound No.	R_1	R_2	R_3	A_1	A_2	A_3	K_D(mM)
1	H	OH	H	C	C	C	1.1
2	CH_2OH	H	H	C	C	C	3.2
3	Br	H	OH	C	C	C	1.3
4	H	H	H	N	N	C	1.6
5	CHO	H	H	C	C	C	1.7
6	OCH_3	NH_2	H	C	C	C	0.4
7	H	H	H	N	C	C	0.2

8	$OCOCH_3$	H	H	C	C	C	0.3
9	OH	H	OH	C	C	C	0.16
10	H	H	H	N	C	N	0.4
11	OH	H	H	C	C	C	0.3
12	OH	H	CN	C	C	C	0.02

The data in Table 1 show the utility of a process of the present invention in determining dissociation or binding constants between a ligand and a target molecule.

Another advantage of an NMR screening assay of the present invention is the ability to correlate observed chemical shifts from the two-dimensional $^{15}N/^{1}H$ NMR correlation spectra with other spectra or projections of target molecule configuration. The results of a representative such correlation are shown in FIG. 9, which depicts regions within the polypeptide at which binding with the substrate molecule is most likely occurring. In this Figure, the apparent binding regions in stromelysin are shown for Compound 1 (from Table 1).

Compounds from the database were screened in a similar manner for binding to the DNA-binding domain of the E2 protein. Those compounds had the structure II below, where R_1-R_4 and A are defined in Table 2.



NMR experiments were performed at 29°C on a Bruker AMX500 NMR spectrometer equipped with a triple resonance probe and Bruker sample changer. The ^{15}N-/^{1}H HSQC spectra were acquired as 80 x 1024 complex points using sweep widths of 2000 Hz (^{15}N,t_1) and 8333 Hz (^{1}H, t_2). A delay of 1 second between scans and 4 scans per free induction decay were employed in the data collection. All NMR spectra were processed and analyzed on Silicon Graphics computers.

FIGs. 2 and 3 show representative two-dimensional $^{15}N/^{1}H$ NMR correlation spectra before and after exposure of the DNA-binding domain of E2 to a first and second test compound, respectively.

It can be seen from FIG. 2 that the first test compound caused chemical shifts of ^{15}N-sites such as those designated I15, Y21, R22 and L23. Those sites correspond to an isoleucine (Ile) residue at position 15, a tyrosine residue (Tyr) at position 21, an arginine (Arg residue at position 22 and a leucine (Leu) residue at position 23 of SEQ ID NO. 6.

It can be seen from FIG. 3 that the second test compound caused chemical shifts in the particular ^{15}N-sites designated I6, G11, H38, and T52. Those sites correspond to an isoleucine (Ile) residue at position 6, a glycine (Gly) residue at position 11, a histidine (His) residue at position 38 and a threonine (Thr) at position 52 of SEQ ID NO. 6.

FIGs. 7 and 8 show the correlation between those NMR binding data and a view of the NMR-derived three-dimensional structure of the DNA-binding domain of E2.

Several structurally similar compounds caused chemical shift changes of the protein signals when screened at a concentration of 1 mM. Two distinct sets of amide resonances were found to change upon the addition of the compounds: one set of signals corresponding to amides located in the ß-barrel formed between the two monomers and a second set corresponding to amides located near the DNA-binding site.

For example, compounds containing two phenyl rings with a carboxylic acid attached to the carbon linking the two rings only caused chemical shift changes to the amides in the DNA-binding site. In contrast, benzophenones and phenoxyphenyl-containing compounds only bound to the ß-barrel. Other compounds caused chemical shift changes of both sets of signals but shifted the signals in each set by different amounts, suggesting the presence of two distinct binding sites.

By monitoring the chemical shift changes as a function of ligand concentration, binding constants for the two binding sites were also measured. The results of those studies are summarized below in Table 2.

Table 2

Comp. No.	A	R_1	R_2	R_3	R_4	DNA K_D(mM)	β-barrel K_D(mM)	Filter binding assay
13	CO	H	H	H	OH	>50	0.6	-
14	O	H	H	H	CH_2OH	>50	2.0	-
15	-[a]	H	H	COO	H	2.0	>50	+
16	-[a]	Cl	Cl	COO	H	0.1	>50	+
17	-[a]	H	H	CH_2COO	H	4.2	4.9	+
18	-[a]	H	H	CH=CHCOO	H	1.2	6.2	+
19	O	H	H	$CH_2CH_2CH(CH_3)$ -CH_2COO	H	0.5	0.2	+
20	O	H	H	$COCH_2CH_2COO$	H	2.7	4.8	+

[a] a dash (-) for A indicates no atom (i.e. byphenyl linkage)

Uniformly ^{15}N-labeled Ras-binding domain of the RAF protein was prepared as described in Example 1 and screened using two-dimensional $^{15}N/^{1}H$ NMR correlation spectral analysis in accordance with the NMR procedures described above. The results of a representative study are shown in FIG. 5, which depicts two-dimensional $^{15}N/^{1}H$ NMR correlation spectra both before and after exposure to a test compound.

Uniformly ^{15}N-labeled FKBP was prepared as described in Example 1 and screened using two-dimensional $^{15}N/^{1}H$ NMR correlation spectral analysis in accordance with the NMR procedures described above. The results of a representative study are shown in FIG. 6, which depicts two-dimensional $^{15}N/^{1}H$ NMR correlation spectra both before and after exposure to a test compound.

Example 3

Comparison of NMR, Enzymatic, Filter Binding and Gel Shift Screening Assays

Studies were performed to compare binding constants of ligands to various biomolecules, determined by the NMR method of the present invention, to similar results obtained from prior art methods.

In a first study, binding constants were determined, both by the NMR method of the present invention, and by a prior art enzymatic assay. The target molecule was the catalytic domain of stromelysin prepared in accordance with the

WHAT IS CLAIMED IS:

1. A process of screening compounds to identify compounds that are ligands that bind to a specific target molecule comprising the steps of:

a) generating a first two-dimensional $^{15}N/^{1}H$ NMR correlation spectrum of a ^{15}N-labeled target molecule;

b) exposing the labeled target molecule to one or a mixture of chemical compounds;

c) generating a second two-dimensional $^{15}N/^{1}H$ NMR correlation spectrum of the labeled target molecule that has been exposed to one or a mixture of compounds in step (b); and

d) comparing said first and second two-dimensional $^{15}N/^{1}H$ NMR correlation spectra to determine differences between said first and said second spectra, the differences identifying the presence of one or more compounds that are ligands which have bound to the target molecule.

2. The process of claim 1 wherein the ^{15}N-labeled target molecule is exposed to a mixture of chemical compounds in step (b), further comprising the steps subsequent to step d) of

e) exposing the ^{15}N-labeled target molecule individually to each compound of said mixture,

f) generating a two-dimensional $^{15}N/^{1}H$ NMR correlation spectrum of the labeled target molecule that has been individually exposed to each compound; and

g) comparing each spectrum generated in step f) to said first spectrum to determine differences in any of those compared spectra, the differences identifying the presence of a compound that is a ligand which has bound to the target molecule.

3. The process of claim 1 wherein the differences in the two-dimensional $^{15}N/^{1}H$ NMR correlation spectra are chemical shifts at particular ^{15}N-labeled sites in the target molecule and chemical shifts in protons attached to those ^{15}N-labeled sites.

4. The process of claim 1 wherein the target molecule is a polypeptide.

5. A process of determining the dissociation constant between a target molecule and a ligand that binds to that target molecule comprising the steps of:

a) generating a first two-dimensional $^{15}N/^{1}H$ NMR correlation spectrum of a ^{15}N-labeled target molecule;

b) exposing the labeled target molecule to various concentrations of a ligand;

c) generating a two-dimensional $^{15}N/^{1}H$ NMR correlation spectrum at each concentration of ligand from step (b);

d) comparing each spectrum from step (c) both to the first spectrum from step (a) and to all other spectra from step (c) to quantify differences in those spectra as a function of changes in ligand concentration; and

e) calculating the dissociation constant between the target molecule and the ligand from those differences according to the equation:

$$K_D = \frac{([P]_o - x)([L]_o - x)}{x}$$

where $[P]_O$ is the total molar concentration of target molecule;

$[L]_O$ is the total molar concentration of ligand; and

x is the molar concentration of the bound species determined according to the equation:

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

where δ_{obs} and δ_{free} are the chemical shift values for the target molecule determined at each concentration of ligand and for the target molecule in the absence of ligand, respectively, and Δ is the difference between the chemical shift at saturating amounts of ligand and δ_{free}.

6. The process of claim 5 wherein the target molecule is a polypeptide.

7. The process of claim 5 further comprising the step of binding the labeled target molecule to a second ligand before step (a).