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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIRECCIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente N°. 08-125.888

Solicitud de Patente de Invención titulada: "DERIVADOS DE AMINO-TIAZOL Y
SUS USOS COMO AGENTES ANTIBACTERIANOS"

Solicitante: NOVARTIS AG.

Nuestra Ref.: P2008/000179

RESPUESTA AL OFICIO No. 07822, NOTIFICADO POR FIJACIÓN EN LISTA No. 256

DE FECHA 18 DE MAYO DE 2012

(PRIMER EXAMEN DE FONDO)

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad solicitante, me permito manifestar lo siguiente ante el auto emanado por ese Despacho, dentro del plazo legal de prórroga previsto en el artículo 45 de la Decisión 486, en los siguientes términos:

1. En primer lugar, con el ánimo de brindar mayor claridad a la materia reivindicada, me permito presentar un **Capítulo Reivindicatorio Modificado**, que reemplaza en su totalidad al Capítulo Reivindicatorio presentado al momento de radicar la presente solicitud.

Así entonces, el Capítulo Reivindicatorio Modificado adjunto contiene los siguientes cambios:

- se eliminaron las Reivindicaciones originales 1-15, 25-31, 33-42 y 44;

- se reenumeran las Reivindicaciones originales 16-24, 32, 43 y 45, como nuevas Reivindicaciones 1-12, respectivamente;
- en la nueva Reivindicación 1 (antigua Reivindicación 16) se limitó drásticamente la materia reclamada de tal forma que:
 - R se limita a $N(R^1)_2$ y $N(R^1)$ -A-G-Q-J;
 - R^{4b} se limita a solamente alquilo;
 - R^9 se elimina;
 - R^1 se restringe estrictamente a lo reclamado en la antigua Reivindicación 11;
 - R^{8a} se limita drásticamente a H, alquil C_{1-6} cicloalquil C_{3-7} , alquil C_{1-6} sustituido con J, y R^{4b} ;
 - se complementan las definiciones de los términos químicos “alquilo”, “cicloalquilo”, “heterociclo”, “arilo”, “heteroarilo”, “halógeno”, “sustituido”, “alcoxilo”, “alquenilo” y “alquinilo”;
- en la nueva Reivindicación 5 (antigua Reivindicación 20), se limitó drásticamente el radical R^1 a aquellos grupos que están dentro del alcance de la nueva Reivindicación 1 (antigua Reivindicación 16);
- en la nueva Reivindicación 6 (antigua Reivindicación 21), se limitó drásticamente el radical R^{13} a aquellos grupos que están dentro del alcance de la nueva Reivindicación 1 (antigua Reivindicación 16);
- en la nueva Reivindicación 10 (antigua Reivindicación 32), se limitan drásticamente los compuestos reclamados a aquellos compuestos que están dentro del alcance de la nueva Reivindicación 1 (antigua Reivindicación 16).

Valga anotar que las modificaciones efectuadas al Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486, ya que éstas de ninguna manera implican una ampliación de la protección correspondiente a la divulgación contenida en la solicitud inicial, éstas por el contrario, corresponden a una drástica limitación de la materia reivindicada.

2. a- La Examinadora argumenta que las Reivindicaciones 33-42 y 44, reclaman materia no patentable.

b- Considero superada la anterior objeción en tanto que las Reivindicaciones 33-42 y 44, fueron retiradas del Capítulo Reivindicatorio Modificado adjunto.

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3. a- La Examinadora señala que las Reivindicaciones 1, 5, 9, 16 y 25 no están sustentadas en la descripción, porque hacen referencia a “enantiómeros”, “esteroisómeros”, “rotámeros”, “tautómeros”, “diastereoisómeros”, “antropisómeros”, “racematos”, “N-óxido de piridina” de los compuestos I, III, V, VI y VIII, de los cuales no hay evidencia que se puedan formar en los compuestos reivindicados. La Examinadora sugiere eliminar estos términos.

Así mismo, la Examinadora argumenta que las Reivindicaciones 1, 5, 9, 16 y 25 no son claras, ni concisas, por la definición de la invención en términos que no definen los átomos de carbono y heteroátomos que los conforman, por ejemplo “alquilo”, “alquil-arilo”, “arilo”, “cicloalquilo”, “heteroarilo”, entre otros, los cuales no permiten distinguir la invención del estado de la técnica.

La Examinadora señala que las reivindicaciones no son claras porque hacen referencia a los sustituyentes R^a y R^b , los cuales no aparecen en las estructuras de los compuestos reclamados. La Examinadora solicita hacer la corrección respectiva.

Finalmente, la Examinadora argumenta que el Capítulo Reivindicatorio no es conciso, ni está sustentando en la descripción, porque no hay suficientes ejemplos que soporten los compuestos de fórmulas I, III, V, VI y VIII con los sustituyentes tal como se encuentran allí definidos. La Examinadora sugiere restringir la presente solicitud a los compuestos que se encuentren sustentados en los ejemplos.

b- i) En primer lugar, llamo respetuosamente la atención de la Examinadora sobre las drásticas limitaciones realizadas en el Capítulo Reivindicatorio Modificado adjunto, tal como se explican en el punto 1 del presente memorial. En particular, en la nueva Reivindicación 1 (antigua Reivindicación 16), se incluyeron definiciones suficientes de expresiones químicas como “alquilo”, “cicloalquilo”, “heterociclo”, “arilo”, “heteroarilo”, “halógeno”, “sustituido”, “alcoxilo”, “alqueno” y “alquino”. Cabe señalar que también se limitó drásticamente el alcance de los radicales, R^{4b} , R^1 y R^9 en la nueva Reivindicación 1, y de R^{13} en la nueva Reivindicación 6. Adicionalmente, las referencias a los sustituyentes R^a y R^b originalmente se encontraban definidas en la Reivindicación original 16 (nueva Reivindicación 1).

Teniendo en cuenta las sugerencias de la Examinadora, y en un esfuerzo por brindar mayor claridad a la materia reivindicada, se retiraron las antiguas Reivindicaciones 1-15, 25-31, 33-42 del Capítulo Reivindicatorio Modificado adjunto, de tal forma que las nuevas Reivindicaciones 1-12 reclaman únicamente compuestos de fórmula VI.

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ii) Con respecto a los términos “enantiomeros”, “esteroisomeros”, “rotámeros”, “tautomeros”, “diastereoisomeros”, “antropisomeros”, “racematos”, “N-oxido de piridina”, me permito respetuosamente argumentar que el uso de estos términos y otros análogos que indican la posibilidad de obtener la invención en diversas modalidades, es una práctica aceptada a nivel mundial en el ámbito de patentes, ya que como la Examinadora debe saberlo, una patente de invención no se limita a una única modalidad, sino que cubre un concepto inventivo global que abarca diferentes modalidades (diversas materializaciones de la invención) ligadas por un efecto técnico común, que finalmente delimita el alcance de la invención. De hecho, la misma SIC ha concedido repetidamente el privilegio de patente para solicitudes que incluyen los términos “tautomero”, “isómeros”, “esteroisómeros”, “enantiomeros”, “rotameros”, “racematos”, (por ejemplo las patentes: CO03-003.340, CO04-068.146, CO06-068.394, CO05-020.516, CO06-070.683, CO02-101.292, CO05-040.051, CO04-012.905, CO04-079.404, CO05-127.734, CO06-029.042, CO06-060.442, CO06-112.546, CO07-015.793 y CO06-127.752). Así las cosas, insisto respetuosamente que los términos “enantiomeros”, “esteroisomeros”, “rotámeros”, “tautomeros”, “diastereoisomeros”, “antropisomeros”, “racematos”, “N-oxido de piridina” en relación con los compuestos de fórmula VI de la presente invención, hacen parte del concepto inventivo reclamado y son perfectamente entendibles por cualquier persona medianamente versada en la materia, de tal manera que a la luz del Capítulo Descriptivo es perfectamente claro el alcance y los límites de la protección solicitada, entendiendo qué moléculas están cobijadas por las reivindicaciones y cuáles no.

Teniendo en cuenta lo anterior, considero superada las anteriores objeciones, en tanto que el Capítulo Reivindicatorio Modificado adjunto fue modificado conforme a las observaciones realizadas por la Examinadora.

4. a- La Examinadora señala que la presente solicitud no cumple con el requisito de unidad de invención dado que se refiere a por lo menos 2 grupos inventivos, correspondientes a:

1. Compuestos de Fórmula VI, donde el sustituyente del anillo tiazol que no pertenece a la unidad macrocíclica, se une a éste a través de un átomo que no es nitrógeno.
2. Compuestos de Fórmulas I, III, V, VI y VIII, donde el sustituyente sobre el anillo tiazol que no pertenece a la unidad macrocíclica, se une por un átomo de nitrógeno.

La Examinadora concluye que los anteriores compuestos no comparten características técnicas esenciales comunes.

b- Considero superada la anterior objeción, en tanto que las Reivindicaciones 1-15, 25-31 y 33-42 fueron retiradas del Capítulo Reivindicatorio Modificado adjunto. Así las cosas, el objeto de la presente solicitud se limita únicamente a los compuestos de Fórmula VI de la nueva Reivindicación 1 (antigua Reivindicación 16).

5. a- La Examinadora indica que el artículo “Components of the GE2270 Complex Produced by *Planobispora rosea* ATCC 53773”¹ (D1), afecta la novedad de la presente invención.

La Examinadora argumenta que el compuesto de fórmula VI, donde el sustituyente del anillo tiazol que no pertenece a la unidad macrocíclica se une a éste por medio de un átomo que no es nitrógeno, se encuentra divulgado en D1, que enseña derivados de GE2270, útiles como agentes antibacteriales.

Adicionalmente, la Examinadora señala que D1 divulga el factor A de los GE2270, en el cual el sustituyente sobre el anillo tiazol que no pertenece a la unidad macrocíclica, se une por un átomo que no es nitrógeno y el sustituyente es un anillo de oxazol, lo que coincide dentro del alcance de la presente solicitud, donde R es –(heteroarilo)-A-G-Q-J.

La Examinadora concluye que las características esenciales del compuesto de la presente solicitud habían sido divulgadas previamente en D1, el cual da a conocer antibióticos factores de GE2270, cuya fórmula estructural es idéntica al compuesto de fórmula VI de la presente invención.

b- i) En primer lugar, nuevamente llamo respetuosamente la atención de la Examinadora sobre el Capítulo Reivindicatorio Modificado adjunto, en el cual se limitó el alcance de la nueva Reivindicación 1 (antigua Reivindicación 16), de manera que R ahora significa únicamente $N(R^1)_2$ y $N(R^1)$ -A-G-Q-J. Es decir, el sustituyente –(heteroarilo)-A-G-Q-J fue eliminado del Capítulo Reivindicatorio Modificado adjunto.

¹ J Antibiot (Tokyo). 1995 Sep;48(9):1039-42. Components of the GE2270 complex produced by *Planobispora rosea* ATCC 53773. Selva E, Ferrari P, Kurz M, Tavecchia P, Colombo L, Stella S, Restelli E, Goldstein BP, Ripamonti F, Denaro M.

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Así las cosas, los compuestos reclamados en la presente solicitud reclaman características técnicas esenciales totalmente diferentes que las divulgadas por el arte previo citado por la Examinadora, ya que en ningún momento el sustituyente R de la presente solicitud se une al anillo tiazol por medio de un átomo que no es nitrógeno. Todo lo contrario, el sustituyente R de la nueva Reivindicación 1 (antigua Reivindicación 16) se une al anillo tiazol **necesariamente** por un átomo de nitrógeno, en contraste con los compuestos divulgados por D1, en los cuales el sustituyente sobre el anillo tiazol que no pertenece a la unidad macrocíclica se une por un átomo que **no** es nitrógeno.

En este punto vale la pena recordar que para fundamentar una afectación válida contra la novedad de una invención, el arte previo debe divulgar explícitamente todas y cada una de las características de la invención.² Es más, el Tribunal Andino de Justicia (TAJ) ha determinado que para establecer una objeción válida contra la novedad, la divulgación del arte previo debe ser lo suficientemente detallada para que una persona versada en la materia pueda usar dicha información para reproducir y explotar la invención.³ En el presente caso, los compuestos específicos divulgados en D1 son completamente diferentes a los aquí reclamados por tanto, la materia aquí reclamada es novedosa sobre D1.

Nada en el arte previo citado por la Examinadora anticipa o enseña los compuestos de la presente solicitud. Los compuestos de fórmula VI reclamados en las Reivindicaciones 1-12 revisten de novedad.

ii) Ahora bien, con el ánimo de brindar mayor evidencia sobre la novedad y nivel inventivo de la presente invención, me permito poner a consideración de la Examinadora dos artículos del Journal of Medicinal Chemistry, los cuales contienen evidencia experimental relacionada con los efectos sorprendentes e inesperados de los compuestos de la presente solicitud.

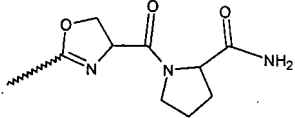
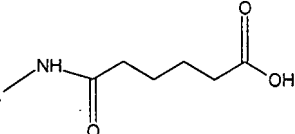
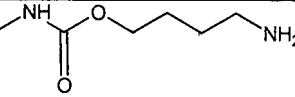
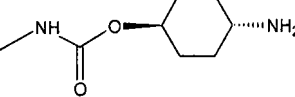
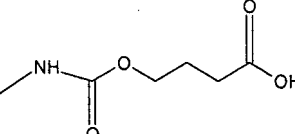
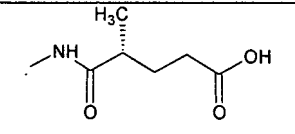
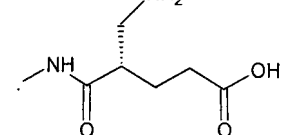
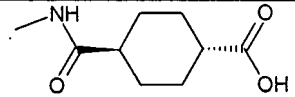
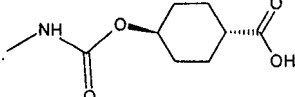
En el primer artículo de La Marche et al.⁴(A1), se evidencian las concentraciones mínimas inhibitorias en µg/mL y los valores de dosis efectivas 50 en mg/Kg, para los compuestos aquí reclamados y los compuestos divulgados por D1. Por ejemplo, en las páginas 8102-8104 del

² Manual Andino de Patentes. (2004). Sección 9.4. (disponible en <http://www.comunidadandina.org/public/patentes.pdf>).

³ Proceso 06-IP-89 (<http://intranet.comunidadandina.org/Documentos/Procesos/209-IP-2005.doc>); y Proceso 36-IP-2004 (<http://www.notinet.com.co/serverfiles/servicios/archivos/30jul04/ca36-04.htm>)

⁴ Journal of Medicinal Chemistry. Antibacterial Optimization of 4-Aminothiazolyl Analogues of the Natural Product GE2270 A: Identification of the Cycloalkylcarboxylic Acids. LaMarche, M., Leeds, J., Amaral, K. ACS. 2011.

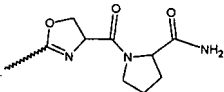
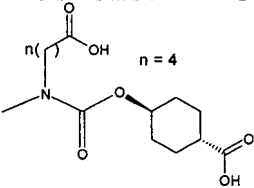
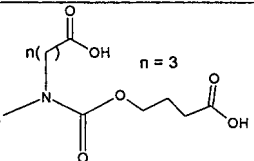
citado artículo, se aprecian resultados para los compuestos de la presenta solicitud. Así, en la siguiente tabla se realiza una comparación con compuestos divulgados por D1, así:

Sustituyente R	Concentración Mínima Inhibitoria (µg/mL)				Dosis Efectiva 50 (mg/Kg)	
	Dosis Efectiva 50 (mg/Kg)					
	<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>S. aureus</i>	<i>E. faecalis</i>
D1 	1	No Reporta	2	>128	No Reporta	No Reporta
	0.06	0.125	0.125	4	10.6	5.5
	0.5	1	1	8	6.48	N.A
	0.5	0.5	1	8	19.3	<3.3
	0.03	0.06	0.06	1	5.4	3.1
	0.25	0.5	0.25	4	10.6	11.1
	0.5	1	1	>32	9.5	>60
	0.06	0.06	0.125	4	5.2	0.6
	0.06	0.03	0.125	2	4.3	0.2

Tal como se puede evidenciar en la tabla anterior, los resultados evidencian diferencias muy significativas entre los compuestos divulgados por D1 y los reclamados en la presente solicitud. Estos resultados evidencian efectos sorprendentes e inesperados, en tanto que revelan concentraciones mínimas inhibitorias **1600%** por debajo de las reveladas por los compuestos de

D1. Cabe recordar que la concentración mínima inhibitoria es una característica de vital importancia de los antibióticos, ya que a menor concentración mínima inhibitoria mayor potencia del antibiótico. De la misma forma, los valores de dosis efectiva 50 para los compuestos de la presente solicitud, evidencian efectos sorprendentes e inesperados, ya que demuestran una excelente potencia in vivo, así como una mínima cantidad de compuesto evidencia un mayor efecto bacteriostático sobre dos de los principales microorganismos gram positivos patógenos conocidos en la actualidad.

En el segundo artículo de La Marche et al.⁵ (A2), se evidencian las concentraciones mínimas inhibitorias en µg/mL y los valores de dosis efectivas 50 en mg/Kg para los compuestos aquí reclamados en relación con la inhibición específica sobre algunos microorganismos y en especial sobre *Clostridium difficile*. Por ejemplo, en las páginas 2380-2382 del citado artículo, se aprecian los resultados de los compuestos de la presenta solicitud. Así, en la siguiente Tabla se realiza una comparación con los divulgados por D1, así:

Sustituyente R de la Presente Solicitud	Concentración Mínima Inhibitoria (µg/mL)					Dosis Efectiva 50 (mg/Kg)	
	Dosis Efectiva 50 (mg/Kg)						
	<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>C. difficile</i>	<i>S. aureus</i>	<i>E. faecalis</i>
D1 	1	No Reporta	2	>128	No Reporta	No Reporta	No Reporta
	<0.06	<0.06	0.125	1	0.03	6.9	2.2
	<0.06	0.06	0.25	1	0.06	2.6	2.2

⁵ Journal of Medicinal Chemistry. Discovey of LFF571: An investigational agent for Clostridium difficle infection. LaMarche, M., Leeds, J., Amaral, K. ACS. 2012.

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Los resultados obtenidos para los compuestos de la presente solicitud evidencian un resultado sorprendente e inesperado, en especial para la concentración mínima inhibitoria para *Clostridium difficile*, la cual se encuentra en **0.03 µg/mL**, lo que sin duda representa un efecto sorprendente e inesperado, ya que anualmente hay como mínimo 15.000 muertes debido a infecciones generadas por *C. difficile*; además, el costo estimado para el tratamiento de infecciones por este microorganismo en los Estados Unidos está alrededor de los \$3 mil millones de dólares.⁶

Por otro lado, según los artículos mencionados A1 y A2, los valores de solubilidad en agua de los compuestos de la presente solicitud son totalmente inesperados y sorprendentes en comparación con los divulgados en D1. Estos resultados se enseñan específicamente en la Tabla 2 del artículo A2, en donde se evidencia que la solubilidad en agua llega a valores de 12 mg/mL, lo que representa una ventaja técnica para este tipo de antibióticos glucopéptidos ya que dichos antibióticos, por su estructura orgánica-hidrofóbica, presentan valores de solubilidad en agua inferiores a 0.001 mg/mL. Cabe resaltar que los valores de solubilidad en agua son de gran importancia en el desarrollo de nuevos medicamentos en vista de la necesidad biológica de disolverse en agua para poder circular en el plasma. El estado del arte enseña que este tipo de antibióticos a lo sumo alcanzan valores de solubilidad en agua cercanos a 0.001 mg/mL y la persona del oficio normalmente versada en la materia no esperaría obtener valores diferentes en la investigación ordinaria de este tipo de moléculas. Es decir, estos resultados evidencian efectos sorprendentes e inesperados en tanto que, revelan valores de solubilidad en agua **12.000 veces** por encima de los revelados por los compuestos de D1.

Nada en el arte previo citado por la Examinadora sugiere o motiva que un sustituyente R que se une al anillo tiazol **necesaria y exclusivamente** por un átomo de nitrógeno, pudiera generar efectos sorprendentes e inesperados como lo son, valores de solubilidad muy por encima por los divulgados por el arte previo, ni mucho menos concentraciones mínimas inhibitorias muy por debajo de las reveladas por D1. Adicionalmente, a la luz de las enseñanzas de D1, no existía motivación alguna para esperar que los compuestos reclamados en la presente solicitud pudieran resultar efectivos contra *Clostridium sp.*

En conclusión, los compuestos de la presente invención proporcionan inesperadamente una mayor actividad inhibitoria sobre microorganismos gran positivos, en especial sobre *Clostridium*

⁶ Journal of Medicinal Chemistry. Discovey of LFF571: An investigational agent for *Clostridium difficile* infection. LaMarche, M., Leeds, J., Amaral, K. ACS. 2012.

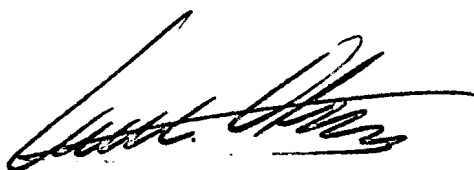
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sp, en contraste con los compuestos representativos divulgados en D1, los cuales presentan actividad normal sobre algunos microorganismos gram positivos. Este efecto no podía predecirse de la información divulgada en el arte previo, puesto que allí no se proveen datos ni indicaciones que sugieran que los compuestos específicos de la presente invención presentarían sorprendentemente una mayor actividad inhibitoria sobre microorganismos gram positivos, ni menos un tipo de microorganismo específico (*Clostridium sp*) que no se divulgaba en D1.

6. Por todo lo anterior, considero que las objeciones presentadas por la Examinadora en su primer examen de fondo, han sido resueltas y por consiguiente solicito respetuosamente que el Despacho tenga por cumplidos los requisitos de patentabilidad y proceda con la concesión de la patente.

Atentamente,



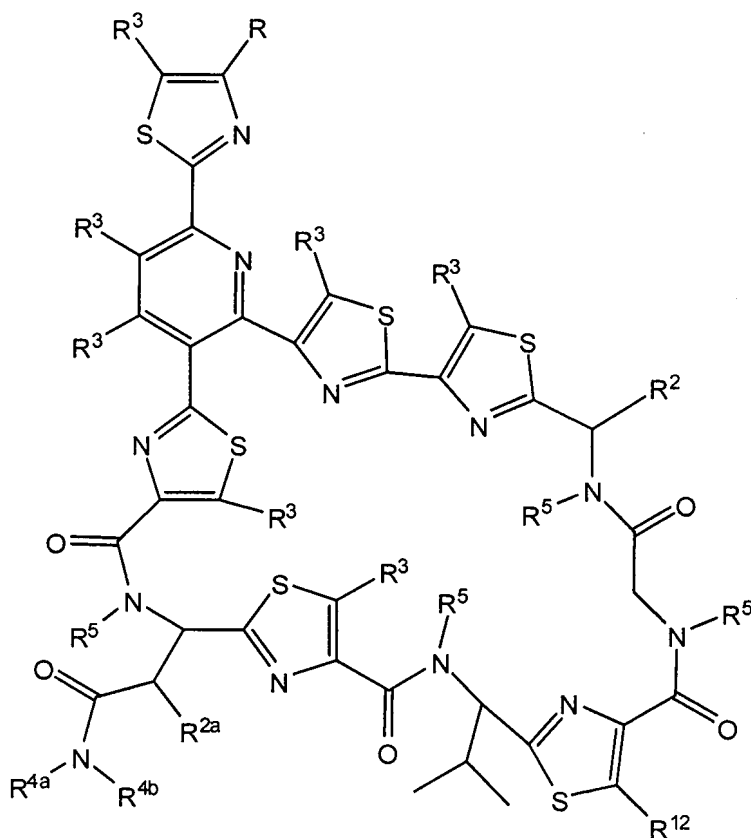
CARLOS R. OLARTE
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T.P. 74.295

Anexo:

- Capítulo Reivindicatorio Modificado.
- Artículos científicos A1 y A2.

CAPITULO REIVINDICATORIO MODIFICADO (Primer Examen de Fondo)

- Un compuesto de la Fórmula VI:



VI

y las sales farmacéuticamente aceptables, enantiómeros, estereoisómeros, rotámeros, tautómeros, diaestereómeros, atropisómeros, o racematos del mismo, incluyendo el N-óxido de piridina del mismo;

en donde:

R se selecciona a partir del grupo que consiste en $N(R^1)_2$ y $N(R^1)$ -A-G-Q-J;

A es un enlace, o se selecciona a partir del grupo que consiste en -C(O)-, -C(O)C(O)-, -C(O)O-, -C(O)N(R^{8a})-, -S(O)₂-, -S(O)-, -S(O)₂N(R^{8a})-, -S(O)N(R^{8a})-, -C(=NR⁸)N(R^{8a})-, -C(=NR⁸)N(R^{8a})C(O)-, -C(=NR⁸)-;

G está ausente, se selecciona a partir del grupo que consiste en -[C(R^a)(R^b)]_x-, -[C(R^a)(R^b)]_x-C(R^a)=C(R^a)-[C(R^a)(R^b)]_y-, -[C(R^a)(R^b)]_x-O-[C(R^a)(R^b)]_y-, y -[C(R^a)(R^b)]_x-N(R^{8a})-[C(R^a)(R^b)]_y-, o se selecciona a partir del grupo que consiste en -(cicloalquilo)-, -(heterociclo)-, -(arilo)-, y (heteroarilo)-, cada uno de los cuales tiene de 0 a 4 sustituyentes;

Q está ausente, se selecciona a partir del grupo que consiste en -C(O)-[C(R^a)(R^b)]_x-, -C(O)-[C(R^a)(R^b)]_x-C(R^a)=C(R^a)-[C(R^a)(R^b)]_y-, -C(O)-[C(R^a)(R^b)]_x-O-[C(R^a)(R^b)]_y-, and -C(O)-{N(R^{8a})-[C(R^a)(R^b)]_y}_p-, o se selecciona a partir del grupo que

consiste en -(cicloalquilo)-, -(heterociclo)-, -(arilo)-, -(heteroarilo)-, -C(O)-(cicloalquilo)-, -C(O)-(heterociclo)-, -C(O)-(arilo)-, y -C(O)-(heteroarilo)-, cada uno de los cuales tiene de 0 a 4 sustituyentes;

J se selecciona a partir del grupo que consiste en H, alquil C₁₋₄, halogeno, alcoxi C₁₋₄, hidroxilo, amino, mono- y di- alquilamino C₁₋₄, trialquilamonio C₁₋₄, N(R^{8a})C(O) alquil C₁₋₄, CO₂H, C(=O)N(R^{8a})₂, CH₂CO₂H, CH₂C(=O)N(R^{8a})₂, CO₂- alquil C₁₋₄, C(O) alquil C₁₋₄, P(O)(OH)₂, P(O)(O-alquil C₁₋₄)₂, alquilenos C₀₋₄-(cicloalquil) y alquilenos C₀₋₄-(heterociclo), cada heterociclo puede estar sustituido de 0 a 4 veces;

R^a se selecciona a partir del grupo que consiste en H, alquilo, F, CO₂H, CO₂-alquilo, -N(R¹)₂, -OR¹, -(CH₂)₀₋₄-J, y -R^{4b};

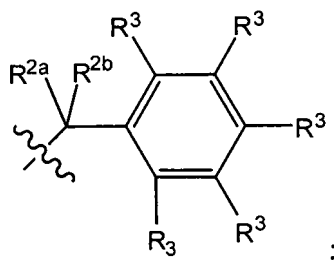
R^b se selecciona a partir del grupo que consiste en H, alquilo, y F;

p es de 0, 1, 2, ó 3;

x e y se seleccionan cada uno independientemente en cada presentación, a partir de los enteros de 0 a 10;

R¹ se selecciona independientemente en cada presentación a partir de -H, alquilo sustituido con J, y -R^{4b};

R² es hidrógeno, alquil C₁₋₆, hidroxialquil C₁₋₆, alcoxiC₁₋₆alquil C₀₋₆, cicloalquil C₃₋₇alquil C₀₋₄, arilalquil C₀₋₄, o un residuo de la Fórmula:



R^{2a} se selecciona a partir del grupo que consiste en H, alquilo de 1 a 6 átomos de carbono, OH, OR^{4a}, OC(O)R^{4a}, OC(O)N(R^{8a})₂, y N(R^{8a})₂;

R^{2b} es H o alquilo de 1 a 6 átomos de carbono, o R^{2a} y R^{2b}, tomados en combinación, forman un grupo oxo o imino;

R³ y R¹² se seleccionan cada uno independientemente a partir del grupo que consiste en H, halógeno, OR^{4b}, -G-J, y N(R^{8a})₂;

R^{4a} se selecciona a partir del grupo que consiste en H y alquilo;

R^{4b} se selecciona a partir del grupo que consiste en alquilo;

R⁵ se selecciona a partir del grupo que consiste en H, alquilo, y R^{4b};

R⁸ se selecciona a partir del grupo que consiste en H, CN, NO₂, alquil C₁₋₆, cicloalquil C₃₋₇, y SO₂- alquil C₁₋₆;

R^{8a} se selecciona a partir del grupo que consiste en H, alquil C₁₋₆ cicloalquil C₃₋₇, alquil C₁₋₆ sustituido con J, y R^{4b};

en donde

“alquilo” significa grupos alquilo de cadena recta seleccionados entre, metilo, etilo, propilo, butilo, pentilo, hexilo, heptilo, octilo, nonilo, y decilo, y grupos alquilo de cadena ramificada seleccionados entre isopropilo, tert-butilo, e isobutilo;

“cicloalquilo” se refiere a uno o más anillos saturados y/o parcialmente saturados, en donde todos los miembros de los anillos son átomos de carbono, y representa ciclopropilo, ciclobutilo, ciclopentilo, ciclohexilo, cicloheptilo, ciclo-octilo, adamantilo, decahidro-naftalenilo, octahidro-indenilo, y las variantes parcialmente saturadas de los anteriores, en particular ciclohexenilo;

“heterociclo” o “heterociclilo” significa un heterociclo aromático o no aromático de 5 a 10 miembros que contiene de 1 a 4 heteroátomos seleccionados a partir del grupo que consiste en O, N, y S, e incluye los grupos bicíclicos;

“arilo” significa grupos seleccionados entre, fenilo, pirrol, furano, tiofeno, tiazol, isotiazol, imidazol, triazol, tetrazol, pirazol, oxazol, isoxazol, piridina, pirazina, piridazina, y pirimidina; “arilo” además se selecciona entre grupos bicíclicos y tricíclicos, los cuales son, naftaleno, benzoxazol, benzodioxazol, benzotiazol, benzoimidazol, benzotiofeno, metilendioxifenilo, quinolina, isoquinolina, antrilo, fenantrilo, naftiridina, indol, benzofurano, purina, deazapurina, o indolizina;

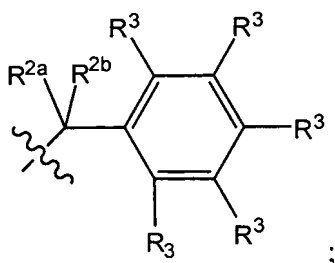
“heteroarilo” representa un anillo monocíclico o bicíclico estable de hasta 7 átomos en cada anillo, en donde cuando menos un anillo es aromático y contiene de 1 a 4 heteroátomos seleccionados a partir del grupo que consiste en O, N, y S;

“halo” o “halógeno” representa fluor, bromo, cloro, y yodo;

“sustituido” representa las fracciones que tienen sustituyentes que reemplazan a un átomo de hidrógeno sobre uno o más átomos de una molécula los cuales se seleccionan entre, halógeno, hidroxilo, alquilcarboniloxilo, arilcarboniloxilo, alcoxicarboniloxilo, ariloxicarboniloxilo, carboxilato, alquilcarbonilo, alcoxicarbonilo, aminocarbonilo, alquiltiocarbonilo, alcóxilo, fosfato, fosfonato, fosfinato, ciano, alquil-amino, dialquilamino, arilamino, diarilamino, alquilarilamino, alquilcarbonilamino, arilcarbonilamino, carbamoilo, ureido, amidino, imino, oxima, sulfidrilo, alquiltio, ariltio, tiocarboxilato, sulfatos, sulfonato, sulfamoilo, sulfonamido, nitro, trifluorometilo, azido, o heterociclilo, o cualquier combinación de los mismos; y en donde

“alcóxilo” significa grupos alquilo, alquenilo, y alquinilo sustituidos e insustituidos covalentemente enlazados a un átomo de oxígeno en donde “alquilo” es como se define arriba, en donde “alquenilo” representa grupos alifáticos insaturados análogos en longitud y en posible sustitución a los alquilos descritos anteriormente, pero que contienen cuando menos un doble enlace, y en donde “alquinilo” representa grupos alifáticos insaturados análogos en longitud y en posibles sustitución a los alquilos descritos anteriormente, pero que contienen cuando menos un triple enlace.

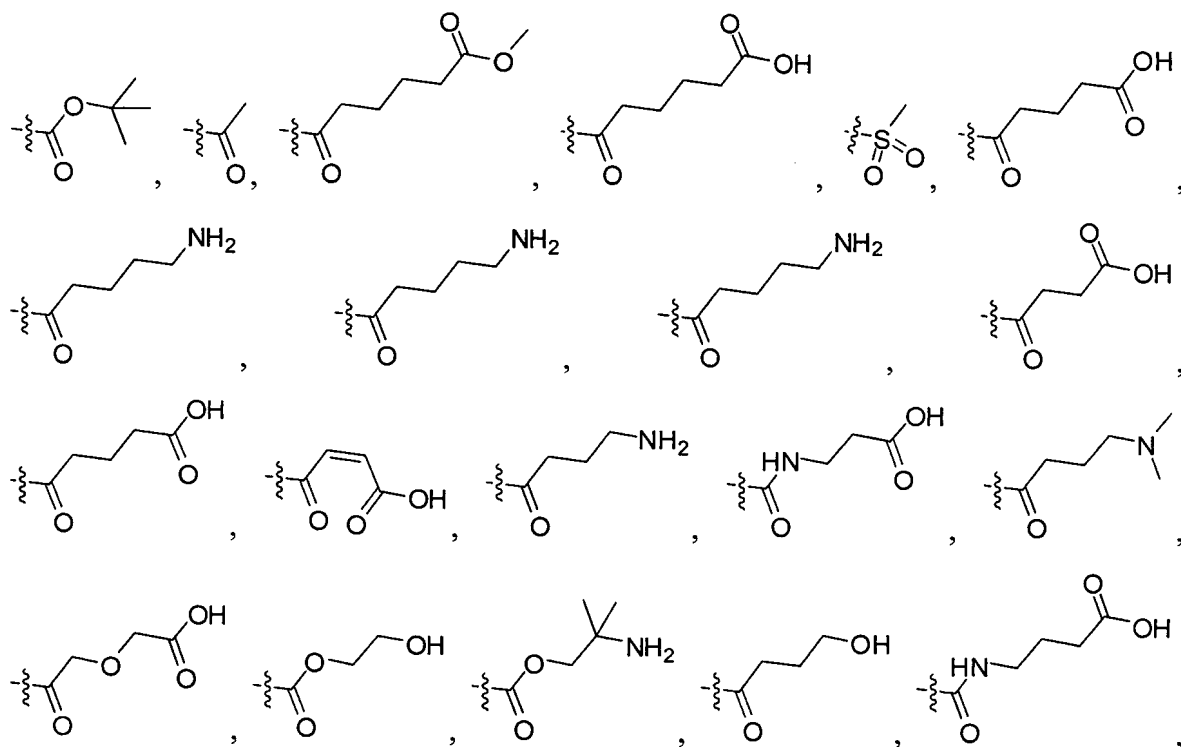
2. El compuesto de la Reivindicación 1, en donde R^2 es un residuo de la Fórmula:

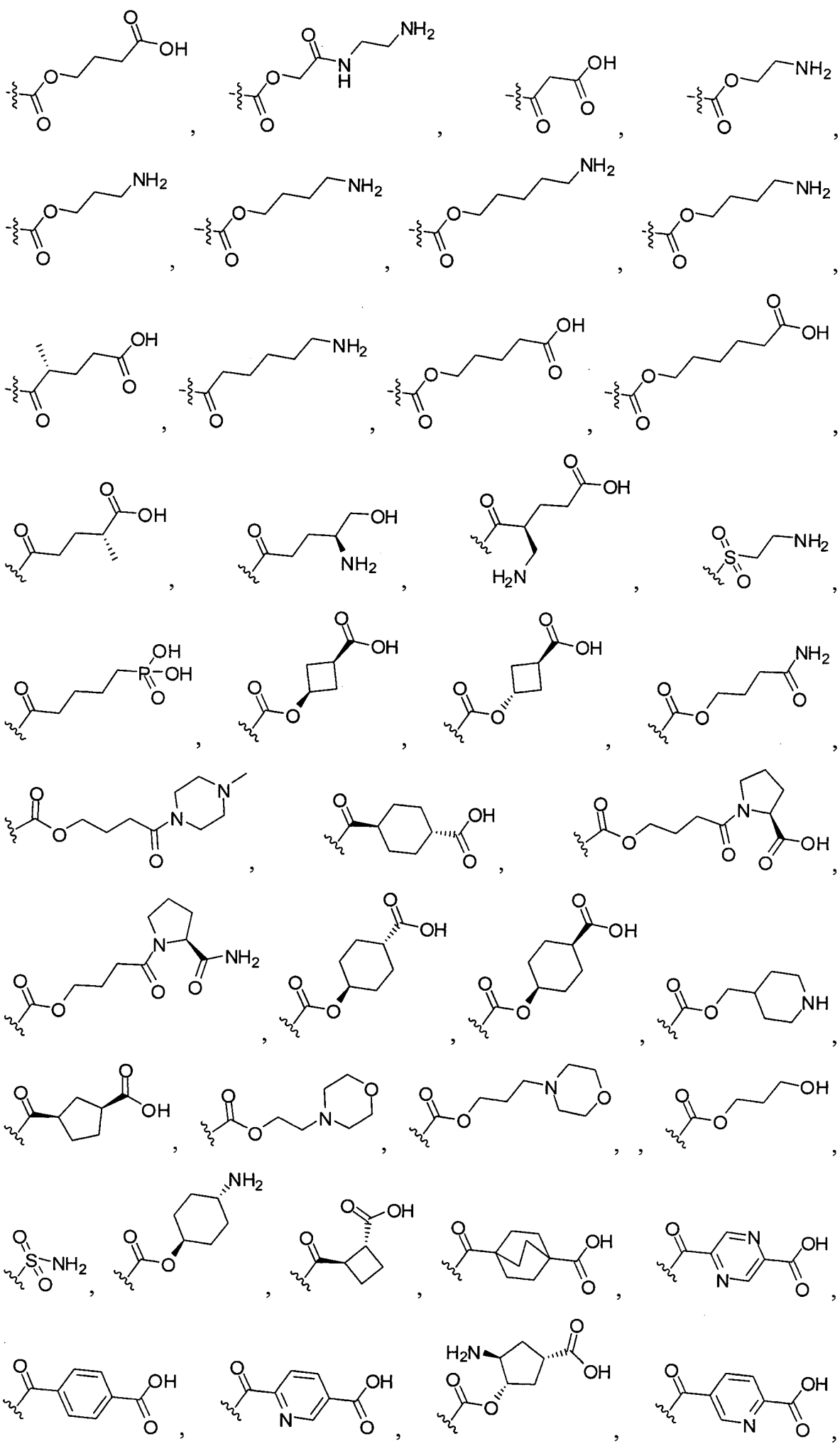


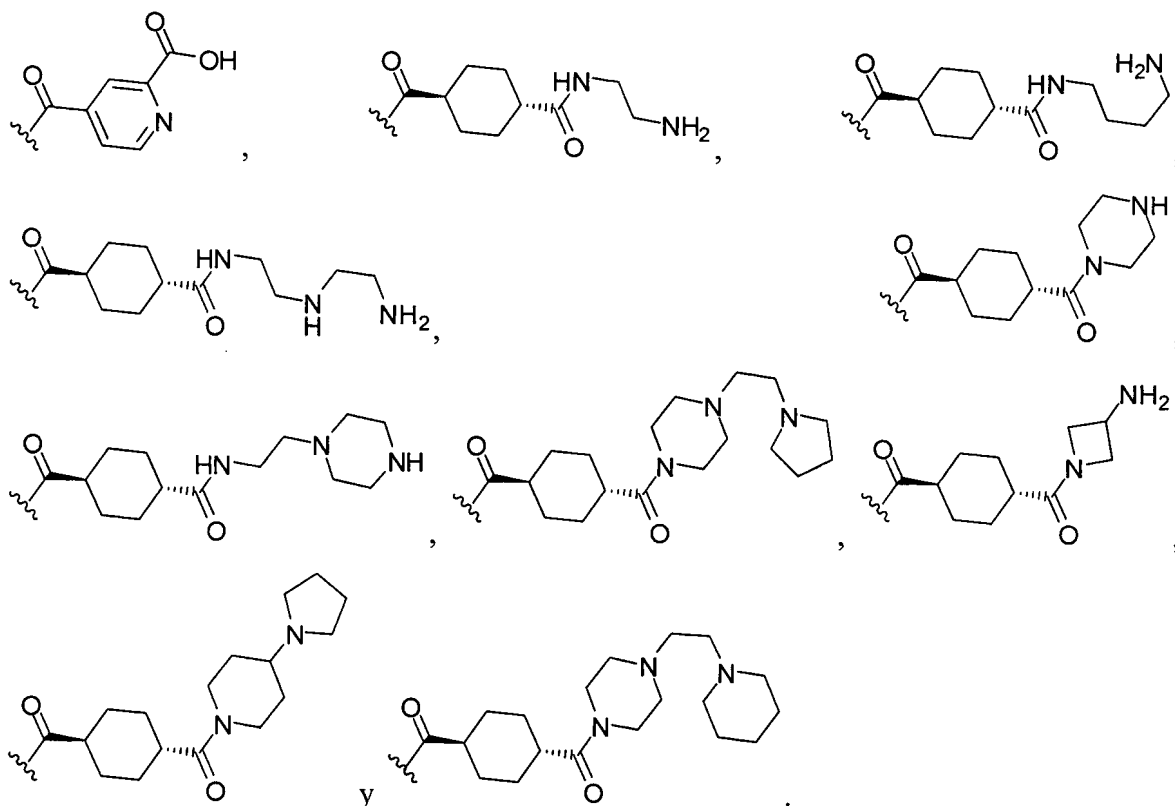
R^{2a} se selecciona a partir del grupo que consiste en H, alquil C_{1-6} , OH, OR^{4a} , $OC(O)R^{4a}$, $OC(O)N(R^{8a})_2$ y $N(R^{8a})_2$; y

R^{2b} es H o alquil C_{1-6} , o R^{2a} y R^{2b} , tomados en combinación, forman un grupo oxo o imino.

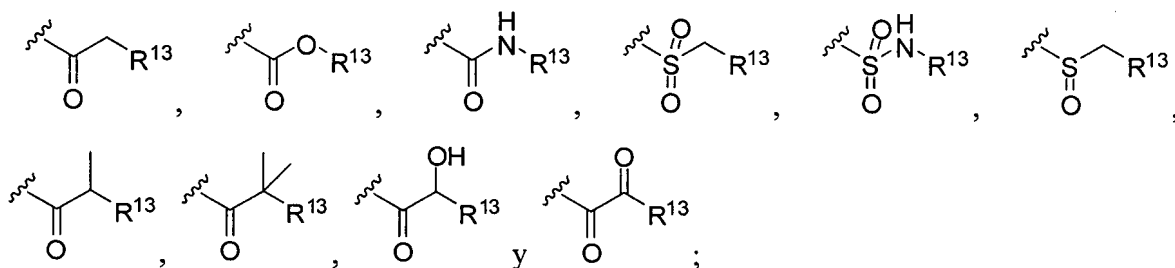
3. El compuesto de la Reivindicación 1, en donde R^{2b} , R^{4b} , y R^5 son H, y R^{4a} es CH_3 .
4. El compuesto de la Reivindicación 1, en donde R^{2b} , R^{4b} , y R^5 son H, R^{4a} es CH_3 , y R^{12} es CH_2-O-CH_3 .
5. El compuesto de la Reivindicación 1, en donde R^1 es $N(R^1)-A-G-Q-J$, R^1 es H, A-G-Q-J forman juntos H, o forman juntos un grupo funcional seleccionado a partir del grupo que consiste en:



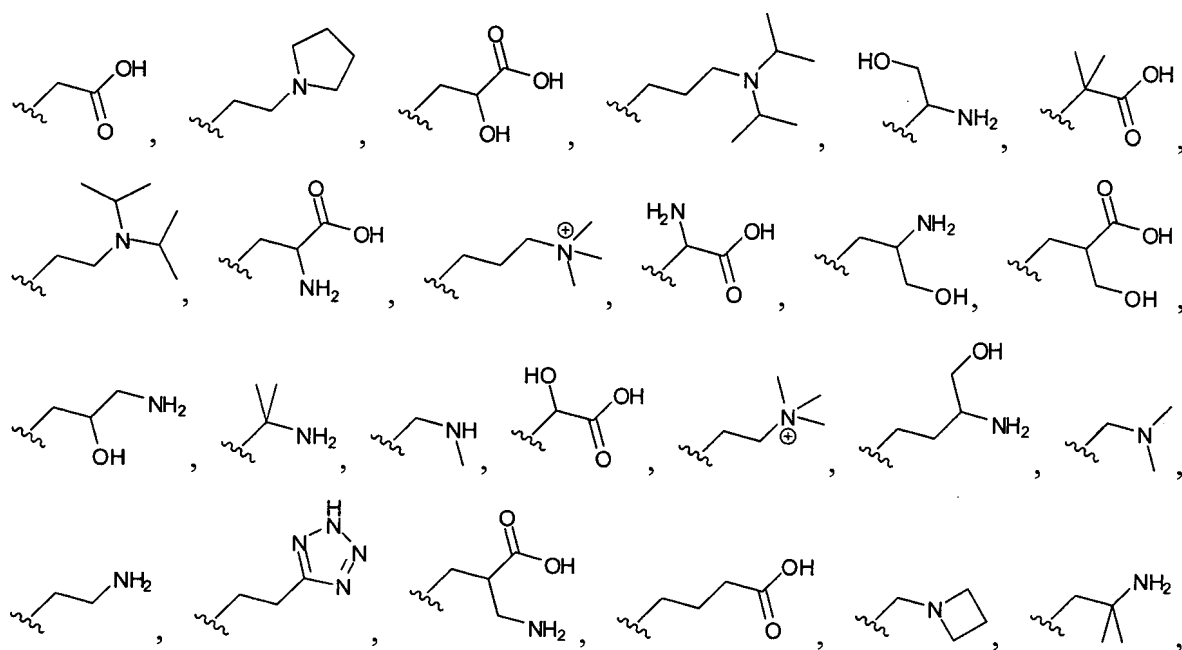


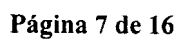


6. El compuesto de la Reivindicación 1, en donde R es N(R¹)-A-G-Q-J, R¹ es H, y A-G-Q-J forman juntos un grupo funcional seleccionado a partir del grupo que consiste en:

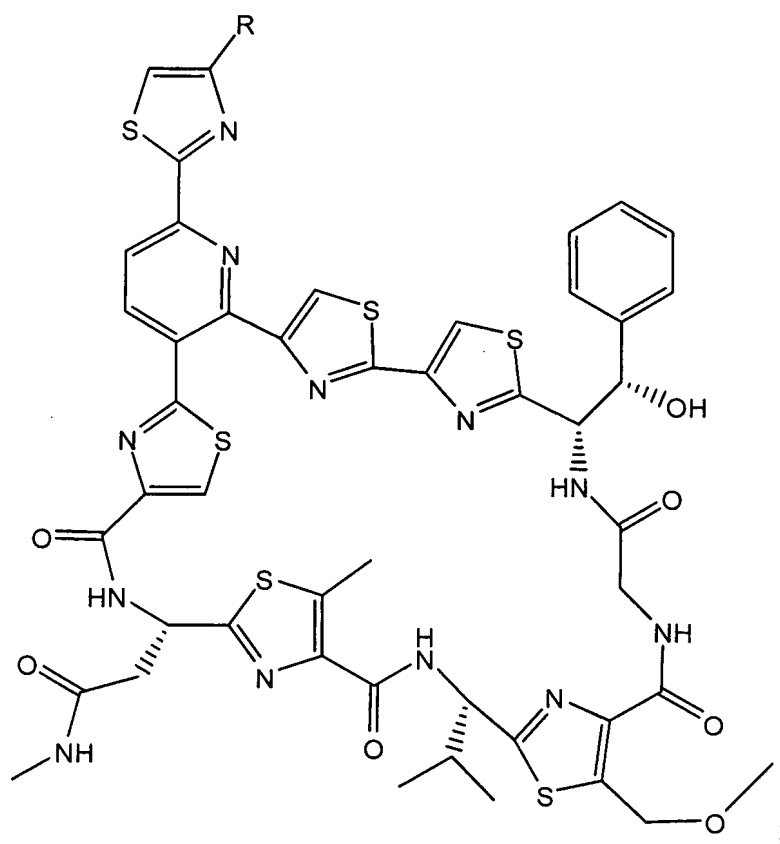


en donde R¹³ se selecciona a partir del grupo que consiste en hidrógeno, hidroximetilo, y amino-metilo, o a partir del grupo que consiste en:





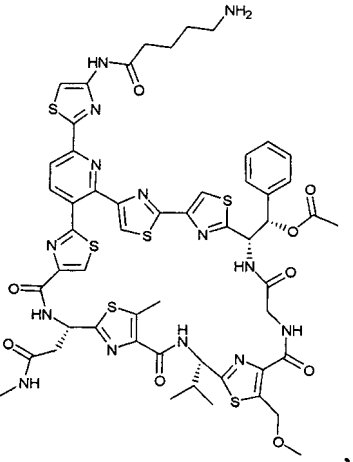
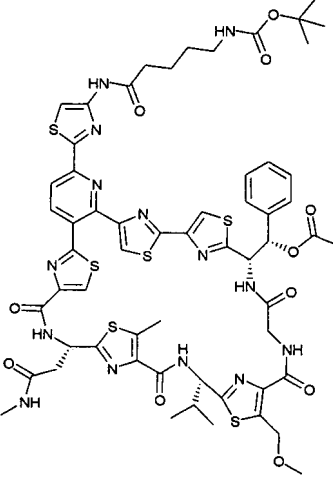
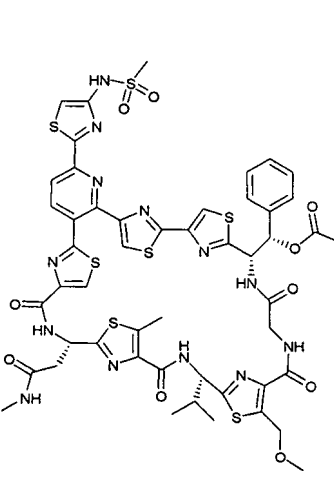
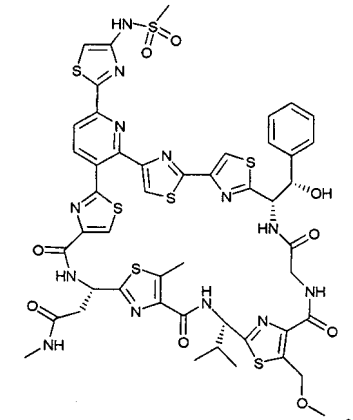
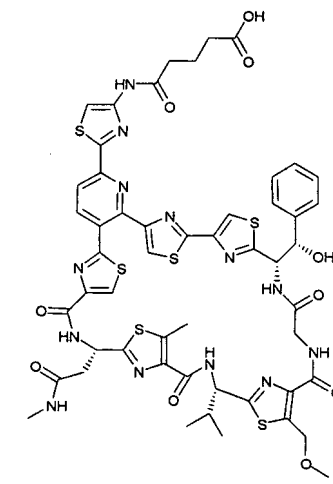
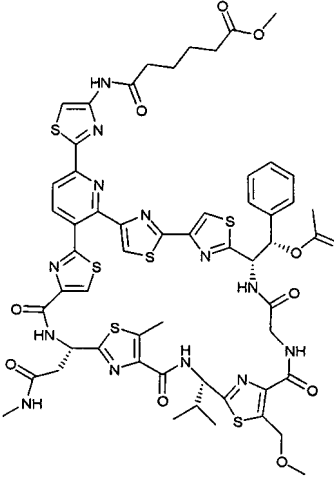
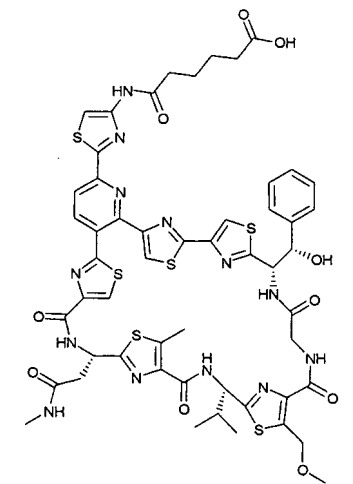
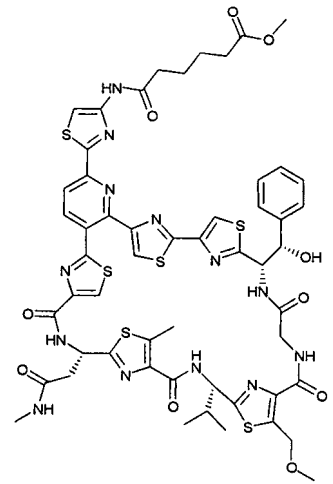
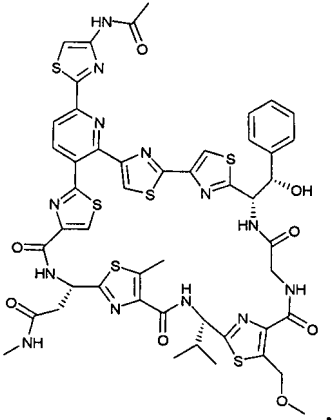
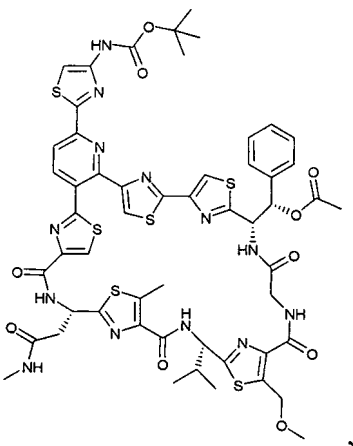
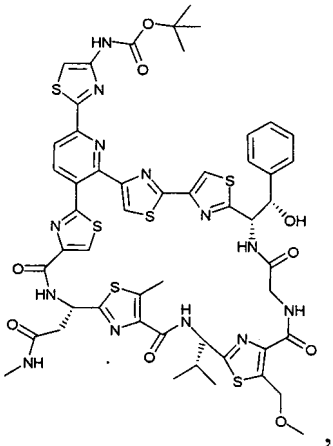
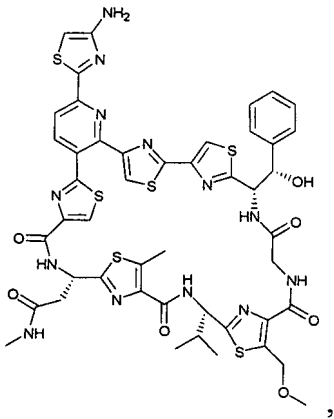
7. El compuesto de la Reivindicación 1, en donde R^{2a} es OH u OAc, y R^{2b} es H.
8. El compuesto de la Reivindicación 1, en donde A es C(O), C(O)O, ó C(O)NH;
G es cicloalquilo de 4 a 7 átomos de carbono;
Q está ausente; y
J es CO₂H ó CO₂-alquilo de 1 a 4 átomos de carbono.
9. El compuesto de la Reivindicación 1, en donde la Fórmula VI está representada por un compuesto de la Fórmula VII:

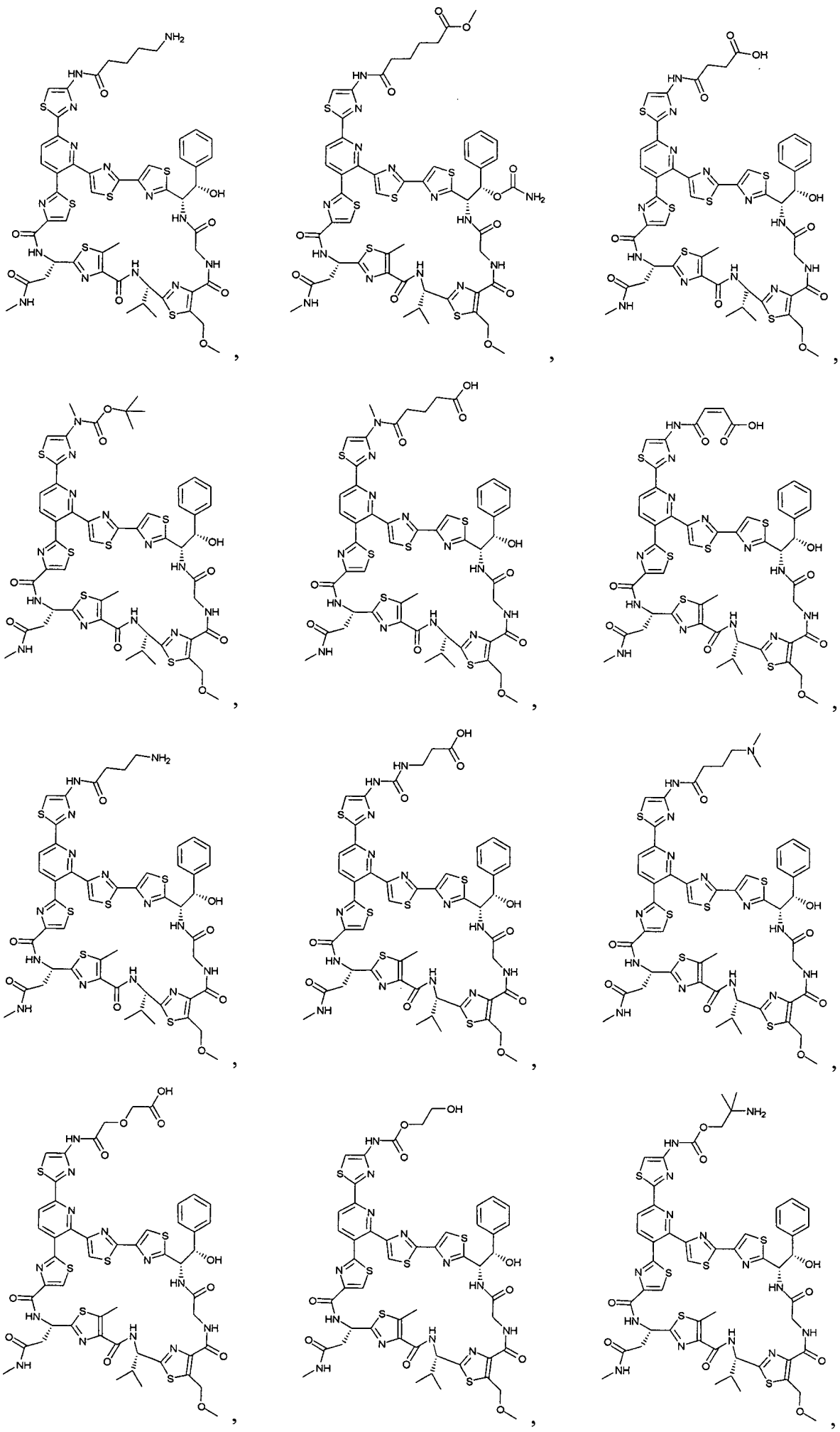


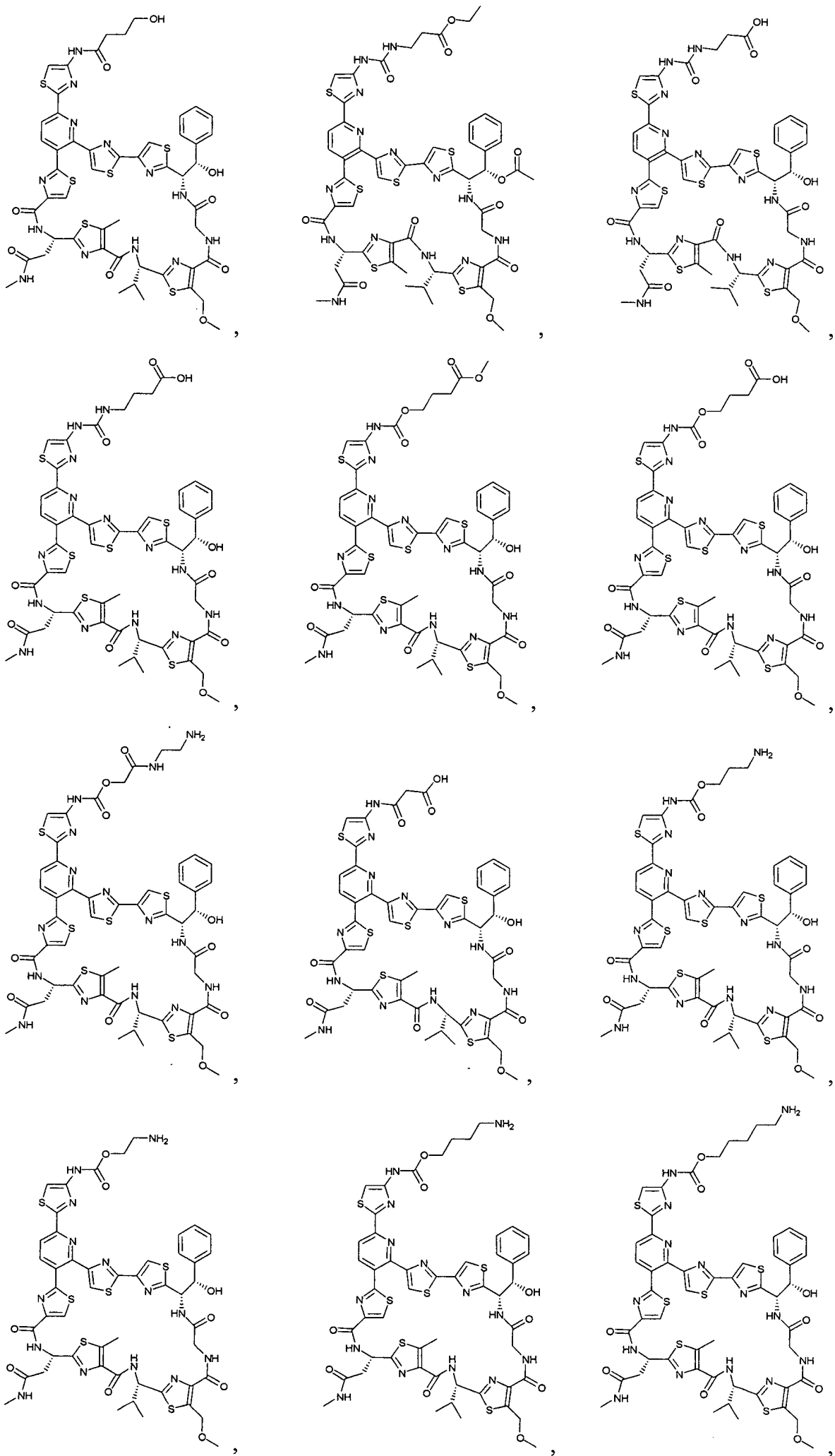
VII

y las sales farmacéuticamente aceptables del mismo.

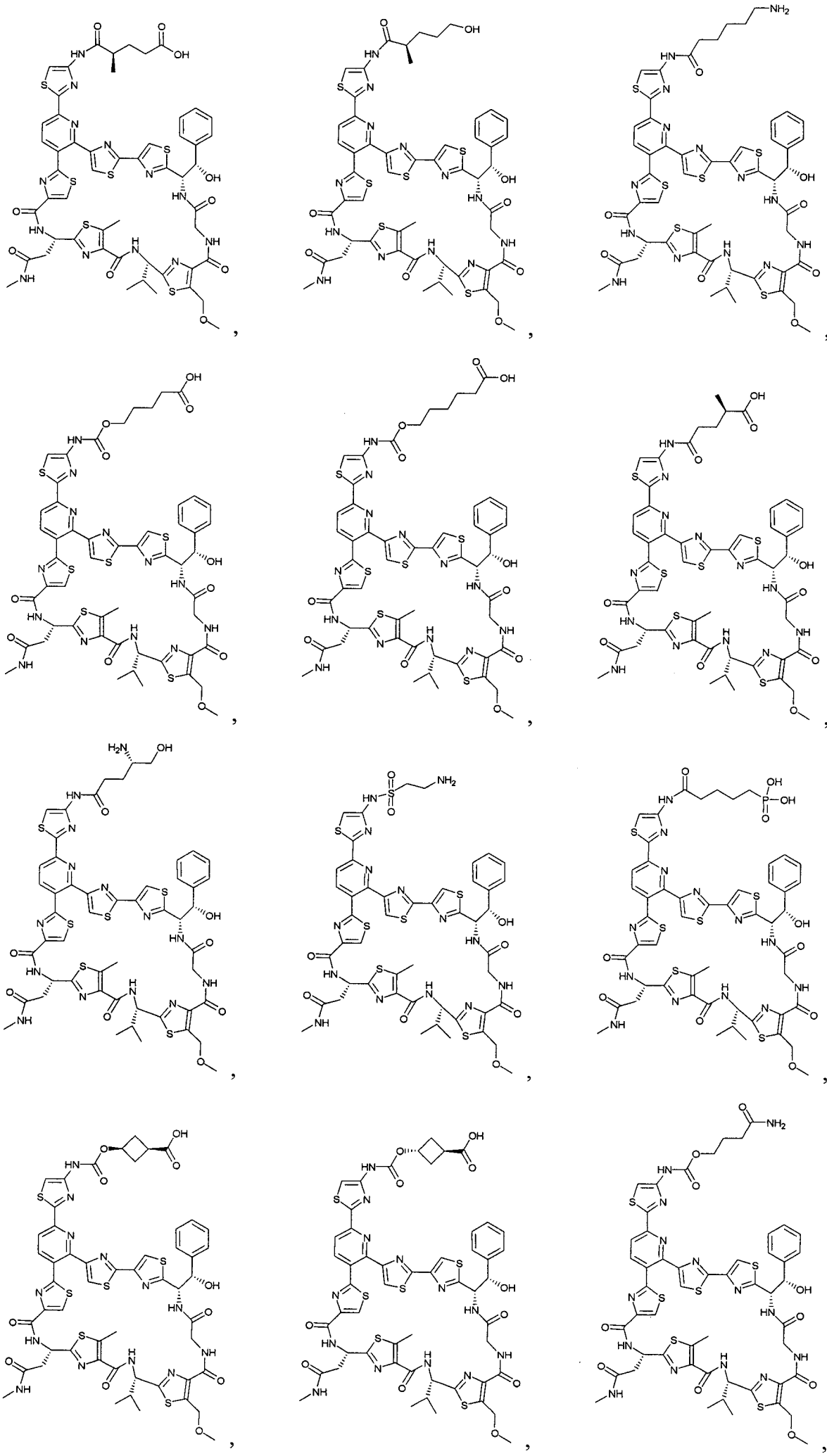
10. Un compuesto de acuerdo con la Reivindicación 1, el cual se selecciona a partir del grupo que consiste en:

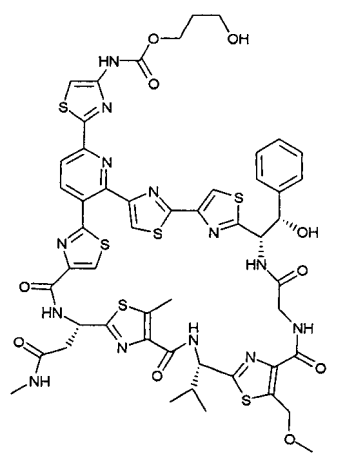
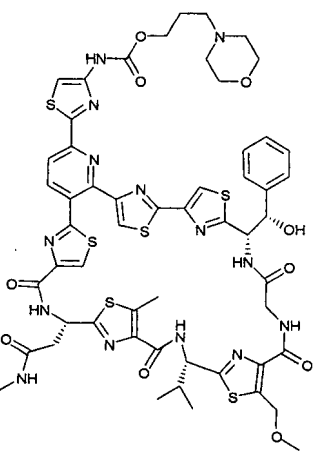
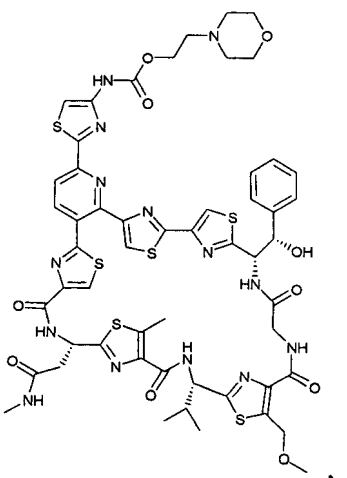
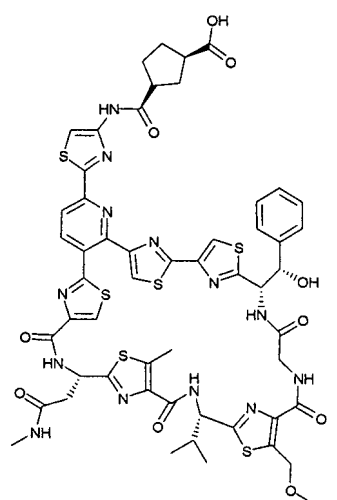
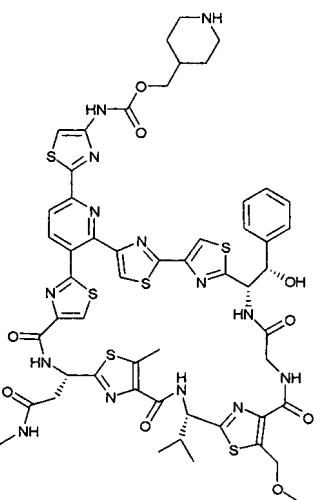
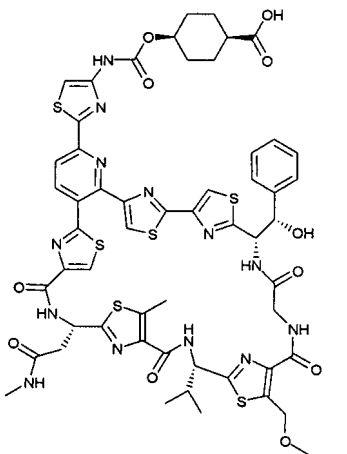
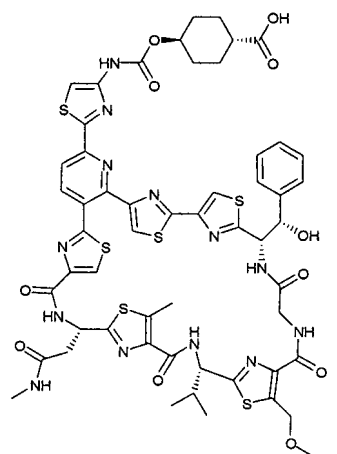
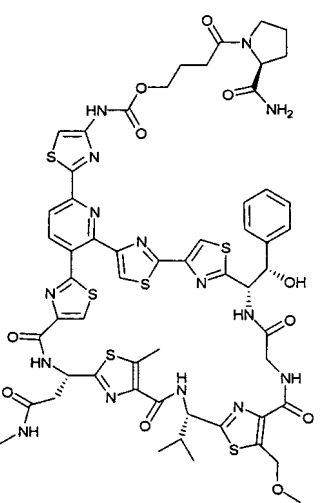
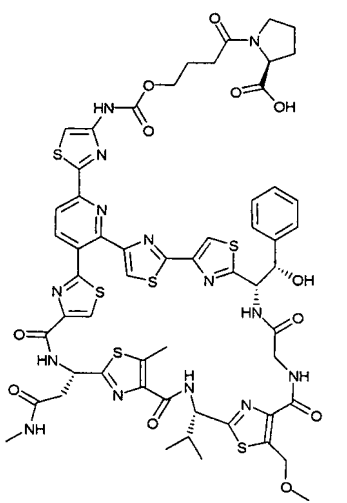
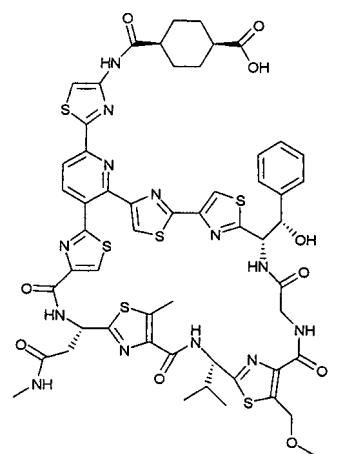
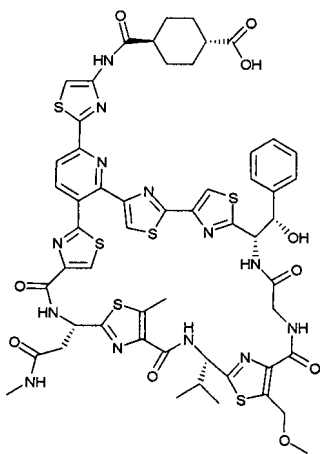
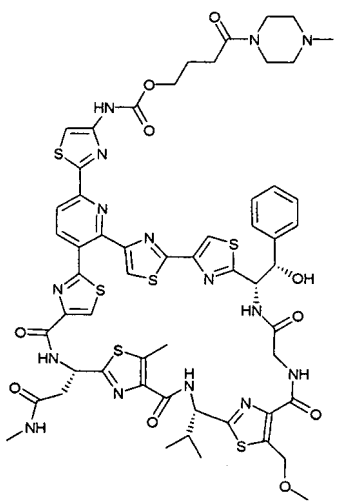


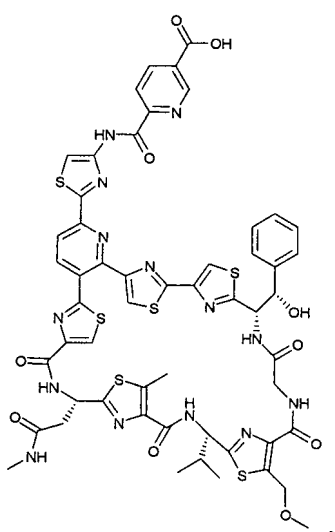
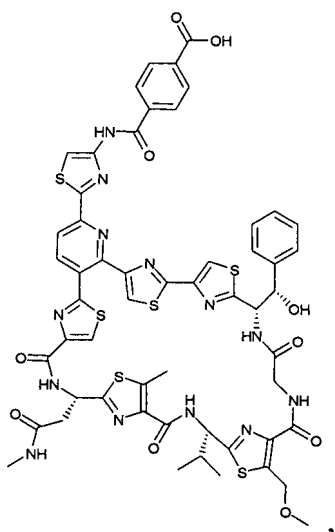
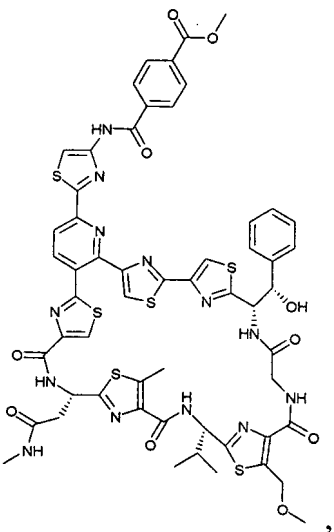
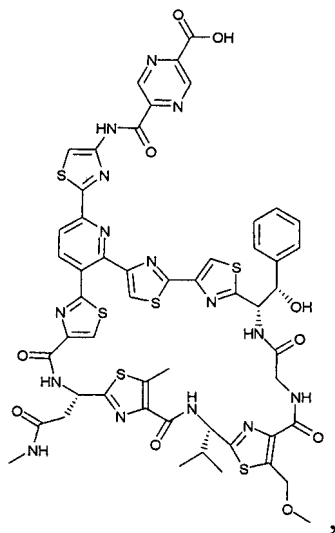
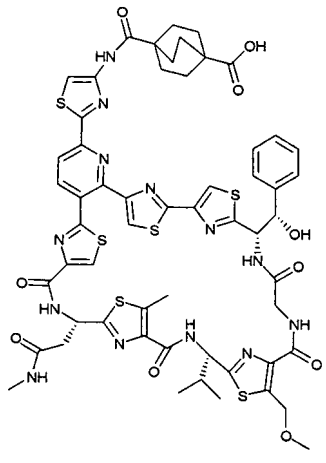
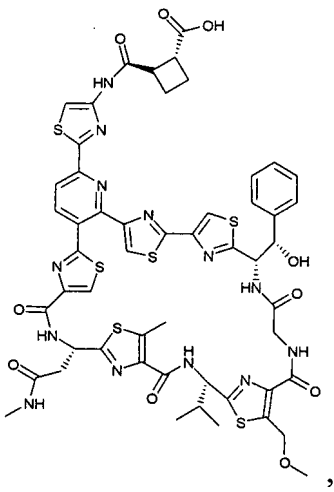
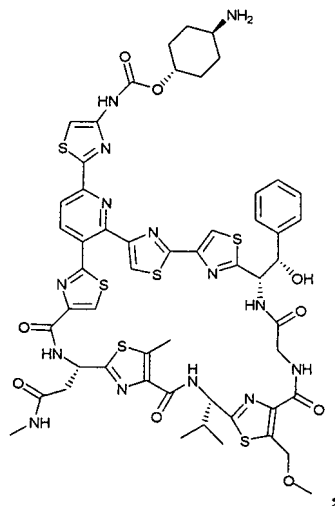
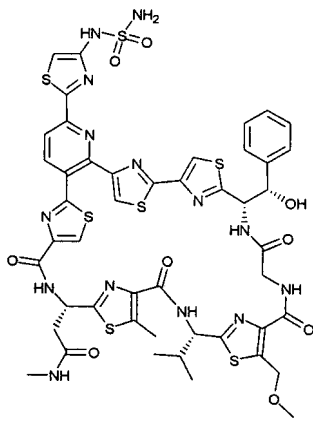
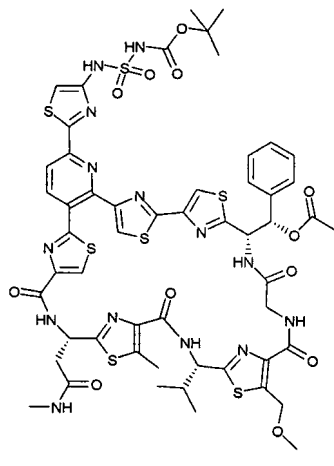




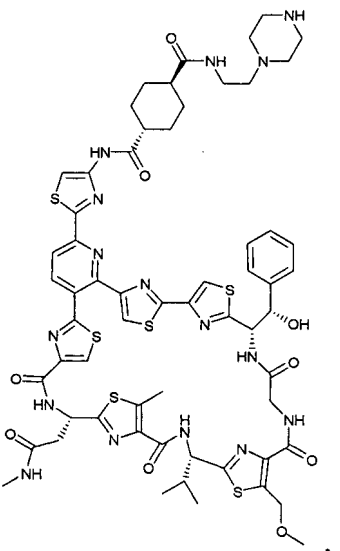
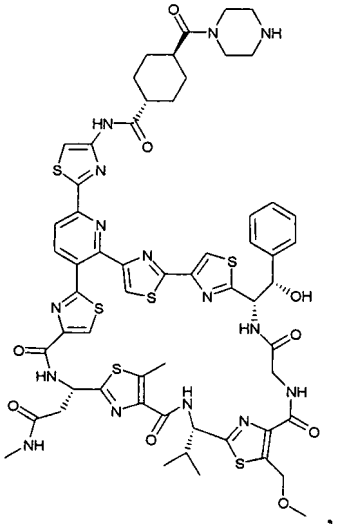
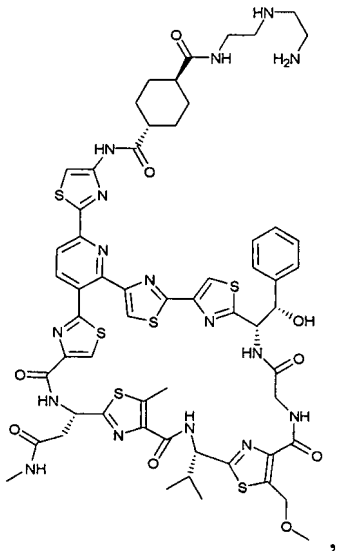
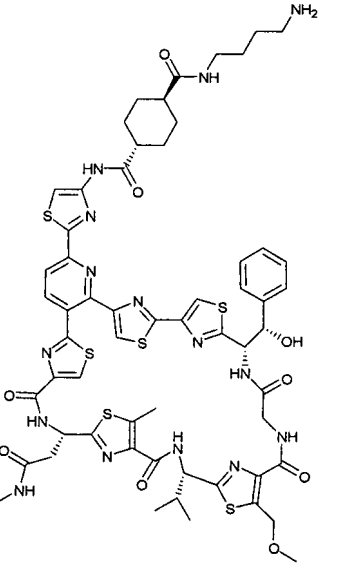
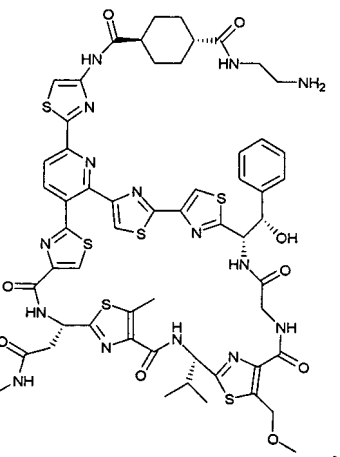
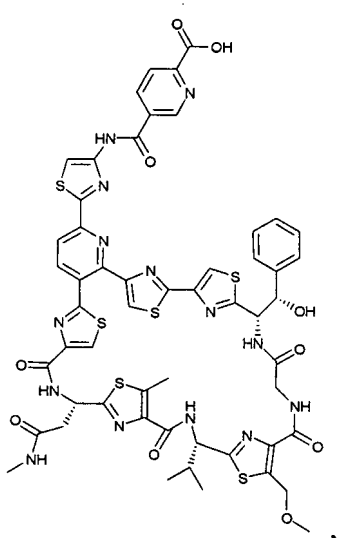
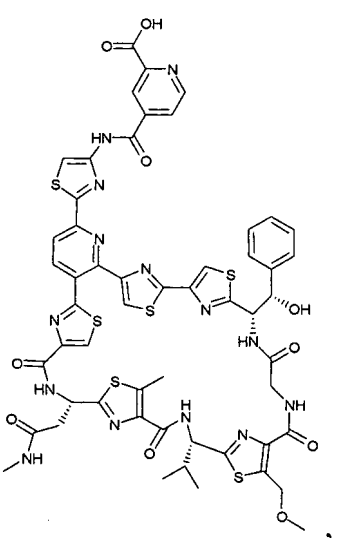
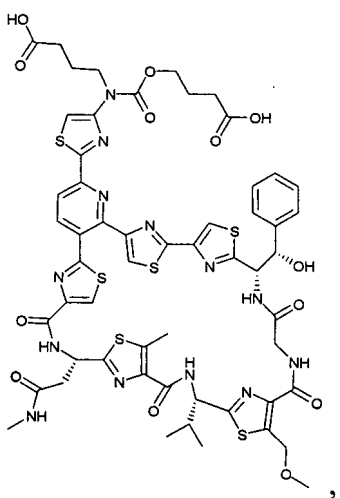
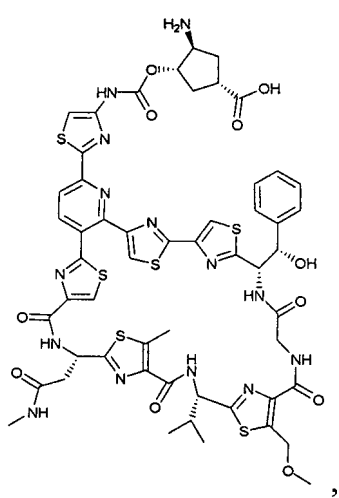
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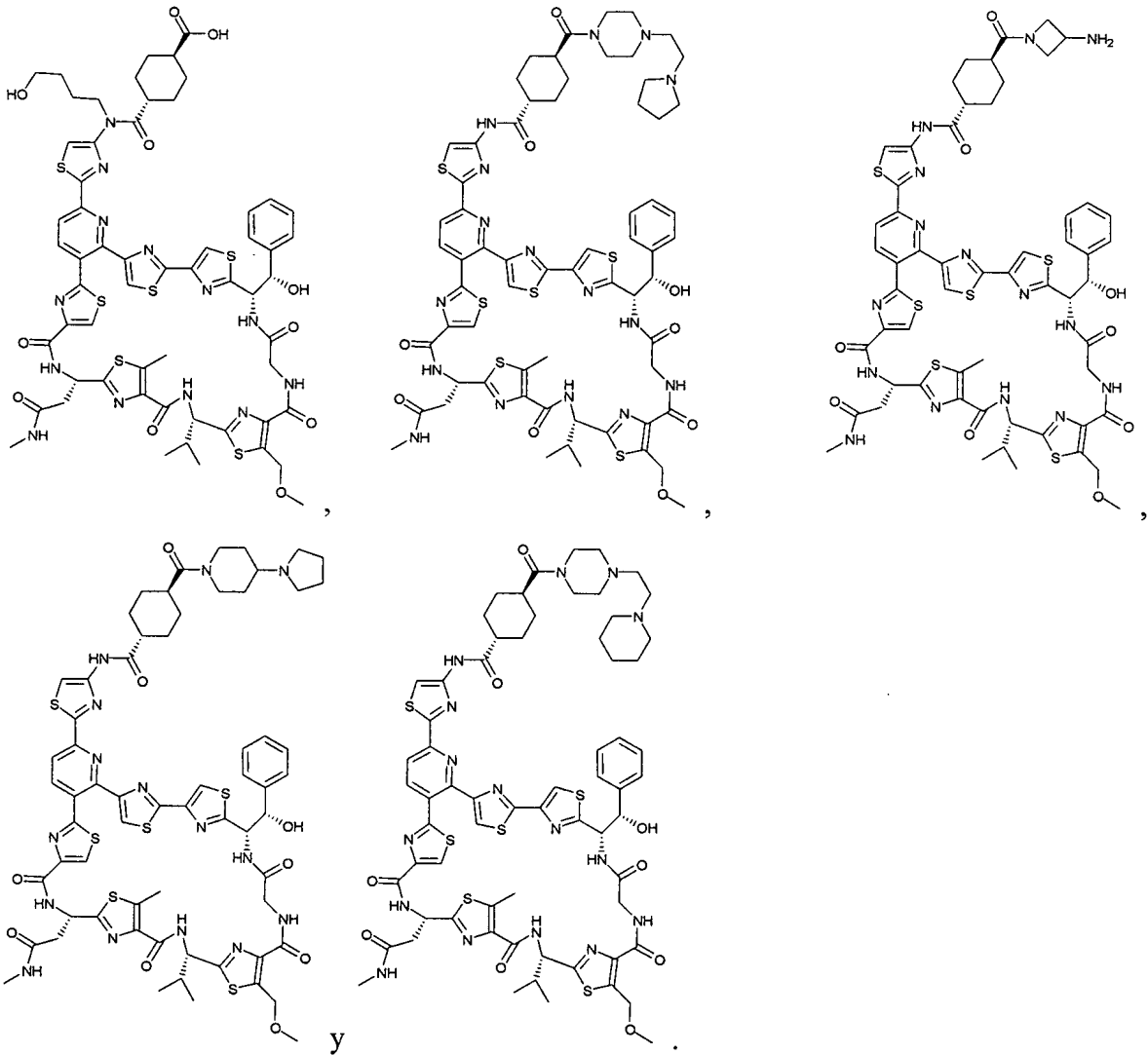






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11. Un tratamiento para infección bacteriana empacado, el cual comprende un compuesto de cualquiera de las Reivindicaciones 1 a 10, empacado con instrucciones para utilizar una cantidad efectiva del compuesto para tratar una infección bacteriana.

12. Una composición farmacéutica, la cual comprende un compuesto de cualquiera de las Reivindicaciones 1 a 10, y cuando menos un vehículo o diluyente farmacéuticamente aceptable.

Antibacterial Optimization of 4-Aminothiazolyl Analogues of the Natural Product GE2270 A: Identification of the Cycloalkylcarboxylic Acids

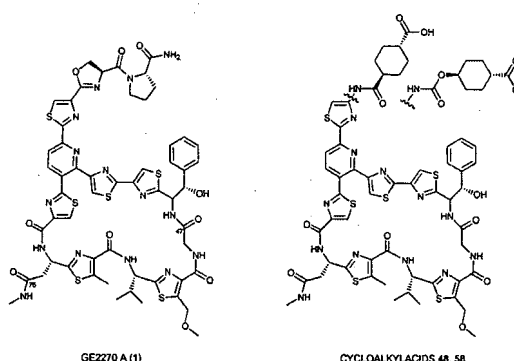
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Supporting Information

ABSTRACT: 4-Aminothiazolyl analogues of the antibiotic natural product GE2270 A (1) were designed, synthesized, and optimized for their activity against Gram positive bacterial infections. Optimization efforts focused on improving the physicochemical properties (e.g., aqueous solubility and chemical stability) of the 4-aminothiazolyl natural product template while improving the in vitro and in vivo antibacterial activity. Structure–activity relationships were defined, and the solubility and efficacy profiles were improved over those of previous analogues and 1. These studies identified novel, potent, soluble, and efficacious elongation factor-Tu inhibitors, which bear cycloalkylcarboxylic acid side chains, and culminated in the selection of development candidates amide 48 and urethane 58.



INTRODUCTION

Clinical resistance to marketed drugs is becoming increasingly common.¹ Therefore, the discovery of antibiotics that act via novel mechanisms of action remains a pressing unmet medical need in infectious disease care. In particular, treating Gram positive skin and soft tissue infections caused by methicillin resistant *S. aureus*, vancomycin resistant enterococci, and group A streptococci remain distinct clinical challenges. In 1991, Selva and co-workers from Lepetit Research Institute reported the structure and antibiotic activity of GE2270 A (1, Figure 1). This thiopeptide-based natural product was isolated from a fermentation broth of *Planobispora rosea* and found to inhibit the prokaryotic chaperone elongation factor Tu (EF-Tu).² The in vitro antibiotic profile against methicillin resistant staphylococci, vancomycin resistant enterococci, and group A streptococci was exquisite, with minimum inhibitory concentrations below 1 $\mu\text{g/mL}$.

Previously, we described lead finding activities associated with 1 concerning the identification, decomposition, and subsequent stabilization of 4-aminothiazolyl analogues.³ Because of promising initial in vitro results, the 4-aminothiazolyl template was selected for further medicinal chemistry optimization. Specifically, a substantial increase in aqueous

solubility would be necessary in order to formulate the compounds for infused intravenous drug delivery, as the aqueous solubility of 1 was undetectable in our assays (detection limit, 5 ng/mL). The improved aqueous solubility and formulatability would allow for in vivo efficacy evaluation in murine infection models (vide infra), an important preclinical milestone for this novel antibacterial chemical template, and also allow for comparison to the standards of care for soft tissue infections (i.e., linezolid, daptomycin).

As part of the strategy to further chemically stabilize the 4-aminothiazole macrocyclic appendage while increasing intrinsic aqueous solubility and improving cellular antibacterial activity, a variety of linkers, spacers, and termini were examined (Tables 1–5). Four organisms comprised our antibiotic activity characterization efforts: *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, and *Streptococcus pyogenes*.⁴ In addition, the antibacterial activity of all analogues against *Streptococcus pneumonia* (not shown) did not differ significantly compared to *Streptococcus pyogenes*. In order to confirm that the novel analogues were acting via inhibition of EF-Tu, all

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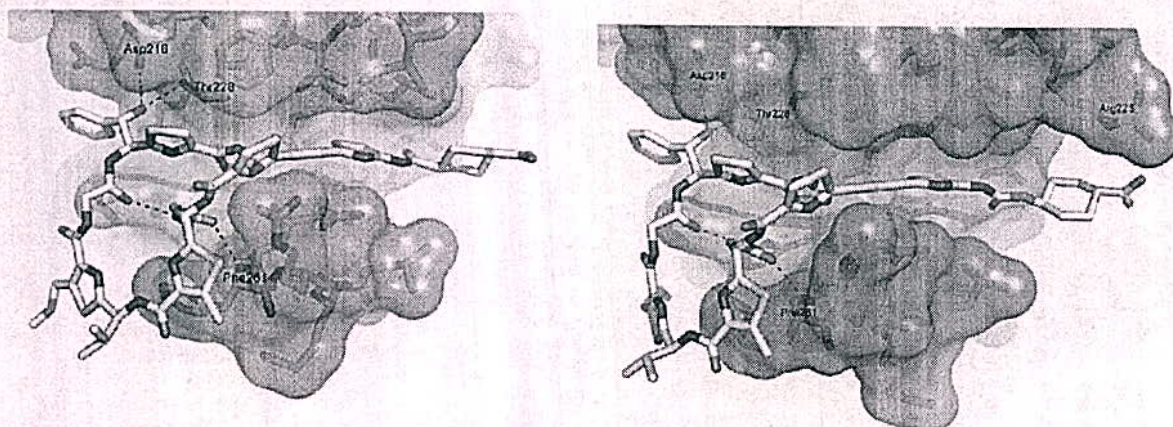


Figure 1. Cocrystal structures: *E. coli* EF-Tu and 48 (left) and 58 (right).

Table 1. Structure–Activity Relationships of the Linker Region

Linker–Spacer–Terminus	Compound	MIC assay: $\mu\text{g/mL}$				
		<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	G258D mut.
	(1)	0.5	0.25	0.25	1	
	(7)	1	1	2	>32	>32
	(8)	2	4	2	>32	>32
	(9)	4	>32	4	>32	>32
	(3)	>128	>128	>128	>128	>32
	(10)	2	4	4	>32	>32
	(11)	1	2	1	8	>32

compounds (Tables 1–5) were monitored for antibacterial activity against a 1-resistant mutant of *S. aureus* with a nucleotide change in the *tufA* gene (encoding EF-Tu). The mutation conferred a single amino acid substitution (G258D) in the thiopeptide binding pocket of the protein.⁵

RESULTS

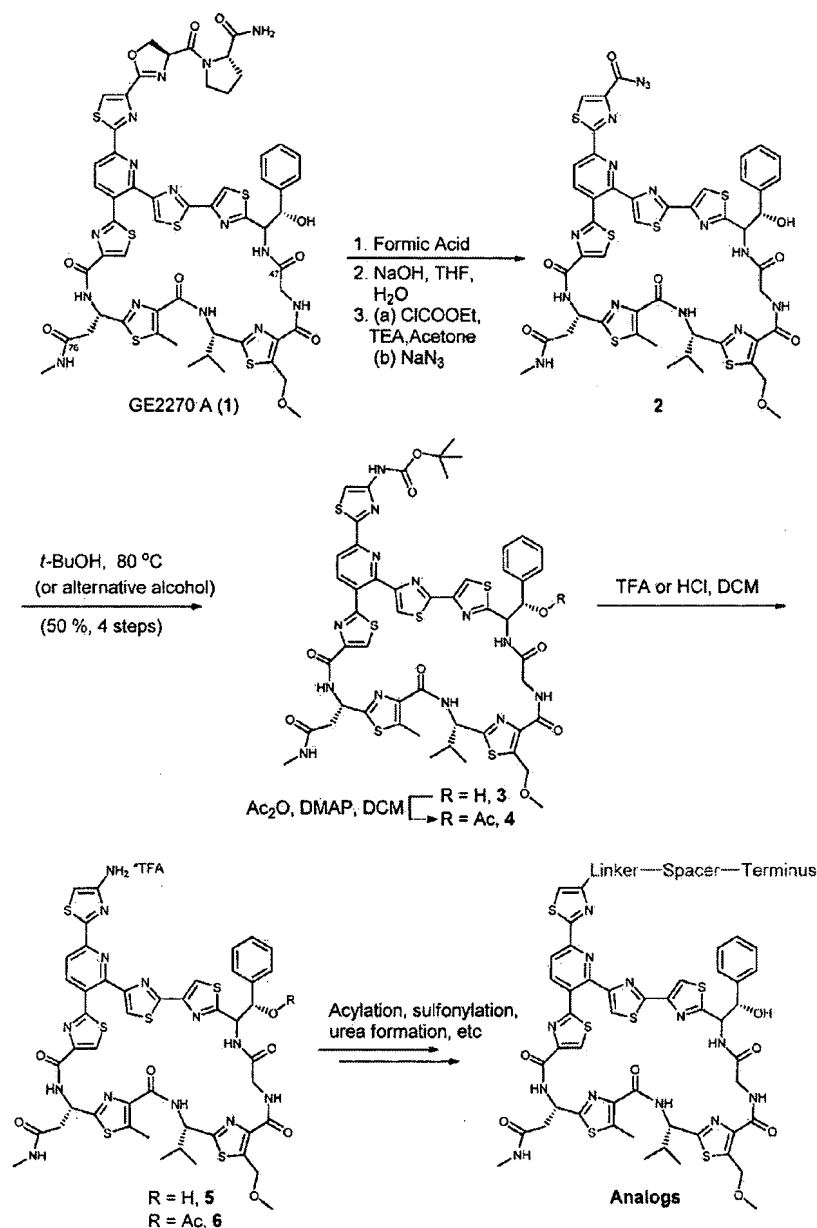
1 (Scheme 1) was first subjected to acid-catalyzed rearrangement and basic hydrolysis,⁶ which afforded the acid in high yield (quant.). The resulting 4-thiazolylcarboxylic acid was next activated with ethyl chloroformate to form the ethyl acylcarbonate, which was displaced in situ by azide. This three-step, one-pot process (50% yield) furnished the acylazide and also set the stage for the Curtius rearrangement. Alternatively, direct acylazide formation from the carboxylate utilizing diethylphosphorylazide led to variable, unreproducible yields. The acylazide was heated in *tert*-butyl alcohol (80 °C) or other alcohols (vide infra). Subsequent deprotection under acidic conditions (TFA or HCl in DCM) afforded 4-aminothiazole 5. Unfortunately basic workup, chromatography, or storage of the 4-aminothiazole free base proved difficult, as hygroscopicity ultimately led to chemical decomposition.³

However, functionalizing the amine via acylation, sulfonylation, urea formation, or imidazole formation stabilized the 4-aminothiazole. This approach enabled the design, synthesis, and biological evaluation of analogues that varied at the 4-aminothiazolyl side chain in three respects: the linker, the terminus, and the spacing between the linker and the terminus (Scheme 1).

During the initial optimization phase, a variety of linkers were evaluated for chemical stability and antibacterial activity. Amide, urethane, imidazole, sulfonamide, and urea linkers all stabilized the 4-aminothiazole (Table 1),⁷ but the antibacterial activity was variable. Compounds 7–11 displayed moderate to weak activity (MIC of 1 to >32 $\mu\text{g/mL}$). Universally, compounds 7–11 were weakly active against *S. pyogenes*. Compound 11 maintained the highest and broadest spectrum of activity. The structurally related boc-protected aminothiazole was inactive (MIC for 3, >128).

Analogues with varying termini were next designed, synthesized, and evaluated for antibacterial activity (Table 2). Nonpolar termini such as trifluoromethyl, carboxy ester, carboxamide, and PEGylated functional groups (compounds 13–16) maintained moderate (>1–4 $\mu\text{g/mL}$) to good (<1 $\mu\text{g/mL}$)

Scheme 1. Synthesis of 4-Aminothiazolyl Analogues



mL) MICs, although without activity against *S. pyogenes* (MIC > 32 $\mu\text{g/mL}$) and mixed results against *S. aureus* (MIC of 0.5 to >32 $\mu\text{g/mL}$). By use of an in vitro bacterial cell extract assay measuring inhibition of protein synthesis in the absence of the cell membrane, these nonionizable molecules gave mixed results (2.4 to >32 μM).⁸ This was presumed to be the result of poor solubility of the compounds lacking ionizable functional groups (vide infra). Therefore, analogues substituted with more polar termini were explored: hydroxyl, amino, carboxylic acid, and phosphoric acid functionalities (compounds 17–21). Hydroxyl and amino functional groups maintained moderate antibacterial activity (MICs for 17–19, 0.5–4 $\mu\text{g/mL}$), including against *S. pyogenes*. The carboxylic acid containing analogue (20) displayed the most potent protein synthesis inhibition (IC_{50} = 1.2 μM) in cell extracts and antibacterial activity (MIC of 0.06–4 $\mu\text{g/mL}$). Carboxylic acid 20 was studied in a mouse sepsis model of infection⁹ and displayed

potent antibacterial activity against *S. aureus* and *E. faecalis* sepsis models (ED_{50} of 10.5 and 5.5 mg/kg, respectively). The carboxylate functional group proved useful for in situ salt formation during formulation.¹⁰ Incorporating the more polar phosphoric acid terminal group eliminated antibacterial activity altogether (21, MIC > 32 $\mu\text{g/mL}$); however, 21 inhibited protein synthesis in cell extracts (IC_{50} = 1.5 μM). Therefore, the observed MIC values for 21 may be due to poor cellular penetration related to the polarity of the phosphoric acid moiety. In summary, a range of termini functional groups including nonpolar, hydroxyl, amino, and acid moieties displayed moderate to excellent antibacterial activity in vitro, and carboxylate 20 displayed encouraging antibacterial activity in vivo.

These results (Table 2) prompted further evaluation of two additional series of derivatives, amines (Table 3) and carboxylic acids (Tables 4 and 5). Alternative functional groups to

Table 2. Structure–Activity Relationships of the Terminus

Linker–Spacer–Terminus			MIC assays: µg/mL				
	Compound	Extract IC ₅₀ (µM)	<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	ED ₅₀ (mg/kg) <i>Staph, Ent</i>
	(13)	>32	4	2	>32	>32	
	(14)	>32	1	1	>32	>32	
	(15)	2.4	0.25	0.25	0.5	>32	
	(16)	10.8	0.5	0.5	4	>32	
	(17)	>16	0.5	0.5	1	>32	
	(18)	1.9	0.5	1	1	4	
	(19)	2.6	0.5	1	1	4	
	(20)	1.2	0.06	0.125	0.125	4	10.6, 5.5
	(21)	1.5	>32	>32	>32	>32	

Table 3. Structure–Activity Relationships of the Spacer Region (Amines)

Linker–Spacer–Terminus			MIC assays: µg/mL				ED ₅₀ (mg/kg)
	Compound	<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>S. aureus., E. faecalis</i>	
	(22)	1	1	1	16		
	(23)	1	1	1	16		
	(24)	0.5	1	1	8	6.48, ND	
	(25)	0.5	0.5	1	8		
	(26)	1	1	2	8		
	(27)	1	0.5	1	8		
	(28)	0.5	0.5	1	8	19.3, <3.3	
	(29)	0.5	0.5	1	>32		

examine the spacing between the linker and terminus were designed, synthesized, and evaluated for antibacterial activity in vitro and in vivo. The functional groups selected included acyclic, branched acyclic, carbocyclic, heterocyclic, heteroacyclic, aromatic, and heteroaromatic groups. In the amine subseries (Table 3), varying the spacing length from three to six atoms (urethanes 22–25), branching of the spacing moiety (26–28), and the tertiary amine 29 proved inconsequential

(MIC of 0.5 to >32 µg/mL). Furthermore, all of the amines consistently inhibited prokaryotic protein synthesis in cell extracts as before (IC₅₀ of 0.9–3.4 µM, data not shown) and displayed no antibacterial effect on the 1-resistant mutant of *S. aureus* (data not shown). Two amines (24 and 28) were studied in a mouse sepsis model of infection and displayed in vivo activity (ED₅₀ of 3.3–19.3 mg/kg), albeit variable. Apparently, the spacing length and substitution pattern within

Table 4. Structure–Activity Relationships of the Spacer Region (Acyclic Acids)

Linker–Spacer–Terminus		MIC assay: $\mu\text{g/mL}$					ED ₅₀ (mg/kg) <i>S. aureus</i> , <i>E. faecalis</i>
	Compound	<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>		
	(20)	0.06	0.125	0.125	4	10.6, 5.5	
	(30)	0.25	0.5	0.25	8		
	(31)	0.25	0.5	0.25	4		
	(32)	1	2	1	8		
	(33)	1	4	1	16		
	(34)	0.03	0.06	0.06	1	5.4, 3.1	
	(35)	0.25	0.5	0.25	4	10.6, 11.1	
	(36)	0.125	0.25	0.25	4		
	(37)	1	1	4	>32		
	(38)	0.5	2	0.5	4		
	(39)	1	2	1	16		
	(40)	>32	>32	>32	>32		
	(41)	8	32	8	8		
	(42)	0.5	1	1	>32	9.5, >60	

the amine subseries have little or no effect on antibacterial activity in vitro and in vivo.

Next, the spacing within the carboxylate subseries (e.g., compound 20, Table 4) was explored. First, the spacing length was examined. Straight-chain alkyl spacers ranging from one to four atoms were synthesized and evaluated for antibacterial activity; the four atom analogue (e.g., 20) between the amide and the carboxylate terminus (totaling eight atoms for the entire side chain) proved superior in MIC assays. Longer spacing lengths failed to provide any advantage (data not shown). In addition, urethanes and ureas were revisited using the optimized linker-spacing–termini length of eight atoms; urethane 34 emerged as one of the most potent derivatives within the 4-aminothiazole class. Further studies included methyl and gem-dimethyl substituents on the linker (35–37); these analogues retained moderate to potent antibacterial activity, respectively (MICs for 35 and 36, 0.25–4 $\mu\text{g/mL}$; MIC for 37, 1 to >32 $\mu\text{g/mL}$). More conformationally restricted functional groups, which included a heteroatom and

a cis-olefin, also retained moderate activity (MIC for 38, 0.5–4 $\mu\text{g/mL}$; MIC for 39, 1–16 $\mu\text{g/mL}$). Incorporating a second ionizable group, for example, an amine (40–42), provided varying results. The activity appeared sensitive to the location of the amino group on the spacer and its proximity to the carboxylate moiety. Placing the amino group further from the terminal acid proved superior, which is supported by comparing 42 (MIC of 0.5 to >32 $\mu\text{g/mL}$) to α -amino acids 40 and 41 (MIC of 8 to >32 $\mu\text{g/mL}$). Importantly, all carboxylates in Table 4 inhibited protein synthesis in cell extracts (IC₅₀ of 1.2–7.8 μM) but displayed no antibacterial effect on the 1-resistant mutant of *S. aureus* (data not shown). Importantly, in the mouse sepsis infection model, the most potent carboxylates (20, 34, 35, and 42) and urethane 34 demonstrated potent antibiotic activity, which is consistent with the in vitro results.

Since the carboxylate analogues containing conformational restrictions (e.g., 35–39) retained antibacterial activity, we decided to further probe structure–activity relationships utilizing carbocyclic and aromatic functional groups. Toward

Table 5. Structure–Activity Relationships of the Spacer Region (Cyclic Acids)

Linker–Spacer–Terminus	Compound	MIC assays: $\mu\text{g/mL}$				ED ₅₀ (mg/kg)
		<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>S. aureus</i> , <i>E. faecalis</i>
	(45)	0.25	0.5	0.25	4	
	(46)	0.25	0.25	0.25	4	
	(47)	0.125	0.25	0.25	4	
	(48)	0.06	0.06	0.125	4	5.2, 0.6
	(49)	0.125	0.25	0.25	4	
	(50)	0.125	0.125	0.125	4	
	(51)	2	8	8	>32	
	(52)	4	8	4	>32	
	(53)	0.125	<0.06	0.25	4	4.4, 0.8
	(54)	0.25	0.25	0.5	8	13.9, 2.2
	(55)	0.06	0.03	0.06	1	2.9, 1.0
	(56)	0.06	0.06	0.125	2	
	(57)	0.125	0.125	0.125	1	
	(58)	0.06	0.03	0.125	2	4.3, 0.2
	(59)	1	4	2	2	

this end, a series of analogues with different cyclic spacing moieties bearing amide and urethane linkers were designed, synthesized, and evaluated for antibacterial activity (Table 5). Multiple four-, five-, and six-membered carbocyclic-linked amides were investigated along with their stereochemical isomers and bridged congeners, and many retained very potent antibacterial activity (MICs for 45–49, 0.06–4 $\mu\text{g/mL}$). The trans-cyclohexylamide analogue (48) provided the best antibacterial profile of the carbocyclic-linked amides, with MICs in the 0.06–4 $\mu\text{g/mL}$ range. In addition to the saturated cycloalkyl congeners, aromatic functional groups (50–54) were evaluated. The phenyl linked analogue retained potent antibacterial activity (50, 0.125–4 $\mu\text{g/mL}$), while heteroaromatic groups (51–54) produced mixed results; the location of the nitrogen atom appeared to play a role in the SAR. This observation is consistent with previous findings within the acyclic subseries (Table 4). Pyridine-based analogues with an ortho-acid in both 1,4 and 1,3 acid–amide systems diminished

the antibacterial activity (51 and 52, 2 to >32 $\mu\text{g/mL}$), while the meta-acid retained potent antibacterial activity (53, 0.06–4 $\mu\text{g/mL}$). Within the urethane subseries (55–59), similar cycloalkyl groups (cis/trans cyclobutyl and cycloalkyl) were profiled and produced results similar to those of the amide series, albeit with modestly improved MIC activity (56–58, 0.03–2 $\mu\text{g/mL}$). Subsequent addition of an amino substituent conferred a loss of antibacterial activity (59, 1–4 $\mu\text{g/mL}$). Importantly, all carboxylates in Table 5 inhibited protein synthesis in cell extracts and also displayed no antibacterial effect against the 1-resistant mutant of *S. aureus* (not shown). In vivo studies in a mouse sepsis model confirmed the antibiotic activity of the most potent carboxylates (48, 53, 54, 55, and 58). Interestingly, amide 48 and urethane 58 exhibited exquisite in vivo effects (e.g., enterococci ED₅₀ < 1 mg/kg), which was consistent with their excellent potency in vitro and warranted additional characterization (vide infra).

Several compounds from these lead optimization studies were examined in cocrystallographic studies with EF-Tu derived from *Escherichia coli*, including amide 48 and urethane 58.¹⁰ A number of cocrystal structures were solved with varying linkers, spacers, and termini. The cocrystal structures of amide 48 and urethane 58 (Figure 1) confirmed that the analogues bind within domain 2 of EF-Tu and revealed that the macrocyclic conformations of 48 and 58 resemble 1 in its bound conformation.¹¹ Additionally, similar interactions were observed between 48/58 and EF-Tu compared to 1 including H-bonding with EF-Tu at several locations (Thr228, Asp216, Asn273, Phe261). Intramolecular H-bonding across the macrocycle from the secondary amide (donor) to the glycine carbonyl (acceptor) was also observed, which may contribute to conformational stabilization. In the aminothiazolyl region, the amide linker (48) and the urethane linker (58) both orient the cyclohexylcarboxylic acid side chain toward solvent as well as Arg223. We speculate that both the cyclohexane functional motif and the appropriate linker–terminus spacing orients the carboxylate favorably for interaction with Arg223. Further exploitation of this interaction will be the subject of future communications.

In order to further characterize 48 and 58 in vivo, we next embarked on comprehensive solubility and formulation studies. The solubilities of 48 and 58 were first analyzed at various pH values (Table 6). This revealed a pH dependency (as expected

also evaluated cosolvents and found that alcoholic and PEG based cosolvents delivered formulations with concentrations exceeding 5 mg/mL. In addition, a variety of surfactants were screened (e.g., Cremophor, Solutol, and VitE TPGS) and increased solubility. Considering the nature of the carboxylic acid functional group, counterions were also screened, and as expected, a variety of basic modulators (i.e., NaOH, NH₄OH, lysine, etc.) increased solubility via in situ salt formation. Taken together, the final formulation for both 48 and 58 achieved up to 20 mg/mL with >1 week solution stability and consisted of 15% v/v NaOH (0.1 N), 5% v/v PEG-300, 25% saline (0.9%), 55% v/v water for injection with a final pH of 7.2–7.8. Thus, the carboxylic acid moiety proved crucial in identifying a developable formulation suitable for intravenous infusion because of its ability to form in situ salts with counterions.

Next, we evaluated the pharmacokinetics of 48 in male Sprague–Dawley rats (Figure 2). Three doses were adminis-

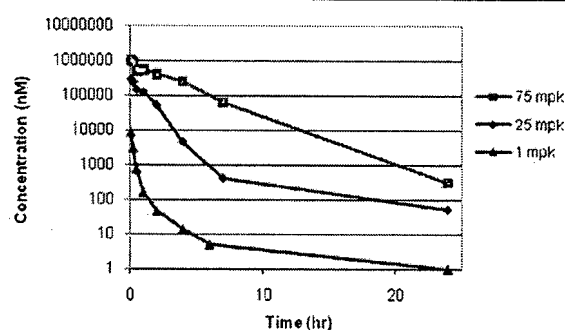


Figure 2. Pharmacokinetic data for 48 at 1, 25, and 75 mg/kg in Sprague–Dawley rats (iv bolus).

Table 6. Solubility Data for 48 and 58^a

vehicle	48	58
pH		
pH 1.0	BLD (1.1)	BLD (1.1)
pH 4.63	BLD (4.7)	BLD (4.7)
pH 7.4	0.3 (7.3)	0.001 (7.4)
pH 12.0	2.441 (9.3)	2.0 (10.7)
cosolvent		
ethanol	6.7	9.6
PEG400	>15.7	>7.6
PG	>14.8	>7.4
surfactant		
10% Cremophor	1.5	1.1
1% Pluronic F-68	0.9	BLD
20% Solutol HS 15	2.6	1.7
1% Tween 80	0.9	0.3
10% VitE TPGS	1.9	3.0
counterion		
0.1 M NH ₄ OH	8.9	3.0
0.1 M L-arginine	4.7	3.0
0.1 M glutamine	9.5	2.0
0.1 M lysine	9.5	1.8
0.1 M KOH	4.1	1.9
0.1 M NaOH	5.7	1.0

^aSolubility in mg/mL. Values in parentheses indicate the final pH. BLD = below limit of detection.

for carboxylic acid-containing compounds) where solubility in buffer increased at higher pH. For example, at low pH (1–4), the solubility was below the limit of detection, while at higher pH (7–10) the solubility increased to the mg/mL range. We

tered via iv (bolus) at 1, 25, and 75 mg/kg (Figure 1). High C_{max} (5 μ M, 320 μ M, and >1 mM, respectively) and AUC (3, 343, and 2864 μ M·h, respectively) were achieved, while V_{dss} (<0.1 L/kg, all doses) and CL (6.7, 1.0, 0.4 mL min⁻¹ kg⁻¹, respectively) were consistently low. High plasma protein binding was observed for both compounds (>99%). We speculate that the decrease in CL at higher doses was due to saturation of clearance mechanisms. Most noteworthy was the nonlinearity observed in overall exposure (AUC/dose: 3, 13.7, 38.2 μ M·h/mg/kg, respectively). The pharmacokinetics of 58 did not differ significantly (data not shown). In addition, other experiments assessed the oral bioavailability of the compounds, which proved very low ($F < 0.1\%$).

Because of the excellent in vitro profile, solubility, and efficacy in the sepsis model of infection, 48 was subsequently profiled in the neutropenic thigh infection model (Figure 3).¹² In this experiment, animals were pretreated with cyclophosphamide to induce neutropenia, infected with *S. aureus* (1×10^6 cfu/mouse) in the left thigh, then treated 2 h after infection with 48 or positive control (daptomycin, linezolid). After 12 or 24 h the thighs were excised, homogenized, and evaluated for bacterial colony forming units. Toward this end, compound 48 was dosed at 20, 40, 80, 160, and 320 mg/kg intraperitoneally, with linezolid (po, 20, 80, 320 mg/kg) and daptomycin (ip, 32 mg/kg) as positive controls. At the end of the experiment, animals that were administered vehicle in lieu of antibiotic had approximately 10^8 colony forming units in the thigh. Compound 48 prevented the increase in initial bacterial load at doses of 80–320 mg/kg at both 12 and 24 h. At 40 mg/kg, compound 48 was bacteriostatic only at the 12 h time point.

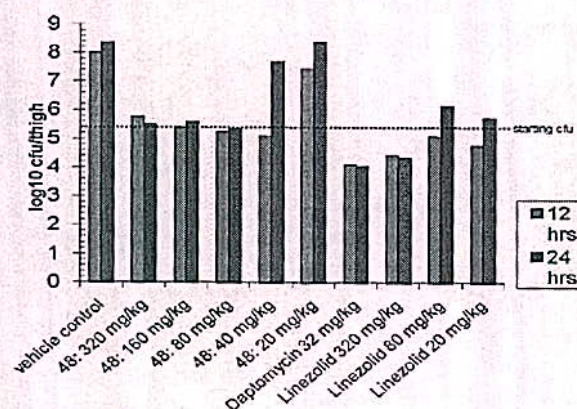


Figure 3. Bacteriostatic effect of 48 in a *S. aureus* thigh model of infection at 12 and 24 h.

In contrast, linezolid was bacteriostatic at 12 h at 80 mg/kg but had a slightly inferior effect compared to 48 at 24 h. Daptomycin proved superior to both 48 and linezolid, showing significant reduction at both 12 and 24 h at 32 mg/kg. These experiments were also performed with 58 and did not differ significantly.

DISCUSSION AND CONCLUSIONS

In summary, 4-aminothiazolyl analogues of the antibiotic natural product 1 were designed, synthesized, and evaluated for Gram positive bacterial growth inhibition. 4-Aminothiazole analogues with varying linker topologies, termini, and spacing between the macrocycle and the side chain terminus were evaluated for antibacterial activity. A trans-cycloalkylcarboxylic acid 4-aminothiazole side chain improved the antibacterial effect in vitro and in mouse sepsis and thigh models of infection. Cocrystallographic studies revealed the inhibitors' binding location and conformation in addition to important interactions between the cyclohexylcarboxylic acid functional motif and EF-Tu. In pharmacokinetic studies, 48 had low V_{dss} , low CL, and nonlinear exposure when dose was escalated and had minimal oral bioavailability. In a mouse thigh model of infection 48 proved bacteriostatic at doses greater than 80 mg/kg which was compared with the clinical standards of care (linezolid and daptomycin). On the basis of the superior antibacterial activity and the ionizable nature of their carboxylate side chains that facilitated formulation development,¹³ amide 48 and urethane 58 were selected for further preclinical profiling as development candidates.¹⁴ Taken together, these data supported further chemical scale-up, process optimization, and evaluation of 48 and 58 in rodent and nonrodent safety studies. Finally, the 4-aminothiazolyl-based chemical template described herein is a unique scaffold for antibacterial drug discovery. Indeed, in order to combat increasing clinical resistance to marketed antibiotics, the discovery of innovative chemical scaffolds that address underexploited antibacterial mechanisms of action remains a pressing need in infectious disease care. Thus, the biological activity exhibited by the cycloalkylcarboxylic acid class of thiopeptides is a significant and promising lead for antibiotic drug discovery. These development candidate identification efforts also impart further relevance to natural-product-based, industrial drug discovery. The details of these continued efforts will be the subject of additional correspondence.

EXPERIMENTAL SECTION

Compound Synthesis and Characterization. Synthetic procedures and compound characterization data are found in the Supporting Information. Compound purity was assessed by two distinct 20 min HPLC runs to confirm >95% purity.

MIC Assays. MIC assays were conducted according to the Clinical and Laboratory Standards Institute (CLSI): Wikler, M. A.; Cockerill, F. R.; Bush, K.; et al. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard—Eighth Edition*; CLSI M07-A8; 2009; Vol. 29, No. 2; bacterial strains included *E. faecalis* (ATCC 29212), *E. faecium* (Prof. Chopra, University of Leeds, U.K., strain 7130724), *S. aureus* (MRSA from Prof. Willinger, isolated from a pharyngeal smear, AKHVienna A4.018), and *S. pyogenes* (ATCC BAA-595); Wikler, M. A.; Cockerill, F. R.; Bush, K.; et al. *Performance Standards for Antimicrobial Susceptibility Testing, Nineteenth Informational Supplement*; CLSI M100-S19, 2009; Vol. 29, No. 3.

In Vitro Transcription/Translation Assays. The *E. coli* S30 extract system for circular DNA (Promegacatalog no. L1020) was used per recommendation of the manufacturer with slight modifications. Briefly, 3.5 μ L of 286 ng/ μ L template DNA (pBESTluc) was mixed with 1.0 μ L each of 1 mM "methionineminus" and "cysteine minus" amino acid mixes, 8 μ L of S30 premix, 6 μ L of S30 extract, and 0.5 μ L of the test agents at 40X final concentration in a total volume of 20 μ L. The reaction mixtures were incubated for 2 h at 37°C in a 384-well flat-bottom white plate (Corning, catalog no. 3704). The formation of luciferase was measured by adding equal volume (20 μ L) of Steady-Glo luciferase reagent (Promega catalog no. 27104), and emitted light was detected with a luminometer (Molecular Devices, LMaxII plate reader). Kirromycin and puromycin, which are known to be protein synthesis inhibitors, were used as positive controls. Ampicillin was used as a negative control. The rabbit reticulocyte TnT Quick coupled transcription/translation system (Promega catalog no. L1170) was used as recommended by the manufacturer except that the assay volumes were scaled down from 50 to 20 μ L. Briefly, the assay components consisted of 16 μ L of TnT Quick mastermix, 3 μ L of pT7luc at 167 ng/ μ L, 0.5 μ L of methionine, and 0.5 μ L of the test compound at 40X final concentration in a final volume of 20 μ L. The reaction mixtures were incubated at 37 °C for 75 min. Luminescence was detected as described above.

Selection for Spontaneous, Single-Step Mutants. Multiplex strains of *S. aureus*, *E. faecalis*, or *E. faecium* were streaked from glycerol stocks onto blood agar plates and incubated overnight at 37 °C. Duplicate sterile tubes containing either 5 mL Mueller Hinton or brain heart infusion broth were inoculated with three to five isolated colonies and incubated overnight with shaking at 37 °C. Saturated cultures were diluted $1/_{50}$ in duplicate flasks containing 50 mL of fresh broth and incubated with shaking at 37 °C for 4.5 h for *S. aureus* or 7 h for enterococci. The culture densities were approximately 10¹⁰ cfu/mL. The duplicate cultures were concentrated 10-fold by centrifugation in 10 mL aliquots at 3500g for 20 min using a Sorvall Legend RT centrifuge. An amount of 1 mL of each 10-fold concentrated cell suspension was spread onto selection plates containing test agents. Selection plates were incubated for 48 h at 37 °C. Resistance frequency was defined as the number of colonies selected divided by the total cfu plated. Representative colonies were restreaked for isolated colonies on plates containing the selecting agent at 2 $\times$ MIC against the isogenic parent. The restreaked cultures were suspended in Mueller Hinton or brain heart infusion broth + 20% glycerol and frozen at -80 °C.

Genetic Characterization of Mutants. PCR primers specific for *S. aureus* tufA, *E. faecalis* tuf, and *E. faecium* tufA and tufB were used to amplify tuf genes from representative single-step mutants and isogenic parental strains. Amplified fragments were sequenced at Agencourt (Beverly, MA), and sequences were aligned with ClustalW.

Mouse Systemic Infection Model. The studies conducted were approved by the Institutional Animal Care and Use Committee of the Novartis Institutes for Biomedical Research Inc., Cambridge, MA. Animals were maintained under controlled conditions with free access to food and water. Female CD1 mice (21–25 g, Charles

Table 7

parameter	48	58
	Data Collection (Highest Resolution Shell)	
resolution range (Å)	8.00–2.15 (2.28–2.15)	43–2.45 (2.60–2.45)
total observations	36 399	30 572
unique reflections	39 701	32 122
completeness (%)	88.6	94.8
<i>I</i> / σ	23.8 (8.0)	25.8 (4.0)
<i>R</i> _{sym}	0.098	
	Refinement	
<i>R</i> _{work} / <i>R</i> _{free}	0.211/0.273	0.224/0.298
no. protein atoms	5766	5996
no. heterogen atoms (Mg/GDP/compd)	168	170
no. solvent atoms	111	145
average <i>B</i> -factor (Å ²)	38.5	50.6
	Root Mean Squared Deviation from Ideal Value	
bond length (Å)	0.011	0.011
bond angle (deg)	1.4	1.2

RiverLaboratories, Wilmington, MA) were used for infections with *S. aureus* (ATCC 49951) and *E. faecalis* (NB04025), a clinical isolate from the Novartis bacterial collection that is resistant to erythromycin, tetracycline, and gentamicin, courtesy of Dr. B. Willinger, Vienna General Hospital, Austria). Lethal infections were induced by intraperitoneal injection of a freshly prepared bacterial suspension of 1×10^8 cfu/mouse in either 50% sterile rat fecal extract (*E. faecalis*) or 0.9% NaCl (*S. aureus*). The injected bacterial dose corresponded to 10–100 times the minimal lethal dose as determined from previous lethal dose titration studies. Therapy for *E. faecalis* infections was initiated immediately following the bacterial inoculation, while for *S. aureus* infections, therapy was initiated 1 and 5 h after inoculation. Compounds were administered via tail vein bolus injection at several dose levels to groups of six mice each. Following inoculation the mice were observed for 5 days. In addition, body temperature was monitored by electronic microtransponders (Bio Medic Data Systems, Inc., Seaford, DE) that were implanted in mice subcutaneously prior to infection. The 50% effective dose (ED₅₀), the dose providing protection to 50% of mice, and the 95% confidence limits (95% CI) were calculated from the survival data at day 5 by probit analysis using the program Systat (SPSS Inc.).

Mouse Thigh Infection Model. The studies conducted were approved by the Institutional Animal Care and Use Committee of the Novartis Institutes for Biomedical Research Inc., Cambridge, MA. Animals were maintained under controlled conditions with free access to food and water. Female CD1 mice (21–25 g, Charles River Laboratories, Wilmington, MA) were used for infections with *S. aureus* (ATCC 01001). Inoculum was prepared by adding 20 μ L from the ampule to 25 mL of Mueller Hinton + cations, then incubated with shaking for 16 h at 36 °C. The culture was diluted 1:50, and the OD₆₀₀ was determined. The cfu/mL was determined for overnight culture and each subsequent dilution and was diluted 1:50 appropriately to achieve the target inoculum cfu/mL of 1.5×10^7 (7.5×10^5 cfu/thigh). Pretreatment (to induce neutropenia) included dosing 4 days prior to the study (150 mg/kg cyclophosphamide dosed intraperitoneally; 600 mg was dissolved in 20 mL saline to achieve 30 mg/mL; application volume of 0.5 mL/100 g) and then 1 day prior to the study (100 mg/kg cyclophosphamide dosed intraperitoneally; 400 mg was dissolved in 20 mL saline to achieve 20 mg/mL; application volume of 0.5 mL/100 g). The left thigh was subsequently infected with 0.05 mL of inoculum (intramuscular injection). The animals (four per group) were then treated intraperitoneally with compound 48 at 320, 160, 80, 40, 20 mg/kg; treated with daptomycin (intraperitoneally) at 32 mg/kg; treated with linezolid (per os) at 80, 20 mg/kg; treated with vehicle (intraperitoneally). Colony forming units were then evaluated by excising the thigh muscle and storing on ice, homogenizing in 5 mL of 0.9% NaCl. Samples were serially diluted 1:10 (0.5 + 4.5 mL) in 0.9% NaCl,

and an amount of 100 μ L was pipetted onto blood agar plates for counting using the ProtoCOL system.

Solubility Measurements. An amount of 1–2 mg of compound was weighed into 1 mL glass tubes, and a fixed volume of each vehicle was added to yield approximately 20 mg/mL compound (48 or 58). After initial mixing using brief vortexing and sonication (5–10 min), samples were equilibrated by shaking for 24 h at room temperature. After equilibration, the vials were visually examined. If a clear solution was obtained, solubility was reported as $>X$ mg/mL (where *X* is the starting concentration in that sample) and the pH of this sample was recorded. Suspensions or solutions with visible particles were filtered through 0.22 μ m PVDF membrane filters. Their pH was recorded, and dissolved drug concentration was analyzed using a RP-HPLC assay.

Rat Pharmacokinetic Studies. Intravenous PK studies (*N* = 2 or 3) were performed with male Sprague–Dawley rats weighing 220–270 g and approximately 6–10 weeks old, which were obtained from Harlan Research Laboratories (South Easton, MA), each bearing dual implanted jugular vein cannula. The rats were fasted overnight before use and for 8 h after dosing. Blood samples were taken into K₂EDTA coated tubes and then centrifuged to yield plasma sample for analysis by LC–MS/MS. Bioanalyses of rat plasma, from both in-life and protein binding experiments, were performed by LC–MS/MS using a system with the following configuration: Agilent liquid chromatograph (Santa Clara, CA), LEAP Technologies CTC–PAL autosampler (Carrboro, NC), and Applied Biosystems API 4000 mass spectrometer (Framingham, MA). LC was performed in gradient mode with reversed phase C18 columns (2.1 mm $\times$ 30–50 mm $\times$ 3.5–5 μ m particle size). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. Gradients were run from 5% B to 95% B in $\sim$ 3.5 min. Plasma samples were protein precipitated with acetonitrile containing glyburide as the internal standard (Sigma–Aldrich, St. Louis, MO).

Protein Binding. Protein binding was determined in triplicate using the rapid equilibrium dialysis device (Pierce Biotechnology, Rockford IL) according to manufacturer specifications and protocol.

Crystallization and X-ray of Compound 48. To form the EF–Tu/48 complex, 10 mg/mL protein (227 μ M) *E. coli* EF–Tu protein in a buffer containing 50 mM Tris, pH 8, and 50 mM NaCl were incubated with 1 mM compound 1 for 1 h at 4 °C. The sample was centrifuged at 20 000g to remove any resulting precipitant. Crystallization was carried out using 300 nL of the protein sample plus 300 nL of crystallization solution containing 0.1 M Bis–Tris, pH 6.5, 22% PEG 3350, 0.2 M ammonium acetate, using a sitting drop format and equilibrated against a reservoir of the crystallization solution. The crystals were flash frozen with liquid nitrogen after being stabilized in a cryobuffer containing 0.1 M Bis–Tris, pH 6.5, 22% PEG 3350, 0.2 M ammonium acetate, 20% ethylene glycol.

X-ray Data Collection. Initial data from a single crystal were collected on a ADSC Quantum 210 CCD detector using synchrotron radiation ($\lambda = 1 \text{ \AA}$) at the IMCA-CAT beamline 17-ID of the Argonne Photon Source. Data were collected using ϕ rotations of 0.5° , and 180° of total data were collected.

X-ray Data Processing, Structure Determination, and Refinement. Data were processed using the HKL2000 Suite, version 0.95. Data-processing statistics are shown in Table 7. Crystals were diffracted to $2.15 \text{ \AA}$ resolution in the space group $C222(1)$ with a unit cell of $a = 97.246 \text{ \AA}$, $b = 100.376 \text{ \AA}$, $c = 156.483 \text{ \AA}$, $\alpha = \beta = \gamma = 90^\circ$. Data collection statistics are shown in Table 7. The structure of the EF-Tu/GDP/48 complex was determined by molecular replacement as implemented in PHASER (20), using *E. coli* EF-Tu protein as a search model (1D8T). The resulting molecular replacement solution contained two EF-Tu/48 protein molecules in the asymmetric unit. Refinement was carried out with CNX (21) using a single round of rigid-body refinement, following several cycles of simulated annealing refinement, B -factor refinement, and model building with the COOT software package. Water molecules, GDP, and the Mg^{2+} ion were added prior to addition of the ligand 48. Refinement produced a final model with excellent geometry (rmsd bond length of $0.011 \text{ \AA}$, rmsd bond angle of 1.4°) and R_{work} and R_{free} of 21.1% and 27.3%, respectively.

Crystallization and X-ray of Compound 58. To form the EF-Tu/58 complex, 10 mg/mL protein ($227 \mu\text{M}$) *E. coli* EF-Tu protein in a buffer containing 50 mM Tris, pH 8, and 50 mM NaCl were incubated with 1 mM compound 1 for 1 h at 4°C . The sample was centrifuged at $20000g$ to remove any resulting precipitant. Crystallization was carried out using 300 nL of the protein sample plus 300 nL of crystallization solution containing 0.1 M Hepes, pH 7.5, 22% PEG 3350, 0.2 M MgCl_2 , using a sitting drop format and equilibrated against a reservoir of the crystallization solution. The crystals were flash frozen with liquid nitrogen after being stabilized in a cryobuffer containing 0.1 M Hepes, pH 7.5, 22% PEG 3350, 0.2 M MgCl_2 , 20% ethylene glycol.

X-ray Data Collection. Initial data from a single crystal were collected on a ADSC Quantum 210 CCD detector using synchrotron radiation ($\lambda = 1 \text{ \AA}$) at the IMCA-CAT beamline 17-ID of the Argonne Photon Source. Data were collected using ϕ rotations of 0.5° , and 180° of total data were collected.

X-ray Data Processing, Structure Determination, and Refinement. Data were processed using the HKL2000 Suite, version 0.95. Data-processing statistics are shown in Table 7. Crystals were diffracted to $2.45 \text{ \AA}$ resolution in the space group $P2(1)2(1)2(1)$ with a unit cell of $a = 80.372 \text{ \AA}$, $b = 82.216 \text{ \AA}$, $c = 129.9 \text{ \AA}$, $\alpha = \beta = \gamma = 90^\circ$. Data collection statistics are shown in Table 7. The structure of the EF-Tu/GDP/58 complex was determined by molecular replacement as implemented in PHASER, using *E. coli* EF-Tu protein as a search model (1D8T). The resulting molecular replacement solution contained two EF-Tu/58 protein molecules in the asymmetric unit. Refinement was carried out with CNX using a single round of rigid-body refinement, following several cycles of simulated annealing refinement, B -factor refinement, and model building with the COOT software package. Water molecules, GDP, and the Mg^{2+} ion were added prior to addition of the ligand. Refinement produced a final model with excellent geometry (rmsd bond length of $0.011 \text{ \AA}$, rmsd bond angle of 1.2°) and R_{work} and R_{free} of 22.4% and 29.8%, respectively.

ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures and compound characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS USED

MIC, minimum inhibitory concentration; EC_{50} , 50% effective dose; G+, Gram positive; MRSA, methicillin resistant *Staphylococcus aureus*; VRE, vancomycin resistant enterococci; *S. aureus*, *Staphylococcus aureus*; *E. faecalis*, *Enterococcus faecalis*; *E. faecium*, *Enterococcus faecium*; *S. pyogenes*, *Streptococcus pyogenes*; EF-Tu, elongation factor Tu

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(13) Carboxylate containing compounds could generally be formulated with 5% PEG. **48** and **58** were formulated up to 20 mg/mL using 5% PEG300, 0.1 M NaOH, and pH 7.4 buffer.

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This paper was published on the Web on November 1, 2011 without the author's corrections. The correct version was reposted on November 3, 2011.

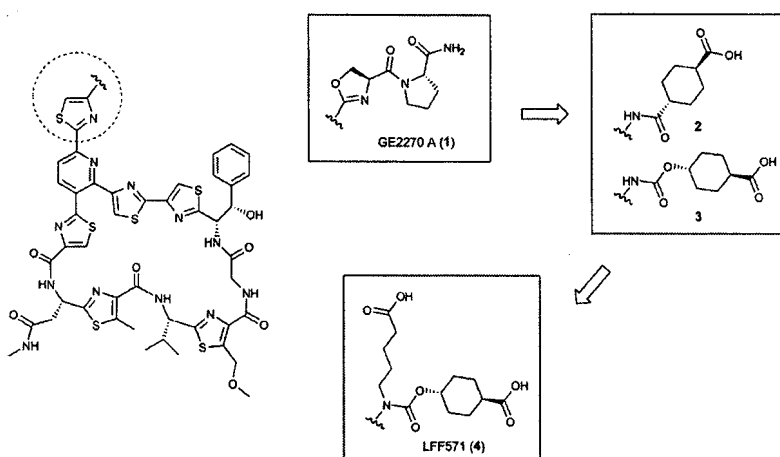
Discovery of LFF571: An Investigational Agent for *Clostridium difficile* Infection

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S Supporting Information



ABSTRACT: *Clostridium difficile* (*C. difficile*) is a Gram positive, anaerobic bacterium that infects the lumen of the large intestine and produces toxins. This results in a range of syndromes from mild diarrhea to severe toxic megacolon and death. Alarming, the prevalence and severity of *C. difficile* infection are increasing; thus, associated morbidity and mortality rates are rising. 4-Aminothiazolyl analogues of the antibiotic natural product GE2270 A (1) were designed, synthesized, and optimized for the treatment of *C. difficile* infection. The medicinal chemistry effort focused on enhancing aqueous solubility relative to that of the natural product and previous development candidates (2, 3) and improving antibacterial activity. Structure–activity relationships, cocrystallographic interactions, pharmacokinetics, and efficacy in animal models of infection were characterized. These studies identified a series of dicarboxylic acid derivatives, which enhanced solubility/efficacy profile by several orders of magnitude compared to previously studied compounds and led to the selection of LFF571 (4) as an investigational new drug for treating *C. difficile* infection.

INTRODUCTION

C. difficile is a spore forming, anaerobic, Gram positive bacterium. *C. difficile* infection results from spore germination in the gut of infected persons and subsequent toxin production, resulting in severe inflammation and diarrhea in infected patients. *C. difficile* infections often follow broad spectrum antibiotic use that disturbs normal and beneficial gut flora.¹ The prevalence and severity of *C. difficile* infections appear to be rising, in part due to changes in hospital environments, rising numbers of patients with risk factors for disease (e.g., elderly patients), an increasing use of broad spectrum antibiotics, and a higher proportion of bacterial

isolates with increased toxin production.^{2,3} Annually in the U.S., there are at least 50 000 admissions, >250 000 cases, and more than 15 000 deaths due to *C. difficile* infection.⁴ The estimated annual U.S. cost for managing *C. difficile* infections is conservatively estimated at \$3.2 billion.⁵

Therapies for *C. difficile* infection exist, but they are inadequate (e.g., efficacy, tolerability). Vancomycin (5, Figure 1), metronidazole (not shown), and fidaxomicin⁶ (6) are used for

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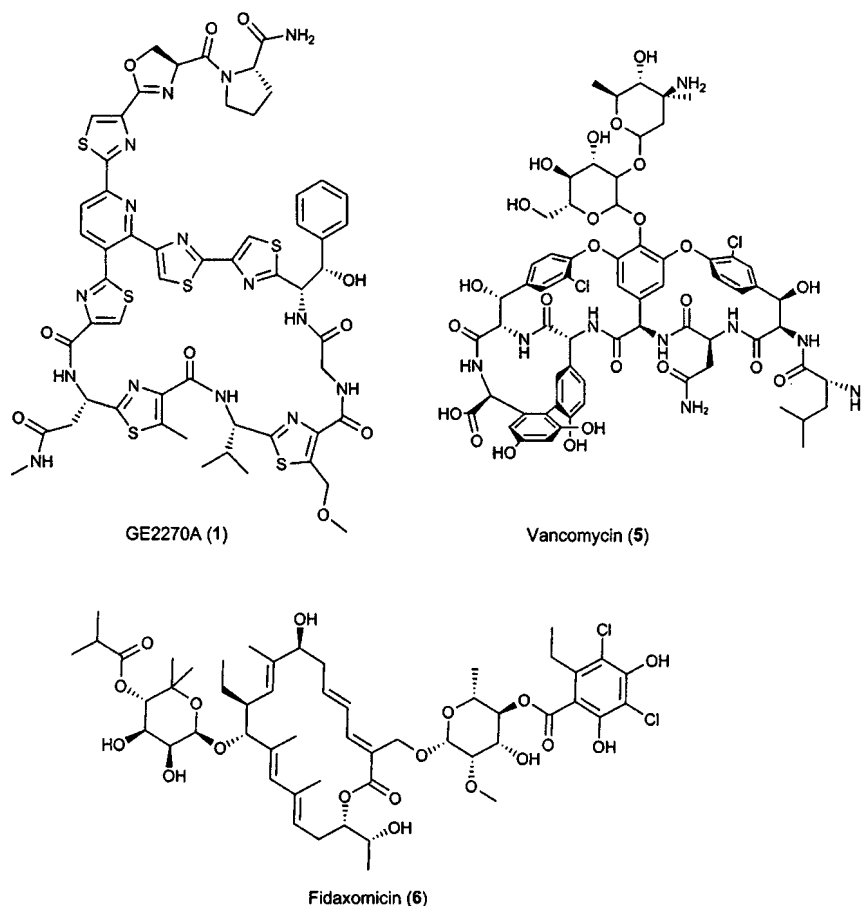


Figure 1. Structures of 1 and natural product-based marketed *C. difficile* antibiotics 5 and 6.

treating *C. difficile* infection. While they have comparable initial response rates, 5 and metronidazole treatments encounter significant disease relapse.⁷

The discovery of new anti-infective chemical entities is a high priority in research and development.⁸ There exists a pressing need for antibacterial chemical templates that act via novel mechanisms of action and are potent against clinically resistant organisms and recalcitrant infections. Historically, natural products have been excellent sources for antibacterial therapies and medicinal chemistry discovery starting points. Indeed many marketed antibiotics are natural products themselves or derivatives thereof (e.g., 5, 6).⁹

In 1991, Selva and co-workers from Lepetit Research Institute reported the structure and activity of the natural product antibiotic 1. This thiopeptide-based secondary metabolite was isolated from a fermentation broth of *Planobispora rosea* and found to inhibit the prokaryotic chaperone elongation factor Tu (EF-Tu).¹⁰ Like 5 and 6, 1 is a macrocyclic natural product of high molecular weight (1291 Da). However, key structural differences relative to 5 and 6 include the presence of aromatic thiazole rings, the dearth of solubilizing functional groups (e.g., amines, carboxylic acids, sugars), and the mechanism of action (EF-Tu). The in vitro antibiotic activity of 1 against *C. difficile*, as well as a range of Gram positive pathogens including methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE), and group A streptococci, is excellent, with minimum inhibitory concentrations (MIC) in the sub $\mu\text{g/mL}$ range.

Although the in vitro antimicrobial activities of 1 are excellent, the aqueous solubility was unmeasurable in our assays ($<0.005 \text{ nM}$).¹¹ Also, EF-Tu is located in the cytoplasm, which presented a particularly formidable drug discovery hurdle, as soluble derivatives would also need to cross the cell membrane in order to exert their antibacterial effect, unlike 5 which targets the cell wall itself. Thus, an increase in aqueous solubility with the retention of cellular antimicrobial activity represented an arduous challenge for the optimization effort.

Previously, we described lead finding and optimization activities associated with 4-aminothiazolyl analogues of 1.¹² On the basis of in vitro and in vivo potency, two distinct carboxylic acid based antibacterials (2 and 3) were further investigated as preclinical development candidates for the treatment of infections caused by *C. difficile* and other Gram positive organisms.¹³ A compelling feature of these molecules included the combination of potent antibacterial activity coupled with improved aqueous solubility through the addition of a single carboxylic acid functional group. Cocrystallography with EF-Tu guided our optimization efforts, which focused on amide and urethane-based aminothiazolyl linkers to orient carboxylic acid containing side chains toward Arg223 of domain II of EF-Tu (Figure 2). Since monocarboxylate-containing compounds (2, 3) retained antibacterial activity and significantly increased aqueous solubility, we investigated additional solubilizing functional groups and general structure–activity relationship expansion of this novel class of antibacterials. Given the proximity of Arg223 and Arg262 within domain II of EF-Tu to the

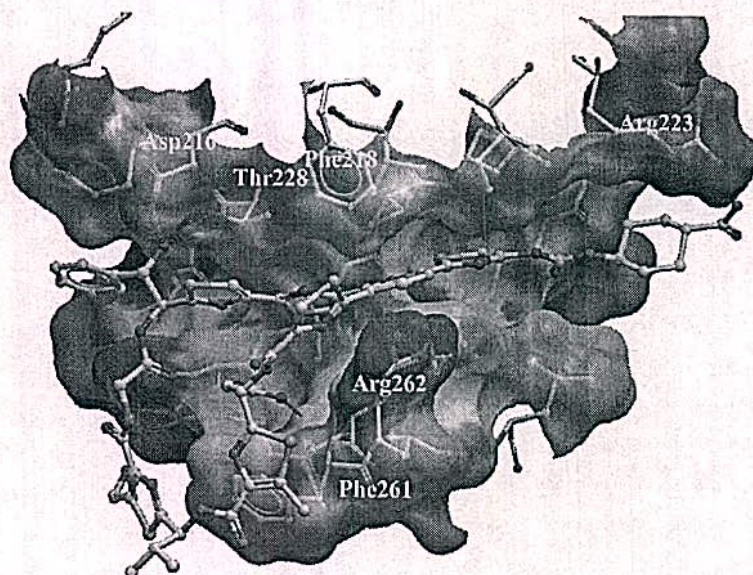


Figure 2. Cocrystal of *E. coli* EF-Tu and carboxylic acid 3.

monocarboxylate-based ligands, we envisioned additional interactions using additional carboxylates. Thus, we designed, synthesized, and evaluated di- and tricarboxylic acid bearing analogues of 1. Herein, we report the structure-guided design, synthesis, structure–activity relationships, and biological evaluation of di- and tricarboxylic acid analogues of 1. These efforts led to the initiation of clinical investigations of the safety and efficacy of LFF571 (4) for the treatment of *C. difficile* infections in humans.

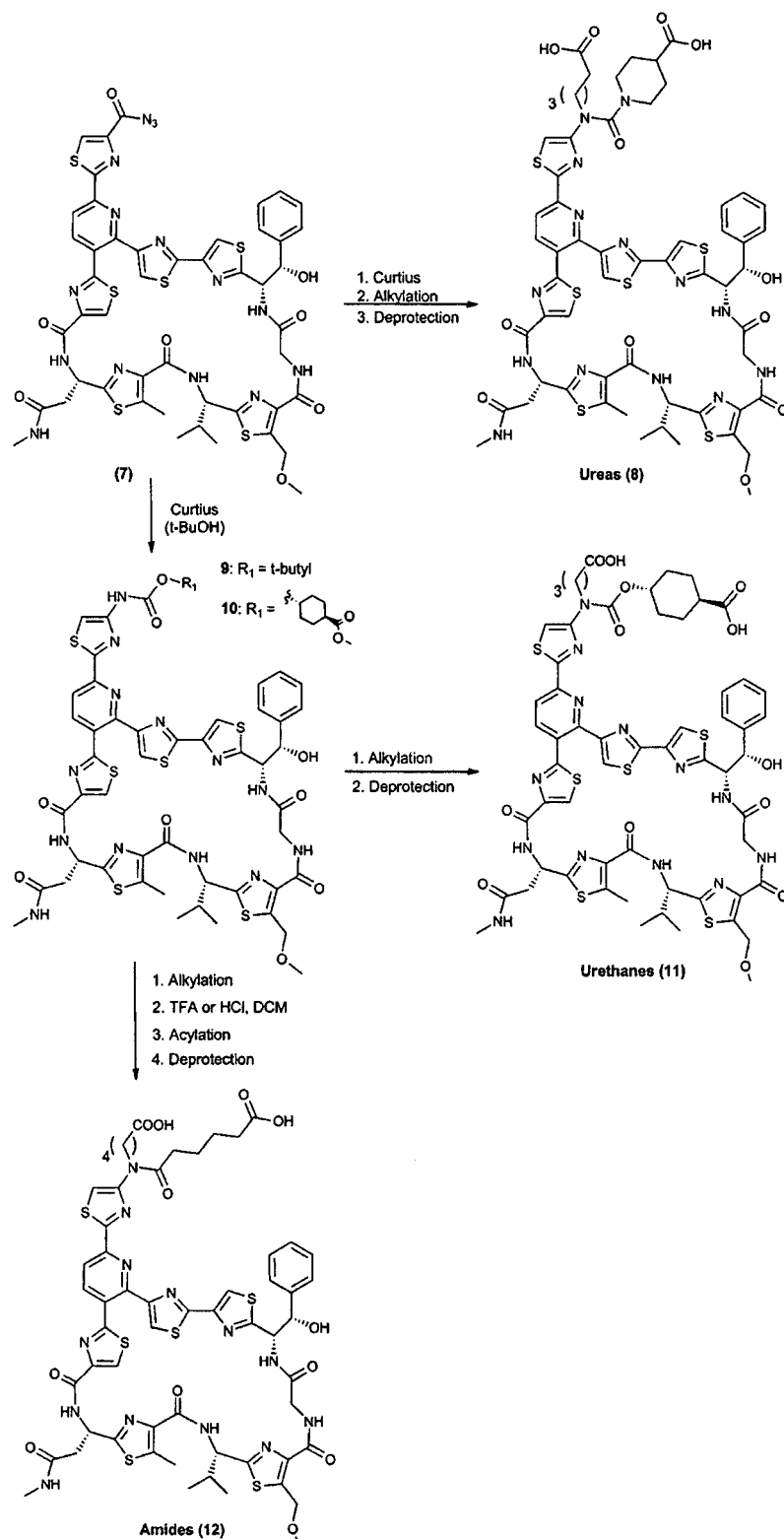
RESULTS

1 was first subjected to a previously reported acid-catalyzed rearrangement, basic hydrolysis,¹⁴ and acyl derivatization which afforded acylazide 7 in high overall yield (not shown).³ In order to evaluate amide, urethane, and urea-linked diacid containing analogues, three synthetic routes were employed (Scheme 1). For urea derivatives (e.g., 8), the azide was heated in the presence of secondary amines and the resulting tertiary urea esters were selectively alkylated on the internal nitrogen. The ester protecting groups were then hydrolyzed, which afforded the ureas (8). For urethane derivatives (e.g., 11), various alcohols were utilized in the Curtius reaction (e.g., 9, 10), followed by selective alkylation of the urethane nitrogen and ester hydrolysis (e.g., 11). We speculate that the pseudobenzyl nature of the urethane and urea nitrogens allowed for selective manipulation over other functional groups (amides) within the structurally complex macrocycle. For amide-based diacid derivatives (e.g., 12), direct alkylation of the amide nitrogen failed, so the acylazide was heated in *tert*-butyl alcohol (80 °C), which furnished the boc-protected aminothiazole (9). Selective alkylation proceeded at the urethane nitrogen, followed by boc removal, acylation of the secondary amine, and final ester hydrolysis (e.g., 12). Alternatively, multiple carboxylic acid functional groups could be installed in a single side chain during the Curtius reaction (*vide infra*).

In order to further improve the aqueous solubility while retaining antibacterial activity, di- and tricarboxylic acid analogues derived from amides, ureas, and urethanes were evaluated for minimum inhibitor concentrations (MIC, Tables 1–3). Five

organisms comprised our antibacterial panel: *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *C. difficile*.¹⁵ *Streptococcus pneumonia* was also evaluated (not shown) but did not differ significantly from *S. pyogenes*. Select compounds were also evaluated in surface plasmon resonance experiments to determine the K_d and also in an *in vitro* transcription/translation assay measuring protein synthesis inhibition in cell extracts.¹⁶ These assays, in conjunction with the MICs, assisted in the evaluation of cellular uptake of compounds that lost antibacterial activity. Methyl derivatization of the amide linker retained moderate antibacterial activity in three organisms (MIC of 13: 2 to >64 $\mu\text{g/mL}$) and also first demonstrated a method for selective alkylation of the boc protected urethane nitrogen, which revealed a new area for medicinal chemistry exploration. Moderate antibacterial activity was also realized with amides terminating in succinic acid (MIC of 14, 15: 4 to >32 $\mu\text{g/mL}$), with a slight stereochemical preference. Thus, to our surprise, retention of moderate antibacterial activity was permissible while incorporating multiple ionizable functional groups. Unfortunately, all efforts to improve antibacterial activity by separating the carboxylic acid functional groups failed (16–26). These results included varying the tether lengths of the second amide side chain (16, 17) and adding carbocycles, which had previously improved antibacterial activity (18–20).⁴ Urea-based diacids also initially failed to retain even moderate antibacterial activity, especially when the internal nitrogen was substituted (21–23). However, when carboxylates were positioned on a piperidine-derived urea, moderate activity was retained (MIC of 24: 0.25–4 $\mu\text{g/mL}$). Heterocyclic derivatives also failed to retain moderate antibacterial activity (25, 26). Of the five target organisms, *C. difficile* was the most sensitive to the compounds. In all cases, the compounds inhibited protein synthesis in bacterial cell extracts (30–90% at 2 μM , not shown) which compares equally to 1 (IC_{50} = 2.0 μM). This indicated that the observed SAR results from variable cell penetration and not reduced pathway inhibition. Furthermore, the dicarboxylate analogues that retained moderate activity (14, 15) were profiled in aqueous solubility assays and displayed a dramatic increase in solubility

Scheme 1. Synthesis of 4-Aminothiazolyl Diacid Analogues

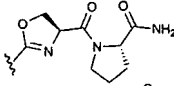
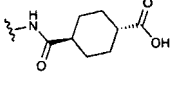
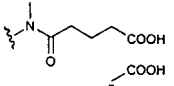
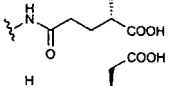
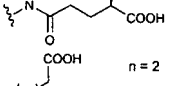
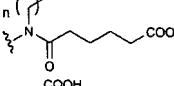
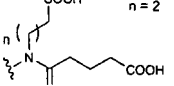
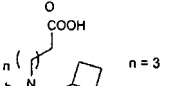
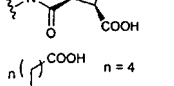
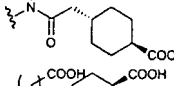
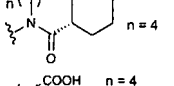
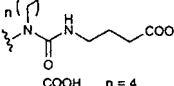
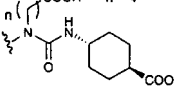
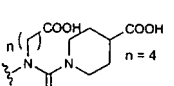
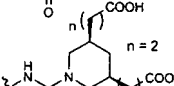
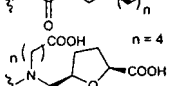


in neutral buffer (>15 mg/mL). Thus, at the outset of the diacid investigation, compounds containing a second carboxylic acid displayed improved aqueous solubility by over an order of magnitude compared to monoacid containing congeners

(2: ~0.3 mg/mL); however, the antibacterial activity of the diacids required further improvement.

In the urethane subseries (Table 2), derivatizing the urethane nitrogen with carboxylic acid containing functional groups

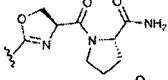
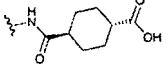
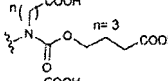
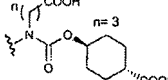
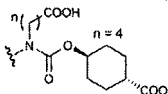
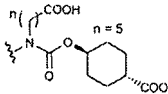
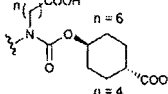
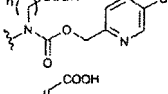
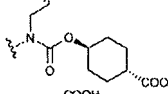
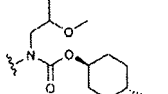
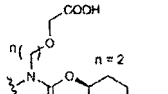
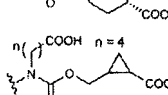
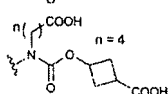
Table 1. SAR of Amide and Urea Diacids

Compound	MIC assay: $\mu\text{g/mL}$					aq solubility pH 7.4 (mg/mL)
	<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>C. difficile</i>	
 1	0.5	0.25	0.25	1	0.015	<0.001
 2	0.06	0.06	0.125	4	0.06	0.3
 13	2	4	2	>64	n/a	
 14	4	8	8	>32	4	15.1
 15	16	32	32	>32	4	17.6
 16	32	32	16	>32	2	
 17	>32	>32	>32	>32	>4	
 18	16	32	32	>32	4	
 19	8	>32	4	32	0.25	
 20	32	>32	16	>32	2	
 21	>32	>32	32	>32	4	
 22	16	>32	16	32	1	
 23	>32	>32	>32	>32	>4	
 24	2	4	4	4	0.25	
 25	>32	>32	>32	>32	>4	
 26	16	32	32	>32	4	

retained very potent antibacterial activity (MIC of 27–30, 4: 0.03–2 $\mu\text{g/mL}$). In addition, carbocyclic derivatives showed improved potency by an order of magnitude in three out of five target organisms (MIC of 28–30, 4: 0.03–2 $\mu\text{g/mL}$), which is

consistent with previous optimization studies of the monoacid subseries.⁴ This finding contrasts with the above amide-based diacid analogues, which failed to retain potent antibacterial activity (Table 1). Unfortunately, pyridine containing derivatives

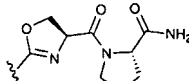
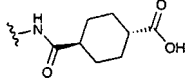
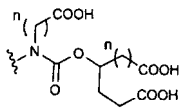
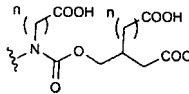
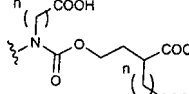
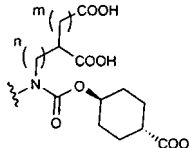
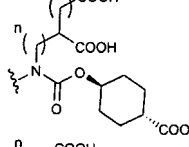
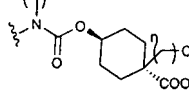
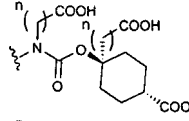
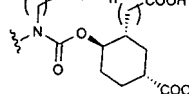
Table 2. SAR of Urethane Diacids

Compound	MIC assay: $\mu\text{g/mL}$					ED ₅₀ (mpk) Staph., Ent.	aq solubility pH 7.4 (mg/mL)
	<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>C. difficile</i>		
	1	0.5	0.25	0.25	1	0.015	<0.001
	2	0.06	0.06	0.125	4	0.06	5.2, 0.6
	27	0.5	0.5	1	2	0.03	11.0
	28	<0.06	0.06	0.25	1	0.06	2.6, 2.2
	4	<0.06	<0.06	0.125	1	0.03	6.9, 2.2
	29	<0.06	<0.06	0.125	1	0.06	9.6, 0.8
	30	<0.06	0.125	0.25	2	0.06	6.6, 1.0
	31	1	1	2	4	0.125	
	32	0.25	0.5	0.5	2	0.03	
	33	1	1	1	4	0.06	
	34	2	2	4	4	0.125	
	35	0.125	0.5	0.25	2	0.03	
	36	0.25	0.25	0.5	2	0.03	

(31), unsaturated congeners (32), heteroatom incorporation (33, 34), and other carbocyclics (35, 36) failed to improve the antibacterial activity further, although all retained good to excellent potency (MIC of 0.03–4 $\mu\text{g/mL}$). As before, *C. difficile* was the most sensitive target organism. In all cases, the compounds inhibited protein synthesis in bacterial cell extracts (67–86% at 2 μM , not shown) which compares equally to 1 (IC₅₀ = 2.0 μM). This indicated that the SAR results from variable cell penetration and not reduced pathway inhibition. The K_d values of select compounds were determined with EF-Tu-GDPNP. Compound 1 had a K_d of 3 nM, while compound

2 had a higher K_d (43 nM). Compounds 27, 29, and 4 were equivalent (8 nM, 6 nM, 6 nM, respectively), indicating that differences in MIC (e.g., in staphylococcus and enterococci) are most likely the result of variable cell penetration. The most potent urethane diacid analogues (4, 27–30) were subsequently profiled in a mouse sepsis model of infection utilizing *S. aureus* and *E. faecalis*.¹⁷ The effective doses (ED₅₀) for all five compounds evaluated in both infection models ranged from 0.8 to 11.0 mg/kg. Importantly, the in vivo results correlated with the in vitro MIC findings (*E. faecalis* ED₅₀ less than *S. aureus* ED₅₀). In addition, two of the most potent diacid congeners (4, 27) were

Table 3. SAR of Urethane Triacids

		MIC assay: $\mu\text{g/mL}$				
<u>Compound</u>		<i>E. faecalis</i>	<i>E. faecium</i>	<i>S. aureus</i>	<i>S. pyogenes</i>	<i>C. difficile</i>
	1	0.5	0.25	0.25	1	0.015
	2	0.06	0.06	0.125	4	0.06
	37	16	32	32	>32	2
	38	8	8	8	32	1
	39	8	16	16	32	0.5
	40	8	8	16	8	0.25
	41	0.25	0.5	1	1	0.06
	42	0.5	1	1	4	0.03
	43	4	8	8	16	n/a
	44	4	4	8	4	0.25

profiled in solubility assays and retained very high aqueous solubility at neutral pH ($>10\text{ mg/mL}$). On the basis of the potent in vitro and in vivo antibacterial results as well as the substantial aqueous solubility, urethane diacids **4** and **27** were subsequently selected for further characterization as development candidates.

Since the addition of a second carboxylic acid functional group retained antibacterial activity and improved aqueous solubility over that of the monoacid class and the natural product itself, we next evaluated triacid analogues of **1** in order to determine whether a third anionic group would retain antibacterial activity/cell penetration. Accordingly, the third carboxylic acid functional group was explored at various points within the most potent diacid structural templates (e.g., **4**, **27**). Similar to the mono- and diacid subseries, in the triacid

subseries, the antibacterial activity was dependent on the location of the third carboxylate functional group. Indeed, moderate antibacterial activity was achieved utilizing acyclic scaffolds containing three carboxylates (Table 3, MIC of **37–39**: 0.5 to $>32\text{ }\mu\text{g/mL}$). Again, improved antibacterial activities were observed in the carbocyclic subseries (e.g., MIC of **40–44**: 0.25 – $8\text{ }\mu\text{g/mL}$), albeit reduced from the most potent diacid and monoacid-containing analogues. In particular, maximizing the distance between the carboxylates improved antibacterial activity (e.g., **40**, **41**), and placement within the cyclohexyl ring also affected activity because 4,4-disubstituted cyclohexanes (**42**) proved to be superior to 1,1-disubstituted (**43**) and 1,2-disubstituted (**44**) analogues. In all cases, the compounds inhibited protein synthesis in bacterial cell extracts (50–79% at $2\text{ }\mu\text{M}$, not shown), which compares equally to **1** ($\text{IC}_{50} = 2.0\text{ }\mu\text{M}$).

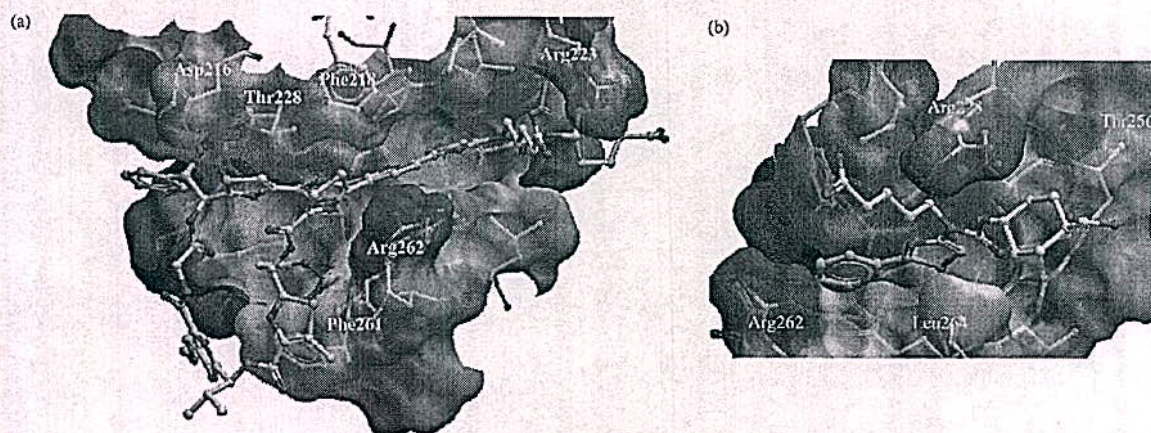


Figure 3. (a) Cocystal of *E. coli* EF-Tu (domain 2 shown) and 4. (b) Protein environment surrounding the diacid side chain of 4.

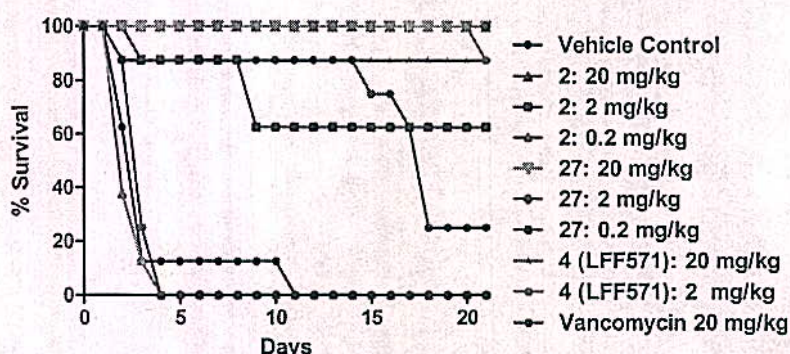


Figure 4. Characterization of 4, 27, 2, and 5 in the golden Syrian hamster model of *C. difficile* infection (eight animals per dose group).

This indicated that the SAR results from variable cell penetration and not reduced pathway inhibition. Although some compounds displayed potent activities across the panel of organisms tested (e.g., 41, 42), no additional benefit was realized, especially given the increased structural and synthetic complexity compared to the diacid series.

Cocystal studies of several diacid congeners and *E. coli* EF-Tu were conducted in order to better understand the observed structure–activity relationships.¹⁸ The homology between *E. coli* EF-Tu and *C. difficile* EF-Tu is high (86% within 5 Å of the binding site). The macrocyclic conformation of 4 was quite similar compared to previous studies (e.g., 2, 3), with intermolecular H-bond interactions noted between the hydroxyphenylalanine moiety of with two residues, Thr228 and Asp216. Further interactions included an additional intermolecular hydrogen bond between the amide side chain of 4 and Phe261. Phe218 also formed a T-stack interaction with the pyridine ring of 4. The urethane aminothiazolyl linker favorably positions the two acid-containing side chains out to solvent accessible areas and in proximity to Arg 223 and Arg226 (Figure 3b).

4 was evaluated in rat pharmacokinetic studies and displayed consistent results with previous experiments.¹⁰ Importantly, very low oral bioavailability was observed ($F < 0.1\%$) resulting from low absorption, which was ideal for the dosing requirements for treating *C. difficile* infection (i.e., oral non-absorbed route), a pathogen that infects the lumen of the large intestine. Therefore, compounds 4 and 27 and a previous development candidate 2 were evaluated in a golden Syrian

hamster model of *C. difficile* infection (Figure 4), which is used to study treatments for the initial infection and relapse stages of *C. difficile* infection.¹⁹ In the experiment, clindamycin was administered on day –1, which cleared the gut flora of the animals, increasing their susceptibility to *C. difficile* infection. On day 0, approximately 1×10^7 CFU of *C. difficile* were administered via oral gavage. 5, 27, 2, 4, or vehicle control (saline) was administered orally once a day from days 1 to 5, and the animals were observed from days 6 to 21. For compound 2, a dose dependent effect was observed with 0%, 62.5%, and 100% survival in the 0.2, 2, and 20 mg/kg groups, respectively. For compound 27, there was 0% survival for the 0.2 mg/kg group and 100% for the 2 and 20 mg/kg groups. For compound 4, a similar effect was observed for the 2 and 20 mg/kg groups. Importantly, all three thiopeptides proved to be superior at lower doses (2 mg/kg) to 5 (20 mg/kg, 25% survival). Also importantly, animals that were successfully treated with 27, 2, 4, or 5 had no detectable levels of *C. difficile* toxins A and B in their cecal contents when measured at the end of the study (via ELISA, day 21). From these experiments and others, we consider a difference of one animal between dose groups and vehicle controls as statistically insignificant. All three thiopeptides (2, 27, 4) had very limited systemic exposure in the infected hamsters using sparse PK sampling (Table 4), compared to very high exposure in the gut as measured by cecum sampling. Thus, in both uninfected rats and infected hamsters, the thiopeptides had very limited systemic exposure while maintaining high exposure in the intestinal tract, which is an ideal profile (i.e., oral nonabsorbed) for treating *C. difficile*

Table 4. Summary of Pharmacokinetics in *C. difficile* Infected Hamsters after 5 Days of Oral Dosing with 20 mg/kg Test Compounds

compd	cecum (μM)		plasma (nM)	
	1 h	3 h	1 h	3 h
2	18	39	BLQ	BLQ
27	129 ^a	72	63.9	BLQ
4	61	208 ^a	44.8	5.2

^a2/3 of animals were above the limit of quantitation.

infection. To our knowledge, this study represents the first evaluation for the thiopeptide class in the golden Syrian hamster model of *C. difficile* infection.

DISCUSSION AND CONCLUSIONS

In summary, diacid- and triacid-containing analogues of the antibiotic natural product 1 were designed, synthesized, and evaluated for Gram positive bacterial growth inhibition. Several cycloalkylurethane-based diacid and triacids retained antibacterial activity in vitro across a panel of Gram positive organisms. 4 was selected for further characterization as a development candidate on the basis of excellent in vitro and in vivo potency and very high aqueous solubility. Cocystallographic studies of 4 demonstrated a similar macrocyclic conformation compared to previous analogues, and intermolecular protein interactions within domain 2 of EF-Tu rationalized the observed structure–activity relationships. 4 was further characterized in oral PK studies, which demonstrated very low absorption, an ideal characteristic for its intended use as an oral nonabsorbed treatment for *C. difficile* infection. In the golden Syrian hamster model of *C. difficile* infection, 4 and other thiopeptides proved to be superior to 5 at lower doses. Taken together, these studies culminated in the selection of 4 for clinical development. Currently, a clinical study evaluating 4 for treating of *C. difficile* infections is ongoing.

EXPERIMENTAL SECTION

Compound Synthesis and Characterization. Synthetic procedures and compound characterization data are found in the Supporting Information. Compound purity was assessed by two distinct 20 min HPLC runs to confirm >95% purity.

MIC Assays. MIC assays were conducted according to broth microdilution methods described by the Clinical and Laboratory Standards Institute (CLSI, M07-A8, 2009).^{15a} Bacterial strains included *E. faecalis* (ATCC 29212), *E. faecium* (Prof. Chopra, University of Leeds, U.K., strain 7130724), *S. aureus* (MRSA from Prof. Willinger, isolated from a pharyngeal smear, AKH Vienna A4.018), *S. pyogenes* (ATCC BAA-595), *C. difficile* (ATCC 43255).^{15b}

Mouse Systemic Infection Model. The studies conducted were approved by the Institutional Animal Care and Use Committee of the Novartis Institutes for BioMedical Research Inc., Cambridge, MA. Animals were maintained under controlled conditions with free access to food and water. Female CD1 mice (21–25 g, Charles River Laboratories, Wilmington, MA) were used for infections with *S. aureus* (ATCC 49951) and *E. faecalis* (NB04025, a clinical isolate from the Novartis bacterial collection that is resistant to erythromycin, tetracycline, and gentamicin, courtesy of Dr. B. Willinger, Vienna General Hospital, Austria). Lethal infections were induced by intraperitoneal injection of a freshly prepared bacterial suspension of 1×10^8 CFU/mouse in either 50% sterile rat fecal extract (*E. faecalis*) or 0.9% NaCl (*S. aureus*). The injected bacterial dose corresponded to 10–100 times the minimal lethal dose as determined from previous lethal dose titration studies. Therapy for *E. faecalis* infections was initiated immediately following the bacterial inoculation, while for *S. aureus*

infections, therapy was initiated 1 and 5 h after inoculation. Compounds were administered via tail vein bolus injection at several dose levels to groups of six mice each. Following inoculation the mice were observed for 5 days. In addition, body temperature was monitored by electronic microtransponders (Bio Medic Data Systems, Inc. Seaford, DE) that were implanted in mice subcutaneously prior to infection. The 50% effective dose (ED_{50}), the dose providing protection to 50% of mice, and the 95% confidence limits (95% CI) were calculated from the survival data at day 5 by probit analysis using the program Systat (SPSS Inc.).

Solubility Measurements. An amount of 1–2 mg of compound was weighed into 1 mL glass tubes, and a fixed volume of each vehicle was added to yield approximately 20 mg/mL compound. After initial mixing using brief vortexing and sonication (5–10 min), samples were equilibrated by shaking for 24 h at room temperature. After equilibration, the vials were visually examined. If a clear solution was obtained, solubility was reported as $>X$ mg/mL (where X is the starting concentration in that sample) and the pH of the sample was recorded. Suspensions or solutions with visible particles were filtered through 0.22 μm PVDF membrane filters. Their pH was recorded, and dissolved drug concentration was analyzed using a RP-HPLC assay.

In Vitro Transcription/Translation Assays. The *E. coli* S30 extract system for circular DNA (Promega catalog no. L1020) was used per recommendation of the manufacturer with slight modifications. Briefly, 3.5 μL of 286 ng/ μL template DNA (pBESTluc) was mixed with 1.0 μL each of 1 mM “methionineminus” and “cysteine minus” amino acid mixes, 8 μL of S30 premix, 6 μL of S30 extract, and 0.5 μL of the test agents at 40 $\times$ final concentration in a total volume of 20 μL . The reaction mixtures were incubated for 2 h at 37 $^{\circ}\text{C}$ in a 384-well flat-bottom white plate (Corning, catalog no. 3704). The formation of luciferase was measured by adding equal volume (20 μL) of Steady-Glo luciferase reagent (Promega catalog no. 27104), and emitted light was detected with a luminometer (Molecular Devices, LMaxd plate reader). Kirromycin and puromycin, which are known to be protein synthesis inhibitors, were used as positive controls. Ampicillin was used as a negative control. The rabbit reticulocyte TnT Quick coupled transcription/translation system (Promega catalog no. L1170) was used as recommended by the manufacturer except that the assay volumes were scaled down from 50 to 20 μL . Briefly the assay components consisted of 16 μL of TnT Quick mastermix, 3 μL of pT7luc at 167 ng/ μL , 0.5 μL of methionine, and 0.5 μL of the test compound at 40 $\times$ final concentration in a final volume of 20 μL . The reaction mixtures were incubated at 37 $^{\circ}\text{C}$ for 75 min. Luminescence was detected as described above.

In Vitro Biotinylation of *E. coli* EF-Tu. EF-Tu proteins were first buffer exchanged to 1 $\times$ PBS by dialysis and then treated with EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce). The amount of the cross-linking reagent, the reaction time, and the temperature were controlled to allow only one molecule of biotin to cross-link with one molecule of EF-Tu. Under these conditions, about 25–30% of protein was 1:1 biotinylated. The rest of EF-Tu was not biotinylated (Figure S1) and was separated from the biotinylated EF-Tu immobilized on streptavidin (SA) sensor chip by flowing buffer over the chip surface.

Determination of Equilibrium Binding Constant (K_D) and Kinetic Binding Parameters of Thiopeptides by SPR. Biotinylated EF-Tu was immobilized onto a streptavidin (SA) sensor chip preconditioned with 40 mM NaOH and 1 M NaCl. Immobilization was controlled to achieve maximum response unit (R_{max}) of 50–100. For standard direct binding studies of thiopeptide binding to the GTP-bound form of EF-Tu, a running buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 μM GDPNP, 1 mM MgCl_2 , 1 mM TCEP, 0.05% P20 (GE Healthcare), and 3% DMSO was used. SPR experiments were performed on a Biacore T-100 instrument (GE Healthcare). Instrument parameters used for EF-Tu/TP interactions were as follows: flow rate 40 $\mu\text{L}/\text{min}$; contact (association) time = 240 s; dissociation time = 600–900 s depending on TP's dissociation rate. All binding studies were conducted at room temperature. SPR data were analyzed using BiaEvaluation software (GE Healthcare) to obtain K_D .

Rat Pharmacokinetic Studies. $N = 2$ or 3, iv PK studies were performed with male Sprague–Dawley rats weighing 220–270 g that

are approximately 6–10 weeks old, obtained from Harlan Research Laboratories (South Easton, MA), each bearing dual implanted jugular vein cannula. The rats were fasted overnight before use and for 8 h after dosing. Blood samples were taken into K₂-EDTA coated tubes and then centrifuged to yield plasma sample for analysis by LC–MS/MS. Bioanalysis of rat plasma, from both in-life and protein binding experiments, were performed by LC–MS/MS using a system with the following configuration: Agilent liquid chromatograph (Santa Clara, CA), LEAP Technologies CTC-PAL autosampler (Carrboro, NC), and Applied Biosystems API 4000 mass spectrometer (Framingham, MA). LC was performed in gradient mode with reversed phase C18 columns (2.1 mm × 30–50 mm × 3.5–5 μm particle size). The mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. Gradients were run from 5% B to 95% B in ~3.5 min. Plasma samples were protein precipitated with acetonitrile containing glyburide as the internal standard (Sigma-Aldrich, St. Louis, MO).

Oral Dosing Solution Formulations. *Compound 2.* The concentration used was 10 mg/mL. Ingredients for the amount for 1 mL (percentage) were as follows: 0.1 N NaOH [250 μL (25% v/v)], PEG-400 [200 μL (20% v/v)], 10% Cremophor EL in purified water [250 μL (25% v/v)], purified water [297 μL (29.7% v/v)], 1 N HCl [3 μL (0.3% v/v)]. The compound was weighed in a vial, and to it was added 0.1 N NaOH to completely wet the compound. The mixture was stirred for 60 min at 37 °C, and to it was added PEG-400. The mixture was stirred for 5 min to obtain a yellow solution. To it was added 10% Cremophor EL solution followed by purified water, and the mixture was stirred for 5 min (a clear solution results). The pH of the solution was adjusted to 7.5–8.0 using 1 N HCl. If the pH is above 8, it was adjusted with an additional amount of 1 N HCl. Final formulation is a light-yellow clear solution with pH of 7.5–8.0. Solution is physically stable for at least 24 h at room temperature. Final concentration of Cremophor EL in formulation is 2.5%. To prepare lower concentrations, 10 mg/mL solution was diluted with pH 7.4 phosphate buffered saline (0.15 M PBS). The diluted solutions are stable for 24 h at room temperature.

Compound 27. The concentration was 10 mg/mL. Ingredients (amount for 1 mL (percentage) were as follows: 0.1 N NaOH [150 μL (15% v/v)], PEG-400 [200 μL (20% v/v)], 10% Cremophor EL in purified water [250 μL (25% v/v)], purified water [400 μL (40% v/v)]. The compound was weighed in a vial, and to it was added 0.1 N NaOH. The mixture was sonicated/stirred for 20–30 min to completely dissolve the compound. To it was added PEG-400, and the mixture was stirred for 5 min. The solution remained clear. To it was added 10% Cremophor EL solution followed by purified water, and the mixture was stirred for 5 min. The solution remained clear. The final pH of the solution was measured and recorded. Final formulation is a light-yellow clear solution with pH of 7.0–8.0. Solution is physically stable for at least 24 h at room temperature. Final concentration of Cremophor EL in formulation is 2.5%. To prepare lower concentrations, 10 mg/mL solution was diluted with pH 7.4 phosphate buffered saline (0.15 M PBS). The diluted solutions are stable for 24 h at room temperature.

Compound 4. The concentration was 10 mg/mL. Ingredients (amount for 1 mL) were as follows: 0.1 N NaOH [100 μL (10% v/v)], PEG-400 [200 μL (20% v/v)], 10% Cremophor EL in purified water [250 μL (25% v/v)], purified water [450 μL (45% v/v)]. The compound was weighed in a vial. To it was added 0.1 N NaOH, and the mixture was sonicated/stirred for 20–30 min to completely dissolve the compound. To it was added PEG-400, and the mixture was stirred for 5 min. The solution remained clear. To it was added 10% Cremophor EL solution followed by purified water, and the mixture was stirred for 5 min. The solution remained clear. The final pH of the solution was measured and recorded. Final formulation is a light-yellow clear solution with pH of 7.0–8.0. Solution is physically stable for at least 24 h at room temperature. Final concentration of Cremophor EL in formulation is 2.5%. To prepare lower concentrations, the 10 mg/mL

solution was diluted with pH 7.4 phosphate buffered saline (0.15 M PBS). The diluted solutions are stable for 24 h at room temperature.

Hamster Pharmacokinetics. The pharmacokinetics of compounds 2, 27, and 4 were determined following 20 mg/kg oral dosing in infected hamsters using sparse plasma and cecal sampling. Six hamsters were administered 0.6 mL of each compound at 3.3 mg/mL in appropriate vehicle once a day for 5 days. At 1 and 3 h after the fifth dose, three hamsters at each time point were euthanized by CO₂ inhalation and both plasma and cecal samples taken. Blood samples were obtained by cardiac puncture and centrifuged to obtain plasma (K₂-EDTA). The plasma was harvested within 30 min after drawing the blood and then stored at –80 °C. Cecal contents were weighed and then frozen at –80 °C after collection. For quantification of thiopeptides in cecal samples, cecal tissue weight was recorded by subtracting an average tube weight. To each sample, phosphate buffered saline (PBS) with 25% acetonitrile was added at 1.5× volume of the tissue weight (1:2.5 dilution). Samples were sonicated for 10 min and homogenized with an Omni-TH tissue homogenizer for 30–60 s. Next the homogenized samples were centrifuged for 10 min at 4000 rpm. An amount of 5 μL of the resulting cecal supernatant was further diluted with 45 μL of control cecal homogenate supernatant (1:10 dilution) resulting in a final dilution of 1:25. In addition, a 10 μL aliquot of control hamster plasma was added to each cecal sample and standard. The samples were then extracted by precipitation of proteins with acetonitrile (200 μL) containing 100 ng/mL internal standard (glyburide). Samples were subsequently vortexed for 5 min followed by centrifugation at approximately 4000 rpm for 5 min. The resulting supernatant (~150 μL) was transferred into a clean 96-well plate, and 50 μL of water was added and vortexed for 2 min.

Hamster Model of *C. difficile* Infection. Hamsters were kept under controlled conditions with 12 h dark and 12 h light cycles, 68–72 °F constant temperature, 50% relative humidity, and 10–15 exchanges of fresh HEPA filter air per hour. Animals were kept in 7.5 in. wide by 6 in. deep cages (Alternative Design Manufacturing, Silom Springs, AR) with sterilized Bed O-Cob bedding (corn cob) and free access to water and standard rodent chow (Harlan). Animal were hamster-golden Syrian (*Mesocricetus auratus*), Michigan-206 Golden Syrian wild-type from Harlan, male, 90–110 g, 5–6 weeks old. Studies described were approved by the Institutional Animal Care and Use Committee (IACUC) of the Novartis Institutes for BioMedical Research Inc., Cambridge under protocol number OS 20,061.

C. difficile ATCC 43255 was received from American Type Culture Collection and stored at –80 °C in Brucella broth supplemented with 20% glycerol. On day –1, all animals received a single subcutaneous injection of clindamycin (10 mg/kg). At 24 h after clindamycin pretreatment, hamsters were infected by oral gavage with approximately 10⁶ CFU of the *C. difficile* culture. All culture work was completed within 30 min, and all cultures were back in anaerobic conditions by this time. Briefly, strains were resuspended from overnight plates (in reinforced clostridial medium + 1% oxyrase (Oxyrase, Inc., Mansfield, OH) and diluted to 1 × 10⁷ CFU/mL (OD₆₀₀ = 1.1–1.3 nm, dilute 1:100). Hamsters were immediately infected with 0.75 mL inoculum (~7.5 × 10⁶ CFU/hamster final). An aliquot of inoculum was diluted 1:1000 and spiral plated (100 μL, slow deposition) on TSAB or RCMA (×2) and incubated at 37 °C anaerobically for determination of infection titer. Hamsters were administered the test compound (8 animals per dose level) starting 24 h after infection. Single antibiotic doses were administered orally and formulated as described above. Antibiotics were administered 1 time per day and continued for up to 5 days. The control group was administered the solution vehicle alone. Animals were observed two times a day for the duration of the experiment. General observations included signs for mortality and morbidity and for the presence of diarrhea (“wet tail”), overall appearance (activity, general response to handling, touch, ruffled fur) and recorded. Animals judged to be in a moribund state will be euthanized. Criteria used to assign a moribund state are extended periods (5 days) of weight loss, progression to an emaciated state, prolonged lethargy (more than 3 days), signs of paralysis, skin erosions or trauma, hunched posture, and a distended abdomen. Observations continued, with any deaths or euthanasia recorded for up to 21 days postinfection (for relapse).

Any animal that died during the observation period was necropsied, and the contents of their cecums were removed, diluted with an equal volume of PBS, and frozen at -80°C until processing (efficacy study). All surviving animals were euthanized by CO_2 inhalation and sampled in a similar manner as above.

Cecum homogenates are assayed for the presence of *C. difficile* toxins A and B using the Wampole *C. difficile* Tox A/B II ELISA assay kit (VWR catalog no. 89013-262) in accordance with the manufacturer's directions.

Crystallization and X-ray of Compound 4-EF-Tu Complex. To form the EF-Tu-NVP-4 complex, 10 mg/mL protein (227 μM), *E. coli* EF-Tu protein in a buffer containing 50 mM Tris, pH 8, and 50 mM NaCl were incubated with 1 mM compound 4 for 1 h at 4°C . The sample was centrifuged at 20000g to remove any resulting precipitant. Crystallization was carried out using 300 nL of the protein sample plus 300 nL of crystallization solution containing 0.1 mM Tris, pH 8.23, 18% PEG3350, 0.2 M MgSO_4 , using a sitting drop format and equilibrated against a reservoir of the crystallization solution. The crystal was flash frozen with liquid nitrogen after being stabilized in a cryo-buffer containing 0.1 mM Tris, pH 8.23, 18% PEG3350, 0.2 M MgSO_4 , 15% ethylene glycol.

X-ray Data Collection. Initial data from a single crystal of the EF-Tu-4 complex were collected on a ADSC Quantum 210 CCD detector using synchrotron radiation ($\lambda = 1 \text{ \AA}$) at the IMCA-CAT beamline 17-ID of the Argonne Photon Source. Data were collected using φ rotations of 0.5° , and 180° of total data were collected.

X-ray Data Processing, Structure Determination, and Refinement. Data from the EF-Tu-4 complex were processed using the HKL2000 Suite, version 0.95.²⁰ Data processing statistics are shown in Table 5. Crystals were diffracted to 2.7 Å resolution in the space group $P2(1)2(1)2$ with a unit cell of $a = 83.303 \text{ \AA}$, $b = 132.473 \text{ \AA}$, $c = 37.433 \text{ \AA}$, $\alpha = \beta = \gamma = 90^{\circ}$. Data collection statistics are shown in Table 5.

Table 5

Data Collection Parameters (Highest Resolution Shell)	
resolution range (Å)	50–2.7 (2.87–2.7)
total observations	70876
unique reflections	11365
completeness (%)	93.5 (77.2)
I/σ	20.0 (2.8)
R_{sym}	0.099 (0.416)
Refinement Parameters	
$R_{\text{work}}/R_{\text{free}}$	0.233/0.315
protein atoms	2995
heterogen atoms (Mg/GDP/compd)	92
solvent atoms	89
average B-factor ($\text{\AA}^2$)	47.4
Root Mean Squared Deviations from Ideal Value	
bond lengths (Å)	0.007
bond angles (deg)	1.4

The structure of the EF-Tu-GDP-4 complex was determined by the molecular replacement as implemented in PHASER,²¹ using *E. coli* EF-Tu protein as a search model (1D8T). The resulting molecular replacement solution contained one EF-Tu-GDP-4 protein complex in the asymmetric unit. Refinement was carried out with CNX²² using a single round of rigid-body refinement, following several cycles of simulated annealing refinement, B-factor refinement, and model building with the COOT software package.²³ Water molecules, GDP, and the Mg^{2+} ion were added prior to addition of the ligand 4. Refinement produced a final model with excellent geometry (rmsd bond lengths of 0.007 Å, rmsd bond angles of 1.4°), and R-factors of R_{work} and R_{free} of 23.3% and 31.5%, respectively.

■ ASSOCIATED CONTENT

● Supporting Information

Detailed synthetic procedures and compound characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

MIC, minimum inhibitory concentration; EC_{50} , 50% effective dose; G+, Gram positive; MRSA, methicillin resistant *Staphylococcus aureus*; VRE, vancomycin resistant enterococci; *S. aureus*, *Staphylococcus aureus*; *E. faecalis*, *Enterococcus faecalis*; *E. faecium*, *Enterococcus faecium*; *S. pyogenes*, *Streptococcus pyogenes*; EF-Tu, elongation factor Tu

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