

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO  
DIRECCIÓN DE NUEVAS CREACIONES

2020  
Bogotá, D.C.,

Abogado  
ALVARO CORREA ORDOÑEZ  
office.bogota@bakermckenzie.com

ASUNTO      Radicación      08 001 924  
                 Trámite          011  
                 Evento          379  
                 Actuación        519  
                 Oficio  
                 Folios                19

E 150251

Patente de Invención ☒      Patente de Modelo de Utilidad ☐  
Via Nacional ☐  
Via 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

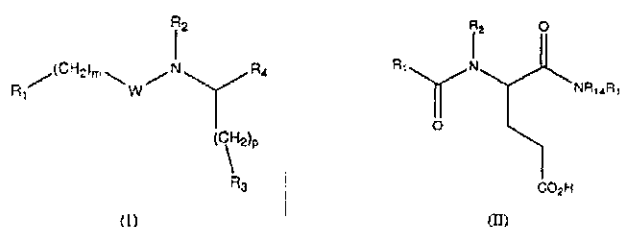
Se acepta la reivindicación de prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

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 agreanasa.

##### 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 R<sub>1</sub>, R<sub>3</sub>, R<sub>5</sub>, R<sub>6</sub>, R<sub>7</sub>, R<sub>8</sub>, R<sub>9</sub>, R<sub>10</sub>, R<sub>11</sub>, R<sub>12</sub>, R<sub>13</sub>, R<sub>14</sub> y R<sub>15</sub> 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 <sub>1</sub>  | Fenilo, bifenilo, 9H-fluoreno, 9-oxo-9H-fluoreno, tienil, piridin, furoil,                                                                                                                                                                                                                                                                                                                                      |
| R <sub>2</sub>  | H                                                                                                                                                                                                                                                                                                                                                                                                               |
| R <sub>3</sub>  | -CO <sub>2</sub> H                                                                                                                                                                                                                                                                                                                                                                                              |
| R <sub>4</sub>  | -CO <sub>2</sub> Et, -CO <sub>2</sub> H, -CONH <sub>2</sub> , -CONR <sub>9</sub> R <sub>10</sub> , -CH <sub>2</sub> -O-                                                                                                                                                                                                                                                                                         |
| R <sub>6</sub>  | AlquilC <sub>1-2</sub> , alquenilC <sub>2-3</sub> , alquiniilC <sub>2</sub> , cicloalquilC <sub>3-6</sub> , fenilo, -CH <sub>2</sub> -O-, -C(O)-, -O-alquilC <sub>1-4</sub> , -O-arilC <sub>6</sub> , -O-alquilC <sub>1</sub> -arilC <sub>6</sub> , alquilC <sub>1</sub> -O-, -NH-, furil, benzodioxol, piridin, tiazolil, tienil, dihidrobenzofuranil, naftil, 9-hidroxi-9H-fluorenil, -OH, -NHC(O)-, halógeno |
| R <sub>7</sub>  | H, alquilC <sub>1-2</sub> , ariloC <sub>6</sub> , pirimidin, piridin,                                                                                                                                                                                                                                                                                                                                           |
| R <sub>8</sub>  | H, alquilC <sub>1-2</sub>                                                                                                                                                                                                                                                                                                                                                                                       |
| R <sub>9</sub>  | H, AlquilC <sub>1-2</sub>                                                                                                                                                                                                                                                                                                                                                                                       |
| R <sub>10</sub> | 2,3-dihidro-1H-2-il-indano, alquilC <sub>1-7</sub> , cicloalquilC <sub>5-8</sub> , alquilC <sub>1-2</sub> -OH, adamantil, naftilo                                                                                                                                                                                                                                                                               |
| R <sub>12</sub> | AlquilC <sub>1-2</sub> , fenil, -O-alquilC <sub>1-3</sub> , cicloalquilC <sub>3</sub> , benzodioxol, adamantil, naftilo, furilo, tienilo, morfolina, pirimidin, -NO <sub>2</sub> , -NH <sub>2</sub> , -OH, -CF <sub>3</sub> , halógeno                                                                                                                                                                          |
| R <sub>13</sub> | AlquilC <sub>1-4</sub> , alquenilC <sub>2</sub> , ariloC <sub>6</sub> , -OalquilC <sub>1-2</sub> , cicloalquilC <sub>4-6</sub> , arilo sustituido con NH <sub>2</sub> , naftil, -OH, -CF <sub>3</sub> , 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 R<sub>1</sub>, R<sub>5</sub>, R<sub>6</sub>, R<sub>7</sub>, R<sub>8</sub>, R<sub>11</sub>, R<sub>12</sub>, R<sub>13</sub>, R<sub>14</sub> y R<sub>15</sub> 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 metaioproteinasas 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 R<sub>1</sub> 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<br>(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 $\alpha$ -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.

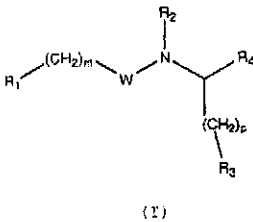
D3 divulga (Pág. 2290, Fl. 822) un procedimiento de síntesis de [N-(4amino-4deoxi-10-metilpteroil)- $\alpha$ -glutamil]-L-alanina dihidrato (MTX- $\alpha$ -alanina)

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)



o una sal farmacéuticamente aceptable de éste.

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

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

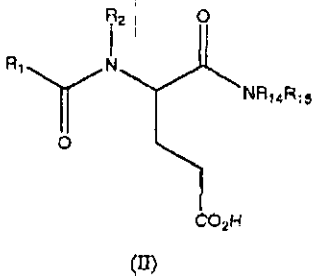
Las características **esenciales** del compuesto de la fórmula (I) de la reivindicación 1 habían sido divulgadas previamente en D1 y D2. El documento D1 enseña derivados de ácido L-glutámico inhibidores de metaloproteinasa como los compuestos 2b, 7, 8 y 9 que coinciden con la compuestos de la fórmula (I) cuando W: -C(O)-, R<sub>1</sub>: heteroarilo tricíclico, R<sub>2</sub>: H, R<sub>3</sub>: -COHNH, R<sub>4</sub>: -CONR<sub>9</sub>R<sub>10</sub>, -COOH o -(CH<sub>2</sub>)<sub>n</sub>OR<sub>7</sub>, R<sub>5</sub>: alquilC<sub>1-6</sub>, R<sub>9</sub>: H, R<sub>10</sub>: alquilC<sub>1-6</sub> sustituido con R<sub>12</sub>, 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)-, R<sub>1</sub>: arilo tricíclico, R<sub>2</sub>: H, R<sub>3</sub>: -COOH, R<sub>4</sub>: -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:

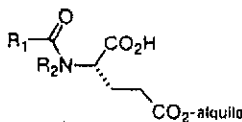
| Características <b>esenciales</b>                                                                | D1                                    |
|--------------------------------------------------------------------------------------------------|---------------------------------------|
| Un compuesto de fórmula:<br><br>(II)                                                             | Un compuesto de fórmula:<br><br>X: OH |
| R <sub>1</sub> : fenilo sustituido con R <sub>5</sub><br>R <sub>5</sub> : alquilC <sub>1-6</sub> |                                       |
| R <sub>2</sub> : H                                                                               | H                                     |
| R <sub>14</sub> : H                                                                              | H                                     |
| R <sub>15</sub> : alquilC <sub>1-6</sub>                                                         | -CH <sub>3</sub>                      |

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 metaloproteinasas como los compuestos 2b, 7, 8 y 9 que coinciden con la compuestos de la fórmula (I) cuando W -C(O)-, R<sub>1</sub>: fenilo, R<sub>2</sub>: H, R<sub>5</sub>: alquilC<sub>1-6</sub>, R<sub>14</sub>: H y R<sub>15</sub>: alquilC<sub>1-6</sub> (Pág. 4529, Fl. 816; Pág. 4534, Fl. 818).

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

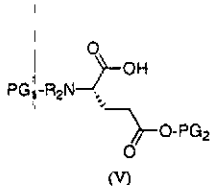
Las reivindicaciones 32 y 34 consisten en un método para sintetizar un compuesto que comprende:

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

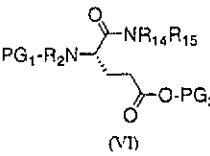


Con una amina, bajo condiciones efectivas para proporcionar un compuesto que tiene fórmula (IV):

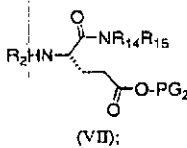




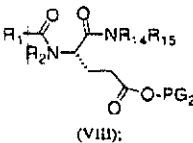
Con una amina, bajo condiciones efectivas para proporcionar un compuesto que tiene fórmula (VI):



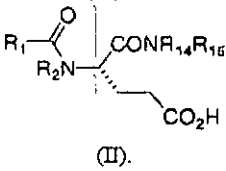
b) Hidrolizar el grupo protector amina de un compuesto de la fórmula (VI), bajo condiciones efectivas para proporcionar un compuesto que tiene la fórmula (VII):



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

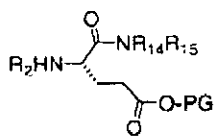
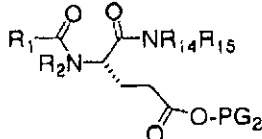
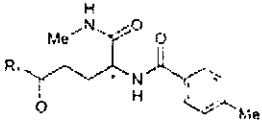
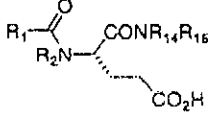
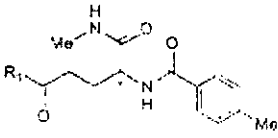


d) Tratar el compuesto de la fórmula (VIII) bajo condiciones efectivas para la hidrólisis del ácido carboxílico protegido, suministrando por lo tanto un compuesto que tiene la fórmula (II):



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

| Características <b>esenciales</b>                                                                                                                         | D1                                                                                                                                                                                         |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Un método para sintetizar un compuesto (II) de fórmula<br><br>(II)                                                                                        | Un método para sintetizar un compuesto de fórmula 2a o 22<br><br>que comprende:                                                                                                            |
| Tratar un compuesto de la fórmula (IV)<br><br>(V)<br>Con una amina, bajo condiciones efectivas para proporcionar un compuesto de fórmula (VI)<br><br>(VI) | Tratar el compuesto de fórmula 19<br><br>R <sub>1</sub> : O <sup>t</sup> Bu, OBzl<br>R <sub>2</sub> : OBzl, Boc<br>con NH <sub>2</sub> Me para proporcionar un compuesto de fórmula 20<br> |

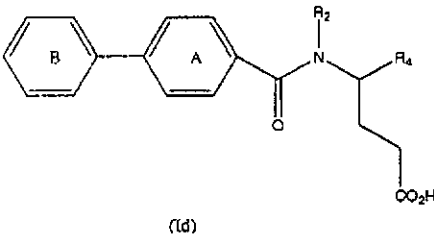
| Características <b>esenciales</b>                                                                                                                                                                                                                                                                            | D1                                                                                                                                                                                                       |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>Hidrolizar el grupo protector amina de un compuesto de la fórmula (VI). bajo condiciones efectivas para proporcionar un compuesto que tiene la fórmula (VII)</p> <div></div> <p>(VII);</p>                               | <p>Hidrolizar el grupo protector</p>                                                                                                                                                                     |
| <p>Tratar el compuesto de la fórmula (VII) con un ácido carboxílico, bajo condiciones efectivas para proporcionar un compuesto que tiene la fórmula (VIII);</p> <div></div> <p>(VIII);</p>                                  | <p>Tratar el compuesto con cloruro de 4-metilbenzoil para obtener el compuesto 21</p> <div></div>                      |
| <p>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):</p> <div></div> <p>(II).</p> | <p>Tratar el compuesto con TFA-H2O o H2, Pd-C, EtOH para obtener el compuesto 2a o 22</p> <div></div> <p>R1: -OH</p> |

Las características **esenciales** del método de las reivindicaciones 33 y 35 habían sido divulgadas previamente en D1, el cual da a conocer un método para sintetizar el compuesto 2a o 22 que comprende tratar el compuesto 19 con NH<sub>2</sub>Me para proporcionar un compuesto de fórmula 20, hidrolizar el grupo protector, tratar el compuesto con cloruro de 4-metilbenzoil para obtener el compuesto 21 y tratar el compuesto 21 con TFA-H<sub>2</sub>O o H<sub>2</sub>, Pd-C, EtOH para obtener el compuesto 2a o 22 como se muestra en la tabla (Pág. 4529, esquema 1, Fl. 816)

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

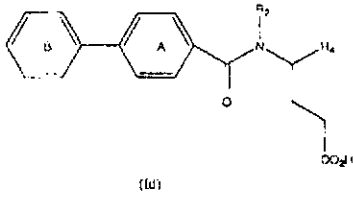
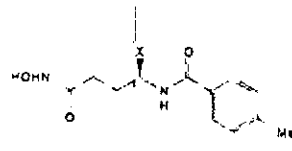
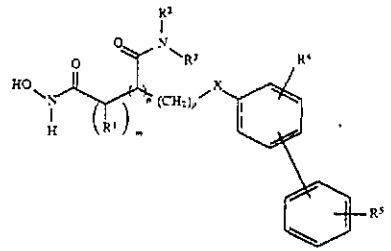
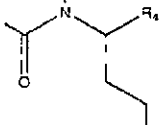
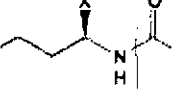
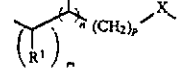
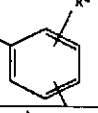
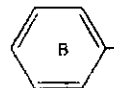
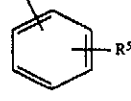
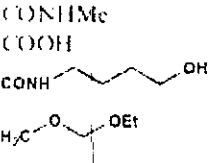
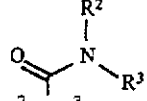
**Nivel Inventivo (Art18 D486)**

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



o una sal farmacéuticamente aceptable de éste.

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

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

Expediente 08 001 924 1er Art 45

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.<br>Bioorganic & Medicinal<br>Chemistry, Vol. 13,<br>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,<br>Biochemistry, Vol. 28,<br>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

Kanji Takahashi, Masahiro Ikura, Hiromu Habashita, Minoru Nishizaki, Tsuneoyuki Sugitara, Shingo Yamamoto, Shingo Nakatani,\* Koji Ogiwara, Hiroyuki Ohno, Hisao Nakai and Masaki Toda

*Ahnase Research Institute, One Pharma-chemical Co., Ltd. 3-4-1 Sakurai, Shimamoto, Mishima, Osaka 518-8533, Japan*  
 Received 8 March 2005; accepted 13 April 2005  
 Available online 23 May 2005

**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-methylamide (Acet-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 **4**.  
 © 2005 Elsevier Ltd. All rights reserved.

## 1. Introduction

Matrix metalloproteinases (MMPs), a family of zinc-containing endopeptidases, have been shown to play a central role in various physiological and pathological 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, arteriosclerosis, 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.<sup>1</sup>

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.<sup>2</sup> 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 <sup>15</sup>N-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.<sup>1</sup>

\* Corresponding author. Tel.: +81 75 964 1151; fax: +81 75 964 9314; e-mail: s-nakatani@ons.co.jp

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 act 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 MMPs 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,<sup>3</sup> 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 methylaniline 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 **2b**. The carboxylic acids **2a** and **22** were converted to hydroxamic acids (**2b** and **5**) by the condensation with *O*-benzylhydroxylamine followed by catalytic hydrogenation.

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

| Compound  | MMP-1           | MMP-2 | MMP-9            | MMP-3           |
|-----------|-----------------|-------|------------------|-----------------|
| <b>2a</b> | >100            | >100  | NT <sup>a</sup>  | >100            |
| <b>2b</b> | 19              | 2.4   | 18               | 39 <sup>a</sup> |
| <b>3</b>  | >100            | >100  | >100             | >100            |
| <b>4</b>  | 59 <sup>a</sup> | 18    | 100 <sup>a</sup> | NT              |
| <b>5</b>  | >100            | >100  | NT               | >100            |
| <b>6</b>  | >100            | >100  | NT               | >100            |

<sup>a</sup> Inhibition percentage at 100 μM.

<sup>b</sup> Not tested

Table 2. Effect of the chemical modification of (1) carboxylic acid moiety of L-glutamic acid analog on the activity profiles

| Compound  | V                                                      | MMP-1 | MMP-2 | MMP-9 | MMP-3            |
|-----------|--------------------------------------------------------|-------|-------|-------|------------------|
| <b>2b</b> | CONHMe                                                 | 19    | 2.4   | 18    | 39 <sup>a</sup>  |
| <b>7</b>  | COOH                                                   | NT    | 14    | NT    | >100             |
| <b>8</b>  | CONHCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> OH | 23    | 1.4   | NT    | 119 <sup>a</sup> |
| <b>9</b>  | H <sub>2</sub> C(OCH <sub>3</sub> ) <sub>2</sub>       | 4.3   | >1    | >1    | 84 <sup>a</sup>  |

<sup>a</sup> Inhibition percentage at 100 μM.

Table 3. Effect of (2) C-1' substitution of L-glutamic acid analogs on the activity profiles

| Compound  | X                  | MMP-1           | MMP-2 | MMP-9           | MMP-3 |
|-----------|--------------------|-----------------|-------|-----------------|-------|
| <b>4</b>  | H                  | 99 <sup>a</sup> | 38    | 20 <sup>a</sup> | NT    |
| <b>10</b> | CH <sub>3</sub>    | 6.1             | 50    | NT              | NT    |
| <b>11</b> | t-Bu               | 58 <sup>a</sup> | 3.3   | 11              | 54    |
| <b>12</b> | i-Pr               | 69 <sup>a</sup> | 2.2   | 88              | >100  |
| <b>13</b> | CH <sub>2</sub> OH | 9.53            | 4.18  | 3.3             | 3.7   |

<sup>a</sup> Inhibition percentage at 100 μM.

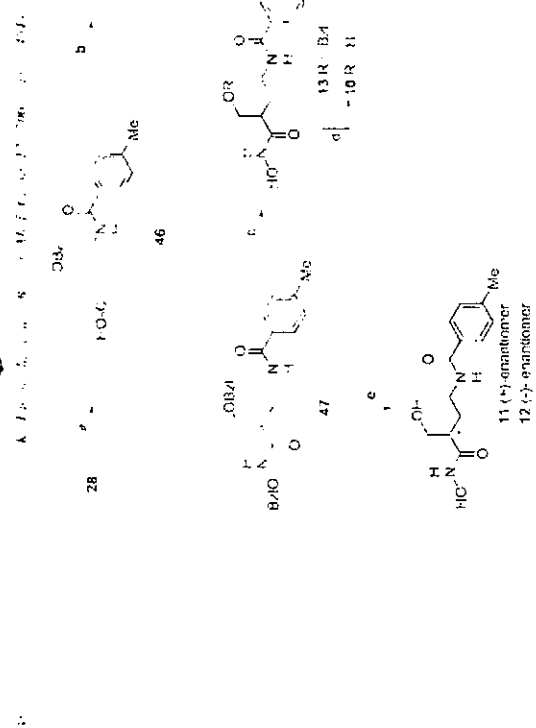
Compound **3** was prepared as outlined in Scheme 2. *N*-Acylation of **25**, which was prepared from **24**, with a glutamic anhydride followed by formation of the *N*-methanamide by the conventional procedures afforded **26b**. Without *N*-protection in **26a**, an exclusive intramolecular cyclization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

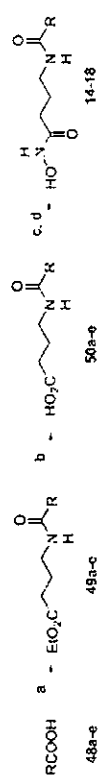
Compound **6** was also synthesized from **19b** as outlined in Scheme 4. Condensation of **19b** with 4-methyl aniline afforded **40**, catalytic hydrogenation of which gave **31**. Condensation of **31** with *O*-benzylhydroxylamine produced **32**. Acidic deprotection of **32** resulted in **33**, which was converted to **34** according to the usual *N*-acylation. 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**

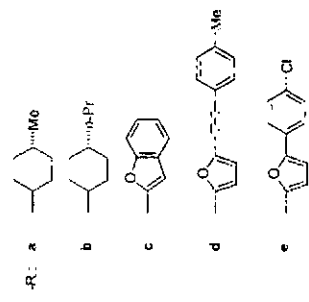




Scheme 7. Synthesis of 10-13. Reagents: (a) LDA, EtOCH<sub>2</sub>CH<sub>2</sub>Cl, HMPA, Et<sub>3</sub>N; (b) HCl, EtOH; (c) NaOH, EtOH; (d) HCl, EtOH; (e) HCl, EtOH.



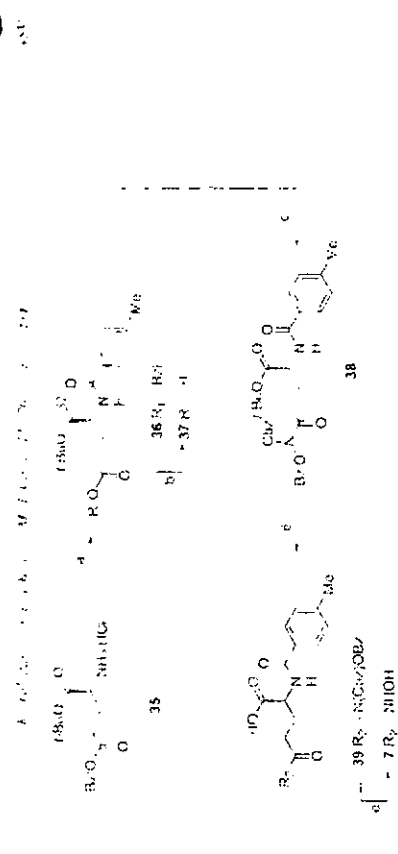
Scheme 8. Synthesis of 14-18. Reagents: (a) LDA, EtOCH<sub>2</sub>CH<sub>2</sub>Cl, HMPA, Et<sub>3</sub>N; (b) HCl, EtOH; (c) NaOH, EtOH; (d) HCl, EtOH; (e) HCl, EtOH.



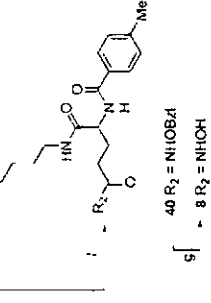
Scheme 9. Synthesis of 19-23. Reagents: (a) LDA, EtOCH<sub>2</sub>CH<sub>2</sub>Cl, HMPA, Et<sub>3</sub>N; (b) HCl, EtOH; (c) NaOH, EtOH; (d) HCl, EtOH; (e) HCl, EtOH.

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 Valacyl 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 a docking study. As shown in Figure 2, compound 2b seemed to fit the active site of MMP-1.

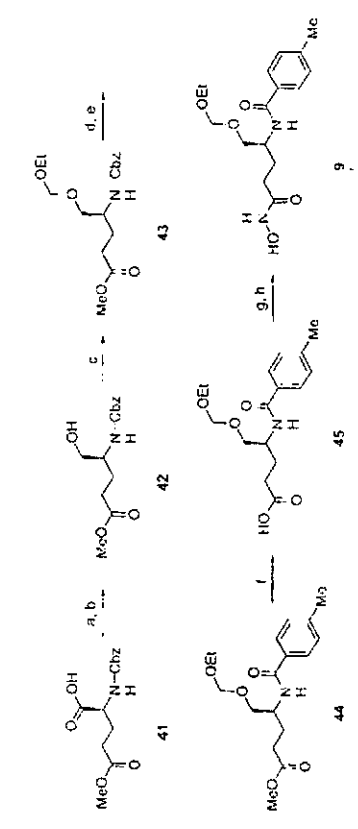
A search of the standard LUDI library was carried out to identify candidate second fragments. This search resulted in a list of 83 small molecules. Although N-acetyl-L-alanine, N-methylamide (Ac-L-Ala NHMe) had a relatively low LUDI score, we tested it because of the ease of synthesis and the enrichment of the α-methyl group. A docking study of Ac-L-Ala NHMe with MMP-1 suggested the formation of key hydrogen bonds with Pro238 and Leu181



Scheme 5. Synthesis of 7 and 8. Reagents: (a) LDA, EtOCH<sub>2</sub>CH<sub>2</sub>Cl, HMPA, Et<sub>3</sub>N; (b) HCl, EtOH; (c) NaOH, EtOH; (d) HCl, EtOH; (e) HCl, EtOH.



Scheme 6. Synthesis of 9. Reagents: (a) LDA, EtOCH<sub>2</sub>CH<sub>2</sub>Cl, HMPA, Et<sub>3</sub>N; (b) HCl, EtOH; (c) NaOH, EtOH; (d) HCl, EtOH; (e) HCl, EtOH.



Scheme 7. Synthesis of 10-13. Reagents: (a) LDA, EtOCH<sub>2</sub>CH<sub>2</sub>Cl, HMPA, Et<sub>3</sub>N; (b) HCl, EtOH; (c) NaOH, EtOH; (d) HCl, EtOH; (e) HCl, EtOH.

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).

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



Figure 1. Docking study of N-4-Ala-MMP-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  $\mu$ 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 glutamic 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 2S-configuration of 2b to a 2R-configuration gave 5, with loss of inhibitory activity against MMP-1, MMP-2, and MMP-3 at 100  $\mu$ M. This indicated that the 2S-configuration of 2b plays a role in supporting the interaction with the target enzyme, while the 2R-configuration of 5 seems to disturb interaction with the enzyme. In order to confirm the importance of hydrogen bonds, a retro-amide analogue of 2b was synthesized and evaluated for

inhibition of MMP-1, -2, -9, and -3. Compound 6 did not inhibit the MMPs at 100  $\mu$ M. At all, these results supported the concept that compound 2b showed its activity through hydrogen bonding interaction of the benzamide moiety (Table 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 hydrophobic substituent was investigated. As shown in Table 1, conversion to the carboxylic acid moiety gave 7, which showed reduced MMP-2 inhibitory activity. Replacement of the methyl group with a 1-hydroxybutyl moiety resulted in 8, with retention of the inhibitory activity against MMP-1 and MMP-2. In order to reduce the peptide nature and maintain hydrophobicity, the N-methylformamide moiety was replaced with a 1-ethoxymethylmethyl 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  $\alpha$ -position of 4-aminobutyric acid analog 4 was investigated. Fortunately, introduction of a hydroxymethyl moiety at the  $\alpha$ -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 benzoxymethyl moiety at the  $\gamma$ -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 supported that the benzamide moiety of 2b could be located in the S1' cavity of 1A enzyme, as shown in Figure 1. Although the MMPs have a similar environment around the active site, there are slight differences of the environment in the S1' pocket, for example, the S1' cavity of MMP-2, -3, and -9 are known to be deeper and narrower than that of MMP-1. 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 1-methylbenzamide moiety of 4 with a *tert*-butylmethylmethylmethyl moiety gave 14, which showed increased activity against MMP-1 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 benzoturan-2-yl moiety provided 16, with loss of inhibitory activity against MMP-1, -2, and -3, conversion to the 5-(4-methylphenylmethylmethylmethyl) moiety of the 5-(4-chlorophenylmethylmethylmethyl) 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.<sup>14</sup> 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 MMPs 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

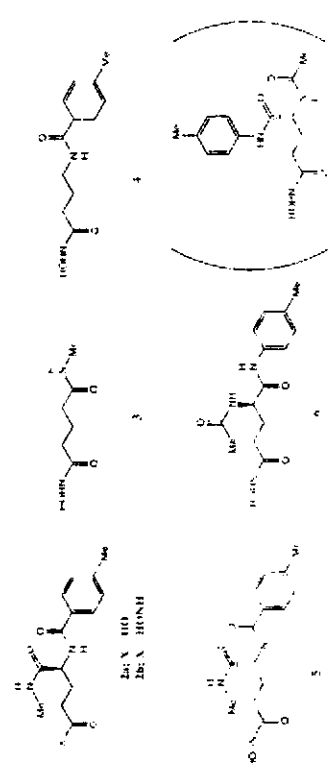


Chart 1. New chemical leads for MMPs.

The title compound was obtained quantitatively as a colorless oil from 30 according to the analogous procedures as described for the preparation of 22.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , MeOH; AcOH, 18:2:1)  $\delta$  7.73 (d,  $J = 8.4$  Hz, 2H), 7.40 (m, 1H), 7.31 (d,  $J = 8.4$  Hz, 1H), 3.47 (m, 2H), 2.41 (t,  $J = 7.3$  Hz, 2H), 2.38 (s, 3H), 1.94 (m, 2H).

#### 5.16. $N^2$ -(*tert*-Butoxycarbonyl)- $N^5$ -benzyloxy- $N^4$ -(4-methylphenyl)- $\alpha$ -glutamine (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 (CHCl<sub>3</sub>, MeOH, 10:1).

#### 5.17. $N^5$ -Benzyloxy- $N^4$ -(4-methylphenyl)- $\alpha$ -glutamine (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 Et<sub>2</sub>O to give 33 (2.74 g, 96% yield) as a white powder. TLC  $R_f$  0.24 (CHCl<sub>3</sub>, MeOH; AcOH, 18:2:1).

#### 5.18. $N^2$ -Acetyl- $N^5$ -benzyloxy- $N^4$ -(4-methylphenyl)- $\alpha$ -glutamine (34)

To a stirred solution of 33 (3.78 mg, 1.00 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) were added *N,N*-diisopropylethylamine (4.38 mL, 2.2 mmol) and acetic anhydride (0.694 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 H<sub>2</sub>O, 1 N HCl, H<sub>2</sub>O, saturated NaHCO<sub>3</sub>, H<sub>2</sub>O, and dried under reduced pressure. The obtained powder was dissolved in MeOH. The solution was evaporated to give a solid, which was triturated with Et<sub>2</sub>O to give 34 (3.30 mg, 83% yield) as a white powder. TLC  $R_f$  0.51 (CHCl<sub>3</sub>, MeOH, 10:1).

#### 5.19. $N^2$ -Acetyl- $N^5$ -hydroxy- $N^4$ -(4-methylphenyl)- $\alpha$ -glutamine (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 (CHCl<sub>3</sub>, MeOH, 10:1); MS (APCI, neg. 40 V)  $m/z$ : 292 (M<sup>+</sup>); IR (KBr) 3269, 2900, 1657, 1607, 1538, 1450, 1381, 1287, 1257, 1109, 1086, 1021, 981, 818, 797, 686, 610, 514 cm<sup>-1</sup>;  $^1\text{H}$  NMR (200 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.41 (s, 1H), 9.94 (s, 1H), 8.70 (s, 1H), 8.16 (d,  $J = 7.5$  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 (3m, 2H); optical rotation [ $\alpha$ ]<sub>D</sub><sup>20</sup> +18.2° (c 0.50, MeOH); IRMS (FAB) calcd for C<sub>14</sub>H<sub>15</sub>N<sub>2</sub>O<sub>5</sub>: 294.1454, Found: 294.1453.

#### 5.20. 5-Benzyloxy-1-*tert*-butyl-*N*-(4-methylbenzoyl)- $\alpha$ -glutamate (36)

To a stirred solution of 35 (10.0 g, 30.3 mmol) in CHCl<sub>3</sub> (50 mL) were added *N,N*-diisopropylethylamine

(21 mL, 90.9 mmol) and benzyl chloroformate (14.3 mL, 100 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 h and diluted with 1 N HCl, and brine, and dried over MgSO<sub>4</sub>. Removal of the volatiles in vacuo provided an oily solid, which was triturated with Et<sub>2</sub>O to give 28 (2.06 g, 9% yield) as colorless crystals. TLC  $R_f$  0.58 (CHCl<sub>3</sub>, MeOH; AcOH, 18:2:1);  $^1\text{H}$  NMR (200 MHz, CDCl<sub>3</sub>)  $\delta$  7.73 (d,  $J = 8.4$  Hz, 2H), 7.40 (m, 1H), 7.31 (d,  $J = 8.4$  Hz, 1H), 3.47 (m, 2H), 2.41 (t,  $J = 7.3$  Hz, 2H), 2.38 (s, 3H), 1.94 (m, 2H).

#### 5.12. $N$ -(4-(Benzyloxyamino)-4-oxobutyl)- $\alpha$ -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 (CHCl<sub>3</sub>, MeOH, 10:1);  $^1\text{H}$  NMR (200 MHz, CDCl<sub>3</sub>)  $\delta$  9.24 (s, 1H), 7.68 (d,  $J = 8.2$  Hz, 2H), 7.37 (m, 5H), 7.23 (d,  $J = 8.2$  Hz, 2H), 6.60 (m, 1H), 4.90 (s, 2H), 3.46 (dt,  $J = 6.9, 5.8$  Hz, 2H), 2.39 (s, 3H), 2.17 (m, 2H), 1.92 (m, 2H).

#### 5.13. $N$ -(4-(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 (CHCl<sub>3</sub>, MeOH, 10:1); MS (APCI, neg. 40 V)  $m/z$ : 235 (M<sup>+</sup>); IR (KBr) 1674, 1613, 1608, 1564, 1510, 1458, 1436, 1360, 1332, 1313, 1258, 1233, 1197, 1178, 1101, 1024, 969 cm<sup>-1</sup>;  $^1\text{H}$  NMR (200 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  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); IRMS (FAB) calcd for C<sub>12</sub>H<sub>17</sub>N<sub>2</sub>O<sub>5</sub>: 237.1239, Found: 237.1241.

#### 5.14. Benzyloxy- $N^2$ -(*tert*-butoxycarbonyl)- $N^4$ -(4-methylphenyl)- $\alpha$ -glutamine (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 (5.36 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 NaHCO<sub>3</sub>, and brine. The organic layer was dried over MgSO<sub>4</sub>. 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);  $^1\text{H}$  NMR (200 MHz, CDCl<sub>3</sub>)  $\delta$  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), 2.12 (m, 1H), 2.02 (m, 1H), 1.44 (s, 9H).

amine (34.8 mL, 100 mmol) and benzyl chloroformate (14.3 mL, 100 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 2 h and diluted with 1 N HCl, and brine, and dried over MgSO<sub>4</sub>. Removal of the volatiles in vacuo provided an oily solid, which was triturated with Et<sub>2</sub>O to give 25 (21.9 g, 85% yield) as a white powder. TLC  $R_f$  0.24 (EtOAc-*n*-hexane, 1:1);  $^1\text{H}$  NMR (200 MHz, CDCl<sub>3</sub>)  $\delta$  7.35 (m, 3H), 5.18 (s, 2H), 4.87 (s, 2H).

#### 5.9. 5-[(Benzyloxy)carbamoyl]amino-5-oxopentanoic acid (26a) and benzyloxy- $N$ -(5-methylamino)-5-oxopentanoic acid (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. Glutamic anhydride (1.26 g, 11.0 mmol) and 4-(dimethylamino)pyridine (50 mg, 4.2 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 MgSO<sub>4</sub>, and filtered. 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 (CHCl<sub>3</sub>, MeOH; AcOH, 18:2:1). To a stirred mixture of 26a (1.11 g, 2.99 mmol), methylaniline (40% in MeOH, 0.50 g, 5.97 mmol), and HOBr (686 mg, 4.48 mmol) in DMF (5 mL) was added Et<sub>3</sub>NHCl (859 mg, 1.48 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, and NaHCO<sub>3</sub>, and brine, and dried over MgSO<sub>4</sub>. Removal of the volatiles in vacuo provided a white powder, which was triturated with Et<sub>2</sub>O to give 26b (68 mg, 7% yield from 25) as a colorless silky powder.  $^1\text{H}$  NMR (CDCl<sub>3</sub>, 200 MHz)  $\delta$  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-*n*-hexane, 1:1); IR (KBr) 3224, 2942, 1646, 1562, 1459, 1413, 1291, 1165, 1092, 1071, 1023, 604, 439 cm<sup>-1</sup>;  $^1\text{H}$  NMR (200 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  10.30 (s, 1H), 9.30 (s, 1H), 9.30 (s, 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); IRMS (FAB) calcd for C<sub>5</sub>H<sub>11</sub>N<sub>2</sub>O<sub>2</sub>: 161.0926, Found: 161.0926.

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

To a stirred suspension of 4-aminobutyric acid (13 g, 11.0 mmol) in THF (21 mL) were added 1 N NaOH

(11.6 mL, 16.7 mmol) and 4-methylbenzoyl chloride (4.4 mL, 33 mmol) at 0 °C. After stirring at room temperature for 3 h, saturated NaHCO<sub>3</sub> 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 NaHCO<sub>3</sub>, and brine. The organic layer was dried over MgSO<sub>4</sub>. Removal of the volatiles in vacuo provided a solid, which was triturated with *n*-hexane to give 36 (7.6 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)- $\alpha$ -glutamine (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.  $^1\text{H}$  NMR (200 MHz, CDCl<sub>3</sub>)  $\delta$  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- $N^5$ -benzyloxy- $N^4$ -(benzyloxy)carbamoyl- $N^2$ -(4-methylbenzoyl)- $\alpha$ -glutamine (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 NaHCO<sub>3</sub>, brine, dried over MgSO<sub>4</sub>. 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);  $^1\text{H}$  NMR (200 MHz, CDCl<sub>3</sub>)  $\delta$  7.72 (d,  $J = 8.0$  Hz, 2H), 7.38 (m, 5H), 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 [ $\alpha$ ]<sub>D</sub><sup>20</sup> +6.84° (c 0.655, CHCl<sub>3</sub>).

#### 5.23. $N^5$ -Benzyloxy- $N^4$ -(benzyloxy)carbamoyl- $N^2$ -(4-methylbenzoyl)- $\alpha$ -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-CHCl<sub>3</sub> (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 (CHCl<sub>3</sub>, MeOH; AcOH, 18:2:1).

#### 5.24. $N^5$ -Hydroxy- $N^4$ -(4-methylbenzoyl)- $\alpha$ -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-*n*-hexane, 1:1); MS (APCI, neg.

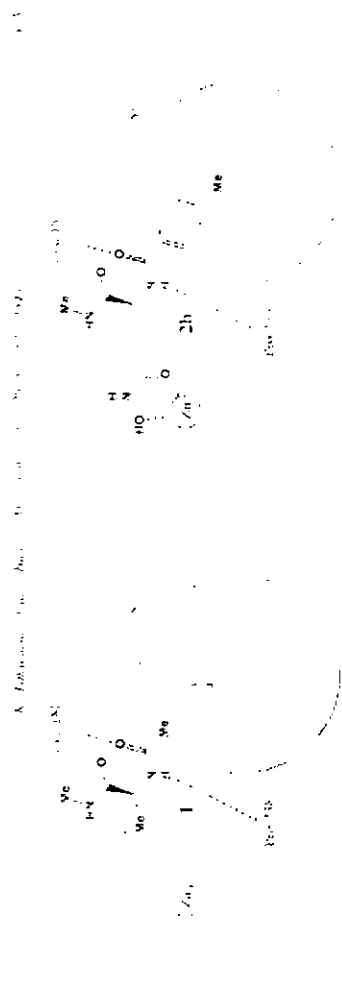


Figure 3. Molecular design of a) glutamine acid amide, 2b

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 <sup>1</sup>H NMR spectra were obtained with a Varian Gemini-200 or MERCURY-400 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 Voyager FT-30 spectrometer. IR spectra were measured using a Perkin Elmer FTR 1700X or JASCO FTR-430 spectrometer. Column chromatography was carried out using silica gel (Merck silica gel 60 (0.063–0.200 mm), Wako Gel C-200, Fuji Silysia F-1060), or Fuji Silysia BW-235. TLC was also performed on silica gel (Merck TLC plate, silica gel 60 F-254). The following abbreviations for solvents and reagents are used: THF, tetrahydrofuran; EtOAc, ethylacetate; MeOH, methanol; EtOH, ethanol; DMF, N,N-dimethylformamide; CHCl<sub>3</sub>, dichloromethane; CHCl<sub>3</sub>/chloroform; EDC-HCl, 1-ethyl-3-(3-dimethylamino)propyl-3-carbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole; HMPA, hexamethylphosphoramide; TFA, trifluoroacetic acid; DMSO, dimethylsulfoxide; AcOH, acetic acid; F<sub>3</sub>CO<sub>2</sub>, diethyl-ether; BuLi, butyllithium; FIFC, fluorescent isothiocyanate; MQAc, 7-methoxyoctanamide-4-acetyl; Dpa, N-(2,4-dinitrophenyl)-1,2,3-diaminopropanol; Dnp, 2,4-dinitrophenyl.

100 MHz), CDCl<sub>3</sub> δ: 74.4 (d, J = 8.0 Hz, 2H), 73.3 (m, J = 6.0 Hz, 1H), 73.2 (s, J = 8.0 Hz, 2H), 66.1 (s, 2H), 59.9 (d, J = 6.6 Hz, 1H), 58.4 (m, J = 9.0 Hz, 3H), 56.5 (m, 4H), 2.40 (s, 3H), 1.43 (s, 9H).

**5.3. Benzyl N<sup>2</sup>-(tert-butyloxycarbonyl)-N<sup>1</sup>-methyl-D-glutamate (20b) and benzyl N<sup>2</sup>-(4-methylbenzoyl)-D-glutamate (21b)**

The amide 20b was obtained as a white powder in 80% yield from 5-benzyl-N<sup>1</sup>-(tert-butyloxycarbonyl)-D-glutamate (19b) according to the analogous procedures as described for the preparation of 20a. Compound 20b: <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.26 (s, 2H), 7.23 (s, 3H), 7.20 (s, 3H), 6.61 (s, 2H), 6.58 (m, 1H), 5.99 (d, J = 6.6 Hz, 1H), 5.84 (m, J = 9.0 Hz, 3H), 5.65 (m, 4H), 2.40 (s, 3H), 1.43 (s, 9H).

**5.4. N<sup>1</sup>-Methyl-N<sup>2</sup>-(4-methylbenzoyl)-D-glutamine (2a)**

A solution of 21a (399 mg, 1.49 mmol) in a mixed solvent of TFA-H<sub>2</sub>O (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. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.38 (s, 3H), 6.24 (br s, 1H), 5.65 (m, 1H), 5.10 (s, 2H), 4.20 (d, 1H), 2.81 (d, J = 5.0 Hz, 3H), 2.49 (s, 3H), 2.17 (s, 2H), 1.44 (s, 9H).

**5.5. N<sup>1</sup>-Methyl-N<sup>2</sup>-(4-methylbenzoyl)-D-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 (344 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. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.26 (s, 2H), 7.23 (s, 3H), 7.20 (s, 3H), 6.61 (s, 2H), 6.58 (m, 1H), 5.99 (d, J = 6.6 Hz, 1H), 5.84 (m, J = 9.0 Hz, 3H), 5.65 (m, 4H), 2.40 (s, 3H), 1.43 (s, 9H).

1H), 7.26 (s, 2H), 7.20 (s, 3H), 7.23 (s, 3H), 6.61 (s, 2H), 6.58 (m, 1H), 5.99 (d, J = 6.6 Hz, 1H), 5.84 (m, J = 9.0 Hz, 3H), 5.65 (m, 4H), 2.40 (s, 3H), 1.43 (s, 9H).

**5.6. N<sup>2</sup>-(benzyloxyl)-N<sup>1</sup>-methyl-N<sup>2</sup>-(4-methylbenzoyl)-D-glutamate (23a) and N<sup>2</sup>-hydroxy-N<sup>1</sup>-methyl-N<sup>2</sup>-(4-methylbenzoyl)-D-glutamate (2b)**

To a stirred solution of 2a (2.57 mg, 0.924 mmol) in DMF (1 mL) were added D-benzylhydroxylamine hydrochloride (1.77 mg, 9.24 mmol), hydroxybenzotriazole monohydrate (1.0 mg, 9.24 mmol), methylamine (0.185 mL, 1.77 mmol), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide 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<sub>3</sub>, and brine, and dried over MgSO<sub>4</sub>. Removal of the volatiles in vacuo provided 23a (205 mg, 58% yield) as a white powder. Compound 23a: <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.26 (s, 2H), 7.23 (s, 3H), 7.20 (s, 3H), 6.61 (s, 2H), 6.58 (m, 1H), 5.99 (d, J = 6.6 Hz, 1H), 5.84 (m, J = 9.0 Hz, 3H), 5.65 (m, 4H), 2.40 (s, 3H), 1.43 (s, 9H).

**5.7. N<sup>2</sup>-(benzyloxyl)-N<sup>1</sup>-methyl-N<sup>2</sup>-(4-methylbenzoyl)-D-glutamate (23b) and N<sup>2</sup>-hydroxy-N<sup>1</sup>-methyl-N<sup>2</sup>-(4-methylbenzoyl)-D-glutamate (2b)**

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: <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ: 7.26 (s, 2H), 7.23 (s, 3H), 7.20 (s, 3H), 6.61 (s, 2H), 6.58 (m, 1H), 5.99 (d, J = 6.6 Hz, 1H), 5.84 (m, J = 9.0 Hz, 3H), 5.65 (m, 4H), 2.40 (s, 3H), 1.43 (s, 9H).

**5.8. Benzyl benzyloxycarbamate (25)**

To a stirred suspension of 24 (16.0 g, 160 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (100 mL) were added N,N-dimethylpropyl-

# Carboxypeptidase-Mediated Release of Methotrexate from Methotrexate- $\alpha$ -Peptides<sup>1</sup>

Ulrike Kuehnert,<sup>2</sup> Ute Lohrmann, Yolanda D. Montejano, Karin S. Vitos, and E. M. Huennekens<sup>3</sup>  
*Division of Biochemistry, Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, California 92037*

Received July 29, 1978; Revised Manuscript Received October 25, 1978

**ABSTRACT:** Methotrexate (MTX),  $\alpha$ -peptides containing representative neutral (alanine), acidic (aspartic acid), and basic (arginine) amino acids were synthesized by a region-specific 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-(mercaptoethyl)-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 (ID<sub>50</sub> values of  $2.0 \times 10^{-6}$  M and  $2.4 \times 10^{-4}$  M, respectively), but in the presence of CP-A the ID<sub>50</sub> of the peptide improved to  $8.5 \times 10^{-6}$  M. Similar results were obtained with MTX-Asp/CP-A and MTX-Ala/CP-B combinations. MTX-Arg showed good cytotoxicity (ID<sub>50</sub> of  $5.0 \times 10^{-4}$  M), due to CP-N activity in the fetal bovine serum of the culture medium; inclusion of CP-B lowered the ID<sub>50</sub> to that of MTX. Possible clinical uses of MTX peptides are discussed.

Methotrexate (MTX), 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 polyglutamated (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 & Zevy, 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  $\alpha$ -carboxyl appears to be more critical than the  $\gamma$ -carboxyl (Sirotnik et al., 1979).

The above considerations suggest that MTX  $\alpha$ -peptides (i.e., derivatives in which amino acids are joined via an amide linkage to the  $\alpha$ -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,

<sup>1</sup> Manuscript BC-8-3113 from the Research Institute of Scripps Clinic supported by an Outstanding Investigator grant (CA-29336) from the National Cancer Institute, National Institutes of Health, and by a grant (CA-3117) from the American Cancer Society. A preliminary account of this work has been presented elsewhere (Kuehnert et al., 1988).  
<sup>2</sup> Recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. Present address: Abteilung Pharmachemie, Boehringer Ingelheim KG, D-6507 Ingelheim, West Germany.

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 Pipet 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*-(methylamino)benzoic acid, triphenylphosphine, diethylphosphite, isobutyrochloroformate, Pd on charcoal, and Celite (Aldrich); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, *N*-Cbz-L-Glu  $\gamma$ -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

<sup>3</sup> Abbreviations: MTX, methotrexate; MTX-Ala, -Asp, and -Arg, peptides in which the indicated amino acids are linked covalently to the  $\alpha$ -carboxyl of MTX; CP-A, -B, -N, and G, carboxypeptidase A, B, N, and G; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; ID<sub>50</sub>, concentration producing 50% cell kill.

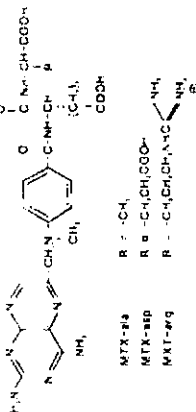


FIGURE 1. MTX  $\alpha$ -peptides.

propagant, pancreatic CP-A (bovine, type 1, 50 units/mg of protein), pancreatic CP-B (porcine, 200 units/mg of protein), trypsin, chymotrypsin, plasmin, subtilisin, bromelain, telase, aminopeptidase, proctase E from *Streptomyces griseus*, and pancreatic proctase type 1 (Sigma); folic acid and proleptase A from *Bacillus subtilis* (Serva); di-2-(mercaptoethyl)-3-(guanidinoethyl)thiopropionic acid and uridine (Calbiochem); DEAE-Trisacryl (IBF Biochemicals); C<sub>18</sub> silica gel (J. T. Baker); and nylon filters (pore size 0.2  $\mu$ m, NaGel).

Carboxypeptidase G<sub>2</sub> 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. Kervar (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 CoCl<sub>2</sub>, 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-methylpteroyl 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. (1983)]. Yield: 18.2 g (70%). TLC (system A) *R*<sub>f</sub> = 0.70. Vigorous oxygenation was required during the condensation step to minimize formation of the 6-methylpteridine (*R*<sub>f</sub> = 0.58) as a secondary product. Treatment of the 6-(hydroxymethyl)pteridine (12.25 g, 63.7 mmol) with B<sub>2</sub> in the presence of triphenylphosphine provided the 6-bromo derivative. The latter, without isolation, was then reacted with *p*-(methylamino)benzoic acid in the presence of diisopropylethylamine to produce 4-amino-4-deoxy-10-methylpteroyl acid (Rosowsky et al., 1985). The product, recovered by lyophilization, was obtained as the semihydrate. Yield: 21.8 g (95%). TLC (system B) *R*<sub>f</sub> = 0.57. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  7.3 (s, 1 H, 7-H), 8.20 (broad, 2 H, NH<sub>2</sub>), 7.78 (d, *J* = 9 Hz, 2 H, phenyl), 7.27 (broad, 2 H, NH<sub>2</sub>), 6.87 (d, *J* = 9 Hz, 2 H, phenyl), 4.87 (s, 2 H, CH<sub>2</sub>), 3.23 ppm (s, 3 H, N-CH<sub>3</sub>).

*N*-Carboxy-L-glutamic acid  $\alpha$ -methyl  $\gamma$ -tert-butyl diester hydrochloride, *N*-Cbz-Glu  $\gamma$ -tert-butyl ester (16.85 g, 50 mmol) was converted to the  $\alpha$ -methyl  $\gamma$ -tert-butyl diester by treatment with CH<sub>2</sub>I<sub>2</sub>, 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% Pd on charcoal) using the method of Kligge and Garbar (1962). After filtration, the solution (at room temperature) was evaporated to dryness under vacuum. The residue was dissolved in 30 mL of CH<sub>2</sub>(OH)-ethyl acetate (1:1) 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*<sub>f</sub> = 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 (diester hydrochloride), a white, amorphous powder, was obtained in a yield of 6.1 g (80%). [Rosowsky et al. (1981) reported a comparable yield of the free base obtained as an oil; mp 124–125 °C (Singer and Glibin (1963), 125–126 °C).

$\gamma$ -tert-Butyl *N*-(4-Amino-4-deoxy-10-methylpteroyl)-L-glutamate (MTX  $\gamma$ -tert-Butyl Ester). The procedure of Rosowsky et al. (1981) was modified in the following manner: 4-Amino-4-deoxy-10-methylpteroyl acid (4.96 g, 14.8 mmol, containing 0.5 equiv of H<sub>2</sub>O) 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  $\alpha$ -methyl  $\gamma$ -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 CHCl<sub>3</sub>. The solution was extracted twice with 500 mL of 1% NH<sub>4</sub>OH (allowing time for the slow separation of emulsions), and the organic fraction was dried over Na<sub>2</sub>SO<sub>4</sub>. Removal of the solvent in vacuum yielded a brown oil (8.0 g). The latter was dissolved in CH<sub>2</sub>Cl<sub>2</sub>/acetone/CH<sub>3</sub>OH (7:3:1) 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*<sub>f</sub> = 0.46. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  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, NH<sub>2</sub>), 6.83 (d, 2 H, *J* = 9 Hz, phenyl), 6.59 (broad, 2 H, NH<sub>2</sub>), 4.79 (broad, 2 H, ArCH<sub>2</sub>), 4.6–4.2 (m, 1 H,  $\alpha$ -CH), 3.63 (s, 3 H, COOCH<sub>3</sub>), 3.20 (s, 3 H, NCH<sub>3</sub>), 2.4–1.8 (m, 4 H, CH<sub>2</sub>CH<sub>2</sub>), 1.33 ppm (s, 9 H, COO-*t*-Bu).

The  $\alpha$ -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)<sub>2</sub>·8H<sub>2</sub>O (1.57 g, 5 mmol) was added to 40 mL of H<sub>2</sub>O, and (B) MTX  $\alpha$ -methyl  $\gamma$ -tert-butyl ester (2.55 g, 5 mmol) was added to 40 mL of CH<sub>2</sub>OH. (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. Na<sub>2</sub>SO<sub>4</sub> (780 mg), dissolved in 5 mL of warm H<sub>2</sub>O, was added to the solution, and after 5 min, the fine precipitate of BaSO<sub>4</sub> was removed by filtration through Celite. The precipitate was washed with a minimal amount of CH<sub>3</sub>OH, 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 CH<sub>3</sub>OH and ether, dried in air, and then dried in high vacuum over P<sub>2</sub>O<sub>5</sub>. The product, MTX  $\gamma$ -tert-butyl ester, was obtained as a yellow, amorphous powder. Yield: 2.3 g (90%). TLC (system C) *R*<sub>f</sub> = 0.45. <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$  8.60 (s,

819  
821

