Bioconjugate Chemistry

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Derivatization of a Bioorthogonal Protected Trisaccharide Linker—Toward Multimodal Tools for Chemical Biology

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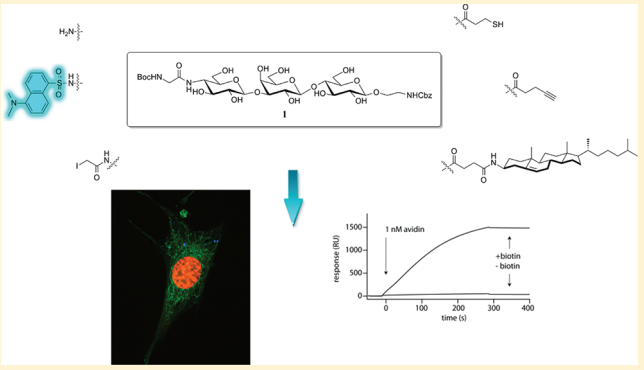
Supporting Information

ABSTRACT: When cross-linking biomolecules to surfaces or to other biomolecules, the use of appropriate spacer molecules is of great importance. Mimicking the naturally occurring spacer molecules will give further insight into their role and function, possibly unveil important issues regarding the importance of the specificity of carbohydrate-based anchor moieties, in e.g., glycoproteins and glycosylphosphatidylinositols. Herein, we present the synthesis of a lactoside-based trisaccharide, potentially suitable as a heterobifunctional bioorthogonal linker molecule whereon valuable chemical handles have been conjugated. An amino-derivative having thiol functionality shows promise as novel SPR-surfaces. Furthermore, the trisaccharide has been conjugated to a cholesterol moiety in combination with a fluorophore which successfully assemble on the cell surface in lipid microdomains, possibly lipid-rafts. Finally, a Cu^{I}-catalyzed azide−alkyne cycloaddition reaction (CuAAC) confirms the potential use of oligosaccharides as bioorthogonal linkers in chemical biology.



INTRODUCTION

To investigate biomolecules *in situ*, their functionality and possible applications, the choice of linker molecule is often focused on availability,[1,2] (i.e., commercially available or easily accessible via chemical synthesis) and bioorthogonality.[3] The understanding of the specific role of spacers, as well as the excavation of suitable bioavailable spacers have been an intensive area of research.[4−9] The use of oligo(ethylene) glycols (OEGs) as linker molecules have been shown to be fruitful in many fields.[10−13] However, OEGs have a rather high degree of variability in comparison to the naturally occurring oligosaccharides as glycosylphosphatidylinositols or lipopolysaccharides. Our interest is to investigate how a more rigid, rod-like[14] carbohydrate-based linker can be adequate as a cross-linking derivative. In spite of carbohydrates' abundance and biological significance, their use as biofunctional molecules in nanotechnology lags behind those of protein and nucleic acids,[15] mainly due to their complexity and challenging availability.[16] In surface plasmon resonace (SPR), however, a caboxymethylated polysaccharide (dextran) is often used to form a three-dimensional matrix on the sensor surface, enabled for conjugation.[17] The synthesized trisaccharide **1** (Figure 1)[18] has been utilized to generate a variety of cross-linking derivatives presenting reactive groups, e.g., (i) amino group for biotinylation,[19] (ii) thiol group for assembly upon gold surfaces,[20−22] (iii) alkyne residue for "click-chemistry" conjugation,[23−25] (iv) Dansyl for fluorescence microscopy, and (iv) *N*-cholesterol moiety as a lipid anchor.[26] Employing a orthogonally protected molecule enables conjugation and elongation in a great variety of sequences, independent of each other, thereby serving as an ideal framework for heterobifunctional cross-linking. In this work, we report such a heterobifunctional rod-like trisaccharide cross-linking molecule for applications in chemical biology. We foresee that future applications of these derivatives will entail, e.g., glyco-lipid−protein or glyco-lipid−fluorophore conjugates with variable linker lengths (tri-, penta-, and heptasaccharide moieties) to probe, e.g., for protein stability as a function of spacer length in relation to the cell membrane, biomembrane organization, and/or glyco-lipid distribution dependence.

RESULTS AND DISCUSSION

Synthesis. Starting from the known 3-β-Azido-5-cholestene **2**,[27] (Scheme 1) lipid-anchor derivative **5** was synthesized by reducing the azido-group to the corresponding amine using standard $NaBH_4/NiCl_2$ condition. The crude aminosteroid was further coupled to the mono-*tert*-butyl-succinate **3**[28] to give compound **4**. The *tert*-butyl was deprotected by refluxing with formic acid in diethyl ether giving the lipid anchor **5**.

The synthesis of the different conjugates commences from trisaccharide **1**, previously synthesized.[18] The thioacetylated derivative **7** (Scheme 2) was synthesized from **1** using catalytic hydrogenolysis followed by coupling with 3-acetylthio-

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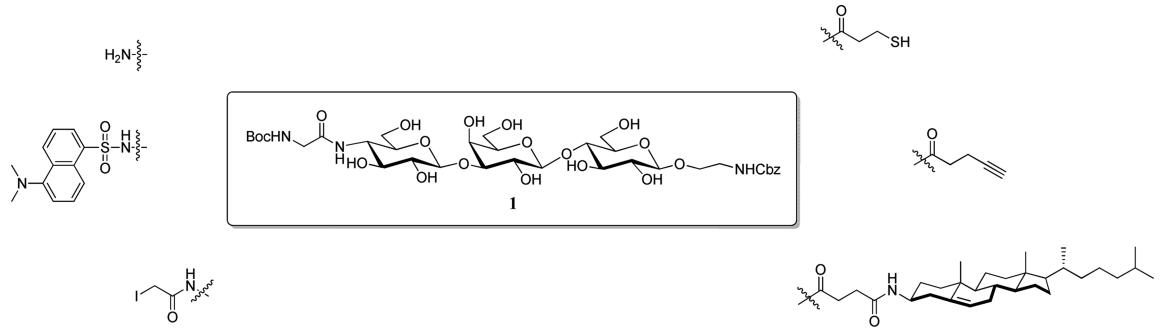
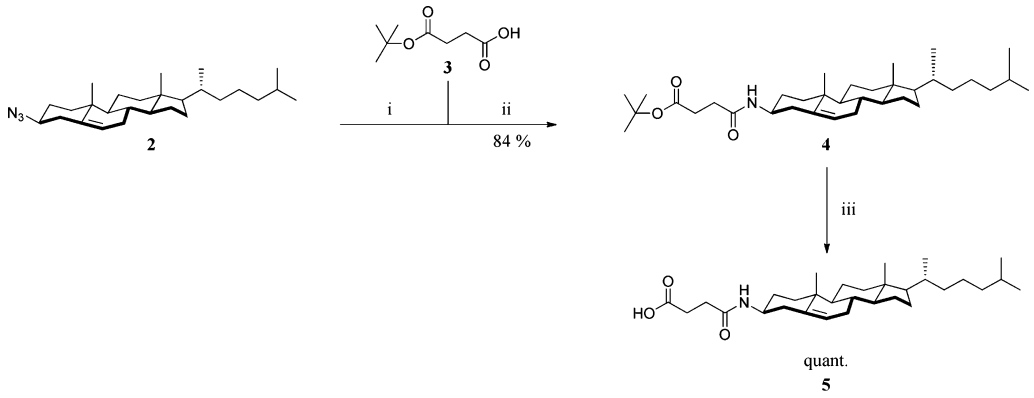


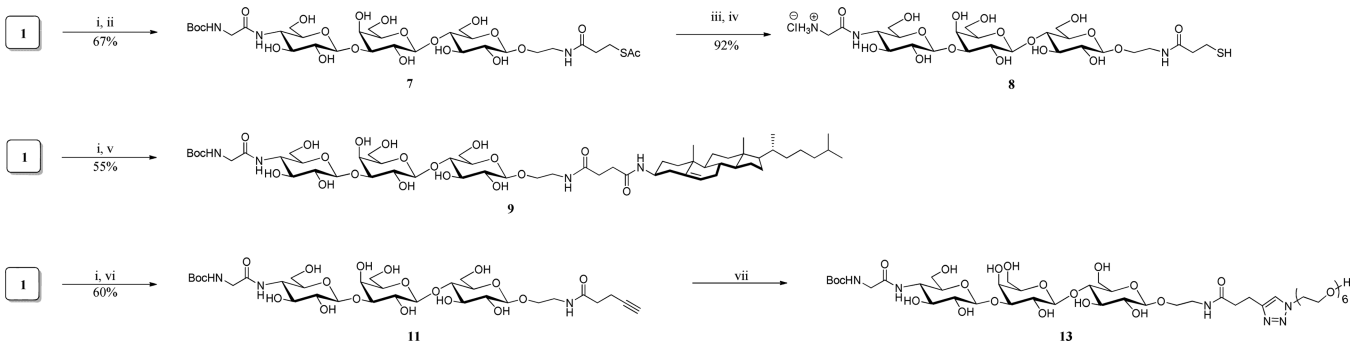
Figure 1. Target cross-linking trisaccharide, having chemically tailored properties to address a diversity of biophysical applications or biological systems.

Scheme 1[a]



[a]Reagents and conditions: (i) $NaBH_4$, $NiCl_2{\cdot}6H_2O$ (cat.), $EtOH/CH_2Cl_2$; (ii) **3**,[28] DIPEA TBTU, DMF; (iii) HCOOH, Et_2O, reflux.

Scheme 2[a]



[a]Reagents and conditions: (i) Pd/C (10%), H_2 (g), MeOH; (ii) **6**,[29] $NaHCO_3$ (s), $MeOH/H_2O$ (1:1); (iii) NaOMe, MeOH; (iv) HCl 1 M (aq.); (v) **5**, 0.5 M HOAt (DMF), *N*-methylmorpholine, EDC·HCl, MeOH; (vi) **10**,[30] $NaHCO_3$ (s), $MeOH/H_2O$ (1:1); (vii) **12**, $CuSO_4{\cdot}5H_2O$, sodium ascorbate.

propionic acid *N*-hydroxysuccinimide ester **6**.[29] A two-step deprotection procedure comprising a Zemplén deacetylation followed by an acid deprotection of the *N*-Boc using 1 M HCl (aq.) yielded the target compound **8**. The lipid-anchoring derivative **9** was synthesized by hydrogenolysis and subsequent coupling with steroid derivative **5** using the same method as optimized earlier.[18] It should be noted that the amphiphilic character of compound **9** is reflected by its low solubility in solvents for standard purifications. In our experience, a highly concentrated solution can only be acquired using either MeOH or a combination of solvent, i.e., chloroform/MeOH/H_2O (7:4:1).

The alkyne derivative **11** was synthesized using protocols in accordance as for the earlier mentioned compounds by hydrogenolysis followed by coupling with 4-pentynoic acid *N*-hydroxysuccinimide ester **10**.[30] In comparison, yields for compound **7** and **11** were improved using the preactivated N-hydroxysuccinimide ester rather than the corresponding carboxylic acids.

A Cu^I-catalyzed azide−alkyne cycloaddition (CuAAC) with azidohexa(ethylene) glycol **12**,[32] was performed on a 2 mg scale; hence, characterization data are incomplete. The following compounds **14** and **16** have been synthesized on a 1−2 mg scale (Scheme 3), i.e., full characterization data not available, as a possible lipid raft dye or for protein conjugation

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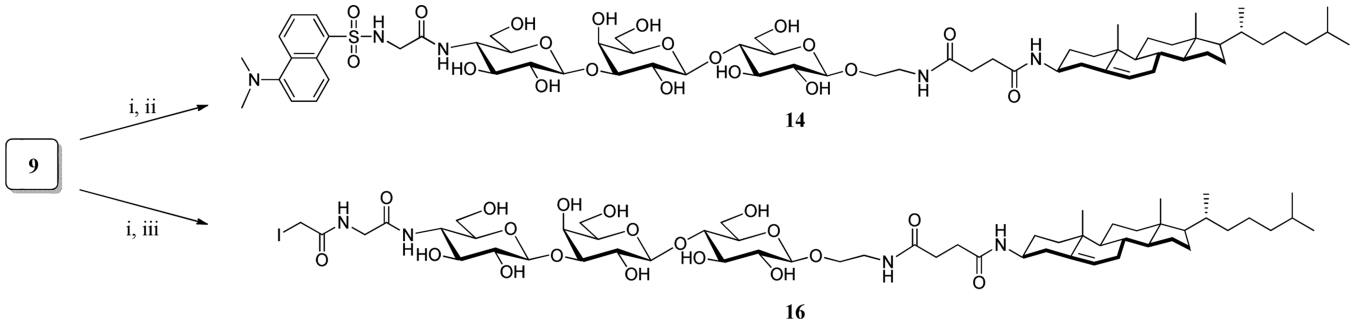
Scheme 3[a]



[a]Reagents and conditions: (i) 1 M HCl (aq.), MeOH; (ii) Dansyl chloride, $NaHCO_3$ (s), $MeOH/H_2O$ (1:1); (iii) **15**,[31] $NaHCO_3$ (s), $MeOH/H_2O$ (1:1).

studies, respectively. From intermediate **9**, the dansyl structure **14**, as well as the 2-iodoacetamido derivative **16**, was synthesized. The two-step conversion was commenced by a deprotection of **9** followed by subsequent coupling with dansyl chloride (→**14**) or iodoacetic acid *N*-hydroxysuccinimide ester **15**[31] to give target compound **16**.

Application of 8 for Surface Plasmon Resonance. The intention to improve and refine biosensors is of great interests. We set out to synthesize a bifunctional trisaccharide having both amine as well as thiol functionality **8** (Scheme 2). This derivative was successfully assembled onto gold surfaces and able to perform as a novel biosensor surface. The thiol moiety in compound **8** enabled formation of self-assembled monolayers (SAMs) on gold surfaces. Biotin-NHS was coupled to the terminal amine group in order to render a flexible biosensor surface. Infrared reflection absorption spectroscopy (IRAS) was employed to confirm the derivatization of the SAMs with biotin. No significant changes were observed in the fingerprint region after biotinylation, but a slight increase in the amide I band (1657 cm^{-1}) and the appearance of a new vibrational mode at 1711 cm^{-1} originating from the biotin C═O stretch indicate successful coupling (Figure 2). The ellipsometric thickness of SAMs of compound **8** was 15.6 ± 0.5 Å. After biotinylation, the thickness increased to 16.7 ± 0.2 Å.

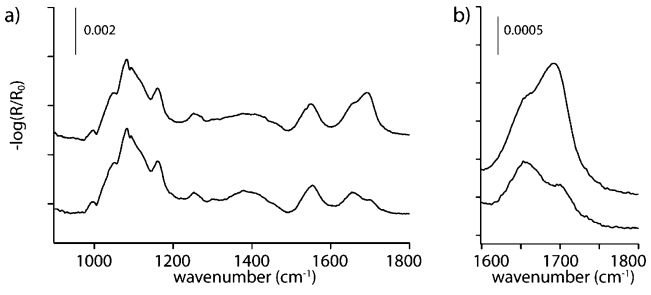


Figure 2. (a) IRAS spectra of the fingerprint and amide regions before (bottom) and after (top) biotinylation. (b) Magnified view of the amide I region.

Compound **8** was subsequently immobilized on SPR-gold substrates. After biotinylation, a 5 min injection of 1 nM avidin at a flow rate of 20 μL/min resulted in the binding of 1500 RU (Figure 3) corresponding to 1.5 ng/mm^2 (+biotin). No binding was observed in the reference channel pretreated with acetic acid *N*-hydroxysuccinimide[33] (−biotin). The surface was saturated with avidin after a second 5 min injection of 10 nM

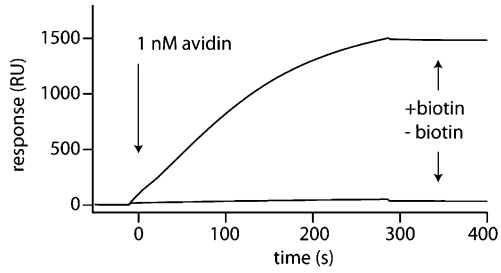


Figure 3. Sensorgram showing the binding of avidin to the biotinylated SAM.

avidin, resulting in an additional increase of 500 RU in the biotinylated channel (not shown).

Application of 14 for Selective Binding to Lipid Microdomains in Living Cells. To generate a molecular tool for studies of glyco-conjugated lipids and for understanding their molecular distributions, we have synthesized a cholesterol containing glycoside **9** (Scheme 2).[34] It was further derivatized with either a fluorophore (Dansyl) containing derivative **14**, for the monitoring of lipid-microdomains (lipid rafts) or an iodoacetamide derivative **16** (Scheme 3), for site-specific conjugation to cysteine residues in proteins.[35,36] It was shown by Peterson and co-workers that having an amide bond compared to a secondary amine on the *N*-cholesterol residue essentially abolished the internalization.[26] Hence, derivatives **14** and **16** should be predicted to accumulate on the cell surface.

To investigate the binding and distribution of **14** in live cells, we stained human fibroblasts with the probe. When adding **14** to living cells for 18 h of incubation, the probe clearly binds to a compartment of the cell (Figure 4a). Comparing with different compartments of the cell, such as the nucleus, mitochondria, and α-tubulin, the probe seems to attach exclusively to the cell membrane. The staining was uneven and punctuated, likely reflecting binding to lipid-microdomains (lipid-rafts). The staining pattern was very similar to that reported previously for lipid-rafts on fibroblasts.[37] An analogous staining procedure was also performed using free Dansyl (5-dimethylamino)-1-naphthalenesulfonamide (DNSA) instead of **14**. No cellular staining was observed from DNSA even though a 15-fold higher staining concentration than that used for **14** was employed (not presented herein). It is most plausible that the cholesterol tag of **14** interacts through hydrophobic partitioning with the target and hence shows specificity toward the membrane lipid bilayer. Lipid rafts are compartments on the cell surface enriched in monosialotetrahexosylganglioside

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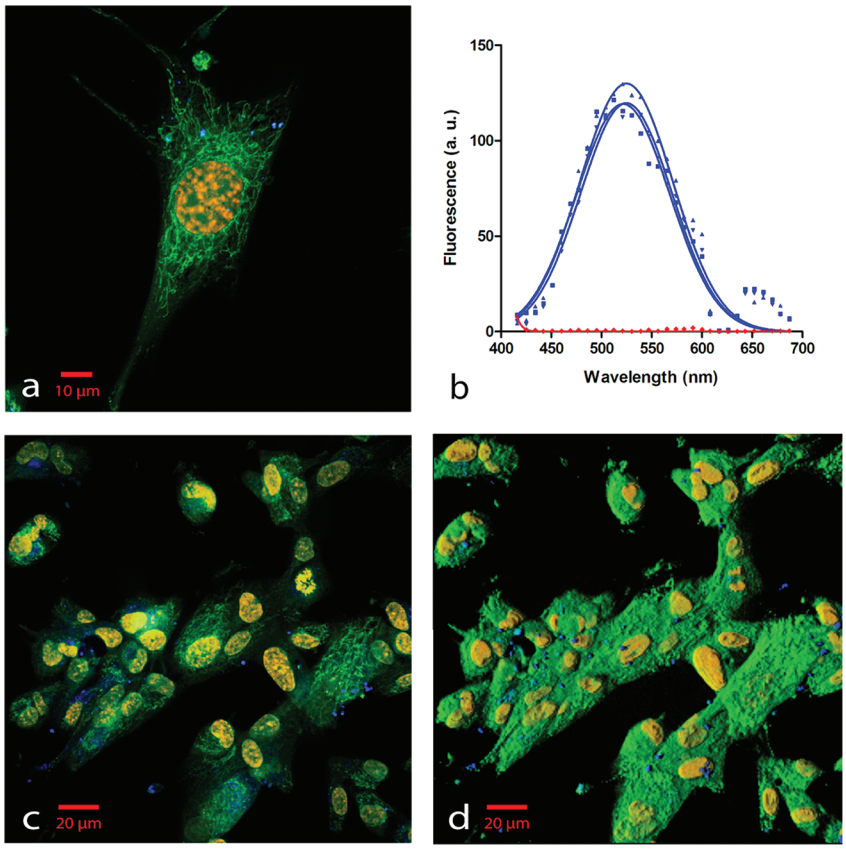

Figure 4. Compound **14** stains lipid-microdomains in living cells. Cultured lung fibroblasts were incubated with 0.3 μM of **14** for 18 h. (a) Single fibroblast with small punctuate microdomains visible at the top of the cell (blue), counterstaining of cells were performed using Mitotracker (green) and ToPro3 (red). (b) Fluorescence emission spectra of punctuate microdomains from three regions of interest showing emission peaks at 521.6–524.8 nm (blue). Background emission was very low (red). (c) Confocal microscope image showing clustered fibroblasts stained as in (a). (d) Computer-generated surface enhanced (3D) image of (c).

(GM1), cholesterol, and GPI-anchored proteins.[38] Hence, specificity of **14** toward lipid-rafts can be assumed and is possibly also reinforced through glycolic interactions with GM1. The cell staining of **14** was weaker in the antitubulin antibody staining protocol (not presented herein), which could be a result of the probe being washed away during several incubation steps which were performed in the presence of the detergent Triton-X.

With fluorescence microspectroscopy, the emission spectrum of the punctuate microdomains where **14** was accumulated showed typical Dansyl emission spectra with emission peaks at 523.7 ± 1.5 nm (Figure 4b). This emission peak distribution of compound **14** reflects a Stokes shift of the probe to reflect a slightly hydrophobic environment.[39] This shows that the probe, when enriched within lipid microdomains on the cell surface, is partially shielded from water. Using confocal scanning fluorescence microscopy, we found that the fluorescently tagged compound **14** showed prevalent labeling of microdomains in clustered fibroblasts (Figure 4c,d), which was more pronounced than for solitary cells (c.f., Figure 4a and Figure 4c,d). This could indicate that lipid rafts are up-regulated during cell–cell interactions.

Application of 11 for Click Chemistry. Finally, to ensure the possible usefulness of derivative **11** (Scheme 2), bearing an alkyne functionality for further "click chemistry" conjugations, a test reaction (2 mg) was performed with hexa(ethylene) glycol **12** having an azido-functionality.[32] The result displayed complete consumption of the alkyne derivative **11** (according to MALDI-TOF) which establishes the potential use of oligosaccharides as spacer molecules as an alternative to the commonly used oligo(ethylene) glycols.

CONCLUSION

In conclusion, an orthogonally protected trisaccharide has been demonstrated as a general spacer/linker molecule by conjugation with different chemical functionalities. It has been illustrated that defined carbohydrate-based compounds have potential use in biosensing applications, i.e., novel SPR surfaces. The result from live cell incubation experiments indicates that the trisaccharide-based linker incorporates into cell surfaces, and was specifically enriched in microdomains. These findings also show promise for conjugating biomolecules onto cell surfaces, thereby mimicking glycoproteins. Using compound **14** as an example of lipid-raft specificity, this opens up the possibility of using compound **16** as either a "fishing hook" for protein conjugation in living cells or a tool for directed interaction of a recombinant protein toward these cellular structures. Furthermore, a successful "click chemistry" cycloaddition confirms the potential of using oligosaccharides as unique bioorthogonal cross-linking derivatives.

EXPERIMENTAL SECTION

Synthesis. *General.* CH_2Cl_2 and toluene was distilled over calcium hydride and collected onto predried 4 Å MS. Thin layer chromatography (TLC) was carried out on Merck 60 F_{254}

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plates and visualized by UV light and/or developed with PAA [EtOH (95%, 744 mL), H_2SO_4 (conc., 27.6 mL), AcOH (100%, 8.4 mL), *p*-anisaldehyde (20.4 mL)]. Flash column chromatography (FC) was carried out on silica gel Merck 60 (40−63 μm). Reverse-phase chromatography (RP) was carried out on Merck LiChroprep (RP-18). Gel permeation was performed using Sephadex LH-20. Dialysis was performed using Spectra/Por MWCO = 500 or MWCO = 1000. Proton nuclear magnetic resonance (^{1}H) and carbon nuclear magnetic resonance (^{13}C) was recorded on a Varian 300 MHz spectrometer; multiplicities are quoted as singlet (s), doublet (d), doublet of doublets (dd), triplet (t), apparent doublet (ad), apparent triplet (at). ESI-MS (recorded at Medivir AB, Huddinge, Sweden) was performed on a Water Synapt HDMS instrument equipped with electrospray interface. Matrix-assisted laser desorption ionization − time of flight (MALDI-TOF) mass spectroscopy was recorded on a Voyager-DE STR Biochemistry Workstation in a positive mode using using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix. Optical measurements were recorded at 20 °C with a Perkin-Elmer 141 polarimeter. FT-IR was recorded on a Perkin-Elmer Spectrum 1000 using KBr pellets; appearances are quoted as strong (s), medium (m), and weak (w). Melting points were recorded on a Stuart melting point apparatus.

***tert*-Butyl-*N*-succinyl-3β-amino-5-cholestene (4).** The 3-β-Azido-5-cholestene[27] **2** (1.00 g, 2.43 mmol) was dissolved in EtOH/CH_2Cl_2 (20 mL, 3:1) whereupon $NaBH_4$ (180 mg, 4.86 mmol) and $NiCl_2{\cdot}6H_2O$ (cat.) were added. After 1 h, the solution was diluted with CH_2Cl_2 and washed with NaCl (sat. aq.), dried, and concentrated. Without further purification, the amine was dissolved in DMF (10 mL) whereupon mono-*tert*-butyl-succinate **3**[28] (850 mg, 4.86 mmol), DIPEA (1.70 mL, 9.72 mmol), and TBTU (1.56 g, 4.86 mmol) were added. After 1 h, the solution was diluted with toluene and washed sequentially with NaCl (sat. aq.), $NaHCO_3$ (sat. aq.), dried, and concentrated. FC (toluene/EtOAc 4:1) followed by crystallization from MeOH afforded **4** (1.11 g, 2.04 mmol, 84%) as white needles. R_f = 0.57 (toluene/EtOAc 2:1); mp = 146−148 °C (MeOH); $[\alpha]_D$ − 35 (c 1, $CHCl_3$); IR (KBr) $\nu_{max}cm^{-1}$: 2934 (s), 1732 (s), 1644 (s), 1547 (s), 1366 (m), 1153 (s); NMR: ^{13}C (75.4 MHz, $CDCl_3$) δ 11.9, 18.8, 19.4, 22.6, 22.9, 28.1, 28.2, 28.3, 29.2, 31.1, 31.6, 31.9, 31.9, 35.9, 39.6, 42.4, 49.7, 50.2, 56.2, 56.8, 80.7, 121.9, 140.4, 170.8, 172.4 (overlaps occur in spectra); ^{1}H (300 MHz, $CDCl_3$): δ 0.67 (s, 3H), 0.85 (d, 3H, *J* = 0.6 Hz), 0.87 (d, 3H, *J* = 0.6 Hz), 0.91 (d, 3H, *J* = 6.3 Hz), 0.94−1.58 (m, 33H), 1.76−1.86 (m, 3H), 1.90−2.11 (m, 3H), 2.25−2.31 (m, 1H), 2.38 (t, 2H, *J* = 6.7 Hz), 2.56 (t, 2H, *J* = 6.7 Hz), 3.60−3.74 (m, 1H), 5.33−5.35 (m, 1H), 5.54 (d, 1H, *J* = 8.1 Hz); ESI-MS: $[M+H]^+$ calcd for $C_{35}H_{60}NO_3$, 542.4495; found 542.4505.

***N*-Succinyl-3β-amino-5-cholestene (5).** The *tert*-butyl-succinyl-3β-amino-5-cholestene **4** (0.50 g, 0.92 mmol) was added to a solution of formic acid/Et_2O (1:1, 20 mL) and heated to reflux. After 1 h, the reaction mixture was evaporated and coconcentrated subsequently with toluene affording **5** (0.44 g, 0.92 mmol, quant.) as a white solid. R_f = 0.66 (EtOAc/MeOH 4:1); $[\alpha]_D$ − 20 (c 0.1, chloroform/MeOH/H_2O 7:4:1); IR (KBr) $\nu_{max}cm^{-1}$: 2963 (m), 2936 (s), 1731 (s), 1625 (s), 1555 (s), 1187 (m), 1158 (s), 822 (w); NMR: ^{13}C (75.4 MHz, $CDCl_3$/CD_3OD 9:1) δ 11.9, 18.8, 19.4, 21.0, 22.6, 22.8, 23.9, 24.3, 28.1, 28.3, 28.9, 29.9, 31.1, 31.9, 31.9, 35.9, 36.3, 36.6, 37.9, 39.0, 39.6, 39.8, 42.4, 49.9, 50.2, 56.2, 56.8, 122.0, 140.3, 171.7, 175.3; ^{1}H (300 MHz, $CDCl_3$/CD_3OD 9:1): δ 0.68 (s, 3H), 0.86 (d, 3H, *J* = 1.3 Hz), 0.88 (d, 3H, *J* = 1.3 Hz), 0.92 (d, 3H, *J* = 6.4 Hz), 0.95−1.63 (m, 24H), 1.78−1.88 (m, 3H), 1.92−2.06 (m, 2H), 2.10−2.15 (m, 1H), 2.23−2.30 (m, 1H), 2.44 (d, 2H, *J* = 6.8 Hz), 2.63 (d, 2H, *J* = 6.8 Hz), 3.58−3.68 (m, 1H), 5.34−5.37 (m, 1H); ESI-MS: $[M+H]^+$ calcd for $C_{31}H_{52}NO_3$, 486.3869; found 486.3844.

***N*-(3-Acetylthio-propanoyl)-2-aminoethyl (4-Deoxy-4-*N*-tert-butyloxycarbonyl-glycyl-glucopyranosyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (7).** To a solution of **1** (0.15 g, 0.18 mmol) in MeOH (5 mL), 10% Pd/C was added and stirred for 4 h under H_2(g) atomsphere (1 atm) followed by filtration though Celite and evaporation. The residue was dissolved in MeOH/H_2O (10 mL) whereupon $NaHCO_3$ (s) (0.15 g, 0.18 mmol) and 3-acetylthio-propionic acid *N*-hydroxysuccinimide ester **6**[29] (46 mg, 0.19 mmol) were added and stirred overnight. The solution was neutralized with Dowex-H^+, filtered, and evaporated. RP (H_2O → H_2O/MeOH 1:1) gave **7** (0.10 g, 0.12 mmol, 67%) as a white solid. R_f = 0.69 (chloroform/MeOH/H_2O 7:4:1); $[\alpha]_D$ - 10 (c 0.1, H_2O); IR (KBr) $\nu_{max}cm^{-1}$: 2933 (w), 1693 (s), 1546 (m), 1368 (w), 1160 (w), 1072 (s); NMR: ^{13}C (75.4 MHz, CD_3OD): δ 25.9, 28.7, 30.5, 36.6, 40.6, 44.8, 53.2, 62.0, 62.5, 62.6, 69.6, 69.6, 71.6, 74.6, 74.8, 75.9, 76.3, 76.4, 76.7, 80.8, 84.5, 104.3, 104.7, 105.6, 158.5, 173.5, 173.7, 197.2, (several overlaps occur in spectra); ^{1}H (300 MHz, CD_3OD): δ 1.46 (s, 9H), 2.32 (s, 3H), 2.50 (t, 2H, *J* = 7.0 Hz), 3.11 (t, 2H, *J* = 7.0 Hz), 3.25−3.90 (m, 23H), 4.10 (ad, 1H, *J* = 3.0 Hz), 4.33 (d, 1H, *J* = 8.0 Hz), 4.43 (d, 1H, *J* = 7.6 Hz), 4.56 (d, 1H, *J* = 7.8 Hz); ESI-MS: $[M+H]^+$ calcd for $C_{32}H_{56}N_3O_{20}S$, 834.3100; found 834.3096.

***N*-(3-Thio-propanoyl)-2-aminoethyl (4-deoxy-4-*N*-glycyl-glucopyranosyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl-ammonium hydrochloride (8).** To a solution of **7** (50 mg, 60 μmol) in MeOH (5 mL), NaOMe (7 mg, 0.12 mmol) was added. After 1.5 h, the mixture was neutralized with Dowex-H^+, filtered, and evaporated. RP (H_2O → H_2O/MeOH (1:1)) gave the thiol which was used subsequently in the following step. The deacetylated compound was dissolved in H_2O (5 mL) whereupon 1 M HCl (0.1 mL) was added. After 1 h, the solution was evaporated and coconcentrated repeatedly with MeOH giving the title compound **8** (40 mg, 55 mmol, 92%) as an off-white solid. R_f = not available; $[\alpha]_D$ + 6 (c 0.1, H_2O); IR (KBr) $\nu_{max}cm^{-1}$: 2882 (w), 1692 (s), 1643 (s), 1648 (s), 1566 (m), 1434 (m), 1158 (w), 1071 (s), 1021 (s), 950 (s); NMR: ^{13}C (75.4 MHz, CD_3OD): δ 19.8, 39.2, 39.6, 40.4, 51.8, 60.3, 60.9, 61.0, 68.3, 68.4, 70.2, 73.2, 73.4, 74.2, 74.7, 75.0, 75.2, 79.0, 82.7, 102.7, 103.0, 103.9, 167.0, 173.2 (overlap occur in spectra); ^{1}H (300 MHz, CD_3OD): δ 2.54 (t, 2H, *J* = 6.7 Hz), 2.76 (t, 2H, *J* = 6.7 Hz), 3.33−3.98 (m, 23H), 4.12−4.13 (m, 1H), 4.39 (d, 1H, *J* = 7.8 Hz), 4.46 (d, 1H, *J* = 7.6 Hz), 4.63 (d, 1H, *J* = 7.7 Hz); ESI-MS: $[M+H]^+$ calcd for $C_{25}H_{46}N_3O_{17}S$, 692.2470; found 692.2491.

3β-Amino-5-cholestene-*N*,*N*-succinyl-2-*N*-aminoethyl (4-Deoxy-4-*N*-tert-butyloxycarbonyl-glycyl-glucopyranosyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranoside (9). Compound **1** (42 mg, 50 μmol) was dissolved in MeOH (10 mL) followed by the addition of Pd/C (10%, cat.). The mixture was stirred under H_2 pressure (1 atm) for 2 h, then filtered through Celite and evaporated. The residue was dissolved in MeOH (10 mL), followed by the addition of **5** (26 mg, 53 μmol), 0.5 M (DMF) HOAt (10 μL, 5 μmol), *N*-methylmorpholine (6 μL, 53 μmol), and stirred for 10 min

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whereupon EDC·HCl (10 mg, 53 μmol) were added and stirred overnight. The mixture was evaporated and the obtained residue was purified on a sephadex LH-20 column using MeOH to give **9** (32 mg, 27 μmol, 55%) as a white solid. R_f = 0.14 (EtOAc/MeOH/H_2O 7:1:0.5); $[\alpha]_D$ − 31 (c 0.1, chloroform/MeOH/H_2O 7:4:1); IR (KBr) $\nu_{max}cm^{-1}$: 2933 (s), 1643 (s), 1547 (s), 1367 (m), 1160 (m), 1074 (s); NMR: ^{13}C (75.4 MHz, $CDCl_3/CD_3OD/D_2O$ 7:4:1): δ 11.4, 18.2, 18.7, 20.6, 22.0, 22.2, 23.4, 23.9, 27.6, 27.8, 28.1, 29.3, 31.1, 31.2, 31.4, 31.5, 35.4, 35.8, 36.2, 37.6, 38.3, 39.0, 39.1, 39.4, 42.0, 43.3, 49.5, 49.9, 51.3, 55.8, 56.4, 60.2, 60.7, 60.8, 68.0, 68.2, 69.8, 72.7, 72.8, 73.8, 74.3, 74.6, 74.8, 74.9, 77.4, 78.8, 80.0, 82.6, 102.4, 102.7, 103.7, 121.3, 140.2, 156.8, 172.1, 173.4 (overlaps occur in spectra); ^1H (300 MHz, $CDCl_3/CD_3OD/D_2O$ 7:4:1): δ 0.41 (m, 3H), 0.57−1.35 (m, 40H), 1.46−1.76 (m, 6H), 1.82−1.97 (m, 3H), 2.12−2.22 (m, 4H), 2.34−2.49 (m, 1H), 2.96−3.72 (m, 23H), 3.81 (ad, 1H, *J* = 2.9 Hz), 4.05−4.1 (m, 2H), 4.29 (d, 1H, *J* = 7.8 Hz), 5.06 (m, 1H); ESI-MS: $[M+H]^+$ calcd for $C_{58}H_{99}N_4O_{20}$, 1171.6775; found 1171.6777.

***N*-(4-Pentynoyl)-2-aminoethyl (4-Deoxy-4-*N*-*tert*-butyloxycarbonyl-glycyl-glucopyranosyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranoside (11).** Compound **1** (38 mg, 45 μmol) was dissolved in MeOH (2 mL) whereupon 10% Pd/C (cat.) was added. The solution was stirred for 3 h under H_2 (g) pressure (1 atm) followed by filtration though Celite and evaporation. The residue was dissolved in MeOH/H_2O (2 mL, 1:1) whereupon $NaHCO_3$(s) (10 mg, 0.12 mmol) and 4-pentynoic acid *N*-hydroxysuccinimide ester **10**[30] (10 mg, 47 μmol) were added and stirred overnight followed by concentration. RP (H_2O → H_2O/MeOH 1:1) gave **11** (21 mg, 26 μmol, 60%) as a white solid. R_f = 0.73 (Chloroform/MeOH/H_2O 7:4:1); $[\alpha]_D$ + 3 (c 0.1, H_2O); IR (KBr) $\nu_{max}cm^{-1}$: 2933 (w), 1702 (s), 1692 (s), 1660 (s), 1552 (s), 1369 (m), 1159 (m), 1073 (s); NMR: ^{13}C (75.4 MHz, CD_3OD): δ 15.7, 28.7, 36.0, 40.6, 44.8, 53.2, 61.9, 62.5, 62.6, 69.6, 69.7, 71.6, 74.6, 74.7, 75.8, 76.2, 76.4, 76.7, 76.8, 80.7, 81.0 84.4, 104.3, 104.6, 105.5, 158.6, 173.6, 174.2 (overlaps occur in spectra); ^1H (300 MHz, CD_3OD): δ 1.45 (s, 9H), 2.37−2.49 (m, 4H), 3.24−3.94 (m, 24H), 4.09 (ad, 1H, *J* = 2.9 Hz), 4.32 (d, 1H, *J* = 7.7 Hz), 4.42 (d, 1H, *J* = 7.6 Hz), 4.55 (d, 1H, *J* = 7.8 Hz); ESI-MS: $[M+H]^+$ calcd for $C_{32}H_{54}N_3O_{19}$, 784.3274; found 784.3236.

***N*-[(4-Propanoyl)-2-aminoethyl [(4-deoxy-4-*N*-*tert*-butyloxycarbonyl-glycyl-glucopyranosyl)-(1→3)-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl]-1*H*-1,2,3-triazol-1-yl-hexa(ethylene) glycol (13).** The alkyne-derivative **11** (2 mg, 3 μmol) was dissolved in H_2O/MeOH (1.5 mL, 2:1) whereupon azidohexa(ethylene) glycol **12**[32] (1 mg, 3 μmol), $CuSO_4·5H_2O$ (6 mg, 0.02 mmol), and sodium ascorbate (2 mg, 0.01 mmol) were added and stirred overnight. According to MALDI-TOF, complete consumption of alkyne **11** was detected to give compound **13**. MALDI-TOF (CHCA): $[M + Na]^+$ calcd for $C_{44}H_{78}NaO_{25}$, 1113.50; found 1113.32.

3β-Amino-5-cholestene-*N*,*N*-succinyl-2-*N*-aminoethyl [4-Deoxy-4-*N*-(5-(dimethylamino)naphthalene-1-sulfonyl)-glycyl-glucopyranosyl]-(1→3)-(β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranoside (14). To a solution of **9** (1 mg, 0.9 μmol) in MeOH (2 mL), 1 M HCl (aq.) (10 μL) was added. After 15 min, the solution was evaporated and co-concentrated repeatedly with MeOH. Without further purification the amine was dissolved in H_2O/MeOH (2 mL, 1:1) whereupon $NaHCO_3$ (s) (2 mg, 0.02 mmol) and Dansylchloride (3 mg, 0.01 mmol) were added and stirred overnight. The mixture was diluted in H_2O (15 mL) and purified with dialysis (MWCO = 1000) over 3 days in the dark (4 × 2 L, H_2O) followed by evaporation. R_f = 0.80 (chloroform/MeOH/H_2O 7:4:1); ESI-MS: $[M+H]^+$ calcd for $C_{65}H_{102}N_5O_{20}S$, 1304.6761; found 1304.6731.

3β-Amino-5-cholestene-*N*,*N*-succinyl-2-*N*-aminoethyl [4-deoxy-4-*N*-(2-iodoacetyl)-glycyl-glucopyranosyl]-(1→3)-(β-D-galactopyranosyl)-(1→4)-(β-D-glucopyranoside (16). To a solution of **9** (1 mg, 1 μmol) in MeOH (2 mL), 1 M HCl (aq.) (10 μL) was added. After 15 min the solution was evaporated and co-concentrated repeatedly with MeOH. Without further purification the amine was dissolved in H_2O/MeOH (2 mL, 1:1) whereupon $NaHCO_3$ (s) (2 mg, 0.02 mmol) and iodoacetic acid *N*-hydroxysuccinimide ester **15**[31] (3 mg, 0.01 mmol) were added and stirred overnight. The mixture was diluted in H_2O (4 mL) and purified with dialysis (MWCO = 500) over 3 days in the dark (4 × 2 L, H_2O) followed by evaporation. R_f = 0.74 (chloroform/MeOH/H_2O 7:4:1); ESI-MS: $[M+H]^+$ calcd for $C_{55}H_{92}IN_4O_{19}$, 1239.532; found 1239.5408.

Gold Surface Preparation and Characterization. SAMs for IRAS and ellipsometry were prepared on gold-coated pieces of silicon wafers. A wafer was cut into 40 × 20 mm^2 pieces, cleaned in a mixture of H_2O, 30% H_2O_2, and 25% NH_3 in a 5:1:1 volume ratio, at 80 C for 10 min (TL1-cleaning), rinsed with Milli-Q (MQ) water, and then blown dry with nitrogen gas. The pieces were mounted in a custom-built electron-beam evaporation system with a base pressure of about 10^{-9} Torr, where a 25 Å Ti layer was deposited immediately before a 2000 Å gold layer. Gold-coated glass slides for SPR were obtained from GE Healthcare. Gold surfaces were TL1-cleaned before 24 h incubation in 100 μM aqueous solutions of the oligosaccharide. After incubation, the SAMs were rinsed with water, sonicated for 2 min, rinsed again, and finally dried before characterization.

IRAS spectra were recorded using a Bruker IFS 66 system, equipped with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector unit and a grazing angle (85°) infrared reflection accessory. The measurement chamber was continuously purged with nitrogen gas during the measurements to reduce the amount of interfering water. 3000 scans were collected at a resolution of 2 cm^{-1}. Before Fourier transformation a three-term Blackmann-Harris apodization function was applied to the interferrograms. Deuterated hexadecanthiol ($HS(CD_2)_{15}CD_3$) immobilized on gold was used as a reference to record the background spectrum.

Null-ellipsometric measurements were conducted on an automatic Rudolph Research AutoEL III ellipsometer with a He−Ne laser light source operating at 632.8 nm at an angle of incidence of 70°. An optical model based on isotropic optical constants for the peptide layer $N_{pep} = n + ik = 1.50$, with *n* = refractive index and *k* = extinction coefficient, was used for the evaluation of the film thickness. Results from at least five measurement spots for each sample were averaged.

Sensorgrams were recorded by a Biacore 3000 instrument (GE Healthcare, Sweden) operating at a wavelength of 760 nm and equipped with four flow channels. The temperature was 25 °C and HBS-EP (10 mM hepes, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4, from GE Healthcare) was used as a running buffer. Avidin from hen egg white (Thermo Scientific) and (+)-Biotin *N*-hydroxysuccinimide ester (Sigma Aldrich) were used.

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Cell Biology. *Cell Culture.* MRC-5 cells (human lung fibroblast, ATCC CCL-171) were maintained in BIOAMF 2, complete medium, (Biological Industries; Beit Haemek, Israel) at 37 °C in humidified air with 5% CO_2 (g). These cells were seeded in a 12 well plate on round glass coverslips with a diameter of 18 mm.

Reagents. MitoTracker Orange CMTMRos, Alexa Fluor 488 antirabbit IgG (H+L) and ToPro3 were purchased from Invitrogen. Rabbit polyclonal antibody for alpha Tubulin (ab18251) was purchased from Abcam and Fluorescence Mounting Medium was from DAKO.

*Staining of Cells with **14** and Mitotracker.* Derivative **14** (96 μM, 50% DMSO in deionized water) was diluted with cell culture medium to 0.3 μM and added to the cells for 18 h. MitoTracker Orange CMTMRos (1 mM in DMSO) was diluted with cell culture medium to 150 nM. The diluted solution with compound **14** was discarded and the mitochondria were stained with the mitotracker solution for 30 min. After three washings with PBS (pH 7.3, Dubecco without Ca/Mg), the cells were fixed in 4% formaldehyde in 37 °C. The fixed cells were washed three times in PBS. ToPro3 (1 mM in DMSO) was diluted with PBS to a final concentration of 15 μM. The nucleus was stained with this solution for 15 min. After washing two times with deionized water, the coverslips were mounted on glass slides with Fluorescence Mounting Medium and dried overnight. The coverslips were sealed with nail polish before further analysis. An analogous staining procedure was also performed using 5 μM free Dansyl (5-Dimethylamino)-1-naphthalenesulfonamide (SigmaAldrich CAS: 1431−39−6) instead of **14**.

*Staining of Cells with **14** and Antialpha-Tubulin.* The staining with derivative **14** was performed equivalent to the procedure with the mitochondria staining, except that the staining concentration of **14** was 0.6 μM. The cells were washed three times in PBS and fixed in 4% formaldehyde at 37 °C. After washing two times in PBS, the cells were permeabilized for 30 min in 0.2% Triton X-100 in PBS. Three washings in PBS for five minutes were performed followed by incubation in blocking solution containing 10% normal goat serum in PBS. The diluted primary antibody, alpha Tubulin, (1:5000 in blocking solution) was added and incubated for one hour. After three washings in PBS for five minutes, the secondary antibody, Alexa Fluor 488 antirabbit IgG (H+L) (1:1000 in blocking buffer), was added and incubated for one hour. The cells were washed three times in PBS for five minutes. The nucleus was stained with ToPro3 and the cells were mounted as previously described in the mitotracker staining procedure.

Cellular Imaging. The fluorescence from the cell preparations was recorded with a confocal laser scanning microscope, LSM 780. The lasers used were at 405 nm (**14**), 550 nm (mitotracker), and 633 nm (ToPro3). Filters were set up at 375−539 nm, 543−630 nm, and 638−735 nm.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure 4 and NMR spectra of synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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