N-Hexyl-4-aminobutyl glycosides for investigating structures and biological functions of carbohydrates†

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The potential applications of *N*-hexyl-4-aminobutyl glycosides in the mass spectrometric investigation of glycan structure and in the investigation of glycan functions were studied. Under collision-induced dissociation (CID) conditions, sodiated glycosides carrying *N*-hexyl-4-aminobutyl groups effectively produced a hemiacetal species (C-ions), which is important in mass-spectrometry-based structural investigation. The usefulness of *N*-hexyl-4-aminobutyl glycosides in biological analysis was also confirmed by obtaining a binding constant for the binding of dipyrromethene boron difluoride C3-labeled *N*-hexyl-4-aminobutyl β-lactoside with an *Erythrina cristagalli* lectin, and by visualizing cellular organelles using a more hydrophobic BODIPY-labeled compound.

Introduction

The investigation of the detailed structure–function relationship of glycoconjugates is an extremely important research topic. Carbohydrates carry multiple hydroxyl groups, each of which is a candidate for the glycosylation reactions. Furthermore, a glycosylation reaction at each of these positions results in the formation of a pair of anomers. Because of these reasons, oligosaccharides have the potential to form an extremely large number of structures.[1] Thus, unnatural oligosaccharides might have novel biological functions. Some of the interesting examples can be highlighted *e.g.* (1) the discovery of some unnatural inhibitors against E-selectin consisting of anomeric isomers[2] and (2) the discovery of unusual substrates of a fucosidase from a small combinatorial library.[3] Thus, we expect that a combinatorial oligosaccharide library will be a pool of new substances that show potential for use as inhibitors, substrates and so on. In order for efficient screening, glycans are required to be tagged for detection purposes.

Fluorescent probes are very important in the investigation of molecular functions and localization of the molecules.[4,5] In order to successfully synthesize and investigate the functions of oligosaccharides, a suitable aglycone with a functional group that can be easily converted into a biological probe is required. Thus far, carboxyl and amino groups have been the most frequently used functional groups, and they have been used with certain spacer elements.[6] The azide group is becoming popular because it allows chemistry to be carried out in aqueous solution.[7-9]

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† Electronic supplementary information (ESI) available: Energy-resolved mass spectrometry (ERMS) for compounds **1**(α,β) and **8**(α,β). ^{1}H-NMR, COSY and HSQC spectra of compounds **1**(α,β), **9** and **10**. LC-MS chromatograph and MS/MS spectra for compounds **9** and **10**. See DOI: 10.1039/b909556j

We have been interested in synthesizing a combinatorial trisaccharide library in order to find a novel functional oligosaccharide.[3,10] Further, we have been attempting to develop a new method based on mass spectrometry (MS) for determining oligosaccharide structures. This method involves the use of a combinatorial oligosaccharide library as the source of structural information.[11,12] Thus the search for a multipurpose aglycone that can be used in both functional and structural investigations has become particularly important.

MS-based structural analysis is one of the current focus areas because of the advantages it offers with respect to sample consumption. Further, collision-induced dissociation (CID) can be used to obtain more detailed structural information through an analysis of the fragmentation process.[13] In the course of our search for a useful aglycone that produces glycone-oriented ion species under CID conditions, we discovered the aminobutyl glycosides produce hemiacetal species called C-ions. The C-ions produced were found to retain the original stereochemistry of their glycosidic bonds.[14–16] Furthermore, C-ions are often produced from a variety of naturally occurring oligosaccharides, and thus, any previously obtained C-ion, including ones from a combinatorial library, can be used as a reference whose fragmentation profile can be compared with that produced from an unknown sample. Since aminobutyl glycosides carry the amino functional group, it is expected that these glycosides can also be used as a molecular probe by introducing fluorescent tags as well.

Molecular probes that can be used for either (1) investigating the binding affinity toward a target protein or the histochemistry by staining cellular organelles, or (2) producing an important C-ion species under CID conditions during MS/MS, have been developed; a probe that can be used to achieve both these objectives has not yet been developed. Multipurpose molecules that help achieve both of the above objectives must be given a great deal of attention in order to investigate the functions and structures of oligosaccharides (Fig. 1).

Here, we present a glycoside carrying an improved aglycone, namely, an *N*-hexyl-4-aminobutyl group, which is useful in

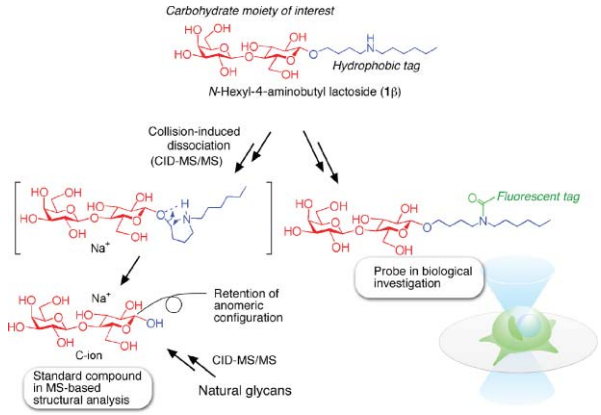


Fig. 1 An illustration of the utility of multipurpose glycosides. A glycoside carrying a novel hydrophobic aglycone (1β) is useful in (1) simple purification on a reverse-phase cartridge column (SPE), (2) the MS-based structural investigation under CID conditions of glycans obtained from natural sources, and (3) the investigation of biological functions and histochemistry of the glycan after incorporation of a fluorescent tag.

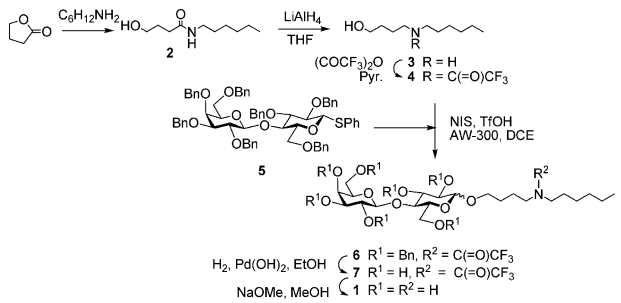
MS-based investigation and produces hemiacetal species in a good yield; further, after fluorescent tagging, this glycoside is also useful in the investigation of binding affinity with proteins and in the visualization of organelles using a fluorescence microscope.

Results and discussion

Synthesis of *N*-hexyl-4-aminobutyl lactoside

We aimed to synthesize *N*-hexyl-4-aminobutyl lactoside **1**. Lactose was chosen because it was present at the core part of the glycosphingolipids and thus it might be used as a primer molecule[17,18] after suitable modification at the secondary amino function of the aglycone moiety.

The first step in this synthesis was the preparation of a suitably protected amino alcohol as a glycosyl acceptor **4**. The reduction of the amide **2**, a condensation product of γ-butyrolactone and *n*-hexylamine, yielded the desired amine **3**. Compound **3** was protected with a trifluoroacetamido (TFA) group **4** (Scheme 1). The hydrophobic nature of the aglycone was also expected to be beneficial for solid-phase extraction (SPE) after removal of the protecting groups on the hydroxyl groups.



Scheme 1 Synthesis of **1**.

The reaction of **4** with a thioglycoside **5**[19] was carried out using *N*-iodosuccinimide (NIS)/trifluoromethanesulfonic acid (TfOH)[20] to afford the corresponding lactoside **6** as an anomeric mixture in a good yield. Non-neighbouring-protective group was used here to obtain α- and β-anomers because we planed to examine the behavior of both anomers in MS analysis. At this step, individual anomers (**6α** and **6β**) were separated by silica gel column chromatography. The *O*-benzyl groups of **6α** and **6β** were cleaved by hydrogenation (H_2–Pd on charcoal) to afford **7α** and **7β**, respectively. Compounds **1α** and **1β**, produced by deprotection of the TFA group of **7α** and **7β** with methanolic sodium methoxide, were purified by a simple cartridge of an ODS column. The calculated log P (partition coefficient) values for *n*-octyl lactoside, **7**, and **1** were –0.96, –0.89, and –1.88, respectively.[21] This may suggest that the glycosides carrying the *N*-hexyl-4-aminobutyl group can be used as substitutes for octyl glycosides permitting enzymatic "SepPak assays" for radio-labeled compounds,[22] and therefore, are useful for isolation of products from chemo-enzymatic synthesis. The presence of an amino group in an aglycone is also advantageous because synthetic compounds can be acylated to tag the disaccharide with a fluorophore for example, thus permitting a variety of experiments based on tracking the fluorophore in protein-binding assays or in cell-uptake experiments *etc.* (See sections below.)

The aglycone produces hemiacetal ions that allow assignment of anomeric configuration of glycosides based on mass spectrometry

We performed CID experiments using compounds **1α** and **1β** to examine the efficiency of these compounds in producing the corresponding C-ion species, which had been shown to retain their original anomeric configurations (for the nomenclature of ions, see ref. 23).[14,15] Such C-ions can be used to determine the anomeric configurations of a corresponding sugar unit in a glycan structure obtained from biological sources.[24,25] This was shown by the determination of a β-glucosyl linkage in a ganglioside GM3 as an example.[14] Although aminobutyl glycosides were shown to be useful in generating C-ion species under CID conditions, further improvements in the yield of C-ions, capability for rapid isolation after synthesis, and utility in biological investigations were required. Thus, sodiated precursor ions ($[M + Na]^+$)[26] were collided with He gas in an ion-trap mass spectrometer to produce a series of product ions. The MS/MS spectra of the products indicated that the C-ion (sodiated lactose equivalent) was indeed the main product of the dissociation reactions of both the anomers. In order to observe the difference between the dissociation of alkylated and non-alkylated 4-aminobutyl glycosides, the intensities of the fragment ions produced by the dissociation of **1** (α and β) were compared with previously reported intensities of fragment ions produced by the dissociation of 4-aminobutyl lactosides **8** (α and β) (Fig. 2).[14,15] The intensities of the C-ion species produced from **1α** and **1β** (Fig. 2, panel a and b) are much greater than those of the C-ion species produced from **8α** and **8β** (Fig. 2, panel c and d). This result suggests that the nucleophilicity of the nitrogen atom increases because of the addition of the alkyl group and is advantageous for the generation of C-ion under CID conditions. We have previously investigated the dissociation mechanism of 4-aminobutyl glycoside, which produced the C-ion species, and found that the dissociation reaction proceeded *via* a five-membered transition state.[27] The C_2-ions (*m*/*z* 365) produced from **8α** and **8β** (see Fig. 2c and 2d) were further subjected to CID conditions to produce a series of fragment ions, respectively (Fig. 3a and 3b). The most abundant

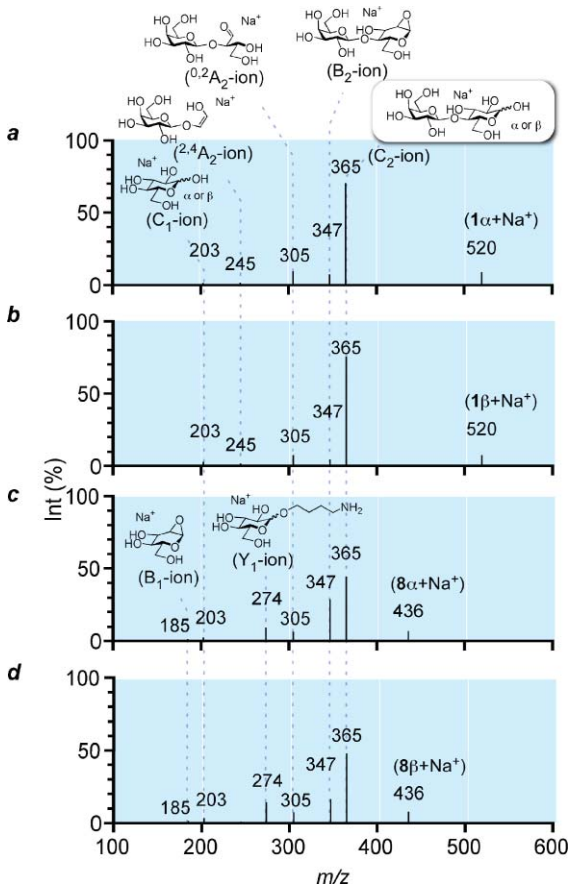


Fig. 2 MS/MS spectra obtained for dissociations of sodiated **1α/β** and **8α/β**. (a) **1α** yielded C_2-ion (sodiated lactose) exclusively. The spectra were obtained at an end-cap radio frequency amplitude (CID energy) of 1.02 V. (b) Dissociation of **1β** proceeded in a manner similar to that of **1α**; CID energy: 1.04 V. (c) Dissociation of **8α**; CID energy: 1.28 V. (d) Dissociation of **8β**; CID energy: 1.34 V. The CID energies were adjusted so that the intensities of the precursor ions became 10 to 20% of the sum of the heights of individual signals. In this manner all spectra can be compared and provide information about the heights of signals of interest among the different spectra.

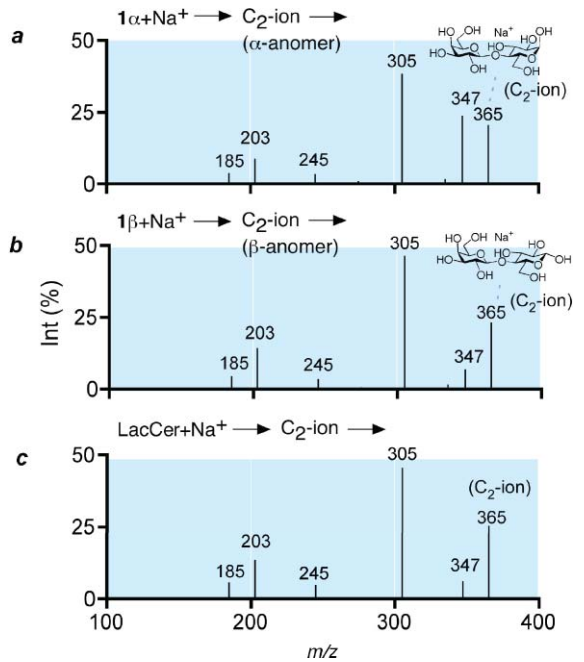
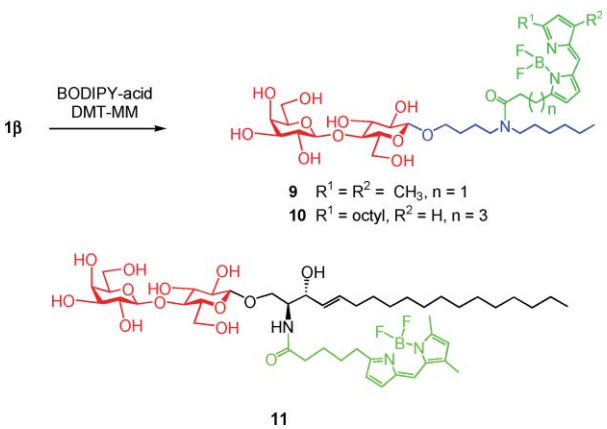


Fig. 3 MS/MS spectra obtained for dissociations of sodiated α- and β-lactose (C_2-ions). (a) C_2-ion (*m/z* 365) produced from **8α** was further subjected to CID conditions; CID energy: 1.00 V. (b) MS/MS spectrum of C_2-ion (*m/z* 365) produced from **8β**; CID energy: 1.08 V. (c) MS/MS spectrum of C_2-ion (*m/z* 365) produced from lactosyl ceramide; CID energy: 1.08 V. See Fig. 2 for the assignments of fragment ions. The CID energies were adjusted so that the intensities of the precursor ions became *ca.* 25% of the sum of the heights of individual signals.

ions in both spectra were $^{0,2}A_2$-ions (*m/z* 305), however, B_2-ion (*m/z* 347) and C_1-ion (*m/z* 203) were the second most abundant ions in the fragmentation of α- and β-lactoses, respectively. This difference is of extreme importance in determining the anomeric configurations of glycosidic linkages in a glycan structure as it was shown that the dissociation of a C-ion generated from ganglioside GM3 (Fig. 3c) resulted in a comparable MS/MS spectrum to that obtained for the corresponding β-hemiacetal. (Fig. 3b)[14,15]

The aglycone can be tagged to allow measurement of binding constants in protein–carbohydrate interaction

To examine the usefulness of a glycoside carrying an *N*-hexyl-4-aminobutyl group in the binding assay, the *N*-hexyl-4-aminobutyl lactoside **1β** was reacted with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propionic acid (BODIPY–FL C3-acid) in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) in THF[28] to afford a fluorescent molecule **9** (88%) (Scheme 2). The fact that the



Scheme 2 Conversion of **1β** into fluorescent probes **9** and **10**; also shown is the structure of **11**.

coupling reagent DMT-MM could be used in aqueous media as well was very promising.[29]

The binding between **9** and an *Erythrina cristagalli* lectin (ECA) was observed using a three-dimensional nanostructured protein hydrogel (3D NPH).[30] We performed the assay using the 3D NPH anticipating the future need for small-scale binding assays. The ECA-containing 3D-protein matrix, prepared by mixing ECA and a copolymer of *N*-acrylmorpholine and *N*-acyloxysuccinimide in a phosphate buffer, was found to enable the measurement of lactoside–ECA binding events on amino-functionalized glass using a few picomoles of **9**. The Ka value for **9** (0.38 mM^{-1})

obtained in this experiment was smaller than that reported for lactose (Ka = 3.8 mM^{-1}); the latter value was determined by isothermal titration microcalorimetry (Fig. 4).[31] This difference could be because of the β-fixed anomeric configuration and, possibly, the added hydrophobic aglycone moiety in the current investigation. It was shown that the newly introduced aglycone modified with a fluorescent tag was successfully used to evaluate binding constants in a lectin binding assay. Furthermore, the method based on 3D NPH itself should be widely applicable to the assay of fluorescently tagged molecules. Also, it should be noted that different fluorophores can easily be introduced to this type of glycoside after chemical or chemoenzymatical synthesis using the SPE isolation method described earlier.

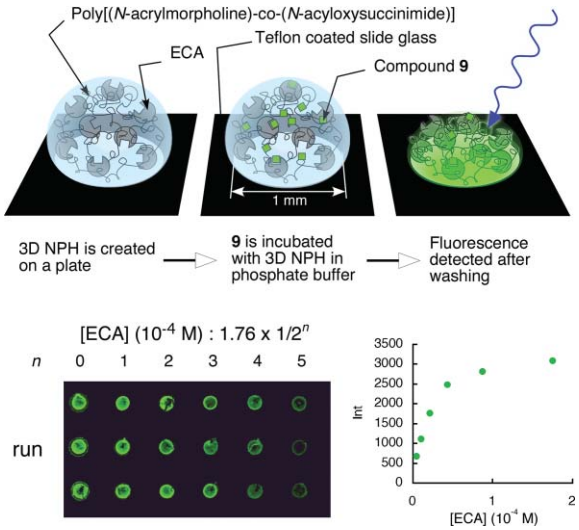


Fig. 4 An assay based on three-dimensional nanostructured protein hydrogel (3D NPH). The processes of creating the 3D NPH on partially Teflon-coated slide glass, incubating the 3D NPH with **9** and scanning the bound complex are shown in the upper diagram. The scan results, a photograph and a plot, for the binding between compound **9** and ECA captured in 3D NPH are also shown.

The aglycone can be tagged for cellular uptake

We further considered the incorporation of fluorescent tags with greater hydrophobicity, as to adjust the partition coefficient might allow observation of the cellular uptake of the molecule. Compound **11** is a well-known fluorescent molecule that is taken up by the cell and allows the Golgi apparatus to be visualized.[32] The log P value for **11** is 2.02, while that for **9** is –0.48. Thus, the 4,4-difluoro-5-octyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl group was chosen for introduction at the amino group. The calculated log P value for **10** was 2.7, which is close to that for **11**. A clear advantage of the newly introduced aglycone having an amino functionality is that it can be suitably modified before use according to the purpose for which it will be used. Thus, the cultured PC-12 (rat adrenal pheochromocytoma cell line) D cells[33] were treated with a complex of **10** and bovine serum albumin (BSA) to investigate whether cellular uptake of the glycoside had taken place. It was found that the cellular uptake of **10** took place in a manner similar to that of **11**, where the molecules accumulated to some extent at the Golgi apparatus (Fig. 5) but

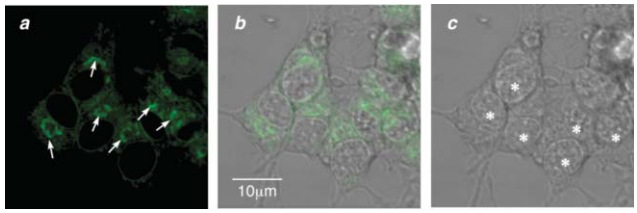


Fig. 5 Images of PC-12D cells. (a) Images of a region *ca.* 5 μm from the bottom of the cells; the excitation wavelength and emission wavelengths were 488 nm and 520 nm, respectively. The BODIPY-tagged compound **10** was internalized by endocytosis and incubated for 120 min; then, the cells were observed. Arrows indicate Golgi apparatus. (b) Image obtained by merging the fluorescent image (a) with the differential interference contrast (DIC) image (c). (c) DIC image. The asterisks indicate nuclei.

were scattered over the membranes under the culture conditions. The detailed localization pattern of **10** was slightly different from that of **11**.[34] Nevertheless, it is expected that the glycosides carrying the new aglycone could be used not only in histochemical analysis but also in investigating glycan processing. It should be noted that no obvious damage to the cultured cells was observed during the experiments. The results suggest that this type of molecule might be used as the "primers" in library synthesis based on cellular functions.[16–18]

Conclusion

The *N*-hexyl-4-aminobutyl glycoside was not only found to be a more effective source of a hemiacetal species (C-ions), which is highly useful in MS-based structural investigation, than 4-aminobutyl glycoside but was also found to be transformed easily into fluorescent molecules, and thus be useful for the structural and biological analysis of oligosaccharides. A molecule with a fluorescent tag was successfully used in a lectin binding assay. It was also suggested that different derivatization of the secondary amino function would enable experiments with a specific purpose to be carried out. It was experimentally shown that the uptake of a molecule with a fluorescent tag by cultured cells took place in a manner similar to that of BODIPY-labeled lactosyl sphingosine. We believe that this type of functional glycoside may widen the utility of synthetic oligosaccharides. Finally, it should be stressed that a library containing the aglycone should be of extreme importance because it would provide a variety of C-ion species useful in MS-based structural analysis and it could be used in various biological investigations.

Methods

General methods for synthesis

Thin-layer chromatography (TLC) was performed on Merck Art. 5715, Kieselgel 60 F_{254}/0.25-mm-thick plates. Preparative TLC was performed on Merck Art. 5745, Kieselgel 60 F_{254}/0.50-mm-thick plates. Visualization was carried out using UV light and a 1% $Ce(SO_4)_2$–1.5% $(NH_4)_6MoO_{24}{\cdot}4H_2O$–10% H_2SO_4 solution or a 0.5% ninhydrin *n*-BuOH solution followed by heating. Silica gel column chromatography was performed using Wakogel C–300 (Wako Pure Chemical Industries, Ltd.). Optical rotations were measured in a 1.0-dm tube using a Horiba SEPA–200

polarimeter. Melting points were measured with Yanaco MP-S3 micro melting point apparatus. IR spectra were obtained with a HORIBA FT-720 FREEXACT-II spectrometer with attenuated total reflection (ATR) method. ^{1}H NMR (500 MHz) spectra were recorded (Avance 500 spectrometer, Bruker Biospin Inc.) in a deuterated solvent using $(CH_3)_4Si$ (0.00 ppm) or the solvent peak (HDO: 4.79 ppm or CD_3OD: 3.31 ppm) as the internal standard. High resolution mass spectra (HRMS) were obtained on a liquid chromatography/mass spectrometry-ion trap-time-of-flight (LCMS–IT–TOF) spectrometer coupled with ESI interface (an IT–TOF mass spectrometer with a reflectron; Shimadzu Corp.) using sodium trifluoroacetate as the external standard for instrument adjustment.

γ-Hydroxybutanoicacid hexylamide 2

A mixture of γ-butyrolactone (1.0 g, 11.62 mmol) and *n*-hexylamine (1.2 g, 11.62 mmol) at 100 °C in a sealed tube was stirred for 20 h. The reaction mixture was subjected to silica gel column chromatography (EtOAc) to afford **2** (2.2 g, 99%) as a powder: R_f 0.25 (EtOAc); mp 43 °C (from Et_2O/*n*–hexane); IR ν_{max} (ATR)/cm^{-1} 3290 (OH), 1628 (C=O); ^{1}H NMR ($CDCl_3$) δ 5.71 (br s, 1H, N*H*), 3.70 (q, 2H, J = 5.3 Hz, HOCH_2), 3.24 (q, 2H, J = 7.1 Hz, NHCH_2), 3.12 (br s, 1H, O*H*), 2.35 {t, 2H, J = 6.5 Hz, CH_2(C=O)}, 1.88 (m, 2H, HOCH$_2$CH_2), 1.52–1.47, 1.34–1.26 {each m, 8H, NHCH$_2$(CH_2)$_4$CH$_3$}, 0.89 {t, 3H, J = 6.6 Hz, NHCH$_2$(CH$_2$)$_4$CH_3}. ^{13}C NMR ($CDCl_3$) δ 173.46, 62.32, 39.70, 34.07, 31.42, 29.46, 28.10, 26.54, 22.51, 13.98. Anal. Calcd for $C_{10}H_{21}NO_2$: C, 64.13; H, 11.30; N, 7.48. Found: C, 64.23; H, 11.09; N, 7.67.

N-Hexyl-*N*-trifluoroacetyl-4-aminobutanol 4

Lithium aluminium hydride (1.57 g, 41.3 mmol) was added to a solution of **2** in THF (30 mL), and the resulting mixture was stirred for 20 h at reflux. After confirming the disappearance of **2** by TLC, the reaction mixture was treated with H_2O and filtered through a Celite pad; then, the filtrate was concentrated. The residue was dissolved in EtOAc, washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. To a DCM (5 mL) solution of the residue containing amino alcohol **3** were added pyridine (5 mL) and trifluoroacetic anhydride (3.3 mL, 23.73 mmol) at 0 °C, and the mixture was stirred for 3 h at room temperature. After the addition of an excess amount of MeOH, the mixture was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (*n*-hexane:EtOAc = 2:1) to afford compound **4** (1.21 g, 55%) as an oil: R_f 0.48 (*n*-hexane:EtOAc = 1:1); IR ν_{max} (ATR)/cm^{-1} 3417 (OH), 1678 (C=O); ^{1}H NMR ($CDCl_3$) δ 3.69 (t, 2H, J = 6.3 Hz, CH$_2$CH_2OH), 3.43–3.33 {m, 4H, CH_2N(C=OCF$_3$)CH_2–}, 1.87 (br s, 1H, OH), 1.75–1.67 (m, 2H, CH_2CH$_2$CH$_2$OH), 1.64–1.54 (m, 4H, CH_2CH$_2$OH, CH_2CH$_2$CH$_2$CH$_2$CH$_3$), 1.33–1.27 (m, 6H, CH_2CH_2CH_2CH$_3$), 0.91–0.87 (m, 3H, CH$_2$CH_3). ^{13}C NMR ($CDCl_3$) δ 156.63 (q, J = 5.6 Hz, 35.6 Hz, *C*(=O)CF$_3$), 116.53 (q, J = 284.1 Hz, *C*F$_3$), 62.3, 62.07, 47.68, 47.66, 47.31, 47.29, 47.26, 46.92, 46.62, 31.40, 31.27, 29.46, 29.38, 28.66, 26.73, 26.41, 26.18, 25.25, 23.32, 22.46, 13.95, 13.91. Anal. Calcd for $C_{12}H_{22}F_3NO_2$: C, 53.52; H, 8.23; N, 5.20. Found: C, 53.50; H, 8.50; N, 5.05.

N-Hexyl-*N*-trifluoroacetyl-4-aminobutyl 4-*O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-2,3,6-tri-*O*-benzyl-α-D-glucopyranoside 6α and *N*-Hexyl-*N*-trifluoroacetyl-4-aminobutyl 4-*O*-(2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl)-2,3,6-tri-*O*-benzyl-β-D-glucopyranoside 6β

A mixture of thioglycoside **5**[19] (87 mg, 81.6 μmol), *N*-hexyl-*N*-trifluoroacetyl-4-aminobutanol **4** (61 mg, 0.225 mmol), and AW-300 (*ca*. 1 g) in DCE (2 mL) was stirred for 1 h under a N_2 atmosphere. NIS (36 mg, 0.153 μmol) and TfOH (15 μL) were added to this solution at 0 °C. The reaction was monitored by TLC and stopped with sat. $NaHCO_3$. After filtration through a Celite pad, the filtrate was poured into a sat. $NaHCO_3$ solution containing $Na_2S_2O_4$, and the products were extracted with DCM. The organic layer was washed with H_2O, dried ($MgSO_4$), and concentrated *in vacuo*. The residue was subjected to silica-gel column chromatography (*n*-hexane:EtOAc = 3:1–1:1) to obtain the corresponding lactosides (**6β**: 49 mg, **6α**: 39 mg, 94%). **6α**: R_f 0.33 (*n*-hexane:EtOAc = 3:1); $[\alpha]_D^{26}$ +14.6° (*c* 1.29 in $CHCl_3$); IR ν_{max} (ATR)/cm^{-1} 1685 (C=O); ^{1}H NMR ($CDCl_3$) δ 7.37–7.12 (m, 35H, Ph), 5.04 (d, 1H, J = 10.6 Hz, benzyl), 4.97 (d, 1H, J = 11.4 Hz, benzyl), 4.82–4.79 (m, 2H, benzyl), 4.76 (d, 1H, J = 11.3 Hz, benzyl), 4.73 (d, 1H, J = 11.1 Hz, benzyl), 4.71 (d, 1H, J = 12.7 Hz, benzyl), 4.69 (d, 1H, $J_{1,2}$ = 3.8 Hz, H–1), 4.66, 4.66 (each d, 1H, J = 11.9 Hz, benzyl), 4.60, 4.60 (each d, 1H, J = 12.1 Hz, benzyl), 4.55 (d, 1H, J = 11.4 Hz, benzyl), 4.51, 4.51 (each d, 1H, J = 12.0 Hz, benzyl), 4.35–4.28 (m, 3H, H–1′, benzyl), 4.22 (d, 1H, J = 11.8 Hz, benzyl), 3.90 (m, 1H, H–4), 3.90 (d, 1H, $J_{3',4'}$ = 2.2 Hz, H–4′), 3.84–3.80 (m, 2H, H–3,6a), 3.74 (br t, 1H, $J_{1,2}$ = $J_{2,3}$ = 8.6 Hz, H–2′), 3.68–3.62 (m, 2H, H5, OCH_2), 3.54–3.48 (m, 2H, H–6′a,6b), 3.48 (dd, 1H, $J_{2,3}$ = 9.6 Hz, H–2), 3.42–3.25 (m, 8H, H–3′,5′,6′b, –OCH_2, NCH_2), 1.70–1.50 {m, 6H, OCH$_2$(CH_2)$_2$CH$_2$NCH$_2$CH_2(CH$_2$)$_3$CH$_3$}, 1.33–1.20 {m, 6H, NCH$_2$CH$_2$(CH_2)$_3$CH$_3$}, 0.90–0.85 {m, 3H, NCH$_2$CH$_2$(CH$_2$)$_3$CH_3}. ^{13}C NMR ($CDCl_3$) δ 156.54 (q, J = 18.3 Hz), 139.40, 139.35, 139.00, 138.98, 138.86, 138.83, 138.53, 138.51, 138.48, 138.45, 138.10, 138.04, 137.99, 128.33, 128.30, 128.25, 128.12, 128.09, 127.99, 127.90, 127.87, 127.83, 127.78, 127.75, 127.71, 127.67, 127.62, 127.57, 127.53, 127.49, 127.48, 127.36, 127.33, 126.98, 126.96, 116.54 (q, J = 287.7 Hz), 102.86, 102.84, 97.16, 97.05, 82.45, 80.23, 80.21, 79.90, 79.88, 79.11, 79.08, 75.31, 75.10, 75.07, 74.67, 73.57, 73.52, 73.43, 73.36, 73.08, 73.07, 73.01, 72.98, 72.46, 70.30, 70.20, 68.10, 68.01, 67.49, 67.37, 47.60, 47.22, 56.88, 46.64, 31.42, 31.29, 28.65, 26.73, 26.60, 26.41, 26.18, 25.59, 23.72, 22.47, 13.96, 13.91. Anal. Calcd for $C_{73}H_{84}F_3NO_{12}$: C, 71.61; H, 6.91; N, 1.14. Found: C, 71.34; H, 6.82; N, 1.18.

6β: R_f 0.40 (*n*-hexane:EtOAc = 3:1); $[\alpha]_D^{26}$ +3.9° (*c* 1.35 in $CHCl_3$); IR ν_{max} (ATR)/cm^{-1} 1689 (C=O); ^{1}H NMR ($CDCl_3$) δ 7.67–7.10 (m, 35H, Ph), 5.02, 5.02 (each d, 1H, J = 10.6 Hz, benzyl), 4.97 (d, 1H, J = 11.4 Hz, benzyl), 4.85 (d, 1H, J = 10.8 Hz, benzyl), 4.82–4.70 (m, 5H, benzyl), 4.67 (d, 1H, J = 12.0 Hz, benzyl), 4.55 (d, 1H, J = 11.5 Hz, benzyl), 4.52, 4.51 (each d, 1H, J = 12.2 Hz, benzyl), 4.42 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H–1′), 4.37 (d, 1H, J = 12.1 Hz, benzyl), 4.35 (d, 1H, $J_{1,2}$ = 7.8 Hz, H–1), 4.33 (d, 1H, J = 11.9 Hz, benzyl), 4.23 (d, 1H, J = 11.8 Hz, benzyl), 3.96–3.89 (m, 2H, H–4, OCH_2–), 3.92 (d, 1H, $J_{3',4'}$ = 3.5 Hz, H–4′), 3.80–3.70 (m, 3H, H–2′,6a,6b), 3.56 (dd, 1H, $J_{2',3'}$ = 9.1 Hz, H–3′), 3.54–3.49 (m, 2H, H–6′a, OCH_2), 3.42–3.19 (m, 9H, H–2,3,5,5′,6′b, NCH_2),

1.76–1.48 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.33–1.20 {m, 6H, $NCH_2CH_2(CH_2)_3CH_3$}, 0.92–0.85 {m, 3H, $NCH_2CH_2(CH_2)_3CH_3$}. ^{13}C NMR ($CDCl_3$) δ 156.53 (q, J = 15.6 Hz), 139.05, 139.02, 138.99, 138.97, 138.74, 138.73, 138.64, 138.58, 138.45, 138.43, 138.28, 138.24, 138.01, 128.33, 128.30, 128.25, 128.23, 128.20, 128.17, 128.10, 128.01, 127.97, 127.92, 127.79, 127.76, 127.72, 127.68, 127.66, 127.64, 127.52, 127.51, 127.48, 127.43, 127.40, 127.37, 127.29, 127.05, 127.04, 116.55 (q, J = 288.7 Hz), 103.46, 103.44, 102.75, 102.73, 82.93, 82.46, 81.68, 79.88, 76.65, 75.33, 75.18, 75.17, 75.08, 75.06, 74.91, 74.65, 73.50, 73.47, 73.33, 73.01, 72.91, 72.89, 72.48, 69.17, 68.97, 68.28, 68.23, 67.99, 47.46, 47.43, 47.22, 47.20, 46.82, 46.52, 31.38, 31.25, 28.59, 26.94, 26.77, 26.70, 26.38, 26.15, 25.57, 23.68, 22.45, 13.95, 13.91. Anal. Calcd for $C_{73}H_{84}F_3NO_{12}$: C, 71.61; H, 6.91; N, 1.14. Found: C, 71.54; H, 6.89; N, 1.26.

N-Hexyl-*N*-trifluoroacetyl-4-aminobutyl 4-*O*-β-D-galactopyranosyl-β-D-glucopyranoside 7β

A mixture of compound **6β** (37 mg, 30.6 μmol) and 20% $Pd(OH)_2$/C (*ca.* 5 mg) in EtOH (1 mL) was stirred for 20 h under a H_2 atmosphere. The reaction mixture was filtered, and the filtrate was concentrated *in vacuo* to afford the de-*O*-benzyl product **7β** (13 mg, 72%): R_f 0.20 (EtOAc:MeOH = 3:1); $[\alpha]_D^{26}$–4.5° (*c* 1.03 in MeOH); m.p. 151–154 °C (crystallized from EtOH); IR ν_{max} (ATR)/cm^{-1} 3371 (OH), 1682 (C=O); ^{1}H NMR (CD_3OD): δ 4.36 (d, 1H, $J_{1',2'}$ = 7.6 Hz, H–1′), 4.29 (d, 1H, $J_{1,2}$ = 7.9 Hz, H–1), 3.95–3.81 {m, 3H, H–6a,6b, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 3.81 (d, 1H, $J_{3',4'}$ = 3.1 Hz, H–4′), 3.78 (dd, 1H, $J_{5',6'a}$ = 7.6 Hz, $J_{6'a,6'b}$ = 11.5 Hz, H–6′a), 3.69 (dd, 1H, $J_{5',6'b}$ = 4.6 Hz, H–6′b), 3.59–3.38 {m, 11H, H–2′,3,3′,4,5,5′, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 3.24 (dd, 1H, $J_{2,3}$ = 8.8 Hz, H–2), 1.78–1.50 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.37–1.29 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 0.92 {m, 3H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}. ^{13}C NMR ($CD_3OD$) δ 157.87 (q, J = 35.0 Hz), 118.09 (q, J = 286.9 Hz), 105.09, 104.20, 104.17, 80.70, 80.61, 77.08, 76.49, 76.44, 74.80, 74.74, 74.73, 72.55,70.28, 70.21, 70.08, 62.48, 61.95, 61.90, 48.12, 47.94, 32.63, 32.54, 29.71, 27.87, 27.77, 27.71, 27.54, 27.31, 26.58, 24.62, 23.62, 23.61, 14.36, 14.33. Anal. Calcd for $C_{24}H_{42}F_3NO_{12}$: C, 48.56; H, 7.13; N, 2.36. Found: C, 48.43; H, 7.09; N, 2.54.

N-Hexyl-*N*-trifluoroacetyl-4-aminobutyl 4-*O*-β-D-galactopyranosyl-α-D-glucopyranoside 7α

Compound **7α** was obtained from **6α** in the same manner as **7β** was obtained from **6β** (97%): R_f 0.18 (EtOAc:MeOH = 3:1); $[\alpha]_D^{26}$ +56.7° (*c* 1.19 in MeOH); IR ν_{max} (ATR)/cm^{-1} 3345 (OH), 1682 (C=O); ^{1}H NMR (CD_3OD) δ 4.78 (d, 1H, $J_{1,2}$ = 3.7 Hz, H–1), 4.35 (d, 1H, $J_{1',2'}$ = 7.6 Hz, H–1′), 3.88–3.65, 3.60–3.40 {each m, 18H, H–2,2′,3,3′,4,4′,5,5′,6a,6′a,6b,6′b, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.80–1.57 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.38–1.28 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 0.93-0.90 {m, 3H $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}. ^{1}H NMR ($D_2O$): δ 4.84 (d, 1H, $J_{1,2}$ = 3.8 Hz, H–1), 4.38 (d, 1H, $J_{1',2'}$ = 7.7 Hz, H–1′), 3.89–3.36 {m, 18H, H–2,2′,3,3′,4,4′,5,5′,6a,6′a,6b,6′b, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.70–1.50 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.26–1.18 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 0.79 {br s, 3H $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}. ^{13}C NMR ($CD_3OD$) δ 157.88 (q, J = 35.0 Hz), 118.08 (q, J = 287.2 Hz), 105.11, 105.08, 99.96, 99.93, 80.99, 80.90, 77.08, 74.83, 74.79, 73.48, 73.47, 73.22, 73.17, 72.55, 72.17, 72.12, 70.29, 68.71, 68.56, 62.50, 61.87, 61.84, 48.09, 47.99, 32.62, 32.52, 29.71, 27.75, 27.55, 27.52, 27.29, 26.69, 24.78, 23.61, 23.60, 14.34, 14.31. Anal. Calcd for $C_{24}H_{42}F_3NO_{12}$: C, 48.56; H, 7.13; N, 2.36. Found: C, 48.48; H, 7.20; N, 2.41.

N-Hexyl-4-aminobutyl 4-*O*-β-D-galactopyranosyl-β-D-glucopyranoside 1β

A MeOH (0.20 mL) solution of compound **7β** (10 mg, 20.5 μmol) containing NaOMe (0.16 mL, 1 M in MeOH) was stirred for 5 h at 25 °C. After evaporation of the solvent, the residue was applied to a SepPak C–18 column (H_2O to MeOH) to obtain **1β** (8.0 mg, 89%): $[\alpha]_D^{25}$ +3.9° (*c* 1.36 in H_2O); IR ν_{max} (ATR)/cm^{-1} 3356 (OH); ^{1}H NMR (CD_3OD): δ 4.36 (d, 1H, $J_{1',2'}$ = 7.6 Hz, H–1′), 4.29 (d, 1H, $J_{1,2}$ = 7.8 Hz, H–1), 3.93–3.90 {m, 1H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 3.89 (dd, 1H, $J_{5,6a}$ = 2.4 Hz, $J_{6a,6b}$ 12.1 Hz, H–6a), 3.84 (dd, 1H, $J_{5,6b}$ = 4.2 Hz, H–6b), 3.81 (d, 1H, $J_{3',4'}$ = 2.9 Hz, H–4′), 3.78 (dd, 1H, $J_{5',6'a}$ = 7.6 Hz, $J_{6'a,6'b}$ = 11.5 Hz, H–6′a), 3.69 (dd, 1H, $J_{5',6'b}$ = 4.6 Hz, H–6′b), 3.61–3.53 {m, 4H, H–2′,4,5′, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 3.52 (t, 1H, $J_{2,3} = J_{3,4}$ = 8.9 Hz, H–3), 3.84 (dd, 1H, $J_{2',3'}$ = 9.8 Hz, H–3′), 3.40 (ddd, 1H, $J_{4,5}$ = 9.4 Hz, H–5), 3.24 (dd, 1H, H–2), 2.66–2.54 {m, 4H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.69–1.49 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.39–1.28 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 0.95–0.89 {m, 3H $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}.^{13}C NMR ($CDCl_3$) δ 105.07, 104.23, 80.51, 77.09, 76.45, 76.44, 74.78, 74.75, 72.55, 70.51, 70.29, 62.71, 62.50, 61.83, 50.56, 50.20, 49.62, 32.91, 30.78, 30.06, 28.40, 28.11, 26.66, 23.67, 14.40. HRMS (ESI) *m*/*z* Calcd for $[C_{22}H_{43}NO_{11}Na]^+$: 520.2734. Found: 520.2729.

N-Hexyl-4-aminobutyl 4-*O*-β-D-galactopyranosyl-α-D-glucopyranoside 1α

Compound **1α** was obtained from **7α** in the same manner as **1β** was obtained from **7β** (quant.): $[\alpha]_D^{25}$ +102.1° (*c* 0.65 in H_2O); IR ν_{max} (ATR)/cm^{-1} 3332 (OH); ^{1}H NMR (D_2O): δ 4.84 (d, 1H, $J_{1,2}$ = 3.2 Hz, H–1), 4.38 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H–1′), 3.85–3.56 {m, 12H, H–3,3′,4,4′,5,5′,6a,6′a,6b,6′b, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 3.54 (dd, 1H, $J_{2,3}$ = 9.6 Hz, H–2), 3.47 (dd, 1H, $J_{2',3'}$ = 8.6 Hz, H–2′), 2.83–2.75 {m, 4H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.66–1.47 {m, 4H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 1.30–1.18 {m, 8H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}, 0.80 {br t, 3H, $OCH_2(CH_2)_2CH_2NCH_2CH_2(CH_2)_3CH_3$}. ^{13}C NMR ($CDCl_3$) δ 105.09, 99.93, 80.98, 77.10, 74.84, 73.49, 73.22, 72.58, 72.15, 70.30, 68.92, 62.51, 61.89, 50.59, 50.25, 32.89, 30.01, 28.23, 28.08, 26.91, 23.66, 14.38. HRMS (ESI) *m*/*z* Calcd for $[C_{22}H_{43}NO_{11}Na]^+$: 520.2734. Found: 520.2730.

N-Hexyl-*N*-{4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propanoyl}-4-aminobutyl 4-*O*-β-D-galactopyranosyl-β-D-glucopyranoside 9

DMT-MM (23.4 mg, 84.6 μmol) and H_2O (0.50 mL) were added to a solution of **1β** (12.4 mg, 24.9 μmol) and BODIPY–FL C–3 acid (14.6 mg, 50.0 μmol) in THF (1.99 mL). After the mixture was

stirred for 19 h at room temperature, it was concentrated, and the residue was purified by PTLC (EtOAc:MeOH:H_2O = 8:1.5:0.5) to afford **9** (15.8 mg, 82%). R_f 0.17 (EtOAc:MeOH:H_2O = 8.5:1.5:1); ^{1}H NMR (CD_3OD): δ 7.46 (s, 1H, BODIPY), 7.01 (d, 1H, J = 3.8 Hz, BODIPY), 6.32 (d, 0.4H, J = 4.0 Hz, BODIPY), 6.30 (d, 0.6H, J = 4.0 Hz, BODIPY), 6.23 (s, 1H, BODIPY), 4.36 (d, 0.6H, $J_{1',2'}$ = 7.6 Hz, H–1′), 4.34 (d, 0.4H, $J_{1',2'}$ = 7.7 Hz, H–1′), 4.29 (d, 0.6H, $J_{1,2}$ = 7.8 Hz, H–1), 4.22 (d, 0.4H, $J_{1,2}$ = 7.8 Hz, H–1), 3.94–3.75 {m, 5H, H–4′,6a,6b,6′a, OCH_2}, 3.69 (dd, 1H, $J_{5',6'b}$ = 4.6 Hz, $J_{6'a,6'b}$ = 11.4 Hz, H–6′b), 3.60–3.45 (m, 6H, H–2′,3,3′,4,5′, OCH_2), 3.42–3.38 (m, 0.6H, H–5), 3.37–3.18 {m, 7.4H, H–2,5, $OCH_2(CH_2)_2CH_2NCH_2(CH_2)_4CH_3$, CH_2CH_2–BODIPY}, 2.78 (q, 2H, J = 7.1 Hz, CH_2CH_2–BODIPY), 2.52, 2.29 (each s, 6H, BODIPY), 1.70–1.20 {m, 12H, $OCH_2(CH_2)_2CH_2NCH_2(CH_2)_4CH_3$}, 0.90, 0.84 {each t, 3H, J = 7.0 Hz, $OCH_2(CH_2)_2CH_2NCH_2(CH_2)_4CH_3$}. HRMS (ESI) m/z Calcd for $[C_{36}H_{56}BF_2N_3O_{12}Na]^+$: 794.3817. Found: 794.3826.

N-Hexyl-*N*-{4,4-difluoro-5-octyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl}-4-aminobutyl 4-*O*-β-D-galactopyranosyl-β-D-glucopyranoside 10

DMT-MM (2.1 mg, 7.42 μmol) and H_2O (0.05 mL) were added to a solution of **1β** (3.3 mg, 6.63 μmol) and C8–BODIPY–C5 acid (1.0 mg, 2.47 μmol) in THF (0.20 mL). After the mixture was stirred for 5 h at room temperature, it was concentrated, and the residue was purified by PTLC (EtOAc:MeOH = 8:2) to afford **10** (2.2 mg, quant). R_f 0.28 (EtOAc:MeOH = 8:2); ^{1}H NMR (CD_3OD): δ 7.39, 7.38 (each s, 1H, BODIPY), 7.11 (d, 1H, J = 4.1 Hz, BODIPY), 6.45, 6.44 (each t, 1H, J = 3.6 Hz, BODIPY), 6.42 (d, 1H, J = 4.1 Hz, BODIPY), 4.36, 4.35 (each d, 1H, $J_{1',2'}$ = 7.6 Hz, H–1′), 4.28 4.27 (each d, 1H, $J_{1',2'}$ = 7.7 Hz, H–1′), 3.94–3.83 (m, 3H, H–6a,6b, OCH_2), 3.81 (br d, 1H, $J_{3',4'}$ = 3.3 Hz, H–4′), 3.78 (br dd, 1H, $J_{5',6'a}$ = 7.5 Hz, $J_{6'a,6'b}$ = 11.4 Hz, H–6′a), 3.70 (dd, 1H, $J_{5',6'b}$ = 4.6 Hz, H–6′b), 3.65–3.46 (m, 6H, H–2′,3,3′,4,5′, OCH_2), 3.40–3.22 {m, 6H, H–2,5, $OCH_2(CH_2)_2CH_2NCH_2(CH_2)_4CH_3$}, 2.99, 2.94 (each t, 4H, J = 7.3 Hz, CH_2–BODIPY), 2.45, 2.41 {m, 2H, NC(=O)$CH_2(CH_2)_2CH_2$–BODIPY}, 1.85–1.27 {m, 24H, $OCH_2(CH_2)_2CH_2NCH_2(CH_2)_4CH_3$, NC(=O)$CH_2(CH_2)_2CH_2$–BODIPY}, 0.93–0.87 {m, 6H, $OCH_2(CH_2)_2CH_2NCH_2(CH_2)_4CH_3$, BODIPY–$CH_2(CH_2)_6CH_3$}. HRMS (ESI) m/z Calcd for $[C_{44}H_{72}BF_2N_3O_{12}Na]^+$: 906.5069. Found: 906.5078.

Mass spectral analysis (ESI–QIT–MS)

Samples were analyzed using a quadrupole ion trap mass spectrometer (QIT–MS) coupled with an electrospray interface (Bruker esquire 3000$^+$, Bruker Daltonics GmbH, Bremen, Germany). Samples dissolved in MeOH (0.002–0.02 μmol mL^{-1}) were introduced into the ion source by infusion (flow rate: 120 μL/hour). The parameters in the analysis were as follows: (1) "dry temperature": 250 °C, (2) nebulizer gas (N_2): 10 psi, (3) dry gas (N_2): 4.0 L min^{-1}, (4) "Smart frag": off, (5) Scan range: m/z 50–600 or 50–700, (6) Compound stability: 300%, (7) ICC target: 5000, (8) maximum acquisition time: 200 ms, (9) average: 10 spectra, (10) "cut-off": 27.6%, and (11) collision gas: He (6.0×10^{-6} mbar).

In our CID experiments, we raised the amplitude of the end-cap radio frequency in 0.02 V increments until the precursor ion could no longer be detected (energy-resolved mass spectrometry).[35–38] The sodiated compounds **1** (α and β) and **8** (α and β) were subjected to the abovementioned CID conditions. From a series of spectra thus obtained, spectra showing *ca.* 7% of the precursor ions were selected to enable quasi-quantitative comparison of MS/MS spectra.

Observation of binding of 9 with *Erythrina cristagalli* lectin (ECA)

The ECA was obtained from Sigma-Aldrich Inc. and stored as a solution in a sodium carbonate–bicarbonate buffer (pH 9.6, 0.05 M). Assays were performed on a micro-well slide (1.0-mmϕ type1 APS(aminopropylsilyl)-coated glass; Matsunami Glass Ind., Ltd.). To a solution of ECA (10 μL; the amount of ECA was varied in the range 1.76×10^{-4}–5.50×10^{-6} M) was added a solution of poly[(*N*-acrylmorpholine)-*co*-(*N*-acyloxysuccinimide)][30] (1.0 μL, 1% in H_2O). The mixture was deposited on each well and was kept under wet conditions for 2 h at 37 °C. The 3D-NPH areas were rinsed with 1 M ethanolamine and H_2O (three times). A PBS solution of **9** (2.4 μM, 1.0 μL) was deposited in each well after the well was dried. Each well was incubated for 5 min, rinsed three times with PBS, and then rinsed with H_2O. Fluorescence was detected at 513 nm by using the Affymetrix 428TM Array Scanner.

Incubation of 10 with PC-12D cells

PC-12D cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum, 5% fetal bovine serum, penicillin G (100 U/mL), streptomycin sulfate (100 μg mL^{-1}), and glutamine at 37 °C with 5% CO_2. Compound **10** was complexed with defatted BSA, as described.[39] Subconfluent cells on collagen-coated glass coverslips were rinsed and then incubated for 2 h at 37 °C in D-MEM containing 20 μM **10**/BSA complexes, 25 mM HEPES, N-2 Supplement (Invitrogen), and glutamine. After incubation, the cells were observed by fluorescent microscopy (Fluoview FV1000; Olympus).

Confocal microscopy

Laser-scanning confocal microscopy was performed using an Olympus Fluoview FV1000 confocal microscope with an UPlanSApo oil-immersion objective lens (60×, NA = 1.35; Olympus). Laser lines from an argon laser with a wavelength of 488 nm were used for excitation. The excitation laser beams were passed through a dichroic mirror (DM405/488), and the fluorescence emission was separated with beam splitters (SDM560) and passed through a barrier filter (500–530 nm). The laser unit, confocal microscope, and detection units were connected to a computer and controlled using Olympus Fluoview software (version 1.5).

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24 Amongst various ion species generated from a precursor under CID conditions, it was found that C- and Y-ions were of particular importance. The reason is that these ion species were found stable and eligible to be treated as "pure" ions under a given low energy CID conditions (ref. 16). This is quite important especially for the C-ion species considering the possibility of anomerization exists. Let us now consider a case that an obtained fragment was a mixture of ions with same m/z values (structural isomers). Fragment ions were generated from its precursor ion, the structure of which determines the dissociation reaction pathways. Therefore, the ratio of the isomeric fragments depends on the structure of a precursor. In this regards, MS/MS analysis of "a fragment peak" consisting of isomeric structures cannot be used for the spectral matching without caution in general. The analysis of some particular ions such as C- and Y-ions are important for the further MS/MS analysis in MS^n studies. The C-ions corresponding to the α- or β-lactoses are two of many important ion species, and are thus chosen. In fact, it was shown to provide the stereochemistry of a glycosidic linkage of glucose and ceramide in the structural investigation of GM3 (ref. 14). Having a larger array of compounds, such as those in a combinatorial library capable of producing important ion species, would be therefore very useful in the future investigation of totally unknown glycan structures.
25 We confirmed that some other disaccharides such as (1→3)- and (1→4)-linked Gal–GlcNAc, of which the reducing anomeric configurations were fixed as α and β, showed the same phenomena (ref. 15). It was possible to elucidate the partial structure of glycan structure based on the comparison of the CID spectra of these individual ions and those obtained from natural glycans (data not shown). Also, synthetic trisaccharides including branching structures produced C-ions as well and were used as standards (data not shown).
26 In the current investigation, sodiated ions were analyzed. We investigated the effect of metal ions, which revealed that adducts of Li^+ gave promising results with somewhat different peak intensities of individual fragment ions. K^+-adducts produced C-ions but MS/MS of the C-ion with K^+ resulted in dissociation of K^+. Rb^+ and Cs^+ dissociated from the precursor ions under the CID conditions without any visible fragments (data not shown). The protonated molecules did not generate C-ion species (ref. 14).
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