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Non-Natural Glycosphingolipids and Structurally Simpler Analogues Bind HIV-1 Recombinant Gp120

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Abstract—Interactions of recombinant gp120 (rgp120) with *non-natural* glycosphingolipids (GSLs) and structurally simpler analogues have been studied using a competitive adhesion assay. Conjugates of cellobiosyl ceramide and melibiosyl ceramide were synthetically prepared as water-soluble GSL analogues. These ligands were screened against a panel of biologically relevant analogues, and the results show that their interactions with rgp120 are comparable to natural cellular receptors. Glycolipid interactions with rgp120 were probed further by the synthesis and testing of structurally simpler analogues that were obtained by reductive amination of lactose, cellobiose, and melibiose with a biotinylated amino ethylene glycol moiety. RGp120 did not recognize conjugates lacking a lipid component. However, palmitoylation of the secondary amino alditols yielded compounds with comparable rgp120 affinity to the natural cellular receptor, galactosyl ceramide (GalCer). Taken together, the SAR showed that both a hydrophobic and a hydrophilic component are required for rgp120 recognition. Moreover, structural variability in the carbohydrate headgroup did not significantly alter rgp120 recognition indicating that this interaction is not highly specific.

Introduction

Glycosphingolipids are the principal glycolipids in mammalian cells.[1] They are present on the outer leaflet of plasma membranes where they are anchored through ceramide (**1**) functionalities, which are *N*-acylated sphingosines (**2**) or sphingolipids (Fig. 1). The carbohydrate marker is attached to the terminal hydroxyl of ceramide. The structures and distribution of GSLs vary widely and they serve a number of different biological functions.[2] GalCer (**3**) is a simple GSL with a β-linkage to ceramide. It has been identified as an essential receptor component for gp120, the cell-surface glycoprotein of HIV that initiates viral infection into host cells. The principal receptor for gp120 is a T-cell associated protein called CD4. However, HIV is known to infect CD negative cells such as neuronal cells,[3] colonic epithelial cells,[4] and vaginal epithelial cells through a recognition process involving GalCer.[5]

Several investigations aimed at identifying other natural GSLs that are recognized by gp120 have been reported. Glucosyl ceramide (GlcCer **4**), lactosyl ceramide (LacCer **5**), and psychosine (**6**) were all studied as potential ligands for gp120. The extent to which these GSLs were recognized by gp120 differed depending upon the binding assay used to probe the interaction. For example, in high performance thin layer chromatography (HPTLC), GlcCer and LacCer were weakly bound by gp120, while psychosine was recognized nearly as well as GalCer.[7] In contrast, liposomal studies showed that gp120 bound GlcCer and LacCer but not psychosine.[7]

It was reasoned that contradicting assay results emanated from differences in ligand presentation geometries inherent in the assays, which invalidated cross comparisons.[8] To circumvent the difficulties associated with lipid-based ELISA and other assay methods, an assay that could be uniformly applied to a variety of GSLs was developed.[9] A water-soluble biotinylated ethylene

Abbreviations: GSL, glycosphingolipid; GSLs, glycospingolipids; bGalCer, biotinylated galactosyl cermide; bGlcCer, biotinylated glucosyl ceramide; bLacCer, biotinylated lactosylceramide; bCelCer, biotinylated cellbiosyl ceramide; bMelCer, biotinylated melibiosyl ceramide.

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glycol linker bearing an acid functionality was synthetically prepared and conjugated to amino-analogues of GSLs. These ligands were irreversibly adhered to microtiter plates coated with NeutrAvidin™, a deglycosylated analogue of avidin. Horseradish peroxidase-tagged recombinant gp120 (rgp120) was added to each well, and rgp120 recognition of the ligands was measured. The plating efficiencies of the ligands were also measured and the data were normalized allowing direct comparison of binding affinities. The adhesion assay results showed that rgp120 bound biotinylated GalCer [bGalCer (**7**)] and biotinylated GlcCer [bGlcCer (**8**)] similarly and it recognized biotinylated LacCer [bLacCer (**9**)] to an even greater extent (Fig. 2). Since the ELISA could not be measured under equilibrium conditions, it was not possible to quantify the binding affinities; only a qualitative assessment was realized. However, recently rgp120 recognition of GalCer, GlcCer, and LacCer was quantified under equilibrium conditions using total internal reflection microscopy.[10] The data between the two studies were closely correlated validating the use of the adhesion assay as a rapid screening tool.

Results and Discussion

In a continued search for compounds that mimic gp120/GalCer interactions without interfering with healthy cellular interactions, GSLs that are not natural cell surface components were targeted. Determining the relative affinities of different disaccharide head groups was of particular interest, since bLacCer bound gp120 so well. A cellobiose analogue (**10**) (Fig. 2), which differs from lactose at the 4″OH, was chosen to probe the specificity requirements of the terminal sugar. Furthermore, cellobiose analogues were deemed attractive targets since humans lack the enzyme required to cleave the interresidue linkage, which could lead to improved pharmacokinetics.[11] α-Melibiosyl ceramide (**11**) was targeted in order to determine if an α-linkage would be tolerated, and whether the regiochemical positioning of sugars (1–6 versus 1–4) was important. In this report, we describe the synthesis of these non-natural GSLs and their relative binding affinities for rgp120. Structure activity studies were further expanded to include structurally simpler analogues that were obtained by reductive amination of lactose, cellobiose, and melibiose with a biotinylated amino ethylene glycol moiety.

Reaction of the tetrafluorophenyl biotinylated tetraethylene glycol ester **12**[12] with D-sphingosine (**13**) afforded biotinylated ceramide (bCer) **14** in 90% yield (Scheme 1). Cellobiose and α-melibiose were readied for glycosylation in a three-step procedure. Each disaccharide was per-*O*-acetylated by the action of acetic anhydride in pyridine and the resulting anomeric acetates were regioselectively removed using the method of Flandor.[13] The final step involved activation of the anomeric hydroxyls to facilitate glycosidic bond formation using the trichloroacetimidate strategy developed by Schmidt.[14] The trichloroacetimidates were then coupled to **14** to yield the per-*O*-acetylated ceramide derivatives (Scheme 2). Glycosylation of per-*O*-acetylated cellobiosyl trichloroacetimidate resulted in a 6% yield, and the yield of the melibiosyl derivative was slightly higher (18% yield). The reducing anomeric centers in the isolated products were determined to be the beta

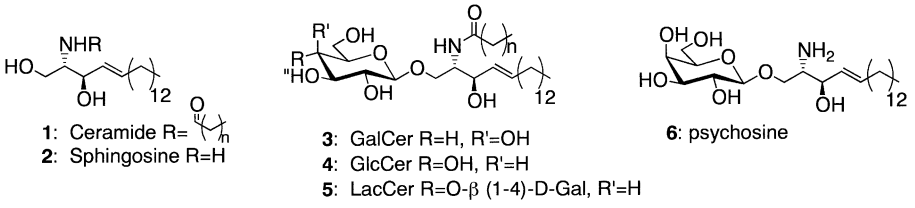


Figure 1. Structures of naturally occurring glycosphingolipds.

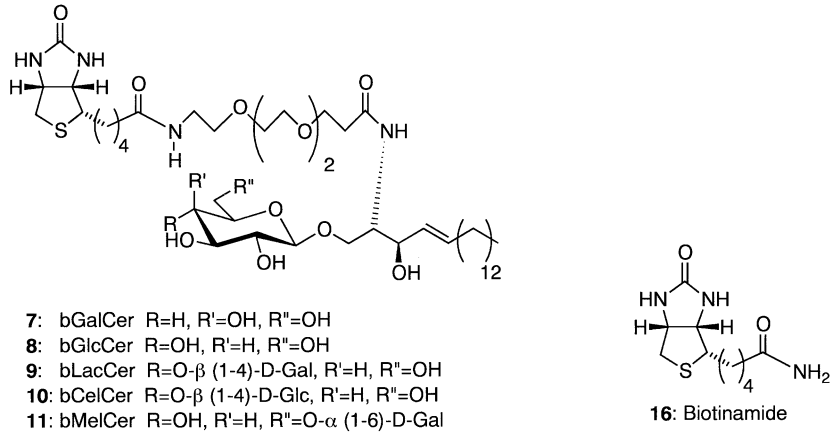
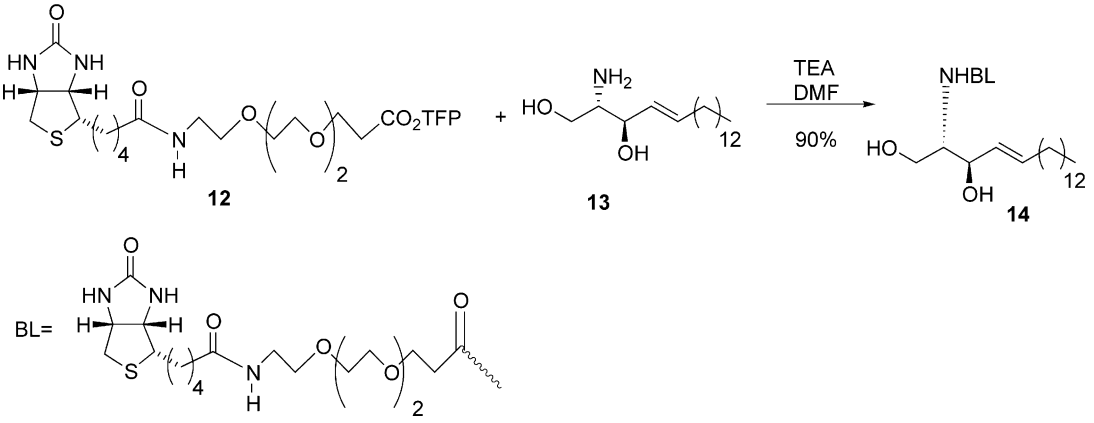


Figure 2. Structures of biotinylated glycosphingolipids.

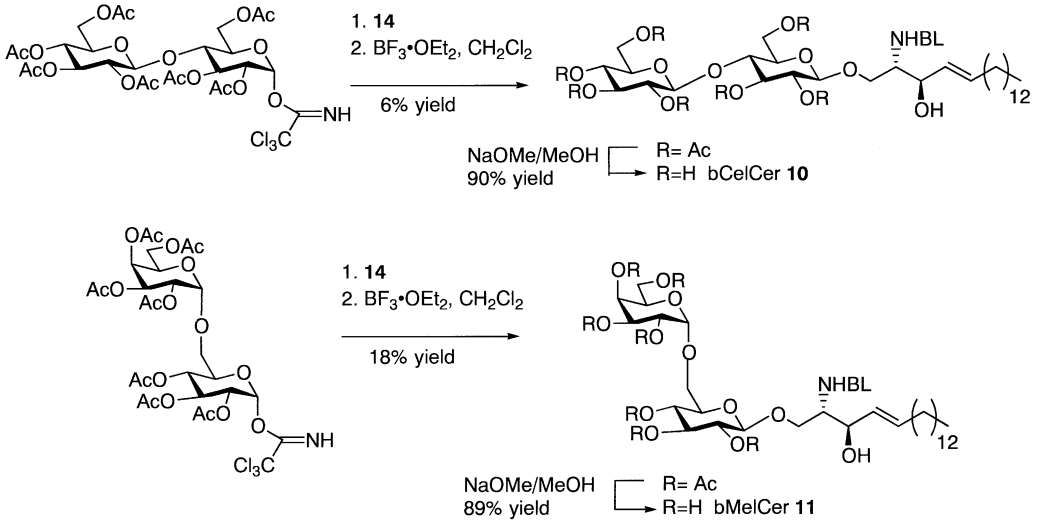
configuration based upon the chemical shift of the anomeric carbon resonances at δ 100.60 and 100.56 for bCelCer and bMelCer, respectively. The alpha glycosides would be expected to appear upfield at approximately 96 ppm as is the case for the alpha glycosidic linkage in bMelCer, which appears at δ 96.42 ppm. Deacetylation in sodium methoxide and methanol provided the target compounds, **10** [biotinylated cellobiosyl ceramide (bCelCer)] and **11** [biotinylated melibiosyl ceramide (bMelCer)] in 90 and 89% yields, respectively.

Glycosylations of ceramides are notoriously poor, due to unfavorable hydrogen bonding interactions.[15–17] Improved yields have been realized using a number of methods, which eliminate the presence of the amide linkage. In one procedure, the amide was replaced by an azide functionality.[18] Another method involved the use of Schiff base-protected amines, which provided ceramides after further manipulations.[19] While strategies utilizing azido- or imino-protected amines as glycosyl acceptors in the formation of sphingosine derivatives may have provided enhanced yields, resources were not invested in these endeavors. The non-natural GSLs were primarily synthesized as tools for establishing SAR. The decision to limit the synthetic protocols to as few steps as possible was consistent with these goals.

The non-natural GSLs, **10** and **11** were evaluated in the adhesion assay in order to compare their binding affinities for rgp120 with natural analogues (**7**, **8**, and **9**). A simple primary amide, biotinamide (**16**) (Fig. 2), and bCer (**14**) were also tested as a non-specific ligands.[9] The adhesion assay was performed in two ways. First the compounds were evaluated as previously described. Plating efficiencies (PE) of the complete ligand panel ranged from 88 to 97%, providing a high level of confidence that absorbance differences were due to variances in binding affinities, and not derived from the number of ligands present in the well. The extent of rgp120 binding was monitored by absorbance changes over 30 min and the end point absorbance values were recorded. The data obtained from these experiments were normalized with respect to plating efficiencies for each ligand. Averaged end point results from four separate wells are plotted in Figure 3. The error bars represent the standard deviations between absorbance readings in each of the wells. The data in Figure 3 are representative of several different experiments.



Scheme 1.



Scheme 2.

In an alternative assay construct, the ligands were evaluated in a competitive mode. Biotinylated GalCer (**7**) was adhered to NeutrAvidin™ coated microtiter plates. After a blocking step (to eliminate nonspecific binding), bGalCer coated wells were incubated with rgp120. The plates were rinsed with PBS to remove unbound rgp120 and then incubated with a solution of ligand for 4 h. The plates were washed and the amount of bound rgp120 was measured using TMB (3,3′,5,5′-tetramethylbenzidine) as the peroxidase substrate. The hypothesis was that a ligand in solution, capable of binding to rgp120, should compete with plated bGalCer for the bound rgp120. In this case, the amount of rgp120 bound to the plate would decrease in proportion to its affinity for the ligand in solution that it was exposed to. A set of bGalCer coated wells that, after rgp120 incubation were exposed to PBS alone, served as controls against which the decrease in binding was calculated. A series of ligands were tested at varying concentrations to provide a dose–response curve. Using the dose–response curve, EC_{50} values for decrease in rgp120 bound to plated bGalCer upon exposure to ligand solutions were calcuated. As can be seen in Table 1, the glycolipids showed micromolar effective concentrations and the results from the competition assay correlated well with the classical adhesion data shown in Figure 3.

The ELISA data provided important information concerning the panel of biotinylated GSLs. First, bLacCer (**9**) gave consistently higher end point absorbancies than the other compounds tested. Secondly, gp120 recognized all of the GSLs with greater affinity than the simple biotinylated ceramide (**14**), which defined the carbohydrate functionality as an important recognition element. Furthermore, the results showed that rgp120 bound bCelCer and bMelCer to the same extent as bGalCer, an *N*-acyl analogue of the natural cellular receptor GalCer.

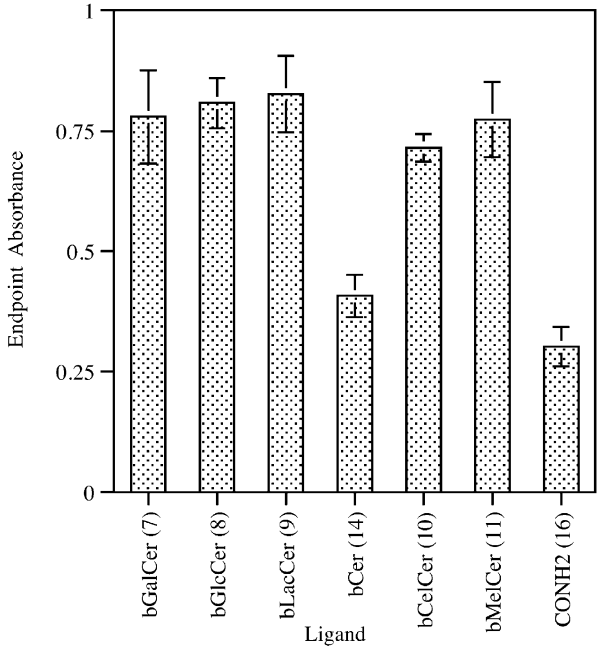


Figure 3. Adhesion assay data reported as end point reading at 30 min. The data were normalised to reflect the plating efficiencies of each ligand. The error bars are the standard deviations between the readings in four separate wells. The data are representative of multiple experiments.

Since the structure of the non-reducing sugar and the regio-and stereochemistry of the interresidue glycosidic linkage did not appreciably affect activity, the SAR studies were expanded to include more synthetically accessible compounds. SAR of simplified GalCer analogues is an active area of research. Bertozzi and Bednarski[20] studied *C*-glycosides and determined that a critical alkyl chain length was required for activity. Rico-Lattes and co-workers synthesized analogues derived from reductive amination of carbohydrates followed by fatty acid conjugation.[21] The same investigators generated other derivatives attempting to improve both anti-HIV activity and cytotoxicity profiles. One lipid chain was terminated with a carboxylate functionality in order to increase water solubility and the optimal chain length of both lipids was probed. It was concluded that the palmitoyl fatty acid chain length was optimal.

In the current study, neutral GalCer analogues were targeted because charged compounds were known to interact with basic amino acids positioned in the V3 loop of gp120.[22] These electrostatic interactions could obscure important hydrophobic and/or hydrophilic interactions, which would invalidate direct comparisons to GalCer. The synthesis of the compounds shown in Scheme 3 was fashioned after the work of Fantini and co-workers.[23] A biotinylated diamino tetraethylene glycol linker was required for reductive amination of lactose, cellobiose, and melibiose, which were slated for subsequent lipid conjugation. These three disaccharides were chosen for two reasons: To have a direct comparison to bLacCer, bCelCer, and bMelCer; and because after reductive amination, they would yield non-reducing sugar headgroups (β-galactose, β-glucose, and α-galactose, respectively). Comparison of the latter compounds to bGalCer and bGlcCer would enhance the SAR.

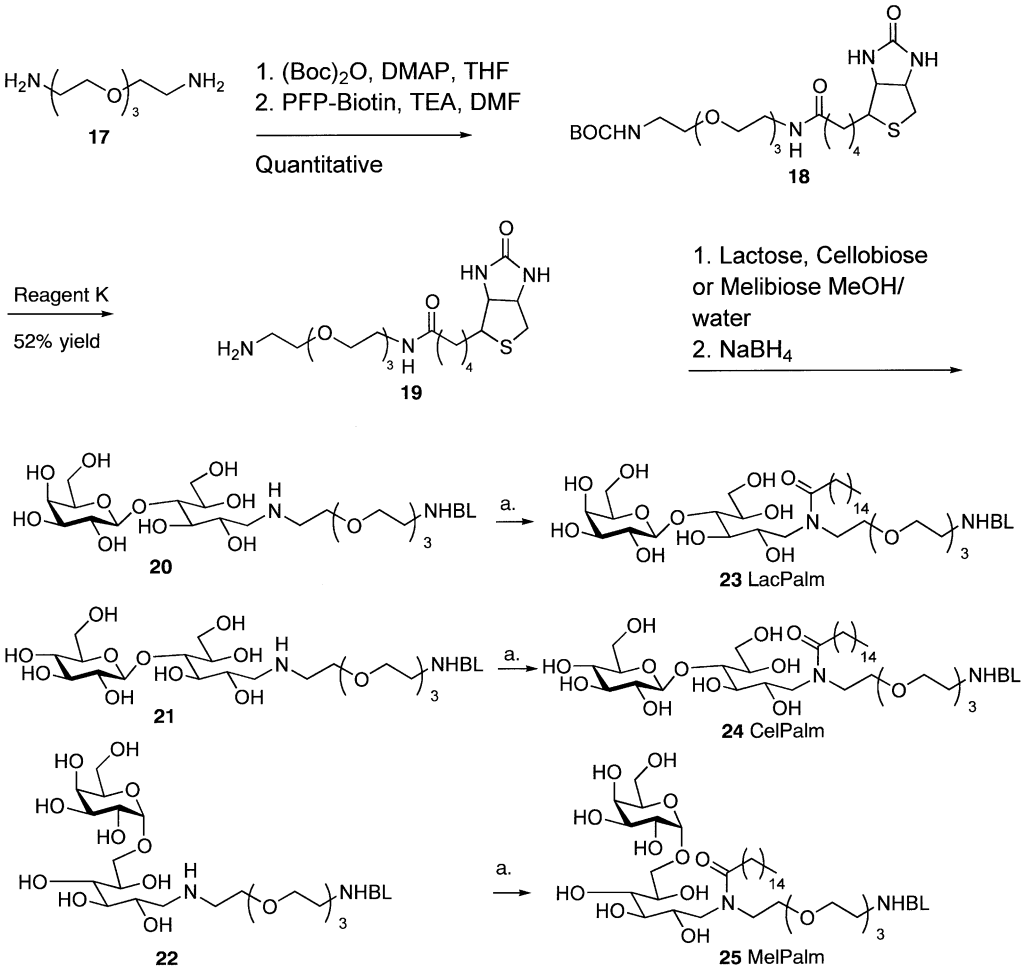
The synthesis of the amino-terminated TEG linker began with commercially available jeffamine **17**, which was monoprotected with *t*-butoxycarbonyl anhydride

Table 1. EC_{50} values derived from competitive adhesion assay calculated by a non-linear regression analysis using variable slope dose–response curves

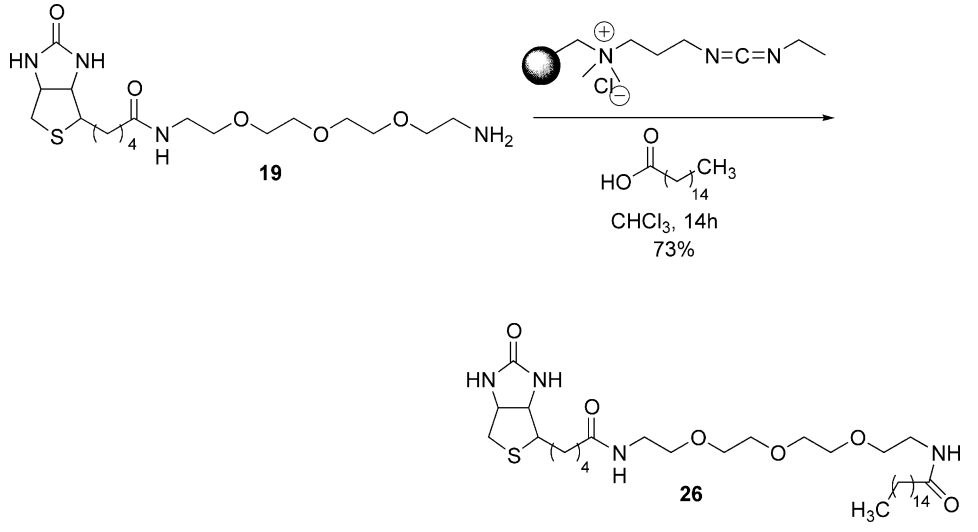
Ligand	EC_{50} mM
$CONH_2$ (**16**)	$>108 \pm 22.9$
bCer (**14**)	6.21 ± 0.960
bGalCer (**7**)	0.096 ± 0.014
bGlcCer (**8**)	0.018 ± 0.011
bLacCer (**9**)	0.019 ± 0.013
bCelCer (**10**)	0.059 ± 0.018
bMelCer (**11**)	0.027 ± 0.014

Compounds **14** and **16** do not display sigmoidal dose–response and their EC_{50} values were calculated by linear regression curve fitting.

(Scheme 3). Biotinylation of the resulting amine was achieved by a two-step, one-pot procedure. An activated biotin acid was generated by the action of 2,3,4,5,6-pentafluorophenyl trifluoroacetate (PFP-TFA) on biotin. Compound **17** was added to the crude pentafluorophenyl ester and a quantitative yield of *N*-Boc-*N*-biotinyl jeffamine (**18**) was realized. Subsequent reaction with Reagent K provided the biotinylated amino linker (**19**) in 52% yield. The disaccharides were diluted in methanol and water and reacted with **19** in the presence



Scheme 3. (a) $PFPOCO(CH_2)_{14}CH_3$, TEA, DMF. The yield of 23 was 19% from lactose, 24 was 33% from cellobiose, and 25 was 37% from melibiose.



Scheme 4.

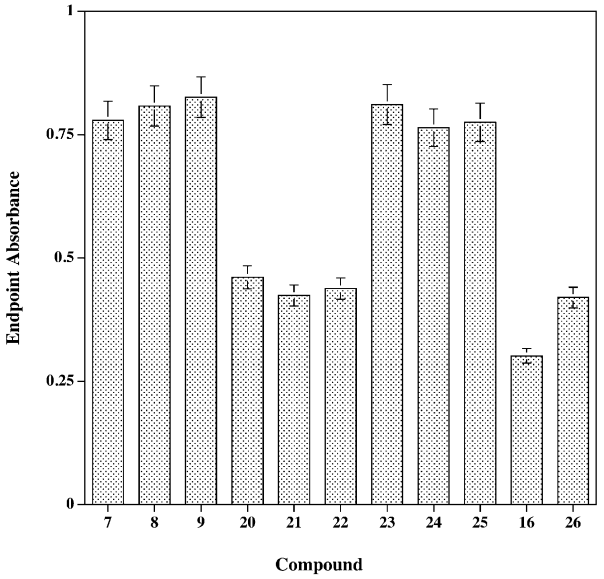


Figure 4. Adhesion assay data reported as end point reading at 30 min. The data were normalized to reflect the plating efficiencies of each ligand. The error bars are the standard deviations between the readings in four separate wells. The data are representative of multiple experiments.

Table 2. EC_{50} values derived from competitive adhesion assay calculated from dose–response curve, plotted using a logarithmic curve fit program

Ligand	EC_{50} mM
Linker (**26**)	3.68±1.40
bLac (**20**)	7.70±2.80
bCel (**21**)	11.45±1.64
bMel (**22**)	2.71±0.50
bLacPalm (**23**)	0.065±0.024
bCelPalm (**24**)	0.038±0.019
bMelPalm (**25**)	0.052±0.002

of $NaBH_4$ to give the amines (**20**, **21**, and **22**). Compounds **20**–**22** were evaluated in the adhesion assay to confirm that a lipid chain was required for rgp120 recognition of these ligands as well. As indicated in Figure 4, these compounds did not show significantly greater binding than the linkers, just as was seen for the ethanolamine derivatives.[9]

To complete the SAR, **21**–**22** were condensed with palmitic acid that had been activated by the action of PFP-TFA. The biotinylated linker **19** was also palmitoylated to form **26** (Scheme 4) as a control measure for non-specific interactions in the adhesion assays. The purified compounds (**23**–**26**) were assayed in both the adhesion and competitive assays and the results are shown in Figure 4 and Table 2, respectively.

Conclusion

The biological data for the simpler analogues clearly show that both a carbohydrate functionality and a hydrophobic chain are required for activity. The fact that the trends between the adhesion assay and the competitive assay are not completely correlated may reflect differences in off rates. The adhesion assay is not performed at equilibrium so observed differences in rgp120 recognition of ligands may be due to differences in the reversible binding event. Similarly, the competition assay allows different ligands to compete for recognition of gp120 which is reversibly bound by plated bGalCer. Differences in EC_{50} values may reflect differences in the reversible binding of competing ligands. Further quantitative measurements of these interactions is necessary before differences in these assays can be completely understood. Nonetheless, it is clear that glycolipids serve as recognition elements for rgp120, and the interactions are not highly specific.

Experimental

General procedures

All materials were obtained from commercial sources and used without additional purification, unless otherwise noted. Solvents were distilled prior to use as reaction media. All glassware utilized in water-sensitive reactions was flame-dried before use. ^{1}H NMR spectra were recorded at 250 or 500 MHz on Bruker superconducting FT spectrometers. ^{1}H NMR data are reported in the order of chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet, br = broad). ^{13}C NMR spectra were recorded at 62.5 or 125 MHz and were proton decoupled. All spectra are reported in parts per million relative to the residual solvent peak. Mass spectrometry was performed at The University of Arizona, Mass Spectrometry Facility, Tucson, AZ, USA.

D-Biotin-12-amido-4,7,10-trioxadodecyl sphingosinamide (bCer, 14). Five millilgrams (0.017 mmol) of D-sphingosine (**13**, Matreya) was dissolved in 266 μL of DMF. 7.1 μL (0.051 mmol) of TEA was added, followed by 11.1 mg (0.019 mmol) of compound **12**. The reaction was stirred at room temperature overnight under argon, then evaporated. The crude product was purified using silica gel chromatography in 9:1 chloroform/methanol. Twelve milligrams (100%) of an off-white powder was obtained. The NMR spectra indicate the presence of a small quantity of the *O*-linked product, estimated to be about 10%. ^{1}H NMR (250 MHz, DMSO-d_6) δ 0.85 (m, 3H, CH_3), 1.14–1.59 (m, 28H, $(CH_2)_{14}$), 1.94 (m, 2H, $CH_2C{=}C$), 2.06 (t, 2H, J = 7.4 Hz, CH_2CONH), 2.32 (t, 2H, J = 6.6 Hz, CH_2CONH), 2.57 (d, 1H, J = 12.7 Hz, CHS), 2.81 (dd, 1H, J = 5.0, 14.9 Hz, CHS), 3.09–3.21 (m, 3H), 3.42–3.65 (m, 14H), 3.87 (m, 1H), 4.12 (m, 1H), 4.49 (t, 1H, J = 7.7 Hz), 4.83 (d, 1H, J = 5.4 Hz), 5.34–5.60 (m, 2H, H–C=C–H), 6.37 (s, 1H, NH), 6.44 (s, 1H, NH), 7.54 (d, 1H, J = 8.7 Hz, CONHCH), 7.86 (t, 1H, J = 5.3 Hz, $CONHCH_2$); ^{13}C NMR δ 13.97, 22.11, 25.26, 28.04, 28.20, 28.60, 28.72, 28.83, 28.99, 29.07, 31.31, 31.73, 35.09, 36.18, 55.43, 59.19, 60.31, 61.03, 66.97, 69.19, 69.52, 69.56, 69.72, 71.19, 130.81, 131.24, 162.71, 169.90, 172.12. High-resolution mass spectrum (FAB+), calcd for $C_{37}H_{69}N_4SO_8$ $(MH)^+$: 729.4836. Found: 729.4814.

β-D-Glucoopyranosyl-(1→4)-*O*-β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*,4*E*)-3-hydroxy-2-*N*-(12-D-biotinamide-4,7,10-trioxadodecyl)-sphingenine (10). Cellobiose (1.0 g, 2.92 mmol, J.T. Baker) was weighed directly into a flame-dried flask. DMAP (35.4 mg, 0.29 mmol, Novabiochem) was added to the flask, followed by 6 mL of pyridine. The flask was stirred in an ice bath under argon, and acetic anhydride (5.5 mL, 58.4 mmol, Aldrich) was added dropwise over a period of 10 min via an addition funnel. The reaction mixture was stirred at room temperature for 1 day, until TLC in 1:1 hexanes/ethyl acetate indicated nearly a complete reaction. The flask was placed on ice and 6 mL of methanol was added. The solvent was evaporated, and the residue was dissolved in ethyl acetate and washed with 3×0.1 M HCl, 1×water, 1×brine. The organic phase was dried over anhydrous Na_2SO_4. A white foam product (1.87 g, 83%) resulted. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.89–2.03 (m, 24H, $(COCH_3)_8$), 3.58–3.75 (m, 3H), 4.01 (m, 2H), 4.29 (dd, 1H, J=4.5, 12.5 Hz), 4.42 (m, 1H), 4.83 (t, 1H, J=8.1 Hz), 4.92–5.19 (m, 5H), 5.59 (d, 1H, J=8.2 Hz). The peracetylated disaccharide was used without further purification in the following manner. 25 mL of a 7:3 mixture of THF/MeOH was stirred at 0 °C with NH_3 (g) bubbled through for 10 min. This solution was transferred to a flask containing peracetylated cellobiose (500 mg, 0.737 mmol), and the mixture was stirred from 0 °C to room temperature for 1.5 h, until TLC in 2:1 ethyl acetate/hexanes indicated a complete absence of the peracetylated disaccharide. The solution was evaporated, and the resultant crude oil was purified in 2:1 ethyl acetate/hexanes, to yield 308 mg (66%) of an α/β mixture of a clear colorless oil (1-hydroxy-hepta-*O*-acetyl cellobiose). ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.90–2.12 (m, 21H, $(COCH_3)_7$) 3.58–3.71 (m, 2H), 3.94–4.01 (m, 2H), 4.20 (d, 1H, J=3.8 Hz), 4.30 (dd, 1H, J=4.2, 12.6 Hz), 4.44 (m, 2H), 4.72 (m, 1H), 4.85 (app t, 1H, J=8.1 Hz), 5.03 (m, 2H), 5.28 (t, 1H, J=3.7 Hz), 5.41 (t, 1H, J=9.5 Hz); ^{13}C NMR δ 20.64, 20.75, 20.85, 20.96, 21.00, 21.11, 61.72, 61.95, 62.10, 67.96, 68.23, 69.49, 71.47, 71.77, 72.0, 72.18, 73.05, 73.13, 73.36, 76.60, 76.69, 76.72, 77.23, 77.43, 77.74, 90.08 (α C-1), 95.27 (β C-1), 100.79 (β-C-1′), 100.89 (β-C-1′), 169.21, 169.48, 169.87, 169.90, 170.39, 170.44, 170.56, 170.64, 170.70, 170.75, 170.80. High-resolution mass spectrum (FAB^+) calcd for $C_{26}H_{37}O_{18}$ $(MH)^+$: 637.1980. Found: 637.1976. The 1-hydroxy-hepta-*O*-acetyl cellobiose product (308 mg, 0.484 mmol) was dissolved in 5 mL of CH_2Cl_2 in a N_2 atmosphere. CCl_3CN (169.5 μL, 1.69 mmol, Aldrich), was added next via a syringe, followed by the addition of 14 mg (0.581 mmol) of NaH (Aldrich). The solution turned a clear yellow immediately upon the addition of NaH. The reaction was stirred at room temperature under N_2, with the color deepening to a brownish-amber. After 5 h, the reaction was shown to be complete by TLC in 2:1 ethyl acetate/hexanes. Purification was achieved by silica gel chromatography utilizing a gradient from 2:1 ethyl acetate/hexanes to 1.5:1 of the same. A fluffy white solid product (155.4 mg, 85%) resulted after evaporation in vacuo from benzene. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.88–2.13 (m, 21H, $(COCH_3)_7$), 3.63 (br d, 1H, J=9.4 Hz), 3.80 (t, 1H, J=9.6 Hz), 4.03 (app dt, 4H, J=4.5, 11.5 Hz), 4.35 (dd, 1H, J=4.0, 12.6 Hz), 4.48 (app t, 2H, J=7.8 Hz), 4.89 (t, 1H, J=8.0 Hz), 5.04 (m, 2H), 5.48 (t, 1H, J=9.6 Hz), 6.44 (d, 1H, J=3.8 Hz, H-1), 8.62 (s, 1H, NH); ^{13}C NMR δ 20.61, 20.69, 20.75, 20.81, 20.87, 20.95, 61.53, 61.68, 67.84, 69.42, 69.81, 70.0, 71.10, 71.44, 71.81, 72.15, 73.01, 73.20, 76.25, 76.52, 76.72, 76.87, 77.23, 77.38, 77.74, 93.0 (α C-1), 101.08 (β C-1′), 128.47, 161.11, 169.23, 169.44, 169.51, 169.60, 170.2, 170.32, 170.36, 170.41, 170.64. High-resolution mass spectrum (FAB^+) calcd for $C_{28}H_{36}NO_{18}Cl_3Na$ $(MNa)^+$: 802.0896. Found: 802.0892. The trichloracetimidate (64.3 mg, 0.082 mmol) and **14** (100 mg, 0.137 mmol) were weighed directly into a flame-dried flask, then azeotroped 4× with dry, distilled benzene to ensure dryness. Flame-dried AW-300 molecular sieves (1 g, Aldrich) were introduced. The residue was dissolved in 3.4 mL of freshly distilled 1,2-dichloroethane, then placed in a methanol bath at ∼10 °C under an argon atmosphere. BF_3 etherate (21 μL, 0.164 mmol, Aldrich) was introduced once the solution had chilled to −10 °C. This temperature was maintained for 1.75 h, at which time the temperature was raised to 0 °C. After an additional 24 h, the temperature was increased again to 10 °C. At this time, an additional 0.5 equiv of BF_3 etherate was added. After a total reaction time of 48 h, the reaction was diluted with $CHCl_3$, filtered through Celite, then washed 1× with a 5% solution of $NaHCO_3$, and 1× with H_2O. The organic layer was dried over anhydrous Na_2SO_4, then filtered and evaporated. Purification was achieved by silica gel chromatography in 12:1 $CHCl_3$/MeOH. Further purification was necessary, and was accomplished by normal-phase HPLC on a semi-preparative silica gel column (Rainin-Varian Microsorb column, 5 μm particle size, 100 Å pore size, 10 250 mm) in 12:1 $CHCl_3$/MeOH. A yellowish amorphous solid (7.3 mg, 6%) resulted. ^{1}H NMR (500 MHz, CDCl=) δ 0.86 (t, 3H, J=6.7 Hz, CH_3), 1.23 (s, 20H, $(CH_2)_{10}$), 1.32 (m, 2H), 1.44 (m, 2H), 1.53–1.68 (m, 4H, $(CH_2)_2$ biotin), 1.99–2.11 (m, 25H, $(COCH_3)_7$, CH_2CONH, CH_2C=C), 2.20 (t, 2H, J=7.2 Hz, CH_2CONH), 2.46 (m, 2H), 2.72 (d, 1H, J=12.6 Hz, CHS), 2.91 (dd, 1H, J=5.0, 12.8 Hz, CHS), 3.08 (s, 1H), 3.15 (app q, 1H, J=7.5, 12.0 Hz, CHS), 3.40 (m, 2H), 3.53 (t, 2H, J=5.0 Hz, CH_2–O), 3.61 (m, 10H, (CH_2–O)5), 3.66 (m, 2H, CH_2–O), 3.75 (m, 3H), 3.96 (dd, 1H, J=5.1, 10.4 Hz), 4.03 (m, 2H), 4.09 (m, 1H), 4.17 (app t, 1H, J=5.0 Hz), 4.31 (m, 1H), 4.35 (dd, 1H, J=4.3, 12.4 Hz), 4.45 (d, 1H, J=7.9 Hz), 4.50 (m, 2H), 4.83–4.88 (m, 2H), 5.04 (t, 1H, J=9.8 Hz), 5.13 (m, 2H), 5.43 (dd, 1H, J=6.3, 15.4 Hz, C=C–H), 5.63 (s, 1H, NH), 5.70 (m, 1H, C=C–H), 6.61 (br s, 1H, NH), 6.71 (dd, 1H, J=7.8 Hz, NH); ^{13}C NMR δ 14.11, 20.54, 20.58, 20.66, 20.73, 20.85, 22.68, 25.43, 28.03, 29.28, 29.32, 29.35, 29.54, 29.66, 29.70, 31.91, 32.40, 35.72, 37.02, 39.14, 40.51, 53.36, 55.29, 60.05, 61.56, 61.77, 67.26, 67.78, 68.50, 69.90, 70.04, 70.27, 70.39, 70.43, 70.48, 71.51, 71.59, 71.93, 72.30, 72.66, 72.81, 72.90, 75.92, 76.38, 100.60, 100.73, 128.37, 133.86, 163.08, 169.06, 169.30, 169.73, 169.87, 170.24, 170.45, 170.50, 171.62, 173.07. High-resolution mass spectrum (FAB^+) calcd for $C_{63}H_{103}N_4SO_{25}$ $(MH)^+$: 1347.6632. Found: 1347.6619. The glycosylated product (5.0 mg, 0.0037 mmol) was dissolved in MeOH. A catalytic amount of

NaOMe (Avocado) was added, and the reaction was stirred at room temperature overnight. The reaction was diluted further with MeOH, and Dowex H+ was stirred in, until the solution achieved a neutral pH. The MeOH was removed in vacuo, then the residue was taken up into a small quantity of H_2O and freeze-dried. An off-white fluffy solid (3.6 mg, 90%) resulted. No further purification was necessary. ^{1}H NMR (500 MHz, 4:1 $CDCl_3/CD_3OD$, externally referenced to tetramethylsilane in $CDCl_3$) δ 0.76 (t, 3H, $J=6.5$ Hz, CH_3), 1.14 (s, 22H, $(CH_2)_{11}$), 1.32 (t, 2H, $J=7.8$ Hz), 1.56 (m, 4H, $(CH_2)_2$ biotin), 1.92 (m, 2H, $CH_2C{=}C$), 2.11 (t, 2H, $J=7.1$ Hz, CH_2CONH), 2.38 (t, 2H, $J=6.3$ Hz, CH_2CONH), 2.62 (d, 1H, $J=12.8$ Hz, CHS), 2.80 (dd, 1H, $J=4.9$, 12.9 Hz, CHS), 3.05 (m, 1H, CHS), 3.20 (m, 2H), 3.28 (m, 4H, incl. CD_3OD signal), 3.43–3.57 (m, 12H), 3.61 (m, 4H), 3.91 (br s, 1H), 4.01 (app q, 1H), 4.17 (d, 1H, $J=7.8$ Hz, H-1 or H-1), 4.20 (app dd, 1H, $J=4.2$, 6.7 Hz, CHNH biotin), 4.31 (d, 1H, $J=7.8$ Hz, H-1 or H-1), 4.39 (app t, 1H, $J=7.3$ Hz, CHNH biotin), 5.34 (dd, 1H, $J=6.9$, 15.7 Hz, C=C–H), 5.59 (app dd, 1H, $J=8.5$, 15.5 Hz, C=C–H). It appears as though some peaks are obscured by the HOD signal at 3.75; ^{13}C NMR δ 13.86, 22.52, 25.35, 27.97, 28.22, 29.15, 29.18, 29.20, 29.40, 29.51, 29.54, 31.77, 32.25, 35.50, 36.35, 38.96, 40.16, 53.27, 55.48, 60.04, 60.08, 60.97, 61.84, 67.13, 69.54, 69.71, 69.75, 69.83, 70.00, 70.11, 72.02, 72.92, 73.13, 73.38, 74.53, 74.85, 76.16, 76.38, 78.91, 102.83, 103.00, 128.76, 134.04, 164.15, 172.14, 174.42. High-resolution mass spectrum (FAB^+) calcd for $C_{49}H_{89}N_4SO_{18}$ $(MH)^+$: 1053.5893. Found: 1053.5884.

β-D-Galactopyranosyl-α-(1→6)-*O*-(β-D-glucopyranosyl)-(1→1)-(2*S*,3*R*,4*E*)-3-hydroxy-2-*N*-(12-D-biotinamide-4,7,10-trioxadodecyl)-sphingenine (11). α-Melibiose (Aldrich, 500 mg, 1.46 mmol) and 14.3 mg (0.117 mmol) of DMAP (Novabiochem) were both weighed directly into the reaction flask. 3 mL of pyridine was added to dissolve the solids, and the flask was then placed in an ice bath at 0 °C under an argon atmosphere. Acetic anhydride (2.8 mL, 29.2 mmol, Aldrich) was added dropwise via an addition funnel over a period of 15 min. After the addition of acetic anhydride was complete, the reaction was stirred from 0 °C to room temperature overnight. Once TLC in 2:1 ethyl acetate/hexanes indicated a complete reaction, the flask was placed on ice and 3 mL of methanol was added. The solution was stirred at 0 °C for 0.5 h, then the solution was evaporated. The residue was taken up in ethyl acetate and washed 3×0.1 M HCl, then 1×with water, and 1×with brine. The organic phase was dried over anhydrous Na_2SO_4. A white foam solid product, the peracetylated disaccharide (934 mg, 94%), resulted in an approximate 5:1 α/β-ratio. This was used without further purification. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.87–2.08 (m, 24H, $(COCH_3)_8$), 3.50 (dt, 1H, $J=2.4$, 11.5 Hz), 3.61 (dd, 1H, $J=4.5$, 11.6 Hz), 3.96 (m, 2H), 4.09 (app q, 1H, $J=6.6$ Hz), 4.88–5.07 (m, 5H), 5.20 (dd, 1H, $J=3.3$, 10.8 Hz), 5.36 (m, 2H), 6.16 (d, 1H, $J=3.6$ Hz); ^{13}C NMR δ 20.41, 20.60, 20.62, 20.70, 20.82, 20.99, 61.60, 61.71, 65.60, 65.77, 66.32, 66.44, 67.38, 68.06, 68.24, 68.47, 69.22, 69.84, 70.18, 70.44, 72.80, 73.41, 76.72, 77.23, 77.74, 88.90, 91.56, 96.02, 96.33, 168.84, 169.25, 169.32, 169.67, 169.83, 170.08, 170.17, 170.20, 170.47, 170.55. High-resolution mass spectrum (FAB^+) calcd for $C_{28}H_{39}O_{19}$ $(MH)^+$: 679.2086. Found: 679.2092. A 7:3 mixture of THF:MeOH (30 mL) was stirred at 0 °C with NH_3 (g) bubbled into the solution for a period of 10 min. This solution was added to a flask containing peracetylated melibiose. After 1 h at 0 °C, the solution was stirred from 0 °C to room temperature. After 2 h, TLC in 2:1 ethyl acetate/hexanes indicated a complete conversion to the 1-hydroxy-hepta-*O*-acetyl disaccharide. After evaporation, the crude oil was purified by silica gel chromatography in 2:1 ethyl acetate/hexanes. An off-white foam product (391 mg, 69%) resulted. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.87–2.10 (m, 21H, $(COCH_3)_7$), 3.47–3.64 (m, 2H), 3.91–4.02 (m, 2H), 4.14 (m, 2H), 4.59 (app br s, 1H), 4.70 (dd, 1H, $J=3.3$, 10.2 Hz), 4.85–4.98 (m, 2H), 5.06 (d, 1H, $J=3.7$ Hz), 5.19 (app dt, 1H, $J=3.3$, 10.8 Hz), 5.30 (br d, 1H, $J=3.0$ Hz), 5.41 (t, 1H, $J=9.5$ Hz); ^{13}C NMR δ 20.62, 20.68, 20.77. 20.97, 61.69, 65.99, 66.91, 67.46, 67.91, 67.95, 68.22, 69.0, 69.17, 69.95, 71.20, 72.63, 72.82, 76.73, 77.23, 77.43, 77.75, 89.80, 95.04, 96.25, 169.53, 169.69, 170.03, 170.21, 170.31, 170.77, 170.87, 171.10. High-resolution mass spectrum (FAB^+) calcd for $C_{26}H_{37}O_{18}$ $(MH)^+$: 637.1980. Found: 637.1982. The 1-hydroxy-hepta-*O*-acetyl derivative of melibiose (378 mg, 0.594 mmol) was dissolved in CH_2Cl_2 under argon. CCl_3CN (209 μL, 2.08 mmol, Aldrich) was introduced via a syringe. This was followed by the addition of NaH (17.1 mg, 0.713 mmol, Aldrich). The solution immediately bubbled upon the addition of the NaH, and turned from a clear colorless solution, to a clear yellow one. The reaction was stirred at room temperature for 5 h, as TLC in 2:1 ethyl acetate/hexanes had indicated a nearly complete reaction. After evaporation of the solvent, the crude residue was purified by silica gel chromatography in 1:1 ethyl acetate/hexanes. A clear yellow oil (129 mg, 28%) resulted. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.90–2.05 (m, 21H, $(COCH_3)_7$), 3.49 (dd, 1H, $J=1.9$, 11.0 Hz), 3.66 (dd, 1H, $J=5.9$, 11.1 Hz), 3.99 (d, 2H, $J=6.6$ Hz), 4.12 (m, 1H), 4.26 (t, 1H, $J=6.5$ Hz), 4.96–5.09 (m, 4H), 5.18 (dd, 1H, $J=3.3$, 10.1 Hz), 5.37 (d, 1H, $J=3.2$ Hz), 5.49 (t, 1H, $J=9.7$ Hz), 6.38 (d, 1H, $J=3.7$ Hz, H-1), 8.83 (s, 1H, NH); ^{13}C NMR δ 20.54, 20.71, 20.75, 20.82, 20.89, 61.80, 65.45, 66.38, 67.58, 68.03, 68.25, 68.50, 69.86, 70.01, 70.69, 76.71, 77.22, 77.42, 77.73, 92.64, 95.85, 160.51, 169.57, 169.95, 170.05, 170.30, 170.42, 179.54. High-resolution mass spectrum (FAB^+) calcd for $C_{28}H_{36}NO_{18}Cl_3Cs$ $(MCs)^+$: 912.0052. Found: 912.0070. The trichloroacetimidate (129 mg, 0.165 mmol) and **14** (124 mg, 0.170 mmol) were weighed directly into a flame-dried flask. The mixture of compounds was azeotroped 3× with dry, distilled benzene to ensure dryness. AW-300 molecular sieves (Aldrich, 1 g) were added next. The flask was placed under an argon atmosphere, then the dry solids were dissolved in 5 mL of 1,2-dichloroethane. The flask was placed in a methanol bath at 10 °C and allowed to equilibrate at this temperature before 42 μL (0.330 mmol) of BF_3 etherate (Aldrich) was added via a syringe. After 1 h, the temperature of the bath was raised to 0 °C. After an additional 16 h at 0 °C, the bath temperature was raised to 10 °C, and allowed to react at this

temperature for 10 h, before diluting the reaction mixture with $CHCl_3$ and filtering through Celite. The solution was washed first with $1\times5\%$ $NaHCO_3$, followed by $1\times H_2O$. The organic layer was subsequently dried over anhydrous Na_2SO_4, then filtered and evaporated. The crude residue was first purified by silica gel chromatography in 9:1 $CHCl_3$/MeOH, then further purified by normal phase silica gel HPLC (Rainin-Varian Microsorb column, 5 μm particle size, 100 Å pore size, 10 250 mm) in 12:1 $CHCl_3$/MeOH. 28 mg (13%) of a yellow amorphous solid resulted. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.85 (t, 3H, $J=6.8$ Hz, CH_3), 1.22 (s, 20H, $(CH_2)_{10}$), 1.33 (m, 2H, CH_2), 1.43 (m, 2H, CH_2 biotin), 1.60–1.74 (m, 4H, $(CH_2)_2$ biotin), 1.96–2.05 (m, 19H, $(COCH_3)_5$, CH_2CONH, $CH_2C{=}C$), 2.12 (m, 6H, $(COCH_3)_2$), 2.20 (t, 2H, $J=7.6$ Hz, CH_2CONH), 2.43–2.54 (m, 1H), 2.72 (d, 1H, $J=12.9$ Hz, CHS), 2.88 (dd, 1H, $J=4.6$, 12.9 Hz, CHS), 3.12 (app q, 1H, $J=6.9$, 12.1 Hz, CHS), 3.40 (app t, 2H, $J=4.6$ Hz), 3.53 (t, 2H, $J=4.6$ Hz, CH_2–O), 3.60 (s, 10H, (CH_2–O)5), 3.65–3.77 (m, 2H, CH_2–O), 3.97 (m, 1H), 4.03–4.11 (m, 2H), 4.16 (app t, 1H, $J=5.3$ Hz), 4.21 (t, 1H, $J=6.1$ Hz), 4.29 (app t, 1H, $J=4.6$ Hz), 4.47 (m, 2H), 4.87 (m, 1H), 4.91–5.06 (m, 1H), 5.18 (m, 2H), 5.27–5.40 (m, 2H), 5.44 (m, 2H), 5.70 (dt, 1H, $J=6.9$, 15.3 Hz, C=C–H), 6.18 (d, 2H, $J=13.0$ Hz, $(NH)_2$), 6.80 (m, 2H, $(NH)_2$); ^{13}C NMR δ 14.09, 20.61, 20.64, 20.67, 20.72, 20.88, 20.93, 22.65, 25.50, 28.05, 28.10, 29.21, 29.27, 29.32, 29.50, 29.53, 29.63, 29.67, 31.88, 32.37, 35.80, 36.91, 39.12, 40.47, 53.34, 53.64, 55.43, 60.11, 60.94, 61.76, 61.89, 65.36, 66.47, 67.27, 67.38, 67.42, 68.08, 68.25, 68.46, 68.72, 69.91,70.04, 70.17, 70.25, 70.37, 71.03, 71.23, 72.46, 72.70, 72.80, 76.64, 96.42, 100.56, 125.66, 128.67, 133.80, 137.86, 163.53, 169.28, 169.55, 169.94, 170.15, 170.51, 170.95, 171.14, 171.52, 173.20. High-resolution mass spectrum (FAB^+) calcd for $C_{63}H_{103}N_4SO_{25}$ $(MH)^+$: 1347.6632. Found: 1347.6581. The glycosylated product (19.4 mg, 0.014 mmol) was dissolved in MeOH. A catalytic amount of NaOMe (Avocado) was added, and the reaction was stirred at room temperature overnight. The reaction was diluted further with MeOH, and Dowex H^+ was stirred in, until the solution achieved a neutral pH. The MeOH was removed in vacuo, then the residue was taken up into a small quantity of H_2O and freeze-dried. 13.5 mg (89%) of an off-white fluffy solid resulted. No further purification was necessitated. ^{1}H NMR (500 MHz, 4:1 $CDCl_3/CD_3OD$, externally referenced to tetramethylsilane in $CDCl_3$) δ 0.74 (t, 3H, $J=6.6$ Hz, CH_3), 1.12 (s, 20H, $(CH_2)_{10}$), 1.21 (m, 2H, CH_2), 1.30 (app t, 3H, $J=7.5$ Hz, CH_2), 1.52 (m, 4H, $(CH_2)_2$), 1.89 (m, 2H, $CH_2C{=}C$), 2.09 (t, 2H, $J=7.1$ Hz, CH_2CONH), 2.34 (t, 2H, $J=6.0$ Hz, CH_2CONH), 2.60 (d, 1H, $J=12.9$ Hz, CHS), 2.78 (dd, 1H, $J=4.9$ Hz, 12.8 Hz, CHS), 3.03 (app q, 1H, $J=7.4$, 12.0 Hz, CHS), 3.15 (t, 1H, $J=8.3$ Hz), 3.22–3.36 (m, 6H, incl. CD_3OD peak), 3.41 (m, 2H, CH_2–O), 3.50 (app s, 10H, (CH_2–O)5), 3.56–3.67 (m, 8H), 3.73 (app d, 2H, $J=5.8$ Hz), 3.84 (br s, 2H, incl. the HOD peak), 3.97 (m, 2H, incl. the HOD peak), 4.17 (m, 2H, C*H*NH biotin, H-1), 4.37 (app t, 1H, $J=6.8$ Hz, C*H*NH biotin), 4.76 (s, 1H, H-1), 5.28 (app dd, 1H, $J=6.8$, 15.3, C=C–H), 5.60 (app dt, 1H, $J=6.8$, 15.3 Hz, C=C–H); ^{13}C NMR δ 13.82, 22.49, 25.34, 27.94, 28.22, 29.15, 29.18, 29.35, 29.39, 29.52, 31.75, 32.23, 35.50, 36.48, 39.00, 40.16, 53.32, 55.48, 60.06, 61.33, 61.40, 61.79, 63.24, 67.03, 69.07, 69.29, 69.36, 69.44, 69.54, 69.76, 69.84, 70.07, 70.17, 70.26, 70.55, 71.85, 73.27, 73.40, 74.54, 76.25, 78.19, 98.38, 103.55, 125.66, 128.83, 133.93, 137.26, 163.14, 164.21, 171.90, 171.96, 174.29. High-resolution mass spectrum (FAB^+) calcd for $C_{49}H_{89}N_4SO_{18}$ $(MH)^+$: 1053.5893. Found: 1053.5911.

D-Biotin-11-amino-3,6,9-trioxaundecylate (19). Compound **17** (Huntsman) was weighed (1.0 g, 5.21mmol) directly into a flask. This was dissolved in 100 mL of THF. 64 mg of DMAP (0.52 mmol) was then added. *t*-Butoxycarbonyl anyhdride (1.137g, 5.21 mmol, Novabiochem) was added via an addition funnel as a solution in 25 mL of THF over a period of 1 h. The reaction solution became increasingly cloudy as the addition of the anhydride proceeded. After the addition was complete, the solution again became clear. After 2 h at room temperature, the reaction mixture was evaporated. Purification was achieved by silica gel chromatography in 12:1 $CHCl_3$/MeOH. 687 mg (45%) of the monoprotected product resulted. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.22 (s, 9H, $(CH_3)_3$), 2.64 (br s, 2H, CH_2NH_2), 3.07 (br q, 2H, $J=5.2$ Hz, CH_2NHCO), 3.30 (q, 4H, $J=4.9$, 10.0 Hz, $(CH_2$–$O)_2$), 3.43 (m, 8H, (CH_2–O)8), 5.42 (br s, 1H, NH); ^{13}C NMR δ 28.21, 40.11, 41.50, 70.00, 70.30, 73.21, 78.61, 149.47, 155.85. High-resolution mass spectrum (FAB^+) calcd for $C_{13}H_{29}N_2O_5$ $(MH)^+$: 293.2076. Found: 293.2072.

Biotinylation. D-Biotin (237 mg, 0.97 mmol, Sigma) was weighed directly into the reaction flask. This was dissolved in 10 mL of DMF. 406 μL of TEA (2.91 mmol) was added next. The reaction flask was placed under an argon atmosphere in an ice bath. After the solution had clarified, 2,3,4,5,6-pentafluorophenyl trifluoroacetate (PFP-TFA, Aldrich) was added via syringe (201 μL, 1.17 mmol). The reaction was then stirred from 0 °C to room temperature under argon. After 4 h, TLC in 9:1 $CHCl_3$/MeOH indicated that all of the biotin had been converted to the intermediate pentafluorophenyl ester. The monoprotected linker was dissolved in 2 mL of DMF and added to the flask containing PFP-biotin via a syringe. An additional 3 mL of DMF was used to rinse the flask, and this was added to the reaction flask as well. TLC indicated that the reaction went to completion almost instantaneously. The reaction was stirred overnight at room temperature under argon to ensure a complete reaction. The reaction mixture was then evaporated and subjected to silica gel chromatography in 9:1 $CHCl_3$/MeOH. 503 mg (100%) of a yellowish amorphous solid (**18**) resulted. ^{1}H NMR (250 MHz, $CDCl_3$) δ 1.27 (br s, 11H, $(CH_3)_3$, CH_2 biotin), 1.51 (m, 4H, $(CH_2)_2$ biotin), 2.06 (t, 2H, $J=7.4$ Hz, CH_2CONH), 2.58 (d, 1H, $J=12.7$ Hz, CHS), 2.73 (dd, 1H, $J=5.0$, 13.2 Hz, CHS), 2.98 (br q, 1H, $J=4.5$ Hz, CHS), 3.14 (app q, 2H, $J=5.0$ Hz, CH_2NHCO), 3.26 (br q, 2H, $J=4.7$, 9.5 Hz, CHS), 3.35–3.47 (m, 12H, $(CH_2$–$O)_6$), 4.14 (m, 1H, C*H*NH biotin), 4.33 (m, 1H, C*H*NH biotin), 5.15 (br t, 1H, NH carbamate), 6.22 (s, 1H, NH biotin), 6.82 (s, 1H, NH biotin), 6.92 (br t, 1H, CH_2NHCO); ^{13}C NMR δ 25.53, 28.01, 28.30, 35.81, 38.95, 40.15, 40.36, 55.70, 60.13, 61.65, 69.79, 69.88,

69.98, 70.18, 77.43, 78.95, 155.92, 164.37, 173.38. High-resolution mass spectrum (FAB^+) calcd for $C_{23}H_{43}N_4SO_7$ $(MH)^+$: 519.2852. Found: 519.2844.

Deprotection. Reagent K[25] was prepared separately in a glass-stoppered flask. This solution was pipetted into the reaction flask containing the biotinylated protected linker (503 mg, 0.970 mmol), and was stirred at room temperature for 1.5 h before evaporating. The crude yellow oil was purified by silica gel chromatography with a gradient of $CHCl_3$/MeOH/TEA starting at 4:1:0.1 and ending with 100% MeOH. 213 mg (52%) of a yellowish amorphous solid resulted. ^1H NMR (250 MHz, 4:1 $CDCl_3/CD_3OD$, external $CHCl_3$ reference) δ 1.17 (app q, 2H, $J=7.0$, 14.1 Hz, CH_2 biotin), 1.43 (m, 4H, $(CH_2)_2$ biotin), 1.95 (t, 2H, $J=7.4$ Hz, CH_2CONH), 2.47 (d, 1H, $J=12.8$ Hz, CHS), 2.57 (t, 2H, $J=5.1$ Hz, CH_2NH$_2$), 2.64 (dd, 1H, $J=4.9$, 12.9 Hz, CHS), 2.89 (app q, 1H, $J=7.7$, 12.1 Hz, CHS), 3.15 (app t, 2H, CH_2NHCO), 3.27 (q, 4H, $J=6.4$, 11.5 Hz, $(CH_2–O)_2$), 3.38 (s, 8H, $(CH_2–O)_4$), 4.04 (app dd, 1H, $J=4.5$, 7.7 Hz, C*H*NH biotin), 4.24 (app dd, 1H, $J=5.0$, 7.7 Hz, C*H*NH biotin), 6.02 (br s, 1H, NH), 6.28 (br s, 1H, NH), 7.37 (br t, 1H, CH2N*H*CO); ^{13}C NMR δ 25.57, 28.14, 28.45, 35.71, 39.04, 39.16, 40.36, 41.11, 55.72, 60.16, 61.85, 69.76, 69.95, 70.00, 70.34, 72.63, 164.41, 174.15, 174.23. High-resolution mass spectrum (FAB^+) calcd for $C_{18}H_{35}N_4SO_5$ $(MH)^+$: 419.2328. Found: 419.2329.

General procedure for the reductive amination of disaccharides

The reductively aminated disaccharides were obtained using the procedure of Fantini and coworkers. 1.61 equiv of compound **19** was weighed directly into a flask and dissolved in a minimal amount of methanol. Into a separate flask, 1.0 equiv of one of the disaccharides (lactose, cellobiose, or melibiose) was weighed, then dissolved in a minimal amount of deionized water. The disaccharide solution was added to the flask containing compound **19**, and this was stirred at room temperature overnight. The reaction mixture was then heated for 6 h at 55 °C. Next, 1.1 equiv of $NaBH_4$ was added to the reaction as a solution in MeOH. The reaction was then stirred at room temperature overnight before evaporating the solvent. A crude purification was achieved for compounds **20**, **21**, and **22** on a Biogel P-2 (Biorad) 1.5×50 cm column with detection at 222 nm. Subsequent purification of compounds **21** and **22** was achieved by gradient reversed-phase HPLC from 0.1% trifluoroacetic acid (TFA) in water to 100% acetonitrile, utilizing a Vydac C-18 semipreparative column with a Perkin-Elmer Binary LC Pump and an LC 90 UV Spectrophotometric Detector. All products were fluffy white solids after lyophilization from water.

11-D-Biotinamide-*N*-[3,6,9-trioxaundecane]-1-amino-1-deoxylactitol (20). 13.2 mg resulted. Yield: 33%. ^1H NMR (500 MHz, D_2O) δ 1.45 (m, 2H, CH_2 biotin), 1.59–1.80 (m, 4H, $(CH_2)_2$ biotin), 2.32 (t, 2H, $J=7.2$ Hz, CH_2CONH), 2.83 (d, 1H, $J=13.1$ Hz, CHS), 3.04 (dd, 1H, $J=5.0$, 13.1 Hz, CHS), 3.24 (t, 1H, $J=12.1$ Hz), 3.80 (m, 3H), 3.44 (m, 3H), 3.59 (app dq, 1H, $J=1.6$, 7.8 Hz), 3.67 (app dt, 2H, $J=1.3$, 5.4 Hz), 3.71 (ddd, 1H, $J=1.7$, 3.4, 10.0 Hz), 3.73–3.84 (m, 12H, $(CH_2–O)_6$), 3.87 (m, 3H), 3.92 (m, 2H), 3.98 (m, 2H), 4.27 (m, 1H), 4.46 (m, 1H, C*H*NH biotin), 4.57 (d, 1H, $J=7.8$ Hz, H-1′), 4.65 (app dt, 1H, $J=0.9$, 5.0 Hz, C*H*NH biotin); ^{13}C NMR (externally referenced to 3-(trimethylsilyl)propionic 2,2,3,3-*d*4 acid, sodium salt) δ 28.02, 30.58, 30.74, 38.35, 41.73, 42.57, 50.05, 52.46, 58.26, 63.16, 64.13, 64.80, 64.98, 68.16, 70.57, 71.59, 71.78, 72.23, 72.49, 72.54, 73.36, 73.77, 73.85, 75.31, 78.22, 81.44, 105.73, 168.24, 179.87. High-resolution mass spectrum (FAB^+) calcd for $C_{30}H_{57}N_4SO_{15}$ $(MH)^+$: 745.3541. Found: 745.3532.

11-D-Biotinamide-*N*-[3,6,9-trioxaundecane]-1-amino-1-deoxycellitol (21). 5.6 mg resulted. Yield: 19%. ^1H NMR (500 MHz, D_2O) δ 1.45 (m, 2H, CH_2 biotin), 1.60–1.76 (m, 4H, $(CH_2)_2$ biotin), 2.30 (t, 2H, $J=7.2$ Hz, CH_2CONH), 2.81 (d, 1H, $J=13.1$ Hz, CHS), 3.02 (dd, 1H, $J=5.0$, 13.1 Hz, CHS), 3.24 (dd, 1H, $J=10.2$, 12.6 Hz), 3.34–3.44 (m, 8H), 3.49 (dd, 1H, $J=2.1$, 5.6 Hz), 3.53 (app t, 1H, $J=9.3$ Hz), 3.65 (t, 2H, $J=5.4$ Hz), 3.72–3.80 (m, 12H, $(CH_2–O)_6$), 3.84 (app t, 2H, CH_2–O), 3.88 (d, 1H, $J=3.2$ Hz), 3.91 (dd, 1H, $J=2.2$, 7.7 Hz), 3.95 (m, 1H), 4.23 (dt, 1H, $J=3.7$, 6.4 Hz), 4.45 (dd, 1H, $J=4.5$, 8.2 Hz, C*H*NH biotin), 4.62 (m, 2H, C*H*NH biotin, H-1); ^{13}C NMR (externally referenced to 3-(trimethylsilyl)propionic 2,2,3,3-*d*4 acid, sodium salt) δ 27.99, 30.55, 30.71, 38.32, 41.70, 42.55, 50.00, 52.42, 58.23, 63.13, 63.42, 64.73, 64.96, 68.12, 70.52, 71.75, 72.20, 72.34, 72.45, 72.50, 72.51, 73.26, 73.73, 76.11, 78.31, 78.82, 81.51, 105.16, 168.23, 179.86. High-resolution mass spectrum (FAB^+) calcd for $C_{30}H_{57}N_4SO_{15}$ $(MH)^+$: 745.3541. Found: 745.3531.

11-D-Biotinamide-*N*-[3,6,9-trioxaundecane]-1-amino-1-deoxymelitol (22). 11.1 mg resulted. Yield: 37%. ^1H NMR (500 MHz, D_2O) δ 1.47 (m, 2H, $(CH_2$ biotin), 1.61–1.80 (m, 4H, $(CH_2)_2$ biotin), 2.32 (t, 2H, $J=7.3$ Hz, CH_2CONH), 2.84 (d, 1H, $J=13.0$ Hz, CHS), 3.04 (dd, 1H, $J=5.0$, 13.1 Hz, CHS), 3.17–3.41 (m, 5H), 3.44 (t, 2H, $J=5.5$ Hz), 3.77 (t, 2H, $J=4.9$ Hz), 3.79–4.02 (m, 20H), 4.04 (d, 2H, $J=2.8$ Hz), 4.48 (dd, 1H, $J=4.5$, 7.9 Hz, C*H*NH biotin), 4.66 (dd, 1H, $J=4.7$, 7.9 Hz, C*H*NH biotin), 5.03 (d, 1H, $J=3.6$ Hz, H-1); ^{13}C NMR (internally referenced to CH_3CN) 24.67, 27.23, 27.40, 35.01, 38.40, 39.23, 54.90, 59.80, 60.69, 61.63, 61.98, 68.04, 68.20, 68.43, 68.79, 68.90, 69.07, 69.12, 69.18, 70.34, 70.41, 70.47, 72.46, 97.93, 164.88, 176.50. High-resolution mass spectrum (FAB^+) calcd for $C_{30}H_{57}N_4SO_{15}$ $(MH)^+$: 745.3541. Found: 745.3569.

General procedure for the amidation of reductively aminated disaccharides

Palmitic acid (1.2 equiv, Matreya, Inc.) was weighed into a flame-dried flask, then dissolved in approximately 0.5–1.0 mL of CH_2Cl_2. The flask was placed under argon. TEA was added (3.0 equiv), followed by PFP-TFA (1.4 equiv, Aldrich). The reaction was allowed to stir at room temperature. After 0.5 h, TLC in 9:1 $CHCl_3$/MeOH indicated complete conversion to the

PFP intermediate. Next, approximately 1.5 mL of DMF was added and compounds **20**, **21**, or **22** were introduced into the flask. The reaction was stirred overnight at room temperature, after which TLC indicated the disappearance of the UV-active intermediate. The solvent was evaporated, and the crude residues were purified by gradient reversed-phase HPLC from 0.1% trifluoroacetic acid (TFA) in water to 100% acetonitrile, utilizing a Vydac C-18 semipreparative column with a Perkin-Elmer Binary LC Pump and an LC 90 UV Spectrophotometric Detector. Off-white grainy solid products **23**, **24**, and **25** resulted after lyophilization from water.

11-D-Biotinamide-*N*-[3,6,9-trioxaundecane]-*N*-palmitoyl-1-amino-1-deoxylactitol (23). Yield: 2.7 mg (30%). ^{1}H NMR (500 MHz, 4:1 $CDCl_3/CD_3OD$, externally referenced to 0.01% tetramethylsilane in $CDCl_3$) δ 0.90 (t, 3H, $J=6.8$ Hz, CH_3), 1.29 (br s, 26 H, $(CH_2)_{13}$), 1.45 (m, 2H, CH_2 biotin), 1.57–1.77 (m, 4H, $(CH_2)_2$ biotin), 2.23 (t, 2H, $J=7.2$ Hz, $CH_2C{=}O$), 2.48 (app q, 2H, $J=7.3$, 15.3 Hz, $CH_2C{=}O$), 2.71 (d, 1H, $J=12.8$ Hz, CHS), 2.93 (dd, 1H, $J=5.1$, 12.8 Hz, CHS), 3.21 (m, 1H, CHS), 3.37 (t, 2H, $J=5.4$ Hz), 3.45–3.57 (m, 8H), 3.62–3.70 (m, 10H), 3.72–3.79 (m, 5H), 3.82–3.93 (m, 3H), 4.07 (m, 1H), 4.19 (m, 1H), 4.31 (m, 1H, C*H*NH biotin), 4.48 (m, 2H, H-1, C*H*NH biotin); ^{13}C NMR δ 13.82, 22.45, 25.16, 25.31, 27.92, 29.13, 29.27, 29.31, 29.38, 29.41, 29.44, 29.48, 31.70, 33.11, 35.49, 38.95, 40.10, 48.13, 49.16, 55.41, 60.00, 61.08, 61.77, 62.25, 68.99, 69.52, 69.60, 69.80, 70.08, 70.25, 70.32, 70.35, 70.63, 71.39, 72.12, 73.24, 75.32, 103.92, 174.27, 175.28, 175.63. High-resolution mass spectrum (FAB^+) calcd for $C_{46}H_{87}N_4SO_{16}$ $(MH)^+$: 983.5838. Found: 983.5828.

11-D-Biotinamide-*N*-[3,6,9-trioxaundecane]-*N*-palmitoyl-1-amino-1-deoxycellitol (24). Yield: 2.6 mg (33%). ^{1}H NMR (500 MHz, 4:1 $CDCl_3/CD_3OD$, externally referenced to 0.01% tetramethylsilane in $CDCl_3$) δ 0.80 (app t, 3H, $J=6.8$ Hz, CH_3), 1.18 (m, 26H, $(CH_2)_{13}$), 1.36 (m, 2H, CH_2 biotin), 1.35–1.66 (m, 4H, $(CH_2)_2$ biotin), 2.15 (t, 2H, $J=7.2$ Hz, $CH_2C{=}O$), 2.34 (m, 2H, $CH_2C{=}O$), 2.66 (d, 1H, $J=12.9$ Hz, CHS), 2.85 (dd, 1H, $J=4.6$, 12.7 Hz, CHS), 3.10 (m, 1H, CHS), 3.21 (t, 2H, $J=8.0$ Hz), 3.33 (m, 10H, incl MeOD signal), 3.41–3.48 (m, 6H), 3.56 (app s, 10H, $(CH_2$–$O)5$), 3.61–3.68 (m, 4H), 3.74–3.86 (m, 4H), 3.96 (m, 1H), 4.24 (dd, 1H, $J=4.6$, 7.9 Hz, C*H*NH biotin), 4.42 (m, 2H, H-1, C*H*NH biotin); ^{13}C NMR δ 13.78, 22.46, 25.16, 25.32, 27.94, 28.24, 29.15, 29.31, 29.41, 29.49, 31.72, 33.12, 33.21, 35.51, 38.97, 40.11, 48.18, 49.21, 50.17, 55.42, 60.01, 61.25, 61.78, 62.33, 68.77, 68.86, 69.02, 69.54, 69.72, 69.80, 70.03, 70.11, 70.26, 70.38, 70.94, 71.63, 71.72, 73.63, 76.23, 76.46, 81.83, 103.36, 174.16, 175.28, 175.67. High-resolution mass spectrum (FAB^+) calcd for $C_{46}H_{87}N_4SO_{16}$ $(MH)^+$: 983.5838. Found: 983.5828.

11-D-Biotinamide-*N*-[3,6,9-trioxaundecane]-*N*-palmitoyl-1-amino-1-deoxymelitol (25). Yield: 1.4 mg (18%). ^{1}H NMR (500 MHz, 4:1 $CDCl_3/CD_3OD$, externally referenced to 0.01% tetramethylsilane in $CDCl_3$) δ 0.72 (t, 3H, $J=6.8$ Hz, CH_3), 1.10 (br s, 26H, $(CH_2)_{13}$), 1.28 (m, 2H, CH_2 biotin), 1.44–1.58 (m, 4H, $(CH_2)_2$ biotin), 2.07 (t, 2H, $J=6.8$ Hz, $CH_2C{=}O$), 2.26 (app q, 2H, $J=5.9$, 13.4 Hz, $CH_2C{=}O$), 2.58 (d, 1H, $J=12.8$ Hz, CHS), 2.76 (dd, 1H, $J=5.0$, 13.0 Hz, CHS), 3.02 (br dd, 1H, $J=6.3$, 8.4 Hz, CHS), 3.20 (m, 4H), 3.39 (t, 2H, $J=5.0$ Hz), 3.48 (br d, 16H), 3.59–3.67 (m, 6H), 3.73 (app t, 2H, incl HOD signal), 4.16 (dd, 1H, $J=4.4$, 7.6 Hz, C*H*NH biotin), 4.35 (dd, 1H, $J=5.2$, 7.7 Hz, C*H*NH biotin), 4.75 (app t, 1H, $J=3.5$ Hz, H-1); ^{13}C NMR δ 14.05, 22.72, 25.46, 25.59, 28.17, 28.46, 29.41, 29.57, 29.75, 31.98, 33.40, 35.73, 39.23, 40.37, 55.70, 60.27, 61.91, 62.04, 68.64, 69.28, 69.55, 69.83, 70.06, 70.48, 70.63, 72.19, 72.70, 98.92, 174.49, 175.52, 176.21. High-resolution mass spectrum (FAB^+) calcd for $C_{46}H_{87}N_4SO_{16}$ $(MH)^+$: 983.5838. Found: 983.5823.

Palmitoylated biotinamidojeffamine (26). Polymer bound EDC (P-EDC; 180 mg ~0.179 mmol) was swelled in 1 mL of $CHCl_3$.[24] A solution of palmitic acid (20.2 mg, 0.078 mmol) in $CHCl_3$ (0.5 mL) and biotinamido jeffamine **19** (30 mg, 0.07 mmol) in $CHCl_3$ (0.5 mL) were sequentially added to the P-EDC suspension. The reaction mixture was stirred gently and progress of the reaction monitored by TLC. After 14 h the reaction mixture was filtered and resin thoroughly washed with alternating washes of $CHCl_3$ and MeOH. Combined organic extracts were dried (Na_2SO_4), concentrated and the crude reaction product was purified by passing through a short plug of silica gel to yield **26** (73%). ^{13}C 173.36, 173.25, 164.01, 70.35, 70.30, 70.11, 70.03, 69.99, 69.91, 61.75, 60.17, 55.57, 40.46, 39.09, 36.66, 35.91, 31.87, 29.65, 29.61, 29.50, 29.39, 29.32, 28.17, 28.08, 25.74, 25.56, 22.64, 14.07. ESI-MS 657.3 (MH^+).

Adhesion assay experimental procedures

Assay ligand dilutions. Freeze-dried biotinylated analytes were weighed out to the tenth of a microgram using a Cahn 21 Automatic Electrobalance. Stock solutions of these compounds were prepared at 1 mg/mL concentrations in 20% DMSO/80% PBS buffer. DMSO was first added, and the compounds allowed to fully dissolve prior to addition of PBS buffer. The solutions were thoroughly mixed prior to further dilution in PBS for assaying, and they were stored at −20 °C between uses. The dilution concentration of all compounds utilized in the assays was 20 μg/mL.

Plating efficiency assay. The plating efficiency of each compound was tested to ensure comparable coverage on NeutrAvidin™-coated eight-well strip plates (Pierce Biochemicals). In this assay, each compound was plated into four wells at 150 μL each, and incubated for 4 h at 25 °C and 1000 rpm in a Jitterbug™ microplate incubator (Boekel). Two blank wells were incubated with 150 μL PBS, and 2–8 wells were incubated with 150 μL PBS each for HRP-biotin determination in the second incubation. After 4 h, each well was rinsed with 3×200 μL of PBS. Next, a 1:4000 dilution of HRP-Biotin was added to all wells excluding the blanks in 100 μL quantities. The blank wells were incubated with 100 μL of PBS. The second incubation was for 1 h at 25 °C and

1000 rpm. After the incubation, the wells were rinsed as before. A TMB (3,3′,5,5′-tetramethylbenzidine, Sigma) solution was prepared (1 mg TMB in 500 μL of DMSO and 9.5 mL of 0.05M phosphate–citrate buffer with sodium perborate). The TMB solution was added to each well, including the blanks, at a volume of 100 μL/well. The plate was immediately read on a BioRad model 550 microplate reader every min for 20 min at 630 nm, with a reference wavelength of 490 nm, and 5 s of shaking between each reading. All wells for each analyte were averaged, and had the TMB blank automatically subtracted through the use of the BioRad Microplate Manager software package. The standard deviations for each of the ligands was determined based upon the original data set. The four wells containing only HRP-biotin at the absorbance maximum served as a 100% measure of the sites filled by HRP-biotin. All other analyte wells were compared to the HRP-biotin wells at the same data point, and were based on these results. The percentage of NeutrAvidin™ sites filled was ascertained for each compound. The results given for this assay represent the mean of four separate experiments.

Adhesion assay. The compounds (see ELISA Ligand Dilutions) were plated in 150 μL volumes into four wells each of NeutrAvidin™-coated eight-well strip plates. The plate was incubated for 4 h at 25 °C and 1000 rpm on a Jitterbug™ microplate incubator (Boekel). An additional four wells each of both a TMB blank (see Plating Efficiency Assay), and a gp120 blank were incubated with 150 μL of PBS in this incubation. Following the first incubation, the wells were rinsed 3×200 μL PBS. Next, 150 μL/well, excluding the TMB and gp120 wells, of compound **16** was incubated for 1 h at 25 °C and 1000 rpm, to ensure that all remaining open NeutrAvidin™ sites were blocked. The TMB and gp120 wells were incubated instead with 150 μL of PBS, as before. After the second incubation, the wells were rinsed as in the first incubation. The third incubation comprised of adding 100 μL of a 1:4 dilution (from a 500 μL vial containing 0.333 μg gp120) of gp120-HRP (Intracel) into every well, except the TMB wells. The TMB wells were incubated with 100 μL of PBS each. The incubation was for 1 h at 25 °C at 1000 rpm. The wells were then rinsed with 5×200 μL of PBS. A solution of TMB reagent (1 mg tablet) was prepared in 500 μL of DMSO and 9.5 mL of phosphate–citrate buffer containing sodium perborate). Then 100 μL of the TMB reagent was added to each well. The plate absorbances were read every min for 30 min at a test wavelength of 630 nm, a reference wavelength of 490 nm, with a 5 s shake in between each reading, on a BioRad model 550 microplate reader. The absorbance readings were normalized to reflect the plating efficiencies by dividing the values by the plating efficiency. All wells for each analyte were averaged, and had the TMB blank automatically subtracted through the use of the BioRad Microplate Manager software package. The standard deviations were derived from the unaveraged data set. The wells containing only gp120 served as a measure of non-specific binding interactions between the glycoprotein and the plate, and were minimal. This assay was repeated two separate times, with the average of these experiments reported.

Competition assay. A solution of bGalCer (**7**) was prepared at 1 mg/mL concentration in 20% DMSO/80% PBS buffer. The solution was stored at −20 °C between uses. A 20 μg/mL solution of bGalCer was made from the stock solution using PBS buffer. NeutrAvidin™ coated polystyrene strip plates (Pierce Biochemicals) were used for the competitive assay. 150 μL of the 20 μg/mL solution of **7** was added to each well for plating with GalSph. The plating was achieved by shaking the plate at 25 °C at 1000 rpm for 4 h using a Jitterbug microplate incubator. Additionally, in each assay plate three wells were plated with blocking agent, biotinamide (**16**) (using 150 μL of the 20 μg/mL solution of biotinamide) served as the negative control. Also, in each assay plate three wells served as a TMB blank[9]. Following the first incubation, the plates were washed with 3×200 μL of PBS buffer. Next, 150 μL a of 20 μg/mL solution of blocking agent biotinamide (**16**) was added to each well (excluding TMB blank wells, which were incubated with 150 μL of PBS buffer) and the plate was incubated for 1 h at 25 °C/1000 rpm, to block off any open unbound NeutrAvidin™ sites. After the second incubation, the plates were rinsed with 3×200 μL of PBS buffer. Next 100 μL of a solution of HRP-rgp120 (obtained after a 1:3 dilution with PBS from a stock 500 μL vial containing 0.333 μg of HRP-rgp120) was added to all wells excluding the TMB blank wells (which were incubated with 100 μL of PBS buffer). The plate was incubated for 1 h at 25 °C/1000 rpm. The plate was then rinsed with 5×200 μL of PBS buffer and then the competition step was performed. 150 μL of the test ligand solution was added to each well and the plate was incubated for 4 h at 25 °C/1000 rpm. Each ligand to be assayed in the competitive mode was tested at six concentrations (1, 10, 20, 40, 80, and 100 μg/mL, the solutions of the test ligands were prepared from the corresponding stock solutions of 1 mg/mL in 20% DMSO/80% PBS buffer). In each assay plate, the test ligand solution at each concentration was added to three separate wells coated with **7**; in addition three bGalCer coated wells were exposed to PBS in place of test ligand during this final competition step. The amount of HRP-rgp120 bound in these plates served as a reference for calculating the decrease in HRP-rgp120 bound after exposure to test ligand solutions. After the 4 h incubation period, the plates were washed with 5×200 μL of PBS buffer. TMB reagent was prepared by dissolving a TMB tablet (1 mg tablet, Sigma) in 0.5 mL of DMSO and 9.5 mL of phosphate–citrate buffer. 100 μL of the TMB reagent was added to each well. The plate absorbances were read every min for 30 min at 630 nm (with 5 s shake before each reading) using a Bio-Rad model 550 microplate reader. Each well reading had the TMB blank reading automatically subtracted through the use of the Biorad Microplate manager software package. At the end of the 30 min reading period the enzymatic reaction in each well was immediately quenched by addition of 100 μL of 2 N H_2SO_4 solution in each well. The absorbance of the resulting yellow solutions were read at 430.

Calculation of EC_{50} values. The EC_{50} value represents the concentration of the compound to induce a 1/2 maximal displacement of rgp120 bound to plated bGalCer (**7**). The EC_{50} values in Table 1 were calculated by a non linear regression analysis using variable slope sigmoidal dose response curves (GraphPad Prizm, version 3.0 GraphPad Software Inc., San Diego, CA, USA). Compounds **14** and **16** do not display a sigmoidal dose response and their EC50 values were calculated by linear regression curve fitting (CA-Cricket Graph III, Computer Associates International Inc., Islandia, NY, USA). EC_{50} values reported in Table 2 were calculated from dose–response curves plotted using logarithmic curve fit program (CA-Cricket Graph III).

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