Glycoconjugate Journal 15, 549–553 (1998)

Conjugation of 5-azido-3-oxapentyl glycosides with thiolated proteins through the use of thiophilic derivatives

Violeta Fernández-Santana, Raul González-Lio, Jorge Sarracent-Pérez, Vicente Verez-Bencomo*

Laboratory of Synthetic Antigens, Facultad de Química, Universidad de la Habana, Ciudad de La Habana, Cuba 10400

5-azido-3-oxa-pentyl β-D-galactopyranoside was prepared from diethylene glycol monochlorohydrin and used as a model of oligosaccharide hapten. After deprotection, a series of amides bearing thiophilic groups had been obtained through the terminal amino function and essayed in coupling reactions with thiolated BSA. Also several Lewis human blood group oligosaccharides had been conjugated with thiolated BSA demonstrating the usefulness of the methodology.

***Keywords*: spacer-arm glycosides; neoglycoprotein; conjugation; Lewis human blood group**

Introduction

Spacer-arm derivatives of oligosaccharides are frequently used for coupling to proteins. Semi-artificial neoglycoproteins have been prepared in this way as vaccines for the production of carbohydrate-specific antibodies [1, 2] in animals, or even as a therapeutic tool for humans [3, 4]. However, very often after a multistep synthesis, a precious oligosaccharide was coupled to a protein [5] with a yield not exceeding 20%.

The success of the overall strategy has been established frequently by the correct choice of spacer. In all cases the compatibility of the protective group with the synthesis and the efficiency of the coupling to a protein decided the success of a given strategy.

In the present paper we describe the synthesis of 5-azido-3-oxa-pentyl β-D-galactopyranoside and the development of conditions for their high yield conjugation to BSA.

Materials and methods

General methods

NMR spectra were recorded at 25 °C on a BRUKER AC-250F spectrometer. ^{1}H and ^{13}C assignments were made on the basis of homo- and heteronuclear correlation experiments. Chemical shifts (δ) are given in ppm relative to the signal for internal tetramethylsilane. In oligosaccharides the signals are represented as follows: for galactose, f and f′ for the two fucose moieties. All compounds characterized were purified by column chromatography on Kieselgel 60 (Fluka, < 230 mesh ASTM) and fractions were monitored by thin layer chromatography (TLC) on Kieselgel 60 F_{254} (Merck). Detection was effected by charring with sulfuric acid. Evaporation was conducted under reduced pressure at 50 °C (bath).

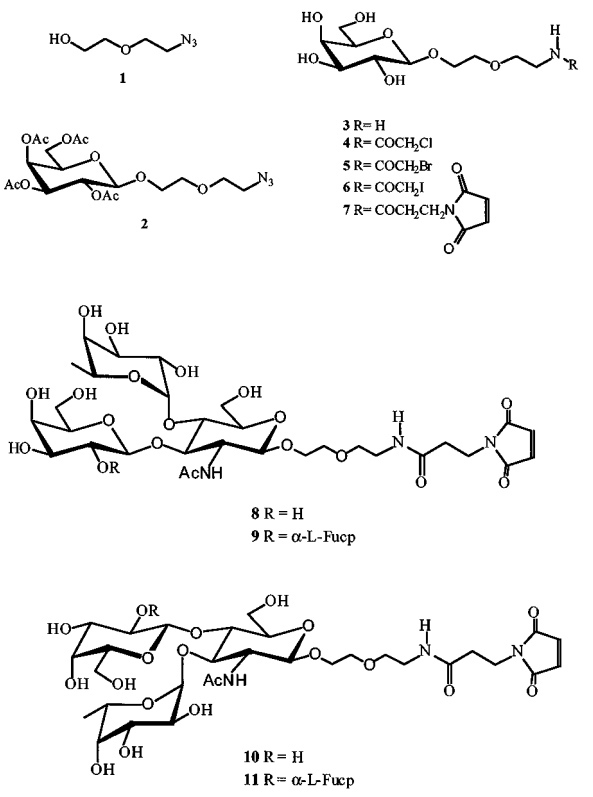
5-Azido-3-oxapentanol (**1**)

To a solution of diethyleneglycol monochlorohydrin (5 ml, 47 mmol) in 2-butanone (25 ml) NaN_3 (4.5 g, 69 mmol), Bu_4NI (2.5 g, 6 mmol), and dicyclohexano-18-crown-6 (10 mg) was added and the mixture was boiled under reflux for 24 h. ^{13}C NMR spectroscopy of a supernatant showed the absence of a signal at δ 42.7 ppm and 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 85–95 °C and 2×10^{-3} mbar, compound **1** (4.6 g, 88%); ^{1}H NMR ($CDCl_3$) δ 3.71–3.6 (m, 6 H, CH_2O), 3.41 (t, 2 H, CH_2N_3); ^{13}C NMR ($CDCl_3$) δ 72.37 and 69.84 (CH_2O), 61.56 (CH_2OH), 50.62 (CH_2N_3).

5-Azido-3-oxapentyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranoside (**2**)

To a stirred solution of **1** (1.9 g, 14.5 mmol) and $Hg(CN)_2$ (4.05 g, 15.7 mmol) in dry CH_3CN (25 ml) active molecular sieves (10 g, 4 Å) was added and, after 10 min, acetobromogalactose (5 g, 12.1 mmol). The mixture was stirred until TLC (hexanes : EtOAc/2 : 1 v/v) indicated the disappearance of the starting material and a new spot at $R_F = 0.45$. It was then diluted with CH_2Cl_2 (50 ml) washed with aqueous

* To whom correspondence should be addressed. Tel.: 537 700481; Fax: 537 336075; E-mail: vicente@karin.fmq.uh.edu.cu



Scheme 1

10% potassium iodide (20 ml), aqueous saturated $NaHCO_3$ (20 ml), water (20 ml), dried (Na_2SO_4), filtered and concentrated. Column chromatography (hexanes: EtOAc/9:1 v/v) of the residue afforded **2** (4.38 g, 78%); $R_F = 0.45$ (hexanes: EtOAc/2:1 v/v); ^{1}H NMR ($CDCl_3$) δ 5.39 (dd, 1 H, H-4), 5.22 (dd, 1 H, $J_{2-3} = 14.3$ Hz, H-2), 5.05 (dd, 1 H, $J_{3-4} = 3.3$ Hz, H-3), 4.09 (d, 1 H, $J_{1-2} = 7.5$ Hz, H-1), 4.16 (m, H-6), 3.92 (m, H-5), 3.65 (m, CH_2O spacer), 3.40 (t, 2 H, CH_2N_3), 2.2–1.98 (4s 12 H, CH_3COO); ^{13}C NMR ($CDCl_3$) δ 170.2–169.4 (C=O), 101.2 (C-1), 70.8 (C-5), 70.5 (C-3), 68.9 (C-2), 66.8 (C-4), 61.1 (C-6), 70.3, 70.1 and 68.7 (CH_2O spacer), 50.67 (CH_2N_3), 20.6-20.4 (CH_3COO); Anal. Calcd for $C_{18}H_{27}O_{11}N_3$: C, 46.96; H, 5.90; N, 9.10. Found: C, 47.14; H, 5.98; N, 8.95.

5-Amino-3-oxapentyl β-D-galactopyranoside (**3**)

NaOMe 0.1 M (pH > 8.5) was added to a solution of **2** (4 g, 8.6 mmol) in dry MeOH (40 ml). After 16 h, TLC (EtOAc: MeOH/1:1 v/v) indicated complete conversion of **2**. The reaction was neutralized with Dowex-50 (H^+) resin, filtered and evaporated to 5 ml, then water (5 ml) was added and the resulting solution was hydrogenolyzed in the presence of 5% Pd-C and hydrogen at atmospheric pressure for 24 h. TLC. (MeOH : EtOAc : H_2O/2 : 2 : 1 v/v, ninhydrin) showed a single spot ($R_F = 0.30$). After centrifugation, the solution was concentrated to afford **3** as syrup (2.0 g, 95%). $R_F = 0.30$ (MeOH : EtOAc : H_2O/2 : 2 : 1 v/v, ninhydrin); $[\alpha]_D = 28^\circ$ (c = 1, H_2O); ^{1}H NMR ($CDCl_3$) δ 4.36 (d, 1 H, J_{1-2} 7.50 Hz, H-1), 3.98 (m, H-6), 3.87 (dd, H-4), 3.77 (m, H-6), 3.73 (m, CH_2O spacer), 3.66 (m, H-5), 3.63 (dd, H-3), 3.54 (dd, 1 H, H-2), 2.85 (t, 2H, CH_2NH_2); ^{13}C NMR ($CDCl_3$) δ 104.1 (C-1), 76.4 (C-5), 73.9 (C-3), 72.8 (C-2), 72.0, 70.7 and 70.0 (CH_2O spacer), 69.8 (C-4), 62.2 (C-6), 40.9 (CH_2NH_2).

Synthesis of amides **4**, **5**, **6** and **7**

The derivatives of chloroacetic, bromoacetic, iodoacetic or β-maleimidopropionic acid were obtained following the reaction of the respective acid with *N*,*N*-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide in dioxane as previously described [6].

N-hydroxysuccinimide chloroacetate: m.p. 61-3 °C; ^{1}H NMR ($CDCl_3$) δ 4.43 (s, 2 H, $ClCH_2$), 2.90 (s, 4 H, CH_2CH_2).

N-hydroxysuccinimide bromoacetate: m.p. 90-2 °C; ^{1}H NMR ($CDCl_3$) δ 4.05 (s, 2 H, $BrCH_2$), 2.82 (s, 4 H, CH_2CH_2).

N-hydroxysuccinimide iodoacetate: m.p. 135-7 °C; ^{1}H NMR ($CDCl_3$) δ 3.95 (s, 2 H, ICH_2), 2.86 (s, 4 H, CH_2CH_2).

N-hydroxysuccinimide β-maleimidopropionate: m.p. 160-3 °C. Lit [7]: M.p. 160-3 °C; ^{1}H NMR (d-acetone) δ 7.02 (s, 2 H, CH=CH), 3.93 (t, 2 H, $CH_2\beta$), 3.10 (t, 2 H, $CH_2\alpha$), 2.82 (s, 4 H, NHS).

The corresponding *N*-hydroxysuccinimide derivative (0.02 mmol) to a solution of **3** (5 mg, 0.018 mmol) in DMF (0.5 ml) was added. After 4 h, TLC (MeOH : EtOAc : H_2O : AcOH/1 : 1 : 1 : 0.2 v/v, ninhydrin) indicated a total conversion of **3** ($R_F = 0.3$) into the corresponding amide **4**, **5**, **6** and **7** ($R_F = 0.75$). The solution was concentrated, dried *in vacuo*, resuspended in D_2O (0.5 ml) and centrifuged (10 min, 3500 rpm). The following compounds were obtained:

5-chloroacetamido-3-oxapentyl β-D-galactopyranoside (**4**)

^{1}H NMR (D_2O) δ 4.40 (d, 1 H, $J_{1-2} = 7.7$ Hz, H-1), 4.13 (s, 2 H, CH_2Cl), 4.02 (m, 1 H, H-6), 3.90 (dd, 1 H, H-4), 3.77 (m, 1 H, H-6), 3.74 (m, 2 H, CH_2O spacer), 3.65 (m, 1 H, H-5), 3.60 (dd, 1 H, J_{3-4} 3.38, H-3), 3.51 (dd, 1 H, J_{2-3} 6.34, H-2), 3.45 (t, 2 H, CH_2NH); ^{13}C NMR (D_2O) δ 104.1 (C-1), 76.4 (C-5), 73.9 (C-3), 72.0 (C-2), 70.8, 69.9 and 69.8 (CH_2O spacer), 69.8 (C-4), 62.2 (C-6), 43.4 (CH_2Cl), 40.5 (CH_2NH).

5-bromoacetamido-3-oxapentyl β-D-galactopyranoside (**5**)

^{1}H NMR (D_2O) δ 4.41 (d, 1 H, $J_{1-2} = 7.7$ Hz, H-1), 4.03 (m, 1 H, H-6), 3.91 (s, 2 H, CH_2Br), 3.90 (d, 1 H, H-4), 3.83 (m, 1 H, H-6), 3.74 (m, 2 H, CH_2O spacer), 3.68 (m, 1 H, H-5), 3.63 (dd, 1 H, H-3), 3.53 (dd, 1 H, H-2), 3.42 (t, 2 H, CH_2NH); ^{13}C NMR (D_2O) δ 104.1 (C-1), 76.4 (C-5), 73.9 (C-3), 72.0

(C-2), 70.9, 69.9 and 69.4 (CH_2O spacer), 69.4 (C-2), 62.2 (C-6), 40.7 (CH_2NH), 29.3 (CH_2Br).

5-iodoacetamido-3-oxapentyl β-D-galactopyranoside (**6**)

^1H NMR (D_2O) δ 4.41 (d, 1 H, $J_{1-2} = 7.7$ Hz, H-1), 4.12 (m, 1 H, H-6), 3.92 (dd, 1 H, H-4), 3.82 (m, 1 H, H-6), 3.78 (s, 2 H, CH_2I), 3.74 (m, 2 H, CH_2O spacer), 3.66 (m, 1 H, H-5), 3.63 (dd, 1 H, H-3), 3.52 (dd, 1 H, H-2), 3.38 (t, 2 H, CH_2NH); ^{13}C NMR (D_2O) δ 104.1 (C-1), 76.4 (C-5), 73.9 (C-3), 72.0 (C-2), 70.8, 69.9 and 69.8 (CH_2O spacer), 69.6 (C-4), 62.2 (C-6), 40.6 (CH_2NH), 1.05 (CH_2I).

5-β-maleimidopropionamido-3-oxapentyl β-D-galactopyranoside (**7**)

^1H NMR (D_2O) δ 6.69 (s, 2 H, CH = CH), 4.45 (d, 1 H, $J_{1-2} = 7.7$ Hz, H-1), 4.07 (m, 1 H, H-6), 3.96 (dd, 1 H, H-4), 3.89 (m, H-6), 3.81 (t, 2 H, $CH_2\beta$), 3.79 (m, 2 H, CH_2O spacer), 3.76 (m, 1 H, H-5), 3.61 (dd, 1 H, H-3), 3.58 (dd, 1 H, H-2), 3.37 (t, 2 H, CH_2NH), 2.56 (t, 2 H, $CH_2\alpha$); ^{13}C NMR (D_2O) δ 135.6 (C=C), 104.1 (C-1), 76.4 (C-5), 73.9 (C-3), 71.9 (C-2), 70.8, 70.6 and 69.9 (CH_2O spacer), 69.8 (C-4), 62.2 (C-6), 40.1 (CH_2NH), 35.8 and 35.6 (CH_2 α and β).

Conjugation of galactose derivatives with proteins

A solution of *N*-hydroxysuccinimide dithiopropionate (3.62 mg, 9 μmol, m.p. 114 °C) in DMF (0.1 ml) was added, under N_2 to a solution of BSA (20 mg, 0.3 μmol) in PBS (pH 8, with EDTA 5 mM, 4 ml). After 2 h, dithiotreitol (19.3 mg, $\rightarrow 25$ mmol l^{-1}) was added under N_2 atmosphere and the mixture was stirred at room temperature for 1 h. The resulting solution was dialyzed against PBS solution (pH = 7.2; 2×2 l) and finally filtered at pH 7.2 using N_2 as a pressure source, over a polysulfone membrane, 10 000 Da cutoff. The protein and SH contents was analyzed by the Lowry [8] and Ellman [9] methods respectively. A 20–25 molar substitution was usually attained.

To a solution of BSA-SH_{21-23} (6 mg) in PBS (pH = 7.2, EDTA 5 mM, 2 ml) was added, under N_2, a solution of the galactose derivative to be coupled (**4**, **5**, **6** or **7**) previously dissolved in PBS (pH = 7.2, 0.4 ml). After several h, (the reaction time and the proportion of reagents are shown in Table 1) the resulting solution was filtered over a polysulfone membrane against PBS (pH 7.4) and the efficiency of the procedure was estimated by the protein and carbohydrate contents that were determined using the Lowry and phenol-sulfuric acid method [10], respectively.

Synthesis of 5-β-maleimidopropionamido derivatives of Lewis human blood group oligosaccharides

To a solution of the free amine (8 μmol) in deaminated DMF (0.5 ml) was added the *N*-hydroxysuccinimide 5-β-maleimidopropionate (9.6 μmol) and the solution stirred at room temperature. After 2 h, the solution was concentrated, dried *in vacuo*, resuspended in D_2O (0.5 ml) centrifuged (10 min, 3500 rpm) and analyzed by ^1H NMR spectroscopy. The following compounds were obtained:

Table 1

No.	Compound	Gal/SH (mol/mol)	Incorporation (Gal/BSA)	Yield (%)	Reaction time (h)
1	**4**	2	4	4.5	48
2	**5**	2	10	18.5	24
3	**6**	2	14	31.3	16
4	**7**	2	20	40.0	0.5
5	**7**	1.1	20	73.0	0.5

5-β-maleimidopropionamido-3-oxapentyl 2-acetamido-2-deoxy-4-*O*-α-L-fucopyranosyl-3-*O*-β-D-galactopyranosyl-2-deoxy-β-D-glucopyranoside (**8**)

^1H NMR (D_2O) δ 6.85 (s, 2 H, CH=CH), 4.90 (d, 1 H, $J_{1-2} = 3.6$ Hz, H-1f), 4.54 (d, 1 H, $J_{1-2} = 8.4$ Hz, H-1), 4.54 (d, 1 H, $J_{1'-2'} = 7.5$ Hz, H-1′), 3.32 (t, 2 H, CH_2NH), 2.50 (t, 2 H, $CH_2\alpha$), 2.21 (CH_3CON), 1.14 (d, 3 H, H-6f).

5-β-maleimidopropionamido-3-oxapentyl 2-acetamido-2-deoxy-4-*O*-α-L-fucopyranosyl-3-*O*-(2-*O*-α-fucopyranosyl)-β-D-galactopyranosyl-β-D-glucopyranoside (**9**)

^1H NMR (D_2O) δ 6.84 (s, 2 H, CH=CH), 5.18 (d, 1 H, $J_{1-2} = 3.5$ Hz, H-1f), 5.06 (d, 1 H, $J_{1'-2'} = 3.5$ Hz, H-1f′), 4.58 (d, 1 H, $J_{1-2} = 7.8$ Hz, H-1), 4.48 (d, 1 H, $J_{1'-2'} = 7.8$ Hz, H-1′), 3.36 (t, 2 H, CH_2NH) 2.52 (t, 2 H, $CH_2\alpha$), 2.04 (CH_3CON), 1.35 and 1.32 (2d, 6 H, H-6f, 6f′).

5-β-maleimidopropionamido-3-oxapentyl 2-acetamido-2-deoxy-3-*O*-α-L-fucopyranosyl-4-*O*-β-D-galactopyranosyl-2-deoxy-β-D-glucopyranoside (**10**)

^1H NMR (D_2O) δ 6.85 (s, 2 H, CH=CH), 5.22 (d, $J_{1-2} = 3.9$ Hz, 1 H, H-1f), 4.63 (d, $J_{1'-2'} = 8.3$ Hz 1 H, H-1′), 4.56 (d, $J_{1-2} = 7.8$ Hz, 1 H, H-1), 3.30 (t, 2 H, CH_2NH), 2.48 (t, 2 H, $CH_2\alpha$), 2.12 (s, 3 H, CH_3CON), 1.36 (d, 3 H, H-6f).

5-β-maleimidopropionamido-3-oxapentyl 2-acetamido-2-deoxy-3-*O*-α-L-fucopyranosyl-4-*O*-(2-*O*-α-fucopyranosyl)-β-D-galactopyranosyl-β-D-glucopyranoside (**11**)

^1H NMR (D_2O) δ 6.86 (s, 2 H, CH = CH), 5.28 (d, $J_{1-2} = 3.9$ Hz, 1 H, H-1f), 4.62 (d, $J_{1'-2'} = 8.3$ Hz 1 H, H-1′), 4.48 (d, $J_{1-2} = 7.8$ Hz, 1 H, H-1), 3.26 (t, 2 H, CH_2NH), 2.42 (t, 2 H, $CH_2\alpha$) 2.06 (s, 3 H, CH_3CON), 1.34 (d, 6 H, H-6f, 6f′).

Conjugation of oligosaccharide derivatives **8**, **9**, **10** and **11** with BSA

To a solution of BSA-SH_{21-23} (6 mg) in PBS (pH = 7.2, EDTA 5 mM, 2 ml) was added under N_2, a solution of the

Table 2

No.	Compound	Le/SH (mol/mol)	Incorporation (Le/BSA)	Yield (%) Le	Reaction time (h)
6	**8**	1.2	16	72.0	2
7	**9**	1.2	15	67.7	2
8	**10**	1.2	14	63.0	2
9	**11**	1.2	11	50.0	2

oligosaccharide derivative to be coupled (**8**, **9**, **10** or **11)** that had previously been dissolved in PBS (pH 7.2, 0.4 ml). After 2 h, the resulting solution was filtered over a polysulfone membrane against PBS, (pH = 7.4) and the efficiency of the procedure was estimated by the protein and carbohydrate contents that were determined using the Lowry and phenol-sulfuric acid method [10], respectively. The results are shown in Table 2.

Results and discussion

The functional group at the terminal position in the spacer is of paramount importance as it decides the success of the coupling to a protein or a matrix. Several spacers containing an amino group [11] were prepared, although the direct coupling of oligosaccharides through an amino group was not very successful. The amino group could be transformed very easily to a wide range of derivatives with increasing coupling potential.

From a large number of amino-protective groups we chose the azido, based on its compatibility with most of the transformations frequently employed in oligosaccharide synthesis. Diethylene glycol was selected for the spacer's chain [12–18], because of the adequate balance between hydrophobicity and inertness and also by the availability.

Previous syntheses [12, 14, 16,] of monoazido di- or triethylene glycol introduce first a protective group at one end followed by tosylation or mesylation and replacement with the azido group. We found a new straightforward route to 5-azido-3-oxa-pentanol **1** from commercially available diethylene glycol monochlorohydrin that could be transformed directly by the action of NaN_3, Bu_4NI and dicyclohexano-18-crown-6 in butanone.

The reaction of acetobromogalactose with **1** in the presence of $Hg(CN)_2$ afforded the corresponding β-galactoside in 78% yield. After deacetylation, the azido group was reduced with hydrogen in the presence of 5% Pd-C to gave the model compound **3** in 95% yield.

The reaction of the free amine with the *N*-hydroxysuccinimide ester of several acids proceeds smoothly in deaminated DMF to afford derivatives with the following thiophilic substituents: chloro- (**4**), bromo- (**5**) and iodoacetamido- (**6**) or β-maleimidopropionamido- (**7**).

The reaction of BSA with the *N*-hydroxysuccinimide dithiopropionate followed by treatment with dithiothreitol allows the introduction of 20–25 thiol groups per mol of the protein. The reaction of thiol functionalized BSA with galactosides bearing spacers with thiophilic groups was studied under several conditions and Gal/SH ratios. As can be seen from Table 1, the efficiency of the coupling alkylation reaction increased from chloro to iodo in the halogen series, as expected. The Michael addition of **7** was more effective even than the alkylation with the iodoacetamido derivative **6**.

To check the usefulness of the procedure, Lewis human blood group oligosaccharides were first treated with the *N*-hydroxysuccinimide β-maleimidopropionate and the corresponding derivatives **8**, **9**, **10** and **11** were conjugated with BSA. The conjugation proceeded smoothly giving substituted BSA in an oligosaccharide-based yield ranging from 50–70%.

The coupling of other important synthetic oligosaccharides to Meningococcal Outer Membrane Protein Complex or Tetanus Toxoid is also straightforward and represents an excellent alternative for vaccine development [19, 20].

In conclusion a mild high yielding procedure was established for coupling synthetic oligosaccharides to protein through a spacer amino group.

References

1 Bundle DR, Gidney MAJ, Kassam N, Rahman AFR (1982) *J Immunology* **129**: 678–82.
2 Martensson S, Brodin T, Carlstrom AS, Dahmen J, Frejd T, Gunnarsson A, Jansson U, Magnusson G, Lundblad A (1986) *Glycoconjugate J* **3**: 163–74.
3 McLean GD, Reddish M, Koganty RR, Wong T, Gandhi S, Smolenski M, Samuel J, Nabholtz JM, Longenecker BM (1993) *Cancer Immunol Immunother* **36**: 215–22.
4 Peeters CCAM, Evenberg D, Hoogerhout P, Kayhty H, Saarinen L, van Boeckel CAA, van Marel GA, van Boom JH, Poolman JT (1992) *Infect Immun* **60**: 1826–33.
5 Magnusson G, Chernyak AY, Kihlberg J, Kononov LO (1994) *Neoglycoconjugates*: *Preparation and Applications* pp 53–143, San Diego, CA: Academic Press.
6 Anderson GW, Zimmerman JE, Callahan FM (1964) *J Am Chem Soc* **86**: 1839–42.
7 Keller O, Rudinger J, (1975) *Helvetica Chim Acta* **58**: 531–41.
8 Collecnnik, Capla (1957) *Methods in Enzymology*, **73**: 448–50.
9 Ellman GL (1958) *Arch Biochem Biophys* **74**: 443.
10 Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) *Anal Biochem* **28**: 350–6.
11 Roy R, Laferriere CA (1990) *J Chem Soc Chem Commun* 1709–11.
12 Amuam-Zollo PH, Sinay P (1986) *Carbohydr Res* **150**: 199–212.
13 Fernandez-Santana VF, Marino-Albernas JR, Verez-Bencomo V, Perez-Martinez CS (1989) *J Carbohydr Chem* **8**: 531–7.
14 Szurmai Z, Szabo L, Liptak A (1989) *Acta Chimica Hungarica* **126**: 259–69.

15 Verez Bencomo V, Campos Valdes MT, Mariño Albernas JR, Fernandez Santana V, Hernandez Rensoli M, Perez Martinez CS (1991) *Carbohydr Res* **271**: 263–7.
16 Bertozzi CR, Bednarski MD (1991) *J Org Chem* **56**: 4326–9.
17 Nilsson S, Bengtsson M, Norberg T (1991) *J Carbohydr Chem* **10**: 1–22.
18 Pozsgay V (1993) *Glycoconjugate J* **10**: 133–41.
19 Adan-Padron A, Cuello-Perez M, Cabrera-Blanco O, Hernandez-Escandell G, Chiu-Machado I, Esnard-Bolaños S, Alamino-Perez M, Gonzalez Lio R, Fernandez-Santana V, Sierra-Gonzalez G, Verez-Bencomo V (1996) XVIII International Carbohydrate Symposium, Milan, Italy, July 21–26, p 590.
20 Verez-Bencomo V, Adán Padrón A, Figueroa Pérez S, Fernández Santana V, González Lio R, Alamino Pérez M, Cejas Ortega PJ, Alonso ME, Cuello Pérez M, Cabrera Blanco O, Sierra Gonzalez G (1997) *Biotecnología Aplicada* **14**: 56–7.

Received 14 January 1997, revised and accepted 12 December 1997