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Synthesis and Evaluation of Hydrophilic Linkers for Antibody−Maytansinoid Conjugates

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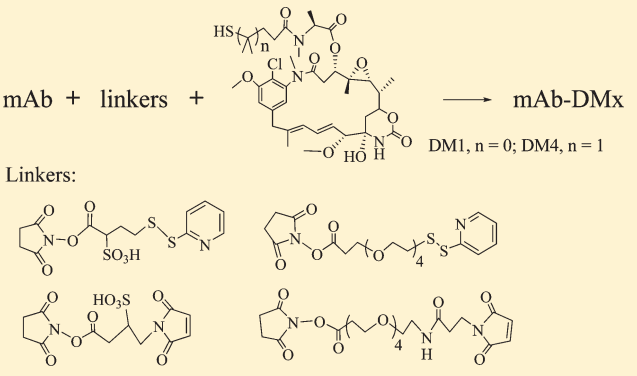
Supporting Information

ABSTRACT: The synthesis and biological evaluation of hydrophilic heterobifunctional cross-linkers for conjugation of antibodies with highly cytotoxic agents are described. These linkers contain either a negatively charged sulfonate group or a hydrophilic, noncharged PEG group in addition to an amine-reactive *N*-hydroxysuccinimide (NHS) ester and sulfhydryl reactive termini. These hydrophilic linkers enable conjugation of hydrophobic organic molecule drugs, such as a maytansinoid, at a higher drug/antibody ratio (DAR) than hydrophobic SPDB and SMCC linkers used earlier without triggering aggregation or loss of affinity of the resulting conjugate. Antibody−maytansinoid conjugates (AMCs) bearing these sulfonate- or PEG-containing hydrophilic linkers were, depending on the nature of the targeted cells, equally to more cytotoxic to antigen-positive cells and equally to less cytotoxic to antigen-negative cells than conjugates made with SPDB or SMCC linkers and thus typically displayed a wider selectivity window, particularly against multidrug resistant (MDR) cancer cell lines in vitro and tumor xenograft models in vivo.



INTRODUCTION

The efficacy of chemotherapeutic drugs is often limited by their side effects on normal tissues such as, for example, acute bone marrow or mucosal toxicity, or chronic cardiac and neurological toxicity.[1] Since the discovery of monoclonal antibodies (mAb) 3 decades ago,[2] treatments for a few types of tumors, notably non-Hodgkin's lymphoma and HER2-positive breast cancer, have advanced considerably because of the development of mAbs that selectively target tumor-associated antigens expressed in their malignancies. The antitumor activity of these antibodies, coupled with their favorable pharmacodynamic and pharmacokinetic properties, and the lack of serious nontargeted toxicities have led to their becoming mainstays in the treatment of NHL and HER2-positive breast cancers.[3]

Antibodies have been extensively explored as vehicles for delivery of various cytotoxic agents: first as conjugates with radionuclides for imaging and cancer therapy,[4] later as conjugates with bacterial and plant toxins,[5] and currently, with small-molecule cytotoxic compounds.[6] The small-molecule cytotoxic compounds, a new class of anticancer agents termed antibody−drug conjugates (ADCs),[6−8] are in various stages of preclinical and clinical development. These include ADCs prepared with highly potent cytotoxic agents such as maytansinoids (DM1 and DM4),[9,10] calicheamicin derivatives,[11,12] CC-1065 derivatives,[13,14] and auristatins.[15,16] As these studies progress, emerging clinical data are beginning to validate the potential of the ADC technology platform as an important modality in the future of cancer treatment where targeted therapy becomes the norm.[10,15]

The link between the antibody and the cell-killing agent plays a critical role in the development of ADCs, as the nature of the linker significantly affects the potency, selectivity, and the pharmacokinetics of the resulting conjugate.[9,13,16−20] ADCs are thought to act via a multistep sequence of events: binding to the cell surface, endocytosis, trafficking (within an endosome) to a lysosome, proteolytic degradation of the conjugate, and diffusion of the released cytotoxic agent across the lysosomal or endosomal membrane toward its intracellular target and its interaction with the target, which ultimately causes cell death. Therefore, the linker should be sufficiently stable while in circulation to allow delivery of the intact ADC to the tumor sites but, on the other hand, sufficiently labile to allow release of the cytotoxic effector from the ADC once inside the targeted tumor cell. Four types of linkers (Scheme 1) have been used for preparation of antibody−drug conjugates that have currently entered the clinic: (a) acid-labile linkers, exploiting the acidic endosomal and lysosomal intracellular microenvironment;[17,20] (b) linkers cleavable by lysosomal proteases;[16,18] (c) chemically stable thioether linkers that release a lysyl adduct after proteolytic

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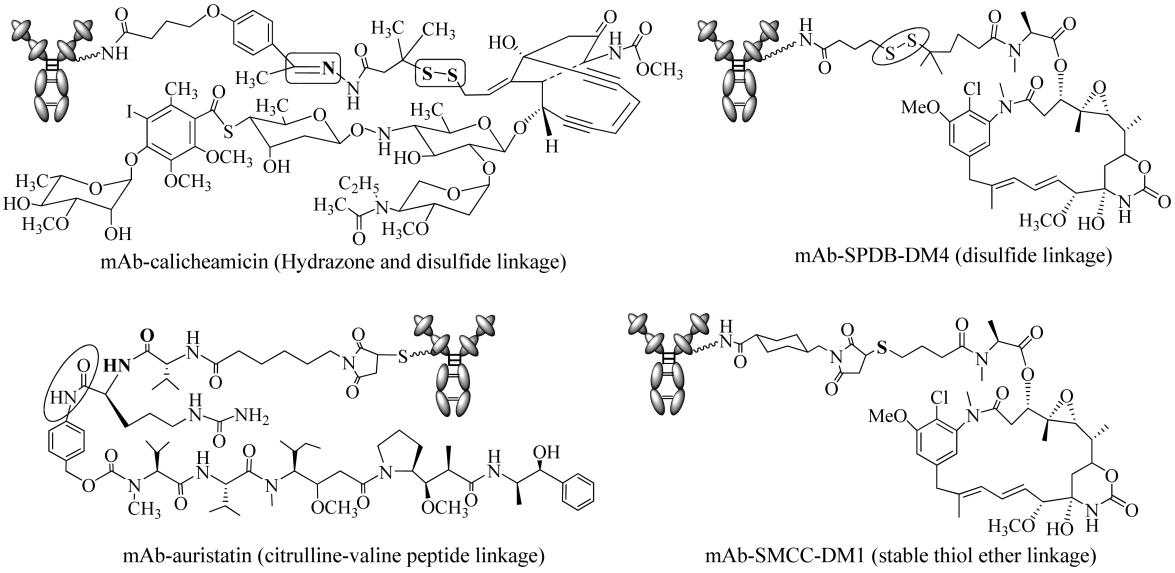
Scheme 1. Chemical Structures of Some Antibody−Cytotoxic Drug Conjugates[a]



[a] The linkers used for each drug are indicated in parentheses, and the labile bonds are in circles.

degradation of the antibody inside the cell;[21,22] and (d) disulfide-containing linkers,[7,9] which are cleaved upon exposure to an intracellular thiol.

One common feature of many linkers used to prepare ADCs is their hydrophobicity. For example, the commercially available cross-linkers SMCC and SPDP are hydrophobic. Typically, ADCs reported in the literature contain no more than 3−4 drug molecules per antibody molecule.[6] Attempts to attain higher drug/antibody ratios (DAR) often failed, particularly if both the cytotoxic agent and the linker were hydrophobic, because of ADC aggregation,[13,18,23,24] loss of affinity for the target antigen,[25] and rapid clearance from circulation.[18,26] Conjugates with high DAR could be beneficial in situations where the target antigen density on the cell surface is low and offers a means of boosting potency.

Another factor that may limit the activity of ADCs is the emergence of drug resistant tumor cells.[27,28] The occurrence of multidrug resistance (MDR) cancer is often attributed to the increased expression or activity of drug transporters, most often P-glycoprotein (Pgp),[29] which facilitate efflux of certain, mostly hydrophobic, compounds. It would be desirable to develop a linker that would reduce such transporter-mediated efflux of the effector drug.[30]

Herein we describe the synthesis and properties of highly water-soluble hydrophilic linkers suitable for conjugation of a thiol-containing cytotoxic agent. These linkers contain either a negatively charged α-sulfonic acid group or a polar short polyethylene glycol (PEG) chain to increase their solubility. The chemically reactive groups in these linkers are *N*-hydroxysuccinimide (NHS) ester on one end and a (pyridin-2-yl)disulfanyl, (nitropyridin-2-yl)disulfanyl group, or maleimido group on the other end. The NHS ester reacts with amino groups of lysine residues on an antibody to form a stable amide bond. The (pyridin-2-yl)disulfanyl group or maleimido group of the linker reacts with the sulfhydryl group of the cytotoxic agent, forming a conjugate containing a disulfide or a thioether, respectively. These novel linkers enabled us to construct antibody-maytansinoid conjugates (AMCs) with a DAR of up to 9, that did not aggregate, that retained the affinity of the antibody, and that were markedly more potent than conjugates with DAR of 3−4. In addition, conjugates with a standard DAR load of 3−4 made using these hydrophilic linkers showed superior activity toward Pgp-expressing MDR cells in vitro than conjugates with standard nonhydrophilic linkers.

RESULTS

Synthesis of Hydrophilic Linkers. The formation of protein aggregates poses a significant problem during the preparation of ADCs, especially when both the linker and cytotoxic agent are hydrophobic.[13,23] Even with relatively hydrophilic cytotoxic agents, such as DM1 and DM4, the AMCs made with the somewhat hydrophobic linkers SPDB and SMCC[9] could only be prepared at a DAR of up to 4−5 depending on the antibody. When AMCs with higher maytansinoid loads were attempted, such conjugates tended to form aggregates, resulting in precipitation. We set out to make and evaluate hydrophilic linkers to test if similar conjugates made with such linkers would maintain monomeric homogeneity at higher maytansinoid loads. To this end, we synthesized two sets of hydrophilic linkers, one set comprising cleavable disulfide linkers containing either a negatively charged sulfonate group or a noncharged PEG group and the other set comprising noncleavable maleimido-bearing linkers containing either a negatively charged sulfonate group or a noncharged PEG group.

The first approach was to attempt synthesis of a simple hydrophilic linker containing a sulfonate anion using α-lipoic acid (**1**) as the starting material (Scheme 2). It has been reported that α-lipoic acid (**1**) can be oxidized with hydrogen peroxide in acetone to form the cyclic thiosulfonate (**3**).[31] We hypothesized that following antibody modification with compound **6**, the exchange reaction between the disulfide of the linker and the free thiol of the cytotoxic agent would generate a conjugate bearing a sulfonate anion (**7**). However, NMR and MS analyses of crude reaction mixtures formed by oxidation of lipoic acid (**1**) by peroxides indicated the presence of a range of oxidized

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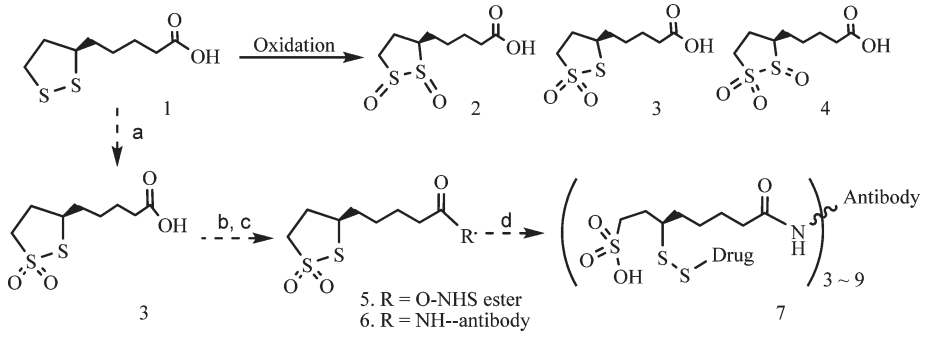
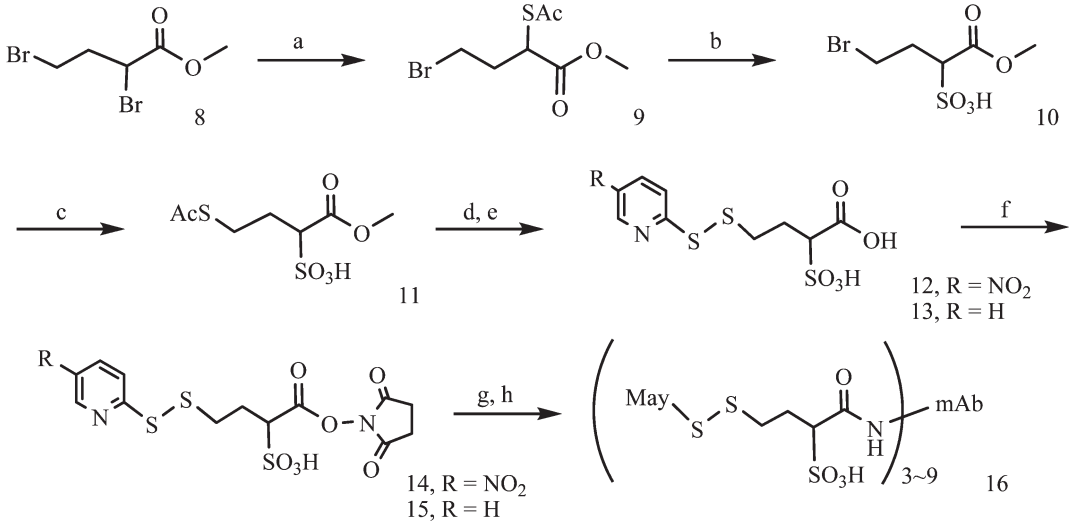
Scheme 2. Original Strategy for Synthesis of the Sulfonate Hydrophilic Linker Starting from Lipoic Acid[a]



[a] Conditions: (a) [O]; (b) NHS/EDC; (c) mAb; (d) drug−SH.

Scheme 3. Synthesis of Disulfide-Linked Antibody−Cytotoxic Agent Conjugates via a Sulfonate Soluble Linker[a]



[a] Conditions: (a) HSAc, DIPEA, THF, −20 °C, 96%; (b) H_2O_2/HOAc, 90%; (c) HSAc, DIPEA, DMA, 90%; (d) NaOH; (e) $(SPyR)_2$, pH 7.5; (f) NHS, EDC, DMA, 82%; (g) antibody; (h) maytansinoid−SH.

compounds, such as disulfoxide (**2**) and sulfone−sulfoxide (**4**), with less than 10% of the desired thiosulfonate (**3**). Attempts to optimize the reaction conditions by varying the amount of oxidants and solvents were unsuccessful.

This result prompted us to devise a different synthetic strategy (Scheme 3) where the desired products would be stable and isolable during the synthetic process. The first α-sulfonate heterobifunctional linker **14** was developed, starting from **10** and **11** which were synthesized from the readily available dibromide **8**. The key to this strategy was the difference in substitution reaction rates for the thioacetate with the α-bromo and γ-bromo substituent of starting material **8**. A practical, reproducible, and readily scalable synthetic route and efficient purification of all precursor molecules of linker **14** were then developed, as shown in Scheme 3. The selective displacement of the α-bromo substituent by 1 equiv of thioacetic acid in THF with DIPEA at −20 °C gave methyl 2-(acetylthio)-4-bromobutanoate (**9**) in nearly quantitative yield. Oxidation of compound **9** with hydrogen peroxide in acetic acid at room temperature provided the sulfonic acid **10** in over 90% yield after crystallization. The γ-bromo moiety of the sulfonic acid **10** was then substituted with thioacetic acid using DIPEA in THF/DMA at higher temperatures (room temperature to 50 °C) to afford compound **11** in over 90% yield. Hydrolysis of the two ester groups of compound **11** by sodium hydroxide, followed by the addition of an excess of 2,2′-dithiobis(5-nitropyridine) gave the expected 4-((5′-nitropyridin-2′-yl)disulfanyl)-2-sulfobutanoic acid (**12**) in over 75% yield following reverse phase chromatography. The desired nitropyridinyl linker **14** was prepared by condensing the sulfobutanoic acid **12** with *N*-hydroxysuccimide in DMA using the carbodiimide coupling agent EDC to give an 82% yield after silica chromatography and crystallization.

We also synthesized a similar linker bearing a pyridyldithio group **15** which was expected to have a different reaction rate toward thiol−disulfide exchange and higher stability in mildly basic conditions. The synthesis was similar to that of the nitropyridin-2-yldisulfanyl linker **14**. The two ester groups of compound **11** were subsequently hydrolyzed by 1 M NaOH, followed by neutralization to pH 7.5 with H_3PO_4 (Scheme 3). The free thiol was reacted with 2,2′-dithiodipyridine to afford the pyridyl disulfide compound **13** in 80% overall yield. The final linker **15** was transformed into the NHS ester by EDC coupling in DMA in 83% yield after silica gel chromatography and crystallization.

We also synthesized a linker wherein the hydrophilic element was a short PEG for use in the formation of AMCs. The

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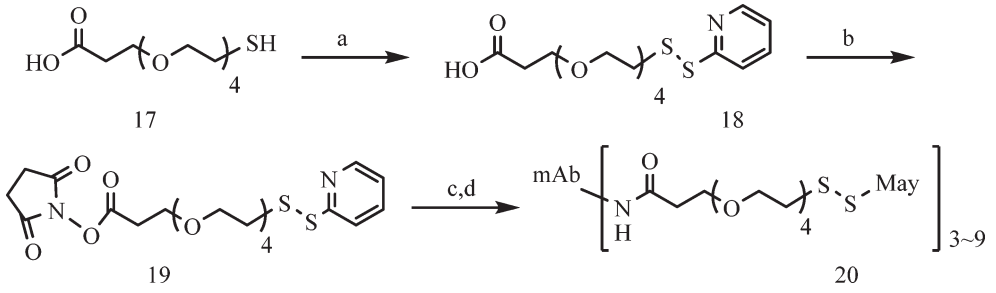
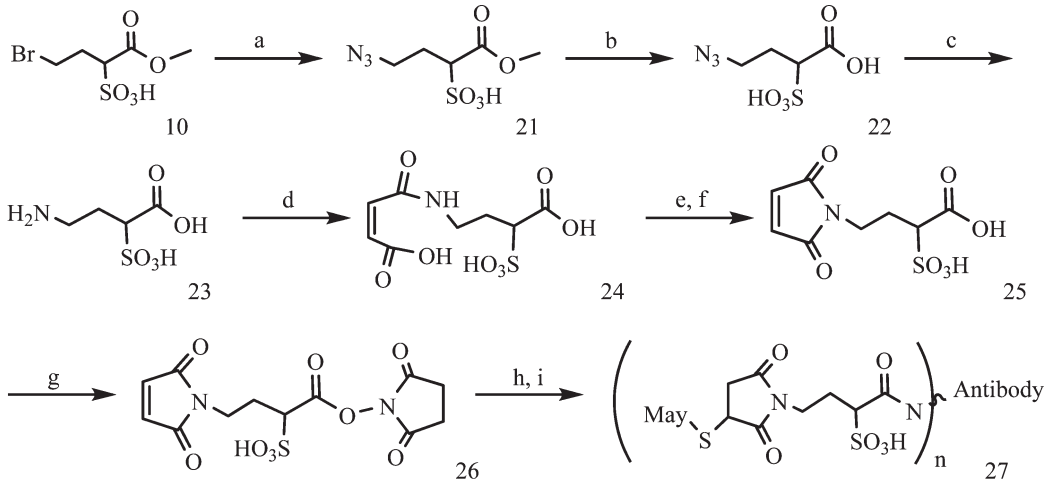
Scheme 4. Synthesis of AMC Bearing a PEGylated Disulfide Linker, 15-(2-Pyridyldithio)-4,7,10,13-tetraoxapentadecanoic Acid *N*-Hydroxysuccinimide Ester 19[a]



[a] Conditions: (a) $(SPy)_2$, DME, 64%; (b) NHS, EDC, DCM, 38%; (c) antibody; (d) maytansinoid−SH.

Scheme 5. Synthesis of a Noncleavable Charged Linker and AMC[a]



[a] Conditions: (a) NaN_3, DMA, 90%; (b) 1 M HCl, HAc, 100 °C, 95%; (c) H_2/Pd/C, H_2O, 95%; (d) maleic anhydride, DMA, 83%; (e) HMDS, ZnCl, DMA, Tol; (f) CH_3OH, HCl (cat.), 85%; (g) NHS, EDC, DMA, 80%; (h) antibody; (i) maytansinoid−SH.

PEG-containing linker **19** was synthesized from commercially available 1-mercapto-3,6,9,12-tetraoxapentadecan-15-oic acid **17**. Compound **17** was readily converted to the pyridin-2-yldisulfanyl compound **18** with 2,2′-dithiodipyridine in 1,2-dimethoxyethane in 64%, followed by transformation of compound **18** to the NHS ester compound **19** by EDC-mediated coupling with *N*-hydroxysuccinimide in DCM in 38% yield (Scheme 4).

The maleimido-bearing linker **26** which would offer a thioether linked noncleavable AMC was synthesized as outlined in Scheme 5. The synthesis started from the bromide **10**, which was converted to the azido compound **21** using sodium azide in DMA, resulting in a 90% yield. Hydrolysis of the ester with 1 M HCl at 100 °C provided the carboxylic acid compound **22** in 95% yield. Hydrogenation of the azido group in water afforded the amino compound **23** in quantitative yield which was used directly for next step without purification. The amino group of compound **23** was coupled with maleic anhydride in DMA to provide compound **24** in 90% yield. The maleic acid **24** was then treated with hexamethyldisilazane (HMDS) and a Lewis acid ($ZnCl_2$) in a mixture of toluene and DMA at 100−120 °C for 8 h using a Dean−Stark trap to remove the byproduct water. Addition of a few drops of 1 M HCl in methanol afforded 85% of the desired maleimido acid **25**. The proposed mechanism of this step is depicted in Scheme 6. In the first step, both the carboxylic acid and the sulfonic acid groups of compound **24** reacted with HMDS using a Lewis acid to form the TMS esters **25a**. However, the amido group preferentially attacked the enamido TMS ester **25b** to form a five-membered maleimido ring **25c** upon coordination with $ZnCl_2$. The addition of HCl in methanol resulted in hydrolysis of the TMS esters to form the desired compound **25**. We applied the previously reported Ac_2O/AcOH/reflux,[32,33] EDC/HOBt,[33] and cyanuric chloride[34] methods to complete the desired maleimido ring formation; however, all these methods resulted in poor yields and the generation of several inseparable cyclic ring or linear byproducts, which probably arose from reaction with the acid groups of compound **24**. The HMDS/$ZnCl_2$ methodology gave the maleimido compound **25** in reasonably high yields. The final linker **26** was prepared through coupling of the carboxylic acid **25** to *N*-hydroxysuccinimide with EDC in DMA and purified by silica gel chromatography. Unexpectedly, the modification reaction of **26** with the antibody's lysine ε-amino groups under neutral conditions (pH 6.5−7.5) was very slow and had to be conducted over 24 h, resulting in extensive NHS ester hydrolysis. Modification under mildly basic conditions (pH 8.0) accelerated the reaction, but some hydrolysis of the maleimide was observed. Therefore, the sulfonate group of linker **26** was moved further away from the carbonyl group to the third carbon position of the linker (**36**).

Scheme 7 outlines the synthesis of the noncleavable linker **36** bearing a 3-sulfonate group. The synthesis started from

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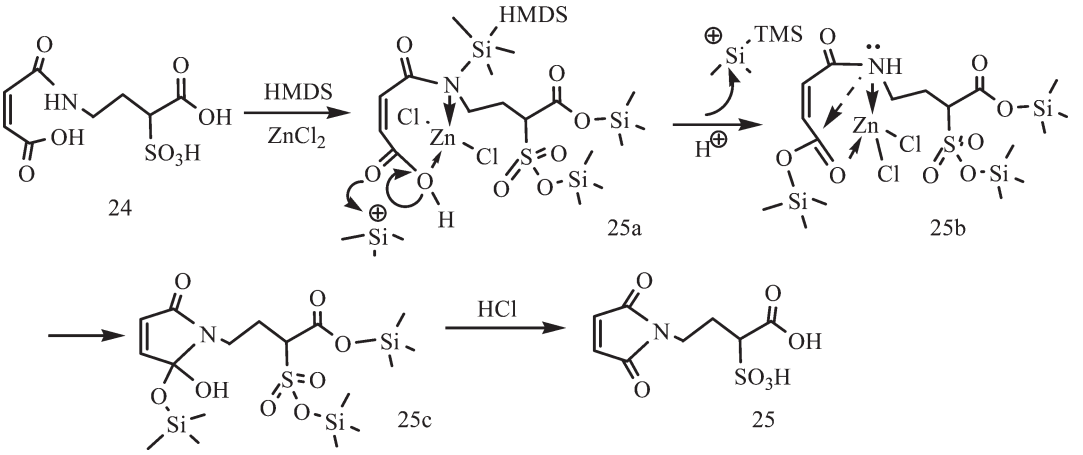
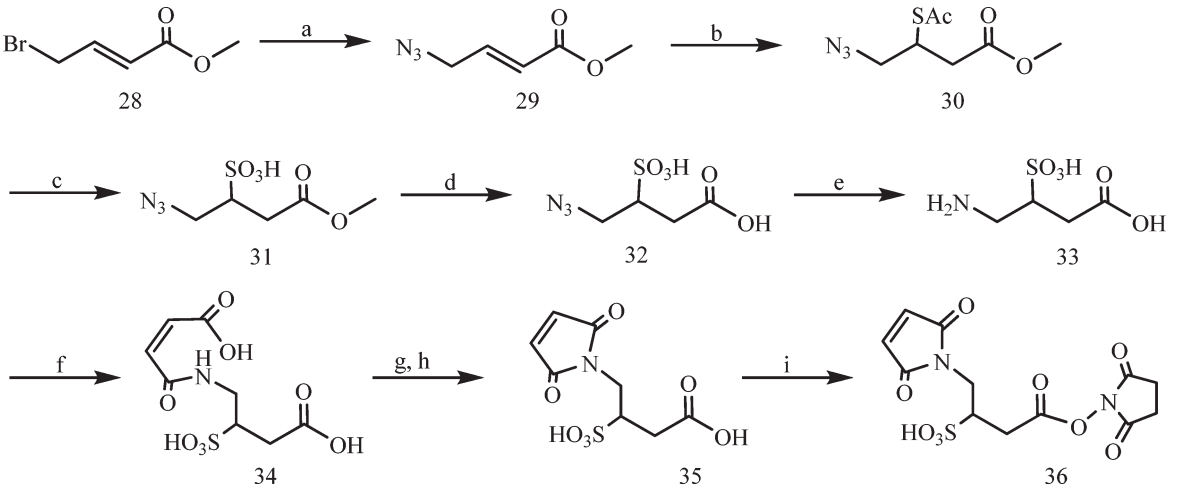
Scheme 6. Mechanism of the Formation of Five-Membered Ring of Maleimic Compound 25



Scheme 7. Synthesis of Succinimidyl 4-*N*-Maleimido-3-sulfobutyrate 36[a]



[a] Conditions: (a) NaN_3, DMF, 0 °C, 80%; (b) HSAc, Et_3N, THF, 81%; (c)H_2O_2, HAc, 93%; (d) HCl, HAc, 110 °C, 85%; (e) Pd/C/H_2, 90%; (f) maleic anhydride, DMA, 83%; (g) HMDS, ZnCl, DMA, Tol; (h) CH_3OH, HCl (cat.), 75%; (i) NHS, EDC, DMA, 75%.

commercially available (*E*)-methyl 4-bromobut-2-enoate **28**, which was reacted with sodium azide in DMA to give (*E*)-methyl 4-azidobut-2-enoate **29** in 80% yield. Michael addition of thioacetic acid to the azido compound **29** in the presence of DIPEA in THF afforded compound **30** in 81% yield. Oxidation of **30** with hydrogen peroxide in acetic acid provided compound **31** in 93% yield, which was followed by de-esterification at reflux in 1 M HCl and HOAc to give the acid compound **32** in 85% yield. Hydrogenation of compound **32** in water produced amino compound **33**, which was readily reacted with maleic anhydride in DMA to afford the enoic acid compound **34** in 83% yield over two steps. Similar to the mechanism outlined in Scheme 6, the five-membered maleimido ring of compound **35** was formed in 75% yield when compound **34** was treated with excess HMDS and $ZnCl_2$ in a mixture of DMA and toluene, with the aid of a Dean−Stark trap, followed by hydrolysis in acidic methanol. The final coupling of compound **35** with NHS in the presence of EDC in DMA gave succinimidyl 4-*N*-maleimido-3-sulfobutyrate **36** in 75% yield.

An alternative synthesis of the enoic acid compound **34** is depicted in Scheme 8. Michael addition of the commercially available (*Z*)-ethyl 3-cyanoacrylate **37** with HSAc in the presence of DIPEA in THF gave ethyl 3-(acetylthio)-3-cyanopropanoate (**38**) in 65% yield. Oxidation of compound **38** with hydrogen peroxide in acetic acid afforded 93% of the ethyl 3-(acetylthio)-3-cyanopropanoate (**39**). Reduction of compound **39** with Raney Ni generated the unstable 1-amino-3-ethoxy-3-oxopropane-1-sulfonic acid, which was readily converted in 85% yield in situ to 1-(*tert*-butoxycarbonylamino)-4-ethoxy-4-oxobutane-2-sulfonic acid **40** in the presence of BOC anhydride. De-esterification of compound **40** with LiOH gave 90% of the acid **41**. Removal of the BOC group of compound **41** in a 4:1 (v/v) mixture of concentrated hydrochloric acid and dioxane followed by coupling with maleic anhydride in DMA provided 83% of the enoic acid compound **34** in two steps. This synthetic route gave an overall yield of 38%, the same as that of Scheme 7.

In the practice of conjugation, an ideal method is direct ("one step") reaction of the maytansinoid-linker moieties with an antibody. Therefore, compounds **43**, **44**, **48**, **50**, and **51** are preferred to be isolated (Scheme 9), when the thiol-bearing maytansinoids are first reacted with the sulfhydryl-reactive

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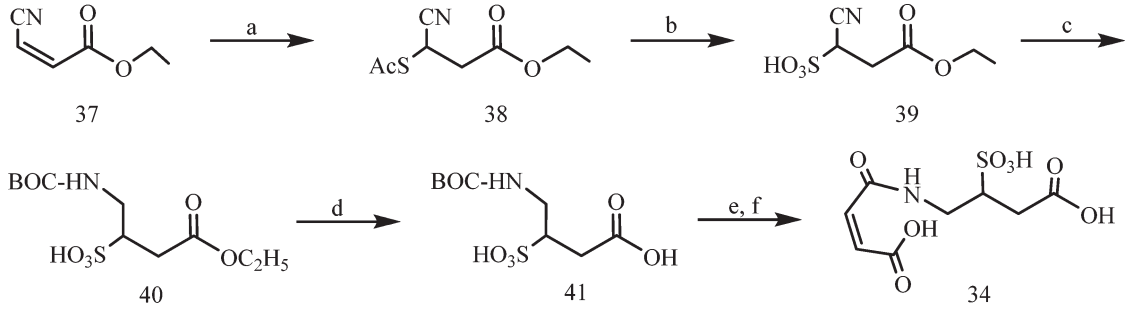
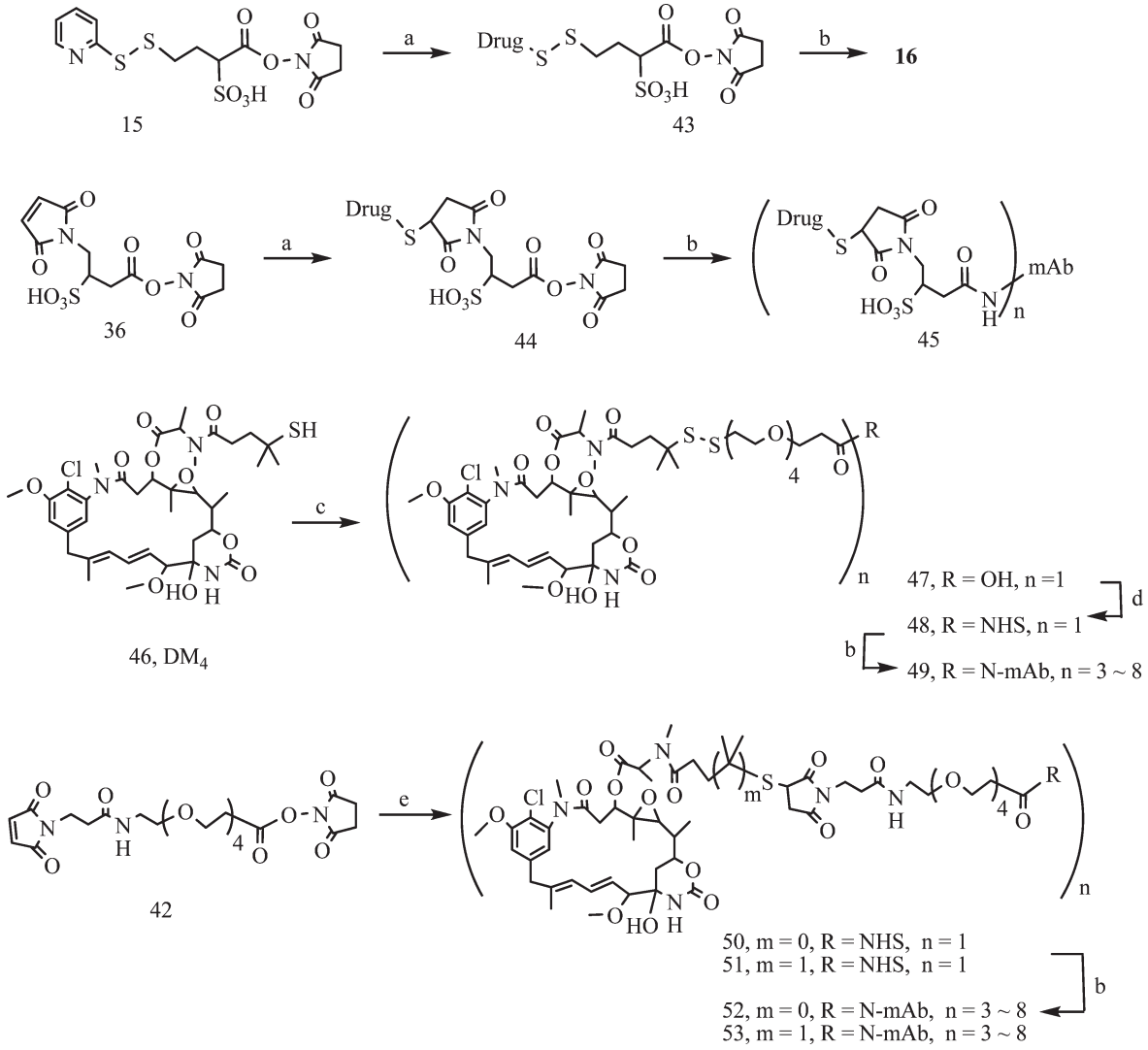
Scheme 8. Synthesis of (*Z*)-4-(3-Carboxy-2-sulfopropylamino)-4-oxobut-2-enoic Acid 34[a]



[a] Conditions: (a) HSAc, DIPEA, THF, 0 °C, 65%; (b) H_2O_2, HAc, 93%; (c) Raney Ni, H_2, BOC_2O, DMA, 85%; (d) LiOH, 90%; (e) 20% HCl, dioxane; (f) maleic anhydride, DMA, 83%.

Scheme 9. Synthesis of Maytansinoid−Linkers for One-Step Antibody Conjugation[a]



[a] Conditions: (a) drug−SH (DM4), pH 4.0−6.5; (b) mAb, pH 6.5−8.0; (c) **18**, DME, 4-methylmorpholine; (d) NHS/EDC, 54%; (e) DM1 or DM4, pH 4.0−6.5.

groups of the hydrophilic linkers (**15**, **19**, **36**, and **42**), respectively, under mild acidic conditions (pH 4.0−6.5). Initial attempts to isolate the "one-step" maytansinoid (DM4) linker reagents **43**, **44**, and **48** were not successful because the thiol−disulfide exchange reactions between the hindered thiol of DM4 and the linker **15**, **19**, or **36** under mild acidic conditions (pH 4.0−6.5) were slow (required at least 2 h), and were accompanied by hydrolysis of NHS on the linker. Therefore,

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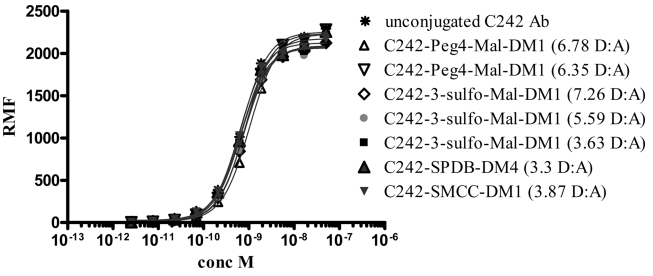
the sulfonate compound **43** and **44**, prepared from reaction of linkers **15** and **36** with an excess of maytansinoids (1.5 equiv) in a pH 6.0 buffer, were used directly without isolation for conjugation with an antibody in a pH 8.0 buffer. Compound **48** could be prepared through coupling DM4 and PEG acid **18** to generate compound **47**, followed by esterification with NHS and chromatographic purification.

Conjugation Using Hydrophilic Linkers. Three antibodies, huC242, huMy9-6, and B38.1, were used to evaluate the utility of the linkers in conjugation with maytansinoids. The first two are humanized IgG1 antibodies that target CanAg expressed on the surface of human colorectal, pancreatic, and gastric cancer cells, and CD33 expressed on myeloid leukemia cells, respectively. B38.1 is a chimeric human IgG1 targeting EpCAM expressed in various malignancies. The different targets were chosen to represent a wide range of antigen expression, expressed as antigen molecules bound per cell (ABC, or antibody binding capacity). CD33 is expressed at low levels (10000−20000 ABC), EpCAM at moderate levels (typically 500000−900000 ABC), and CanAg at very high levels ($\geq 4 \times 10^6$ ABC). Two maytansinoids, DM1 and DM4,[9] were used in the evaluation. The conjugation of DM1 and DM4 via these linkers to these three antibodies generally was accomplished by the previously reported two-step procedure.[9] First, the monoclonal antibody was reacted with the linker, resulting in displacement of the *N*-hydroxysuccinimidyl group of the linker by lysine residues on the antibody under aqueous conditions to introduce a sulfhydryl-reactive group. The modified antibody was then treated with a thiol-bearing maytansinoid, resulting in either disulfide exchange with the pyridyldithio group of linkers **14**, **15**, and **26** or reaction with the maleimido moiety of linkers **26**, **36**, or **42** in a Michael-type addition reaction to form a stable thioether bond. Since the α-sulfonate group presented steric constraints, the modification reaction with linkers **14**, **15**, and **26** was performed at pH 8. At this pH both the nitropyridinyl disulfides of linker **14** and maleimide of linker **26** were unstable; therefore, linkers **15** (2-sulfo-SPDB linkage) and **36** (3-sulfo-Mal linkage) were preferred for use in conjugation. Linker **15** was preferred for conjugation because of its ability to readily modify the antibody at pH 8 without any loss of the thiopyridyl group over 12 h.

Alternatively, the "one-step" procedure was used for AMCs with these hydrophilic linkers **15**, **19**, **36**, and **42**. AMCs with sulfonate linker **15** or **36** were made via first reaction of the linker **15** or **36** with an excess (1.5 equiv) of the thiol-bearing maytansinoids (DM1 or DM4) under mild acid condition (pH 4.0−6.5) for 1−2 h, followed by direct addition of an antibody at an adjusted pH 7.5−8.0 buffer. AMCs bearing PEG linker **19** or **42** were made through direct addition of **48**, **50**, or **51** to a buffer (pH ∼7.5) containing an antibody, respectively. AMCs obtained by this "one-step" method showed, by MS, the expected profile with no evidence of unconjugated linkers appended on the antibody (data not shown).

Antibody conjugates of the maytansinoids DM1 and DM4 using these hydrophilic linkers with DAR varying from 1.2 to 9.1 were obtained through careful control of the linker and maytansinoid excess used in two-step conjugations or through control of the molar ratios of the one-step reagents (up to 14) in the one-step conjugation. All these conjugates consisted predominantly (more than 95%) of the monomeric species, as assessed by analytical ultracentrifugation (AUC) and by size-exclusion chromatography (SEC). The amount of maytansinoid in the final conjugate in the free form (or noncovalently associated with the antibody) was very low (≤0.1% of the total maytansinoid) or was undetectable, as determined by precipitation of the protein with acetone, followed by analyses of the supernatants by UV, HPLC, or LC−MS. Mass spectrometric characterization of conjugates prepared using these linkers gave the expected molar DAR distribution profile,[35] and the average maytansinoid load correlated with that determined spectrophotometrically by UV absorbance. Importantly, all AMCs prepared with these linkers (**15**, **19**, **36**, and **42**) retained the binding affinity of the antibody moiety to its target antigen. A typical example is shown in Figure 1: huC242-DMx conjugates prepared with maytansinoids using the linker **36** or **42**, with DAR of 3.6−7.3, comparable to that of the huC242-DMx conjugates made with SPDB and SMCC at DAR of 3.3−3.9, retained similar affinity of the antibody, all with apparent K_d between 0.6 to 1.0 nM.



Conjugate	MAR	Kd, M
C242 Ab		6.87E-10
C242-Peg4-Mal-DM1	6.78	1.03E-09
C242-Peg4-Mal-DM1	6.35	8.30E-10
C242-3-sulfo-Mal-DM1	7.26	7.76E-10
C242-3-sulfo-Mal-DM1	5.59	7.53E-10
C242-3-sulfo-Mal-DM1	3.63	6.74E-10
C242-SPDB-DM4	3.3	7.02E-10
C242-SMCC-DM1	3.87	6.22E-10

Figure 1. Binding of huC242-DMx conjugates analysis by flow cytometry on COLO 205 cells at 10 000 cells/well in 3 h at 0 °C.

In Vitro Activity of Maytansinoid Conjugates with Different Linkers. First, we compared the cytotoxicity of various conjugates of the anti-CD33 antibody (huMy9-6) with DM4 for the CD33-positive HL60 QC cells (about 15 000 ABC) and for the CD33-negative Ramos cells. These conjugates were made with various linkers: two previously used linkers, the disulfide-containing linker SPDB and the noncleavable linker SMCC, and four linkers with increased hydrophilicity, two disulfide-containing linkers, **15** (2-Sulfo-SPDB) and **19** (PEG_4-SPDB), and two noncleavable linkers, **36** (3-Sulfo-Mal) and **42** (PEG_4-Mal). Conjugates with various DAR values were made. Conjugates with high DAR values could only be made with the hydrophilic linkers **15**, **19**, **36**, and **42**.The results of these experiments are listed in Table 1 as IC_{50} values. Several conclusions can be drawn from the data presented in Table 1: (i) All conjugates killed antigen-expressing HL60/QC cells in an antigen-dependent manner, since their cytotoxicity toward the antigen-negative Ramos cells was much lower. (ii) The potency of the conjugates made with each hydrophilic linker increased with an increase of DAR. For instance, the IC_{50} of huMy9-6-2-sulfo-SPDB-DM4 decreased from 4 nM at 1.2 DAR to 0.5 nM at 3.4 DAR to 0.03 nM at 6.2 DAR (here and below, concentrations given are those of the antibody moiety). (iii) The specificity ((IC_{50} for killing antigen-negative cells)/(IC_{50} for killing antigen-positive cells)) of these conjugates has improved dramatically from 4- to 6-fold at DAR below 2, to 500 or higher at DAR above 6. (iv) The

Table 1. Cytotoxicity of huMy9-6-DMx Conjugates with Different Maytansinoid/Ab Ratios (DAR) for HL60/QC Cells (Ag+) and Ramos Cells (Ag−)

		IC_{50} vs HL60/QC		IC_{50} vs Ramos	
linker−DMx	DAR	concn of the antibody moiety (pM)	concn of the DM4 moiety (pM)	concn of the antibody moiety (pM)	specificity ratio[a]
2-sulfo-SPDB (**15**)-DM4	1.2	4200 ± 800	5040 ± 940	>25000 ± 2900	>6*
	2.5	1500 ± 400	3800 ± 900	>20000 ± 1400	>13*
	3.4	560 ± 300	1820 ± 870	>17000 ± 1400	>30*
	3.8	260 ± 100	999.4 ± 160	>17000 ± 1200	>68*
	4.2	150 ± 40	643.0 ± 147	>17000 ± 1200	>118**
	4.9	70 ± 20	346.9 ± 77.4	>15000 ± 1200	>210**
	5.7	40 ± 3	231.9 ± 15.4	15500 ± 1000	390*
	6.2	31 ± 2	191.6 ± 9.3	15500 ± 600	500*
	6.6	27 ± 1	184.1 ± 7.9	15500 ± 600	573*
	7.7	23 ± 1	180.2 ± 8.5	14300 ± 400	620*
	9.1	22 ± 1	200.2 ± 10.9	5200 ± 400	235*
PEG4-SPDB (**19**)-DM4	1.8	5350 ± 510	9630 ± 920	>25500 ± 1000	>5*
	3.1	1230 ± 250	6910 ± 560	25500 ± 1000	21*
	4.6	95.5 ± 20.3	439.3 ± 93.4	11000 ± 600	115*
	5.7	41.4 ± 3.2	236.2 ± 18.2	8700 ± 500	210*
	6.0	34.7 ± 7.1	208.2 ± 42.6	8200 ± 500	238*
	6.5	27.4 ± 2.2	178.1 ± 14.3	9300 ± 1000	340*
	6.8	18.5 ± 2.1	125.8 ± 14.	4500 ± 700	242*
	7.1	14.1 ± 2.0	100.1 ± 14.2	4300 ± 700	303*
3-sulfo-Mal (**36**)-DM1	3.4	2020 ± 1010	6930 ± 3420		20**
	5.9	155 ± 40	914.5 ± 236		111**
PEG4-Mal (**42**)-DM1	4.1	90.0 ± 26.0	369 ± 106.6		115**
	8.4	18.0 ± 4.2	151.2 ± 35.3		237**
SPDB-DM4	3.9	389 ± 102	1510 ± 400		16**
SMCC-DM1	3.9	320 ± 240	1250 ± 940		14**

[a] Definition of specificity ratio: (*) IC_{50} of the ADC vs Ramos (Ag−)/IC_{50} of the ADC vs HL60/QC (Ag+); (**) (IC_{50} of the ADC vs HL60/QC with the presence of 1 μM of unconjugated My9-6)/(IC_{50} of the ADC vs HL60/QC).

conjugates made with the hydrophilic linkers in the MAR range from 3 to 4, had a better specificity window, and in general, had a higher potency than similar conjugates made with either SPDB or SMCC. In this system, the conjugates are ranked by their potencies as follows: PEG4-Mal (**42**) > PEG4-SPDB (**19**) ≥ 2-sulfo-SPDB (**15**) > SMCC ≥ SPDB > 3-sulfo-Mal (**36**).

Next, we examined the potency of maytansinoid conjugates of the chimeric IgG1 anti-EpCAM antibody B38.1 toward three different colon cancer cell lines, COLO 205 (~900000 ABC), a transfected COLO 205 that expresses p-glycoprotein (mdr), and HCT-15 (~700000 ABC) that naturally express P-glycoprotein (Pgp). As shown in Figure 2, the AMCs bearing the three reducible disulfide linkers (SPDB, **15**, and **19**) or the three nonreducible thioether linkers (SMCC, **36**, and **42**) showed comparable potency toward COLO 205 cells (Figure 2a) with IC_{50} of ~100 pM. When tested against two different multidrug-resistant cell lines, COLO 205/MDR and HCT-15 (Figure 2b and Figure 2c), the AMC made using the hydrophilic sulfo-SPDB linker **15** displayed 10- to 20-fold higher potency than the conjugate made with **19** or the SPDB linker. Among the nonreducible thioether linked conjugates, AMCs made with hydrophilic linkers **36** and **42** were 4-fold more potent than the AMC prepared with the SMCC linker against COLO 205/MDR cells. In the MDR HCT-15 cell line, the AMC made with hydrophilic linker **42** resulted in 2- and 3-fold greater potency when compared to conjugates prepared with **36** and SMCC linkers, respectively. Notably, the IC_{50} (~100 pM) of the AMC having linker **42** on the two multidrug resistant cell lines was similar to the IC_{50} of all the AMCs on the COLO 205 cell line that does not express Pgp. Overall, AMCs made with linker **36** displayed the highest potency among all of the AMCs tested, in particular against MDR cells. AMCs containing a reducible PEG_4 linkage (**19**) in B38.1-DM4 conjugates did not show an activity advantage against MDR cell lines possibly because the disulfide group in the hydrophilic metabolite is readily reduced by an intracellular thiol, such as glutathione, to release the hydrophobic maytansinoid thiol.

Finally, we assessed the potency of maytansinoid conjugates of the huC242 antibody which binds to CanAg expressed in very high amounts on the COLO 205 colon cancer cell line (estimated to be $\geq 4 \times 10^6$ ABC). AMCs utilizing all linkers, and at a range of DAR values from 3.3 to 7.3, had similar potency on COLO205 cells with IC_{50} of about 10 pM (data not shown). Assays were performed under conditions simulating lower antigen density by addition of an excess of nonconjugated antibody to the AMC. For example, adding a 50-fold excess of nonconjugated huC242 antibody to each preparation of AMC would simulate a binding curve to COLO 205 cells that saturates at

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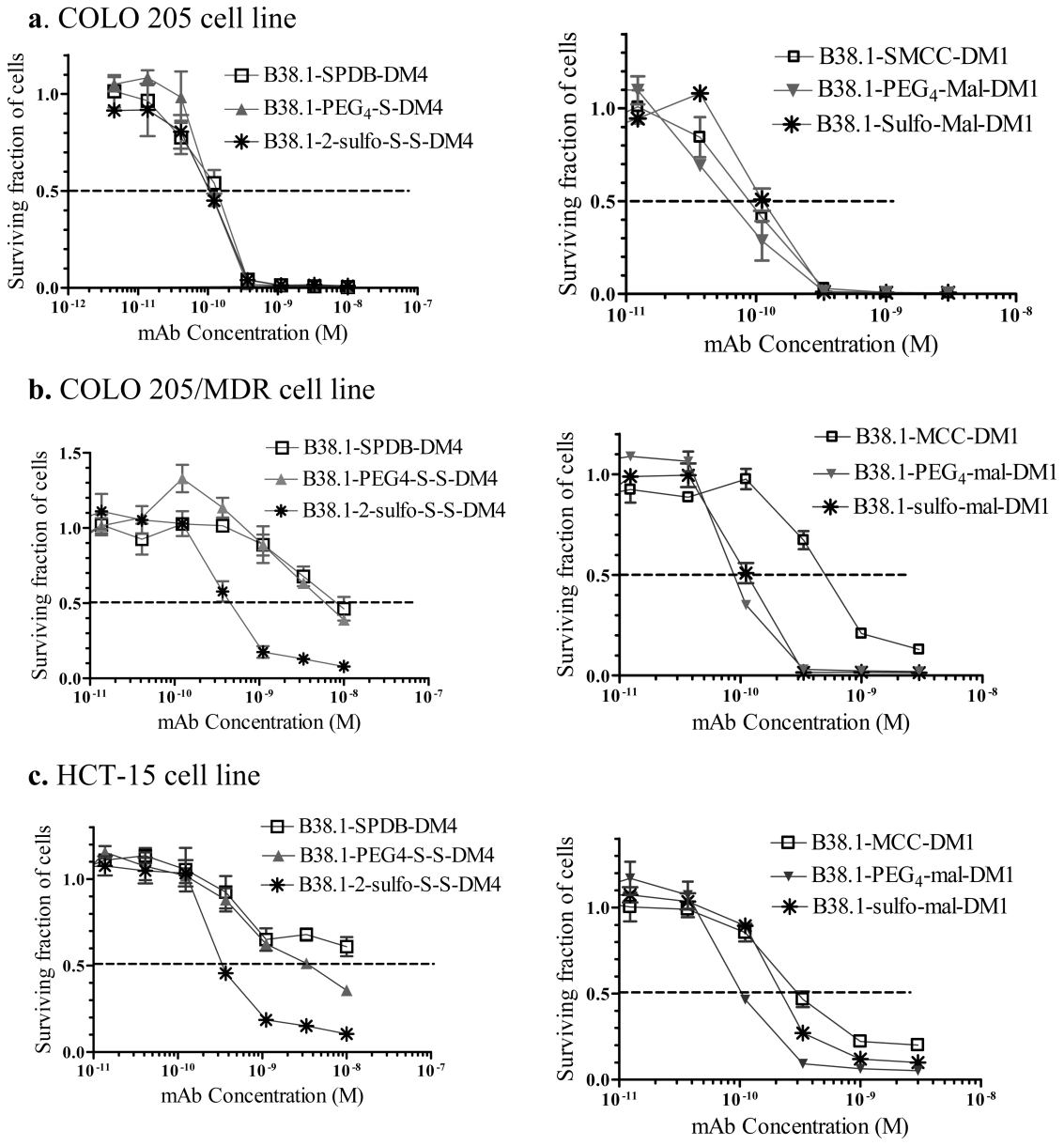

Figure 2. Activity of reducible (left) and nonreducible (right) anti-EpCAM-DMx conjugates prepared with hydrophilic linkers from the hydrophilic linker series with similar ~3.5 ratio of DMx/mAb.

about 80 000 rather than 4×10^6 conjugate molecules bound per cell. Figure 3 and Table 2 show the results of cytotoxicity studies done with AMCs made with the two hydrophilic linkers (**36** and **42**) and two standard linkers (the SPDB cleavable linker and the SMCC noncleavable linker) for comparison. Under the simulated low-antigen conditions (50-fold excess of nonconjugated antibody), the huC242-AMCs prepared with DAR of 5.6−7.3 with hydrophilic linkers **36** and **42** were highly potent with IC_{50} of about 10 pM. By comparison, the huC242-SMCC-DM1 conjugate had an IC_{50} of 27 pM, while the huC242-SPDB-DM4 conjugate had an IC_{50} of 237 pM. These conjugates had DAR of 3.3 and 3.9, respectively, and as mentioned above, because of their hydrophobicity, AMCs with higher DAR in the range of 6−7 could not be made successfully with these linkers. An AMC with linker **36** was made with a DAR of 3.6; its potency was similar to that of huC242-SMCC-DM1 at a similar DAR (both noncleavable linker). Thus, the 2- to 3-fold improvement in potency for the conjugates with higher maytansinoid loading is likely due to the 2- to 3-fold greater amount of maytansinoid delivered per binding event.

Figure 3b shows the results of potency assays done under the same conditions (simulated low-antigen expression) with the same set of AMCs on the COLO205 cell line that had been transfected with the P-glycoprotein gene. The two conjugates made with the hydrophobic linkers SPDB and SMCC showed little activity (IC_{50} not reached at 3000 pM), whereas the AMCs made with hydrophilic linkers **36** and **42** were still highly active on this multidrug resistant cell line, with IC_{50} in the range 7−20 pM.

In Vivo Activity of Maytansinoid Conjugates with Different Linkers. The therapeutic effects of AMCs prepared via traditional SPDB, SPDP, and SMCC linkers have been

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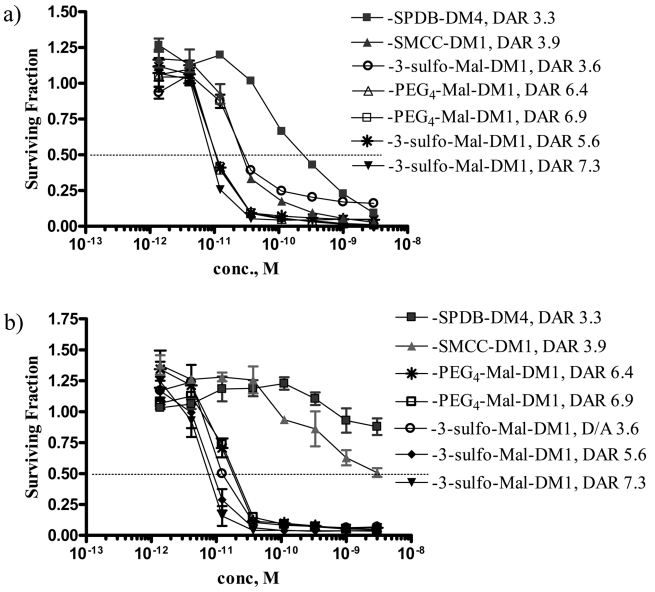

Figure 3. In vitro cytotoxicity of the huC242-DM1 conjugates (3.3−7.3 DAR) toward (a) COLO205 and (b) COLO205-MDR cells upon dilution with 50-fold excess unconjugated huC242 antibody.

Table 2. Cytotoxicity of huC242-DMx Conjugates with Different Maytansinoid/Ab Ratios (DAR) against COLO205 and COLO205-MDR Cells upon Dilution with 50-Fold Excess Unconjugated huC242 Antibody

linker−DMx	DAR	IC_{50} vs COLO205 (pM)	IC_{50} vs COLO205MDR + 50-fold mAb (pM)
3-sulfo-Mal (**36**)-DM1	3.6	28.1	12.1
	5.6	10.4	8.7
	7.3	8.6	7.4
PEG4-Mal (**42**)-DM1	6.4	10.6	18.5
	6.9	10.4	17.5
SPDB-DM4	3.3	237	>5000
SMCC-DM1	3.9	26.7	3000

previously reported.[9] The established COLO 205 MDR human tumor xenograft model in SCID mice was used to examine the in vivo antitumor activity of huB38.1-DMx conjugates prepared with the hydrophilic linkers **15** and **42** in comparison with two of the previously reported hydrophobic linkers. Animals were inoculated with COLO 205/MDR cells, and tumor xenografts were allowed to grow to a mean tumor volume of 138 + 20 mm^3 (8 days). All AMCs used in this study had DAR of approximately 3.5, and animals were dosed with a single injection of 10 mg/kg (antibody dose) (~170 μg/kg conjugated DMx). In the absence of treatment, the mean tumor volume increased to 500 mm^3 in 17 days and 1200 mm^3 in 43 days. Treatment with the hydrophilic linker conjugates B38.1-PEG_4-Mal-DM1 (noncleavable linker) and B38.1-2-sulfo-SPDB-DM4 (cleavable linker) profoundly suppressed the in vivo tumor growth for 20 and 30 days, respectively, as shown in Figure 4. In contrast, conjugates using the nonhydrophilic linkers B38.1-SPDB-DM4 (cleavable linker) and B38.1-SMCC-DM1 (noncleavable linker) were less effective in this multidrug resistant model, only slowing the rate of tumor growth for a net tumor growth delay of about 16 and 14 days, respectively.

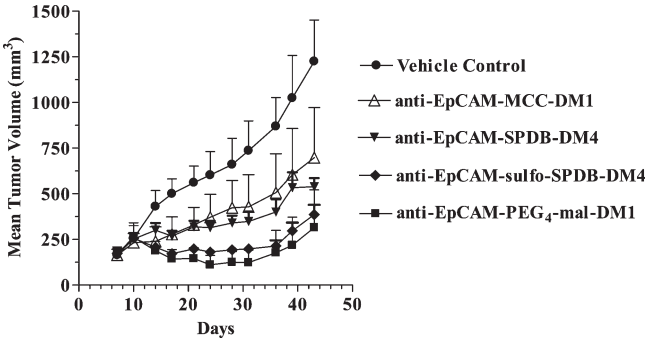

Figure 4. In vivo efficacy of anti-EpCAM-DMx conjugates (~3.5 DMx/Ab) against multidrug resistant COLO205-MDR tumors in SCID mice (single iv injection, 170 μg/kg DMx based dose (10 mg/kg mAb dose)).

DISCUSSION

In the design of antibody−drug conjugates, the linker connecting the cytotoxic agent to the targeting antibody plays a critical role in conjugate potency and efficacy.[7,12,17,21] Over the past 2 decades, linkers have been designed with the goal of achieving high stability in the extracellular environment, yet able to readily release the cell-killing agent upon internalization of the conjugate into the target tumor cell.[21,36] However, conjugation of hydrophobic cytotoxic drugs using linkers that are themselves hydrophobic can lead to conjugate aggregation or loss in the binding affinity of the antibody, particularly at higher (>5 D/A) drug loads.[7,13,23] We reasoned that enhancing the hydrophilicity of the linkers would enable preparation of ADCs with higher drug loads, resulting in a greater concentration of the cytotoxic agent delivered to the target cell and thus improved antitumor activity.

In these novel linkers, a charged sulfonate group or a short polyethyleneglycol spacer was introduced to enhance their hydrophilicity. The synthesis of linkers containing a sulfonic acid substituent at the C2 position was achieved by taking advantage of the preferred substitution reaction of the α-bromo over the γ-bromo substituent in starting material **8** by thioacetate. Although the reaction rate for displacement of the α-bromo substituent by nucleophiles to give the desired product is known to be considerably faster than that of the γ-bromo substituent,[37] conducting the reaction at cold temperature (−20 °C) ensured that the desired product was exclusively obtained. In the next step, the oxidation reaction of the thioacetate group proceeded smoothly with the γ-bromo substituent and the methyl ester being unaffected under the reaction conditions in a mixture of water and acetic acid at room temperature. The subsequent displacement reaction of the γ-bromo substituent by thioacetate proceeded best under warm temperature and mild basic conditions. Higher temperature (>60 °C) or strong basic conditions (pH > 11) resulted in a competing elimination reaction instead of substitution. The other key step was the synthesis of sulfonated maleimido linkers **26** and **36** wherein the formation of the five-membered maleimido ring was aided by coordination of the Lewis acid, $ZnCl_2$ (Scheme 6). The competing cyclization reaction, involving the second carboxylic group and the sulfonic acid group in this molecule, was prevented by addition of excess of HMDS and DIPEA which served to stabilize the coordination reaction. Coordination of the maleic acid group by Zn^{2+} rendered it more electrophilic and facilitated nucleophilic attack

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by the amide nitrogen, resulting in intramolecular cyclization and formation of the maleimide ring. With this strategy for the construction of the maleimide, two different approaches for the precursor **34** starting from **28** or **37** for the synthesis of 3-sulfo-Mal linker **36** were explored (Schemes 7 and 8). Both approaches provided reasonable yields. In the last NHS ester formation step, the possibility of ester formation on both the sulfonate and carboxyl groups had to be considered. We exploited the difference in pK_a values of the sulfonic acid and the carboxylic acid moieties (1.8 and 4.8, respectively) and conducted the coupling reaction with NHS under mildly acidic conditions. These conditions maximized the difference in the coupling rates and gave predominantly the carboxylic acid ester. Thus, the precursors **12**, **13**, **25**, and **35** were prepared in their protonated carboxylic acid forms (purified with C-18 column eluted with acidic solution followed by crystallization), avoiding the need to add an acid catalyst in the NHS ester formation reactions, although the time to achieve complete reaction was long (>6 h). Addition of a catalyst, such as DIPEA or DMAP, to speed up the reaction resulted in the formation of the undesired sulfonate NHS.

These hydrophilic linkers bearing either a short PEG spacer or a sulfonate substituent (e.g., **15**, **19**, **36**, and **42**) have enabled the preparation of conjugates with as many as nine maytansinoids linked per antibody molecule without affecting the monomer content or the binding affinity of the conjugate to the target antigen. AMCs prepared with the hydrophilic disulfide linkers **15** and **19** showed a greater than stoichiometric enhancement of in vitro potency with increasing maytansinoid loading under conditions of low target density on the cell surface. For example, the potency of the huMy9-6-DM4 conjugates was increased over 10-fold when DAR was doubled from 3 to 6, without a concomitant increase in nonspecific cytotoxicity (Table 1). Although the correlation between drug loading and cellular cytotoxicity is known, only a stoichiometric increase in potency has been reported.[25,26] For example, Hamblett and co-workers compared the activities of antiCD30 antibody-MMAE (monomethyl auristatin E) conjugates with DAR of 2, 4, and 8 found that the potency was directly dependent on drug loading, the potency increasing about 2-fold (range 1- to ~3-fold) when doubling the DAR.

An important advantage of the incorporation of hydrophilic linkers in AMCs is their potential to overcome multidrug resistance (MDR). The active efflux of a broad range of anticancer drugs by MDR proteins can seriously limit the efficacy of cancer chemotherapy.[28,30] Chemotherapeutic drugs are exported in both ATP-dependent and ATP-independent manners and even against significant concentration gradients.[38] The ATP-dependent group belongs to the ATP-binding cassette (ABC) transporter family, which includes Pgp, MRP, BCRP, among others.[28,39,40] All of these MDR proteins are involved in diverse physiological processes and are responsible for the uptake and efflux of a multitude of substances from cancer cells. Since the early 1990s, several inhibitors of MDR transporters have been developed for coadministration with cancer therapeutic agents in clinical trials: examples include verapamil, cyclosporine, valspodar (PSC-833), and others.[28,39] Unfortunately, most of them had unfavorable pharmacological properties (poor specificity, low bioavailability at tumor site); many of them were also potassium and calcium channel blockers, and such agents also inhibit clearance of the cytotoxic drug by the efflux pumps in normal cells, thus increasing toxicity. These limitations have precluded their use in the clinic.

Here, we explored whether AMCs with hydrophilic linkers could overcome multidrug resistance. Previous reports have shown that AMCs are efficiently metabolized in cells to generate a lysine−linker−maytansinoid metabolite, wherein the lysine residue is derived from lysosomal degradation of the antibody component of the conjugate.[22] Thus, AMCs with hydrophilic linkers are expected to generate a more polar lysine−hydrophilic linker−maytansinoid metabolite inside the cell, which would be anticipated to be a poor substrate for MDR efflux pumps. Our results indicate that AMCs with hydrophilic linkers are indeed more potent toward MDR cells in vitro and in vivo compared to AMCs with standard hydrophobic linkers. For example, incorporation of the sulfonate moiety enhances the activity of AMCs with reducible (**15**) and nonreducible (**36**) linkages in B38.1-DM1 conjugates against MDR cell lines (Figure 2), suggesting that the polar lysine−sulfo-linker−maytansinoid metabolites generated inside the target cells either are poor MDR protein substrates or have limited accessibility to an MDR transporter (the binding of a substrate to MDR protein occurs only after diffusion of the substrate into the lipid bilayer of the plasma membrane).[41,42] B38.1-DMx conjugates with a nonreducible hydrophilic PEG_4 linker (**42**) also displayed enhanced potency toward MDR cells. A recent report indicates that the hydrophilic maytansinoid metabolite derived with an AMC with the PEG_4 linker is retained to a greater extent inside MDR cells compared to an AMC with a standard linker, which provides an explanation for the higher activity of the former versus the latter.[42]

The enhancement of activity toward MDR cell lines, as well as the opportunity afforded by hydrophobic linkers to increase potency of an AMC in cases where the target antigen has relatively low expression (~10000−100000 ABC), was further demonstrated with a third antibody/antigen target system utilizing the huC242 antibody that targets CanAg. Incorporation of the sulfonate or PEG functionalities into the nonreducible linkers **36** and **42** resulted in as much as a 100-fold greater cytotoxicity (compared with huC242-DMx conjugates prepared with SPDB and SMCC linkers) against both COLO 205 and COLO 205/MDR cells under experimental conditions to stimulate a relatively low maximal ABC value of ~80000 (Figure 3).

The results from the in vivo studies further support the findings in vitro. Conjugates prepared with either the sulfonate (**15**) or PEG linkers (**42**) demonstrated enhanced conjugate efficacy against the COLO205MDR xenograft model as exemplified by using the B38.1 antibody targeting EpCAM expressed on this multidrug resistant colon cancer cell line. Taken together, the results of in vitro and in vivo studies utilizing three different antibody/target antigen systems suggest that incorporating hydrophilic linkers into AMCs provides two benefits. First, the ability to deliver a higher concentration of maytansinoid into target cells per antibody binding event using AMCs with high DAR is made possible by using hydrophilic linkers. The increase in potency can be superstoichiometric, since just doubling the maytansinoid load from 3.4 to 6.6 with the sulfo-SPDB linker (**15**) increases the specific potency of the CD33-targeting AMC by 10-fold (see Table 1), and in the case of the sulfo-Mal nonreducible linker (**36**), AMC potency can be increased by greater than 100-fold upon increasing the DAR from 3.4 to 5.9. Such superstoichiometric enhancement of potency may be important in cases where the target antigen density is low (such as is the case for CD33). Second, AMCs with hydrophilic linkers generate metabolites that may be poorly effluxed out of the tumor cells by MDR transporters, resulting in greater activity

toward multidrug resistant cells. The biological mechanisms have been further explored for linker **42**, confirming that resistance to pgp-enhanced efflux is the mechanism by which increased potency of the linked maytansinoid was obtained.[42]

Although the ideal linker for all antibody−drug conjugates may not exist, we believe the modifications for functionality of the linker in ADCs can have a significant impact on conjugate activity, especially in enhancing the therapeutic index and mitigating the systemic toxicity. We have developed a broad portfolio of novel linkers with differing chemical attributes for evaluation of the optimal linker−drug combination for a given antibody/target antigen pair. Following these principles, there are ongoing efforts toward the evaluation and development of hydrophilic linkers in the design of antibody−drug conjugates with the goal of achieving better efficacy against a broad range of tumor cell lines.

■ MATERIALS AND METHODS

All other reagents were obtained from the Aldrich Chemical Co., unless otherwise stated. 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 values relative to that of an internal tetramethylsilane (Me_4Si) standard. UV spectra were recorded on a Beckman DU-640 spectrophotometer. Optical rotations were determined using a Rudolph AUTOPOL IV polarimeter. Low resolution mass spectra were obtained on a Bruker Daltonics ion-trap mass spectrometer, and high resolution mass spectra were obtained by continuous infusion electrospray mass spectrometry using a Waters QTOF-API-US mass spectrometer. Before HRMS analysis, the samples were mixed with calibration solution containing NaI and CsI in 50% isopropanol. For measurements, several spectra were summed; the summed spectra were smoothed and centered using a lock mass value from the calibration standard peaks that were close to the m/z value of the analyte. Elemental analysis was performed by Micro-Analysis, Inc. (Wilmington, DE). High performance liquid chromatography (HPLC) was performed on either a Beckman or Varian instrument (analytical or preparative scale) equipped with a diode array detector. The linkers used for antibody conjugation were over 95% pure as indicated by HPLC (see Supporting Information).

Methyl 2-(Acetylthio)-4-bromobutyrate (9). To a solution of methyl 2,4-dibromobutyrate (**8**) (10.0 g, 38.4 mmol) in 100 mL of dry THF at −20 °C was added dropwise a mixture of thioacetic acid (2.75 mL, 38.5 mmol) and DIPEA (8.50 mL, 48.9 mmol) in 50 mL of dry THF in 1.5 h. After being stirred overnight at −20 °C, then at 0 °C for 2 h under Ar, the mixture was concentrated, diluted with EtOAc/hexane, washed with 1.0 M NaH_2PO_4, dried over $MgSO_4$, filtered, evaporated, and purified by SiO_2 chromatography (1:12 to 1:10 EtOAc/hexane) to afford 9.5 g (96%) of the title compound. ^{1}H NMR ($CDCl_3$) 4.38 (1H, t, J = 7.1 Hz), 3.74 (s, 3H), 3.40 (m, 2H), 2.57−2.47 (m, 1H), 2.37 (s, 3H), 2.36−2.21 (m, 1H); ^{13}C NMR 193.24, 171.36, 53.15, 44.45, 34.67, 30.46, 29.46; ESI MS m/z+ 276.9 (M + Na), 278.9 (M + 2 + Na); HRMS m/z+ ($C_7H_{11}BrO_3S$ + Na), calcd 276.9510, found 276.9521.

***R,S*-Methyl 4-Bromobutyrate 2-Sulfonic Acid (10).** A solution of methyl 2-(acetylthio)-4-bromobutyrate (**9**) (9.20 g, 36.3 mmol) in acetic acid (80 mL) was treated with hydrogen peroxide (40 mL, 30% in water). The mixture was stirred overnight, evaporated, and coevaporated with water, diluted with water, neutralized with $NaHCO_3$ to pH 5, and washed with 1:1 hexane/EtOAc. The aqueous solution was evaporated, dissolved in ethanol, concentrated, and crystallized with ethanol/toluene/hexane to afford 8.6 g (90% yield) of the title compound. Mp = 275−283 (dec); ^{1}H NMR (D_2O) 4.12 (dd, 1H, J = 4.8, 9.3 Hz), 3.83 (s, 3H), 3.64 (m, 1H), 3.53 (m, 1H), 2.54 (m, 2H); ^{13}C NMR 172.16, 66.73, 55.66, 33.39, 32.70; ESI MS m/z− 260.8 (M − 1); HRMS calcd for ($C_5H_9BrO_5S$ − H) 258.9276, found 258.9268.

4-(Acetylthio)-1-methoxy-1-oxobutane-2-sulfonic Acid (or Methyl 4-(Acetylthio)-2-sulfobutyrate) (11). To methyl 2-sulfo-4-bromobutyrate (**10**) (5.0 g, 19.2 mmol) in a mixture of DMA (20 mL) and THF (60 mL) were added thioacetic acid (3.0 mL, 40.2 mmol) and DIPEA (9.0 mL, 51.7 mmol) in THF (50 mL). The mixture was stirred overnight at 35 °C, evaporated, and coevaporated with 3 × 100 mL of water, then neutralized to pH 6.5 with $NaHCO_3$. The mixture was redissolved in methanol, filtered through Celite, concentrated, and purified by SiO_2 chromatography, eluent CH_3OH/CH_2Cl_2/HCOOH 37.5:250:1 to 50:250:1), to afford 4.4 g (90% yield) of the title compound. ^{1}H NMR(D_2O) 3.95 (dd, 1H, J = 4.1, 10.3 Hz), 3.83 (s, 3H), 3.74 (m, 2H), 3.22 (dd, 2H, J = 7.4, 14.9 Hz), 2.39 (s, 3H); ^{13}C NMR 203.88, 172.91, 67.32, 56.17, 29.04, 20.61; ESI MS m/z− 254.8 (M − H); HRMS m/z− ($C_7H_{12}O_6S_2$ − H) calcd 254.9997, found 255.004.

4-((5-Nitropyridin-2-yl)disulfanyl)-2-sulfobutanoic Acid (12). To 4-(acetylthio)-1-methoxy-1-oxobutane-2-sulfonic acid (**11**) (3.0 g, 11.7 mmol) in water (100 mL) was added NaOH (3 M, 50 mL). After being stirred under argon for 3 h, the mixture was neutralized with 1 M H_3PO_4 to pH 7.2 under Ar and then added dropwise to a solution of 1,2-bis(5-nitropyridin-2-yl)disulfane (15.0 g, 48.3 mmol) in DMA (200 mL). After being stirred for 4 h under argon the mixture was concentrated, diluted with water, filtered, evaporated, and purified by loading onto a C-18 4.0 cm × 20 cm column, eluted with water/methanol (98:2), and then crystallized with EtOH/hexane to afford 3.1 g (75% yield) of the title compound. Mp = 288−291 °C (dec); ^{1}H NMR (DMF-d_7) 9.29 (d, 1H, J = 2.2 Hz), 8.63 (dd, 1H, J = 2.7, 8.9 Hz), 8.17 (d, 1H, J = 8.9 Hz), 3.73 (t, 1H, J = 7.2 Hz), 3.22−3.17 (m, 1H), 3.15−3.10 (m, 1H), 2.41−2.33 (m, 2H); ^{13}C NMR 170.92, 169.10, 146.04, 143.67, 133.65, 120.72, 64.22, 37.82, 29.26; ESI MS m/z− 352.8 (M − H); HRMS m/z− ($C_9H_{10}N2O_7S_3$ − H) calcd 352.9572, found 352.9576.

4-(Pyridin-2-yldisulfanyl)-2-sulfobutanoic Acid (13). To 4-(acetylthio)-1-methoxy-1-oxobutane-2-sulfonic acid (**11**) (1.50 g, 5.85 mmol) was added an aqueous solution of NaOH (0.5 M, 100 mL). After being stirred under Ar for 3 h, the mixture was concentrated to ∼50 mL and neutralized with 1 M H_3PO_4 to pH 7.2 under Ar. The mixture was added dropwise to a solution of 2, 2′-dithiodipyridine (5.0 g, 22.65 mmol) in DMA (60 mL). After being stirred for 4 h under Ar, the mixture was concentrated, diluted with water, filtered, evaporated, and purified on a C-18 4.0 cm × 20 cm column eluted with water/methanol (99:1 to 90:10) to afford 1.32 g (73% yield) of the title compound. ^{1}H NMR (DMF-d_7) 8.39 (dd, 1H, J = 3.5, 4.8 Hz), 7.86 (m, 2H), 7.25 (m, 1H), 3.59 (dd, 1H, J = 5.2, 9.4 Hz), 2.90 (m, 2H), 2.28 (m, 2H); ^{13}C NMR 172.60, 159.16, 148.93, 138.09, 121.03, 119.38, 67.49, 36.39, 28.666; ESI MS m/z− 307.8 (M − H). HRMS m/z− ($C_9H_{11}NO_5S_3$ − H) calcd 307.9721, found 307.9727.

1-(2,5-Dioxopyrrolidin-1-yloxy)-4-((5-nitropyridin-2-yl)-disulfanyl)-1-oxobutane-2-sulfonic Acid (or Succinimidyl 4-(5-Nitropyridin-2-yl)disulfanyl)-2-sulfobutyrate (14)). To 4-((5-nitropyridin-2-yl)disulfanyl)-2-sulfobutanoic acid (**12**) (220 mg, 0.62 mmol) in DMA (15 mL) was added NHS (100 mg, 0.87 mmol) and EDC (480 mg, 2.50 mmol). The mixture was stirred under argon overnight, evaporated, and purified by SiO_2 chromatography, eluting with CH_2CH_2/CH_3OH/HCOOH (10000:1000:1 to 10000:1500:1) to afford 227 mg (82% yield) of the title compound. ^{1}H NMR (DMSO-d_6) 9.25 (d, 1H, J = 5.2 Hz), 8.57 (dd, 1H, J = 2.5, 8.9 Hz), 8.04 (t, 1H, J = 8.0 + 8.9 Hz), 3.86 (dd, 1H, J = 4.9, 9.7 Hz), 3.13−3.12 (m, 2H), 2.76 (s, 4H), 2.36−2.30 (m, 1H), 2.25−2.21 (m, 1H); ^{13}C NMR 166.96, 165.01, 144.93, 142.26, 132.63, 119.61, 61.00, 35.03, 29.30, 25.39; ESI MS m/z− 449.8 (M − H); HRMS m/z− ($C_{13}H_{13}N_3O_9S_3$ − H) calcd

449.9736, found 449.9739. Anal. Calcd for $C_{13}H_{13}N_3O_9S_3$: C, 34.59; H, 2.90; N, 9.31. Found: C, 34.80; H, 3.17; N, 9.30.

Succinimidyl 4-(Pyridin-2-yl)disulfanyl)-2-sulfobutyrate (15). To 4-(pyridin-2-yldisulfanyl)-2-sulfobutanoic acid (**13**) (680 mg, 2.20 mmol) in DMA (50 mL) were added NHS (300 mg, 2.60 mmol) and EDC (1.35 g, 7.03 mmol). The mixture was stirred under Ar overnight, evaporated, and purified by SiO_2 chromatography, eluting with $CH_2CH_2/CH_3OH/HCOOH$ (10000:1000:1 to 10000:1500:1) to afford 720 mg (80% yield) of the title compound. ^{1}H NMR (DMSO-d_6) 8.40 (dd, 1H, J = 3.5, 4.7 Hz), 7.85 (m, 2H), 7.24 (m, 1H), 3.58 (dd, 1H, J = 5.1, 9.4 Hz), 2.94−2.90 (m, 2H), 2.74 (s, 4H), 2.31−2.27 (m, 2H); ^{13}C NMR 168.16, 161.11, 147.91, 139.22, 121.63, 119.31, 66.80, 36.30, 28.36, 25.42; ESI MS $m/z-$ 404.8 (M − H); HRMS $m/z-$ ($C_{13}H_{14}N_2O_7S_3$ − H) calcd 404.9885, found 404.9881. Anal. Calcd for $C_{13}H_{14}N_2O_7S_3$: C, 38.41; H, 3.47; N, 6.89. Found: C, 38.57; H, 3.52; N, 6.99.

15-(2-Pyridyldithio)-4,7,10,13-tetraoxapentadecanoic Acid (18). To a solution of aldrithiol-2 (1.17 g, 5.31 mmol) in 5.0 mL of 1,2-dimethoxyethane was added a solution of 3-(2-thiotetraethylene glycol)propionic acid (17) (Quanta Biodesign, 490 mg, 1.73 mmol) dissolved in 1.0 mL of 1,2-dimethoxyethane. The reaction mixture was stirred at room temperature for 3.5 h and then concentrated and purified by silica chromatography, eluting with 5% methanol in methylene chloride, to yield 431.6 mg (63.7% yield) of the desired product. ^{1}H NMR ($CDCl_3$) 2.615 (2H, t), 2.986 (2H, t), 3.569−3.626 (4H, m), 3.634 (8H, d, J = 3.2 Hz), 3.738 (4H, dt), 7.101 (1H, m), 7.667 (1H, m), 7.781 (1H, d, J = 8.4 Hz), and 8.466 ppm (1H, m); HRMS $m/z-$ ($C_{16}H_{25}NO_6S_2$ + Na) calcd 414.1021, found 414.1049.

15-(2-Pyridyldithio)-4,7,10,13-tetraoxapentadecanoic Acid *N*-Hydroxysuccinimide Ester (19). A 10 mL round bottomed flask was charged with 15-(2-pyridyldithio)-4,7,10,13-tetraoxapentadecanoic acid (431 mg, 1.10 mmol), methylene chloride (50 mL), *N*-hydroxysuccinimide (3.6 mg, 0.31 mmol), and 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (6.8 mg, 0.036 mmol), and the reaction was allowed to proceed for 2 h at room temperature with stirring. The product was purified by silica chromatography, eluting with 7% 1,2-dimethoxyethane in methylene chloride to give 206.3 mg (38.4% yield) of the desired product. ^{1}H NMR ($CDCl_3$) δ 2.81 (4H, s), 2.86 (2H, t, J = 6.4 Hz), 2.98 (2H, t, J = 6.3 Hz), 3.53−3.55 (2H, m), 3.59−3.60 (m, 2H), 3.61 (10H, d, J = 2.5 Hz), 3.62 (2H, t, J = 6.2 Hz), 3.81 (2H, t, J = 6.4 Hz), 7.10 (1H, dd, J = 5.0, 7.2 Hz), 7.71 (1H, dt, J = 15.5, 8.0, and 1.5 Hz), 7.81 (1H, d, J = 8.0 Hz), 8.43 (1H, dd, J = 4.1, 0.7 Hz); ^{13}C NMR 25.79, 32.37, 38.82, 53.62, 65.93, 68.99, 70.64, 70.70, 70.87, 70.94, 120.41, 121.01, 148.79, 160.49, 166.91, 169.11; ESI MS: $m/z+$ 511.1 $(M + Na)^+$; HRMS $m/z-$ ($C_{20}H_{28}N_2O_8S_2$ + Na) calcd 511.1185, found 511.1182. Anal. Calcd for $C_{20}H_{28}N_2O_8S_2$: C, 49.17; H, 5.78; N, 5.73. Found: C, 49.19; H, 5.91; N, 5.77.

Methyl 4-Azido-2-sulfobutyrate (21). A mixture of methyl 4-bromo-2-sulfobutyrate (**10**) (1.07 g, 4.11 mmol) and sodium azide (0.70 g (10.7 mmol) in DMF (50 mL) was stirred overnight. The mixture was evaporated and purified by silica chromatography and eluted with 1:5:0.01 CH_3OH/CH_2Cl_2/HAc and crystallized with CH_3OH/toluene/hexane to afford 1.00 g (95%) of the title compound. Mp = 267 −272 °C (dec). ^{1}H NMR (DMF-d_7): 12.06 (br, 1H), 3.65 (s, 3H), 3.59 (dd, 1H, J = 5.4, 8.9 Hz), 3.47 (m, 2H), 2.24 (m, 2H). ^{13}C NMR 171.10, 64.29, 52.24, 50.64, 21.35. ESI MS $m/z+$ 267.9 (M + 2Na−H), $m/z-$ 222.0 (M − H). HRMS $m/z-$ ($C_5H_9N_3O_5S$ − H) calcd 222.0185, found 222.0179.

4-Azido-2-sulfobutyric Acid (22). Methyl 4-azido-2-sulfobutyrate (**21**) (1.00 g, 4.08 mmol) in a mixture of HCl (50 mL, 1.0 M) and HOAc (5 mL) was heated at 100 °C for 6 h. The mixture was evaporated and coevaporated with 3 × 50 mL of water and crystallized with water/acetone to afford 1.0 g (99%) of the title compound. ^{1}H NMR (DMF-d_7): 3.60 (m, 2H), 3.52 (m, 1H), 2.24 (m, 2H). ^{13}C NMR 170.96, 63.04, 50.66, 29.12. ESI MS $m/z-$ 207.7 (MW − H); HRMS $m/z-$ ($C_4H_7N_3O_5S$ − H) calcd 208.0028, found 208.0021.

4-Amino-2-sulfobutyric Acid (23). 4-Azido-2-sulfobutyric acid (**22**) (500 mg, 2.40 mmol), water (20 mL), and Pd/C (110 mg, 10% Pd, 50% water based) were placed into a 250 mL hydrogenation shaking bottle. After evacuation, 20 psi of hydrogen was let into the bottle. The mixture was shaken for 8 h, then filtered through Celite, washed with DMF, evaporated, and coevaporated with dry DMF to afford 476 mg (91% HCl salt) of the title product. ESI MS $m/z-$ 181.8 (MW − H). This product was used directly without further purification.

(*Z*)-4-(3-Carboxy-3-sulfopropylamino)-4-oxobut-2-enoic Acid (24). The above 4-amino-2-sulfobutyric acid, HCl salt (**23**) (476 mg, 2.16 mmol) in dry DMF (20 mL) was treated with maleic anhydride (232 mg, 2.36 mmol). The mixture was stirred under argon overnight, evaporated, and purified on a self-packed C-18, 1.0 cm × 25 cm column, eluted with water. The fractions containing product were pooled, evaporated, and crystallized with H_2O/acetone to afford 552 mg (83%, in two steps) of the title product. ^{1}H NMR (DMF-d_7): 9.70 (br, 1H), 6.73 (d, 1H, J = 12.8 Hz), 6.32 (d, 1H, J = 12.8 Hz), 3.69 (m, 1H), 3.47 (m, 2H), 2.27 (m, 2H). ^{13}C NMR 171.47, 167.32, 165.87, 135.44, 133.07, 63.82, 39.13, 27.62. ESI MS $m/z-$ 279.8 (MW − H); HRMS $m/z-$ ($C_8H_{11}NO_8S$ − H) calcd 280.0127, found 280.0111.

4-*N*-Maleimido-2-sulfobutanoic Acid (25). (*Z*)-4-(3-Carboxy-3-sulfopropylamino)-4-oxobut-2-enoic acid (**24**) (310 mg, 1.10 mmol) in a mixture of dry DMA (5 mL) and dry toluene (20 mL) was heated. After the temperature reached 80 °C, HMDS (hexamethyldisilazane) (1.40 mL, 6.71 mmol) and $ZnCl_2$ (1.85 mL, 1.0 M in diethyl ether, 1.85 mmol) were added. The mixture was continually heated to 115−125 °C, and toluene was collected through Dean−Stark trap. The reaction mixture was fluxed at 120 °C for 6 h. During this period, 2 × 20 mL of dry toluene was added to keep the mixture volume around 8−10 mL. Then the mixture was cooled, and 1 mL of 1:10 HCl (conc)/CH_3OH was added. The mixture was evaporated, and the sample was purified by silica chromatography and eluted with CH_3OH/CH_2Cl_2/HAc (1:5:0.01 to 1:4:0.01) to afford 245 mg (85%) of the title product. ^{1}H NMR (DMF-d_7): 10.83(br, 1H), 6.95 (s, 2H), 3.65 (m, 1H), 3.54 (m, 2H), 2.27 (m, 2H). ^{13}C NMR 173.61, 172.04, 135.47, 64.18, 37.1, 27.89. ESI MS $m/z-$ 261.8 (MW − H). HRMS $m/z-$ ($C_8H_9NO_7S$ − H) calcd 262.0021, found 262.0037.

Succinimidyl 4-*N*-Maleimido-2-sulfobutyrate (26). 4-*N*-Maleimido-2-sulfobutanoic acid (**25**) (240 mg, 0.91 mmol) in DMA (10 mL) was added to NHS (220 mg, 1.91 mmol) and EDC (500 mg, 2.60 mmol). The mixture was stirred under argon overnight, evaporated, and purified on SiO_2 chromatography, eluting with CH_2CH_2/CH_3OH/HAc (10000:1000:1 to 10000:2000:1), then crystallized with DMA/EtOAc/hexane to afford 262 mg (80% yield) of the title compound. ^{1}H NMR (DMF-d_7) 6.99 (s, 1H), 3.83 (m, 1H), 3.64 (m, 2H), 2.75 (s, 4H), 2.34 (m, 2H); ^{13}C NMR 171.97, 171.82, 166.64, 135.58, 62.00, 36.66, 26.62; ESI MS $m/z-$ 358.9 (M − H); HRMS $m/z-$ ($C_{12}H_{12}N_2O_9S$ − H) calcd 359.0185, found 359.0171. Anal. Calcd for $C_{12}H_{12}N_2O_9S$: C, 40.00; H, 3.36; N, 7.78. Found: C, 40.22; H, 3.42; N, 7.87.

(*E*)-Methyl 4-Azidobut-2-enoate (29). To the solution of NaN_3 (2.80 g, 43.01 mmol) in 100 mL of DMF at 0 °C was added methyl 4-bromocrotonate (**28**) (5.00 mL, 85%, 36.10 mmol). After being stirred at 0 °C for 30 min, the mixture was stirred at room temperature for 4 h, evaporated, suspended with EtOAc/hexane (1:1), filtered, evaporated, and chromatographically purified on a silica column, eluting with ethyl acetate/hexane (1:25 to 1:10) afforded 4.08 g (80% yield) of the title product. ^{1}H NMR ($CDCl_3$) 6.88 (m, 1H), 6.06 (ddd, 1H, J = 1.7, 3.4, 15.6 Hz), 3.97 (dd, 2H, J = 1.2, 4.96 Hz), 3.73 (s, 3H); ^{13}C NMR 166.23, 140.86, 123.49, 51.95, 51.36; ESI MS $m/z+$ 182.5 (M + Na + H_2O), 305.5 (2M + Na); HRMS $m/z+$ ($C_5H_7N_3O_2S$ + Na + H_2O) calcd 182.0542, found 182.0555.

Methyl 3-(Acetylthio)-4-azidobutanoate (30). To the solution of (*E*)-methyl 4-azidobut-2-enoate (**29**) (4.00 g, 28.37 mmol) in 60 mL of THF at 0 °C were added a mixture of thioacetic acid (3.0 mL, 42.09 mmol) and DIPEA (8.0 mL, 45.92 mmol) in 60 mL of THF over 20 min. After being stirred at 0 °C for 1 h, the mixture was allowed to warm to room temperature, then stirred at room temperature overnight, and evaporated to dryness. The residue was redissolved in CH_2Cl_2, washed with $NaHCO_3$ (sat.) and 1 M NaH_2PO_4/NaCl (sat.), pH 4, successively, dried over $MgSO_4$, filtered, evaporated, and chromatographed on silica. The product eluted with an ethyl acetate/hexane gradient (1:8 to 1:4) to afford 4.98 g (81%) of the title product. ^{1}H NMR ($CDCl_3$) 3.66 (m, 1H), 3.62 (s, 3H), 3.40 (dd, 1H, J = 7.5, 12.7 Hz), 3.31 (m, 1H), 2.78 (m, 1H), 2.60 (m, 1H), 2.32 (s, 3H); ^{13}C NMR (DMF-d_7) 192.20, 172.48, 56.56, 53.60, 51.31, 34.58, 30.56; ESI MS m/z+ 240.0 (M + Na), 255.9 (M + K); HRMS m/z+ ($C_7H_{11}N_3O_3S$ + Na) calcd 240.0419, found 240.0406.

Azido-4-methoxy-4-oxobutane-2-sulfonic Acid (31). To methyl 3-(acetylthio)-4-azidobutanoate (**30**) (4.00 g, 18.43 mmol) in acetic acid (75 mL) was added 30% H_2O_2 (25 mL). The mixture was stirred overnight, evaporated, and coevaporated with EtOH/toluene, and purified on SiO_2, eluting with CH_3OH/CH_2Cl_2/HAc (100:800:1 to 100:500:1) to afford 3.85 (93%) g of the title compound. ^{1}H NMR (CD_3OD) 3.78 (dd, 1H, J = 5.0, 12.7 Hz), 3.62 (s, 3H), 3.44 (dd, 1H, J = 7.5, 12.7 Hz), 3.33 (m, 1H), 2.84 (dd, 1H, J = 5.6, 16.5 Hz), 2.57 (dd, 1H, J = 7.5, 16.5 Hz); ^{13}C NMR (DMF-d_7) 173.37, 57.31, 52.54, 52.49, 34.51; ESI MS m/z− 221.7 (M + H); HRMS m/z− ($C_5H_9N_3O_5S$ − H) calcd 222.0185, found 222.0176.

4-Azido-3-sulfobutanoic Acid (32). Azido-4-methoxy-4-oxobutane-2-sulfonic acid (3.80 g, 17.04 mmol) in a mixture of 1.0 M HCl (150 mL) and acetic acid (8 mL) was refluxed at 120 °C overnight, evaporated, and successively coevaporated with water and ethanol/toluene, and purified on SiO_2, eluting with CH_3OH/CH_2Cl_2/HOAc (100:500:1 to 100:400:1) to afford 3.02 (85%) g of the title compound. ^{1}H NMR (CD_3OD) 3.77 (dd, 1H, J = 5.1, 12.8 Hz), 3.45 (dd, 1H, J = 7.0, 12.8 Hz), 3.31 (m, 1H), 2.86 (dd, 1H, J = 4.7, 16.7 Hz), 2.51 (dd, 1H, J = 8.4, 16.7 Hz); ^{13}C NMR (DMF-d_7) 173.98, 67.50, 59.78, 27.82; ESI MS m/z− 207.7 (M − H); HRMS m/z− ($C_4H_7N_3O_5S$ − H) calcd 208.0028, found 208.0055.

4-Amino-3-sulfobutanoic Acid (33). In a 500 mL hydrogenation bottle were placed 4-azido-3-sulfobutanoic acid (**32**) (3.00 g, 14.35 mmol) in methanol (150 mL) and 0.32 g of Pd/C (10% Pd, 50% wet). After evacuating the air, 30 psi of H_2 was introduced, and the mixture was shaken overnight, filtered through Celite, evaporated, and coevaporated with dry EtOH to afford about 2.50 g (95%) of 4-amino-3-sulfobutanoic acid. ^{1}H NMR (CD_3OD) 3.24 (m, 1H), 3.17 (m, 1H), 2.90 (dd, 1H, J = 2.6, 16.5 Hz), 2.33 (dd, 1H, J = 10.1, 16.5 Hz); ESI MS m/z− 181.60 (M − H). The resulting compound was unstable and was used directly without further purification.

(*Z*)-4-(3-Carboxy-2-sulfopropylamino)-4-oxobut-2-enoic Acid (34). To the solution of 4-amino-3-sulfobutanoic acid (**33**) (~2.50 g, 13.66 mmol) in 100 mL of DMA was added maleic anhydride (1.48 g, 15.10 mmol), and the mixture was stirred overnight, evaporated, purified on C-18 column (2 cm × 30 cm) eluted with 1% HOAc in water and crystallized with MeOH/acetone/toluene to afford 3.34 g (83%) of (*Z*)-4-(3-carboxy-2-sulfopropylamino)-4-oxobut-2-enoic acid. ^{1}H NMR (CD_3OD) 6.33 (d, 1H, J = 12.6 Hz), 6.10 (d, 1H, J = 12.6 Hz), 3.64 (dd, 1H, J = 5.8, 14.0 Hz), 3.54 (m, 1H), 3.30 (m, 1H), 2.78 (dd, 1H, J = 4.9, 16.8 Hz), 2.39 (m, 1H); ^{13}C NMR 173.52, 168.68, 167.98, 135.59, 127.79, 57.31, 40.56, 34.52; ESI MS m/z− 279.7 (M − H); HRMS m/z− ($C_8H_{11}NO_8S$ − H) calcd 280.0127, found 208.0169.

4-(2,5-Dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-3-sulfobutanoic Acid (35). (*Z*)-4-(3-Carboxy-2-sulfopropylamino)-4-oxobut-2-enoic acid (**34**) (450 mg, 1.60 mmol) in a mixture of 10 mL of dry DMA and 50 mL of dry toluene was heated. After the temperature reached 80 °C, HMDS (hexamethyldisilazane, 1.80 mL, 8.63 mmol) and $ZnCl_2$ (3.2 mL, 1.0 M in diethyl ether) were added. The mixture was heated to 115−125 °C, and toluene was collected through a Dean−Stark trap. The reaction mixture was fluxed at 120 °C for 6 h. During this period, 2 × 20 mL of dry toluene was added to keep the mixture volume around 8−10 mL. Then the mixture was cooled, 1 mL of 1:10 HCl (conc)/CH_3OH was added, evaporated, and purified on SiO_2 eluted with 1:5:0.01 CH_3OH/CH_2Cl_2/HAc to afford 315 mg (75%) of the title product. ^{1}H NMR (DMF-d_7) 6.96 (s, 2H), 4.04 (dd, 1H, J = 4.3, 13.8 Hz), 3.47 (m, 1H), 3.23 (dd, 1H, J = 7.4, 14.7 Hz), 2.99 (dd, 1H, J = 3.3, 16.8 Hz), 2.35 (dd, 1H, J = 8.1, 16.9 Hz); ^{13}C NMR 173.58, 172.18, 135.54, 54.61, 40.24, 32.43; ESI MS m/z− 261.70 (M − H); HRMS m/z− ($C_8H_9NO_7S$ − H) calcd 262.0021, found 262.0040.

1-(2,5-Dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-4-(2,5-dioxopyrrolidin-1-yloxy)-4-oxobutane-2-sulfonic Acid (36). 4-(2,5-Dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)-3-sulfobutanoic acid (**35**) (110 mg, 0.418 mmol), EDC (240 mg, 1.25 mmol), and *N*-hydroxysuccinimide (58 mg, 0.504 mmol) were stirred in 10 mL of DMA overnight and then evaporated and purified on SiO_2, eluting with CH_3OH/CH_2Cl_2/HAc (100:900:1 to 100: 600:1) to afford 112 mg (75%) of the title product. ^{1}H NMR (DMF-d_7) 6.93 (s, 2H), 4.06 (dd, 1H, J = 4.8, 13.1 Hz), 3.80 (dd, 1H, J = 10.7, 13.9 Hz), 3.35 (dd, 1H J = 3.3, 17.8 Hz), 3.25 (m, 1H), 3.10 (dd, 1H, J = 2.2, 16.4 Hz), 2.87 (m, 4H); ^{13}C NMR 172.27, 170.88, 169.29, 135.55, 55.28, 40.22, 32.69, 26.66; ESI MS m/z− 261.70 (M − H); HRMS m/z− ($C_{12}H_{12}N_2O_9S$ − H) calcd 359.0185, found 359.0208. Anal. Calcd for $C_{12}H_{12}N_2O_9S$: C, 40.00; H, 3.36; N, 7.78. Found: C, 40.36; H, 3.27; N, 7.67.

Ethyl 3-(Acetylthio)-3-cyanopropanoate (38). To (*Z*)-ethyl 3-cyanoacrylate (**37**) (5.01 g, 40.00 mmol) in 80 mL of THF at −20 °C was added a solution of thioacetic acid (5.0 mL, 70.15 mmol) and DIPEA (16.0 mL, 92.03 mmol) in 20 mL of THF in 30 min. The mixture was kept at −20 °C for 4 h and then at room temperature overnight. The mixture was concentrated, diluted with CH_2Cl_2, washed with saturated $NaHCO_3$, dried over $MgSO_4$, filtered, evaporated, and purified by SiO_2 chromatography (1:4 EtOAc/hexane) to afford 5.22 g (65%) of the title compound. R_f = 0.25 (1:4 EtAC/hexane); ^{1}H NMR ($CDCl_3$), 4.44 (m, 1H), 4.11 (dd, 2H, J = 7.1, 14.3 Hz), 3.38 (m, 1H), 3.15 (m, 1H), 2.17 (s, 3H), 1.19 (t, 3H, J = 7.2 Hz); ^{13}C NMR 194.12, 173.21, 119.82, 61.35, 33.52, 30.08, 14.62; MS m/z+ 225.9 (M + Na), m/z− 201.7 (M − H); HRMS m/z− ($C_8H_{11}NO_3S$ + Na) calcd 224.0357, found 224.0262.

Cyano-3-ethoxy-3-oxopropane-1-sulfonic Acid (39). Ethyl 3-(acetylthio)-3-cyanopropanoate (**38**) (2.00 g, 9.95 mmol) in acetic acid (40 mL) was treated with H_2O_2 (12 mL, 30%). The mixture was stirred overnight, evaporated, and purified on silica gel chromatography, eluting with methanol/dichloromethane/acetic acid (1:8:0.01 to 1:5:0.01) to afford 1.72 g (84%) of the title compound. ^{1}H NMR (DMSO), 4.63 (m, 1H), 4.12 (dd, 2H, J = 7.1, 14.3 Hz), 3.27 (m, 1H), 3.05 (m, 1H), 1.28 (t, 3H, J = 7.2 Hz); ^{13}C NMR 173.15, 113.85, 61.38, 48.32, 26.33, 14.15; ESI MS m/z− 205.8 (MW − H); HRMS m/z− ($C_6H_9NO_5S$ − H) calcd 206.0123, found 206.0151.

1-(*tert*-Butoxycarbonylamino)-4-ethoxy-4-oxobutane-2-sulfonic Acid (40). In a hydrogenation bottle was added cyano-3-ethoxy-3-oxopropane-1-sulfonic acid (**39**) (2.50 g, 12.06 mmol), ethanol (80 mL), fresh filtered Raney Ni (0.40 g), and BOC anhydride (3.30 g, 15.12 mmol). After the air inside the bottle was evacuated by vacuum, 20 psi of hydrogen was introduced into the bottle. The bottle was shaken overnight, and the reaction mixture was filtered through Celite, evaporated, and purified on silica gel, eluting with methanol/dichloromethane/acetic acid (1:6:0.01) to afford 3.18 g (85%) of the title compound. ^{1}H NMR (DMSO), 6.82 (s, 1H), 4.26 (m, 1H), 4.11 (dd, 2H, J = 7.1, 14.3 Hz), 3.53 (dd, 1H, J = 4.2, 13.4 Hz), 3.36 (m, 1H), 2.86 (m, 1H), 2.51 (m, 1H), 1.38 (s, 9H), 1.22 (t, 3H, J = 7.2 Hz); ^{13}C NMR 173.35, 155.72, 80.44, 62.05, 52.55, 41.61, 34.50, 28.85, 14.52; ESI

MS $m/z-$ 309.8 (M − H); HRMS $m/z-$ ($C_{11}H_{21}NO_7S$ − H) calcd 310.0960, found 310.0904.

4-(*tert*-Butoxycarbonylamino)-3-sulfobutanoic Acid (41). To 1-(*tert*-butoxycarbonylamino)-4-ethoxy-4-oxobutane-2-sulfonic acid (**40**) (402 mg, 1.29 mmol) in a mixture of THF/H_2O (1:2, 60 mL) was added lithium hydroxide monohydrate (2.0 g, 47.6 mmol). The mixture was stirred under Ar overnight, concentrated, and purified on C-18 column (2 cm × 30 cm), eluting with a gradient from 100% water to 10% methanol in water to afford 328 mg (90%) of the title compound. ^{1}H NMR (DMSO), 6.78 (s, 1H), 4.03 (m, 1H), 3.57 (dd, 1H, J = 4.2, 13.4 Hz), 3.41 (m, 1H), 2.89 (m, 1H), 2.61 (m, 1H), 1.39 (s, 9H); ^{13}C NMR 174.21, 155.82, 79.85, 59.95, 42.06, 32.52, 28.88, 14.55; ESI MS 281.8 (M − H); HRMS $m/z-$ ($C_9H_{17}NO_7S$ − H) calcd 282.0647, found 282.0630.

(*Z*)-4-(3-Carboxy-2-sulfopropylamino)-4-oxobut-2-enoic Acid (34). 4-(*tert*-Butoxycarbonylamino)-3-sulfobutanoic acid (**41**) (321 mg, 1.13 mmol) was stirred in a mixture of HCl (conc)/dioxane (1:4, 15 mL) for 30 min, evaporated, and coevaporated with ethanol/toluene (1:1, 4 × 20 mL) to dryness. To the dried material were added maleic anhydride (121 mg, 1.23 mmol) and DMA (20 mL), and the mixture was stirred overnight, evaporated, and run through a C-18 column eluted with water, and crystallized with EtOH/hexane to afford 263 mg (83%) of the title compound. ESI MS 279.8 (M − H). The NMR data are the same through the route with 4-azido-3-sulfobutanoic acid (**32**).

Succinimidyl-[(*N*-maleimidopropionamido)tetraethyleneglycol] Ester (42, NHS-PEG$_4$-maleimide). An amount of 100 mg of the compound purchased from Quanta Biodesign was coevaporated with CH_2Cl_2 and benzyne (1:2, 2 × 50 mL) to remove the water before being checked by NMR and elemental analysis. ^{1}H NMR ($CDCl_3$) 2.47 (2H, t, J = 7.0 Hz), 2.80 (s, 4H), 2.85 (2H, t, J = 6.4 Hz), 3.36−3.39 (m, 2H), 3.49 (2H, t, J = 5.2 Hz), 3.56−3.64 (m, 12H), 3.78 − 3.82 (m, 4H), 6.66 (2H, s); ^{13}C NMR 25.77, 32.32, 34.49, 34.69, 39.44, 65.89, 69.82, 70.39, 70.62, 70.65, 70.68, 70.79, 70.88, 134.38, 166.92, 169.20, 169.95, 170.69; HRMS $m/z+$ ($C_{22}H_{32}N_3O_{11}$) calcd 514.2037, found 514.2018. Anal. Calcd for $C_{22}H_{31}N_3O_{11}\cdot{}^1/_3H_2O$): C, 50.86; H, 6.14; N, 8.09. Found: C, 50.75; H, 6.16; N, 7.92.

15-(DM4-dithio)-4,7,10,13-tetraoxapentadecanoic Acid (47). A solution of $N^{2'}$-deacetyl-$N^{2'}$-(4-mercapto-4-methyl-1-oxopentyl)maytansine (DM4, 18.6 mg, 0.0239 mmol) and 15-(2-pyridyldithio)-4,7,10,13-tetraoxapentadecanoic acid (14.0 mg, 0.0358 mmol) was prepared in 0.75 mL of 1,2-dimethoxyethane. 4-Methylmorpholine (6.0 mg, 0.0597 mmol) was added to the reaction vessel, and the reaction proceeded for 24 h at room temperature with stirring. Upon reaction completion, the crude reaction mixture was dried in vacuo and used without further purification.

15-(DM4-dithio)-4,7,10,13-tetraoxapentadecanoic Acid *N*-Hydroxysuccinimide Ester (48). The crude 15-(DM4-dithio)-4,7,10,13-tetraoxapentadecanoic acid was dissolved in 2.0 mL of methylene chloride and combined with *N*-hydroxysuccinimide (3.6 mg, 0.31 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (6.8 mg, 0.036 mmol). The mixture was stirred for 2.5 h, and the product was purified by silica chromatography, eluting with 4% methanol in methylene chloride. The solvent was removed under vacuum to give 15.0 mg (54.2% yield) of the desired product. ESI MS: m/z found, 1179.3 $(M + Na)^+$; calcd, 1179.4.

23-(2,5-Dioxopyrrolidin-1-yloxy)-2,3-dimethyl-4,23-dioxo-11,14,17,20-tetraoxa-7,8-dithia-3-azatricosan-1-oic Acid, Maytansinol Ester. DM1-MAL-PEG$_4$-NHS (50). A solution of $N^{2'}$-deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)maytansine (DM1, 28.1 mg, 0.0381 mmol) was prepared in 0.50 mL of THF, and succinimidyl-[(*N*-maleimidopropionamido)tetraethyleneglycol] ester (**42**, NHS-PEG$_4$-maleimide, Quanta Biodesign, 39.1 mg, 0.0762 mmol) was added in 1.5 mL of 2:1 potassium phosphate buffer (50 mM, pH 6) and THF. The reaction proceeded for 1 h with stirring at room temperature, and TLC analysis indicated that the reaction was complete. The crude reaction mixture was purified by silica chromatography, eluting with 6% ethanol in methylene chloride. The solvent was removed under vacuum to give 9.6 mg (20.1% yield) of the desired product. ^{1}H NMR ($CDCl_3$, reference 7.26 ppm) δ 0.792 (3H, s), 1.192−1.274 (3H, m), 1.290 (3H, s), 1.311 (2H, d, J = 2.4 Hz), 1.452 (1H, m), 1.560 (1H, d, J = 13.6 Hz), 1.631 (3H, s), 2.167 (1H, dd, J = 11.6 and 2.6 Hz), 2.351 (1H, dd, J = 14.8 and 3.8 Hz), 2.437 (2H, m), 2.565−2.639 (2H, m), 2.787−2.902 (10H, m), 2.919−3.142 (5H, m), 3.192 (3H, s), 3.348 (3H, d, J = 1.6 Hz), 3.399 (2H, m), 3.484 (1H, d, J = 8.8), 3.523 (2H, m), 3.645 (13H, m), 3.708 (2H, m), 3.767 (1H, m), 3.833 (2H, t), 3.980 (3H, s), 4.272 (1H, t), 4.770 (1H, d, J = 12 Hz), 5.354 (1H, m), 5.644 (1H, m), 6.236 (1H, s), 6.362 (1H, broad s), 6.415 (1H, dd, J = 11.2 and 4 Hz), 6.625 (1H, s), 6.658 (1H, dd, J = 6.4 and 4.6 Hz), and 6.822 (1H, dd, J = 4 and 1.6 Hz). ESI MS: m/z found, 1273.5 $(M + Na)^+$, calcd 1273.5. HRMS: found, 1273.4637 $(M + Na)^+$; calcd, 1273.4605.

24-(2,5-Dioxopyrrolidin-1-yloxy)-2,3,7,7-tetramethyl-4,24-dioxo-12,15,18,21-tetraoxa-8,9-dithia-3-azatetracosan-1-oic Acid, Maytansinol Ester. DM4-MAL-PEG$_4$-NHS (51). In a 10 mL round-bottom flask, DM4 (340.8 mg, 0.437 mmol) and Mal-PEG$_4$-NHS (**42**) (247 mg, 0.480 mmol) were combined and dissolved in *N*,*N*-dimethylacetamide (2 mL). The reaction flask was equipped with a stir bar and the solution stirred as the reactants went into solution. *N*,*N*-Diisopropylethylamine (0.076 mL, 0.437 mmol) was then added. The flask was equipped with a septa, and the mixture was stirred at room temperature for ∼30 min. The crude reaction mixture appeared clear and dark orange. Upon reaction completion (as determined by HPLC) the reaction volume was reduced in vacuo to give a crude orange oil. No further workup was performed. The crude material was dissolved in a minimal volume of CH_2Cl_2 and purified by SiO_2 chromatography, eluting with 6% EtOH in CH_2Cl_2. Then 5 mL fractions were collected and checked for product by TLC. Pure-product-containing fractions were combined and concentrated in vacuo under a high vacuum (using an individual pump). ^{1}H NMR ($CDCl_3$, reference 7.26 ppm) δ 0.796 (3H, s), 1.200−1.360 (14H, m), 1.424−1.489 (1H, m), 1.578 (1H, d, J = 13.6 Hz), 1.633 (3H, s), 1.798−2.094 (2H, m), 2.171 (1H, d, J = 13.2), 2.259−2.326 (1H, m), 2.426−2.472 (2H, m), 2.520 (1H, m), 2.577 (1H, m), 2.836 (4H, s), 2.879−2.906 (5H, m), 3.010 (1H, dd, J = 6 and 3.6 Hz), 3.073−3.167 (2H, m), 3.205 (3H, m), 3.345 (3H, s), 3.399 (2H, m), 3.486 (1H, d, J = 9.2 Hz), 3.536 (2H, t), 3.643 (12H, s), 3.648 (3H, s), 3.769 (2H, m), 3.838 (2H, t, J = 6.4 Hz), 3.978 (3H, s), 4.277 (1H, t, J = 10.4 Hz), 4.775 (1H, m), 5.345 (1H, m), 5.657 (1H, m), 6.246 (1H, s), 6.418 (2H, m), 6.646 (1H, dd, J = 1.2 and 6 Hz), 6.696 (1H, s), and 6.824 (1H, s). ESI MS: found 1315.6 $(M + Na)^+$, calcd 1315.5; found 1329.4 $(M + Cl)^-$, calcd 1329.3. HRMS: found 1315.5103 $(M + Na)^+$, calcd 1513.5075.

Preparation of Conjugates. Three antibodies, huC242, huMy9-6, and B38.1, were used to evaluate the utility of the linkers in conjugation with maytansinoids. The first two are humanized IgG1 antibodies that target CanAg expressed on the surface of human colorectal, pancreatic, and gastric cancer cells and CD33 expressed on myeloid leukemia cells, respectively. B38.1 is a chimeric human IgG1 targeting EpCAM expressed on various malignancies. The linker (**14**, **15**, **20**, **26**, **36**, or **42**) was dissolved in DMA (*N*,*N*-dimethylacetamide) at approximately 20 mM. For the antibody modification reaction, a solution of an antibody (8 mg/mL) was treated with 5−50 equiv of linker in a sodium phosphate buffer (pH 8.0 for linker **14**, **15**, and **26**; pH 6.5−7.0 for **20**, **36**, and **42**) with stirring in the presence of 5% (v/v) DMA. The reaction was allowed to proceed at ambient temperature for 10 min to 24 h. Unreacted linker was removed from the antibody by gel filtration using a Sephadex G25 column equilibrated in 150 mM potassium phosphate buffer containing 100 mM NaCl at pH 6.5 or pH 7.4. The extent of incorporation of S-Py linker was assessed by

release of pyridine-2-thione using 50 mM DTT and measuring the absorbance at 343 nm as described below (ε_{343} = 8080 M^{-1} cm^{-1} for free pyridine-2-thione). For the S-Py-NO_2 linker, modification was assessed directly by measuring the absorbance at 325 nm (ε_{325} = 10 964 M^{-1} cm^{-1} for the 4-nitropyridyl-2-dithio group linked to antibody). For the conjugation reaction, thiol-containing maytansinoid (either DM1 or DM4) was dissolved in DMA at approximately 10 mM. The maytansinoid (0.8- to 1.7-fold molar excess relative to the number of linker molecules) was slowly added with stirring to the antibody which was at 2.5 mg/mL in sodium phosphate (pH 6.5−7.0) in a final concentration of 3% (v/v) DMA. The reaction was allowed to proceed at ambient temperature for 2−12 h. Conjugated antibody was purified using a Sephadex G25 column equilibrated with sodium phosphate buffer (pH 6.5). The number of molecules of maytansinoid incorporated per antibody molecule was assessed by measuring A_{252} and A_{280} of the conjugate as described below.

Measurement of Releasable Pyridine-2-Thione and Antibody Concentration of Linker-Modified Antibodies. The molar ratio of pyridine-2-thione linked per mole of antibody is determined by measuring the A_{280} of the modified antibody and then the increase at A_{343} following the addition of DTT (50 μL of 1 M DTT/mL of sample). The concentration of DTT-released pyridine-2-thione is then calculated using an ε_{343} of 8080 M^{-1} cm^{-1}. The concentration of antibody can then be calculated using an ε_{280} of 194 712 M^{-1} cm^{-1} after subtracting the contribution of pyridine-2-thione absorbance at 280 nm (A_{343nm} post-DTT × 5100/8080) from the total A_{280nm} measured before DTT addition. The molar ratio of pyridine-2-thione/antibody can then be calculated. The mg/mL (g/L) concentration of antibody is calculated using a molecular weight of 147 000 g/mol.

Measurement of Antibody-Linked 5-Nitropyridyl-2-dithio Groups and Antibody Concentration of a Linker Modified Antibody. The molar ratio of the 4-nitropyridyl-2-dithio groups linked per mole of antibody is calculated by measuring the A_{280} and A_{325} of the modified antibody without DTT treatment. The number of antibody-bound 4-nitropyridyl-2-dithio groups is calculated using an ε_{325nm} of 10 964 M^{-1} cm^{-1}. The concentration of antibody can then be calculated using an ε_{280nm} of 194 712 M^{-1} cm^{-1} after subtracting the contribution of the 5-nitropyridyl-2-dithio group absorbance at 280 nm (A_{325nm} × 3344/10964) from the total $A_{280\ nm}$ measured. The molar ratio of 4-nitropyridyl-2-dithio groups/antibody can then be calculated. The mg/mL (g/L) concentration of antibody is calculated using a molecular weight of 147 000 g/mol

Calculating Antibody and DMx Component Concentrations of an AMC. The antibody and DMx both absorb at the two wavelengths used to measure each component separately, i.e., 280 and 252 nm. The components are quantified using the following algebraic expressions which account for the contribution of each component at each wavelength (C_{Ab} is the molar concentration of antibody, and C_D is the molar concentration of DM1):

$$\text{total } A_{280} = 194712C_{Ab} + 5700C_D \quad (1)$$

$$\text{total } A_{252} = (194712 \times 0.37)C_{Ab} + (4.7 \times 5700)C_D \quad (2)$$

Each equation is solved for C_{Ab}:

$$C_{Ab} = (A_{280} - 5700C_D)/194712 \quad (1a)$$

$$C_{Ab} = (A_{252} - 26790C_D)/72043 \quad (2a)$$

An equality is set up (eq 1a = eq 2a) and solved for C_D:

$$C_D = (A_{252} - 0.37A_{280})/24681$$

Once the C_D is calculated, the value is used to solve for C_{Ab} in eq 1a (or eq 2a) above. The ratio of DMx/antibody (DAR) can then be calculated. The mg/mL (g/L) concentration of antibody is calculated using a molecular weight of 147 000 g/mol. The concentration of DM1 is calculated using a molecular weight of 736.5 g/mol (linked DM1), and the concentration of DM4 is calculated using a molecular weight of 778.5 g/mol (linked DM1).

General One-Step Conjugation Procedure. An amount of 1.0 equiv of linker (**14**, **15**, **26**, or **36**, ∼2 mM) in DMA was added to PBS buffer (pH 6.0) containing 1.5 equiv of DM1 or DM4, and the mixtures were incubated for 45−120 min at 4−20 °C. Then 0.1−0.25 equiv of mAb in a buffer solution (∼pH 8) was added to the linker−drug mixture and the final pH adjusted to 7.0−8.0. After incubating for 2−24 h at room temperature, the mixture was purified using a Sephadex G25 column equilibrated with PBS, pH 6.5. The number of maytansinoid molecules incorporated was determined as described above. For conjugation of AMC with PEG linkers, the maytansinoid−linker moiety (**48**, **50**, or **51**, 2−10 equiv) was added to a PBS solution (pH ∼7) containing an antibody. After incubating for 2−24 h at room temperature, the mixture was purified using a Sephadex G25 column equilibrated with PBS, pH 6.5.

Analytical (MS) Assay of the Conjugate. AMCs at 1 mg/mL were deglycosylated with PNGase F (New England BioLabs, Ipswitch, MA) using 2 units/μg of protein. The samples were incubated overnight at 37 °C, after which they were analyzed using a TSK gel Super 3000 SW size-exclusion column (4.6 mm × 30 cm, 4 μm particle size, 250 Å pore size) from Tosoh Biosciences (Montgomeryville, PA), installed on a UPLC chromatographic system (Waters, Milford, MA). The column was maintained at 60 °C and operated with a mobile phase composition of 50% acetonitrile, 1% formic acid, 0.02% TFA with a flow rate of 0.25 mL/min. The outlet of the column was split and $^1/_5$ of the flow was directed toward the electrospray source of a LCT time-of-flight mass spectrometer (Waters). The raw mass spectra were acquired using an m/z range between 1000 and 4500. The spectra originating from the immunoconjugates were summed and deconvoluted using MaxEnt1 deconvolution algorithm (Waters).

In Vitro Cytotoxicity Assays. The cell lines used in the cytotoxicity assays were HCT-15 (ATCC CCL-225), a human colon tumor multidrug resistant cell line; HL60/QC, a variant of the human promyelocytic leukemia cell line HL60; COLO 205 (ATCC CCL-222), a human colon tumor cell line; Ramos (ATCC CRL-1923), a human Burkitt's lymphoma cell; and COLO205/MDR, a clone of the COLO 205 cell line retrovirally transduced with the mdr1 gene.[42] HCT-15 cells were grown in RPMI-1640 (catalog no. 30-2001, ATCC, Manassas, VA) supplemented with 10% fetal bovine serum and gentamycin. All other cell lines were grown in RPMI-1640 (catalog no. 11875-085, Invitrogen), supplemented with 10% fetal bovine serum and gentamycin. The cells were maintained at 37 °C in a humidified atmosphere that contained 6% CO_2.

The cytotoxicity study was performed using a WST-8 dye-based cell survival assay as previously described.[42]

In Vivo Evaluation. SCID mice were inoculated subcutaneously in the right flank with COLO 205/MDR cells (1.5 × 10^6 cells/mouse) in 0.1 mL of serum-free medium. The tumors were grown for 8 days to an average size of about 100 mm^3, and the animals were then randomly divided into several groups (5−6 animals per group). The first group of mice served as the control group and was treated with the phosphate-buffered saline vehicle. The remaining groups were treated with a single injection of the following conjugates at a maytansinoid dose of 170 μg/kg: (a) B38.1-SMCC-DM1, (b) B38.1-PEG4-Mal-DM1, (c) B38.1-SPDB-DM4, and (d) B38.1-2-sulfo-SPDB-DM4 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.

■ ASSOCIATED CONTENT

Supporting Information. HPLC purity data and UV chromatographic profiles for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

ADC, antibody−cytotoxic drug conjugate; BCRP, breast cancer resistance protein; DAR or D/A, drug/antibody ratio; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMA, dimethylacetamide; DME, 1,2-dimethoxyethane; DMx, DM1 and/or DM4; DTT, dithiothreitol; ε, molar absorbance coefficient; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; GSH, reduced glutathione; mAb, monoclonal antibody; MDR, multidrug resistance; MOAT, multispecific organic anion transporter; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NHS, *N*-hydroxysuccinimide; PBS, phosphate buffer saline; Pgp, P-glycoprotein; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SPDB, *N*-succinimidyl 4-(2-pyridyldithio)butanoate; SPDP, *N*-succinimidyl 4-(2-pyridyldithio)propanoate

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