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SYNTHESIS OF THE TETRASACCHARIDE REPEATING UNIT OF THE O-SPECIFIC POLYSACCHARIDE FROM *SALMONELLA MUENSTER* AND *SALMONELLA MINNEAPOLIS*.

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Abstract—The tetrasaccharide β-D-Man-(1→4)-α-L-Rha-(1→3)-D-Gal-(4←1)-α-D-Glc the repeating unit of the O-specific polysaccharide chain of the lipopolysaccharides from *Salmonella muenster* and *S. minneapolis* was obtained by glycosylation of benzyl 2,6-di-O-benzyl-4-O-(2,3,4-tri-O-benzyl-6-O-benzoyl-α-D-glucopyranosyl)-β-D-galactopyranoside with 3-O-acetyl-4-O-(2,3,4,6-tetra-O-acetyl-β-D-mannopyranosyl)- β-L-rhamnopyranose-1,2-(methylorthoacetate), followed by removal of protecting groups. The structure of the synthetic tetrasaccharide was confirmed by methylation analysis, CrO_3–AcOH oxidation and ^{13}C-NMR.

The polysaccharide chains of the lipopolysaccharides of gram-negative bacteria which are responsible for O-antigen specificity are block polymers of regular structure, with repeating oligosaccharide units. The structure of O-specific polysaccharides is unique, for each serological group of bacteria securing a high degree of immunological specificity.[1] Moreover, it has been shown quite recently,[2] that O-specific polysaccharides of different bacteria, possessing identical serological behavior have identical chemical structures.

The synthesis of fragments of O-specific polysaccharides and related structural analogs with subsequent biological and immunochemical study, would undoubtedly be of importance for biochemical, immunochemical and taxonomic studies of gram-negative bacteria. The synthetic programm of this laboratory includes the synthesis of biological repeating units of O-specific polysaccharides from *Salmonella* species.

Recently we described the synthesis of repeating units of O-specific polysaccharides from *S. anatum*[3] and *S. senftenberg*.[4] Now we report the synthesis of tetrasaccharide repeating unit of the O-specific polysaccharides from *S. muenster* and *S. minneapolis*[5] β-D-Man(1→4)-α-L-Rha-(1→3)-D-Gal-(4←1)-α-D-Glc **1**.

The synthesis of tetrasaccharide **1** was carried out by a 2+2 scheme, analogous to that used for the synthesis of tetrasaccharide from *S. senftenberg*[4], i.e. through the preparation of the α-D-Glc-(1→4)-D-Gal fragment, followed by glycosylation of this disaccharide at OH-3 of the galactopyranose residue by an appropriate derivative of the second disaccharide β-D-Man-(1→4)-L-Rha. The synthesis of the last disaccharide was accomplished earlier by Kochetkov *et al.*[3] A most complicated point of the synthetic scheme was synthesis of a α-D-Glc-(1→4)-D-Gal derivative bearing free hydroxyl group or appropriate protecting group at C_3 of galactose unit. For construction of α-glycosidic linkage for disaccharide, which also presents some additional difficulties, we tried to use glycosylation of galactose derivative by 6-O-p-nitrobenzoate derivative of glucose,[6] or glycosylation in the presence of tetraethylammonium bromide (TEAB).[7] Our first attempts to use for this synthesis selective glycosylation of readily accessible galactopyranose derivative with two free OH-groups at C_3 and C_4 were unsuccessful. In contrast to our previous data showing on approximately equal reactivity of the C_3 and C_4 hydroxyl groups in benzyl 2,6-di-O-acetyl-β-D-galactopyranoside **2** towards glycosylation with disaccharide bromide,[3] the reaction of **2** with 2,3,4-tri-O-benzyl-6-O-p-nitrobenzoyl-β-D-glucopyranosyl bromide **4** in the presence of 2,4,6-collidine[6] gave 1→3 linked disaccharide as the only product of glycosylation. The replacement of **2** with benzyl 2,6-di-O-benzyl-β-D-galactopyranoside **3** produced the same result. The attempts of glycosylation of **2** or **3** with 2,3,4,6-tetra-O-benzyl-β-D-glucopyranosyl bromide **5** in the presence of TEAB[7] also gave rise to the 1→3 linked disaccharide as the main product.

It should be noted that our previous experiments[3] were conducted in boiling benzene, whereas in present experiments reactions were performed at room temperature. It seems possible that appearence of a portion of ^1C conformer of **2** due to shift of conformational equilibrium with increasing temperature may be responsible for enhanced reactivity of OH-4 group under these conditions.

As a result of these experiments the glycosylation of less accessible galactopyranose derivative with a single free OH-group at C-4 remain as the only alternative.

Such a derivative was obtained through selective benzoylation of **3** with 1 mole of benzoyl chloride.

The monobenzoylated product, benzyl 2,6-di-O-benzyl-3-O-benzoyl-β-D-galactopyranoside **6** was obtained after chromatography as a crystalline form in 60% yield. The structure of **6** was confirmed by methylation with CH_2N_2 in the presence of $Et_2O{\cdot}BF_3$[8] followed by removal of benzoate and benzyl groups, reduction with $NaBD_4$, acetylation and identification of acetate 4-O-methylgalactitol-1-d by GLC-MS.[9] Glycosylation of **6** with bromide **4** under conditions described in Ref. 6 and with bromide **5** by methods mentioned in Ref. 7 was not successful. Only glycosylation of **6** by bromide **4** in the presence of CF_3SO_3Ag[10] gave the desired disaccharide derivative **7** in 60% yield. (Bromide **4** was used as a glycosylating agent as it may be expected that the presence of p-nitrobenzoyl group at C-6 should increase the stereoselectivity, probably due to 1.6-participation.[11] The experience of this work allows to suggest, that this method can be recommended as one of the most convenient routes for α-glucoside synthesis.)

Debenzoylation of 7 gave 8 in high yield, which was converted after hydrogenolysis to α-D-Glc-(1→4)-D-Gal 10. This disaccharide was found to be homogeneous and differed from α-D-Glc-(1→3)-D-Gal 11, as shown by ion-exchange chromatography in borate buffer.[12]

The structure of 10 was confirmed by ^{13}C NMR spectrum (see Table 1). Selective benzoylation of 8 produced high yield of monobenzoate 9 with free hydroxyl group at position 3 of galactopyranose moiety. Its structure was proved by methylation with CH_2N_2 in the presence of $Et_2O{\cdot}BF_3$, followed by removal of benzoate and benzyl groups, $NaBD_4$ reduction, hydrolysis and identification of acetates of 3-O-methylgalactitol-1-*d* and glucose in a ratio 1:1 by GLC-MS.

The condensation of two disaccharides to complete synthesis of tetrasaccharide 1 could be achieved most successfully by orthoester method of glycosylation.[4]

Glycosylation of monobenzoate 9 by disaccharide orthoester 12 in the presence of 4Å molecular sieve[4] gave tetrasaccharide derivative 13 with 35% yield. PMR data of 13 were in accord with the expected structure. After removal of protecting groups from 13 the free tetrasaccharide 1 was obtained.

Ion-exchange chromatography in borate buffer of the tetrasaccharide showed homogeneity of the product. This fact proves anomeric purity of the tetrasaccharide, as ion-exchange chromatography is known to permit separation of isomeric tetrasaccharides with α- and β-rhamnosyl-galactose glycosidic bonds.[4] Reduction of the tetrasaccharide by $NaBH_4$ in borate buffer[13] gave rise to glycosyl-galactitol 16, the latter was also homogeneous in ion-exchange chromatography. Methylation analysis of 16 followed by GLC-MS gave the acetates of 2,3,4,6-tetra-O-methylmannitol, 2,3-di-O-methylrhamnitol, 2,3,4,6-tetra-O-methylglucitol, 1,2,5,6-tetra-O-methylgalactitol in a 1:1:1:1-ratio.

When the acetate of the tetrasaccharide 1 was subjected to oxidation with CrO_3 in AcOH,[14] with subsequent hydrolysis and sugar analysis, only the mannose residue was destroyed. The result confirms the configuration of β-mannose, α-rhamnose and α-glucose glycosidic lincages. The additional confirmation of the tetrasaccharide structure was obtained by ^{13}C NMR spectrum (see Table 1).

The assignments of β-mannopyranose, 4-O-substituted α-L-rhamnopyranose and α-glucopyranose ring carbon atom signals were found unequivocally through comparison with ^{13}C NMR spectra of the model compounds 10 and 14. The most complicated problem was the assignment of the signals in 3,4-di-O-substituted galactopyranose residue of 1, due to difficulties of identification of the C-3 and C-4 signals in 1 using the disaccharides 10 and 11 spectra only, explained by mutual influence of substituents on chemical shifts of substituted carbon atoms, when galactose residue is simultaneously substituted at C-3 and C-4.

In order to overcome this difficulty 3,4-di-O-methyl-galactose 15 has been specially synthesized, and its ^{13}C NMR spectrum was recorded and interpreted. It became clear from this spectrum, that signals of carbon atoms, participated in glycosydic bonds in galactopyranose residue of 1, may be arranged as $C_{4\beta} \approx C_{4\alpha} < C_{3\alpha} < C_{3\beta}$ (in the order of increasing of chemical shifts).

Certainly, the absolute values of chemical shifts C-4 and C-3 carbon atoms of galactopyranose in 1 and 15 will differ due to different α-effects of methylation and glycosylation.[15]

Thus, the physico-chemical, and espesially ^{13}C NMR data completely corroborate both the structures of the key intermediate compounds, and of the tetrasaccharide 1.

EXPERIMENTAL

Melting points were determined with a Kofler apparatus and are uncorrected. PMR spectra were recorded with a Varian DA-60-IL spectrometer using Me_4Si as internal standard. ^{13}C NMR spectra were obtained using a Bruker WP-60 spectrometer with the frequency of 15.05 MHz on carbon. The pulse width was 3 μsec (30°), the number of transients ≈ 10,000 for disaccharides (concentration ≈ 80 mg/ml) and 90,000 for tetrasaccharide (concentration ≈ 30 mg/ml). Spectra were recorded with a spectral width 3750 Hz and digitization into 8/4K data points. Substances were dissolved in D_2O with CH_3OH as the internal standard (50.15 ppm). All chemical shifts are expressed in δ_{ac}. GLC-MS was carried out by Varian MAT 111(GNOM) device. Optical rotations were determined with a Perkin–Elmer 141 polarimeter. Solutions were concentrated *in vacuo* at 40°. Ion-exchange chromatography of neutral carbohydrates was carried out with a Technicon carbohydrate analyzer on the columns 13×0.5 cm (A) and 20×0.5 cm (B) of Durrum DA×4 resin with 0.5 M sodium-borate buffer (pH 8.54) at 55° and 20 ml/h. The orcinol-sulphuric acid reagent was used to monitor separations.

Table 1.

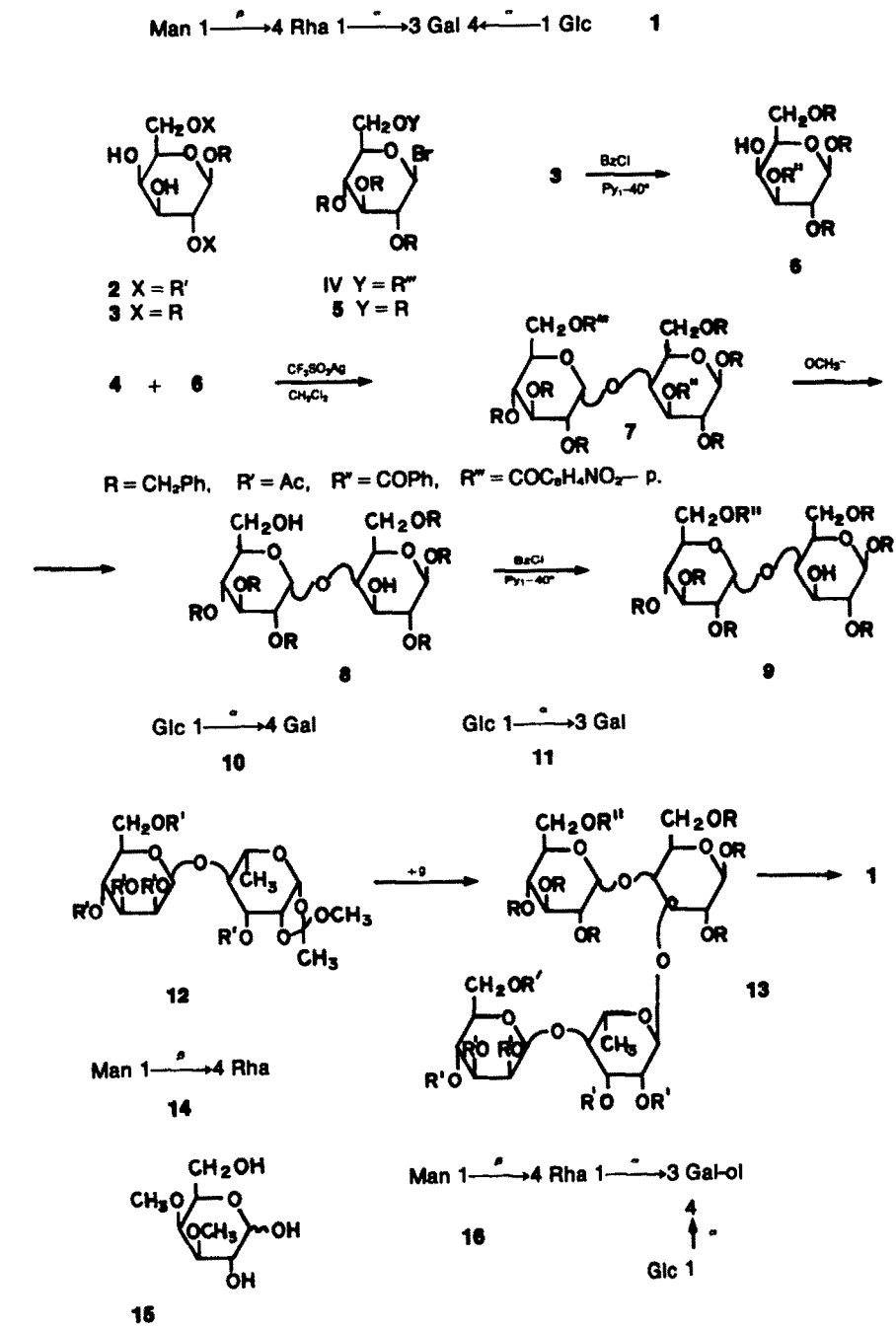
	Glc 1 $\xrightarrow{\alpha}$ 3 Gal 11		Glc 1 $\xrightarrow{\alpha}$ 4 Gal 10		Man 1 $\xrightarrow{\beta}$ 4 Rha 14			Man 1 $\xrightarrow{\beta}$ 4 Rha 1 $\xrightarrow{\alpha}$ 3 Gal 4 $\xleftarrow{\alpha}$ 1 Glc 1					3,4-di-O-Me--Gal 15
	Glc	Gal	Glc	Gal	Man		Rha		Man	Rha	Glc	Gal	Gal‡
	α	β*	α	β*	β	α	β	β	α	α	α	β	α	β
c1	96.55	97.7	101.4	97.9	101.75	95.05	94.5	101.8	102.85 102.7	101.3 **	93.6	97.8	93.4	97.7
c2	73.0	71.5	73.6†	73.1	71.8	72.2	71.8	71.9	71.5	73.7	69.9	73.3	69.0	72.4
c3	74.1	78.8	73.95†	73.1	74.25	71.2	74.0	74.4	71.5	73.8	78.3	80.75	80.5	84.0
c4	70.7	66.3	70.55	78.6	67.95	80.8	80.4	68.2	79.35	70.5	76.5	76.5	76.7	76.5
c5	72.6	76.1	73.1	76.3	77.3	68.2	72.8	77.5	68.2	73.3	72.8	76.1	71.95	75.9
c6	61.65	62.2	61.4	61.4	62.2	18.3	18.3	62.35	18.2	61.5	61.75	61.6	62.2	61.9

*The great predominance of β-anomer in mutarotating mixture.

**The splitting of c-1 signal: α-Rha 1→3α-D-Gal 102.7 and α-Rha 1→3-βD-Gal 102.85.

†Attribution could be reversible.

‡O-Me(4) 61.1; -O-Me(3) 58.1(α) and 58.35(β).



TLC was performed on silica gel LSL 5/40 μ (Chemapol), PLC on silica gel containing 5% of gypsum, column chromatography on silica gel L 100/160 μ (Chemapol). Solvent systems for TLC acetone-chloroform 5:95(1), acetone-chloroform 2:98(2), benzene-ethyl acetate 9:1(3), benzene-ethyl acetate 7:3(4), toluene-ethyl acetate 6:4(5). Methylation analysis of oligosaccharides was performed as described in Ref. [16]

Benzyl 2,6-di-O-benzyl-β-D-galactopyranoside 2. Benzyl chloride (30 ml) was added dropwise to a colled soln of benzyl 3,4-O-isopropylidene-β-D-galactopyranoside (7.6 g)[17] in 200 ml of dimethylsulfinyl anion (obtained from 200 ml of DMSO and 2.5 g NaH). The mixture was kept overnight at r.t., then diluted with 200 ml of $CHCl_3$ and washed with H_2O (5 × 500 ml), concentrated and DMSO removed *in vacuo* (1 mm, 100°). The residue (homogeneous in system 1) was dissolved in 150 ml of $CHCl_3$ containing 10% of CF_3COOH (v/v), and kept for 70 min at r.t. The soln was evaporated, CF_3COOH removed by co-evaporation with toluene. Crystallysation of the resulting product from benzene-light petroleum ether gave 8.77 g of 3 (30%), m.p. 108–109° $[\alpha]_D^{20}$ −17.2° (c2 $CHCl_3$), Calc. for: $C_{27}H_{30}O_6$: C, 72.0; H, 6.7. Found: C, 71.9; H, 6.7%.

Benzyl 2,6-di-O-benzyl-3-O-benzoyl-β-D-galactopyranoside 6 Benzoyl chloride (0.6 ml) was added dropwise to a cooled stirred soln (−40°) of 3 (2 g) in 50 ml of pyridine (distilled over CaH_2). The solution was stirred for 60 min at −40°, and kept overnight at −5°. Pyridine was evaporated, the residue dissolved in 100 ml of $CHCl_3$. The soln was washed with H_2O (3 × 200 ml), evaporated, and column chromatography of the residue (benzene→ether) gave 1.5 g of 6 (60%) R_f 0.8 (system 1), m.p. 91–92° (benzene-light petroleum ether), $[\alpha]_D^{20}$ +48° (c3, $CHCl_3$), Calc. for $C_{34}H_{34}O_7$: C, 72.8; H, 6.15 Found: 72.85; H, 6.16% Methylation of 6 by CH_2N_2, followed by hydrogenolysis, $NaBD_4$-reduction and acetylation gave the acetate of 4-O-methylgalactitol-1-*d* identified by GLC-MS.

3-O-α-D-Glucopyranosyl-D-galactopyranose 11. The soln of 2,3,4 - tri - O - benzyl - 6 - O - p - nitrobenzoyl - β - D - glucopyranosyl bromide (4, 2.0 g)[18] and benzyl 2,6-di-O-acetyl-β-

D-glactopyranoside (2, 0.8 g)[17] in CH_3NO_2 (12 ml) and 2,4,6-collidine (4 ml) was kept at r.t. for 72 h with exculsion of moisture. The soln was diluted with 100 ml of $CHCl_3$, washed with H_2O (3 × 200 ml), 2 M AcOH (2 × 100 ml), and evaporated. PLC of the residue (system 2) gave 500 mg of benzyl 2,6 - di - O - acetyl - 3 - O - (2,3,4 - tri - O - benzyl - 6O - p - nitrobenzoyl - α - D - glucopyranosyl) - β - D - galactopyranoside, 25%, R_f 0.4 (system 2), m.p. 137–138° (EtOH), $[\alpha]_D^{20}$ + 39° (c2, $CHCl_3$). Calc. for $C_{51}H_{51}O_{16}N$: C, 65.8; H, 5.5; N, 1.5. Found: C, 66.0; H, 5.4; N, 1.5% Methylation analysis gave acetate of 2,4,6-tri-O-methylgalactitol, identified by GLC.

A methanolic soln of the disaccharide derivative was deacylated with 2 M methanolic sodium methylate, and then deionized with KU-2(H^+) resin, filtered, and concentrated. The residue was debenzylated in EtOH over 10% palladium-on-charcoal to give 130 mg of **11**. Ion-exchange chromatography in borate buffer shows **11** to be homogeneous with R_t 100 min (column B), $[\alpha]_D^{20}$ + 105° (c9 H_2O). ^{13}C NMR spectrum, see Table 1.

Benzyl 2,6 - di - O - benzyl - 3 - O - benzoyl - 4 - O - (2,3,4 - tri - O - p - nitrobenzoyl - α - D - glucopyranosyl) - β - D - galactopyranoside **7**. Solution of bromide **4**(1 g) in CH_2Cl_2(20 ml, distilled over P_4O_{10}) was added dropwise during 5 min in the darkness to the stirred soln of **6** (600 mg), CF_3SO_3Ag (500 mg) and tetramethylurea (1 ml) in CH_2Cl_2(20 ml). The mixture was stirred in the darkness for 72 h at r.t. filtered, washed with 2 × 20 ml of H_2O and concentrated. The column chromatography of the residue (toluene–ethylacetate) gave **6** (750 mg, 60%), R_f 0.7 (system 3), $[\alpha]_D^{20}$ + 87° (c2 $CHCl_3$), syrup. Calc. for $C_{68}H_{65}O_{15}N$: C, 72.0; H, 5.7; N, 1.2. Found: C, 72.1; H, 5.8; N, 1.3%.

Benzyl 2,6 - di - O - benzyl - 4 - O - (2,3,4 - tri - O - benzyl - 6 - O - p - nitrobenzoyl - α - D - glucopyranosyl) - β - D - galactopyranoside **8**. A methanolic soln of **6** (700 ml) was boiled for 30 min with 2 M methanolic sodium methoxide, then deionized with KU-2 (H^+) resin, filtered and concentrated, PLC of the residue gave **8** (400 mg 75%), R_f 0.4 (system 4); $[\alpha]_D^{20}$ + 43° (c2, $CHCl_3$), syrup. Calc. for $C_{54}H_{58}O_{11}$: C, 73.4; H, 6.7 Found: C, 73.0; H, 6.7%.

Benzyl 2,6-di-O-benzyl-4-O-(2,3,4-tri-O-benzyl-6-O-benzoyl-α-D-glucopyranosyl)-β-D-galactopyranoside **9**. Benzoyl chloride (0.6 ml) was added to a stirred cooled (−40°) soln of **8** (1.8 g) in pyridine (50 ml, freshly distilled over CaH_2). Solution was stirred for 1 h at −40°, and kept overnight at −5°. Pyridine was evaporated, residue was dissolved in 200 ml of $CHCl_3$, the solution was washed with 2 × 200 ml of H_2O, and evaporated. Column chromatography of resulting syrup (benzene–ethyl acetate) gave **9**, (1.8 g, 90%), R_f 0.8 (system 4), $[\alpha]_D^{20}$ + 47.5° (c2 $CHCl_3$) syrup. Calc. for $C_{61}H_{62}O_{12}$: C, 74.2; H, 6.4. Found: C, 74.5; H, 6.5%.

Methylation of **9** with CH_2N_2[18] followed by removal of benzoate and benzyl groups, $NaBD_4$ reduction, hydrolysis and acetylation gave acetates of glucose and 3-O-methygalactitol-1-*d* identified by GLC-MS..

4-O-α-D-Glucopyranosyl-D-galactopyranose **10**. The derivative **8**(300 mg) was debenzylated over 10% palladium-on-charcoal to give **10** (80 mg), $[\alpha]_D^{20}$ + 74.5° (c8 H_2O). Ion-exchange chromatography in borate buffer shows **10** to be homogenous with R_t 60 min (column B). ^{13}C NMR spectrum, see Table 1.

Benzyl 2,6 - di - O - benzyl - 3 - O - [2,3 - di - O - acetyl - 4 - O - (2,3,4,6 - tetra - O - acetyl - β - D - mannopyranosyl) - α - L - rhamnopyranosyl] - 4 - O - (2,3,4 - tri - O - benzyl - 6 - O - benzoyl - α - D - glucopyranosyl) - β - D - galactopyranoside **13**. **9** (120 mg) was glycosylated as described in Ref. 4 with 1,2 - O - methyl - orthoacetyl - 3 - O - acetyl - 4 - O - (2,3,4,6 - tetra - O - acetyl β - D - mannopyranosyl) - β - L - rhamnopyranose (**12**, 200 mg).[4] After reaction was finished (control by TLC), 100 ml of $CHCl_3$ were added, the soln was washed with 3 × 100 ml H_2O, and evaporated. PLC of the residue gave **13** (100 mg, 35%), R_f 0.55 (system 5), $[\alpha]_D^{20}$ + 15.5° (c2, $CHCl_3$), syrup. Calc, for $C_{85}H_{94}O_{27}$: C, 80.0; H, 4.95. Found: C, 80.0; H, 5.0%. PMR data (CCl_4): 8.0–7.0 (34 H aromatic), 2.0(18 H O-Ac), 1,3(d, 3 H, J = 5 Hz, rhamnose C-Me).

3-O-[4-O-(β-D-mannopyranosyl)-α-L-rhamnopyranosyl]-4-O-(α - D - glucopyranosyl) - D - galactopyranose **1**. A methanolic solution **13** (60 mg) was deacylated with 2 M methanolic sodium methoxide, and then deionized with KU-2(H^+) resin filtered and concentrated. The product was debenzylated in 20 ml of EtOH over 10% palladium-on-charcoal to give **1**, (30 mg, 85%), $[\alpha]_D^{20}$ + 35.1° (c3, H_2O). Ion-exchange chromatography in borate buffer shows **1** to be homogeneous with R_t 50 min (column A). PC showed a single spot with R_{Glc} 0.28 (BuOH–Py–H_2O 6:4:3, Filtrak FN-11). ^{13}C NMR spectrum, see Table 1. Sodium borohydride (20 mg) was added to the solution of **1** (5 mg) and H_3BO_3 (20 mg) in 2 ml of H_2O. Solution was kept for 16 h at r.t., then was deionized with KU-2(H^+) resin, fitered and co-evaporated with methanol up to dry. Ion-exchange chromatography in borate buffer showed the obtained glycosyl-galactitol **16** to be homogeneous with R_f 207 min (column B). Methylation analysis of **16** gave the acetates of 1,2,5,6-tetra-O-methylgalactitol, 2,3-di-O-methyl-rhamnitol, 2,3,4,6-tetra-O-methylmannitol and 2,3,4,6-tetra-O-methylglucitol in 1:1:1:1-molar ratio.

Oxidation of the acetate of tetrasaccharide **1**. Tetrasaccharide **1** (1 mg) was acetylated with acetic anhydride, and then oxidized with CrO_3–AcOH.[14] The product was hydrolysed (2M HCl, 16 h, 100°). Only mannose was destroyed by this procedure.

3-O-β-D-Mannopyranosyl-L-rhamnopyranose **14**. Obtained by the method described in Ref. 19; $[\alpha]_D^{20}$ − 46° (c2, H_2O).

3,4-Di-O-methylgalactopyranose **15**. Obtained by hydrolysis (2 M HCl, 16 h, 100°) of methyl-3,4-di-O-methyl-α-D-galactopyranoside.[20]

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