

CAVELIER

ABOGADOS

EDIFICIO SISKI
CARRERA 4 No. 72 - 35
BOGOTÁ 8, COLOMBIA

www.cavelier.com
cavelier@cavelier.com

Teléfono: (57-1) 347 3611
Telefax: (57-1) 211 8650
(57-1) 700 4814
675 7743

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 08-077425- -00005-0000

Fecha: 2012-09-21 16:35:46 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 444 RESPUESTAREQUE Folios: 18 46

Señor Director
DIRECCION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

Trámite : 011
Evento : 378
Actuación : 444

Referencia : Expediente No. 08.077.425
Asunto : Solicitud de Patente para: **AGENTES CITOTÓXICOS QUE
COMPRENDEN NUEVOS DERIVADOS DE TOMAIMICINA
Y SU USO TERAPEUTICO**
Solicitante : **SANOFI-AVENTIS**
Clase : C07D 487/004
Fecha de Solicitud : 25 de julio de 2008

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, de conformidad con la sustitución de poder otorgada a mí por el doctor Emilio FERRERO, me refiero al oficio No. 11177 notificado por fijación en lista del 08 de junio de 2012.

2. Mediante oficio No. 11177 se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las observaciones realizadas en el oficio son las siguientes:

2.1 De la solicitud de patente

2.1.1 Reivindicaciones relativas a usos

El uso de las reivindicaciones 40-41 no es patentable de acuerdo con el Artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina.

2.1.2 Reivindicaciones

De manera resumida el Examinador ha expuesto lo siguiente:

- La reivindicación 1 no es concisa, porque presenta una excesiva complejidad, lo cual dificulta determinar la extensión del objeto que se busca proteger, particularmente en la definición de los radicales. Se sugiere limitar la materia reclamada a una razonable generalización de los ejemplos. Lo anterior también aplica a las reivindicaciones dependientes 2-25 y 32-38.
- En la reivindicación 1, si bien es cierto que para la persona del oficio versada en la materia los términos “hidratos”, “sales hidratadas” son conocidos, no se evidencia en la descripción que estos puedan llegar a formarse. Además el término “estructuras polimórficas cristalinas de estos compuestos” no permite delimitar el alcance de la invención.
- La reivindicación 13 no es concisa ya que los términos que definen el enlazador son géneros funcionales.
- Las reivindicaciones 32-38 mencionan un proceso de preparación de un compuesto de acuerdo con las reivindicaciones precedentes 1-25, pero este no se define mediante características como alguna etapa o condición específica.

2.1.3 Unidad de Invención

De manera resumida el Examinador ha expuesto lo siguiente:

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 2 grupos inventivos:

1. Compuestos de fórmula (I) y proceso de preparación de dichos compuestos (reivindicaciones 1-25, 32-38 y 40-41).
2. Un conjugado que contiene uno o mas derivados de tomamicina unidos químicamente a un agente de unión a las células a través de un enlazador que comprende un grupo de unión (reivindicaciones 26-31 y 38).

Se requiere al solicitante definir el alcance de la solicitud restringiendo el capítulo reivindicatorio al grupo inventivo de interés y/o presentando la solicitud divisional correspondiente al grupo inventivo soportado.

2.2 Análisis de patentabilidad

2.2.1 Novedad

Para la evaluación de este requerimiento se emplea los documentos D1: WO 2005085250, D2: WO 2005110423, D3: WO 2005040170, D4: WO 2004087716, D5: WO 2005085259, D6: "DNA binding potencial and cytotoxicity of newly designed pyrrolobenzodiazepine dimers linked through a piperazine side-armed-alkane spacer" y D7: "Design, synthesis and in vitro aytotoxic studies of novel bis-pyrrolo[2,1][1,4] benzodiazepine-pyrrole and imidazole polyamide conjugates". El Examinador afirma que la reivindicación 1 y sus reivindicaciones dependientes 2-25, 32-38 y 40-41 carecen de novedad a la luz de las enseñanzas de estos documentos.

3. Respuesta a las objeciones

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desea presentar las siguientes modificaciones, aclaraciones y argumentos:

3.1 Modificación de una solicitud en trámite

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por 31 reivindicaciones.

Así, siguiendo las amables sugerencias del Examinador se presenta un nuevo pliego reivindicatorio compuesto por treinta y un (31) reivindicaciones, en el cual se han realizado las siguientes modificaciones:

- Se han incluido las antiguas reivindicaciones 15 y 24 en la definición del enlazador de la nueva reivindicación 1.
- Se ha limitado X y X' a solo oxígeno, como se encontraba en la antigua reivindicación 6.
- Se ha limitado tanto R₁ y R₂ como R'₁ y R'₂ a grupos alquénilo.
- El grupo T se ha limitado a fenilo o piridilo.
- Se ha especificado que el enlazador comprende sustituyentes que contienen tio-, sulfuro o disulfuro tal y como se mencionaba en la antigua reivindicación 14,
- Las reivindicaciones referentes al proceso de fabricación de los compuestos reclamados se han definido en términos de las etapas del proceso y no por las características técnicas del compuesto obtenido.
- Se han eliminado las antiguas reivindicaciones 40-41.

CAVELIER

ABOGADOS

- Los términos “hidratos, sales hidratadas, estructuras cristalinas polimórficas, isómeros ópticos, racematos, diastómeros y enantiómeros” han sido eliminados de las nuevas reivindicaciones 1 y 18.
- Se ha especificado el número de átomos de carbono en los sustituyentes y la naturaleza del heteroátomo en los grupos heteroariles y heterocíclicos.

Como se puede evidenciar, el capítulo reivindicatorio ha sido limitado de acuerdo con los compuestos ejemplificados en la descripción. En particular, se ha limitado la definición del grupo T, X, X', R₁, R₂, R'₁, R'₂, W, W', Y, Y' y el enlazador. Por lo cual la reivindicación 1 es concisa y comprende una generalización adecuada de la materia soportada en los ejemplos.

Por lo anterior, es posible establecer que el pliego reivindicatorio define claramente los compuestos reivindicados, los conjugados que los comprenden y el proceso de fabricación de los mismos, y sus métodos de preparación.

Así entonces, consideramos que las modificaciones efectuadas son suficientes para aclarar la materia reivindicada y las mismas deben tenerse por sustentadas en la descripción:

Respecto a la objeción de unidad de invención hecha por el Examinador, se aclara que los derivados de tomamicina usados en los conjugados son los compuestos de fórmula (I) de acuerdo a la reivindicación 1. Debido a que estos compuestos son novedosos (como se explicará a continuación) se debe entender que existe unidad inventiva entre los compuestos de fórmula (I) y los conjugados que los contienen, al tener al menos una característica esencial novedosa en común.

En consecuencia, se han atendido las recomendaciones del Examinador con respecto a las objeciones claridad, concisión y unidad inventiva. Por lo tanto, se solicita atentamente tener en cuenta este nuevo pliego reivindicatorio para el análisis de patentabilidad de la solicitud.

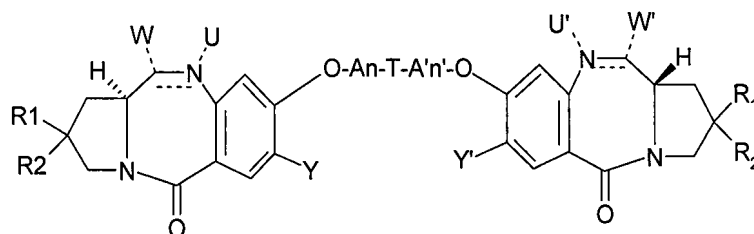
3.2 Argumentos a favor de la patentabilidad de la presente solicitud

3.2.1 Novedad

Siguiendo los lineamientos establecidos para el análisis de novedad, es posible establecer que una anterioridad afecta la novedad de una solicitud cuando revela todas y cada una de las características técnicas del producto.

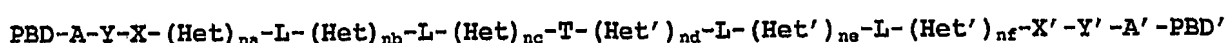
El Examinador considera que los documentos citados D1 a D7 son anterioridades que afectan la novedad de la presente invención. Respetuosamente manifestamos nuestro desacuerdo por las razones mencionadas a continuación:

La presente reivindicación 1 reclama los compuestos de formula (I):



En donde A y A' son seleccionados independientemente entre un grupo alquilo (C1-C12) o un alquenoilo (C1-C12) opcionalmente sustituidos y T es un fenilo o piridilo sustituido con uno o varios enlazadores.

El documento D1 revela compuestos de fórmula:



en donde X y X' son ambos NH ó C(=O), y T es -NH-Q-NH- ó -C(=O)-Q-C(=O)-. Q es un radical divalente derivado de un grupo alquilo (C1-C7), un grupo heterociclo (C3-C20), o un grupo arilo (C5-C20), opcionalmente sustituidos.

En el caso en que los compuestos de D1 presenten algún grupo (Het) o (Het') (es decir en el caso que cualquiera de n_a , n_b , n_c , n_d , n_e , n_f sea diferente de 0), los compuestos de formula (I) de la presente invención serian novedosos frente a los compuestos de D1 ya que como se mencionó anteriormente, A y A' se han especificado para ser seleccionados entre grupos alquilo o alquenoilo. Es decir, las definiciones de A y A' no incluyen las definiciones de Het y Het de D1, el cual indica que Het y Het' son seleccionados independientemente del grupo amino-heteroarileno-carbonilo.

Para el caso contrario, en donde el compuesto de D1 no presente grupos Het ni Het' (es decir, todos los n_a , n_b , n_c , n_d , n_e , n_f son igual a 0), el dímero de dicho compuesto seria cualquiera de formula:

- -NH-C(=O)-Q-C(=O)-NH-
- -C(=O)-NH-Q-NH-C(=O)-

dadas las definiciones de X, x', y T para el documento D1. Es claro que el dímero de D1 difiere de la parte -O-A_n-T-A'_n-O- del compuesto de fórmula (I) de la presente invención, dadas las nuevas definiciones de los grupos.

En síntesis, para el documento D1, los compuestos reclamados son novedosos en cualquier caso sobre los compuestos de esta anterioridad.

Con respecto a los documentos D2 a D7, ninguna de estas anterioridades revela compuestos que tengan un grupo T sustituido por un enlazador tal y como se define en la reivindicación 1, mucho menos revelan la posibilidad de formar conjugados mediante la unión del producto al enlazador en el grupo T.

Por lo anterior, el objeto del capítulo reivindicatorio es novedoso frente a las referencias citadas, dado que ninguna menciona dímeros de tomamicina con un grupo enlazador tal y como se define en la reivindicación 1.

Se desea poner de presente que de acuerdo con la Guía para tramitar solicitudes de patente de su Despacho solamente un documento es necesario para objetar la novedad de una invención, cuando el mismo revela todas y cada una de las características técnicas esenciales de la misma. Por esta razón, parece superfluo que se citen 7 documentos diferentes para sustentar con cada uno de ellos la ausencia de novedad de la presente invención. Adicionalmente, esta situación es además inconveniente para el solicitante pues no puede anticipar posibles combinaciones entre siete diferentes documentos que puedan llevar al Examinador a objetar el nivel inventivo de la invención.

Por lo anterior, si esa fuera la decisión de su Despacho, respetuosamente solicitamos que se profiera una nueva acción oficial que específicamente señale los dos documentos en los que basaría dicha objeción, de tal manera que garanticemos no solo un examen de patentabilidad adecuado a la Guía de su Despacho sino conforme al debido proceso del solicitante.

3.2.2 Nivel Inventivo

A pesar de lo anterior y de que el Examinador no objetó el nivel inventivo de la presente solicitud, mi representada desea suministrar la siguiente información con el fin de demostrar que la invención también cumple con el requerimiento de nivel inventivo. Lo anterior, repetimos, sin perjuicio de que de considerarse procedente una objeción en este sentido se deba trasladar la misma mediante una nueva acción oficial, so pena de violarse el tenor literal y espíritu del artículo 45 de la Decisión 486:

El artículo 18 de la Decisión 486 señala lo siguiente:

“Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica”.

Consecuentemente, una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo, para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que, a partir de un conocimiento medio en dicho campo, le permitan llegar de manera obvia a la invención reivindicada.

Teniendo en cuenta lo anterior, se quiere presentar los siguientes argumentos que demuestran el nivel inventivo de la solicitud:

En primer lugar, y tal como se ha mencionado en los argumentos a favor de la novedad de la invención, ninguno de los documentos citados revela o sugiere compuestos con un grupo T sustituido por un enlazador, a diferencia de los compuestos de fórmula (I) de la nueva reivindicación 1. Debido a que no hay sugerencias en dichas anterioridades, para una persona medianamente versada en la materia no sería obvio o deducible a partir del estado del arte, la preparación de derivados de tomamicina que contengan las características del grupo T de los compuestos de fórmula (I), ni tan poco podría deducir con base a las enseñanzas de D1 a D7 que los dímeros que contienen O-A_n-T-A'_n-O- serían eficientes para la formación de conjugados con elementos de unión a las células.

Una persona con conocimientos en el arte sabría que hay una gran cantidad de elementos enlazadores conocidos con el fin de generar la unión entre agentes citotóxicos y elementos de unión a las células como los anticuerpos. Sin embargo, la dificultad se encuentra en la selección del enlazador apropiado que permita la liberación del agente citotóxico de manera eficiente cuando el anticuerpo o cualquier otro elemento de unión reconozca con gran especificidad la célula objetivo. Por tal motivo, la incorporación del enlazador específicamente

definido en la reivindicación 1 no resulta obvia debido a la gran cantidad de opciones de enlazadores conocidos y a los diferentes resultados que presentan estos enlazadores.

Tampoco resulta obvio el hecho de que los compuestos derivados de tomaimicina de formula (I) puedan generar conjugados que liberen de manera eficiente el compuesto en las células objetivo. Para demostrar este hecho se presentan como anexos a este documento, dos artículos que concuerdan en la dificultad de elección del enlazador apropiado para agente citotóxico.

El primer artículo, *Chari et al* (1992), se refiere a la conjugación de maitansina, otro agente citotóxico. Este artículo revela que la elección del enlazador apropiado no es obvia para la persona versada en la materia, en particular menciona: "la mayoría de enlazadores que han sido usados para la conjugación de drogas con anticuerpos no liberan eficientemente la droga activa en el interior de la célula" (página 127, columna izquierda).

El segundo artículo presentado, *Widdison et al* (2006), muestra lo impredecibles que resultan la preparación de conjugados de tomaimicina. En este artículo, el enlazador tuvo que ser optimizado para cada droga. Como se evidencia en las Figuras 3 y 4 del mismo artículo, los resultados de las pruebas a los conjugados preparados con distintos grupos enlazadores en células tumorales de colon muestran las diferencias que presenta el producto final en cuanto a actividad y potencia de los conjugados, lo cual ratifica el hecho de que la elección de enlazadores específicos como los presentados en la invención no resulta obvia.

Por último, ninguno de los documentos citados por el Examinador menciona la posibilidad de que los compuestos reclamados derivados de tomaimicina formen conjugados que puedan unirse específicamente a células tumorales.

Dada la ausencia de información en los documentos citados sugiriendo los compuestos reclamados con los enlazadores definidos en la reivindicación 1 para la formación de conjugados, una persona medianamente versada en la materia no hubiera podido obtener los compuestos de la reivindicación 1 mediante modificaciones a los compuestos conocidos en el estado del arte. Por lo anterior, las reivindicaciones modificadas presentan nivel inventivo.

3.3 Conclusión

Con las modificaciones introducidas al pliego reivindicatorio y los argumentos de novedad acá presentados, se superan las objeciones formuladas por el Examinador en el oficio No. 11177.

4. Puesto que se ha dado respuesta a todas las objeciones formuladas por el Examinador, se solicita atentamente proceder al otorgamiento de la solicitud de patente. En todo caso, en el evento que ese Despacho considere que alguna de las reivindicaciones no cumple con el requisito de patentabilidad, solicito que el privilegio de patente se otorgue de forma parcial. Adicionalmente, respetuosamente recordamos al Examinador que la patente no podrá ser negada con base en objeciones diferentes a las expuestas en la acción oficial No. 11177, toda vez que al no haber sido trasladadas a mi representada, dicha decisión violaría tanto el tenor literal del artículo 45 de la Decisión 486 como el derecho constitucional al debido proceso. En este evento, y con base en el inciso 2 del artículo 45, respetuosamente solicitamos al Examinador expedir una nueva acción oficial que permita a mi representada contra argumentar las mismas o modificar la solicitud con el fin de cumplir los requisitos de patentabilidad.

5. Anexos

- Nuevo pliego reivindicatorio conformado por 31 reivindicaciones.
- Formulario de modificaciones en solicitud pendiente.
- Comprobante de pago de 21 reivindicaciones adicionales después de la décima reivindicación por concepto de 630.000 pesos.
- Chari et al, Immunoconjugates containing novel maytansinoids: Promising anticancer drugs, Cancer Research 52, 127-131, Enero 1, 1992.
- Wayne et al, Semisynthetic Maytansine Analogues for the targeted treatment of cancer. Journal of Medical Chemistry, 2006, Vol 49, 4932-4408.

Señor Director,

Luiz Clemencia de Páez

LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

COLO-14775-0841-071-5
CNV\CNVID-226066\WPC14775
No. M-2012-226066\CNV



Espacio reservado para el adhesivo de radicación

**DIRECCION DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES**

1. Identificación del Trámite

- ☐ Error material
- ☒ Patente de Invención
- ☐ Patente de Modelo de Utilidad
- ☐ Registro de Diseño Industrial
- ☐ Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural

Persona Jurídica

SANOFI-AVENTIS

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa Micro ☐ Pequeña ☐ Mediana ☐ Otra ☐

Documento de identificación: ☐ C.C. ☐ C.E. ☐ NIT ☐ Otro Número

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Francia	174 Avenue de France	75013 Paris
Dirección electrónica	No. Fax	Número telefónico
clientes@cavelier.com	2 11 86 50	3 47 36 11

- ☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del apoderado:

Apellidos y Nombre
De Páez Luz Clemencia

Documento de identificación
C.C. 35.456.344

Tarjeta profesional
T.P.A. 23.555

Dirección y domicilio		Ciudad
Carrera 4ª No. 72 –35		Bogotá
Dirección electrónica	No. Fax	Número telefónico
cavelier@cavelier.com	2 11 86 50	3 47 36 11

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general

4. Datos del trámite

Corrección:

Expediente No 08.077.425

Aparece

Debe ser

Modificación:**Modificación al capítulo reivindicatorio.**

Presento nuevo capítulo reivindicatorio compuesto por 31 reivindicaciones y pido respetuosamente que el examen de patentabilidad sea realizado sobre estas nuevas reivindicaciones presentadas.

Respetuosamente manifiesto que a pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud, beneficiándose de la fecha de prioridad inicialmente reivindicada.

**Modificación al capítulo descriptivo.****Modificación al resumen.****Modificación a los dibujos.****Modificación al solicitante.****5. Anexos**

Poder, si fuere el caso.



Documento que contiene las modificaciones o correcciones.



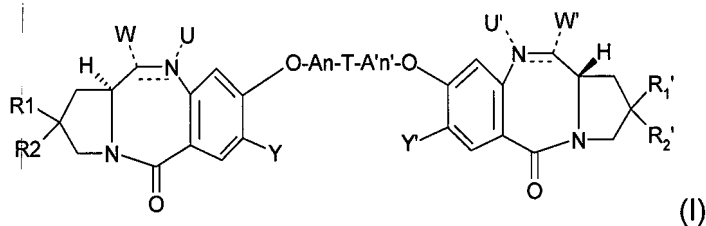
Comprobante de pago por 21 reivindicaciones adicionales después de la décima reivindicación por \$630.000.

6. Firma	
Nombre y apellidos	Firma
LUZ CLEMENCIA DE PAEZ	<i>LuZ Clemencia De PaeZ</i>
C.C. 35.456.344 DE Usaquén	Tarjeta Profesional 23.555

✍ CNV\NCA\WPC14775\ID-226190

Reivindicaciones Modificadas

1. Compuestos de formula (I):



en donde:

--- representa un enlace sencillo opcional;

==== representa un enlace sencillo o un doble enlace;

con la condición de que cuando ---- representa un enlace sencillo, U y U', iguales o diferentes, representan independientemente H y W y W', iguales o diferentes, se seleccionan independientemente entre el grupo que consiste en OH, -OR, -OCOR, -OCOOR, -OCONRR', -NRCONRR', -OCSNHR, -SH, -SR, -SOR, -SOOR, -SO₃⁻, -NRSOOR, -NRR', -NROR', -NRCOR, -N₃, un ciano, un halo, un trialquilo(C1-C12) o triarilfosfonio(C6-C10), un grupo derivado de aminoácido;

y cuando === representa un doble enlace, U y U' están ausentes y W y W' representan H;

R₁ y R₂ y R₁' y R₂' forman juntos un grupo que contiene un doble enlace =B y =B' respectivamente; en donde

B y B' son iguales o diferentes y se eligen independientemente entre alqueno(C2—C12) que está opcionalmente sustituido con uno o más CN, R, CF₃, OR, arilo(C6-C10), S(O)_qR;

A, A' son iguales o diferentes y se eligen independientemente entre alquilo(C1-C12) o alqueno(C2-C12), cada uno opcionalmente sustituido con uno o más CN, NRR', CF₃, OR, S(O)_qR, arilo(C6-C10), alquilo(C1-C12), alqueno(C2-C12);

Y, Y' son iguales o diferentes y se eligen independientemente entre O(C1-C12)alquilo;

T es fenilo o piridilo, sustituido por uno o mas enlazador(es) y opcionalmente sustituido con uno o más CN, NRR', CF₃, R, OR, S(O)_qR;

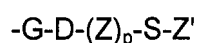
n, n', iguales o diferentes, son 0 ó 1;

q es 0, 1 ó 2;

R, R' son iguales o diferentes y se eligen independientemente entre H, (C1-C12)alquilo, (C6-C12)arilo, estando cada uno de ellos opcionalmente sustituido con CN, NRR', CF₃, R, OR, S(O)_qR, (C6-C10)arilo;

en donde dicho enlazador contiene sustituyentes que comprenden grupos tiol-, sulfuro- o disulfuro- y

*es de formula:



en la que

- G es un enlace sencillo o un doble enlace, -O-, -S- o -NR-;
- D es un enlace sencillo o -E-, -E-NR-, -E-NR-F-, -E-O-, -E-O-F-, -E-NR-CO-, -E-NR-CO-F-, -E-CO-, -CO-E-, -E-CO-F-, -E-S-, -E-S-F-, -E-NR-C-S-, -E-NR-CS-F-;
- E y F son iguales o diferentes y se eligen independientemente entre - (OCH₂CH₂)_i (C1-C12)alquil(OCH₂CH₂)_j- lineal o ramificado, -(C1-C12)alquil(OCH₂CH₂)_i-(C1-C12)alquil-, -(OCH₂CH₂)_i-, -(OCH₂CH₂)_i(C3-C10)cicloalquil(OCH₂CH₂)_j-, -(OCH₂CH₂)_iheterocíclico(OCH₂CH₂)_j-, -(OCH₂CH₂)_i(C6-C10)aril(OCH₂CH₂)_j-, -(OCH₂CH₂)_iheteroaril(OCH₂CH₂)_j-, (C1-C12)alquil-(OCH₂CH₂)_i(C1-C12)alquil(OCH₂CH₂)_j-, -(C1-C12)alquil-(OCH₂CH₂)_i-, -(C1-C12)alquil-(OCH₂CH₂)_i(C3-C10)cicloalquil(OCH₂CH₂)_j-, -(C1-C12)alquil(OCH₂CH₂)_iheterocíclico(OCH₂CH₂)_j-, -(C1-C12)alquil-(OCH₂CH₂)_iaril(OCH₂CH₂)_j-, -(C1-C12)alquil(OCH₂CH₂)_iheteroaril(OCH₂CH₂)_j-, -(C3-C10)cicloalquil-(C1-C12)alquil-, -(C1-C12)alquil-(C3-C10)cicloalquil-, -heterocíclico-(C1-C12)alquil-, -(C1-C12)alquil-heterocíclico-, -(C1-C12)alquil-(C6-C10)aril-, -(C6-C10)aril-(C1-C12)alquil-, -(C1-C12)alquil-heteroaril-, -heteroaril-(C1-C12)alquil-;
- i y j, iguales o diferentes, son números enteros y se eligen independientemente entre 0, 1 a 2000;
- Z es -(C1-C12)alquil- lineal o ramificado;
- p es 0 ó 1;
- Z' representa H, un grupo protector de tiol, R₂₀ O SR₂₀, donde R₂₀ representa H, metilo, (C1-C12)alquilo, (C3-C10)cicloalquilo, (C6-C10)arilo,

heteroarilo o un grupo heterociclico, con la condición de que cuando Z' es H, dicho compuesto esté en equilibrio con el compuesto correspondiente formado por ciclación intramolecular producida por la adición del grupo tiol -SH al enlace imina -NH= de uno de los restos PBD.

*o es elegido entre la siguiente lista:

- (CR₁₃R₁₄)_t(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
- (CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_y(OCH₂CH₂)_ySZ';
- (CR₁₃R₁₄)_t(NR₁₉CO)(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
- (CR₁₃R₁₄)_t(OCO)(R₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
- (CR₁₃R₁₄)_t(CO)(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
- (CR₁₃R₁₄)_t(CONR₁₉)(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
- (CR₁₃R₁₄)_t-fenil-CO(CR₁₅R₁₆)_uSZ';
- (CR₁₃R₁₄)_t-fural-CO(CR₁₅R₁₆)_uSZ';
- (CR₁₃R₁₄)_t-oxazolil-CO(CR₁₅R₁₆)_uSZ';
- (CR₁₃R₁₄)_t-tiazolil-CO(CR₁₅R₁₆)_uSZ';
- (CR₁₃R₁₄)_t-tienil-CO(CR₁₅R₁₆)_uSZ';
- (CR₁₃R₁₄)_t-imidazolil-CO(CR₁₅R₁₆)_uSZ';
- (CR₁₃R₁₄)_t-morfolino-CO(CR₁₅R₁₆)_uSZ';
- (CR₁₃R₁₄)_t-piperazino-CO(CR₁₅R₁₆)_uSZ';
- (CR₁₃R₁₄)_t-M-metil-piperazino-CO(CR₁₅R₁₆)_uSZ';
- (CR₁₃R₁₄)_t-fenil-QSZ';
- (CR₁₃R₁₄)_t-fural-QSZ';
- (CR₁₃R₁₄)_t-oxazolil-QSZ';
- (CR₁₃R₁₄)_t-tiazolil-QSZ';
- (CR₁₃R₁₄)_t-tienil-QSZ';
- (CR₁₃R₁₄)_t-imidazolil-QSZ';
- (CR₁₃R₁₄)_t-morfolino-QSZ';
- (CR₁₃R₁₄)_t-piperazino-QSZ';
- (CR₁₃R₁₄)_t-N-metil-piperazino-QSZ';
- O(CR₁₃R₁₄)_t(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
- O(CR₁₃R₁₄)_t(NR₁₉CO)(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
- O(CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
- O-fenil-QSZ';
- O-fural-QSZ';
- O-oxazolil-QSZ';
- O-tiazolil-QSZ';
- O-tienil-QSZ';

-O-imidazolil-QSZ';
 -O-morfolino-QSZ';
 -O-piperazino-QSZ';
 -O-N-metil-piperazino-QSZ';
 -OCO(CR₁₃R₁₄)_t(NR₁₉CO)_v(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
 -OCO(CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
 -OCONR₁₂(CR₁₃R₁₄)_t(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
 -OCO-fenil-QSZ';
 -OCO-furil-QSZ';
 -OCO-oxazolil-QSZ';
 -OCO-tiazolil-QSZ';
 -OCO-tienil-QSZ';
 -OCO-imidazolil-QSZ';
 -OCO-morfolino-QSZ';
 -OCO-piperazino-QSZ';
 -OCO-N-metil-piperazino-QSZ';
 -CO(CR₁₃R₁₄)_t(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
 -CO(CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
 -CONR₁₂(CR₁₃R₁₄)_t(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
 -CO-fenil-QSZ';
 -CO-furil-QSZ';
 -CO-oxazolil-QSZ';
 -CO-fenil-QSZ';
 -CO-tiazolil-QSZ';
 -CO-tienil-QSZ';
 -CO-imidazolil-QSZ';
 -CO-morfolino-QSZ';
 -CO-piperazino-QSZ';
 -CO-piperidino-QSZ';
 -CO-N-metil-piperazino-QSZ';
 -NR₁₉(CR₁₃R₁₄)_t(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
 -NR₁₉CO(CR₁₃R₁₄)_t(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
 -NR₁₉(CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_t(OCH₂CH₂)_ySZ';
 -NR₁₉CO(CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_t(OCH₂CH₂)_ySZ';
 -NR₁₉CONR₁₂(CR₁₃R₁₄)_t(CR₁₅R₁₆)_u(OCH₂CH₂)_ySZ';
 -NR₁₉CONR₁₂(CR₁₃R₁₄)_t(CR₁₇=CR₁₈)(CR₁₅R₁₆)_t(OCH₂CH₂)_ySZ';
 -NR₁₉CO-fenil-QSZ';
 -NR₁₉CO-furil-QSZ';
 -NR₁₉CO-oxazolil-QSZ';

$-NR_{19}CO\text{-tiazolil-QSZ'}$;
 $-NR_{19}CO\text{-tienil-QSZ'}$;
 $-NR_{19}CO\text{-imidazolil-QSZ'}$;
 $-NR_{19}CO\text{-morfonilo-QSZ'}$;
 $-NR_{19}CO\text{-piperazino-QSZ'}$;
 $-NR_{19}CO\text{-piperidino-QSZ'}$;
 $-NR_{19}CO\text{-N-metil-piperazino-QSZ'}$;
 $-NR_{19}\text{-fenil-QSZ'}$;
 $-NR_{19}\text{-furil-QSZ'}$;
 $-NR_{19}\text{-oxazolil-QSZ'}$;
 $-NR_{19}\text{-tiazolil-QSZ'}$;
 $-NR_{19}\text{-tienil-QSZ'}$;
 $-NR_{19}\text{-imidazolil-QSZ'}$;
 $-NR_{19}\text{-morfonilo-QSZ'}$;
 $-NR_{19}\text{-piperazino-QSZ'}$;
 $-NR_{19}\text{-piperidino-QSZ'}$;
 $-NR_{19}\text{-N-metil-piperazino-QSZ'}$;
 $-NR_{19}CONR_{12}\text{-fenil-QSZ'}$;
 $-NR_{19}CONR_{12}\text{-oxazolil-QSZ'}$;
 $-NR_{19}CONR_{12}\text{-tiazolil-QSZ'}$;
 $-NR_{19}CONR_{12}\text{-tienil-QSZ'}$;
 $-NR_{19}CONR_{12}\text{-piperidino-QSZ'}$;
 $-S(O)_q(CR_{13}R_{14})_t(CR_{15}R_{16})_u(OCH_2CH_2)_ySZ'$;
 $-S(O)_q(CR_{13}R_{14})_t(CR_{17}=CR_{18})(CR_{15}R_{16})_t(OCH_2CH_2)_ySZ'$;
 $-SCONR_{12}(CR_{13}R_{14})_t(CR_{15}R_{16})_u(OCH_2CH_2)_ySZ'$;
 $-SCO\text{-morfolino-QSZ'}$;
 $-SCO\text{-piperazino-QSZ'}$;
 $-SCO\text{-piperidino-QSZ'}$;
 $-SCO\text{-N-metil-piperazino-QSZ'}$;

en donde:

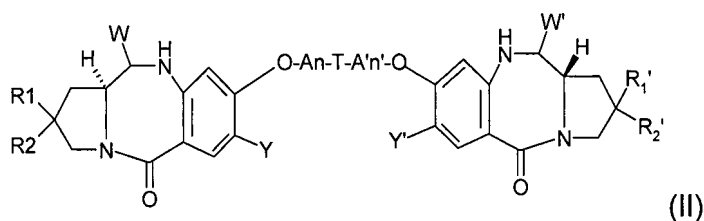
- Z' es H, un grupo protector de tiol, R_{20}' o SR_{20}' donde R_{20}' representa alquilo(C1-C12), arilo(C6-C10), un grupo heterocíclico o heteroarilo,
- Q es un enlace directo o un grupo alquilo lineal o alquilo ramificado que tiene 1-10 átomos de carbono o un espaciador de polietilenglicol con 2 a 20 unidades de repetición etileno oxi;
- R_{19} y R_{12} son iguales o diferentes y son alquilo lineal, alquilo ramificado o alquilo cíclico que tiene de 1 a 10 átomos de carbono, o un grupo arilo(C6-C10) o heterocíclico simple o sustituido, y R_{12} puede ser además H,

- R_{13} , R_{14} , R_{15} y R_{16} son iguales o diferentes y son H o un alquilo lineal o ramificado que tiene de 1 a 4 átomos de carbono,
- R_{17} y R_{18} son H o alquilo (C1-C12),
- u es un número entero de 1 a 10 y también puede ser 0,
- t es un número entero de 1 a 10 y también puede ser 0,
- y es un número entero de 1 a 20 y puede ser también 0.

en donde:

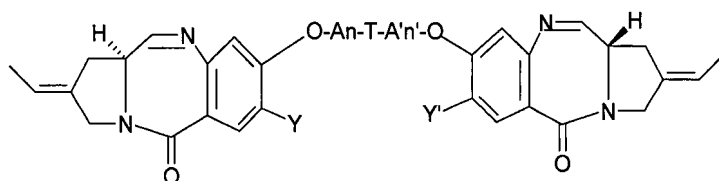
- el término "heteroaril" o "heterociclo aromático" se refiere a un anillo hetero-, mono-, bi- aromático o multicíclico de 5 a 14 miembros.
- El término "heterocilo" o "heterocíclico" se refiere a un anillo mono-, bi-, o multicíclico no aromático de 3 a 14 miembros, saturado, o parcialmente insaturado o insaturado, en donde al menos un miembro del anillo es un heteroátomo elegido entre nitrógeno, oxígeno y azufre.

2. Compuestos de acuerdo a la reivindicación 1, de formula (II):



3. Compuestos de acuerdo a cualquiera de las reivindicaciones 1 ó 2, en donde $B=B'=CH_2$ o $CH-CH_3$.

4. Compuestos de acuerdo a cualquiera de las reivindicaciones 1 ó 2 de formula:



5. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde $n=n'=1$.

6. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde $A=A'$.

7. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde $A=A'$ = alquilo(C1-C12) linear sin sustituir.
8. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde $Y=Y'$.
9. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde $Y=Y'=O$ Alquilo(C1-C12).
10. Compuesto de acuerdo a la reivindicación 8 donde $Y=Y'=Ome$.
11. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde G es un enlace sencillo o -O- o -NR-.
12. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde G es -O-.
13. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde D es un enlace sencillo o -E-, -E-NR-, -E-CO-, -CO-E, -E-NR-CO-.
14. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde D es -E-NR-CO-.
15. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde E es un alquil(C1-C12) lineal o ramificado, $-(OCH_2CH_2)_i$, o alquil(C1-C12)-heterocíclico.
16. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde Z es $-(CH_2)_2-C(CH_3)_2-$.
17. Compuestos de acuerdo a cualquiera de las reivindicaciones anteriores donde p es 1.
18. Compuestos seleccionados de la lista que comprende:
 - 8,8'-{5-[3-(4-metil-4-metildisulfanil-pentanoilamino)propiloxi]-1,3-benzenodilbis(metilenooxi)}-bis[(S)-2-et-(E)-ilideno-7-metoxi-1,2,3,11a-tetrahidro-5H-pirrol[2,1-c][1,4]benzodiazepin-5-ona] ;

- 8,8'-[5-acetiltiometil-1,3-bencenodiilbis(metilenooxi)]-bis[(S)-2-metileno-7-metoksi-1,2,3,11a-tetrahidro-5H-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[3-(2-acetiltioetil)-1,5-pentanodiilbis(oxi)]-bis[(S)-2-metileno-7-metoksi-1,2,3,11a-tetrahidro-5H-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[5-(N-4-mercapto-4,4-dimetilbutanoil)amino-1,3-bencenodiilbis(metilenoxi)]-bis[7-metoksi-2-metileno-1,2,3,11a-tetrahidro-5H-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[5-(N-4-metilditio-4,4-dimetilbutanoil)-amino-1,3-bencenodiilbis(metilenoxi)]-bis[7-metoksi-2-metileno-1,2,3,11a-tetrahidro-5H-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[5-(N-metil-N-(2-mercapto-2,2-dimetiletil)amino-1,3-bencenodiil(metilenoxi)]-bis[7-metoksi-2-metileno-1,2,3,11a-tetrahidro-5H-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[5-(N-metil-N-(2-metilditio-2,2-dimetiletil)amino-1,3-bencenodiil(metilenoxi)]-bis[7-metoksi-2-metileno-1,2,3,11a-tetrahidro-5H-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(4-(2-(4-mercapto-4-metil)-pentanamido-etoxi)-piridin-2,6-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoksi-1,2,3,11a-tetrahidro-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(4-(2-(4-metil-4-metildisulfanil)-pentanamido-etoxi)-piridin-2,6-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoksi-1,2,3,11a-tetrahidro-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(1-(2-(4-metil-4-metildisulfanil)-pentanamido-etoxi)-benceno-3,5-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoksi-1,2,3,11a-tetrahidro-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(4-(3-(4-metil-4-metildisulfanil)-pentanamido-propoxi)-piridin-2,6-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoksi-1,2,3,11a-tetrahidro-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(4-(4-(4-metil-4-metildisulfanil)-pentanamido-butoxi)-piridin-2,6-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoksi-1,2,3,11a-tetrahidro-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(4-(3-[4-(4-metil-4-metildisulfanil-pentanoil)-piperazin-1-il]-propil)-piridin-2,6-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoksi-1,2,3,11a-tetrahidro-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(1-(3-[4-(4-metil-4-metildisulfanil-pentanoil)-piperazin-1-il]-propil)-benzeno-3,5-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoksi-1,2,3,11a-tetrahidro-pirrolo[2,1-c][1,4]benzodiazepin-5-ona] ;

- 8,8'-[(4-(2-{2-[2-(4-metil-4-metildisulfanil-pentanoilamino)-etoxi]-etoxi}-etoxi)-piridin-2,6-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoxi-1,2,3,11a-tetrahidro-pirrol[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(1-(2-{2-[2-(2-{2-[2-(4-metil-4-metildisulfanil-pentanoilamino)-etoxi]-etoxi]-etoxi}-etoxi)-etoxi]-etoxi)-benceno-3,5-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoxi-1,2,3,11a-tetrahidro-pirrol[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(1-(2-{2-[2-(4-metil-4-metildisulfanil-pentanoilamino)-etoxi]-etoxi}-etoxi)-benceno-3,5-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoxi-1,2,3,11a-tetrahidro-pirrol[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(4-(2-{2-[2-(2-{2-[2-(4-metil-4-metildisulfanil-pentanoilamino)-etoxi]-etoxi]-etoxi}-etoxi)-etoxi]-etoxi)-piridin-2,6-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoxi-1,2,3,11a-tetrahidro-pirrol[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(1-(2-[metil-(2-metil-2-metildisulfanil-propil)-amino]-etoxi)-benceno-3,5-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoxi-1,2,3,11a-tetrahidro-pirrol[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(4-(3-[metil-(4-metil-4-metildisulfanil-pentanoil)-amino]-propil)-piridin-2,6-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoxi-1,2,3,11a-tetrahidro-pirrol[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(4-(3-[metil-(2-metil-2-metildisulfanil-propil)-amino]-propil)-piridin-2,6-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoxi-1,2,3,11a-tetrahidro-pirrol[2,1-c][1,4]benzodiazepin-5-ona] ;
- 8,8'-[(1-(4-metil-4-metildisulfanil)-pentanamido)-benceno-3,5-dimetil)-dioxi]-bis[(S)-2-et-(E)-ilideno-7-dimetoxi-1,2,3,11a-tetrahidro-pirrol[2,1-c][1,4]benzodiazepin-5-ona]

19. Compuestos seleccionados de la lista que comprende:

- 8,8'-[4-(3-tert-butoxicarbonilaminopropiloxi)-2,6-piridinadiilbis(metilenoxi)]-bis[(S)-2-et-(E)-ilideno-7-metoxi-1,2,3,11a-tetrahidro-5H-pirrol[2,1-c][1,4]benzodiazepin-5-ona];
- 8,8'-[5-(3-aminopropiloxi)-1,3-bencenodiilbis(metilenoxi)]-bis[(S)-2-et-(E)-ilideno-7-metoxi-1,2,3,11a-tetrahidro-5H-pirrol[2,1-c][1,4]benzodiazepin-5-ona];
- 8,8'-[5-(N-metil-3-tert-butoxicarbonilaminopropil)-1,3-bencenodiilbis(metilenoxi)]-bis[(S)-2-et-(E)-ilideno-7-metoxi-1,2,3,11a-tetrahidro-5H-pirrol[2,1-c][1,4]benzodiazepin-5-ona]

20. Un conjugado que comprende uno o más compuestos de acuerdo a cualquiera de las reivindicaciones 1 a 19, unidos covalentemente a un agente de unión a las

células a través de un grupo de unión del enlazador(es), donde dicho grupo de unión es un grupo tiol, sulfuro o disulfuro.

21. Un conjugado de acuerdo con la reivindicación 20, donde dicho agente de unión a las células ha sido modificado por un agente modificante para mejorar la reactividad del agente de unión a las células hacia el grupo de unión del enlazador.
22. Un conjugado de acuerdo con la reivindicación 20 ó 21, en el que dicho agente de unión a las células se elige entre anticuerpos o un fragmento de un anticuerpo que contiene al menos un sitio de unión, linfoquinas, hormonas, factores de crecimiento, o moléculas transportadoras de nutrientes.
23. Un conjugado de acuerdo con cualquiera de las reivindicaciones 20 a 22, en el que dicho agente de unión a las células se elige entre anticuerpos monoclonales; anticuerpos quiméricos; anticuerpos humanizados; anticuerpos totalmente humanos; anticuerpos monocatenarios; fragmentos de anticuerpos; interferones; péptidos; linfoquinas; hormonas; hormonas esteroideas; factores de crecimiento y factores estimuladores de colonias; o vitaminas.
24. Proceso de preparación de un conjugado de acuerdo con cualquiera de las reivindicaciones 20 a 23, que comprende la etapa de reaccionar un compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 19 en donde T comprende un grupo tiol, sulfuro, disulfuro, o un precursor de los mismos, con un agente de unión a las células que comprende una función reactiva hacia tiol o disulfuro, de tal modo que el compuesto y el agente de unión a las células se unan por medio de un enlace sulfuro o disulfuro.
25. Proceso para la preparación de un conjugado en donde un agente de unión a las células que comprende un grupo tiol libre o protegido, se hace reaccionar con un compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 19 que comprende un grupo tiol- o disulfuro-, y en donde el agente de unión a las células es un péptido o un anticuerpo modificado por un reactivo de entrecruzamiento.
26. Proceso de acuerdo con la reivindicación 25, en donde dicho reactivo de entrecruzamiento es N-succinimidil 3-(2-piridilditio)propionato, N-succinimidil 4-(2-piridilditio)pentanoato, 4-succinimidil-oxicarbonil- α -metil- α -(2-piridilditio)-tolueno, N-succinimidil-3-(2-piridilditio) butirato, succinimidil piridil-ditiopropionato, ácido 4-(2-piridilditio)butanoico N-hidrosuccinimida éster, succinimidil 4-[N-

maleimidometil]ciclohexano-1-carboxilato, N-sulfosuccinimidil-3-(2-(5-nitro-piridilditio) butirato, 2-iminotiolano, o anhídrido S-acetilsuccinico.

27. Proceso de acuerdo con la reivindicación 25, en donde:

- el grupo tiopiridilo de un anticuerpo monoclonal modificado con succinimidil piridil-ditiopropionato es desplazado por el tratamiento con un compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 19 que contiene un grupo tiol-, generando un conjugado de enlace disulfuro;
- el grupo aril-tiol de un compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 19 es desplazado por grupos sulfhidrilo previamente introducidos a un anticuerpo, generando un conjugado de enlace disulfuro.

28. Proceso de acuerdo con la reivindicación 25, en donde un compuesto de acuerdo con cualquiera de las reivindicaciones 1 a 18 que contiene un grupo tiol, se une mediante un enlace tioeter, a un anticuerpo o a un agente de unión a las células modificado con N-succinimidil 4-(maleimidometil)ciclohexanocarboxilato, N-succinimidil-4-(N-maleimidometil)-ciclohexano-1-carboxi-(6-amidocaproato), N-succinimidil-4-(iodoacetil)-aminobenzoato, N-succinimidil iodoacetato, N-succinimidil bromoacetato o N-succinimidil 3-(bromo-acetamido)propionato.

29. Proceso de acuerdo con cualquiera de las reivindicaciones 25 a 28, en donde el conjugado es purificado por una técnica de cromatografía estándar o por diálisis o diafiltración.

30. Proceso de acuerdo con la reivindicación 29, en donde la técnica cromatográfica es HPLC, cromatografía de exclusión por tamaño, cromatografía de adsorción, cromatografía de intercambio iónico, cromatografía de interacción hidrofóbica o cromatografía de afinidad, cromatografía sobre hidroxiapatita.

31. Una composición farmacéutica que comprende un molécula de conjugado como se define en cualquiera de las reivindicaciones 20 a 23 o un compuesto como se define en cualquiera de las reivindicaciones 1 a 19 junto, junto con un vehículo farmacéuticamente aceptable.

Immunoconjugates Containing Novel Maytansinoids: Promising Anticancer Drugs

Ravi V. J. Chari,¹ Bridget A. Martell, Jonathan L. Gross, Sherrilyn B. Cook, Sudhir A. Shah, Walter A. Blättler, Sara J. McKenzie, and Victor S. Goldmacher¹

ImmunoGen, Inc., Cambridge, Massachusetts 02139 [R. V. J. C., B. A. M., J. L. G., S. B. C., S. A. S., W. A. B., V. S. G.], and Applied bioTechnology, Cambridge, Massachusetts 02142 [S. J. M.]

ABSTRACT

The potential of immunoconjugates of cytotoxic drugs for the treatment of cancer has not yet been realized owing to the difficulty of delivering therapeutic concentrations of these drugs to the target cells. In an effort to overcome this problem we have synthesized maytansinoids that have 100- to 1000-fold higher cytotoxic potency than clinically used anticancer drugs. These maytansinoids are linked to antibodies via disulfide bonds, which ensures the release of fully active drug inside the cells. The conjugates show high antigen-specific cytotoxicity for cultured human cancer cells (50% inhibiting concentration, 10 to 40 pM), low systemic toxicity in mice, and good pharmacokinetic behavior.

INTRODUCTION

Numerous attempts to target tumors with conventional antineoplastic drugs conjugated to monoclonal antibodies have met with limited success. Most frequently, such antibody-drug conjugates are insufficiently cytotoxic, e.g., the conjugates are even less potent than the nonconjugated drugs (1-3). In contrast, a number of antibody conjugates with single chain ribosome-inactivating protein toxins or with isolated A-chains of two chain ribosome-inactivating protein toxins, such as ricin, have been prepared (4, 5) that are at least 10³-fold more cytotoxic than conjugates between antibodies and antineoplastic drugs (1-3). Conjugates between antibodies and protein toxins are also much more potent than the corresponding nonconjugated toxins.

One reason for the different efficacy of these two classes of immunoconjugates may lie in the different modes of action of their toxic moieties. Protein toxins act catalytically, and it has been demonstrated that a single toxin molecule in the cytoplasm of a cell can cause the death of that cell (6, 7). In contrast, anticancer drugs currently in clinical use, such as doxorubicin, methotrexate, and the *Vinca* alkaloids, act stoichiometrically, and relatively much higher intracellular concentrations are needed to achieve comparable cytotoxicities. Intracellular concentrations of drug necessary to kill the target cells are difficult to achieve with antibody-drug conjugates for the following reasons: (a) a majority of commonly used anticancer drugs are only moderately cytotoxic; (b) antigen targets are present on cell surfaces often in only limited numbers (8); (c) the internalization processes for antigen-antibody complexes are frequently inefficient; and (d) most linkers that have been used for the conjugation of drugs to antibodies (1-3, 9-14) do not efficiently release active drug inside the cell.

We describe here an approach that seeks to overcome these problems by replacing the current anticancer drug candidates with compounds of 100- to 1000-fold higher cytotoxicity, and by conjugating these compounds with antibodies via disulfide-containing linkers that can be cleaved inside the cell to release active drug.

Received 8/7/91; accepted 10/22/91.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom requests for reprints should be addressed, at ImmunoGen, Inc., 148 Sidney Street, Cambridge, MA 02139.

MATERIALS AND METHODS

Maytansine and Maytansinoids. Maytansine (1) is a product of Takeda Chemical Industries, Ltd. (Japan). Ansamitocin P-3, a precursor that was used to synthesize maytansinoid 2 (Fig. 2) was supplied by Takeda Chemical Industries of Japan. Synthesis of maytansinoid 2 was performed as follows. Maytansine or ansamitocin P-3 was reduced with lithium aluminum hydride to give the same C-3 alcohol maytansinol as previously described (15). Maytansinol was esterified with *N*-methyl-*N*-(methylthiopropionyl)-L-alanine in the presence of dicyclohexylcarbodiimide and zinc chloride to give maytansinoid 2, which was purified by column chromatography on silica gel eluting with 6% methanol in chloroform. This synthesis will be described in detail elsewhere.²

Antibodies. Murine monoclonal antibody A7 that binds to an antigen expressed on human colon cancer cell lines (16) was a generous gift of Dr. Takahashi (Kyoto University, Kyoto, Japan). The production of the murine monoclonal antibody TA.1 that binds to the HER-2/*neu* oncogene protein has been described elsewhere (17). Murine monoclonal antibodies 5E9 (anti-human transferrin receptor) and anti-B4 (anti-CD19) were purified by methods described previously (8, 18).

Conjugation of Maytansinoids with Antibodies. In order to generate antibody-maytansinoid conjugates, the antibody was modified with *N*-succinimidyl-3-(2-pyridyldithio)propionate to introduce dithiopyridyl groups, or with succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate to introduce maleimido groups. May-SS-Me 2 was reduced to May-SH 3 by the following procedure. A solution of 2 (2 mM) in a mixture of ethanol (0.3 ml) and 50 mM potassium phosphate buffer (0.2 ml; pH 7.5) was treated with dithiothreitol (3 mM) at 4°C for 4 h under nitrogen. The freshly prepared May-SH 3 was purified by high-pressure liquid chromatography and characterized by high-resolution nuclear magnetic resonance analysis. The product (1.2 equivalents per pyridyldithio group or maleimido group introduced into the antibody) was mixed with the modified antibody solution in 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM EDTA. The course of the reaction was followed by monitoring the increase in absorbance at 343 nm due to release of thiopyridyl group. After completion of the reaction (in approximately 40 min) the conjugate was purified by gel filtration on a Sephadex G-25 column. Modification of the antibody to a different extent with *N*-succinimidyl-3-(2-pyridyldithio)propionate gave 1 to 6 dithiopyridyl groups per antibody molecule (as assayed by liberation of thiopyridyl group using dithiothreitol), or with *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate gave 1 to 6 maleimido groups (as determined by reaction with [¹⁴C]cysteine). Conjugates containing 1 to 6 maytansinoid molecules per antibody molecule were prepared by this method, as calculated from absorbances at 252 and 280 nm, using extinction coefficients of $E_{280\text{ nm}} = 5700\text{ M}^{-1}\text{ cm}^{-1}$ and $E_{252\text{ nm}} = 28044\text{ M}^{-1}\text{ cm}^{-1}$ for drug and $E_{280\text{ nm}} = 224000\text{ M}^{-1}\text{ cm}^{-1}$ and $E_{252\text{ nm}} = 87360\text{ M}^{-1}\text{ cm}^{-1}$ for antibody.

Cells and Cell Culture. Human cell lines SK-BR-3 (HTB 30), Nalmwa (CRL 1432), KB (CCL 17), SW-620 (CCL 227), A-498 (HTB 44), HT-29 (HTB 38), NIH:Ovar-3 (HTB 161), and LoVo (CCL 229) were purchased from the American Type Culture Collection (Rockville, MD).

Cytotoxicity Assays. The methods for the determination of the surviving fractions of adherent and nonadherent cell cultures (by colony-forming ability and by back-extrapolation of the exponential growth curves) have been described previously (19, 20).

Pharmacological Studies. Four mice were given injections i.v. of 2 mg/kg (by antibody) of the A7-SS-May conjugate. The volume of the

² Manuscript in preparation.

injection samples was 150 μ l. Following the injections, 75- μ l samples of blood were drawn from the retroorbital plexus of the animals at various time intervals, and the pooled serum from the four animals was diluted 1:4 in PBS³, containing 0.5% bovine serum albumin. The resulting samples (100 μ l) were incubated with 1×10^6 HT-29 cells for 30 min at 0°C, washed in AB buffer, and then incubated with either FITC-labeled goat anti-mouse IgG antiserum (Sigma Chemical Co., St. Louis, MO), diluted 1:50 (v/v) with 0.5% bovine serum albumin/PBS at 4°C for 30 min, for detection of the A7 moiety, or with 0.1 μ M FITC-labeled murine monoclonal anti-maytansine antibody (purified, IgG1, developed at ImmunoGen), for detection of the maytansinoid moiety. The cells were then washed with AB buffer, fixed with 1% formaldehyde in PBS, and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Standard curves for the quantitation of A7 and maytansinoid were generated with A7(-SS-May)₂ samples dissolved in a 1:4 (v/v) mixture of murine serum and AB buffer.

Competition Binding Assays. Competition of binding between a trace amount of FITC-labeled TA.1 antibody and various concentrations of unmodified TA.1 and TA.1-May conjugates to the *neu* antigen on SK-BR-3 cells was analyzed as follows. Cells (7×10^4) were incubated with mixtures of 6 nM FITC-labeled TA.1 and different concentrations of a conjugate for 30 min at 0°C in 25 μ l of AB buffer, and then washed in ice-cold AB buffer. The mean fluorescence of the cells was then measured on a flow cytometer (using a linear scale), the background (cell autofluorescence) was subtracted, and the resulting value was then compared with a control (FITC-labeled nonconjugated antibody).

RESULTS AND DISCUSSION

We have chosen a highly cytotoxic drug, maytansine, for the preparation of immunoconjugates. Maytansine kills cells by interfering with the formation of microtubules and depolymerization of already formed microtubules (21). *In vitro* screening revealed that maytansine (1) (21, 22) is about 100- to 1000-fold more toxic for a range of human cancer cell lines than are most other anticancer drugs. For example, maytansine is highly cytotoxic for Namalwa cells (Fig. 1), with an IC_{50} of 4×10^{-10} M. Despite this high potency, maytansine was ineffective in human clinical trials (23) because of its high systemic toxicity, which resulted in a low therapeutic index. We reasoned that the nonspecific toxicity of maytansine could be lowered and the therapeutic index increased by the targeted delivery of this drug in conjugated form. In order to exploit the cytotoxic potential of maytansine in the conjugate, however, it is necessary to release the drug at the target cell in fully active form.

Two types of cleavable linkers have been widely used in the preparation of antibody-drug conjugates: acid-labile linkers (24–28) and peptidase-sensitive linkers (29). However, with few exceptions (30, 31), the cytotoxic potency of the resulting conjugates for cultured cell lines is very low, much lower than the cytotoxicity of the unconjugated drug, suggesting that internalization and release of drug molecules from these conjugates is inefficient. In addition, release into the medium of even a small fraction of a potent drug from a relatively nonpotent conjugate abolishes any hope of a specific cytotoxic effect. Internalization processes are poorly understood and difficult to influence, but the release of drugs may be improved by changing the design of the linker between the drug candidate and the antibody molecule.

On the basis of the promising results that have been obtained with antibody-toxin conjugates, where the conjugating linkage

³ The abbreviations used are: PBS, phosphate-buffered saline; AB buffer, minimum essential medium modified for suspension cultures supplemented with 2.5% pooled human serum of AB type; FITC, fluorescein isothiocyanate; IC_{50} , the inhibiting concentration of a drug that leaves a surviving fraction of 0.5 i.e., kills 50% of cells.

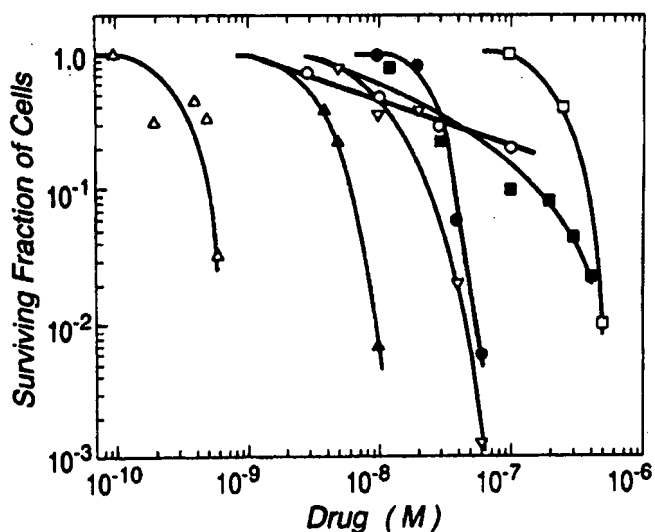


Fig. 1. Comparison of the cytotoxicity of various anticancer drugs for cultured human cells. The Burkitt's lymphoma cell line Namalwa was exposed to different drugs for 24 h at 37°C and the surviving fractions of cells were determined by a direct cytotoxicity assay. The symbols represent maytansine (Δ), actinomycin D ($\blacktriangle$), colchicine (∇), daunomycin ($\bullet$), vinblastine ($\circ$), methotrexate ($\blacksquare$), and mitomycin C ($\square$).

is a disulfide bond (5), we asked whether disulfide linkers would produce antibody-maytansinoid conjugates of comparable potency. Methotrexate linked to poly-D-lysine is the only reported drug conjugate where cleavage of a disulfide bond between the toxic and the targeting moiety releases the active drug, but this is only moderately cytotoxic (32). To obtain a highly cytotoxic drug that has a thiol "handle," we have synthesized a new maytansinoid, 2 (Fig. 2). This novel compound 2 contains a methyl disulfide group and is 3- to 10-fold more cytotoxic than native maytansine (Table 1). The corresponding thiol-containing derivative 3 is as cytotoxic ($IC_{50} = 1 \times 10^{-11}$ M for SK-BR-3 cells after a 24-h exposure) as the disulfide-containing derivative 2.

We therefore prepared antibody conjugates of the maytansi-

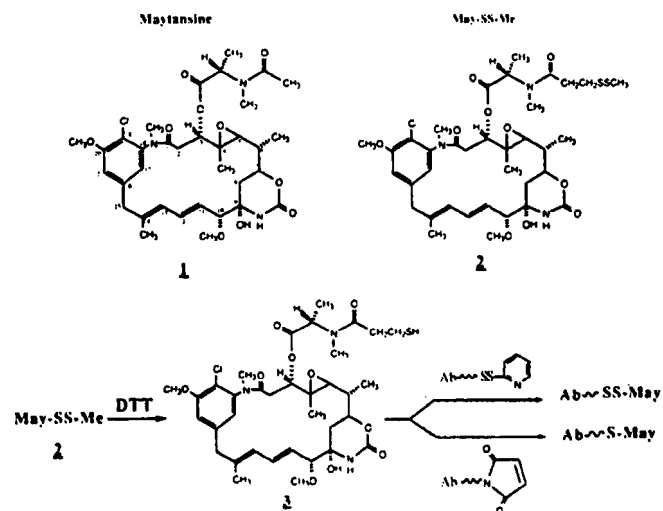


Fig. 2. Maytansinoids and their conjugation to antibodies. In order to generate antibody-drug conjugates the antibody was modified with SPDP [*N*-succinimidyl-3-(2-pyridyldithio)-propionate] to introduce dithio-pyridyl groups, or with SMCC [succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate] to introduce maleimido groups. May-SS-Me 2 was reduced to May-SH 3 (see "Materials and Methods") and reacted with the modified antibodies.

IMMUNOCONJUGATES OF MAYTANSINOIDS

Table 1 Comparison of *in vitro* cytotoxicity of maytansine and May-SS-Me (2)^a

Cell line	IC ₅₀ (M)	
	Maytansine	May-SS-Me (2)
KB (human epidermoid cancer)	3.4 × 10 ⁻¹¹	1.1 × 10 ⁻¹¹
SK-BR-3 (human breast cancer)	1.1 × 10 ⁻¹⁰	1.1 × 10 ⁻¹¹ (1.0 × 10 ⁻¹²) ^b
SW-620 (human colon cancer)	1.0 × 10 ⁻¹⁰	9.0 × 10 ⁻¹²
A-498 (human renal cancer)	>10 ⁻¹⁰	1.0 × 10 ⁻¹⁰
NIH:Ovar-3 (human ovarian cancer)	1.0 × 10 ⁻¹⁰	5.0 × 10 ⁻¹¹
HT-29 (human colon cancer)	ND ^c	2.5 × 10 ⁻¹¹
LoVo (human colon cancer)	ND	4.0 × 10 ⁻¹¹

^a Cytotoxicity of these drugs at different concentrations has been measured after a 24-h exposure of cells to the drugs, using a clonogenic assay, and logarithm of surviving fractions *versus* drug concentration plots have been generated similar to the plots shown in Fig. 3c. Values for IC₅₀ were determined from these plots.

^b After a 72-h exposure.

^c ND, not done.

noid 3 and the murine monoclonal antibody TA.1 (Fig. 2), using linkers containing either a disulfide bond or a noncleavable thioether bond. The TA.1 antibody binds to the HER-2/*neu* oncogene protein (also known as *c-erb-2*) that is expressed at high levels on human breast tumor cells (17). Conjugates with different molar ratios of covalently bound drug to antibody (ranging from one to six molecules of drug per molecule of antibody) have been prepared. In a competition binding assay, it was shown that the specific affinity of the antibody moiety is fully preserved in all conjugates (Fig. 3a).

The specific cytotoxicity of TA.1(-SS-May)₄ was tested *in vitro* on the human breast cancer cell line SK-BR-3, which expresses 3 × 10⁵ *neu* surface antigens per cell. Exposure of the target cells for 24 h to various concentrations of the drug conjugate showed a concentration-dependent cytotoxic effect, with an IC₅₀ of 1.6 × 10⁻¹¹ M (Fig. 3b), demonstrating that the drug conjugate achieved a degree of cytotoxicity similar to that of the free drug. The cytotoxic effect of TA.1(-SS-May)₄ could be abolished by an excess of nonconjugated TA.1 antibody, indicating that the killing of cells depends upon the specific antibody-antigen interaction. In addition, this conjugate was at least 1000-fold less cytotoxic towards *neu*-negative KB cells (Fig. 3b).

The cytotoxicity of TA.1(-SS-May)_n conjugates (where *n* is an average number of maytansinoid molecules per antibody) could be increased by linking more drug molecules per antibody molecule, and it reached its maximum value at *n* = 4 (Table 2). Treatment of SK-BR-3 cells with TA.1(-SS-May)₄ for 72 h was even more effective in killing cells (IC₅₀ = 3 × 10⁻¹² M; 99.9% of cells were killed at 0.1 nM concentration; Fig. 3c). Again, the cytotoxicity of TA.1 (-SS-May)₄ could be abolished by an excess of nonconjugated TA.1 antibody. Also, the cytotoxicity of anti-B4(-SS-May)₆, a conjugate with an antibody of the same isotype but that does not bind to the target cells, was more than 300-fold lower with an IC₅₀ greater than 1 × 10⁻⁹ M (Fig. 3c). A TA.1-May conjugate in which the drug molecules are linked via a noncleavable thioether bond was 200-fold less potent under the same conditions (Fig. 3c). Similar results have been obtained with maytansinoid conjugates of the A7 antibody (16) directed against human colon cancer cell lines (Fig. 3d), and the antibody 5E9 directed against the human transferrin receptor (data not shown).

Pharmacological studies in mice showed that, following an i.v. injection, A7(-SS-May)₆ is cleared slowly from the blood of mice (Fig. 4). The difference in the time course of clearance of the maytansinoid moiety of the conjugate and the antibody moiety of the conjugate indicates that the conjugate slowly dissociates. Free drug is then quickly cleared (in a separate

experiment we determined that the half-life for maytansine in the circulation is less than 20 min, data not shown). An alternative explanation is that the fraction of the conjugate that contains more drug molecules per antibody is cleared faster than the rest of the conjugate. We are currently developing a more stable disulfide-containing linker.

The conjugate A7(-SS-May)₆ was not toxic for the animals: 8 of 8 mice survived when given a dose of 8 mg/kg (by antibody) of the maytansinoid conjugate (containing 200 μg/kg of may-

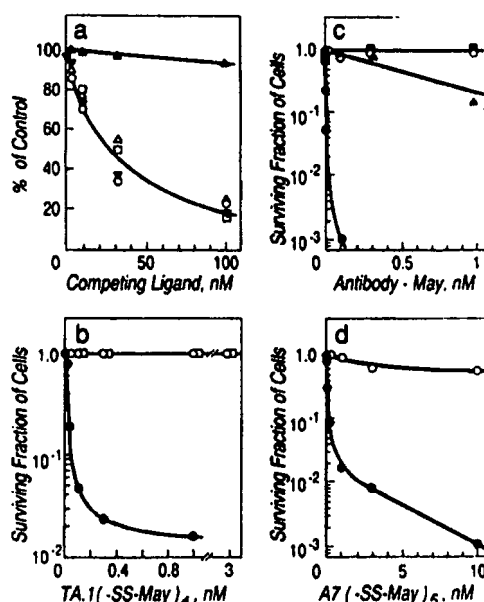


Fig. 3. Interaction of antibody-maytansinoid conjugates with cultured cells. (a) Competition of binding between a trace amount of FITC-labeled TA.1 antibody and unmodified TA.1 and TA.1-May conjugates to the *neu* antigen on SK-BR-3 cells (see "Materials and Methods"). Briefly, cells were incubated with mixtures of FITC-labeled TA.1 and different concentrations of an antibody or a conjugate. The mean fluorescence of the cells was then measured on a flow cytometer, the background (cell autofluorescence) was subtracted, and the resulting value was then compared with a control (FITC-labeled nonconjugated antibody). The symbols represent competition by antibody TA.1 (○), by conjugates TA.1(-SS-May)_n with *n* = 2 (○), *n* = 4 (Δ), *n* = 6 (□), or with a nonbinding antibody (anti-B4) of the same isotype (Δ). (b) Cytotoxicity of TA.1(-SS-May)₄. Cytotoxicity was measured in the presence (○) or in the absence of 1 μM nonconjugated TA.1 (●) on antigen-positive SK-BR-3 cells and compared to the cytotoxicity measured on antigen-negative KB cells (□). Cells were exposed to the conjugate for 24 h at 37°C, and the surviving fractions of cells were then determined by using a clonogenic assay. (c) Cytotoxicity of TA.1(-SS-May)₄ in the presence (○) or in the absence of 1 μM nonconjugated TA.1 (●), TA.1(noncleavable linker-May)₄ (Δ), and anti-B4(-SS-May)₆ (■) for SK-BR-3 cells after an extended (72 h) exposure. The surviving fractions of cells were determined by a clonogenic assay. (d) Cytotoxicity of A7(-SS-May)₅ for HT-29 cells. We determined with a binding experiment using a sample of radioiodinated A7 antibody that HT-29 cells express on the average 8 × 10⁵ A7 antigens per cell. The methods used in these experiments are described in Ref. 8. Cells were incubated with the conjugate for 72 h in the presence (○) or in the absence (●) of 1 μM A7 antibody. The surviving fractions of cells were then determined by a clonogenic assay.

Table 2 Comparison of *in vitro* cytotoxicity of TA.1(-SS-May)_n conjugates for the SK-BR-3 cell line after a 24-h exposure^a

Conjugate	IC ₅₀ (M)
TA.1(-SS-May) ₁	4.6 × 10 ⁻¹⁰
TA.1(-SS-May) ₂	2.1 × 10 ⁻¹⁰
TA.1(-SS-May) ₄	1.2 × 10 ⁻¹¹
TA.1(-SS-May) ₆	2.0 × 10 ⁻¹¹

^a Cytotoxicity of these conjugates at different concentrations has been measured after a 24-h exposure of cells to the conjugates, using a clonogenic assay, and logarithm of surviving fractions *versus* drug concentration plots have been generated similar to the plots shown in Fig. 3c. Values for IC₅₀ were determined from these plots. *n* is an average number of maytansinoid molecules per antibody molecule.

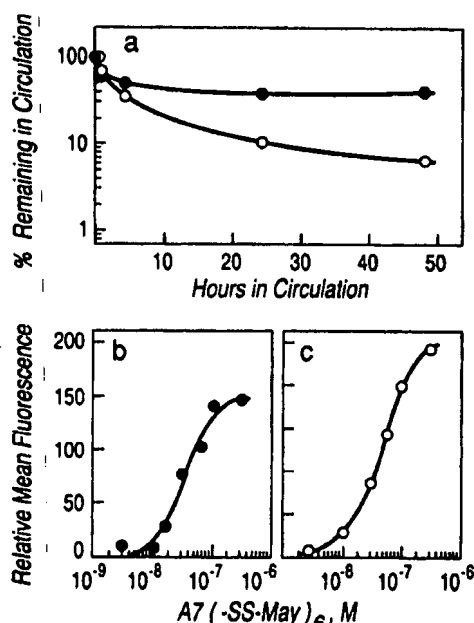


Fig. 4. Clearance and degradation of A7(-SS-May)₆ during circulation in CD-1 mice after i.v. injection. (a) Four mice were given injections of 2 mg/kg (by antibody) of conjugate; 75- μ l samples of blood were drawn from the animals and then analyzed for the presence of the A7 and the maytansinoid moiety as described in "Materials and Methods." Symbols designate A7 moiety ($\bullet$), and maytansinoid moiety ($\circ$). Standard curves for the quantitation of A7 (b), and maytansinoid (c) were generated with A7(-SS-May)₆ samples dissolved in a 1:4 (v/v) mixture of murine serum and AB buffer.

tansinoid) in a single bolus injection (the highest dose tested). These injections effected peak serum concentrations of about 0.1 mg/ml (0.6 μ M). Free maytansine has a 10% lethal dose of 400 μ g/kg in mice (23).

The high specific cytotoxicity of maytansinoid conjugates toward tumor cell lines in conjunction with their low systemic toxicity indicates that these potent conjugates may possess a therapeutic index sufficient for the effective treatment of human cancer.

We believe that the approach described here is not limited to maytansinoids, and that it opens the door for the clinical use of other highly cytotoxic drugs that are too toxic to be used alone. We also envision that conjugates of small drugs may be made with fragments of antibodies, further reducing the size of the resulting therapeutic agents and thereby offering the potential for better penetration of solid tumors. The development of "humanized" antibodies will offer an opportunity to produce drug conjugates that would be less immunogenic than similar conjugates of murine antibodies.

ACKNOWLEDGMENTS

We are grateful to Dr. Emil Frei III (Dana-Farber Cancer Institute) for helping us to procure maytansine. We wish to thank Takeda Chemical Industries of Japan for a generous supply of Ansamitocin P-3. We also wish to thank Dr. Takahashi (Kyoto University, Kyoto, Japan) for a sample of the antibody A7. We would like to thank our colleagues at ImmunoGen, Diane Yachimiak, Gail Raymond, Dr. Sergey Preobrazhensky, Patricia Halloran, and Arikha Moses for technical assistance, Mary Hoffee and Susan Camper for generating the anti-maytansinoid monoclonal antibody, and Drs. John M. Lambert and Albert Collinson for helpful discussions and comments on the manuscript.

REFERENCES

- Sela, M., and Hurwitz, E. Conjugates of antibodies with cytotoxic drugs. In: C.W. Vogel (ed.), *Immunoconjugates*, pp. 189-216. New York: Oxford University Press, 1987.
- Ghose, T. L., Blair, A. H., Vaughan, K., and Kulkarni, P. Antibody-directed drug targeting in cancer therapy. In: E. P. Goldberg (ed.), *Targeted Drugs*, pp. 1-22. New York: John Wiley & Sons, 1983.
- Pietersz, G. A. The linkage of cytotoxic drugs to monoclonal antibodies for the treatment of cancer. *Bioconjugate Chem.*, 1: 89-95, 1990.
- Pastan, I., Willingham, M. C., and Fitzgerald, D. J. P. Immunotoxins. *Cell*, 47: 641-648, 1986.
- Lambert, J. M., Blättler, W. A., McIntyre, G. D., Goldmacher, V. S., and Scott, C. F. Immunotoxins containing single chain ribosome-inactivating proteins. In: A. E. Frankel (ed.), *Immunotoxins*, pp. 175-209. Norwell, MA: Kluwer Academic Publishers, 1988.
- Yamaizumi, M., Mekada, E., Uchida, T., and Okada, Y. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell*, 15: 245-250, 1978.
- Eiklid, K., Olsnes, S., and Pihl, A. Entry of lethal doses of abrin, ricin, and modeccin into the cytosol of HeLa cells. *Exp. Cell Res.*, 126: 321-326, 1980.
- Goldmacher, V. S., Scott, C. F., Lambert, J. M., McIntyre, G. D., Blättler, W. A., Collinson, A. R., Stewart, J. K., Chong, L. D., Cook, S., Slayter, H. S., Beaumont, E., and Watkins, S. Cytotoxicity of gelonin and its conjugates with antibodies is determined by the extent of endocytosis. *J. Cell. Physiol.*, 141: 222-234, 1989.
- Kanellos, J., Pietersz, G. A., and McKenzie, I. F. C. Studies of methotrexate-monomonal antibody conjugates for immunotherapy. *J. Natl. Cancer Inst.*, 75: 319-332, 1985.
- Hurwitz, E., Kashi, R., Aaron, R., Wilchek, M., and Sela, M. The covalent linkage of two nucleotide analogs to antibodies. *J. Med. Chem.*, 28: 137-140, 1985.
- Durrant, L. G., Garnett, M. C., Gallego, J., Armitage, N. C., Ballantyne, K. C., Marksman, R. A., Hardcastle, J. D., and Baldwin, R. W. Sensitivity of newly established cell lines to cytotoxic drugs and monoclonal antibody drug conjugates. *Br. J. Cancer*, 56: 722-726, 1987.
- Endo, N., Takeda, Y., Umemoto, N., Kishida, K., Watanabe, K., Saito, M., Kato, Y., and Hara, T. Nature of linkage and mode of action of methotrexate conjugated with anti-tumor antibodies. Implications for future preparation of conjugates. *Cancer Res.*, 48: 3330-3335, 1988.
- Laguzza, B. C., Nichols, C. L., Briggs, S. L., Cullinan, G. J., Johnson, D. A., Starling, J. J., Baker, A. L., Bumol, T. F., and Corvalan, J. R. F. New antitumor monoclonal antibody-VincA conjugates LY 203725 and related compounds. Design, preparation and representative *in vivo* activity. *J. Med. Chem.*, 32: 548-555, 1989.
- Aboud-Pirak, E., Hurwitz, E., Bellot, F., Schlessinger, J., and Sela, M. Inhibition of human tumor growth in nude mice by a conjugate of doxorubicin with monoclonal antibodies to epidermal growth factor receptor. *Proc. Natl. Acad. Sci. USA*, 86: 3778-3781, 1989.
- Kupchan, S. M., Sneden, A. T., Branfman, A. R., Howie, G. A., Rebhun, L. I., McIvor, W. E., Wang, R. W., and Schnaitman, T. C. Structural requirements for antileukemic activity among the naturally occurring and semisynthetic maytansinoids. *J. Med. Chem.*, 21: 31-37, 1978.
- Takahashi, T., Yamaguchi, T., Kitamura, K., Suzuyama, H., Honda, M., Yokota, T., Kotonagi, H., Takahashi, M., and Hashimoto, Y. Clinical application of monoclonal antibody-drug conjugates for immunotargeting chemotherapy of colorectal carcinoma. *Cancer (Phila.)*, 61: 881-888, 1988.
- McKenzie, S. J., Marks, P. J., Lam, T., Morgan, J., Panicali, D. L., Trimpe, K. L., and Carney, W. P. Generation and characterization of monoclonal antibodies specific for the human *neu* oncogene product p185. *Oncogene*, 4: 543-548, 1989.
- Scott, C. F., Goldmacher, V. S., Lambert, J. M., Jackson, J. V., and McIntyre, G. D. An immunotoxin composed of a monoclonal anti-transferrin receptor antibody linked by a disulfide bond to the ribosome inactivating protein gelonin: potent *in vitro* and *in vivo* effects against human tumors. *J. Natl. Cancer Inst.*, 79: 1163-1172, 1987.
- Goldmacher, V. S., Tinnel, N. L., and Nelson, B. C. Evidence that pinocytosis in lymphoid cells has a low capacity. *J. Cell Biol.*, 102: 1312-1319, 1986.
- Scott, C. F., Goldmacher, V. S., Lambert, J. M., Chari, R. V. J., Bolender, S., Gauthier, M. N., and Blättler, W. A. The antileukemic efficacy of an immunotoxin composed of a monoclonal anti-Thy-1 antibody disulfide linked to the ribosome-inactivating protein gelonin. *Cancer Immunol. Immunother.*, 25: 31-40, 1987.
- Remillard, S., Rebhun, L. I., Howie, G. A., and Kupchan, S. M. Antimitotic activity of the potent tumor inhibitor maytansine. *Science (Washington DC)*, 189: 1002-1005, 1977.
- Kupchan, S. M., Komoda, Y., Branfman, A. R., Sneden, A. T., Court, W. A., Thomas, G. J., Hints, H. J. P., Smith, R. M., Karim, A., Howie, G. A., Verma, A. K., Nagao, Y., Dailey, R. G., Zimmerly, V. A., and Sumner, W. C. The maytansinoids. Isolation, structure elucidation, and chemical interrelation of novel ansa macrolides. *J. Org. Chem.*, 42: 2348-2357, 1977.
- Issell, B. F., and Crooke, S. T. Maytansine. *Cancer Treat. Rev.*, 5: 199-207, 1978.
- Yang, H. M., and Reisfeld, R. A. Pharmacokinetics and mechanism of action of a doxorubicin-monomonal antibody 9.2.27 conjugate directed to a human melanoma proteoglycan. *J. Natl. Cancer Inst.*, 80: 1154-1159, 1988.

IMMUNOCONJUGATES OF MAYTANSINOIDS

25. Dillman, R. O., Johnson, D. E., Shawler, D. L., and Koziol, J. A. Superiority of an acid-labile daunorubicin-monoclonal antibody immunoconjugate compared to free drug. *Cancer Res.*, **48**: 6097-6102, 1988.
26. Ding, L., Yu, L., Xie, G., Gong, D., Vergidis, R., and Diener, L. Application of target-specific drug immunoconjugates to experimental bone marrow replacement therapy in mice. *Cancer Res.*, **50**: 1538-1543, 1990.
27. Greenfield, R. S., Kaneko, T., Daves, A., Edson, M. A., Fitzgerald, K. A., Olech, L. J., Grattan, J. A., Spitalny, G. L., and Braslawsky, G. R. Evaluation *in vitro* of Adriamycin immunoconjugates synthesized using an acid-sensitive hydrazone linker. *Cancer Res.*, **50**: 6600-6607, 1990.
28. Braslawsky, G. R., Edson, M. A., Pearce, W., Kaneko, T., and Greenfield, R. S. Antitumor activity of Adriamycin (hydrazone-linked) immunoconjugates compared with free Adriamycin and specificity of tumor cell killing. *Cancer Res.*, **50**: 6608-6614, 1990.
29. Trouet, A., Masquelier, M., Baurain, R., and Campeneere, D. D-D. A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate: *In vitro* and *in vivo* studies. *Proc. Natl. Acad. Sci. USA*, **79**: 626-629, 1982.
30. Tsukada, Y., Bischof, W. K. D., Hibi, N., Hirai, H., Hurwitz, E., and Sela, M. Effect of a conjugate of daunomycin and antibodies to rat α -fetoprotein on the growth of α -fetoprotein producing tumor cells. *Proc. Natl. Acad. Sci. USA*, **79**: 621-625, 1982.
31. Yang, H. M., and Reisfeld, R. A. Doxorubicin conjugated with a monoclonal antibody directed to a human melanoma-associated proteoglycan suppresses the growth of established tumor xenografts in nude mice. *Proc. Natl. Acad. Sci. USA*, **85**: 1189-1193, 1988.
32. Shen, W.-C., Ryser, H.-P., and LaManna, L. Disulfide spacer between methotrexate and poly(D-lysine). *J. Biol. Chem.*, **260**: 10905-10908, 1985.

Semisynthetic Maytansine Analogues for the Targeted Treatment of Cancer

Wayne C. Widdison, Sharon D. Wilhelm, Emily E. Cavanagh, Kathleen R. Whiteman, Barbara A. Leece, Yelena Kovtun, Victor S. Goldmacher, Hongsheng Xie, Rita M. Steeves, Robert J. Lutz, Robert Zhao, Lintao Wang, Walter A. Blättler, and Ravi V. J. Chari*

ImmunoGen, Inc., 128 Sidney Street, Cambridge, Massachusetts 02139

Received March 20, 2006

Maytansine, a highly cytotoxic natural product, failed as an anticancer agent in human clinical trials because of unacceptable systemic toxicity. The potent cell killing ability of maytansine can be used in a targeted delivery approach for the selective destruction of cancer cells. A series of new maytansinoids, bearing a disulfide or thiol substituent were synthesized. The chain length of the ester side chain and the degree of steric hindrance on the carbon atom bearing the thiol substituent were varied. Several of these maytansinoids were found to be even more potent in vitro than maytansine. The targeted delivery of these maytansinoids, using monoclonal antibodies, resulted in a high, specific killing of the targeted cells in vitro and remarkable antitumor activity in vivo.

Introduction

Maytansine (**1**) was originally isolated from the bark of the African shrub *Maytenus ovatus* by Kupchan and colleagues¹ and is the first compound of a class of benzoansamcrolide antibiotics named maytansinoids.² Maytansinoids are antimetabolic agents that bind to tubulin and inhibit microtubule assembly.³ They bind to the same site on tubulin as the *Vinca* alkaloids do, and similar inhibitory constants were measured for both drug classes.⁴ However, many maytansinoids are 100 to 1000-times more cytotoxic than vincristine and vinblastine toward cancer cell lines in vitro.⁵ Maytansine was extensively tested in preclinical and clinical settings.⁶ Several phase I and phase II clinical trials that were performed with patients suffering from diverse types of cancers established the toxicity profile in humans, but failed to demonstrate therapeutic benefits at tolerable doses. Further development stopped, and maytansine became one of the many cytotoxic agents that were discontinued for the lack of a sufficiently large therapeutic window.

Although, maytansine was not effective in human clinical trials, the unusually high cytotoxic activity of the maytansinoids makes them attractive candidates for tissue-specific delivery. Tumor-reactive monoclonal antibodies are large proteins that bind with high affinity to receptors that are selectively expressed on the surface of cancer cells. Although these antibodies display high specificity for the tumor, a majority of them are not potent enough to be therapeutically useful. Imaging studies with radiolabeled antibodies have demonstrated the localization of antibodies at the tumor site in cancer patients; however, the concentration of antibodies achieved at the tumor was quite low.⁷ The in vitro cytotoxic inhibitory concentrations (IC₅₀ values) of maytansinoids are even lower than the antibody concentrations achieved at the tumor, making them suitable candidates for targeted delivery using antibodies. The selective delivery to tumor tissue has the effect of changing the in vivo distribution and pharmacokinetics of the cytotoxic agent. Humanized monoclonal antibodies typically have a circulation half-life of about two weeks in humans and are largely found in the vascular and extravascular plasma space. Thus, linking a small drug molecule to the antibody will prevent penetration into healthy

tissue and can extend the in vivo half-life of the drug (typically, minutes to a few hours for the unconjugated drug) to as long as two weeks, thereby accumulating a proportionally larger amount at the malignant tissue than at the healthy tissues.⁸ Thus, this method of delivery should effectively contribute to a wider therapeutic window for the maytansinoids and make them effective anticancer agents. Preclinical evaluations of the first set of antibody–maytansinoid conjugates have demonstrated that this targeted delivery approach greatly improves the tumor specificity and the antitumor activity of the maytansinoid.^{9,10} On the basis of this promising preclinical data, the first antibody–maytansinoid conjugate has undergone a phase I clinical evaluation in patients with colorectal and pancreatic cancers.^{11,12}

Delivery of the maytansinoid by a monoclonal antibody requires that the maytansinoid is stably linked to the antibody. However, once the antibody has bound to the antigen on the cancer cells and the antibody–antigen complex is internalized into the cell, the cytotoxic agent needs to be released inside the cell to enable it to efficiently arrive at the target and inactivate it. If one considers that the cytoplasm of cells contains millimolar concentrations of the thiol-containing tripeptide, glutathione, whereas the concentration of glutathione in human blood is about 1000-fold lower (generally in the micromolar range), then a disulfide bond appears to have ideal chemical characteristics.¹³ It is thermodynamically very stable (bond energy of about 65 kcal/mol) but kinetically labile in the presence of sulfhydryl groups. The strength of the disulfide bond can be manipulated to achieve maximal stability during circulation in the blood stream, while allowing for efficient cleavage inside the target cell. This is achieved by the introduction of methyl substituents on the carbon atoms geminal to the disulfide link, conferring varying degrees of steric hindrance. We, therefore, set out to synthesize maytansinoid analogues that contained sulfhydryl groups and showed equal or enhanced cytotoxic activity relative to that of the parent compound, maytansine. Some of these maytansinoids were linked to a monoclonal antibody, and the effect of disulfide bond strength correlated with antitumor activity.

Structure–activity studies with maytansinoids had identified the C3 *N*-acyl-*N*-methyl-L-alanyl ester side chain, the C4–C5 epoxide moiety, the C9 carbinol function, and the position of

* Corresponding author. Tel: (617) 995-2500. Fax: (617) 995-2510. E-mail: ravi.chari@immunogen.com.

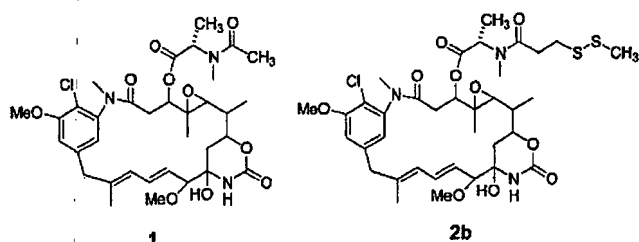


Figure 1. Structures of maytansine and disulfide-containing maytansinoid.

the conjugated C11 and C13 double bonds as critical elements for activity.¹⁴ This left the phenyl ring and the *N*-acyl group as chemically modifiable entities. Although the *N*-acyl group is required for biological activity, studies by Kupchan et al.¹⁵ have shown that the nature of the acyl group can be varied without a significant loss in activity. Thus, we initially replaced the *N*-acetyl group in maytansine (1) with a 3-methyldithiopropionyl group, thus producing disulfide-containing maytansinoid 2b (DM1SMe), (Figure 1). On the basis of the promising *in vitro* potency data of this compound, we then synthesized a variety of disulfide and thiol-containing maytansinoids bearing different *N*-acyl-*N*-methyl-L-alanyl ester moieties at the C3 position. A representative set of these maytansinoids were then conjugated to monoclonal antibodies to provide conjugates with varying disulfide bond strengths. The synthesis of these new maytansinoids and the results from their biological testing in free form or as conjugates of monoclonal antibodies is reported herein.

Biosynthetically, maytansinoids are complex polyketides. Consequently, soon after the initial isolation from plants, a bacterial actinomycetes strain *Actinosynnema pretiosum* was isolated, which produces ansamycin antibiotics called ansami-

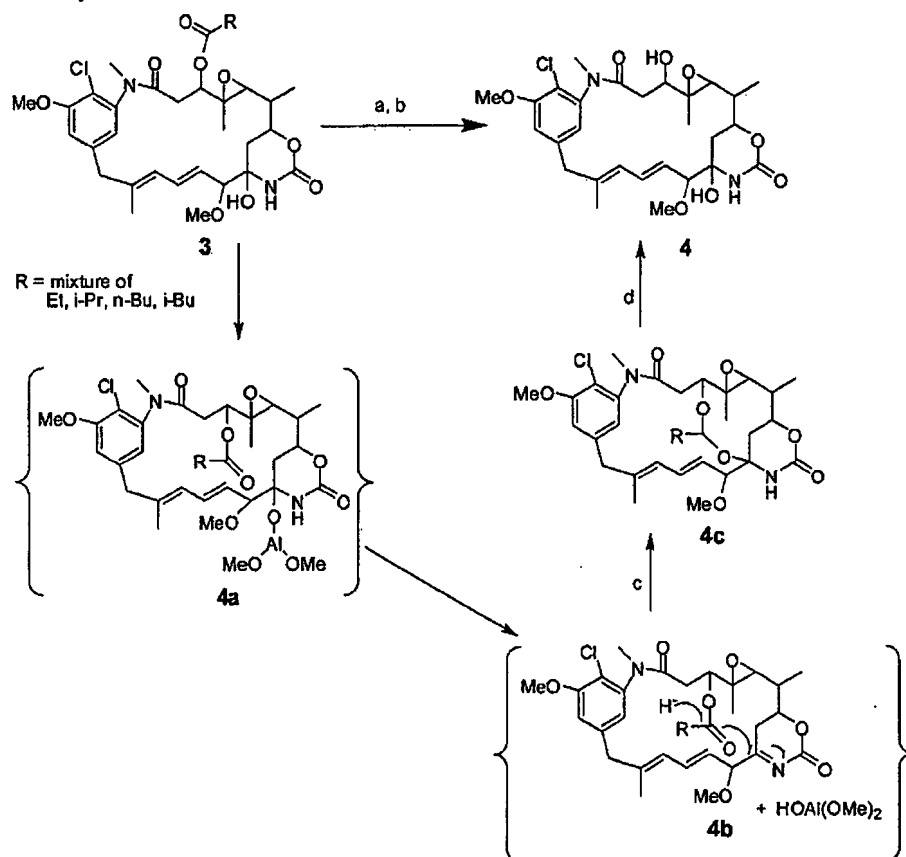
tocins that are maytansinoids with C3 alkanolic acid esters.^{16,17} Because ansamitocins lack the C3 *N*-acyl-*N*-methyl-L-alanyl side chain, they have diminished cytotoxicity; however, they constitute a ready starting material for the preparation of maytansinoid analogues with novel C3 side chains.

Results and Discussion

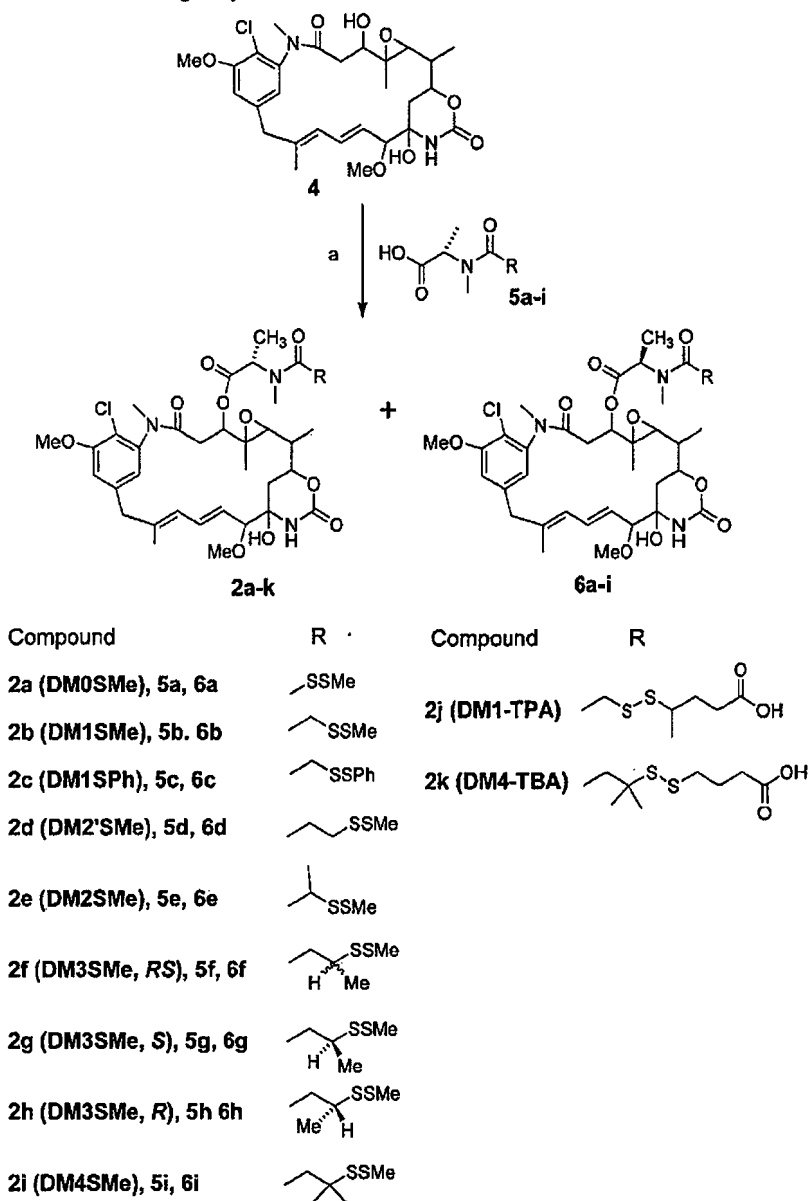
Chemistry. The chemistry followed the scheme originally designed by Kupchan et al.,^{5,15} who first reported the chemical conversion of maytansine to the C3 hydroxy compound, maytansinol, and its subsequent conversion to different C3 esters. Esterification of maytansinol with alkanolic acids gave the bacterial ansamitocins. Kawai et al.,¹⁸ have described the esterification of maytansinol with a variety of *N*-acyl-*N*-methyl-L-alanyl compounds to produce highly active C3 ester analogues of maytansine.

The synthetic route for the preparation of disulfide-containing maytansinoid esters is shown in Schemes 1 and 2. Maytansinol (4) was synthesized from ansamitocins (3), which in turn were obtained from the fermentation of the microorganism *Actinosynnema pretiosum*. Ansamitocins were isolated as a mixture of C3 esters of maytansinol, including propanoate, butyrate, isobutyrate, and pentanoate esters. The predominant species (~80%) was the C3 isobutyrate ansamitocin P-3. Because the C3 ester group in maytansinoids is susceptible to elimination under mild basic conditions (pH > 9) to give the α,β -unsaturated maytansinoid maysine,¹⁹ ester hydrolysis was achieved through a reductive cleavage process. The previously described method¹⁵ for the reductive cleavage of the C3 ester used lithium aluminum hydride and gave low yields of maytansinol with several side products. Several other reducing agents, including DIBAL, NaBH₄, Red-Al, or Red-Al + 1 eq methanol, gave poor yields

Scheme 1. Synthesis of Maytansinol^a



^a (a) LiAlH(OMe)₃, THF, -30 to -40 °C; (b) pH 12, 30 min (c) pH 6-7, (d) pH 2, 2 h or pH 12, 30 min.

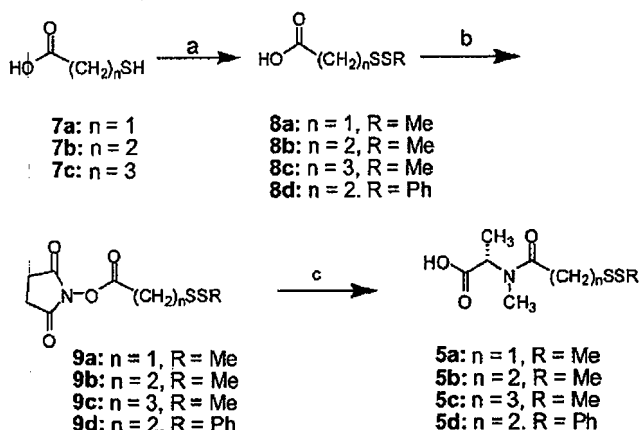
Scheme 2. Synthesis of Disulfide-Containing Maytansinoids^a

^a Reaction conditions: (a) DCC/ZnCl₂/CH₂Cl₂, rt.

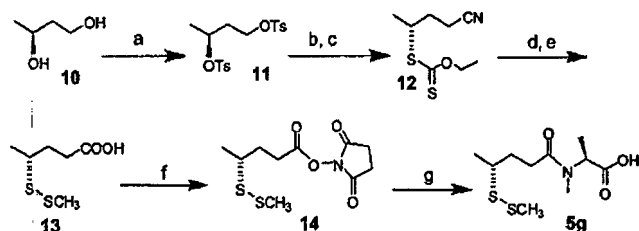
and multiple side products. Attempted enzymatic cleavage of the ester using a wide panel of commercially available esterases and lipases was also unsuccessful, with no detectable hydrolysis. However, the ester group in the ansamitocins was efficiently cleaved using the mild reducing agent lithium trimethoxyaluminum hydride, under controlled temperature (−30 to −40 °C), to give maytansinol in good yields. Higher reaction temperatures led to epoxide opening, whereas colder reaction temperatures resulted in a high proportion of unreacted starting material. Maytansinol was obtained after quenching the reaction mixture at −40 °C with water, which resulted in a pH of about 12, and holding at this pH for 30 min, followed by subsequent neutralization with formic acid. Interestingly, when the reaction mixture was neutralized with acid to a pH between 6 and 7, the very stable acetal intermediate **4c** (presumably formed via **4a**, **4b**) was obtained. This intermediate could be isolated and stored. It was converted to maytansinol (**4**) either by acidification and holding at pH 2 for 2 h or by adding a base and holding at basic pH (~11) for 30 min (Scheme 1).

Mercaptocarboxylic acids of varying linear chain lengths were converted into methylthio or arylthio carboxylic acids by

reacting with methyl methanethiosulfonate or aryl disulfides, respectively (Scheme 3). The dithio-carboxylic acids were converted into their *N*-hydroxysuccinimide esters, followed by reaction with *N*-methyl-L-alanine to form the desired C3 *N*-acyl-*N*-methyl-L-alanine side chains for reaction with maytansinol (**4**). The two enantiomeric 4-(methylthio)pentanoic acids (compounds **13** and **18**) were prepared from the corresponding optically pure 1,3-dihydroxybutanes as shown in Schemes 4 and 5. The conversion of the 1,3-dihydroxybutanes to the ditosylates, followed by the displacement of the primary tosyl group by cyanide, and the substitution of the secondary tosyl group with a xanthate moiety gave compounds **12** and **17**. Base hydrolysis, followed by disulfide exchange with methyl methanethiosulfonate gave compounds **13** and **18**, which were then converted to their respective *N*-hydroxysuccinimide esters **14** and **19**. Acylation of *N*-methyl-L-alanine with the *N*-hydroxysuccinimide ester of *S*- and *R*-4-(methylthio)pentanoic acids gave the two diastereomeric acyl *N*-methyl-L-alanyl side chains **5g** and **5h** for condensation with maytansinol. To synthesize a maytansinoid ester bearing a sterically hindered thiol substituent, the *N*-acyl-*N*-methyl-L-alanine compound **5i**, bearing two methyl

Scheme 3. Synthesis of *N*-Acyl-*N*-methyl-L-alanines^a

^a Reaction conditions: (a) $\text{CH}_3\text{SSO}_2\text{CH}_3$, (PhSSPh for **8d**); (b) *N*-hydroxysuccinimide, EDC; (c) *N*-methyl-L-alanine, triethylamine.

Scheme 4. Synthesis of *N*-Methyl-*N*-[(4-*S*)-methylthio]-1-oxopentyl]-*S*-alanine^a

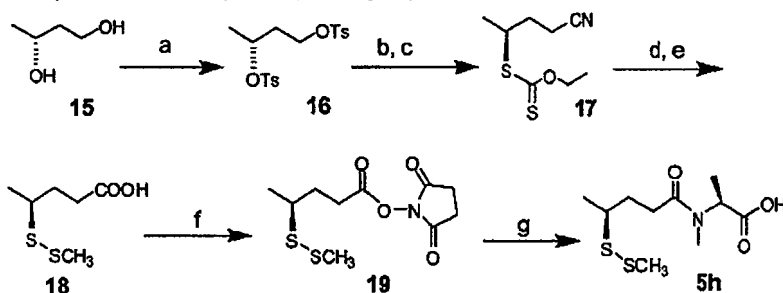
^a Reaction conditions: (a) TsCl, Py; (b) NaCN; (c) KSSCOEt; (d) NaOH; (e) $\text{CH}_3\text{SSO}_2\text{CH}_3$; (f) *N*-hydroxysuccinimide, EDC; (g) *N*-methyl-L-alanine, triethylamine.

substituents on the carbon bearing the disulfide bond, was synthesized from isobutylene sulfide using the steps shown in Scheme 6. Ring opening of episulfide **20** with lithiated acetonitrile gave nitrile **21**. The hydrolysis of the nitrile, followed by disulfide exchange with methylmethanethiolsulfonate gave carboxylic acid **23**. The condensation of **23** with *N*-hydroxysuccinimide in the presence of EDC gave hydroxysuccinimide ester **24**, followed by the reaction with *N*-methyl-L-alanine to give **5i**.

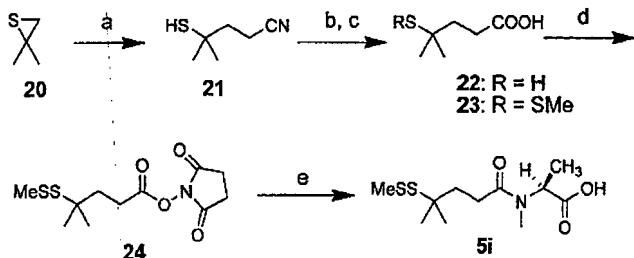
Disulfide-containing maytansinoid esters (**2a–i**), in which both the chain length and degree of branching in the acyl group were varied, were prepared by condensing maytansinol (**4**) with the *N*-acyl-*N*-methyl-L-alanyl derivatives described above in the presence of the coupling reagent 1,3-dicyclohexylcarbodiimide (DCC) along with catalytic amounts of zinc chloride (Scheme 2). In all cases, two diastereomeric products (**2a–i** and **6a–i**) containing the L- and D-aminoacyl side chain, respectively, were obtained, despite the fact that enantiomerically pure acylamino acids were used in the reaction. This indicates that epimerization

at the α -carbon of the *N*-methyl-L-alanine moiety takes place during the reaction. In addition, unreacted maytansinol (typically 10–20%) was also recovered. The addition of another aliquot of the carboxylic acid, DCC, and ZnCl_2 did not result in any further reaction. Several other coupling agents, solvents, and reaction conditions were tried but generally gave poorer yields. Of the Lewis acids investigated, zinc chloride gave the best yields. The substitution of zinc chloride with acyl transfer catalysts such as (dimethylamino)pyridine or hydroxybenzotriazole gave little or no product. The diastereomeric maytansinoid esters were readily separated by normal phase HPLC, using a cyano-bonded silica column. On this column, the two diastereomers had retention times that were about 10 min apart, enabling a facile separation. In some cases, the diastereomers could also be separated by preparative TLC on silica gel. In one case, the maytansinoid ester could not be isolated in a reasonable yield. In this case, the maytansinoid DM2SMe (**2e**) was found to be unstable under the reaction conditions and underwent rapid decomposition, presumably because of facile β -elimination of the methylthio substituent to give the substituted alkene, which is the thermodynamically favored product.

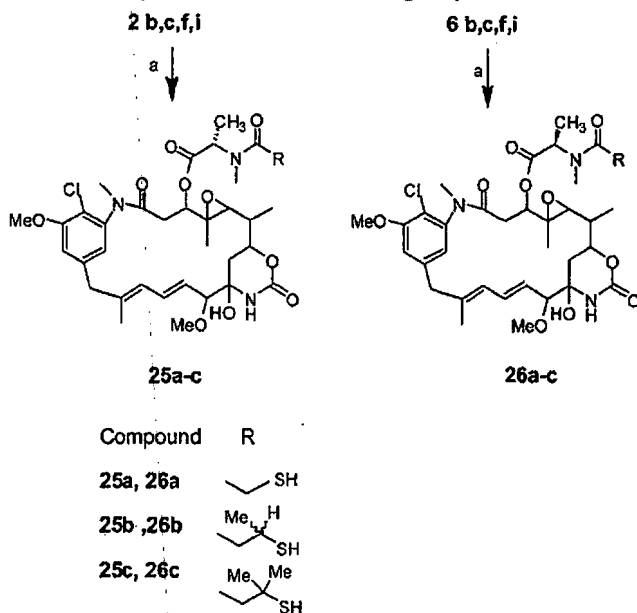
Because maytansinoids are sensitive to bases, nucleophiles, and strong reducing agents, the reduction of disulfide-containing maytansinoids to the thiol compounds had to be achieved using very mild and disulfide-specific reagents. The reduction of the disulfide-containing maytansinoids (**2b,c,e,f,i**) proceeded smoothly upon treatment, in neutral buffered aqueous solution, with a small excess (2–3 equiv) of dithiothreitol (DTT), a reducing agent commonly used by biochemists for the scission of disulfide bonds in proteins (Scheme 7). Initial attempts to obtain pure thiol-containing drugs by reverse phase HPLC using a water–acetonitrile mobile phase resulted in a considerable loss of product because of the rapid oxidation of the thiols to dimeric maytansinoids that had low solubility in aqueous solutions. The thiol-containing maytansinoids could be efficiently purified using nonaqueous solvents on a normal phase cyano-bonded HPLC column to provide mercapto-L-aminoacyl-maytansinoids **25a** (DM1), **25b** (DM3), and **25c** (DM4) (Scheme 7). Similarly, the reduction of the disulfide-containing-D-aminoacyl maytansinoids (**6b,c,f,i**) gave the thiol-containing maytansinoids **26a–c**. The water soluble reducing agent tris-(2-carboxyethyl)-phosphine (TCEP) could also be used instead of DTT with similar results. Once purified, using nonaqueous solvents, the thiol-containing drugs were stable upon extended storage in dry form. For a subsequent reaction with antibodies, the thiol-containing maytansinoids had to be reconstituted in a water-miscible solvent that would keep the drug in solution in the predominantly aqueous milieu (<5% organic solvent) and also compatible with proteins. Dimethylacetamide (DMA^a) was found to be the ideal solvent, once it was re-purified and freed

Scheme 5. Synthesis of *N*-Methyl-*N*-[(4-*R*)-methylthio]-1-oxopentyl]-*S*-alanine^a

^a Reaction conditions: (a) TsCl, Py; (b) NaCN; (c) KSSCOEt; (d) NaOH; (e) $\text{CH}_3\text{SSO}_2\text{CH}_3$; (f) *N*-hydroxysuccinimide, EDC; (g) *N*-methyl-L-alanine, triethylamine.

Scheme 6. Synthesis of *N*-Methyl-*N*-[4-methyl-(4-methyldithio)-1-oxopentyl]-*S*-alanine^a

^a Reaction conditions: (a) CH₃CN, ^tBu-Li; (b) NaOH; (c) CH₃SSO₂CH₃; (d) *N*-hydroxysuccinimide, EDC; (e) *N*-methyl-L-alanine.

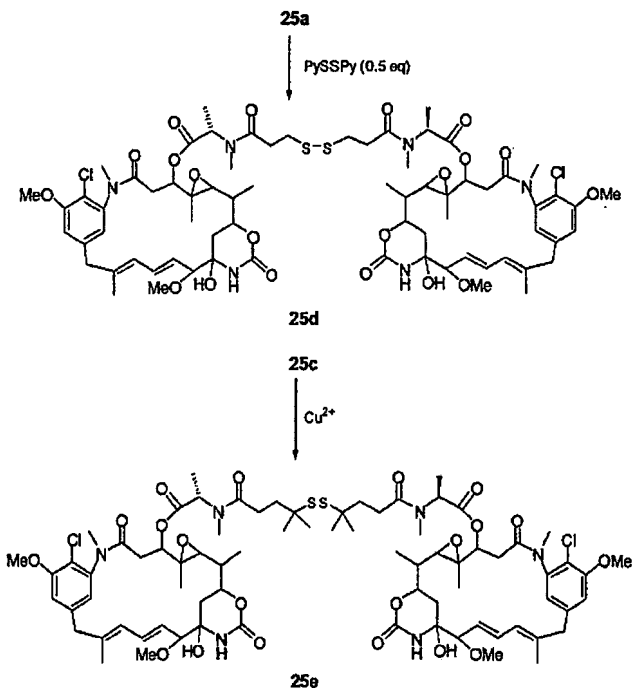
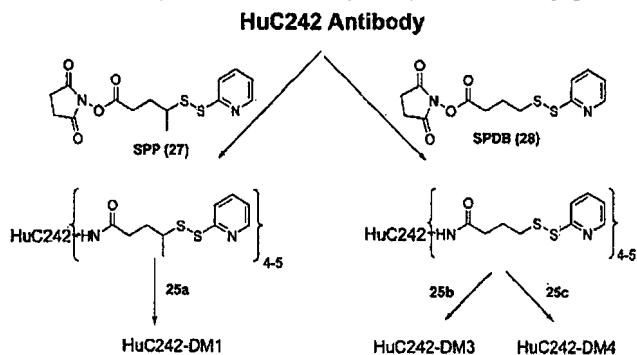
Scheme 7. Synthesis of Thiol-Containing Maytansinoids^a

^a Reaction conditions: (a) dithiothreitol/RT.

of metal ions. This was achieved by the careful glass-distillation of high-grade commercial DMA.

Maytansinoids **2j** (DM1-TPA) and **2k** (DM4-TBA) were sometimes obtained as byproducts of the conjugation reaction of thiol-containing maytansinoids with antibodies. To determine the potency of these compounds, they were independently synthesized from the corresponding thiol-containing maytansinoids **25a** and **25c**, respectively, by a disulfide exchange with the appropriate pyridyldithioalkanoic acids. The disulfide dimers **25d** and **25e** of DM1 and DM4, respectively, were also sometimes obtained as byproducts of the conjugation reaction. These two dimers were synthesized either by disulfide exchange or by metal-ion-catalyzed oxidation of the parent thiol-containing maytansinoids **25a** and **25c**, respectively (Scheme 8).

Antibody Conjugates. The antibody used in this study was huC242, a humanized version of the murine monoclonal antibody C242²⁰ that binds to the CanAg antigen that is highly expressed on the surface of several human solid tumors, including colon, pancreatic, gastric, and some lung tumors. This antibody displayed limited cross-reactivity with human normal tissues. The conjugation of the maytansinoid to the monoclonal antibodies was accomplished in two steps (Scheme 9). First, the monoclonal antibody was modified either with *N*-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP, **27**) or with *N*-

Scheme 8. Synthesis of Maytansinoid Dimers^a**Scheme 9.** Preparation of Antibody–Maytansinoid Conjugates^a

succinimidyl 4-(2-pyridyldithio)butanoate (SPDB, **28**) to introduce an active disulfide-containing group. The treatment with these bifunctional cross-linking agents resulted in the formation of stable amide bonds with the ϵ -amino group of lysine residues to generate a modified antibody bearing, on an average, four to five linked pyridyldithio groups per antibody molecule. Modification with SPP results in the introduction of a sterically hindered center on the carbon atom bearing the disulfide moiety, whereas the treatment with SPDB results in the introduction of a nonhindered disulfide. In the second step, the treatment with a thiol-containing maytansinoid led to disulfide exchange with the pyridyldithio group to give antibody conjugates containing, on the average, three to four maytansinoid drugs linked via the newly formed disulfide bonds. The strength of the disulfide bond was varied by using maytansinoids **25b** and **25c** that bear sterically hindered thiols or by utilizing the SPP cross-linker, which would introduce a methyl substituent on the antibody side of the disulfide link. For example, the huC242-DM1 conjugate cantuzumab mertansine contains one methyl substituent on the antibody side of the disulfide link. The huC242-DM3 conjugate bears one methyl substituent on the drug side of the disulfide link, whereas the huC242-DM4 conjugate bears two methyl substituents on the drug side of the disulfide link. A structural representation of these conjugates is shown in Figure 2. The conjugates were monomeric, soluble in neutral

^a Abbreviations: DMA, dimethylacetamide; SPP, *N*-succinimidyl 4-(2-pyridyldithio)pentanoate; SPDB, *N*-succinimidyl 4-(2-pyridyldithio)butanoate.

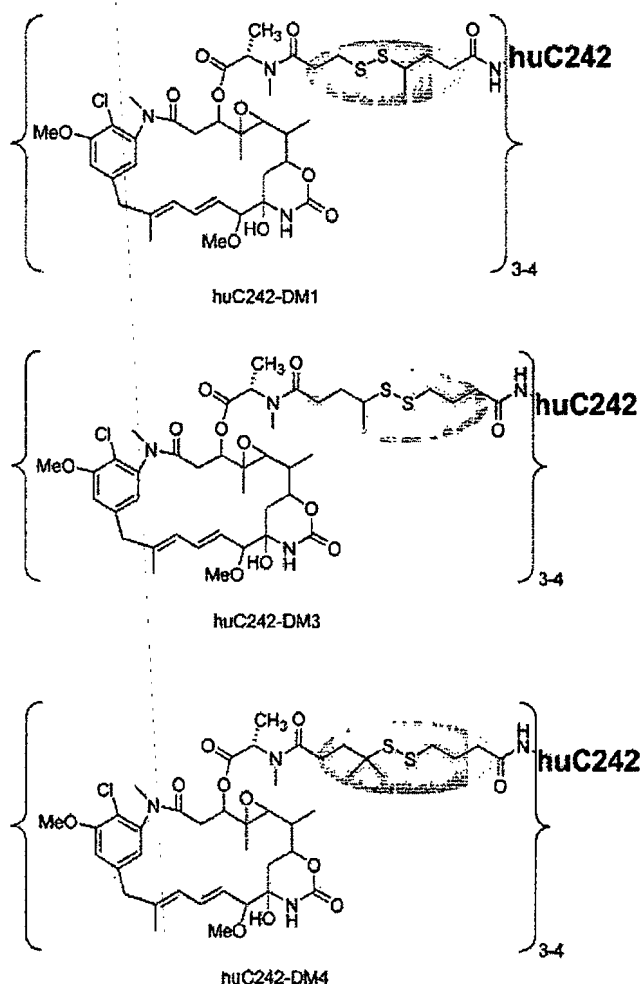


Figure 2. Structural representation of maytansinoid conjugates with the huC242 antibody. The differences in the linker region are shadowed.

buffered aqueous solution, and stable for up to two years upon storage at 4 °C.

Biological Studies. Free Drugs. The disulfide-containing maytansinoid drugs and thiol-containing maytansinoid drugs were evaluated for their ability to suppress the proliferation of human tumor cell lines in vitro. KB (human epidermoid carcinoma) and SK-BR-3 (human breast tumor) cells were exposed to the compounds for 24 or 72 h, and the surviving fractions of cells were measured using a clonogenic plating efficiency assay. The IC_{50} values were then calculated from these data. The L-aminoacyl maytansinoid **2b** (DM1SMe), bearing a methyldithio-propanoyl substituent in the C3 ester side chain, was found to be highly potent with IC_{50} values of 0.029 and 0.014 nM toward KB and SK-BR-3 cells, respectively (Table 1). This methyldithio-maytansinoid displayed cytotoxicity similar to that of the parent drug maytansine toward KB cells and increased potency compared to that of maytansine toward SK-BR-3 cells. The potency of **2b** was then compared to that of maytansine on a wider panel of human tumor cell lines (see Table 1). Compound **2b** was potent toward SW-620 (colon adenocarcinoma) with an IC_{50} value of 0.040 nM, about 3-fold more potent than maytansine. Both **2b** and maytansine were less potent toward HCT-15 (colon carcinoma) cells with IC_{50} values of 0.13 and 0.75 nM, respectively. The lower potency of these drugs toward HCT-15 cells line can be explained by the reported expression of the multidrug resistance gene (*mdr1*/P-glycoprotein) in this cell line.²¹ Compound **2b** was about twice as cytotoxic (IC_{50} = 0.05 nM) as maytansine against the ovarian cancer line OVCAR3. Thus, the replacement of the acetyl group

Table 1. Comparison of the in Vitro Potency of Maytansine (**1**) and Disulfide-Containing Maytansinoid, DM1SMe (**2b**) after 72 h of Exposure to the Drugs

cell line	IC_{50} (nM)	
	maytansine (1)	maytansinoid 2b
KB	0.034 ± 0.005	0.029 ± 0.003
Sk-Br-3	0.030 ± 0.005	0.014 ± 0.006
A-431	0.040	<0.030
A-549	0.160 ± 0.040	0.120
SW-620 ^a	>0.100	0.040 ± 0.004
Ovcar-3 ^a	0.100 ± 0.020	0.050 ± 0.010
HCT-15	0.750	0.130
MCF-7	0.044 ± 0.002	<0.03
A-375	0.037	0.030 ± 0.015

^a 24 h exposure.

Table 2. Cytotoxicity of Various Maytansinoids in Vitro toward Tumor Cell Lines (72 h of Exposure)

compd	IC_{50} (nM)	
	KB cells	SK-Br-3 cells
4	>3	1.2
1	0.034	0.030
2a	0.190	nd
2b	0.029	0.014
2c	0.100	nd
2d	0.0085	0.038
2f	0.011	0.0042
2g	0.040	0.032
2h	0.042	0.027
2i	0.0011	0.0032
2j	1.80	3.40
2k	23.0	7.10
6b	3.0	6.0
6d	2.0	nd
6f	1.20	0.22
6g	>3.0	>3.0
6h	2.40	2.20
6i	>0.30	>0.30
25a	1.10	1.10
25b	0.077	0.031
25c	0.290	0.120
25d	0.140	0.100
25e	0.0016	0.0012
26a	>3.0	>3.0
26c	>1.0	3.3

in the C3 side chain of maytansine with a methyldithiopropionyl group not only preserved the cytotoxic activity of maytansine but, in some cases, even enhanced its potency toward cancer cell lines.

We then examined the effect of the chain length of the acyl group on potency (Table 2). Maytansinoid **2d**, bearing the methyldithiobutanoyl side chain (one methylene longer than **2b**), displayed similar potency to that of **2b**. In contrast, the methyldithioacetyl maytansinoid **2a** (one methylene unit shorter than **2b**) was about 10-fold less cytotoxic than **2b**, suggesting that a minimum of two methylene groups are required in the *N*-acyl side chain to achieve high potency. The phenyldithio analogue **2c** was also about 10-fold less potent than the corresponding methyldithio-maytansinoid **2b**. Maytansinoids bearing a branched L-aminoacyl side chain, incorporating one methyl substituent (DM3SMe, **2f,g,h**) or two methyl substituents (DM4SMe, **2i**) on the carbon atom bearing the methyldithio substituent, were highly potent, with IC_{50} values ranging from 0.0042 to 0.011 nM for **2f** and 0.011 to 0.0032 nM for **2i**. Maytansinoid **2f** bears a new chiral center in the acyl side chain. Maytansinoids **2g** and **2h** bearing the *R* and *S* configurations in the acyl side chains, respectively, were found to display potencies similar to that of **2f**, which bears the racemic acyl side chain. Although the stereochemistry in the acyl side chain

was not important for activity, the stereochemistry of the *N*-methylalanyl moiety had a dramatic influence on potency. Thus, the maytansinoids **6b,d,f,g,h,i** bearing the unnatural *N*-methyl-D-alanyl moiety were about 100-fold less cytotoxic than their corresponding L-epimers toward both cell lines.

In all cases, the mercapto-containing maytansinoids were less potent than their corresponding methyldisulfide analogues. Thus, maytansinoid **25a** (DM1), bearing the mercaptopropanoyl side chain, had an IC_{50} value of 1.1 nM toward KB and SK-BR-3 cells, rendering it about 30- and 100-fold less potent than **2b** toward these cell lines. This decrease in potency may be attributed to the instability of thiol-containing maytansinoids such as **25a** under cell culture conditions wherein the oxidation of **25a** to its disulfide dimer **25d** was observed. However, dimer **25d** was independently synthesized and found to be only about 3- to 10-fold less potent than **2b**, suggesting that **25a** might be inactivated additionally by some other mechanism. For example, the thiol group could react with a cell culture component such as cystine to provide a charged maytansinoid carboxylate, which is incapable of internalization into the cell, resulting in diminished potency. Maytansinoids **25b,c** bearing sterically hindered thiols were 3- to 12-fold more potent than **25a**, which is consistent with the predicted lower reactivity and consequently, the greater stability of these compounds in the cell culture milieu. The dimer (**25e**) of DM4 was the most potent maytansinoid tested, with IC_{50} values of 0.0012 and 0.0016 nM toward the two cell lines tested. Maytansinoids **2j** and **2k** bear a terminal carboxyl group that renders them at least 100-fold less potent than the parent compounds **2b** and **2i**, suggesting that the charged carboxylate residue might interfere with the internalization into cells, resulting in diminished potency.

Antibody-Drug Conjugates. Cytotoxicity in Vitro. The conjugation of the huC242 antibody, via disulfide bonds, with thiol-containing maytansinoids DM1 (**25a**), DM3 (**25b**), and DM4 (**25c**) gave the conjugates labeled huC242-DM1, huC242-DM3, and huC242-DM4, respectively (Figure 2). In these conjugates, the antibody modifying agent and the maytansinoids were chosen with the goal of producing conjugates of varying disulfide bond stability. All three conjugates displayed high potency in vitro toward the antigen-expressing COLO 205 cell line, with IC_{50} values (in protein concentration) of 1.1×10^{-11} , 1.3×10^{-11} , and 3.8×10^{-11} M for huC242-DM4, huC242-DM3, and huC242-DM1, respectively. Thus, the disulfide bond strength does not appear to play an important role in determining the magnitude of in vitro potency toward target cells. Importantly, all three conjugates were about 200-fold less cytotoxic toward antigen-negative A-375 cells, demonstrating the specificity of the cytotoxic effect. Figure 3 shows an example of how an antibody can confer target specificity to an otherwise nonspecifically cytotoxic drug. Both the COLO 205 and A-375 cell lines were killed equally well by the nonconjugated maytansinoid **2i** (DM4SMe). In contrast, the conjugated maytansinoid huC242-DM4 kills the target COLO 205 cells at a 1000-fold lower concentration than the nontarget A-375 cell, establishing that the linkage to antibodies confers tumor specificity to the otherwise indiscriminately toxic maytansinoid drugs.

Antitumor Activity in Vivo. The antitumor activity of the three huC242-maytansinoid conjugates, huC242-DM1, huC242-DM3, and huC242-DM4, were compared in human colon tumor xenograft models established with COLO 205 cells. We have previously shown¹⁰ that C242-DM1 is curative in this model and that the free DM1 and the unconjugated C242 antibody show little or no antitumor effect. To compare the

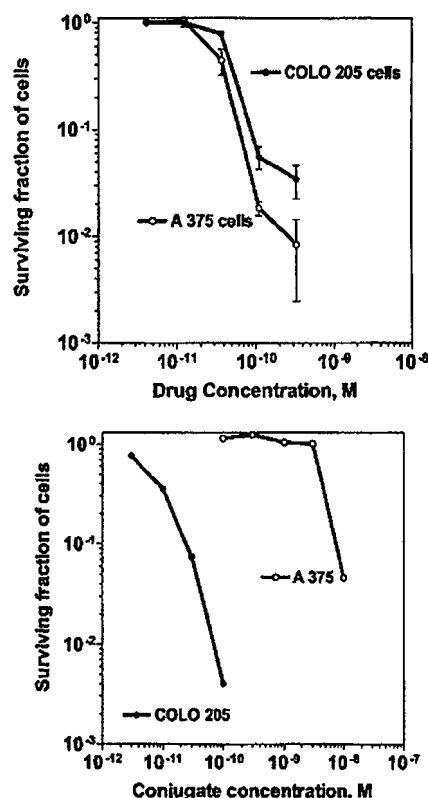


Figure 3. Top panel: in vitro potency of unconjugated drug DM4SMe (**2i**) toward COLO 205 and A-375 cells. Bottom panel: in vitro potency of huC242-DM4 conjugate toward target COLO 205 cells and nontarget A-375 cells.

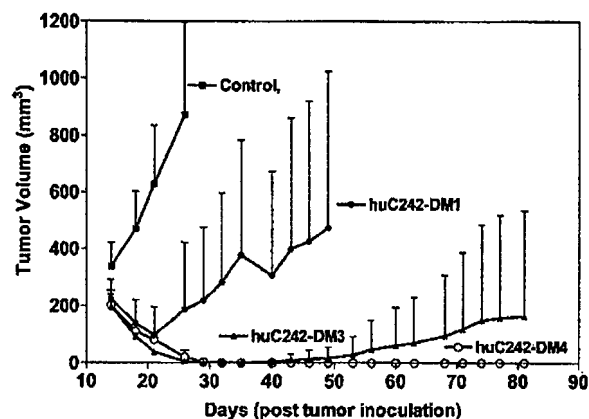


Figure 4. Comparison of the antitumor activity of huC242-maytansinoid conjugates in a human colon tumor xenograft model established subcutaneously with COLO 205 cells.

relative efficacy of the three conjugates, we selected a treatment regimen that used a low dose of the conjugate (~20% of the maximum tolerated dose) that would result in a delay in tumor growth but would not be curative. The results are shown in Figure 4. The tumors in the control group of mice grew to a size of nearly 900 mm³ in 24 days. The treatment with huC242-DM1 (linked maytansinoid dose of 75 μ g/kg, qd $\times$ 5) resulted in a tumor growth delay of 20 days, whereas the treatment with huC242-DM3, at an equivalent dose, resulted in a greater therapeutic effect and caused complete tumor regressions lasting 45 days. Treatment at an equivalent dose of the huC242-DM4 conjugate was even more efficacious, resulting in cures of all of the treated animals. Interestingly, the antitumor activity of huC242-DM3 is superior to that of huC242-DM1, although both conjugates would have been predicted to display similar stability in vivo. The huC242-DM3 conjugate bears one methyl

group on the carbon atom bearing the disulfide bond on the drug side of the conjugate, whereas huC242-DM1 bears one methyl substituent at the carbon atom on the antibody side of the disulfide link. However, cleavage of the disulfide bond in huC242-DM3 releases the thiol-containing maytansinoid DM3, which is about 10-fold more potent than DM1, the active component released from huC242-DM1, which could explain the greater efficacy observed with the DM3 conjugate.

The greater antitumor activity of huC242-DM4 compared to that of huC242-DM1 can be attributed to at least two factors: (a) the greater stability of the disulfide bond between the antibody and the drug in huC242-DM4, which results in a longer circulation time of the intact conjugate and thus potentially greater accumulation at the tumor site, and (b) the greater stability of the released DM4 drug, which bears a thiol substituent at a tertiary carbon center. Studies to determine the parameters contributing to the different *in vivo* activities of the conjugates are ongoing.

Conclusions

To harness the potent cytotoxic activity of maytansinoids for the treatment of cancer, we prepared analogues of maytansine that can be covalently linked to monoclonal antitumor antibodies for targeted treatment. Such analogues not only have to maintain the activity of maytansine but also have to be able to readily form a stable chemical bond with a protein in aqueous solution. For manufacturing reasons, bond formation must be efficient and occur in high yield. Sulfhydryl groups or their respective thiolate anions react readily in aqueous solutions with maleimido moieties in a Michael-type addition reaction to form thioethers and with disulfide groups in a disulfide exchange reaction to form new disulfide bonds. We, therefore, prepared and tested maytansinoid compounds with reactive sulfhydryl groups, which during synthesis and for some biological testing were protected in the form of simple disulfide compounds.

Thus, we have synthesized a series of new maytansinoids bearing a disulfide or thiol substituent. The series was chosen so as to evaluate the influence of the chain length of the ester side chain and the degree of steric hindrance on the carbon atom bearing the thiol substituent on *in vitro* cytotoxicity. Several of these maytansinoids were found to be even more potent than the parent drug maytansine in killing human tumor cells *in vitro*. Antibody conjugates bearing varying degrees of steric hindrance around the disulfide link between the maytansinoid and antibody were shown to be highly active in selectively killing target cells to which the antibody was capable of binding. Nontarget cells were not affected even at a 100-fold higher concentration, demonstrating that the high potency of the maytansinoids could be directed toward the specific killing of target cells. Remarkable antitumor efficacy was observed in a xenograft model. These studies, in part, have led to the development of huC242-DM4 for evaluation as an antitumor agent in human clinical trials in patients expressing the antigen for C242.

Experimental Section

All reagents were obtained from the Aldrich Chemical Co., unless otherwise stated. Maytansine (NCI 158358/26/0) was obtained from the National Cancer Institute. Ansamitocin P-3 was produced by fermentation of an *Actinosynnema pretiosum* strain derived from several rounds of mutagenesis of the parent ATCC 31565 strain. Human tumor cell lines used in biological assays were obtained from the American Type Culture Collection (ATCC). Melting points were determined on an electrothermal melting point apparatus. Proton magnetic resonance (¹H NMR) spectra were obtained on a Bruker Avance spectrometer operating at 400 MHz. The chemical

shifts are reported in δ values relative to that of an internal tetramethylsilane (Me₄Si) standard. UV spectra were recorded on a Beckman DU-640 spectrophotometer. The optical rotations were determined using a Rudolph AUTOPOL IV polarimeter. Mass spectra were obtained on a Bruker Daltonics spectrometer or a Waters QTOF-API-US spectrometer. Elemental analyses were performed by Atlantic Microlabs, Atlanta, GA. High performance liquid chromatography (HPLC) was performed on a Beckman or Varian instrument (analytical or preparative scale) equipped with a diode array detector.

Synthesis of Maytansinol (4). To a 200 mL flask was added a 1 M solution of lithium aluminum hydride (45 mmol, 45 mL) in THF. The solution was placed under an argon atmosphere and cooled to -40 °C. A mixture of anhydrous methanol (5.4 mL, 135 mmol) in THF (25 mL) was added dropwise. The temperature was maintained between -30 and -40 °C. After the addition was complete, the reaction mixture was stirred at this temperature for an additional period of 10 min to give lithium trimethoxyaluminum hydride (LiAlH(OMe)₃). In a separate 250 mL flask, ansamitocin (3.2 g, 5.0 mmol) was dissolved in anhydrous THF (30 mL), and the solution was placed under an argon atmosphere and cooled in a dry ice-acetone bath to -40 °C. The solution of LiAlH(OMe)₃ was transferred via a cannula over 10 min into the chilled solution of ansamitocins. The reaction temperature was maintained between -30 and -35 °C under argon and stirred for 2 h. Deionized water (22 mL) was added dropwise while the reaction mixture was maintained at -30 and -35 °C. After 30 min, 88% formic acid (1.8 mL) and ethyl acetate (50 mL) were added. The mixture was allowed to warm up to room temperature. The precipitate was removed by vacuum filtration, and the filtrate was concentrated under vacuum. The residue was dissolved in a minimum volume of dichloromethane and loaded onto a silica gel column packed in dichloromethane. The column was eluted with dichloromethane/methanol (95:5, v/v). The fractions containing the desired product were pooled and evaporated under reduced pressure to give 2.3 g of maytansinol (81% yield) as a white solid: mp 205–207 °C; ¹H NMR (CDCl₃) δ 0.82 (3H, s), 1.28 (3H, d, *J* = 6 Hz), 1.52–1.54 (1H, m), 1.67 (3H, s), 1.80 (br s), 2.07–2.29 (4H, m), 2.56 (1H, d, *J* = 11 Hz), 2.95 (br s), 3.10 (1H, d, *J* = 12 Hz), 3.19 (3H, s), 3.41 (3H, s), 3.44–3.51 (3H, m), 3.97 (3H, s), 4.34 (1H, t, *J* = 11 Hz), 5.50 (1H, dd, *J* = 9 and 15 Hz), 6.13 (1H, d, *J* = 11 Hz), 6.37 (1H, s), 6.42 (1H, dd, *J* = 11 and 15 Hz), 6.79 (1H, d, *J* = 1.5 Hz), 7.02 (1H, d, *J* = 1.5 Hz); HRMS calcd for C₂₈H₃₃ClN₂O₈ (M + H)⁺ *m/z* = 565.2317; found, 565.2329. HPLC analysis was performed on a Kromasil analytical C-8 column (length: 100 mm, i.d.: 4.6 mm), at a flow rate of 1 mL/min, eluting with a gradient of water and acetonitrile (30% to 45% CH₃CN over 30 min). Under these conditions, maytansinol eluted as a sharp peak with a retention time of 11.15 min.

Acetal 4c. A solution of ansamitocins (3.0 g, 4.72 mmol) in THF (15 mL) was cooled to -35 °C, and a solution of 0.67 M LiAl(OMe)₃H (56 mL, 37.7 mmol) in THF was added dropwise by syringe using a syringe pump. The temperature of the reaction was maintained between -30 and -40 °C throughout the addition. After addition was complete, the reaction was stirred for 2 h at between -34 and -37 °C. A solution of 88% formic acid (1.85 mL, 2.16 g, 41.5 mmol) in deionized water (23 mL) was added dropwise to the flask at a rate that did not produce excessive frothing, followed by the addition of ethyl acetate (60 mL). The cooling bath was removed, and the mixture was allowed to warm up to room temperature. The pH of the mixture was checked with pH paper and found to be approximately 6. Precipitated aluminum-based byproducts were removed by vacuum filtration, and the solvent was removed from the filtrate by rotary evaporation under vacuum. Butyl acetate (10 mL) was added to the residue, and the solvent was then evaporated to remove the residual water. The residue was purified by silica chromatography using dichloromethane/methanol 95:5 (v/v), giving a later eluting band (maytansinol) and an early eluting band. The maytansinol band was collected, and the solvent was removed by rotary evaporation to give 1.55 g of maytansinol (58%). The solvent was removed from the earlier eluting band,

and the material was dissolved in a minimum volume of acetonitrile and then purified by preparative reverse-phase HPLC. The solvent was removed by rotary evaporation to give 440 mg (15% yield) of **4c**, $R = -CH(CH_3)_2$; ^1H NMR ($CDCl_3$) δ 0.78 (s, 3H), 0.97 (d, 3H, $J = 6.9$), 1.04 (d, 3H, $J = 6.7$), 1.23 (m, 1H), 1.28 (d, 3H, $J = 6.4$), 1.54 (m, 1H), 1.66 (s, 3H), 1.72 (m, 2H), 2.03 (dd, 1H, $J = 14$, 3.6 Hz), 2.3 (d, 1H, $J = 14$), 2.49 (dd, 1H, $J = 11.7$, 14), 2.92 (d, 1H, $J = 9.5$ Hz), 3.14 (s, 3H), 3.12 (m, 1H), 3.37, (s, 3H), 3.52 (m, 3H), 3.65 (m, 1H), 3.75 (m, 1H), 3.97 (s, 1H), 4.31 (m, 2H), 5.52 (dd, 1H, $J = 16$, 8.7 Hz), 6.13 (d, 1H, $J = 11$ Hz), 6.34 (s, 1H), 6.45 (dd, 1H, $J = 16$, 11 Hz), 6.80 (d, 1H, $J = 1.5$ Hz), 6.92 (d, 1H, $J = 1.5$ Hz); MS m/z 619.3 ($M + H$) $^+$.

3-(Methyldithio)propanoic Acid (8b). To a stirred solution of 3-mercaptopropanoic acid (**7b**) (5.00 g, 0.047 mol) in water (150 mL) cooled in an ice bath was added methyl methanethiolsulfonate (6.54 g, 0.052 mol) in absolute ethanol (75 mL). The reaction mixture was stirred overnight at room temperature. The mixture was then diluted with saturated aqueous NaCl (400 mL) and extracted with ether (3 $\times$ 150 mL), and the combined ether extracts were then washed with saturated NaCl. The solution was dried over Na_2SO_4 , concentrated, and then distilled to afford a colorless liquid (6.47 g, 90%); bp (1 mm) = 105 $^{\circ}C$; ^1H NMR ($CDCl_3$) δ 2.32 (3H, s), 2.80 (4H, m), 11.20 (1H, s).

4-(Methyldithio)butanoic Acid (8c). To a stirred solution of bis-(3-carboxypropyl)disulfide (1.00 g, 4.20 mmol) in methanol (20 mL) was added a solution of dithiothreitol (0.647 g, 4.20 mmol) in H_2O (20 mL). A solution of 10 M NaOH (0.842 mL, 8.42 mmol) was then added and the mixture was allowed to stir overnight at room temperature to effect complete reduction. Methyl methanethiolsulfonate (1.17 g, 9.24 mmol) was added, and the reaction mixture was stirred for another 3 h. The mixture was then diluted with saturated aqueous NaCl (150 mL), acidified (aqueous HCl), and extracted with ethyl ether (3 $\times$ 100 mL). The combined organic layers were washed with saturated NaCl, dried (Na_2SO_4), concentrated, and chromatographed on silica gel, eluting with dichloromethane/ethyl acetate to give 0.867 g (56%) of a colorless liquid; ^1H NMR ($CDCl_3$) δ 2.12 (2H, m), 2.40 (3H, s), 2.74 (2H, m), 11.18 (1H, s).

3-Phenyldithio)propanoic Acid (8d). To a stirred solution of diphenyl disulfide (3.0 g, 13.8 mmol) in a mixture of ether (10 mL) and methanol (20 mL) under a nitrogen atmosphere at room temperature was added a solution of 3-mercaptopropanoic acid (**7b**) (0.49 g, 4.6 mmol) in ether (5 mL), followed by a solution of 10 M NaOH (0.46 mL, 4.6 mmol). The reaction mixture was stirred at room temperature for 20 h and then stripped of the solvents under reduced pressure. The product was purified by column chromatography on silica gel, eluting with ethyl acetate/hexane. The product was obtained as a white solid (0.56 g, 56.6%); mp 57–59 $^{\circ}C$; ^1H NMR ($CDCl_3$, TMS) δ 2.6–3.0 (4H, m), 7.12–7.60 (5H, m), 10.6 (1H, s).

R-1,3-Di-O-(para-toluenesulfonyl)butane (11). A solution of R-(–)-1,3-butanediol (**10**, 2.00 g, 22.22 mmol) in a mixture of dry pyridine (40 mL) and dry toluene (60 mL) was treated with *p*-toluenesulfonyl chloride (12.70 g, 66.84 mmol) under argon at 0 $^{\circ}C$. After stirring at 0 $^{\circ}C$ for 5 min followed by stirring at room temperature for 2 h, the mixture was evaporated under vacuum, redissolved in ethyl acetate, and washed with 0.1 M aqueous $NaHCO_3$, followed by saturated aqueous NaCl. The organic layer was dried over $MgSO_4$ and filtered, and the solvent was evaporated. Purification by chromatography on silica gel, eluting with 1:2 (v/v) ethyl acetate/hexane gave 6.51 g (74%) of the title compound ($R_f = 0.40$, 1:1 EtOAc/hexane) as a white solid; mp 56–58 $^{\circ}C$; ^1H NMR ($CDCl_3$) δ 7.76 (dd, 4H, $J = 1.0$, 8.0 Hz), 7.35 (dt, 4H, $J = 0.4$, 8.0 + 8.0 Hz), 4.70 (m, 1H), 4.03 (m, 1H), 3.94 (m, 1H), 2.46 (s, 6H), 1.92 (m, 2H), 1.26 (d, 3H, $J = 6.3$ Hz); ^{13}C NMR 145.17, 133.00, 130.11, 128.12, 127.91, 76.28, 66.21, 36.08, 21.86, 21.06; MS: m/z 420.99 ($M + Na$) $^+$.

S-4-(O-Ethylxanthic)pentanenitrile (12). A solution of R-1,3-di-O-*p*-toluenesulfonyl-butane (**11**, 4.80 g, 12.06 mmol) in dry DMSO (50 mL) was treated with NaCN (0.65 g, 13.26 mmol). After stirring at room temperature under argon for 18 h, the reaction

mixture was diluted with ethyl acetate, washed successively with cold 1.0 M NaH_2PO_4 at pH 7.5, water, and 1.0 M aqueous NaH_2PO_4 at pH 4.0. The organic layer was separated and dried over $MgSO_4$, filtered, and then evaporated to give 2.63 g of crude R-3-O-*p*-toluenesulfonyl-pentanenitrile. MS: m/z 275.80 ($M + Na$) $^+$. The product was used directly without further purification. To the solution of crude of R-3-O-*p*-toluenesulfonyl-pentanenitrile (2.63 g) in ethanol (15 mL) was added potassium O-ethylxanthate (4.55 g) in ethanol (50 mL). After stirring overnight under argon, the mixture was concentrated, diluted with ethyl acetate, and filtered through a short silica column. The eluant was concentrated and purified by chromatography on silica gel, eluting with 1:4 (v/v) EtOAc/hexane, to give 1.54 g (63%, 2 steps) of the title compound ($R_f = 0.40$ (1:4 EtOAc/hexane)) as a colorless oil. ^1H NMR ($CDCl_3$) δ 4.67 (dd, 2H, $J = 7.1$, 14.2 Hz), 3.86 (ddd, 1H, $J = 7.0$, 14.0, 21.9 Hz), 2.50 (t, 2H, $J = 7.3 + 7.6$ Hz), 2.06 (m, 2H), 1.44 (m, 6H); ^{13}C NMR 213.04, 119.16, 70.28, 44.57, 32.10, 20.20, 15.21, 13.93; MS: m/z 226.51 ($M + Na$) $^+$, 242.51 ($M + K$) $^+$.

S-(+)-4-(Methyldithio)pentanoic Acid (13). To a solution of S-4-O-Ethylxanthic-pentanenitrile (**12**, 1.95 g, 9.61 mmol) in a mixture of ethanol (10 mL) and water (150 mL) was added NaOH (5 g). The reaction mixture was refluxed overnight under argon. The mixture was cooled to room temperature and diluted with water (150 mL) and extracted with 1:1 EtOAc/hexane (2 $\times$ 100 mL). The aqueous layer was acidified with H_3PO_4 to pH 2.5–3.0 and extracted with EtOAc (6 $\times$ 75 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and evaporated to dryness to give the crude S-4-mercaptopentanoic acid. This crude product was used directly in the next step without further purification. To a solution of crude S-4-mercaptopentanoic acid (1.2 g) in a mixture of ethanol (50 mL) and 0.5 M NaH_2PO_4 buffer (75 mL) at pH 7.0 was added dropwise methyl methanethiolsulfonate (1.47 g, 11.65 mmol) in dry THF (5 mL) over 45 min at 0 $^{\circ}C$. After stirring under argon at 0 $^{\circ}C$ for 30 min followed by stirring at room temperature for 2 h, the mixture was concentrated and extracted with dichloromethane (2 $\times$ 50 mL). The aqueous layer was acidified with H_3PO_4 to pH 2.5–3.0 and extracted with EtOAc (4 $\times$ 100 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and evaporated. The residue was purified by chromatography over silica gel, eluting with 1:100:400 HOAc/EtOAc/hexane to give 1.43 g (83%) of the desired product ($R_f = 0.32$, 1:100:400 HOAc/EtOAc/hexane) as a white solid; mp 290–293 $^{\circ}C$ (dec); ^1H NMR ($CDCl_3$) δ 2.91 (ddd, 1H, $J = 6.8$, 13.7, 20.5 Hz), 2.53 (t, 2H, $J = 7.7 + 7.4$ Hz), 2.42 (s, 3H), 1.94 (m, 2H), 1.36 (d, 3H, $J = 6.8$ Hz); ^{13}C NMR 179.18, 45.35, 31.58, 30.73, 24.70, 21.05; MS: (m/z) 202.92 ($M + Na$) $^+$; $[\alpha]^{25}_D = +41.35$ ($c = 2$, CH_3OH).

S-1,3-Di-O-(para-toluenesulfonyl)butane (16). A solution of S-(–)-1,3-butanediol (**15**, 2.00 g, 22.22 mmol) in a mixture of dry pyridine (40 mL) and dry toluene (60 mL) was treated with *p*-toluenesulfonyl chloride (12.70 g, 66.84 mmol) under argon at 0 $^{\circ}C$. After stirring at 0 $^{\circ}C$ for 5 min followed by stirring at room temperature for 2 h, the mixture was evaporated under vacuum. The residue was redissolved in ethyl acetate and washed with 0.1 M aqueous $NaHCO_3$ and saturated NaCl. The organic layer was separated, dried over $MgSO_4$, filtered, and evaporated. The residue was purified by chromatography over silica gel, eluting with 1:2 ethyl acetate/hexane to give 6.25 g (71%) of the title compound ($R_f = 0.40$ (1:1 EtOAc/hexane)). Recrystallization from ether/hexane afforded a white solid; mp 56–58 $^{\circ}C$; ^1H NMR ($CDCl_3$) δ 7.76 (dd, 4H, $J = 1.0$, 8.0 Hz), 7.35 (dt, 4H, $J = 0.4$, 8.0 + 8.0 Hz), 4.70 (m, 1H), 4.03 (m, 1H), 3.94 (m, 1H), 2.46 (s, 6H), 1.92 (m, 2H), 1.26 (d, 3H, $J = 6.3$ Hz); ^{13}C NMR 145.17, 133.00, 130.11, 128.12, 127.91, 76.28, 66.21, 36.08, 21.86, 21.06; MS: m/z 420.99 ($M + Na$) $^+$.

R-4-O-(Ethylxanthic)pentanenitrile (17). A solution of S-1,3-di-O-(*p*-toluenesulfonyl)butane (**16**, 6.25 g, 15.70 mmol) in 60 dry DMSO (50 mL) was treated with NaCN (0.85 g). The reaction mixture was stirred under argon for 18 h at room temperature. The reaction mixture was then diluted with ethyl acetate, washed sequentially with cold 1.0 M NaH_2PO_4 buffer at pH 7.5, water, and 1.0 M NaH_2PO_4 buffer at pH 4.0. The organic layer was dried

over MgSO_4 , filtered, and evaporated to give 3.62 g of crude *S*-3-*O*-(*p*-toluenesulfonyl)pentanenitrile. The product was used directly without further purification. To a solution of crude *S*-3-*O*-(*p*-toluenesulfonyl)pentanenitrile (3.62 g) in ethanol (50 mL) was added potassium *O*-ethylxanthate (5.72 g) in ethanol (100 mL). After stirring overnight under argon, the mixture was concentrated, diluted with ethyl acetate, and filtered through a short column of silica gel. The eluant was concentrated, and the residue was purified by chromatography over silica gel, eluting with 1:4 EtOAc/hexane to give 2.0 g (62%, 2 steps) of the title compound ($R_f = 0.40$ (1:4 EtOAc/hexane)) as a colorless liquid. ^1H NMR (CDCl_3) δ 4.67 (dd, 2H, $J = 7.1, 14.2$ Hz), 3.86 (ddd, 1H, $J = 7.0, 14.0, 21.9$ Hz), 2.50 (t, 2H, $J = 7.3 + 7.6$ Hz), 2.06 (m, 2H), 1.44 (m, 6H); ^{13}C NMR 213.04, 119.16, 70.28, 44.57, 32.10, 20.20, 15.21, 13.93; MS: m/z 226.51 ($\text{M} + \text{Na}$) $^+$, 242.51 ($\text{M} + \text{K}$) $^+$.

***R*-(−)-4-(Methyldithio)pentanoic Acid (18).** A solution of *R*-4-*O*-(ethylxanthic)pentanenitrile (17, 2.0 g, 9.85 mmol) in a mixture of ethanol (10 mL) and water (200 mL) was treated with NaOH (6.0 g). The reaction mixture was refluxed overnight under argon. The mixture was diluted with water (150 mL) and extracted with 1:1 EtOAc/hexane (2 $\times$ 100 mL). The aqueous layer was acidified with H_3PO_4 to pH 2.5–3.0 and extracted with EtOAc (6 $\times$ 75 mL). The organic layers were combined, dried over MgSO_4 , filtered, and evaporated to dryness to give crude *R*-4-mercaptopentanoic acid. This crude product was used directly in the next step without further purification. To a solution of 1.60 g of the crude *R*-4-mercaptopentanoic acid in a mixture of ethanol (50 mL) and 0.5 M NaH_2PO_4 buffer at pH 7.0 (75 mL) was added dropwise a solution of methyl methanethiolsulfonate (1.96 g, 15.53 mmol) in dry THF (7 mL) over 45 min at 0 $^\circ\text{C}$. The reaction mixture was stirred under argon at 0 $^\circ\text{C}$ for 30 min and then at room temperature for 2 h. The mixture was concentrated and extracted with dichloromethane (2 $\times$ 50 mL). The aqueous layer was acidified with H_3PO_4 to pH 2.5–3.0 and extracted with EtOAc (4 $\times$ 100 mL). The organic layers were combined, dried over MgSO_4 , filtered, and evaporated. The residue was purified by chromatography over silica gel, eluting with HOAc/EtOAc/hexane (1:100:400) to give 1.65 g (93%) of the title compound ($R_f = 0.32$ (HOAc/EtOAc/hexane, 1:100:400). Recrystallization from ether/hexanes afforded a white solid; mp 157–160 $^\circ\text{C}$; ^1H NMR (CDCl_3) δ 2.91 (ddd, 1H, $J = 6.8, 13.7, 20.4$ Hz), 2.53 (t, 2H, $J = 7.7 + 7.4$ Hz), 2.42 (s, 3H), 1.96 (m, 2H), 1.36 (d, 3H, $J = 6.8$ Hz); ^{13}C NMR 179.46, 45.67, 31.91, 31.07, 25.02, 21.36; MS: 202.9 ($\text{M} + \text{Na}$) $^+$, 203.9; $[\alpha]_D^{25} = -39.16$ ($c = 2$, CH_3OH).

4-Mercapto-4-methylpentanoic Acid (22). Compound 21 was prepared by the method previously described.²² A 500 mL flask was equipped with a stir bar and a 150 mL addition funnel. The system was placed under an argon atmosphere, and anhydrous THF (150 mL) and *n*-BuLi (2.5 M, 75 mL, 18.7 mmol) in hexanes were added via a cannula, and the solution was cooled in a -78 $^\circ\text{C}$ dry ice/acetone bath. Acetonitrile (9.4 mL, 18 mmol) was added dropwise via a syringe over approximately 5 min. The reaction mixture was stirred for 30 min, while a white precipitate of lithiated acetonitrile was formed. Isobutylene sulfide (15.0 g, 17 mmol) was dissolved in anhydrous THF (100 mL) and added dropwise over approximately 30 min via the addition funnel. The cooling bath was removed, and the reaction was allowed to stir at room temperature for 3 h. The flask was cooled in an ice/water bath as 0.5 M HCl (38 mL) was added dropwise. The THF layer was retained, and the aqueous layer was washed twice with 75 mL of ethyl acetate. The THF and ethyl acetate layers were combined, dried over approximately 20 g of anhydrous sodium sulfate, and transferred to a 250 mL flask. The solvent was removed by rotary evaporation under reduced pressure to give crude 21. Ethanol (30 mL) was added, and the contents were stirred. A solution of NaOH (8 g) in water (30 mL) was slowly added. The flask was equipped with a reflux condenser and placed under an argon atmosphere. The reaction was refluxed overnight and then cooled to room temperature. Water (60 mL) was added, and the mixture was extracted twice with 25 mL portions of a 2:1 mixture of ethyl acetate and hexane. The aqueous layer was acidified to pH 2 with

concentrated HCl and then extracted with ethyl acetate (3 $\times$ 75 mL). The organic layers were dried over anhydrous Na_2SO_4 , and the solvent was removed by rotary evaporation under vacuum to give 10 g of product 22 (39% yield). The material was used without further purification. ^1H NMR (CDCl_3) δ 1.38 (6H, s), 1.87–1.93 (2H, m), 2.08 (1H, s), 2.51–2.57 (2H, m).

4-Methyl-4-(methyldithio)pentanoic Acid (23). A solution of 4-mercapto-4-methylpentanoic acid (22) (6.0 mL, 40 mmol) was dissolved in deionized water (50 mL) in a 250 mL flask. The solution was magnetically stirred as sodium carbonate (6.4 g, 60 mmol) was added to the acid at a rate that would not cause excessive frothing. The flask was equipped with a 100 mL addition funnel, which was charged with a solution of methyl methanethiolsulfonate (7.5 g, 60 mmol) dissolved in absolute ethanol (30 mL). The flask was cooled in an ice/water bath, and the system was maintained under an argon atmosphere. The methyl methanethiolsulfonate solution was added dropwise to the flask as rapidly as possible but without causing excessive frothing. The cooling bath was removed, and the reaction mixture was allowed to stir at room temperature for an additional 3 h. The solvent was removed by rotary evaporation under vacuum, until approximately 20 mL remained. Then, saturated sodium bicarbonate (10 mL) and deionized water (30 mL) were added. The mixture was washed with ethyl acetate (3 $\times$ 25 mL), and the aqueous layer was separated and adjusted to approximately pH 2 with 5 M HCl and then extracted with ethyl acetate (2 $\times$ 120 mL). The organic layers were combined and washed with a solution composed of saturated NaCl (16 mL) and 1 M HCl (4 mL). The organic layer was then dried over 14 g of anhydrous sodium sulfate and filtered, and the solvent was removed by rotary evaporation under vacuum to give 5.4 g of product 23 (70% yield) as a white solid. ^1H NMR (CDCl_3) δ 1.54 (6H, s), 2.15–2.21 (2H, m), 2.64 (3H, s), 2.69–2.72 (2H, m); MS: m/z 217.1 ($\text{M} + \text{Na}$) $^+$.

General Method for the Synthesis of *N*-Hydroxysuccinimide Esters 9a–d, 14, 19, and 24. (Methyldithio)-alkanoic acid (15 mmol) was dissolved in dichloromethane (20 mL) and stirred magnetically as *N*-hydroxysuccinimide (2.65 g, 23 mmol) was added followed by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC, 4.4 g, 23 mmol). The mixture was stirred under an argon atmosphere for 2 h. The reaction mixture was poured into a 125 mL separatory funnel, 40 mL of ethyl acetate was added, and the solution was washed with 50 mM potassium phosphate buffer at pH 6.0 (2 $\times$ 20 mL) and saturated sodium chloride (20 mL). The organic layer was dried over anhydrous Na_2SO_4 , and the solvent was removed by rotary evaporation under vacuum to give the desired product, which was used without further purification.

General Method for the Synthesis of *N*-Acyl-*N*-methyl-L-alanines (5a–i). *N*-Methyl-L-alanine (18.0 mmol) was dissolved in a mixture of dimethoxyethane (25 mL) and deionized water (25 mL) in a 125 mL flask equipped with a magnetic stir bar. Triethylamine (6.9 g, 36 mmol) was added, and the solution was vigorously stirred as the *N*-hydroxysuccinimidyl ester (9a–d, 14, 19, 24) (18 mmol) dissolved in 40 mL of the same solvent mixture was added dropwise over approximately 5 min. After 2 h, the reaction mixture was concentrated to approximately 40 mL by rotary evaporation under vacuum. Deionized water (10 mL) and 1 M HCl were added to give a pH of approximately 2. The mixture was poured into a separatory funnel and extracted with ethyl acetate (2 $\times$ 50 mL). The organic layers were combined and washed with saturated sodium chloride solution (10 mL). The organic layer was dried over 8.0 g of anhydrous Na_2SO_4 , and the solvent was removed by rotary evaporation under vacuum. The residue was taken up in a minimum volume of ethyl acetate and purified by chromatography on silica (silica: 40 micron flash grade, silica bed: 24 $\times$ 3.0 cm, mobile phase: hexanes/ethyl acetate/acetic acid, 50:48:2). The fractions containing the desired product were combined, and the solvent was removed under vacuum. Residual acetic acid was removed by dissolving the residue in a minimum volume of ethyl acetate and precipitating the product by the rapid but dropwise addition of hexane with stirring. Hexane was added until the product

was no longer detected in the supernatant by TLC analysis. The precipitate was vacuum-dried for 4 h to give the desired products **5a–i**.

N-Methyl-N-[(2-methyldithio)-1-oxoethyl]-L-alanine (5a). Pale yellow solid (19% yield); ^1H NMR (CDCl_3) δ 1.458 (3H, d, J = 7.6), 2.446 (3H, s), 3.069 (3H, s), 3.666 (2H, s), 5.185 (1H, m); MS: m/z 246.0 ($\text{M} + \text{Na}$) $^+$.

N-Methyl-N-[(3-methyldithio)-1-oxopropyl]-L-alanine (5b). White solid (60% yield); mp 103–104 $^\circ\text{C}$; ^1H NMR (CDCl_3) δ 1.449 (3H, d, J = 7.6), 2.422 (3H, s), 2.821 (2H, m), 2.989 (2H, m), 3.017 (3H, s), 5.194 (1H, m). Anal. ($\text{C}_8\text{H}_{15}\text{NO}_3\text{S}_2$) C, H, N. MS: m/z 259.9 ($\text{M} + \text{Na}$) $^+$.

N-Methyl-N-[(3-phenyldithio)-1-oxopropyl]-L-alanine (5c). White solid (60% yield); mp 96–97 $^\circ\text{C}$; ^1H NMR (CDCl_3) δ 1.42 (2H, d), 2.7–3.0 (7H, m), 5.24 (1H, q), 7.24–7.60 (5H, m).

N-Methyl-N-[4-methyldithio-1-oxobutyl]-L-alanine (5d). White crystals (44% yield); mp 92–93 $^\circ\text{C}$; ^1H NMR (CDCl_3) δ 1.439 (3H, d, J = 7.2), 2.089 (2H, t, J = 7.2), 2.405 (3H, s), 2.517 (2H, t, J = 7.2), 2.779 (2H, m), 2.989 (3H, s), 5.123 (1H, m); MS: m/z 290.0 ($\text{M} + \text{K}$) $^+$.

N-Methyl-N-[(4-*R,S*)-methyldithio-1-oxopentyl]-L-alanine (5f). White solid (60% yield). ^1H NMR δ 1.35 (3H, d, J = 7), 1.41 (3H, d, J = 7), 1.94–2.03 (2H, m), 2.43 (3H, s), 2.50–2.55 (2H, m), 2.83–2.93 (1H, m), 2.98 (3H, s), 5.14 (1H, q, J = 7). MS: m/z 288.1 ($\text{M} + \text{Na}$) $^+$.

N-Methyl-N-[(4-*S*)-methyldithio-1-oxopentyl]-S-alanine (5g). White solid (62% yield); ^1H NMR δ 1.36 (3H, d, J = 7), 1.42 (3H, d, J = 7), 1.93–1.98 (2H, m), 2.40 (3H, s), 2.50–2.53 (2H, m), 2.90–2.95 (1H, m), 2.99 (3H, s), 5.14 (1H, q, J = 7). MS: m/z 288.1 ($\text{M} + \text{Na}$) $^+$.

N-Methyl-N-[(4-*R*)-methyldithio-1-oxopentyl]-S-alanine (5h). White solid (60% yield); ^1H NMR δ 1.35 (3H, d, J = 7), 1.42 (3H, d, J = 7), 1.93–2.00 (2H, m), 2.43 (3H, s), 2.50–2.55 (2H, m), 2.88–2.95 (1H, m), 3.00 (3H, s), 5.14 (1H, q, J = 7). MS: m/z 288.1 ($\text{M} + \text{Na}$) $^+$.

N-Methyl-N-[4-methyl-(4-methyldithio)-1-oxopentyl]-L-alanine (5i). White solid (51% yield) mp 77–79 $^\circ\text{C}$; ^1H NMR (CDCl_3) δ 1.32 (6H, s), 1.42 (3H, d, J = 7 Hz), 1.90–1.97 (2H, m), 2.40 (3H, s), 2.42–2.49 (2H, m), 2.9 (3H, s), 5.15 (1H, q, J = 7 Hz). MS: m/z 302.0 ($\text{M} + \text{Na}$) $^+$.

General Procedure for the Esterification of Maytansinol to Give Maytansinoids 2a–i and 6a–i. In a typical experiment, maytansinol (**4**, 25 mg, 0.044 mmol) and carboxylic acids **5a–i** (0.264 mmol) were dissolved in dichloromethane (3 mL) and stirred under an argon atmosphere. A solution of dicyclohexylcarbodiimide (DCC, 57.2 mg, 0.277 mmol) in dichloromethane (0.7 mL) was added. After 1 min, a solution of 1 M ZnCl_2 in diethyl ether (0.055 mL, 0.055 mmol) was added. The mixture was stirred at room temperature for 2 h, then ethyl acetate (5 mL) was added, and the mixture was vacuum filtered through coarse filter paper. The filtrate was washed with a solution of saturated aqueous sodium bicarbonate (2 mL) followed by a saturated sodium chloride solution (1 mL). The organic layer was separated, dried over anhydrous sodium sulfate, and filtered, and the solvent was removed under reduced pressure. The residue was purified by silica chromatography using a mixture of dichloromethane and methanol to remove the unreacted maytansinol. The fractions containing the desired product were combined, and the solvent was removed under vacuum to give a mixture of diastereomeric maytansinoids bearing the *N*-methyl-*N*-acyl-*L*- and *D*-alanine eaters. The residue was taken up in a minimum volume of ethyl acetate and purified by one of three ways: (1) preparative TLC on silica, (2) HPLC on an analytical Kromasil cyano-bonded silica column (4.6 mm $\times$ 250 mm), using an isocratic elution at a flow rate of 1 mL/min, with hexane/ethyl acetate/2-propanol (68:24:8, v/v/v), or (3) HPLC using a preparative (50 cm $\times$ 250 cm, 10 micron) Kromasil or Diazem cyano-bonded silica column using as mobile phase a 68:8:24 mixture of hexane, 2-propanol, and ethyl acetate. The flow rate was 118 mL/min. Under either HPLC condition, typically, the desired product *L*-aminoacyl maytansinoid (**2a–i**) eluted about 8–10 min before the corresponding *D*-aminoacyl isomer (**6a–i**).

***N*'-Deacetyl-*N*'-[2-(methyldithio)-1-oxoethyl]maytansine (D-M0SMe, **2a**).** Maytansinol (**4**) was reacted with *N*-methyl-*N*-[(2-methyldithio)-1-oxoethyl]-*L*-alanine (**5a**) as described above. The desired maytansinoid **2a**, and the corresponding *D*-aminoacyl isomer **6a** were isolated by purification using preparative TLC, eluting with 5% methanol in ethyl acetate to give a white solid. **6a** ^1H NMR δ 0.796 (3H, s), 1.252 (1H, m), 1.306 (3H, d, J = 6.4 Hz), 1.322 (3H, d, J = 6.8 Hz), 1.344 (3H, d, J = 10.8 Hz), 1.414 (2H, m), 1.553 (1H, d, J = 14.4 Hz), 1.640 (3H, s), 2.182 (1H, dd, J = 12.4, 2.8 Hz), 2.416 (3H, s), 2.624 (1H, dd, J = 12, 2.4 Hz), 2.914 (3H, s), 3.022 (1H, d, J = 9.6 Hz), 3.099 (1H, d, J = 12.4 Hz), 3.210 (3H, s), 3.357 (3H, s), 3.499 (1H, d, J = 9.2 Hz), 3.571 (1H, d, J = 14 Hz), 3.980 (3H, s), 4.266 (1H, dt, J = 12, 1.6 Hz), 4.787 (1H, dd, J = 9.2, 2.8 Hz), 5.379 (1H, m), 5.674 (1H, dd, J = 9.2, 6 Hz), 6.287 (1H, s), 6.422 (1H, dd, J = 11.2, 4 Hz), 6.678 (1H, d, J = 1.6 Hz), 6.691 (1H, d, J = 8.8 Hz) and 6.822 (1H, d, J = 1.6 Hz). HRMS calcd for $\text{C}_{35}\text{H}_{48}\text{ClN}_3\text{O}_{10}\text{S}_2\text{Na}$ ($\text{M} + \text{Na}$) $^+$, 792.2367; found, 792.2350.

6a. ^1H NMR δ 0.861 (3H, s), 1.273 (1H, m), 1.275 (3H, d, J = 6.4 Hz), 1.428–1.478 (2H, m), 1.519 (3H, d, J = 7.6 Hz), 1.689 (3H, s), 1.765 (1H, d, J = 13.6 Hz), 2.214 (1H, dd, J = 11.2, 3.2 Hz), 2.487 (3H, s), 2.667 (1H, dd, J = 12, 2.4 Hz), 2.856 (1H, d, J = 9.6 Hz), 3.114 (3H, s), 3.168 (3H, s), 3.203 (1H, d, J = 13.2 Hz), 3.340 (3H, s), 3.458 (1H, d, J = 9.2 Hz), 3.508 (1H, d, J = 12.8 Hz), 3.636 (2H, J = 6 Hz), 3.995 (3H, s), 4.293 (1H, t), 4.888 (1H, dd, J = 8.8, 3.2 Hz), 5.126 (1H, m), 5.869 (1H, dd, J = 9.6, 6 Hz), 6.230 (1H, d, J = 10.8 Hz), 6.331 (1H, s), 6.423 (1H, dd, J = 11.2, 4 Hz), 6.801 (1H, d, J = 1.2 Hz) and 6.852 (1H, d, J = 1.6 Hz). HRMS calcd for $\text{C}_{35}\text{H}_{48}\text{ClN}_3\text{O}_{10}\text{S}_2\text{Na}$ ($\text{M} + \text{Na}$) $^+$, 792.2367; found, 792.2350.

***N*'-Deacetyl-*N*'-(3-methyldithio-1-oxopropyl)-maytansine (DM1SMe, **2b**) and Its *D*-Alanil Isomer *D*-DM1SMe (**6b**).** Maytansinol (**4**) was reacted with *N*-methyl-*N*-[(3-methyldithio)-1-oxopropyl]-*L*-alanine (**5b**) as described above. Purification by column chromatography over silica gel, eluting with a mixture of dichloromethane/ethyl acetate/methanol to give a mixture of DM1SMe (**2b**) and the *D*-aminoacyl isomer *D*-DM1SMe (**6b**). The two diastereomers were separated by HPLC on a Kromasil cyano preparative HPLC column (250 mm $\times$ 50 mm, 10 micron particle size). The column was equilibrated in a mixture of hexanes/2-propanol/ethyl acetate (17:2:6, v/v/v) at a flow rate of 150 mL/min. Under these conditions, the desired *L*-DM1SMe (**2b**) had a retention time of 42 min, whereas the *D*-DM1SMe isomer (**6b**) elutes at 56 min. Optically pure products were obtained by this method (ee > 99.9%). HPLC analysis using a Vydac analytical C-18 column (length: 100 mm, i.d.: 4.6 mm, particle size: 3 microns) at a flow rate of 1 mL/min, eluting with a gradient of water and acetonitrile (0–3 min, 35% CH_3CN ; 3–13 min, 35–65% CH_3CN). Under these conditions *L*-DM1SMe (**2b**) eluted with a retention time of 10.76 min.

DM1SMe (2b). White solid (32% yield); ^1H NMR (CDCl_3) δ 0.84 (3H, s), 1.11–1.23 (1H, m), 1.31 (3H, d, J = 6 Hz), 1.35 (3H, d, J = 7 Hz), 1.46–1.52 (1H, m), 1.68 (3H, s), 1.97 (1H, d, J = 9 Hz), 2.24 (1H, dd, J = 12, 15 Hz), 2.30 (3H, s), 2.65 (1H, dd, J = 12, 15 Hz), 2.73–2.86 (2H, m), 2.90 (3H, s), 2.92–3.03 (2H, m), 3.08 (1H, d, J = 9 Hz), 3.14 (1H, d, J = 12 Hz), 3.28 (3H, s), 3.39 (3H, s), 3.54 (1H, d, J = 9 Hz), 3.72 (1H, d, J = 13 Hz), 4.02 (3H, s), 4.31 (1H, t, J = 11 Hz), 4.82 (1H, dd, J = 3, 12 Hz), 5.45 (1H, q, J = 7 Hz), 5.69 (1H, dd, J = 9, 15 Hz), 6.25 (1H, s), 6.47 (1H, dd, J = 11, 15 Hz), 6.67 (1H, d, J = 1.5 Hz), 6.77 (1H, d, J = 11 Hz), 6.85 (1H, d, J = 1.5 Hz). HRMS calcd for $\text{C}_{36}\text{H}_{51}\text{ClN}_3\text{O}_{10}\text{S}_2$ ($\text{M} + \text{H}$) $^+$, 784.2704; found, 784.2693; extinction coefficient $\epsilon_{280\text{ nm}}$ (ethanol) = 5545 $\text{M}^{-1}\text{cm}^{-1}$, $\epsilon_{252\text{ nm}}$ = 25 700 $\text{M}^{-1}\text{cm}^{-1}$.

***D*-DM1SMe (6b).** White solid (30% yield); mp 170–171 $^\circ\text{C}$; ^1H NMR (CDCl_3) δ 0.85 (3H, s), 1.11–1.20 (1H, m), 1.22–1.29 (3H, m), 1.49 (3H, d, J = 7.5 Hz), 1.69 (3H, s), 2.20 (1H, dd, J = 3, 14 Hz), 2.40 (3H, s), 2.65 (1H, dd, J = 12, 14 Hz), 2.79–2.84 (3H, m), 2.92–2.96 (2H, m), 3.04 (3H, s), 3.15 (3H, s), 3.33 (3H, s), 3.43 (1H, d, J = 9 Hz), 3.50 (1H, d, J = 12 Hz), 3.98 (3H, s), 4.28 (1H, m), 4.90 (1H, dd, J = 3, 12 Hz), 5.17 (1H, q, J = 7 Hz),

5.88 (1H, dd, $J = 9$ Hz), 6.22–6.26 (2H, m), 6.41 (1H, dd, $J = 11$, 15 Hz), 6.78 (1H, d, $J = 1.6$ Hz), 6.84 (1H, d, $J = 1.6$ Hz). HRMS calcd for $C_{36}H_{51}ClN_3O_{10}S_2$ ($M + Na$)⁺, 806.2524; found, 806.2518.

***N*²-Deacetyl-*N*²-[2-(phenyldithio)-1-oxopropyl]maytansine (DMISPh, 2c) and Its *D*-Alanyl Isomer *D*-DMISPh (6c).** Maytansinol (4) was reacted with *N*-methyl-*N*-[(2-phenyldithio)-1-oxopropyl]-L-alanine (5c) as described above. The product was purified by preparative TLC of the residue on silica gel, and eluting twice with 5% MeOH/CHCl₃ gave two strongly UV absorbing bands with $R_f = 0.5$ and 0.6. The two products were isolated by extraction with ethyl acetate. The higher R_f band was found to be the *D*-aminoacyl ester 6c of maytansinol, and the lower band was the *L*-aminoacyl ester 2c. The products were separated and further purified by HPLC on a Waters Radialpak C-18 column, eluting at a flow rate of 1.5 mL/min with a linear gradient of acetonitrile/water (70–90% acetonitrile/10 min). Under these conditions, both isomers had an identical retention time of 6.0 min.

DMISPh (2c). ¹H NMR (CDCl₃) δ 0.82 (3H, s), 1.11–1.25 (1H, m), 1.33 (3H, d, $J = 3$ Hz), 1.61 (3H, s), 1.63 (3H, d, $J = 14$ Hz), 2.19 (1H, dd, $J = 3$, 15 Hz), 2.61 (1H, dd, $J = 12$, 15 Hz), 2.78 (3H, s), 2.68–3.03 (2H, m), 3.07 (1H, d, $J = 9$ Hz), 3.20 (3H, s), 3.38 (3H, s), 3.53 (1H, d, $J = 9$ Hz), 3.63 (1H, d, $J = 13$ Hz), 3.68 (3H, s), 4.01 (3H, s), 4.30 (1H, t, $J = 11$ Hz), 4.79 (1H, dd, $J = 3$, 8 Hz), 5.43 (1H, q, $J = 7$ Hz), 5.68 (1H, dd, $J = 9$, 15 Hz), 6.23 (1H, s), 6.45 (1H, dd, $J = 12$, 15 Hz), 6.60 (1H, d, $J = 1.5$ Hz), 6.75 (1H, d, $J = 12$ Hz), 6.77 (1H, d, $J = 1.5$ Hz), 7.22–7.40 (5H, m). HRMS calcd for $C_{41}H_{52}ClN_3O_{10}S_2Na$ ($M + Na$)⁺, 868.2680; found, 868.2670.

***D*-DMISPh (6c).** ¹H NMR (CDCl₃) δ 0.89 (3H, s), 1.31 (3H, d, $J = 6.4$ Hz), 1.48–1.53 (m, 1H), 1.51 (3H, d), 1.72 (3H, s), 2.10 (1H, d, $J = 6$ Hz), 2.15 (1H, dd, $J = 3$, 15 Hz), 2.69 (1H, dd, $J = 12$, 15 Hz), 2.81–2.89 (2H, m), 2.96 (3H, s), 3.02 (1H, d, $J = 7$ Hz), 3.20 (3H, s), 3.36 (3H, s), 3.47 (1H, d, $J = 9$ Hz), 3.55 (1H, d, $J = 13$ Hz), 3.68 (3H, s), 4.04 (3H, s), 4.31 (1H, t, $J = 11$ Hz), 4.93 (1H, dd, $J = 3$, 12 Hz), 5.20 (1H, q, $J = 7$ Hz), 5.90 (1H, dd, $J = 9$, 15 Hz), 6.25 (1H, s), 6.27 (1H, d, $J = 12$ Hz), 6.45 (1H, dd, $J = 12$, 15 Hz), 6.83 (1H, d, $J = 1.5$ Hz), 6.89 (1H, d, $J = 1.5$ Hz), 7.27–7.49 (5H, m). HRMS calcd for $C_{41}H_{52}ClN_3O_{10}S_2Na$ ($M + Na$)⁺, 868.2680; found, 868.2672.

***N*²-Deacetyl-*N*²-[3-(methylthio)-1-oxobutyl]maytansine (*D*-M2SMe, 2d).** Maytansinol (4) was reacted with *N*-methyl-*N*-3-(methylthio)-1-oxobutyl-L-alanine (5d) as described above. The reaction mixture was purified by preparative TLC on silica gel, eluting twice with 7% MeOH in CHCl₃. Two new UV absorbing bands ($R_f = 0.65$, 0.75) were obtained. The products were isolated by extraction with ethyl acetate. The higher R_f band was determined to be the *D*-aminoacyl ester 6d (31%), whereas the lower R_f band was the desired *L*-aminoacyl ester 2d (44%). Both isomers were further purified by HPLC using a Waters Radialpak C-18 column, eluting at a flow rate of 2 mL/min with a linear gradient of acetonitrile/water (50–80% acetonitrile/10 min). Under these conditions, the *D*-aminoacyl ester had a retention time of 7.4 min, whereas the *L*-aminoacyl isomer had a retention time of 7.6 min. The diastereomeric purity of 2d and 6d were determined by HPLC analysis using a Kromasil cyano column (250 mm $\times$ 4.6 mm, 10 micron particle size) at a flow rate of 1.50 mL/min, eluting with an isocratic mixture of ethyl acetate/hexane/2-propanol (24:66:10/v/v). Under these conditions, 2d eluted with a retention time of 11.96 min, and its *D*-alanyl 6d isomer eluted with a retention time of 17.30 min.

2d. ¹H NMR (CDCl₃) δ 0.803 (3H, s), 1.265 (2H, m), 1.280 (3H, d, $J = 2.4$ Hz), 1.309 (3H, d, $J = 2.4$ Hz), 1.638 (1H, d, $J = 13.6$ Hz), 1.643 (3H, s), 1.760 (1H, m), 2.051 (2H, m), 2.179 (1H, dd, $J = 11.2$, 2.8 Hz), 2.318 (3H, s), 2.365–2.536 (2H, m), 2.611 (1H, dd, $J = 12.4$, 2 Hz), 2.719 (2H, m), 2.852 (3H, s), 3.033 (1H, d, $J = 9.6$ Hz), 3.110 (1H, d, $J = 12.4$ Hz), 3.219 (3H, s), 3.355 (3H, s), 3.498 (1H, d, $J = 10.8$ Hz), 3.657 (1H, d, $J = 12.4$ Hz), 3.983 (3H, s), 4.284 (1H, t), 4.872 (1H, dd, $J = 8.8$, 2.8 Hz), 5.660 (1H, dd, $J = 8.8$, 5.6 Hz), 6.197 (1H, s), 6.430 (1H, dd, $J = 11.2$, 4 Hz), 6.648 (1H, d, $J = 2$ Hz), 6.740 (1H, d, $J = 11.6$ Hz) and

6.820 (1H, d, $J = 2$ Hz). HRMS calcd for $C_{37}H_{52}ClN_3O_{10}S_2Na$ ($M + Na$)⁺, 820.2680; found, 820.2667.

6d. ¹H NMR (CDCl₃) δ 0.874 (3H, s), 1.193–1.429 (3H, m), 1.290 (3H, d, $J = 6.4$ Hz), 1.314 (3H, d, $J = 6.8$ Hz), 1.696 (3H, s), 1.777 (1H, d, $J = 13.6$ Hz), 2.058 (2H, m), 2.209 (1H, dd, $J = 11.2$, 3.2 Hz), 2.420 (3H, s), 2.529 (2H, m), 2.662 (1H, dd, $J = 12$, 3.6 Hz), 2.727–2.801 (2H, m), 2.826 (1H, d, $J = 9.6$ Hz), 3.044 (3H, s), 3.167 (3H, s), 3.204 (1H, d, $J = 11.2$ Hz), 3.342 (3H, s), 3.439 (1H, d, $J = 9.2$ Hz), 3.514 (1H, d, $J = 7.6$ Hz), 3.999 (3H, s), 4.351 (1H, t), 4.933 (1H, dd, $J = 8.8$, 3.2 Hz), 5.082 (1H, m), 5.844 (1H, dd, $J = 9.6$, 5.6 Hz), 6.231 (1H, d, $J = 8.8$ Hz), 6.242 (1H, s), 6.434 (1H, dd, $J = 11.2$, 4.4 Hz), 6.799 (1H, d, $J = 2$ Hz) and 6.851 (1H, d, $J = 2$ Hz). HRMS calcd for $C_{37}H_{52}ClN_3O_{10}S_2Na$ ($M + Na$)⁺, 820.2680; found, 820.2658.

***N*²-Deacetyl-*N*²-[4-(*R,S*)-(methylthio)-1-oxopentyl]maytansine (DM3SMe, 2f).** Maytansinol (4) was reacted with *N*-methyl-*N*-[(4-(*R,S*)-methylthio-1-oxopentyl)]-S-alanine (5f) as described above. The reaction mixture was purified by silica chromatography using a mixture of dichloromethane and methanol to remove unreacted maytansinol. The fractions containing the desired product were combined, and the solvent was removed under vacuum to give a mixture of the diastereomers 2f and 6f. The residue was purified on a 50 cm $\times$ 250 cm, 10 micron Diazem CN column using as mobile phase a 68:8:24 mixture of hexane, 2-propanol, and ethyl acetate. The flow rate was 118 mL/min. The desired product 2f eluted with a retention time of 11 min, and the undesired diastereomer 6f had a retention time of 19 min.

2f. White solid (36% yield). ¹H NMR (CDCl₃) δ 0.80 (3H, s), 1.19–1.23 (1H, m), 1.28–1.36 (9H, m), 1.42–1.46 (1H, m), 1.53–1.63 (2H, m), 1.64 (3H, s), 1.80–1.89 (1H, m), 1.90–2.09 (1H, m), 2.18 (1H, dd, $J = 3$ and 14 Hz), 2.32 (3H, s), 2.33–2.42 (1H, m), 2.49–2.62 (2H, m), 2.88 (3H, s), 3.04 (1H, d, $J = 9$ Hz), 3.11 (1H, d, $J = 11$ Hz), 3.23 (3H, s), 3.35 (3H, s), 3.49 (1H, d, $J = 9$ Hz), 3.63 (1H, d, $J = 12$ Hz), 3.98 (3H, s), 4.27 (1H, t, $J = 10$ Hz), 4.79 (1H, dd, $J = 3$ and 12 Hz), 5.41 (1H, q, $J = 7$ Hz), 5.66 (1H, dd, $J = 9$ and 15 Hz), 6.21 (1H, s), 6.42 (1H, dd, $J = 11$ and 15 Hz), 6.65 (1H, d, $J = 1.5$ Hz), 6.73 (1H, d, $J = 11$ Hz), 6.81 (1H, d, $J = 1.5$ Hz). HRMS calcd for $C_{38}H_{54}ClN_3O_{10}S_2Na$ ($M + Na$)⁺, 834.2837; found, 834.2819.

6f. White solid (30% yield). ¹H NMR δ 0.869 (3H, s), 1.200–1.315 (2H, m), 1.283 (3H, d, $J = 6.8$ Hz), 1.373 (3H, dd, $J = 5.6$, 1.2 Hz), 1.498 (3H, d, $J = 7.6$ Hz), 1.600 (1H, d, $J = 14.4$ Hz), 1.691 (3H, s), 1.772 (1H, m), 1.929 (2H, m), 2.203 (1H, dd, $J = 11.6$, 3.2 Hz), 2.421 (3H, s), 2.518 (2H, m), 2.654 (1H, t), 2.814 (1H, dd, $J = 8$, 1.6 Hz), 2.902 (1H, m), 3.038 (3H, d, $J = 3.2$ Hz), 3.164 (3H, s), 3.200 (1H, d, $J = 13.2$ Hz), 3.339 (3H, s), 3.436 (1H, d, $J = 8$ Hz), 3.507 (1H, d, $J = 12.8$ Hz), 3.996 (3H, s), 4.309 (1H, t), 4.936 (1H, dd, $J = 4.8$, 4 Hz), 5.093 (1H, m), 5.851 (1H, m), 6.233 (1H, d, $J = 11.2$ Hz), 6.269 (1H, s), 6.429 (1H, dd, $J = 10.8$, 4.4 Hz), 6.792 (1H, s) and 6.848 (1H, s). HRMS calcd for $C_{38}H_{54}ClN_3O_{10}S_2Na$ ($M + Na$)⁺, 834.2837; found, 834.2830.

***N*²-Deacetyl-*N*²-[4-(*S*)-(methylthio)-1-oxopentyl]maytansine (2g).** Maytansinol (4) was coupled with *N*-methyl-*N*-[(4-(*S*)-methylthio)-1-oxopentyl]-S-alanine (5g) using DCC and zinc chloride in dichloromethane as described above for the synthesis of 2f. A mixture of 2 diastereomers bearing the *N*-methyl-*L*-alanyl moiety (2g, *S,S*) and the *N*-methyl-*D*-alanyl moiety (6g, *R,S*) were obtained. The diastereomers were separated by HPLC on a Kromasil cyano column (4.6 mm $\times$ 250 mm), using an isocratic elution at a flow rate of 1 mL/min with hexane/ethyl acetate/2-propanol (68:24:8, v/v/v). Under these conditions, the isomer 2g (*S,S*) eluted at 24.5 min. The peak for the other isomer 6g (*R,S*) was well separated and eluted at 34.6 min.

2g. ¹H NMR (CDCl₃) δ 0.798 (3H, s), 1.197–1.267 (2H, m), 1.292 (3H, d, $J = 7.2$ Hz), 1.283 (3H, d, $J = 2$ Hz), 1.300 (3H, d, $J = 1.2$ Hz), 1.462 (1H, m), 1.536 (1H, d, $J = 8$ Hz), 1.640 (3H, s), 1.834–2.020 (2H, m), 2.176 (1H, dd, $J = 11.6$, 2.8 Hz), 2.326 (3H, s), 2.373–2.522 (2H, m), 2.603 (1H, dd, $J = 12.4$, 2 Hz), 2.847 (3H, s), 2.871–2.903 (1H, m), 3.028 (1H, d, $J = 9.6$ Hz), 3.108 (1H, d, $J = 12.4$ Hz), 3.223 (3H, s), 3.351 (3H, s), 3.499 (1H, d, $J = 9.2$ Hz), 3.639 (1H, d, $J = 12.8$ Hz), 3.983 (3H, s),

4.279 (1H, t), 4.627 (1H, dd, $J = 8.8, 3.2$ Hz), 5.388 (1H, m), 5.658 (1H, dd, $J = 8.8, 6.4$ Hz), 6.207 (1H, s), 6.426 (1H, dd, $J = 11.2, 4.4$ Hz), 6.639 (1H, d, $J = 1.6$ Hz), 6.737 (1H, d, $J = 11.2$ Hz) and 6.818 (1H, d, $J = 2$ Hz). MS: m/z 834.2 ($M + Na$)⁺.

6g. ¹H NMR (CDCl₃) δ 0.867 (3H, s), 1.251–1.383 (3H, m), 1.284 (3H, d, $J = 6$ Hz), 1.374 (3H, d, $J = 6.8$ Hz), 1.498 (3H, d, $J = 7.6$ Hz), 1.691 (3H, s), 1.763 (1H, d, $J = 14$ Hz), 1.932 (2H, m), 2.206 (1H, dd, $J = 11.2, 3.2$ Hz), 2.421 (3H, s), 2.518 (2H, m), 2.653 (1H, dd, $J = 12.4, 2.4$ Hz), 2.817 (1H, d, $J = 9.6$ Hz), 2.911 (1H, m), 3.034 (3H, s), 3.165 (3H, s), 3.200 (1H, d, $J = 13.2$ Hz), 3.339 (3H, s), 3.438 (1H, d, $J = 9.2$ Hz), 3.508 (1H, d, $J = 12.8$ Hz), 3.996 (3H, s), 4.311 (1H, t), 4.929 (1H, dd, $J = 8.8, 3.2$ Hz), 5.095 (1H, m), 5.856 (1H, dd, $J = 9.2, 6$ Hz), 6.236 (1H, d, $J = 13.2$ Hz), 6.253 (1H, s), 6.428 (1H, dd, $J = 11.2, 4$ Hz), 6.794 (1H, d, $J = 1.6$ Hz) and 6.849 (1H, d, $J = 1.2$ Hz). MS: m/z 834.2 ($M + Na$)⁺.

***N*'-Deacetyl-*N*'-(4-(*R*)-methylthio-1-oxopentyl)maytansine (2h).** Maytansinol (4) was coupled with *N*-methyl-*N*-[(4-(*R*)-methylthio)-1-oxopentyl]-*S*-alanine (5h) using DCC and zinc chloride in dichloromethane as described above. A mixture of 2 diastereomers bearing the *N*-methyl-*S*-alanyl moiety (2h, *S,R*) and the *N*-methyl-*R*-alanyl moiety (6h, *R,R*) were obtained. The diastereomers were separated by HPLC on a Kromasil cyano column (4.6 mm $\times$ 250 mm), using an isocratic elution at a flow rate of 1 mL/min, with hexane/ethyl acetate/2-propanol (68:24:8, v/v/v). Under these conditions, isomer 2h (*S,R*) eluted at 23.9 min. The peak for the other isomer 6h (*R,R*) was well separated and eluted at 33.7 min.

2h. ¹H NMR (CDCl₃) δ 0.796 (3H, s), 1.230 (2H, m), 1.290 (3H, d, $J = 5.6$ Hz), 1.297 (3H, d, $J = 1.2$ Hz), 1.305 (3H, d, $J = 6.4$ Hz), 1.447 (1H, m), 1.575 (1H, d, $J = 13.6$ Hz), 1.637 (3H, s), 1.815–2.002 (2H, m), 2.170 (1H, dd, $J = 11.2, 3.2$ Hz), 2.303 (3H, s), 2.357–2.547 (2H, m), 2.606 (1H, dd, $J = 12, 2.4$ Hz), 2.846 (3H, s), 3.027 (1H, d, $J = 9.6$ Hz), 3.108 (1H, d, $J = 12.8$ Hz), 3.215 (3H, s), 3.350 (3H, s), 3.494 (1H, d, $J = 9.6$ Hz), 3.648 (1H, d, $J = 12.8$ Hz), 3.978 (3H, s), 4.273 (1H, t), 4.775 (1H, dd, $J = 9.2, 2.8$ Hz), 5.384 (1H, m), 5.665 (1H, m), 6.227 (1H, s), 6.634 (1H, d, $J = 1.6$ Hz), 6.734 (1H, d, $J = 11.2$ Hz) and 6.816 (1H, d, $J = 1.6$ Hz). MS: m/z 834.2 ($M + Na$)⁺.

6h. ¹H NMR (CDCl₃) δ 0.867 (3H, s), 1.280 (3H, d, $J = 6.4$ Hz), 1.359 (2H, m), 1.384 (3H, d, $J = 6.4$ Hz), 1.446 (1H, m), 1.495 (3H, d, $J = 7.2$ Hz), 1.689 (3H, s), 1.780 (1H, d, $J = 13.4$ Hz), 1.886–1.964 (2H, m), 2.200 (1H, dd, $J = 11.6, 2.8$ Hz), 2.419 (3H, s), 2.470–2.564 (2H, m), 2.651 (1H, dd, $J = 12, 2.4$ Hz), 2.808 (1H, d, $J = 9.6$ Hz), 2.893 (1H, m), 3.040 (3H, s), 3.161 (3H, s), 3.196 (1H, d, $J = 13.2$ Hz), 3.338 (3H, s), 3.432 (1H, d, $J = 9.2$ Hz), 3.504 (1H, d, $J = 12.8$ Hz), 3.993 (3H, s), 4.303 (1H, t), 4.938 (1H, dd, $J = 8.8, 3.2$ Hz), 5.091 (1H, m), 5.842 (1H, m), 6.234 (1H, d, $J = 12.8$ Hz), 6.250 (1H, s), 6.428 (1H, dd, $J = 10.8, 4.4$ Hz), 6.788 (1H, d, $J = 1.6$ Hz) and 6.847 (1H, d, $J = 1.6$ Hz). MS: m/z 834.2 ($M + Na$)⁺.

***N*'-Deacetyl-*N*'-(4-methyl-4-(methylthio)-1-oxopentyl)maytansine (DM4-SMe, 2i) and Its *D*-Alanyl Isomer *D*-DM4SMe.** Maytansinol (4) was reacted with *N*-methyl-*N*-[4-methyl-4-(methylthio)-1-oxopentyl]-*L*-alanine (5i), as described above. The reaction mixture was purified by silica chromatography using a mixture of dichloromethane and methanol to remove the unreacted maytansinol. The fractions containing the desired product were combined, and the solvent was removed under vacuum to give a mixture of diastereomers 2i and 6i. The residue was taken up in a minimum volume of ethyl acetate and purified on a 50 cm $\times$ 250 cm, 10 micron Diazem CN column using as mobile phase a mixture of hexane, 2-propanol, and ethyl acetate at a ratio of 68:8:24. The flow rate was 118 mL/min. Under these conditions, the desired product 2i eluted with a retention time of 11 min, and the undesired diastereomer 6i had a retention time of 19 min.

2i (DM4SMe). White solid (36% yield); ¹H NMR (CDCl₃) δ 0.80 (3H, s), 1.28–1.36 (13H, m), 1.42–1.46 (2H, m), 1.53–1.63 (2H, m), 1.64 (3H, s), 1.75–1.85 (1H, m), 1.90–2.10 (1H, m), 2.18 (1H, dd, $J = 3$ and 14 Hz), 2.31 (3H, s), 2.40–2.49 (1H, m), 2.50–2.65 (1H, m), 2.85 (3H, s), 3.04 (1H, d, $J = 9$ Hz), 3.11

(1H, d, $J = 11$ Hz), 3.23 (3H, s), 3.35 (3H, s), 3.49 (1H, d, $J = 9$ Hz), 3.63 (1H, d, $J = 12$ Hz), 3.98 (3H, s), 4.27 (1H, t, $J = 10$ Hz), 4.79 (1H, dd, $J = 3$ and 12 Hz), 5.41 (1H, q, $J = 7$ Hz), 5.66 (1H, dd, $J = 9$ and 15 Hz), 6.21 (1H, s), 6.42 (1H, dd, $J = 11$ and 15 Hz), 6.65 (1H, d, $J = 1.5$ Hz), 6.73 (1H, d, $J = 11$ Hz), 6.81 (1H, d, $J = 1.5$ Hz). HRMS ($M + H$)⁺ calcd for C₃₉H₅₇ClN₃O₁₀S₂ ($M + H$)⁺, 826.3174; found, 826.3150; extinction coefficient (ethanol) $\epsilon_{280\text{ nm}} = 5140\text{ M}^{-1}\text{ cm}^{-1}$, $\epsilon_{252\text{ nm}} = 24\text{ 700 M}^{-1}\text{ cm}^{-1}$.

6i (D-DM4SMe). White solid; mp 165–167 °C; ¹H NMR (CDCl₃) δ 0.869 (3H, s), 1.265 (3H, d, $J = 7.2$ Hz), 1.327 (6H, s), 1.495 (3H, d, $J = 7.6$ Hz), 1.590 (2H, m), 1.690 (3H, s), 1.785 (1H, m), 1.915 (2H, m), 2.201 (1H, dd, $J = 17.6$ and 11.6 Hz), 2.422 (3H, s), 2.452 (2H, m), 2.646 (1H, d, $J = 26.8$ and 2.4 Hz), 2.800 (1H, d, $J = 9.6$ Hz), 3.041 (3H, s), 3.165 (3H, s), 3.195 (1H, d, $J = 13.2$ Hz), 3.340 (3H, s), 3.431 (1H, d, $J = 9.2$ Hz), 3.508 (1H, d, $J = 12.8$ Hz), 3.995 (3H, s), 4.313 (1H, t), 4.960 (1H, dd, $J = 15.2$ and 8.8 Hz), 5.117 (1H, q, $J = 7.6$ Hz), 5.838 (1H, dd, $J = 24.4$ and 6 Hz), 6.233 (1H, d, $J = 10$ Hz), 6.246 (1H, s), 6.428 (1H, dd, $J = 26$ and 4 Hz), 6.785 (1H, d, $J = 1.6$) and 6.847 (1H, d, $J = 1.6$ Hz). HRMS calcd C₃₉H₅₆ClN₃O₁₀S₂Na ($M + Na$)⁺, 848.2993; found, 848.2975.

***N*'-Deacetyl-*N*'-(3-mercapto-1-oxopropyl)-maytansine (DM1, 25a).** A solution of *N*'-deacetyl-*N*'-(3-methylthio-1-oxopropyl)-maytansine (2b) (1.95 g, 2.5 mmol) in a mixture of ethyl acetate (140 mL) and methanol (210 mL) was stirred at room temperature under an argon atmosphere and treated with a solution of dithiothreitol (0.95 g, 6.2 mmol) in 0.05 M potassium phosphate buffer (140 mL) at pH 7.5 containing 2 mM ethylenediaminetetraacetic acid (EDTA). The progress of the reaction was monitored by HPLC and was complete in three hours. The reaction mixture was treated with a solution of 0.2 M potassium phosphate buffer (250 mL) at pH 6.0 containing 2 mM EDTA and then extracted with ethyl acetate (3 $\times$ 600 mL). The organic layers were combined, washed with brine (100 mL), and then dried over sodium sulfate. Evaporation of the solvent gave a residue of crude thiol-containing maytansinoid 25a. The crude residue was purified by HPLC using a preparative Diazem cyano HPLC column (250 mm $\times$ 50 mm, 10 micron particle size) that was equilibrated in a mixture of hexanes/2-propanol/ethyl acetate (78.0:5.5:16.5, v/v/v) and run at a flow rate of 150 mL/min. The desired product 25a eluted as a peak centered at 16 min. The fractions containing the product were evaporated to give 25a as a white solid (76% yield); mp 190–192 °C (dec); ¹H NMR (CDCl₃) δ 0.84 (3H, s), 1.33 (3H, d, $J = 5$ Hz), 1.35 (3H, d, $J = 5$ Hz), 1.60 (3H, s), 1.68 (3H, s), 2.22 (1H, dd, $J = 3$ and 14 Hz), 2.60–2.82 (2H, m), 2.88 (3H, s), 3.08–3.20 (2H, m), 3.25 (3H, s), 3.39 (3H, s), 3.55 (1H, d, $J = 9$ Hz), 3.71 (1H, d, $J = 12$ Hz), 4.02 (3H, s), 4.32 (1H, t, $J = 10$ Hz), 4.81 (1H, dd, $J = 3$ and 12 Hz), 5.45 (1H, q, $J = 7$ Hz), 5.67 (1H, dd, $J = 9$ and 15 Hz), 6.25 (1H, s), 6.47 (1H, dd, $J = 11$ and 15 Hz), 6.70 (1H, d, $J = 1.5$ Hz), 6.75 (1H, d, $J = 11$ Hz), 6.86 (1H, d, $J = 1.5$ Hz). HRMS calcd for C₃₅H₄₉ClN₃O₁₀S ($M + H$)⁺ 738.2827; found, 738.2820; $[\alpha]^{25}_D = -113.2$, ($c = 0.306$, CHCl₃); extinction coefficient (methanol): $\epsilon_{280\text{ nm}} = 5422\text{ M}^{-1}\text{ cm}^{-1}$, $\epsilon_{252\text{ nm}} = 25\text{ 800 M}^{-1}\text{ cm}^{-1}$.

HPLC Analysis. HPLC analysis was performed using a Waters symmetry shield analytical C-8 column (length: 150 mm, i.d.: 3.9 mm, particle size: 5 microns) operating at 40 °C and at a flow rate of 1 mL/min, eluting with a linear gradient of water (containing 0.05% TFA) and acetonitrile (containing 0.05% TFA) from 35% to 45% acetonitrile over 30 min. Under these conditions, DM1 (25a) eluted with a retention time of 12.13 min. The diastereomeric purity of 25a was determined by HPLC analysis using a Diazem cyano column (length: 250 mm, i.d.: 4.6 mm, particle size: 5 microns) at a flow rate of 1 mL/min, eluting with an isocratic mixture of ethyl acetate/hexane/2-propanol (24:68:8 v/v respectively). Under these conditions, 25a eluted with a retention time of 13.70 min. There was no detectable signal for the *D*-isomer 26a, which elutes at 17.3 min.

D-DM1 (26a). A solution of *D*-DM1SMe (6b) (20 mg, 0.027 mmol) in a mixture of ethyl acetate (0.5 mL) and methanol (1.0 mL) was stirred at room temperature under an argon atmosphere

and treated with a solution of dithiothreitol (6.1 mg, 0.039 mmol) in 0.05 M potassium phosphate buffer (0.5 mL) at pH 7.5 containing 2 mM ethylenediamine-tetraacetic acid (EDTA). The progress of the reaction was monitored by HPLC and was complete in 2 h. The reaction mixture was then extracted with ethyl acetate (3 × 1 mL). The organic layers were combined and dried over sodium sulfate. Evaporation of the solvent gave a residue of crude thiol-containing maytansinoid **26a**. The crude residue was purified by HPLC using a semipreparative Diazem cyano HPLC column (250 mm × 10 mm, 10 micron particle size) that was equilibrated in a mixture of hexanes/2-propanol/ethyl acetate (65:10:25, v/v/v) and run at an initial flow rate of 2 mL/min. After 2 min, the flow rate was increased to 4.5 mL/min for the remainder of the run. The desired product eluted as a peak centered at 15 min. The fractions containing the product were evaporated to give **26a** as a white solid; mp 187–192 °C (dec); ¹H NMR (CDCl₃) δ 0.85 (3H, s), 1.26–1.29 (4H, m), 1.50 (3H, d, *J* = 7.6 Hz), 1.69 (3H, s), 2.21 (1H, dd, *J* = 3 and 14 Hz), 2.65 (1H, dd, *J* = 12 Hz and 14 Hz), 2.79–2.84 (3H, m), 2.92–2.96 (6H, m), 3.03 (3H, s), 3.16 (3H, s), 3.34 (3H, s), 3.45 (1H, d, *J* = 9 Hz), 3.51 (1H, d, *J* = 12 Hz), 4.00 (3H, s), 4.04 (9H, m), 4.29 (1H, m), 4.88 (1H, dd, *J* = 3 and 12 Hz), 5.20 (1H, q, *J* = 7 Hz), 5.90 (1H, dd, *J* = 9 and 15 Hz), 6.23–6.27 (2H, m), 6.43 (1H, dd, *J* = 11 and 15 Hz), 6.80 (1H, d, *J* = 1.6 Hz), 6.85 (1H, d, *J* = 1.6 Hz). HRMS calcd for C₃₅H₄₈ClN₃O₁₀SNa (*M* + *H*)⁺; found, 760.2637; [α]_D²⁵ = –135.0, (*c* = 0.294, CHCl₃). HPLC analysis: The diastereomeric purity of **26a** was determined as described above for **26a**. The isomer **26a** eluted with a retention time of 17.25 min. There was no detectable signal for the L-isomer, which elutes at 13.7 min.

N²-Deacetyl-N^{2'}-(4-mercapto-1-oxopentyl)maytansine (DM3, **25b).** DM3-SMe (**2f**, 12 mg, 0.015 mmol) was dissolved in 1.0 mL of a 1:1 mixture of ethyl acetate and methanol. A solution of dithiothreitol (18 mg, 0.117 mmol) in 0.50 mL of 50 mM phosphate buffer at pH 7.5 was then added. The reaction solution was magnetically stirred under an argon atmosphere for 3 h, then 1 mL of 200 mM phosphate buffer at pH 6.0 was added, and the mixture was extracted three times with 2 mL portions of ethyl acetate. The organic layers were combined and washed with 1 mL of saturated sodium chloride solution and then dried over 1 g of anhydrous sodium sulfate. The solvent was removed under vacuum, and the residue was taken up in a minimum of ethyl acetate and purified on a 50 cm × 250 cm, 10 micron Diazem CN column using as mobile phase a 70:8:22 mixture of hexane, 2-propanol, and ethyl acetate. The flow rate was 22 mL/min. The desired product eluted with a retention time of 10 min. The fractions containing the pure product were combined, and the solvent was removed under vacuum to give 11 mg of product **25b** (97% yield). ¹H NMR (CDCl₃) δ 0.80 (3H, s), 1.19–1.23 (1H, m), 1.28–1.36 (9H, m), 1.42–1.46 (1H, m), 1.53–1.63 (2H, m), 1.64 (3H, s), 1.80–1.89 (1H, m), 1.90–2.09 (1H, m), 2.18 (1H, dd, *J* = 3 and 14 Hz), 2.33–2.42 (1H, m), 2.49–2.62 (2H, m), 2.88 (3H, s), 3.04 (1H, d, *J* = 9 Hz), 3.11 (1H, d, *J* = 11 Hz), 3.23 (3H, s), 3.35 (3H, s), 3.49 (1H, d, *J* = 9 Hz), 3.63 (1H, d, *J* = 12 Hz), 3.98 (3H, s), 4.27 (1H, t, *J* = 10 Hz), 4.79 (1H, dd, *J* = 3 and 12 Hz), 5.41 (1H, q, *J* = 7 Hz), 5.66 (1H, dd, *J* = 9 and 15 Hz), 6.21 (1H, s), 6.42 (1H, dd, *J* = 11 and 15 Hz), 6.65 (1H, d, *J* = 1.5 Hz), 6.73 (1H, d, *J* = 11 Hz), 6.81 (1H, d, *J* = 1.5 Hz). HRMS calcd for C₃₇H₅₂ClN₃O₁₀SNa (*M* + Na)⁺, 788.2960; found, 788.2957.

N²-Deacetyl-N^{2'}-(4-mercapto-4-methyl-1-oxopentyl)maytansine (DM4, **25c).** DM4SMe (**2i**) (12 mg, 0.015 mmol) was dissolved in 1.0 mL of 1:1 ethyl acetate/methanol. A solution of dithiothreitol (18 mg, 0.117 mmol) in 0.50 mL of 50 mM phosphate buffer at pH 7.5 was then added. The solution was magnetically stirred under an argon atmosphere for 3 h, then 1 mL of 200 mM phosphate buffer at pH 6.0 was added, and the mixture was extracted three times with 2 mL portions of ethyl acetate. The organic layers were combined and washed with 1 mL of saturated sodium chloride solution and then dried over 1 g of anhydrous sodium sulfate. The solvent was removed under vacuum, and the residue was taken up in a minimum of ethyl acetate and purified on a 50 cm × 250 cm, 10 micron Diazem CN column using as mobile phase a mixture of

hexane, 2-propanol, and ethyl acetate at a ratio of 70:8:22. The flow rate was 22 mL/min. The desired product eluted with a retention time of 10 min. The fractions containing product were combined, and the solvent was removed under vacuum to give 11 mg of **25c** (97% yield) as a white solid; mp 185–187 °C (dec); ¹H NMR (CDCl₃) δ 0.80 (3H, s), 1.19–1.23 (1H, m), 1.28–1.36 (12H, m), 1.42–1.46 (2H, m), 1.53–1.63 (2H, m), 1.64 (3H, s), 1.75–1.85 (1H, m), 1.90–2.10 (1H, m), 2.18 (1H, dd, *J* = 3 and 14 Hz), 2.40–2.49 (1H, m), 2.50–2.65 (2H, m), 2.88 (3H, s), 3.04 (1H, d, *J* = 9 Hz), 3.11 (1H, d, *J* = 11 Hz), 3.23 (3H, s), 3.35 (3H, s), 3.49 (1H, d, *J* = 9 Hz), 3.63 (1H, d, *J* = 12 Hz), 3.98 (3H, s), 4.27 (1H, t, *J* = 10 Hz), 4.79 (1H, dd, *J* = 3 and 12 Hz), 5.41 (1H, q, *J* = 7 Hz), 5.66 (1H, dd, *J* = 9 and 15 Hz), 6.21 (1H, s), 6.42 (1H, dd, *J* = 11 and 15 Hz), 6.65 (1H, d, *J* = 1.5 Hz), 6.73 (1H, d, *J* = 11 Hz), 6.81 (1H, d, *J* = 1.5 Hz). HRMS calcd for C₃₈H₅₄ClN₃O₁₀SNa (*M* + Na)⁺, 802.3101; found, 802.3116. [α]_D²⁵ = –106.7, (*c* = 0.057, CHCl₃), extinction coefficient (ethanol): ε_{280 nm} = 5180 M^{–1} cm^{–1}, ε_{252 nm} = 26 200 M^{–1} cm^{–1}. HPLC analysis: purity was determined by HPLC analysis using a Vydac analytical C-18 column (250 mm × 4.6 mm, 3 micron particle size) at a flow rate of 1.00 mL/min in a gradient of water and acetonitrile, eluting with 37–58% acetonitrile over 25 min. Under these conditions L-DM4 (**25c**) eluted with a retention time of 11.8 min. The purity of L-DM4 was 98.8%. The diastereomeric purity of **25c** was determined by HPLC analysis using a Diazem cyano column (50 mm × 4.6 mm, 5 micron particle size) at a flow rate of 1.00 mL/min, eluting with a isocratic mixture of ethyl acetate/hexane/2-propanol (20:75:5 v/v/v). Under these conditions, **25c** eluted with a retention time of 4.52 min. There was no detectable signal for the D-alanyl isomer, which elutes at 5.42 min, indicating that **25c** was a diastereomerically pure compound.

D-DM4 (26c). A solution of D-DM4SMe (**6i**) (40.0 mg, 0.048 mmol) in a mixture of ethyl acetate (0.2 mL) and methanol (0.5 mL) was stirred at room temperature under an argon atmosphere and treated with a 0.50 mL solution containing dithiothreitol (59.7 mg, 0.387 mmol) in 0.050 M potassium phosphate buffer (0.5 mL) at pH 7.5 and 2 mM ethylenediamine-tetraacetic acid (EDTA). The progress of the reaction was monitored by HPLC and was complete after stirring overnight. The reaction mixture was treated 0.05 M potassium phosphate buffer (1.0 mL) at pH 6.0 containing 2 mM EDTA, and the product was extracted with ethyl acetate (3 × 1 mL). The organic layers were combined and dried over sodium sulfate. Evaporation of the solvent gave a residue of crude thiol-containing maytansinoid **26c**. The crude residue was purified by HPLC using a preparative Diazem cyano HPLC column (250 mm × 10 mm, 10 micron particle size) that was equilibrated in a mixture of hexanes/2-propanol/ethyl acetate (65:10:25, v/v/v) and run at an initial flow rate of 118 mL/min. The desired product **26c** eluted as a peak centered at 12.0 min. The fractions containing the product were evaporated to give **26c** as a white solid.

¹H NMR (CDCl₃) δ 0.866 (3H, s), 1.282 (3H, d, *J* = 6.4 Hz), 1.409 (6H, s), 1.500 (3H, d, *J* = 6.4 Hz), 1.625 (2H, m), 1.689 (3H, s), 1.766 (1H, m), 1.875 (2H, m), 2.205 (1H, dd, *J* = 17.6 and 11.2 Hz), 2.578 (2H, m), 2.646 (1H, dd, *J* = 26.8 and 2.8 Hz), 2.811 (1H, d, *J* = 9.6 Hz), 3.168 (3H, s), 3.196 (1H, d, *J* = 12.4 Hz), 3.341 (3H, s), 3.431 (1H, d, *J* = 9.2 Hz), 3.511 (1H, d, *J* = 13.2 Hz), 3.995 (3H, s), 4.300 (1H, t), 4.946 (1H, dd, *J* = 15.2 and 8.8 Hz), 5.156 (1H, q, *J* = 7.6 Hz), 5.836 (1H, dd, *J* = 24.4 and 6 Hz), 6.243 (1H, d, *J* = 10.4 Hz), 6.246 (1H, s), 6.426 (1H, dd, *J* = 26.4 and 4 Hz), 6.790 (1H, d, *J* = 1.6 Hz) and 6.848 (1H, d, *J* = 1.6 Hz). HRMS calcd for C₃₈H₅₄ClN₃O₁₀SNa (*M* + Na)⁺, 802.3101; found, 802.3099; [α]_D²⁵ = –90.4, (*c* = 0.052, CHCl₃). HPLC analysis: purity was determined by HPLC analysis using a Vydac analytical C-18 column (250 mm × 4.6 mm, 3 micron particle size) at a flow rate of 1.00 mL/min with a gradient of water and acetonitrile, eluting with 20–50% acetonitrile over 30 min. Under these conditions, D-DM4 (**26c**) eluted with a retention time of 17.85 min. The purity of **26c** was 97.8%. The diastereomeric purity of **26c** was determined as described above for DM4 (**25c**). The isomer eluted with a retention time of 5.42 min. There was no

detectable signal for the 25c isomer, which elutes at 4.52 min, indicating that 26c was a diastereomerically pure compound.

Synthesis of *N*²-Deacetyl-*N*²-[3-(3-carboxy-1-methyl-propyl-dithio)-1-oxopropyl]-maytansine (DM1-TPA, 2j). A solution of 4-(2-pyridyldithio)pentanoic acid (24 mg, 0.10 mmol) and DM1 (25a, 30 mg, 0.041 mmol) in glass distilled methanol (5 mL) was vigorously stirred, and 3 mL of an aqueous buffer (200 mM KH₂PO₄, 2 mM EDTA at pH 7.6) was added dropwise. The reaction was stirred overnight, and the product was purified by HPLC using a Vydac C-18 column (10 × 250 mm) operating at 30 °C and a flow rate of 4.75 mL/min, eluting with a linear gradient of acetonitrile (15% to 85% over 30 min) in 40 mM ammonium acetate buffer at pH 7.2. DM1-TPA (2j) eluted with a retention time of 12 min. The product was collected as the ammonium salt was taken up in ethyl acetate (15 mL). The solution was washed with 1 M HCl (4 mL) followed by saturated sodium chloride (3 mL). The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed under vacuum to give 15 mg (37% yield) of product 2j. ¹H NMR (400 MHz, CDCl₃) δ 0.81 (3H, s), 1.21–1.37 (11H, m), 1.44–1.48 (1H, m), 1.61 (1H, m), 1.65 (3H, s), 1.77–1.93 (3H, m), 2.20 (1H, d, *J* = 12 Hz), 2.39–2.49 (2H, m), 2.60 (1H, d, *J* = 12 Hz), 2.70 (1H, t, *J* = 9 Hz), 2.78–3.00 (7H, m), 3.12 (1H, d, *J* = 14 Hz), 3.22 (3H, s), 3.36 (3H, s), 3.48 (1H, d, *J* = 9 Hz), 3.64 (1H, d, *J* = 12 Hz), 3.98 (3H, s), 4.31 (1H, t, *J* = 10 Hz), 4.82 (1H, d, *J* = 12 Hz), 5.15–5.20 (1H, m), 5.63 (1H, dd, *J* = 9 and 15 Hz), 6.39–6.45 (2H, m), 6.59 (1H, t, *J* = 12 Hz), 6.67 (1H, d, *J* = 9 Hz), 6.82 (1H, s). HRMS calcd for C₄₀H₅₅ClN₃O₁₂S₂Na (M + Na)⁺, 892.2891; found, 892.2955. HPLC analysis was performed using a Vydac analytical C-18 column (length: 100 mm, i.d.: 4.6 mm, particle size: 3 microns) at a flow rate of 1 mL/min, eluting with a gradient of water and acetonitrile as follows: 0 min: 5% CH₃CN, 5 min: 5% CH₃CN, 5–65 min: 5–95% CH₃CN. Under these conditions DM1-TPA eluted with a retention time of 23.75 min.

Synthesis of *N*²-Deacetyl-*N*²-[4-methyl-4-(3-carboxy-propyl-dithio)-1-oxopentyl]-maytansine (DM4-TBA, 2k). A solution of 4-(2-pyridyldithio)butanoic acid (41.1 mg, 0.179 mmol) and DM4 (25c, 70.0 mg, 0.09 mmol) in glass distilled methanol (7 mL) was vigorously stirred, and 5 mL of an aqueous buffer (50 mM KH₂PO₄, 2 mM EDTA at pH 7.5) was added dropwise. The reaction was monitored by HPLC and was complete after 3 h. The solvent was evaporated, and the residue was purified by column chromatography over silica gel, eluting with a mixture of dichloromethane/methanol (97:3, v/v containing 0.1% glacial acetic acid) to give 45 mg (57% yield) of the purified product DM4-TBA (2k). ¹H NMR (CDCl₃) 0.800 (3H, s), 1.239 (6H, s), 1.302 (3H, d, *J* = 2 Hz), 1.303 (3H, d, *J* = 15.6 Hz), 1.414–1.513 (1H, m), 1.601 (2H, m), 1.642 (3H, s), 1.813–1.991 (2H, m), 1.932 (2H, t), 2.188 (1H, dd, *J* = 17.2 and 11.2 Hz), 2.346–2.530 (2H, m), 2.415 (2H, t), 2.636 (1H, dd, *J* = 18 and 12 Hz), 2.667 (2H, t), 2.875 (3H, s), 3.001 (1H, d, *J* = 9.6 Hz), 3.121 (1H, d, *J* = 12.4 Hz), 3.219 (3H, s), 3.356 (3H, s), 3.492 (1H, d, *J* = 8.8 Hz), 3.641 (1H, d, *J* = 12.8 Hz), 3.981 (3H, s), 4.293 (1H, t), 4.791 (1H, dd, *J* = 15.2 and 8.8 Hz), 5.324 (1H, q, *J* = 6.8 Hz), 5.667 (1H, dd, *J* = 24.4 and 6.4 Hz), 6.399 (1H, s), 6.421 (1H, dd, *J* = 26.4 and 4 Hz), 6.500 (1H, d, *J* = 1.6 Hz), 6.674 (1H, d, *J* = 11.2 Hz) and 6.826 (1H, d, *J* = 1.6 Hz). HRMS calcd for C₄₂H₆₀ClN₃O₁₂S₂Na (M + Na)⁺, 920.3186; found, 920.3192. HPLC analysis was performed using a Vydac analytical C-18 column (length: 100 mm, i.d.: 4.6 mm, particle size: 3 microns) at a flow rate of 1 mL/min, eluting with a linear gradient of 40 mM ammonium acetate buffer at pH 7.2 and acetonitrile as follows: 0 min: 5% CH₃CN, 5 min: 5% CH₃CN, 5–35 min: 5–35% CH₃CN. Under these conditions DM4-TBA eluted with a retention time of 18.40 min.

Synthesis of *N*²-Deacetyl-*N*²-(3-mercapto-1-oxopropyl)-maytansine dimer (DM1 dimer, 25d). A solution of 25a (26.4 mg, 0.035 mmol) in a 1:1 (v/v) mixture of ethanol and 0.05 M potassium phosphate buffer at pH 7.5 (5 mL) was treated with a solution of 2,2'-dithiodipyridine (3.9 mg, 0.017 mmol) in ethanol (1 mL). The reaction mixture was stirred at room temperature for 2 h. Most of the ethanol in the reaction mixture was removed by evaporation

under reduced pressure. The resulting aqueous layer was extracted with ethyl acetate (3 × 5 mL), and the combined organic layer was washed with saturated sodium chloride solution (1 × 5 mL) and then dried over sodium sulfate and filtered. The solvent was evaporated under reduced pressure, and the residue was purified by HPLC using a Metachem Inertsil ODS column, eluting with a gradient of water and acetonitrile to provide dimer 25d as a white solid (13.7 mg, 52% yield). ¹H NMR (CDCl₃) δ 0.75 (3H, s), 1.23–1.26 (1H, m), 1.28 (3H, d, *J* = 6 Hz), 1.30 (3H, d, *J* = 7 Hz), 1.42–1.52 (1H, m), 1.66 (3H, s), 2.15 (1H, m), 2.59 (1H, dd, *J* = 6 and 14 Hz), 2.71–2.82 (2H, m), 2.84 (3H, s), 3.00 (1H, d, *J* = 9 Hz), 3.09 (1H, d, *J* = 12 Hz), 3.18 (3H, s), 3.35 (3H, s), 3.48 (1H, d, *J* = 9 Hz), 3.61 (1H, d, *J* = 13 Hz), 3.97 (3H, s), 4.26 (1H, t, *J* = 11 Hz), 4.76 (1H, dd, *J* = 3 and 12 Hz), 5.33 (1H, q, *J* = 7 Hz), 5.61 (1H, dd, *J* = 9 and 15 Hz), 6.28 (1H, s), 6.41 (1H, dd, *J* = 11 and 15 Hz), 6.61 (1H, d, *J* = 1.6 Hz), 6.65 (1H, d, *J* = 11 Hz), 6.81 (1H, d, *J* = 1.5 Hz). HRMS: calcd for C₇₀H₉₄Cl₂N₆O₂₀S₂Na (M + Na)⁺, 1495.5239, found 1495.5242; extinction coefficients (methanol): ε_{280 nm} = 11 195 M⁻¹ cm⁻¹, ε_{252 nm} = 68 035 M⁻¹ cm⁻¹. HPLC analysis was performed using a Vydac analytical C-18 column (length: 100 mm, i.d.: 4.6 mm, particle size: 3 microns) at a flow rate of 1 mL/min, eluting with a gradient of water and acetonitrile as follows: 0 min 40% CH₃CN, 5 min 40% CH₃CN, 5–30 min 40–60% CH₃CN. Under these conditions, DM1 dimer eluted with a retention time of 13.69 min.

Synthesis of *N*²-Deacetyl-*N*²-(4-mercapto-4-methyl-1-oxopentyl)-maytansine Dimer (DM4 Dimer, 25e). A solution of 25c (100.0 mg, 0.128 mmol) in a 1:1 (v/v) mixture of dichloromethane and ethyl acetate (4 mL) was treated with a solution of CuCl₂ (85.76 mg, 0.640 mmol) in 0.50 M HCl (3 mL). The reaction mixture was stirred at room temperature for 2 h. The product was extracted with ethyl acetate (5 mL), and the aqueous phase was separated. An additional extraction of the aqueous phase with ethyl acetate (3 mL) was combined with the original mixture and washed with brine (2 mL) and dried over anhydrous Na₂SO₄ and filtered. The solvent was evaporated under reduced pressure to give the L-DM4-dimer 25e (11.7 mg, 12% yield). ¹H NMR (CDCl₃) δ 0.796 (6H, s), 1.136 (6H, d, *J* = 4 Hz), 1.227 (2H, m), 1.298 (12H, s), 1.299 (6H, d, *J* = 12 Hz), 1.463 (2H, m), 1.633 (6H, s), 1.670–1.842 (4H, m), 2.171 (2H, dd, *J* = 17.2 and 11.2 Hz), 2.262–2.456 (4H, m), 2.596 (2H, dd, *J* = 26.4 and 2 Hz), 2.842 (6H, s), 3.028 (2H, d, *J* = 9.6 Hz), 3.105 (2H, d, *J* = 12.4 Hz), 3.209 (6H, s), 3.353 (6H, s), 3.489 (2H, d, *J* = 8.8 Hz), 3.634 (2H, d, *J* = 12.8 Hz), 3.966 (6H, s), 4.277 (2H, t), 4.766 (2H, dd, *J* = 14.8 and 9.2 Hz), 5.366 (2H, q, *J* = 6.8 Hz), 5.644 (2H, dd, *J* = 24.4 and 6.4 Hz), 6.346 (2H, s), 6.417 (2H, dd, *J* = 26.4 and 4 Hz), 6.623 (2H, d, *J* = 1.6 Hz), 6.708 (2H, d, *J* = 11.2 Hz) and 6.816 (2H, d, *J* = 1.6 Hz). HRMS calcd for C₇₆H₁₀₆Cl₂N₆O₂₀S₂Na (M + Na)⁺, 1579.6178; *m/z*: found, 1579.6180. HPLC analysis was performed using a Vydac analytical C-18 column (length: 100 mm, i.d.: 4.6 mm, particle size: 3 microns) at a flow rate of 1 mL/min, eluting with a gradient of water and acetonitrile (0 min: 5% CH₃CN, 5 min: 5% CH₃CN, 5–65 min: 5–95% CH₃CN. HPLC analysis was performed using a Vydac analytical C-18 column (length: 100 mm, i.d.: 4.6 mm, particle size: 3 microns) at a flow rate of 1 mL/min, eluting with a gradient of water and acetonitrile (37–58% CH₃CN over 25 min). Under these conditions, the DM4 dimer eluted with a retention time of 22.48 min.

***N*-Succinimidyl 4-(2-pyridyldithio)pentanoate (SPP, 27).** A solution of 2,2'-dithiodipyridine (300 g, 1.36 mol) in a mixture of ethanol (1 L) and glacial acetic acid (42 mL) was placed in a flask and stirred under an argon atmosphere. A solution of 4-mercapto-pentanoic acid (101.8 g, 0.76 mol) in ethyl acetate (400 mL) was added dropwise over 15 min, and the reaction was stirred for an additional 2 h. The solvent was removed by rotary evaporation, and the residue was purified by column chromatography over silica gel, eluting with a mixture of hexanes/ethyl acetate/acetic acid (4:1:0.1) to give 4-(2-pyridyldithio)pentanoic acid (40 g, 21.4% yield). ¹H NMR in CDCl₃ δ 1.34 (3H, d, *J* = 7.0 Hz), 1.72–1.95 (2H, m), 2.52–2.63 (2H, m), 2.91–3.02 (1H, m), 7.08–7.13 (1H, m), 7.62–7.77 (2H, m), 8.46–8.49 (1H, m), 11.5 (1H, br s).

A solution of 4-(2-pyridyldithio)pentanoic acid (30 g, 123 mmol) in dichloromethane (525 mL) was treated with *N*-hydroxysuccinimide (14.3 g, 124 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC, 31.8 g, 165 mmol). The contents were stirred at room temperature for 2 h after which ethyl acetate (750 mL) was added. The solution was washed with 0.5% aqueous acetic acid (3 × 300 mL) and then with saturated aqueous NaCl (150 mL). The organic layer was separated and dried over anhydrous sodium sulfate and filtered, and the solvent was evaporated. The residue was purified by column chromatography over silica gel, eluting with hexanes/ethyl acetate (1:1). The fractions containing the pure product were combined, and the solvent was removed by rotary evaporation. The resulting oil (31 g) was taken up in a minimum volume of warm ethanol and stirred as ethyl ether (350 mL) was added, followed by hexanes (100 mL). The resulting precipitate was collected by vacuum filtration and dried in a vacuum oven at 30 °C for 12 h, to give SPP (27) as a white solid (18.7 g, 45% yield); mp 48–9 °C; ¹H NMR (CDCl₃) δ 1.36(3H, d, *J* = 7.0 Hz), 1.97–2.8 (2H, m), 2.73–2.86 (2H, m), 2.84 (4H, br s), 3.02–3.11 (1H, m), 7.08–7.13 (1H, m), 7.62–7.77 (2H, m), 8.46–8.49 (1H, m); Anal. (C₁₄H₁₆N₂O₄S₂) C, H, N, S.

***N*-Succinimidyl 4-(2-pyridyldithio)butanoate (SPDB, 28).** A solution of 2,2'-dithiopyridine (3.8 g, 17.3 mmol) in a mixture of acetic acid (0.5 mL) and ethyl alcohol (20 mL) was stirred under an argon atmosphere. A solution of 4-mercaptobutyric acid (7c, 1.39 g, 11.6 mmol) in ethyl acetate (10 mL) was added dropwise over approximately 2 min. The contents were stirred for 2 h, and the solvent was evaporated under reduced pressure. The residue was taken up in a minimum volume of ethyl acetate and purified by column chromatography on silica gel, eluting with a mixture of hexanes/ethyl acetate/acetic acid (65:33:2). The fractions containing the pure product were combined, and the solvent was removed under vacuum to give 1.5 g (6.55 mmol, 56% yield) of 4-(2-pyridyldithio)butanoic acid. ¹H NMR (CDCl₃) δ 2.0–2.1(2H, m), 2.505(1H, t, *J* = 7 Hz), 2.508 (1H, t, *J* = 7), 2.74 (1H, t, *J* = 7 Hz), 2.86 (1H, t, *J* = Hz), 7.10–7.13 (1H, m), 7.67 (1H, dt, *J* = 1, *J* = 5), 7.73 (1H, d, *J* = 8 Hz), 8.49 (1H, dd, *J* = 0.7, *J* = 5 Hz), 11.81 (1H, br s).

A flask containing a stir bar was charged with 4-(2-pyridyldithio)butanoic acid (1.1 g, 4.8 mmol) in dichloromethane (20 mL). *N*-Hydroxysuccinimide (0.64 g, 5.6 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC, 1.1 g, 5.6 mmol) were then added. The contents were stirred at room temperature for 2 h after which ethyl acetate (35 mL) was added. The reaction mixture was washed with 0.5 M HCl (3 × 10 mL) and then with saturated aqueous NaCl (1 × 10 mL). The organic layer was separated and dried over anhydrous sodium sulfate and filtered. The solvent was evaporated, and the residue was purified by column chromatography on silica gel, eluting with a mixture of hexanes/ethyl acetate/acetic acid (71:28.8:0.2). The fractions containing the pure product were combined, and the solvent was removed under vacuum. The resulting oil was taken up in a minimum volume of warm reagent-grade ethanol and stirred while ethyl ether (30 mL) was added, followed by hexanes (9.0 mL). The resulting precipitate was collected by filtration and dried in a vacuum oven at 30 °C for 12 h to give 0.50 g of SPDB (28) as a white solid (~31% yield); mp 47–48 °C; ¹H NMR in CDCl₃ δ 2.16 (2H, tt, *J* = 7, *J* = 15 Hz), 2.795 (2H, t, *J* = 7 Hz), 2.83(4H, br s), 2.91(1H, t, *J* = 7 Hz), 7.09 (1H, m), 7.65–7.67 (2H, m), 8.48 (1H, d, *J* = 5 Hz); Anal. (C₁₃H₁₄N₂O₄S₂) C, H, N, S.

Preparation of Antibody Conjugates with Maytansinoids 25a, 25b, and 25c. A solution of huC242 antibody (8 mg/mL) in aqueous buffer (50 mM potassium phosphate, 50 mM sodium chloride, 2 mM ethylenediaminetetraacetic acid disodium salt) at pH 6.5 was incubated for 2 h with a 7- to 10-fold molar excess of either *N*-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP, 27, or *N*-succinimidyl 4-(2-pyridyldithio)butanoate (SPDB, 28). The reaction mixture was purified by passage through a Sephadex G25 gel filtration column to remove low molecular weight material. The concentration of the antibody was determined spectrophotometrically using the known extinction coefficients for the antibody

$\epsilon_{280\text{ nm}} = 217\,560\text{ M}^{-1}\text{ cm}^{-1}$. The number of pyridylthio groups introduced per antibody molecule was determined using a spectrophotometric assay. An aliquot of the modified antibody was treated with an excess (>20 equiv) of dithiothreitol and the release of pyridine-2-thione determined using the known extinction coefficients of $\epsilon_{343\text{ nm}} = 8080\text{ M}^{-1}\text{ cm}^{-1}$ and $\epsilon_{280\text{ nm}} = 5100\text{ M}^{-1}\text{ cm}^{-1}$ for pyridine-2-thione.

The modified antibody was diluted to 2.5 mg/mL in aqueous buffer (50 mM potassium phosphate, 50 mM sodium chloride, 2 mM ethylenediaminetetraacetic acid disodium salt) at pH 6.5. The SPP-modified antibody was treated with 1.7 equiv of DM1 (25a), whereas the SPDB-modified antibody was treated with a 1.7 equiv of either DM3 (25b) or DM4 (25c) dissolved in glass-distilled DMA (final concentration of DMA was 3% v/v). The reaction mixture was incubated for 18 h at room temperature. The reaction mixture was purified by passage through a Sephadex G25 gel filtration column to remove the unconjugated drug and other low molecular weight species. The concentration of the conjugate was determined spectrophotometrically using the known extinction coefficients for the antibody ($\epsilon_{280\text{ nm}} = 217\,560\text{ M}^{-1}\text{ cm}^{-1}$ and $\epsilon_{252\text{ nm}} = 80\,062\text{ M}^{-1}\text{ cm}^{-1}$) and the drugs. The resulting conjugate was monomeric and contained, on the average, 3.2–3.5 maytansinoid molecules linked per antibody molecule.

Cytotoxicity Assays in Vitro. The cell lines used in cytotoxicity assays were (1) KB (ATCC CCL-17), a cell line of human epithelial origin, (2) SK-BR-3 (ATCC HTB-30), a cell line established from a human breast adenocarcinoma, (3) COLO 205 (ATCC CCL-222), a human colon tumor cell line, and (4) A-375 (ATCC CRL 1619), a human melanoma cell line. The cell lines were grown in Dulbecco's modified Eagles Medium (DMEM, Biowhittaker, Walkersville, MD) with L-glutamine supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 50 µg/mL of gentamycin sulfate (Life Technologies, Rockville, MD). The cells were maintained at 36–37.5 °C in a humidified atmosphere that contained 6% CO₂.

The cytotoxicity study was performed using a clonogenic assay as previously described.^{23,24} The test cell lines were plated into 6-well culture dishes at a constant number of 1000 cells per well. The cells were incubated with varying concentrations (0 to 3 nM) of the various maytansinoids (free or conjugated to antibodies) for 72 h. The medium was then aspirated from the plates and replaced with fresh medium. The cultures were allowed to grow and form colonies for a total of 7–10 days after plating. The cultures were then fixed and stained with 0.2% crystal violet in 10% formalin/PBS, and the colonies were counted. Plating efficiency of nontreated cells (medium alone) was determined by dividing the number of colonies counted by the number of cells plated. The surviving fraction of cells exposed to the drugs was determined by dividing the number of colonies in wells that were exposed to the drug by the number of colonies in the control wells.

Antitumor Activity in Vivo. The in vivo efficacy of conjugates of maytansinoids DM1 (25a), DM3 (25b), and DM4 (25c) with the huC242 antibody was evaluated in a human colon tumor xenograft model established with COLO 205 cells. Five-week-old female SCID mice (20 animals) were inoculated subcutaneously in the right flank with COLO 205 human colon carcinoma cells (1.5 × 10⁶ cells/mouse) in 0.1 mL of serum-free medium. The tumors were grown for 11 days to an average size of 100 mm³. The animals were then randomly divided into four groups (5 animals per group). The first group of mice served as the control group and were treated with the phosphate-buffered saline vehicle. The remaining three groups were treated with either huC242–DM1 (DM1 dose of 75 µg/kg, qd × 5), huC242–DM3 (DM3 dose of 75 µg/kg, qd × 5), or huC242–DM4 conjugate (DM4 dose of 75 µg/kg, qd × 5), administered intravenously. Tumor sizes were measured twice weekly, and the tumor volumes were calculated using the formula tumor volume = 1/2(length × width × height). The weight of the animals was also measured twice per week.

Supporting Information Available: Analytical data (elemental analysis and HRMS and HPLC purity data) for target compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Kupchan, S. M.; Komoda, Y.; Court, W. A.; Thomas, G. J.; Smith, R. M.; Karim, A.; Gilmore, C. J.; Haliwanger, R. C.; Bryan, R. F. Maytansine, a novel antileukemic ansa macrolide from *Maytenus ovatus*. *J. Am. Chem. Soc.* 1972, 94, 1354–1356.
- (2) Kupchan, S. M.; Brannan, A. R.; Sneden, A. T.; Verma, A. K.; Dailey, R. G., Jr.; Komoda, Y.; Nagao, Y. Novel maytansinoids. Naturally occurring and synthetic antileukemic esters of maytansinol. *J. Am. Chem. Soc.* 1975, 97, 5294–5295.
- (3) Remillard, S.; Rebhun, L. I.; Howie, G. A.; Kupchan, S. M. Antimitotic activity of the potent tumor inhibitor maytansine. *Science* 1975, 189, 1002–1005.
- (4) Huang, A. B.; Lin, C. M.; Hamel, E. Maytansine inhibits nucleotide binding at the exchangeable site of tubulin. *Biochem. Biophys. Res. Commun.* 1985, 128, 1239–1246.
- (5) Kupchan, S. M.; Komoda, Y.; Brannan, A. R.; Sneden, A. T.; Court, W. A.; Thomas, G. J.; Hintz, H. P.; Smith, R. M.; Karim, A.; Howie, G. A.; Verma, A. K.; Nagao, Y.; Dailey, R. G., Jr.; Zimmerly, V. A.; Sumner, W. C., Jr.; The maytansinoids. Isolation, structural elucidation, and chemical interrelation of novel ansa macrolides. *J. Org. Chem.* 1977, 42, 2349–2357.
- (6) Issell, B. F.; Crooke, S. T. Maytansine. *Cancer Treat. Rev.* 1978, 5, 199–207.
- (7) Sedlacek, H.-H.; Seemann, G.; Hoffmann, D.; Czech, J.; Lorenz, P.; Kolar, C.; Bosslet, K.; Antibodies as carriers of cytotoxicity. In *Contributions to Oncology*; Sedlacek, H.-H., et al., Eds.; Basel: New York, 1992; pp 1–145.
- (8) Xie, H.; Audette, C.; Hoffee, M.; Lambert, J. M.; Blättler, W. A. Pharmacokinetics and biodistribution of the antitumor immunoconjugate, cantuzumab mertansine (huC242-DM1), and its two components in mice. *J. Pharmacol. Exp. Ther.* 2004, 310, 1073–1082.
- (9) Chari, R. V. J.; Martell, B. A.; Gross, J. L.; Cook, S. B.; Shah, S. A.; Blättler, W. A.; McKenzie, S. J.; Goldmacher, V. S. Immunoconjugates containing novel maytansinoids: promising anticancer drugs. *Cancer Res.* 1992, 52, 127–131.
- (10) Liu, C.; Tadayoni, B. M.; Bourret, L. A.; Mattocks, K. M.; Derr, S. M.; Widdison, W. C.; Kodersha, N. L.; Ariniello, P. D.; Goldmacher, V. S.; Lambert, J. M.; Blättler, W. A.; Chari, R. V. J. Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. *Proc. Natl. Acad. Sci. U.S.A.* 1996, 93, 8618–8623.
- (11) Tolcher, A. W.; Ochoa, L.; Hammond, L. A.; Patnaik, A.; Edwards, T.; Takimoto, C.; Smith, L.; de Bono, J.; Schwartz, G.; Mays, T.; Jonak, Z. L.; Johnson, R.; DeWitte, M.; Martino, H.; Audette, C.; Maes, K.; Chari, R. V. J.; Lambert, J. M.; Rowinsky, E. K. Cantuzumab mertansine, a maytansinoid immunoconjugate directed to the CanAg antigen: a phase I, pharmacokinetic, and biologic correlative study. *J. Clin. Oncol.* 2003, 21, 211–222.
- (12) Helft, P. R.; Schilsky, R. L.; Hoke, F. J.; Williams, D.; Kindler, H. L.; Sprague, E.; DeWitte, M.; Martino, H. K.; Erickson, J.; Pandite, L.; Russo, M.; Lambert, J. M.; Howard, M.; Ratain, M. J. A phase I study of cantuzumab mertansine administered as a single intravenous infusion once weekly in patients with advanced solid tumors. *Clin. Cancer Res.* 2004, 10, 4363–4468.
- (13) Meister, A.; Anderson, M. E. *Annu. Rev. Biochem.* 1983, 52, 711–760.
- (14) Cassidy, J. M.; Chan, K. K.; Floss, H. G.; Leistner, E. Recent developments in the maytansinoid antitumor agents. *Chem. Pharm. Bull. (Tokyo)* 2004, 52, 1–26.
- (15) Kupchan, S. M.; Sneden, A. T.; Brannan, A. R.; Howie, G. A.; Rebhun, L. I.; McIvor, W. E.; Wang, R. W.; Schnaitman, T. C. Structural requirements for antileukemic activity among the naturally occurring and semisynthetic maytansinoids. *J. Med. Chem.* 1978, 21, 31–37.
- (16) Higashide, E.; Asai, M.; Ootsu, K.; Tanida, S.; Kozai, Y.; Hasegawa, T.; Kishi, T.; Sugino, Y.; Yoneda, M. Ansamitocin, a group of novel maytansinoid antibiotics with antitumor properties from *Nocardia*. *Nature* 1977, 29, 721–722.
- (17) Asai, M.; Mizuta, E.; Izawa, M.; Haibaba, K.; Kishi, T. Isolation, chemical characterization and structure of ansamitocin, a new antitumor antibiotic. *Tetrahedron* 1978, 35, 1079–1085.
- (18) Kawai, A.; Akimoto, H.; Kozai, Y.; Ootsu, K.; Tanida, S.; Hashimoto, N.; Nomura, H. Chemical modification of ansamitocins. III. Synthesis and biological effects of 3-acyl esters of maytansinol. *Chem. Pharm. Bull. (Tokyo)* 1984, 32, 3341–3351.
- (19) Kupchan, S. M.; Komoda, Y.; Brannan, A. R.; Dailey, R. G.; Zimmerly, V. A. Novel maytansinoids. Structural interrelations and requirements for antileukemic activity. *J. Am. Chem. Soc.* 1974, 96, 3706–3708.
- (20) Lindholm, L.; Holmgren, J.; Svennerholm, L.; Fredman, P.; Nilsson, O.; Persson, B.; Myrvold, H.; Lagergard, T. Monoclonal antibodies against gastrointestinal tumour-associated antigens isolated as monosialogangliosides. *Int. Arch. Allergy Appl. Immunol.* 1983, 71, 178–181.
- (21) Mickley, L. A.; Bates, S. E.; Richert, N. D.; Currier, S.; Tanaka, S.; Foss, F.; Rosen, N.; Fojo, A. T. Modulation of the expression of a multidrug resistance gene (*mdr1/P-glycoprotein*) by differentiating agents. *J. Biol. Chem.* 1989, 264, 18031–18040.
- (22) Goff, D. A.; Carroll, S. F. Substituted 2-iminothiolanes: Reagents for the preparation of disulfide cross-linked conjugates with increased stability. *Bioconjugate Chem.* 1990, 1, 381–386.
- (23) Goldmacher, V. S.; Tinnel, N. L.; Nelson, B. C. Evidence that pinocytosis in lymphoid cells has a low capacity. *J. Cell Biol.* 1986, 102, 1312–1319.
- (24) Goldmacher, V. S.; Anderson, J.; Blättler, W. A.; Lambert, J. M.; Senter, P. D. Antibody-complement-mediated cytotoxicity is enhanced by ribosome-inactivating proteins. *J. Immunol.* 1985, 135, 3648–3651.

JM060319F

46 19

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
NIT : 800.176.089-2
- / -

 Industria y Comercio SUPERINTENDENCIA	RECIBO DE CAJA	No. 12 - 0088174
		Bogotá D.C., Agosto 28 de 2012 - 08:36:20

RECIBIDO DE : CAVELIER ABOGADOS NI 860.041.367

*** Soporte del Pago ***					
TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR. PAGO
RECIBO DE CA	999999999	81516	09/08/2012		20.000.00
RECIBO DE CA	999999999	81517	09/08/2012		20.000.00
RECIBO DE CA	999999999	85530	22/08/2012		590.000.00

*** Conceptos Pagados ***			
CANT. RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
21 50005-01-01 SOLICITUDES	1607 REIVINDICACION UNITARIA ADICIONAL A LAS 10 INICIALES	30.000.00	630.000.00
			<u>\$630.000.00</u>
			=====

SON: **SEISCIENTOS TREINTA MIL PESOS MONEDA CORRIENTE**

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 08-077425- -00005-0000
Fecha: 2012-09-21 16:35:46 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 444 RESPUESTAREQUE Folios: 19

COLO: 14775- 841-071-5