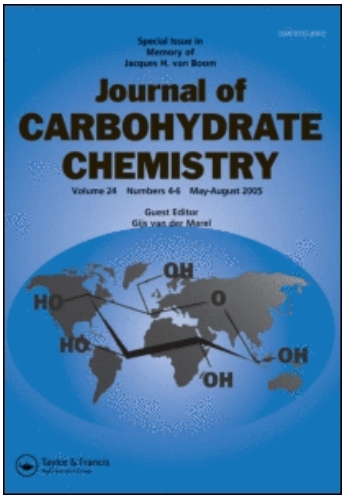

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SYNTHESIS OF NEOGLYCOPROTEINS AS ARTIFICIAL ANTIGENS

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ABSTRACT

The synthesis of various allyl glycosides of L-rhamnose containing ether and/or ester substituents are reported. Ozonolysis of allyl glycosides followed by reductive amination with ε-amino groups of lysine residues in bovine serum albumin using sodium cyanoborohydride at pH 7.8 provided different structural neoglycoproteins as artificial antigens.

INTRODUCTION

Carbohydrates as glycoproteins are not only widely distributed but are also decisive factors in post translational biological selectivity especially in biological recognition.[2-4] The presence of complex carbohydrates as integral constituents of membranes and cell walls[5-7] had led to the antigenic specificity of carbohydrates which is due to the structural variability of the non-reducing terminus comprising one, two or three additional glycosyl units.[8] The work on the targeted synthesis of complex carbohydrates is of interest since the oligosaccharides containing rhamnose are widespread in the lipopolysaccharide *O*-chains of bacteria which serve as the

recognition markers for the immune system.[7] An additional objective has been to achieve conjugation of synthesized hapten to protein via the simplest type of linker arm, consistent with binding of the carbohydrate moiety to antibodies and with the retention of potentially removable groups such as *O*-acyl substituents which are lost in the case of the methoxycarbonyl octyl arm of the Lemieux type[9] undergoing the hydrazide formation. In this connection, the method of Bernstein and Hall[10] was found to be more suitable and in which the ozonolysis of allyl glycosides was followed by reductive amination to protein with the formation of an *N*-glycosyloxyethyllysine residue.

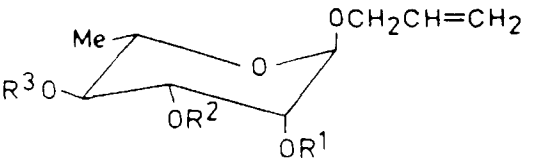
RESULTS AND DISCUSSION

Attention was first drawn to the synthesis of allyl-α-**L**-rhamnopyranoside (**1**), $[\alpha]_D$-49° (lit[11] $[\alpha]_D$-56°), for the preparation of structurally different neoglycoproteins. Isopropylidenation, methylation followed by deisopropylidenation of **1** yielded allyl 4-*O*-methyl-α-**L**-rhamnopyranoside, $[\alpha]_D$-84° (*c* 1.0, methanol) (lit[12] $[\alpha]_D$ -78.5°, methanol).

Regioselective methoxybenzylation[13] of the 2,3-dibutylstannylene derivative of the 4-*O*-methyl derivative of **1** with 4-methoxybenzyl chloride (MPMCl) and tetra-*n*-butyl ammonium bromide gave allyl 3-*O*-(4-methoxybenzyl)-4-*O*-methyl-α-**L**-rhamnopyranoside (**2**).

Acetylation of **2** yielded allyl 2-*O*-acetyl-3-*O*-(4-methoxybenzyl)-4-*O*-methyl-α-**L**-rhamnopyranoside (**3**). Demethoxybenzylation of **3** with ceric ammonium nitrate gave allyl 2-*O*-acetyl-4-*O*-methyl-α-**L**-rhamnopyranoside (**4**). Allyl 2-*O*-benzoyl-3-*O*-(4-methoxybenzyl)-4-*O*-methyl-α-**L**-rhamnopyranoside (**5**) was synthesized from **2** with benzoyl chloride followed by demethoxybenzylation with ceric ammonium nitrate yielding allyl 2-*O*-benzoyl-4-*O*-methyl-α-**L**-rhamnopyranoside (**6**). Likewise, allyl 3-*O*-(4-methoxybenzyl)-2,4-di-*O*-methyl-α-**L**-rhamnopyranoside (**7**) was synthesized from **2** with sodium hydride and methyl iodide. Compound **7** was deprotected

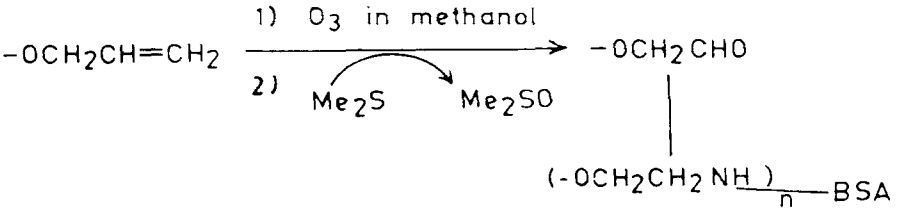
SCHEME I



	R^1	R^2	R^3
1	H	H	H
2	H	MBn	Me
3	Ac	MBn	Me
4	Ac	H	Me
5	Bz	MBn	Me
6	Bz	H	Me
7	Me	MBn	Me
8	Me	H	Me
9	Me	Me	Me
10	Ac	Ac	Ac
11	Bz	Bz	Me
12	CMe_2		Bn
13	H	H	Bn
14	CMe_2		Ac
15	H	H	Ac
16	H	MBn	Ac
17	Bz	MBn	Ac
18	Bz	H	Ac

MBn = 4-methoxybenzyl
Bz = Benzoyl
Bn = Benzyl
CMe_2 = Isopropyl

SCHEME II



Compound number **1**, **2**, **4**, **6**, **9**, **10**, **11**, **13**, **15** and **18** in Scheme I were conjugated to protein.

at *O*-3 with ceric ammonium nitrate to give allyl 2,4-di-*O*-methyl- α -**L**-rhamnopyranoside (**8**). Allyl 2,3,4-tri-*O*-methyl- α -**L**-rhamnopyranoside (**9**) and allyl 2,3,4-tri-*O*-acetyl- α -**L**-rhamnopyranoside (**10**), $[\alpha]_D$-56° (lit[14] $[\alpha]_D$-53°), were synthesized from **1** using conventional methylating and acetylating procedures respectively. Allyl 2,3-di-*O*-benzoyl-4-*O*-methyl-α-**L**-rhamnopyranoside (**11**) was prepared from the 4-*O*-methyl derivative of **1** using benzoyl chloride. Allyl 4-*O*-benzyl-2,3-*O*-isopropylidene- α -**L**-rhamnopyranoside (**12**), $[\alpha]_D$-49° (lit[15] $[\alpha]_D$-58°), was prepared from 2,3-*O*-isopropylidene derivative[15] of **1** using sodium hydride and benzyl bromide. Deketalation of **12** with aq acetic acid yielded allyl 4-*O*-benzyl- α -**L**-rhamnopyranoside (**13**), $[\alpha]_D$-62° (lit[16] $[\alpha]_D$-72.5°).

Acetylation of the 2,3-*O*-isopropylidene derivative of **1** gave allyl 4-*O*-acetyl-2,3-*O*-isopropylidene- α -**L**-rhamnopyranoside (**14**). Deisopropylidenation of **14** gave allyl-4-*O*-acetyl- α -**L**-rhamnopyranoside (**15**). Compound **15** was subsequently converted to allyl 4-*O*-acetyl-2-*O*-benzoyl-α-**L**-rhamnopyranoside (**18**) via the formation of allyl 4-*O*-acetyl-3-*O*-(4-methoxybenzyl)- α -**L**-rhamnopyranoside (**16**), followed by benzoylation to allyl 4-*O*-acetyl-2-*O*-benzoyl-3-*O*-(4-methoxybenzyl)- α -**L**-rhamnopyranoside (**17**) and finally demethoxybenzylation of **17** with ceric ammonium nitrate gave **18**.

The above compounds, viz. **1**, **2**, **4**, **6**, **9**, **10**, **11**, **13**, **15** and **18** were used as a carbohydrate hapten during conjugation with bovine serum albumin (BSA) (a protein) by the Bernstein and Hall method.[10]

The neoglycoproteins (NGPs) of the compounds **4**, **6**, **10**, **11**, **15** and **18** containing *O*-acyl substituents were found to retain the *O*-acyl groups[17] confirming that the Bernstein and Hall method is superior to Lemieux method of conjugation.

EXPERIMENTAL

General Procedures. Solvents and reagents were purified and dried according to standard procedures.[18] All compounds

were dried over phosphorus pentaoxide in a high vacuum prior to use. Evaporations were conducted under diminished pressure at <40 °C. Melting points were taken on an electrically heated Toshniwal CL-03001 melting point apparatus. Column chromatography was performed with silica gel (60-120 mesh) using light petroleum-ethyl acetate (9:1) unless otherwise stated. UV absorbance (λ_{max} 490 nm) were recorded on Bausch and Lomb spectronic 2000 model. ^1H NMR were recorded in $CDCl_3$ at Bruker AM 300 FT NMR, EM 360 L or Perkin-Elmer R-32 spectrometer with Me_4Si as internal standard at ambient temperature. NMR spectra are reported in sufficient detail only to substantiate the chemical changes effected. Resonances for allyl group are not cited individually since all showed δ_H 4.12-4.26 (m, 2H, OCH_2), 5.18-5.34 (m, 2H, $=CH_2$) and 5.82-5.96 (m, 1H, CH). Optical rotations were determined on a Perkin-Elmer 241 polarimeter at ambient temperature using 1% concentration of the compounds in chloroform unless otherwise stated.

Neoglycoproteins (Scheme II). The procedure of Bernstein and Hall[10] was applicable to the synthesis of all NGPs. Allyl glycoside (~30 mg) in methanol (~30 mL) was ozonized for 10 min at -78 °C, then treated with dimethylsulphide (0.2 mL) for 2 h and concentrated to a syrupy aldehyde. The product (~ 29 mg) and bovine serum albumin (~35 mg) in 0.2 m sodium phosphate buffer (2 mL, pH 7.8) were incubated with sodium cyanoborohydride (35 mg) at 37 °C for 72 h. The resulting solution was then purified by passing it through a column of Sephadex G-25 equilibrated in and eluted with sodium phosphate buffer at pH 7.8. The carbohydrate rich fractions that gave a positive phenol sulphuric acid test[19] were dialyzed against distilled water. These compounds gave a band at 280 nm in their UV spectra. The NGPs were finally freeze dried (~ 38 mg).

The analysis of the degree of glycosylation of the various NGPs showed that in general, 18-49 moles of carbohydrate were carried per mol of bovine serum albumin, which has a total of 59 lysine residues.

Allyl 3-*O*-(4-Methoxybenzyl)-4-*O*-methyl-α-L-rhamnopyranoside (2). To a stirred solution of the 4-*O*-methyl derivative of **1** (2 g, 9.1 mmol) in benzene (30 mL), was added dibutyltin oxide (2.48 g, 9.99 mmol) and the reaction mixture was refluxed for 2 h with continuous removal of water. After concentration of the solution to 75%, 4-methoxy benzyl chloride (0.94 mL, 9.34 mmol) and tetra-*n*-butyl ammonium bromide (3.01 g, 9.34 mmol) were added and the mixture was refluxed overnight and concentrated. The residue was subjected to column chromatography to give **2** (2.9 g, 94%) as a crystalline compound: mp 65-67 °C; $[\alpha]_D$ -61.5°; ^{1}H NMR δ 1.30 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6), 3.56, 3.80 (2s, 6H, 2MeO), 4.64 (s, 2H, OCH_2Ph), 4.82 (s, 1H, H-1), 6.85-7.38 (m, 4H, Ph-H).

Anal. calcd for $C_{18}H_{26}O_6$: C, 63.88; H, 7.74. Found: C, 63.62; H, 7.55.

Allyl 2-*O*-Acetyl-3-*O*-(4-methoxy benzyl)-4-*O*-methyl-α-L-rhamnopyranoside (3). Acetic anhydride (5 mL) was added to **2** (800 mg) in pyridine (5 mL) at room temperature and the solution stirred overnight. The mixture was concentrated with coevaporation of toluene under reduced pressure. Column chromatography gave **3** as a syrup (800 mg, 89%): $[\alpha]_D$-29°; ^{1}H NMR δ 1.30 (d, 3H, $J_{5,6}$ = 6Hz, Me-6), 2.20 (s, 3H, AcO), 3.50, 3.80 (2s, 6H, 2MeO), 4.76 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1), 6.76-7.40 (m, 4H, Ph-H).

Anal. calcd for $C_{20}H_{28}O_7$: C, 63.14; H, 7.41. Found: C, 63.08; H 7.26.

Allyl 2-*O*-Acetyl-4-*O*-methyl-α-L-rhamnopyranoside (4). A solution of **3** (600 mg, 1.5 mmol) and ceric ammonium nitrate (1.73 g, 2 meq) in acetonitrile : water (9:1, 10 mL) was stirred at room temperature for 2 h. The reaction mixture was then diluted with dichloromethane, washed with aq sodium hydrogen carbonate, dried and concentrated. The residue was chromatographed to give **4** as a viscous syrup (205 mg, 50%): $[\alpha]_D$-63°; ^{1}H NMR δ 1.32 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6), 2.18 (s, 3H, AcO), 3.61 (s, 3H, MeO), 4.76 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1).

Anal. calcd for $C_{12}H_{20}O_6$: C, 55.37; H, 7.74. Found: C, 55.19; H 7.63.

Allyl 2-O-Benzoyl-3-O-(4-methoxybenzyl)-4-O-methyl-α-L-rhamnopyranoside (5). Benzoyl chloride (0.04 mL, 1.5 meq) was added to **2** (800 mg, 2.36 mmol) in dry pyridine (10 mL) at 0 °C. The reaction mixture was stirred overnight at room temperature. Usual workup and concentration followed by column chromatography yielded **5** (1 g, 96%) as a crystalline product: mp 80-82 °C; $[\alpha]_D$ +50°; ^{1}H NMR δ 1.35 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6), 3.56, 3.76 (2s, 6H, 2MeO), 4.52, 4.72 (2d, 2H, J_{AB} = 11 Hz, OCH_2-Ph) 4.88 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1), 6.76-8.14 (m, 9H, 2Ph-H).

Anal. calcd for $C_{25}H_{30}O_7$: C, 67.85; H, 6.83. Found: C, 67.67; H, 6.73.

Allyl 2-O-Benzoyl-4-O-methyl-α-L-rhamnopyranoside (6). A solution of **5** (500 mg, 1.13 mmol) and ceric ammonium nitrate (1.24 g, 2 meq) in acetonitrile:water (9:1, 10 mL) was stirred at room temperature for 2 h as in **3**. Column chromatography gave **6** as a crystalline product (200 mg, 55%): mp 40-42 °C; $[\alpha]_D$ - 13.5°; ^{1}H NMR δ 1.38 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6), 3.61 (s, 3H, MeO), 4.89 (s, 1H, $J_{1,2}$ = 1.3 Hz, H-1), 7.44-8.00 (m, 5H, Ph-H).

Anal. calcd for $C_{17}H_{22}O_6$: C, 63.34; H, 6.87. Found: C, 63.15; H, 6.69.

Allyl 3-O-(4-Methoxybenzyl)-2,4-di-O-methyl-α-L-rhamnopyranoside (7). Sodium hydride (257 mg, 2.5 meq) was added to **2** (800 mg, 2.36 mmol) in tetrahydrofuran (30 mL) and the mixture was stirred at room temperature for 1 h and then cooled to 0 °C. Methyl iodide (0.58 mL, 4 meq) was added and the mixture was stirred at room temperature over a period of 3 h. Excess sodium hydride was destroyed by the addition of methanol, the solvents were evaporated and the residue was taken in chloroform. The chloroform solution was washed with aq sodium chloride and once with water, dried, filtered and concentrated. The resulting syrup was chromatographed to give **7** (810 mg, 97%) as a viscous syrup: $[\alpha]_D$-69°; ^{1}H NMR δ 1.30 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6) 3.48, 3.50, 3.80 (3s, 9H, 3MeO), 6.70-7.38 (m, 4H, Ph-H).

Anal. calcd for $C_{19}H_{28}O_6$: C, 64.75; H, 7.95. Found: C, 64.59; H, 7.88.

Allyl 2,4-Di-*O*-methyl-α-L-rhamnopyranoside (8). A solution of **7** (700 mg, 1.98 mmol) and ceric ammonium nitrate (2.49 g, 2 meq) in acetonitrile: water (9:1, 10 mL) was stirred at room temperature for 2 h as in **3**. Column chromatography over silica gel with light petroleum-ethyl acetate (8:2) gave **8** as a viscous syrup (300 mg, 57%): $[\alpha]_D$-59°; ^{1}H NMR δ 1.20 (d, 3H, $J_{5,6}$ = 6Hz, Me-6), 3.35, 3.42 (2s, 6H, 2MeO), 4.72 (s, 1H, H-1).

Anal. calcd for $C_{11}H_{20}O_5$: C, 56.88; H, 8.67. Found: C, 56.70; H, 8.50.

Allyl 2,3,4-Tri-*O*-methyl-α-L-rhamnopyranoside (9). Sodium hydride (160 mg, 7.5 meq) was added to **1** (100 mg, 0.49 mmol) in tetrahydrofuran (30 mL). The mixture was stirred at room temperature for 1 h and then cooled to 0 °C. Methyl iodide (0.3 mL, 12 meq) was added and the mixture was allowed to reach to room temperature as in **2**. The residue was chromatographed to give **9** as a viscous syrup (80 mg, 66%): $[\alpha]_D$ -32°; ^{1}H NMR δ 1.32 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6), 3.42, 3.50, 3.56 (3s, 9H, 3MeO), 4.82 (s, 1H, H-1).

Anal. calcd for $C_{12}H_{22}O_5$: C, 58.51; H, 9.00. Found: C, 58.46; H 8.80.

Allyl 2,3-Di-*O*-benzoyl-4-*O*-methyl-α-L-rhamnopyranoside (11). Benzoyl chloride (0.35 mL, 3 meq) was added dropwise to a stirred solution of the 4-*O*-methyl derivative of **1** (200 mg, 0.91 mmol) in dry pyridine at 0 °C. The reaction mixture was stirred overnight at room temperature as in **2**. The residue was chromatographed to give **11** (300 mg, 77%) as a crystalline product: mp 108-110 °C; $[\alpha]_D$+2°; ^{1}H NMR δ 1.36 (d, 3H, $J_{5,6}$ = 6Hz, Me-6), 3.51 (s, 3H, MeO), 4.93 (s, 1H, H-1), 7.30-8.00 (m, 10H, 2Ph-H).

Anal. calcd for $C_{24}H_{26}O_7$: C, 67.59; H, 6.14. Found: C, 67.40; H, 6.08.

Allyl 4-*O*-Acetyl-2,3-*O*-isopropylidene-α-L-rhamnopyranoside (14). Conventional acetylation of the 2,3-*O*-isopropylidene derivative of **1** (3 g, 12.2 mmol) with 1:1

pyridine: acetic anhydride (10 mL) gave **14** as in **2**. Column chromatography gave **14** (2.9g, 82%) as a viscous syrup $[\alpha]_D$ -18°; ^{1}H NMR δ 1.20 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6), 1.40, 1.65 (2s, 6H, 2Me), 2.12 (s, 3H, AcO), 4.90 (s, 1H, H-1).

Anal. calcd for $C_{14}H_{22}O_6$: C, 58.72; H, 7.74. Found: C, 58.66; H, 7.63.

Allyl 4-O-Acetyl-α-L-rhamnopyranoside (15). A solution of **14** (2.5 g, 8.7 mmol) in acetic acid : water (4:1, 10 mL) was warmed at 60 °C for 0.5 h. The reaction mixture was then concentrated by co-evaporation with toluene. The residue was chromatographed on silica gel with light petroleum-ethyl acetate (7:3) to give **15** as a crystalline product (2.05 g, 95%): mp 50-52 °C; $[\alpha]_D$-80°; ^{1}H NMR δ 1.23 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6), 2.13 (s, 3H, AcO), 4.75 (s, 1H, H-1).

Anal. calcd for $C_{11}H_{18}O_6$: C, 53.65; H, 7.36. Found: C, 53.52; H, 7.28.

Allyl 4-O-Acetyl-3-O-(4-methoxybenzyl)-α-L-rhamnopyranoside (16). A stirred solution of **15** (2 g, 8.13 mmol) in benzene (30 mL) containing dibutyltin oxide (2.22 g, 8.92 mmol) was refluxed for 2 h with continuous removal of water. After the concentration of the solution to 75%, 4-methoxybenzyl chloride (1.84 mL, 8.34 mmol) and tetra-*n*-butylammonium bromide (2.68 g, 8.34 mmol) were added as in the 4-*O*-methyl derivative of **1**. The residue was chromatographed over silica gel with light petroleum-ethyl acetate (8:2) to give **16** (2.2 g, 74%) as a viscous syrup: $[\alpha]_D$-31.5°; ^{1}H NMR δ 1.20 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6), 1.98 (s, 3H, AcO), 4.80 (s, 1H, H-1), 6.75-7.35 (m, 4H, Ph-H).

Anal. calcd for $C_{19}H_{26}O_7$: C, 62.28; H, 7.15. Found: C, 62.15; H, 7.00.

Allyl 4-O-Acetyl-2-O-benzoyl-3-O-(4-methoxybenzyl)-α-L-rhamnopyranoside (17). Benzoyl chloride (0.9 mL, 1.5 meq) was added to **16** (2 g, 5.4 mmol) in dry pyridine (10 mL) at 0 °C. The reaction mixture was stirred overnight at room temperature as in **2**. Column chromatography gave pure viscous syrup **17** (2.2g, 86%): $[\alpha]_D$ + 44.5°; ^{1}H NMR δ 1.20 (d, 3H,

$J_{5,6}$ = 6Hz, Me-6), 2.00 (s, 3H, AcO), 3.75 (s, 3H, MeO), 4.20-4.60 (q, 2H, J_{AB} = 12 Hz, OCH_2-Ph), 4.90 (s, 1H, H-1), 6.70-8.20 (m, 9H, Ph-H).

Anal. calcd for $C_{26}H_{30}O_8$: C, 66.37; H, 6.42. Found: C, 66.23; H, 6.32.

Allyl 4-O-Acetyl-2-O-benzoyl-α-L-rhamnopyranoside (18). A solution of **17** (2 g, 4.2 mmol) and ceric ammonium nitrate (4.6 g, 2 meq) in acetonitrile: water (9:1, 10 mL) was stirred at room temperature for 2 h as in **3**. The residue was chromatographed with light petroleum-ethyl acetate (8:2) to give crystalline **18** (1.2 g, 81%): mp 105-108 °C; $[\alpha]_D$ +20°; ^1H NMR δ 1.30 (d, 3H, $J_{5,6}$ = 6 Hz, Me-6), 2.15 (s, 3H, AcO), 4.97 (s, 1H, H-1), 7.45-8.08 (m, 5H, Ph-H).

Anal. calcd for $C_{18}H_{22}O_7$: C, 61.70; H, 6.32. Found: C, 61.55; H, 6.18.

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