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Bifunctional Ligands that Target Cells Displaying the $\alpha_v\beta_3$ Integrin

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*Strategies to eliminate tumor cells have long been sought. We envisioned that a small molecule could be used to decorate the offending cells with immunogenic carbohydrates and evoke an immune response. To this end, we describe the synthesis of bifunctional ligands possessing two functional motifs: one binds a cell-surface protein and the other binds a naturally occurring human antibody. Our conjugates combine an RGD-based peptidomimetic, to target cells displaying the $\alpha_v\beta_3$ integrin, with the carbohydrate antigen galactosyl-α(1–3)galactose [Galα(1–3)Gal or α-Gal]. To generate such bifunctional ligands, we designed and synthesized RGD mimetics **1b** and **2c**, which possess a free amino group for modification. These compounds were used to generate bifunctional derivatives **1c** and **2d**, with dimethyl squarate serving as the linchpin; thus, our synthetic approach is modular. To evaluate the binding of our peptidomimetics to the target $\alpha_v\beta_3$-displaying cells, we implemented a cell-adhesion assay. Results from this assay indicate that the designed, small-molecule ligands inhibit $\alpha_v\beta_3$-dependent cell adhesion. Additionally, our most effective bifunctional ligand exhibits a high degree of selectivity (4000-fold) for $\alpha_v\beta_3$ over the related $\alpha_v\beta_5$ integrin, a result that augurs its utility in specific cell targeting. Finally, we demonstrate that the bifunctional ligands can bind to $\alpha_v\beta_3$-positive cells and recruit human anti-Gal antibodies. These results indicate that both the integrin-binding and the anti-Gal-binding moieties can act simultaneously. Bifunctional conjugates of this type can facilitate the development of new methods for targeting cancer cells by exploiting endogenous antibodies. We anticipate that our modifiable $\alpha_v\beta_3$-binding ligands will be valuable in a variety of applications, including drug delivery and tumor targeting.*

Introduction

Methods to deliver biologically active compounds selectively to unwanted cells are needed. Cell-targeting agents have a wide range of potential therapeutic applications, including diagnostic imaging[1] and the destruction of cellular pathogens.[2,3] One especially attractive use of cell targeting is the selective delivery of cancer chemotherapeutic agents[4,5]—an objective that has prompted studies since Ehrlich described the "magic bullet" concept in 1906.[6] In the typical approach, a targeting moiety that recognizes a cancer-associated epitope is used to direct a cytotoxic drug or protein toxin. A major problem with this strategy is that the toxic agent often causes undesirable side effects.[7] Specifically, protein toxins and small-molecule anticancer agents can destroy not only the target cells but also normal ones. We envisioned an alternative strategy that relies on redirecting endogenous antibodies to cancer cells.

We sought to harness the natural human immune response against the α-Gal epitope to test our hypothesis.[8] This carbohydrate antigen is well known to be immunogenic; indeed, it serves as the major barrier in xenotransplantation.[9] Attempts to transplant porcine donor organs into primates have revealed the importance of hyperacute rejection as a complication. This rejection is mediated by complement, which is recruited when primate anti-Gal antibodies bind to the surface-display of α-Gal on the porcine donor cells.[10] Human cells do not display the α-Gal epitope, unlike most mammalian and bacterial cells.[11] Presumably, it is exposure to these foreign cells that elicits the high level of anti-Gal antibody found in humans. We reasoned that exploiting this known response to reject tumor cells could afford an attractive anticancer strategy. To test our hypothesis, we required bifunctional conjugates that contain, in addition to the α-Gal epitope, a targeting moiety that recognizes an appropriate cell-surface receptor relevant for cancer (Figure 1). Although many biomarkers are upregulated on tumor cells, we sought a receptor that can be targeted with ligands that bind with both high affinity and high specificity. To this end, we examined small-molecule inhibitors of the $\alpha_v\beta_3$ integrin.

Integrins are heterodimeric cell-adhesion receptors that facilitate communication between a cell and its surroundings.[12] Integrins comprise two separate polypeptide chains, and the

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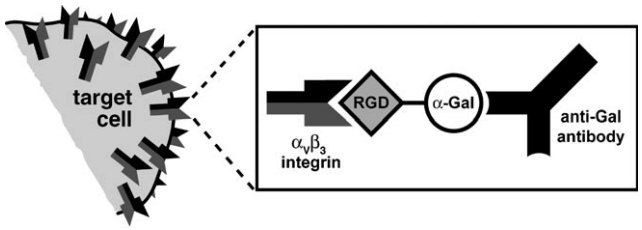


Figure 1. Schematic description of anti-Gal antibody recruitment to the target cell surface by a bifunctional ligand. One portion of the conjugate is designed to selectively bind to cells displaying the $\alpha_v\beta_3$ integrin, while the other displays the α-Gal carbohydrate epitope, which can interact with anti-Gal antibodies.

complex of these α- and β-subunits dictates integrins' binding specificity and ultimate biochemical function. The $\alpha_v\beta_3$ integrin mediates the attachment of cells to the extracellular matrix and has been implicated in tumor-induced angiogenesis, tumor invasion, and metastasis.[13,14] This integrin is upregulated on both cancer cells and tumor-associated blood vessels; however, $\alpha_v\beta_3$ is absent or present only at low levels on most normal tissues.[15] Experiments with arginine-glycine-aspartic acid (RGD) peptide conjugates suggest that integrins can serve as cell-surface receptors for recruiting anti-Gal antibodies.[16] Given its location on the cell surface and its role in cancer, we reasoned that $\alpha_v\beta_3$ would serve as an excellent target receptor.

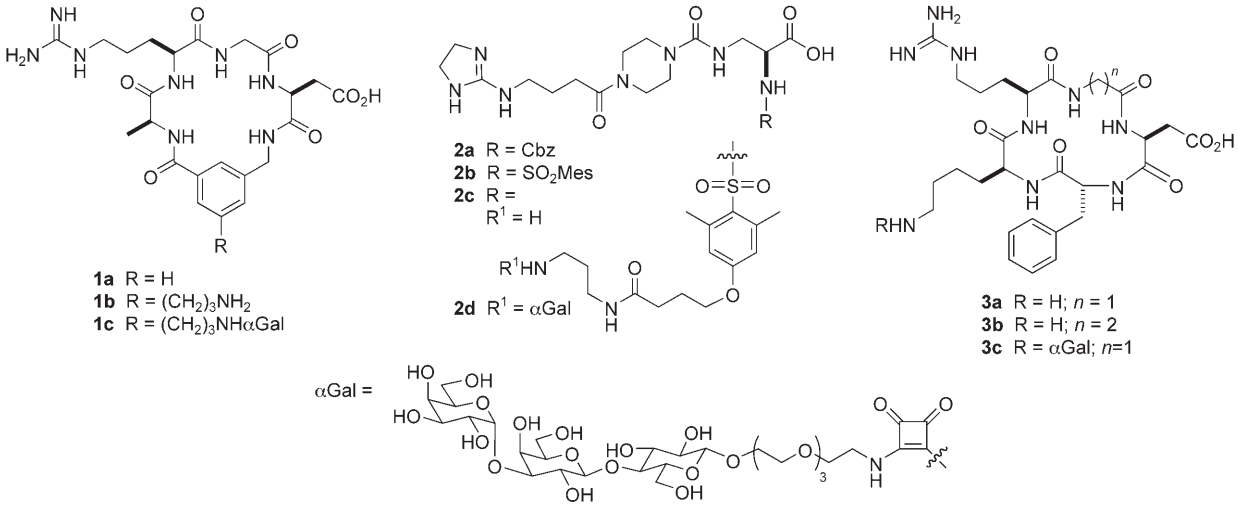
Because they can be readily generated, the most common integrin ligands used are peptides. Linear peptide sequences containing the RGD motif are known to bind integrins, and these have been employed as cell-targeting agents.[17] Because of the low affinity and promiscuity of such linear peptides, however, their utility for selective cell targeting is limited. Peptide derivatives, such as cyclic Arg-Gly-Asp-D-Phe-Lys [c(-RGDfK-); **3a**, Scheme 1], have been used as tumor-homing agents due to their selectivity for $\alpha_v\beta_3$ over the closely related $\alpha_{IIb}\beta_3$ integrin.[18–20] Although more discriminating than its linear counterparts, this derivative is also a ligand for the $\alpha_v\beta_5$ integrin, a receptor highly expressed on many normal cell types. For our studies, we required integrin ligands that could be used for the construction of bifunctional conjugates and that are selective for the $\alpha_v\beta_3$ integrin. Although there are a few examples,[21–25] nonpeptidic derivatives that satisfy these criteria are rare. Several uses have been described for peptidomimetics that exhibit selectivity in integrin targeting.[26] In one example, an integrin-binding small molecule was modified so that it could be conjugated to a variety of antibodies for tumor targeting.[27–30] This ligand and others equipped with handles for modification, however, bind the closely related integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. We set out to expand the toolkit of integrin ligands by generating compounds with the desired attributes that are selective for $\alpha_v\beta_3$.

Here, we report the modular synthesis of novel functionalized $\alpha_v\beta_3$ integrin ligands. We generated several bifunctional conjugates by appending the α-Gal trisaccharide to these ligands, thereby highlighting the benefits of our modular synthetic strategy. We also implement an integrin-dependent cell-adhesion assay to assess the inhibitory potencies of these compounds. Our results indicate that these peptidomimetics maintain their binding affinity and possess high specificity for $\alpha_v\beta_3$. Moreover, the modular assembly method that we employ should facilitate the development of bifunctional conjugates for a variety of cell-targeting applications.

Results and Discussion

Bifunctional conjugate design

The importance of the $\alpha_v\beta_3$ integrin has fueled the discovery of numerous small-molecule ligands.[31,32] As a starting point for our studies, we utilized potent $\alpha_v\beta_3$ antagonists with well-characterized integrin-selectivity profiles. We selected two inhibitors: the cyclic RGD peptide mimetic **1a** and the non-peptidic compound **2a** (Scheme 1). Compound **1a** inhibits binding of purified $\alpha_v\beta_3$ to an immobilized RGD-containing ligand with an IC_{50} value of 20 nM; it preferentially binds $\alpha_v\beta_3$ over the platelet integrin $\alpha_{IIb}\beta_3$ with 1000-fold greater activity.[33,34] Com-



Scheme 1. Target functionalized $\alpha_v\beta_3$ integrin ligands (**1b**, **1c**, **2c**, **2d**, **3c**) and the parent compounds (**1a**, **2b**, **3a**) from which they are derived.

pound **2a** is a potent inhibitor ($IC_{50} = 1.1$ nM) and is at least 400-fold more selective for $\alpha_v\beta_3$ over even more closely related integrins, including $\alpha_v\beta_5$.[35]

To generate the bifunctional conjugates, we devised a modular synthetic strategy. One facile method for linking two compounds is through the use of squaric acid esters.[36] Because the rate of formation of the squaric acid diamide is slower than formation of the monoamide, dimethyl squarate can be used to form a conjugate from two different amine-containing compounds. Thus, we needed an α-Gal derivative and an integrin-binding ligand—each bearing a free amino group. The former can be synthesized readily, as the amine can be appended through an anomeric substituent. To generate an integrin-binding moiety with the desired features, we analyzed the available structural and functional data.

To install a substituent that would preserve the integrin binding and the selectivity of the prototype ligands, we analyzed the structure of the $\alpha_v\beta_3$ integrin complex with the cyclic peptide ligand c(RGDf-*N*[*Me*]V) (Figure 2A).[37] Determination of this structure by X-ray crystallography revealed that, while the critical RGD motif contacts both subunits of the protein, the remaining two residues (D-Phe and *N*-methyl-Val) are solvent exposed. As long as they do not alter the conformation of the RGD-mimicking moiety, structural modifications of this exposed region should be permitted (Figure 2B). Accordingly, amine-bearing compound **1b** should maintain integrin binding (Scheme 1). Similarly, **2a** analogues that bear an appropriate substituent at the amine group α to the carboxylic acid should be accommodated.[38] Specifically, compound **2a** can be elaborated by introducing a functionalized mesityl sulfonamide to provide compound **2b**; studies optimizing RGD-mimetic activity had revealed that other $\alpha_v\beta_3$ inhibitors with arylsulfonamide groups at corresponding positions have excellent potencies.[39] If derivative **2b** possesses the predicted potency, we envisioned introducing a linker via this aryl sulfonamide substituent, as in compound **2c**. With these blueprints, we set out to build the target integrin ligands.

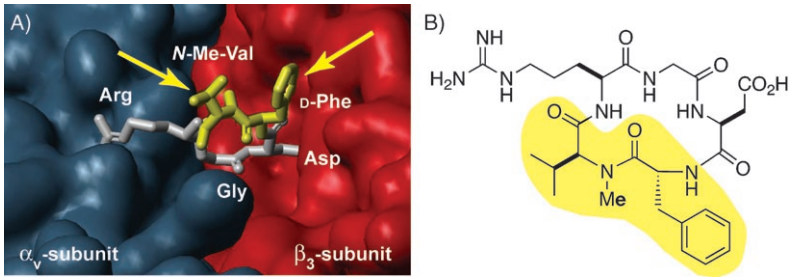
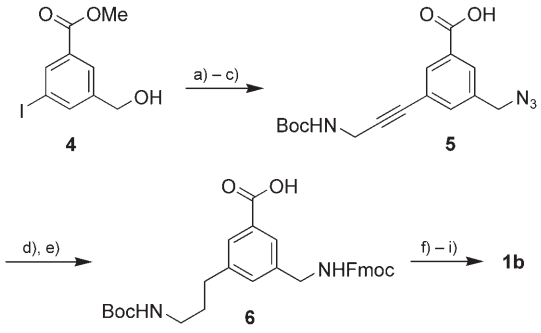


Figure 2. Structural model used to guide the design of integrin-binding compounds. A) Structure of the extracellular domain of $\alpha_v\beta_3$ integrin bound to a cyclic peptide containing the RGD recognition motif as determined by X-ray crystallographic analysis by Xiong et al.[37] The yellow arrows indicate the solvent-exposed regions of the molecule. B) Chemical structure of c(RGDf-*N*[Me]V). The residues highlighted in yellow correspond to parts of the compound that might be modified chemically without perturbing binding to the receptor.

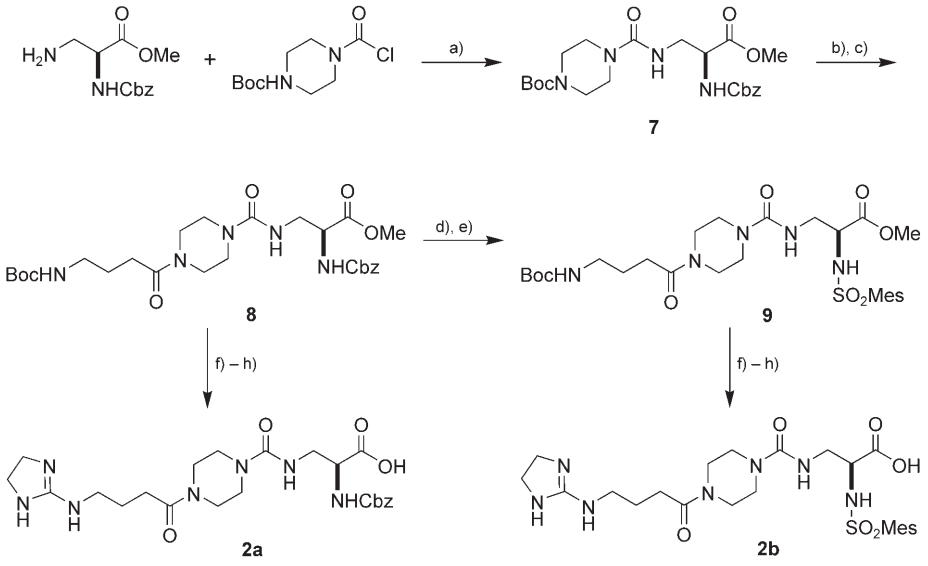
Synthesis of peptidomimetic integrin ligands

Our initial efforts focused on the synthesis of cyclic peptide **1b**, which could be assembled either by solid-phase methods or in solution.[40,41] Guided by a report,[42] we sought to generate the relevant linear peptide using solid-phase synthesis and then cyclize it in solution. Accordingly, a route to non-natural amino acid **6** was required (Scheme 2). We reasoned that the aminopropyl side chain could be introduced by Pd-catalyzed cross coupling. The requisite aryl iodide **4** was synthesized in high yield from the known aniline derivative by using the Sandmeyer reaction.[43] Subsequent introduction of the alkynyl side chain under modified Sonogashira conditions provided the trisubstituted aromatic ring system in excellent yield.[44] Conversion of the benzylic alcohol to the azide group under standard conditions and subsequent hydrolysis of the methyl ester provided intermediate **5**. This compound then was transformed into the desired Fmoc-protected amino acid **6** by dual reduction of the azide and alkynyl functionalities with Pearlman's catalyst. Subsequent protection of the benzylic amine was effected under standard conditions. With amino acid **6** in hand, the desired protected peptide sequence was synthesized and cleaved under standard Fmoc solid-phase peptide synthesis (SPPS) conditions. The crude peptide was then cyclized with benzotriazole-1-yl-oxytrispyrrolidinophosphonium hexafluorophosphate (PyBOP) in dimethylformamide (DMF). The protecting groups were removed with trifluoroacetic acid (TFA), and the product was purified by high performance liquid chromatography (HPLC) to provide ligand **1b** in 11 steps and 22% overall yield.

In addition to preparing cyclic peptide **1b**, we also sought to generate sulfonamide-containing inhibitors **2b** and **2c**. We envisioned that the former would be a valuable comparator in assessing the relative potency of **2c** as an $\alpha_v\beta_3$ integrin ligand (vida infra). In their initial studies, DeGrado and co-workers synthesized ligand **2a** on a solid support as part of a larger



Scheme 2. Synthesis of the non-natural amino acid **6** and its use in generating a cyclic RGD mimetic **1b**: a) $PdCl_2(PPh_3)_2$, CuI, THF, Et_3N, *N*-Boc-propargyl amine, 98%; b) MsCl, Et_3N, toluene, NaN_3, Bu_4NBr, H_2O; c) LiOH, THF, H_2O, 85% over 2 steps; d) $Pd(OH)_2/C$, MeOH; e) Fmoc-OSu, Et_3N, ACN/H_2O, 65% over 2 steps; f) standard Fmoc SPPS; g) 1% TFA, CH_2Cl_2; h) PyBOP, DIPEA, DMF (1.5 mM); i) TFA, TIS, H_2O, 45% over 4 steps.



Scheme 3. Synthesis of the nonpeptidic RGD mimetic **2a** and its sulfonamide analogue **2b**: a) Et_3N, CH_2Cl_2, 92%; b) HCl, dioxane, MeOH; c) *N*-Boc-4-aminobutyric acid, EDCI, DMAP, Et_3N, CH_2Cl_2, 89% over 2 steps; d) H_2, $Pd(OH)_2$/C, MeOH, $CHCl_3$; e) $ClSO_2Mes$, Et_3N, CH_2Cl_2, 88% over 2 steps; f) HCl, dioxane, MeOH; g) 2-methylthio-2-imidazoline hydroiodide, MeOH, Et_3N, Δ; h) LiOH, H_2O, 82% for **2a** and 70% for **2b** over 3 steps.

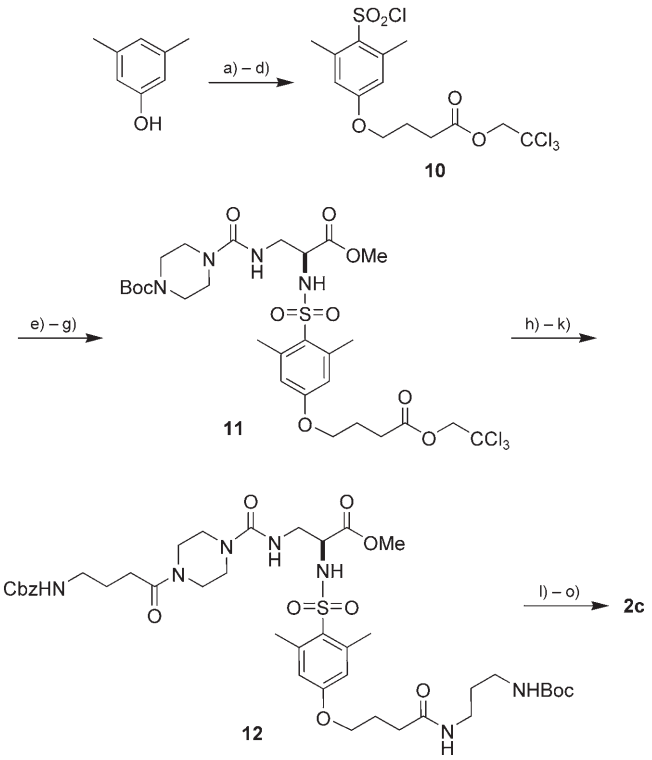
combinatorial library.[35] For our studies, however, we required quantities larger than those conveniently prepared by SPPS. We therefore developed an iterative, solution-phase route (Scheme 3).

Our route began with the commercially available diaminopropionic acid, which we esterified to form the known methyl ester derivative.[38] Initial attempts to introduce the urea linkage by using the coupling agent employed in the solid-phase route, *para*-nitrophenyl chloroformate, proved unsuccessful. Treatment with a known piperazine-derived chloroformamide,[45] however, provided the desired intermediate **7** in high yield. The cyclic secondary amine was liberated by acid-induced cleavage of the Boc protecting group, and this product was subjected to amide bond-forming conditions to afford compound **8** in excellent overall yield. After removal of the Boc group, the arginine mimic was introduced by using 2-methylthio-2-imidazoline hydroiodide; hydrolysis of the methyl ester provided known compound **2a** (Scheme 3).

Compound **8** could readily be elaborated to generate sulfonamide **2b**. To this end, the Cbz protecting group was removed by hydrogenolysis, and the resulting amine was treated with 2-mesitylenesulfonyl chloride to provide **9** in high yield. The desired compound **2b** was generated in three additional steps: removal of the Boc-protecting group, introduction of the guanidine group as above, and cleavage of the methyl ester (Scheme 3).

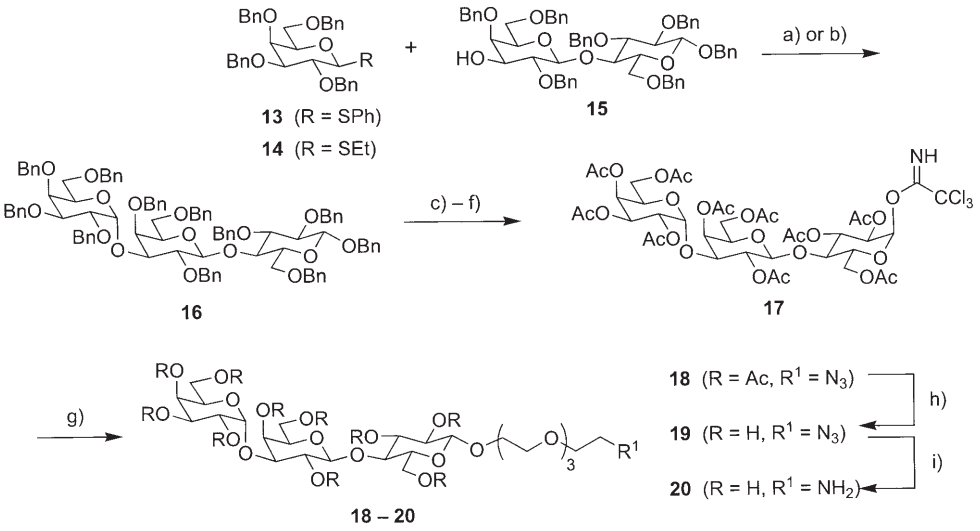
To embed a linker within the aryl sulfonamide group of **2c**, we assembled aryl sulfonyl chloride **10** (Scheme 4). We subjected the commercially available 3,5-dimethylphenol to alkylation with 4-bromoethyl butyrate, and the resulting ethyl ester was converted to the acid under standard hydrolytic conditions. We explored the direct conversion of this intermediate to the corresponding sulfonyl chloride with chlorosulfonic acid; however, the desired product was not isolated. As a result, we masked the acid group to generate the 2,2,2-trichloroethyl ester; this protecting group was selected because of its stability to acids and compatibility with our synthetic route. Indeed, treatment of the ester with chlorosulfonic acid readily generated protected sulfonyl chloride **10**.

The presence of the linker within the aryl sulfonamide of compound **2c** necessitated some changes to the route used to assemble **2b** (Scheme 4). Compound **10** was modified with *N*-β-Boc-protected diaminopropionic acid derivative to generate the expected sulfonamide. After removal of the Boc group, the free amine could be modified with the aforementioned pi-



Scheme 4. Synthetic route for the preparation of linker-functionalized compound **2c**: a) ethyl-4-bromobutyrate, K_2CO_3, KI, DMF; b) NaOH, EtOH/H_2O, 77% over 2 steps; c) EDCI, DMAP, $HOCH_2CCl_3$, CH_2Cl_2, 95%; d) $ClSO_2Cl$, CH_2Cl_2, 51%; e) NH_2-Dap(Boc)-OMe, Et_3N, CH_2Cl_2, 81%; f) 4 N HCl, dioxane; g) Boc-protected piperazine-derived chloroformamide,[45] Et_3N, CH_2Cl_2, 84% over 2 steps; h) 4 N HCl, dioxane; i) Cbz-4-aminobutyric acid, EDCI, DMAP, Et_3N, CH_2Cl_2, 94% over 2 steps; j) Zn, THF, 1 M KH_2PO_4; k) Boc-$NH(CH_2)_3NH_2$, EDCI, NHS, Et_3N, CH_2Cl_2, 83% over 2 steps; l) H_2, $Pd(OH)_2$/C, MeOH/$CHCl_3$, 100%; m) 2-methylthio-2-imidazoline hydroiodide, MeOH, Et_3N, Δ; n) LiOH, H_2O; o) TFA, 54% over 3 steps.

perazine-derived chloroformamide to produce compound **11**. Removal of the Boc protecting group under acidic conditions afforded the free secondary amine. Initially, we synthesized a compound in which the 4-aminobutyric acid moiety was protected with a Boc group (i.e., in analogy to compound **8**). Unfortunately, cleavage of the Boc group en route to introduction of the guanidine derivative led to a complex product mixture. A switch in protecting group from Boc to Cbz solved the problem. Removal of the 2,2,2-trichloroethyl ester was effected by Zn. Initial attempts to directly couple the resulting acid with a mono-Boc-protected diamine[46] under standard, single-step amide bond-forming reaction conditions provided compound **12**—but only in low yield. Converting the acid to the succinimidyl (NHS) ester prior to coupling greatly increased the yields of the desired product. Hydrogenolysis of the Cbz protecting group efficiently provided the resulting primary amine in quantitative yield. This compound was ultimately converted to the substituted guanidine derivative, and the remaining protecting groups were removed under standard conditions to afford target compound **2c** in 15 steps from 3,5-dimethylphenol (Scheme 4).



Scheme 5. Synthetic route to the α-Gal trisaccharide possessing an oligo(ethylene glycol)-based linker **20**: a) $PhHgOTf$, CH_2Cl_2, 90%; b) $CuBr_2/Bu_4NBr$, 80%; c) H_2, 10% Pd/C, EtOAc/MeOH/H_2O/AcOH; d) Ac_2O, DMAP, pyridine, 92% over 2 steps; e) NH_2NH_2·HOAc, DMF; f) Cl_3CCN, DBU, CH_2Cl_2, 75% over 2 steps; g) H-$(OCH_2CH_2)_4$-N_3, BF_3·OEt_2, CH_2Cl_2, 4 Å MS, 58%; h) cat. NaOMe, MeOH; i) H_2, $Pd(OH)_2$/C, MeOH/$CHCl_3$, 98% over 2 steps.

Preparation of the α-Gal epitope

To generate the bifunctional conjugates, we planned to tether an $\alpha_v\beta_3$ integrin ligand to the α-Gal epitope through a linker at the carbohydrate anomeric position. The disaccharide Galα(1–3)Gal is the minimal structure suggested to be required for anti-Gal antibody recognition. Still, equilibrium-binding studies indicate that this carbohydrate binds only weakly to the anti-Gal antibody ($IC_{80} = 3.3$ mM).[47] In addition, we found that even multivalent presentations of this epitope are poor ligands for anti-Gal antibodies (unpublished results). In contrast, the interaction of the trisaccharide Galα(1–3)Galβ(1–4)Glc for the anti-Gal antibody is at least threefold stronger. Moreover, it appears that this epitope can recruit naturally occurring anti-Gal antibodies.[48] As a linker, we used an oligo(ethylene glycol)-based moiety terminated with an azide group. This structure was added to provide adequate separation between the two recognition motifs, because both the cell-surface receptor and the anti-Gal antibody must bind simultaneously. Studies with surface-bound displays of $\alpha_v\beta_3$ integrin ligands have indicated that linkers that can span approximately 20 Å (at their full extension) are required for efficient interaction with $\alpha_v\beta_3$-positive cells.[49] Lastly, the azide serves as a masked amino group; it can be converted under mild conditions into a substrate for squarate coupling. Thus, the desired trisaccharide **20** was selected for synthesis (Scheme 5).

Several methods for preparing α-galactosyl trisaccharides have been reported.[50–53] The key challenge is to form the alpha linkage efficiently and with excellent stereoselectivity. To exploit the anomeric effect in forming the axial anomer, conditions that result in an S_N1-like mechanism with a late transition state should favor the desired product. We initially followed a previously published protocol describing the high-yielding reaction (>90%) between a 3′-OH group on a lactosyl acceptor and a benzyl-protected galactosyl donor with an anomeric phenyl sulfoxide group.[54] We repeated this procedure and obtained the fully protected trisaccharide in high yield (90%), but only as an inseparable α/β isomeric mixture. Accordingly, we turned our attention to a metal-catalyzed reaction of a phenyl thiogalactoside **13** galactosyl donor. We reasoned that this process should proceed along the desired mechanistic pathway. Indeed, the glycosylation reaction of **13** and **15**[55] in the presence of phenylmercury triflate[56] gave exclusively the α-glycoside **16** in excellent yield (90%).

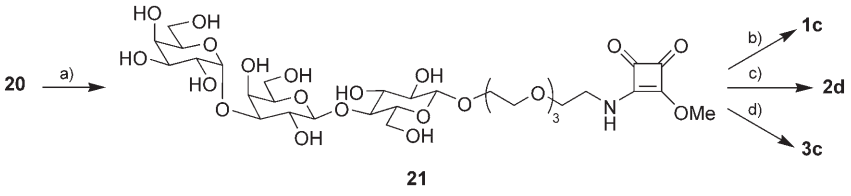
To avoid using a toxic catalyst in the assembly of carbohydrate **16**, several other glycosylation conditions were examined. The most efficient procedure tested employed the donor ethyl thiogalactoside **14** and copper(II) bromide–tetrabutylammonium bromide as a promoter,[57] and led to **16** in 80% yield along with some (10%) recovered disaccharide starting material **15**. Although it remains less efficient than the classical mercury-catalyzed glycosylation reaction, we found this latter method effective.

We appended the anomeric linker after generating the trisaccharide, as this strategy allows for the introduction of different anomeric substituents. We converted compound **16** into an appropriate glycosyl donor, the peracetylated trichloroacetimidate derivative **17**, in four steps. The glycosylation reaction proceeded smoothly to afford compound **18**, which possesses the azide-bearing linker. Removal of the acetate protecting groups had to be carried out at low temperature (4 °C) with a catalytic amount of sodium methoxide (NaOMe) to attain quantitative yields of **19**. At room temperature or under more alkaline reaction conditions, undesired side reactions occurred. The azido sugar **19** was reduced by catalytic hydrogenation to give the desired amine **20** in 8 steps and 35% or 31% overall yield from **13** or **14**, respectively. With access to appropriately functionalized integrin-targeting ligands and the oligosaccharide unit, we turned to assembling bifunctional conjugates.

Bifunctional conjugates

As described, a critical objective of our initial studies was to synthesize bifunctional conjugates that contain a cell-surface-targeting agent and a moiety that could direct the immune response to tumor cells. Because different ligands can serve as the tumor homing agents or the immune system activating components, the modularity of dimethyl squarate-mediated coupling is attractive.[36] This conjugation chemistry is both chemoselective and compatible with unprotected carbohydrate epitopes.[58,59] With regard to integrin ligand coupling, it is known that primary amine groups can be selectively functionalized in the presence of guanidinium groups.[60,61] Still, the utility of dimethyl squarate for assembling this type of complex conjugate was untested; nevertheless, we sought to apply it to the construction of conjugates **1c**, **2d**, and **3c**.

Because amine-bearing unprotected carbohydrates can react selectively with dimethyl squarate, we used trisaccharide **20** as the initial coupling partner. As expected, this compound underwent a chemoselective reaction to provide compound **21** (Scheme 6). To generate the bifunctional ligands, compound **21** was incubated under basic, aqueous conditions with the putative $\alpha_v\beta_3$ integrin ligand **1b** or **2c**. After complete consumption of the integrin ligand, the desired products **1c** and **2d**, respectively, were isolated in high yields. The same synthetic strategy was applied to tether the cyclic RGD peptide, c-(-RGDfK-) **3a**, to the α-Gal moiety, thereby yielding conjugate **3c**. Because the activity of **3a** as an $\alpha_v\beta_3$-targeting ligand has been well characterized, we envisioned that **3c** could be used to calibrate our binding studies.



Scheme 6. Strategy for the modular synthesis of bifunctional conjugates **1c**, **2d**, and **3c** by using a squarate-mediated coupling reaction: a) dimethyl squarate, Et_3N, $MeOH/H_2O$, 69%; b) compound **1b**, 50 mM borate buffer (pH 9), 71%; c) compound **2c**, 50 mM borate buffer (pH 9), 66%; d) compound **3a**, 50 mM borate buffer (pH 9), 45%.

Integrin-binding assay

To ascertain whether our compounds would be useful as cell-surface-targeting agents, a method was needed to evaluate their potency and selectivity for $\alpha_v\beta_3$. To this end, we examined their ability to inhibit the binding of WM115 cells, an $\alpha_v\beta_3$-positive human melanoma cell line, to fibrinogen, a known protein ligand for the $\alpha_v\beta_3$ integrin.[62] By adapting a cell-adhesion assay that had been applied to assess inhibitors of VLA-4 binding to VCAM-1,[63] we devised a high-throughput assay for identifying $\alpha_v\beta_3$ ligands. Briefly, individual V-shaped wells of a microtiter plate were coated with fibrinogen and then blocked. WM115 tumor cells, labeled with a membrane-permeable fluorescein [5-carboxyfluorescein diacetoxymethyl ester (BCECF-AM)], were added to the wells in the presence of various concentrations of compound. After incubation, the plate was gently centrifuged to concentrate the nonadherent cells in the bottoms of the wells; the fluorescence emission from the resulting pellets was measured from below. Each of the known inhibitors (**1a**, **2a**, and **3a**) was capable of preventing adhesion of the cells to fibrinogen, and their IC_{50} values were in the expected (10^{-9} M) range (Table 1). The relative potencies determined with this assay are consistent with those from previous studies,[18,33–35] a result that underscores the utility of this assay. The observed inhibition depends on the structure of the peptidomimetic. Compound **3b**,[49] in which the critical glycine residue has been replaced with β-alanine, was unable to inhibit binding (IC_{50} value $\gg$ 5 μM).

The potent IC_{50} values for the bifunctional compounds (Table 1) support the validity of our attachment strategy. For example, the trisaccharide substituent of conjugate **3c** had minimal effect on its inhibitory potency (compare with **3a**). This result is consistent with previous studies involving modification of compound **3a**.[64,65] In contrast, when the α-Gal epitope was introduced in conjugate **1b** to afford **1c**, the latter was more than tenfold less active. Ultimately, the functionalized derivatives based on ligand **2a** proved to be the most potent. As hypothesized, conjugate **2b**, in which the Cbz group is replaced by a mesitylsulfonamide moiety, is 15-fold more active than **2a**. Further modification of the 4-position of the mesityl group led to minimal changes in the observed potency, as can be seen by the IC_{50} value of 1.3 nM for compound **2c**. Interestingly, the bifunctional compound **2d** is slightly more potent than the corresponding integrin ligand **2a**. These data provide clear evidence that each of the designed bifunctional conjugates can bind to the $\alpha_v\beta_3$ integrin.

Table 1. Inhibition constants (IC_{50} values) of compounds **1a–c**, **2a–d**, and **3a–c** determined in an assay assessing the binding of $\alpha_v\beta_3$-positive WM115 cells to immobilized fibrinogen.

Compound	IC_{50} [nM]	Compound	IC_{50} [nM]
1a	61 ± 30	**1b**	68 ± 100
1c	930 ± 600	**2a**	8.1 ± 2
2b	0.55 ± 0.2	**2c**	1.3 ± 0.4
2d	1.8 ± 0.7	**3a**	24 ± 6
3b	> 5000	**3c**	47 ± 50

In addition to high affinity, our targeting strategy requires that the bifunctional ligands possess high selectivity for the target $\alpha_v\beta_3$ integrin. To assay for specificity over a key related integrin, $\alpha_v\beta_5$, we utilized MCF7 human breast carcinoma cells, which are known to display $\alpha_v\beta_5$. Vitronectin, the natural protein ligand for this receptor,[62] was substituted for fibrinogen in our fluorescence-based cell-binding assay. Under these conditions, we measured a significantly higher IC_{50} value of 7.8 ± 5.7 μM for conjugate **2d**, which represents more than a 4000-fold decrease in potency. This value suggests that compound **2d** is even more selective for $\alpha_v\beta_3$ integrin than the compound (**2a**) upon which it is based. These data suggest that conjugates based on our the potent inhibitor **2d** will exhibit excellent cell-targeting selectivity.

Antibody-binding assay

For our synthetic conjugates to function as designed, they must bind to $\alpha_v\beta_3$-displaying cells and interact simultaneously with anti-Gal antibodies. To evaluate whether they can act in this capacity, we incubated an $\alpha_v\beta_3$-positive cell line, M21 human melanoma cells, with 10 nM of compound **2d** and human serum; the latter serves as a source of anti-Gal IgG. To test for binding of anti-Gal, washed cells were treated with a fluorescein-labeled anti-human IgG secondary antibody and subsequently analyzed by flow cytometry. In the absence of **2d**, the cells displayed no anti-Gal binding; however, cells treated with **2d** exhibited a significant increase in the fluorescence signal (Figure 3). These results indicate that bifunctional ligand **2d** maintains its ability to interact with anti-Gal antibodies when bound to the surface of $\alpha_v\beta_3$-positive cells. Because both the integrin binding domain and the anti-Gal epitope can function simultaneously, our results bode well for using these or related bifunctional ligands as novel tumor-targeting agents.

Conclusions

In summary, we have successfully developed a modular route to bifunctional conjugates that target cells displaying the $\alpha_v\beta_3$ integrin. In devising integrin ligands with the appropriate attributes, we designed and synthesized two $\alpha_v\beta_3$-binding small molecules with excellent potencies. In addition, the selectivity of compound **2d** for $\alpha_v\beta_3$ over related integrins indicates that it is a valuable new addition to the limited set of functionalized non-peptidic ligands that bind this receptor.

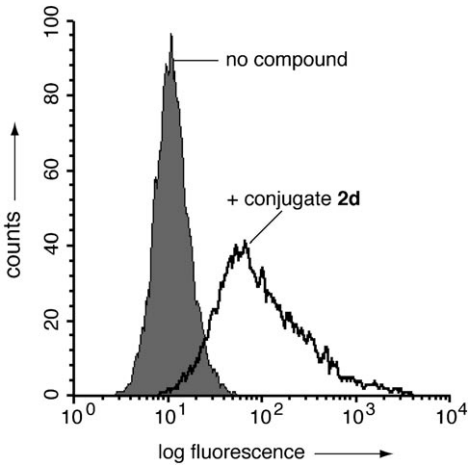


Figure 3. Representative flow cytometry histogram illustrating anti-Gal antibody binding to M21 cells. M21 tumor cells were treated with bifunctional conjugate **2d** and human serum, a source of anti-Gal IgG. Antibody binding was detected by flow cytometry and a fluorophore-labeled secondary anti-human antibody.

The sites for modification integrated into our $\alpha_v\beta_3$ integrin ligands and the dimethyl squarate coupling chemistry that we have employed can be exploited for a variety of purposes. For instance, the handles we have installed can be used to immobilize the integrin ligands, thereby creating surfaces for $\alpha_v\beta_3$-positive cell adhesion or growth.[26,66] Alternatively, these handles can serve as points of attachment to tumor imaging agents. Finally, our functionalized integrin ligands can be used to append protein or small-molecule toxins to create novel antitumor agents.

Experimental Section

General: All materials were obtained from commercial suppliers and used as provided unless otherwise noted. Reaction solvents were purified by distillation by using standard protocols. Tetrahydrofuran (THF), diethyl ether, toluene, and benzene were distilled from sodium metal and benzophenone under an argon atmosphere. Triethylamine and dichloromethane were distilled from calcium hydride. Methanol was distilled from magnesium. Dimethylformamide (DMF) was rendered amine-free by treatment with Dowex 50WX8–200 cation-exchange resin (H^+ form, 1 g L^{-1}). Dimethylsulfoxide (DMSO) was stored over 3 Å molecular sieves. All moisture- and air-sensitive reactions were carried out in flame-dried glassware under an atmosphere of nitrogen. Liquid reagents were introduced by oven-dried glass syringes. To monitor the progress of reactions, thin-layer chromatography (TLC) was performed with Merck (Darmstadt) silica gel 60 F_{254} precoated plates by eluting with the solvents indicated. Analyte visualization was accomplished by busing a multiband UV lamp and charring with one of the following stains: *p*-anisaldehyde, ninhydrin, potassium permanganate, or phosphomolybdic acid. Flash chromatography (FC) was performed on Scientific Adsorbents Incorporated silica gel (32–63 μM, 60 Å pore size) by using distilled reagent-grade hexanes and ACS-grade ethyl acetate or methanol and chloroform. ^1H and ^{13}C NMR spectra were recorded on Bruker AC-300 or Varian Inova-500 spectrometers, and chemical shifts are reported relative to tetramethylsilane (TMS) or residual solvent peaks in parts per

million. Yields were calculated for materials that appeared as a single spot by TLC and homogeneous by ^{1}H NMR. HPLC was performed on a Spectra-Physics UV2000 instrument, with UV absorption at 220 nm and/or 254 nm for analyte detection. Samples were eluted on reversed-phase C18 columns from Vydac (Protein & Peptide $l=220$ mm, i.d. = 5 or 10 mm, 10 or 22 μm particle size). Liquid chromatography/mass spectroscopy (LCMS) measurements were performed on a Shimadzu LCMS 2010.

Biological studies: All chemicals were from Sigma–Aldrich unless otherwise noted. All cell-culture reagents, including minimal essential medium alpha (αMEM), RPMI-1640, fetal bovine serum (FBS), penicillin-streptomycin (pen-strep), L-glutamine (Gln), insulin, and trypsin-EDTA, were from Invitrogen. Tissue culture flasks for adherent cells were obtained from Sarstedt (Newton, NC). The dye 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) was from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA) was from Research Organics (Cleveland, OH). V-shaped 96-well plates were obtained from Nalge Nunc, International (Rochester, NY). Fibrinogen and vitronectin were from CalBiochem (San Diego, CA). FITC-labeled goat anti-human IgG was from Vector Laboratories (Burlingame, CA).

Tumor cells: Human MCF7 breast carcinoma cells and WM115 melanoma cell lines were from American Type Culture Collection (Manassas, VA). M21 cells (sorted for high levels of $\alpha_v\beta_3$ integrin) were kindly provided by Drs. P. M. Sondel and S. C. Helfand (University of Wisconsin–Madison). WM115 cells were grown in αMEM supplemented with 10% FBS, Gln (2 mM), and 100 U antibiotics pen-strep. MCF7 cells were grown as above, but the medium was further supplemented with insulin (0.01 mg mL^{-1}). M21 cells were cultured in RPMI-1640 supplemented with 10% FBS, Gln (2 mM), and 100 U pen-strep. All cells were detached from flasks with 0.05% trypsin-EDTA.

Synthesis of compound 4: The known aniline derivative[43] (1.0 g, 5.7 mmol) was dissolved in a 1:1 (*v*/*v*) mixture of acetone and aqueous H_2SO_4 (3 N, 260 mL), and the solution was cooled to −20 °C. A solution of $NaNO_2$ (9.0 g, 0.13 mol) in H_2O (70 mL) was added dropwise, during which time the mixture became gummy. To this suspension, a solution of urea (1.4 g, 23 mmol) and KI (33.0 g, 200 mmol) in H_2O (50 mL) was added dropwise. Nitrogen gas evolved from the reaction during the course of the addition. The mixture was removed from the ice bath and stirred for 3 h at RT. Saturated aqueous $NaHCO_3$ (200 mL) was added to the mixture, and the acetone was removed under reduced pressure. The resulting solution was extracted with EtOAc (3×100 mL), and the combined organic extracts were washed with saturated aqueous $NaHCO_3$ (2×80 mL) and brine (2×80 mL) and then dried (Na_2SO_4). The solvents were removed under reduced pressure, and the residue was purified by FC (hexane/EtOAc 1:1) to yield **4** as a white solid (1.53 g, 92%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=8.23$ (m, 1H), 7.92 (m, 1H), 7.88 (m, 1H), 4.66 (s, 2H), 3.89 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=165.49$, 143.23, 139.64, 137.01, 131.40, 126.66, 126.47, 93.77, 77.40, 76.97, 76.55, 63.23, 52.34; LRMS (EI): calcd for $C_9H_9IO_3$ $[M]^+$ 292.0; found 292.0.

Synthesis of compound 5: Aryl iodide **4** (490 mg, 1.7 mmol), *N*-Boc-propargyl amine (520 mg, 3.4 mmol), and $PdCl_2(PPh_3)_2$ (36 mg, 0.050 mmol) were dissolved in THF (7 mL); Et_3N (490 μL, 3.4 mmol) was added, and the reaction mixture was stirred for 10 min at RT. CuI (9.5 mg, 0.050 mmol) was then added, and the reaction mixture was stirred for 1.5 h at RT. After this time, the THF was removed under reduced pressure, and the residue was suspended in EtOAc (15 mL). The resulting suspension was washed with 5% aqueous citric acid (2×5 mL), saturated aqueous $NaHCO_3$ (2×5 mL), 1% aqueous sodium diethyldithiocarbamate (2×5 mL), and brine (2×5 mL) and dried (Na_2SO_4). The solvents were removed under reduced pressure, and the residue was purified by FC (hexane/EtOAc 3:1 to 2:1) to yield the alkyne product (534 mg, 98%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=7.89$ (s, 2H), 7.53 (s, 1H), 5.09 (br s, 1H), 4.63 (s, 2H), 4.09 (d, $J=5.1$ Hz, 1H), 3.86 (s, 3H), 3.25 (br s, 1H), 1.43 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=166.39$, 155.49, 141.88, 134.01, 131.64, 130.32, 127.50, 123.23, 86.42, 81.93, 79.94, 63.90, 52.29, 31.04, 28.34; LRMS (ESI): calcd for $C_{34}H_{42}N_2NaO_{10}$-$[2M+Na]^{2+}$ 661.2; found 661.2.

The above alkyne (530 mg, 1.7 mmol) was dissolved in THF (8 mL), Et_3N (960 μL, 6.6 mmol) was added, and the mixture was cooled to 0 °C. Methanesulfonyl chloride (200 μL, 2.50 mmol) was added dropwise, and the reaction mixture was stirred at RT for 1.5 h. A solution of NaN_3 (860 mg, 13 mmol) and Bu_4NBr (54 mg, 0.17 mmol) in water (2 mL) was then added, and the mixture was heated at reflux for 20 min. After cooling, the reaction mixture was diluted with EtOAc (15 mL), washed with 5% aqueous citric acid (2×5 mL), saturated aqueous $NaHCO_3$ (2×5 mL), and brine (2×5 mL) and dried (Na_2SO_4). Removal of the solvents under reduced pressure followed by purification by FC (hexane/EtOAc 7:1) provided the azide as an oil (508 mg, 89%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=8.01$ (t, $J=1.6$ Hz, 1H), 7.89 (t, $J=1.7$ Hz, 1H), 7.52 (t, $J=1.7$ Hz, 1H), 4.92 (br s, 1H), 4.35 (s, 2H), 4.13 (d, $J=5.8$ Hz, 1H), 3.90 (s, 3H), 1.45 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=165.79$, 155.28, 136.17, 135.06, 132.45, 130.87, 128.67, 123.79, 110.06, 87.12, 81.43, 53.78, 52.32, 30.99, 28.27; LRMS (ESI): calcd for $C_{17}H_{20}N_4NaO_4$ $[M+Na]^+$ 367.1; found 367.1.

This azide intermediate (500 mg, 1.45 mmol) was dissolved in THF/MeOH (10 mL, 10:1), and a solution of LiOH (120 mg, 2.9 mmol) in H_2O (3 mL) was added. The mixture was stirred for 3 h, then THF and MeOH were removed under reduced pressure. HCl (1 N) was added to the remaining liquid, and the mixture was extracted with EtOAc (3×10 mL). The combined organic layers were washed with brine (3×10 mL) and dried (Na_2SO_4). The solvent was removed under reduced pressure to yield **5** (461 mg, 96%) as a white solid. ^{1}H NMR (300 MHz, CD_3OD): $\delta=7.95$ (t, $J=1.4$ Hz, 1H), 7.91 (t, $J=1.4$ Hz, 1H), 7.56 (t, $J=1.4$ Hz, 1H), 4.42 (s, 2H), 4.07 (s, 2H), 1.45 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD): $\delta=168.88$, 158.38, 138.69, 136.58, 133.68, 133.29, 130.36, 125.61, 88.94, 82.16, 81.10, 55.02, 31.79, 29.14; LRMS (ESI): calcd for $C_{16}H_{17}N_4O_4$ $[M-H]$ 329.1; found 329.1.

Synthesis of amino acid 6: Azide **5** (460 mg, 1.39 mmol) was dissolved in MeOH/$CHCl_3$ (30 mL, 30:1) and solid $Pd(OH)_2/C$ (120 mg) was added. The reaction mixture was placed under 1 atm of H_2 for 3 h and then filtered through Celite. Removal of solvent under reduced pressure provided the crude amine which was used directly in the next reaction. ^{1}H NMR (300 MHz, CD_3OD): $\delta=7.97$ (br s, 1H), 7.89 (br s, 1H), 7.26 (br s, 1H), 4.19 (s, 2H), 3.06 (t, $J=6.8$ Hz, 2H), 2.72 (t, $J=6.8$ Hz, 2H), 1.81 (p, $J=7.5$, 2H), 1.43 (s, 9H); LRMS (ESI): calcd for $C_{16}H_{25}N_2O_4$ $[M+H]^+$ 309.2; found 309.2.

The crude amine was dissolved in water (2 mL), and Et_3N was added to adjust the solution to pH 8. The mixture was cooled to 0 °C, a solution of 9-fluorenylmethyloxycarbonyl-*N*-hydroxysuccinimide (Fmoc-OSu; 520 mg, 1.5 mmol) dissolved in acetonitrile (6 mL) was then added, and the pH of the solution was readjusted to 8. After 1.3 h at RT, the pH was adjusted to 5 with 1 N HCl; the acetonitrile was removed under reduced pressure. The remaining solution was acidified to pH 2, washed with EtOAc and CH_2Cl_2 (3× 10 mL each), and the organic layers were dried (Na_2SO_4). The solvents were removed under reduced pressure, the resulting residue

was dissolved in MeOH, the mixture was adsorbed onto silica gel, and the resulting mixture was placed atop a silica gel column with CH_2Cl_2. Elution with hexane/EtOAc (2:1) with 1% acetic acid provided **6** (480 mg, 65% over two steps) as a white solid, which contained a trace of fluorenyl by-products. ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD): δ = 7.72 (m, 4H), 7.57 (d, J = 7.7 Hz, 2H), 7.27 (m, 5H), 4.31 (m, 3H), 4.15 (t, J = 6.5 Hz, 1H), 3.03 (t, J = 7.1 Hz, 2H), 2.62 (t, J = 7.4 Hz, 2H), 1.75 (t, J = 7.4 Hz, 2H), 1.39 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD): δ = 168.33, 156.98, 156.45, 143.34, 141.88, 140.69, 138.97, 131.49, 130.42, 128.02, 127.06, 126.45, 125.74, 127.46, 19.26, 78.52, 66.25, 43.79, 43.67, 39.25, 32.13, 30.77, 27.52; LRMS (ESI): calcd for $C_{31}H_{33}N_2O_6$ $[M+H]^+$ 529.2; found 529.3.

Solid-phase synthesis of peptide 1b: Synthesis was performed manually in a 10 mL polyethylene syringe containing a polypropylene frit. Fmoc-Gly-Sasrin resin (0.69 mmol g^{-1} loading, 114 mg, 0.0786 mol; Bachem) was swelled in and washed with CH_2Cl_2 and DMF prior to use. To effect cleavage of the Fmoc group, a solution of piperidine in DMF (20%, 4 mL) was drawn up into the syringe, and the vessel was agitated for 5 min. The resin was washed with DMF (2×), and the process was repeated. The resin was washed with DMF (3×), CH_2Cl_2 (3×), MeOH (1×), and DMF (3×), and the success of the cleavage was assessed by Kaiser test. Next, the desired amino acid (4 equiv), PyBOP (4 equiv) and HOBt (4 equiv) were dissolved in a minimal amount of DMF. *N*,*N*-diisopropylethylamine (DIPEA; 4 equiv) was added, and the solution was drawn into the syringe. The reaction vessel was agitated for 2 h, the resin was washed with DMF (3×), CH_2Cl_2 (3×), MeOH (1×), and DMF (3×), and the success of the coupling was assessed again by the Kaiser test. This process was repeated for each amino acid residue. After final Fmoc cleavage, the resin was first washed with CH_2Cl_2 (3×). A 1% TFA solution (5 mL) was drawn into the syringe and agitated for 15 min and then expelled into a mixture of CH_2Cl_2 and Et_3N. This process was repeated (5×), and the resin was washed with CH_2Cl_2 (2×). Concentration of the cleavage solutions under reduced pressure provided the crude, side-chain-protected linear peptide (58 mg). Identity of the product was confirmed by LCMS (ESI): calcd for $C_{48}H_{74}N_9O_{13}S$ $[M+H]^+$ 1016.5; found 1016.4.

To effect cyclization in solution, a portion of the crude peptide (43 mg, 0.042 mmol) was dissolved in distilled DMF (30 mL, 0.0015 mM), and PyBOP (26 mg, 0.050 mmol) and DIPEA (23 μL, 0.13 mmol) were added. The reaction mixture was stirred for 12 h at RT, a second portion of PyBOP (26 mg, 0.050 mmol) and DIPEA (23 μL, 0.13 mmol) was added, and the reaction mixture was stirred for an additional 12 h. Water was added, and the solvents were removed under high vacuum. The identity of the product was confirmed by LCMS (ESI): calcd for $C_{48}H_{74}N_9O_{13}S$ $[M+H]^+$ 998.5; found 998.4.

The crude cyclized product was dissolved in a TFA deprotection cocktail (TFA/triisopropylsilane (TIS)/H_2O, 95:2.5:2.5, 5 mL), and the solution was stirred for 2 h. The majority of the TFA was removed under a stream of N_2 gas, and the remainder was precipitated into cold diethyl ether with filtration through a plug of glass wool. The resulting solid was collected and purified by HPLC to yield **16** (13 mg, 45%) as the mono TFA salt. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.59 (s, 1H), 7.54 (s, 1H), 7.31 (s, 1H), 4.63 (d, J = 16.3 Hz, 1H), 4.49 (m, 2H), 4.32 (q, J = 7.3 Hz, 1H), 4.23 (d, J = 16.5 Hz, 1H), 4.21 (d, J = 18 Hz, 1H), 3.73 (d, J = 17.1 Hz, 1H), 3.13 (m, 2H), 3.00 (dd, J = 17.1, 6.5 Hz, 1H), 2.94 (t, J = 7.9 Hz, 2H), 2.76 (t, J = 8.8 Hz, 2H), 2.67 (dd, J = 17.1, 7.4 Hz, 1H), 2.05 (m, 1H), 1.99 (p, J = 7.9 Hz, 2H), 1.72–1.55 (m, 3H), 4.54 (d, J = 7.4 Hz, 3H), 1.36 (m, 2H); ^{13}C NMR (125 MHz, CD_3OD): δ = 176.46, 174.59, 174.21, 172.97, 172.00, 171.41, 158.77, 142.23, 141.43, 135.68, 137.71, 126.65, 125.58, 53.88, 53.41, 52.47, 43.10, 43.02, 42.02, 40.36, 35.84, 33.34, 30.26, 229.49, 26.52, 17.52; HRMS (ESI): calcd for $C_{26}H_{40}N_9O_{17}$ $[M+H]^+$ 590.3051; found 590.3069.

Synthesis of compound 7: Methyl *N*-α-benzyloxycarbonyl-L-2,3-diaminopropionate (200 mg, 0.7 mmol) was dissolved in CH_2Cl_2 (3.5 mL), Et_3N (300 μL, 2.10 mmol) was added, and the suspension was cooled to 0 °C. The known chloroformamide[45] (250 mg, 0.90 mmol) was then added, and the solution was stirred overnight at RT. The reaction mixture was then diluted with EtOAc (20 mL), washed with 5% aqueous citric acid (2×10 mL), saturated aqueous $NaHCO_3$ (2×10 mL), and brine (2×10 mL), and dried (Na_2SO_4). The solvents were removed under reduced pressure, and the resulting residue was purified by FC (hexane/EtOAc 2:3 to 0:1) to yield **7** (294 mg, 92%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.32 (m, 5H), 6.42 (d, J = 7.3 Hz, 1H), 5.59 (t, J = 5.6 Hz, 1H), 5.07 (m, 2H), 4.37 (m, 1H), 3.71 (s, 3H), 3.61 (m, 2H), 3.32 (m, 8H), 1.46 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.84, 157.56, 156.41, 154.42, 135.99, 128.37, 128.07, 127.93, 80.03, 66.95, 54.85, 52.57, 43.36, 43.09, 28.23; LRMS (ESI): calcd for $C_{22}H_{32}N_4NaO_7$ $[M+Na]^+$ 487.2; found 487.2.

Synthesis of compound 8: Compound **7** (715 mg, 1.54 mmol) was dissolved in 4 N HCl/dioxane (8 mL), and the resulting solution was stirred for 15 min at RT during which time an oil precipitated. The solution was sparged with nitrogen to remove HCl, and the solvent was removed under reduced pressure to yield the crude amine. This compound, *N*-Boc-4-aminobutyric acid (370 mg, 1.8 mmol) and 4-dimethylamino pyridine (DMAP; 12 mg, 0.18 mmol) were dissolved in CH_2Cl_2 (7.5 mL). The solution was cooled to 0 °C, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI; 345 mg, 1.80 mmol) was added. The reaction mixture was stirred for 5 min at 0 °C, and Et_3N (780 μL, 5.4 mmol) was then added. After 6 h at RT, the reaction mixture was diluted with EtOAc (50 mL), washed with 5% aqueous citric acid (2×20 mL), saturated aqueous $NaHCO_3$ (2×20 mL), and brine (2×20 mL) and dried (Na_2SO_4). The solvents were removed under reduced pressure, and the resulting residue was purified by FC (MeOH/CH_2Cl_2 5:95 to 10:90) to yield **8** (732 mg, 89%) as a white, foamy solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.30 (m, 5H), 6.34 (d, J = 7.2 Hz, 1H), 5.57 (t, J = 5.0 Hz, 1H), 5.05 (m, 2H), 4.9 (t, J = 5.6 Hz, 1H), 4.35 (m, 1H), 3.70 (s, 3H), 3.67–3.49 (m, 4H), 3.35 (s, 4H), 3.24 (m, 2H), 3.12 (q, J = 6.2 Hz, 2H), 6.2 (t, J = 7.2 Hz, 2H), 1.77 (p, J = 6.9 Hz, 2H), 1.39 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.09, 170.94, 157.63, 156.38, 156.02, 136.04, 128.33, 128.02, 127.86, 78.97, 66.81, 55.04, 52.46, 44.87, 43.49, 43.23, 42.70, 40.83, 39.97, 30.26, 28.23, 25.16; LRMS (ESI): calcd for $C_{26}H_{39}NaO_8$ $[M+Na]^+$ 572.3; found 572.1.

Synthesis of compound 9: Compound **8** (186 mg, 0.338 mmol) was dissolved in MeOH/$CHCl_3$ (40:1, 7 mL). $Pd(OH)_2$/C (50 mg) was added, and the reaction mixture was placed under 1 atm of H_2 for 7 h. The solution was filtered through Celite, and the solvents were removed under reduced pressure. The crude amine was dissolved in CH_2Cl_2 (3.5 mL), Et_3N (150 μL, 1.0 mmol) was then added followed by 2-mesitylenesulfonyl chloride (89 mg, 0.41 mmol). The reaction mixture was stirred at RT for 3.5 h, diluted with EtOAc (20 mL), washed with 5% aqueous citric acid (2×10 mL), saturated aqueous $NaHCO_3$ (2×10 mL), and brine (2×10 mL), and dried (Na_2SO_4). The solvents were removed under reduced pressure, and the resulting material was purified by FC (5:95 MeOH/CH_2Cl_2) to yield **9** (17.8 mg, 88%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.92 (s, 2H), 6.13 (d, J = 7.8 Hz, 1H), 5.44 (t, J = 5.5 Hz, 1H), 5.5 (t, J = 5.5 Hz, 1H), 3.90 (td, J = 7.7, 3.7 Hz, 1H), 3.69 (ABX_2, J_{AB} = 13.4 Hz, J_{AX_1} = 6.3 Hz, J_{AX_2} = 3.4 Hz, 1H), 3.61 (m, 2H), 3.54 (s, 3H), 3.49–3.31 (m, 8H), 3.16 (q, J = 6.2 Hz, 2H), 2.60 (s, 6H), 2.36 (t, J =

6.9, 2H), 2.27 (s, 3H), 1.82 (p, $J=6.5$ Hz, 2H), 1.42 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=171.32$, 170.50, 157.60, 156.24, 142.72, 139.29, 133.09, 132.11, 79.19, 55.73, 53.90, 45.16, 43.68, 43.60, 43.52, 41.16, 40.27, 30.57, 28.51, 25.44, 22.65, 21.03; LRMS (ESI): calcd for $C_{22}H_{32}N_4NaO_7$ $[M+Na]^+$ 620.3; found 620.3.

Synthesis of compound 2a: Intermediate **8** (72 mg, 0.13 mmol) was dissolved in MeOH (2 mL). A solution of 4 N HCl/dioxane (1 mL) was then added, the reaction mixture was stirred at RT for 1.5 h, and the solvents were removed under reduced pressure. The residue and 2-methylthio-2-imidazoline hydroiodide (48 mg, 0.20 mmol) were dissolved in MeOH/Et_3N (1:1, 1.4 mL). The resulting solution was heated to reflux for 2.25 h, and the progress of the reaction was monitored by LCMS (1 μL reaction diluted into 100 μL 0.4% aqueous formic acid). The solvents were removed under reduced pressure, and the residue was subsequently dissolved in MeOH/H_2O (3.3:1, 2.6 mL) containing LiOH (27 mg, 0.65 mmol). The reaction mixture was stirred for 1.5 h and neutralized with 1 N HCl, and the solvents were removed under reduced pressure. The resulting reside was purified by HPLC to provide **2b** (54 mg, 82%) as a white solid. ^{1}H NMR (300 MHz, CD_3OD): $\delta=7.32$ (m, 5H), 5.11 (AB, $J_{AB}=12.3$ Hz, 1H), 5.05 (AB, $J_{AB}=12.3$ Hz, 1H), 4.35 (dd, $J=8.1$, 4.5 Hz, 1H), 3.68 (m, 5H), 3.55 (m, 2H), 3.45 (m, 5H), 3.33 (m, 2H), 3.20 (t, $J=7.0$ Hz, 2H), 2.46 (t, $J=6.5$ Hz, 2H), 1.86 (p, $J=7.2$ Hz, 2H); ^{13}C NMR (75 MHz, CD_3OD): $\delta=174.10$, 173.43, 161.78, 160.36, 158.81, 138.45, 129.76, 129.32, 129.14, 67.95, 56.42, 46.42, 44.91, 44.34, 43.47, 42.71, 30.72, 25.76; HRMS (ESI): calcd for $C_{23}H_{33}N_7NaO_6$ $[M+Na]^+$ 504.2571; found 504.2588.

Synthesis of compound 2b: Intermediate **9** (51 mg, 0.085 mmol) was dissolved in MeOH (2 mL). A solution of 4 N HCl/dioxane (1 mL) was then added, the reaction mixture was stirred at RT for 2 h, and the solvents were removed under reduced pressure. The resulting residue and 2-methylthio-2-imidazoline hydroiodide (31 mg, 0.13 mmol) were dissolved in MeOH/Et_3N (1:1 0.9 mL). The resulting solution was heated to reflux for 4 h, a further portion of 2-methylthio-2-imidazoline hydroiodide (20 mg, 0.082 mmol) was added, and the reaction mixture was heated for an addition 2 h, the progress of the reaction was monitored by LCMS (1 μL reaction diluted into 100 μL 0.4% aqueous formic acid). The solvents were removed under reduced pressure, and the residue was subsequently dissolved in MeOH/H_2O (3.3:1, 1.9 mL) containing LiOH (17 mg, 0.41 mmol). The reaction mixture was stirred for 1.5 h and neutralized with 1 N HCl, and the solvents were removed under reduced pressure. The resulting reside was purified by HPLC to provide **2b** (33 mg, 70%) as a white solid. ^{1}H NMR (500 MHz, CD_3OD): $\delta=6.89$ (s, 2H), 3.98 (dd, $J=9.0$, 4.6 Hz, 1H), 3.70 (s, 4H), 3.57 (m, 5H), 3.44 (m, 2H), 3.35 (t, $J=4.7$ Hz, 2H), 3.21 (m, 3H), 2.61 (s, 6H), 2.48 (t, $J=6.9$ Hz, 2H), 2.27 (s, 3H), 1.87 (t, $J=7.2$ Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD): $\delta=173.47$, 161.78, 160.06, 143.83, 140.77, 135.76, 133.14, 57.05, 46.41, 44.89, 44.79, 44.34, 43.48, 42.69, 30.73, 25.76, 23.50, 21.22; HRMS (ESI): calcd for $C_{24}H_{38}N_7O_6S$ $[M+H]^+$ 552.2604; found 552.2626.

Synthesis of compound 10: 3,5-Dimethylphenol (2.0 g, 16 mmol) and ethyl 4-bromobutyrate (3.1 mL, 21 mmol) were dissolved in distilled DMF (55 mL). Potassium carbonate (2.5 g, 18 mmol) and potassium iodide (270 mg, 1.63 mmol) were added, and the resulting suspension was heated to 65 °C for 20 h. The reaction mixture was then cooled to RT and poured into ice, and the resulting solution was extracted with Et_2O (3×50 mL). The combined organic layers were washed with 5% aqueous citric acid (2×40 mL), saturated aqueous $NaHCO_3$ (2×40 mL), and brine (2×40 mL) and dried (Na_2SO_4). The solvent was removed under reduced pressure to yield the alkylated product as a viscous oil (3.4 g). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.60$ (br s, 1H), 6.53 (br s, 2H), 4.15 (q, $J=7.2$ Hz, 2H), 3.98 (t, $J=6.2$ Hz, 2H), 2.51 (t, $J=7.4$ Hz, 2H), 2.29 (d, $J=0.6$ Hz, 6H), 2.09 (p, $J=7.1$ Hz, 2H), 1.28 (t, $J=4.4$ Hz, 3H).

The crude material isolated above (3.4 g, 14 mmol) was dissolved in EtOH/H_2O (1:2, 71 mL) containing NaOH (1.7 g, 43 mmol), and the reaction mixture was stirred for 3 h at RT. The EtOH was removed under reduced pressure, and the remaining liquid was washed with Et_2O (2×40 mL). The aqueous layer was then acidified to pH 3 with concentrated HCl and extracted with EtOAc (3×50 mL). The combined organic layers were washed with brine (2×40 mL) and dried (Na_2SO_4), and the solvent was removed under reduced pressure. The resulting solid was recrystallized from hexane/EtOAc to yield the acid intermediate as a colorless solid (2.6 g, 77% two steps). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.60$ (m, 1H), 6.53 (br s, 2H), 4.00 (t, $J=6$ Hz, 2H), 2.59 (t, $J=7.2$ Hz, 2H), 2.29 (s, 6H), 2.11 (p, $J=6.2$ Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=179.76$, 158.56, 138.96, 122.35, 122.02, 66.04, 30.43, 24.19, 21.20; LRMS (ESI): calcd for $C_{12}H_{15}O_3$ $[M-H]$ 207.1; found 207.1.

This acid (5.2 g, 25 mmol) and 2,2,2-trichloroethanol (2.6 mL, 27 mmol) were dissolved in CH_2Cl_2 (125 mL), and the solution was cooled to 0 °C. EDCI (5.3 g, 27 mmol) and DMAP (300 mg, 2.74 mmol) were added, and the reaction was stirred at 0 °C for 10 min and at RT overnight. The solution was diluted with EtOAc (200 mL), and the organic layer was washed with 5% aqueous citric acid (2×60 mL), saturated aqueous $NaHCO_3$ (2×60 mL), and brine (2×60 mL) and then dried (Na_2SO_4). The solvents were removed under reduced pressure, and the product was purified by FC (hexane/EtOAc 5:1) to yield the protected intermediate as a clear oil (8.0 g, 95%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.60$ (br s, 1H), 6.52 (br s, 2H), 4.76 (s, 2H), 4.01 (t, $J=5.9$ Hz, 2H), 2.69 (t, $J=7.4$ Hz, 2H), 2.28 (d, $J=0.5$ Hz, 6H), 2.16 (p, $J=7.1$ Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta=171.64$, 158.76, 139.19, 122.61, 112.24, 94.95, 73.95, 66.19, 30.56, 24.55, 21.43; LRMS (ESI): calcd for $C_{14}H_{17}Cl_3NaO_3$ $[M+Na]^+$ 361.0; found 361.0.

The above masked intermediate (1.2 g, 0.55 mmol) was dissolved in CH_2Cl_2 (5.5 mL), and the solution was cooled to 0 °C. Chlorosulfonic acid (720 μL, 11 mmol) was added over 5 min, and the mixture was allowed to stir at 0 °C for 5 min and then at RT for an 15 min. An additional portion of chlorosulfonic acid (400 μL) was then added over 15 min, and the reaction mixture was stirred for an additional 10 min. The solution was then poured into ice and extracted with ethyl acetate (3×50 mL), then the combined organic layers were dried (Na_2SO_4). The solvents were removed under reduced pressure, and the resulting residue was purified through a plug of silica gel (CH_2Cl_2) to yield sulfonyl chloride **10** as an oil (800 mg, 51%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.68$ (s, 2H), 4.75 (s, 2H), 4.11 (t, $J=6$ Hz, 2H), 2.90 (s, 6H), 2.68 (t, $J=7.5$ Hz, 2H), 2.19 (p, $J=6.2$ Hz, 2H). This material was used without further purification in the next step.

Synthesis of compound 11: Compound **10** (260 mg, 0.595 mmol) was dissolved in CH_2Cl_2 (2.3 mL), and methyl *N*-β-*tert*-butyloxycarbonyl-L-2,3-diaminopropionate (117 mg, 0.459 mmol) and Et_3N (270 μL, 1.87 mmol) were then added. After 4.5 h at RT, the reaction mixture was diluted with ethyl acetate (10 mL), and the organic layer was washed with 5% aqueous citric acid (2×10 mL), saturated aqueous $NaHCO_3$ (2×10 mL), and brine (2×10 mL) and dried (Na_2SO_4). The solvents were removed under reduced pressure, and the resulting residue was purified by flash FC (hexane/EtOAc 3:1 to 1:1) to yield the desired sulfonamide as a foamy solid (230 mg, 81%). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta=6.59$ (s, 2H), 5.88 (d, $J=8.2$ Hz, 1H), 5.11 (t, $J=6.0$ Hz, 1H), 4.73 (s, 2H), 4.02 (t, $J=6.1$ Hz, 2H), 3.86

(m, 1H), 3.54 (s, 3H), 3.42 (t, J=6.5 Hz, 2H), 2.64 (t, J=7.2 Hz, 2H), 2.50 (s, 6H), 2.13 (p, J=6.5 Hz, 2H), 1.37 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=171.28, 170.40, 160.35, 155.92, 141.78, 128.21, 116.44, 94.74, 73.85, 66.31, 55.40, 52.74, 43.02, 30.21, 28.15, 24.15, 23.24; LRMS (ESI): calcd for $C_{23}H_{33}Cl_3NaO_9S$ [M+Na]$^+$ 641.1; found 641.0.

The sulfonamide isolated above (312 mg, 0.504 mmol) was dissolved in 4 N HCl/dioxane (2.5 mL), and the reaction mixture was stirred at RT for 1 h. After this time, the mixture was sparged with a stream of N_2 gas to remove excess HCl; the dioxane was then removed under reduced pressure to yield the crude deprotected amine as an oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ=6.68 (s, 2H), 4.79 (s, 2H), 4.46 (brs, 4H), 4.21 (m, 1H), 4.10 (t, J=6.1 Hz, 2H), 3.50 (s, 3H), 3.33 (ABX, J_{AB}=13.3 Hz, J_{AX}=8.5 Hz, J_{BX}=4.7, 2H), 2.70 (t, J=7.2 Hz, 2H), 2.65 (s, 6H), 2.19 (p, J=6.5 Hz, 2H).

The crude amine was dissolved in CH_2Cl_2 (2.5 mL), Et_3N (220 μL, 1.52 mmol) was added, and the solution was cooled to 0 °C. The known chloroformamide[45] (160 mg, 0.645 mmol) was then added, and the solution was stirred overnight at RT. The reaction mixture was diluted with EtOAc (10 mL), washed with 5% aqueous citric acid (2×10 mL), saturated aqueous $NaHCO_3$ (2×10 mL), and brine (2×10 mL), and dried (Na_2SO_4). Removal of the solvent under reduced pressure followed by purification by FC (hexane/EtOAc 3:2 to 0:1) provided compound **11** as a foam (308 mg, 84% two steps). ^{1}H NMR (300 MHz, $CDCl_3$): δ=6.56 (s, 2H), 6.23 (d, J=7.9 Hz, 1H), 5.48 (t, J=5.6 Hz, 1H), 4.71 (s, 2H), 4.0 (t, J=6.0 Hz, 2H), 3.86 (td, J=7.6, 4.6 Hz, 1H), 3.62 (ddd, J=13.7, 6.2, 3.7 Hz, 1H), 3.51 (s, 3H), 3.33 (m, 10H), 2.63 (t, J=7.4 Hz, 2H), 2.56 (s, 6H), 2.12 (p, J=7.0 Hz, 2H), 1.41 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=171.36, 170.53, 160.52, 157.69, 154.61, 141.92, 128.07, 116.57, 94.92, 80.09, 73.97, 66.45, 55.72, 52.79, 43.53, 43.33, 30.31, 28.40, 24.27, 23.34; LRMS (ESI): calcd for $C_{28}H_{41}Cl_3N_4NaO_{10}S$ [M+Na]$^+$ 730.2; found 730.1.

Synthesis of compound 12: Intermediate **11** (825 mg, 1.12 mmol) was dissolved in 4 N HCl/dioxane (7 mL), and the solution was stirred at RT for 1 h. After this time, it was sparged with a stream of N_2 gas to remove excess HCl, and the dioxane was removed under reduced pressure to yield the deprotected amine. CbzNH-$(CH_2)_3$-COOH (330 mg, 1.39 mmol) was added to the crude amine, and the mixture was dissolved in CH_2Cl_2 (6 mL), then cooled to 0 °C. EDCI (270 mg, 1.41 mmol) and DMAP (12 mg, 0.10 mmol) were added, and the reaction mixture was stirred at 0 °C for 10 min and at RT for 5 h. The mixture was then diluted with EtOAc (15 mL), washed with 5% aqueous citric acid (2×15 mL), saturated aqueous $NaHCO_3$ (2×15 mL), and brine (2×15 mL) and dried (Na_2SO_4). The solvents were removed under reduced pressure and purification by FC (EtOAc to MeOH/CH_2Cl_2 5:95) yielded the product (897 mg, 94%). ^{1}H NMR (300 MHz, $CDCl_3$): δ=7.33 (m, 5H), 6.61 (s, 2H), 6.00 (brt, J=7.9 Hz, 1H), 5.37 (brs, 1H), 5.21 (brs, 1H), 5.07 (s, 2H), 4.75 (s, 2H), 4.04 (t, J=6.0 Hz, 2H), 3.88 (td, J=7.6, 3.7 Hz, 1H), 3.73 (m, 1H), 3.59 (m, 2H), 2.59 (s, 3H), 3.39 (m, 8H), 3.24 (q, J=6.3, 2H), 2.67 (t, J=7.3 Hz, 2H), 2.61 (s, 6H), 2.36 (t, J=7.1 Hz, 2H), 2.16 (p, J=7.0 Hz, 2H), 1.85 (p, J=6.8 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=171.26, 171.08, 170.38, 160.48, 157.45, 156.50, 141.83, 136.59, 128.41, 127.97, 116.54, 94.81, 73.91, 66.39, 55.48, 52.77, 46.57, 44.94, 43.47, 43.36, 40.99, 40.64, 30.34, 30.23, 25.00, 24.18, 23.25; LRMS (ESI): calcd for $C_{28}H_{41}Cl_3N_4NaO_{10}S$ [M+Na]$^+$ 753.2; found 753.2.

The above intermediate (592 mg, 0.695 mmol) was dissolved in THF (40.5 mL), and KH_2PO_4 was added (1 M, 7.5 mL). Zn dust (13 g) was added, and the reaction mixture was stirred for 2 h at RT. It was then acidified with 1 N HCl and filtered through Celite, and the combined washings were extracted with ethyl acetate (3×50 mL). The extractions were combined, washed with brine (2×50 mL), and dried (Na_2SO_4). Removal of the solvents under reduced pressure yielded crude acid (587 mg). ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD): δ=7.22 (m, 5H), 6.51 (s, 2H), 4.96 (m, 2H), 3.91 (t, J=6.0 Hz, 2H), 3.81 (dd, J=8.2, 4.3 Hz, 1H), 3.45 (m, 3H), 3.27 (m, 8H), 3.09 (t, J=6.6, 2H), 2.49 (s, 6H), 2.37 (t, J=7.4 Hz, 2H), 2.27 (t, H=7.2 Hz, 2H), 1.96 (p, J=6.6 Hz, 2H), 1.70 (p, J=7.0 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD): δ=175.17, 171.65, 170.40, 160.35, 157.63, 156.78, 141.55, 136.27, 128.14, 127.96, 127.72, 127.55, 116.21, 76.53, 66.50, 66.23, 55.26, 52.25, 44.78, 42.50, 40.87, 40.01, 29.96, 24.78, 24.05, 22.81; LRMS (ESI): calcd for $C_{33}H_{45}N_5O_{11}S$ [M−H] 718.3; found 718.3.

A portion of the acid isolated above (153 mg, 0.212 mmol), EDCI (48 mg, 0.25 mmol), and NHS (29 mg, 0.25 mmol) were dissolved in CH_2Cl_2 (2 mL), and the solution was stirred for 4 h. BocNH-$(CH_2)_3NH_2$[46] (53 mg, 0.25 mmol) and Et_3N (36 μL, 0.63 mmol) were then added, and the mixture was stirred overnight. After dilution with EtOAc (8 mL), the organic layer was washed with 5% aqueous citric acid (2×5 mL), saturated aqueous $NaHCO_3$ (2×5 mL), and brine (2×5 mL) and dried (Na_2SO_4). The solvent was removed under reduced pressure, and FC purification (MeOH/CH_2Cl_2 5:95 to 10:90) yielded **12** (152 mg, 83%); ^{1}H NMR (300 MHz, $CDCl_3$): δ=7.31 (m, 5H), 6.59 (s, 2H), 6.56 (brt, J=5.1 Hz, 1H), 6.15 (d, J=7.3 Hz, 1H), 5.45 (t, J=5.1 Hz, 1H), 5.36 (brt, J=5.3 Hz, 1H), 5.06 (m, 2H), 5.02 (t, J=6.3 Hz, 1H), 3.98 (t, J=5.9 Hz, 2H), 3.89 (td, J=7.7, 3.6 Hz, 1H), 3.66 (ddd, J=13.3, 5.4, 2.9 Hz, 1H), 3.57 (m, 5H), 3.40 (m, 3.43–3.18 (m, 12H), 3.11 (q, J=5.8 Hz, 2H), 2.58 (s, 6H), 2.35 (t, J=7.8 Hz, 4H), 2.08 (p, J=6.6 Hz, 2H), 1.83 (p, J=6.8 Hz, 2H), 1.57 (p, J=6.4 Hz, 2H), 1.41 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=172.58, 171.33, 170.70, 160.79, 157.58, 156.73, 141.88, 136.68, 136.68, 128.54, 128.12, 128.05, 127.94, 116.68, 79.40, 67.16, 66.58, 55.81, 52.89, 45.06, 43.43, 43.25, 41.12, 40.71, 37.17, 36.02, 32.65, 30.39, 30.16, 28.44, 25.15, 25.04, 23.37; LRMS (ESI): calcd for $C_{41}H_{61}N_7NaO_{12}S$ [M+Na]$^+$ 898.4; found 898.3.

Synthesis of ligand 2c: Compound **12** (58 mg, 0.066 mmol) was dissolved in MeOH/$CHCl_3$ (3 mL, 40:1), and solid $Pd(OH)_2$/C (14 mg) was added. The suspension was placed under 1 atm of H_2 for 11 h. The reaction mixture was then filtered through Celite, and the solvent was removed under reduced pressure to afford the monoprotected amine derivative (52 mg, quantitative). ^{1}H NMR (300 MHz, CD_3OD): δ=7.84 (brt, J=4.4 Hz, 1H), 6.55 (s, 2H), 3.86 (m, 3H), 3.46–3.29 (m, 8H), 3.27 (m, 2H), 3.17 (m, 3H), 3.05 (m, 2H), 2.88 (m, 4H), 2.45 (s, 8H), 2.12 (t, J=7.6 Hz, 2H), 1.90 (p, J=5.5 Hz, 2H), 1.81 (p, J=6.6 Hz, 2H), 1.48 (p, J=6.5 Hz, 2H), 1.27 (s, 9H); ^{13}C NMR (75 MHz, CD_3OD): δ=175.56, 172.86, 172.38, 162.18, 159.71, 158.59, 143.28, 130.24, 117.70, 80.10, 68.51, 46.26, 56.98, 52.98, 44.50, 44.59, 43.80, 42.56, 40.66, 38.90, 38.06, 37.94, 33.66; LRMS (ESI): calcd for $C_{33}H_{56}N_7O_{10}S$ [M+H]$^+$ 742.4; found 742.3.

This amine (26 mg, 0.033 mmol) and 2-methylthio-2-imidazoline hydroiodide (12 mg, 0.049 mmol) were dissolved in MeOH and Et_3N (400 μL, 1:1). The reaction mixture was heated at reflux, and the progress of the reaction was monitored by LCMS (1 μL of reaction mixture diluted into 100 μL 0.4% aqueous formic acid). After approximately 4 h, the solvents were removed under reduced pressure. The resulting residue was dissolved in a mixture of MeOH and H_2O (600 μL, 1:2) that contained LiOH (4 mg, 0.1 mmol). The solution was stirred for 5 h, and progress was again monitored by LCMS. The reaction was then neutralized with 1 N HCl, and the solvents were removed under high vacuum. TFA (1.5 mL) was then added, and the reaction mixture was stirred for 1.25 h. Most of the TFA was removed under a stream of N_2 gas, and the product was

triturated with cold Et_2O. The crude mixture was purified by HPLC to yield **2c** as a white solid (14 mg, 54%). ^{1}H NMR (500 MHz, CD_3OD): δ=6.69 (s, 2H), 4.03, (t, J=5.9 Hz, 2H), 3.93 (dd, J=8.8, 4.6 Hz, 1H), 3.70 (s, 4H), 3.63–3.52 (m, 5H), 3.45 (t, J=4.9 Hz, 2H), 3.37 (t, J=5.1 Hz, 2H), 3.27, (t, J=6.3 Hz, 2H), 3.23 (t, J=4.7 Hz, 1H), 3.21 (t, J=6.9 Hz, 2H), 2.92 (t, J=7.1 Hz, 2H), 2.62 (s, 6H), 2.49 (t, J=6.8 Hz, 2H), 2.39 (t, J=7.4 Hz 2H), 2.06 (p, J=6.4 Hz, 2H), 1.87 (p, J=6.8 Hz, 2H), 1.82 (p, J=7.5 Hz, 2H); ^{13}C NMR (125 MHz, CD_3OD): δ=176.51, 173.49, 162.30, 161.80, 160.07, 143.44, 130.54, 117.82, 68.50, 57.14, 46.44, 44.94, 44.80, 44.48, 44.37, 43.51, 42.74, 38.51, 37.22, 33.54, 30.76, 29.10, 26.66, 25.79, 23.92; HRMS (ESI): calcd for $C_{30}H_{50}N_9O_8S$ $[M+H]^+$ 696.3503; found 696.3510.

Synthesis of trisaccharide 16: A suspension of **15** (14.58 g, 15 mmol), phenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-galactopyranoside **13** (14.22 g, 22.5 mmol) and molecular sieves (4 Å, 15 g) in CH_2Cl_2 (200 mL) was stirred at RT for 1 h under Ar. A suspension of phenyl mercury triflate (10 g, 23.4 mmol) and 4 Å molecular sieves (5 g) in CH_2Cl_2 (150 mL) was stirred for 15 min, transferred to the previous suspension of sugars and kept at RT for 1 h. Filtration through Celite followed by purification by flash chromatography (hexane/EtOAc 100:10→100:15) gave **16** as an oil (20.3 g, 90%). ^{1}H NMR (500 MHz, $CDCl_3$): 7.45–7.05 (m, 55H), 5.20 (d, J=3.3 Hz, H-C(1″)), 5.09 (d, J=11.6 Hz, PhCH), 5.02 (d, J=10.7 Hz, PhCH), 4.92 (d, J=12.2 Hz, PhCH), 4.89 (d, J=11.4 Hz, PhCH), 4.88 (d, J=11.0 Hz, PhCH), 4.85 (d, J=12.2 Hz, PhCH), 4.83 (d, J=10.8 Hz, PhCH), 4.75 (d, J=10.8 Hz, PhCH), 4.73 (d, J=11.1 Hz, PhCH), 4.68 (d, J=11.5 Hz, PhCH), 4.67 (d, J=11.0 Hz, PhCH), 4.64 (d, J=11.8 Hz, PhCH), 4.62 (d, J=11.9 Hz, PhCH), 4.58 (d, J=12.0 Hz, PhCH), 4.49 (d, J=11.4 Hz, PhCH), 4.44 (brd, J=8.0 Hz, 2H; H-C(1), H-C(1′)), 4.43 (d, J=11.5 Hz, PhCH), 4.34 (d, J=11.4 Hz, PhCH), 4.33 (d, J=12.0 Hz, PhCH), 4.32 (d, J=12.0 Hz, PhCH), 4.28 (d, J=12.0 Hz, PhCH), 4.27 (t, J=5.0 Hz, H-C(5″)), 4.24 (d, J=12.0 Hz, PhCH), 4.20 (d, J=11.9 Hz, PhCH), 4.11 (dd, J=3.3, 10.0 Hz, H-C(2″)), 3.96 (brt, J=9.0 Hz, H-C(4)), 3.92 (dd, J=2.8, 10.0 Hz, H-C(3″)), 3.91 (d, J=2.5 Hz, H-C(4″)), 3.78 (dd, J=7.8, 9.0 Hz, H-C(2′)), 3.67 (d, J=3.0 Hz, H-C(4′)); 3.77–3.65 (m, 3H; H-C(3′), 2H-C(6)); 3.56–3.30 (m, 7H; H-C(3), H-C(2), 2H-C(6″), 2H-C(6′), H-C(5′)), 3.26 (ddd, J=2.0, 4.0, 10.0 Hz, H-C(5)); ^{13}C NMR (75 MHz, $CDCl_3$): 139.22 (s), 138.99 (s), 138.72 (s), 138.56 (s), 138.55 (s), 138.53 (s), 138.27 (s, 2C), 138.14 (s, 2C), 137.46 (s), 128.17–127.00 (several d), 102.83 (d) and 102.36 (d, C(1), C(1′)), 95.72 (d, C(1″)), 82.91 (d, C(3)), 81.62 (d, C(2)), 79.09 (d, C(3′)), 78.89 (d, C(3″)), 78.02 (d, C(2′)), 76.46 (d, C(4)), 76.36 (d, C-(2″)), 75.38 (t, $PhCH_2$), 75.09 (d, C(5)), 74.98 (t, $PhCH_2$), 74.83 (d, C(4′)), 74.68 (t, $PhCH_2$), 74.53 (t, $2PhCH_2$), 74.20 (t, $2PhCH_2$), 73.20 (t, $PhCH_2$), 72.96 (t, $PhCH_2$), 72.91 (d, C(4″), C(5′)), 72.32 (t, $PhCH_2$), 70.83 (t, $PhCH_2$), 69.12 (d, C(5″)), 68.86 (t, C(6″)), 68.20 (t, C(6)), 68.02 (t, C(6′)). FAB-MS: calcd for $C_{95}H_{98}O_{16}$ $[M+H+Na]^{2+}$ 1519.7; found 1519.

Synthesis of compound 17: A suspension of **16** (16.8 g) and solid 10% Pd/C (6.0 g) in EtOAc/MeOH/H_2O/AcOH (5:5:2:1, 130 mL) was shaken under H_2 (345 kPa) for 36 h. The mixture was filtered through Celite, and the solid was washed with H_2O and pyridine. The combined filtrate was concentrated. The residue was dried and dissolved in pyridine (100 mL) and treated with Ac_2O (50 mL) and 4-dimethylaminopyridine (200 mg) for 12 h. The sample was subjected to evaporation, then coevaporation with toluene and finally FC (hexane/EtOAc 4:6) to afford the peracetylated acceptor (10.0 g, 92%). ^{1}H NMR (300 MHz, $CDCl_3$): 6.27 (d, J=3.6 Hz, 0.4H; H-C(1α)); 5.68 (d, J=8.2 Hz, 0.6H; C(1β)); FAB-MS: *m/z* (%): 989 (100) $[M+Na]^+$, 947 (45), 619 (10), 331 (35).

Hydrazine acetate (350 mg) was added to a solution of the above intermediate (2.0 g, 2.07 mmol) in DMF (15 mL) at 55 °C, and the mixture was stirred for 5 min before water (20 mL) was added. The resulting solution was extracted with EtOAc (10×). The organic phase was concentrated and purified by FC (hexane/EtOAc 3:7 eluent) to give saccharide intermediate with a free reducing end (1.72 g, 89%). FAB-MS: *m/z* (%): 947.2 (100) $[M+Na]^+$, 176 (30).

1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU; 0.300 mL, 1.97 mmol) was added to a solution of this compound (1.70 g, 1.83 mmol) and trichloroacetonitrile (2 mL, 20.0 mmol) in CH_2Cl_2 (10 mL) at −5 °C. The reaction mixture was kept at 0 °C for 2 h, then the resulting trichloracetimidate product was isolated and purified by FC (hexane/EtOAc 3:2) to yield **17** (1.48 g, 75%) as a foam. ^{1}H NMR (300 MHz, $CDCl_3$): 8.67 (s, NH), 6.49 (d, J=3.9 Hz, H-C(1)), 5.58 (t, J=9.6 Hz, H-C(3)), 5.47 (brd, J=3.0 Hz, H-C(4′)), 5.35 (brd, J=3.0 Hz, H-C(4″)), 5.28 (dd, J=3.0, 10.0 Hz, H-C(3″)), 5.26 (brs, H-C(1″)), 5.20 (dd, J=7.9, 10.3 Hz, H-C(2′)), 5.14–5.08 (m, H-C(2″)), 5.07 (dd, J=3.8, 10.1 Hz, H-C(2)), 4.47 (d, J=8.0 Hz, H-C(1)), 4.46 (dd, J=2.0, 12.0 Hz, 1H; H-C(6)), 4.24–4.05 (m, 7H), 3.92–3.79 (m, 3H), 2.17 (s, 3H), 2.15 (s, 3H), 2.14 (s, 3H), 2.10 (s, 3H), 2.08 (s, 6H), 2.07 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H), 1.96 (s, 3H); FAB-MS: *m/z* (%): 1092.1 (100) $[M+Na]^+$, 989 (55).

Synthesis of compound 18: A suspension of **17** (1.45 g, 1.357 mmol), H-$(OCH_2CH_2)_4$-N_3 (220 μL) and molecular sieves (4 Å, 1.93 g) in CH_2Cl_2 (30 mL) was stirred at room temperature for 1 h, cooled in an ice–acetone bath, and treated with BF_3·OEt_2 (0.8 mL, 6.5 mmol). The reaction mixture was stirred at RT for 2 h, treated with Et_3N for 10 min, and purified directly by FC (hexane/EtOAc 3:7) to give **18** (790 mg, 58%) as an oil. ^{1}H NMR (300 MHz, $CDCl_3$): 5.45 (brd, J=2.9 Hz, H-C(4″)), 5.32 (brd, J=3.0 Hz, H-C(4′)), 5.26 (dd, J=3.3, 10.5 Hz, H-C(2″)), 5.24 (d, J=3.0 Hz, H-C(1″)), 5.21 (t, J=9.4 Hz, H-C(3)), 5.16 (dd, J=8.0, 10.1 Hz, H-C(2′)), 5.09 (dd, J=3.1, 10.0 Hz, H-C(3″)), 4.92 (dd, J=7.9, 9.4 Hz, H-C(2)), 4.56 (d, J=7.7 Hz, H-C(1)), 4.50 (dd, J=2.0, 12.1 Hz, H-C(6)), 4.42 (d, J=7.9 Hz, H-C(1′)), 4.23–4.00 (m, 6H), 4.00 (ddd, J=3.3, 4.8, 13.3 Hz, 1H; $OCH_2CH_2N_3$), 3.83 (dd, J=3.0, 9.0 Hz, H-C(3′)), 3.80 (brt, J=5.0 Hz, H-C(5′)), 3.81 (t, J=9.4 Hz, H-C(4)), 3.68 (ddd, J=3.3, 8.1, 13.5 Hz, 1H; $OCH_2CH_2N_3$), 3.63 (ddd, J=2.0, 5.0, 10.0 Hz, H-C(5)), 3.46 (ddd, J=3.5, 8.5, 13.4 Hz, 1H; CH_2N_3), 3.26 (ddd, J=3.3, 4.7, 13.2 Hz, 1H; CH_2N_3), 2.12 (s, 3H), 2.11 (s, 6H), 2.08 (s, 3H), 2.04 (s, 3H), 2.03 (s, 6H), 2.02 (s, 6H), 1.92 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): 170.2 (s), 170.1 (s), 170.0 (s), 169.94 (s), 169.91 (s), 169.7 (s), 169.5 (s), 169.5 (s), 168.6 (s), 100.8 (d, C(1′)), 100.1 (d, C(1)), 93.1 (d, C(1″)), 75.7 (d, C(3′)), 72.64 (d, C(3)), 72.61 (d, C(5)), 72.5 (d, C(4)), 71.3 (d, C(2)), 70.5 (d, C(5′)), 69.5 (d, C(2′)), 68.4 (t, OCH_2), 67.4 (d, C(4″)), 66.9 (d, C(3″)), 66.6 (d, C(5″)), 66.2 (d, C(2″)), 64.4 (d, C(4′)), 61.5 (t, C(6)), 61.0 (t, C(6′)), 60.8 (t, C(6″)), 50.23 (t, CH_2N_3), 20.57(q), 20.56 (q), 20.52 (q), 20.47 (q, 2C), 20.44 (q), 20.39 (q), 20.37 (q), 20.34 (q), 20.36 (q); FAB-MAS: *m/z* (%): 1016 (100) $[M+Na]^+$, 619 (15), 331 (25), 169 (25).

Synthesis of azido sugar 19: A solution of **18** (897 mg, 0.83 mmol) in MeOH (30 mL) was treated with a solution of NaOMe (0.8 M, 1 mL) at RT for 12 h. The mixture was neutralized with Amberlite IR-120 and filtered. The filtrate was concentrated to give **19** as a solid (580 mg, 99%). ^{1}H NMR (300 MHz, D_2O + ca. 0.1% MeOH): 4.95 (d, J=3.8 Hz, H-C(1″)), 4.33 (d, J=7.7 Hz, 2H; H-C(1), H-C(1′)), 4.05–3.35 (m, 32H), 3.32 (brt, J=4.4 Hz, 2H; CH_2N_3); ^{13}C NMR (75 MHz, D_2O + ca. 0.1% MeOH): 103.03 (d) and 102.27 (d, C(1), C(1′)), 95.60 (d, C(1″)), 78.77 (d), 77.37 (d), 75.21 (d), 74.90 (d), 74.54 (d), 72.96 (d), 70.98 (d), 69.85 (t), 69.84 (t), 69.78 (t), 69.74 (t), 69.64 (t), 69.47 (d), 69.39 (t), 69.30 (d), 68.89 (t), 68.38 (d), 64.98 (d), 61.16 (t) and 61.14 (t) and 60.31 (t, C(6), C(6′), C(6″)), 50.31 (t, CH_2N_3); MALDI-MS: calcd for $C_{26}H_{47}N_3O_{19}Na$: 728.3 $[M+Na]^+$; found 728.

Synthesis of amino sugar 20: A suspension of **19** (580 mg, 0.82 mmol) and 20% $Pd(OH)_2/C$ (200 mg) in MeOH (15 mL) and AcOH (0.2 mL) was kept under H_2 (354 kPa) for 8 h. The suspension was filtered, and the filtrate was evaporated to give **20** (520 mg, 98%) as an oil. ^{1}H NMR (300 MHz, D_2O + ca. 0.1% MeOH): 4.93 (d, J=3.6 Hz, H-C(1″)), 4.32 (d, J=7.9 Hz) and 4.31 (d, J=7.5 Hz, H-C(1), H-C(1′)), 4.05–3.35 (m, 32H), 3.01 (t, J=4.8 Hz, 2H; CH_2N); ^{13}C NMR (75 MHz, D_2O + ca. 0.1% MeOH): 103.00 (d) and 102.20 (d, C(1), C(1′)), 95.56 (d, C(1″)), 78.70 (d), 77.31 (d), 75.18 (d), 74.88 (d), 74.54 (d), 72.92 (d), 70.96 (d), 69.77 (t, 3C), 69.66 (d), 69.59 (t), 69.53 (t), 69.43 (d), 69.27 (d), 68.88 (t), 68.35 (d), 66.49 (t), 64.94 (d), 61.15 (t), 61.06 (t) and 60.23 (t, C(6), C(6′), C(6″)), 39.25 (t, CH_2NH_2); MALDI-MS: calcd for $C_{26}H_{49}NO_{19}$: 680.3 $[M+H]^+$; found 680.

Synthesis of carbohydrate 21: Trisaccharide **20** (27 mg, 0.034 mmol) was dissolved in a mixture of MeOH and H_2O (2:1, 1 mL). Dimethylsquarate (7 mg, 0.05 mmol) and Et_3N (6 μL, 0.4 mmol) were added to this solution, and the mixture was stirred for 24 h. Removal of the solvents under reduced pressure and purification of the residue by FC ($MeOH/CH_2Cl_2/H_2O$ 1.5:3:0.2) provided **21** (19 mg, 69%) as a white solid. ^{1}H NMR (300 MHz, CD_3OD/D_2O, ca. 1:2 mixture of rotamers about vinylogous amide): δ=4.98 (d, J=3.7 Hz, 1H), 4.34 (dd, J=7.5, 2.7 Hz, 2H), 4.22 and 4.21 (s, 3H total, rotamers), 4.02 (m, 2H), 2.85–2.26 (m, 30H), 3.19 (m, 2H); ^{13}C NMR (75 MHz, CD_3OD/D_2O, ca. 1:2 mixture of isomers about vinylogous amide bond): δ=189.98, 184.70, 184.63, 178.85, 178.57, 174.59, 174.52, 104.12, 103.34, 96.70, 79.95, 78.53, 76.22, 75.94, 75.60, 73.99, 72.00, 70.81, 70.55, 70.36, 69.84, 69.44, 66.05, 62.13, 61.99, 61.37, 45.23, 44.99; LRMS (MALDI): calcd for $C_{31}H_{51}NNaO_{22}S$: 812.3 $[M+Na]^+$; found 812.2.

Synthesis of conjugate 1c: Peptide **1b** (1.8 mg, 2.6 μmol) and saccharide **21** (2.5 mg, 3.1 μmol) were dissolved in borate buffer (160 μL, 50 mM, pH 9), and the mixture was stirred at RT for 30 h. A solution of HOAc in H_2O (0.2 M, 50 μL) was added, and the reaction mixture was purified by HPLC to yield **1c** (2.5 mg, 71%). ^{1}H NMR (500 MHz, D_2O): δ=7.47 (s, 1H), 7.37 (s, 1H), 7.30 (s, 1H), 5.12 (s, J=4 Hz, 1H), 4.64 (t, J=6.7 Hz, 1H), 4.54 (m, 2H), 4.48 (d, J=7.4 Hz, 1H), 4.44 (d, J=7.2 Hz, 1H), 4.34–4.14 (m, 5H), 4.03–3.91 (m, 4H), 3.88–3.82 (m, 2H), 3.81–3.51 (m, 28H), 3.30 (m, 1H), 3.17 (m, 2H), 2.98 (ABX, J_{AB}=17 Hz, J_{AX}=6.3 Hz, 1H), 2.81 (m, 2H), 2.76 (ABX, J_{AB}=17 Hz, J_{BX}=7.4 Hz, 1H), 2.04 (m, 2H), 1.97 (m,1H), 1.70–1.51 (m, 3H), 1.54 (d, J=7.2 Hz, 3H); LRMS (MALDI): m/z calcd for $C_{56}H_{87}N_{10}O_{28}$: 1347.6 $[M+H]$; found 1347.3; HRMS (ESI): calcd for $C_{56}H_{87}N_{10}NaO_{28}$: 685.2795 $[M+H+Na]^{2+}$; found 685.2875.

Synthesis of conjugate 2d. Inhibitor **2c** (2.1 mg, 2.6 μmol) and saccharide **21** (2.7 mg, 3.4 μmol) were dissolved in borate buffer (160 μL, 50 mM, pH 9) and mixed at RT for 29 h. A solution of AcOH in H_2O (0.2 M, 50 μL) was added, and the mixture was purified by HPLC to yield **2d** (2.5 mg, 66%). ^{1}H NMR (500 MHz, D_2O): δ=6.76 (s, 2H), 5.14 (d, J=3.5 Hz, 1H), 4.50 (dd, J=9.7, 8.2 Hz, 1H), 4.18 (m, 2H), 4.08–3.92 (m, 7H), 3.88–3.47 (m, 36H), 3.39–3.13 (m, 12H), 2.54 (s, 6H), 2.51 (t, J=8.2 Hz, 2H), 2.42 (t, J=6.7 Hz, 2H), 2.08 (m, 2H), 1.86 (p, J=7.2 Hz, 2H), 1.72 (p, J=6.7 Hz, 2H); LRMS (MALDI): calcd for $C_{60}H_{97}N_{10}O_{29}S$ 1453.6 $[M+H]^+$; found 1453.6; HRMS (ESI): calcd for $C_{60}H_{97}N_{10}NaO_{29}S$ 738.3021 $[M+H+Na]^{2+}$; found 738.3005.

Synthesis of conjugate 3c: Peptide **3a** (3.5 mg, 5.8 μmol) and saccharide **21** (4.8 mg, 6.0 μmol) were dissolved in borate buffer (350 μL, 50 mM, pH 9), and the mixture was stirred at RT for 3 days. A solution of HOAc in H_2O (0.2 M, 200 μL) was added, and the product was purified by HPLC to yield **3c** (3.5 mg, 44%). ^{1}H NMR (500 MHz, D_2O): δ=7.34 (t, J=6.8 Hz, 2H), 7.28 (t, J=6.8 Hz, 1H), 7.23 (d, J=6.8 Hz, 2H), 5.14 (d, J=3.9 Hz, 1H), 4.76 (dd, J=7.8, 6.4 Hz, 1H), 4.61 (dd, J=10, 6.5 Hz, 1H), 4.5 (dd, J=7.4, 3.9 Hz, 2H), 4.35 (dd, J=8.7, 5.7 Hz, 1H), 4.19 (m, 3H), 4.08–3.47 (m, 34H), 3.33 (m, 1H), 3.18 (m, 2H), 3.06 (ABX, J_{AB}=13.1 Hz, J_{AX}=6.2 Hz, 1H), 2.96 (ABX, J_{AB}=13.1 Hz, J_{BX}=6.2 Hz, 1H), 2.91 (ABX, J_{AB}=16.9 Hz, J_{AX}=7.7 Hz, 1H), 2.72 (ABX, J_{AB}=17.0 Hz, J_{BX}=6.2 Hz, 1H) 1.86 (m, 1H), 1.66 (m, 2H), 1.51 (m, 5H), 1.02 (m, 1H); LRMS (MALDI): m/z calcd for $C_{57}H_{89}N_{10}O_{28}$: 1361.6 $[M+H]$; found: 1361.3; HRMS (ESI): m/z calcd for $C_{57}H_{89}N_{10}NaO_{28}$: 692.2864 $[M+H+Na]^{2+}$; found: 692.2873.

Integrin-binding assay: WM115 cells were trypsinized and resuspended at 1.25×10^6 cells per mL in PBS, and BCECF-AM (2.5 μg mL^{-1}) was added for 30 min at 37 °C. Cells were washed and diluted to 4×10^5 cells per mL in "binding buffer", which consisted of BSA (1.5%), glucose (5 mM), $MgCl_2$ (1.5 mM), and $MnCl_2$ (1.5 mM) in Tris-buffered saline (TBS), pH 7.2, for 60 min at 4 °C. Cells were then diluted to 5×10^3 cells per mL. Previously, V-bottom 96-well microtiter plates had been coated with fibrinogen (100 μL, 1 μg mL^{-1}) overnight at 4 °C. The solution was aspirated, and the plates were blocked with a "blocking buffer", which consisted of BSA (1.5%) and Tween-20 (0.5%) in Na_2CO_3 (25 mM, pH 9.6), for 2 h at RT. This solution was removed, and the wells were washed with binding buffer (3×). BCECF-AM-labeled cells (5000 cells per well) in binding buffer were added to washed wells with or without compound. The plates were incubated for 15 min at 37 °C and then centrifuged at 1830 g for 10 min in an Allegra 6KR centrifuge (Beckman Coulter, Fullerton, CA). Nonadherent cells were quantified on an EnVision 2100 plate reader (Perkin–Elmer, Boston, MA) set in bottom reading mode.

Each experiment was performed in triplicate and contained a minimum of eight concentrations of compounds in addition to wells coated with fibrinogen that contained no compound and untreated wells blocked with BSA. The percent inhibition was defined as:

$$\frac{FI_{inhibitor}-FI_{fibrinogen}}{FI_{no\ fibrinogen}-FI_{fibrinogen}}$$

where $FI_{inhibitor}$ is the fluorescent signal in the presence of fibrinogen and inhibitor, $FI_{fibrinogen}$ is the signal with no inhibitor present (minimum signal) and $FI_{no\ fibrinogen}$ is the signal in the absence of fibrinogen (maximum signal). For each experiment, the maximal percent inhibition was normalized to 100 percent. IC_{50} values were determined by averaging the percent inhibitions for at least three separate experiments and fitting the resulting curve with the following equation:

$$y = \frac{FI_{max}-FI_{min}}{1+(x/IC_{50})^{slope}+FI_{max}}$$

Fits were performed in ProFit by using individual y errors (standard deviation) and assuming a 5% error in the x values. Initial fits were obtained by using a Monte Carlo fitting routine for a minimum of 80000 iterations. Final fits, including errors, were obtained by using a Levenberg–Marguardt fitting routine.

Anti-Gal antibody binding: Near confluent M21 cells were harvested, washed, counted, and resuspended at a density of 4×10^5 cells per mL for activation in binding buffer for 60 min at 4 °C. Cells were then diluted to 2×10^5 cells per mL and incubated with compound **2d** (10 nM) on ice for 60 min. Cells were washed with binding buffer and resuspended in a 20% solution of heat-inactivated human serum (HIHS) obtained from a healthy donor with signed consent. After a 30–60 min incubation on ice, cells were washed and incubated again at 4 °C with fluorescein-conjugated goat anti-

human IgG antibody (5 μg mL^{-1}) for 30 min. Finally, propidium iodide (5 μg mL^{-1}) was added to identify dead cells, and the population was immediately analyzed for fluorescence by using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data were analyzed by using CellQuest software (Becton Dickinson, San Jose, CA). Omitting the bifunctional conjugate allowed the background fluorescence intensity to be assessed. Binding experiments were repeated in triplicate.

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[1] M. Rudin, R. Weissleder, *Nat. Rev. Drug Discovery* **2003**, *2*, 123 – 131.
[2] M. A. Lindorfer, C. S. Hahn, P. L. Foley, R. P. Taylor, *Immunol. Rev.* **2001**, *183*, 10 – 24.
[3] J. E. Gestwicki, L. E. Strong, C. W. Cairo, F. J. Boehm, L. L. Kiessling, *Chem. Biol.* **2002**, *9*, 163 – 169.
[4] P. A. Trail, H. D. King, G. M. Dubowchik, *Cancer Immunol. Immunother.* **2003**, *52*, 328 – 337.
[5] V. Guillemard, H. U. Saragovi, *Curr. Cancer Drug Targets* **2004**, *4*, 313 – 326.
[6] A. M. Silverstein, *Nat. Immunol.* **2004**, *5*, 1211 – 1217.
[7] G. P. Adams, L. M. Weiner, *Nat. Biotechnol.* **2005**, *23*, 1147 – 1157.
[8] U. Galili, *Alpha-Gal and Anti-Gal: Alpha-1,3-Galactosyltransferase, Alpha-Gal Epitopes, and the Natural Anti-Gal Antibody, Vol. 32*, Kluwer/Plenum, New York, **1999**.
[9] U. Galili, L. Wang, D. C. LaTemple, M. Z. Radic, *Subcell. Biochem.* **1999**, *32*, 79 – 106.
[10] T. E. Mollnes, A. E. Fiane, *Mol. Immunol.* **2003**, *40*, 135 – 143.
[11] U. Galili, S. B. Shohet, E. Kobrin, C. L. M. Stults, B. A. Macher, *J. Biol. Chem.* **1988**, *263*, 9858 – 9866.
[12] R. O. Hynes, *Cell* **2002**, *110*, 673 – 687.
[13] B. P. Eliceiri, D. A. Cheresh, *Curr. Opin. Cell Biol.* **2001**, *13*, 563 – 568.
[14] B. Felding-Habermann, *Clin. Exp. Med.* **2003**, *20*, 203 – 213.
[15] P. C. Brooks, R. A. F. Clark, D. A. Cheresh, *Science* **1994**, *264*, 569 – 571.
[16] A. Janczuk, J. Li, W. Zhang, X. Chen, Y. Chen, J. Fang, J. Wang, P. G. Wang, *Curr. Med. Chem.* **1999**, *6*, 155 – 164.
[17] L. D. D'Andrea, A. Del Gatto, C. Pedone, E. Benedetti, *Chem. Biol. Drug Des.* **2006**, *67*, 115 – 126.
[18] R. Haubner, R. Gratias, B. Diefenbach, S. L. Goodman, A. Jonczyk, H. Kessler, *J. Am. Chem. Soc.* **1996**, *118*, 7461 – 7472.
[19] M. Pfaff, K. Tangemann, B. Muller, M. Gurrath, G. Muller, H. Kessler, R. Timpl, J. Engel, *J. Biol. Chem.* **1994**, *269*, 20 233 – 20 238.
[20] K. Temming, R. M. Schiffelers, G. Molema, R. J. Kok, *Drug Resist. Updates* **2005**, *8*, 381 – 402.
[21] M. E. Duggan, L. T. Duong, J. E. Fisher, T. G. Hamill, W. F. Hoffman, J. R. Huff, N. C. Ihle, C. T. Leu, R. M. Nagy, J. J. Perkins, S. B. Rodan, G. Wesolowski, D. B. Whitman, A. E. Zartman, G. A. Rodan, G. D. Hartman, *J. Med. Chem.* **2000**, *43*, 3736 – 3745.
[22] J. D. Hood, M. Bednarski, R. Frausto, S. Guccione, R. A. Reisfeld, R. Xiang, D. A. Cheresh, *Science* **2002**, *296*, 2404 – 2407.
[23] T. D. Harris, S. Kalogeropoulos, T. Nguyen, S. Liu, J. Bartis, C. Ellars, S. Edwards, D. Onthank, P. Silva, P. Yalamanchili, S. Robinson, J. Lazewatsky, J. Barrett, J. Bozarth, *Cancer Biother. Radiopharm.* **2003**, *18*, 627 – 641.
[24] C. A. Burnett, J. Xie, J. Quijano, Z. Shen, F. Hunter, M. Bur, K. C. P. Li, S. N. Danthi, *Bioorg. Med. Chem.* **2005**, *13*, 3763 – 3771.
[25] S. Biltresse, M. Attolini, J. Marchand-Brynaert, *Biomaterials* **2005**, *26*, 4576 – 4587.
[26] A. Meyer, J. Auemheimer, A. Modlinger, H. Kessler, *Curr. Pharm. Des.* **2006**, *12*, 2723 – 2747.
[27] C. Rader, S. C. Sinha, M. Popkov, R. A. Lerner, C. F. Barbas III, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 5396 – 5400.
[28] M. Popkov, C. Rader, B. Gonzalez, S. C. Sinha, C. F. Barbas III, *Int. J. Cancer* **2004**, *119*, 1194 – 1207.
[29] L. S. Li, C. Rader, M. Matsushita, S. K. Das, C. F. Barbas III, R. A. Lerner, S. C. Sinha, *J. Med. Chem.* **2004**, *47*, 5630 – 5640.
[30] F. Guo, S. K. Das, B. M. Mueller, C. F. Barbas III, R. A. Lerner, S. C. Sinha, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11 009 – 11 014.
[31] W. H. Miller, R. M. Keenan, R. N. Willette, M. W. Lark, *Drug Discovery Today* **2000**, *5*, 397 – 408.
[32] B. Cacciari, G. Spalluto, *Curr. Med. Chem.* **2005**, *12*, 51 – 70.
[33] A. C. Bach II, J. R. Espina, S. A. Jackson, P. F. W. Stouten, J. L. Duke, S. A. Mousa, W. F. DeGrado, *J. Am. Chem. Soc.* **1996**, *118*, 293 – 294.
[34] J. Luna, T. Tobe, S. A. Mousa, T. M. Reilly, P. A. Campochiaro, *Lab Instrum.* **1996**, *75*, 563 – 573.
[35] J. W. Corbett, N. R. Graciani, S. A. Mousa, W. F. DeGrado, *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1371 – 1376.
[36] L. F. Tietze, M. Arlt, M. Beller, K. H. Gluesenkamp, E. Jaehde, M. F. Rajewsky, *Chem. Ber.* **1991**, *124*, 1215 – 1221.
[37] J.-P. Xiong, T. Stehle, R. Zhang, A. Joachimiak, M. Frech, S. L. Goodman, M. A. Arnaout, *Science* **2002**, *296*, 151 – 155.
[38] M. S. Egbertson, B. Bednar, R. A. Bednar, G. D. Hartman, R. J. Gould, R. J. Lynch, L. M. Vassallo, S. D. Young, *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1415 – 1420.
[39] D. G. Batt, J. J. Petraitis, G. C. Houghton, D. P. Modi, G. A. Cain, M. H. Corjay, S. A. Mousa, P. J. Bouchard, M. S. Forsythe, P. P. Harlow, F. A. Barbera, S. M. Spitz, R. R. Wexler, P. K. Jadhav, *J. Med. Chem.* **2000**, *43*, 41 – 58.
[40] J. N. Lambert, J. P. Mitchell, K. D. Roberts, *J. Chem. Soc. Perkin Trans. 1* **2001**, 471 – 484.
[41] J. S. Davies, *J. Pept. Sci.* **2003**, *9*, 471 – 501.
[42] D. Boturyn, P. Dumy, *Tetrahedron Lett.* **2001**, *42*, 2787 – 2790.
[43] P. Chand, Y. S. Babu, S. Bantia, N. M. Chu, L. B. Cole, P. L. Kotian, W. G. Laver, J. A. Montgomery, V. P. Pathak, S. L. Petty, D. P. Shrout, D. A. Walsh, G. W. Walsh, *J. Med. Chem.* **1997**, *40*, 4030 – 4052.
[44] S. Thorand, N. Krause, *J. Org. Chem.* **1998**, *63*, 8551 – 8553.
[45] K. D. Rice, A. R. Gangloff, E. Y. L. Kuo, J. M. Dener, V. R. Wang, R. Lum, W. S. Newcomb, C. Havel, D. Putnam, L. Cregar, M. Wong, R. L. Warne, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2357 – 2360.
[46] P. G. Mattingly, *Synthesis* **1990**, 366 – 368.
[47] U. Galili, L. M. Khushi, *Transplantation* **1996**, *62*, 256.
[48] K. P. Naicker, H. Li, A. Heredia, H. Song, L.-X. Wang, *Org. Biomol. Chem.* **2004**, *2*, 660 – 664.
[49] M. Kantlehner, D. Finsinger, J. Meyer, P. Schaffner, A. Jonczyk, B. Diefenbach, B. Nies, H. Kessler, *Angew. Chem.* **1999**, *111*, 587 – 590; *Angew. Chem. Int. Ed.* **1999**, *38*, 560 – 562.
[50] J. W. Fang, J. Li, X. Chen, Y. N. Zhang, J. Q. Wang, Z. M. Guo, W. Zhang, L. B. Yu, K. Brew, P. G. Wang, *J. Am. Chem. Soc.* **1998**, *120*, 6635 – 6638.
[51] C. Gege, W. Kinzy, R. R. Schmidt, *Carbohydr. Res.* **2000**, *328*, 459 – 466.
[52] R. J. Hinklin, L. L. Kiessling, *J. Am. Chem. Soc.* **2001**, *123*, 3379 – 3380.
[53] Y. H. Wang, Q. Y. Yan, J. P. Wu, L. H. Zhang, X. S. Ye, *Tetrahedron* **2005**, *61*, 4313.
[54] A. K. Sarkar, K. L. Matta, *Carbohydr. Res.* **1992**, *233*, 245.
[55] K. H. Jung, M. Hoch, R. R. Schmidt, *Liebigs Ann. Chem.* **1989**, 1099.
[56] P. J. Garegg, C. Henrichson, T. Norberg, *Carbohydr. Res.* **1983**, *116*, 161 – 165.
[57] S. Sato, M. Mori, Y. Ito, T. Ogawa, *Carbohydr. Res.* **1986**, *155*, C6 – C10.
[58] V. P. Kamath, P. Diedrich, O. Hindsgaul, *Glycoconjugate J.* **1996**, *13*, 315 – 319.
[59] P. I. Kitov, D. R. Bundle, *J. Chem. Soc. Perkin Trans. 1* **2001**, 838 – 853.

[60] H. G. Lerchen, J. Baumgarten, K. von dem Bruch, T. E. Lehmann, M. Sperzel, G. Kempka, H. H. Fiebig, *J. Med. Chem.* **2001**, *44*, 4186 – 4195.
[61] N. Zanatta, A. M. C. Squizani, L. Fantinel, F. M. Nachtigall, H. G. Bonacorso, M. A. P. Martins, *Synthesis* **2002**, 2409 – 2415.
[62] E. F. Plow, T. A. Haas, L. Zhang, J. Loftus, J. W. Smith, *J. Biol. Chem.* **2000**, *275*, 21 785 – 21 788.
[63] M. Weetall, R. Hugo, C. Friedman, S. Maida, S. West, S. Wattanasin, R. Bouhel, G. Weitz-Schmidt, P. Lake, *Anal. Biochem.* **2001**, *293*, 277 – 287.
[64] S. Liu, D. S. Edwards, M. C. Ziegler, A. R. Harris, S. J. Hemingway, J. A. Barrett, *Bioconjugate Chem.* **2001**, *12*, 624 – 629.
[65] N. Nasongkla, X. Shuai, H. Ai, B. D. Weinberg, J. Pink, D. A. Boothman, J. M. Gao, *Angew. Chem.* **2004**, *116*, 6483 – 6487; *Angew. Chem. Int. Ed.* **2004**, *43*, 6323 – 6327.
[66] B. P. Orner, R. Derda, R. L. Lewis, J. A. Thomson, L. L. Kiessling, *J. Am. Chem. Soc.* **2004**, *126*, 10 808.

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