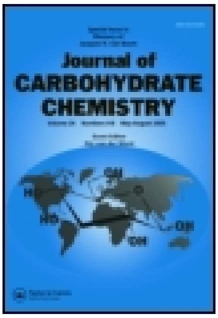

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Synthesis of Le^X and Le^Y Oligosaccharides with Azido-Type Spacer-Arms. Comparison of 3- and 4-Methoxybenzyl Groups as Key Temporary Protective Groups

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SYNTHESIS OF LE^X AND LE^Y OLIGOSACCHARIDES WITH AZIDO-TYPE SPACER-ARMS. COMPARISON OF 3- AND 4-METHOXYBENZYL GROUPS AS KEY TEMPORARY PROTECTIVE GROUPS[1]

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ABSTRACT

5-Azido-3-oxa-1-pentanol was prepared from 2-(2-chloroethoxy)ethanol and used as a spacer in the chemical synthesis of the trisaccharide β-D-Gal-(1→4)-[α-L-Fuc-(1→3)]-GlcNAc and the tetrasaccharide α-L-Fuc-α-(1→2)-β-D-Gal-(1→4)-[α-L-Fuc-(1→3)]-GlcNAc that represent the epitopes defining the human blood groups Le^x and Le^y. The classical 4-methoxybenzyl group and the remarably acid-stable 3-methoxybenzyl group were compared as temporary protective groups for position 3 at the glucosamine unit to circumvent the problems associated with the simultaneous presence of allyl and azido groups. The resulting oligosaccharides were coupled to proteins with high efficiency.

INTRODUCTION

Spacer-arm derivatives of oligosaccharides are frequently used for coupling to proteins. Several neoglycoproteins have been prepared in this way as immunogens for the production of carbohydrate-specific antibodies[2,3] in animals or even as possible therapeutic tools for humans.[4,5] However, after a multistep synthesis, very often a precious oligosaccharide is coupled to a protein with a yield not exceeding 20%, based on the oligosaccharide.

We[6] and others[7,8] recently developed the use of 5-azido-3-oxa-1-pentanol for the preparation of spacer-armed oligosaccharides. The reduction of the azido group to an amine group followed by derivatization as a maleimide allowed almost quantitative coupling between the oligosaccharide and protein by using only a slight molar excess of the oligosaccharide. Other thiophilic groups introduced into the spacer through the terminal amino function were also coupled efficiently.

In an attempt to prepare the Le^x and Le^y haptens using this spacer, the classical allyl temporary protective group strategy failed owing to the problems associated with the selective removal of the allyl group in the presence of an azido function. In the present paper, we describe the synthesis of type 2 Lewis oligosaccharides and compare the use of 4-methoxybenzyl and the more acid resistant 3-methoxybenzyl group[9] in the place of allyl.

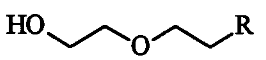
RESULTS AND DISCUSSION

2-(2-Chloroethoxy)ethanol (**1**) could be transformed directly by the action of sodium azide, tetrabutylammonium iodide and dicyclohexano-18-crown-6 in butanone into 5-azido-3-oxa-1-pentanol (**2**) in excellent yield. The reaction of 2-deoxy-2-acetamido-3,4,6-tri-*O*-α-D-glucopyranosyl chloride with **2** in the presence of mercury(II) cyanide proceeds smoothly to give crystalline glucosaminide **3** that was deacetylated and transformed into a key benzylidenated intermediate **4**, isolated by crystallization.

In previous syntheses of Le^x and Le^y, different strategies[10] were employed but most use the allyl group as a temporary protective group[11] for position 3. In our case, the presence of allyl and azido groups in the same molecule caused attempts at selective removal of the allyl group[12] to give low yields and complex product mixtures. In search of a better protecting group, we first tried the 4-methoxybenzyl group as previously reported.[13] Preliminary results pointed to the advantage of a more acid stable group in our sequence. We decided to assess the performance of both the 4- and the 3-methoxybenzyl groups during the syntheses of type 2 Lewis oligosaccharides.

The benzylidene intermediate **4** was methoxybenzylated using the corresponding methoxybenzyl bromides[14] in the presence of barium oxide-barium hydroxide[15] in DMF.

Both crystalline derivatives **5** and **6** were then treated with sodium cyanoborohydride and HCl/ether in tetrahydrofuran[16] to afford the 6-*O*-benzyl derivatives **7** and **8**. The yields were similar in the two cases, but for the 4-methoxybenzyl group, the acidity had to be controlled very carefully. The structures of **7** and **8** were ascertained by ^{13}C NMR spectroscopy (see Table 1); the signals corresponding to C-6 were almost unaffected by substitution (**7**, 68.5 ppm → 68.7 ppm and **8**, 68.5 ppm →68.6 ppm) while those corresponding to C-4 were shielded (**7**, 82.1 ppm → 75.5 ppm; **8**, 82.6 ppm →75.2ppm).



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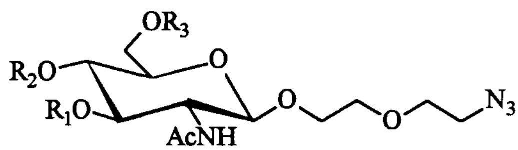
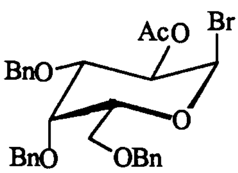
2 R = N_3



3 $R_1 = R_2 = R_3$ = Ac
4 R_1 = H, R_2, R_3 = PhCH
5 R_1 = mMBn, R_2, R_3 = PhCH
6 R_1 = pMBn, R_2, R_3 = PhCH
7 R_1 = mMBn, R_2 = H, R_3 = Bn
8 R_1 = pMBn, R_2 = H, R_3 = Bn



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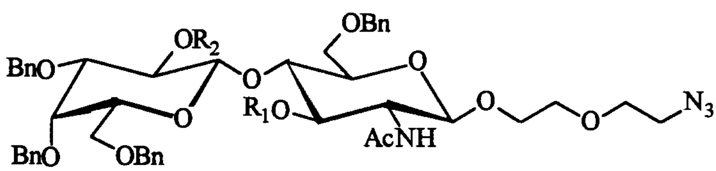


10 R_1 = mMBn, R_2 = Ac
11 R_1 = pMBn, R_2 = Ac
12 R_1 = H, R_2 = Ac
13 $R_1 = R_2$ = H

Galactosylations of **7** and **8** were performed with 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-galactopyranosyl bromide[11e] in dichloromethane in the presence of silver triflate.[17]

Table 1. ^{13}C NMR spectral data[a] for compounds **3-14**

compd	Glucosamine						Galactose					
	C1	C2	C3	C4	C5	C6	C1`	C2`	C3`	C4`	C5`	C6`
3	100.9	54.0	72.4	70.8	71.5	62.0						
4	101.5	56.1	70.4	81.2	66.0	67.9						
5	100.9	56.2	77.6	82.1	65.8	68.5						
6	100.8	57.2	76.4	82.6	66.0	68.5						
7	101.7	55.6	83.0	75.1	72.3	68.7						
8	101.2	56.2	81.5	75.2	72.6	68.6						
10	101.3	52.7	79.1	76.0	75.2	68.5	100.9	72.2	80.7	73.2	73.8	69.5
11	101.2	51.4	77.5	75.1	75.1	68.4	100.6	72.2	80.6	73.4	73.9	69.0
12	101.5	55.8	73.1	80.4	74.1	68.5	100.7	71.1	80.0	72.1	73.6	68.8
13	101.3	56.5	73.1	82.4	74.0	68.2	104.4	71.1	82.0	73.2	73.8	68.8
14	99.6	56.3	73.3	72.2	75.8	68.5	99.8	72.0	80.3	72.9	72.4	68.2
Fuc	97.0	75.2	79.6	78.2	66.6	16.2						
15	99.3	59.6	73.6	73.1	75.4	68.4	100.2	72.8	83.8	73.3	72.3	67.8
Fuc	98.0	75.3	79.8	78.2	66.7	16.2	97.8	75.7	78.9	78.0	66.4	16.1

a. Chemical shifts for protective groups are as follows: CH_2N_3 50.1-50.8 ppm; 3-methoxybenzyl 140.5, 113.5, 160.3, 113.9, 129.7 and 120.4 ppm; 4-methoxybenzyl 134.7, 131.4, 114.2 and 159.8 ppm; benzylidene 101.2, 127.4-129.3 ppm; Bn 72.0-74.6 and 127.4-129.3 ppm; CH_3CONH 23.2-23.4 and 170.1-170.4 ppm; CH_3CO 20.9 and 169.3 ppm.

These conditions led to disaccharide **10** in 60% yield. However, disaccharide **11** was obtained[18] in acceptable yield only when *N,N*-diisopropylethyamine was added to prevent the acid cleavage of the 4-methoxybenzyl group. The structures of disaccharides **10** and **11** were confirmed by the presence in their ^1H NMR spectra of doublets at 4.55 ppm (J 7.7 Hz) and 4.48 ppm (J 7.0 Hz), respectively.

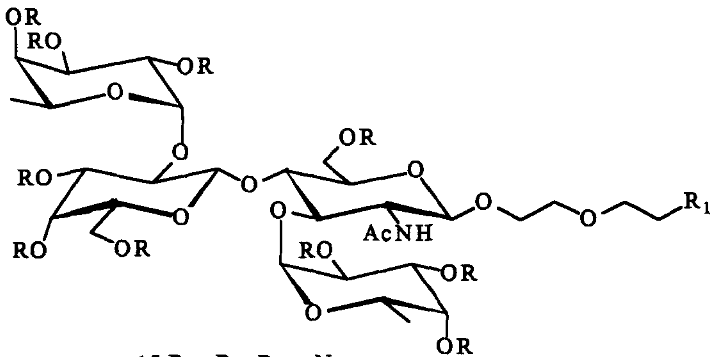
In previous reactions, even if the yields were only slightly different, the two groups displayed distinctive behavior under acidic conditions. The 3-methoxybenzyl group is as stable as a benzyl or allyl group, while the 4-methoxybenzyl group was as acid sensitive as an acetal.

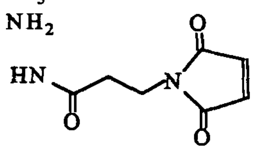
Oxidative removal of *O*-methoxybenzyl groups from disaccharides **10** and **11** with DDQ in dichloromethane-water proceeded better for **11** as expected. It was also possible to remove the 3-methoxybenzyl group from **10** albeit in a lower yield as these conditions led to partial removal of some benzyl groups on the galactose unit. The remarkable acid stability of the latter group deserves future study to improve the selectivity of deprotection. The structure of **12** was established based upon the absence of the signal corresponding to the methoxybenzyl group and by the shielding of the C-3 signal (79.1 ppm → 73.1 ppm) in the ^{13}C NMR spectrum.

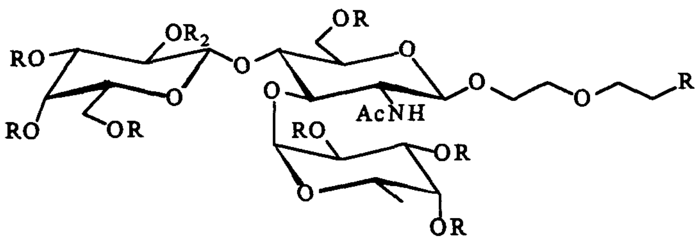
Fucosylation of **8** was performed with tri-*O*-benzyl-α-L-fucopyranosyl bromide[19,20] as the donor using the halide-ion catalyzed reaction[21] to give the α-L-linked trisaccharide **14** in 82% yield. The disaccharide **12** was first deacetylated and then the acceptor **13** was di-α-L-fucosylated in the same way to afford the Lewis^y tetrasaccharide **15** in 60% yield.

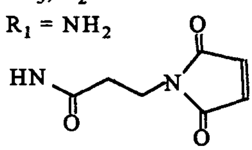
After hydrogenolysis, the spacer amino group of the corresponding Le^x and Le^y free oligosaccharides (**16** and **17**) reacted with the *N*-hydroxysuccinimide derivative of β-maleimidopropionic acid following the procedure previously described for model oligosaccharides[22] to give the corresponding β-maleimidopropionamide derivatives **18** and **19**. The ^{1}H NMR spectra showed complete transformation of the free amino group into the corresponding amides (δ 3.14→3.26). Small amounts of β-maleimidopropionic acid, that were very difficult to remove, were detected by the presence of triplets at 2.44 ppm in the ^{1}H NMR spectra. Compounds **18** and **19** are stable in aqueous solution at pH < 6.5 but hydrolysed slowly at pHs above this value. However, the reactions with the BSA thiol-groups were several times faster than hydrolysis at pH 7.2 and proceeded smoothly with only one equivalent of oligosaccharide per SH-group.

Incorporations ranging from 13 to 20 mol of oligosaccharide per mol of BSA were usually obtained that represent yields between 50-80 % based on the oligosaccharide. The presence of the β-maleimidopropionic acid did not affect the rate of linkage formation to the oligosaccharide. The use of this and other neoglycoproteins as immunogens for the preparation of monoclonal antibodies is now in progress.



15 $R = Bn, R_1 = N_3$
16 $R = H, R_1 = NH_2$
18 $R = H, R_1 =$ 



14 $R = Bn, R_1 = N_3, R_2 = Ac$
17 $R = R_2 = H, R_1 = NH_2$
19 $R = H, R_1 =$ 

EXPERIMENTAL

General procedures. Optical rotations were measured at 25 °C with a POLAMAT A automatic polarimeter, using a 5 cm 5 mL cell. NMR spectra were recorded at 25 °C with a BRUKER AC-250F spectrometer. Chemical shifts (δ) are given in ppm relative to the signal for internal tetramethylsilane for ^{1}H NMR specrtra and are referenced to the central line of $CDCl_3$, δ 77.03, for ^{13}C NMR spectra. Assignments were made on the basis of homonuclear and heteronuclear correlation experiments. The following notations are used for identification of monosaccharide units in the NMR spectra: ' for Gal; f and f' for the Fuc unit linked to GlcNAc or Gal, respectively.

All compounds were purified by column chromatography on Kieselgel 60 (Fluka, < 230 mesh ASTM) and fractions were monitored by TLC on Kieselgel 60 F_{254} (Merck). Detection was effected by charring with sulfuric acid after examination under UV light. Evaporations were conducted under reduced pressure at 40 °C (bath).

5-Azido-3-oxa-1-pentanol (2).[23] To a solution of 2-(2-chloroethoxy)ethanol (**1**) (5 mL, 47 mmol) in 2-butanone (25 mL) was added sodium azide (4.5 g, 69 mmol), tetrabutylammonium iodide (2.5 g, 6 mmol) and dicyclohexano-18-crown-6 (10 mg). The mixture was refluxed at 90 °C for 24 h, when ^{13}C NMR spectroscopy of the supernatant liquid showed the absence of a signal at δ 42.7 and the presence of a strong signal at δ 50.0 ppm. The mixture was filtered, the solids were rinsed with acetone and the combined solutions were concentrated. Distillation of the residue gave, at 60-80 °C and 0.2 mbar, compound **2** (4.8 g, 78.6%): ^1H NMR ($CDCl_3$) δ 3.71-3.6 (m, 6H, CH_2O) and 3.41 (t, 2H, CH_2N_3); ^{13}C NMR ($CDCl_3$) δ 72.37 and 69.84 (CH_2O), 61.56 (CH_2OH) and 50.62 (CH_2N_3).

5-Azido-3-oxapentyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyrannoside[24] (3). A solution of 2-acetamido-2-deoxy-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride (5 g, 15.1 mmol) and compound **2** (2.33 g, 17.7 mmol) in anhydrous dichloromethane (20 mL) containing 0.4 nm molecular sieves (5 g) and drierite (5 g) was stirred for 1 h under a nitrogen atmosphere. Mercury(II) cyanide (3.45 g, 13.6 mmol) was added and the mixture was stirred for 48 h. Then, the mixture was diluted with dichloromethane (50 mL), filtered through Celite and the filtrate washed with aqueous 10% potassium iodide (40 mL), saturated aqueous sodium hydrogen carbonate (40 mL), water, dried (Na_2SO_4), filtered and concentrated. Crystallisation from ethyl acetate/diethyl ether afforded **4** (4.84 g, 77.5%): mp 100-102 °C; $[\alpha]_D$ +54.0° (*c* 1.0, chloroform); R_F 0.54 (dichloromethane/acetone, 4:1 v/v); ^1H NMR ($CDCl_3$) δ 6.35 (δ, 1H, J = 10.2 Hz, NH), 5.21-5.28 (m, 2H, H-3, 4), 4.78 (d, 1H, $J_{1,2}$ = 7.6 Hz, H-1), 4.35 (dd, $J_{6a,6b}$ = 10.3 Hz, $J_{5,6a}$ = 5.3 Hz, 1H, H-6a), 4.15 (dd, 1H, H-6b), 3.95 (m, 1H, H-2), 3.75 (m, 1H, H-5), 3.65 (m, 6H, CH_2 spacer), 3.42 (t, 2H, CH_2N_3) and 2.1-1.95 (3s, 12H, CH_3CON and CH_3COO).

Anal. Calcd. for $C_{18}H_{28}O_{10}N_4$ (460.64): C, 46.95; H, 6.12, N, 12.17. Found: C, 47.26; H, 5.92; N 12.11.

5-Azido-3-oxapentyl 2-Acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (4). To a solution of compound **4** (3.8 g, 8.2 mmol) in dry methanol (50 mL), was added sodium methoxide (0.1 M) to pH 9. After 1 h, the reaction was neutralised with Dowex-50 (H^+) resin, filtered and concentrated. A solution of the residue (2.56 g, 7.6 mmol) and anhydrous zinc chloride (2.7 g, 7.6 mmol) in benzaldehyde (16 mL, 146.2 mmol) was stirred for 24 h. The mixture was then poured into ice-water (250 mL) and hexane (100 mL) with vigorous stirring. The solid was filtered, rinsed thoroughly with hexane and crystallised from absolute ethanol to afford **4** (2.6 g, 65%): mp 230-232 °C; $[\alpha]_D$ -72.0° (*c* 1, DMSO); R_F 0.27 (dichloromethane/acetone, 4:1 v/v); ^{1}H NMR (DMSO-d_6) δ 7.84 (d, 1H, J = 8.5 Hz, NH), 7.47-7.37 (m, 5H, Ph), 5.61 (s, 1H, PhC*H*), 5.32 (d, 1H, $J_{3,OH}$ = 5.4 Hz, OH-3), 4.54 (d, 1H, $J_{1,2}$ = 7.9 Hz, H-1), 4.25 (dd, 1H, $J_{5,6a}$= 4.6 Hz, $J_{6a,6b}$ = 10.0 Hz, H-6a), 3.74 (m, 1H, H-6b), 3.58 (m, 2H, H-2,3), 3.41 (m, 3H, H-4, C*H*$_2$N$_3$), 3.34 (m, 1H, H-5) and 1.84 (s, 3H, C*H*$_3$CON).

Anal. Calcd for $C_{19}H_{26}O_7N_4$ (422.43): C, 54.01; H, 6.20; N, 13.26. Found: C, 53.65; H, 6.58; N, 13.33.

5-Azido-3-oxapentyl 2-Acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(3-methoxybenzyl)-β-D-glucopyranoside (5). To a solution of **4** (2.5 g, 5.35 mmol), barium oxide (4.1 g, 26.75 mmol) and $Ba(OH)_2.8H_2O$ (740 mg, 2.3 mmol) in *N,N*-dimethylformamide (20 mL) was added 3-methoxybenzyl bromide (1.83 mL, 12.6 mmol) and the mixture was stirred for 15 min at rt. Dichloromethane (50 mL) was then added and the resulting mixture was refluxed for 1 h and then filtered through Celite. The filtrate was washed with 2% hydrochloric acid (25 mL), saturated aqueous sodium hydrogen carbonate (25 mL) and water (25 mL), then dried and concentrated. After the addition of toluene (10 mL) the solid was filtered, rinsed thoroughly with toluene and recrystallized from ethyl acetate to afford **5** (2.6 g, 85%): mp 207-209 °C; $[\alpha]_D$ -15.6° (*c* 0.78, chloroform); R_F 0.69 (dichloromethane/ acetone 5:1 v/v); ^{1}H NMR (CDCl$_3$) δ 7.5-7.2 (m, 6H, Ph), 6.65 (m, 3H, H-2, 4, 6 PhOMe), 6.35 (d, 1H, J = 8.5 Hz, NH), 5.65 (s, 1H, PhC*H*), 4.95 (d, 1H, J = 8.3 Hz, H-1), 4.70 (AB, C*H*$_2$PhOCH$_3$), 4.36 (dd, 1H, $J_{5,6a}$= 4.1 Hz, $J_{6a,6b}$ = 10.25 Hz, H-6a), 4.10 (t, $J_{2,3}$ = 9.3 Hz, 1H, H-3), 3.95 (m, 1H, H-2), 3.73 (m, 5H, H-4, H-6b, C*H*$_3$O), 3.50 (m, 1H, H-5), 3.38 (t, 2H, J = 4.9 Hz, C*H*$_2$N$_3$) and 1.95 (s, 3H, C*H*$_3$CON).

Anal. Calcd for $C_{27}H_{34}O_8N_4$ (542.59): C, 59.77; H, 6.32; N, 10.33. Found: C, 59.30; H, 6.58; N, 10.20.

5-Azido-3-oxapentyl 2-Acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(4-methoxybenzyl)-β-D-glucopyranoside (6). Compound **6** was obtained from **4** as described above for the preparation of **5**: yield 83.2%; mp 207-209 °C; $[\alpha]_D$ -19.8° (*c* 0.8, chloroform); R_F 0.69 (dichloromethane/acetone 5:1 v/v); ^{1}H NMR ($CDCl_3$) 7.55-7.45 (m, 5H, Ph), 7.30 (d, 2H, H-2, 6 $PhOCH_3$), 6.70 (d, 2H, H-3, 5 $PhOCH_3$) 5.75 (d, J = 8.5 Hz, 1H, NH), 5.58 (s, 1H, C*H*Ph), 4.95 (d, J = 8.3 Hz, 1H, H-1), 4.70 (AB pattern, C$H_2$$PhOCH_3$), 4.36 (dd, $J_{5,6a}$ = 4.1 Hz, $J_{6a,6b}$ = 10.25 Hz, 1H, H-6a), 4.15 (t, $J_{2,3}$ = 9.3 Hz, 1H, H-3), 3.95 (m, 1H, H-2), 3.70 (m, 5H, H-4, H-6b, CH_3O), 3.50 (m, 1H, H-5), 3.40 (t, J = 4.9 Hz, 2H, CH_2N_3) and 1.95 (s, 3H, CH_3CON).

Anal. Calcd for $C_{27}H_{34}O_8N_4$ (542.59): C, 59.77; H, 6.32; N, 10.33. Found: C, 59.15; H, 6.31; N, 10.27.

5-Azido-3-oxapentyl 2-Acetamido-6-*O*-benzyl-2-deoxy-3-*O*-(3-methoxybenzyl)-β-D-glucopyranoside (7). A mixture of compound **5** (1g, 1.7 mmol), sodium cyanoborohydride (1.07 g, 17 mmol) and 0.3 nm molecular sieves (2 g) in dry tetrahydrofuran (20 mL) was stirred for 15 min at rt. Then the mixture was cooled to 0 °C and dry diethyl ether saturated with hydrogen chloride was added at 0 °C until the evolution of gas stopped. The cooling bath was removed and the reaction mixture was further stirred for 20 min. Cold water (5 mL) was added, then the suspension was diluted with dichoromethane (50 mL) and filtered through Celite. The filtrate was washed with 1 % aqueous potasium permanganate (3 x 20 mL), saturated aqueous sodium hydrogen carbonate (20 mL) and water (20 mL), then dried and concentrated. Column chromatography (dichloromethane/acetone 5:1 v/v) of the residue afforded **6** (600 mg, 60 %) as a colorless solid. An analytical sample was obtained by recrystallization from ethyl acetate: mp 96-98 °C; $[\alpha]_D$ -10.9° (*c* 6.2, chloroform); R_F 0.48 (dichloromethane/acetone 4:1 v/v); ^{1}H NMR (C_6D_6) δ 7.45-7.35 (m, 6H, Ph), 6.90 (m, 3H, H-2,4,6 PhOMe), 6.62 (d, 1H, J = 7.7 Hz, NH), 4.97 (AB pattern, 2H, CH_2Ph), 4.69 (d, 1H, $J_{1,2}$ = 8.3 Hz, H-1), 4.41 (s, 2H, PhCH_2), 4.15 (m, 1H, H-2), 3.88 (m, 3H, H-3, 4, 6a), 3.70 (m, 1H, H-5), 3.52 (s, 3H, OCH_3), 3.45 (m, 1H, H-6b), 3.23 (t, 2H, H-C$H_2$$CH_2N_3$), 2.87 (t, 2H, J = 4.9 Hz, CH_2N_3) and 1.82 (s, 3H, CH_3CON).

Anal. Calcd for $C_{27}H_{36}O_8N_4$ (544.60): C, 59.54; H, 6.66; N, 10.29. Found: C, 58.98; H, 6.71; N, 10.08.

5-Azido-3-oxapentyl 2-Acetamido-6-*O*-benzyl-2-deoxy-3-*O*-(4-methoxybenzyl)-β-D-glucopyranoside (8). A mixture of compound **6** (1g, 1.7 mmol), sodium cyanoborohydride (1.07 g, 17 mmol) and 0.3 nm molecular sieves (2 g) in dry tetrahydrofuran (20 mL) was stirred for 15 min at rt. Then the mixture was cooled to 0 °C and dry diethyl ether saturated with hydrogen chloride was added at 0 °C until the evolution of gas stopped and the solution was stirred for 10 min at 0 °C. Cold water (5 mL) was added, and the suspension was diluted with dichoromethane (50 mL) and filtered through Celite. The filtrate was washed with 1% aqueous potasium permanganate (3 x 20mL) saturated aqueous sodium hydrogen carbonate (20 mL) and water (20 mL), dried and concentrated. Column chromatography (dichloromethane/acetone 5:1 v/v) of the residue afforded **8** (561 mg, 56%) as a colorless solid. An analytical sample was obtained by recrystallization from ethyl acetate: mp 88-90 °C; $[\alpha]_D$ -8.18° (*c* 8.8, chloroform); R_F 0.48 (dichloromethane/acetone 4:1 v/v); ^{1}H NMR (C_6D_6) δ 7.45-7.38 (m, 5H, Ph), 7.30 (d, 2H, H-2, H-6 $PhOCH_3$), 6.80 (d, 2H, H-3, 5 $PhOCH_3$) 5.92 (d, J = 7.7 Hz, 1H, NH), 4.87 (AB, 2H, CH_2Ph), 4.65 (d, $J_{1,2}$ = 8.3 Hz, 1H, H-1), 4.41 (s, 2H, $PhCH_2$), 3.82(m, 4H, H-2, 3, 4, 6a), 3.65 (m, 1H, H-5), 3.35 (s, 3H, OCH_3), 3.35 (m, 2H, H-6b), 3.23 (t, 2H, $CH_2CH_2N_3$), 2.87 (t, J = 4.9 Hz, 2H, CH_2N_3) and 1.82 (s, 3H, CH_3CON).

Anal. Calcd for $C_{27}H_{36}O_8N_4$ (544.60): C, 59.54; H, 6.66; N, 10.29. Found: C, 59.78; H, 6.86; N, 10.16.

5-Azido-3-oxapentyl 2-Acetamido-4-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-6-*O*-benzyl-2-deoxy-3-(3-methoxybenzyl)-β-D-glucopyranoside (10). A solution of **6** (200 mg, 0.35 mmol) and silver triflate (300 mg, 1.17 mmol) in dry dichloromethane (3 mL) containing molecular sieves (1.5 g) was stirred under nitrogen for 15 min at rt and then a solution of the bromide **9** (647 mg, 1.17 mmol) in dichloromethane (3 mL) was added. After being stirred for 6 h under nitrogen at rt, the mixture was diluted with dichloromethane (10 mL) and filtered trough Celite. The filtrate was washed with water (5 mL), saturated sodium hydrogen carbonate (5 mL) and water (5 mL), then dried and concentrated. Column chromatography (dichloromethane/acetone 8:1 v/v) of the residue afforded **10** as a syrup (210 mg, 60%): $[\alpha]_D$ -47.0° (*c* 1.0, chloroform); R_F 0.65 (dichloromethane/acetone 4:1 v/v); ^{1}H NMR (C_6D_6) δ 7.50-7.30 (m, 21H, Ph), 6.80 (m, 3H,H-2,4,6 PhOMe), 6.60 (d, J = 9.1 Hz, 1H, NH), 5.72 (dd, $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$= 10.1 Hz, 1H, H-2'), 4.78 (d, $J_{1,2}$ = 6.2 Hz, 1H, H-1), 4.55 (d, 1H, H-1'), 4.40 (m, 1H, H-2), 4.20 (m, 1H, H-4), 3.87 (m, 4H, H-6a, 6a`, 3, 4'), 3.61 (m, 1H, H-5), 3.54 (s, 3H, OCH_3), 3.37 (m, 2H, H-6b, 5'), 2.90 (t, J = 4.9 Hz, 2H, CH_2N_3), 1.91 (s, 3H, CH_3CON) and 1.87 (s, 3H, Ac).

Anal. Calcd for $C_{56}H_{66}O_{14}N_4$ (1019.16): C, 66.00; H, 6.53; N, 5.50. Found: C, 66.17; H, 6.59; N, 5.31.

5-Azido-3-oxapentyl 2-Acetamido-4-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-6-*O*-benzyl-2-deoxy-3-(4-methoxybenzyl)-β-D-glucopyranoside (11). A solution of **6** (100 mg, 0.18 mmol), silver triflate (162 mg, 0.63 mmol) and *N,N*-di-isopropylethylamine (32 μL, 0.18 mmol) in dry dichloromethane (3 mL) containing 0.4 nm molecular sieves (1.5 g) was stirred under nitrogen for 15 min at rt and then a solution of bromide **9** (350 mg, 0.63 mmol) in dichloromethane (3 mL) was added. After being stirred for 6 h at rt, the mixture was diluted with dichloromethane (10 mL) and filtered through Celite. The filtrate was washed with water (5 mL), saturated sodium hydrogencarbonate (5 mL) and water (5 mL), then dried and concentrated. Column chromatography (dichloromethane/acetone 8:1 v/v) of the residue afforded **11** (106 mg, 57%) as a syrup; $[\alpha]_D$ -66.3° (*c* 1.17, chloroform); R_F 0.65 (dichloromethane/acetone 4:1 v/v); ^{1}H NMR (C_6D_6) δ 7.50-7.40 (m, 20H, Ph), 7.25 (d, 2H, H-2, 6PhOCH$_3$), 6.75 (d, 2H, H-3,5 PhOCH$_3$) 6.21 (d, J = 8.9 Hz, 1H, NH), 5.80 (dd, $J_{1',2'}$ = 7.7 Hz, $J_{2',3'}$ = 10.1 Hz, 1H, H-2'), 4.85 (AB pattern, 2H, CH_2Ph), 4.80 (d, $J_{1,2}$= 6.5 Hz, 1H, H-1), 4.48 (d, 1H, H-1'), 4.48 (m, 1H, H-2), 4.35 (m, 1H, H-4), 3.95 (m, 2H, H-6a,3), 3.87 (m, 1H, H-4`), 3.72 (dd, 1H, H-6a`), 3.57 (m, 2H, H-6b, 6b`), 3.43 (s, 3H, OCH_3), 3.42 (m, 2H, H-5, 5'), 2.90 (t, J = 5.0 Hz, 2H, CH_2N_3), 1.92 (s, 3H, CH_3CON) and 1.90 (s, 3H, Ac).

Anal. Calcd for $C_{56}H_{66}O_{14}N_4$ (1019.16): C, 66.00; H, 6.53; N, 5.50. Found: C, 65.37; H, 6.68; N, 5.65.

5-Azido-3-oxapentyl 2-Acetamido-6-*O*-benzyl-4-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-2-deoxy-β-D-glucopyranoside (12). a) A solution of **11** (100 mg, 0.098 mmol) and DDQ (46 mg, 0.19 mmol) in dichloromethane/water 50:1 (4 mL) was stirred for 20 min at rt. Dichloromethane (5 mL) was added and the solution was washed with saturated aqueous sodium hydrogen carbonate (3 mL), water (3 mL), dried and concentrated. Column chromatography (dichloromethane/acetone 6:1 v/v) of the residue afforded **12** as a syrup (74 mg, 83%); **b) 10** (214 mg, 0.21 mmol) was treated with DDQ (150 mg, 0.63 mmol) in dichloromethane/water 50:1 (4 mL) for 5 h at rt. Workup and column chromatography as described in (a) afforded **12** (80 mg, 42%); $[\alpha]_D$ +36.6° (*c* 0.6, chloroform); R_F 0.30 (dichloromethane/acetone 4:1 v/v); ^{1}H NMR ($CDCl_3$) δ 7.45-7.37 (m, 20H, Ph), 5.77 (d, J= 8.2 Hz, 1H, NH), 5.32 (dd, $J_{1,2}$ = 7.8 Hz, $J_{2,3}$ = 9.8 Hz, 1H, H-2'), 4.67(d, $J_{1,2}$ = 6.2 Hz, 1H, H-1), 4.35 (d, 1H, H-1'), 4.00-3.30 (m, 13H, H-2, 3, 3', 4, 4', 5, 5', 6a, 6b, 6', 6'b, CH_2N_3), 1.99 (s, 3H, CH_3CON) and 1.97 (s, 3H, Ac).

Anal. Calcd for $C_{48}H_{58}O_{13}N_4$ (899.01): C, 64.13; H, 6.50; N, 6.23. Found: C, 63.65; H, 6.68; N, 6.09.

5-Azido-3-oxapentyl 2-Acetamido-4-*O*-(3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (13). To a solution of compound **12** (70 mg, 0.078 mmol) in dry methanol (1 mL), was added a 0.1 M methanolic solution of sodium methoxide (0.1 mL). After 16 h, the reaction was neutralized with Dowex-50 (H^+) resin, filtered and concentrated to afford **13** (64 mg, 96%) as a syrup: $[\alpha]_D$ +18.4° (*c* 2.24, chloroform); R_F 0.45 (dichloromethane/acetone 4:1 v/v); ^{1}H NMR ($CDCl_3$) δ 7.45-7.37 (m, 20H, Ph), 6.18 (d, J = 7.7 Hz, 1H, NH), 4.69 (d, $J_{1,2}$ = 7.4 Hz, 1H, H-1), 4.26 (d, $J_{1',2'}$ = 7.8 Hz, 1H, H-1'), 3.91 (m, 1H, H-2'), 3.82 (m, 1H, H-3), 3.75 (m, 1H, H-4'), 3.68 (m, 1H, H-2), 3.54 (m, 3H, H-5,5',4), 3.34 (dd, $J_{2',3'}$ = 10 Hz, $J_{3',4'}$ = 3 Hz, 1H, H-3'), 3.30 (t, J = 5 Hz, 2H, CH_2N_3) and 1.90 (s, 3H, CH_3CON).

Anal. Calcd for $C_{46}H_{56}O_{12}N_4$ (856.97): C, 64.47; H, 6.59; N, 6.54. Found: C, 64.65; H, 6.78; N, 5.37.

5-Azido-3-oxapentyl 2-Acetamido-4-*O*-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-β-D-galactopyranosyl)-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (14). A solution of **12** (28 mg, 31.7 μmol), tetraethylammonium bromide (7 mg, 32 μmol) in dichloromethane (0.5 mL) containing molecular sieves (100 mg) was stirred for 15 min at rt and then a solution of 2,3,4-tri-*O*-tribenzyl-α-L-fucosyl bromide (41 mg, 82 μmol) in dichloromethane (1 mL) was added. After being stirred for 2 d at rt, the mixture was diluted with dichloromethane (5 mL) and filtered through Celite. The filtrate was washed with water (3 mL), saturated aqueous sodium hydrogen carbonate (3 mL) and water (3 mL), then dried and concentrated. Column chromatography (dichloromethane/acetone 10:1 v/v) of the residue afforded **14** (34 mg, 82 %) as a syrup; $[\alpha]_D$ -93.0° (*c* 0.64, chloroform); R_F 0.50 (dichloromethane/acetone 8:1 v/v); ^{1}H NMR ($CDCl_3$) δ 7.45-7.28 (m, 50H, Ph), 5.88 (d, J = 6.9 Hz, 1H, NH), 5.02 (d, $J_{1,2}$ = 3.6 Hz, 1H, H-1f), 4.90 (d, J=3 Hz, 1H, H-1), 4.47(d, 1H, J = 6 Hz, H-1'), 4.37(m, 1H, H-5f), 3.98 (m, 2H, H-4', 2f), 3.86 (m, 2H, H-3, 3f), 3.74 (m, 2H, 6'a, 6a), 3.60 (m, 3H, H-4, 6b, 6'b), 3.50 (m, 2H, H-2, 5'), 3.30 (m, 5H, 5, 3', 4f, CH_2N_3), 1.75 (s, 3H, Ac) and 1.09 (d, J = 6.3 Hz, 3H, C-6f).

Anal. Calcd for $C_{75}H_{86}O_{17}N_4$ (1315.52): C, 68.48; H, 5.59. N, 4.26. Found: C, 68.65; H, 6.86; N, 4.93.

5-Azido-3-oxapentyl 2-Acetamido-4-*O*-(3,4,6-tri-*O*-benzyl-2-*O*-[2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl]-β-D-galactopyranosyl)-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fuco-pyranosyl)-6-*O*-benzyl-2-deoxy-β-D-glucopyranoside (15). A solution of **13** (70 mg, 81.7 μmol), tetraethylammonium bromide (3.5 mg, 16.9 μmol) in dichloromethane (1 mL) containing molecular sieves (200 mg) was stirred for 15 min at rt and then a solution of 2,3,4-tri-*O*-tribenzyl-α-L-fucosyl bromide (41 mg, 82 μmol) in dichloromethane (1 mL) was added. After being stirred for 2 d at rt, the mixture was diluted with dichloromethane (5 mL) and filtered through Celite. The filtrate was washed with water (3 mL), saturated aqueous sodium hydrogen carbonate (3 mL) and water (3 mL), then dried and concentrated. Column chromatography (hexane/ ethyl acetate 1:1 v/v) of the residue afforded **15** as a syrup (80 mg, 58%); $[\alpha]_D$ -48.0° (*c* 1.56, chloroform); R_F 0.35 (hexane/ethyl acetate 1:1 v/v); ^{1}H NMR ($CDCl_3$) δ 7.45-7.28 (m, 50H, Ph), 5.77 (d, J = 6.9 Hz, 1H, NH), 5.67 (d, $J_{1,2}$ = 3.4 Hz, 1H, H-1f'), 5.15 (d, $J_{1,2}$ = 8.6 Hz, 1H, H-1), 4.91 (d, J = 3 Hz, 1H, H-1f), 4.58 (q, 1H, H-5f), 4.47 (d, J = 7.7 Hz, 1H, H-1'), 4.20 (m, 2H, H-3, 5f'), 4.00 (m, 5H, 4', 2f', 2f, 2', 4), 3.83 (m, 3H, 6a, 3f, 3f'), 3.67 (m, 4H, 6'a, 4f', 6b, 6'b), 3.53 (dd, $J_{2,3}$ = 8.2 Hz, $J_{3,4}$ = 2.4 Hz, 1H, 3'), 3.35 (t, 2H, J = 5 Hz, CH_2N_3), 3.25 (m, 3H, 5', 5, 4f), 1.97 (s, 3H, Ac); 1.28 (d, 3H, H-6f') and 1.10 (d, 3H, H-6f).

Anal. Calcd for $C_{100}H_{112}O_{20}N_4$ (1689.00): C, 71.11; H, 6.62. N, 3.32 Found: C, 70.62; H, 6.81, N, 3.20.

5-Amino-3-oxapentyl 2-Acetamido-2-deoxy-3-*O*-(α-L-fucopyranosyl)-4-*O*-(2-*O*-[α-L-fucopyranosyl]-β-D-galactopyranosyl)-β-D-glucopyranoside (16). A solution of compound **15** (78 mg, 0.046mmol) in ethyl acetate/methanol/water/acetic acid 5:5:1:0.1 v/v (1 mL) containing 10 % palladium on carbon (40 mg) was stirred overnight under H_2 at rt. The mixture was filtered and the solid washed with water (25 mL). The filtrate and washings were concentrated under reduced pressure and lyophilized to afford **16** as a colorless powder (32 mg, 90%); $[\alpha]_D$ -102° (*c* 0.27, water); R_F 0.23 (ethyl acetate/ methanol/ water/ acetic acid 5:5:1:0.1 v/v); ^{1}H NMR (D_2O) δ 5.32 (d, $J_{1,2}$ = 2.5 Hz, 1H, H-1f'), 5.16 (d, $J_{1,2}$ = 3.9 Hz, 1H, H-1f), 4.91 (q, 1H, H-5f), 4.66(d, $J_{1,2}$ = 8.3 Hz 1H, H-1'), 4.57(d, J=7.8 Hz, 1H, H-1), 4.32 (q, 1H, H-5f'), 4.10 (dd, $J_{6,6a}$ = 10.5 Hz, $J_{5,6a}$ = 2.4 Hz, 1H,6a), 3.99 (m, 2H, H-4, 3f), 3.91 (m, 9H, H-4', 3', 3, 2, 6b, 3f', 4f', 4f, 2f'), 3.80 (m, 3H, H-6'a, 6'b, 2f), 3.72 (dd, $J_{2,3}$ = 8.5 Hz, 1H, H-2), 3.56(m, 2H, H-5', 5), 3.14 (t, 2H, CH_2NH_2), 2.10 (s, 3H, CH_3CON), 1.36 (d, 3H, H-6f') and 1.33 (d, 3H, H-6f); ^{13}C NMR ($CDCl_3$) δ 170.5 (C=0), 101.8 (C-1), 101.1 (C-1'), 100.2 (C-1f'), 99.2 (C-1f), 77.3 (C-2'), 76.5 (C-3), 75.8 (C-5), 75.7 (C-5'), 74.6 (C-4), 74.4 (C-3'), 73.0 (C-4f), 72.6 (C-4f'), 70.4 (C-3f'), 69.8 (C-3f), 69.5 (C-4'), 69.0 (C-2f), 68.8 (C-2f'), 67.5 (C-5f, 5f'), 62.1 (C-6'), 60.9 (C-6), 56.8 (C-2), 40.3 (CH_2NH_2), 23.4(CH_3CON) and 16.4 (C-6f, 6f').

Anal. Calcd for $C_{30}H_{54}O_{20}N_2$ (761.65): C, 47.24; H, 7.14; N, 3.67. Found: C, 47.36; H, 7.20; N, 3.78.

5-Amino-3-oxapentyl 2-Acetamido-2-deoxy-3-*O*-(α-L-fucopyranosyl)-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (17). A solution of compound **14** (20mg, 0.014 mmol) was dissolved in a dry solution of methanolic sodium methoxide 0.01 M (1 mL) and stirred for 16 h at rt. The solution was then neutralised with Amberlite IR 120 (H^+) resin. The resulting mixture was filtered and the filtrate concentrated to a syrupy residue that was hydrogenolised over 10 % palladium on carbon (15 mg) in ethyl acetate/ methanol/water/acetic acid 5:5:1:0.1 v/v (0.5 mL) overnight at rt. The resulting mixture was filtered and the solid washed with water (20 mL). The filtrate was concentrated under reduced pressure and lyophilized to afford **17** as a colorless powder (8.2 mg, 89%); R_F 0.29 (ethyl acetate/methanol/water/acetic acid 5:5:1:0.1 v/v); ^{1}H NMR (D_2O) δ 5.18 (d, $J_{1,2}$ = 3.9 Hz, 1H, H-1f), 4.91 (q, 1H, H-5f), 4.59(d, $J_{1,2}$ = 8.3 Hz 1H, H-1'), 4.55 (d, J=7.8 Hz, 1H, H-1), 4.09 (dd, $J_{6,6'}$ = 10.5 Hz, $J_{5,6}$ = 2.4 Hz, 1H, H-6), 3.99 (m, 2H, H-4, 3f), 3.91 (m, 9H, H-4', 3', 3, 2, 6a, 4f), 3.80 (m, 3H, H-6b, 6'a, 2f), 3.56(m, 3H, H-5', 2', 5), 3.14 (t, 2H, CH_2NH_2), 2.12 (s, 3H, CH_3CON) and 1.34 (d, 3H, H-6f); ^{13}C NMR ($CDCl_3$) δ 170.6 (C=0), 101.9 (C-1), 101.0 (C-1'), 99.5 (C-1f), 76.7 (C-3), 75.8 (C-5), 75.6 (C-5'), 74.4 (C-3'), 74.6 (C-4), 73.0 (C-4f), 71.3 (C-2'), 69.8 (C-3f), 69.5 (C-4'), 69.0 (C-2f), 67.3 (C-5f), 61.8 (C-6'), 61.2 (C-6), 55.8 (C-2), 40.2 (CH_2NH_2), 23.4 (CH_3CON) and 16.3 (C-6f).

Anal. Calcd for $C_{24}H_{44}O_{17}N_2$(632.62): C, 45.57; H, 7.01. N, 4.43 Found: C, 45.01; H, 7.45, N, 4.35.

Coupling reaction between 16 or 17 and BSA. To a solution of the free oligosaccharide **16** or **17** (4 μmol) in *N,N*-dimethylformamide (distilled in *vacuo* and pumped for 30 min before use, 0.5 mL) was added the *N*-hydroxysuccinimide derivative of β-maleimidopropanoic acid[25] (1.3 mg, 5μmol). After 2 h, the solution was concentrated and dried *in vacuo*. The residue was resuspended in D_2O (0.5 mL) and centrifuged to remove the excess of reagent. ^{1}H NMR (D_2O) δ 6,89 (s, 2H, HC=CH), 3.42 (t, 2H, CH_2N), 2.52 (t, 2H, CH_2CO). The maleimido derivative **18** or **19** could be stored in a lyophilised form at 0°C or used directly in the coupling reaction.

Compound **18** or **19** was added to a solution[26] of BSA-SH_{23} in PBS (pH 7.2, 0.4 mL). After 2 h, the process was complete as evidence by negative Ellman test[27] and the resulting solution was dyafiltrated against PBS (pH = 7.4). The protein and carbohydrate contents were determined by the Lowry[28] and phenol-sulfuric acid methods,[29] respectively. Several assays gave carbohydrate to protein ratios between 13-20 oligosaccharide units per BSA molecule.

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