

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

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Bogotá, D.C.,

Abogado
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (Fase Nacional) ☒ Capítulo I ☐ Capítulo II ☒

No. Solicitud Internacional PCT/US2006/027066
Fecha de Presentación Internacional 11/Julio/2006

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 comunica:

1. Documentos estudiados

Descripción:	Folios 287 a 656	10/Enero/2008
Reivindicaciones:	Folios 657 a 715	10/Enero/2008
Dibujos:	Folios 717 a 718	10/Enero/2008
Prioridad:	US 60/697,590	11/Julio/2005

2. Análisis de la Prioridad

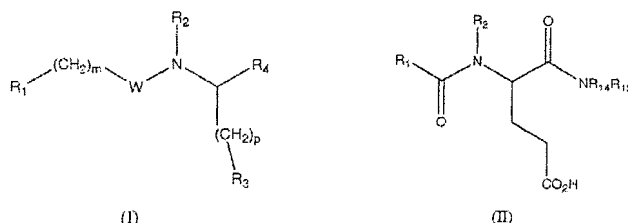
Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Inhibidores de Agrecanasa Glutamato".

El problema planteado en esta solicitud consiste en la necesidad de suministrar inhibidores de metaloproteinasas, que incluyen inhibidores de MMP y agrecanasas.

Para resolver este problema técnico la solicitud en estudio proporciona un compuesto de la fórmula (I), (II) y métodos para su preparación.



El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07C 237/22, 237/42, 235/84, 231/16, 323/41, 259/06; C07D 317/58, 333/24, 213/56, 277/30; A61K 31/166, 31/36, 31/381, 31/4409, 31/426.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 27 – 31 no es patentable de acuerdo con el Art citado.

5. De la Solicitud de Patente

Título

El título no es claro, se sugiere especificar que los compuestos son derivados de glutamato con actividad inhibitoria de agrecanasa.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

En la reivindicación 1 no es claro a que se refiere el solicitante con “un grupo de ácido mimético”, se sugiere realizar las aclaraciones necesarias.

La reivindicación 20 se encuentra como independiente, sin embargo, ésta no cuenta con ninguna característica esencial que la haga diferente de la reivindicación 1, adicionalmente, varias reivindicaciones independientes de una misma categoría son un claro indicio de falta de concisión, por lo cual se sugiere dejar esta reivindicación como dependiente de la reivindicación principal 1.

En el paso a) de la reivindicación 33, la estructura de la fórmula (V) se encuentra cortada. Se sugiere allegar una copia legible de la misma.

La frase “bajo condiciones efectivas” de las reivindicaciones 32 - 35, es imprecisa, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirla por las condiciones específicas en cada uno de las etapas del procedimiento.

Las reivindicaciones 34 y 35 que se encuentran encabezadas como un compuesto, se definen por una secuencia lógica de etapas, por lo tanto se sugiere encabezarla como un método o procedimiento que es el real objeto de la reivindicación. Adicionalmente, se recuerda al solicitante que una reivindicación de producto, sólo puede ser definida en términos de su proceso de fabricación si: el producto es nuevo e inventivo y no se puede definir por sus características esenciales.

Las reivindicaciones 1, 4, 5, 20, no son concisas porque las definiciones de R₁, R₃, R₅, R₆, R₇, R₈, R₉, R₁₀, R₁₁, R₁₂, R₁₃, R₁₄ y R₁₅ incluyen géneros funcionales como arilo, arilo bicíclico, arilo tricíclico y cicloalquilo, sin definir el numero de carbonos y heteroarilo, heteroarilo bicíclico, heteroarilo tricíclico, heterocicloalquilo sin definir el numero de carbonos, número de heteroátomos ni los heteroátomos presentes;

adicionalmente la gran cantidad de posibilidades en cuanto a sustituyentes no permite definir el alcance de la invención y da lugar a un sinnúmero de compuestos que no tienen soporte en la descripción. Se sugiere restringir la solicitud a una razonable generalización del tipo de compuestos que ha sido sintetizado ó probado, es decir, que se encuentran debidamente soportados. En la descripción se encuentra:

Sustituciones	Soporte de los ejemplos presentados
W	-C(O)-
R ₁	Fenilo, bifenilo, 9H-fluoreno, 9-oxo-9H-fluoreno, tienil, piridin, furoil,
R ₂	H
R ₃	-CO ₂ H
R ₄	-CO ₂ Et, -CO ₂ H, -CONH ₂ , -CONR ₉ R ₁₀ , -CH ₂ -O-
R ₆	AlquilC ₁₋₂ , alquenilC ₂₋₃ , alquiniilC ₂ , cicloalquilC ₃₋₆ , fenilo, -CH ₂ -O-, -C(O)-, -O-alquilC ₁₋₄ , -O-arilC ₆ , -O-alquilC ₁ -arilC ₆ , alquilC ₁ -O-, -NH-, furil, benzodioxol, piridin, tiazolil, tienil, dihidrobenzofuranil, naftil, 9-hidroxi-9H-fluorenil, -OH, -NHC(O)-, halógeno
R ₇	H, alquilC ₁₋₂ , ariloC ₆ , pirimidin, piridin,
R ₈	H, alquilC ₁₋₂
R ₉	H, AlquilC ₁₋₂
R ₁₀	2,3-dihidro-1H-2-il-indano, alquilC ₁₋₇ , cicloalquilC ₅₋₈ , alquilC ₁₋₂ -OH, adamantil, naftilo
R ₁₂	AlquilC ₁₋₂ , fenil, -O-alquilC ₁₋₃ , cicloalquilC ₃ , benzodioxol, adamantil, naftilo, furilo, tienilo, morfolina, pirimidin, -NO ₂ , -NH ₂ , -OH, -CF ₃ , halógeno
R ₁₃	AlquilC ₁₋₄ , alquenilC ₂ , ariloC ₆ , -OalquilC ₁₋₂ , cicloalquilC ₄₋₆ , arilo sustituido con NH ₂ , naftil, -OH, -CF ₃ , halógeno
m	0, 1,
p	2

Las reivindicaciones 32 – 35 no so concisas porque se refieren a los pasos del proceso como hidrólisis del éster de alquilo, hidrolizar el grupo protector amina, hidrólisis del ácido carboxílico protegido sin definir cada una de las etapas con los compuestos que participan y las condiciones de cada una de las etapas. Adicionalmente, las fórmulas (III), (IV), (V), (VI), (VII) y (VIII) en sus radicales R₁, R₅, R₆, R₇, R₈, R₁₁, R₁₂, R₁₃, R₁₄ y R₁₅ se encuentran definidas por géneros funcionales como arilo, arilo bicíclico, arilo tricíclico y cicloalquilo, sin definir el numero de carbonos; y heteroarilo, heteroarilo bicíclico, heteroarilo tricíclico, heterocicloalquilo sin definir el numero de carbonos, número de heteroátomos ni los heteroátomos presentes. Adicionalmente, la cantidad de posibilidades en cuanto a sustituyentes dan lugar a un sinnúmero de compuestos que no se encuentran soportados en la descripción. Se sugiere restringir la solicitud a una razonable generalización del tipo de compuestos que se han utilizado en los métodos de síntesis, es decir, que se encuentran debidamente soportados.

Descripción (Art 28 D 486)

En la tabla 1 (Págs. 352 -362, Fls. 637 – 647) no es claro el significado del símbolo “@”, ni de la abreviatura “no inh”. Se solicita realizar las aclaraciones necesarias.

Se requiere al Solicitante hacer las aclaraciones necesarias.

6. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el requisito de unidad de invención porque el concepto común inventivo que se refiere a compuestos de fórmula (I) o (II) como inhibidores

de metaloproteinasas ya se encontraba divulgado en el documento D1, por lo tanto se encuentran por lo menos 6 grupos inventivos.

Los grupos inventivos están definidos por las siguientes características **esenciales**:

- 1° Compuestos de fórmula (I), (Ia) o (II) cuando R₁ es fenilo (Divulgados en D1)
- 2° Compuestos de fórmula (Ib)
- 3° Compuestos de fórmula (Ic)
- 4° Compuestos de fórmula (Id)
- 5° Proceso de obtención de compuestos de fórmula (II) de las reivindicaciones 32 y 34 (Divulgado en el documento D3)
- 6° Proceso de obtención de compuestos de fórmula (II) de las reivindicaciones 33 y 35 (Divulgado en el documento D1)

Se sugiere presentar las solicitudes divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 11/Julio/2005 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	TAKAHASHI K. ET AL, Bioorganic & Medicinal Chemistry, Vol. 13, Págs. 4527 - 4543	Novel Matrix Metalloproteinase Inhibitors: Generation of Lead Compounds by the in Silico Fragment-Based Approach	23/Mayo/2005 (Disponible en internet)
D2	WO 01/90077	Sulfonamide and Carbamide Derivatives of 6(5H)Phenanthridinones and Their Uses	29/Noviembre/2001
D3	KUEFNER, U. ET AL, Biochemistry, Vol. 28, Págs. 2288 - 2297	Carboxypeptidase-Mediated Release of Methotrexate from Methotrexate α -Peptides	1989
D4	US 5,665,777	Biphenyl Hydroxamate Inhibitors of Matrix Metalloproteinases	09/Septiembre/1997

D1 divulga (Pág. 4527, resumen, Fl. 815) derivados de ácido L-glutámico inhibidores de metaloproteinasa y el procedimiento para su síntesis.

D2<http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=0190077A1&KC=A1&FT=D&ND=&date=20011129&DB=&locale=en> EP divulga (Pág. 1, líneas 10 – 31; Pág. 19, compuesto 25) compuestos inhibidores de PARP útiles para el tratamiento de patologías como la osteoartritis y composiciones de los mismos.

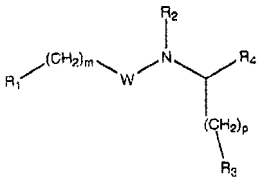
D3 divulga (Pág. 2290, Fl. 822) un procedimiento de síntesis de [N-(4amino-4deoxi-10-metilpteroil)- α -glutamil]-L-alanina dihidrato (MTX- α -alanina)

D4<http://worldwide.espacenet.com/publicationDetails/biblio?DB=worldwide.espacenet.com&II=0&ND=3&adjacent=true&locale=en> EP divulga (Col. 1, líneas 6 - 45; Col. 40 – 41, reivindicación 1) compuestos inhibidores de metaloproteinasas para el tratamiento de patologías como la artritis reumatoide y la osteoartritis.

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en un compuesto de la fórmula (I)



(I)

o una sal farmacéuticamente aceptable de éste.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2
Un compuesto de fórmula: (I)	Un compuesto de fórmula: 	Un compuesto de fórmula:
W: -C(O)-	-C(O)-	-C(O)-
R ₁ : fenilo sustituido con R ₅ , heteroarilo tricíclico, R ₅ : alquilC ₁₋₆		
R ₂ : H	H	H
R ₃ : -COHNH	 R ₂ : NHOH	-COOH
R ₄ : -CONR ₉ R ₁₀ , -COOH, -(CH ₂) _n OR ₇ R ₉ : H R ₁₀ : alquilC ₁₋₆ sustituido con R ₁₂	 X:	-COOH
m: 0	0	0
p: 2	2	2

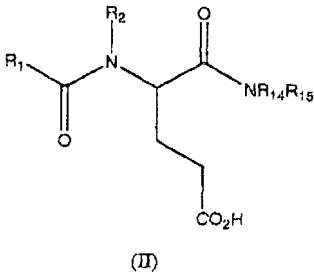
Las características **esenciales** del compuesto de la fórmula (I) de la reivindicación 1 habían sido divulgadas previamente en D1 y D2. El documento D1 enseña derivados de ácido L-glutámico inhibidores de metaloproteinasa como los compuestos 2b, 7, 8 y 9 que coinciden con la compuestos de la fórmula (I) cuando W: -C(O)-, R₁: heteroarilo tricíclico, R₂: H, R₃: -COHNH, R₄: -CONR₉R₁₀, -COOH o -(CH₂)_nOR₇, R₅: alquilC₁₋₆, R₉: H, R₁₀: alquilC₁₋₆ sustituido con R₁₂, m: 0 y p: 2 (Pág. 4527, resumen, Fl. 815; Pág. 4528, tabla 2, Fl. 815). El documento D2 revela un compuesto que concuerda con el compuesto de la fórmula (I) cuando W: -C(O)-, R₁: arilo tricíclico, R₂: H, R₃: -COOH, R₄: -COOH m: 0 y p: 2 (Pág. 19, compuesto 25)

El documento D2 también da a conocer composiciones del compuesto (Pág. 86, reivindicaciones 6 – 9).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 – 3, 7, 8, 13, 15, 25 y 26 no mencionan alguna característica que haga nuevo al objeto de la reivindicación 1, por lo cual se considera que no son nuevas.

La reivindicación 20 consiste en un compuesto de la fórmula (II)



o una sal farmacéuticamente aceptable de éste.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

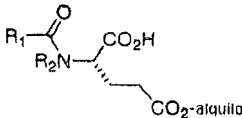
Características esenciales	D1
Un compuesto de fórmula: (II)	Un compuesto de fórmula: X: OH
R ₁ : fenilo sustituido con R ₅ R ₅ : alquilC ₁₋₆	
R ₂ : H	H
R ₁₄ : H	H
R ₁₅ : alquilC ₁₋₆	-CH ₃

Las características **esenciales** de la composición de la reivindicación 1 habían sido divulgadas previamente en D1, el cual enseña derivados de ácido L-glutámico inhibidores de metaloproteinasa como los compuestos 2b, 7, 8 y 9 que coinciden con la compuestos de la fórmula (I) cuando W -C(O)-, R₁: fenilo, R₂: H, R₅: alquilC₁₋₆, R₁₄: H y R₁₅: alquilC₁₋₆ (Pág. 4529, Fl. 816; Pág. 4534, Fl. 818).

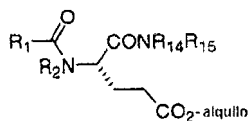
En consecuencia, la reivindicación 20 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones 32 y 34 consisten en un método para sintetizar un compuesto que comprende:

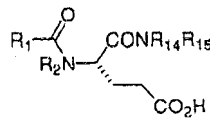
a) Tratar un compuesto de la fórmula (III):



Con una amina, bajo condiciones efectivas para proporcionar un compuesto que tiene fórmula (IV):

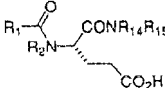
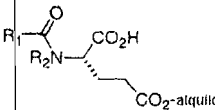
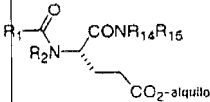
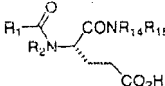
 $(IV);$

- b) Tratar el compuesto de la fórmula (IV) bajo condiciones efectivas para la hidrólisis del éster de alquilo, suministrando por lo tanto un compuesto que tiene la fórmula (II):



III.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

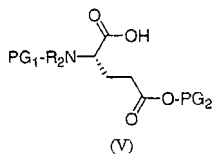
Características esenciales	D3
Un método para sintetizar un compuesto que comprende:	Un método para sintetizar el compuesto [N-(4amino-4deoxi-10-metilpteroil)-α-glutamil]-L-alanina dihidrato (MTX-α-alanina) que comprende:
 (II).	Tratar un compuesto de la fórmula (III)
	Tratar MTX y-ter-butil éster con trietilamina para proporcionar el compuesto di-ter-butil éster de MTX-α-Ala
Con una amina, bajo condiciones efectivas para proporcionar un compuesto de fórmula (IV)	Tratar el di-ter-butil éster de MTX-α-Ala con THF y HCl para obtener MTX-α-Ala
 (IV).	Tratar el compuesto de la fórmula (IV) bajo condiciones efectivas para la hidrólisis del éster de alquilo, suministrando un compuesto de fórmula (II)
 (II).	Tratar el di-ter-butil éster de MTX-α-Ala con THF y HCl para obtener MTX-α-Ala

Las características **esenciales** del método de la reivindicación 16 habían sido divulgadas previamente en D3, el cual da a conocer un método para sintetizar el compuesto [N-(4-amino-4-deoxi-10-metilpteroil)-α-glutamil]-L-alanina dihidrato (MTX-α-alanina) que comprende tratar MTX γ-ter-butil éster con trietilamina para proporcionar el compuesto di-ter-butil éster de MTX-α-Ala, el cual es tratado con THF y HCl para obtener MTX-α-Ala (Pág. 2290, Fl. 822)

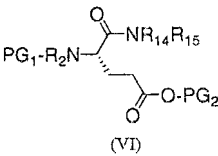
En consecuencia, las reivindicaciones 32 y 34 no son nuevas, según lo divulgado en el documento D3.

Las reivindicaciones 33 y 35 consisten en un método para sintetizar un compuesto que comprende:

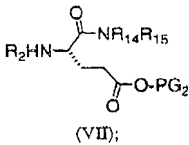
- a) Tratar un compuesto de la fórmula (V):



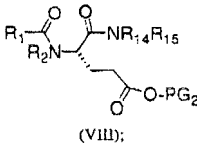
Con una amina, bajo condiciones efectivas para proporcionar un compuesto que tiene fórmula (VI):



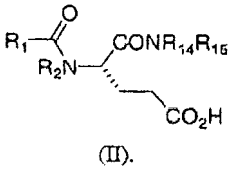
b) Hidrolizar el grupo protector amina de un compuesto de la fórmula (VI), bajo condiciones efectivas para proporcionar un compuesto que tiene la fórmula (VII):



c) Tratar el compuesto de la fórmula (VII) con un ácido carboxílico, bajo condiciones efectivas para proporcionar un compuesto que tiene la fórmula (VIII):

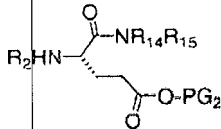
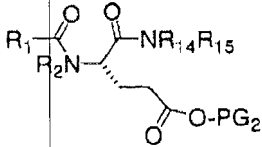
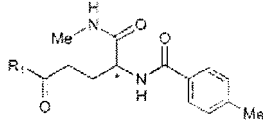
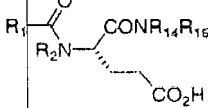
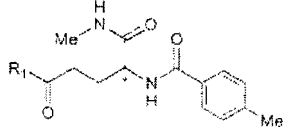


d) Tratar el compuesto de la fórmula (VIII) bajo condiciones efectivas para la hidrólisis del ácido carboxílico protegido, suministrando por lo tanto un compuesto que tiene la fórmula (II):



Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1
Un método para sintetizar un compuesto (II) de fórmula (II)	Un método para sintetizar un compuesto de fórmula 2a o 22
que comprende:	que comprende:
Tratar un compuesto de la fórmula (IV) (V) Con una amina, bajo condiciones efectivas para proporcionar un compuesto de fórmula (VI) (VI)	Tratar el compuesto de fórmula 19 R₁: O^tBu, OBzl R₂: OBzl, Boc con NH₂Me para proporcionar un compuesto de fórmula 20

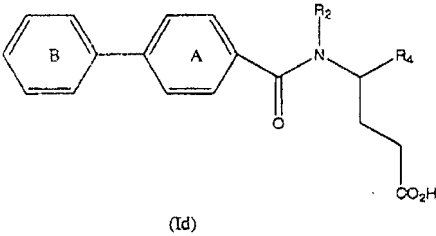
Características esenciales	D1
Hidrolizar el grupo protector amina de un compuesto de la fórmula (VI), bajo condiciones efectivas para proporcionar un compuesto que tiene la fórmula (VII)  (VII);	Hidrolizar el grupo protector
Tratar el compuesto de la fórmula (VII) con un ácido carboxílico, bajo condiciones efectivas para proporcionar un compuesto que tiene la fórmula (VIII):  (VIII);	Tratar el compuesto con cloruro de 4-metilbenzoil para obtener el compuesto 21 
Tratar el compuesto de la fórmula (VIII) bajo condiciones efectivas para la hidrólisis del ácido carboxílico protegido, suministrando por lo tanto un compuesto que tiene la fórmula (II):  (II).	Tratar el compuesto con TFA-H2O o H2, Pd-C, EtOH para obtener el compuesto 2a o 22  R1: -OH

s características **esenciales** del método de las reivindicaciones 33 y 35 habían lo divulgadas previamente en D1, el cual da a conocer un método para sintetizar el mpuesto 2a o 22 que comprende tratar el compuesto 19 con NH₂Me para proporcionar un compuesto de fórmula 20, hidrolizar el grupo protector, tratar el mpuesto con cloruro de 4-metilbenzoil para obtener el compuesto 21 y tratar el mpuesto 21 con TFA-H₂O o H₂, Pd-C, EtOH para obtener el compuesto 2a o 22 mo se muestra en la tabla (Pág. 4529, esquema 1, Fl. 816)

i consecuencia, las reivindicaciones 33 y 35 no son nuevas, según lo divulgado en documento D1.

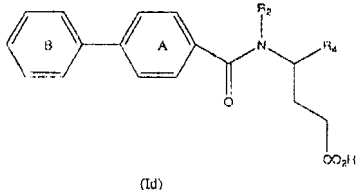
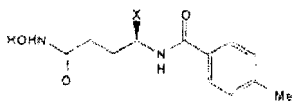
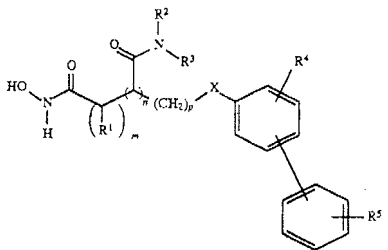
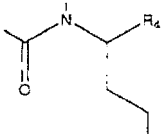
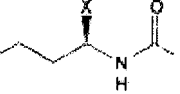
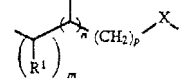
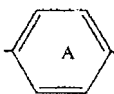
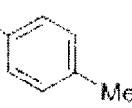
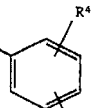
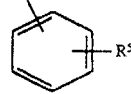
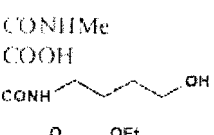
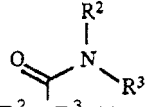
vel Inventivo (Art18 D486)

reivindicación 16 consiste en el compuesto de la reivindicación 1 de la fórmula):



una sal farmacéuticamente aceptable de éste.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D5
Un compuesto de fórmula:  (Id)	Un compuesto de fórmula: 	Un compuesto de fórmula: 
		 p: 0 n: 2 R ¹ : H X: NH
		
	No se menciona	
-CO ₂ H	-COOH	-CONH-OH
R ₂ : H	H	H
R ₄ : -CONR ₉ R ₁₀ , -COOH, -(CH ₂) _n OR ₇ R ₉ y R ₁₀ : H, alquilC ₁₋₆ , aril, heteroaril opcionalmente sustituidos con R ₁₂ ; o R ₉ y R ₁₀ juntos forman un grupo cíclico de 5 – 7 miembros que contiene hasta 3 heteroátomos seleccionados de N, O o S.	 X: 	 R ² y R ³ : H, alquilC ₁₋₆ , fenil, fenil sustituido, piridil, piridil sustituido; o R ² y R ³ tomados junto con el átomo de nitrógeno forman un heterociclo saturado de 5 – 6 miembros que puede tener un heteroátomo adicional seleccionado de -NR ⁸ , -O- o -S-

Los documentos D1 y D4 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 16 porque divulgan compuestos inhibidores de metaloproteinasas. El documento D1 da a conocer compuestos inhibidores de metaloproteinasas de la matriz que corresponden con la compuestos de la fórmula (Id) cuando R₂: H, R₄: -CONR₉R₁₀, -COOH o -(CH₂)_nOR₇, R₉: H y R₁₀: alquilC₁₋₆ sustituido con R₁₂ y da a conocer que estos compuestos son derivados del ácido L-glutámico o del ácido 4-aminobutírico (Pág. 4527, resumen, Fl. 815; Pág. 4528, tabla 2, Fl. 815; Pág. 4535, Fl. 819). El documento D4 enseña compuestos inhibidores de metaloproteinasas de la matriz que concuerdan con la fórmula (Id) cuando R₂: H, R₄: -CONR₉R₁₀, -COOH o -(CH₂)_nOR₇, R₉ y R₁₀: H, alquilC₁₋₆, aril, heteroaril opcionalmente sustituidos con R₁₂, o R₉ y R₁₀ juntos forman un grupo cíclico de 5 – 7 miembros que contiene hasta 3 heteroátomos seleccionados de N, O o S (Col. 1, líneas 6 -14; Col. 40 – 41, reivindicación 1).

La diferencia entre la invención, definida en la reivindicación 16 y los compuestos del documento D1 es el anillo fenilo B.

El efecto que logra el incluir el anillo fenilo B es la inhibición de metaloproteinasas

como las agrecanasas.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar el estado de la técnica para obtener compuestos inhibidores de metaloproteinasas alternativos”.

Sin embargo, la utilización de compuestos que incluyen el radical bifenilo y son inhibidores de la metaloproteinasas ya está divulgada en el documento D4 (Col. 1, líneas 6 -14; Col. 40 – 41, reivindicación 1). Adicionalmente el documento D1 también se enseña el uso de radicales bifenilo en los compuestos inhibidores de metaloproteinasas (Pág. 4534, Fl. 818).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el anillo fenilo B para formar un bifenilo según el documento D4 en los compuestos del documento D1 para llegar así al objeto de la reivindicación 16 de la solicitud. Por lo cual se considera obvia.

Conclusión:

La reivindicación 16 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

9. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	TAKAHASHI K. ET AL, Bioorganic & Medicinal Chemistry, Vol. 13, Págs. 4527 - 4543	1 – 3, 7, 8, 13, 20, 25, 26, 33, 35	Novedad
		6, 11, 16, 21	Nivel Inventivo
D2	WO 01/90077	1 – 3, 7, 8, 13, 15, 20, 25, 26	Novedad
D3	KUEFNER, U. ET AL, Biochemistry, Vol. 28, Págs. 2288 - 2297	32, 34	Novedad
D4	US 5,665,777	6, 11, 16, 21	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 (60) días contados a partir de la fecha de 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.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha **28 AGO 2012**

Elaborado por: Jeny Paola Villate Becerra *P. Villate*
Revisado por: Consuelo Leguizamón *CL*

Expediente 08 001 924 1er Art 45

Novel matrix metalloproteinase inhibitors: Generation of lead compounds by the in silico fragment-based approach

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Abstract—Generation of structurally new matrix metalloproteinase inhibitors was successfully carried out using an in silico technique. In order to identify the small fragment interacting with residues in the S1' pocket of MMP-1 through hydrogen bonds, we performed in silico screening using the LUDI program. As a result, acetyl-L-alanyl-L-N-methylamide (Ac-L-Ala-NHMe) was selected to link with another fragment, hydroxamic acid that interacted with catalytic zinc. By this approach, the L-glutamic acid derivative **2b** was discovered to be a new type of matrix metalloproteinase inhibitor. Further transformation to reduce its peptide nature and improve activity yielded nonpeptidic lead compounds as inhibitors of MMP-1, -2, -3, and -9.

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1. Introduction

Matrix metalloproteinases (MMPs), a family of zinc-containing endopeptidases, have been shown to play a central role in various physiological and pathophysiological events, such as embryonic development, blastocyst implantation, nerve growth, ovulation, morphogenesis, angiogenesis, tissue resorption, and tissue remodeling. In addition, the overexpression or dysregulation of MMPs is associated with several disease processes, such as tumor metastasis, arthritis, atherosclerosis, aneurysm, breakdown of the blood brain barrier, periodontal disease, skin ulcers, corneal ulcers, gastric ulcers, and liver fibrosis. In patients with these diseases, MMPs are considered to be a potential drug target. In fact, a number of MMP inhibitors (MMPIs) have been developed, several of which have shown some efficacy in clinical trials.¹

Inhibition of MMPs is related to the coordination between the inhibitor molecule (generally as an anion) and the catalytic metal ion, with or without substitution of the metal-bound water molecule. MMPIs should have

a zinc-binding moiety attached to a substructure that interacts with the substrate recognition sites of the enzyme. The strongest known class of MMPIs is the hydroxamates, which have been shown to bind bidentately to the catalytic Zn(II) of the enzyme, causing it to adopt a distorted trigonal bipyramidal geometry.² The hydroxamate anion forms a short and strong hydrogen bond with the carboxylate moiety of Glu 219, which is oriented toward the unprimed binding regions, whereas the NH hydroxamate participates in a hydrogen bond with the carbonyl oxygen of Ala 182. Thus, several strong interactions occur at the zinc site without any significant unfavorable contacts.

To discover the substructure interacting with the substrate recognition sites of MMPs, NMR-based screening has proven to be a valuable approach. For this approach, individual small compounds are mixed with a ¹⁵N-labeled protein to evaluate their binding affinity. By this method, 4-cyanobiphenyl was found to show weak binding to the S1' pocket in the case of stromelysin (MMP-3), and then was connected with hydroxamic acid through an appropriate linker to successfully produce potent inhibitors of MMP-3. Although this NMR-based fragment approach is a powerful method of discovering lead compounds, a significant amount of isotope-labeled protein is required for screening.³

In silico virtual screening of small molecules based on the three-dimensional structure of the target protein is an attractive way to identify candidates that may interact with a specific site. As structural information about the target protein is established in more detail, this in silico approach becomes increasingly reliable. In the case of MMPs, detailed three-dimensional structural information about the binding sites of MMPIs is available from X-ray crystallography, NMR experiments, and structure activity relationship (SAR) data. This situation prompted us to explore virtual screening with the de novo program, LUDI,⁴ in order to identify new substructures that might interact with amino acids around the S1' pocket of MMPs. Here, we report the results of our in silico fragment-based approach and the preliminary SAR data for the identified lead compounds.

2. Chemistry

Synthesis of the test compounds listed in Tables 1–4 is described in Schemes 1–8. Synthesis of compounds **2a,b** and **5** is outlined in Scheme 1. Condensation of the optically pure glutamic acid derivatives **19a,b** with methylamine afforded **20a,b**, respectively. Catalytic hydrogenation of **20a** provided **21a**, while acidic deprotection of **20b** gave **21b**. Acidic deprotection of **21a** resulted in **2a**, while catalytic hydrogenation of **21b** produced **22**. The carboxylic acids **2a** and **22** were converted to hydroxamic acids (**2b** and **5**) by the condensation with *O*-benzylhydroxamine followed by catalytic hydrogenation.

Table 1. Activity profiles of newly designed chemical leads (Chart 1)

Compound	IC ₅₀ (μM)			
	MMP-1	MMP-2	MMP-9	MMP-3
2a	>100	>100	NT ^a	>100
2b	19	2.4	38	39%
3	>100	>100	>100	>100
4	59%	18	40%	NT
5	>100	>100	NT	>100
6	>100	>100	NT	>100

^a Inhibition percentage at 100 μM.

^b Not tested.

Table 3. Effect of the C1 substitution of 4-aminobiphenyl acid analogs on the activity profiles

Compound: X	IC ₅₀ (μM)			
	MMP-1	MMP-2	MMP-9	MMP-3
4	H	59%	38	39%
10	CH ₃ OH	6.4	1.9	NT
11	(+)-CH ₃ OH	88%	2.2	34
12	(-)-CH ₃ OH	69%	72	38
13	CH ₃ OBu	9.53	0.18	1.2

^a Inhibition percentage at 100 μM.

Compound **3** was prepared as outlined in Scheme 2. *N*-Acylation of **25**, which was prepared from **24**, with a glutaric anhydride followed by formation of the *N*-methylamide by the conventional procedures afforded **26b**. Without *N*-protection in **26a**, an exclusive intramolecular cyclization took place in the following *N*-methylamide formation reaction. Deprotection of **26b** with a catalytic hydrogenation resulted in **3**.

Compound **4** was synthesized as outlined in Scheme 3. *N*-Acylation of 4-aminobiphenyl acid with 4-methylbenzoylchloride in the presence of sodium hydroxide gave **28**, which was converted to the corresponding hydroxamic acid **4** by the usual method.

Compound **6** was also synthesized from **19b** as outlined in Scheme 4. Condensation of **19b** with 4-methyl aniline afforded **30**, catalytic hydrogenation of which gave **31**. Condensation of **31** with *O*-benzylhydroxamine produced **32**. Acidic deprotection of **32** resulted in **33**, which was converted to **34** according to the usual *N*-acetylation. Deprotection of **34** by catalytic hydrogenation produced the hydroxamate **6**.

Synthesis of **7–8** is described in Scheme 5. *N*-Acylation of a protected glutamic acid **35** with 4-methylbenzoyl chloride afforded **36**, deprotection of which by catalytic hydrogenation provided the carboxylic acid **37**. Condensation of **37** with **25** gave **38**. Acidic deprotection of **38**

Table 2. Effect of the chemical modification of C1 carboxylic acid moiety of L-glutamic acid analog on the activity profiles

Compound	X	IC ₅₀ (μM)			
		MMP-1	MMP-2	MMP-9	MMP-3
2b	CONHMe	19	2.4	38	39%
7	COOH	NT	14	NT	>100
8	CONH(CH ₂) ₄ OH	23	3.4	NT	41%
9	H ₂ C=O-CH ₂ -OEt	4.3	>1	>1	84%

^a Inhibition percentage at 100 μM.

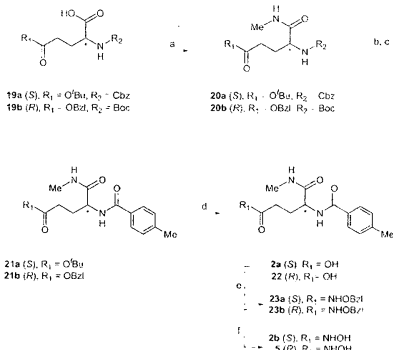
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doi:10.1016/j.bmc.2005.04.051

Table 4. *In vitro* inhibitory activity of the MMP-13 inhibitors (MMPi) against MMP-13 (EC₅₀ values).

Inhibitor	R	MMP-13	MMP-13	MMP-13	MMP-13
4		59.1 ^a	N	40.1 ^a	NT
14		9.6	67.1 ^a	NT	>100
15		67.1 ^a	6.8	NT	63.1 ^a
16		>100	19.1 ^a	NT	>100
17		>100	7.8	NT	13
18		>100	3.5	NT	1.0

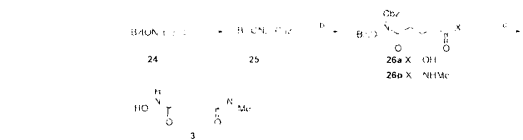
^aInhibition percentage at 100 μM.



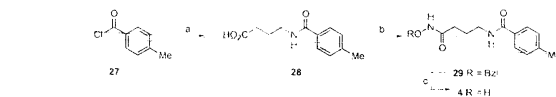
Scheme 1. Synthesis of **2a,b** and **5**. Reagents: (a) (i) ClCO^tBu, NMM, THF; (ii) NtMe; (b) H₂, Pd/C, MeOH or 4 N HCl/dioxane; (c) 4-methylbenzoyl chloride, pyridine or 4-methylbenzoyl chloride, Et₃N, CH₂Cl₂; (d) TEA, H₂O or H₂, Pd/C, EtOH; (e) HCl/NH₂OBz, EDC, HOBT, Et₃N, DMF; (f) H₂, Pd/C, MeOH.

resulted in **39**, catalytic hydrogenation of which led to **7**. Condensation of **39** with 4-amino-1-butanol afforded **40**, after which deprotection by the usual method produced **8**.

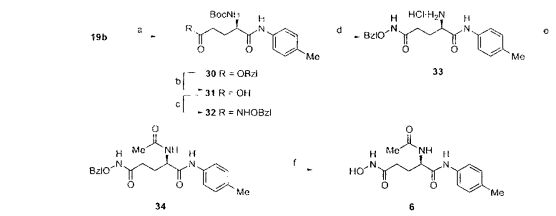
Synthesis of **9** is outlined in Scheme 6. A commercially available protected glutamic acid **41** was converted to **45** by the following sequence of reactions. Formation of an activated ester of **41**, followed by sodium borohy-



Scheme 2. Synthesis of **3**. Reagents: (a) (i) H₂, Pd/C, MeOH; (ii) glutaric anhydride, DMAP, CH₂Cl₂; (iii) H₂NMe, EDC, HOBT, Et₃N, DMF; (iv) H₂, Pd/C, MeOH.



Scheme 3. Synthesis of **4**. Reagents: (a) 4-aminobutyric acid, NaOH aq, THF; (b) HCl/NH₂OBz, EDC, HOBT, Et₃N, DMF; (c) H₂, Pd/C, MeOH.



Scheme 4. Synthesis of **6**. Reagents: (a) 4-methylamine, EDC, HOBT, Et₃N, DMF; (b) H₂, Pd/C, EtOAc; (c) HCl/NH₂OBz, EDC, HOBT, Et₃N, DMF; (d) 4 N HCl/dioxane; (e) Ac₂O, Et₃N; (f) H₂, Pd/C, MeOH.

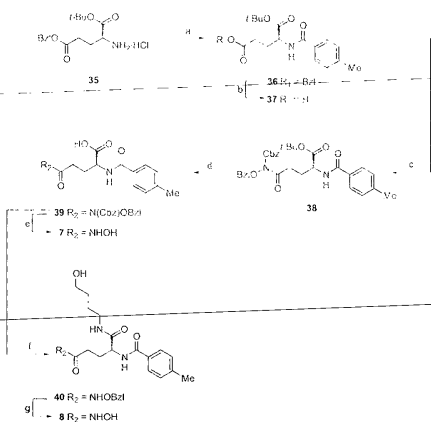
dride reduction, gave **42**, after which *O*-alkylation with ethoxycetylchloride provided **43**. Catalytic hydrogenation of **43**, followed by *N*-acylation with 4-methylbenzoyl chloride, afforded **44**, after which alkaline hydrolysis resulted in **45**. Condensation of **45** with *O*-(2-methoxypropane-2-yl)hydroxylamine followed by acidic deprotection produced **9**.

Synthesis of **10–13** is described in Scheme 7. *ε*-2-Alkylation of a 4-amino-*N*-benzoyl-butyric acid **28** with benzoyloxymethylchloride afforded **46**, condensation of which with *O*-benzyl hydroxylamine provided **47**. Partial hydrogenolysis of **47** gave **13**, which was converted to an alcohol **10** by treatment with boron tribromide. After optical resolution of **47** using a chiral HPLC column, the resulting two enantiomers were deprotected by catalytic hydrogenation to produce **11** and **12**, respectively.

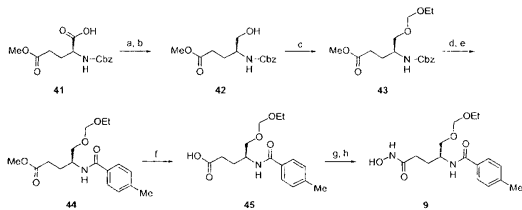
Compounds **14–18** were synthesized as described in Scheme 7. Condensation of carboxylic acids **48a–e** with 4-aminobutyric acid ethyl ester afforded **49a–e**, alkaline hydrolysis of which gave **50a–e**, respectively. Condensation of **50a–e** with *O*-(2-methoxypropane-2-yl)hydroxylamine, followed by acidic deprotection, produced **14–18**, respectively.

3. In silico generation of lead compounds

In order to design new MMP inhibitors, we selected the strategy of connecting two fragments by an appropriate linker. The first fragment was intended to interact with the catalytic Zn(H) ion. Based on information about known MMPis, we selected formic acid and *N*-formyl hydroxylamine as candidate fragments. The second fragment was expected to form hydrogen bonds around



Scheme 5. Synthesis of 7 and 8. Reagents: (a) 4-methylbenzoyl chloride, Pr_2NEt , CHCl_3 ; (b) H_2 , Pd/C, EtOAc; (c) 25, EDC, DMAP, DMF; (d) TFA; (e) H_2 , Pd/C, MeOH; (f) 4-amino-1-butanol, EDC, HOBT, Et₃N, DMF; (g) H_2 , Pd/C, MeOH.

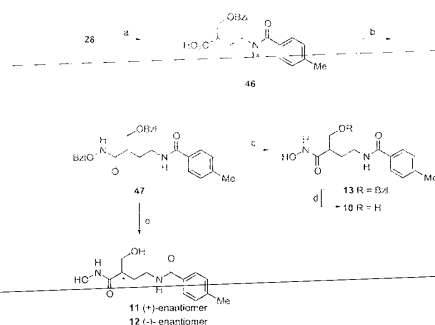


Scheme 6. Synthesis of 9. Reagents: (a) HONs, DCC, THF; (b) NaBH₄, THF; (c) EtOAc, Pd/C, EtOAc; (d) H_2 , Pd/C, MeOH; (e) 4-methylbenzoyl chloride, Et₃N, CHCl_3 ; (f) NaOH aq, MeOH; (g) *O*-(2-methoxypropyl)-2-ylhydroxylamine, EDC, HOBT, DMF; (h) HCl, MeOH.

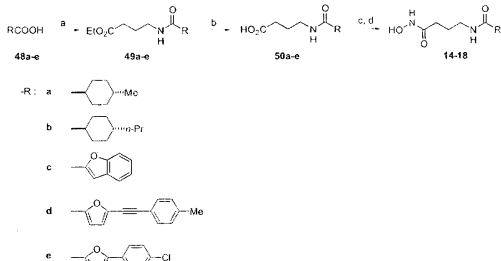
the S1' pocket and to be involved in the van der Waals interaction with the pocket. LUDI was used as a de novo program to detect fragments with the possibility of showing a second interaction with collagenase (MMP-1).⁵

From the X-ray crystallography and the well-established SAR data on inhibition of MMPs by numerous inhibitors, detailed three-dimensional structural information

is available about the binding sites of MMPs. Most MMPs bind to the same enzyme pocket and occupy common subregions. Around the S1' pocket, they all show a common interaction with Pro238 and Leu181. Each MMP1 donates a hydrogen bond to a proline residue (Pro238) and accepts a hydrogen bond from a leucine residue (Leu181). We reasoned that a novel inhibitor should have the ability to form these two key hydrogen bonds. It should also contain a lipophilic S1'



Scheme 7. Synthesis of 10-13. Reagents: (a) LDA, BuOCH_2Cl , HMPA, THF; (b) HCl/NH₄OH, EDC, HOBT, Pr_2NEt , DMF; (c) H_2 , Pd/C, MeOH; (d) BBr₃, CH_2Cl_2 ; (e) HPLC separation then, H_2 , Pd/C, MeOH.



Scheme 8. Synthesis of 14-18. Reagents: (a) 4-aminobutyric acid ethyl ester hydrochloride, EDC, HOBT, Et₃N, DMF; (b) NaOH aq, MeOH; (c) *O*-(2-methoxypropyl)-2-ylhydroxylamine, EDC, HOBT, DMF; (d) HCl, MeOH.

pocket to satisfy the steric and lipophilic requirements of the enzyme pocket.

A search of the standard LUDI library was carried out to identify candidate second fragments. This search resulted in a list of 83 small molecules. Although *N*-acetyl-L-alanine *N*-methylamide (Ac-L-Ala-NHMe) had a relatively low LUDI score, we tested it because of the ease of synthesis and the orientation of the α -methyl group. A docking study of Ac-L-Ala-NHMe with MMP-1⁵ suggested the formation of key hydrogen bonds with Pro238 and Leu181

(Fig. 1). As the methyl moiety of Ala was suggested to be directed towards the zinc site, we decided to connect this methyl group with the first fragment using a simple linker (methylene group). It was also thought that the methyl moiety of *N*-acetyl should be located in the lipophilic S1' pocket. However, this methyl group was too small to fill the cavity, so we replaced it with a 4-methylphenyl group. Based on the above-mentioned analysis, we designed the glutamic acid derivatives 2a,b and performed a docking study. As shown in Figure 2, compound 2b seemed to fit the active site of MMP-1.

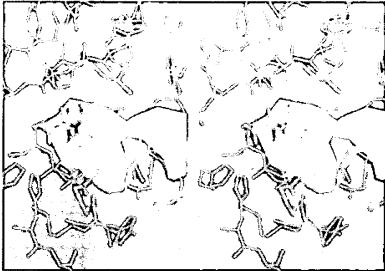


Figure 1. Docking study of Ac-L-Ala-NHMe 1 with MMP-1.

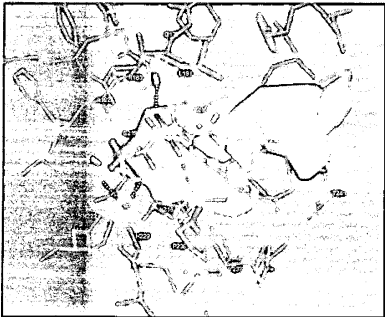


Figure 2. Docking study of 2b with MMP-1.

Based on the promising outcome of in silico simulation, compounds **2a,b** were synthesized, and their inhibition of MMP-1, -2, -9, and -3 was measured in vitro. The carboxylic acid **2a** did not show any significant inhibitory activity at 100 μ M, but the hydroxamic acid **2b** did demonstrated moderate to weak inhibitory activity (Table 1).

4. Structure–activity relationships

As shown in Table 1, the effect of chemical modification of the two amide moieties of **2b** on the activity profile was investigated in order to confirm the results of in silico modeling. The glutaric acid derivative **3** showed complete

loss of inhibitory activity, but the 4-aminobutyric acid derivative **4** still showed weak inhibition of MMP-1, MMP-2, and MMP-9. This result indicated that the benzamide moiety is more important for interaction with the target enzyme than the formamide moiety. Conversion of the 2*S*-configuration of **2b** to a 2*R*-configuration gave **5**, with loss of inhibitory activity against MMP-1, MMP-2, and MMP-3 at 100 μ M. This indicated that the 2*S*-configuration of **2b** plays a role in supporting the interaction with the target enzyme, while the 2*R*-configuration of **5** seems to disturb interaction with the enzyme. In order to confirm the importance of hydrogen bonds, a retro-inverse analogue of **2b** was synthesized and evaluated for

inhibition of MMP-1. **6**, **7**, and **8**, its analogue **6** did not inhibit the MMPs at 100 μ M at all. These results supported the concept that compound **2b** showed its activity through hydrogen bond interactions at the benzamide moiety (Chart 1).

Based on the above information, further optimization was conducted without chemical modification of the hydroxamate moiety. The docking study of **2b** with MMP-1 indicated that the *N*-methylformamide moiety might not interact with the enzyme and could be located in the aqueous environment. Accordingly, its replacement of this part with a hydrophilic substituent was investigated. As shown in Table 2, conversion to the carboxylic acid moiety gave **7**, which showed reduced MMP-2 inhibitory activity. Replacement of the methyl group with a 4-hydroxybutyl moiety resulted in **8**, with retention of the inhibitory activity against MMP-1 and MMP-2. In order to reduce the peptidic nature and maintain hydrophilicity, the *N*-methylformamide moiety was replaced with a 1-ethoxymethyl moiety. The resulting compound **9** showed an increase of inhibitory activity against MMP-1 and MMP-3.

Hydroxamate-containing compounds are known to be susceptible to hydrolysis to form the corresponding carboxylic acids in vivo. One promising approach to prevent such hydrolysis is the introduction of a substituent near the hydroxamate moiety, so the effect on the activity profile of incorporation of a hydroxymethyl group at the α -position of 4-aminobutyric acid analog **4** was investigated. Fortunately, introduction of a hydroxymethyl moiety at the α -position of **4** gave **10**, which showed an increase of inhibitory activity against MMP-1 and MMP-2. (+)-Isomer **11** showed more potent inhibitory activity against all of the tested isoforms than (–)-isomer **12**. Introduction of a benzyl-oxymethyl moiety at the γ -position of **4** afforded **13**, which showed a significant increase of inhibitory activity against all the MMP isoforms tested. As a result, the carbon next to hydroxamate was also found to be another site for further chemical modification as illustrated by **13**.

A docking study showed that the benzamide moiety of **2b** might be located in the S1' cavity of the enzyme, as shown in Figure 3. Although all MMPs have a similar environment around the active site, there are slight differences of the environment in the S1' pocket, for example, the S1' cavities of MMP-1, -3, and -9 are known to be deeper and narrower than that of MMP-13. To ascertain whether the benzamide moiety of **2b** was located in the S1' cavity, **14**, **18** were synthesized and their inhibitory activity was evaluated. Replacement of the 4-methylbenzoyl moiety of **4** with a *trans*-4-methylcyclohexanoyl moiety gave **14**, which showed increased activity against MMP-3 and reduced activity against MMP-2. Conversion of the methyl group of **14** to an *n*-propyl group afforded **15**, which showed a decrease of activity against MMP-1 and an increase of activity against MMP-2. Replacement of the 4-methylbenzoyl moiety of **4** with a benzotriazin-2-yl moiety provided **16**, with loss of inhibitory activity against MMP-1, -2, and -3, conversion to the 5-(4-methylphenylethynyl)furan-2-yl moiety or the 5-(4-chlorophenyl)furan-2-yl moiety resulted in **17** or **18** and both showed a significant increase of inhibitory activity against MMP-2 and MMP-3. A similar trend was observed in the case of substrate-based inhibitors.¹⁶ While the selective MMP-1 inhibitors had short and bulky substituents such as cyclohexyl or isobutyl groups at the P1' site, the selective MMP-2 or MMP-3 inhibitors had long substituents like biphenyl or 4-phenylbutyl groups at that site. These results strongly suggested that the benzamide moiety was located in the S1' cavity.

In summary, we succeeded in the generation of structurally new lead compounds that acted as MMPis using the in silico fragment approach. As shown in Figure 3, **2b** was designed by replacing the *N*-acetyl residue of compound **1** with a 4-methylbenzoyl residue, followed by connection of the methyl moiety of the Ala unit of compound **1** with an *N*-formylhydroxylamine by a simple linker, methylene. Compounds **2b**, **8**, and **11** inhibited MMP-2 selectively, while **9** and **14** inhibited MMP-1 selectively relative to the other MMP isoforms. Compound **13** inhibited all of the MMP isoforms although its inhibitory activity against MMP-1 and MMP-2 was

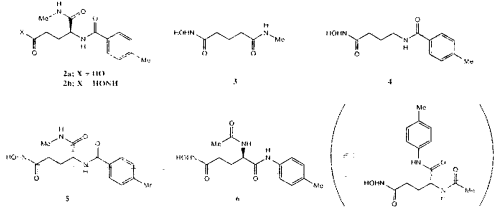


Chart 1. New chemical leads for MMPis

amine (34.8 mL, 200 mmol) and benzyl chloroformate (143 mL, 100 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 h and diluted with EtOAc. The resulting suspension was washed with 1 N HCl, and brine, and dried over MgSO₄. Removal of the volatiles in vacuo provided a white solid, which was triturated with *n*-hexane to give **25** (21.9 g, 85% yield) as a white powder: TLC *R_f* = 0.24 (EtOAc/*n*-hexane, 1:4); ¹H NMR (200 MHz, CDCl₃): δ 7.35 (m, 1H), 5.18 (s, 2H), 4.87 (s, 2H).

5.9. 5-[(benzyloxy)l(benzyloxy)carbonyl]amino-5-oxopentanoic acid (26a**) and benzyl *N*-[5-(methylamino)-5-oxopentanoyl]benzyloxycarbamate (**26b**)**

To a stirred solution of glutaric anhydride (1.26 g, 11.0 mmol) in acetonitrile (10 mL) were added **25** (2.57 g, 10.0 mmol) and 4-(dimethylamino)pyridine (12 mg, 1.0 mmol) at room temperature. The mixture was stirred for 40 h at room temperature. Glutaric anhydride (1.26 g, 11.0 mmol) and 4-(dimethylamino)pyridine (12 mg, 1.0 mmol) were added to it. The mixture was stirred for 3 h at room temperature, and concentrated under reduced pressure. The residue was acidified with 1 N HCl, and extracted with EtOAc. The extract was washed with brine, dried over MgSO₄, and filtrated. Concentration under reduced pressure gave a white powder, which was triturated with hexane to afford **26a** as a white powder (4.57 g): TLC *R_f* = 0.67 (CHCl₃/MeOH, 18:2:1). To a stirred mixture of **26a** (1.11 g, 2.99 mmol), methylaniline (40% in MeOH, 0.50 g, 5.98 mmol), *N,N*-(diisopropyl)ethylamine (1.56 mL, 8.97 mmol), and HOBt (686 mg, 4.48 mmol) in DMF (5 mL) was added EDCHCl (859 mg, 4.48 mmol) at 0 °C. The mixture was stirred for 64 h at an ambient temperature, and diluted with EtOAc. The suspension was washed with brine, 1 N HCl, brine, satd NaHCO₃, and brine, and dried over MgSO₄. Removal of the volatiles in vacuo provided a white powder, which was triturated with EtO to give **26b** (68 mg, 7% yield from **25**) as a colorless silky powder: ¹H NMR (CDCl₃, 200 MHz): δ 7.41 (m, 5H), 7.34 (m, 5H), 5.54 (m, 1H), 5.27 (s, 2H), 4.90 (s, 2H), 2.85 (t, *J* = 7.0 Hz, 2H), 2.80 (d, *J* = 5.2 Hz, 3H), 2.24 (t, *J* = 6.6 Hz, 2H), 2.02 (m, 2H).

5.10. *N*-Hydroxy-*N*'-methylpentanediamide (3**)**

The title compound was obtained as colorless crystals in 81% yield from **26** according to the analogous procedures as described for the preparation of **2b**. TLC *R_f* = 0.61 (EtOAc/AcOH/H₂O, 3:1:1); MS (APCI, neg. 40 V) *m/z*: 159 (M⁺); IR (KBr) 3224, 2942, 1646, 1562, 1459, 1413, 1291, 1165, 1092, 1071, 1023, 604, 439 cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆): δ 10.30, 9.30 (br, 1H), 9.30, 8.30 (m, 1H), 7.69 (m, 1H), 2.55 (d, *J* = 4.8 Hz, 3H), 2.05 (t, *J* = 7.3 Hz, 2H), 1.94 (t, *J* = 7.7 Hz, 2H), 1.70 (t, *J* = 7.7, 7.3 Hz, 2H); HRMS (FAB) calcd for C₄H₁₁N₂O₂: 161.0926. Found 161.0926.

5.11. 4-[(4-Methylbenzoyl)amino]butanoic acid (28**)**

To a stirred suspension of 4-aminobutyric acid (1.13 g, 11.0 mmol) in THF (21 mL) were added 1 N NaOH

(21 mL, 22 mmol) at 0 °C and 4-methylbenzoyl chloride **27** (1.25 g, 10.0 mmol). After starting at room temperature for 3 h, the reaction mixture was acidified with 2 N HCl and extracted with EtOAc. The organic layer was washed with brine and dried over MgSO₄. Removal of the volatiles in vacuo provided a solid, which was washed with diethyl ether to give **28** (1.30 g, 90% yield) as colorless crystals: TLC *R_f* = 0.58 (CHCl₃/MeOH, 18:2:1); ¹H NMR (200 MHz, CDCl₃): δ 7.73 (d, *J* = 8.4 Hz, 2H), 7.40 (m, 1H), 7.1 (d, *J* = 8.4 Hz, 2H), 3.47 (m, 2H), 2.41 (t, *J* = 7.1 Hz, 2H), 2.38 (s, 3H), 1.94 (m, 2H).

5.12. *N*-(4-(benzyloxy)amino)-4-oxobutyl-4-methylbenzamide (29**)**

The title compound was obtained as a white powder in 76% yield from **28** according to the analogous procedures as described for the preparation of **23a**: TLC *R_f* = 0.52 (CHCl₃/MeOH, 10:1); ¹H NMR (200 MHz, CDCl₃): δ 9.24 (s, 1H), 7.68 (td, *J* = 8.2 Hz, 2H), 7.37 (m, 3H), 7.23 (d, *J* = 8.2 Hz, 2H), 6.66 (m, 1H), 4.90 (s, 2H), 3.46 (dt, *J* = 6.0, 5.8 Hz, 2H), 2.39 (s, 3H), 2.17 (m, 2H), 1.92 (m, 2H).

5.13. *N*-(4-(hydroxyamino-4-oxobutyl)-4-methylbenzamide (4**)**

The title compound was obtained as colorless crystals in 85% yield from **29** according to the analogous procedures as described for the preparation of **2b**: TLC *R_f* = 0.23 (CHCl₃/MeOH, 10:1); MS (APCI, neg. 40 V) *m/z*: 235 (M⁺); IR (KBr) 1674, 1613, 1608, 1564, 1510, 1458, 1436, 1360, 1332, 1313, 1258, 1233, 1197, 1178, 1101, 1024, 969 cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆): δ 10.39 (s, 1H), 8.70 (s, 1H), 8.40 (t, *J* = 5.2 Hz, 1H), 7.74 (d, *J* = 8.1 Hz, 2H), 7.25 (d, *J* = 8.1 Hz, 2H), 3.24 (td, *J* = 6.6, 5.2 Hz, 2H), 2.35 (s, 3H), 2.02 (t, *J* = 7.7 Hz, 2H), 1.74 (m, 2H); HRMS (FAB) calcd for C₁₂H₁₇N₂O₃: 237.1239. Found 237.1244.

5.14. Benzyl *N*²-(*tert*-butoxycarbonyl)-*N*¹-(4-methylphenyl)-*n*-glutamate (30**)**

To a stirred solution of **19b** (6.75 g, 20.0 mmol) in DMF (20 mL) were added *p*-methylaniline (4.31 g, 30.0 mmol), triethylamine (8.36 mL, 60.0 mmol), 1-hydroxybenzotriazole monohydrate (3.67 g, 24.0 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (4.60 g, 24.0 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 16 h. The solution was diluted with EtOAc, and washed sequentially with 1 N HCl, saturated NaHCO₃, and brine. The organic layer was dried over MgSO₄. Removal of the volatiles in vacuo provided brown oil, which was recrystallized from EtOAc/*n*-hexane (1:4) to give **30** (8.14 g, 98% yield) as colorless crystals: TLC *R_f* = 0.54 (EtOAc/*n*-hexane, 2:3); ¹H NMR (200 MHz, CDCl₃): δ 8.30 (br s, 1H), 7.35 (m, 7H), 7.11 (d, *J* = 8.0 Hz, 2H), 5.38 (d, *J* = 8.0 Hz, 1H), 5.16 (d, *J* = 13.0 Hz, 1H), 5.13 (d, *J* = 13.0 Hz, 1H), 4.28 (m, 1H), 2.62 (m, 1H), 2.50 (m, 1H), 2.31 (s, 3H), 2.22 (m, 1H), 2.02 (m, 1H), 1.44 (s, 9H).

5.15. *N*²-(*tert*-Butoxycarbonyl)-*N*¹-(4-methylphenyl)-*n*-glutamine (31**)**

The title compound was obtained quantitatively as a colorless oil from **30** according to the analogous procedures as described for the preparation of **22**: TLC *R_f* = 0.57 (CHCl₃/MeOH/AcOH, 18:2:1).

5.16. *N*²-(*tert*-Butoxycarbonyl)-*N*²'-benzyloxy-*N*¹-(4-methylphenyl)-*n*-glutamate (32**)**

The title compound was obtained as a white powder in 98% yield from **31** according to the analogous procedures as described for the preparation of **23a**: TLC *R_f* = 0.41 (CHCl₃/MeOH, 10:1).

5.17. *N*²-Benzyloxy-*N*¹-(4-methylphenyl)-*n*-glutamate (33**)**

A solution of **32** (3.25 g, 7.56 mmol) in 4 N HCl in 1,4-dioxane (20 mL) was stirred at room temperature for 3 h. The reaction mixture was evaporated to give an oily residue, which was triturated with Et₂O to give **33** (2.74 g, 96% yield) as a white powder: TLC *R_f* = 0.24 (CHCl₃/MeOH/AcOH, 18:2:1).

5.18. *N*²-Acetyl-*N*²'-benzyloxy-*N*¹-(4-methylphenyl)-*n*-glutamate (34**)**

To a stirred solution of **33** (378 mg, 1.00 mmol) in CH₂Cl₂ (5 mL) were added *N,N*-(diisopropyl)ethylamine (0.35 mL, 2.2 mmol) and acetic anhydride (0.094 mL, 1.0 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 16 h. The mixture was concentrated under reduced pressure to give a powder, which was washed with H₂O, 1 N HCl, H₂O, saturated NaHCO₃, H₂O, and dried under reduced pressure. The obtained powder was dissolved in MeOH. The solution was evaporated to give a solid, which was triturated with Et₂O to give **34** (320 mg, 83% yield) as a white powder: TLC *R_f* = 0.51 (CHCl₃/MeOH, 10:1).

5.19. *N*²-Acetyl-*N*²'-hydroxy-*N*¹-(4-methylphenyl)-*n*-glutamate (6**)**

The title compound was obtained as a white powder in 89% yield from **34** according to the analogous procedures as described for the preparation of **2b**: TLC *R_f* = 0.13 (CHCl₃/MeOH, 10:1); MS (APCI, neg. 40 V) *m/z*: 292 (M⁺); IR (KBr) 3269, 2900, 1657, 1607, 1538, 1450, 1381, 1287, 1257, 1109, 1056, 1021, 981, 818, 797, 686, 610, 514 cm⁻¹; ¹H NMR (200 MHz, DMSO-*d*₆): δ 10.41 (s, 1H), 9.94 (s, 1H), 8.70 (s, 1H), 8.16 (d, *J* = 7.8 Hz, 1H), 7.48 (d, *J* = 8.4 Hz, 2H), 7.10 (d, *J* = 8.4 Hz, 2H), 4.37 (m, 1H), 2.25 (s, 3H), 2.00 (m, 2H), 1.87 (s, 3H), 1.95, 1.70 (m, 2H); optical rotation [α]_D²⁵ +18.2° (c 0.50, MeOH); HRMS (FAB) calcd for C₁₄H₁₅N₂O₄: 294.1454. Found: 294.1453.

5.20. 5-Benzyl 1-*tert*-butyl *N*-(4-methylbenzoyl)-*n*-glutamate (36**)**

To a stirred solution of **35** (10.0 g, 30.3 mmol) in CHCl₃ (50 mL) were added *N,N*-(diisopropyl)ethylamine

(11.6 mL, 66.7 mmol) and 4-methylbenzoyl chloride (443 mL, 33.4 mmol) at 0 °C. After stirring at room temperature for 17 h, saturated NaHCO₃ was added to the reaction mixture with vigorous stirring. After stirring for 30 min, the reaction mixture was then diluted with EtOAc and washed sequentially with brine, 1 N HCl, brine, saturated NaHCO₃, and brine. The organic layer was dried over MgSO₄. Removal of the volatiles in vacuo provided a solid, which was triturated with *n*-hexane to give **36** (11.6 g, quantitative yield) as a white powder: TLC *R_f* = 0.19 (EtOAc/*n*-hexane, 1:4).

5.21. 1-*tert*-Butyl *N*-(4-methylbenzoyl)-*n*-glutamate (37**)**

The title compound was obtained as colorless crystals from **36** in 76% yield according to the analogous procedures as described for the preparation of **22**: ¹H NMR (200 MHz, CDCl₃): δ 7.70 (d, *J* = 8.2 Hz, 2H), 7.22 (d, *J* = 8.2 Hz, 2H), 6.97 (d, *J* = 7.5 Hz, 1H), 4.71 (m, 1H), 2.47 (m, 2H), 2.38 (s, 3H), 2.28 (m, 1H), 2.05 (m, 1H), 1.49 (s, 9H).

5.22. *tert*-Butyl *N*²-benzyloxy-*N*¹-(benzyloxy)carbonyl]-*N*²-(4-methylbenzoyl)-*n*-glutamate (38**)**

To a stirred solution of **37** (643 mg, 2.00 mmol) in DMF (10 mL) were added **25** (515 mg, 2.00 mmol), 4-(dimethylamino)pyridine (24 mg, 0.20 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (422 mg, 2.20 mmol) at 0 °C. The mixture was stirred at room temperature for 16 h, and diluted with EtOAc. The suspension was washed with brine, 1 N HCl, brine, saturated NaHCO₃, brine, dried over MgSO₄. Removal of the volatiles in vacuo provided a colorless oil, which was purified by silica gel chromatography with EtOAc/*n*-hexane (3:7) to give **38** (992 mg, 88% yield) as a colorless syrup: TLC *R_f* = 0.27 (EtOAc/*n*-hexane, 3:7); ¹H NMR (200 MHz, CDCl₃): δ 7.72 (d, *J* = 8.0 Hz, 2H), 7.38 (m, 3H), 7.30 (m, 5H), 7.20 (d, *J* = 8.0 Hz, 2H), 6.89 (d, *J* = 7.8 Hz, 1H), 5.25 (s, 2H), 4.89 (d, *J* = 10.0 Hz, 1H), 4.85 (d, *J* = 10.0 Hz, 1H), 4.72 (m, 1H), 2.94 (m, 2H), 2.38 (s, 3H), 2.30 (m, 1H), 2.16 (m, 1H), 1.49 (s, 9H); optical rotation [α]_D²⁵ +6.84° (c 0.655, CHCl₃).

5.23. *N*²-Benzyloxy-*N*²'-[(benzyloxy)carbonyl]-*N*²-(4-methylbenzoyl)-*n*-glutamine (39**)**

A solution of **38** (980 mg, 1.75 mmol) in trifluoroacetic acid (5 mL) was stirred at room temperature for 3 h. The reaction mixture was evaporated to give a crude oil, which was purified by silica gel chromatography with AcOH/MeOH/CHCl₃ (5:10:85) to give a solid, which was triturated with diisopropylether to give **39** (495 mg, 56% yield) as a white powder: TLC *R_f* = 0.30 (CHCl₃/MeOH/AcOH, 18:2:1).

5.24. *N*²-Hydroxy-*N*²-(4-methylbenzoyl)-*n*-glutamine (7**)**

The title compound was obtained as a white powder in 95% yield from **39** according to the analogous procedures as described for the preparation of **2b**: TLC *R_f* = 0.43 (EtOAc/AcOH/H₂O, 8:1:1); MS (APCI, neg.

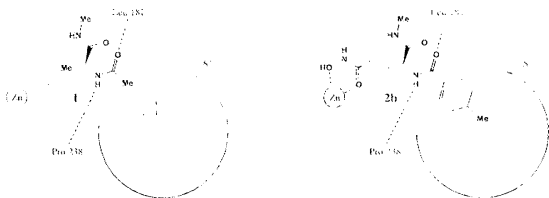


Figure 3. Molecular design of a L-glutamic acid analog 2b.

stronger than that against MMP-9 and MMP-3. These new L-glutamic acid or 4-aminobutyric acid-based structures should undergo diverse chemical modifications to identify MMP isoform-selective inhibitors because of cost-effectiveness and good functionality. Further optimization of these compounds will be reported in the near future.

5. Experimental

Analytical samples were homogeneous as confirmed by thin-layer chromatography (TLC), and yielded spectroscopic data consistent with the assigned structures. All ^1H NMR spectra were obtained with a Varian Gemini-200 or Mercury-300 spectrometer. The chemical shift values are reported in ppm (δ) and coupling constants (J) in hertz (Hz). Fast atom bombardment (FAB) and electron ionization (EI) mass spectra were obtained with a JEOL JMS-DX303HF or JMS-700 spectrometer. Atmospheric pressure chemical ionization (APCI) mass spectra were determined by a Hitachi M-1200H spectrometer. Matrix-assisted laser desorption/ionization (MALDI) mass spectra were obtained on a PerSeptive Biosystems VoyagerTM Elite spectrometer. IR spectra were measured using a Perkin-Elmer FTIR 1760X or JASCO FTIR-430 spectrometer. Column chromatography was carried out using silica gel [Merck silica gel 60 (0.063–0.200 mm), Wako Gel C200, Fuji Silysia FL60D, or Fuji Silysia BW-235]. TLC was also performed on silica gel (Merck TLC plate, silica gel 60 F254). The following abbreviations for solvents and reagents are used: THF, tetrahydrofuran; EtOAc, ethylacetate; MeOH, methanol; EtOH, ethanol; DMF, *N,N*-dimethylformamide; CH_2Cl_2 , dichloromethane; CHCl_3 , chloroform; EDC-HCl, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole; HMPA, hexamethylphosphoramide; TFA, trifluoroacetic acid; DMSO, dimethylsulfoxide; AcOH, acetic acid; Et₂O, diethyl ether; BuLi, butyllithium; FITC, fluorescein isothiocyanate; MOCAc, 7-methoxycoumarin-4-acetyl; Dpa, *N*-3-(2,4-dinitrophenyl)-2,3-diaminopropyl; Dnp, 2,4-dinitrophenyl.

5.1. *tert*-Butyl *N*²-[(benzyloxy)carbonyl]-*N*¹-methyl-L-glutamate (20a)

To a stirred solution of 5-*tert*-butyl *N*-carboxybenzoyl-L-glutamate (19a) (1.00 g, 2.97 mmol) in THF (6 mL) were added *N*-methylmorpholine (0.40 mL, 3.6 mmol) and isobutyl chloroformate (0.50 mL, 3.6 mmol) at -20°C . After stirring at -20°C for 15 min, excess amount of aqueous methylamine solution (40%) was added to the mixture, and then it was warmed to room temperature. The reaction mixture was diluted with EtOAc and washed with 1 N HCl, saturated NaHCO_3 , and brine. The organic layer was dried over MgSO_4 . Removal of the volatiles in vacuo provided a solid, which was purified by silica gel chromatography with MeOH/ CHCl_3 (3:97) to give the title compound 20a in quantitative yield as a white powder: TLC R_f = 0.73 (CHCl_3 /MeOH, 9:1); MS (APCI, pos. 20 V) m/z : 351 ($\text{M}+\text{H}^+$); ^1H NMR (200 MHz, CDCl_3): δ 7.38–7.31 (m, 5H), 6.24 (br s, 1H), 5.65 (m, 1H), 5.10 (s, 2H), 4.20–4.12 (m, 1H), 2.81 (d, J = 5.0 Hz, 3H), 2.49–2.24 (m, 2H), 2.17–1.85 (m, 2H), 1.44 (s, 9H).

5.2. *tert*-Butyl *N*¹-methyl-*N*²-(4-methylbenzoyl)-L-glutamate (21a)

Catalytic hydrogenation of 20a (1.18 g, 3.37 mmol) in MeOH (12 mL) was conducted at room temperature for 21 h in the presence of 5% palladium on carbon (50 mg) at atmospheric pressure. Removal of the catalyst by filtration followed by evaporation of the solvent afforded the amine in 98% yield as a white amorphous powder: TLC R_f = 0.29 (CHCl_3 /MeOH, 9:1). To a stirred solution of the above amine (500 mg, 2.31 mmol) in pyridine (3 mL) was added 4-methylbenzoyl chloride (0.30 mL, 2.8 mmol) at 0°C . After stirring at room temperature overnight, the reaction mixture was diluted with EtOAc. The resulting solution was washed with 1 N HCl, saturated NaHCO_3 , and brine, and dried over MgSO_4 . Removal of the volatiles in vacuo provided an oily residue, which was purified by silica gel chromatography with MeOH/ CHCl_3 (3:97) to give 21a (499 mg, 65% yield) as a white powder: TLC R_f = 0.46 (CHCl_3 /MeOH, 9:1); MS (APCI, pos. 20 V) 355 ($\text{M}+\text{H}^+$); ^1H NMR

(200 MHz, CDCl_3): δ 7.41 (d, J = 8.0 Hz, 2H), 7.37 (d, J = 6.6 Hz, 1H), 7.24 (d, J = 8.0 Hz, 2H), 6.61–6.55 (m, 3H), 4.39 (q, J = 6.6 Hz, 1H), 2.84 (d, J = 4.9 Hz, 3H), 2.65–2.08 (m, 4H), 2.40 (s, 3H), 1.43 (s, 9H).

5.3. Benzyl *N*²-(*tert*-butoxycarbonyl)-*N*¹-methyl-L-glutamate (20b) and benzyl *N*¹-methyl-*N*²-(4-methylbenzoyl)-L-glutamate (21b)

The amide 20b was obtained as a white powder in 80% yield from 5-benzyl *N*-(*tert*-butoxycarbonyl)-L-glutamate (19b) according to the analogous procedures as described for the preparation of 20a. Compound 20b: TLC R_f = 0.20 (EtOAc/ *n*-hexane, 1:1). A solution of 20b (3.34 g, 10.0 mmol) in 4 N HCl/dioxane (50 mL) was stirred at room temperature for 1 h. The reaction mixture was evaporated to give the amine hydrochloride quantitatively as a yellow oil. To a stirred solution of the above amine hydrochloride (10.0 mmol) in CH_2Cl_2 (50 mL) were added triethylamine (3.4 mL, 24 mmol) and 4-methylbenzoyl chloride (1.39 mL, 10.5 mmol) at 0°C . After stirring at room temperature overnight, the solution was washed with 1 N HCl, saturated NaHCO_3 , and brine, and dried over MgSO_4 . Removal of the volatiles in vacuo provided 21b (2.39 g, 65% yield) as a white powder: TLC R_f = 0.50 (CHCl_3 /MeOH, 30:1); MS (EI, pos.) m/z : 368 (M^+); ^1H NMR (200 MHz, CDCl_3): δ 7.90–7.70 (m, 2H), 7.40–7.10 (m, 3H), 6.60–6.30 (br, 1H), 5.12 (s, 2H), 4.80–4.50 (m, 2H), 2.81 (d, J = 4.8 Hz, 3H), 2.40 (s, 3H), 2.80–2.10 (m, 3H).

5.4. *N*¹-Methyl-*N*²-(4-methylbenzoyl)-L- α -glutamine (2a)

A solution of 21a (499 mg, 1.49 mmol) in a mixed solvent of TFA/ H_2O (3 mL/0.3 mL) was stirred at room temperature for 1 h. The reaction mixture was evaporated to give 2a (416 mg, 100% yield) as a white powder: TLC R_f = 0.40 (CHCl_3 /MeOH, 9:1); MS (FAB, pos.) m/z : 279 ($\text{M}+\text{H}^+$); IR (KBr) 3343, 2944, 1647, 1527, 1501, 1454, 1415, 1290, 1210, 946, 897, 834, 761, 653, 566, 534, 484 cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO}-d_6$): δ 12.13 (br s, 1H), 8.33 (d, J = 7.8 Hz, 2H), 7.86–7.78 (m, 3H), 7.26 (d, J = 8.1 Hz, 2H), 4.40–4.25 (m, 1H), 2.59 (d, J = 4.8 Hz, 3H), 2.36 (s, 3H), 2.30–2.23 (m, 2H), 2.05–1.82 (m, 2H); optical rotation $[\alpha]_D^{25}$ +5.65° (c 0.54, MeOH); HRMS (FAB) calcd for $\text{C}_{13}\text{H}_{19}\text{N}_3\text{O}_4$: 279.1345. Found: 279.1339.

5.5. *N*¹-Methyl-*N*²-(4-methylbenzoyl)-L- α -glutamine (22)

Catalytic hydrogenation of 21b (2.38 g, 6.46 mmol) in EtOH (65 mL) was conducted at room temperature for 1 h in the presence of 10% palladium on carbon (34 mg) at atmospheric pressure. Removal of the catalyst by filtration followed by evaporation of the solvent afforded 22 (1.63 g, 91% yield) as a white powder: TLC R_f = 0.20 (CHCl_3 /MeOH, 5:1); MS (FAB, pos.) 279 ($\text{M}+\text{H}^+$); IR (KBr) 3339, 3260, 1735, 1698, 1651, 1531, 1305, 1416, 1306, 1210, 947, 870, 835, 761, 692, 534 cm^{-1} ; ^1H NMR (200 MHz, CDCl_3 + CD_3OD): δ 7.73–7.60 (m, 2H), 7.22–7.14 (m, 2H), 4.60–4.52 (m,

1H), 2.36 (s, 3H), 2.36–2.30 (m, 2H), 2.34 (s, 3H), 2.22–1.83 (m, 4H).

5.6. *N*¹-(Benzyloxy)-*N*¹-methyl-*N*²-(4-methylbenzoyl)-L-glutamate (23a) and *N*¹-hydroxy-*N*¹-methyl-*N*²-(4-methylbenzoyl)-L-glutamate (2b)

To a stirred solution of 2a (257 mg, 0.924 mmol) in DMF (1 mL) were added *O*-benzylhydroxylamine hydrochloride (177 mg, 1.11 mmol), triethylamine (0.155 mL, 1.11 mmol), and 1-[3-(dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (213 mg, 1.11 mmol) at 0°C . After stirring at room temperature for 1 h, the reaction mixture was diluted with EtOAc. The resulting suspension was washed with 1 N HCl, saturated NaHCO_3 , and brine, and dried over MgSO_4 . Removal of the volatiles in vacuo provided 23a (205 mg, 58% yield) as a white powder. Compound 23a: TLC R_f = 0.44 (CHCl_3 /MeOH, 9:1). Catalytic hydrogenation of 23a (205 mg, 0.699 mmol) in a mixed solvent of MeOH/ CHCl_3 (2:1, 3 mL) was conducted at room temperature in the presence of 5% palladium on carbon (2 mg) at atmospheric pressure. Removal of the catalyst by filtration followed by evaporation of the solvent afforded a solid. The resulting solid was purified by silica gel chromatography with a gradient from MeOH/ CHCl_3 (3:97) to MeOH/ CHCl_3 (1:9) to give 2b (77 mg, 49% yield) as a white amorphous powder. Compound 2b: TLC R_f = 0.69 (CHCl_3 /MeOH, 4:1); MS (FAB, pos.) m/z : 294 ($\text{M}+\text{H}^+$); IR (KBr) 3267, 1636, 1538, 1189, 838, 753, 433 cm^{-1} ; ^1H NMR (200 MHz, $\text{DMSO}-d_6$): δ 10.37 (br s, 1H), 8.69 (m, 1H), 8.43 (d, J = 7.6 Hz, 1H), 7.85–7.79 (m, 3H), 7.25 (d, J = 8.0 Hz, 2H), 4.40–4.25 (m, 1H), 2.59 (d, J = 4.6 Hz, 3H), 2.06 (s, 3H), 2.10–1.85 (m, 4H); optical rotation $[\alpha]_D^{25}$ +7.96° (c 0.515, MeOH); HRMS (FAB) calcd for $\text{C}_{13}\text{H}_{20}\text{N}_3\text{O}_4$: 294.1454. Found: 294.1457.

5.7. *N*²-(Benzyloxy)-*N*¹-methyl-*N*²-(4-methylbenzoyl)-L-glutamate (23b) and *N*¹-hydroxy-*N*¹-methyl-*N*²-(4-methylbenzoyl)-L-glutamate (5)

The hydroxamate 23b was obtained as a white powder in 61% yield from 22 according to the analogous procedures as described for the preparation of 23a. Compound 23b: TLC R_f = 0.69 (CHCl_3 /MeOH, 5:1). The hydroxamic acid 5 was obtained as a white powder in 94% yield from 23b according to the analogous procedures as described for the preparation of 2b. Compound 2b: TLC R_f = 0.30 (CHCl_3 /MeOH, 10:1); MS (APCI, neg. 20 V) m/z : 292 ($\text{M}-\text{H}^-$); IR (KBr) 3322, 1652, 1615, 1537, 1506, 1449, 1413, 1384, 1302, 1257, 1184, 1075, 1031, 834, 752, 686, 600 cm^{-1} ; ^1H NMR (200 MHz, CD_3OD): δ 7.72 (d, J = 8.0 Hz, 2H), 7.20 (d, J = 8.0 Hz, 2H), 4.50–4.44 (m, 1H), 2.67 (s, 3H), 2.31 (s, 3H), 2.22–1.85 (m, 4H); optical rotation $[\alpha]_D^{25}$ -5.76° (c 0.545, MeOH); HRMS (FAB) calcd for $\text{C}_{13}\text{H}_{20}\text{N}_3\text{O}_4$: 294.1454. Found: 294.1456.

5.8. Benzyl benzyloxycarbamate (25)

To a stirred suspension of 24 (16.0 g, 190 mmol) in CH_2Cl_2 (100 mL) were added *N,N*-diisopropylethyl-

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Carboxypeptidase-Mediated Release of Methotrexate from Methotrexate α -Peptides¹

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ABSTRACT: Methotrexate (MTX) α -peptides containing representative neutral (alanine), acidic (aspartic acid), and basic (arginine) amino acids were synthesized by a regiospecific route. Purity and authenticity of MTX-Ala, MTX-Asp, and MTX-Arg were established by TLC, HPLC, elemental analysis, and NMR and absorbance spectra. These peptides were hydrolyzed by carboxypeptidases to yield MTX and the amino acids. Reactions were monitored by using a ninhydrin assay for the amino acids and HPLC and spectrophotometric assays for MTX. Pancreatic carboxypeptidase A (CP-A) hydrolyzed MTX-Ala and, at a much slower rate, MTX-Asp and MTX-Arg. MTX-Ala was also a substrate for pancreatic carboxypeptidase B (CP-B), marginal activity was observed with this enzyme and MTX-Arg. Human serum hydrolyzed only MTX-Arg; biphasic inhibition of this activity by 2-(mercaptomethyl)-3-(guanidinoethyl)thiopropionate was consistent with the known presence of two types of endogenous carboxypeptidase (CP-N). Cytotoxicity of the MTX peptides toward L1210 cells in culture was enhanced considerably in the presence of the appropriate carboxypeptidases. MTX-Ala was much less toxic than MTX (ID₅₀ values of 2.0×10^{-6} M and 2.4×10^{-8} M, respectively), but in the presence of CP-A the ID₅₀ of the peptide improved to 8.5×10^{-8} M. Similar results were obtained with MTX-Asp/CP-A and MTX-Ala/CP-B combinations. MTX-Arg showed good cytotoxicity (ID₅₀ of 5.0×10^{-8} M), due to CP-N activity in the fetal bovine serum of the culture medium; inclusion of CP-B lowered the ID₅₀ to that of MTX. Possible clinical uses of MTX peptides are discussed.

Methotrexate (MTX),¹ which is used extensively in cancer chemotherapy, enters eukaryotic cells via an active transport system whose primary substrate is 5-methyltetrahydrofolate [reviewed by Henderson (1986)]. After internalization, MTX is polyglutamylated (which enhances its retention in the cells), and the cytotoxicity of MTX polyglutamates results primarily from their potent inhibition of dihydrofolate reductase [reviewed by Huennekens et al. (1987)]. A membrane-associated folate-binding protein in L1210 mouse leukemia cells, which mediates the transport process, has been solubilized and partially characterized (Henderson & Zevely, 1984; Price & Freisheim, 1987). Substrate specificity of the transport system has been obtained from studies on the uptake or inhibitory activity of various folate compounds. It has been found, for example, that derivatization of the carboxyl groups via amide or ester formation greatly impairs internalization of the substrate; in this respect the α -carboxyl appears to be more critical than the γ -carboxyl (Sirotnak et al., 1979).

The above considerations suggest that MTX α -peptides (i.e., derivatives in which amino acids are joined via an amide linkage to the α -carboxyl of MTX) might have chemotherapeutic potential. These compounds would not be taken up readily by cells, but, if they could be hydrolyzed enzymatically, free MTX would be available for uptake and cell kill. Ideally,

it would be desirable for release of the drug to occur only in the vicinity of tumor cells, thereby sparing normal cells from concomitant destruction. This might be accomplished by linking the enzyme to a monoclonal antibody targeted to the tumor.

The alanine, aspartic acid, and arginine derivatives of MTX (MTX-Ala, MTX-Asp, and MTX-Arg), whose structures are shown in Figure 1, were selected as model peptides of MTX containing neutral, acidic, and basic amino acids. The present report describes the synthesis of these compounds via the general procedures developed by Rosowsky et al. (1981) and Piper et al. (1982), their susceptibility to hydrolysis by carboxypeptidases, and the cytotoxicity of the free drug, generated in this manner, for L1210 cells in culture.

EXPERIMENTAL PROCEDURES

Materials

The following were obtained from the indicated commercial sources: 2,4,5,6-tetraaminopyrimidine sulfate hydrate, dihydroxyacetone, *p*-(methylanino)benzoic acid, triphenylphosphine, diethylphosphite, isobutylchloroformate, Pd on charcoal, and Celite (Aldrich); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, *N*-Cbz-L-Glu γ -*tert*-butyl ester, L-Ala *tert*-butyl ester hydrochloride, L-Asp di-*tert*-butyl ester hydrochloride, L-Arg methyl ester dihydrochloride, hippuryl-Phe, hippuryl-Arg, ninhydrin, ninhydrin with acrosol

¹ Abbreviations: MTX, methotrexate; MTX-Ala, -Asp, and -Arg, peptides in which the indicated amino acids are linked covalently to the α -carboxyl of MTX; CP-A, -B, -N, and G₁, carboxypeptidase A, B, N, and G₁; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; ID₅₀, concentration producing 50% cell kill.

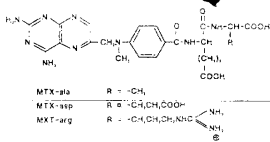


FIGURE 1: MTX α -peptides.

propellant, pancreatic CP-A (bovine; type I, 50 units/mg of protein), pancreatic CP-B (porcine; 300 units/mg of protein), trypsin, chymotrypsin, plasmin, subtilisin, bromelain, leucine aminopeptidase, pronase E from *Streptomyces griseus*, and pancreatic protease type I (Sigma); ficin and proteinase A from *Bacillus subtilis* (Serva); DL-2-(mercaptomethyl)-3-(guanidinoethyl)thiopropionate and urokinase (Calbiochem); DEAE-Trisacryl (BF Biochemicals); C₁₈ silica gel (J. T. Baker); and nylon filters (pore size 0.2 μ m, Nalgae). Carboxypeptidase G₁ isolated from *Pseudomonas* sp. strain RS-16 (Sherwood et al., 1985) was kindly provided by Dr. J. R. Bertino (Memorial Sloan Kettering Cancer Center). MTX and a cloned human dihydrofolate reductase were gifts from Dr. S. Kerwar (Lederle Laboratories) and Dr. J. Freisheim (Medical College of Ohio), respectively.

Diethyl phosphorocyanidate was prepared by the procedure of Rosowsky et al. (1981), except that the reaction mixture was stirred for 12 h after addition of CNBr. Human serum was obtained by centrifuging whole blood (obtained from the General Clinical Research Center of this institution) at 2500 rpm and 5 °C for 5 min. The supernatant was dialyzed against three changes of 0.85% NaCl and stored frozen. Just prior to use, the serum was diluted 1:4 with saline, made 0.5 mM in CoCl₂, and kept at 25 °C for 2 h. The activated serum was then centrifuged (3000 rpm, 10 min, 5 °C) and used immediately.

Synthesis of MTX Peptides

4-Amino-4-deoxy-10-methylptericoic Acid. 2,4,5,6-Tetraaminopyrimidine sulfate hydrate (35.0 g, 0.137 mol) was reacted with dihydroxyacetone (37.0 g, 0.203 mol) to produce 2,4-diamino-6-(hydroxymethyl)pteridine [according to the procedure of Rosowsky et al. (1985)]. Yield: 18.2 g (70%). TLC (system A) *R*_F = 0.70. Vigorous oxygenation was required during the condensation step to minimize formation of the 6-methylpteridine (*R*_F = 0.58) as a secondary product. Treatment of the 6-(hydroxymethyl)pteridine (12.25 g, 63.7 mmol) with Br₂ in the presence of triphenylphosphine provided the 6-bromo derivative. The latter, without isolation, was then reacted with *p*-(methylanino)benzoic acid in the presence of diisopropylethylamine to produce 4-amino-4-deoxy-10-methylptericoic acid (Rosowsky et al., 1985). The product, recovered by lyophilization, was obtained as the semihydrate. Yield: 21.8 g (95%). TLC (system B) *R*_F = 0.57. ¹H NMR (DMSO-*d*₆) δ 7.3 (s, 1 H, 7-H), 8.20 (broad, 2 H, NH₂), 7.78 (d, *J* = 9 Hz, 2 H, phenyl), 7.27 (broad, 2 H, NH₂), 6.87 (d, *J* = 9 Hz, 2 H, phenyl), 4.87 (s, 2 H, CH₂), 3.23 ppm (s, 3 H, NCH₃).

***N*-Carboxbenzoxy-L-glutamic Acid α -Methyl γ -*tert*-Butyl Diester Hydrochloride.** *N*-Cbz-Glu γ -*tert*-butyl ester (16.85 g, 50 mmol) was converted to the α -methyl γ -*tert*-butyl diester by treatment with CH₃I, using the procedure of Rosowsky et al. (1981). Yield (17.2 g) (97%). An aliquot (8.68 g, 24.7 mmol) of this intermediate (yellow oil) was used without

further purification. Cbz was removed by hydrogenation (10 psi, 12 h, Pd on charcoal), using the method of Klieger and Gibian (1962). After filtration, the solution (at room temperature) was evaporated to dryness under vacuum. The residue was dissolved in 30 mL of CH₃OH/ethyl acetate/ether (1:4:1), and the solution was filtered and cooled to -20 °C. The pH was adjusted to 6.0 with a saturated solution of HCl in absolute ether, and after 1 h, the precipitate was collected by filtration, washed with absolute ether (-20 °C), and dried under vacuum. TLC (system D) *R*_F = 0.77. The filtrate was cooled again to -20 °C, the pH adjusted to 5.3 with HCl-ether, and additional precipitate was recovered by the above procedure. The combined product (disaster hydrochloride), a white, amorphous powder, was obtained in a yield of 6.3 g (80%). (Rosowsky et al. (1981) reported a comparable yield of the free base obtained as an oil, mp 124-125 °C [Klieger and Gibian (1962), 125-126 °C].

γ -*tert*-Butyl *N*-(4-Amino-4-deoxy-10-methylpteroyl)-L-glutamate (MTX γ -*tert*-Butyl Ester). The procedure of Rosowsky et al. (1981) was modified in the following manner. 4-Amino-4-deoxy-10-methylptericoic acid (4.96 g, 14.8 mmol, containing 0.5 equiv of H₂O) was added to a stirred solution of diethyl phosphorocyanidate (3.62 g, 22.2 mmol) and triethylamine (3.10 mL, 22.2 mmol) in 500 mL of absolute DMF. After the mixture was stirred at room temperature for 3 h, Glu α -methyl γ -*tert*-butyl diester hydrochloride (3.76 g, 14.8 mmol) and triethylamine (4.1 mL, 29.6 mmol) were added and stirring was continued for 72 h. The solvent was removed in vacuum (at 45 °C), and the residue was dissolved in 800 mL of CHCl₃. The solution was extracted twice with 500 mL of 1% NH₄OH (allowing time for the slow separation of emulsions), and the organic fraction was dried over Na₂SO₄. Removal of the solvent in vacuum yielded a brown oil (8.0 g). The latter was dissolved in CH₂Cl₂/acetone/CH₃OH (7:7:1) and chromatographed on silica gel. Appropriate fractions were pooled and evaporated to dryness to obtain a yellow, amorphous powder. Yield: 5.66 g (73%). TLC (system E) *R*_F = 0.46. ¹H NMR (DMSO-*d*₆) δ 8.60 (s, 1 H, 7-H), 8.28 (m, 1 H, CONH), 7.75 (d, 2 H, *J* = 9 Hz, phenyl), 7.6-7.4 (m, 2 H, NH₂), 6.83 (d, 2 H, *J* = 9 Hz, phenyl), 6.59 (broad, 2 H, NH₂), 4.79 (broad, 2 H, ArCH₂N), 4.6-4.2 (m, 1 H, α -CH), 3.63 (s, 3 H, COOCH₃), 3.20 (s, 3 H, NCH₃), 2.4-1.8 (m, 4 H, CH₂CH₃), 1.33 ppm (s, 9 H, COO-*t*-Bu).

The α -methyl group in the above product was removed by hydrolysis in base with the following modification (which provides a granular, rather than gelatinous, product) of the procedure of Rosowsky et al. (1981). (A) Ba(OH)₂·8H₂O (1.57 g, 5 mmol) was added to 40 mL of H₂O, and (B) MTX α -methyl γ -*tert*-butyl ester (2.55 g, 5 mmol) was added to 40 mL of CH₃OH. (A) and (B) were each heated to 50 °C (with stirring) for 15 min and then cooled to room temperature. The two solutions ((A) still slightly turbid) were combined and stirred at room temperature. After 30 min, examination by TLC (system E) indicated that hydrolysis had been completed. Na₂SO₄ (780 mg), dissolved in 5 mL of warm H₂O, was added to the solution, and after 5 min, the fine precipitate of BaSO₄ was removed by filtration through Celite. The precipitate was washed with a minimal amount of CH₃OH, and the pH of the filtrate was adjusted to 4.5 with 10% acetic acid. After standing for 12 h at -30 °C, the mixture was filtered through sintered glass to collect the precipitate. The latter was washed with C₂H₅OH and ether, dried in air, and then dried in high vacuum over P₂O₅. The product, MTX γ -*tert*-butyl ester, was obtained as a yellow, amorphous powder. Yield: 2.3 g (90%). TLC (system C) *R*_F = 0.45. ¹H NMR (DMSO-*d*₆) δ 8.60 (s,

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1 H, 7-H), 8.7-6.9 (m, 1 H, CONH), 7.0 (d, 2 H, *J* = 9 Hz, phenyl), 7.2-7.46 (m, 2 H, NH), 6.90 (d, 2 H, *J* = 9 Hz, phenyl), 6.59 (broad, 2 H, NH), 4.78 (m, 2 H, ArCH₂N), 4.6-4.2 (m, 1 H, α -CH), 3.7 (s, 3 H, NCH₃), 2.4-1.7 (m, 4 H, CH₂CH₂), 1.3 ppm (s, 9 H, COO-*t*-Bu).

[N-(4-Amino-4-deoxy-10-methylperoyl)- α -glutamyl]-L-arginine Dihydrate (MTX- α -arginine). MTX- γ -*tert*-butyl ester (1.28 g, 2.5 mmol) was dissolved in 30 mL of absolute DMF. Triethylamine (528 mg, 3.76 μ L, 5.22 mmol) and isobutylchloroformate (360 mg, 342 μ L, 2.64 mmol) were added, and the mixture was stirred (with exclusion of moisture) at room temperature for 15 min; the precipitate of triethylammonium chloride was not removed. *Ala*-*tert*-butyl ester hydrochloride (480 mg, 2.64 mmol) was added, and the mixture was stirred at room temperature for 6 h. Examination by TLC (system C) indicated that all of the MTX-*tert*-butyl ester had reacted. After the mixture was cooled to 4 °C, the precipitate was removed by filtration and washed with dry DMF. The combined filtrates were concentrated under high vacuum (temperature below 40 °C), and the yellow oil was suspended in acetone. The mixture was stirred for 6 h and cooled to 4 °C, and the precipitate was recovered by filtration. The material was suspended in 10 mL of CH₃OH, stirred overnight at room temperature, recovered by filtration, washed with CH₃OH, and dried under vacuum. From the filtrate, additional material was obtained by evaporating the solvent, adding a small amount of CH₃OH, and repeating the procedure. The product, the di-*tert*-butyl ester of MTX- α -Ala, was obtained as a bright yellow, amorphous powder. Yield: 829 mg (52%). TLC (system C) *R_f* = 0.76; (system F) *R_f* = 0.33. Anal. for C₃₃H₄₃N₅O₉ (525.2): calcd C 58.38, H 6.80, N 19.77; found C 57.49, H 6.83, N 19.15. ¹H NMR (DMSO-*d*₆) 8.60 (s, 1 H, 7-H), 8.20 (m, 1 H, NH), 7.97 (m, 1 H, NH), 7.77 (d, 2 H, *J* = 9 Hz, phenyl), 7.7-7.4 (broad, 2 H, NH₂), 6.82 (d, 2 H, *J* = 9 Hz, phenyl), 6.57 (broad, 2 H, NH₂), 4.79 (s, broad, 2 H, ArCH₂N), 4.5-4.13 (m, 1 H, α -CH), 4.1-3.76 (m, 1 H, α -CH), 3.20 (s, 3 H, NCH₃), 2.5-1.7 (m, 4 H, CH₂CH₂), 1.37 (s, 18 H, *t*-Bu), 1.21 ppm (d, 3 H, *J* = 7 Hz, CH₃).

The di-*tert*-butyl ester of MTX- α -Ala (959 mg, 1.5 mmol) was suspended in 15 mL of THF, and 30 mL of 2.5 N HCl was added. The solution was heated to 55 °C for 5 h, cooled to room temperature, and neutralized with 5 N NaOH. THF was removed by evaporation, the solution was adjusted to pH 4.5 with HCl, and the yellow precipitate containing a portion of product was recovered by filtration. The precipitate was washed with H₂O and saved. The combined filtrates were desalted by using C₁₈ reversed-phase silica gel that had been prepared in the following manner: C₁₈ silica gel (15 mL) was placed on a sintered glass funnel (3.5 cm in diameter) and washed with 100 mL of CH₃CN/water (1:1), followed by 200 mL of 10⁻⁴ N HCl. The combined filtrates were applied to the gel in 20-mL portions. The colorless effluent was discarded, and the gel was washed with 40 mL of 10⁻⁴ N HCl. If the filtrate was yellow, it was acidified to pH 4, reappplied to the gel, and washed with an additional 40 mL of dilute acid. The product was eluted with 75% CH₃OH until the gel was almost colorless. (Silica gel can be regenerated by washing it alternatively with 25% CH₃OH and CH₃OH and then drying it in air.) The eluate was evaporated at 25 °C under high vacuum (dry ice-acetone trap), and the remaining H₂O was removed by brief lyophilization; extended lyophilization caused loss of product due to sublimation. The dry material was combined with the previous precipitate and suspended in 3 mL of H₂O. NaOH (1 N) was added dropwise (with

shaking and gentle heating) until the oil dissolved, excess base unpaired the chromatographic space as described below. The solution was applied to DEAE-Trisacryl (2 $\times$ 15 cm), washed previously with 20 volumes of 5% NH₄HCO₃ and 40 volumes of H₂O). The column was washed with 20 mL of H₂O and eluted with a linear gradient (H₂O $\rightarrow$ 2% NH₄HCO₃ 1 L, elution flow rate 0.5 mL/min). Fractions (15 mL) were examined for product by HPLC (see Experimental Procedures). Appropriate fractions were combined, the pH was adjusted to pH 4.5 (because of the direction, no precipitate appeared), and the sample was desalted and dried as described above. The product, MTX- α -Ala, was a yellow, amorphous powder. Yield: 381 mg (69%). TLC (system G) *R_f* = 0.33. Anal. for C₂₃H₂₉N₅O₇ (461.55): calcd C 49.19, H 5.56, N 22.45; found C 49.61, H 5.78, N 22.32. ¹H NMR (DMSO-*d*₆) 8.31 (s, 1 H, 7-H), 7.92-7.70 (m, 2 H, NH₂), 7.44 (d, 2 H, *J* = 9 Hz, phenyl), 7.4-7.1 (m, 2 H, 2 $\times$ NH), 6.56 (d, 2 H, *J* = 9 Hz, phenyl), 6.37 (broad, 2 H, NH₂), 4.53 (m, 2 H, ArCH₂N), 4.31-3.99 (m, 1 H, α -CH), 3.86 (m, 1 H, α -CH), 2.93 (s, 3 H, NCH₃), 2.15-1.87 (m, 2 H, CH₂), 1.57-1.49 (m, 2 H, CH₂), 0.98 ppm (m, 3 H, *J* = 7 Hz, CH₃).

[N-(4-Amino-4-deoxy-10-methylperoyl)- α -glutamyl]-L-aspartic Acid Dihydrate (MTX- α -aspartic Acid). MTX- γ -*tert*-butyl ester (1.28 g, 2.5 mmol) was reacted with Asp di-*tert*-butyl ester hydrochloride (744 mg, 2.64 mmol) via the procedure described above for the synthesis of MTX- α -Ala. The crude product (tri-*tert*-butyl ester of MTX- α -Asp), obtained as an oil, was dissolved in CH₂Cl₂/CH₃OH (10:1) and chromatographed on silica gel (2.5 $\times$ 20 cm; 230-400 mesh) using the above solvent for elution. Fractions (10 mL) were examined for product by TLC (see below). Appropriate fractions were evaporated, and the oil was taken up in 15 mL of CH₃OH and stirred for 12 h. The precipitate was recovered by filtration and dried under vacuum. From the filtrate, additional material could be obtained by repeating the precipitation procedure. The product, tri-*tert*-butyl ester of MTX- α -Asp, was obtained as a bright yellow, amorphous powder. Yield: 1.02 g (55.3%). TLC (system C) *R_f* = 0.9; (system F) *R_f* = 0.52. Anal. for C₃₄H₄₃N₅O₉ (737.86): calcd C 58.60, H 6.97, N 17.09; found C 58.53, H 6.76, N 16.96. ¹H NMR (CDCl₃) 8.64 (s, 1 H, 7-H), 7.94-7.63 (m, 4 H, NH₂, phenyl), 7.38-7.00 (m, 2 H, 2 $\times$ NH), 6.63 (d, 2 H, *J* = 8 Hz, phenyl), 5.55-5.27 (m, 2 H, NH₂), 4.84-4.50 (m, 4 H, ArCH₂N, 2 $\times$ α -CH), 3.19 (s, 3 H, NCH₃), 2.90-2.69 (m, 2 H, CH₂), 2.58-2.30 (m, 2 H, CH₂), 2.26-1.99 (m, 2 H, CH₂), 1.36 ppm (s, 27 H, 3 $\times$ COO-*t*-Bu).

To remove the blocking groups, the tri-*tert*-butyl ester of MTX- α -Asp (1.02 g, 1.38 mmol) was subjected to acid hydrolysis, as described for MTX- α -Ala. The product, MTX- α -Asp, was obtained as a yellow, amorphous powder. Yield: 164 mg (21%). TLC (system G) *R_f* = 0.18. Anal. for C₁₉H₂₃N₅O₇ (405.6): calcd C 47.60, H 5.16, N 20.82; found C 47.16, H 5.41, N 21.11. ¹H NMR (DMSO-*d*₆) 8.35 (s, 1 H, 7-H), 8.0-7.6 (m, 2 H, NH₂), 7.50 (d, 2 H, *J* = 8 Hz, phenyl), 7.4-7.1 (m, 2 H, 2 $\times$ NH), 6.57 (d, 2 H, *J* = 8 Hz, phenyl), 6.40 (broad, 2 H, NH₂), 4.57 (s, broad, 2 H, ArCH₂N), 4.33-3.91 (m, 2 H, 2 $\times$ α -CH), 2.98 (s, 3 H, NCH₃), 2.2-1.5 ppm (2 m, 6 H, 3 $\times$ CH₃).

[N-(4-Amino-4-deoxy-10-methylperoyl)- α -glutamyl]-L-arginine Dihydrate (MTX- α -arginine). MTX- γ -*tert*-butyl ester (1.02 g, 2.0 mmol), triethylamine (616 μ L, 4.4 mmol), and isobutylchloroformate (304 mg, 288 μ L, 2.24 mmol) were dissolved in 12 mL of absolute DMF, and the solution was stirred for 15 min at room temperature. Arg methyl ester hydrochloride (584 mg, 2.24 mmol) was added, and the so-

lution was stirred at room temperature for 4 h, cooled to 4 °C, and filtered. Three volumes of ethyl acetate were added to the filtrate, and after 12 h at -20 °C, the yellow supernatant was discarded, and the oil was suspended in 20 mL of acetone; 6 mL of CH₃OH (3:1) and stirred vigorously for 6 h. The precipitate was filtered and dried under vacuum. The product, the γ -*tert*-butyl (Glu) α -methyl (Arg) diester of MTX- α -Arg-HCl, was obtained as an orange-yellow, amorphous powder; it was used, without further purification, as described below. Yield: 1.35 g (94%). TLC (system B) *R_f* = 0.25. ¹H NMR (DMSO-*d*₆) 8.29 (s, 1 H, 7-H), 8.20-8.00 (m, 1 H, NH), 7.90-7.70 (m, 1 H, NH), 7.59-7.10 (m, 5 H, NH, NH₂, phenyl), 6.96-6.66 (m, 3 H, NH, NH₂), 6.66-6.47 (m, 2 H, phenyl), 6.47-6.29 (m, 2 H, NH₂), 4.54 (broad, 2 H, ArCH₂N), 4.30-3.86 (m, 2 H, 2 $\times$ α -CH), 3.38 (s, 3 H, COOCH₃), 2.97 (s, 3 H, NCH₃), 2.16-1.10 (m, 10 H, 5 $\times$ CH₂), 1.10 ppm (s, 9 H, COO-*t*-Bu).

The diester of MTX- α -Arg was first treated to remove the methyl blocking group. The material (1.35 g, 1.88 mmol) was dissolved in 30 mL of CH₃OH by vigorous stirring at room temperature. Ba(OH)₂·8H₂O (2.64 g, 8.36 mmol) was suspended in 120 mL of H₂O, and the mixture was stirred at 50 °C for 10 min. The two solutions were combined and stirred at room temperature for 60 h. An additional amount (593 mg, 1.88 mmol) of Ba(OH)₂ in 50 mL of warm H₂O (50 °C) was added, and stirring was resumed for another 5 h, or until TLC (system B) indicated complete consumption of the starting material. To hydrolyze the *tert*-butyl group, the solution was then adjusted to pH 1 with 5 N HCl and stirred for 30 min. TLC (system G) verified that both blocking groups had been removed.

Further purification of the product was accomplished via the following procedure. To the above solution, Na₂SO₄ (1.60 g, 11.3 mmol), dissolved in a minimal amount of H₂O, was added, and after 10 min at room temperature, the precipitate was removed by filtration. The filtrate was adjusted to pH 7, and the solution was passed through a nylon filter to remove additional BaSO₄. The pH was adjusted to 4.5 with 1 N HCl, which caused some precipitation of product; this was recovered by filtration, washed with H₂O, and saved. The combined filtrates were desalted over C₁₈ reversed-phase silica gel, as described above for MTX- α -Ala. The product was eluted from the gel with 75% CH₃OH and recovered as a solid after removal of solvent. The dry material was combined with the previous precipitate and suspended in 4 mL of H₂O. NaOH (1 N) was added dropwise until the solid dissolved (see precaution regarding this step in synthesis of MTX- α -Ala). By use of the procedure described previously for the preparation of MTX- α -Ala, the solution was chromatographed on DEAE-Trisacryl (2.5 $\times$ 20 cm), and effluent fractions were monitored by HPLC (system B). In addition to removing impurities, this step also partially separated the D,L- and L,L-diastereomers (see Discussion) of MTX-Arg. Early, intermediate, and late fractions contained the D,L form, the D,L,L mixture, and the L,L form, respectively. Appropriate fractions were combined, and the solutions were desalted over silica gel and taken to dryness (CH₃OH removed by high vacuum; H₂O removed by brief lyophilization) as described previously for MTX- α -Ala. The products were obtained as yellow, amorphous powders. Total yield: 435 mg (34.1%). The more abundant D,L material was used for characterization of the product. TLC (system G) *R_f* = 0.19. Anal. for C₂₀H₂₄CIN₅O₇·2H₂O (683.1): calcd C 45.71, H 5.76, N 24.60; found C 46.39, H 5.47, N 24.32. ¹H NMR (DMSO-*d*₆) 9.4-9.1 (m, 1 H, NH), 9.1-8.7 (m, 2 H, 2 $\times$ NH), 8.6-8.3 (m, 2 H, NH, 7-H),

7.86-7.30 (m, 6 H, 2 $\times$ NH, NH₂, phenyl), 6.94-6.33 (m, 4 H, NH₂, phenyl), 4.77 (broad, 2 H, ArCH₂N), 4.49-4.18 (m, 1 H, α -CH), 3.96-3.70 (m, 1 H, α -CH), 3.44 (s, 3 H, NCH₃), 2.4-1.1 ppm (m, 10 H, 5 $\times$ CH₂).

Methods

Assay Procedures for *In vitro* Hydrolysis of MTX Peptides. (A) **TLC.** MTX peptides (ca. 1 mM) were treated with various proteolytic enzymes. In each instance, the pH and buffer composition were optimal for the enzyme used, and the amount of enzyme was ca. 100-fold greater than that required for complete hydrolysis of a standard substrate. Separate controls (enzyme omitted; substrate omitted) were utilized. Mixtures were incubated at 25-40 °C for 24 h. At various time intervals, aliquots were removed and examined by TLC (system G). *R_f* values: MTX- α -Ala (0.33); MTX-Asp (0.18); MTX-Arg (0.19); MTX (0.34); Ala (0.26); Asp (0.12); and Arg (0.10).

(B) **Ninhydrin.** Hydrolysis of MTX- α -Ala, catalyzed by CP-A, was measured by the following procedure. CP-A (35 μ L, 105 units/mL in 9% LiCl) was added to MTX- α -Ala (1.2 mL, 1.0 mM in 0.025 M Tris-0.5 M NaCl, pH 7.5), and the mixture was incubated at 25 °C. At various time intervals, 50 μ L aliquots were removed and analyzed for Ala according to the ninhydrin method described by Spies (1957). Absorbances at 570 nm were determined by using a Hewlett-Packard spectrophotometer (Model 8450A) and corrected for the zero-time control (enzyme added to substrate, followed immediately by ninhydrin and subsequent processing). Concentrations of the product (Ala) were calculated from a standard curve, and the results are averages of duplicate determinations. One unit of CP-A will hydrolyze 1.0 μ mol of hippuricphenylalanine/min at pH 7.5 and 25 °C.

(C) **Spectrophotometric.** Hydrolysis of MTX- α -Ala by CP-A was measured by the following procedure. MTX- α -Ala (50 μ L, 1.0 mM) and Tris buffer (440 μ L, 0.1 M, pH 7.3, containing 0.2 mM ZnSO₄) were added to a 1-cm quartz cuvette. *A*₃₂₀ of the solution was determined by using a Gilford spectrophotometer (Model 252), and after 5 min, 5 μ L of a solution of CP-G₂ (9 units/mL) was added. At 15 min, 6 μ L of a solution of CP-A (31.4 units/mL) was added, and *A*₃₂₀ was recorded as a function of time. The initial rate was determined from the linear portion (first 5 min) of the curve. A value of $\epsilon_{320} = 8.3$ (Levy & Goldman, 1967) was used to calculate the concentration change for the overall reaction (MTX peptide $\rightarrow$ 4-amino-4-deoxy-10-methylperate). One unit of CP-G₂ will hydrolyze 1 μ mol of MTX/min at pH 7.3 and 30 °C (McCallough et al., 1971).

(D) **HPLC.** Hydrolysis of MTX- α -Ala by CP-A was measured by the following procedure. The mixture for the spectrophotometric assay (see above) was used, except that 0.64 unit of CP-A was present and CP-G₂ was omitted. Reactions in identical samples (at 37 °C) were initiated by addition of CP-A. At various time intervals, one sample was placed in ice to stop the reaction. The samples were heated (90 °C, 1 min) and centrifuged (1 min; Eppendorf microcentrifuge, Model 3414). Aliquots (20 μ L) of the supernatants were analyzed by HPLC (system A).

Cytotoxicity of MTX Peptides. These measurements were performed by using a microculture technique and colorimetric assay, adapted from Moemann (1983) and Finlay et al. (1986). Cytotoxicity of MTX- α -Ala, activated by CP-A, was measured by the following procedure. Late-log 1,1210 cells were suspended (10⁵ cells/135 μ L) in RPMI 1640 medium containing 5% dialyzed fetal bovine serum, 200 units/mL penicillin, and 200 μ g/mL streptomycin. Using a multichannel pipettor, 135

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