Design, Synthesis, and in Vitro Evaluation of Dipeptide-Based Antibody Minor Groove Binder Conjugates

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Antibody−drug conjugates (ADCs) were prepared consisting of DNA minor groove binder drugs (MGBs) attached to monoclonal antibodies (mAbs) through peptide linkers designed to release drugs inside the lysosomes of target cells. The site of linker attachment on the MGB was at the 5-position on the B-ring, since model studies showed that attachment of an electron-withdrawing group (i.e., acyl, carbamoyl) at this position increased the stability of the molecule. Because of the hydrophobic nature of the MGBs, several measures were required to overcome their tendencies to induce mAb aggregation upon conjugation. This is exemplified in the series of ADCs containing the amino-CBI drug **1**. Initial adducts were prepared using the peptide sequence valine−citrulline, attached to a self-immolative *para*-aminobenzyl carbamate spacer. The resulting ADCs were completely aggregated. Removal of the self-immolative spacer, introduction of a more hydrophilic valine−lysine sequence, and incorporation of a tetraethyleneglycol unit between the mAb and the peptide resulted in conjugates that were nonaggregated, even with as many as eight drugs per mAb. These results were extended to include the hydroxy aza-CBI drug **2**, which was linked to the valine-lysine sequence through a *para*-aminobenzyl ether self-immolative spacer. The resulting mAb conjugates were monomeric and released the hydroxy aza-CBI drug upon treatment with human cathepsin B. In vitro cytotoxicity assays established that the mAb−MGB drug conjugates were highly cytotoxic and effected immunologically specific cell kill at subsaturating doses. The results provide a general strategy for MGB prodrug design and illustrate the importance of linker hydrophilicity in making nonaggregated, active mAb−MGB conjugates.

Introduction

The effectiveness of drugs for cancer chemotherapy generally relies on differences in growth rates, biochemical pathways, and physiological characteristics between cancer and normal tissues. Consequently, most standard chemotherapeutics are relatively nonspecific and exhibit dose-limiting toxicities that contribute to suboptimal therapeutic effects. Considerable effort has been directed toward the use of monoclonal antibodies (mAbs) for targeted drug delivery due to their high selectivities for tumor-associated antigens, favorable pharmacokinetics, and relatively low intrinsic toxicities. The mAb−drug conjugates (ADCs) are formed by covalently linking anticancer drugs to mAbs, usually through a conditionally stable linker system. Upon binding to cell surface antigens, mAbs used for most ADCs are actively transported to lysosomes or other intracellular compartments, where enzymes, low pH, or reducing agents facilitate drug release.[1,2]

The only clinically approved ADC is Mylotarg, which is used in the treatment of acute myeloid leukemia.[3,4] This drug is comprised of an anti-CD33 mAb linked to the highly potent DNA minor groove binder calecheamicin through an acid-labile hydrazone linker. Several other ADCs are in various stages of preclinical or clinical development. These include conjugates of doxorubicin,[5,6] a maytansine derivative,[7] potent taxol derivatives,[8] conjugates of CC-1065 and duocarmycin minor groove binders,[9−11] and the auristatin antimitotic drugs.[12,13] Collectively, these studies have led to the identification of such issues as drug potency, linker stability, and efficient release of active drug at the tumor site that affect ADC efficacy, potency, and toxicity. We have recently reported the effects of ADCs consisting of the antimitotic drug monomethylauristatin E (MMAE), which incorporated an enzymatically labile dipeptide linker connected to MMAE through a self-immolative *para*-aminobenzyl carbamate (PABC) spacer.[12,13] Upon treatment with a lysosomal enzyme such as cathepsin B, the bond between the peptide and the PABC group was hydrolyzed, leading to release of MMAE. The conjugates were potent, selective, and highly active in carcinoma and hematologic xenograft models. Most importantly, cures and regressions of established tumors were obtained without toxicity, in some cases as low as $^1/_{60}$ the maximum tolerated dose (MTD).

We wished to extend these findings to include drugs with complementary mechanisms of activity. The cyclopropylindole DNA minor groove binder (MGB) alkylating agents attracted our interest due to their high potencies, mechanisms of activity, circumvention of common MDR mechanisms, and compatibility with the linker strategy developed for MMAE. Representative

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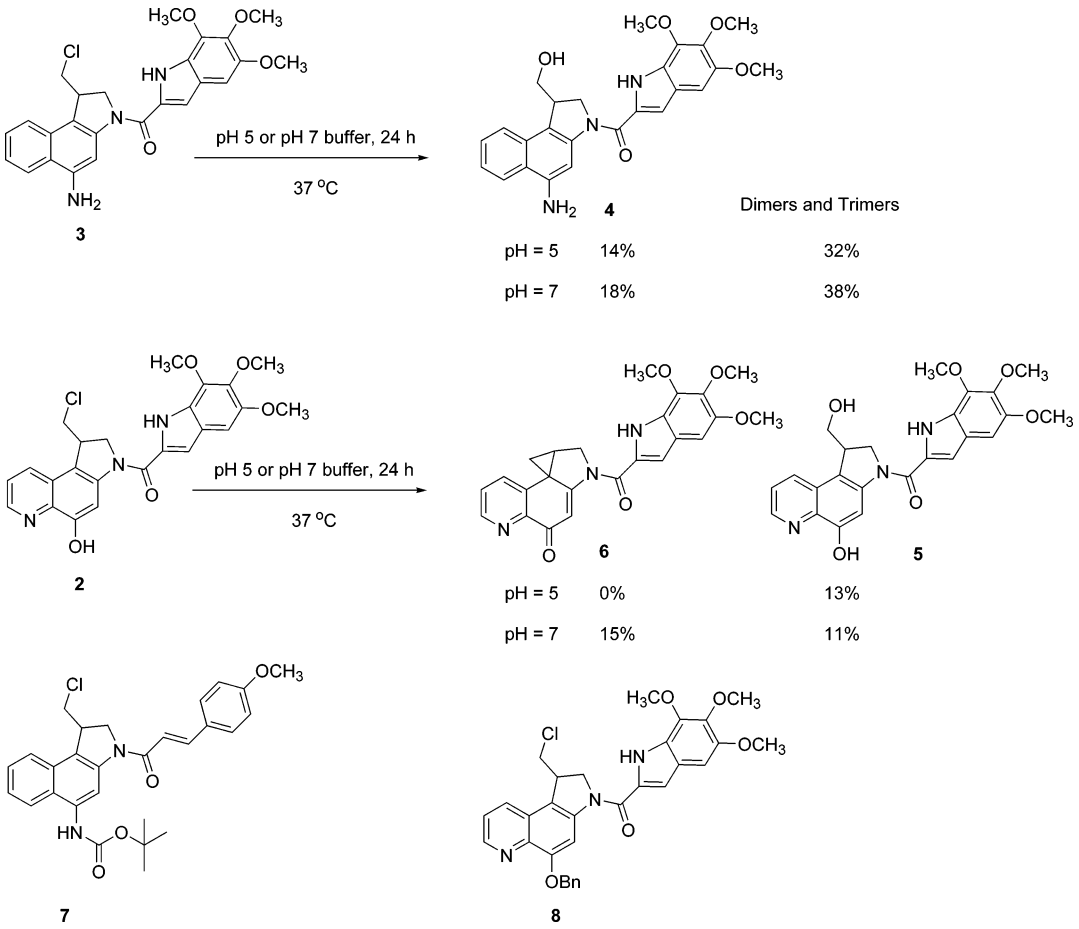
Scheme 1



MGBs include such natural products as CC-1065,[14] the duocarmycins,[15] and synthetic analogues such as the racemic seco chloride amino-CBI compound (**1**)[16,17] and racemic seco chloride hydroxy aza-CBI compound (**2**).[18] It has previously been shown that ADCs of CC-1065 analogues displayed both in vitro and in vivo activity.[9−11] This was in spite of the fact that the reactivity of the alkylating portion of the drug was not attenuated, making the drug susceptible to hydrolysis, alkylation, and inactivation while still attached to the mAb. In addition, the linker between the drug and the mAb involved a sterically hindered disulfide. Linkers of this class are known to have limited stability in serum.[19] The approach we describe here employs a linking strategy that inactivates the MGB while it is conjugated to the mAb and uses a peptide linker with high serum stability. Since this class of drugs is quite hydrophobic, particular attention was focused on developing hydrophilic peptide-linker derivatives that allowed conjugates to be produced without causing mAb aggregation. The resulting monomeric ADCs exhibited potent and specific in vitro cell kill. The approaches described should be broadly applicable to the general class of seco halide CC-1065-like MGBs.

Results

Design of Drug−Linker Constructs. Compounds **2** and **3** proved to be unstable in aqueous solutions when incubated at 37 °C (Scheme 1). The amino-CBI **3** underwent significant decomposition at both pH 5 and 7 (>25% in 24 h), forming the hydroxymethyl compound **4** as well as higher order species, which were assigned as dimers and trimers according to LC−MS analysis. The hydroxy aza-CBI **2** was also susceptible to hydrolysis (**5**) and, in addition, formed the cyclopropyl adduct **6**, through the loss of HCl. Presumably, the hydroxy compound **5** is formed via hydrolysis of the cyclopropyl intermediate **6**. Similarly, compound **4** is likely formed through its corresponding cyclopropyl adduct.

These data prompted us to devise methods of linking the drugs to mAbs in a manner that would significantly attenuate unwanted drug reactivity, since mAb−drug conjugates may circulate in blood for several days before binding to tumor cells.[1,2] Functional groups that inhibit cyclopropyl group formation are known to significantly reduce MGB reactivity and, as a consequence, drug potency.[20,21] To test this with the MGB classes described here, we prepared the *tert*-butyl carbamate **7** from **1** and analyzed this compound, as well as the benzyl ether **8**, to determine their stabilities in aqueous buffers at pH 7. Both of these compounds were highly stable, giving <5% hydrolysis in 24 h. Thus, significant attenuation of MGB chemical reactivity is achieved by alkylation or acylation of the activating heteroatom at the drugs' 5-position.

On the basis of the stability data, three different strategies for linking MGBs to mAbs, as depicted in Figure 1, were explored. Approach A incorporates a *para*-aminobenzyl carbamate (PABC) self-immolative spacer between the enzymatically reactive peptide linker and the drug. This approach has previously been used for the release of doxorubicin,[22,23] MMAE,[12,13] and

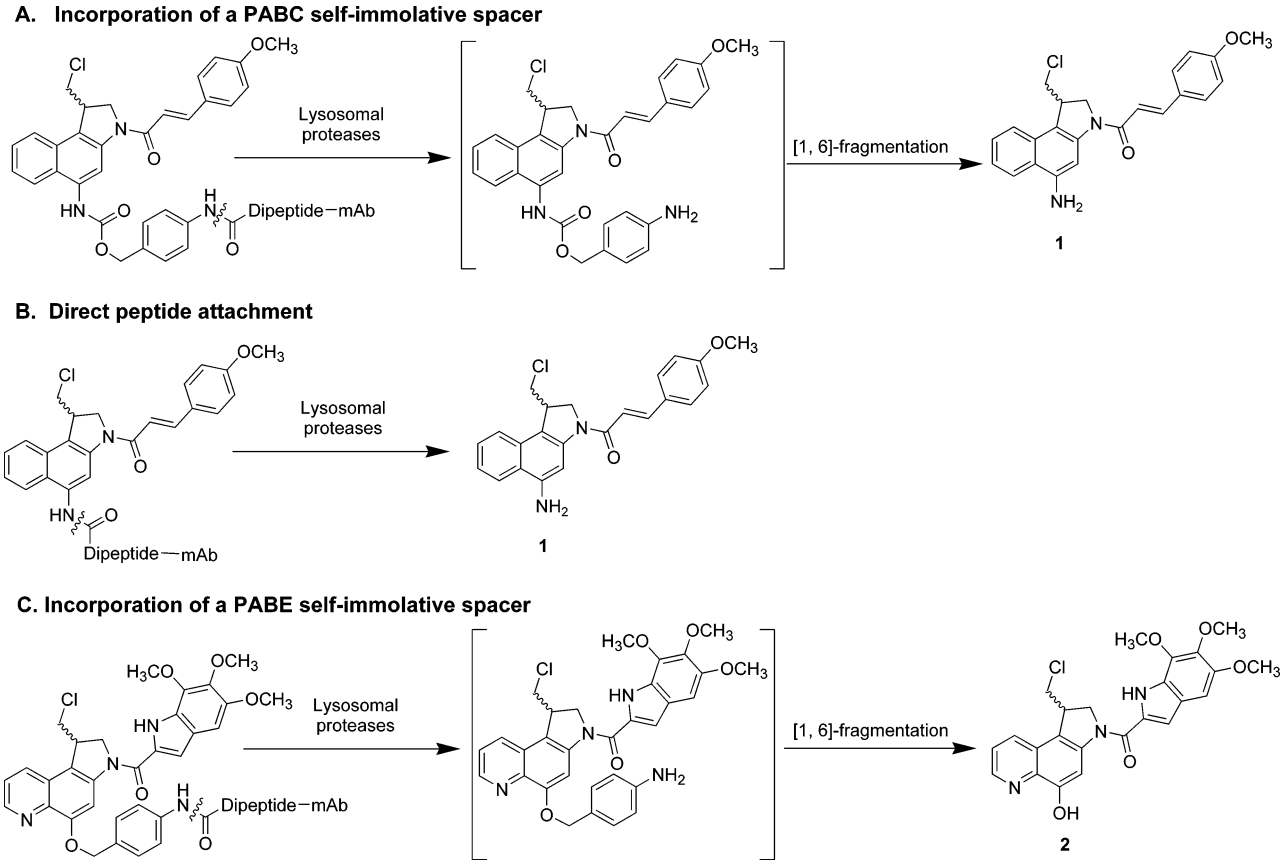


Figure 1. General approaches for the attachment of MGBs to mAbs. A *para*-aminobenzyl carbamate (PABC)-containing self-immolated spacer (A) was used for the release of the amino-CBI **1** through proteolytic cleavage followed by 1,6-elimination. Direct attachment of a dipeptide (B) was anticipated to release **1** directly. The *para*-aminobenzyl ether (PABE) self-immolative spacer was employed (C) for the release of the hydroxy aza-CBI **2**.

camptothecin[24] from ADCs upon treatment with lysosomal preparations or purified lysosomal enzymes. The PABC spacer was incorporated into these ADCs to minimize the effects of drug steric interactions on peptide bond hydrolysis. In approach B, the drug is linked directly to the peptide without the use of a self-immolative spacer. It was reasoned that the aniline amide would provide high drug stability while attached to the mAb and that the drug could be proteolytically cleaved, since the aminonaphthalene leaving group in the amino-CBI class is structurally similar to the PABC spacer aniline in approach A. In approach C, a *para*-aminobenzyl ether (PABE) group is used as a self-immolative spacer between the drug and the peptide. This approach extends previous work from our laboratory showing that *para*-amidobenzyl ethers of phenolic drugs were fragmented upon amide bond hydrolysis—leading to the release of the free phenolic drug.[25] This approach should be broadly applicable to seco halide CC-1065-like MGB drugs, since the vast majority have a phenolic hydroxyl group as the activating heteroatom.[26]

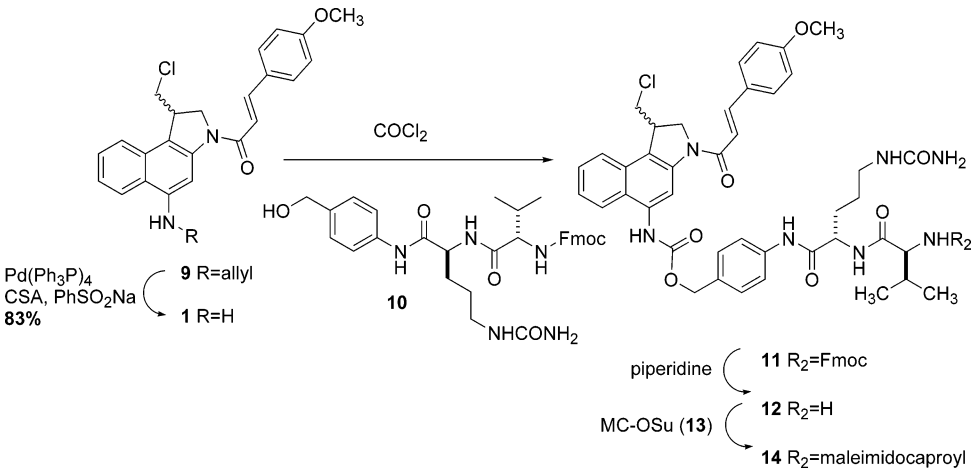
Approach A: PABC Constructs Using Amino-CBI. The synthesis of amino-CBI **1** was carried out by modification of a previously described route.[27] The final step in the synthesis of **1** involves removal of the allyl protecting group from the aniline nitrogen of **9**, a reaction that is reported to occur in low yields (42%) using Grubbs' catalyst. When a palladium catalyst for allyl group removal was used,[28] it was possible to improve the yield of **1** to 83%. Incorporation of the PABC-linker moiety was accomplished as shown in Scheme 2. Briefly, the amino-CBI compound **1** was reacted with excess phosgene to form the corresponding isocyanate in situ, and **10** was added providing the carbamate **11**. Removal of the Fmoc group gave **12**, which was coupled directly to the *N*-hydroxysuccinimide ester of maleimidocaproic acid (MC-OSu, **13**) to afford the final product **14**.

Approach B: Directly Attached Constructs Using Amino-CBI. Dipeptide drug−linkers lacking the *para*-aminobenzyl carbamate were prepared according to the synthetic route shown in Scheme 3. Coupling of the Fmoc-protected amino acid residues **15** and **16** to **1** was accomplished using a HATU/HOAt coupling system[29,30] to provide **17a** and **17b**. The Fmoc group was cleaved, yielding the free amines **18a** and **18b**. A second peptide-coupling reaction, using Fmoc-Val-OSu (**19**), afforded **20a** and **20b**, and amine group deprotection gave the dipeptides **21a** and **21b**. Compounds **21a** and **21b** were reacted with MC-OSu (**13**) to provide **22a** and **22b**, respectively. Deprotection of **22a** in cold TFA afforded the desired product **23**.

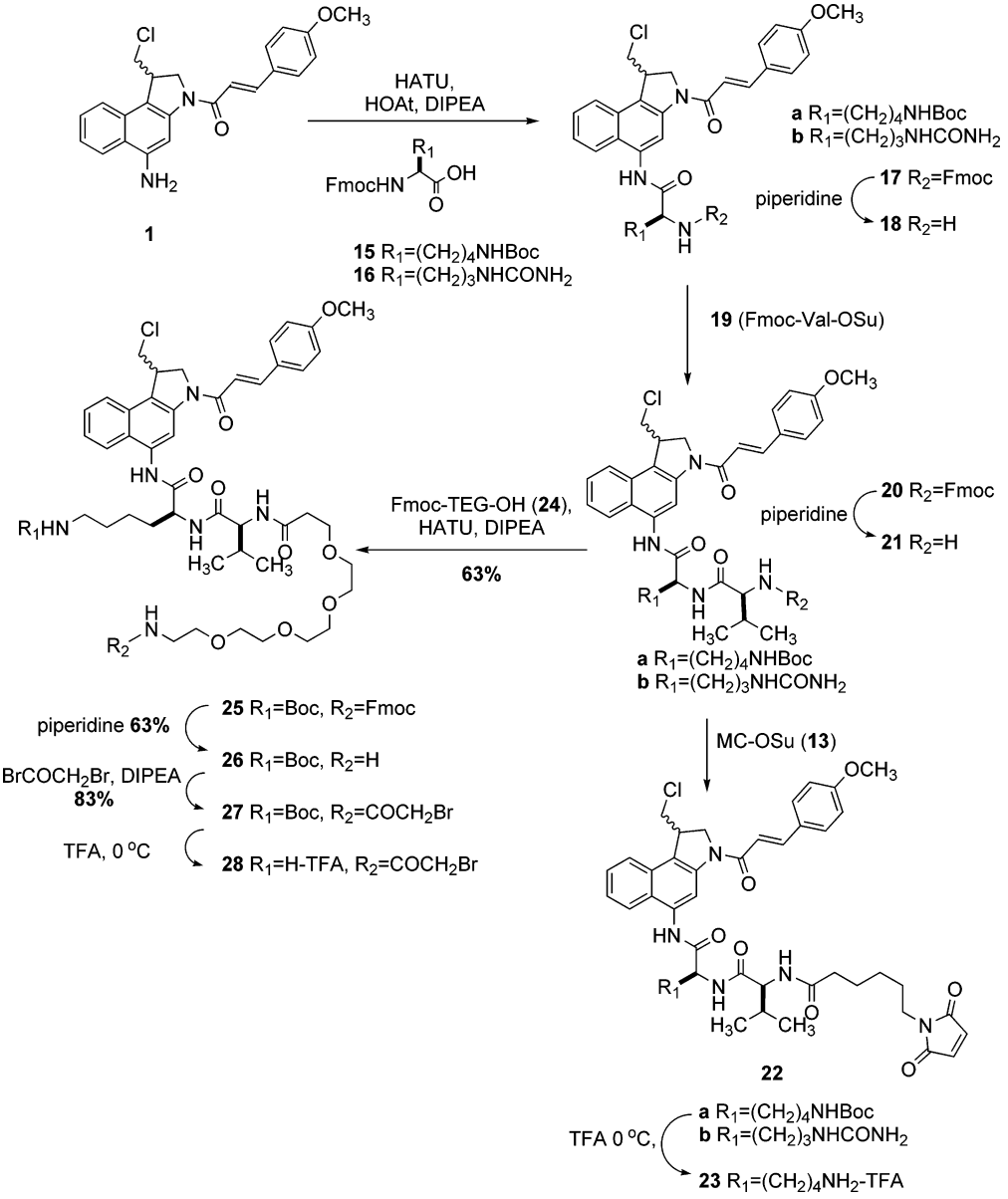
A compound with potentially greater water solubility was formed from **21a** using the tetraethyleneglycol (TEG) acid **24** as a more hydrophilic spacer than maleimidocaproyl. This yielded **25**, which was subsequently deprotected to provide **26**. Treatment of this compound with bromoacetyl bromide gave **27**, and this was followed by removal of the Boc group which afforded **28**.

Ultimately it was discovered that the bromoacetamide functional group used in **28** was less suitable than a

Scheme 2



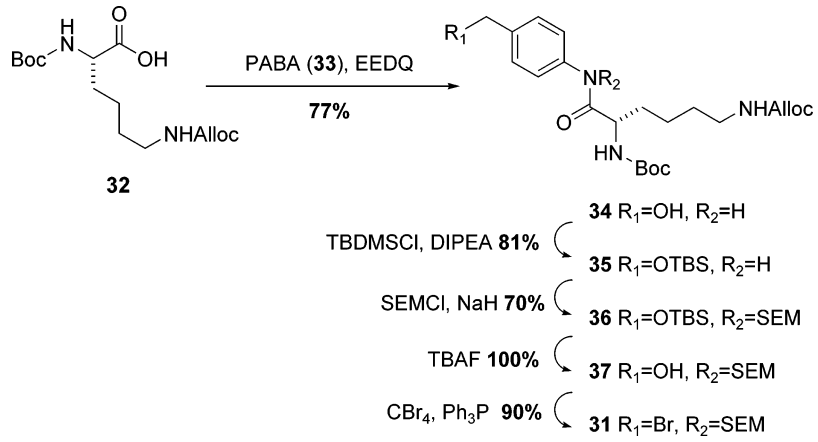
Scheme 3



maleimide for antibody thiol alkylation. This was due to its instability at pH 9, the pH required for mAb alkylation. The maleimide-containing drug−linkers could be reacted with reduced antibodies under essentially neutral conditions and were stable. This resulted in more efficient loading of the mAb with drug-linker.

Approach C: PABE Construct Using Hydroxy Aza-CBI. A third approach for preparing a MGB

Scheme 4



derivative for mAb attachment involved O-alkylation of molecules in the hydroxy aza-CBI family to form ethers. The key step in the synthesis of **29** involved formation of an ether bond through O-alkylation of aza-CBI core **30** with the bromide **31**. The synthesis of bromide **31** began with coupling of the protected lysine acid **32** to *para*-aminobenzyl alcohol (**33**) using EEDQ as a coupling reagent (Scheme 4).[31] Next, the alcohol **34** was converted to the TBS ether **35**.[32] This was followed by alkylation of the anilide nitrogen of **35** with SEMCl and sodium hydride[33] to afford the fully protected adduct **36**. Removal of the TBS protecting group with TBAF afforded the benzyl alcohol **37**, which was converted to the desired benzyl bromide **31** using carbon tetrabromide and triphenylphosphine. The SEM protecting group served to stabilize **31** against base-promoted 1,6-elimination of bromide.

Coupling of **31** to **30** (prepared through hydrogenation of **38**[18]) proceeded smoothly using potassium carbonate as base, to give the key intermediate **39** in good yield (Scheme 5). Both the SEM and Boc protecting groups were removed through treatment of **39** with ice-cold trifluoroacetic acid, resulting in the formation of the hydroxymethyl adduct **40**, as determined by LC−MS analysis. Workup with aqueous ammonia resulted in the formation of the free anilide **41**. The primary amino group proved to be more nucleophilic than the secondary aniline, allowing for selective coupling with Fmoc-Val-OSu (**19**), which afforded **42**. Acylation of the secondary aniline of **42** with **43** led to the trimethoxyindole **44**. Transesterification in acidic methanol afforded the alcohol **45**, which was converted to the corresponding chloride **46** through treatment with carbon tetrachloride and triphenylphosphine.

A lysine residue ϵ-amine protecting group exchange was needed next. This would eliminate the need for purification of the final deprotected molecule. Removal of the Alloc group of **46** with a palladium catalyst gave **47**,[28] and the newly liberated ϵ-amine was capped using Boc-anhydride to afford **48**. Both steps were high-yielding.

Standard peptide coupling chemistry was all that was required to complete the synthesis of **29**. The removal of the Fmoc group of **48** gave **49**, and this was coupled to the tetraethylene glycol (TEG) acid **24** to afford **50** in high yield. The Fmoc group was removed to give **51**, and a reaction with MC-OSu (**13**) afforded the maleimide **52**. Finally, deprotection of the lysine ϵ-amine with cold TFA led to the formation of the target compound **29**.

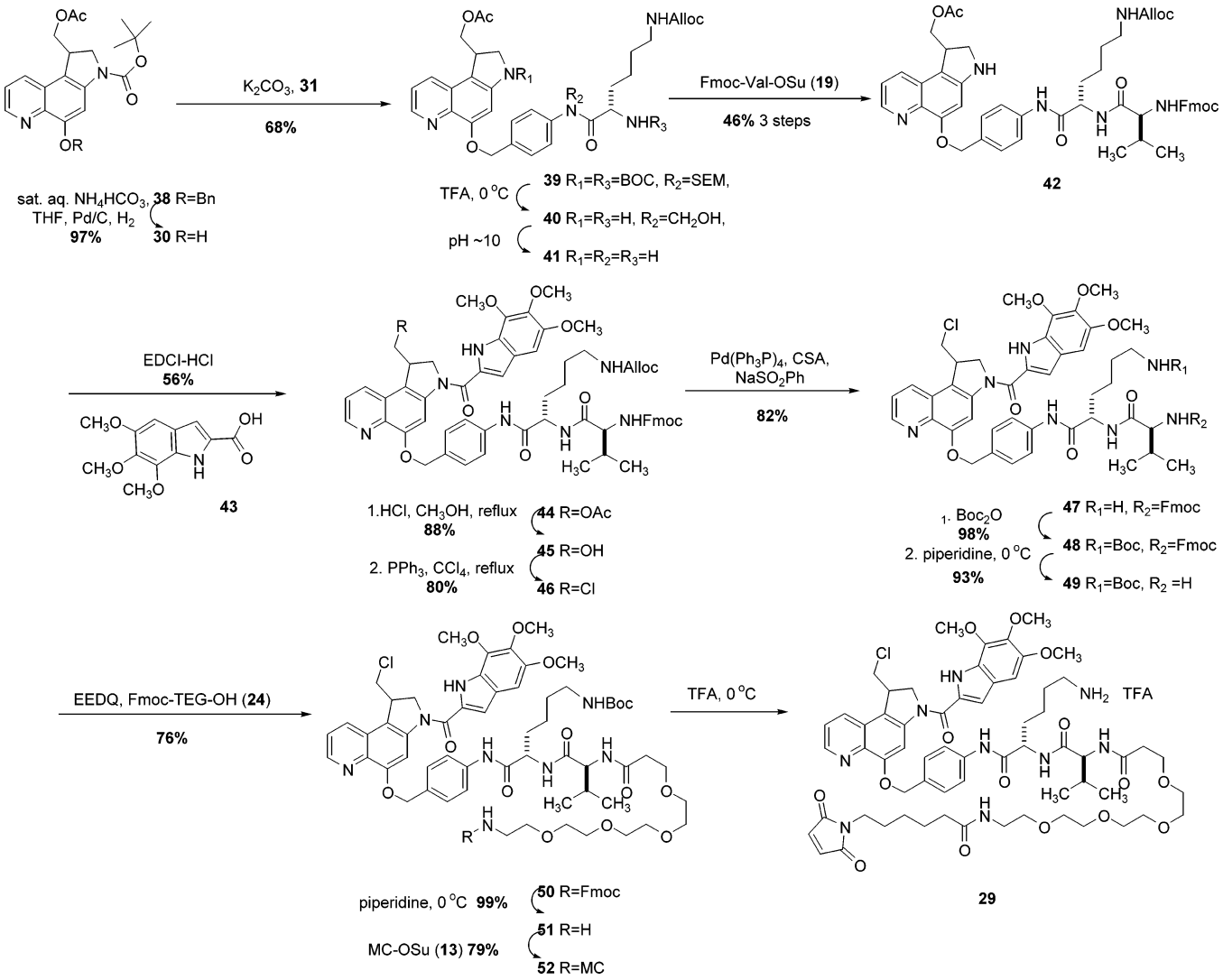
The purity of drug−linkers **14**, **22b**, **23**, **28**, and **29** was assessed by analytical HPLC analysis using both neutral and acidic mobile phases and a diode array detector. Each compound was obtained in high purity as was determined by analysis of each compound's chromatogram at multiple wavelengths.

Preparation of Immunoconjugates. The mAbs used in these investigations were cAC10, a chimeric IgG_1 against the CD30 antigen,[34] 1F6, a murine IgG_1 against the CD70 antigen,[35,36] and cBR96, a chimeric IgG_1 against the Le^y carbohydrate antigen.[5] Conjugates were formed as previously described[12,13] by reducing interchain disulfides under mild conditions with dithiothreitol (DTT), adding the drugs **14**, **22b**, **23**, **28**, and **29**, and purifying the resulting conjugate away from free drug either by size-exclusion or by hydroxyapatite chromatography. The extent of mAb reduction, and subsequent drug incorporation, was a function of how much DTT was used to reduce the interchain disulfides. For the cAC10 and cBR96 mAbs with four interchain disulfides, reduction methods allowed for an average of one to four disulfide bonds to be broken. Under nondenaturing conditions, the mAb chains remain tightly associated, even in the absence of intact disulfides (unpublished results).

The cAC10 conjugate derived from compound **14** was highly aggregated, even with low levels of drug substitution (Table 1). With two drugs per mAb, 48% of the conjugate was aggregated, and this level increased with increasing drug/mAb ratios. As with cBR96−doxorubicin conjugates,[22] the soluble aggregates were noncovalent according to SDS−PAGE (data not shown) and were most likely due to hydrophobic interactions between the drugs.[22] Attempts to circumvent aggregation by altering reaction conditions or using different buffers, cosolvents, and detergents were without success.

Incremental improvements in the aggregation state of the cAC10 mAb conjugates were obtained using drugs that were less hydrophobic than **14**. Conjugates formed from **22b**, a drug lacking the hydrophobic PABC spacer, also had a pronounced tendency to aggregate but to a slightly lesser degree than cAC10-**14**. Aggregation was reduced even further in cAC10−**23** conjugates, in which the citrulline residue in **22b** was replaced with a lysine.

Scheme 5



The aggregate content of cAC10−**23** with four drugs per mAb was only 22% compared to 44% with the corresponding citrulline conjugate cAC10−**22b**.

A significant improvement was realized with **28**, a drug that contains a hydrophilic TEG spacer between the thiol-reactive functionality and the Val-Lys dipeptide. The cAC10−**28** conjugates were formed with as many as eight drugs per mAb, representing maximal substitution of available cysteines, with only 5% aggregated protein. Similar results were obtained with conjugates of **29** which contained the hydrophobic PABE spacer. This further underscores the importance of the hydrophilic TEG linker in the formation of monomeric conjugates with high levels of drug loading.

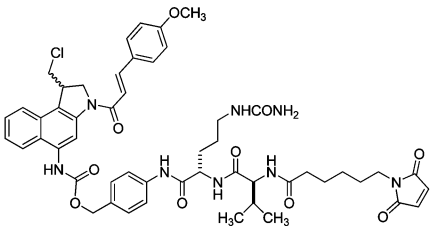
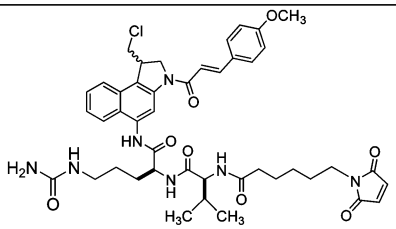
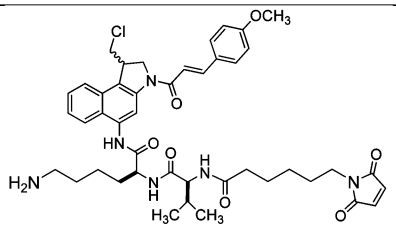
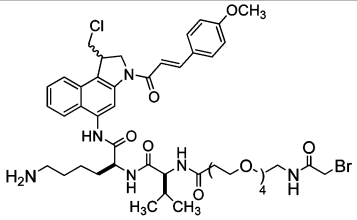
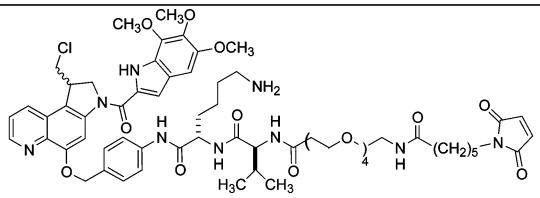
The tendency for a drug−linker to cause mAb aggregation was largely independent of the mAb. For example, conjugation of **14** to cBR96 gave essentially identical results to that described for cAC10. Similar to the cAC10 results, conjugation of compounds **28** and **29** to cBR96 gave little mAb aggregation.

Human Cathepsin B Mediated Cleavage. Exposure of conjugates cAC10−**28** and cAC10−**29** to human cathepsin B resulted in the observed loss of drug from only the cAC10−**29** conjugate (Figure 2). In both cases, drug loss from the conjugate was monitored by the change in 320 nm versus 280 nm absorbances (drug chromophore absorbance versus mAb absorbance) from the conjugates' SEC HPLC trace. After 6 h, approximately 30% of the drug was released from the cAC10−**29** conjugate, whereas no drug release was observed from cAC10−**28**.

The results with cAC10−**29** are consistent with cathepsin B cleavage experiments involving a cysteine-quenched derivative of **29**, in which efficient cleavage was observed (data not shown). In addition, observations previously published from this laboratory showed dipeptide-containing *para*-aminobenzyl ethers (PABE) of phenolic drugs were readily cleaved under the action of cathepsin B.[25] The failure of cAC10−**28** to cleave with cathepsin B is likely due to steric hindrance from the amino-CBI core. This result is in line with studies using a cysteine-quenched, close structural analogue of **28** (contained a phenylalanine−lysine dipeptide as opposed to the valine−lysine sequence in **28**) which was not a substrate for cathepsin B (data not shown). These results were extrapolated to other mAb conjugates of **28** and **29**.

In Vitro Cytotoxicity. Compounds **1** and **2** were highly cytotoxic on a panel of cancer cell lines (Table 2). The IC_{50} values are consistent with previous reports

Table 1. Structures of mAb−MGB ADCs and the Effects of Drug Loading on Aggregation

Compound	Drug-Linker Structure[a]	Free SH/ mAb	Drugs/mAb in monomer	% Aggregate[b]
cAC10-**14**		1.8	1.0	23
		3.0	2.0	48
		3.7	2.5	58
		4.5	2.5	68
		8.0	0	100
cAC10-**22b**		1.8	1.5	6
		3.0	3.3	27
		3.7	3.5	32
		4.5	4.0	44
		8.0	0	100
cAC10-**23**		1.8	1.5	1
		3.0	3.3	9
		3.7	3.5	13
		4.5	4.0	22
		8.0	0	100
cAC10-**28**		8.0	8.0	5
cAC10-**29**		8.0	8.0	0

[a] The drug was attached to mAb sulfhydryl groups through the formation of a maleimido−thioether bond. [b] Aggregates consisted of mAb dimers and higher species as determined by size-exclusion HPLC.

for these molecules[16,18] and are 50- to 200-fold more potent than mitomycin C, a clinically approved minor groove binding agent.

The activities of mAb−**28** and mAb−**29** conjugates against two different cell lines are shown in Figure 3. The cAC10 conjugates (anti-CD30) and 1F6 conjugates (anti-CD70) were specifically cytotoxic against CD30-positive Karpas human anaplastic large cell lymphoma cells (Figure 3A) and Caki-1, a CD70-positive human renal cell carcinoma line (Figure 3B), respectively. The conjugates' cytotoxic effects were immunologically specific, since nonbinding control conjugates were significantly less cytotoxic than corresponding conjugates that bound to cell-surface antigens. On the basis of drug concentration, the binding conjugates and free drugs were nearly equipotent, indicating that the efficiency of drug delivery by the conjugates was not reduced compared to the corresponding free drugs. Neither cAC10 nor m1F6 displays significant cytotoxic properties in their unconjugated form (data not shown).

Additional studies were undertaken in the human small cell lung carcinoma cell line NCI-H69 and a drug resistant variant of this cell line, NCI-H69/LX4 that was selected after prolonged exposure to doxorubicin.[37] FACS analysis indicated that the variant cell line overexpressed P-glycoprotein (P-gp), accounting for its 24-fold resistance to doxorubicin compared to the parental cell line (Table 3). As shown, P-gp did not impart significant resistance to the alkylating agent cisplatin or to **1**. Consistent with this, drug resistant NCI-H69/LX4 cells were sensitive to BR96−**28** and in fact were slightly more sensitive to the conjugate than the parental cell line. While this may reflect the 2.9-fold difference in Lewis-Y expression, the results indicate

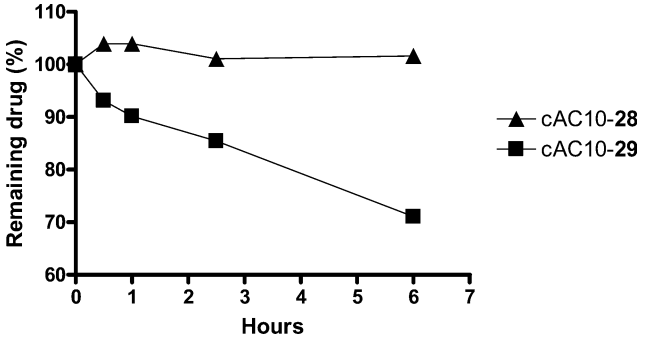


Figure 2. Hydrolysis of cAC10−**28** and cAC10−**29** by human cathepsin B. Conjugates were added to activated enzyme followed by incubation at 37 °C (pH 5). Drug release from the conjugates was monitored by SEC HPLC (320 nm) relative to mAb absorbance (280 nm).

Table 2. Cytotoxic Activities of Amino-CBI (**1**) and Hydroxy Aza-CBI (**2**) on Human Karpas 299 Anaplastic Large Cell Lymphoma and Human Caki-1 Renal Cell Carcinoma Cell Lines[a]

	IC_{50} (nM)			
	Karpas 299 (CD30+)		Caki-1 (CD70+)	
free drug	4 h	96 h	4 h	96 h
1	0.34 (1)	1.17 ± 0.24 (9)	0.59 (1)	0.68 (1)
2	0.28 (1)	0.18 (1)	0.55 (1)	0.45 (1)
mitomycin C	525 (1)	133 ±12 (39)	251 (1)	40 ± 6.2 (14)

[a] Values are the mean ± SEM (number of experiments).

that targeted MGBs might not be susceptible to at least one common form of multidrug resistance.

Discussion

Several recent studies have provided convincing evidence that drug potency can strongly influence ADC activity, particularly in the treatment of solid tumors. This is exemplified by comparing mAb−doxorubicin conjugates[5] with corresponding mAb−auristatin conjugates.[12,13] It was shown that the highly potent auristatin ADCs effected cures and regressions of established tumors at doses that were not only well-tolerated but were $^1/_{100}$ of that needed to achieve similar levels of efficacy with the doxorubicin conjugates. These results prompted us to investigate other highly potent drug classes, specifically the MGBs **1** and **2** described in this paper.

MGBs are of great interest for targeted drug delivery. In general, these drugs are highly potent, amenable to a variety of chemical manipulations, and are active against many cancer cell lines. Previously, Chari and co-workers[9] described mAb−MGB conjugates that were efficacious against metastatic human B-cell lymphoma (Namalwa) at doses just below the MTD. This was in spite of the fact that the ADCs contained a labile

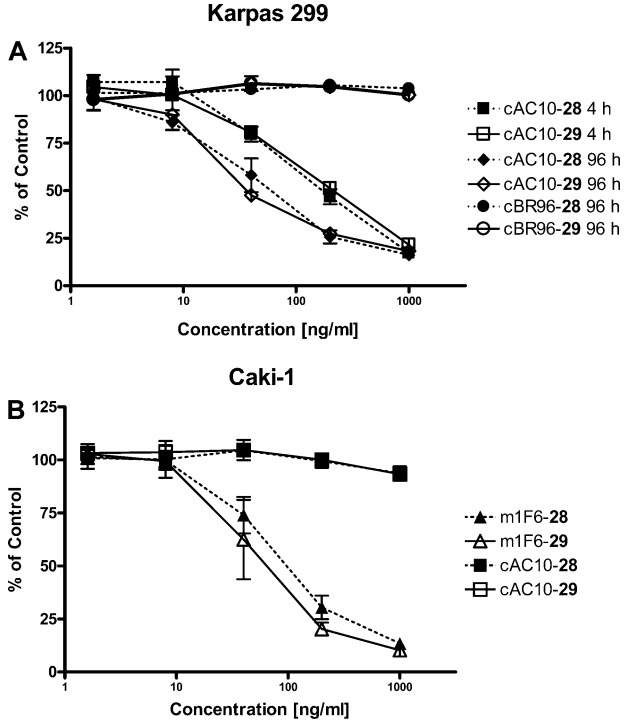


Figure 3. Cytotoxic activities of ADCs on cancer cell lines. (A) Karpas 299 cells (CD30+) were treated with the anti CD30 ADCs, cAC10−**28**, and cAC10−**29**, for 4 and 96 h, and the cytotoxic effects were compared to cells treated with the nonbinding control conjugates cBR96−**28** and cBR96−**29**. (B) Cytotoxic activities of murine 1F6−**28** and 1F6−**29** conjugates on Caki-1 cells (CD70+) were treated for 4 h with the anti-CD70 ADCs, 1F6−**28**, and 1F6−**29**, and the activities were compared to nonbinding control conjugates cAC10−**28** and cAC10−**29**. Results are shown as mean values ± SD.

disulfide linker bridging the drug to the mAb, and the drug was not inactivated while attached to the mAb. In developing newer generation mAb−MGB conjugates, we felt that it was critical to incorporate linker technologies that were considerably more stable than hindered disulfides and to attach the drug in a manner that suppressed reactivity while attached to the mAb carrier.

We have demonstrated that two classes of MGBs, amino-CBIs as represented by **1** and hydroxyl aza-CBI as represented by **2**, were labile in the free forms but were considerably more stable when the activating heteroatoms were modified—either acylated in the case of the **1** or alkylated in the case of **2** (Scheme 1). Consequently, peptide-containing linkers were attached to the activating heteroatoms, leading to ADCs with or without self-immolative spacers. The mode of attachment of the self-immolative spacer to the drugs was either through a carbamate bond to the amino-CBI

Table 3. Cytotoxic Activities of Amino-CBI **1** and CBR96−**28** on MDR^+ Cells[a]

	MFI[b]		IC_{50} (n = 3) (nM)			
cell line	Le^Y	P-gp	Dox[c]	cisplatin	**1**	cBR96−**28**[f]
NCI-H69[d]	224	14	11.3 ± 1.6	683 ± 185	0.87 ±0.61	13.4 ± 5
NCI-H69/LX4[e]	640	78	1800 ± 400	2820 ± 637	2.87 ± 0.64	20.7 ± 6
fold difference	2.9	5.6	74	4.1	3.3	1.54

[a] Values are the mean ± SEM. [b] Mean fluorescence intensity (MFI) was obtained from at least four measurements after a background fluorescence was subtracted. LeY and P-gp expression were determined using cBR96 and MRK-16 mAbs, respectively. [c] Doxorubicin. [d] Human small cell lung carcinoma cell line. [e] Derived from parental NCI-H69 cells upon prolonged drug exposure.[37] [f] Concentration based on drug payload (8 drugs per mAb).

molecules or through an ether bond to molecules in the hydroxy aza-CBI class.

Because of the hydrophobicity of the MGB class of molecules, several modifications from **14** were required to form conjugates that were not aggregated. Incorporation of a hydrophilic peptide linker, together with a TEG chain between the peptide and the mAb, allowed us to attach as many as eight amino-CBI- or hydroxy aza-CBI-based molecules to cAC10, leading to cAC10−**28** and cAC10−**29**, respectively. The conjugates were almost exclusively monomeric and were both highly potent and immunologically specific. The finding that both conjugates were active in cells, while only cAC10−**29** was a cathepsin B substrate, strongly argues for the involvement of enzymes other than cathepsin B in drug release. The activities of cAC10−**28** and cAC10−**29** compared favorably to those of the cAC10−Val-Cit-PABC-MMAE conjugates, which are highly active in several in vivo tumor models.[12] The m1F6−**28** and m1F6−**29** ADCs were also found to be active against Caki-1, a renal cell carcinoma line, indicating that this approach may be applicable to a wide array of tumor types. We note that the work described here comprises a general method for attaching seco halide MGBs in the CC-1065 family to mAbs, since it applies to both hydroxyl- and amino-containing MGB analogues. Furthermore, this is the first application of the PABE technology for ADC formation.[25]

The potential of mAb−MGB conjugates is further underscored by the demonstration that cBR96−**28** is active against a cell line that has high levels of P-gp (Table 3). These results are in stark contrast to Mylotarg, which contains the MGB calicheamicin and is known to be highly susceptible to the MDR phenotype.[3,4,38] This is an important advantage in using drugs in the CC-1065 family, since clinical studies have correlated MDR with resistance to Mylotarg. Taken together, the in vitro results provide a strong basis for ongoing in vivo studies designed to explore the therapeutic potential of mAb−**28** and mAb−**29** conjugates described here.

Experimental Section

Unless otherwise indicated, all anhydrous solvents were commercially obtained and stored in Sure-seal bottles under nitrogen. All other reagents and solvents were purchased as the highest grade available and used without further purification. NMR spectra were recorded on Varian Mercury 400 MHz instrument. Chemical shifts (δ) are reported in parts per million (ppm) referenced to tetramethylsilane at 0.00, and coupling constants (J) are reported in hertz. Low-resolution mass spectral data were acquired on a Micromass ZMD mass spectrometer interfaced with an HP Agilent 1100 high-performance liquid chromatography instrument for LC−MS. Products were eluted on a Phenomonex Synergi 2.0 mm × 150 mm, 4 μm, 80 Å MAX RP column using a linear gradient of mobile phase B (CH_3CN with 0.05% HCO_2H) in A (0.05% aqueous HCO_2H) at 0.4 mL/min. Unless otherwise specified, the reported retention times (t_R) are those from LC−MS. High-resolution (exact mass) data were obtained at the University of Washington Medicinal Chemistry Mass Spectrometry Center on a Bruker APEXIII 47e [FT(ICR)]MS mass spectrometer. Analytical HPLC was conducted on a Waters 2695 instrument using a Waters 2996 PDA and Millenium[32] software. For analytical HPLC, the stationary phase used was a Phenomonex Synergi 4.6 mm × 150 mm, 4 μm, 80 Å MAX RP column. Products were eluted on either acidic linear gradients (designated gradient A) of mobile phase B (CH_3CN with 0.05% HCO_2H; 10−95% over 8 min) in A (0.05% aqueous TFA) or neutral linear gradients (designated gradient N) of mobile phase B (CH_3CN; 10−90% over 10 min, then hold at 90% for 5 min) in A (5.0 mM $NH_4H_2PO_4$) at a flow rate of 1.0 mL/min. For preparative HPLC, the stationary phase used was a Phenomonex Synergi 10 mm × 250 mm, 4 μm, 80 Å semi-preparative column, using the acidic gradient A at a flow rate of 4.6 mL/min. All peaks were monitored at 340 and 215 nm. Radial chromatography was performed on a Chromatotron instrument (Harrison Research, Palo Alto, CA); preparative thin-layer chromatography was performed on Whatman 20 cm × 20 cm, 500 μm, 60 Å silica gel plates, and all other preparative normal phase purifications were done by standard flash silica gel chromatography using Whatman Science 60 Å 230−400 mesh silica gel as adsorbent.

{1-Chloromethyl-3-[3-(4-methoxy-phenyl)acryloyl]-2,3-dihydro-1*H*-benzo[*e*]indol-5-yl}-carbamic Acid 4-{2-[2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-3-methyl-butyrylamino]-5-ureido-pentanoylamino}-benzyl Ester (11). A solution of **1** (331 mg, 0.842 mmol) in dichloromethane (5 mL) was treated with a solution of phosgene in toluene (4 mL, 20 wt %, Fluka). The mixture was stirred in a sealed vessel for 17 h, then concentrated and dried under high vacuum for at least 30 min. A solution of **10** (495 mg, 0.822 mmol) in DMF (5 mL) was stirred with 4 Å molecular sieves (2 g) for 2 h, then combined with the residue obtained above. The resulting solution was heated to 45 °C for at least 2 h, then allowed to cool, filtered, and concentrated in vacuo. The residue so obtained was purified by SiO_2 PTLC (20 cm × 20 cm plate, 1 mm thickness, partially eluted with 20% MeOH/CH_2Cl_2, then fully eluted with 5% MeOH/CH_2Cl_2 and 10% MeOH/CH_2Cl_2) to give **11** (211 mg, 25%) as an amorphous solid. ^{1}H NMR (DMF-d_7) δ 0.96−1.00 (m, 6H), 1.55 (m, 2H), 1.73 (m, 1H), 1.88 (m, 1H), 2.18 (m, 1H), 3.06 (m, 1H), 3.26 (m, 1H), 3.88 (s, 3H), 3.99 (dd, J = 11.5, 8.2 Hz, 1H), 4.12 (dd, J = 11.5, 3.5 Hz, 1H), 4.16 (dd, J = 8.6, 6.5 Hz, 1H), 4.33−4.25 (m, 2H), 4.64 (bs, 4H), 5.20 (s, 2H), 5.67 (bs, 2H), 6.27 (bs, 1H), 7.05 (d, J = 8.8 Hz, 2H), 7.24 (d, J = 15.3 Hz, 1H), 7.47−7.32 (m, 10H), 7.58 (t, J = 7.0 Hz, 2H), 7.85−7.72 (m, 12 H), 7.92 (d, J = 7.6 Hz, 2H), 7.98 (m (obsd), 2H), 8.24 (d, J = 7.4 Hz, 2H), 8.99 (bs, 1H), 9.72 (bs, 1H), 10.21 (s, 1H); LC−MS m/z (ES$^+$), 1042.3 $(M + Na)^+$.

{1-Chloromethyl-3-[3-(4-methoxy-phenyl)-acryloyl]-2,3-dihydro-1*H*-benzo[*e*]indol-5-yl}-carbamic Acid 4-(2-{2-[6-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)hexanoylamino]-3-methyl-butyrylamino}-5-ureido-pentanoylamino)-benzyl Ester (14). Carbamate **11** (195 mg, 0.191 mmol) was dissolved in DMF (7.5 mL) and treated with a solution of 20% piperidine in DMF (4 mL). The solution was stirred for at least 2 min, then partitioned between EtOAc (200 mL) and saturated ammonium bicarbonate (50 mL). The EtOAc layer was washed with water (3 × 75 mL) and brine (75 mL), dried over Na_2SO_4, and concentrated. Purification of the crude product by PTLC (SiO_2, 20 cm × 20 cm plate, 1 mm thickness, eluted with 10% MeOH/CH_2Cl_2) gave amine **12** (51 mg, 33%). The purified amine (51 mg, 0.064 mmol) was dissolved in DMF (1 mL), and the solution was treated with MCOSu (**13**) (34 mg, 0.11 mmol). The mixture was stirred for 17 h, then concentrated to dryness. Purification of the residue by PTLC (SiO_2, 20 cm × 20 cm plate, 1 mm thickness, eluted with 10% MeOH/CH_2Cl_2) gave **14** (58 mg, 91%). ^{1}H NMR (DMF-d_7) δ 0.95−0.98 (m, 6H), 1.26 (m, 1H), 1.49−1.61 (m, 9H), 1.72 (m, 1H), 1.88 (m, 1H), 2.14 (m, 2H), 2.23−2.33 (m, 3H), 3.05 (m, 1H), 3.20 (m, 2H), 3.42 (t, J = 7.0 Hz, 1H), 3.87 (s, 3H), 3.99 (dd, J = 11.1, 8.0 Hz, 1H), 4.11 (dd, J = 11.1, 3.1 Hz, 1H), 4.34 (dd, J = 8.4, 6.3 Hz, 1H), 4.57−4.64 (m, 3H), 5.19 (s, 2H), 5.67 (s, 2H), 6.42 (bs, 1H), 7.00 (s, 2H), 7.05 (d, J = 8.8 Hz, 1H), 7.24 (d, J = 15.2 Hz, 1H), 7.43−7.46 (m, 3H), 7.57 (t, J = 7.22 Hz, 1H), 7.76 (d, J = 15.2 Hz, 1H), 7.83 (m, 2H), 7.98 (m (obs), 1H), 8.24 (d, J = 8.2 Hz, 1H), 8.31 (d, J = 7.6 Hz, 1H), 8.99 (s, 1H), 9.75 (s, 1H), 10.26 (s, 1H); UV λ_{max} 316, 241 nm; anal. HPLC (gradient A) t_R = 6.65 min, 93% AUC_{340}, (gradient N) t_R = 9.72 min, 95% AUC_{340}; LC−MS m/z (ES$^+$) found, 991.1

$(M + H)^+$; HRMS *m/z* for $C_{52}H_{59}ClN_8O_{10}Na^+$ $(M + Na)^+$ calcd, 1013.3940; found, 1013.3940.

(5-Amino-5-{1-chloromethyl-3-[3-(4-methoxy-phenyl)-acryloyl]-2,3-dihydro-1*H*-benzo[*e*]indol-5-ylcarbamoyl}-pentyl)carbamic Acid *tert*-Butyl Ester (17a). To an ice-cold solution of **1** (105 mg, 0.27 mmol) in 2 mL of dichloromethane was added, sequentially, 1-hydroxy-7-azabenzotriazole (HOAt, 44 mg, 0.32 mmol), N^{α}FmocN$^{\epsilon}$Boc-lysine (**15**, 150 mg, 0.32 mmol), DIPEA (0.11 mL, 0.64 mmol), and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) (122 mg, 0.32 mmol). The reaction was allowed to equilibrate to an ambient temperature and was stirred for 24 h. The mixture was diluted with dichloromethane, and the organic phase was washed successively with 0.1 mM citric acid, $NaHCO_3$, and brine. The organic phase was dried and concentrated in vacuo to a residual oil that was purified on flash silica (97:3 $CHCl_3$−MeOH) to give **17a** as a yellow solid (220 mg, 97%): HPLC t_R = 13.50 min; ^{1}H NMR ($CDCl_3$) δ 8.85 (br, 1H), 7.50 (br, 1H), 7.00−7.20 (m, 18 H), 6.90 (d, 1H), 4.90 (br, 1H), 4.00−4.60 (m, 9H), 3.83 (s, 3H), 3.15−3.20 (m, 2H), 1.45−2.20 (m, 4H), 1.40 (s, 9H), 10.10−1.150 (m, 2H); LC−MS *m/z* (ES^+) 843.3 $(M + H)^+$; t_R = 5.28 min. Anal. ($C_{49}H_{51}ClN_4O_7$) C, H, N.

(5-Amino-5-{1-chloromethyl-3-[3-(4-methoxy-phenyl)-acryloyl]-2,3-dihydro-1*H*-benzo[*e*]indol-5-ylcarbamoyl}-pentyl)carbamic Acid *tert*-Butyl Ester (18a). Intermediate **17a** was dissolved in 1 mL of DMF and treated at an ambient temperature with 20% v/v piperidine in DMF (20 mL) for 2.5 h. The reaction mixture was poured into a 200-mL 4:1 brine/ethyl acetate solution, and the organic phase was separated, washed an additional 3 times with brine, dried, and concentrated in vacuo to a residual oil that was purified on flash silica (97:3 $CHCl_3$−MeOH) to give **18a** (108 mg, 67%): HPLC t_R = 6.36 min; ^{1}H NMR ($CDCl_3$) δ 9.00 (br, 1H), 7.40−7.80 (m, 8 H), 6.90−7.00 (m, 3H), 6.80 (br, 1H), 4.60−4.70 (m, 1H), 4.50−4.60 (m, 1H), 4.35−4.45 (m, 1H), 3.80−4.00 (m, 1H), 3.83 (s, 3H), 3.60−3.70 (m, 1H), 3.40−3.50 (m, 1H), 3.15−3.20 (m, 2H), 1.70−2.20 (m, 6H), 1.50−1.60 (m, 2H), 1.40 (s, 9H); LC−MS *m/z* (ES^+) 621.2 $(M + H)^+$, 521.4 $(M - Boc + H)^+$; t_R = 3.96 min. Anal. ($C_{34}H_{41}ClN_4O_5$) C, H, N.

{5-{1-Chloromethyl-3-[3-(4-methoxy-phenyl)acryloyl]-2,3-dihydro-1*H*-benzo[*e*]indol-5-ylcarbamoyl}-5-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methyl-butyrylamino]-pentyl}-carbamic Acid *tert*-Butyl Ester (20a). To a solution of **18a** (41 mg, 66 μmol) in DMF (2 mL) was added Fmoc-Val-OSu (**19**) (87 mg, 0.2 mmol), and the reaction mixture was stirred for 16 h. The reaction mixture was concentrated under reduced pressure and was purified via radial chromatography on a 1 mm plate, eluting with a 25−50% ethyl acetate/hexanes gradient. The major UV-active 254-nm band was collected and concentrated under reduced pressure to give 54 mg (87%) of **20a** as a yellow solid: ^{1}H NMR (DMSO-d_6) δ 0.88 (d, J = 6.5 Hz, 3H), 0.91 (d, J = 6.3 Hz, 3H), 1.26 (m, 1H), 1.36 (s, 9H), 1.38−1.52 (m, 3H), 1.78 (m, 1H), 1.90 (m, 1H), 2.94 (m, 2H), 3.83 (s, 3H), 3.88−4.07 (m, 4H), 4.20−4.40 (m, 5H), 4.51 (m, 3H), 4.624 (m, 1H), 6.53 (bs, 1H), 7.01 (d, J = 8.8 Hz, 2H), 7.06 (d, J = 15.5 Hz, 1H), 7.23 (bs, 1H), 7.30 (t, J = 7.6 Hz, 2H), 7.40 (dd, J = 8.2, 15.3 Hz, 2H), 7.55 (t, J = 7.2 Hz, 1H), 7.64 (d, J = 15.3 Hz, 1H), 7.67 (m, 1H), 7.74 (d, J = 8.4 Hz, 2H), 7.86 (d, J = 7.2 Hz, 2H), 7.92 (8.4 Hz, 1H), 8.01 (d, J = 8.4 Hz, 2H), 8.61 (bs, 1H), 9.88 (bs, 1H); LC−MS *m/z* (ES^+) 942 $(M + H)^+$. Anal. ($C_{54}H_{60}ClN_5O_8$) C, H, N.

(5-(2-Amino-3-methyl-butyrylamino)-5-{1-chloromethyl-3-[3-(4-methoxy-phenyl)acryloyl]-2,3-dihydro-1*H*-benzo-[*e*]indol-5-ylcarbamoyl}-pentyl)carbamic Acid *tert*-Butyl Ester (21a). See the preparation of **18a** for general Fmoc-deprotection procedure. Deprotection of **20a** (54 mg, 57 μmol) gave 33 mg (80%) of **21a**: LC−MS *m/z* (ES^+) 720 $(M + H)^+$; Anal. ($C_{39}H_{50}ClN_5O_6$) C, H, N.

Fmoc-TEG 25. To a mixture of **21a** (29 mg, 40 μmol) and Fmoc-TEG-OH (**24**) (25.3 mg, 52 μmol) in dichloromethane (2 mL) was added DIPEA (21 μL, 0.12 mmol) followed by HATU (23.3 mg, 61 μmol). The reaction mixture was stirred for 1 h. The reaction mixture was directly aspirated onto a 1-mm radial Chromatotron plate and was eluted with a 2−5% methanol/dichloromethane solvent gradient. The major 254-nm UV-active band was collected and concentrated under reduced pressure to give 30 mg (63%) of **25** as an amorphous yellow solid. Material was not characterized but was used directly in the preparation of **26**.

Amine 26. See preparation of **18a** for a general Fmoc-deprotection procedure and method of purification. Deprotection of **25** (30 mg, 25.3 μmol) gave 15 mg (62%) of **26** as an amorphous solid: LC−MS *m/z* (ES^+) 967.6 $(M + H)^+$.

Bromoacetamide 27. To a 0 °C solution of **26** (15.4 mg, 16 μmol) in dichloromethane (4 mL) was added DIPEA (8.3 μL, 48 μmol). This was followed by the addition of 1 mL of a bromoacetyl bromide/dichloromethane solution, prepared by dissolving bromoacetyl bromide (32 μL) into dichloromethane (10 mL). The reaction was stirred for 5 min before being aspirated directly onto a 1-mm radial Chromatotron plate. The product was eluted using a 5−7% methanol/dichloromethane solvent gradient, and the major 254-nm band was collected and concentrated. This gave 14.5 mg (83%) of **27** as a yellow solid: LC−MS *m/z* (ES^+) 1087.5 $(M + H)^+$.

Amine-TFA Salt 28. To a flask containing **27** (14.5 mg, 13.3 μmol) and cooled in an ice−water bath was added TFA (4 mL, precooled in an ice−water bath). The reaction mixture was stirred for 15 min and was concentrated under reduced pressure. The resulting residue was redissolved in dichloromethane (5 mL) and concentrated 2 additional times under reduced pressure to give a solid residue 16.4 mg of **28** as a TFA salt complex: ^{1}H NMR (DMF-d_7) δ 0.99 (m, 6H), 1.58−1.75 (m, 2H), 1.80−1.95 (m, 3H), 2.05−2.21 (m, 2H), 2.50−2.64 (m, 2H), 3.13 (m, 2H), 3.35 (q, J = 5.7 Hz, 2H), 3.51 (t, J = 5.9 Hz, 2H), 3.56 (s, 12H), 3.69 (t, J = 6.5 Hz, 2H), 3.88 (s, 3H), 3.96 (s, 2H), 4.01 (m, 1H), 4.12 (dd, J = 3.1, 11.3 Hz, 1H), 4.35 (dd, J = 1.8, 8.0 Hz, 1H), 4.41 (bs, 1H), 4.66 (bs, 2H), 4.78 (bs, 1H), 7.05 (d, J = 8.8 Hz, 2H), 7.24 (d, J = 15.1 Hz, 1H), 7.45 (t, J = 6.9 Hz, 1H), 7.59 (t, J = 7.2 Hz, 1H), 7.75 (d, J = 15.3 Hz, 2H), 7.83 (d, J = 8.7 Hz, 1H), 8.06 (dd, J = 2.7, 7.8 Hz, 2H), 8.16 (m, 1H), 8.26 (m, 3H), 8.36 (m, 2H), 8.91 (bs, 1H), 10.01 (bs, 1H); UV λ_{max} 317 nm; anal. HPLC (gradient A) t_R = 4.55 min, 93% AUC_{340}, (gradient N) t_R = 10.67 min, 96% AUC_{340}; LC−MS *m/z* (ES^+) 987.4 $(M + H)$; HRMS *m/z* $(M + H)^+$ for $C_{47}H_{65}BrClN_6O_{10}^+$ calcd, 987.3634; found, 987.3597.

Maleimide 22a. To a DMF (0.2 mL) solution of **21a** (10 mg, 14 μmol) was added MCOSu (**13**) (10 mg, 42 μmol), and the mixture was stirred for 16 h at an ambient temperature. The reaction mixture was concentrated under reduced pressure, and the resulting residue was chromatographed on a 1-mm radial Chromatotron plate, eluting with a 1−5% methanol/dichloromethane gradient. A single 254-nm UV-active band was collected and concentrated to give **22a**, 6.4 mg (50%) as an amorphous solid. MS *m/z* (ES^+) 913 $(M + H)^+$.

6-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)hexanoic Acid [1-(1-{1-Chloromethyl-3-[3-(4-methoxy-phenyl)acryloyl]-2,3-dihydro-1*H*-benzo[*e*]indol-5-ylcarbamoyl}-4-ureido-butylcarbamoyl)-2-methyl-propyl]-amide (22b). This compound was prepared in a manner very similar to **22a** and starting from **1**: UV λ_{max} 317 nm; anal. HPLC (gradient A) t_R = 6.28 min, 95% AUC_{340}, (gradient N) t_R = 9.05 min, 95% AUC_{340}; HRMS *m/z* for $C_{44}H_{52}ClN_7O_8Na$ $(M + Na)^+$ calcd, 864.3464; found, 864.3444.

Amine-TFA Salt 23. See procedure for synthesis of **28** for Boc-removal. The conversion of **22a** (6.4 mg, 7 μmol) gave 12.5 mg of **23** as an amorphous TFA salt complex: ^{1}H NMR (DMSO-d_6) δ 0.84 (m, 6H), 1.12−1.28 (m, 3H), 1.33−1.65 (m, 8H), 1.64−1.8 (m, 1H), 1.82−2.03 (m, 2H), 2.05−2.22 (m, 2H), 2.80 (m, 2H), 3.34 (m, 2H), 3.80 (s, 3H), 3.91 (t, J = 10.6 Hz, 1H), 4.01 (d, J = 8.6 Hz, 1H), 4.19 (m, 1H), 4.36 (bs, 1H), 4.44−4.59 (m, 3H), 6.99 (m, 4H), 7.08 (d, J = 15.5 Hz, 1H), 7.39 (t, J = 6.5 Hz, 1H), 7.54 (t, J = 7.4 Hz, 1H), 7.51 (d, J = 15.1 Hz, 1H), 7.67 (bs, 2H), 7.75 (d, J = 8.8 Hz, 2H), 7.84−7.96 (m, 3H), 8.24 (m, 1H), 8.61 (m, 1H), 10.0 (bs, 1H); UV λ_{max} 317 nm; anal. HPLC (gradient A) t_R = 4.68 min, 96% AUC_{340},

(gradient N) t_R = 12.10 min, 95% AUC_{340}; MS *m/z* (ES^+) 813.4 (M + H); HRMS *m/z* $(M + H)^+$ for $C_{44}H_{54}ClN_6O_7^+$ calcd, 813.3743; found, 813.3715.

1-(5-Amino-1-chloromethyl-1,2-dihydro-benzo[*e*]indol-3-yl)-3-(4-methoxy-phenyl)propenone (1). To a solution of **9** (691 mg, 1.6 mmol) in dichloromethane (20 mL) were added $PhSO_2Na$ (524 mg, 3.2 mmol) and camphorsulfonic acid (742 mg, 3.2 mmol). The $(Ph_3P)_4Pd$ (92 mg, 0.08 mmol) was added, and the reaction mixture was stirred under an inert atmosphere and at ambient temperatures. After 15 min, the mixture was directly aspirated onto a 4-mm Chromatotron plate, and the product was eluted as the major yellow band with 25% ethyl acetate/hexanes. The combined fractions were concentrated under reduced pressure to give 547 mg of **1** (87%). This material was identical in all respect to an authentic sample of **1**.[17]

{**1-Chloromethyl-3-[3-(4-methoxy-phenyl)acryloyl]-2,3-dihydro-1*H*-benzo[*e*]indol-5-yl}-carbamic Acid *tert*-Butyl Ester (7).** To a mixture of **1** (100 mg, 0.51 mmol) in THF (2.5 mL) was added Boc_2O (330 mg, 1.53 mmol), and the mixture was heated at reflux for 7 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was dissolved in ethyl acetate (5 mL) and precipitated by the addition of hexanes (30 mL). The resulting light yellow solid was isolated by filtration to give 109 mg (87%) of **7**: LC−MS *m/z* (ES^+) 493 $(M + H)^+$.

[5-*tert*-Butoxycarbonylamino-5-(4-hydroxymethyl-phenylcarbamoyl)pentyl]-carbamic Acid Allyl Ester (34). To a solution of Boc-Lys (Alloc)-OH (**32**) (100 mg, 0.3 mmol) and *para*-aminobenzyl alcohol (**33**) (37 mg, 0.3 mmol) in chloroform (5 mL) was added EEDQ (88 mg, 0.36 mmol). The mixture was stirred at an ambient temperature for 2 h and was poured into a 0.1 N HCl solution (50 mL), and the resulting mixture was extracted with ethyl acetate (3 × 25 mL). The combined extracts were washed with water and brine and were dried over magnesium sulfate, before being filtered and concentrated under reduced pressure. The resulting residue was purified via radial chromatography on a 2-mm plate eluting with a 25% ethyl acetate/hexanes to 100% ethyl acetate gradient. The major 254-nm UV-active band was collected and concentrated under reduced pressure to give 100 mg (77%) of **34** as a viscous oil: ^1H NMR (DMSO-d_6) δ 1.22−1.53 (m, 13H), 1.55−1.71 (m, 2H), 2.98 (q, J = 6.3 Hz, 2H), 4.04 (m, 1H), 4.45 (m, 4H), 4.92 (t, J = 5.5 Hz, 1H), 5.15 (d, J = 10.4 Hz, 1H), 5.26 (dd, J = 1.8, 7.2 Hz, 1H), 5.90 (m, 1H), 6.70 (bs, 1H), 6.98 (bs, 1H), 7.24 (d, J = 8.0 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 9.68 (s, 1H); LC−MS *m/z* (ES^+) 458 $(M + Na)^+$.

{**5-Allyloxycarbonylamino-1-[4-(*tert*-butyl-dimethyl-silanyloxymethyl)phenylcarbamoyl]-pentyl}-carbamic Acid *tert*-Butyl Ester (35).** To a DMF (3 mL) solution of **34** (500 mg, 1.15 mmol) was added DIPEA (1 mL, 5.76 mmol) followed by TBSCl (517 mg, 3.45 mmol). The reaction mixture was stirred for 10 min, before being concentrated under reduced pressure. The residue was dissolved in dichloromethane/hexanes (1:1, 20 mL) and was directly aspirated onto a 4-mm radial Chromatotron plate and eluted with a 10−50% ethyl acetate/hexanes solvent gradient. The major 254-nm UV-active band was collected and concentrated to give 547 mg (87%) of **35** as a clear oil: ^1H NMR ($CDCl_3$) δ 0.05 (s, 6H), 0.89 (s, 9H), 1.42 (s, 9H), 1.48−1.75 (m, 6H), 1.92 (m, 1H), 3.17 (q, J = 6.3 Hz, 2H), 4.13 (bs, 1H), 4.53 (d, J = 5.3 Hz, 2H), 4.66 (s, 2H), 4.81 (m, 1H), 5.17 (m, 2H), 5.26 (dq, J = 1.6, 15.7 Hz, 1H), 5.87 (m, 1H), 7.28 (d, J = 8.4 Hz, 2H), 7.45 (d, J = 8.4 Hz, 2H), 8.26 (bs, 1H); LC−MS *m/z* (ES^+) 572 $(M + Na)^+$.

{**5-*tert*-Butoxycarbonylamino-5-[[4-(*tert*-butyl-dimethyl-silanyloxymethyl)phenyl]-(2-trimethylsilanyl-ethoxymethyl)carbamoyl]-pentyl}-carbamic Acid Allyl Ester (36).** To a solution of **35** (559 mg, 1.0 mmol) in THF (10 mL) was added NaH (40 mg of a 60% dispersion in mineral oil, 1.0 mmol). After 10 min, neat SEMCl (185 μL, 1.12 mmol) was added, and the reaction mixture was stirred for 30 min. The crude reaction mixture was concentrated under reduced pressure, and the resulting oil was dissolved in hexanes and directly aspirated onto a 4-mm radial Chromatotron plate, eluting with 25% ethyl acetate/hexanes. The product (R_f = 0.7 TLC using 50% ethyl acetate/hexanes) eluted as the first band, followed by residual starting material. (Note: the 254-nm UV absorbance of product is greatly diminished relative to starting material.) The product-containing fractions were combined and concentrated under reduced pressure to give 538 mg (78%) of **36** as a clear oil: ^1H NMR ($CDCl_3$) δ 0.00 (s, 9H), 0.12 (d, J = 1.4 Hz, 6H), 0.95−0.97 (m, 11H), 1.08−1.26 (m, 2H), 1.35−1.53 (m, 11H), 2.99 (m, 1H), 3.63 (dd, J = 8.6, 8.6 Hz, 2H), 4.33 (m, 1H), 4.53 (d, J = 5.5 Hz, 2H), 4.76 (s, 2H), 4.96 (d, J = 9.8 Hz, 1H), 5.14 (d, J = 10 Hz, 1H), 5.20 (m, 2H), 5.28 (dd, J = 1.5, 17.2 Hz, 1H), 5.91 (m, 1H), 7.26 (d, J = 8.4 Hz, 1H), 7.40 (d, J = 8.4 Hz, 1H); LC−MS *m/z* (ES^+) 702 $(M + Na)^+$.

{**5-*tert*-Butoxycarbonylamino-5-[(4-hydroxymethyl-phenyl)-(2-trimethylsilanyl-ethoxymethyl)carbamoyl]-pentyl}-carbamic Acid Allyl Ester (37).** To a solution of **36** (530 mg, 0.78 mmol) in THF (20 mL) was added TBAF (1 mL of a 1.0 M solution, 1 mmol). After 15 min, the reaction mixture was concentrated under reduced pressure and redissolved in dichloromethane (3 mL). This mixture was directly aspirated onto a 4-mm radial Chromatotron plate and eluted with 50% ethyl acetate/hexanes to 100% ethyl acetate solvent gradient. A single 254-nm UV-active band (R_f = 0.2, 50% ethyl acetate/hexanes) was collected and concentrated under reduced pressure to give 436 mg (95%) of **37** as a clear oil: ^1H NMR ($CDCl_3$) δ 0.00 (s, 9H), 0.96 (m, 2H), 0.99−1.28 (m, 4H), 1.33−1.54 (m, 11H), 2.85 (m, 2H), 3.63 (dd, J = 8.0, 8.0 Hz, 2H), 4.34 (m, 1H), 4.54 (d, J = 5.2 Hz, 2H), 4.71 (s, 2H), 4.82 (bs, 1H), 4.98 (d, J = 10.0 Hz, 2H), 5.08−5.25 (m, 2H), 5.27 (dd, J = 1.6, 15.7 Hz, 1H), 5.94 (m, 1H), 7.31 (d, J = 8.4 Hz, 2H), 7.47 (d, J = 8.4 Hz, 2H); LC−MS *m/z* (ES^+) 588 $(M + Na)^+$.

{**5-[(4-Bromomethyl-phenyl)-(2-trimethylsilanyl-ethoxymethyl)carbamoyl]-5-tertbutoxycarbonylamino-pentyl}-carbamic Acid Allyl Ester (31).** To a solution of **37** (450 mg, 0.8 mmol) in dichloromethane (25 mL) was added triphenylphosphine (631 mg, 2.4 mmol) followed by carbon tetrabromide (794 mg, 2.4 mmol). The reaction mixture was stirred for 1 h and was directly aspirated onto a 4-mm radial Chromatotron plate and eluted with 50% ethyl acetate/hexanes. Product-containing fractions were concentrated under reduced pressure to give 450 mg (68%) of **31** as a clear oil: ^1H NMR ($CDCl_3$) δ (ppm) 0.00 (s, 9H), 0.94 (m, 2H), 1.05−1.28 (m, 4H), 1.35−1.54 (m, 11H), 2.99 (m, 2H), 3.63 (dd, J = 8.0, 8.0 Hz, 2H), 4.30 (m, 1H), 4.49 (s, 2H), 4.53 (d, J = 5.2 Hz, 2H), 4.73 (bs, 1H), 4.95 (d, J = 10.0 Hz, 2H), 5.14−5.21 (m, 2H), 5.27 (ddd, J = 1.6, 1.6, 17.2 Hz, 1H), 5.91 (m, 1H), 7.30 (d, J = 7.6 Hz, 2H), 7.48 (d, J = 8.4 Hz, 2H); LC−MS *m/z* (ES^+) 650 $(M + Na)^+$.

1-Acetoxymethyl-5-hydroxy-1,2-dihydro-pyrrolo[3,2-*f*]-quinoline-3-carboxylic-acid *tert*-Butyl Ester (30). To a 0 °C solution of **38**[18] (350 mg, 0.78 mmol) in THF (20 mL) and saturated aqueous ammonium bicarbonate (4 mL) was added 10% Pd/carbon (350 mg). The reaction vessel was equipped with a balloon containing hydrogen gas, and the reaction mixture was stirred vigorously for 1.5 h. The reaction mixture was filtered through a plug of Celite, and the plug was washed with ethyl acetate (20 mL) and ethanol (20 mL). The reaction mixture was concentrated to approximately 20 mL, and the material was filtered through a Millipore filter (0.2 μm, syringe filter) to remove residual catalyst and carbon. Concentration gave 271 mg (97%) of **30**, crude yield. The material was used without purification: ^1H NMR ($CDCl_3$) δ (ppm) 1.61 (s, 9H), 2.09 (s, 3H), 3.89 (m, 2H), 4.09 (m, 2H), 4.46 (m, 1H), 7.41 (dd, J = 3.9, 4.5 Hz), 8.16 (d, J = 8.8 Hz, 1H), 8.61 (s, 1H); LC−MS *m/z* (ES^+) 359 $(M + H)^+$.

1-Acetoxymethyl-5-{4-[(6-allyloxycarbonylamino-2-*tert*-butoxycarbonylamino-hexanoyl)-(2-trimethylsilanyl-ethoxymethyl)amino]-benzyloxy}-1,2-dihydro-pyrrolo-[3,2-*f*]quinoline-3-carboxylic Acid *tert*-Butyl Ester (39). To a mixture of **31** (171 mg, 0.273 mmol) and phenol **30** (117 mg, 0.327 mmol) in DMF (2 mL) was added potassium carbonate (42 mg, 0.304 mmol), and the reaction mixture was stirred for 16 h at an ambient temperature. The mixture was concentrated under reduced pressure, and the resulting resi-

due was suspended in dichloromethane (3 mL) and was directly aspirated onto a 2-mm radial Chromatotron plate. The product was eluted with ammonia-saturated dichloromethane, and the fractions were combined and concentrated. The partially pure product was purified a second time on a 1-mm radial Chromatotron plate eluting with a 5% methanol/dichloromethane mixture. The product-containing fractions were combined and concentrated under reduced pressure to give 179 mg (68%) of **39** as a white solid: ^{1}H NMR (DMSO-d_6) δ (ppm) 0.01 (s, 6H), 0.88 (dd, J = 1.3, 9.1 Hz, 2H), 0.092−1.29 (m, 4H), 1.38 (s, 9H), 1.44 (m, 2H), 1.56 (s, 9H), 1.96 (s, 3H), 2.84 (m, 2H), 3.59 (hex, 8.0 Hz, 2H), 3.94−4.17 (m, 5H), 4.36 (dd, J = 4, 10.4 Hz, 1H), 4.43 (d, J = 5.6 Hz, 2H), 4.95 (d, J = 10.4 Hz, 1H), 5.06 (m, 1H), 5.12 (m, 1H), 5.22 (dd, J = 1.4, 17.1 Hz, 1H), 5.34 (s, 2H), 5.87 (m, 1H), 6.71 (m, 1H), 6.84 (m, 1H), 7.40 (d, J = 5.4 Hz, 2H), 7.50−7.58 (m, 3H), 7.61−7.66 (m 3H), 7.87 (bs, 1H), 8.27 (dd, J = 1.6, 8.5 Hz, 1H), 8.75 (t, J = 1.6 Hz, 1H); LC−MS m/z (ES$^+$) 906 (M + H)$^+$. Anal. ($C_{47}H_{67}N_5O_{11}Si$) C, H, N.

Acetic Acid 5-[4-(6-Allyloxycarbonylamino-2-aminohexanoylamino)benzyloxy]-2,3-dihydro-1*H*-pyrrolo[3,2-*f*]quinolin-1-ylmethyl Ester (41). To TFA (20 mL) at 0 °C was added neat **39** (176 mg, 0.194 mmol) followed by stirring for 1 h. The mixture was poured into an ice−water bath, and the pH was adjusted to 9−10 with aqueous ammonium hydroxide. The solution was extracted with ethyl acetate (3 × 50 mL). The combined extracts were washed with water and brine and were dried over magnesium sulfate. Filtration and concentration under reduced pressure afforded 116 mg of crude **41** that was carried on without purification: LC−MS m/z (ES$^+$) 776 (M + H)$^+$.

Acetic Acid 5-(4-{6-Allyloxycarbonylamino-2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methyl-butyrylamino]-hexanoylamino}-benzyloxy)-2,3-dihydro-1*H*-pyrrolo[3,2-*f*]quinolin-1-ylmethyl Ester (42). To a solution of **41** (116 mg, 0.20 mmol) in DMF (3 mL) was added Fmoc-Val-OSu (**19**, 263 mg, 0.61 mmol), and the mixture was stirred at an ambient temperature for 16 h. The reaction mixture was concentrated under reduced pressure, and the resulting residue was dissolved in dichloromethane (5 mL) and directly aspirated onto a 2-mm radial Chromatotron plate. The product was eluted using a 1−5% methanol/dichloromethane gradient and the product-containing fractions were combined and concentrated to give 101 mg (58% for two steps) of **42**. The material was carried forward without characterization.

Acetic Acid 5-(4-{6-Allyloxycarbonylamino-2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methyl-butyrylamino]-hexanoylamino}-benzyloxy)-3-(5,6,7-trimethoxy-1*H*-indole-2-carbonyl)-2,3-dihydro-1*H*-pyrrolo[3,2-*f*]quinolin-1-ylmethyl Ester (44). To a mixture of **42** (101 mg, 0.113 mmol) and trimethoxy indole carboxylic acid **43** (43 mg, 0.169 mmol) in DMA (2 mL) was added EDCI-HCl (52 mg, 0.27 mmol), and the reaction mixture was stirred at an ambient temperature for 3 h. An additional portion of the trimethoxy indole carboxylic acid **43** (33.5 mg, 0.113 mmol) was added, and the reaction mixture was stirred for an additional 1.5 h. An additional portion of EDCI-HCl (22 mg, 0.113 mmol) was added, and the reaction mixture was stirred at an ambient temperature for 16 h. The reaction mixture was poured into saturated aqueous sodium bicarbonate (50 mL), and the mixture was extracted with ethyl acetate (3 × 100 mL). The combined extracts were washed with water and brine and were dried over magnesium sulfate. Filtration and concentration resulted in a residue that was dissolved in dichloromethane (5 mL, with several drops of methanol) and aspirated directly onto a 2-mm radial Chromatotron plate. The product was eluted with a 1−5% methanol/dichloromethane gradient. The product-containing fractions were combined and concentrated to give 52 mg (41%) of **44** as an off-white solid: ^{1}H NMR (DMSO-d_6) δ (ppm) 0.86 (m, 6H), 1.23−1.46 (m, 4H), 1.55−1.77 (m, 2H), 1.91 (s, 3H), 2.00 (m, 1H), 2.95 (q, J = 6.1 Hz, 2H), 3.80 (s, 3H), 3.82 (s, 3H), 3.84−4.92 (m, 4H), 4.06−4.18 (m, 3H), 4.20−4.38 (m, 4H), 4.38−4.45 (m, 3H), 4.65 (m, 1H), 5.11 (d, J = 6.8 Hz, 1H), 5.19−5.23 (m, 3H), 5.86 (m, 1H), 6.92 (bs, 1H), 6.95 (s, 1H), 7.02 (s, 1H), 7.19 (bs, 1H), 7.29 (t, J = 7.2 Hz, 2H), 7.35−7.43 (m, 4H), 7.52−7.63 (m, 3H), 7.63−7.75 (m, 2H), 7.84−7.91 (m, 3H), 8.03 (bs, 1H), 8.33 (dd, J = 1.6, 8.4 Hz, 1H), 8.77 (dd, J = 1.3, 4.1 Hz, 1H), 9.89 (bs, 1H), 11.28 (bs, 1H); LC−MS m/z (ES$^+$) 1130 (M + H)$^+$. Anal. ($C_{63}H_{67}N_7O_{13}$) C, H, N.

(5-[2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-3-methyl-butyrylamino]-5-{4-[1-hydroxymethyl-3-(5,6,7-trimethoxy-1*H*-indole-2-carbonyl)-2,3-dihydro-1*H*-pyrrolo-[3,2-*f*]quinolin-5-yloxymethyl]-phenylcarbamoyl}-pentyl)carbamic Acid Allyl Ester (45). Methanol (10 mL) was treated with acetyl chloride (30 μL), and the resulting mixture was added to **44** (47 mg, 41.6 μmol). The mixture was stirred for 16 h at an ambient temperature and was then heated at 50 °C for 6 h. The reaction mixture was neutralized with DIPEA until the pH was 7. The mixture was concentrated under reduced pressure, and the resulting residue was dissolved in dichloromethane (3 mL) and directly aspirated onto a 1-mm radial Chromatotron plate. The product was eluted with a 0−5% methanol/dichloromethane gradient, and the product-containing fractions were combined and concentrated to give 32 mg (71%) of **45** as a white solid: ^{1}H NMR (DMSO-d_6) δ 0.86 (m, 6H), 1.24−1.47 (m, 4H), 1.58−1.75 (m, 2H), 2.00 (spt, J = 6.7 Hz, 1H), 2.95 (q, J = 6.3 Hz, 2H), 3.48 (m, 1H), 3.70 (m, 1H), 3.78 (s, 3H), 3.81 (s, 3H), 3.85−3.95 (m, 5H), 4.09−4.37 (m, 3H), 4.41 (d, J = 5.2 Hz, 2H), 4.49 (dd, J = 2.5, 11 Hz, 1H), 4.60 (t, J = 9.2 Hz, 1H), 4.84 (dd, J = 5.3, 5.3 Hz, 1H), 5.11 (dd, J = 1.6, 10.6 Hz, 1H), 5.19−5.24 (m, 3H), 5.86 (m, 1H), 6.91 (bs, 1H), 6.96 (s, 1H), 7.04 (d, J = 2.0 Hz, 1H), 7.19 (s, 1H), 7.29 (t, J = 7.4 Hz, 2H), 7.39 (m, 2H), 7.43 (d, J = 9.3 Hz, 2H), 7.51 (dd, J = 4.1, 8.4 Hz, 1H), 7.59 (d, J = 8.6 Hz, 2H), 7.67−7.72 (m, 2H), 7.83−7.88 (m, 3H), 8.15 (bs, 1H), 8.30 (dd, J = 1.8, 6.4 Hz, 1H), 8.75 (dd, J = 1.9, 4.1 Hz, 1H), 9.88 (bs, 1H), 11.22 (bs, 1H); LC−MS m/z (ES$^+$) 1088 (M + H)$^+$.

{5-{4-[1-Chloromethyl-3-(5,6,7-trimethoxy-1*H*-indole-2-carbonyl)-2,3-dihydro-1*H*-pyrrolo[3,2-*f*]quinolin-5-yloxymethyl]-phenylcarbamoyl}-5-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methyl-butyrylamino]-pentyl}carbamic Acid Allyl Ester (46). To a mixture of **45** (32 mg, 29 μmol) in dichloromethane (1.5 mL) was added triphenylphospine (23 mg, 87 μmol) and carbon tetrachloride (25 μL, 260 μmol). The reaction mixture was heated at reflux for 30 min. An additional quantity of both triphenylphospine (23 mg, 87 μmol) and carbon tetrachloride (25 μL, 260 μmol) was added, and the reaction mixture was heated for an additional 1 h. The reaction mixture was directly aspirated onto a 1-mm radial Chromatotron plate, and the product was eluted with a 0−3% methanol/dichloromethane gradient to give 27.4 mg (86%) of **46** as a white solid: ^{1}H NMR (DMSO-d_6) δ 0.86 (t, J = 7.2 Hz, 6H), 1.25−1.43 (m, 4H), 1.57−1.72 (m, 2H), 2.01 (m, 1H), 2.95 (q, J = 7.2 Hz, 2H), 3.80 (s, 3H), 3.82 (s, 3H), 3.88−3.94 (m, 4H), 4.02 (dd, J = 3.5, 10.8 Hz, 1H), 4.21−4.32 (m, 5H), 4.41 (d, J = 5.3 Hz, 2H), 4.50 (d, J = 10.5 Hz, 1H), 4.73 (t, J = 11.0 Hz, 1H), 5.09 (d, J = 10.6 Hz, 1H), 5.19−5.23 (m, 3H), 5.86 (m, 1H), 6.91 (bs, 1H), 6.96 (s, 1H), 7.04 (s, 1H), 7.18 (b s, 1H), 7.29 (t, J = 7.6 Hz, 2H), 7.34 (t, J = 7.0 Hz, 2H), 7.42 (d, J = 8.2 Hz, 2H), 7.53 (dd, J = 4.3, 8.6 Hz, 1H), 7.59 (d, J = 8.6 Hz, 2H), 7.68 (d, J = 7.8 Hz, 2H), 7.80−7.88 (m, 3H), 8.09 (bs, 1H), 8.35 (d, J = 8.6 H, 1H), 8.77 (t, J = 1.6 Hz, 1H), 9.98 (bs, 1H), 11.26 (bs, 1H); LC−MS m/z (ES$^+$) 1106 (M + H)$^+$.

[1-(5-Amino-1-{4-[1-chloromethyl-3-(5,6,7-trimethoxy-1*H*-indole-2-carbonyl)-2,3-dihydro-1*H*-pyrrolo[3,2-*f*]quinolin-5-yloxymethyl]-phenylcarbamoyl}-pentylcarbamoyl)-2-methyl-propyl]-carbamic Acid 9*H*-fluoren-9-ylmethyl Ester (47). To a solution of **46** (8.6 mg, 7.8 μmol) in dichloromethane (300 μL) was added camphor sulfonic acid (4 mg, 16 μmol) and $NaSO_2Ph$ (3 mg, 16 μmol). Tetrakistriphenylphospine palladium (1 mg, 0.78 μmol) was added. After 10 min, the reaction mixture was directly aspirated onto a 1-mm radial Chromatotron plate and eluted with a 1−5% methanol/ammonia-saturated dichloromethane gradient to give a single

254-nm UV-active band, which was collected and concentrated to give 6.5 mg (82%) of **47** as a solid: LC−MS *m/z* (ES^+) 1022 $(M + H)^+$.

{5-{4-[1-Chloromethyl-3-(5,6,7-trimethoxy-1*H*-indole-2-carbonyl)-2,3-dihydro-1*H*-pyrrolo[3,2-*f*]quinolin-5-yloxymethyl]-phenylcarbamoyl}-5-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-3-methyl-butyrylamino]-pentyl}-carbamic Acid *tert*-Butyl Ester (48). To a THF (0.5 mL) solution of **47** (6.5 mg, 6.4 μmol) was added Boc anhydride (50 μL). The reaction mixture was stirred for 10 min and was directly aspirated onto a 1-mm radial Chromatotron plate, and the product was eluted with a 0−3% methanol/dichloromethane gradient. A single band was collected, and the fractions were concentrated to give 7.0 mg (98%) of **48** as an amorphous white solid: ^{1}H NMR (DMSO-d_6) δ 0.85 (d, J = 7.0 Hz 3H), 0.88 (d, J = 8.4 Hz, 3H), 1.25−1.44 (m, 13H), 1.56−1.75 (m, 2H), 1.99 (m, 1H), 2.88 (q, J = 5.7 Hz, 2H), 2.93 (bs, 1H), 3.80 (s, 3H), 3.83 (s, 3H), 3.88−3.94 (m, 5H), 4.04 (dd, J = 3.7, 11.0 Hz, 1H), 4.20−4.33 (m, 4H), 4.42 (m, 1H), 4.50 (d, J = 11.2 Hz, 1H), 4.77 (t, J = 9.0 Hz, 1H), 5.21 (s, 2H), 6.62 (bs, 1H), 6.96 (s, 1H), 7.07 (d, J = 2.0 Hz, 1H), 7.31 (t, J = 7.0 Hz, 2H), 7.38−7.45 (m, 3H), 7.56 (dd, J = 4.1, 8.4 Hz, 2H), 7.61 (d, J = 8.6 Hz, 2H), 7.72 (t, J = 8.0 Hz, 1H), 7.86 (d, J = 7.6 Hz, 1H), 7.97 (d, J = 7.2 Hz, 1H), 8.13 (bs, 1H), 8.39 (dd, J = 1.9, 8.7 Hz, 1H), 8.78 (dd, J = 1.6, 4.1 Hz, 1H), 9.98 (bs, 1H), 11.41 (bs, 1H); LC−MS *m/z* (ES^+) 1122 $(M + H)^+$.

(5-(2-Amino-3-methyl-butyrylamino)-5-{4-[1-chloromethyl-3-(5,6,7-trimethoxy-1*H*-indole-2-carbonyl)-2,3-dihydro-1*H*-pyrrolo[3,2-*f*]quinolin-5-yloxymethyl]-phenylcarbamoyl}-pentyl)carbamic acid *tert*-Butyl Ester (49). To a 0 °C solution of **48** (17 mg, 15 μmol) in dichloromethane (4 mL) was added piperidine (1 mL). The reaction mixture was stirred for 5 min, before being concentrated under reduced pressure. The resulting residue was dissolved in dichloromethane (1 mL) and aspirated directly onto a 1-mm radial Chromatotron plate. The product was eluted with a 1−5% methanol/ammonia-saturated dichloromethane gradient. The second major 254-nm UV-active band observed at 254 nm was collected (first band dibenzofulvene) to give 13.1 mg (88%) of **49** as a solid: ^{1}H NMR (DMSO-d_6) δ 0.78 (d, J = 6.5 Hz, 3H), 0.89 (d, J = 6.8 Hz, 3H), 1.24−1.42 (m, 13H), 1.57−1.75 (m, 2H), 1.95 (m, 1H), 2.89 (q, J = 6.4 Hz, 2H), 2.97 (bs, 2H), 3.02 (m, 1H), 3.80 (s, 3H), 3.83 (s, 3H), 3.90 (dd, J = 7.0, 11.2 Hz, 1H), 3.94 (s, 3H), 4.04 (dd, J = 3.5, 11.2 Hz, 1H), 4.29 (m, 1H), 4.45 (m, 1H), 4.76 (t, J = 10 Hz, 1H), 5.21 (s, 2H), 6.64 (bs, 1H), 6.98 (s, 1H), 7.07 (d, J = 1.6, 1H), 7.45 (d, J = 8.4 Hz, 2H), 7.56 (dd, J = 4.9, 8.4 Hz, 1H), 7.62 (d, J = 8.4 Hz, 2H), 8.03 (bs, 1H), 8.12 (bs, 1H), 8.38 (d, J = 7.2 Hz, 1H), 8.77 (t, J = 1.4 Hz, 1H), 10.04 (bs, 1H), 11.42 (bs, 1H); LC−MS *m/z* (ES^+) 900 $(M + H)^+$.

Tetraethylene Glycol 50. To a solution of **49** (13 mg, 14.4 μmol) and the TEG acid **24** (8.3 mg, 17 μmol) in chloroform (0.5 mL) was added EEDQ (4.2 mg, 17 μmol), and the reaction mixture was stirred at an ambient temperature for 16 h. The mixture was directly aspirated onto a 1-mm radial Chromatotron plate, and the product was eluted with a 1−5% methanol/dichloromethane gradient. A single UV-active band at 254 nm was collected and concentrated to give 17.8 mg (90%) of **50** as an amorphous solid: LC−MS *m/z* (ES^+) 1369 $(M + H)^+$.

Amine 51. See procedure for the synthesis of **49** for a general procedure for Fmoc group deprotection. Compound **50** (17.8 mg, 13 μmol) was deprotected to give 13.1 mg (88%) of **51** as an amorphous solid: ^{1}H NMR (DMSO-d_6) δ 0.84 (d, J = 6.8 Hz, 3H), 0.86 (d, J = 6.8 Hz, 3H), 1.23−1.42 (m, 13H), 1.62 (m, 1H), 1.73 (m, 1H), 2.00 (sept, J = 6.5 Hz, 1H), 2.40 (m, 1H), 2.63 (t, J = 5.1 Hz, 1H), 2.89 (q, J = 5.9 Hz, 2H), 3.34 (t, J = 3.3 Hz, 2H), 3.47−3.51 (m, 16H), 3.62 (m, 2H), 3.80 (s, 3H), 3.82 (s, 3H), 3.88 (dd, J = 3.5, 11.0 Hz, 1H), 3.94 (s, 3H), 4.02 (dd, J = 3.3, 10.8 Hz, 1H), 4.19 (t, J = 8.6 Hz, 1H), 4.26 (m, 1H), 4.35 (m, 1H), 4.49 (d, J = 11 Hz, 1H), 4.73 (t, J = 11.1 Hz, 1H), 6.97 (s, 2H), 6.51 (bs, 1H), 6.97 (s, 1H), 7.04 (s, 1H), 7.43 (d, J = 8.2 Hz, 2H), 7.53 (dd, J = 4.1, 8.4 Hz, 1H), 7.60 (d, J = 8.6 Hz, 2H), 7.70 (d, J = 7.2 Hz, 1H), 7.83 (d, J = 7.2 Hz, 1H), 8.09 (bs, 1H), 8.35 (dd, J = 1.6, 8.5 Hz), 8.77 (d, J = 2.5 Hz, 1H), 9.78 (bs, 1H), 11.41 (bs, 1H); LC−MS *m/z* (ES^+) 1147 $(M + H)^+$.

Maleimide 52. To a solution of **51** (4.9 mg, 4.9 μmol) in DMF (0.2 mL) was added maleimidocaproyl NHS ester **13** (4.2 mg, 12.8 μmol). The mixture was stirred for 16 h at an ambient temperature, and the reaction mixture was concentrated. The resulting residue was purified via radial chromatography on a 1-mm plate. The product was eluted with a 1−5% methanol/dichloromethane gradient, and a single UV-active band at 254 nm was collected to give 5.2 mg (79%) of **52** as a solid. This material was carried forward without characterization.

Amine-TFA Salt 29. See Boc-removal procedure for the synthesis of **28**. Compound **52** (8.5 mg, 6.3 μmol) was deprotected to provide 10.4 mg of **29** as a solid TFA salt complex: ^{1}H NMR (DMSO-d_6) δ 0.83 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 7.0 Hz, 3H), 0.92−1.00 (m, 4H), 1.12−1.20 (m, 3H), 1.28−1.79 (m, 10H), 1.96 (q, J = 7.7 Hz, 1H), 2.03 (t, J = 7.4 Hz, 1H), 2.32−2.40 (m, 1H), 2.74−2.81 (m, 2H), 3.16 (q, J = 5.9 Hz, 2H), 3.33−3.39 (m, 5H), 3.49 (m, 12H), 3.59 (m, 2H), 3.80 (s, 3H), 3.82 (m, 3H), 3.86−3.95 (m, 4H), 4.04 (m, 1H), 4.20 (dd, J = 8.0, 15.3 Hz, 1H), 4.30 (m, 1H), 4.37 (m, 1H), 5.50 (m, 1H), 4.78 (m, 1H), 5.23 (s, 2H), 6.98−7.0 (m, 2H), 7.10 (d, J = 2.2 Hz, 1H), 7.37−7.50 (m, 2H), 7.56−7.67 (m, 4H), 7.80 (t, J = 15.3 Hz, 1H), 7.91 (d, J = 8.6 Hz, 1H), 8.15 (d, J = 8.0 Hz, 1H), 8.45 (m, 1H), 8.78 (dd, J = 1.6, 4.1 Hz, 1H), 10.02 (s, 1H), 11.51 (s, 1H); UV λ_{max} 312, 250 nm; anal. HPLC (gradient A) t_R = 4.33 min, 91% AUC_{340}, (gradient N) t_R = 10.29 min, 93% AUC_{340}; LC−MS *m/z* (ES^+) 1240 $(M + H)^+$; HRMS *m/z* for $C_{63}H_{83}ClN_9O_{15}^+$ $(M + H)^+$ calcd, 1240.5697; found, 1240.5679.

Conjugate Preparation. The mAbs (>5 mg/mL) in PBS containing 50 mM sodium borate, pH 8.0, were treated with dithiothreitol (10 mM final) at 37 °C for 30 min. After gel filtration (G-25, PBS containing 1mM DTPA), thiol determination using 5,5′-dithiobis(2-nitrobenzoic acid) indicated that there were approximately eight thiols per mAb. To the reduced mAb at 4 °C was added the maleimide or bromoacetamide (0.5 M sodium borate buffer pH 9 was required to promote mAb alkylation with bromoacetamide) drug derivatives (1.2 equiv/SH group) in cold DMSO (20% v/v). After 1 h, the reactions were quenched with excess cysteine; the conjugates were concentrated by centrifugal ultrafiltration, gel filtered (G-25, PBS), and sterile filtered. Protein concentration and drug loading were determined by spectral analysis at 280 and 320 nm, respectively. Size-exclusion HPLC was used to determine percent monomer of each conjugate prepared, and RP-HPLC established that there was less than 0.5% unconjugated cysteine-quenched drug.

Cathepsin B Drug Release Assay. Human liver cathepsin B (Calbiochem no. 219364, 5U) as provided (49.1 μL) was constituted into a glycerol (46 μL), 0.5 M sodium acetate (4 μL), and 0.5 M EDTA (0.2 μL) to a final enzyme concentration of 0.275 mg/mL. The specific activity was determined to be 75 (μg/min)/mg.[39] The enzyme solution (6 μL) was diluted into water (168 μL), buffered with 0.5 M sodium acetate pH 5 (60 μL), and treated with 0.1 M DTT (12 μL). The solution was incubated at 37 °C for 15 min. The conjugates cAC10−**28** and cAC10−**29** (56 μL of a 1-mg/mL solution) were added, and the mixtures were incubated at 37 °C. Aliquots (10 μL) were taken at 30 min, 1 h, 2.5 h, and 6 h and analyzed by SEC HPLC. Drug release from the conjugates was determined by measuring the AUC for drug chromophore (320 nm) relative to protein absorbance (280 nm) associated with the eluting conjugate.

Stability Assay. Both compounds **2** and **3** were dissolved in DMSO (1 mM solutions), and 100 μL of each was added to both pH 5 and pH 7 ammonium phosphate buffers (900 μL). The mixtures were vortexed, and all four samples were incubated at 37 °C and monitored by HPLC−MS at various intervals up to and beyond 24 h. Remaining starting materials, and new product formation, were calculated based on percentage of the AUC.

Cell Culture. The $CD30^+$ cell line Karpas 299 was purchased from the DSMZ (Braunschweig, Germany) and grown in RPMI 1640 + 10% FBS (both Invitrogen, Carlsbad, CA).

The cell line pair NCI-H69 and its P-glycoprotein-expressing subline NCI-H69/LX4 were purchased from the ECACC (Salisbury, England) and grown in RPMI 1640 supplemented with 2 mM L-glutamine and 10% FBS. NCI-H69/LX4 growth medium also contained 0.4 μg/mL doxorubicin as directed by the manufacturer. Bulk cultures of cells were routinely passaged 2 times per week, and all assays were initiated while cells were in log phase.

In Vitro Growth Inhibition. For assays to assess the activity of cAC10 mAb-ADCs, cells were collected and plated in 96-well black-sided plates at a density of 10 000 cells/well in 150 μL. Serial dilutions of mAb-ADC were made in medium at a 4× working concentration, and 50 μL of the dilution was transferred from the dilution plate to the cultures. After addition of ADC, cultures were incubated to 96 h at 37 °C. Four hours before the end of the assay, 50 μL of 250 μM resazurin (Sigma, St. Louis, MO) in medium was added to each well (final concentration, 50 μM), and the plates were returned to the incubator. At the completion of the assay, plates were read on a Fusion HT microplate reader (Packard, Meriden, CT) using an excitation wavelength of 525 nm and an emission wavelength of 590 nm. For experiments to determine if the drug was a substrate for P-glycoprotein, the NCI-H69 cell line and the NCI-H69/LX4 subline were used.[37] Cells were collected and plated at 5000 (NCI-H69) or 8000 (NCI-H69/LX4) cells/well in 150 μL of medium. After incubating 24 h to allow surface protein reconstitution, serial dilutions of cBR96 mAb-ADC were added as above, and the cultures were incubated for 6 days at 37 °C. Cultures were then labeled with resazurin as described above. Data from all assays were reduced using GraphPad Prism version 4 for Windows (GraphPad Software, San Diego, CA). The IC_{50} concentrations were determined from four-parameter curve fits and are defined as the concentration that inhibits cellular growth by 50% compared with untreated cultures.

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Supporting Information Available: Elemental analysis results for compounds **17a**, **18a**, **20a**, **21a**, **39**, and **44**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Dubowchik, G. M.; Walker, M. A. Receptor-mediated and enzyme-dependent targeting of cytotoxic anticancer drugs. *Pharmacol. Ther.* **1999**, *83*, 67−123.
(2) Meyer, D. L.; Senter, P. D. Recent advances in antibody drug conjugates for cancer therapy. *Annu. Rep. Med. Chem.* **2003**, *38*, 229−237.
(3) Damle, N. K.; Frost, P. Antibody-targeted chemotherapy with immunoconjugates of calicheamicin. *Curr. Opin. Pharmacol.* **2003**, *3*, 386-390.
(4) Giles, F.; Estey, E.; O'Brien, S. Gemtuzumab ozogamicin in the treatment of acute myeloid leukemia. *Cancer* **2003**, *98*, 2095−2104.
(5) Trail, P. A.; Willner, D.; Lasch, S. J.; Henderson, A. J.; Hofstead, S.; Casazza, A. M.; Firestone, R. A.; Hellstrom, I.; Hellstrom, K. E. Cure of xenografted human carcinomas by BR96-doxorubicin immunoconjugates. *Science* **1993**, *261*, 212−215.
(6) Saleh, M. N.; Sugarman, S.; Murray, J.; Ostroff, J. B.; Healey, D.; Jones, D.; Daniel, C. R.; LeBherz, D.; Brewer, H.; Onetto, N.; LoBuglio, A. F. Phase I trial of the anti-Lewis Y drug immunoconjugate BR96-doxorubicin in patients with lewis Y-expressing epithelial tumors. *J. Clin. Oncol.* **2000**, *18*, 2282−2292.
(7) Liu, C.; Tadayoni, B. M.; Bourret, L. A.; Mattocks, K. M.; Derr, S. M.; Widdison, W. C.; Kedersha, N. L.; Ariniello, P. D.; Goldmacher, V. S.; Lambert, J. M.; Blattler, W. A.; Chari, R. V. Eradication of large colon tumor xenografts by targeted delivery of maytansinoids. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 8618−8623.
(8) Ojima, I.; Geng, X.; Wu, X.; Qu, C.; Borella, C. P.; Xie, H.; Wilhelm, S. D.; Leece, B. A.; Bartle, L. M.; Goldmacher, V. S.; Chari, R. V. Tumor-specific novel taxoid-monoclonal antibody conjugates. *J. Med. Chem.* **2002**, *45*, 5620−5623.
(9) Chari, R. V.; Jackel, K. A.; Bourret, L. A.; Derr, S. M.; Tadayoni, B. M.; Mattocks, K. M.; Shah, S. A.; Liu, C.; Blattler, W. A.; Goldmacher, V. S. Enhancement of the selectivity and antitumor efficacy of a CC-1065 analogue through immunoconjugate formation. *Cancer Res.* **1995**, *55*, 4079−4084.
(10) Suzawa, T.; Nagamura, S.; Saito, H.; Ohta, S.; Hanai, N.; Kanazawa, J.; Okabe, M.; Yamasaki, M. Enhanced tumor cell selectivity of adriamycin-monoclonal antibody conjugate via a poly(ethylene glycol)-based cleavable linker. *J. Controlled Release* **2002**, *79*, 229−242.
(11) Suzawa, T.; Nagamura, S.; Saito, H.; Ohta, S.; Hanai, N.; Yamasaki, M. Synthesis of a novel duocarmycin derivative DU-257 and its application to immunoconjugate using poly-(ethylene glycol)-dipeptidyl linker capable of tumor specific activation. *Bioorg. Med. Chem.* **2000**, *8*, 2175−2184.
(12) Doronina, S. O.; Toki, B. E.; Torgov, M. Y.; Mendelsohn, B. A.; Cerveny, C. G.; Chace, D. F.; DeBlanc, R. L.; Gearing, R. P.; Bovee, T. D.; Siegall, C. B.; Francisco, J. A.; Wahl, A. F.; Meyer, D. L.; Senter, P. D. Development of potent monoclonal antibody auristatin conjugates for cancer therapy. *Nat. Biotechnol.* **2003**, *21*, 778−784.
(13) Francisco, J. A.; Cerveny, C. G.; Meyer, D. L.; Mixan, B. J.; Klussman, K.; Chace, D. F.; Rejniak, S. X.; Gordon, K. A.; DeBlanc, R.; Toki, B. E.; Law, C. L.; Doronina, S. O.; Siegall, C. B.; Senter, P. D.; Wahl, A. F. cAC10-vcMMAE, an anti-CD30-monomethyl auristatin E conjugate with potent and selective antitumor activity. *Blood* **2003**, *102*, 1458−1465.
(14) Boger, D. L.; Johnson, D. S. CC-1065 and the duocarmycins: Unraveling the keys to a new class of naturally derived DNA alkylating agents. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3642−3649.
(15) Yasuzawa, T.; Muroi, K.; Ichimura, M.; Takahashi, I.; Ogawa, T.; Takahashi, K.; Sano, H.; Saitoh, Y. Duocarmycins, potent antitumor antibiotics produced by *Streptomyces* sp. structures and chemistry. *Chem. Pharm. Bull.* **1995**, *43*, 378−391.
(16) Atwell, G. J.; Wilson, W. R.; Denny, W. A. Synthesis and cytotoxicity of amino analogues of the potent DNA alkylating agent seco-CBI-TMI. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1489−1594.
(17) Atwell, G. J.; Milbank, J. J.; Wilson, W. R.; Hogg, A.; Denny, W. A. 5-Amino-1-(chloromethyl)-1,2-dihydro-3H-benz[e]indoles: Relationships between structure and cytotoxicity for analogues bearing different DNA minor groove binding subunits. *J. Med. Chem.* **1999**, *42*, 3400−3411.
(18) Denny, W. A.; Wilson, W. R.; Ware, D. C.; Atwell, G. J.; Milbank, J. B.; Stevenson, R. J. *Anti-Cancer 2,3-Dihydro-1H-pyrroloc[3,2-f]quinoline Complexes of Cobalt and Chromium*; New Zealand, 2002; p 92.
(19) Xie, H.; Audette, C.; Hoffee, M.; Lambert, J. M.; Blattler, W. A. Pharmacokinetics and biodistribution of the antitumor immunoconjugate, cantuzumab mertansine (huC242-DM1), and its two components in mice. *J. Pharmacol. Exp. Ther.* **2004**, *308*, 1073−1082.
(20) Tietze, L. F.; Lieb, M.; Herzig, T.; Haunert, F.; Schuberth, I. A strategy for tumor-selective chemotherapy by enzymatic liberation of seco-duocarmycin SA-derivatives from nontoxic prodrugs. *Bioorg. Med. Chem.* **2001**, *9*, 1929−1939.
(21) Hay, M. P.; Anderson, R. F.; Ferry, D. M.; Wilson, W. R.; Denny, W. A. Synthesis and evaluation of nitroheterocyclic carbamate prodrugs for use with nitroreductase-mediated gene-directed enzyme prodrug therapy. *J. Med. Chem.* **2003**, *46*, 5533−5545.
(22) King, H. D.; Dubowchik, G. M.; Mastalerz, H.; Willner, D.; Hofstead, S. J.; Firestone, R. A.; Lasch, S. J.; Trail, P. A. Monoclonal antibody conjugates of doxorubicin prepared with branched peptide linkers: Inhibition of aggregation by methoxytriethyleneglycol chains. *J. Med. Chem.* **2002**, *45*, 4336−4343.
(23) Dubowchik, G. M.; Firestone, R. A.; Padilla, L.; Willner, D.; Hofstead, S. J.; Mosure, K.; Knipe, J. O.; Lasch, S. J.; Trail, P. A. Cathepsin B-labile dipeptide linkers for lysosomal release of doxorubicin from internalizing immunoconjugates: Model studies of enzymatic drug release and antigen-specific in vitro anticancer activity. *Bioconjugate Chem.* **2002**, *13*, 855−869.
(24) Walker, M. A.; Dubowchik, G. M.; Hofstead, S. J.; Trail, P. A.; Firestone, R. A. Synthesis of an immunoconjugate of camptothecin. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 217−219.
(25) Toki, B. E.; Cerveny, C. G.; Wahl, A. F.; Senter, P. D. Protease-mediated fragmentation of *p*-amidobenzyl ethers: A new strategy for the activation of anticancer prodrugs. *J. Org. Chem.* **2002**, *67*, 1866−1872.
(26) Denny, W. A. DNA minor groove alkylating agents. *Curr. Med. Chem.* **2001**, *8*, 533−544.

(27) Yang, S.; Denny, W. A. A new short synthesis of 3-substituted 5-amino-1-(chloromethyl)-1,2-dihydro-3H-benzo[e]indoles (amino-CBIs). *J. Org. Chem.* **2002**, *67*, 8958−8961.
(28) Honda, M.; Morita, H.; Nagakura, I. Deprotection of allyl groups with sulfinic acids and palladium catalyst. *J. Org. Chem.* **1997**, *62*, 8932−8936.
(29) Carpino, L. A. 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive. *J. Am. Chem. Soc.* **1993**, *115*, 4397−4398.
(30) Carpino, L. A.; El-Faham, A. Effect of tertiary bases on O-benzotriazolyluronium salt-induced peptide segment coupling. *J. Org. Chem.* **1994**, *59*, 695−698.
(31) Belleau, B.; Malek, G. New convenient reagent for peptide syntheses. *J. Am. Chem. Soc.* **1968**, *90*, 1651−1652.
(32) Corey, E. J.; Venkateswarlu, A. Protection of hydroxyl groups as *tert*-butyldimethylsilyl derivatives. *J. Am. Chem. Soc.* **1972**, *94*, 6190−6191.
(33) Zeng, Z.; Zimmerman, S. C. Convenient synthesis of 9-alkyl and 9-arylacridines from [2-(trimethylsilyl) ethoxy] methyl (sem) protected acridone. *Tetrahedron Lett.* **1988**, *29*, 5123−5124.
(34) Wahl, A. F.; Klussman, K.; Thompson, J. D.; Chen, J. H.; Francisco, L. V.; Risdon, G.; Chace, D. F.; Siegall, C. B.; Francisco, J. A. The anti-CD30 monoclonal antibody SGN-30 promotes growth arrest and DNA fragmentation in vitro and affects antitumor activity in models of Hodgkin's disease. *Cancer Res.* **2002**, *62*, 3736−3742.
(35) Hishima, T.; Fukayama, M.; Hayashi, Y.; Fujii, T.; Ooba, T.; Funata, N.; Koike, M. CD70 expression in thymic carcinoma. *Am. J. Surg. Pathol.* **2000**, *24*, 742−746.
(36) Held-Feindt, J.; Mentlein, R. CD70/CD27 ligand, a member of the TNF family, is expressed in human brain tumors. *Int. J. Cancer* **2002**, *98*, 352−356.
(37) Reeve, J. G.; Rabbitts, P. H.; Twentyman, P. R. Amplification and expression of mdr1 gene in a multidrug resistant variant of small cell lung cancer cell line NCI-H69. *Br. J. Cancer* **1989**, *60*, 339−342.
(38) Walter, R. B.; Raden, B. W.; Hong, T. C.; Flowers, D. A.; Bernstein, I. D.; Linenberger, M. L. Multidrug resistance protein attenuates gemtuzumab ozogamicin-induced cytotoxicity in acute myeloid leukemia cells. *Blood* **2003**, *102*, 1466−1473.
(39) Bajkowski, A. S.; Frankfater, A. Specific spectrophotometric assays for cathepsin B1. *Anal. Biochem.* **1975**, *68*, 119−127.

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