Synthesis of the Trisaccharide–Protein Conjugate of the Phenolic Glycolipid of *Mycobacterium tuberculosis* for the Serodiagnosis of Tuberculosis

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Received March 11, 1991

The trisaccharide segment of the phenolic glycolipid (PGL) of *Mycobacterium tuberculosis*, 2-*O*-methyl-3-*O*-[3-*O*-(2,3,4-tri-*O*-methyl-α-L-fucopyranosyl)-α-L-rhamnopyranosyl]-α-L-rhamnopyranose, was synthesized in the form of the *p*-(2-methoxycarbonylethyl)phenyl glycoside by a stepwise condensation. 2,4-Di-*O*-benzyl-3-*O*-acetyl-α-L-rhamnopyranosyl chloride was coupled to *p*-(2-methoxycarbonylethyl)phenyl 4-*O*-benzyl-2-*O*-methyl-α-L-rhamnopyranoside in the presence of silver triflate, and 2,3,4-tri-*O*-methyl-α-L-rhamnopyranosyl chloride was then coupled to the deacetylated disaccharide by the same procedure. The trisaccharide was deblocked and coupled to BSA, giving the neoglycoconjugate TB-NT-P-BSA. TB-NT-P-BSA showed its possibility as a useful tool for the serodiagnosis of tuberculosis.

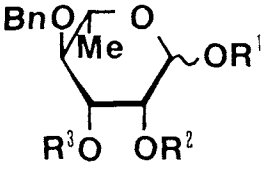
The isolation of phenolic glycolipids (PGL) of *Mycobacterium leprae* and successful syntheses of their haptenic di- and trisaccharide–protein conjugates has shown that these conjugates were very specific and sensitive to leprosy sera, especially to multibacillary cases.[1-8] By using them, powerful and simple tools for the serodiagnosis of leprosy have been developed, and some of them are already in practical use.[9] Similar studies are in progress on *M. kansasii*,[10,11] suggesting the usefulness of serodiagnosis by using synthetic saccharides of PGL of these mycobacteria. In addition to those, some mycobacteria including *M. bovis*[12] and *M. marinum*[13] have PGL, suggesting that PGL is common in most mycobacteria.

For tuberculosis, which is a very important mycobacterial disease, Daffé *et al.* have reported the isolation and structural elucidation of the PGL of *M. tuberculosis*.[14] This PGL carried the phithiocerol core, a common structural component of other PGL, and the unique trisaccharide, 2-*O*-methyl-3-*O*-[3-*O*-(2,3,4-tri-*O*-methyl-α-L-fucopyranosyl)-α-L-rhamnopyranosyl]-L-rhamnopyranose. Torgal-Garcia *et al.* have shown that this PGL was specific and sensitive to the sera of tuberculosis.[15] Therefore, we synthesized the trisaccharide-protein conjugate for use in an immunological assay for the serodiagnosis of tuberculosis. This strategy avoided difficulties like the poor solubility and low reproducibility we encountered when *M. tuberculosis*-PGL itself was used in an immunological assay. It also offers the possibility to develop various methods for serodiagnosis by using a variety of proteins as carriers.

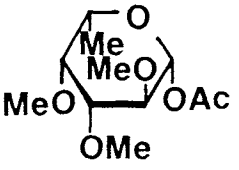
Synthesis of the trisaccharide of *M. tuberculosis* PGL in the form of the *p*-(2-methoxycarbonylethyl)phenyl glycoside, which provides a link arm to hook it to a protein carrier, was carried out by stepwide condensation of the derivatized monosaccharides.

O-Methylation of benzyl 4-*O*-benzyl-α-L-rhamnopyranoside (**1**)[16] with a large excess of methyl iodide and a concentrated sodium hydroxide solution, under phase-transfer catalysis, gave mainly the 2-*O*-methylated derivative (**2**, 67% of the reaction products). Brief acid hydrolysis with 3 M trifluoroacetic acid (TFA) and subsequent acetylation gave the di-*O*-acetyl compounds as anomeric epimers.

The mixture was heated, without separation, at about 120°C with methyl *p*-hydroxyphenylpropionate in the presence of a catalytic amount of *p*-toluenesulfonic acid to give an anomeric mixture of *p*-(2-methoxycarbonylethyl)phenyl α-L- and β-L-rhamnoside (**3** and **4**) in a ratio of about 3 : 1. These were roughly separated into two fractions, and the fraction mainly consisting of **3** was deacetylated with sodium methoxide, and purified by column chromatography to give an α-L-linked reducing-end monosaccharide derivative with a link arm (**5**) in a yield of 23.8% after 4 steps from **2**. The large J_1 value (168.59 Hz) of **5** from the ^{13}C-NMR analysis confirmed the α-L-linkage.[17]



	R^1	R^2	R^3
1	Bn(α)	H	H
2	Bn(α)	Me	H
3	La(α)	Me	Ac
4	La(β)	Me	Ac
5	La(α)	Me	H
6	Bn(α)	Bn	H
7	Ac(α)	Bn	Ac



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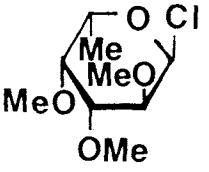

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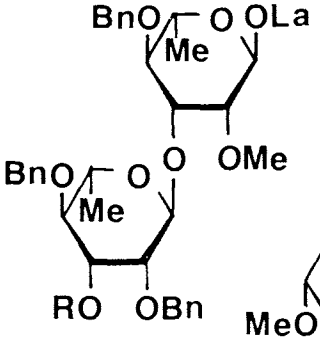
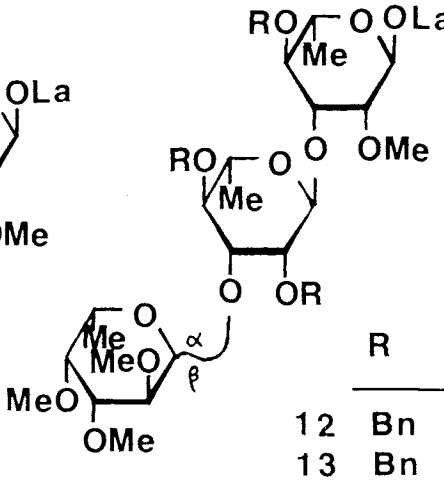
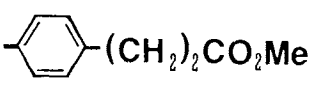


	R
8	Ac
9	H



	R	Fuc-Rha link
12	Bn	α
13	Bn	β
14	H	α

La = 

Benzylation of **1** with a limited amount of benzyl bromide under phase-transfer catalysis mainly gave 2-*O*-benzylated compound **6**. Compound **6** was rather resistant to acid hydrolysis with 3 M TFA, the degree of hydrolysis only being about 65% after 6 hr at 100°C. However, the hydrolysis was stopped after 6 hr to avoid degradation. The hydrolyzate was acetylated to give 2,4-di-*O*-benzylated acetate **7** in a relatively low yield of 39.7%. Compound **7** was chlorinated by heating in trimethylsilyl chloride-dry toluene at about 120°C. As the chloride was not stable enough for purification, it was used for the coupling reaction without further purification. Coupling of the chloride with **5** was carried out by a Koenigs–Knorr reaction with 1,1,3,3-*N*-tetramethylurea and silver triflate, the synthesized disaccharide (**8**) being purified by repeated column chromatography. The same large J_1 and $J_{1'}$ values of 170.24 Hz (99.99 and 95.28 ppm) in the ^{13}C- and coupled ^{13}C–^1H-NMR spectra of **8** showed that each sugar residue was in an α-L-configuration. A brief treatment of **8** with sodium methoxide gave **9**.

Methyl 2,3,4-tri-*O*-methyl-α-L-fucopyranoside, which was synthesized by methylating methyl α-L-fucopyranoside, was refluxed in 3 M TFA and then acetylated to give **10**. Compound **10** was next heated at about 120°C in trimethylsilyl chloride-dry toluene. The ^1H-NMR spectrum of the product suggested that it was mostly the α-L-chloride ($J_{1,2}=3.5$ Hz, **11**) and contained only a small amount of degradation products. The condensation reaction of **9** and **11** in the presence of silver triflate and 1,1,3,3-tetra-*N*-methylurea gave a trisaccharide mixture consisting of *ca.* 70% of α-L-linked trisaccharide (**12**) and 30% of β-L-linked trisaccharide (**13**). These were separated by silica gel column chromatography to give pure **12** (45.1%) and **13** (20.9%).

The ^1H-NMR spectrum of **12** showed three anomeric proton signals at 5.46 ppm (H-1, $J_{1,2}=1.8$ Hz), 5.22 ppm (H-1′, $J_{1',2'}=1.5$ Hz) and 5.22 ppm (H-1″, $J_{1'',2''}=3.7$ Hz) confirming that all three glycosidic linkages were in an α-L configuration. This was strongly supported by the large J values of three anomeric carbons ($J_1=165.13$, $J_{1'}=162.60$, $J_{1''}=169.96$ Hz) in the coupled ^{13}C-^1H-NMR spectrum. The large negative $[\alpha]_D$ value of $-76.7°$ also supported the structure of **12**. The NMR spectrum of **13** showed three anomeric carbon signals at 101.10 (C-1), 100.27 (C-1′) and 95.27 ppm (C-1″). Both the C-1 and C-1′ signals had almost the same δ values as those of **12**, and had large ^{13}C–^1H coupling constants of 169.02 and 164.72 Hz, respectively. The C-1″ signal was in a lower field than that of the α-L-Fuc *p* residue of **12** by 1.44 ppm, and had a small coupling constant of 152.18 Hz. These data indicated that the fucose residue in **13** was in the β-L-configuration. The small $[\alpha]_D$ value of $-4.7°$ also supported the structure of **13**.

Compound **12** was treated with hydrogen gas in the presence of palladium-carbon, giving the expected compound **14** in a good yield. The ^1H-NMR spectrum of **14** agreed with the reported data,[14] and the ^{13}C-NMR spectrum supported the structure of **14**.

Trisaccharide **14** was treated with 0.1 M sodium hydroxide and then coupled to bovine serum albumin (BSA) by the carbodiimide method,[19] giving the neoglycoconjugate TB-NT-P-BSA. When the molar ratio of the trisaccharide to BSA was 93 : 1 in the coupling reaction, the synthesized TB-NT-P-BSA contained 35.6 mol of the trisaccharide per mole mole of BSA. The recovery of the trisaccharide was 29.5%, which is a good figure. GLC-MS analysis of the products after treating with hydrogen chloride in 2-butanol showed that the trisaccharide was incorporated to BSA without degradation. These results proved that the carbodiimide method was suitable for preparing TB-NT-P-BSA. TB-NT-P-BSA was very soluble in water and attached firmly to a microtiter plate, providing easy handling and good reproducibility.

Preliminary results of the serological assay, using an enzyme-linked immunosorbent assay, showed that TB-NT-P-BSA had relatively high activity (71.2%) and specificity (89.4%) against human tuberculosis sera. Although both the activity and specificity are not yet enough and

need to be improved, these data indicate the possibility to develop a useful method for the serodiagnosis of tuberculosis by using TB-NT-P-BSA, as the case for leprosy. An additional important observation is that the aglycon substituent allowed binding of the synthetic trisaccharide to various kinds of protein, which provide a new method in fields other than serodiagnosis. For example, TB-NT-P-KLH (keyhole limpets hemocyanin) had strong antigenicity. Therefore, TB-NT-P-KLH would be a good antigen for preparing a high-titer antiserum or monoclonal antibody to the PGL of *M. tuberculosis*.

Experimental

General procedures. Optical rotation values were determined in chloroform with a Horiba SEPA 2000 polarimeter at 20°C. TLC was conducted on silica gel F_{254} (0.25 mm, Merck) with a solvent system of 100:3 benzene–methanol (A) or benzene–acetone (B, 16:1, C; 8:1; D, 4:1; or E, 2:1). ^{1}H-NMR spectra were recorded with a Hitachi R-24B (60 MHz) or a Hitachi R-2000 (90 MHz) nuclear magnetic resonance spectrometer, while ^{13}C-NMR spectra were recorded with a Hitachi R-2000 (22.5 MHz) nuclear magnetic resonance spectrometer. All NMR spectra were recorded in chloroform-*d* with Me_4Si as a standard. For the ^{1}H- and ^{13}C-NMR data, only the characteristic and important signals are cited. The symbols in parentheses in the ^{13}C-NMR data express the coupling patterns in coupled ^{13}C–^{1}H spectra.

Benzyl 4-O-benzyl-2-O-methyl-α-L-rhamnopyranoside (**2**). Compound **1** (9.2 g, 26.7 mmol), methyl iodide (9 ml, 139.5 mmol), tetrabutylammonium bromide (4.3 g, 14.0 mmol), aq. 20% sodium hydroxide (27 ml) and dichloromethane (270 ml) were shaken at room temperature for 4 days. The organic layer was washed with water four times, dried (Na_2SO_4) and concentrated. The residue was chromatographed on a silica gel column. The fractions obtained by eluting with benzene–acetone (32:1) were collected to give **2** (5.25 g, 14.6 mmol, 54.9%): $[\alpha]_D$ −58.7° (c=15.6), lit.[18] $[\alpha]_D$ −62°; *Rf* 0.53 (solvent A), lit.[18] *Rf* 0.50; IR ν_{max} cm^{-1}: 3700–3200 (νO–H); ^{1}H-NMR δ (60 MHz): 7.33, 7.30 (10H, m, 2Ph–CH_2), 4.93 (1H, d, $J_{1,2}$=1.5 Hz, H-1), 3.40 (3H, s, OMe), 2.68 (1H, d, J=8.5 Hz, OH), 1.32 (3H, d, $J_{5,6}$=6.3 Hz, H-6). *Anal.* Found: C, 70.55; H, 7.52. Calcd. for $C_{21}H_{26}O_5$: C, 70.37; H, 7.31%.

2-(Methoxycarbonylethyl)phenyl 4-O-benzyl-2-O-methyl-α-L-rhamnopyranoside (**5**). Compound **2** (5.25 g, 14.65 mmol) was refluxed in 3 M TFA (125 ml) for 30 min. The solution was concentrated to a small volume with the repeated addition of methanol, neutralized with sat. sodium hydrogen carbonate and then concentrated. The residue was acetylated with 30 ml of acetic anhydride–pyridine (1:1). The usual processing of the reaction mixture gave 5.09 g of a syrup. This syrup, methyl *p*-hydroxyphenylpropionate (4.2 g, 23.3 mmol) and *p*-toluenesulfonic acid (72 mg in 6 ml of acetic acid–acetic anhydride 95:5) were heated together at about 120°C for 1 hr under reduced pressure. The mixture was diluted twice with chloroform, successively washed with water, 10% sodium hydroxide, and water, dried and then concentrated to give 5.11 g of a syrupy residue. This residue was applied to a silica gel column, and the fractions obtained by eluting with benzene–acetone (32:1) gave mainly **3** (2.64 g). This was boiled for 2 min in 20 ml of 0.05 M methanolic sodium methoxide, neutralized with Amberlite IR-120 (H^+) and concentrated. The residue was applied to a silica gel column, and the fractions collected after elution with benzene–acetone (30:1) gave **5** (1.5 g, 3.49 mmol, 23.8% calcd for **2**); $[\alpha]_D$ −73.0° (c=3.4); *Rf* 0.42 (solvent B); IR ν_{max} cm^{-1}: 3700–3250 (νO–H), 1730 (νC=O); ^{1}H-NMR δ (90 MHz): 7.29 (5H, s, Ph–CH_2), 7.02 (4H, m, O–Ph–CH_2), 5.49 (1H, d, $J_{1,2}$=1.5 Hz, H-1), 4.76 (2H, q, J=8.9 Hz, Ph–CH_2), 3.62 (3H, s, COOMe), 3.50 (3H, s, OMe), 2.95–2.56 (5H, m, OH and O–CH_2–CH_2), 1.26 (3H, d, $J_{5,6}$=6.2 Hz, H-6); ^{13}C-NMR δ (22.5 MHz): 173.14 (COOMe), 154.92, 138.48, 134.23, 129.23, 128.31, 127.83, 116.41, 94.84 (d, J_1=168.59 Hz, C-1), 81.93, 80.65, 74.97, 71.43 (Ph–CH_2), 67.99, 58.93 (COOMe), 51.47 (OMe), 35.79 (O–CH_2–CH_2), 30.12 (O–CH_2–CH_2), 19.03 (C-6). *Anal.* Found: C, 66.68; H, 7.16. Calcd. for $C_{24}H_{30}O_7$: C, 66.96; H, 7.02%.

Benzyl 2,4-di-O-benzyl-α-L-rhamnopyranoside (**6**). Compound **1** (11 g, 31.97 mmol), benzyl bromide (8.46 g, 49.4 mmol) and tetrabutylammonium bromide (5.1 g, 24.3 mmol) were shaken in dichloromethane–aq. sodium hydroxide (10%) at room temperature for 4 days. The organic layer was washed with water, concentrated and chromatographed in a silica gel column. Elution with benzene containing 1% of *t*-butanol gave **6** (7.06 g, 16.24 mmol, 50.8%); $[\alpha]_D$ −13.6° (c=1.3, lit.[18] −15°); *Rf* 0.82 (solvent C). *Anal.* Found: C, 74.44; H, 6.64. Calcd. for $C_{27}H_{30}O_5$: C, 74.63; H, 6.96%.

1,3-Di-O-acetyl-2,4-di-O-benzyl-α-L-rhamnopyranose (**7**). Compound **6** (5.6 g, 12.89 mmol) in 3 M TFA (125 ml) was refluxed for 6 hr, neutralized with sat. sodium hydrogen carbonate, and concentrated. The residue was acetylated with acetic anhydride–pyridine (1:1, 30 ml), and then applied to a silica gel column. Elution with benzene containing 2% of *t*-butanol gave **7** (2.19 g, 5.11 mmol, 39.7%); $[\alpha]_D$ 4.9° (c=1.5); *Rf* 0.78 (solvent B); IR ν_{max} cm^{-1}: 1750, 1730 (νC=O); ^{1}H-NMR δ (60 MHz): 7.33, 7.30 (10H, m, 2Ph–CH_2), 6.13 (1H, d, $J_{1,2}$=1.6 Hz, H-1), 2.02, 1.93 (6H, 2OAc), 1.32 (3H, d, $J_{5,6}$=5.7 Hz, H-6). *Anal.* Found: C, 67.46; H, 6.34. Calcd. for $C_{24}H_{28}O_7$:

C, 67.28; H, 6.59%.

p-(2-Methoxycarbonylethyl)phenyl 4-O-benzyl-2-O-methyl-3-O-(3-O-acetyl-2,4-di-O-benzyl-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (**8**). Compound **7** (1.9 g, 4.43 mmol) was heated at about 120°C for 6 hr in a mixture (19 ml) of toluene–trimethylsilyl chloride (10 : 3). The mixture was concentrated with the repeated addition of toluene, treated with charcoal and concentrated. A solution of the residue and **5** (1.4 g, 3.25 mmol) was stirred for 3 hr at room temperature in dichloromethane (10 ml) in the presence of silver triflate (1.2 g, 4.67 mmol) and 1,1,3,3-*N*-tetramethylurea (0.5 ml). The mixture was diluted twice with chloroform, filtered to remove the silver salts, and the filtrate was successively washed with water, sat. sodium hydrogen carbonate and water, and then concentrated. The residue was applied to a silica gel column. Elution with benzene–acetone (36 : 1) gave a fraction which contained mainly **8**. This fraction was applied again to a silica gel column, and the column was washed with benzene–acetone (50 : 1). Further elution with benzene–acetone (40 : 1) gave **8** (1.01 g, 1.26 mmol, 38.8%); $[\alpha]_D$ −33.0° (c = 2.2); *Rf* 0.68 (solvent B); IR ν_{max} cm^{-1}: 1740 (νC=O); ^{1}H-NMR δ (90 MHz): 7.31 (15H, m, 3Ph–CH_2), 7.02 (4H, m, O–Ph–CH_2), 5.48 (1H, d, $J_{1,2}$ = 0.8 Hz, H-1), 5.30 (1H, dd, $J_{2',3'}$ = 2.9 Hz, $J_{3',4'}$ = 9.2 Hz, H-3'), 5.15 (1H, d, $J_{1',2'}$ = 0.9 Hz, H-1'), 3.6 (3H, s, COOMe), 3.5 (3H, s, OMe), 2.84 (2H, d, O–CH_2–CH_2, J = 7.3 Hz), 2.61 (2H, d, O–CH_2–CH_2. J = 7.3 Hz), 1.91 (3H, s, OAc), 1.38 (3H, d, $J_{5',6'}$ = 5.9 Hz, H-6'), 1.31 (3H, d, $J_{5,6}$ = 5.7 Hz, H-6); ^{13}C-NMR δ (22.5 MHz): 173.03 (s, COOMe), 169.93 (COOMe), 154.94, 138.59, 138.34, 137.95, 129.21, 128.24, 127.89, 127.21, 116.42, 99.99 (d, $J_{1'}$ = 170.24 Hz, C-1'), 95.28 (d, J_1 = 170.24 Hz, C-1), 80.24, 80.05, 79.36, 79.05, 76.87, 74.90 (Ph–CH_2), 74.82 (Ph–CH_2), 73.58, 72.72 (Ph–CH_2), 68.87, 68.43, 58.88 (OMe), 51.39 (COOMe), 35.78 (O–CH_2–CH_2), 30.10 (O–CH_2–CH_2), 20.92 (OAc), 18.21 (C-6'), 17.94 (C-6). *Anal.* Found: C, 69.35; H, 6.98. Calcd. for $C_{46}H_{54}O_{12}$: C, 69.16; H, 6.81%.

p-(2-Methoxycarbonylethyl)phenyl 4-O-benzyl-2-O-methyl-3-O-(2,4-di-O-benzyl-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside (**9**). Compound **8** (1.24 g, 1.55 mmol) in 15 ml of 0.1 M methanolic sodium methoxide was boiled for 5 min, neutralized with Amberlite IR-120 (H^+) and concentrated. The residue was applied to a silica gel column. Elution with benzene–acetone (20 : 1) gave **9** (0.90 g, 1.19 mmol, 76.7%); $[\alpha]_D$ −59.6° (c = 4.7); *Rf* 0.53 (solvent B); IR ν_{max} cm^{-1}: 3700–3300 (νO–H), 1740 (νC=O); ^{1}H-NMR δ (90 MHz): 7.31–7.25 (15H, m, 3Ph–CH_2), 7.01 (4H, m, O–Ph–CH_2), 5.45 (1H, d, $J_{1,2}$ = 1.7 Hz, H-1), 5.18 (1H, d, $J_{1',2'}$ = 1.3 Hz, H-1'), 3.64 (3H, s, COOMe), 3.55 (3H, s, OMe), 3.1–2.6 (5H, m, CH_2–CH_2 and OH), 1.41 (3H, d, $J_{5',6'}$ = 6 Hz, H-6'), 1.25 (3H, d, $J_{5,6}$ = 6 Hz, H-6). *Anal.* Found: C, 69.57; H, 6.81. Calcd. for $C_{44}H_{52}O_{11}$: C, 69.82; H, 6.93%.

1-O-Acetyl-2,3,4-tri-O-methyl-β-L-fucopyranose (**10**). Methyl 2,3,4-tri-*O*-methyl-L-fucopyranoside (2.37 g, 10.76 mmol) was refluxed in 30 ml of 3 M TFA for 1 hr and concentrated to about 10 ml with repeated addition of methanol. The solution was neutralized with sat. sodium hydrogen carbonate and then concentrated. The residue was acetylated with acetic anhydride–pyridine (1 : 1) and the acetates were applied to a silica gel column. Elution with benzene–acetone (40 : 1) gave **10** (910 mg, 3.67 mmol, 34.1%); $[\alpha]_D$ −28.7° (c = 10.0); *Rf* 0.47 (solvent B); IR ν_{max} cm^{-1} 1740 (νC=O); ^{1}H-NMR δ (60 MHz): 5.35 (1H, d, $J_{1,2}$ = 7.05 Hz, H-1), 3.57 (3H, s, OMe), 3.52 (6H, 2OMe), 2.10 (3H, s, OAc), 1.28 (3H, d, $J_{5,6}$ = 5.9 Hz, H-6). *Anal.* Found: C, 53.43; H, 8.35. Calcd. for $C_{11}H_{20}O_6$: C, 53.22; H, 8.12%.

Chlorination of **10**. Compound **10** (780 mg, 3.14 mmol) was heated to about 120°C for 5 hr in toluene–trimethylsilyl chloride (8 ml, 4 : 1), and the mixture was concentrated with the repeated addition of toluene. The residue was dissolved in toluene and treated with charcoal to give a fraction consisting mostly of chloride **11**, 2,3,4-tri-*O*-methyl-α-L-fucopyranosyl chloride (680 mg, 3.03 mmol), ^{1}H-NMR δ (60 MHz): 6.23 (d, $J_{1,2}$ = 3.5 Hz, H-1).

p-(2-Methoxycarbonylethyl)phenyl 4-O-benzyl-2-O-methyl-3-O-[2,4-di-O-benzyl-3-O-(2,3,4-tri-O-methyl-α- and β-L-fucopyranosyl)-α-L-rhamnopyranosyl]-α-L-rhamnopyranoside (**12** *and* **13**). Compounds **9** (800 mg, 1.06 mmol) and **11** (680 mg, 3.03 mmol), which was prepared from 780 mg of **10**, were stirred at room temperature overnight in dichloromethane (5 ml) in the presence of silver triflate (600 mg, 2.34 mmol) and 1,1,3,3-tetra-*N*-methylurea (0.25 ml). The mixture was diluted twice with chloroform, successively washed with water and sat. sodium hydrogen carbonate, dried and concentrated. The residue was chromatographed in a silica gel column and elution with benzene–acetone (45 : 1) gave β-L-linked trisaccharide **13** (210 mg, 0.222 mmol, 20.9%). Further elution with benzene–acetone (15 : 1) gave α-L-linked trisaccharide **12** (452 mg, 0.478 mmol, 45.1%). For **12**, $[\alpha]_D$ −76.7° (c = 3.1); *Rf* 0.51 (solvent B); IR ν_{max} cm^{-1}: 1740 (νC=O); ^{1}H-NMR δ (90 MHz): 7.30, 7.27 (15H, m, 3Ph–CH_2), 7.02 (4H, m, O–Ph–CH_2), 5.46 (1H, d, $J_{1,2}$ = 1.8 Hz, H-1), 5.22 (2H, 2d, $J_{1',2'}$ = 1.5 Hz, H-1' and $J_{1'',2''}$ = 3.7 Hz, H-1''), 3.61 (3H, s, COOMe), 3.518, 3.516, 3.48, 3.31 (12H, 4s, 4OMe), 2.78 (2H, d, J = 5.8 Hz, O–CH_2–CH_2), 2.57 (2H, d, J = 5.8 Hz, O–CH_2–CH_2), 1.35 (3H, d, J = 6.2 Hz, H-6 or H-6'), 1.25 (3H, d, J = 5.7 Hz, H-6' or H-6), 0.95 (3H, d, $J_{5'',6''}$ = 6.6 Hz, H-6'' or H-6'); ^{13}C-NMR δ (22.5 MHz): 172.90 (COOMe), 154.98 (O–Ph–CH_2), 139.21, 138.51, 134.30, 129.23, 128.35, 128.15, 128.10, 127.16, 116.47, 99.66 (d, $J_{1''}$ = 165.13 Hz, C-1''), 99.36 (d, $J_{1'}$ = 162.60 Hz, C-1'), 95.27 (d, J_1 = 169.96 Hz, C-1), 80.62, 80.35, 80.07, 79.40, 78.79, 78.03, 77.36, 75.92, 74.79, 74.61, 71.43, 68.81, 66.40, 61.49 (OMe), 59.08 (OMe), 58.74 (OMe), 57.98, 51.33 (COOMe), 35.78

(O–CH$_2$–CH$_2$), 30.15 (O–CH$_2$–CH$_2$), 18.22 (C-6 or C-6′), 17.98 (C-6′ or C-6), 16.48 (C-6″). *Anal.* Found: C, 67.58; H, 7.43. Calcd. for $C_{53}H_{68}O_{15}$: C, 67.36; H, 7.25%. For **13**, $[\alpha]_D$ −67.1° (c=7.0); *Rf* 0.51 (solvent B); IR ν_{max} cm^{-1}: 1735 (νC=O); ^{1}H-NMR δ (90 MHz): 7.27 (15H, m, 3Ph–CH$_2$), 7.04 (4H, m, O–Ph–CH$_2$), 5.47 (1H, d, $J_{1,2}$=1.7 Hz, H-1), 5.22 (1H, d, $J_{1,2}$=1.6 Hz, H-1′), 3.63 (3H, s, COOMe), 3.58, 3.52, 3.50, 3.49 (12H, 4s, 4OMe), 2.80 (2H, d, J=6.1 Hz, O–CH$_2$–CH$_2$), 2.63 (2H, d, J=6.1 Hz, O–CH$_2$–CH$_2$), 1.45–1.20 (9H, m, H-1, 1′ and 1″); ^{13}C-NMR δ (22.5 MHz): 173.08 (COOMe), 155.02 (O–Ph–CH$_2$), 138.90, 138.63, 138.44 (3Ph–CH$_2$), 134.27, 129.23, 128.65, 128.37, 128.13, 127.37, 116.47, 101.10 (d, $J_{1'',}$=152.18 Hz, C-1″), 100.27 (d, $J_{1'}$=169.02 Hz, C-1′), 95.27 (d, J_1=164.79 Hz, C-1), 84.68, 80.74, 79.58, 78.70, 78.55, 77.11, 75.71, 74.88, 72.59, 70.28, 68.75, 61.61, 60.88, 58.80, 58.13, 51.42 (COOMe), 35.82 (O–CH$_2$–CH$_2$), 30.15 (O–CH$_2$–CH$_2$), 17.94 (C-6 and C-6′), 16.70 (C-6″). *Anal.* Found: C, 67.62; H, 7.41. Calcd. for $C_{53}H_{68}O_{15}$: C, 67.36; H, 7.25%.

p-(2-Methoxycarbonylethyl)phenyl 2-O-Methyl-3-O-[3-O-(2,3,4-tri-O-methyl-α-L-fucopyranosyl)-α-L-rhamnopyranosyl]-α-L-rhamnopyranoside (**14**). An ethanolic solution (10 ml) of compound **12** (406 mg, 0.430 mmol) was stirred overnight at about 35°C in the presence of 100 mg of palladium-carbon (10%) under hydrogen gas. The catalyst was filtered and the filtrate concentrated. The residue was applied to a silica gel column. Elution with benzene–acetone (2:1) gave expected trisaccharide **14** (174 mg, 0.258 mmol, 60.0%); $[\alpha]_D$ −118.7° (c=1.5); *Rf* 0.49 (solvent D); IR ν_{max} cm^{-1}: 3650–3250 (νO–H), 1735 (νC=O); ^{1}H-NMR δ (90 MHz): 7.05 (4H, m, O–Ph–CH$_2$), 5.50 (1H, d, $J_{1,2}$=1.7 Hz, H-1), 5.18 (1H, d, $J_{1',2'}$=1.4 Hz, H-1′), 5.12 (1H, d, $J_{1'',2''}$=3.8 Hz, H-1″), 3.66 (6H, COOMe and OMe), 3.57 (3H, s, OMe), 3.51 (6H, 2OMe), 2.87 (2H, d, J=6.8 Hz, O–CH$_2$CH$_2$), 2.7 (2H, d, J=6.8 Hz, O–CH$_2$CH$_2$), 1.40–1.22 (9H, m, H-6, 6′ and 6″); ^{13}C-NMR δ (22.5 MHz); 102.10 (d, $J_{1'',}$=16.28 Hz, C-1″), 100.52 (d, $J_{1'}$=167.40 Hz, C-1′), 95.33 (d, J_1=169.28 Hz, C-1). *Anal.* Found: C, 57.23; H, 7.21. Calcd. for $C_{32}H_{50}O_{15}$: C, 56.96; H, 7.47%.

Coupling of **14** *with BSA* (*TB-NT-P-BSA*). Coupling of **14** with BSA was carried out according to the method of Lönngren *et al.*[19] Compound **14** (38 mg, 56 μmol) was stirred in 1 ml of 0.1 M sodium hydroxide. The pH was adjusted to 4.75 with 0.1 M hydrogen chloride and then BSA (40 mg in 1 ml of water) was added to the solution. A solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (110 mg in 0.5 ml of water) was added dropwise at room temperature, the pH being maintained at 4.75 during the 2 hr course of the reaction. After stirring for additional 4 hr at room temperature, the solution was dialyzed 8 times against double-distilled water. Lyophilization of the solution gave a white powder of TB-NT-P-BSA (42.2 mg). The prepared TB-NT-P-BSA had 35.1 mol of the trisaccharide per one mole of BSA, and the recovery of the trisaccharide was 29.3%.

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