Enzymic Production of Sweet Stevioside Derivatives: Transglucosylation by Glucosidases

Sergei Victrovich LOBOV,* Ryoji KASAI, Kazuhiro OHTANI, Osamu TANAKA** and Kazuo YAMASAKI

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi, Minami-ku, Hiroshima 734, Japan
**Institute of Sugar Beet NPO "Sahsvekla," Klinicheskaya str. 25, Kiev 252110, USSR*
***Suzugamine Women's College, Inokuchi, Nishi-ku, Hiroshima 733, Japan*

Received May 28, 1991

For the purpose of improving sweetness and a further study on the structure–sweetness relationship of steviol glycosides, transglycosylation of stevioside by a variety of commercial glucosidases was investigated. It was revealed that two α-glucosidases gave glucosylated products. Transglucosylation of stevioside by Pullulanase and pullulan exclusively afforded three products, 13-*O*-[β-maltotriosyl-(1→2)-β-D-glucosyl]-19-*O*-β-D-glucosyl-steviol (1), 13-*O*-[β-maltosyl-(1→2)-β-D-glucosyl]-19-*O*-β-D-glucosyl-steviol (2) and 13-*O*-β-sophorosyl-19-*O*-β-maltotriosyl-steviol (3). All of these products have already been obtained by trans-α-1,4-glucosylation of stevioside by the cyclodextrin glucanotransferase starch system, and 1 and 2 have been proven to be tasty and potent sweeteners. Transglucosylation of stevioside by Biozyme L and maltose afforded three new products, 4, 5 and 6, the structures of these compounds being elucidated as 13-*O*-β-sophorosyl-19-*O*-β-isomaltosyl-steviol (4), 13-*O*-[β-isomaltosyl(1→2)-β-D-glucosyl]-19-*O*-β-D-glucosyl-steviol (5) and 13-*O*-[β-nigerosyl-(1→2)-β-D-glucosyl]-19-*O*-β-D-glucosyl-steviol (6). A significantly high quality of taste was evaluated for 4.

Stevioside (13-*O*-β-sophorosyl-19-*O*-β-D-glucosyl-steviol) is the major sweet glycoside of leaves of *Stevia rebaudiana* BERTONI (Compositae) and is utilized as a low-calorie sweetener in Japan. Because stevioside has slight bitterness and an aftertaste, improvements to the taste by means of the enzymic transglycosylation of stevioside and congener (rubusoside: 13,19-di-*O*-β-glucosyl-steviol from *Rubus suavissimus* S. Lee, Rosaceae) have been investigated.[1,2] A significant improvement in the quality of taste was evaluated for a mixture of *trans*-α-1,4-glucosylated products from stevioside by the cyclodextrin glucanotransferase (CGTase) starch system, and this mixture is currently used as a better sweetener than stevioside. Recently, the separation of all of the mono-, di- and tri-α-1,4-glucosylated products from this complex mixture has been achieved, and the relationship between sweetness and the location of transglucosylation was investigated.[1] Transfructofuranosylation by fructosidase has also been reported.[3] The present paper deals with a further study on transglucosylation by a variety of commercial glucosidases.

Results and Discussion

A preliminary investigation of transglucosylation by several commercial glucosidases was conducted by incubating a solution of stevioside, a donor (maltose or pullulan) and an enzyme in acetate buffer (pH 5.45 or 6.05) at 37° or 50°C for 1 to 32 hr. The analysis of each reaction mixture by high-performance liquid chromatography (HPLC, see Table I and the experimental section) revealed that Pullulanase (α-1,6-glucosidase from *Klebsiella* sp.) and Biozyme L (crude β-amylase from *Aspergillus*

Table I. ENZYMIC GLUCOSYLATION OF STEVIOSIDE BY COMMERCIAL GLUCOSIDASES

Enzyme	Origin	pH	Temp. (°C)	Reaction time (hr)	Results[a]	
					Maltose	Pullulan
β-Amylase						
Biozyme A	*Aspergillus* sp.	5.45	50	1–32	–	–
C	*Aspergillus* sp.	5.45	50	1–32	–	–
M	Malt	5.45	50	1–32	–	–
L	*Aspergillus* sp.	6.05	50	1–32	+	–
α-1,6-Glucosidase						
Pullulanase	*Klebsiella* sp.	6.05	50	4–96	+	+
β-Glucanase						
Finzym	*Aspergillus niger*	5.45	37	1–32	–	–
Cereflo	*Bacillus subtilis*	6.05	37	1–32	–	–
α-Glucosidase						
Type I	Baker's yeast	5.45	37	1–32	–	–
Type II	Yeast	5.45	37	1–32	–	–

[a] Reaction detected by HPLC; peaks other than that for stevioside were detected (+), or not (–).

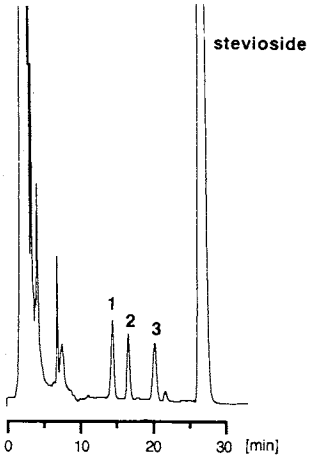


Fig. 1. Chromatogram of Products Transglucosylated with Pullulanase.

HPLC conditions: column, YMC-pack ODS-AM302; eluent, 50% MeOH; flow rate, 0.8 ml/min; column temp., 60°C; chart speed, 2.0 mm/min.

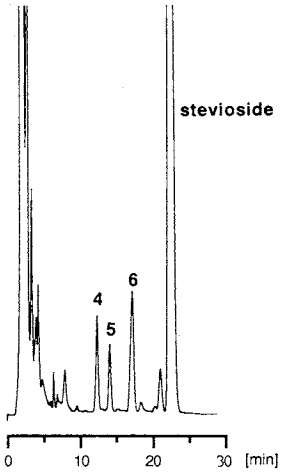


Fig. 2. Chromatogram of Products Transglucosylated with Biozyme L.

HPLC conditions: the same as those in Fig. 1.

sp.) afforded transglycosylated products at 32 hr and 24 hr, respectively (Figs. 1 and 2).

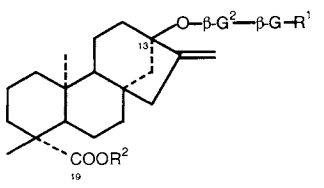
On a preparative scale, a solution of stevioside, pullulan and Pullulanase in acetate buffer (pH 6.05) was incubated at 50°C. The products were chromatographed on highly porous synthetic resin to remove the ions, enzyme and saccharides. The resulting glucoside mixture was separated by HPLC to give three compounds, **1**, **2** and **3** together with the starting material. An inspection of the ^{1}H- and ^{13}C-NMR spectra proved that products **1**, **2** and **3** were respectively identical with 13-*O*-[β-maltotriosyl(1→2)-β-D-glucosyl]-19-*O*-β-D-glucosyl-steviol, 13-*O*-[β-maltosyl(1→2)-β-D-glucosyl]-19-*O*-β-D-glucosyl-steviol and 13-*O*-β-sophorosyl-19-*O*-β-maltotriosyl-steviol. These products had already been isolated from the reaction mixture of the *trans*-α-1,4-glucosylation of stevioside by the CGTase–

starch system,[1] and among a number of products in this *trans*-α-1,4-glucosylation, **1** and **2** were the most tasty sweeteners not only in the intensity of sweetness but also in the quality of taste; the relative sweetness of stevioside to sucrose was ×160, and that of **1** and **2** was ×180 and ×205, respectively. Product **3** showed some bitter aftertaste and its relative sweetness was ×133.

Pullulanase is an enzyme which hydrolyzes the α-1,6-glucosidic linkages in pullulan to produce maltotriose, commercial pullulanase being practically applicable for saccharifying starch to produce glucose and maltose on an industrial scale.[4] The specific transglycosylation of these sugars at the 4-position of the glucose moiety in this present study may have been due to a new function of this enzyme, or caused by contaminating the commercial product.

It is noteworthy that, as already mentioned, transglucosylation of stevioside by CGTase–starch system afforded a complex mixture of a number of mono-, di-, tri- and more glucosylated products, while that by the pullulanase–pullulan system gave mainly **1**, **2** and **3**, although the yield under the present conditions was lower than that by CGTase starch system.

A solution of stevioside, Biozyme L and maltose in an acetate buffer (pH 5.45) was incubated at 50°C. The reaction mixture was separated in a similar manner to that already described to give three products, **4**, **5** and **6** together with the starting material.



	R^1	R^2
Stevioside	-H	-β-G
1	4-α-G⁴-α-G	-β-G
2	4-α-G	-β-G
3	-H	-β-G⁴-α-G 4-α-G
4	-H	-β-G⁶-α-G
Steviolbioside	-H	-H
5	6-α-G	-β-G
5a	6-α-G	-H
6	3-α-G	-β-G
6a	3-α-G	-H

G: D-glucopyranosyl

Negative high-resolution FAB-MS of **4** gave the molecular formula $C_{44}H_{70}O_{23}$ (calculated by M-H). The ^{1}H-NMR spectrum of **4** exhibited four anomeric proton signals at δ 5.99 (1H, d, J=7.9 Hz, 19-*COO*-β-Glc), 5.26 (1H, d, J=7.5 Hz, β-Glc) and 5.12 (1H, d, J=7.7 Hz, β-Glc), 5.33 (1H, d, J=3.7 Hz, α-Glc), and the ^{13}C-NMR spectrum had four anomeric carbon signals at δ 95.5 (19-*COO*-β-Glc), 97.9 (13-*O*-β-Glc), 106.7 (β-Glc) and 100.4 (α-Glc), indicating that **4** must have been a mono-α-glucosylated product of stevioside. Alkaline saponification of **4** gave steviolbioside (13-*O*-β-sophorosyl steviol) that has already been obtained from stevioside by the same method.[2] The location of the α-glucosyl moiety must be on 19-*COO*-β-Glucose. A methylation analysis of **4** indicated the presence of a terminal glucose, 2-linked glucose and 6-linked glucose,[2] demonstrating that α-glucose was bonded to the 6-position of 19-*COO*-β-glucose. This allocation was further confirmed by the ^{13}C-NMR signal due to glucosylated C-6 (δ 67.8 shifted from 62.6). Consequently, **4** can be formulated as 13-*O*-β-sophorosyl-19-*O*-isomaltosyl-steviol (Table II).

Negative high-resolution FAB-MS of **5** gave the molecular formula $C_{44}H_{70}O_{23}$ (calculated by M-H). The ^{1}H-NMR spectrum of **5** exhibited four anomeric proton signals at δ 6.07 (1H, d, J=7.7 Hz, 19-*COO*-β-Glc), 5.22 (1H, d, J=7.3 Hz, β-Glc) and 5.20 (1H, d, J=7.7 Hz, β-Glc), 5.48 (1H, d, J=3.7 Hz, α-Glc), and the ^{13}C-NMR spectrum had four anomeric carbon signals at δ 95.8 (19-*COO*-β-Glc), 97.4 (13-*O*-β-Glc), 106.5 (β-Glc) and 100.4 (α-Glc), indicating that **5** must have been a mono-α-glucosylated product of stevioside. Alkaline saponification of **5** afforded a proaglycone (**5a**).

The ^{1}H-NMR spectrum of **5a** exhibited three anomeric proton signals at δ 5.20 (1H, d, J=7.7 Hz, β-Glc), 5.24 (1H, d, J=7.5 Hz, β-Glc) and 5.52 (1H, d, J=3.7 Hz, α-Glc), and

Table II. ^1H AND ^{13}C-NMR CHEMICAL SHIFTS OF THE SUGAR MOIETIES OF COMPOUNDS **4–6** AND RELATED COMPOUNDS IN C_5D_5N

	4		Steviolbioside		**5**		**5a**		**6**		**6a**	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
13-*O*-Glc												
1	5.26 d (J=7.5 Hz)	97.9	5.27 d (J=7.6 Hz)	97.8	5.22 d (J=7.3 Hz)	97.4	5.24 d (J=7.6 Hz)	97.5	5.20 d (J=7.5 Hz)	97.9	5.20 d (J=7.4 Hz)	98.0
2		84.6		84.7		84.1		84.3		84.5		84.4
3		78.1		78.2		78.3		78.3		78.0		77.9
4		71.4		71.9		70.9		71.0		71.0		71.1
5		77.7		77.8		78.2		78.3		77.7		77.7
6		62.6		62.5		63.0		62.9		62.8		62.9
β-Glc												
1	5.12 d (J=7.7 Hz)	106.7	5.15 d (J=7.8 Hz)	106.7	5.20 d (J=7.7 Hz)	106.5	5.21 d (J=7.7 Hz)	106.6	5.09 d (J=7.7 Hz)	106.3	5.10 d (J=7.9 Hz)	106.3
2		77.0		77.1		76.4		76.5		75.6		75.7
3		78.5		78.5		78.3		78.4		84.6		84.8
4		71.4		71.4		71.6		71.7		70.7		70.8
5		78.0		78.1		75.9		75.8		78.0		78.0
6		62.8		62.9		68.6		68.6		62.7		62.7
α-Glc												
1					5.48 d (J=3.7 Hz)	100.4	5.48 d (J=3.8 Hz)	100.4	5.91 d (J=3.8 Hz)	101.8	5.98 d (J=3.6 Hz)	101.7
2						73.9		74.0		74.2		74.3
3						75.2		75.2		75.2		75.2
4						71.7		71.8		72.1		72.0
5						75.9		76.1		78.0		78.1
6						62.7		62.7		62.0		62.0
19-*O*-Glc												
1	5.99 d (J=7.9 Hz)	95.5			6.07 d (J=7.7 Hz)	95.9			6.09 d (J=7.7 Hz)	95.8		
2		73.9				74.0				73.4		
3		78.9				78.7				79.0		
4		72.1				72.1				72.2		
5		75.4				79.1				79.2		
6		67.8				62.1				61.9		
α-Glc												
1	5.33 d (J=3.7 Hz)	100.4										
2		73.9										
3		75.3										
4		71.9										
5		73.6										
6		62.6										

the ^{13}C-NMR spectrum and three anomeric carbon signals at δ 97.5 (13-*O*-β-Glc), 106.5, (β-Glc) and 100.4 (α-Glc), indicating that **5a** must have been a mono-α-glucosylated product of steviolbioside. The sugar chain on the 13-position should be straight, since the electron impact mass spectrum (EI-MS) of the acetate of **5a** gave the ion peaks at *m/z* 331, 619 and 907, attributable to a peracetylated straight-chain glucotriosyl moiety. The location of the α-glucosyl moiety was determined to be at the 6-position of the terminal β-glucosyl moiety by a methylation analysis of **5a**, which showed the presence of a terminal glucose, 2-linked glucose and 6-linked glucose. This allocation was justified by the presence

of the ^{13}C-NMR signal of glucosylated C-6 (δ 68.6 shifted from 62.8). Consequently, **5a** and **5** can be formulated as 13-*O*-[β-isomaltosyl(1→2)-β-D-glucosyl]-steviol and 13-*O*-[β-isomaltosyl(1→2)-β-D-glucosyl]-19-*O*-β-D-glucosyl-steviol, respectively.

Negative high resolution FAB-MS of **6** gave the molecular formula $C_{44}H_{70}O_{23}$ (calculated by M-H). In a similar manner to that for compound **5**, the structure of **6** was formulated as 13-*O*-[β-nigerosyl (1→2)-β-D-glucosyl]-19-*O*-β-D-glucosyl-steviol (see Table II and the experimental section).

The relative intensity of sweetness to sucrose, and the quality of the taste were determined for **4**, **5** and **6** by a panel of six professional tasters in the same manner as that described in the previous paper.[2] The results are summarized in Table III. Biozyme L is classified as a β-amylase which produces maltose from amylose. The unexpected transglycosylation in this present study might have been caused by α-glucosidase contaminating this commercial enzyme.

The relative intensity of the sweetness of **4** and **5** (especially of the latter) was less than that of stevioside, while **6** tasted bitter. With regard to the quality of taste, a remarkable improvement was observed for **4**, and if appropriate conditions to increase the yield are developed, this compound may be promising for practical use as a better sweetener than stevioside.

As already mentioned, the structure–sweetness relationship in the *trans*-α-1,4-glucosylation of stevioside and its congeners by the CGTase starch system has been investigated.

Table III. Relative Sweetness and Quality of Taste

Compound	RS[a]	QT[b]
4	110	++
5	40	+
6	Bitter	−

[a] Sweetness relative to a 6% aqueous solution of sucrose.

[b] Quality of taste: ++, better; +, slightly better; −, worse.

This present study suggests that an elongation of the 13-*O*-glucosyl moiety up to a total of four glucosyl units resulted in a remarkable improvement to sweetness, while glucosylation at the 19-position sometimes led to a change for the worse in the sweetness. However, the present results reveal that the foregoing structure–sweetness relationship is not valid in the case of glucosylation at positions other than the 4-hydroxyl group; α-glucosylation of the 6-hydroxyl group of the terminal glucosyl unit of the 13-*O*-sophorosyl moiety evidently decreased the intensity of sweetness (in the case of **5**), and that of the 3-hydroxyl group led to a change in taste to bitter (in the case of **6**). α-Glucosylation of the 6-hydroxy group of the 19-*O*-glucosyl moiety resulted in a decrease in the intensity of sweetness, as in the case of α-1,4-glucosylation, while this glucosylation led to a remarkable improvement in the quality of taste. A similar decrease of the intensity and remarkable improvement in the quality of taste has also been observed for the fructosylation of the 19-*O*-glucosyl moiety.[2]

Experimental

Materials and methods. Stevioside was supplied by Maruzen Kasei Co., Ltd. and β-amylases (Biozyme A, C, M, L) and pullulanase were purchased from Amano Pharmaceutical Co., Ltd. β-Glucanases (Cereflo and Finizym) were purchased from Novo Chemical Company, and α-glucosidases (Types I and II) were purchased from Boehringer Company. ^{1}H-NMR and ^{13}C-NMR were recorded in C_5D_5N with a JEOL JNM GX-400 spectrometer at 400 MHz for protons and at 100 MHz for carbon-13. FAB-MS and EI-MS data were recorded with a JEOL JMS SX-102 spectrometer. HPLC was carried out with a Tosoh CCPM pump equipped with a UV detector (210 nm); ODS column, YMC D-ODS-5 (4.6 mm × 15 cm for analysis, 20 mm × 25 cm for preparation); flow rate of the mobile phase, 7.0 ml/min for preparation.

Preliminary test of transglucosylation by various glucosidases. A solution of stevioside (1 g), a donor (1 g of maltose or pullulan) and enzyme (2000 units of several commercial glycosidases) in 50 ml of acetate buffer (pH 5.45 or 6.05) was incubated at 37°C or 50°C for 32 hr. The reaction mixture was analyzed by HPLC after treating with Diaion HP-20 (Mitsubishi Kasei Co., Ltd.) after 1, 2, 4, 8, 16 and 32 hr. HPLC conditions: column, Tosoh TSK gel ODS-120T (4.6 mm × 25 cm); column temperature,

60°C; mobile phase, 50% MeOH; flow rate, 1.0 ml/min; detection, UV 210 nm (see Figs. 1 and 2 and Table I).

Transglucosylation by pullulanase. A solution of stevioside (1 g), pullulan (2.5 g) and pullulanase (1,500 unit) in 50 mM acetate buffer (pH 6.05, 50 ml) was incubated at 50°C for 96 hr. After boiling for 30 min, the mixture was filtered, and then chromatographed on Diaion HP-20 by eluting with H_2O, 40% MeOH and finally MeOH. The MeOH eluate was separated by HPLC in a YMC-pack ODS-5 column using 50% MeOH as the eluent to give three compounds, **1** (36 mg), **2** (24 mg) and **3** (16 mg).

Identification of compound **1**. Colorless needles from MeOH–H_2O, mp 224–225°C (lit. 222–225°C), $[\alpha]_D^{18}$ +35.4° (lit. +38.1°) (MeOH, *c* 0.65). **1** was identified as 13-*O*-[*β*-maltotriosyl(1→2)-*β*-D-glucosyl]-19-*O*-*β*-D-glucosyl-steviol that had previously been obtained by transglucosylation of stevioside with CGTase by comparing its melting point, optical rotation, TLC and HPLC behavior, ^{1}H-NMR and ^{13}C-NMR data.[1)]

Identification of compound **2**. Colorless needles from MeOH–H_2O, mp 214–215°C (lit. 211–214°C), $[\alpha]_D^{18}$ +13.5° (lit. +12.7°) (MeOH, *c* 0.98). **2** was identified as 13-*O*-[*β*-maltosyl(1→2)-*β*-D-glucosyl]-19-*O*-*β*-D-glucosyl-steviol that had previously been obtained by transglucosylation of stevioside with CGTase by comparing its melting point, optical rotation, TLC and HPLC behavior, ^{1}H-NMR and ^{13}C-NMR data.[1)]

Identification of compound **3**. Colorless needles from MeOH–H_2O, mp 224–225°C (lit. 224–227°C), $[\alpha]_D^{18}$ +10.4° (lit. +12.0°) (MeOH, *c* 0.96). **3** was identified as 13-*O*-*β*-sophorosyl-19-*O*-[*β*-maltotriosyl(1→2)-steviol that had previously been obtained by transglucosylation of stevioside with CGTase by comparing its melting point, optical rotation, TLC and HPLC behavior, ^{1}H-NMR and ^{13}C-NMR data.[1)]

Transglucosylation by Biozyme L. A solution of stevioside (1 g), maltose (1 g) and Biozyme L (2,000 units) in 50 mM acetate buffer (pH 5.45, 50 ml) was incubated at 50°C for 24 hr. After boiling for 30 min, the mixture was filtered, and then chromatographed on Diaion HP-20 by eluting with H_2O, 40% MeOH and finally MeOH. The MeOH eluate was separated by HPLC in a YMC-pack ODS-5 column using 50% MeOH as the eluent to give three compounds, **4** (20 mg), **5** (11 mg) and **6** (23 mg).

Compound **4**. Colorless needles from MeOH–H_2O, mp 209–211°C, $[\alpha]_D^{18}$ +90.5° (MeOH; *c* 1.00). Negative FAB-MS $[M-H]^-$: Found, *m/z* 965.4237; $C_{44}H_{70}O_{23}$ −H requires 965.4229. ^{1}H-NMR and ^{13}C-NMR data: see Table II.

Methylation analysis of **4**. To a solution of **4** (10 mg) in anhydrous dimethylsulfoxide (DMSO, 0.5 ml) was added 2 M sodium dimsyl in DMSO (0.5 ml), before the mixture was sonicated for 1 hr. To this mixture was added methyl iodide (0.3 ml), and the mixture was further sonicated for 1 hr. The mixture was diluted with H_2O (10 ml) and extracted with chloroform. The chloroform layer was concentrated to dryness, and a solution of the residue in 90% formic acid (2 ml) was heated in a boiling water bath for 1 hr and then concentrated to dryness. The residue was further hydrolyzed by heating with 1 N sulfuric acid (2 ml) in a boiling water bath for 4 hr. The reaction mixture was neutralized with barium carbonate and filtered. The filtrate was concentrated to 2 ml and sodium borohydride (25 mg) was added. The mixture was left at room temperature for 2 hr, and after treating with Dowex 50 W (H^+ form), the mixture (pH 3.5) was concetrated to dryness. The residue was acetylatd by treating with acetic anhydride (0.5 ml) and anhydrous pyridine (0.5 ml) at 100°C for 1 hr. The mixture of alditol acetates so obtained was analyzed by gas-liquid chromatography-mass spectroscopy (GLC-MS). GLC-MS conditions: column, fused silica capillary column coated with CP-Sil 43CB (0.25 mm × 25 m); column temperature, 190°C; carrier gas, He (2.0 ml/min); ionization voltage, 70 eV. t_R (min): 21.0 (from terminal glucose), 35.3 (from 2-linked glucose) and 43.2 (from 6-linked glucose).

Alkaline saponification. A solution of **4** (20 mg) in a 5% potassium hydroxide solution (1.5 ml) was refluxed for 1 hr. The mixture was deionized with Amberlite MB-3 ion-exchange resin and analyzed by HPLC (70% methanol mobile phase) to give steviolbioside (10 mg) as colorless needles from methanol, mp 193–194°C, $[\alpha]_D^{18}$ −34.0° (MeOH, *c* = 0.5).

Compound **5**. Colorless needles from MeOH–H_2O, mp 224–225°C, $[\alpha]_D^{18}$ +86.5° (MeOH, *c* 0.75); negative FAB-MS $[M-H]^-$: found, *m/z* 965.4216; $C_{44}H_{70}O_{23}$ −H requires 965.4229. ^{1}H-NMR and ^{13}C-NMR data: see Table II.

Alkaline saponification. A solution of **5** (20 mg) in a 5% potassium hydroxide solution (2 ml) was refluxed for 1 hr. The mixture was deionized with Amberlite MB-3 and analyzed by HPLC (70% methanol mobile phase) to give **5a** (16 mg) as a white powder, $[\alpha]_D^{18}$ +95.7° (MeOH, *c* 0.87). ^{1}H-NMR and ^{13}C-NMR data: see Table II. EI-MS of the acetate: *m/z* 331$[GlcAc_4]^+$, 619 $[(Glc–Glc)Ac_7]^+$ and 907 $[(Glc–Glc–Glc)Ac_{10}]^+$.

Methylation analysis of **5a**. By the same procedure as that for **4**, three alditol acetates were identified. t_R (min): 19.6 (from terminal glucose), 33.0 (from 2-linked glucose) and 39.1 (from 6-linked glucose).

Compound **6**. Colorless needles from MeOH–H_2O, mp 232–234°C, $[\alpha]_D^{18}$ +86.5° (MeOH, *c* 0.75); negative

FAB-MS $[M-H]^-$: found, *m/z* 965.4193; $C_{44}H_{70}O_{23}-H$ requires 965.4229. ^{1}H-NMR and ^{13}C-NMR data: see Table II.

Alkaline saponification. A solution of **6** (20 mg) in a 5% potassium hydroxide solution (2 ml) was refluxed for 1 hr. The mixture was deionized with Amberlite MB-3 and analyzed by HPLC (70% methanol mobile phase) to give **6a** (14 mg) as a white powder, $[\alpha]_D^{18}$ +85.6° (MeOH, $c=0.75$). ^{1}H-NMR and ^{13}C-NMR data: see Table II. EI-MS of the acetate: *m/z* 331 $[GlcAc_4]^+$, 619 $[(Glc-Glc)Ac_7]^+$ and 907 $[(Glc-Glc-Glc)Ac_{10}]^+$.

Methylation analysis of **6a**. By the same procedure as that for **4**, three alditol acetates were identified. t_R (min): 18.3 (from terminal glucose), 30.7 (from 2-linked glucose) and 37.0 (from 3-linked glucose).

Acknowledgments. We thank the Nakano Foundation for the sweetness test, some enzymes and financial support. One of the authors (L. S. V) is indebted to the USSR government and the Japan Cultural Association for Overseas Work.

References

1) Y. Fukunaga, T. Miyata, N. Nakayasu, K. Mizutani, R. Kasai and O. Tanaka, *Agric. Biol. Chem.*, **53**, 1603 (1989).
2) K. Ohtani, Y. Aikawa, H. Ishikawa, R. Kasai, S. Kitahata, K. Mizutani, S. Doi, M. Nakaura and O. Tanaka, *Agric. Biol. Chem.*, **55**, 449 (1991).
3) H. Ishikawa, S. Kitahata, K. Ohtani, C. Ikuhara and O. Tanaka, *Agric. Biol. Chem.*, **54**, 3137 (1990).
4) Y. Sakano, in "Handbook of Amylases and Related Enzymes," ed. by The Amylase Research Society of Japan, Pergamon Press, Oxford, 1988, pp. 131–139.