Chemistry of *Stevia rebaudiana* Bertoni

——New Source of Natural Sweeteners——

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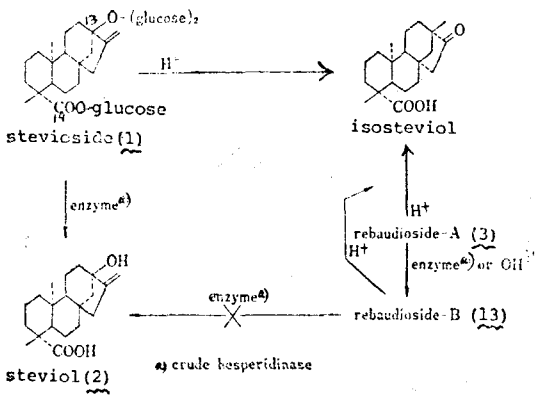
It has been known that sucrose has a profound effect on the developement of fatness, dental caries and other diseases. Since withdrawal of cyclamate and saccharin from the consumer's market in Japan because of their toxicity, researches for new safe and economical sweeteners have been currently of intense scientific and public interests.

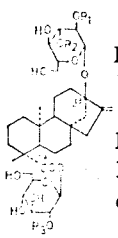
Leaves of *Stevia rebaudiana* (Compositae), a wild herb of Paraguay, South America, has been used as a sweetening agent for tea and coffee by the natives because of the remarkable sweetness.

In 1931, French chemists isolated a major sweet principle of this plant in a crystalline state, designating it stevioside. The chemial structure of stevioside (**1**) was finally established by Mosettig, Fletcher and their co-workers of N.I.H., U.S.A. in 1963[1]. The genuine aglycone of stevioside, named steviol (**2**) is formulated as 13-hydroxy-*ent*-kaurenoic acid and afforded isosteviol, a beyrene-type diterpene ketone during the process of acid hydrolysis.



The genuine aglycone, **2** can be obtained by mean of enzymic hydrolysis, the snake enzyme or crude pectinase. As a part of our studies on the developement of modern procedures for glycoside chemistry, we have searched a glycosidase which has high activity for mild hydrolysis of glycosides with acid unstable aglycones, such as **2**, disclosing that crude hesperidinase is able to hydrolyze **1** completely to give **2** in almost quantitative yield[2]. This crude enzyme preparation is produced by *Aspergillus niger* and used for hydrolysis of hesperidin (flavanol-rhamnoglucoside) in the food industry, production of canned unshumikan (*Citrus unshu Marc.*) in Japan[3].

It has been mentioned that crude extract of leaves of *S. rebaudiana* tastes much sweeter and more pleasant than purified stevioside (**1**). We conducted thin layer chromatography of the crude extract, demonstrating the presence of a number of glycosides other than stevioside (**1**). We isolated these glycosides by column chromatography, designating them rebaudiosides-A



	R_1	R_2	R_3	
Stvioside (**1**)	gluc	H	H	(1963)
Rebaudioside-A (**3**)	gluc	gluc	H	(1975)
Rebaudioside-C (**4**) (=dulcoside-B)	rham	gluc	H	(1977)
Rebaudioside-D (**5**)	gluc	gluc	gluc	(1977)
Rebaudioside-E (**6**)	gluc	H	gluc	(1977)
dulcoside-A (**7**)	rham	H	H	(1977)

gluc: β-D-glucopyranosyl
rham: α-L-rhamnopyranosyl

(3)[4], -C(4),[5], -D(5), and -E(6)[6]. Mitsuhashi et al. also investigated glycosides of this plant, isolating two sweet glycosides, dulcosides A(7) and B[7], later of which is identical with our rebaudioside-C(4).

Besides the study on glycosidases, we have further investigated the modern procedures for structure determination of plant glycosides; ^{13}C NMR and field desorption mass spectroscopy (F.D. Mass).

Mass spectrometry is known to be a highly useful technique for identification and structure determination of natural products. However, for eletcron impact(E.I) or chemical ionization (C.I.) mass spectrometry, samples must be volatile and lessvolatile compounds such as plant oligoglycosides must be converted into a volatile derivative, i.e., trimethylsilyl ether, acetate, or methyl ether. Further, it is also extremely difficult to observe a molecular ion of the derivatives of plant oligoglycosides by E.I. or C.I. mass.

Recently, Schulten, University of Bonn, Kawasaki and Komori, Kyushu University and I reported the application of field desorption (F.D.) mass spectrometry to the chemistry of steroidal and triterpenoid oligoglycosides including ginseng-saponins, finding their molecular molecular ion as a cluster ion (M^++Na or M^+ +K) without any derivatization[8] In continuing this study, Sakamoto, Morimoto, and I, in cooperationing with Schulten, have studied F. D. mass of stevia sweet glycosides. The spectra are shown in Fig. 1-5. It was demonstrated that the spectra are quite simple in contrast to E.I. mass spectra and exhibited strong M^++Na and characteristic fragmentations due to the cleavages of sugar units, being promissing for identification and structure study of the glycosides of this type.[9]

^{13}C NMR spectroscopy is now the most essential tool in natural product chemistry. Our research group elaborated synthese of a number of epimeric pairs of glucosides[10], mannosides[11], rhamnosides[11], and arabinosides[12] and studied the glycosylation shifts, displacements of carbon signals of both sugar and aglycone moieties on glycoside formation, elucidating the novel regualarities between the glycosylation shifts and stereo-structures of sugar and aglycone units.

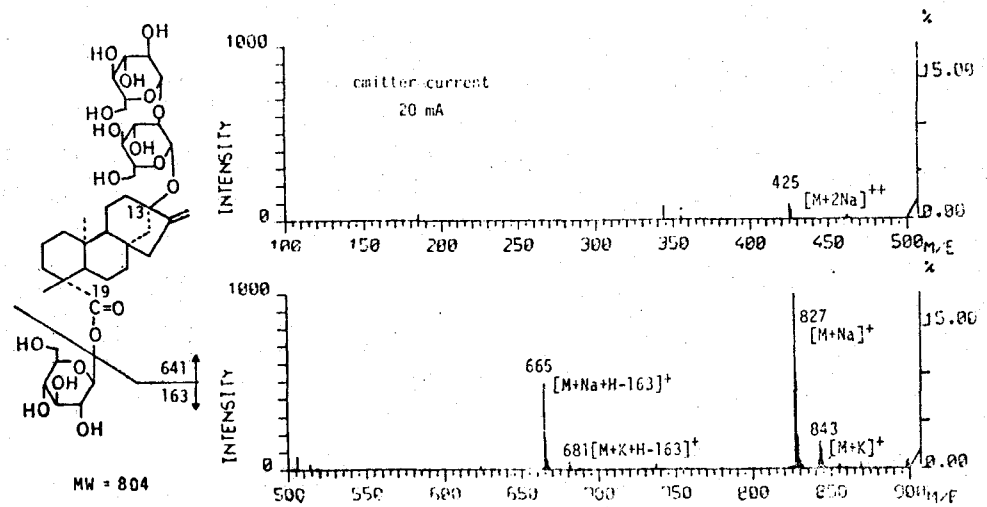


Fig. 1. FD Mass spectrum of stevioside (1)

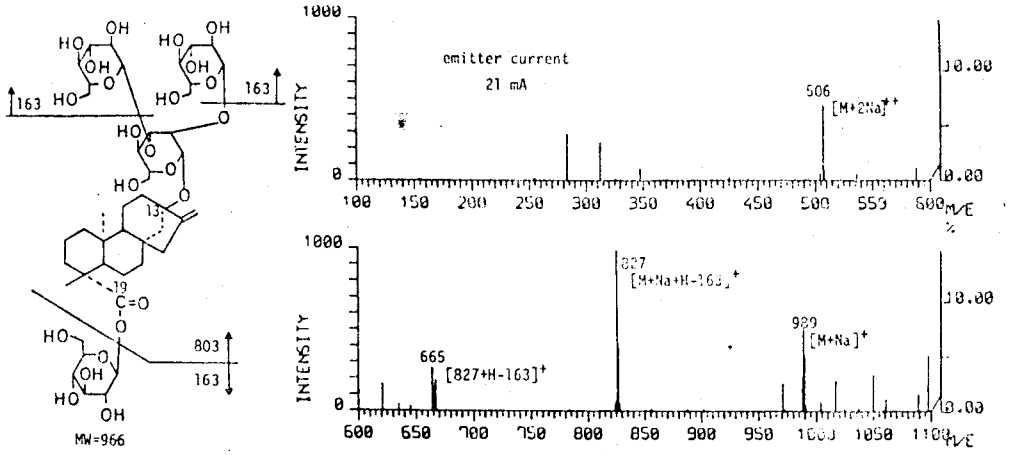


Fig. 2. FD-Mass spectrum of rebaudioside A (3)

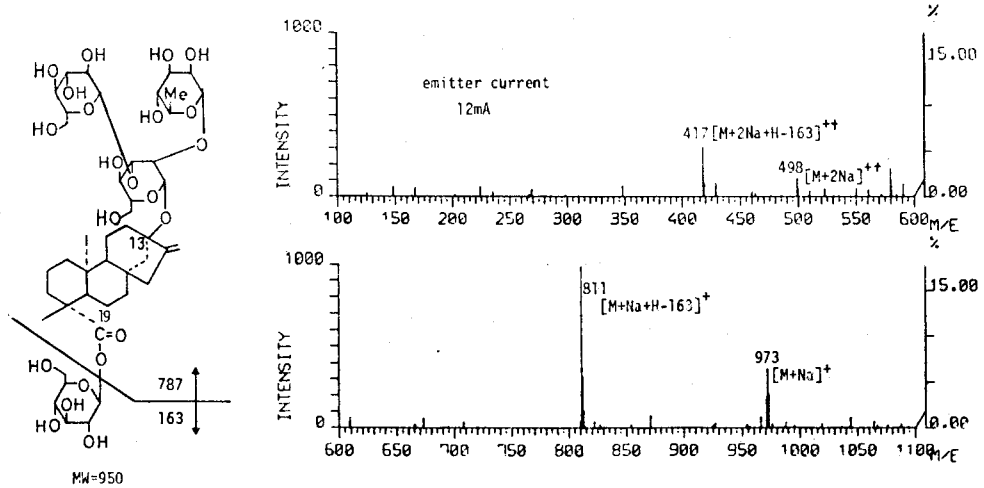


Fig. 3. FD Mass spectrum of rebaudioside C (4)

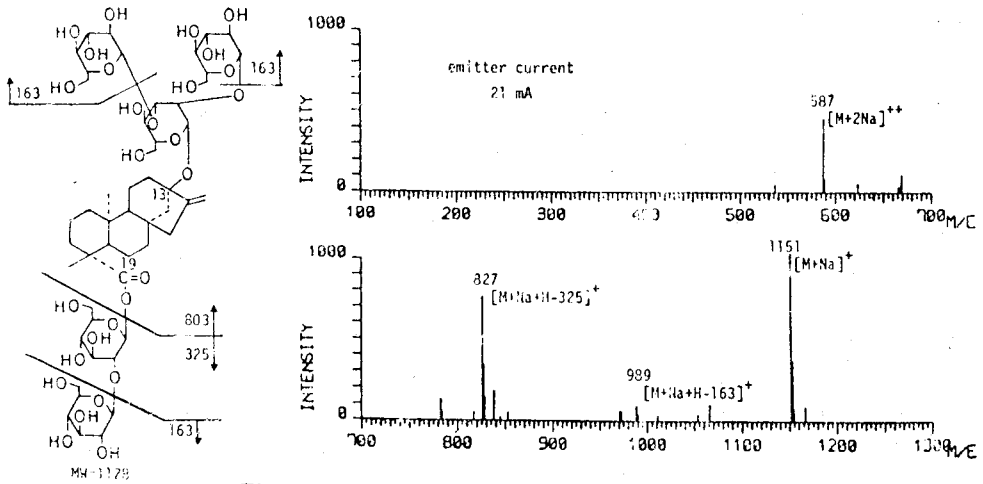


Fig. 4. FD Mass spectrum of rebaudioside D (5)

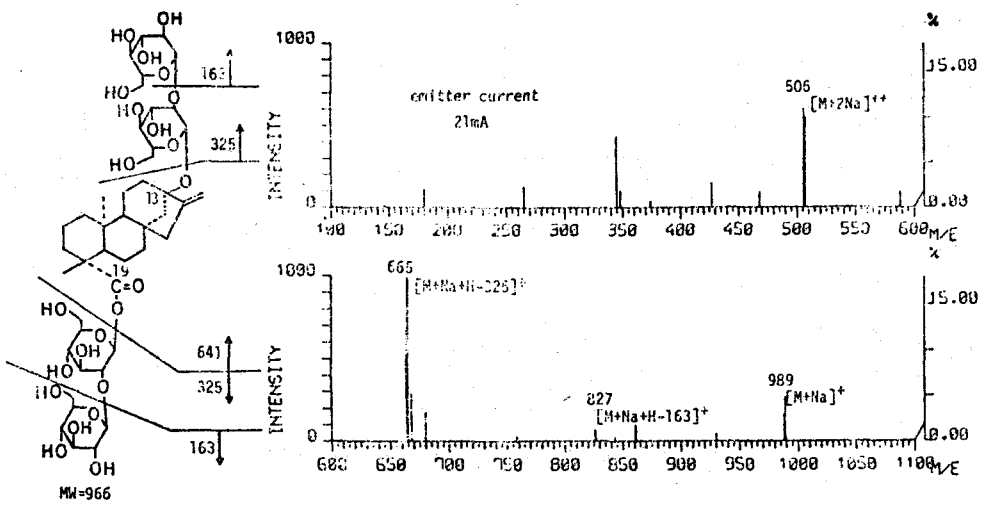


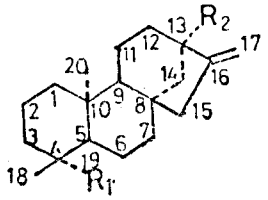
Fig. 5. FD Mass spectrum of rebaudioside-E (**6**)

By means of ^{13}C NMR, we can now determined structure of a genuine aglycone, location of glycoside-linkage in an aglycone and sequence of sugar units. It is also easy to determine an anomeric configuration of glycoside-linkage of rhamnosides and mannosides which is almost impossible to determine by other procedures such as ^{1}H NMR.[11]

We also established the specific glycosylation shifts for ester glycosides as the full-assignments of the carbon signals of kaurene-type diterpenes[13]. By means of these modern procedures, the structures of the new sweet glycosides, **3**-**6** were determined as shown in Chart 2 with minimal consumption of the materials. Table I illustrates ^{13}C NMR assignments and glycosylation shifts of *Stevia* sweet glucosides and their related compounds, *ent*-kaurene (**8**), *ent*-kaurenosic acid (**9**), its methyl ester (**10**) and chemically or enzymicaly derived glucosides, **11**, **12**, and **13**. ^{13}C NMR assignments of **4**-**6** were reported in the papers of their structure determination[5,6].

With regard to the content in the leaves, stevioside(**1**) is a major glucoside (5∼15% in the dried leaves) and rebaudioside-A(**3**) is another major glucoside (3∼6% in the dried leaves). Other glycosides, **4**-**7** are minor components. However, in view of the sweetness, **3**, **5**, and **6** are sweeter and much more delicious than **1** and this is the reason why the crude extract of the leaves tastes more pleasant than purified **1** (*vide suprs*). Now, more than ten food-industries in Japan are undertaking the production of *Stevia*-glucosides as a food additive and the cultivation of this plant is widely carried out not only in Japan but also the countries of South East Asia. On the basis of the above reason, all of the food industries are attempting to produce the fraction with higher content of **3** than **1** and therefore, a plant containing much more **3** in the leaves must be attractive from the view point of food additive production. In this respect, we attempted the the conversion of the major glucoside, **1** into **3**, the better natural sweetener.

Stevicside (**1**) has eleven hydroxyl groups in its molecule and it must be extremely difficult to introduce one glucosyl unit selectively into one hydroxyl group to prepare **3**. We found that Takadiastase, the digestive enzyme mixture produced by Sankyo Co. Ltd., selec-

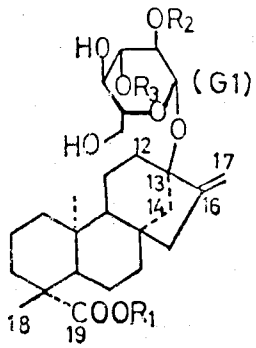


R_1	8 CH_3	9 COOH	10 COOMe	2 COOH
R_2	H	H	H	OH
C-1	40.5 (40.5)	41.1	40.8 (40.9)	41.1
2	18.9[b](18.7)[b]	19.8	19.5 (19.2)	19.8
3	42.2 (42.1)	38.6	38.2 (38.1)	38.6
4	33.3 (33.3)	43.8	43.9 (43.9)	43.9
5	56.2[c](56.3)[c]	57.1	56.9 (57.2)	57.1
6	20.4 (20.3)	22.5	22.2 (21.9)	22.6
7	41.3 (41.3)	41.5	41.4 (41.4)	41.8
8	44.3 (44.3)	44.4	44.4 (44.3)	41.8
9	56.1[c](56.1)[c]	55.2	55.1 (55.2)	54.3
10	39.5 (39.4)	39.9	39.6 (39.5)	39.8
11	18.3[b](18.2)[b]	18.6	18.6 (18.4)	20.8
12	33.5 (33.3)	33.3	33.3 (33.2)	40.7
13	44.3 (44.0)	44.2	44.2 (43.9)	79.8
14	40.0 (39.9)	39.9	39.8 (39.7)	47.4
15	49.4 (49.2)	49.2	49.1 (49.0)	48.1
16	155.8(156.0)	155.7	155.7(155.8)	157.6
17	103.4(102.8)	103.5	103.6(103.1)	102.9
18	33.7 (33.7)	29.3	28.6 (28.8)	29.3
19	21.7 (21.7)	179.9	177.5(178.0)	180.0
20	17.7 (17.6)	16.0	15.5 (15.4)	15.9
		$COOCH_3$	51.1 (51.1)	

δ ppm from internal TMS in C_5D_5N ($CDCl_3$) at 25.15 MHz at 25°.

b, c : Values in any vertical column may be reversed although those given here are prefered.

Table I ^{13}C Chemical Shifts



	R_1	R_2	R_3
11	H	H	H
12	H	Glc (G2)	H
1	Glc (G*)	Glc (G2)	H
13	H	Glc (G2)	Glc (G3)
3	Glc (G*)	Glc (G2)	Glc (G3)

Glc: β-glucopyranosyl

	11	12	1	13	3
C-1	41.0	40.9	40.7	41.0	40.9
2	19.7	19.7	19.2	19.8	19.4
3	38.4	38.5	38.1	38.7	38.3
4	43.8	43.8	43.9	43.8	44.0
5	56.9	56.9	57.3	57.0	57.4
6	22.5	22.4	22.0	22.5	22.2
7	41.6	41.5	41.5	41.8	41.8
8	42.1	42.4	42.5	42.2	42.4
9	54.1	54.1	53.8	54.1	54.0
10	39.7	39.6	39.7	39.8	39.8
11	20.6	20.5	20.6	20.6	20.7
12	38.4	37.2	36.6	37.8	37.3
13	86.4	86.1	85.9	86.8	86.6
14	44.6	44.7	44.3	44.5	44.5
15	48.2	47.9	47.5	47.9	47.9
16	153.7	154.0	154.3	153.7	153.9
17	104.9	104.9	104.5	104.6	104.5
18	29.2	29.2	28.2	29.3	28.3
19	180.0	180.1	177.0	180.2	177.0
20	15.7	15.9	15.4	16.1	15.5
G*-1			95.6		95.6
2			73.8		73.8
3			79.0		78.6
4			70.8		70.8
5			79.0		78.8
6			61.9		62.3
G1-1	99.4	97.6	97.7	97.8	97.9
2	75.3	84.1	84.3	80.5	80.7
3	[77.9]	[77.6]	77.9	88.0	87.8
4	71.5	71.1	[71.3]	69.8	70.5
5	[78.5]	[77.8]	77.9	[78.4]	[78.3]
6	62.5	62.3	62.5	[62.3]	[62.3]
G2-1		106.2	105.5	[104.4]	[104.5]
G3-1				[104.6]	104.5

tively hydrolyzes **1** to give a bioside (**14**) in an almost quantitative yield. This is the key step of the synthesis. The resulting bioside (**14**) was saponified with alkali to afford monoside (**11**) in a yield of 95%. In order to protect its two hydroxly groups, 4' and 6', this monoside (**11**) was converted into a benzylidene derivative (**15**) which was glucosylated by means of the orthoester procedure[14] followed by removal of the protecting group to furnish the preparation of **3** in a fairly high yield (nearly 70% from **1**)[15].

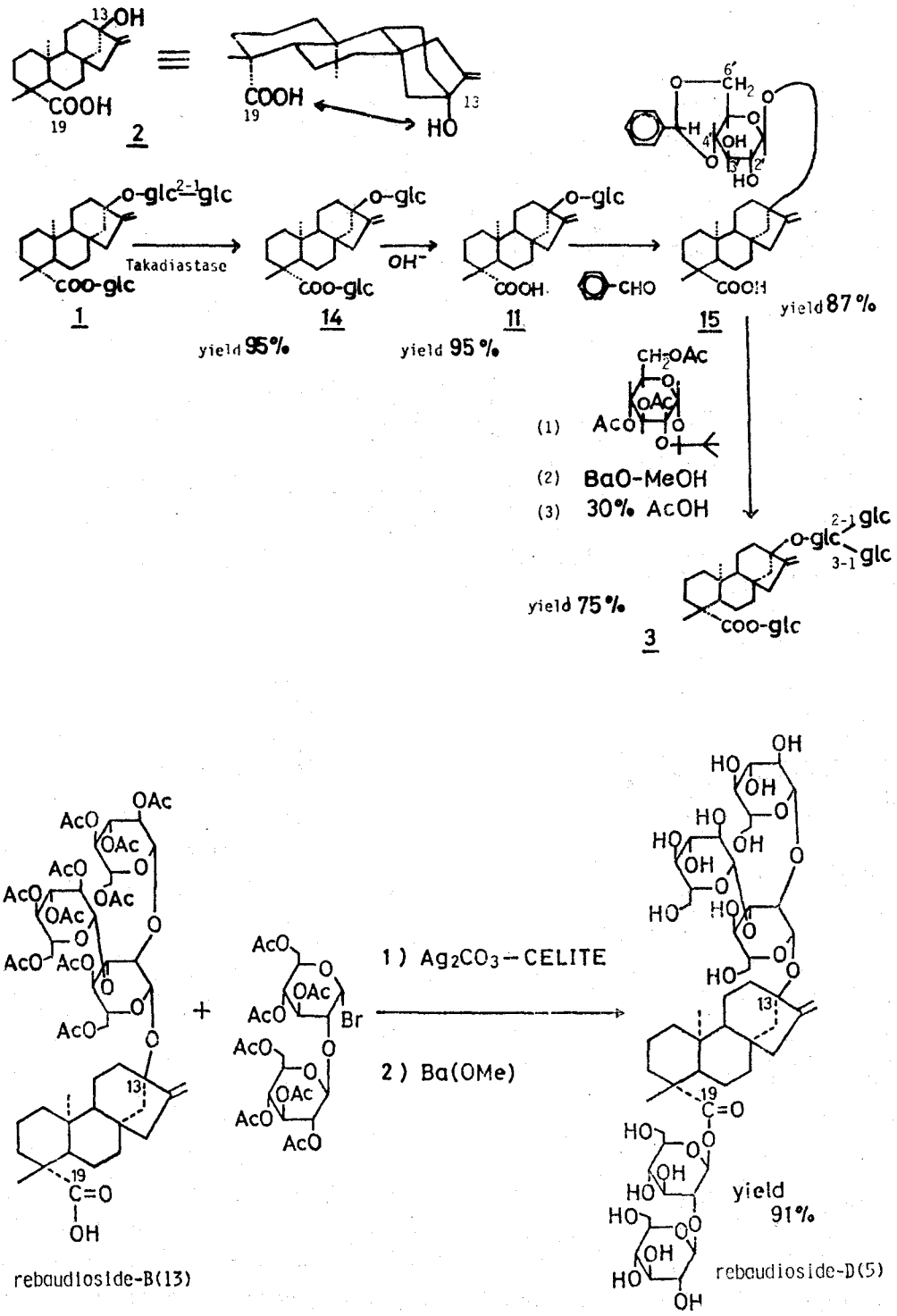


Fig. 6. Synthesis of rebaudioside A,D.

In connection with this synthethic study, we investigated the preparation of glucosyl ester of *ent*-kaurenoic acid (**9**, Table I) disclosing the following stereochemical notice. Silver salt of **9** can be readily glucosylated by the usual way, α-acetobromoglucose method and the acetyl groups of sugar moiety of the resulting β-glucosyl ester were removed by treatment with BaO in methanol at room temperature without any saponifcation of the ester glucosyl linkage. On the other hand, this common procedure for glucosyl ester preparation could not be applied to the preparation of the glucosyl ester of **2**. This can be explainable in term of the stereochemical interaction between the 19-carboxyl group and 13-hydroxyl group of **2**. This stereostructure is significant also for the consideration of structure-sweetness relationship of the glycoside of this type (*vide infra*). The glucosylation of the 19-carboxylic acid of *ent*-kaurenoic acid derivative having a function at its 13-position was achieved by the orthoester procedure[14] or with acetobromosugar in the presence of silver carbonate on Celite[16] By means of the later procedure, rebaudiosides-D (**5**) and -E(**6**) were also prepared from **3** through rebaudioside-B (**13**)[6] which is an artifact formed from **3** by alkaline hydrolysis or by enzymic partial hydrolysis with crude hesperidinase[2].

In further continuing of chemistry of *Stevia* spp., we isolated several new *ent*-kaurene glucosides named paniculosides-I-V (**16-20**) from leaves of *S. paniculata* and *S. ovata* both of which also grow in South America.[17] It should be noted that the leaves of these plants as well as the isolated glucosides (**16-20**) don't taste sweet. Glucosyl ester (**21**) of ent-kaurenoic acid (**9**) itself is of cource not sweet. These evidences indicates that *ent*-kaurenoic acid glucosyl ester homologs without any function at C-13 exhibit no sweetness.

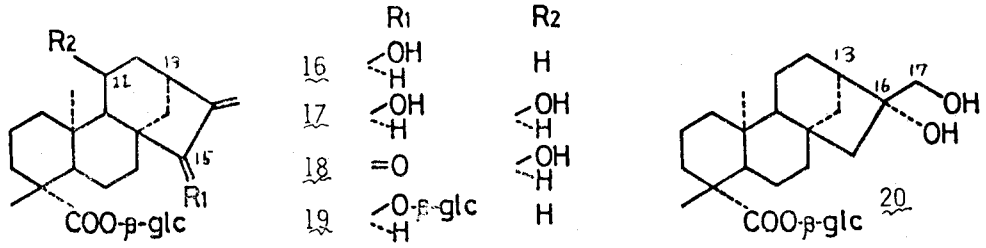
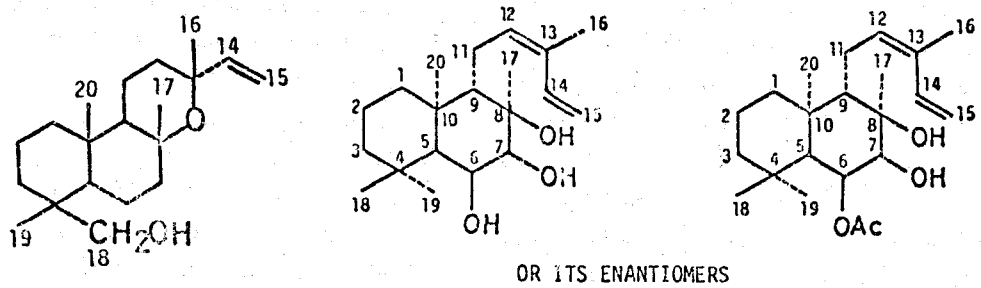


Fig. 7. Diterpene-glucosides of *Stevia paniculata* and *S. ovata* (leaves)

We have synthesized a number of steviol glycosides by chemical or enzymic procedures and conducted also some structure medification of **1**, investigating the structure-sweetness relationship of the glycoside of this type in term of the relative sweetness to sucrose. The relative sweetness of glucosides which have one or two glucose unit only at one of the functions of **2** (19-carboxyl or 13-hydroxyl group) is found to be less than 50-fold. Glucosides with one glucose unit on each function (glucoside (**14**)) or with glucotriosyl unit on 13-hydroxyl group show the relative sweetness between 50~100 fold. The relative sweetness of **1**, **6**, and some other glucosides having more than three glucose units are between 100 and 150 fold. It is notable that glucosides with the branched sugar unit i.e., **3** and **5** are about 150~200 fold sweeter than sucrose, while replacement of a glucose unit by a rhamnose unit decreases of

the sweetness (**4** and **7** are less sweet than **3** and **1**, respectively). Hydrogenation or hydroxylation of the 16(17)-double bond remarkable decreases sweetness.

with regard to constituents other than sweet glycosides, Fujita et al. analyzed essential oil of the leaves by gas-Mass procedure, identifing some mono-and sesqui-terpenes[18]. We isolated, from the ether soluble fraction of the leaves, three diterpenes, **22**, **23**, and **24**, along with fatty acid esters of sterols and lupeol[19]. Of these diterepenes, **22** was identified with jhanol which was previously isolated from *Eupatorium jhani* (Compositae)[20]. The second diterpene, **23** was identical with austroinulin, previously isolated from *Austroeupatorium inulaefolium* (Compositae)[21]. The last diterpene **24** is a new compound and its structure was established to be 6-O-acetyl-austroinulin by means of ^{13}C and ^{1}H NMR spectroscopy.



Jhanol (22)
Previously isolated by Gonzalez et. al (1977) from *Eupatorium jhanii*

Austroinulin (23)
Previously isolated by Bohlmann et. al (1977) from *Austroeupatorium inulaefolium*

6-OAc-austroinulin (24)
(New compound)

Structure of Labdane Type Diterpenes isolated from *Stevia rebaudiana*

Table 11. Result of mutagenesis screening tests of various fractions and compounds isolated from *Stevia rebaudiana* (Compositae) with *Salmonella typhimurium* TA 100 & TA 98 tester strains

Samples	Maximum dose (mg/plate)	Response**
Stevioside (1)	10	neg.
Rebaudioside-A (3)	10	neg.
Jhanol (22)	1	neg.
Austroinulin (23)	10	neg.
6-OAc-austroinulin (24)	1	neg.
MeOH extract	10	neg.
Ether fraction	5	neg.
n-BuOH fraction	10	neg.

** all tests were performed with the tester strains in the presence and absence of S-9 mix.

No significant acute toxicity has been observed for *Stevia* sweet glycosides, though the long term toxicity test has not been conducted as yet. We examined the mutagenesis test against *Salmonella typhimurium* strain TA-98 and 100 for the constituents of Stevia leaves mentioned above, observing no significant activities as shown in Table II.

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