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Note

Identification and synthesis of a trisaccharide produced from lactose by transgalactosylation

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Abstract

Enzymatic transgalactosylation of lactose by means of *Streptococcus thermophilus*, subspecies DN-001065, led to a mixture of D-galactose (~4%), D-glucose (~15%), lactose (~51%), minor disaccharides (6%), trisaccharides (~20%) and tetrasaccharides (3%). The major trisaccharide (~16%) was identified by NMR spectroscopy and chemical synthesis as being the known β-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucose (3′-β-D-galactopyranosyl-lactose). It was purified from a mixture of peracetylated oligosaccharides by column chromatography followed by deacetylation. For the first time, 3′-β-D-galactopyranosyl-lactose has been obtained on the 1 g scale, by resorting to simple techniques and equipment. NMR spectra have been unambiguously assigned.

Keywords: Transgalactosylation; β-D-Galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucose; 3′-β-D-Galactopyranosyl-lactose

1. Introduction

Carbohydrates, because of their particular structural features, comprise a vast number of isomers that may have quite different properties in spite of apparent similarities. These differences may be understood on the basis of chemical reactivity and, particularly as far as one considers life-related aspects, in terms of biocatalyzed transformations and molecular recognition [1]. The fact that some oligosaccharides are not metabolized by humans, but are selectively fermented by bacteria having a beneficial incidence on the health of humans and animals, has led to the concept of Ecological Health Control Products [2], and to the development of gluco- [2], galacto- [3], and fructo- [4] oligosaccharides. Along this line, enzymatic modification of sucrose has wide applications [5], in particular because glycosylation employing biosystems opens new avenues for regio- and stereo-specific preparations of complex oligosaccharides [6]

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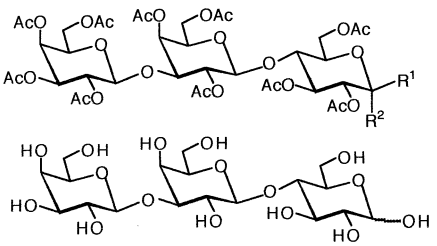
of interest in food products [7]. Some of them, such as the fructo-oligosaccharides Actilight, are resistant to the action of human digestive enzymes, so that they can reach the lower digestive tract without being absorbed. Those that can be utilized there by Bifidobacteria as an energy source, thus favouring their proliferation, are of particular interest. Bacteria of this class contribute to maintaining the state of the intestinal bacterial flora, which is considered as having a major impact on human health. In humans, the dietetic benefits of intestinal Bifidobacteria are as follows: (a) maintenance of a normal intestinal balance; (b) improvement of lactose tolerance and digestibility of milk products; (c) antitumorigenic activity; (d) reduction of serum cholesterol levels; (e) synthesis of B-complex vitamins and (f) enhanced absorption of dietary calcium [8,9]. For these reasons, galacto-oligosaccharides, considered as promoters for the growth of Bifidobacteria and Lactobacilli in the large intestine, have been investigated thoroughly [9,10], also in connection with the modified viscosity of dairy produce [11]. Another reason for studying oligosaccharides stems from attempts made by the industry to bring the composition of infant formula closer to that of human milk [12]. In this context, we became involved in a project aiming at the production of lactose-derived oligosaccharides by enzyme-mediated transgalactosylation [13], with the focus on the structural identification and purification on the gram scale, for standard purposes, of the major trisaccharide present in the crude mixture.

2. Results and discussion

An aqueous mixture of oligosaccharides ($\sim$35% w/w), obtained from lactose by *Streptococcus thermophilus*-induced transgalactosylation using DN-001065 subspecies [Collection Nationale de Cultures de Microorganismes, Institut Pasteur, 25, rue du Docteur Roux, F-75724 Paris, France], was shown by HPLC to contain D-glucose ($\sim$15%) and D-galactose ($\sim$4%), lactose ($\sim$51%) and minor disaccharides ($\sim$6%), various trisaccharides ($\sim$21%) and tetrasaccharides (3%). We focused our attention on the predominant trisaccharide with the following objectives: (i) its isolation from the oligosaccharide mixture for structure determination, (ii) its preparation on the 1 g scale by either chemical synthesis or separation from the raw mixture. Although structural analyses of oligosaccharides rely on both long-known [14] and continuously improving methods [15] with the help of new techniques in mass spectroscopy and high-field NMR [16], we decided to tackle the problem by applying methodologies familiar to the carbohydrate synthetic chemist, which might possibly allow the isolation of the desired trisaccharide in significant amounts from the mixture, with quite simple equipment.

To this end, the aqueous syrupy mixture was first concentrated to dryness under diminished pressure before acetylation under standard conditions (acetic anhydride, pyridine) and the resulting mixture of peracetylated oligosaccharides was subjected to column chromatography on silica gel with solvent mixtures of increasing polarity. From the weights of the collected fractions, the distributions of the different mono-, di-, tri-, and tetrasaccharides were 23.8, 57.2, 17.7 and 1.3%, respectively. The fractions containing the major peracetylated trisaccharide, visible as a single spot on TLC plates, were pooled and examined by NMR spectroscopy. As expected, the isolated material turned out to be an anomeric mixture (α:β ratio $\sim$3:7). Therefore, we preferred to achieve the transformation of **1**α,β stereoselectively, via the corresponding peracetylated bromide **2** [17] into the *O*-methyl glycoside **3**, taking advantage of the anchimeric assistance of the *O*-acetyl group at the C-2 position of the D-glucose unit. The trisaccharide **3**, prepared by this two-step sequence (HBr–AcOH, then MeOH, AgOTf, *sym*-collidine) was a single anomer, as shown, in particular, by the presence of one methoxy resonance at 3.48 ppm. The same two-step protocol was applied to the oligosaccharide mixture, which was therefore converted, without purification of the intermediate bromides, to the corresponding peracetylated *O*-methyl glycosides. Their separation by column chromatography on silica gel afforded homogeneous fractions, which were ac-

curately weighted. The calculated distribution of the *O*-methyl mono-, di-, tri-, and tetra-saccharides (21.3, 59.1, 16.4 and 3.2%) was similar to that found for the peracetates. The observed fit supported the conclusion that the chemical treatments applied were mild enough so as to prevent any cleavage of *O*-glycosidic bonds. Therefore, the structure of the obtained methyl trisaccharide **3**, giving NMR spectra simpler than those of **1**α,β, was determined, to deduce the sugar sequence in the major trisaccharide precursor **4**α,β.



	R^1	R^2
1α	H	OAc
1β	OAc	H
2	H	Br
3	OMe	H

4 α,β

^{1}H and ^{13}C NMR (1D, 2D) spectroscopy led to the conclusion that the isolated peracetylated *O*-methyl trisaccharide **3** was methyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside. The presence of one D-glucopyranose unit and two D-galactopyranose units, which was suggested by the ^{1}H NMR spectrum, was confirmed by COSY, 1D COSY with multistep relays, HSQC and HMBC experiments. The 2D gradient COSY experiment allowed a complete assignment of the protons of the D-glucopyranose unit. However, this task was not straightforward for the two D-galactose units due to the small coupling constant between their H-4 and H-5 protons and because of the similar chemical shifts of the H-4 proton in each D-*galacto* unit. In the sugar series, the 1D TOCSY experiment is generally initiated by selective excitation of the anomeric proton, allowing a complete assignment of the spin system for long spin-lock times. However, in the present case, the magnetization was not transferred from H-4 to H-5 in the D-*galacto* units, probably because of a competition between TOCSY and ROESY effects. Fortunately, a set of multistep relayed 1D COSY experiments was successfully carried out (see Fig. 1). The pulse sequence used, derived from that proposed by Adel et al. [18] was modified so as to include relays. Considering first the anomeric proton H-1, the standard 1D COSY experiment provided the antiphase multiplet H-2 and allowed the measurement of the passive coupling constant. A new transfer period was added to the 1D COSY sequence with a transfer adjusted to $1/2J$ where J was equal to $J_{2,3}$. The success of this pulse sequence stems from the fact that the COSY transfer can occur in spite of the < 1 Hz coupling between H-4 and H-5 in the D-*galacto* units. Hence, this technique, which does not require ^{13}C-labelled molecules [19], appears to be a method of choice for polysaccharide structural determination when superimposed signals cannot be distinguished by classical 2D NMR techniques. Afterwards, a classical gradient HMBC sequence was applied so as to achieve the sequencing of the sugar units by establishing unambiguously which carbon atoms were engaged in the glycosidic bonds. Each pyranosyl unit in **3** was found in the $^4C_1$-D chair conformation and the stereochemistry of the three glycosidic bonds was established as β on the basis of the ~ 8 Hz vicinal couplings observed for each of the anomeric protons. Therefore, the major enzymatically produced trisaccharide **4** was likely to be β-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucose (3′-β-D-galactopyranosyl-lactose). From the isolated amount of the peracetylated *O*-methyl trisaccharide **3**, the proportion of compound **4** in the mixture was estimated to be $\sim 16\%$.

The chemical synthesis of **4** (Scheme 1) was attempted following a known procedure [20], using 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (**5**) [17], and benzyl 4-*O*-(2,6-di-*O*-acetyl-β-D-galactopyranosyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**7**) [20]. However, in our hands, the glycosidation step was found to be unsatisfactory. Therefore, 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose trichloroacetimidate (**6**) [21], prepared from the corresponding peracetates by selective 1-*O*-deacetylation [22], was envisaged as the glycosyl donor. Coupling of **6** and **7** afforded trisaccharide **8** (28% yield), which was converted to **9** by acetylation. In spite of the enhanced reactivity of the equatorial hydroxy group at

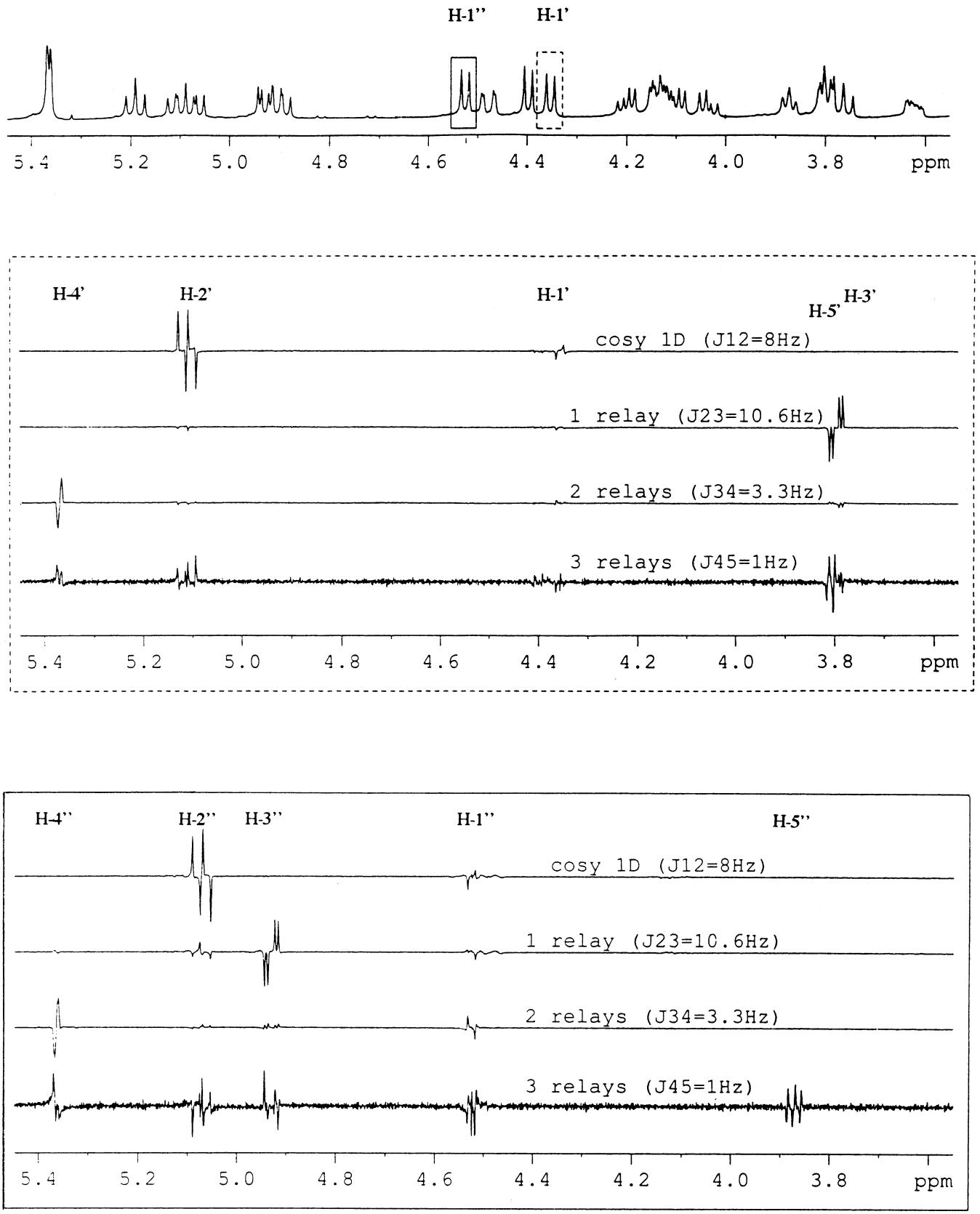
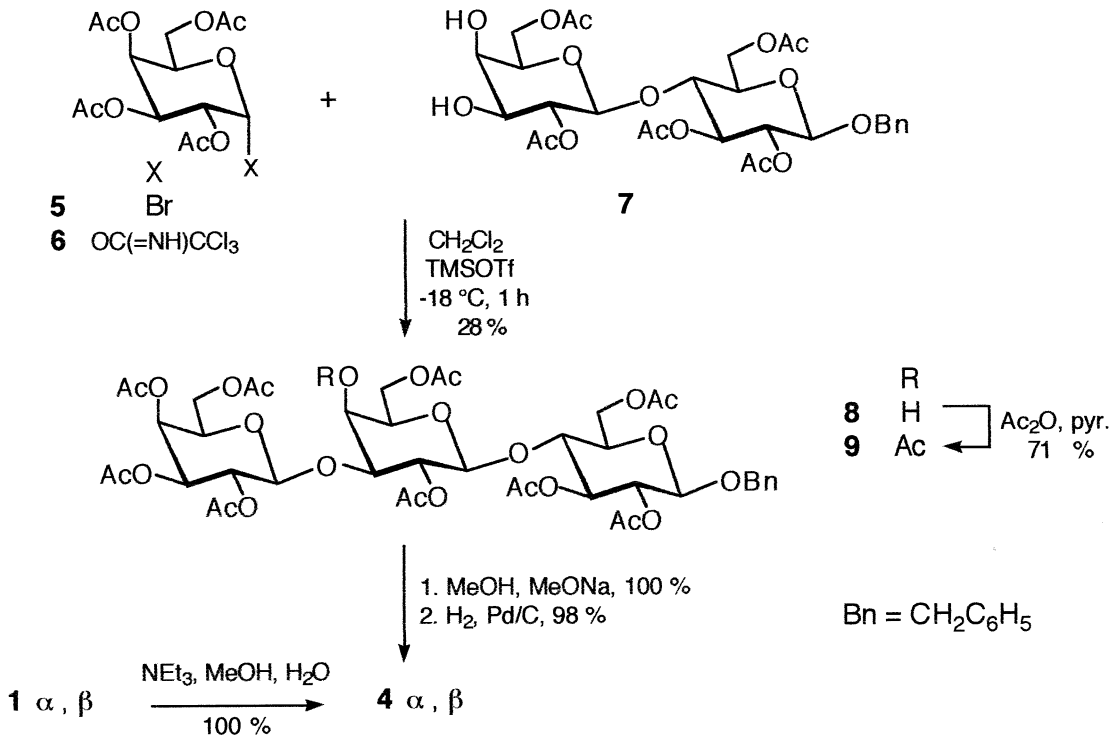


Fig. 1. The ^{1}H NMR spectrum of **3** is shown, in part, at the top. The selectively excited H-1′ and H-1″ protons of the D-Gal*p* units are indicated by rectangles with dashed and normal lines. The corresponding 1D relayed COSY set of spectra, which are respectively represented below, in dashed and normal frames, show, successively, the transfer of magnetization from the anomeric proton to the ring protons, in each unit.



Scheme 1.

C-3′ in **7**, under homogeneous glycosidation conditions [23,24], the coupling was not efficient, as observed recently by others [25]. Compound **4** was obtained from **9** in two high-yielding steps by deacetylation and hydrogenolysis, as previously described [20].

Finally, since the chemical synthesis of **4** required optimization, separation of the mixture of peracetylated oligosaccharides was carried out by column chromatography with mobile phases of increasing polarity. Pure compound **1**α,β was subjected to deacetylation (MeOH, H_2O, NEt_3) to yield quantitatively **4** as a white crystalline material ($\sim$1 g) [20]. The NMR data obtained for **4** were identical to that reported for 3′-β-D-galactopyranosyl-lactose, isolated from the milk of the tammar wallaby and the grey kangaroo [16,26–28], and found as a component of human milk [12].

In conclusion, our work unambiguously establishes that *S. thermophilus*-induced modifications of lactose, using subspecies DN-001065, lead to mixtures containing D-galactose ($\sim$4%) and D-glucose ($\sim$15%), lactose and minor disaccharides ($\sim$57% altogether), trisaccharides ($\sim$20%) and tetrasaccharides ($\sim$3%). The trisaccharide fraction mainly contains the known 3′-β-D-galactopyranosyl-lactose, not mentioned in related investigations [9,11]. This compound results from preferential transfer of a β-D-Gal*p* unit to the 3′ position of lactose [10], which was also reported to enzymatically undergo transfer of β-D-Gal*p* unit to the 4′ [3,9,29], and 6′ [9,30] positions, mainly [10]. For the first time, 3′-β-D-galactopyranosyl-lactose has been obtained on the 1 g scale, by resorting to simple techniques and equipment, and previous assignments, based on comparison, of its ^{13}C NMR spectrum have been unambiguously confirmed.

3. Experimental

General methods.—Melting points were determined with a Büchi capillary apparatus and were not corrected. Optical rotations were determined with a Perkin–Elmer 241 polarimeter. ^1H and ^{13}C NMR spectra were recorded with Bruker AC 200/AM 300/DRX500 instruments for solutions in $CDCl_3$, with Me_4Si as the internal reference or in D_2O. Reactions

were monitored by TLC on Silica Gel 60 F_{254} (E. Merck) plates exposed to H_2SO_4 (10% in 1:1 EtOH–water) spray followed by charring ($\sim$250 °C). Spraying successively a fluorescein solution in abs EtOH (0.1% w/v), then a 1:1 mixture of H_2O_2 (30% in water)–AcOH followed by charring was used to detect bromine-containing compounds, which appeared as pink-coloured spots. Column chromatography was performed using Silica Gel Geduran Si 60 (E. Merck). Solvents were distilled before use. Water was distilled twice.

Enzymatic transgalactosylation of lactose.—The mixture of *trans-galacto*-oligosaccharides was produced by Blédina s.a. (Steenvoorde, France). Enzyme production was based on a reported procedure [31], using *S. thermophilus* strains [13], subspecies DN-001065 [Collection Nationale de Cultures de Microorganismes, Institut Pasteur, 25, rue du Docteur Roux, F-75724 Paris, France] grown for $\sim$6 h at $\sim$50 °C. Then, an aq concd soln of lactose was incubated with the obtained culture medium. After centrifugation for removal of *Streptococcus*, the aq mixture of oligosaccharides ($\sim$35% w/w) was shown by HPLC (25 cm × 4.6 mm Supercosil LC NH_2 5 m column, 3:1 CH_3CN–water as the mobile phase) to contain D-glucose ($\sim$15%) and D-galactose ($\sim$4%), lactose ($\sim$51%) and minor disaccharides ($\sim$6%), various trisaccharides (21%) and tetrasaccharides (3%). The major tri-, and tetrasaccharides amounted to $\sim$18 and 1.5%, respectively.

(2,3,4,6-Tetra-O-*acetyl-β*-D-*galactopyranosyl)-(1→3)-(2,4,6-tri*-O-*acetyl-β*-D-*galactopyranosyl)-(1→4)-1,2,3,6-tetra*-O-*acetyl*-D-*glucopyranose* (**1**).—After concn to dryness of the aq mixture of oligosaccharides resulting from enzymatic modification of lactose, an aliquot (1.19 g) in pyridine (3 mL, 37.1 mmol) and Ac_2O (5.5 mL, 58.3 mmol) was stirred for 4 days at rt. Excess Ac_2O was quenched by addition of ice–water (30 mL). The aq layer was extracted with CH_2Cl_2 (3 × 20 mL); the organic phase was washed with water (3 × 30 mL), dried (Na_2SO_4) and concd. The crude material was purified by chromatography with a column (length: $\sim$50 cm; external diameter: 35 mm) eluted with mixtures of EtOAc–petroleum ether–CH_2Cl_2 (gradient: 3:3:7→6:2:7 v/v) and 1:1 EtOAc–CH_2Cl_2 to give mixtures of peracetylated monosaccharides ($R_f \sim 0.73$ in 6:2:7 EtOAc–petroleum ether–CH_2Cl_2, 106 mg, $\sim$24%), and disaccharides ($R_f \sim 0.53$, 255 mg, $\sim$57%), trisaccharides ($R_f \sim 0.16$, 79 mg, $\sim$18%), and tetrasaccharides ($R_f \sim 0.04$, 6 mg, $\sim$1.3%).

Methyl (2,3,4,6-tetra-O-*acetyl-β*-D-*galactopyranosyl)-(1→3)-(2,4,6-tri*-O-*acetyl-β*-D-*galactopyranosyl)-(1→4)-2,3,6-tri*-O-*acetyl-β*-D-*glucopyranoside* (**3**).—To a solution of a dried mixture of oligosaccharides (833 mg) in Ac_2O (4.2 mL) was added at rt 33% HBr in AcOH (0.83 mL). After stirring for 22 h at rt, another portion of HBr in AcOH (4.2 mL) was added and stirring was maintained for 9 h at rt. The mixture was concd under reduced pressure to a syrup from which traces of Ac_2O were removed by co-evaporation with toluene [17]. The residue (1.7 g) was dissolved in MeOH (5 mL) and the solution, cooled to 0 °C was treated successively with AgOTf (687 mg, 2.67 mmol) and *sym*-collidine (0.29 ml, 2.2 mmol). After stirring for 5 h, the mixture, quenched by adding satd aq Na_2CO_3 and NaCl solns (10 mL each), was filtered, after addition of CH_2Cl_2 (15 mL) to dissolve the organic materials. The organic phase was washed with water (3 × 15 mL), dried with Na_2SO_4 and evaporated. The crude material (1.57 g) was purified by chromatography with a column (length: $\sim$50 cm; external diameter: 25 mm) eluted with EtOAc–petroleum ether–CH_2Cl_2 mixtures (gradient: 4:3:7→7:2:6, v/v) to give peracetylated methyl glycosides: monosaccharides ($R_f \sim 0.71$ in 7:2:6 EtOAc–petroleum ether–CH_2Cl_2, 118 mg, $\sim$21%), disaccharides ($R_f \sim 0.50$, 328 mg, $\sim$59%), trisaccharide **3** ($R_f \sim 0.17$, 91 mg, $\sim$16%), and tetrasaccharides ($R_f \sim 0.02$, 18 mg, $\sim$3%).

3: Mp 164–165 °C; $[\alpha]_D$ − 5.4° (*c* 1, $CHCl_3$). ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 5.36 (dd, 2 H, $J_{3',4'} = J_{3'',4''}$ 3.4, $J_{4',5'} = J_{4'',5''}$ 0.8 Hz, H-4′ and H-4″), 5.18 (dd, 1 H, $J_{2,3}$ 9.3, $J_{3,4}$ 9.2 Hz, H-3), 5.09 (dd, 1 H, $J_{1',2'}$ 8.0, $J_{2',3'}$ 10.2 Hz, H-2′), 5.06 (dd, 1 H, $J_{1'',2''}$ 7.8, $J_{2'',3''}$ 10.6 Hz, H-2″), 4.92 (dd, 1 H, H-3″), 4.88 (dd, 1 H, $J_{1,2}$ 7.9 Hz, H-2), 4.52 (d, 1 H, H-1″), 4.46 (dd, 1 H, $J_{5,6a}$ 2.0, $J_{6a,6b}$ 11.9 Hz, H-6a), 4.39 (d, 1 H, H-1), 4.34 (d, 1 H, H-1′), 4.18 (dd, 1 H, $J_{5'',6''a}$ 6.1, $J_{6''a,6''b}$ 11.2 Hz, H-6″a), 4.14–4.11 (m, 3

H, H-6b, H-6′a, H-6″b), 4.03 (dd, 1 H, $J_{5',6'b}$ 6.7, $J_{6'a,6'b}$ 11.5 Hz, H-6′b), 3.86 (ddd, 1 H, $J_{5'',6''b}$ 7.1 Hz, H-5″), 3.78 (dd, 1 H, H-3′), 3.79–3.76 (m, 2 H, H-5′, H-4), 3.60 (ddd, 1 H, $J_{4,5}$ 7.4, $J_{5,6b}$ 4.9 Hz, H-5), 3.48 (s, 3 H, OCH_3), 2.17, 2.13, 2.13, 2.10, 2.08, 2.06, 2.04, 2.03, 2.02, 1.96 (10 s, 30 H, acetyl). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 170.52, 170.37, 170.33, 170.14, 169.81, 169.75, 169.67, 169.16, 168.51 (C=O), 101.33 (C-1), 100.97 (C-1″), 100.70 (C-1′), 75.87 (C-4), 75.66 (C-3′), 72.64 (C-5), 72.52 (C-3), 71.49 (C-2), 71.25 (C-5′), 70.90 (C-2′), 70.72 (C-5″), 70.55 (C-3″), 68.49 (C-4′), 68.44 (C-2″), 66.63 (C-4″), 62.08 (C-6), 61.63 (C-6′), 60.84 (C-6″), 57.00 (OCH_3), 20.84, 20.73, 20.70, 20.68, 20.65, 20.65, 20.63, 20.58, 20.49, 20.49 ($OCOCH_3$). Anal. Calcd for $C_{39}H_{54}O_{26}$ (938.84): C, 49.89; H, 5.80; O, 44.31. Found: C, 49.93; H, 5.95; O, 44.33.

A mixture of peracetylated tri- and tetrasaccharides (275 mg) was treated with Ac_2O (1.4 mL) and 30% HBr in AcOH (1.7 mL) for 8 h at rt to give a mixture of the corresponding bromides as a gum (296 mg). This was reacted for 5 h at 0 °C with MeOH (0.6 mL) in the presence of silver triflate (84 mg) and *sym*-collidine (35 μL). Trisaccharide **3** (127 mg) was obtained by column chromatography, as before.

Benzyl (2,3,4,6-tetra-O-*acetyl-β*-D-*galactopyranosyl)-(1→3)-(2,4,6-tri*-O-*acetyl-β*-D-*galactopyranosyl)-(1→4)-2,3,6-tri*-O-*acetyl-β*-D-*glucopyranoside* (**9**) *[20].*—To a solution of benzyl 4-*O*-(2,6-di-*O*-acetyl-β-D-galactopyranosyl)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**7**) [20] (514 mg, 0.8 mmol) in anhyd CH_2Cl_2 (15 mL) were added 4 Å molecular sieves (415 mg) and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl trichloroacetimidate (**6**) [21] (591 mg, 1.2 mmol) and stirring was continued for 1 h at rt. The mixture was cooled to −18 °C and treated with Me_3SiOTf (15 μL, 0.08 mmol). After 5 h at −18 °C, the reaction was quenched by addition of a satd aq $NaHCO_3$ soln (10 mL) before warming up to rt. After separation of the aq layer, it was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were washed with water until neutral and dried (Na_2SO_4). After concentration, the residue was purified by column (length: ~50 cm; external diameter: 25 mm) chromatography on silica gel (1:1 CH_2Cl_2–Et_2O) to yield benzyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-(2,6-di-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranoside (**8**) (220 mg, 28%) [20]: ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 170.82, 170.61, 170.33, 170.18, 170.12, 170.00, 169.63, 169.31, 168.62 (C=O), 136.75, 128.47, 128.03, 127.75 (phenyl), 101.55 (C-1), 100.57 (C-1″), 99.19 (C-1′), 79.62 (C-3′), 75.68 (C-4), 72.89 (C-5), 72.45 (C-3), 72.00 (C-2), 71.60 (C-5′), 71.06 (C-2′), 70.77 (CH_2Ph), 70.63 (C-5″), 70.52 (C-3″), 68.38 (C-4′), 68.20 (C-2″), 66.84 (C-4″), 62.71 (C-6), 62.31 (C-6′), 61.19 (C-6″), 20.87, 20.82, 20.75, 20.69, 20.67, 20.63, 20.60, 20.56, 20.54 ($OCOCH_3$).

The product **8** (150 mg), after acetylation with Ac_2O (1.1 mL) and pyridine (0.75 mL) was chromatographed on silica gel (3:1 then 2.5:1.5 CH_2Cl_2–Et_2O) to give **9** (111 mg, 71%) [20]: ^1H NMR (300.13 MHz, $CDCl_3$): δ 7.34–7.25 (m, 5 H, Ph), 5.35 (d, 2 H, $J_{3',4'} = J_{3'',4''}$ 3.2 Hz, H-4′ and H-4″), 5.14 (dd, 1 H, $J_{2,3}$ 9.3, $J_{3,4}$ 9.1 Hz, H-3), 5.08 (dd, 1 H, $J_{1',2'}$ 8.0, $J_{2',3'}$ 10.1 Hz, H-2′), 5.05 (dd, 1 H, $J_{1'',2''}$ 7.8, $J_{2'',3''}$ 10.3 Hz, H-2″), 4.96 (dd, 1 H, $J_{1,2}$ 7.9 Hz, H-2), 4,91 (dd, 1 H, H-3″), 4.86 (d, 1 H, *J* 12.3 Hz, CH_2Ph), 4.59 (d, 1 H, *J* 12.3 Hz, CH_2Ph), 4.51 (d, 1 H, H-1″), 4.48 (d, 1 H, H-1′), 4.46 (m, 1 H, H-6a), 4.34 (d, 1 H, H-1), 4.16 (dd, 1 H, $J_{5'',6''a}$ 6.1, $J_{6''a,6''b}$ 11.1 Hz, H-6″a), 4.14–4.06 (m, 3 H, H-6b, H-6′a, H-6″b), 4.02 (dd, 1 H, $J_{5',6'b}$ 6.7, $J_{6'a,6'b}$ 11.2 Hz, H-6′b), 3.86 (broad t, 1 H, $J_{5'',6''b}$ 6.8 Hz, H-5″), 3.80–3.73 (m, 3 H, H-3′, H-4, H-5′), 3.60 (ddd, 1 H, $J_{4,5}$ 7.6, $J_{5,6a}$ 1.9, $J_{5,6b}$ 5.2 Hz, H-5), 2.16, 2.15, 2.12, 2.08, 2.07, 2.06, 2.02, 2.01, 1.99, 1.96 (10 s, 30 H, acetyl). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 170.55, 170.55, 170.39, 170.37, 170.16, 169.85, 169.82, 169.60, 169.20, 168.54 (C=O), 136.69, 128.48, 128.05, 127.75 (phenyl), 101.07 (C-1), 100.72 (C-1″), 99.09 (C-1′), 75.91 (C-4), 75.79 (C-3′), 72.82 (C-5), 72.59 (C-3), 71.38 (C-2), 71.02 (C-5′), 70.86 (C-2′), 70.77 (CH_2Ph), 70.72 (C-5″), 70.65 (C-3″), 68.63 (C-4′), 68.58 (C-2″), 66.78 (C-4″), 62.22 (C-6), 61.76 (C-6′), 60.96 (C-6″), 20.89, 20.78, 20.73, 20.72, 20.68, 20.68, 20.63, 20.61, 20.53, 20.53 ($OCOCH_3$).

β-D-Galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-*glucose* (**4**).—(a) By chemical synthesis: a solution of **9** (101 mg) in

MeOH (5 ml) containing a catalytic amount of NaOMe was stirred for 48 h at rt. The soln was evaporated under reduced pressure and the residue was dissolved in water. The aq soln was then neutralized with Amberlite IRC-50 (H^+) and evaporated under reduced pressure to give benzyl β-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (60 mg, 100%). A solution of this compound (60 mg) in 80% aq EtOH (26 mL) was hydrogenolysed in the presence of Pd/C (10%) for 24 h. The catalyst was removed by filtration, and the solvent was evaporated under reduced pressure to yield **4** (50 mg, 98%, α/β ratio ~35:65). ^{1}H NMR (300.13 MHz, D_2O) [12]: δ 5.23 (d, 1 H, $J_{1,2}$ 4.7 Hz, H-1 α anomer), 4.68 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1 β anomer), 4.62 (d, 1 H, $J_{1,2}$ 7.1 Hz), 4.52 (d, 1 H, $J_{1,2}$ 7.6 Hz), 4.2–3.5 (m, 18 H). ^{13}C NMR (75.47 MHz, D_2O): δ 104.88 (C-1″), 103.12 (C-1′), 96.34 (C-1 β-anomer), 92.39 (C-1 α anomer), 82.45 (C-3′), 78.93 (C-4 α anomer), 78.81 (C-4 β anomer), 75.62 (C-5″), 75.54 (C-5′), 75.34 (C-5 β anomer), 74.92 (C-3 β anomer), 74.40 (C-2 β anomer), 73.09 (C-3″), 71.97 (C-2 α anomer), 71.74 (C-3 α anomer), 71.60 (C-2″), 70.75 (C-2′), 70.65 (C-5 α anomer), 69.15 (C-4″), 68.98 (C-4′), 61.53 (C-6′, C-6″), 60.69 (C-6 β anomer), 60.57 (C-6 α anomer). The assignment of the carbon resonances of the α- and β-D-glucopyranose units is based on the observed signal intensities and on literature data [16,26–28].

(b) By separation from the mixture followed by deacetylation: (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-1,2,3,6-tetra-*O*-acetyl-D-glucopyranose (**1**) (717 mg, 0.74 mmol), separated from the oligosaccharide mixture as indicated above, was dissolved in a 8:1:1 MeOH–NEt_3–water mixture (100 mL). After stirring for ~12 h at rt, deacetylation was over, as shown by TLC (R_f ~0.08 in 7:5:1 EtOAc–MeOH–water). Concentration under diminished pressure followed by two co-evaporations with water and concentration to dryness led to **4** as a white solid (373 mg, quantitative yield) which crystallized from water–MeOH–EtOH; mp 200–202 °C (dec.); α/β ratio ~45:55; $[\alpha]_D^{23}$ + 48° (*c* 0.75, H_2O) lit. $[\alpha]_D^{25}$ + 26.1° (*c* 0.69, water) [20]. The NMR spectra were identical to those described above. Anal. Calcd for $C_{18}H_{32}O_{16}\cdot H_2O$ (522.45): C, 41.38; H, 6.56; O, 52.06. Found: C, 41.28; H, 6.75; O, 51.78.

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