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Glucosylation of Steviol and Steviol-Glucosides in Extracts from *Stevia rebaudiana* Bertoni

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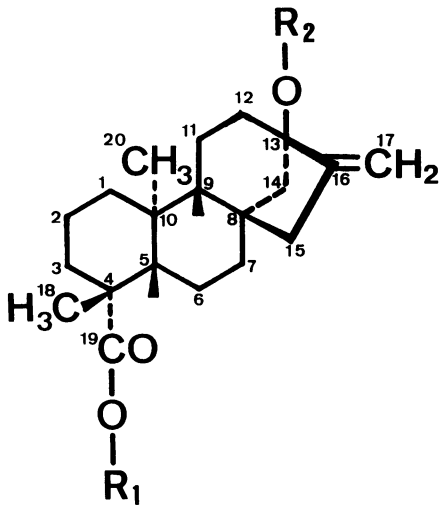
ABSTRACT

To evaluate and characterize stevioside biosynthetic pathway in *Stevia rebaudiana* Bertoni cv Houten, two enzyme fractions that catalyze glucosylation of steviol (*ent*-13-hydroxy kaur-16-en-19-oic acid) and steviol-glucosides (steviol-13-*O*-glucopyranoside, steviolbioside and stevioside), utilizing UDP-glucose as the glucose donor, were prepared from the soluble extracts of *S. rebaudiana* leaves. Enzyme fraction I, passed through DEAE-Toyopearl equilibrated with 50 millimolar K-phosphate pH 7.5, catalyzed the glucosylation to steviol and 19-*O*-methylsteviol, but not to *iso*-steviol and 13-*O*-methylsteviol, indicating that 13-hydroxyl group of the steviol skeleton is glucosylated first from UDP-glucose to produce steviol-13-*O*-glucopyranoside. Enzyme fraction II, eluted from the DEAE-Toyopearl column with 0.15 molar KCl, catalyzed the glucose transfer from UDP-glucose to steviol-13-*O*-glucopyranoside, steviolbioside and stevioside, but not to rubusoside (13,19-di-*O*-glucopyranoside) and rebaudioside A. The reaction products glucosylated from steviol-13-*O*-glucopyranoside, steviolbioside and stevioside were identified to be steviolbioside, stevioside and rebaudioside A, respectively. These results indicate that in the steviol-glucoside biosynthetic pathway, steviol-13-*O*-glucopyranoside produced from the steviol glucosylation is successively glucosylated to steviolbioside, then to stevioside producing rebaudioside A.



	R_1	R_2
Steviol	H	H
Steviolmonoside	H	β-Glc
Rubusoside	β-Glc	β-Glc
Steviolbioside	H	β-Glc-β-Glc(2-1)
Stevioside	β-Glc	β-Glc-β-Glc(2-1)
Rebaudioside-A	β-Glc	β-Glc-β-Glc(2-1) β-Glc(3-1)

Figure 1. Chemical structures of steviol and steviol-glucosides.

One remarkable, sweet constituent of *Stevia rebaudiana* Bertoni, a wild shrub belonging to Compositae family, has been named stevioside, and proven to some 300 times as sweet as sucrose. Stevioside is a glucoside composed of three glucose molecules and an aglycon, steviol (*ent*-13-hydroxy kaur-16-en-19-oic acid), a diterpenoidic carboxylic alcohol (Fig. 1). In addition to stevioside, the leaves of *S. rebaudiana* contain several kinds of the steviol-related glucosides such as steviolbioside, rebaudioside A, and others as the minor constituents. Stevioside and rebaudioside A, the latter is steviol-tetraglucoside, are known to be the main sweet constituents (10). As a consequence of their high content (5–10% of dry weight) in sweet glucosides, *S. rebaudiana* leaves are now becoming important in Japan, Brazil and other countries as a new noncaloric sweetner.

Several works on the relationship between sweetness and chemical structures of steviol-glucosides (2, 3, 5, 9, 11, 13) and the methods for chemical synthesis of stevioside (15) are already reported. On the biosynthesis of steviol, [^{14}C]acetic acid (17) and 17-[^{14}C]kaurene (1) are reportedly incorporated into the steviol skeleton; thus, steviol is believed to be synthesized from kaurene (18), which is also a precursor of gibberellins. The later stage of steviol-glucoside biosynthesis, the enzymatic glucosylations toward steviol and steviol-glucosides, remains to be characterized. In the soluble extracts from the leaves of *S. rebaudiana* Bertoni, we have detected the activities of glucosylation from UDP-glucose toward steviol, steviol-13-*O*-glucopyranoside, steviolbioside and stevioside, and report here some properties of these enzymatic glucosylations and characterization of the reaction products, then discuss on the main biosynthetic pathway of stevioside and rebaudioside A from steviol in *S. rebaudiana* Bertoni.

MATERIALS AND METHODS

Preparation of Steviol and Steviol-Glucosides

Stevioside and rebaudioside A were extracted from *S. rebaudiana* leaves according to the methods in (10), then purified finally by HPLC under the conditions described below. Steviol was prepared by the chemical degradation of stevioside with $NaIO_4$ and KOH, then crystallized in methanol (15). An isomeric *iso*-steviol was obtained by hydrolysis of stevioside with 10% *aq* H_2SO_4 (20). Methylation of steviol with diazomethane (19) or with CH_3I (7) afford 19-*O*-methylsteviol or 13-*O*-methyl-steviol, respectively. Steviolbioside was prepared by saponification of stevioside (20). A specific β-glycosidation which produced rubusoside from stevioside, was performed with a microbial enzyme (H Nishihashi, unpublished data). Saponification of rubusoside afforded steviol-13-*O*-glucopyranoside. Steviol and steviol-glucosides thus prepared were checked for their purity by TLC and HPLC, and then were used as authentic standards or substrate for enzymatic glucosylation.

Plant Material

Stevia (*Stevia rebaudiana* Bertoni cv Houten) plants were field grown, and the leaves were harvested in late summer from fully grown shoots, frozen in liquid nitrogen, and kept at −30°C.

Preparation of Enzyme Fractions

Frozen stevia leaves (about 200 g) were homogenized in a blender in the presence of 600 mL 0.2 M K-phosphate (pH 7.5) containing 5 mM $MgCl_2$, 10 mM EDTA, 20 mM 2-mercaptoethanol, 2 g sodium *iso*-ascorbate, and 10 g polyclar AT. The homogenate was passed through two layers of Miracloth and the filtrate was centrifuged for 30 min at 20,000*g*. The enzyme fraction was precipitated by $(NH_4)_2SO_4$ (30–75% saturation) and redissolved in a minimal volume of 50 mM K-phosphate (pH 7.5) containing 1 mM EDTA and 10 mM 2-mercaptoethanol (buffer A). Excess $(NH_4)_2SO_4$, and a small amount of steviol-glucosides still present in the fraction were removed by filtration through Sephadex G-25, equilibrated and eluted with buffer A. The protein fraction was designated the crude enzyme, and chromatographed on DEAE-Toyopearl 650M column (20 × 2.5 cm i.d.). The column was washed with 200 mL buffer A before the following linear gradient was applied: 300 mL 0 to 0.5 M KCl in buffer A. Two distinct active fractions, passed through the column and eluted with about 0.15 M KCl, were separately concentrated by ultrafiltration (UP-20 filter, ADVANTEC, Japan) and designated enzyme I and enzyme II.

Enzyme Assays

The usual standard reaction mixture for the glucosyltransferase assays contained the following in a total volume of 250 μL: 0.1 M Tris-HCl (pH 7.5), 10 to 40 μM steviol or one of steviol-glucosides, 1 or 2 μM (18.5 or 37 KBq, respectively) UDP-[^{14}C(U)]glucose (11.5 GBq/mmol) purchased from American Radiolabel Chemicals, Inc., and up to 100 μL protein solution. Once the labeled UDP-glucose was added, the reaction was run at 35°C for 60 min, and stopped by adding 200 μL 1-butanol saturated previously with water. After mixing, the organic layer was separated by centrifugation. Under the same experimental conditions, no [^{14}C]glucose nor UDP-[^{14}C]glucose was found to be transferred into 1-butanol layer from the aqueous layer. Aliquots of the butanol extract were taken for radioactivity measurements, and the amount of the glucosylated [^{14}C]glucose was calculated. Other aliquot was subjected to TLC or HPLC analysis.

TLC and HPLC

Steviol-glucosides and the reaction products of glucosyltransferase containing authentic standards were spotted on precoated silicagel 60 F_{254} plates (0.25 mm thick; E. Merck, Darmstadt, FRG) and developed with chloroform-methanol-water (10:6:1). After development, chromatograms were air-dried, sprayed with 50% sulfuric acid then heated at 100°C for 15 min to visualize the glucosides. Labeled compounds on the TLC plates were localized by autoradiography and quantified by Berthold TLC-Multi-Tracemaster LB285.

For the HPLC analysis of stevioside and rebaudioside A, the liquid chromatograph (Tosoh LC system) was equipped with a prepacked TSK Gel-NH_2-60 column (25 cm × 4.6 mm i.d.) and the operating conditions were: ambient temperature; flow rate of eluting solvent, acetonitrile-water (79:21), 1 mL/min; wavelength of UV detector, 210 nm. Radio LC detector (Tosoh RS-8000) was also fitted to analyze [^{14}C]glucose-labeled reaction products. Steviol-13-*O*-glucopyranoside and steviolbioside were separated on TSK gel ODS-80 Tm (15 cm × 4.6 mm i.d.) using 70% methanol as the solvent.

RESULTS

Glucosyltransferases Toward Steviol and Steviol-Glucosides

Although numerous studies have been concerned with the sweet steviol-glucosides, surprisingly little is known about the glucosylation to the steviol skeleton in *S. rebaudiana* leaves which accumulate sweet glucosides in a yield 5 to 10% per dry leaf basis. As we could detect the UDP-glucose dependent glucosylation activities for steviol and steviol-glucosides in the crude extracts (Table I), the extract was applied onto DEAE-Toyopearl column to obtain partially purified enzyme preparations. Two distinct active fractions, enzyme I and II were obtained under the chromatographic conditions cited. Substrate specificities of these enzyme fractions were examined (Table I). A background radioactivity found in the assay without substrate, was subtracted from the radioactivity determined with substrate in all other experiments. Such blank activity was assumed to depend on the steviol and its glucosides present still in the enzyme preparations, because it increased with the amounts of protein added to the reaction mixture. Crude extract acted on steviol, steviolbioside, and stevioside, but not on rebaudioside A. Enzyme I was specific for steviol. Enzyme II was free from the activity toward steviol and could act on steviol-13-*O*-glucopyranoside, steviolbioside and stevioside, but again not on rebaudioside A (Table I).

Table I. *Substrate Specificity of Glucosyltransferases to Steviol and Steviol-Glucosides*

Crude enzyme and enzyme I and II were prepared as described in the text. To the each reaction mixtue (250 μL), crude enzyme (480 μg protein), enzyme I (210 μg protein), or enzyme II (360 μg protein) was added, then the reaction was run for 1 h.

Acceptor	[^{14}C]Glucose Transferred		
	Crude	Enzyme I	Enzyme II
	dpm × 10^{-3}/*h*		
None	2.8	0.3	2.5
Steviol	4.1	22.9	2.1
Steviolmonoside[a]		0.4	12.7
Steviolbioside	5.0	0.5	23.9
Stevioside	3.2	0.8	9.6
Rebaudioside A	2.8	0.5	2.1

[a] Steviol-13-*O*-glucopyranoside.

Among steviol-glucosides, steviolbioside was preferred as the substrate for enzyme II.

Effect of Nucleotides and Nucleotide-Glucoses

The enzymatic glucosylation using UDP-glucose produces the glucoside and UDP. Some UDP-glucose dependent glucosyltransferases are reportedly inhibited by UDP (6, 8, 12). This was the case for glucosylation toward steviol and steviol-glucosides. UDP, in contrast to ADP, strongly inhibited the glucosylation catalyzed by enzyme I and II (Table II). ADP-, CDP-, and GDP-glucose had no effect on the enzyme activity.

Characterization of the Reaction Products

Steviol have two sites, 13-hydroxyl and 19-carboxyl groups, for accepting glucose to produce steviol-glucosides (Fig. 1). To evaluate which group of steviol is glucosylated first, we used 13-*O*-methylsteviol and 19-*O*-methylsteviol as the substrate for enzyme I (Table III). Steviol and 19-*O*-methylsteviol accepted [^{14}C]glucose, but 13-*O*-methylsteviol and a stereoisomer, *iso*-steviol could not serve as substrates. These results indicate that the 13-hydroxyl group of steviol is glucosylated first to produce steviol-13-*O*-glucopyranoside.

Table II. *Influence of Nucleotides and XDP-Glucose on Glucosyltransferases*

Enzymes I and II were assayed with steviol and steviolbioside, respectively. In the control with no additive, 5100 dpm of the [^{14}C] labeled glucoside was formed in a 1-h incubation with enzyme I (360 μg protein), and 8300 dpm of the glucoside with enzyme II (310 μg protein).

Addition		Activity	
		Enzyme I	Enzyme II
	mM	% of control	
UDP	0.1	48	95
	0.5	20	75
	1	7	15
	5	3	3
ADP	1	96	109
ADP-glucose	1	104	114
CDP-glucose	1	102	94
GDP-glucose	1	92	105

Table III. *Reactivity of Steviol Derivatives with Enzyme I*

The reactions were performed with enzyme I (1.3 mg protein) for 1 h in the presence of 40 μM steviol or its derivatives and 13 μM UDP-[^{14}C]glucose.

Acceptor	Activity: [^{14}C]Glucose Transferred
	dpm/h
Steviol	11,400
iso-Steviol	0
19-*O*-Methylsteviol	17,700
13-*O*-Methylsteviol	200

Putative steviol-[^{14}C]glucosides obtained from the enzymatic reactions were analyzed by TLC. Authentic standards (spot numbers 1–5 in Fig. 2) were separated well from each other on the TLC plate. The radioactivity on the plate was scanned and quantified by the TLC-Radioscanner. The product from steviol with UDP-[^{14}C]glucose (lane F) was found to be identical in R_F to authentic steviol-13-*O*-glucopyranoside. This glucoside (lane E) was converted into compound with an identical R_F to that of steviolbioside. The reaction products of stevioside (lane B) and steviolbioside (lane C) showed identical R_Fs with those of rebaudioside A and stevioside, respectively. On the other hand, rubusoside (lane D) could not accept [^{14}C]glucose and rebaudioside A (lane A) appeared to give no distinct reaction product.

Analysis by HPLC of the reaction mixture of steviol-13-*O*-glucopyranoside revealed the presence of radioactive compound (45% of total ^{14}C) with a retention time of 11.6 min, that coeluted with authentic steviolbioside (Fig. 3A). The remaining [^{14}C] was eluted at 2.2 min under the reverse phase conditions, suggesting the presence of [^{14}C]stevioside. About 80% of the radioactivity in the reaction product of steviolbioside appeared as the main peak with the retention time corresponding to stevioside (Fig. 3B). The main product (84% of total ^{14}C) of the reaction with stevioside was identified as rebaudioside A (Fig. 3C).

Under the reaction conditions reported here, we used relatively low concentrations of UDP-glucose (1–2 μM) as compared with those of the glucose acceptor (10–40 μM) to obtain the sole reaction product. In the presence of higher concentrations of UDP-glucose (1 mM) with steviol-13-*O*-glucoside and enzyme II, at least three products corresponding to steviolbioside, stevioside, and rebaudioside A were detected on the TLC plates (data not shown). These results confirm that steviol-13-*O*-glucopyranoside, steviolbioside, and stevioside were glucosylated by the enzyme II from UDP-glucose producing steviolbioside, stevioside, and rebaudioside A, respectively.

DISCUSSION

The chemical structure of stevioside was established in 1955 (14, 20). Although biosynthetic route of steviol skeleton has been reported (1, 17), little information about the biosynthesis

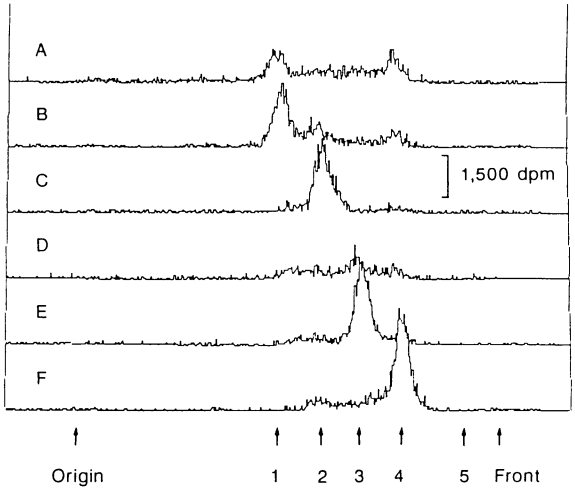


Figure 2. TLC of labeled products synthesized from UDP-[U-^{14}C] glucose by enzyme I or II. The following substrates were used: A, rebaudioside A; B, stevioside; C, steviolbioside; D, rubusoside; E, steviol-13-*O*-glucopyranoside; F, steviol. Steviol was reacted with enzyme I, other glucoside with enzyme II. Numbers indicated by arrows show the position of authentic standards: 1, rebaudioside A; 2, stevioside; 3, steviolbioside; 4, steviol-13-*O*-glucopyranoside; 5, steviol.

of steviol-glucosides from steviol has been reported since that time. This report is concerned with the extraction, separation and characterization of glucosyltransferases involved in the steviol-glucoside biosynthesis in *S. rebaudiana*. Two distinct enzymes are identified based on their separation by DEAE-Toyopearl chromatography and substrate specificity differences. All glucosylation steps from steviol to rebaudioside A were catalyzed by these two enzyme fractions.

Steviol having two sites to accept glucose to form steviol-glucosides is glucosylated first at 13-hydroxyl group, then the glucose bound to this position is glucosylated, leaving still 19-carboxyl group and producing steviolbioside which has been detected as the minor constituent of steviol-glucoside (10) in *S. rebaudiana*. After the formation of bisglucose moiety bound to steviol at 13-position, steviolbioside, the 19-carboxyl group is ready to accept glucose. This finding will be supported further by the results that rubusoside, a steviol-bisglucoside occupied with two glucose molecule at 13 and 19-positions, could not serve as the substrate for the glucosyltransferase (Fig. 2). Thus, the glucosylation to 19-carboxyl group occurs after the formation of bisglucose structure at 13-position of the steviol skeleton. The glucosylation products of steviolbioside and stevioside were found to be stevioside and rebaudioside A, respectively.

ent-Kaurene, the first hydrophobic intermediate in gibberellin biosynthesis is subsequently oxidized by microsomal or chloroplastic mixed function oxidases to yield *ent*-7-α-hydroxy kaurenoic acid (16). Similar mixed function oxidase may act on *ent*-kaurene to yield *ent*-13-hydroxy kaurenoic acid, steviol, which is also a hydrophobic compound. There is no indication on the localization of steviol-glucosides in vacuoles, which is the known organelle to accumulate many plant secondary products (6). If this is true for the accumulation of steviol-glucosides, hydrophobic steviol have to be converted to any hydrophilic derivative. The glucosylation reaction is expected to produce a water soluble reaction product, thus permitting its segregation to the vacuoles (4). The glucosylation to steviol and steviol-glucosides would play a role, physiologically, to produce the water soluble products. However, the reason remains to be solved why stevioside and rebaudioside A have three and four glucose moieties in their molecules.

For the synthesis of rebaudioside A from steviol-13-*O*-glucopyranoside, hydroxyl and carboxyl groups have to be glucosylated. It is very interesting to elucidate whether these different groups are glucosylated by one enzyme or by two individual enzymes. Purified UDP-glucose:flavonoid 3-*O*-glucosyltransferase from *Hippeastrum* petals can act on the hydroxyl groups of flavanones, flavones, flavonoids, and anthocyanidins (6). Partially purified UDP-glucose:IAA glucosyl-

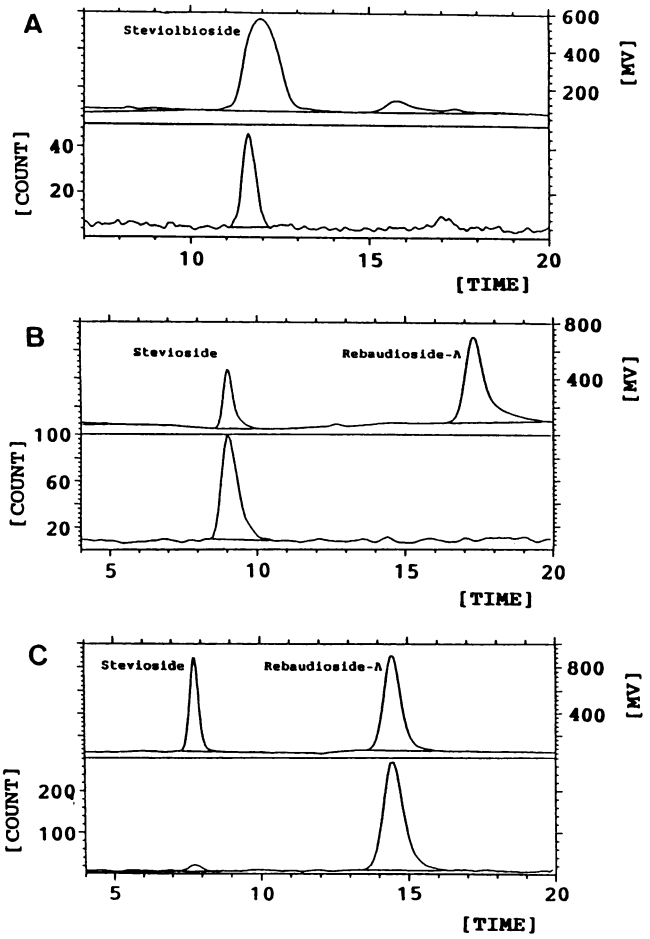


Figure 3. Identification by HPLC of the [^{14}C]glucose labeled reaction products synthesized from UDP-[^{14}C]glucose by enzyme II. The following substrates were used: A, steviol-13-*O*-glucopyranoside; B, steviolbioside; and C, stevioside. The upper parts of the three panels showed the elution profiles of the authentic standards monitored by an absorbance at 210 nm, the lower panels indicated the distribution of [^{14}C] monitored by Radio LC detector.

transferase forming an ester of IAA and glucose is possible to utilize hydroxy acids such as hydroxycinnamic acids and *p*-coumaric acid, which inhibit also IAA-ester formation (12), suggesting that the enzyme can act only on the carboxyl group. However, it remains to be established whether glucosyltransferases forming ether or ester linkage can act on both hydroxyl and carboxyl groups.

LITERATURE CITED

1. **Bennett RD, Lieber ER, Heftmann E** (1967) Biosynthesis of steviol from (−)-kaurene. Phytochemistry **6:** 1107–1110
2. **Darise M, Mizutani K, Kasai R, Tanaka O, Kitahata S, Okada S, Ogawa S, Murakami F, Chen F-H** (1984) Enzymatic transglucosylation of rubusoside and the structure-sweetness relationship of steviol-bisglucosides. Agric Biol Chem **48:** 2483–2488
3. **DuBois GE, Stephenson RA** (1985) Diterpenoid sweeteners. Synthesis and sensory evaluation of stevioside analogues with improved organoleptic properties. J Med Chem **28:** 93–98
4. **Franz G** (1982) Glycosylation of heterosides (Glycosides). *In* FA Loewus, W Tanner, eds, Encyclopedia of Plant Physiology (New Series), Vol 13A, Plant Carbohydrates I. Intracellular Carbohydrates. Springer-Verlag, Berlin, pp 384–393
5. **Fukunaga Y, Miyata T, Nakayasu N, Mizutani K, Kasai R, Tanaka O** (1989) Enzymatic transglucosylation products of stevioside: separation and sweetness-evaluation. Agric Biol Chem **53:** 1603–1607
6. **Hrazdina G** (1988) Purification and properties of a UDPglucose: flavonoid 3-*O*-glucosyltransferase from *Hippeastrum* petals. Biochim Biophys Acta **955:** 301–309
7. **Imanari T, Tamura Z** (1967) Gas chromatography of *O*-glucuronides. Chem Pharm Bull **15:** 1677–1681
8. **Kalinowska M, Wojciechowski ZA** (1988) Substrate specificity of partially purified UDP-glucose:nuatigenin glucosyl-transferase from oat leaves. Plant Sci **55:** 239–245
9. **Kasai R, Kaneda N, Takano O, Yamasaki K, Sakamoto I, Morimoto K, Okada S, Furukawa H** (1981) Sweet diterpene-glycosides of leaves of *Stevia rebaudiana* Bertoni. Synthesis and structure-sweetness relationship of rebaudioside-A, D, E and their related glycosides. Bull Chem Soc Jpn **1981:** 726–735
10. **Kohda H, Kasai R, Yamasaki K, Murakami K, Tanaka O** (1976) New sweet diterpene glucosides from *Stevia rebaudiana*. Phytochemistry **15:** 981–983
11. **Kusama S, Kusakabe I, Nakamura Y, Eda S, Murakami K** (1986) Transglucosylation into stevioside by enzyme system from *Streptomyces* sp. Agric Biol Chem **50:** 2445–2451
12. **Leznicki A, Bandurski RS** (1988) Enzymatic synthesis of indoleacetyl 1-*O*-β-D-glucose. II Metabolic characteristics of the enzyme. Plant Physiol **88:** 1481–1485
13. **Mizutani K, Miyata T, Kasai R, Tanaka O, Ogawa S, Doi S** (1989) Studies on improvement of sweetness of steviol bisglucosides: selective enzymatic transglucosylation of the 13-*O*-glucosyl moiety. Agric Biol Chem **53:** 395–398
14. **Mosetting E, Nes WR** (1955) Stevioside II. The structure of the aglycon. J Org Chem **20:** 884–899
15. **Ogawa T, Nozaki M, Matsui M** (1980) Total synthesis of stevioside. Tetrahedron **36:** 2641–2648
16. **Railton ID, Fellows B, West CA** (1984) *Ent*-kaurene synthesis in chloroplasts from higher plants. Phytochemistry **23:** 1262–1267
17. **Ruddat M, Heftmann E, Lang A** (1965) Biosynthesis of steviol. Arch Biochem Biophys **110:** 496–499
18. **Sembdner G, Gross D, Liebisch H-W, Schneider G** (1980) Biosynthesis and metabolism of plant hormones. *In* J MacMillan, ed, Encyclopedia of Plant Physiology (New Series), Vol 9, Hormonal Regulation of Development I. Molecular Aspects of Plant Hormones. Springer-Verlag, Berlin, pp 299–444
19. **Tamura Z, Imanari T** (1964) Gas chromatography of *O* glucuronides. Chem Pharm Bull **12:** 1386–1388
20. **Wood HB, Allerton R, Diehl HW, Fletcher HG** (1955) Stevioside I. The structure of the glucose moieties. J Org Chem **20:** 875–883