Noncariogenic Intense Natural Sweeteners

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Abstract: There is a definite relationship between the dietary consumption of sucrose and the incidence of dental caries. Noncaloric sucrose substitutes for use in the sweetening of foods, beverages, and medicines may be either synthetic compounds or natural products. In the United States, four potently sweet artificial sweeteners are approved, namely, saccharin, aspartame, acesulfame potassium, and sucralose. Highly sweet plant constituents are used in Japan and some other countries, including the diterpene glycoside stevioside and the protein thaumatin. Recent progress in a research project oriented towards the discovery and evaluation of novel potentially noncariogenic sweeteners from plants has focused on substances in the sesquiterpenoid, diterpenoid, triterpenoid, steroidal saponin, and proanthocyanidin structural classes. The feasibility of using Mongolian gerbil electrophysiological and behavioral assays to monitor the sweetness of plant extracts, chromatographic fractions, and pure isolates has been investigated. An *in vivo* cariogenicity study on the commercially available natural sweeteners stevioside and rebaudioside A has been carried out.
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Key words: plant constituents; sucrose substitutes; isoprenoids; phenols; biological evaluation

1. INTRODUCTION

Food additives constitute a $4.2 billion industry each year in the United States, and food substitutes inclusive of the artificial sweeteners account for about $1.5 billion of this total.[1] With the approval of sucralose by the U.S. Food and Drug Administration in April 1998, there are now four synthetic "high-intensity" sweeteners on the market in this country, with the others being saccharin, aspartame, and acesulfame potassium.[1–3] At present, aspartame has the largest share of the sucrose substitute market in the United States, for which the major use is in soft drinks. This substance was recently given permission for sale as a "general-purpose sweetener," enabling it to be used in all types of

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foods and beverages.[1] However, since the initial approval of aspartame as a sweetener and flavor enhancer for the U.S. market in 1974, there has been a considerable debate concerning its perceived safety, although this dipeptide is a very thoroughly tested food additive.[4] Saccharin has been used as an artificial sweetener in the United States longer than any other compound, but was listed as a carcinogen in the 1970s, so the many products on the market containing this compound must carry a warning label.[3,5] Acesulfame potassium (Ace-K) was approved initially in the U.S. in 1988 for use in dry food products, and, while its range of applications has steadily increased, it is not yet available for use in all food items or diet soft drinks.[1,3] On the other hand, sucralose, a trichlorinated sucrose derivative, has been approved as a nonnutritive sweetener in as many as 15 food categories. This compound is not metabolized by the body, but may be hydrolyzed to two substituted monosaccharides when kept at elevated temperatures in highly acidic foods for long periods.[2] Two other potently sweet synthetic sucrose substances used in countries other than the United States are alitame and cyclamate.[1,3] All of these compounds are noncaloric, and, since they possess a sweetness intensity that is some 50 times or more than that of sucrose, they are categorized as "intense" sweeteners. A number of less highly sweet caloric or "bulk" sucrose substitutes such as crystalline fructose, high-fructose corn syrup, isomalt, lactitol, mannitol, sorbitol, and xylitol are also used as permitted sweeteners in several countries.[3]

While perhaps they are better known as the sources of sweet-tasting monosaccharides, disaccharides, and polyols (i.e., "bulk" sweeteners),[6,7] plants have afforded also about 75 highly sweet compounds to date.[8] These sweet compounds fall mainly within the terpenoid, flavonoid, and protein compound classes, although altogether nine distinct structural groups of potently sweet molecules from plants were included in a recent survey.[8] So far, highly sweet compounds have not been documented as occurring in lower plants, insects, or marine organisms, and the taxonomic distribution of plants found to biosynthesize highly sweet compounds is random within the angiosperm superorders as classified according to Dahlgren.[9]

Several highly sweet plant constituents are used commercially as sucrose substitutes in one or more countries, with the plant secondary metabolites of most widespread interest in this regard being the steviol glycosides, stevioside and rebaudioside A, which are constituents of the South American plant, *Stevia rebaudiana* (Bertoni) Bertoni.[10] Products made from these two *S. rebaudiana* diterpenoid glycosides are widely available in Japan, with stevioside having been approved also as a sweetener in Brazil, and having limited use in Korea.[10] It is possible to improve on the somewhat unpleasant hedonic characteristics of stevioside by the production of "sugar-transformed steviol," in which a bacterial enzyme is used to alter this natural product structurally, by transglycosylation of its saccharide moieties, to afford a pleasant-tasting mixture of sweet substances.[11] Other plant-derived sweeteners used in Japan are the oleanane-type triterpenoid glycoside glycyrrhizin (from the roots of *Glycyrrhiza glabra* L. and other *Glycyrrhiza* species) and the protein thaumatin [a mixture of sweet substances from the fruits of *Thaumatococcus daniellii* (Bennett) Benth.].[12] Ammoniated glycyrrhizin has GRAS (Generally Regarded as Safe) status in the United States, and is used as a flavorant, flavor modifier, and foaming agent. Thaumatin also has GRAS status in the U.S., where it is used as a flavor enhancer, and it is approved as a sweetening agent in other countries, including Australia, Switzerland, and the United Kingdom.[12] Two other potently sweet compounds have minor use in Japan, namely, mogroside A (a cucurbitane-type triterpene glycoside) and phyllodulcin (a dihydroisocoumarin).[12] Two semisynthetic derivatives of plant natural products have use for sweetening purposes; perillartine, based on the monoterpene perillaldehyde (Japan), and neohesperidin dihydrochalcone (Argentina and Belgium), based on the flavanone, neohesperidin.[8,12]

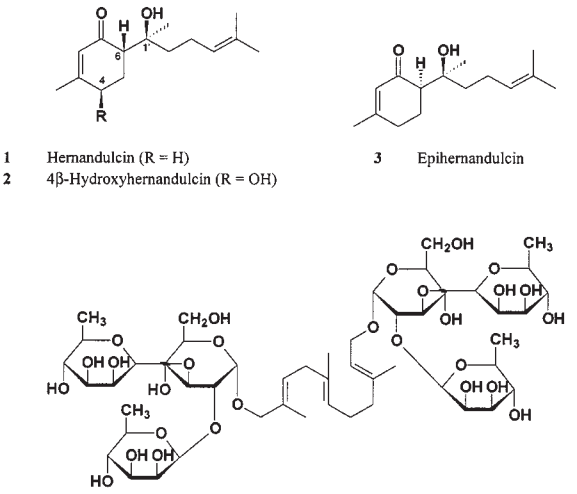
Given the excellent track record of the utilization of plant constituents as "intense" sweetening agents, and because of the great public demand for natural food ingredients, particularly for diabetic and dietetic applications, our group at the College of Pharmacy, University of Illinois at Chicago has worked on the discovery of novel potential noncariogenic sucrose substitutes since the early 1980s. Our collaborative studies on natural sweeteners have included aspects of compound isolation, identification, structure elucidation, synthesis, chromatographic assay development, stability and

solubility testing, *in vitro* metabolism, and the determination of structure–activity relationships, as well as botanical field and literature studies, mutagenicity and acute toxicity testing, human sensory evaluation, the development of electrophysiological and behavioral methods for natural sweetener detection using Mongolian gerbils, and a cariogenicity study on stevioside and rebaudioside A. A wide structural variety of highly sweet substances has been worked on by our team, inclusive of bisabolane and linear sesquiterpenoids; *ent*-kaurene and labdane diterpene glycosides; cucurbitane-, cycloartane-, oleanane-, and secodammarane-type triterpene glycosides; steroidal saponins; phenylpropanoids; a dihydroisocoumarin; dihydroflavonols; and a proanthocyanidin. Several reviews on our work on natural product sweetening agents have appeared in the literature.[13–18] In the following paragraphs, progress made in the last few years in our project on highly sweet compounds of natural origin will be summarized.

2. STRUCTURAL CHARACTERIZATION OF NOVEL PLANT-DERIVED SWEETENERS

A. Sesquiterpenoids and Diterpenoids

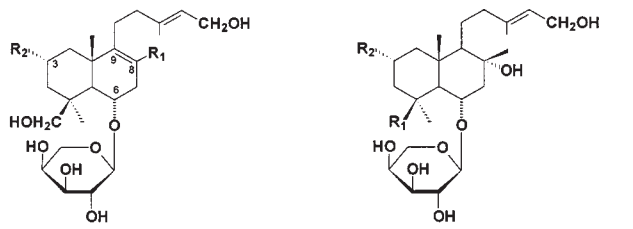
The novel bisabolane sesquiterpenoid, (+)-hernandulcin [**(1),** Fig. 1] was discovered by our group as a minor constituent (0.04% w/w) of the aerial parts of *Lippia dulcis* Trev. (Verbenaceae), a New



1 Hernandulcin (R = H)
2 4β-Hydroxyhernandulcin (R = OH)

3 Epihernandulcin

4 Mukurozioside IIb



		R_1	R_2
5	Gaudichaudioside A	CHO	H
6	Gaudichaudioside B	CH_2OH	H
7	Gaudichaudioside C	CH_2OH	OH

		R_1	R_2
8	Gaudichaudioside D	CH_2OH	H
9	Gaudichaudioside E	CH_3	OH

Figure 1. Sesquiterpenoid and diterpenoid sweeteners and their derivatives.

World species documented as being sweet in the 16th century by the Spanish physician, Francisco Hernández. Natural (+)-hernandulcin was rated about 1000 times sweeter than sucrose on a molar basis, although it was found to possess some bitterness and distinct off- and aftertastes.[19−21] It was established by Mori and Kato by synthesis from (*R*)-(+)-limonene that the absolute configuration of naturally occurring (+)-hernandulcin is the (6*S*,1′*S*)-form.[22] Analog development undertaken by our group led to the conclusion that the tertiary hydroxyl group at C-1′, the keto group at C-1, and the $\Delta^{4',5'}$ olefin in the lipophilic side chain are all essential for the exhibition of a sweet taste by (+)-hernandulcin. The C-1′ OH and the C-1 C=O correspond to Shallenberger's AH,B unit, and the $\Delta^{4',5'}$ double bond to Kier's hydrophobic moiety, two structural units which seem to be necessary for an organic compound to exhibit a sweet taste.[9,23] In our more recent work, a recollection of the leaves and flowers of *L. dulcis* from Panama afforded (+)-hernandulcin in quite high yield (0.15% w/w dry weight), thereby suggesting that **1** occurs at high levels when the plant is flowering.[24] Based in part on our previous identification of camphor in high yield in the original sample of *L. dulcis* we investigated from Mexico,[25] a Puerto Rican group has recently concluded that there are two chemotypes of this species, a hernandulcin **(1)** type and a camphor type.[26]

A second novel sweet substance, (+)-4β-hydroxyhernandulcin [**(2),** Fig. 1], was isolated as a trace constituent from our Panamanian source of *L. dulcis,* and epihernandulcin [**(3),** Fig. 1], the non-sweet 6*R*,1′*S* stereoisomer of **1,** was obtained as a new natural product.[24] 4β-Hydroxyhernandulcin exhibited a similar chromogenic response to hernandulcin when visualized with a sulfuric acid spray reagent on thin-layer chromatography, and the stereochemistry of its C-4 hydroxyl group was determined as a result of a comparison of observed coupling constants between H-4 and H-5, and between H-5 and H-6, with those values predicted by molecular modeling.[24] Although obtained in too low a quantity for either a safety or a sensory evaluation to be performed, 4β-hydroxyhernandulcin is noteworthy since it is only the second sweet compound of the bisabolane type to have been isolated to date. In addition, it may be seen that a C-4 methylene group is not essential for the mediation of a sweet taste in the hernandulcin compound class. Furthermore, the 4β-OH group provides a potential point of attachment for sugars or other polar moieties, in order to synthesize more water-soluble hernandulcin analogs.[24]

The parent compound, hernandulcin [mainly in its (±)-form] has served as a target compound for synthetic organic chemists, and has been synthesized from (*R*)-(+)-limonene,[22,27] from a cyclohexadiene derivative using boron and silicon enolates,[28] by an intramolecular nitrile cycloaddition route from (2*Z*,6*E*)-farnesal oxime,[29] and from an *E*-dienyl carbonate by titanium chloride catalysis.[30] Natural (+)-hernandulcin has been produced from both hairy root and shoot cultures of *Lippia dulcis,* with a yield of as high as 2.9% w/w being produced in the latter case.[31,32] Recently, a Japanese patent has appeared describing the use of hernandulcin in a dentifrice formulation flavored with menthol and stabilized by cyclic ketones.[33]

The fruits of *Sapindus rarak* DC. (Sapindaceae) were collected during a field trip in western Java because of the pronounced sweet taste of the fruit pulp. We performed initially an analysis of the saccharide constituents of *S. rarak,* and concluded that the relatively low levels of sugars and polyols (2.91% w/w) could not account for the sweet taste of the plant.[34] Following initial safety tests (mouse acute toxicity, bacterial mutagenicity) on an initial MeOH-H_2O extract, sweetness was traced to the previously known linear (acyclic) sesquiterpene glycoside, mukurozioside IIb [**(4),** Fig. 1], which occurred in a very high yield (6.3% w/w) in *S. rarak* fruits.[34,35] This compound was found to be innocuous in mouse acute toxicity and bacterial mutagenicity safety tests, and its sweetness potency was about the same as that of sucrose by a human taste panel. In spite of this low sweetness potency, mukurozioside IIb does represent a new type of sweet natural product.[34]

From the aboveground portions of the Paraguayan medicinal plant, *Baccharis gaudichaudiana* DC. (Asteraceae) (a plant recognized as being sweet as a result of botanical field work), a novel prototype natural product sweetener was isolated, namely, the labdane diterpene glycoside, gaudichaudioside A [15,19-dihydroxylabda-8(9), 13(14)*E*-dien-17-al-6α-*O*-α-L-arabinopyranoside] [**(5),** Fig.

1].[36] The structure of this isolate was determined using a combination of one- and two-dimensional NMR methods (COSY, HETCOR, NOESY, COLOC, selective INEPT). At the concentrations tested, this pleasant-tasting water-soluble compound was neither a bacterial mutagen nor acutely toxic for mice, and was rated as about 55 times sweeter than sucrose.[36] Gaudichaudiosides B-E [**(6–9),** Fig. 1], four closely related compounds based on the same skeleton as gaudichaudioside A, were also isolated from *B. gaudichaudiana,* and found to possess transient sweet-leading-to-bitter (sweet–bitter), neutral, entirely bitter, and sweet–bitter tastes, respectively. Hence *B. gaudichaudiana* accumulates a group of closely related labdane diterpenoid glycosides with a wide disparity of taste characteristics.[36] A trihomolabdane diterpenoid obtained from this same plant source was highly bitter,[37] while a number of other *B. gaudichaudiana* diterpenoid constituents were devoid of any taste-modifying properties.[38]

Baccharis gaudichaudiana is one of about 15 species within the section *Caulopterae* (*Alatae*) of the overall genus *Baccharis,* which has a total of 450 to 500 species. Since *B. gaudichaudiana* was once treated as a varietal form of another species in the same section of the genus, namely, *Baccharis articulata* (Lam.) Pers., we examined the latter plant for the presence of gaudichaudiosides A-E. Clerodane rather than labdane diterpenes were isolated and characterized in *B. articulata,* which supports the practice of treating *B. gaudichaudiana* and *B. articulata* as distinct species.[39] The sweet-tasting labdane glycoside gaudichaudioside A was also absent from the constituents of *B. genistelloides* Pers., a further member of the section *Caulopterae* of the genus *Baccharis.* A number of neoclerodane diterpenoids rather than labdane diterpenoids were characterized from the specimen of *B. genistelloides* that we examined.[40]

B. Triterpenoids

The seeds of *Abrus precatorius* L. (Fabaceae) are well known to be toxic, owing to the presence of abrin, a ribosome-inactivating protein.[41] However, the leaves do not appear to contain this type of toxin, and have a long history of human consumption without any reports of harmful effect. For example, *A. precatorius* leaves are used to sweeten betel quid (a stimulant and masticatory) and are sold in marketplaces in Java (Indonesia).[42] Although earlier workers correlated the sweet taste of *A. precatorius* leaves to the presence of the oleanane-type triterpenoid, glycyrrhizin, this substance was found to be absent in our work, and several years ago we reported the isolation and structural characterization of four sweet-tasting cycloartane derivatives, abrusosides A–D [**(10–13),** Fig. 2].[42–45] During the structure determination work, abrusoside A, the most nonpolar compound of this group was hydrolyzed, and its aglycone, abrusogenin, was characterized as (20*S*,22*S*)-3β,22-dihydroxy-9,19-cyclolanost-24-en-26,29-dioic acid lactone, using a combination of NMR procedures. The structure and stereochemistry of abrusogenin methyl ester was confirmed by single-crystal x-ray crystallography.[43] Abrusosides A-D were also isolated from a second species in the small genus *Abrus, A. fruticulosus* Wall. ex W. & A., collected in Thailand.[46] These four sweeteners are the first known highly sweet cycloartane-type triterpene glycosides, being based on a novel carbon skeleton with an unusual lactone ring. The compounds were individually shown to be not acutely toxic for mice, and were not mutagenic in a forward mutation bacterial assay.[42] The water-soluble ammonium salts of abrusosides A-D were rated as being, respectively, 30, 100, 50, and 75 times sweeter than 2% sucrose, and the compounds exhibit a pleasant sweet taste that is not marred by bitterness, although they have a delayed onset of sweet taste.[42] Abrusosides A-D showed little or no breakdown when heated at 180°C for a week, making them suitable candidates for use in baked goods.[17] It is possible to harvest the leaves of *A. precatorius* before the toxic seeds appear, and we have come across products containing the leaves of this plant used as a sweet-tasting beverage in Malaysia and as a medication to treat the tropical disease sprue in Indonesia, thereby suggesting the safety of ingesting small amounts of *A. precatorius* leaves by humans.[17] However, the rather low yields of abrusosides A-D in the dried leaves of *A. precatorius* (<1.0% w/w when combined), and the relative dif-

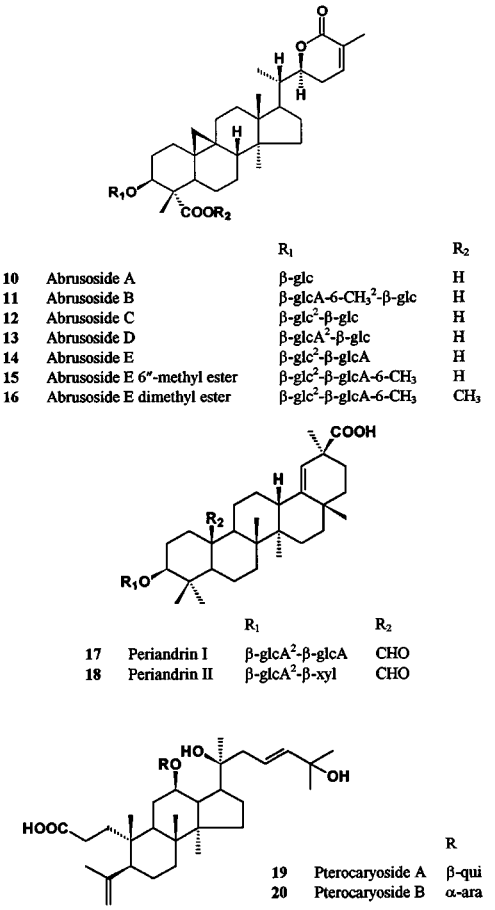


Figure 2. Triterpenoid sweeteners and their derivatives.

ficulty of purifying and separating the individual abrusoside sweeteners, have proven to be obstacles to the commercialization of these compounds as novel high-intensity sweeteners.

In more recent work, a further analog of these cycloartane glycoside sweeteners was isolated and characterized, namely, abrusoside E [**(14),** Fig. 2]. In this compound, the two sugar units are transposed when compared to abrusoside D, but it is only marginally sweet-tasting, and has very poor solubility properties in many solvents.[47] During attempts to find a derivative that could be used to assist in the structural determination of compound **14**, abrusoside E 6″-monomethyl ester [**(15),** Fig. 2] was prepared, and this substance proved to be most highly sweet-tasting compound in the abrusoside series (*ca.* 150 times sweeter than 2% sucrose) obtained to date.[17] This suggests that other synthetic abrusosides may be worth evaluating in terms of their sweetness. It is apparent that a free carboxylic acid unit is necessary for the mediation of a sweet taste by the abrusosides, since abrusoside E dimethyl ester [**(16),** Fig. 2] was totally devoid of any sweet effect.[17]

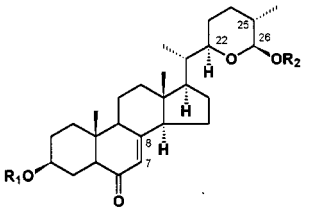
Periandrin I [**(17),** Fig. 2] is the prototype sweet substance isolated from the roots of *Periandra dulcis* Mart. (Fabaceae) (Brazilian licorice), and this oleanane glycoside was structurally characterized in the early 1980s by Hashimoto and co-workers at what is now Kobe Pharmaceutical University.[48] This compound, as well as three structural analogs (periandrins II–IV), was rated as about 90 to 100 times sweeter than sucrose.[8,48] Periandrin V [**(18),** Fig. 2] was isolated and characterized as

the fifth compound in this series in our laboratory, from a lyophilized extract of *P. dulcis.*[49] This glycoside was not soluble in any of the usual NMR solvents, so it was converted to a dimethyl ester for detailed spectroscopic investigation. While periandrins I–IV all possess a diglucuronide saccharide moiety, periandrin V {3β-*O*-[β-D-xylopyranosyl-(1→2)-β-D-glucuronopyranosyl]-25-al-olean-18(19)-30-oic acid} possesses a terminal xylose unit. After preliminary acute toxicity and mutagenicity safety testing, periandrin V was evaluated by a small taste panel, and was rated as possessing about 200 times the sweetness intensity of a 2% w/v sucrose standard. Periandrin V is therefore one of the sweetest natural product terpenoids to have been discovered, and has over twice the sweetness intensity relative to sucrose as periandrins I–IV.[8,48,49]

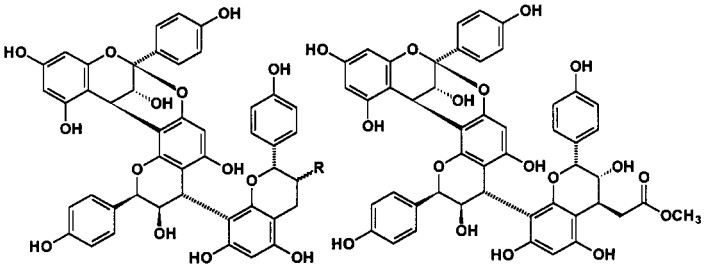
The leaves of the plant *Pterocarya paliurus* Batal. (Juglandaceae) are used by local populations in remote areas of Hubei Province in the People's Republic of China to sweeten foodstuffs, and "tian ye shu," the Chinese name for this plant, translates as "sweet-leaf tree." Two sweet-tasting secodammarane saponins, pterocaryosides A [**(19),** Fig. 2] and B [**(20),** Fig. 2] were isolated from this lead, and were structurally characterized using a combination of specroscopic methods as (23*E*)-(12*R*,20*S*)-12,20,25-trihydroxy-3,4-secodammara-4(28),23-dien-3-oic acid 12-*O*-β-D-quinovopyranoside and (23*E*)-(12*R*,20*S*)-12,20,25-trihydroxy-3,4-secodammara-4(28),23-dien-3-oic acid 12-*O*-α-L-arabinopyranoside, respectively. It was necessary to use the methylated peracetates of these compounds for structural work, due to the relative insolubility of the parent compounds in the usual NMR solvents, and the stereochemistry of these compounds was assigned by comparison to known dammarane derivatives.[50] While our work on *P. paliurus* was in progress, a report appeared in the literature describing several sweet-tasting dammarane derivatives from the same species.[50,51] However, no sweet-tasting saponins based on the intact dammarane skeleton were obtained in our work on *P. paliurus.* Pterocaryosides A and B were not found to be bacterial mutagens and nor were they acutely toxic to mice in preliminary safety tests, and their ammonium salts were rated as being about 50 and 100 times sweeter than 2% sucrose, respectively, by a small taste panel.[50] The compounds exhibited an almost instantaneous sweet response, but both had a persistent, mildly bitter off-taste, with pterocaryoside B being significantly more pleasant to the taste than pterocaryoside A. Pterocaryosides A and B are the first sweet-tasting secodammarane derivatives to have been identified to date, and therefore represent a new sweet-tasting chemotype.[50] Their identification underscores the value of ethnobotanical observations in sweet compound discovery.

C. Steroidal Saponins and Proanthocyanidins

The long-established structure of osladin, a steroidal saponin which is the sweet principle of the rhizomes of the European fern, *Polypodium vulgare* L. (Polypodiaceae), was recently subjected to stereochemical revision [**(21),** Fig. 3] by Prof. M. Nishizawa's group at Tokushima Bunri University, Tokushima, Japan, as a result of synthetic and x-ray crystallographic studies.[52,53] By analogy, the structure of the related compound, polypodoside A [**(22),** Fig. 3], a novel highly sweet steroidal saponin isolated by our group several years ago from the rhizomes of *Polypodium glycyrrhiza* D.C. Eaton (Polypodiaceae)[54] has also been reinvestigated, since the NMR comparison of polypodoside A with Nishizawa's data for osladin suggested a common aglycone stereochemistry.[14,55] Polypodogenin, the aglycone of polypodoside A, is a known compound, and was first identified by Czech workers in 1971. Polypodogenin was established as the $\Delta^{7,8}$-derivative of the aglycone of osladin, and its stereochemistry was determined by partial synthesis from solasodine.[54] Therefore, we have collaborated with Professor Nishizawa on the stereochemical reassignment of polypodoside A. Hence, the synthetic intermediate i (Scheme 1) obtained in earlier work on osladin has been converted to the enone vi obtained from polypodoside A on hydrolysis and silylation. Briefly, lactone i was methylated by treatment with LDA and then with methyl iodide to give a mixture of C-25 epimers. A catalytic amount of triflic acid effected the solvolysis of i to give ii in high yield. Hemi-



		R_1	R_2	Other
21	Osladin	β-glc^2-α-rha	α-rha	7,8-dihydro
22	Polypodoside A	β-glc^2-α-rha	α-rha	-
23	Polypodoside B	β-glc	α-rha	-
24	Polypodoside C	β-glc	α-rha-3-CH_3	-



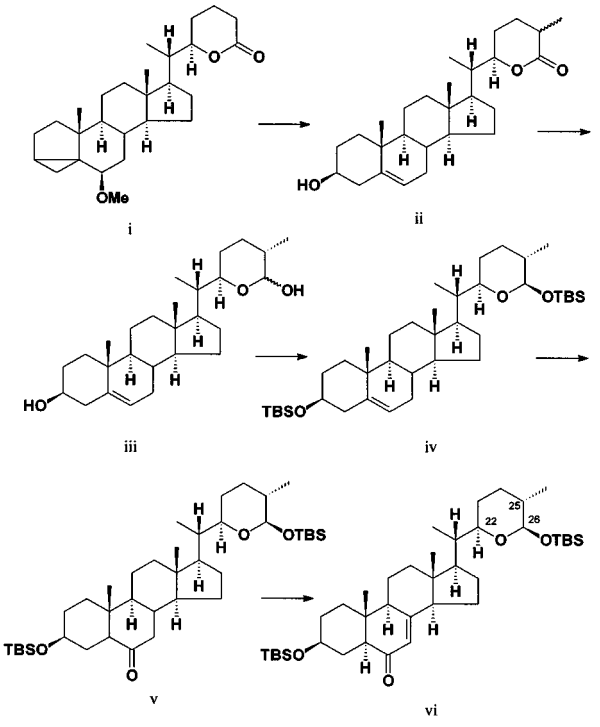
25 Selligueain A (R = β-OH)
26 Epimer of selligueain A (R = α-OH)

27 Selligueain B

Figure 3. Steroidal saponin and proanthocyanidin sweeteners and their derivatives.

acetals prepared from ii by DIBAH reduction were treated with NaOMe in MeOH leading to iii as a mixture of C-26 stereomers. The C-25 stereochemistry of iii was controlled to the desired *S* configuration exclusively. Silylation of iii gave iv, which was then converted to ketone v by hydroboration followed by oxidation. The enolate derived from compound v was quenched with TMSCl to give the corresponding vinyl silyl ether, which, upon palladium acetate-catalyzed dehydrosilylation in the presence of *p*-benzoquinone in benzene–acetonitrile provided the α,β-unsaturated ketone, vi (Scheme 1). This same enone (superimposable spectral data) was obtained from natural polypodoside A by hydrolysis with crude hesperidinase enzyme and silylation. Therefore, polypodoside A has been confirmed as having 22*R* and 25*S* stereochemistry, with the 26*R* stereochemistry inferred from ^{1}H-NMR spectroscopy. This represents a reversal of the stereocenters at C-22, C-25, and C-26 from those published previously.[14,54,55] By biogenetic analogy, the structures of the related compounds polypodosides B and C[56] require revision as [**(23),** Fig. 3] and [**(24),** Fig. 3], respectively. The steroidal saponins osladin and polyodoside A are two of the sweetest natural products known (rated as 500 and 600 times the sweetness intensity of sucrose, respectively).[52–54] In contrast, polypodoside B, with shorter saccharide chain at C-3 than polypodoside A, was barely sweet, while polypodoside C (a methylated derivative of polypodoside B) was devoid of any sweet taste.[14,56]

Work up of a butanol-soluble extract of the mature rhizomes of another fern, *Selliguea feei* Bory (Polypodiaceae), collected near the site of a volcano in West Java, Indonesia, has led to the isolation and characterization of an unexpected sweet-tasting constituent, a novel proanthocyanidin, to which



Scheme 1. Reaction sequence leading to the proof of stereochemistry of polypodogenin, the aglycone of polypodoside A **(22).**

we have accorded the trivial name, selligueain A [**(25),** Fig. 3]. This compound occurred as a major secondary metabolite component of *S. feei* rhizomes (0.69% w/w), and was purified by a combination of silica gel chromatographic steps and by crystallization from methanol. Prior to conducting a taste test, pure selligueain A was demonstrated to lack discernible acute toxicity for mice when administered by oral intubation (1 g/kg and 2 g/kg body weight), and was also inactive in a bacterial forward mutation assay (*Salmonella typhimurium* strain TM677). The compound was rated as possessing about 35 times the sweetness of a 2% w/v sucrose solution. This is the first proanthocyanidin ("condensed tannin") to have been demonstrated in the laboratory to be highly sweet in a defined manner, and is unusual since such compounds are normally astringent when tasted.[57]

The structural characterization procedure for selligueain A was somewhat prolonged and required peracetylation, thiolysis, and desulfurization steps to establish that this substance is the novel doubly linked A unit compound, epiafzelechin-(4β→8,2β→O→7)-epiafzelechin-(4β→8)-afzelechin. Although other workers have indicated that other proanthocyanidins are sweet-tasting, such compounds to not seem to have been evaluated for their sweetness intensities relative to sucrose prior to our work. It appears that there are stringent structural requirements for proanthocyanidins to exhibit a sweet taste, and the epimer of selligueain A (epiafzelechin-(4β→8,2β→O→7)-epiafzelechin-(4β→8)-epiafzelechin) [**(26),** Fig. 3] (kindly donated by Dr. G.-i. Nonaka, Kyushu University, Japan) was astringent without a hint of sweetness.[57] Further isolation work by our group has not afforded any other sweet principle from *S. feei* rhizomes, although selligueain B [**(27),** Fig. 3], was obtained as a second novel proanthocyanidin principle of this species.[58] Recently, Bohlin and co-workers have isolated selligueain A from a second plant source (*Polypodium triseriale* Sw., Polypodiaceae), and shown this compound to be an inhibitor of the proteolytic enzyme, elastase.[59]

3. BIOLOGICAL EVALUATION OF NATURAL SWEETENERS

A. Preliminary Safety Testing and General Sensory Evaluation

In order to isolate and structurally characterize novel natural product sweetening agents in our program to date, it has been necessary to perform activity-guided fractionation on plant extracts and chromatographic cuts using human volunteers to assess the presence or absence of sweetness. However, preliminary safety testing has been conducted on crude extracts, through acute toxicity testing in mice[60] and bacterial mutagenicity testing using a forward mutation assay.[61] Safety testing in a similar manner is also applied to pure sweet compounds prior to their being assessed for sweetness intensity by a small human taste panel constituted by three trained volunteers. Each test compound, which first must be determined as not acutely toxic for mice and inactive as a bacterial mutagen, is dissolved in water (or ammonium hydroxide if it contains one or more carboxylic acid residues), and diluted until its sweetness intensity is perceived to be equivalent to that of 2% w/v sucrose solution. A ratio of the concentrations of the solutions rated as having the same degree of sweetness provides a notion of the sweetness potency of the test substance.[36,42,50,54,57] For sweet natural products which are fairly insoluble in water, these can be dissolved in up to 2% aqueous ethanol before the threshold sweetness comparison is conducted.[34,62]

B. Electrophysiological and Behavioral Assays with the Mongolian Gerbil

To investigate the possibility of circumventing the preliminary safety protocols described above, which are rather inconvenient, costly, and can be wasteful of test materials, we have attempted to use an alternative procedure to monitor sweetness based on a combination of two previously published assays involving the Mongolian gerbil (*Meriones unguiculatus*). In so doing, it was hoped to substitute for the use of human volunteers to monitor plant crude extracts and chromatographic fractions for the presence or absence of sweetness. The two gerbil assays comprise an electrophysiological procedure and a conditioned taste aversion (CTA) test, and will be discussed briefly in turn. This work was performed in collaboration with Professor William Jakinovich, Jr., of the City University of New York, who pioneered the use of both of these gerbil procedures. It has been shown that the gerbil's intact chorda tympani nerve may be electrophysiologically stimulated by sweet monosaccharides, disaccharides, and polyols, in addition to "intensely" sweet substances of synthetic and natural origin such as chlorosucrose, L-cyanosuccinalic acid, dulcin, sodium saccharin, stevioside, and D-tryptophan. This electrophysiological method is performed by exposing and recording data from the exposed chorda tympani nerve with chemical stimulation of the tongue effected using a gravity tube mechanism. It is highly sensitive to sweeteners such as stevioside, where it is applicable to a concentration as low as 2×10^{-5} M.[63–65] The behavioral CTA assay was then used to characterize the taste of electrophysiologically active samples. This method involves the use of animals trained to avoid sweet, salty, bitter, and sour tastes, and the degree of similarity of taste is determined by the amount of flavored experimental fluids that are consumed. The results were assessed statistically through one-way analysis of variance.[65,66]

In a preliminary study using these gerbil methods, it was found that when used in combination the procedure could be used to detect the presence or absence of sweet-tasting terpenoid glycosides of extracts of different polarities from *Abrus precatorius* leaves (sweet cycloartane-type triterpene glycoside constituents), *Stevia rebaudiana* leaves (sweet *ent*-kaurane-type diterpenoid glycoside constituents) and *Thladiantha grosvenorii* (Swingle) C. Jeffrey fruits [now *Siraitia grosvenorii* (Swingle) C. Jeffrey] (sweet cucurbitane-type triterpene glycoside constituents).[67] This study was followed by an evaluation of these methods using several pure terpenoid plant-derived natural sweet-

eners. It was found that the gerbil's chorda tympani nerve did not respond to rebaudiosides B and C and steviolbioside from *S. rebaudiana* in electrophysiological experiments. However, electrophysiological concentration-response curves were obtained for hernandulcin **(1),** mogroside V (a cucurbitane-type triterpenoid sweetener from *S. grosvenorii*), periandrin III (an oleanane-type triterpenoid sweetener from *Periandra dulcis*), and stevioside and rebaudioside A from *S. rebaudiana*. All of these compounds were more effective stimuli in the gerbil than sucrose, and were ranked in the following order of potency electrophysiologically: rebaudioside A = stevioside = periandrin III > hernandulcin > mogroside V > sucrose.[68] However, this did not parallel the order of potency of these sweeteners in the human (hernandulcin > mogroside A > rebaudioside A > stevioside > periandrin III > sucrose).[8] In the subsequent conditioned-avoidance test, gerbil trained to avoid these five stimulatory compounds generalized an avoidance to sucrose but not to hydrochloric acid, and it was concluded that these substances taste like sucrose to gerbils. While these gerbil models do not respond to all classes of substances perceived as sweet by humans, they may have some validity in replacing humans in natural sweetener discovery research, but they are complex experimentally, and require substantial technical expertise.[68]

C. In Vivo *Cariogenicity Study on Stevioside and Rebaudioside A*

Working with colleagues in the Department of Pediatric Dentistry, College of Dentistry, University of Illinois at Chicago, the commercially used natural sweeteners stevioside and rebaudioside A were tested for cariogenicity in albino Sprague–Dawley rats. Rat pups were colonized with *Streptococcus sobrinus*, and four groups used in the experiment were fed for 5 weeks with a diet incorporating either 30% sucrose, 0.5% stevioside, 0.5% rebaudioside A, or no sweetener addition. Using Keyes' technique to evaluate caries production, it was concluded that neither stevioside nor rebaudioside A was cariogenic under the conditions of the study.[69]

4. CONCLUSIONS

Despite the widespread use of artificial sweeteners in the United States, sucrose remains the paramount sweetener throughout the world because of its easy availability and widespread applications. However, there are important health reasons why the consumption of sucrose should be used more sparingly, especially in regard to the demonstrated effects of this disaccharide in causing dental caries. Plants have afforded a number of highly sweet compounds, and although the number of such compounds is not great, several are used commercially in one or more countries, particularly in Japan. In our natural sweetener discovery program in the last few years, we have isolated several new sweet-tasting chemotypes through follow-up investigations of a number of ethnobotanical and other leads. However, it is entirely possible that the most outstanding candidate sweet plants have already been studied scientifically, and that in the future plant-derived sweet compounds will only be obtained through random organoleptic investigations or from species that are used by indigenous populations in remote areas. It is to be hoped that our past studies will lead to a better understanding of the relationship between chemical structure and the elicitation of a sweet taste, and in the rational design of novel synthetic sweeteners.

We are grateful to many people who have contributed in the past to our multidisciplinary studies on natural sweeteners, including the following faculty colleagues: Dr. J. M. Pezzuto (mutagenicity studies) and Dr. S. Das (cariogenicity studies) (both of the University of Illinois at Chicago), Dr. W. Jakinovich, Jr. (H. H. Lehman College, City University of New York) (gerbil electrophysiological and behavioral studies), and Prof. M. Nishizawa (Tokushima Bunri University, Tokushima, Japan) (synthetic studies). In addition, we are grateful to a number of botanists who have brought our attention to candidate sweet-tasting plants, and are thankful for the meticulous efforts of many additional outstanding postdoctorals, technicians, and graduate students who have participated in this sweetener project, and whose names are indicated in the bibliography. Finally, we

are very pleased to be able to contribute to this special journal issue in honor of Dr. Monroe E. Wall, who together with his close colleague, Dr. Mansukh C. Wani, has found success in natural products research as a result of unceasing enthusiasm, commitment, and determination. The outstanding work of Dr. Wall has inspired many younger scientists to devote their research careers to the study of biologically active compounds from natural sources.

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