One-Step Synthesis of Biotinyl Photoprobes from Unprotected Carbohydrates

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A simple and versatile approach for the preparation of carbohydrate photoprobes has been developed. By a single-step reaction at 37 °C, a biotinylated carbene-generating unit was introduced to the reducing end of unprotected carbohydrates. Micromole quantities of *N*-acetyllactosamine, Lewis X trisaccharide, and sialyl Lewis X tetrasaccharide were easily converted to their biotinylated photoreactive analogues, which enabled the nonradioisotopic chemiluminescent detection of the photolabeled products. Thus, a sequence of lectin photoaffinity labeling, from the probe synthesis to the detection of labeled protein, was readily accomplished within one week. Our strategy may be applicable to any aldehyde-bearing ligand.

Introduction

We have developed a general method for the rapid preparation of photoreactive carbohydrate probes. Many biological phenomena are initiated by the recognition of carbohydrate ligands by endogenous receptor proteins.[1] The method of photoaffinity labeling enables the direct probing of the target protein through a covalent bond which is introduced between a ligand and its specific receptor.[2] Recently, phenyldiazirine derivatives which have an attached biotin residue have been developed as convenient nonradioactive reagents of photoaffinity labeling.[3] The method has been successfully applied to identification of the binding-site region of β1,4-galactosyltransferase.[3b,c] The combination of biotinyl photoprobes with mass spectrometric sequencing has become a powerful tool for the rapid analysis of protein−ligand interactions.[4] However, the application of photoaffinity labeling to carbohydrate-binding proteins often relies on multistep syntheses of photoreactive carbohydrate probes involving protection, activation and deprotection reactions.[5] More generally, the modification of oligosaccharides usually has to be performed on a very small scale due to ligand availability limitations.[6] Therefore, the current problems of natural and synthetic carbohydrate availability significantly retards their conversion to photoreactive analogues.

Results and Discussion

Here we report the novel photophore **6** (Scheme 1) for the one-step introduction of a carbene-generating phenyldiazirine into unprotected carbohydrates. The key feature of **6** is an aminooxy group. This is designed for the ligation of the photoreactive group to the terminal aldehyde of carbohydrates and is based on the recently developed chemoselective method.[7] A biotin tag is also pre-installed for enabling the nonradioactive detection and affinity isolation of photolabeled targets based on avidin−biotin technology. This novel photoreactive reagent **6** can be readily prepared as a Boc protected form **7** from a readily available aldehyde **1**.[8]

To demonstrate the effectiveness of our new approach, we coupled **6** to *N*-acetyllactosamine (LacNAc, **8**) under conventional ligation conditions.[7b] HPLC analysis revealed the presence of the three major peaks of products in the ratio of 2:6:1 (Figure 1), which suggests the formation of *E*/*Z* oxime isomers **9** and/or the existence of cyclic α/β anomers **10** (Scheme 2).[8] The isomerization was slow enough for the HPLC isolation of each product, and the structures of **9-*Z***, **9-*E***, and **10-β** were identified from their ^{1}H NMR spectra. According to the previous assignment of glucose oximes, doublets at 6.85 and 7.47 ppm were assigned as the oxime CH protons of *Z*- and *E*-form of **9**, respectively.[9] The doublet at 4.16 ppm was assigned as the anomeric proton of **10-β** based on its *J* value (9.8 Hz). The structural heterogeneity at the reducing end might be a synthetic drawback if this chemistry is considered for the application to stereoselective glycosylation.[10] However, many cell surface carbohydrates usu-

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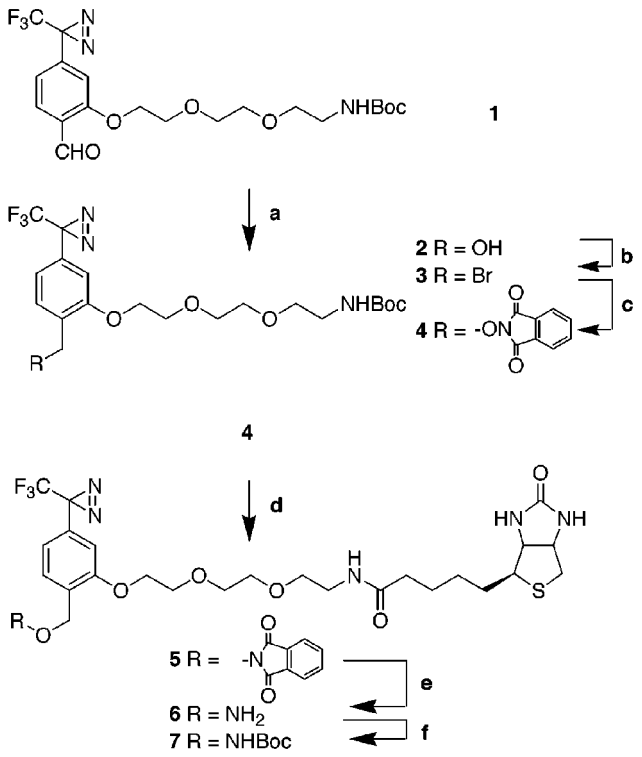
Scheme 1[a]



[a] Reagents: (a) $NaBH_4$, EtOH; (b) Ph_3P, CBr_4, CH_2Cl_2, 0 °C; (c) *N*-hydroxyphthalimide, DMSO, K_2CO_3; (d) (i) TFA, CH_2Cl_2, 0 °C, (ii) biotin *N*-hydroxysuccinimide ester, DMF, Et_3N; (e) NH_2NH_2, MeOH; (f) Boc_2O, $CHCl_3/CH_3CN$ = 1:1, Et_3N.

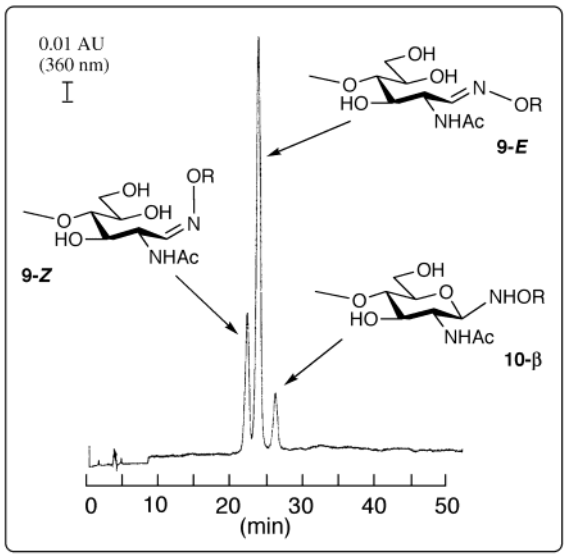


Figure 1. HPLC profile of LacNAc probe isomers at equilibrium. HPLC separation was performed under isocratic conditions with 85% water/acetonitrile at a flow rate of 1 mL/min. Peaks were monitored at 360 nm.

ally exist as an oligomeric chain structure. The binding of receptors to these carbohydrates mainly depends on the sugar structure at the nonreducing terminus,[11] and the configuration at the reducing end is likely to be less important for the binding of photoprobes. In such a case,

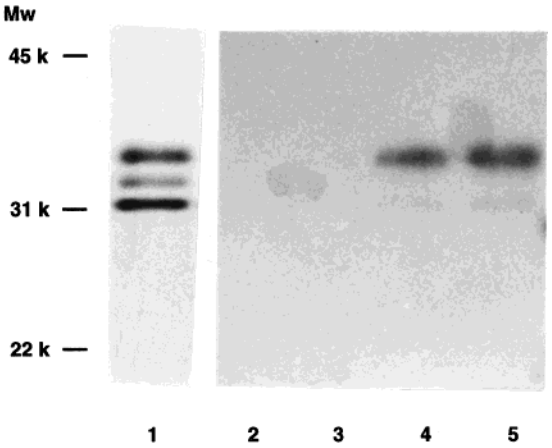
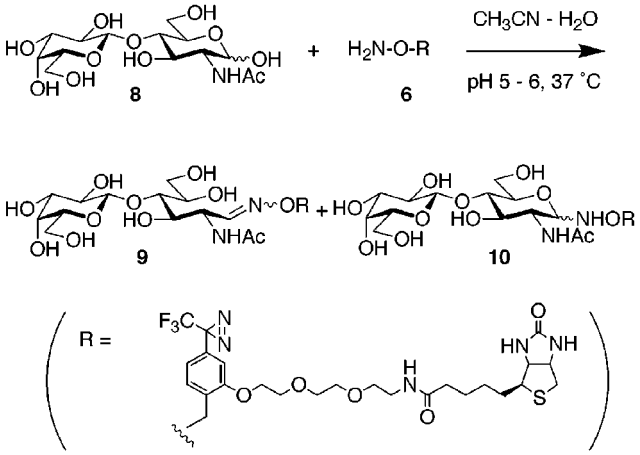


Figure 2. Silver-stained 12% SDS−polyacrylamide gel (lane 1) and the chemiluminescent detection result (lanes 2−5) of RCA lectin photolabeled with the isomeric mixture of LacNAc probe (lanes 2 and 5) and HPLC purified **9-*E*** (lanes 3 and 4), respectively. For the evaluation of specific photolabeling, 0.1 M of LacNAc was incorporated as the inhibitor of the photoprobe binding during the irradiation (lanes 2 and 3).

Scheme 2



the separation of each photoprobe isomer may not be necessary for the application of current method. The technique of reductive amination is widely accepted in the glycoconjugate synthesis as the acyclic type derivatization of reducing terminus.[12]

To examine this expectation, we directly compared the labeling ability of the LacNAc probe mixture with that of purified **9-*E*** using *Ricinus communis* agglutinin (RCA). RCA proteins are composed of two subunits, 30 kDa A and 37 kDa B chains (Figure 2, lane 1). A weak band between A and B is known to be an impurity resulting from the closely related other lectin.[13] Among these RCA proteins, only the RCA B chain possesses an affinity for the terminal β-D-galactosyl residues.[14] After the photolabeling, these bands were electroblotted from polyacrylamide gels onto poly(vinylidene difluoride) membranes for chemiluminescent detection of the photolabeled products (lanes 2−5).[5b] Both LacNAc probes equally and

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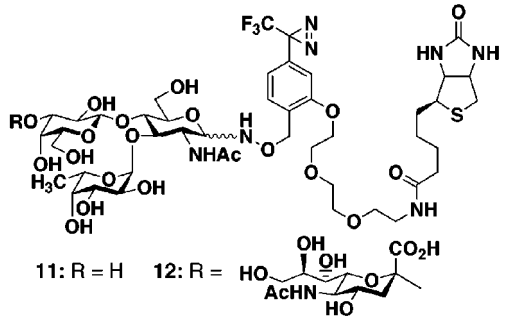
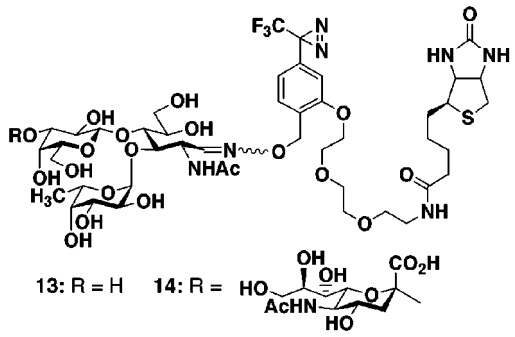
specifically identified the galactose binding 37 kDa subunit of RCA lectin (lanes 4 and 5). No detectable chemiluminescence was observed in the zone corresponding to the band of the A chain. Furthermore, the presence of a large excess of LacNAc efficiently prevented the photoincorporation of both probes by blocking the galactose binding-site in the RCA B chain (lanes 2 and 3).

Other biologically very important antigens,[15] Lewis X trisaccharide (Galβ1−4(Fucα1−3)GlcNAc, **Le^x**) and sialyl Lewis X tetrasaccharide (NeuAcα2−3Galβ1−4(Fucα1−3)GlcNAc, **sLe^x**), were next examined. By simple incubation with **6** at 37 °C, these sugars were also easily converted to the desired photoreactive conjugates. After the HPLC separation of probe peaks, the possible isomers of **Le^x** probe (**11** and **13**) and **sLe^x** probe (**12** and **14**) were not further purified and were directly used for photolabeling.





The **Le^x** binding lectin from *Lotus tetragonolobus*[16] was photolabeled with the **Le^x** probe (Figure 3, lanes 1 and 2) and the α2−3-linked sialic acid residue binding *Maackia amurensis* leucoagglutinin (MAL)[17] was examined with the **sLe^x** probe (lanes 3 and 4), respectively. Chemiluminescent detection again demonstrated that these probes clearly labeled the 27 kDa lotus lectin subunit (Figure 3, lane 2) and the 33 kDa MAL lectin protein (lane 4), respectively. The label incorporation into these bands was diminished in the presence of competitive sugar ligands, methyl α-fucopyranoside (lane 1) and *N*-acetyl-neuraminylα2−3lactose (lane 3), respectively. The result again shows that the isomeric mixtures of photoprobes are sufficiently useful for the identification of these lectins.

In summary, we have developed a rapid and versatile method for the microscale preparation of photoaffinity probes, whereby a biotinylated carbene-generating unit is introduced into various unprotected oligosaccharides. The biotinyl group may be changed to other reporter groups. A sequence of lectin photoaffinity labeling, from the probe synthesis to the detection of labeled protein, was readily accomplished within one week. Our strategy may be applicable to any aldehyde-bearing ligand. The present approach may extend the potential of photoaffinity labeling to become a rapid and more sensitive means for the elucidation of protein structures and binding-sites.

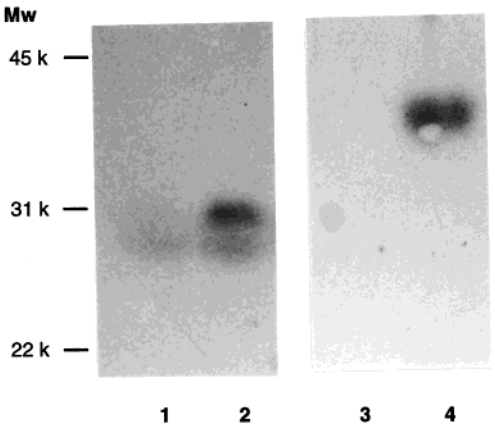


Figure 3. Pattern of chemiluminescent detection of biotin-tagged proteins from lotus lectin (lanes 1 and 2) and MAL lectin (lanes 3 and 4) after photolabeling. For the inhibition of specific photolabeling, 0.1 M of FucαOMe (lane 1) or NeuNAcα2−3lactose (lane 3) was incorporated, respectively, during the photolysis.

Experimental Section

General Experimental Procedures. The diazirine aldehyde **1** was prepared from 3-(3-methoxyphenyl)-3-(trifluoromethyl)-3*H*-diazirine.[8] Other chemical reagents were commercially available and were used without further purification. The reaction flasks containing all diazirine derivatives were protected from light by wrapping with aluminum foil. The fast atom bombardment (FAB) mass spectra were obtained by using 3-nitrobenzyl alcohol as the matrix. Silica gel for column chromatography was Kieselgel 60 (Merck, No. 7734, 70-230 mesh).

2-[2-[2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy]-ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl Alcohol (2). The aldehyde **1** (3.69 g, 8 mmol) was dissolved in EtOH (40 mL) followed by the addition of $NaBH_4$ (303 mg, 8 mmol) at 0 °C. After stirring at 0 °C for 30 min, the solvent was removed and the residue was suspended in H_2O. The pH of this mixture was adjusted to 3 with 1 N HCl at 0 °C and the alcohol **2** was extracted with ethyl acetate. The extract was washed with H_2O, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, and dried over $MgSO_4$. The solvent was removed by evaporation to leave the alcohol as a yellow oil which was used in the next step without further purification: ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 7.26 (d, J = 8.0 Hz, 1H), 6.79 (d, J = 8.0 Hz, 1H), 6.66 (br s, 1H), 6.01 (br, 1H), 5.02 (br, 1H), 4.66 (s, 2H), 4.22 (t, J = 4.8 Hz, 2H), 3.84 (t, J = 4.8 Hz, 2H), 3.7 (m, 2H), 3.6 (m, 2H), 3.51 (t, J = 5.1 Hz, 2H), 3.3 (m, 2H), 1.45 (s, 9H); MS (FAB$^+$) m/z 486 (100) [M + Na]$^+$, 464 (16) [M + H]$^+$; HRMS calcd for $C_{20}H_{29}F_3N_3O_6$ [M + H]$^+$ = 464.2008, found 464.1997.

2-[2-[2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy]-ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl Bromide (3). The crude alcohol **2** (prepared from 8 mmol of **1**), carbon tetrabromide (3.32 g, 10 mmol), K_2CO_3 (1.66 g, 12 mmol) and CH_2Cl_2 (16 mL) were placed in a flask. Triphenylphosphine (3.15 g, 12 mmol) was slowly added to above

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suspension at 0 °C. After stirring for 30 min at 0 °C, the reaction mixture was partitioned between CH_2Cl_2 and H_2O. The organic layer was dried over $MgSO_4$.and the solvent was removed under reduced pressure. The brown residue was chromatographed on silica gel (eluting with ethyl acetate/hexane = 1:1) to give **3** (3.62 g, 86% from **1**) as a yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 7.35 (d, J = 8.1 Hz, 1H), 6.77 (d, J = 8.1 Hz, 1H), 6.65 (br s, 1H), 5.00 (br, 1H), 4.51 (s, 2H), 4.21 (t, J = 4.8 Hz, 2H), 3.91 (t, J = 4.8 Hz, 2H), 3.75 (m, 2H), 3.65 (m, 2H), 3.55 (t, J = 5.1 Hz, 2H), 3.3 (m, 2H), 1.43 (s, 9H); MS (FAB$^+$) m/z 550 (41) [M + Na]$^+$, 548 (40), 528 (12) [M + H]$^+$, 526 (11); HRMS calcd for (^{79}Br) $C_{20}H_{28}BrF_3N_3O_5$ [M + H]$^+$ = 526.1164, found 526.1193.

2-[2-[2-(2-*tert*-Butoxycarbonylaminoethoxy)ethoxy]-ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl-oxyphthalimide (4). A mixture of the bromide **3** (2.63 g, 5 mmol), *N*-hydroxyphthalimide (0.98 g, 6 mmol), K_2CO_3 (0.69 g, 5 mmol), and DMSO (10 mL) was stirred for 12 h at room temperature. Purification on silica gel (hexane/acetone 3:1) gave **4** as a yellow oil (2.59 g, 85%): ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 7.82−7.73 (AB, 4H), 7.54 (d, J = 7.9 Hz, 1H,), 6.81 (d, J = 7.9 Hz, 1H,), 6.66 (br s, 1H), 5.27 (s, 2H), 5.05 (br, 1H), 4.15 (t, J = 4.8 Hz, 2H), 3.86 (t, J = 4.8 Hz, 2H), 3.7 (m, 2H), 3.6 (m, 2H), 3.54 (t, J = 5.2 Hz, 2H), 3.3 (m, 2H), 1.42 (s, 9H); MS (FAB$^+$) m/z 631 (100) [M + Na]$^+$, 609 (9) [M + H]$^+$; HRMS calcd for $C_{28}H_{32}F_3N_4O_8$ [M + H]$^+$ = 609.2172, found 609.2164.

2-[2-[2-(2-Biotinylaminoethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxyphthalim-ide (5). Deprotection of **4** (304 mg, 0.5 mmol) was performed with 1 mL of 50% TFA−CH_2Cl_2 at 0 °C. After stirring at 0 °C for 1 h, the reaction mixture was concentrated to provide a yellow oil. The resulting TFA salt of the deprotected amine was dissolved in DMF (0.5 mL) and the solution was cooled at 0 °C. Triethylamine (174 μL, 1.25 mmol) was added followed by a solution of *d*-biotin *N*-hydroxysuccinimide ester (171 mg, 0.5 mmol) in DMF (2 mL). The reaction flask was wrapped with aluminum foil and the mixture was stirred at room-temperature overnight. The mixture was concentrated under reduced pressure and the residue was dissolved in CH_2Cl_2/MeOH. The organic layer was successively washed with 1 N NaOH, saturated NaCl, 1 N HCl, and saturated NaCl. After drying over $MgSO_4$, the solvent was evaporated in vacuo. Chromatography of the residue on silica gel (eluting with $CHCl_3$/EtOH = 10:1) gave **5** (320 mg, 87%) as a colorless solid: ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 7.83−7.73 (AB, 4H), 7.53 (d, J = 7.9 Hz, 1H), 6.84 (s, 1H), 6.81 (d, J = 7.9 Hz, 1H), 6.64 (s, 1H), 6.61 (s, 1H), 5.64 (s, 1H), 5.26 (s, 2H), 4.5 (m, 1H), 4.3 (m, 1H), 4.15 (t, J = 4.8 Hz, 2H), 3.90 (t, J = 4.8 Hz, 2H), 3.7 (m, 2H), 3.65 (m, 2H), 3.55 (m, 2H), 3.4 (br m, 2H), 3.1 (m, 1H), 2.9 (m, 1H), 2.72 (d, J = 12.5 Hz, 1H), 2.19 (t, J = 7.5 Hz, 2H), 1.8−1.6 (m, 4H), 1.4 (m, 2H); MS (FAB$^+$) m/z 757 (16), [M + Na]$^+$, 735 (62) [M + H]$^+$; HRMS calcd for $C_{33}H_{38}F_3N_6O_8S$ [M + H]$^+$ = 735.2424, found 735.2458.

2-[2-[2-(2-Biotinylaminoethoxy)ethoxy]ethoxy]-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyloxycarbamic Acid *tert*-Butyl Ester (7). Phthaloyl group of **5** (73 mg, 0.1 mmol) was deprotected with 1 mL of 1 M hydrazine in methanol at room temperature. After 30 min, the solvent was evaporated and the residue was dissolved in toluene. The residual hydrazine was removed by azeotropic distillation in vacuo to give crude **6** which was dissolved in 1 mL of $CHCl_3$/CH_3CN (1:1). To this solution, triethylamine (70 μL, 0.5 mmol) and Boc_2O (109 mg, 0.5 mmol) were added and the mixture was stirred at room temperature overnight. The solvent was evaporated and the residue was chromatographed on silica gel (eluting with $CHCl_3$/EtOH = 10:1) to give **7** (64 mg, 95%) as a colorless solid: ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ 8.31 (s, 1H), 7.39 (d, J = 7.9 Hz, 1H), 6.97 (br, 1H), 6.81 (d, J = 7.9 Hz, 1H), 6.64 (s, 1H), 6.58 (s, 1H), 5.66 (s, 1H), 4.91 (s, 2H), 4.5 (m, 1H), 4.3 (m, 1H), 4.16 (t, J = 4.5 Hz, 2H,), 3.87 (t, J = 4.5 Hz, 2H), 3.7 (m, 2H), 3.65 (m, 2H), 3.55 (m, 2H), 3.4 (br m, 2H), 3.1 (m, 1H), 2.9 (m, 1H), 2.70 (d, J = 12.8 Hz, 1H), 2.17 (t, J = 7.3 Hz, 2H,), 1.8−1.6 (m, 4H), 1.45 (s, 9H), 1.4 (m, 2H); UV/VIS (MeOH) λ_{max} (ϵ) = 360 (400), 283 (2600); MS (FAB$^+$) m/z 727 (100), [M + Na]$^+$, 705 (12) [M + H]$^+$; HRMS calcd for $C_{30}H_{44}F_3N_6O_8S$ [M + H]$^+$ = 705.2893, found 705.2853

Ligation of 6 to Oligosaccharides. Deprotection of **7** was performed with TFA and the resulting **6** was incubated with a half equivalent of oligosaccharides. Typically, **7** (7.0 mg, 10 μmol) was treated with 200 μL of 50% TFA−CH_2Cl_2 at 0 °C for 30 min. After evaporation, the residue was dissolved in 80% aq. acetonitrile (250 μL) containing LacNAc **8** (1.9 mg, 5 μmol). After adjusting the pH to 5−6 with diisopropylethylamine, the mixture was incubated at 37 °C for 40 h in the dark. Separation on a silica gel HPLC column (Aquasil SS-1251, 4.6 250 mm, Sensyu Kagaku Co Ltd., Japan) gave the isomeric mixture of the glyco-conjugates. Products were monitored at 360 nm. The yields were determined from the UV spectra of MeOH solutions using the ϵ value of **7** (ϵ^{360} = 400) as standard.

LacNAc Probe (9, 10). HPLC solvent: 10−25% water/acetonitrile in 20 min at a flow rate of 1 mL/min. Peaks eluting at 19−21 min were collected to give a glyco-conjugate mixture: yield 63%. Reaction at pH 2 and pH 9 decreased the yields to 20% and 40%, respectively. Further HPLC separation (isocratic elution with 85% water/acetonitrile at a flow rate of 1 mL/min) gave pure **9-*Z***, **9-*E***, and **10-β**.

9-*Z*: ^{1}H NMR (400 MHz, D_2O, 25 °C) δ 7.35 (d, J = 8.0 Hz, 1H), 6.93 (d, J = 8.0 Hz, 1H), 6.85 (d, J = 5.8 Hz, 1H), 6.76 (s, 1H), 5.13 (s, 2H), 5.07 (dd, J = 7.6, 5.8 Hz, 1H), 4.43 (m, 1H), 4.23 (m, 1H), 4.13 (br, 2H), 3.96 (d, J = 7.6 Hz, 1H), 3.95 (m, 1H), 3.81 (m, 2H), 3.75−3.50 (14H), 3.37 (m, 2H), 3.26 (t, J = 5.2 Hz, 2H), 3.10 (m, 1H), 2.83 (m, 1H), 2.63 (d, J = 13.2, 1H), 2.08 (t, J = 7.2, 2H), 1.91 (s, 3H), 1.47 (m, 4H), 1.22 (m, 2H); HRMS calcd for $C_{39}H_{57}F_3N_7O_{16}S$ [M − H]$^-$ 968.3535, found 968.3594.

9-*E*: ^{1}H NMR (400 MHz, D_2O, 25 °C) δ 7.42 (d, J = 5.6 Hz, 1H), 7.34 (d, J = 8.0 Hz, 1H), 6.92 (d, J = 8.0 Hz, 1H), 6.73 (s, 1H), 5.06 (AB, 2H), 4.85 (dd, J = 9.3, 5.6 Hz, 1H), 4.44 (m, 1H), 4.23 (d, J = 7.8 Hz, 1H), 4.20 (m, 1H), 4.15 (br, 2H), 3.99 (m, 1H), 3.82 (m, 2H), 3.75−3.50 (14H), 3.41 (m, 2H), 3.26 (t, J = 5.2 Hz, 2H), 3.12 (m, 1H), 2.82 (m, 1H), 2.63 (d, J = 12.7, 1H), 2.08 (t, J = 7.2, 2H), 1.85 (s, 3H), 1.47 (m, 4H), 1.22 (m, 2H); HRMS found 968.3528.

10-β: ^{1}H NMR (400 MHz, D_2O, 25 °C) δ 7.34 (d, J = 7.8 Hz, 1H), 6.92 (d, J = 7.8 Hz, 1H), 6.74 (s, 1H), 4.67 (s, 2H), 4.43 (m, 1H), 4.34 (d, J = 7.8 Hz, 1H), 4.22 (m, 1H), 4.16 (d, J = 9.8 Hz, 1H), 4.14 (br, 2H), 3.81 (m, 3H), 3.75−3.50 (15H), 3.43 (m, 2H), 3.26 (t, J = 5.2 Hz, 2H), 3.12 (m, 1H), 2.83 (m, 1H), 2.63 (d, J = 13.2, 1H), 2.08 (t, J = 7.2, 2H), 1.88 (s, 3H), 1.47 (m, 4H), 1.22 (m, 2H); HRMS found 968.3635.

Le^x Probe (Mixture of 11 and 13). These were prepared from 0.5 mg of **Le^x** (0.94 μmol) and from peaks eluting at 23−24 min were collected to give a glyco-conjugate mixture: yield 78%; HPLC solvent: 10−30% water/acetonitrile in 15 min at a flow rate of 1 mL/min: MS (FAB$^-$) m/z 1114 (47) [M − H]$^-$; HRMS calcd for $C_{45}H_{66}F_3N_7O_{20}S$ [M − H]$^-$ 1114.4114, found 1114.4163.

sLe^x Probe (Mixture of 12 and 14). These were prepared from 1.0 mg of **sLe^x** (1.2 μmol) and from peaks eluting at 20−22 min were collected to give a glyco-conjugate mixture: yield 71%; HPLC solvent: 15−35% water/acetonitrile in 15 min at a flow rate of 1 mL/min: MS (FAB$^-$) m/z 1405 (29) [M − H]$^-$; HRMS calcd for $C_{56}H_{84}F_3N_8O_{28}S$ [M − H]$^-$ 1405.5068, found 1405.5143.

Photolabeling of Lectins. Irradiation and analysis were performed following established procedures.[5b] Thus, 50 μL each of photoaffinity labeling sample containing photoprobes (0.1 mM) lectin (0.1 mM) in 0.1 M phosphate buffer (pH 7.6) was also prepared. In the meanwhile, 50 μL each of control sample containing photoprobes (0.1 mM) lectin (0.1 mM) and an inhibitor (0.1M) in 0.1 M phosphate buffer (pH 7.6) was prepared. Carbohydrates used as inhibitors were LacNAc, FucαOMe, and NeuNAcα2−3lactose for RCA, lotus, and MAL lectins, respectively. The photoaffinity labeling and control samples prepared as above were incubated in the dark at 25 °C for 30 min. The samples at 0 °C on ice were then irradiated for 1 h from above with a 30-W long-wavelength UV lamp (Funakoshi XX-15) at the distance of 5 cm.

Chemiluminescent Detection of Photolabeled Products. After irradiation, each of the samples was separated by conventional methods using 12% SDS-polyacrylamide electrophoresis. Following SDS−PAGE, protein bands were electrotransferred onto a PVDF membrane (Immobilon P, Millipore) in 192 mM glycine, 25 mM Tris-HCl, 20% methanol, 0.1% SDS. Running time was 12 h at 12 mA and a constant temperature of 4 °C was maintained. The transferred membrane was blocked for 1 h at room temperature with phosphate-buffered saline with Tween (T-PBS: 0.1 M sodium phosphate, pH 7.3, 0.15 M NaCl, 0.1% Tween-20) containing 2% skimmed milk and then washed with T-PBS (5 min, two times). After soaking the membrane in 1,500 times diluted streptavidin−horseradish peroxidase conjugate (Amersham) for 1 h at room temperature, the membrane was developed using chemiluminescent detection reagents (RENAISSANCE, DuPont NEN) for 1 min. The membrane was then wrapped in a plastic sheet and exposed to Hyperfilm−ECL (Amersham) in the dark. The results are shown in Figures 2 and 3.

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