Further Study on the 1,4-α-Transglucosylation of Rubusoside, a Sweet Steviol-Bisglucoside from *Rubus suavissimus*

Kazuhiro OHTANI, Yoko AIKAWA, Hiroshi ISHIKAWA,* Ryoji KASAI, Sumio KITAHATA,* Kenji MIZUTANI,** Shigeki DOI,** Masaharu NAKAURA** and Osamu TANAKA

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi, Minami-Ku, Hiroshima 734, Japan
** Osaka Municipal Technical Research Institute, 1–6–50, Morinomiya, Jyoto-ku, Osaka 536, Japan*
*** Department of Research and Development, Maruzen Kasei Co., Ltd., Mukaihigashi-cho, Onomichi 722, Japan*

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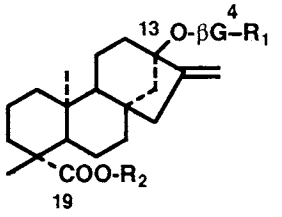
Rubusoside (the β-D-glucosyl ester of 13-*O*-β-D-glucosyl-steviol), which is the major sweet principle of leaves of *Rubus suavissimus* S. Lee, was subjected to 1,4-α-transglucosylation by the cyclodextringlucanotransferase-starch system (the CGTase system). The tri- and tetra-glucosylated products were isolated together with the mono- and di-glucosylated products, which had already been isolated. A prominent increase in intensity of the sweetness was observed for the compounds which were di- and tri-glucosylated at the 13-*O*-glucosyl moiety. This result further substantiated the structure–sweetness relationship for 1,4-α-glucosylated compounds of steviol-glycosides reported previously. For protection of the 19-COO-glucosyl moiety against glucosylation by the CGTase system, the 4-hydroxyl group of the 19-COO-glucosyl moiety was β-galactosylated by the β-galactosidase-lactose system. This galactosylated compound was subjected to a regio-selective glucosylation of the 13-*O*-glucosyl moiety by the CGTase system, which was followed by enzymic elimination of the galactosyl group to furnish an exclusive preparation of the improved sweeteners just mentioned.

Rubusoside (**R**), the β-D-glucosyl ester of 13-*O*-β-D-glucosyl-steviol (=desgluco-stevioside), has been isolated in a high yield as the major sweet principle from leaves of *Rubus suavissimus* S. Lee (Rosaceae), which grows in the southern districts of China.[1] In our first paper,[1] this plant was tentatively assigned as *R. chingii* Hu. However, later on, it was reported that this sweet plant is not identical with *R. chingii*[2] but is a new species named as already mentioned.[3] Rubusoside (**R**) was nearly one hundred-fold as sweet as sucrose. In order to improve the sweetness of **R**, a 1,4-α-transglucosylation of **R** by the cyclodextrin–glucanotransferase–starch system (the CGTase system) has been carried out, and separation of the mono- and di-glucosylated compounds (**RG-1a**, **-1b**, **-2a**, **-2b** and **-2c**, see Chart I) has already been reported.[4] The sweetness evaluation of these compounds revealed that an evident improvement in sweetness was observed for **RG-1a** and **-2a**, which were mono- and di-glucosylated at the 13-*O*-glucosyl moiety. The present paper reports the isolation, structural elucidation and sweetness evaluation of the tri- and tetra-glucosylated compounds from a complex mixture of the glucosylated products in this enzymic transglucosylation. The regio-selective preparation of the improved sweeteners by means of the enzymic process is also described.

Results

A solution of **R**, soluble starch and CGTase[5] (from *Bacillus circulans*) in an acetate buffer (pH 5.4) was incubated at 40°C. The products were chromatographed on highly porous synthetic polymer resin (Diaion HP-20) to remove the ions, enzyme and saccharides. The resulting mixture of glucosylated compounds and

the starting material (**R**) was subjected to chromatography on silica gel and then by preparative high-performance liquid chromatography (HPLC) to give all of tri-glucosylated compounds (**RG-3a**, **-3b**, **-3c** and **-3d**) and three of five tetra-glucosylated compounds (**RG-4a**, **-4b** and **-4e**), together with **RG-1a**, **-1b**, **-2a**, **-2b** and **-2c** and the starting material, **R**. The other two tetra-glucosylated compounds (**RG-4c** and **-4d**) have not been separated as yet, being obtained as a mixture, **RG-4cd**. The optical rotation and the results of an elemental analysis of the newly isolated compounds are summarized in Table I. The $[M-H]^-$ of the negative FAB-MS and anomeric proton signals, as well as the anomeric carbon signals, are shown in Table II.



Compound	R_1	R_2
R	H	βG
RG-1a	αG	βG
RG-1b	H	βG^4αG
RG-2a	αG^4αG	βG
RG-2b	H	βG^4αG^4αG
RG-2c	αG	βG^4αG
RG-3a	αG^4αG^4αG	βG
RG-3b	αG^4αG	βG^4αG
RG-3c	αG	βG^4αG^4αG
RG-3d	H	βG^4αG^4αG^4αG
RG-4a	αG^4αG^4αG^4αG	βG
RG-4b	αG^4αG^4αG	βG^4αG
RG-4c	αG^4αG	βG^4αG^4αG
RG-4d	αG	βG^4αG^4αG^4αG
RG-4e	H	βG^4αG^4αG^4αG^4αG
SM	H	H
SMG-1	αG	H
SMG-2	αG^4αG	H
SMG-3	αG^4αG^4αG	H
SMG-4	αG^4αG^4αG^4αG	H
RGal-1a	H	βG^4βGal

Chart I. G, D-glucopyranosyl; Gal, D-galactopyranosyl

For an elucidation of the position of glucosylation of these products, 13-*O*-mono-, -di-, -tri-, -tetra- and -penta-glucosyl-steviols were prepared from a mixture of transglucosylated products from **R** by the CGTase system, which was treated with alkali to saponify the 19-COO-glycosyl linkage. The resulting products were separated by HPLC to give the 13-*O*-β-D-glucosyl-steviol (steviolmonoside, **SM**), 13-*O*-β-maltosyl-(**SMG-1**), 13-*O*-β-maltotriosyl-(**SMG-2**), 13-*O*-β-maltotetrosyl-(**SMG-3**) and 13-*O*-β-maltopentosyl-(**SMG-4**) steviols shown in Chart I. Of these 13-*O*-glycosylsteviols, **SM**, **SMG-1** and **SMG-2** have already been obtained from **R**, **RG-1a** and **RG-2a**, respectively.[4] The structures of **SMG-3** and **-4** were established from an inspection of the anomeric proton and carbon signals, $[M-H]^-$ of the negative FAB-MS, and by the glycosylation

Table I.

Compound	$[\alpha]_D^{24}$ (c, MeOH)	*Anal.* Calcd. for	Found
RG-3a	+52.0° (1.01)	$C_{50}H_{80}O_{28}\cdot H_2O$	
		C, 52.35; H, 7.21	C, 52.73; H, 7.29
RG-3b	+54.4° (1.03)	$C_{50}H_{80}O_{28}\cdot 2H_2O$	
		C, 51.54; H, 7.27	C, 51.54; H, 7.29
RG-3c	+49.6° (1.03)	$C_{50}H_{80}O_{28}\cdot H_2O$	
		C, 52.35; H, 7.21	C, 52.44; H, 7.29
RG-3d	+51.8° (1.01)	$C_{50}H_{80}O_{28}\cdot H_2O$	
		C, 52.35; H, 7.21	C, 52.39; H, 7.31
RG-4a	+66.4° (1.01)	$C_{56}H_{90}O_{33}\cdot 4H_2O$	
		C, 49.33; H, 7.25	C, 49.66; H, 7.24
RG-4b	+69.5° (1.01)	$C_{56}H_{90}O_{33}\cdot 2H_2O$	
		C, 50.67; H, 7.14	C, 50.58; H, 7.14
RG-4e	+63.8° (1.01)	$C_{56}H_{90}O_{33}\cdot 2H_2O$	
		C, 50.67; H, 7.14	C, 50.99; H, 7.39

Table II.

Compound	$[M-H]^-$	^{1}H-NMR (C_5D_5N) δ	^{13}C-NMR (C_5D_5N) δ
RG-3a	1127	5.07 (1H, d, J=7.7 Hz)	95.9
		5.74 (1H, d, J=3.3 Hz)	99.6
		5.79 (1H, d, J=3.9 Hz)	102.7
		5.92 (1H, d, J=3.5 Hz)	102.8
		6.08 (1H, d, J=7.7 Hz)	103.1
RG-3b	1127	5.25 (1H, d, J=7.9 Hz)	95.6
		5.70 (1H, d, J=3.7 Hz)	99.5
		5.88 (1H, d, J=3.9 Hz)	102.7
		5.91 (1H, d, J=3.7 Hz)	102.8
		5.99 (1H, d, J=8.3 Hz)	102.9
RG-3c	1127	4.95 (1H, d, J=7.9 Hz)	95.8
		5.72 (1H, d, J=3.9 Hz)	99.8
		5.78 (1H, d, J=3.8 Hz)	102.5
		5.80 (1H, d, J=3.8 Hz)	102.8
		5.97 (1H, d, J=8.1 Hz)	103.1
RG-3d	1127	5.12 (1H, d, J=7.9 Hz)	95.6
		5.79 (1H, d, J=3.9 Hz)	99.6
		5.84 (1H, d, J=3.7 Hz)	102.7
		5.93 (1H, d, J=3.9 Hz)	103.0
		6.10 (1H, d, J=8.2 Hz)	103.1
RG-4a	1289	5.06 (1H, d, J=7.7 Hz)	95.9
		5.78 (1H, d, J=3.7 Hz)	99.6
		5.81 (1H, d, J=3.9 Hz)	102.5
		5.83 (1H, d, J=4.0 Hz)	102.6
		5.94 (1H, d, J=3.8 Hz)	102.9
		6.10 (1H, d, J=7.9 Hz)	103.0
RG-4b	1289	5.07 (1H, d, J=7.7 Hz)	95.6
		5.76 (1H, d, J=3.7 Hz)	99.5
		5.79 (1H, d, J=3.7 Hz)	102.6
		5.92 (2H, d, J=3.7 Hz)	102.8×2
		6.02 (1H, d, J=8.4 Hz)	103.0
RG-4e	1289	5.13 (1H, d, J=7.7 Hz)	95.6
		5.81 (1H, d, J=3.5 Hz)	99.6
		5.82 (1H, d, J=3.8 Hz)	102.7
		5.89 (1H, d, J=3.7 Hz)	102.9
		5.96 (1H, d, J=3.9 Hz)	103.0
		6.09 (1H, d, J=8.2 Hz)	103.1

shift of sugar carbon signals, as well as by the regio and anomeric specificity of transglucosylation by the CGTase system.

Anomeric carbon and proton signals and $[M-H]^-$ of the negative FAB-MS indicated that **RG-3a**, **-3b**, **-3c** and **-3d** were tri-1,4-α-D-glucosyl-rubusosides. By alkaline saponification, **RG-3a**, **-3b**, **-3c** and **-3d** yielded **SMG-3**, **-2** and **-1** and **SM**, respectively. Accordingly, the structures of **RG-3a**, **-3b**, **-3c** and **-3d** were assigned to be 13-*O*-β-maltotetrosyl-19-β-D-glucosyl, 13-*O*-β-maltotriosyl-19-*O*-β-maltosyl, 13-*O*-β-maltosyl-19-*O*-β-maltotriosyl and 13-*O*-β-D-glucosyl-19-*O*-β-maltotetrosylsteviols, respectively, as shown in Chart I.

Table III. Sweetness of 1,4-α-Glucosylated Rubusoside (**R**)

$(1,4\text{-}\alpha\text{-Glc})_x\text{-}\beta\text{-Glc-OOC-Steviol-}O\text{-}\alpha\text{-Glc-}(1,4\text{-}\alpha\text{-Glc})_y$

Compound	x	y	A/B	Compound	x	y	A/B
R	0	0	114	**-2a**	0	2	278
RG-1a	0	1	132	**-2b**	2	0	99
-1b	1	0	102	**-2c**	1	1	95
-3a	0	3	214	**-4a**	0	4	115
-3b	1	2	182	**-4b**	1	3	202
-3c	2	1	110	**-4c**	2	2	184
-3d	3	0	58	**-4d**	3	1	
				-4e	4	0	49

A/B, relative sweetness to sucrose.

In a similar manner, **RG-4a**, **-4b** and **-4e** were formulated as 13-*O*-β-maltopentosyl-19-*O*-β-D-glucosyl, 13-*O*-β-maltotetrosyl-19-*O*-β-maltosyl and 13-*O*-β-D-glucosyl-19-*O*-β-maltopentosylsteviols, respectively. The ^{1}H and ^{13}C-NMR spectra of **RG-4cd** indicated that this mixture consisted of 13,19-di-*O*-β-maltotriosyl (**RG-4c**) and 13-*O*-β-maltosyl-19-*O*-β-maltotetrosyl (**RG-4d**)steviols.

The relative intensity of sweetness to sucrose was determined for each compound by a panel of five professional tasters in the same manner as that in the previous paper.[4] The results are summarized in Table III. We have reported the structure–sweetness relationship for a variety of steviol-bisglycosides,[4,6,7] which were obtained from stevioside and **R** by CGTase 1,4-α-transglucosylation. A significant increase in the sweetness was observed for the compounds which had totally three or four 1,4-α-glucosyl units at the 13-position, while further elongation of the 13-*O*-glycosyl chain resulted in a decrease in the intensity of sweetness. It was also disclosed that 1,4-α-transglucosylation at the 19-COO-glucosyl moiety did not always result in an increase of sweetness. These

structure–sweetness correlations were further supported by the present study. The remarkable increase in the intensity of sweetness was observed for compounds **RG-3a**, **-3b** and **-4b**, while the intensity of sweetness of **RG-4a** was not improved. It was also found that an evident decrease of sweetness was observed for **RG-3d** and **-4e**.

Kitahata *et al.* have previously reported that galactose was not an efficient acceptor for transglucosylation by the CGTase system.[8] Previously, the 19-*O*-*β*-glucosyl moiety of stevioside and **R** was chemically replaced by a *β*-galactosyl moiety.[6] Both the products were subjected to transglucosylation by the CGTase system, affording better sweeteners which were glucosylated regio-selectively at the 13-*O*-glycosyl moiety. Recently, it has been found that treating **R** with lactose and the *β*-galactosidase from *Bacillus circulans* for a short time (nearly 30–50 min) mainly yielded a product (**RGal-1a**) which was regio-selectively *β*-galactosylated at the 4-OH group of the 19-COO-glucosyl moiety.[9] In the present paper, using this enzymic protection of the 19-COO-glucosyl moiety against transglucosylation by the CGTase system, a regio-selective elongation of the 13-*O*-glucosyl moiety of **R** was conducted to prepare the improved sweeteners, **RG-1a**, **-2a** and **-3a**. The *β*-galactosyl compound (**RGal-1a**) was subjected to transglucosylation by the CGTase system, and then the galactosyl group was eliminated with *β*-galactosidase, affording **RG-1a**, **-2a** and **-3a** together with a small amount of **RG-4a**.

Experimental

Materials and methods. Rubusoside (**R**) was isolated from the leaves of *R. suavissimus* (Rosaceae) collected in Kwangchow, China by the method reported previously.[1] ^1H and ^{13}C NMR spectra 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 data were recorded with a JEOL JMS SX-102 spectrometer. HPLC was carried out with a Tosoh CCPM pump equipped with a Tosoh RI-8000 differential refractometer as a detector; amide-column, TSK-gel Amide-80 (21.5 mm i.d. × 30 cm); ODS-column, YMC D-ODS-5 (20 mm i.d. × 25 cm) unless otherwise stated; flow rate of the mobile phase, 6.0 ml/min.

1,4-*α*-Transglucosylation of **R**. A solution of **R** (5 g), soluble starch (10 g) and CGTase (1 ml, produced by *Bacillus circulans*, 100 unit/ml) in 1 M acetate buffer (pH 5.4, 1.1 ml) and H_2O (95 ml) was incubated at 40°C for 96 hr. After boiling for 30 min, the mixture was filtered, and chromatographed on Diaion HP-20 (Mitsubishi Kasei Co., Ltd.) by eluting with H_2O, 40% MeOH and then MeOH. The MeOH eluate was subjected to chromatography on silica gel with $CHCl_3$–MeOH–H_2O (30 : 10 : 1, 13 : 7 : 1 and then 10 : 5 : 1, all homogeneous) to give nine fractions designated as Frs. 1–9. Fr. 1 was composed of **R** (941 mg); Frs. 2–9 were respectively separated by HPLC on an amide column; HPLC of Fr. 2 with 80% MeCN gave **R** (160 mg) and a mixture of monoglucosylated compounds (RG-1). Fr. 3 consisted of RG-1. HPLC of Fr. 4 with 80% MeCN afforded RG-1 and two mixtures of diglucosylated compounds (RG-2 and -2′). With 78% MeCN, Fr. 5 yielded RG-1, RG-2 and -2′, and two mixtures of triglucosylated compounds (RG-3 and -3′). With 78% MeCN, Fr. 6 gave RG-2, -2′, -3 and -3′. With 76% MeCN, Fr. 7 afforded RG-2, -2′, -3, -3′ and two mixtures of tetraglucosylated compounds (RG-4 and -4′). With 74% MeCN, Fr. 8 yielded RG-3, -3′, -4 and -4′. HPLC of Fr. 9 with 72% MeCN furnished the separation into RG-3, -3′, -4, -4′ and a mixture of more glucosylated compounds.

RG-1 was further separated into **RG-1a** (520 mg) and **-1b** (790 mg) by HPLC on an amide column with 83% MeCN. The final separation and purification of the di-, tri- and tetra-glucosylated compounds were carried out by HPLC on an ODS column with 66% MeOH. RG-2 gave **RG-2a** (145 mg) and **-2c** (153 mg); RG-2′ afforded **RG-2b** (150 mg); RG-3 afforded **RG-3a** (114 mg), **-3b** (90 mg) and **-3c** (90 mg); RG-3′ yielded **RG-3d** (168 mg); RG-4 gave **RG-4a** (80 mg), **-4b** (72 mg) and a mixture (**RG-4cd**, 80 mg) of **RG-4c** and **-d**; RG-4′ gave **RG-4e** (100 mg).

Preparation of the 13-O-mono-, di-, tri- and tetraglucosylated steviols. A solution of **R** (300 mg), soluble starch (300 mg) and CGTase (20 *μ*l, produced by *B. circulans*, 1500 unit/ml) in a 1 M acetate buffer (300 *μ*l, pH 5.4) and H_2O (30 ml) was incubated at 40°C for 8 hr. After working up by the procedure already given, the reaction products were saponified by heating with 5% KOH (30 ml) at 100°C for 4 hr and then extracted with 1-BuOH (saturated with H_2O). The BuOH layer was washed with H_2O and subjected to HPLC on a column of TSK gel ODS-120T (21.5 mm i.d. × 30 cm) with 70% MeOH to give **SM** (47 mg), **SMG-1** (25 mg), **-2** (28 mg), **-3** (22 mg) and **-4** (17 mg), together with a pentaglucosylated compound (11 mg, negative FAB-MS $[M-H]^-$ *m/z* 1289). These compounds could be distinguished from each other by TLC on a silica gel plate with $CHCl_3$–MeOH–H_2O (6 : 4 : 1, homogeneous), detecting with H_2SO_4. **SM** and **SMG-1** and **-2** were identified by comparing their NMR and other physical constants with those of respective samples reported previously.[4] **SMG-3**, a with powder,

$[\alpha]_D^{17}$ +53.1° (c=1.02, MeOH); anomeric proton signals (each 1H, d): δ 5.09 (J=7.7 Hz), 5.83 (J=3.7 Hz), 5.84 (J=3.5 Hz), 5.98 (J=3.7 Hz); anomeric carbon signals: δ 100.6, 103.8, 104.0, 104.2; negative FAB-MS $[M-H]^-$ Found: m/z 965.4249; Calcd. for $C_{44}H_{70}O_{23}-H$: 965.4230. **SMG-4**, a white powder, $[\alpha]_D^{17}$ +73.5° (c=0.73, MeOH); anomeric proton signals (each 1H, d): δ 5.08 (J=7.9 Hz), 5.84 (J=4.4 Hz), 5.85 (J=4.8 Hz), 5.87 (J=3.8 Hz), 5.95 (J=3.9 Hz); anomeric carbon signals: δ 99.5, 102.6, 102.8, 102.9, 103.1; negative FAB-MS Found $[M-H]^-$ m/z 1127.4759; Calcd. for $C_{50}H_{80}O_{28}-H$: 1127.4760.

Saponification of 1,4-α-glucosylated products from ***R***. A few mg of each product was saponified with 5% KOH in a similar way to that already given, and the products were identified by comparing the TLC data (*vide supra*) with respective authentic samples. **RG-4a** yielded **SMG-4**. **RG-3a** and **RG-4b** afforded **SMG-3**. **RG-3c** gave **SMG-1**. **RG-3d** and **RG-4e** afforded **SM**. A mixture of **RG-4c** and **-4d** (**RG-4cd**) yielded a mixture of **SMG-2** and **-1**.

Selective preparation of ***RG-1a, -2a, -3a and -4a from R***. As reported in the previous paper,[9] incubating **R**, β-galactosidase (produced by *B. circulans*) and lactose at pH 6.0 for 30 min afforded a product which mainly consisted of **RGal-1a**. A solution of this product (200 mg), soluble starch (200 mg) and CGTase (66.8 μl, produced by *B. stearothermophilus*, 1400 unit/ml) in a 1 M acetate buffer (pH 5.4, 400 μl) and H_2O (20 ml) was incubated at 40°C for 8 hr. After boiling for 30 min, to the reaction mixture was added β-galactosidase (20 ml, produced by *B. circulans*, 25 unit/ml), the solution being incubated at 40°C for 30 min. After boiling for 30 min, the mixture was chromatographed on Diaion HP-20 by eluting with H_2O, 40% MeOH and then MeOH. The MeOH eluate was separated by HPLC on an ODS column with 68% MeOH to give **RG-1a** (56 mg), **-2a** (23 mg), **-3a** (13 mg) and **-4a** (8 mg), together with the recovered **R** (74 mg).

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