Semisynthetic Maytansine Analogues for the Targeted Treatment of Cancer

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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 colleagues[1] and is the first compound of a class of benzoansamacrolide antibiotics named maytansinoids.[2] Maytansinoids are antimitotic 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 (IC_{50} 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

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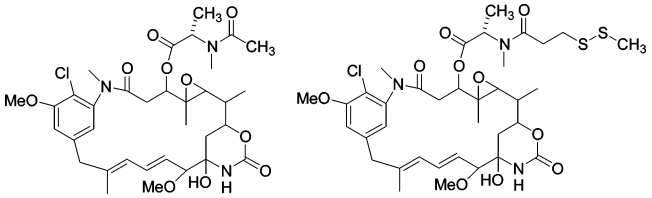
10.1021/jm060319f CCC: $33.50
Published on Web 06/10/2006



1 **2b**

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.

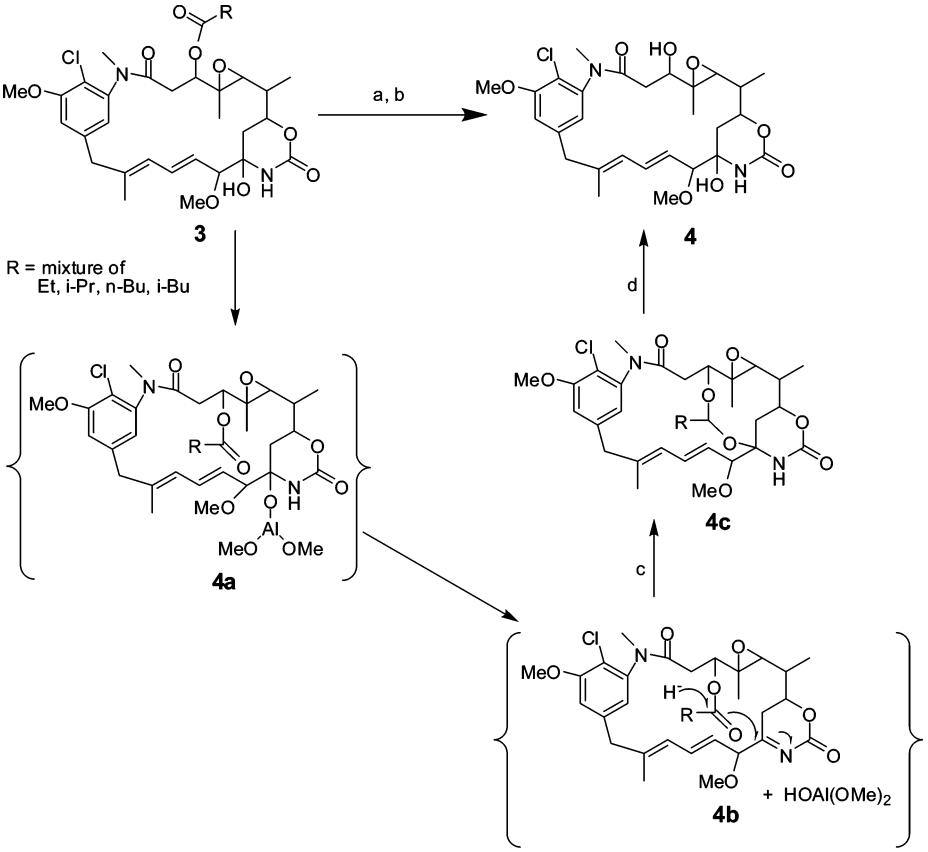
Biosynthetically, maytansinoids are complex polyketides. Consequently, soon after the initial isolation from plants, a bacterial actinomycetes strain *Actinosynnema pretiosum* was isolated, which produces ansamycin antibiotics called ansamitocins that are maytansinoids with C3 alkanoic acid esters.[16,17] 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 at 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 alkanoic 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.

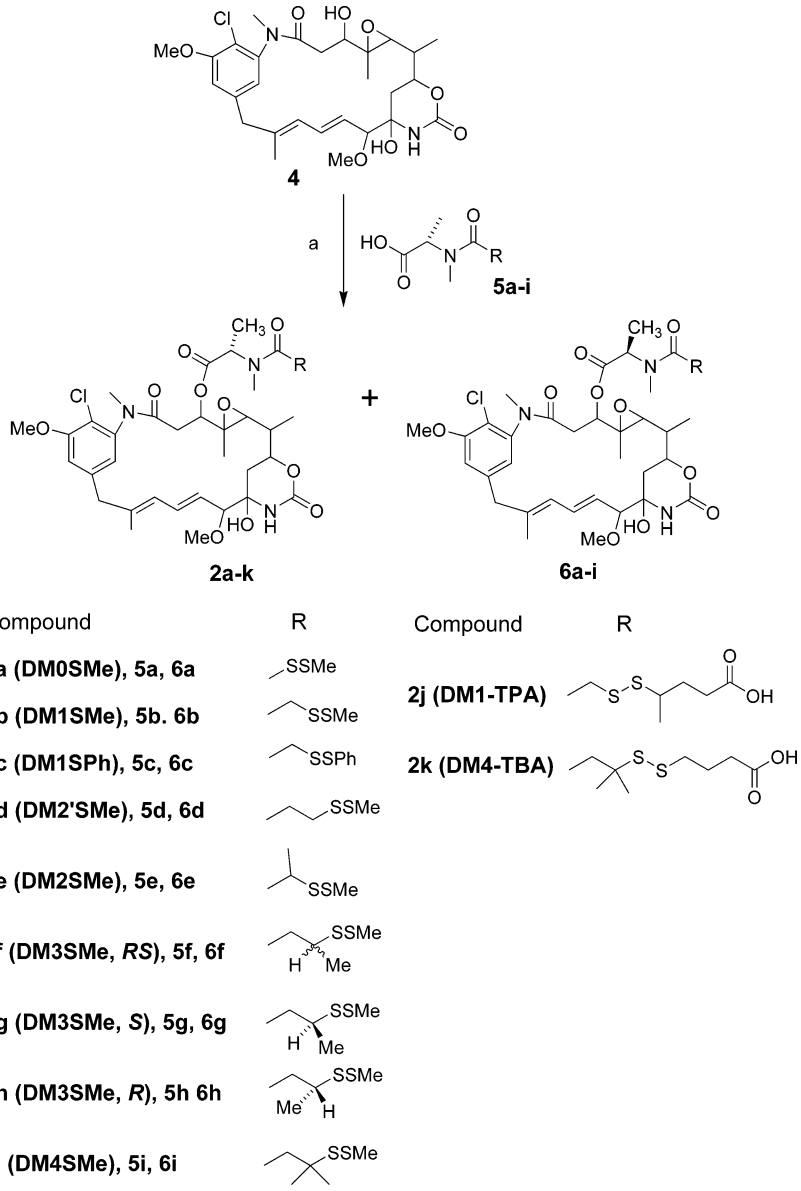
The synthetic route for the preparation of disulfide-containing maytansinoid esters is shown in Schemes 1 and 2. Maytansinol (**4**) was synthesized from ansamitocins (**3**), which in turn were obtained from the fermentation of the microorganism *Actinosynnema pretiosum*. Ansamitocins were isolated 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 method[15] 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, $NaBH_4$, 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]

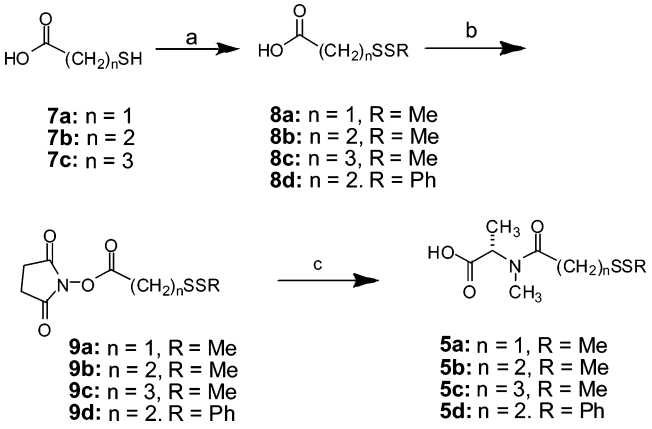


[a] Reaction conditions: (a) DCC/$ZnCl_2$/CH_2Cl_2, rt.

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).

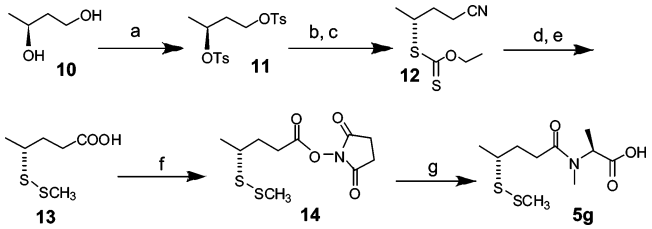
Mercaptocarboxylic acids of varying linear chain lengths were converted into methyldithio or aryldithio carboxylic acids by reacting with methyl methanethiolsulfonate or aryl disulfides, respectively (Scheme 3). The dithio-carboxylic acids were converted into their *N*-hydroxysuccinimide esters, followed 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-(methydithio)pentanoic acids (compounds **13** and **18**) were prepared from the corresponding optically pure 1,3-dihdroxybutanes as shown in Schemes 4 and 5. The conversion of the 1,3-dihdroxybutanes 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 methanethiolsulfonate 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*-hydroxysuccinimde ester of *S*- and *R*-4-(methyldithio)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) $CH_3SSO_2CH_3$, (PhSSPh for **8d**); (b) *N*-hydroxysuccinimide, EDC; (c) *N*-methyl-L-alanine, triethylamine.

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



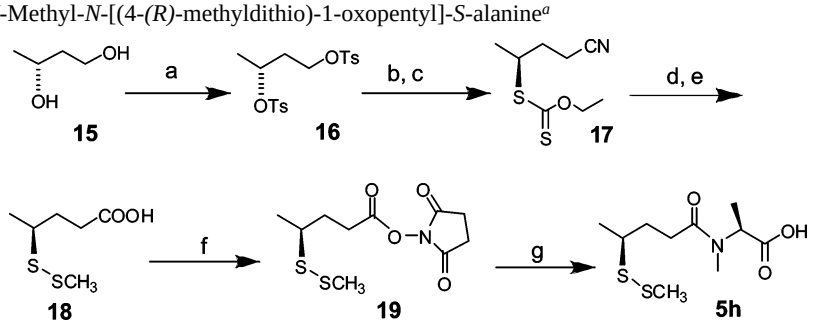
[a] Reaction conditions: (a) TsCl. Py; (b) NaCN; (c) KSSCOEt; (d) NaOH; (e) $CH_3SSO_2CH_3$; (f) *N*-hydroxysuccinimide, EDC; (g) *N*-methyl-L-alanine, triethylamine.

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 methydithio substituent to give the substituted alkene, which is the thermodynamically favored product.

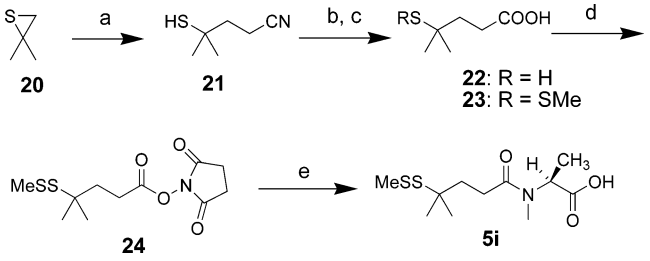
Because maytansinoids are sensitive to bases, nucleophiles, and strong reducing agents, the reduction of disulfide-containing maytansinoids to the thiol compounds had to be achieved using very mild and disulfide-specific reagents. The reduction of the disulfide-containing maytansinoids (**2b**,**c**,**e**,**f**,**i**) 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)*-methyldithio)-1-oxopentyl]-*S*-alanine[a]



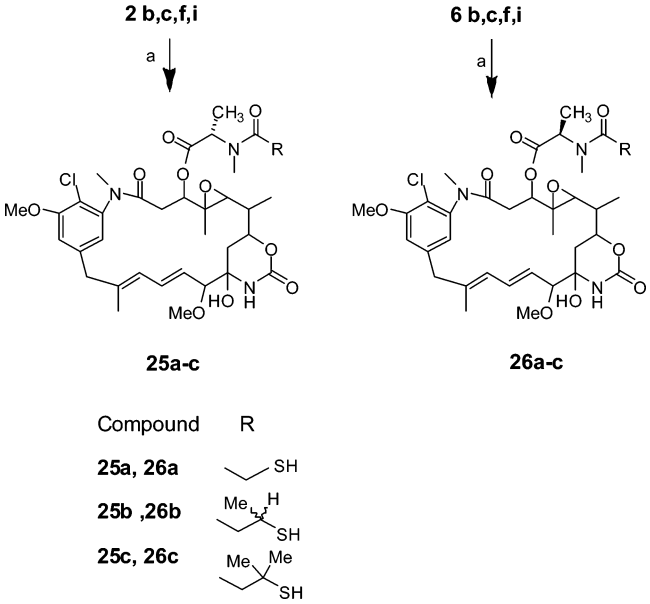
[a] Reaction conditions: (a) TsCl. Py; (b) NaCN; (c) KSSCOEt; (d) NaOH; (e) $CH_3SSO_2CH_3$; (f) *N*-hydroxysuccinimide, EDC; (g) *N*-methyl-L-alanine, triethylamine.

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



[a] Reaction conditions: (a) CH_3CN, ^nBu-Li; (b) NaOH; (c) $CH_3SSO_2CH_3$; (d) *N*-hydroxysuccinimide, EDC; (e) *N*-methyl-L-alanine.

Scheme 7. Synthesis of Thiol-Containing Maytansinoids[a]



[a] Reaction conditions: (a) dithiothreitol/RT.

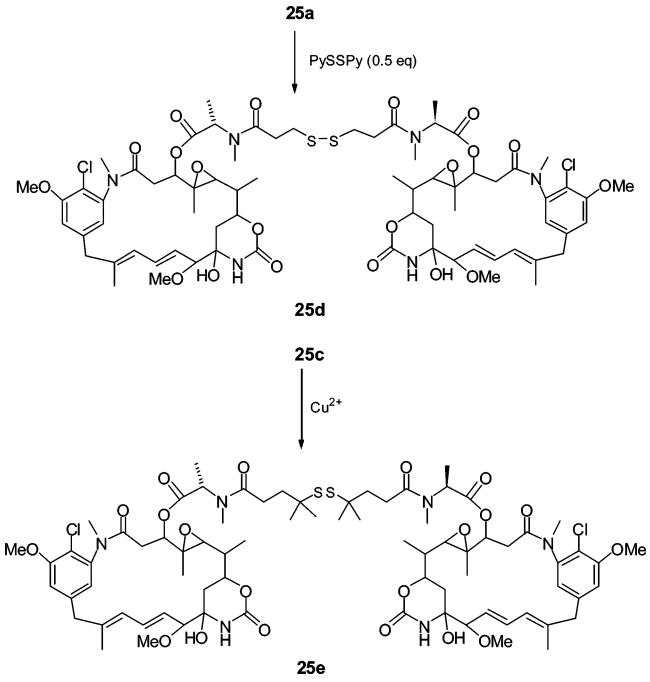
of metal ions. This was achieved by the careful glass-distillation of high-grade commercial DMA.

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).

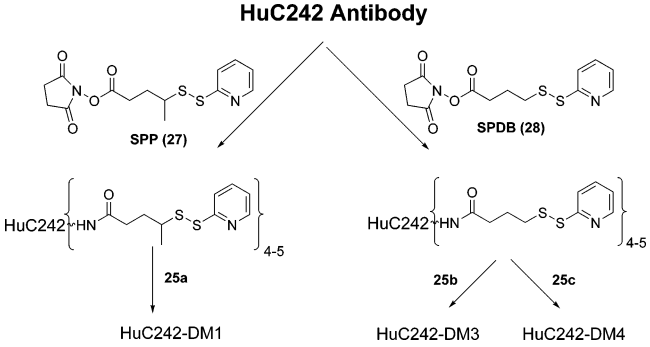
Antibody Conjugates. The antibody used in this study was huC242, a humanized version of the murine monoclonal antibody C242[20] that binds to the CanAg antigen that is highly expressed on the surface of several human solid tumors, including colon, pancreatic, gastric, and some lung tumors. This antibody 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*-

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

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

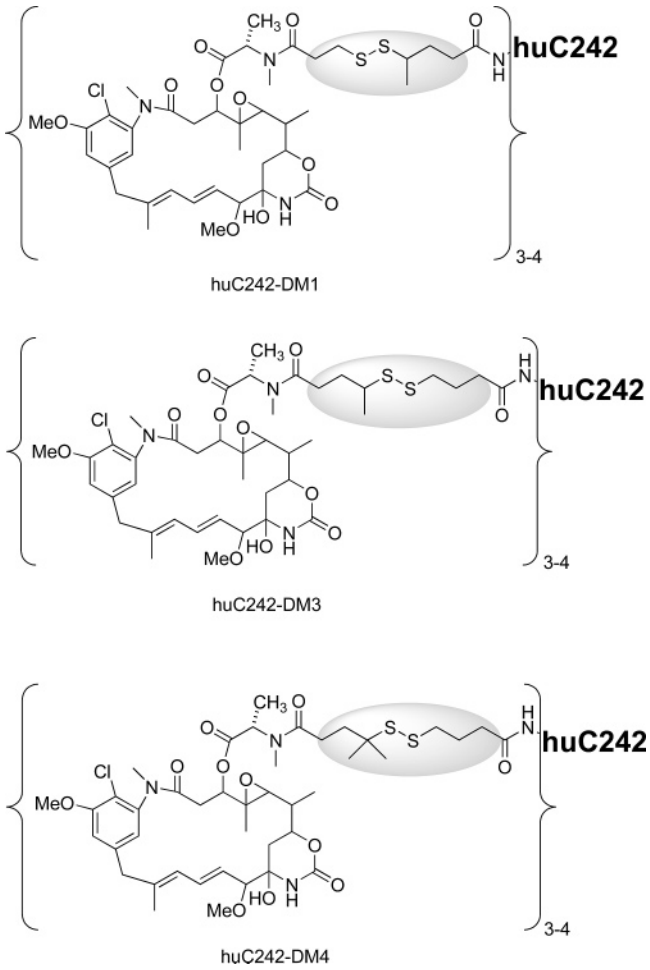


Figure 2. Structural representation of maytansinoid conjugates with the huC242 antibody. The differences in the linker region are shadowed.

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

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

	IC_{50} (nM)	
cell line	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)

	IC_{50} (nM)	
compd	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

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 methydithio-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 methydithio 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 methydisulfide 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 shown[10] 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

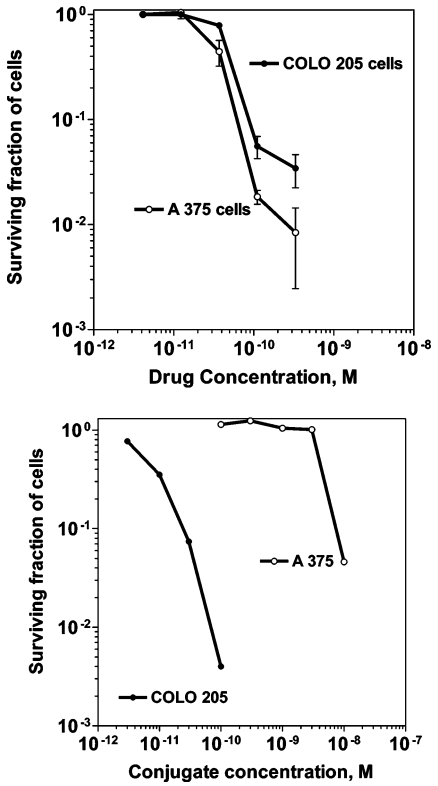


Figure 3. Top panel: in vitro potency of unconjugated drug DM4SMe (**2i**) toward COLO 205 and A-375 cells. Bottom panel: in vitro potency of huC242−DM4 conjugate toward target COLO 205 cells and no-target A-375 cells.

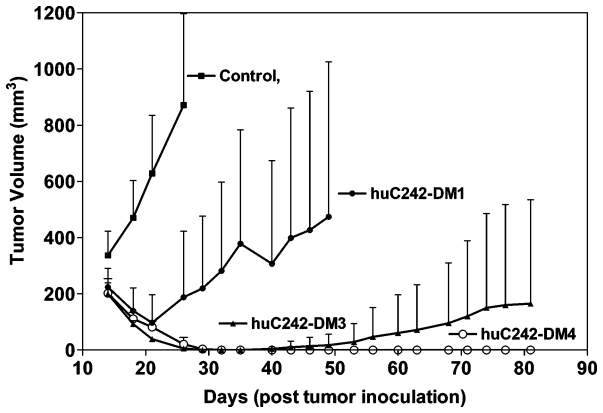


Figure 4. Comparison of the antitumor activity of huC242−maytansinoid conjugates in a human colon tumor xenograft model established subcutaneously with COLO 205 cells.

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 mm^3 in 24 days. The treatment with huC242−DM1 (linked maytansinoid dose of 75 μg/kg, qd × 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 (^{1}H 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 (Me_4Si) 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; ^{1}H NMR ($CDCl_3$) 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 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 $C_{28}H_{33}ClN_2O_8$ $(M + H)^+$ 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% CH_3CN 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 × 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 °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 × 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 °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 °C. After stirring at 0 °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 °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 × 100 mL). The aqueous layer was acidified with H_3PO_4 to pH 2.5−3.0 and extracted with EtOAc (6 × 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_3 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 °C. After stirring under argon at 0 °C for 30 min followed by stirring at room temperature for 2 h, the mixture was concentrated and extracted with dichloromethane (2 × 50 mL). The aqueous layer was acidified with H_3PO_4 to pH 2.5−3.0 and extracted with EtOAc (4 × 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 °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 °C. After stirring at 0 °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 °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. ^{1}H 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) $^+$.

***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 × 100 mL). The aqueous layer was acidified with H_3PO_4 to pH 2.5−3.0 and extracted with EtOAc (6 × 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 °C. The reaction mixture was stirred under argon at 0 °C for 30 min and then at room temperature for 2 h. The mixture was concentrated and extracted with dichloromethane (2 × 50 mL). The aqueous layer was acidified with H_3PO_4 to pH 2.5−3.0 and extracted with EtOAc (4 × 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 °C; ^{1}H 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 $(M + Na)^+$, 203.9; $[\alpha]^{25}_D = -39.16$ ($c = 2$, 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 °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 × 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.^{1}H 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 × 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 × 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. ^{1}H 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 (M + 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 × 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 × 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 × 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); ^{1}H 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 $(M + Na)^+$.

***N*-Methyl-*N*-[(3-methyldithio)-1-oxopropyl]-L-alanine (5b).** White solid (60% yield); mp 103−104 °C; ^{1}H 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. ($C_8H_{15}NO_3S_2$) C, H, N. MS: m/z 259.9 $(M + Na)^+$.

***N*-Methyl-*N*-[(3-phenyldithio)-1-oxopropyl]-L-alanine (5c).** White solid (60% yield); mp 96−97 °C; ^{1}H 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 °C; ^{1}H 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 $(M + K)^+$.

***N*-Methyl-*N*-[(4-*(R,S)*-methyldithio-1-oxopentyl)] -L-alanine (5f).** White solid (60% yield). ^{1}H 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 $(M + Na)^+$.

***N*-Methyl-*N*-[(4-*(S)*-methyldithio)-1-oxopentyl]-*S*-alanine (5g).** White solid (62% yield); ^{1}H 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 $(M + Na)^+$

***N*-Methyl-*N*-[(4-(*R*)-methyldithio)-1-oxopentyl]-*S*-alanine (5h).** White solid (60% yield); ^{1}H 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 $(M + Na)^+$.

***N*-Methyl-*N*-[4-methyl-(4-methyldithio)-1-oxopentyl)-L-alanine (5i).** White solid (51% yield) mp 77−79 °C; ^{1}H NMR ($CDCl_3$) δ 1.32 (6H, s), 1.42 (3H, d, J = 7 Hz), 1.90−97 (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. $(M + Na)^+$.

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 course 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 × 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 × 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*$^{2'}$-Deacetyl-*N*$^{2'}$-[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** ^{1}H 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 $C_{35}H_{48}ClN_3O_{10}S_2Na$ $(M + Na)^+$, 792.2367; found, 792.2350.

6a. ^{1}H 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 $C_{35}H_{48}ClN_3O_{10}S_2Na$ $(M + Na)^+$, 792.2367; found, 792.2350.

***N*$^{2'}$-Deacetyl-*N*$^{2'}$-(3-methyldithio-1-oxopropyl)-maytansine (DM1SMe, 2b) and Its D-Alanyl Isomer D-DM1SMe (6b).** Maytansinol (**4**) was reacted with *N*-methyl-*N*-[(2-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 × 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); ^{1}H 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 $C_{36}H_{51}ClN_3O_{10}S_2$ $(M + H)^+$, 784.2704; found, 784.2693; extinction coefficient $\epsilon_{280\ nm}$ (ethanol) = 5545 M^{-1} cm^{-1}, $\epsilon_{252\ nm}$ = 25 700 M^{-1} cm^{-1}.

D-DM1SMe (6b). White solid (30% yield); mp 170−171 °C; ^{1}H 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-(phenydithio)-1-oxopropyl]maytansine (DMISPh, 2c) and Its D-Alanyl Isomer D-DM1SPh (6c).** Maytansinol (**4**) was reacted with *N*-methyl-*N*-[(2-phenyldithio)-1-oxoproyl]-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/$CHCL_3$ 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.

DM1SPh (2c). ^{1}H NMR ($CDCl_3$) 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 cacld for $C_{41}H_{52}ClN_3O_{10}S_2Na$ $(M + Na)^+$, 868.2680; found, 868.2670.

D-DM1SPh (6c). ^{1}H NMR ($CDCl_3$) 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 (1 H, 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 cacld for $C_{41}H_{52}ClN_3O_{10}S_2Na$ $(M + Na)^+$, 868.2680; found, 868.2672.

***N*$^{2'}$-Deacetyl-*N*$^{2'}$-[3-(methyldithio)-1-oxobutyl]maytansine (D-M2′SMe, 2d).** Maytansinol (**4**) was reacted with *N*-methyl-*N*-3-(methyldithio)-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 $CHCl_3$. 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 × 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. ^{1}H NMR ($CDCl_3$) 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. ^{1}H NMR ($CDCl_3$) 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*)-(methyldithio)-1-oxopentyl]maytansine (DM3-SMe, 2f).** Maytansinol (**4**) was reacted with *N*-methyl-*N*-[(4-*(R,S)*-methyldithio-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 × 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). ^{1}H NMR ($CDCl_3$) 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) ^{1}H 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*)-(methyldithio)-1-oxopentyl]maytansine (2g).** Maytansinol (**4**) was coupled with *N*-methyl-*N*-[(4-*(S)*-methyldithio)-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 × 250 mm), using an isocratic elution at a flow arte 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. ^{1}H NMR ($CDCl_3$) 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. ^{1}H NMR ($CDCl_3$) 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*$^{2'}$-Deacetyl-*N*$^{2'}$-(4-*(R)*-methyldithio-1-oxopentyl)maytansine (2h).** Maytansinol (**4**) was coupled with *N*-methyl-*N*-[(4-*(R)*-methyldithio)-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 × 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. ^{1}H NMR ($CDCl_3$) 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. ^{1}H NMR ($CDCl_3$) 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*$^{2'}$-Deacetyl-*N*$^{2'}$-(4-methyl-4-(methyldithio)-1-oxopentyl)maytansine (DM4-SMe, 2i) and Its D-Alanyl Isomer D-DM4SMe.** Maytansinol (**4**) was reacted with *N*-methyl-*N*-[4-methyl-4-(methyldithio)-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 × 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); ^{1}H NMR ($CDCl_3$) 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 $C_{39}H_{57}ClN_3O_{10}S_2$. $(M + H)^+$, 826.3174; found, 826.3150; extinction coefficient (ethanol) $\epsilon_{280\ nm}$ = 5140 M^{-1} cm^{-1}, $\epsilon_{252\ nm}$ = 24 700 M^{-1} cm^{-1}.

6i (D-DM4SMe). White solid; mp 165−167 °C; ^{1}H NMR ($CDCl_3$) 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 $C_{39}H_{56}ClN_3O_{10}S_2Na$ $(M + Na)^+$, 848.2993; found, 848.2975.

***N*$^{2'}$-Deacetyl-*N*$^{2'}$-(3-mercapto-1-oxopropyl)-maytansine (DM1, 25a).** A solution of *N*$^{2'}$-deacetyl-*N*$^{2'}$-(3-methyldithio-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 × 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 × 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); ^{1}H NMR ($CDCl_3$) 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 $C_{35}H_{49}ClN_3O_{10}S$ $(M + H)^+$ 738.2827; found, 738.2820; $[\alpha]^{25}_D$ = −113.2, (c = 0.306, $CHCl_3$); extinction coefficient (methanol): $\epsilon_{280\ nm}$ = 5422 M^{-1} cm^{-1}, $\epsilon_{252\ nm}$ = 25 800 M^{-1} 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 a 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); ^{1}H NMR ($CDCl_3$) 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 91H, 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 $C_{35}H_{48}ClN_3O_{10}$SNa *m/z*: 760.2647 $(M + H)^+$; found, 760.2637; $[\alpha]^{25}_D = -135.0$, (c = 0.294, $CHCl_3$). 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,

***N*$^{2'}$-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). ^{1}H NMR ($CDCl_3$) 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 $C_{37}H_{52}ClN_3O_{10}$SNa $(M + Na)^+$, 788.2960; found, 788.2957.

***N*$^{2'}$-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); ^{1}H NMR ($CDCl_3$) 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 $C_{38}H_{54}ClN_3O_{10}$-SNa $(M + Na)^+$, 802.3101; found, 802.3116. $[\alpha]^{25}_D = -106.7$, (c = 0.057, $CHCl_3$), extinction coefficient (ethanol): $\epsilon_{280\ nm} = 5180$ M^{-1} cm^{-1}, $\epsilon_{252\ nm} = 26\ 200$ 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.

^{1}H NMR ($CDCl_3$) 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 $C_{38}H_{54}ClN_3O_{10}$SNa $(M + Na)^+$, 802.3101; found, 802.3099; $[\alpha]^{25}_D = -90.4$, (c = 0.052, $CHCl_3$). 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-propyldithio)-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 KH_2PO_4, 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**. ^{1}H NMR (400 MHz, $CDCl_3$) 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 $C_{40}H_{56}ClN_3O_{12}S_2Na$ $(M + Na)^+$, 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% CH_3CN, 5 min: 5% CH_3CN, 5−65 min: 5−95% CH_3CN. 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-propyldithio)-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 KH_2PO_4, 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**). ^{1}H NMR ($CDCl_3$) 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 $C_{42}H_{60}ClN_3O_{12}S_2Na$ $(M + Na)^+$, 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% CH_3CN, 5 min: 5% CH_3CN, 5−35 min: 5−35% CH_3CN, 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). ^{1}H NMR ($CDCl_3$) 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 $C_{70}H_{94}Cl_2N_6O_{20}S_2Na$ $(M + Na)^+$, 1495.5239, found 1495.5242; extinction coefficients (methanol): $\epsilon_{280\ nm}$ = 11 195 M^{-1} cm^{-1}, $\epsilon_{252\ nm}$ = 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% CH_3CN, 5 min 40% CH_3CN, 5−30 min 40−60% CH_3CN. 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 $CuCl_2$ (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 Na_2SO_4 and filtered. The solvent was evaporated under reduced pressure to give the L-DM4-dimer **25e** (11.7 mg, 12% yield). ^{1}H NMR ($CDCl_3$) 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 $C_{76}H_{106}Cl_2N_6O_{20}S_2Na$ $(M + Na)^+$, 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% CH_3CN, 5 min: 5% CH_3CN, 5−65 min: 5−95% CH_3CN. 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% CH_3CN 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-mercaptopentanoic 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). ^{1}H NMR in $CDCl_3$ 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; ^{1}H NMR ($CDCl_3$) 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. ($C_{14}H_{16}N_2O_4S_2$) 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. ^{1}H NMR ($CDCl_3$) 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; ^{1}H NMR in $CDCl_3$ 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. ($C_{13}H_{14}N_2O_4S_2$) 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 $\epsilon_{280\ nm} = 217\,560\ M^{-1}\ cm^{-1}$. 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 $\epsilon_{343\ nm} = 8080\ M^{-1}\ cm^{-1}$ and $\epsilon_{280\ nm} = 5100\ M^{-1}\ cm^{-1}$ 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 ($\epsilon_{280\ nm} = 217\,560\ M^{-1}\ cm^{-1}$ and $\epsilon_{252\ nm} = 80\,062\ M^{-1}\ cm^{-1}$) 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% CO_2.

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 zenograft 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 (1.5×10^6 cells/mouse) in 0.1 mL of serum-free medium. The tumors were grown for 11 days to an average size of 100 mm^3. 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.

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JM060319F