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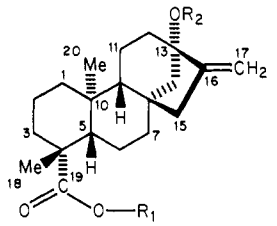
Communications to the Editor

Diterpenoid Sweeteners. Synthesis and Sensory Evaluation of Stevioside Analogues Nondegradable to Steviol

Sir:

As a result of the banning of cyclamate for use as a nonnutritive sweetener in the United States and of the question raised regarding the possible carcinogenicity of saccharin, a great deal of work has been carried out in recent years directed toward the development of alternative sweeteners. Several years ago we became interested in Stevioside (1), a potently sweet diterpenoid glycoside



1, (stevioside), R_1 = β-D-glucosyl; R_2 = β-D-sophorosyl
2, (steviol), $R_1 = R_2$ = H
3, (steviolbioside), R_1 = H; R_2 = β-D-sophorosyl
4, $R_1 = (CH_2)_3SO_3Na$; R_2 = β-D-sophorosyl
5, $R_1 = (CH_2)_3SO_3Na$; R_2 = H
6, $R_1 = R_2 = (CH_2)_3SO_3Na$

found in the Paraguayan shrub *Stevia rebaudiana*. It has been valued as a sweetener by the natives of that region for centuries. More recently, the plant source has been cultivated quite intensively in China, Korea, and Japan. Most of the product of this cultivation is for the Japanese market where a crude extract containing mainly 1 is now approved for food usage. The long history of human exposure and the absence of reported toxicity suggested that stevioside may have use as a nonnutritive sweetener. In 1966, however, Vignais and co-workers[1] reported the results of a study concerned with elucidation of the mode of action of the respiratory toxin atractyligenin. Included in their study were several compounds of related structure, including steviol (2), the aglycon of stevioside. Surprisingly, in cell mitochondria, steviol was found to be an even more potent inhibitor of ATP synthetase than atractyligenin. Clearly, if stevioside was converted to steviol in vivo, significant toxicity may be expected. Recent results reported by Wingard and co-workers[2] suggest the likelihood that stevioside would be largely converted to steviol in vivo and further that the steviol thus produced would subsequently be completely absorbed through the gastrointestinal tract wall. Thus, as a result of a combination of the Vignais and Wingard work, it was hypothesized that stevioside may be expected to exhibit significant acute toxicity. This expectation contrasts with preliminary results of Akashi and Yokoyama[3a] (Tama Biochemical Co., Ltd., Tokyo, Japan) and also of Mitsuhashi[3b] (Department of Pharmacy, Hokkaido University), where LD_{50} determinations of >15000 and >8200 mg/kg, respectively, were obtained on oral administration of stevioside in mice. In summary, at the present time, the safety of stevioside is still uncertain. If, however, metabolism to steviol could be prevented or if a potently sweet analogue could be developed which was not degraded to steviol, safety for use in foods would be anticipated.

Until very recently, very little structure–activity work in the stevioside area had been reported. It had been stated that steviolbioside (3), the carboxylic acid obtained on saponification of 1, was tasteless.[4] In 1979, however, Kamiya and co-workers[5] reported that 3 was in fact sweet and about half as potent as 1. Thus, it is clear that the glucose ester moiety of 1 is not involved in any essential receptor binding interactions. Therefore, we reasoned that it should be possible to replace the glucose ester moiety with other groups of similar polarity without loss of sweetness.[6] For this reason we chose to prepare 4, the sulfopropyl ester of 3. Thus, alkylation of 3 with 1,3-propane sultone[7] (K_2CO_3/DMF/1,3-propane sultone/25 °C) gave the sulfopropyl ester 4 as a dihydrate in 42%[8]

(1) Vignais, P. V.; Duee, E. D.; Vignais, P. M.; Huet, J. *Biochim. Biophys. Acta* **1966**, *118*, 465–483.
(2) Wingard, R. E., Jr.; Brown, J. P.; Enderlin, F. E.; Dale, J. A.; Hale, R. L.; Seitz, C. T. *Experientia* **1980**, *36*, 519–520.
(3) (a) Akashi, H.; Yokoyama, Y. *Shokuhin Kogyo* **1975**, *18*, 34–43. (b) Mitsuhashi, H. In "Safety of Stevia"; Tama Biochemical Co., Ltd.: Tokyo, 1981.
(4) Wood, H. B., Jr.; Allerton, R.; Diehl, H. W.; Fletcher, H. G., Jr. *J. Org. Chem.* **1955**, *20*, 875–883.
(5) Kamiya, S.; Konishi, F.; Esaki, S. *Agric. Biol. Chem.* **1979**, *43*, 1863–1867.
(6) The neohesperidose sugar moiety of the potent sweetener neohesperidin dihydrochalcone has been replaced with a great variety of polar functionalities without adverse effects on either sweetness potency or quality; cf. (a) DuBois, G. E.; Crosby, G. A.; Saffron, P. *Science* **1977**, *195*, 397–399. (b) DuBois, G. E., Crosby, G. A.; Stephenson, R. A.; Wingard, R. E., Jr. *J. Agric. Food Chem.* **1977**, *25*, 763–772. (c) DuBois, G. E.; Crosby, G. A.; Stephenson, R. A. *J. Med. Chem.* **1981**, *24*, 408–428.
(7) **Caution**: 1,3-Propane sultone has been shown to be a potent carcinogen in animals; cf. Druckey, H.; Kruse, H.; Preussmann, R. *Naturwissenschaften* **1968**, *55*, 449. Doak, S. M. A.; Simpson, B. J. E.; Hunt, P. F.; Stevenson, D. E. *Toxicology* **1976**, *6*, 139. Ulland, B.; Finkelstein, M.; Weisburger, E. K.; Rice, J. M.; Weisburger, J. H. *Nature (London)* **1971**, *230*, 460. Reactions employing it should be carried out with extreme caution.

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Table I. Sensory Evaluation of Stevioside and Analogues[a]

compd	judgements	concn, ppm	I_p[b]	P_w[c]	P_M[c]	taste character: sweet	sour	salty	bitter	other	sweet/bitter, other
1	12	500	0.94 (0.06)	190 (10)	440 (30)	62 (10)	0 (0)	0 (0)	30 (10)	8 (5)	62/38
2 (K salt)	1	2000	0.5	25	23	0	0	0	100	0	0/100
3 (Na salt)	12	1000	1.0 (0.1)	100 (10)	190 (20)	65 (9)	0 (0)	0 (0)	35 (10)	0 (1)	65/35
4	12	534	0.84 (0.06)	160 (10)	360 (20)	92 (6)	0 (0)	0 (0)	4 (6)	4 (4)	92/8
5	1	2000	1	50	70	0	0	0	100	0	0/100
6	12	1000	1.1 (0.2)	110 (20)	190 (40)	18 (9)	0 (0)	0 (0)	82 (9)	0 (0)	18/82
saccharin	12	330	0.98 (0.09)	300 (30)	180 (20)	85 (6)	0 (0)	0 (0)	12 (8)	3 (2)	85/15

[a] As detailed in our earlier work,[6c] compounds were required to be ≥95% pure and to show absence of toxicity prior to evaluation by a volunteer human panel of judges. Sample purity was assessed as previously described,[6c] except that 1 was saponified, and 4–6 were converted by ion exchange (Bio-Rad AG MP-50) to acid forms, prior to proton titration. Compounds were screened for mutagenicity with five *Salmonella typhimurium* tester strains, with and without microsomal activation, and were also subjected to limited scale, single oral dose, toxicity testing in mice. Results of these tests were reviewed by an institutional review board, and only compounds showing complete absence of toxicity were evaluated by the human sensory panel. Sensory analysis was conducted by a trained panel of six judges which evaluated each sample once in the morning and once in the afternoon. Sensory data are reported as follows: mean value ($2S_m$). [b] Sample intensity relative to 10% sucrose; compound concentrations were chosen by the panel supervisor to yield solutions having taste intensity comparable to the 10% sucrose reference. [c] P_w = compound potency calculated on a weight basis; P_m = compound potency calculated on a molar basis.

yield after ion exchange[9] and recrystallization from methanol: mp[10a] 183–186 °C; IR[10b] λ 2.90 (OH), 5.86 (C=O), 6.04 (C=CH_2), 8.55 (S=O), 9.30 (S=O) μm; NMR[10c] δ 0.82 (s, 3 H, 20-CH_3), 1.13 (s, 3 H, 18-CH_3), 4.75 (s, 1 H, 17-CH), 4.88 (s, 1 H, 17-CH). Neutralization equivalents:[10e] calcd, 787; found, 791. Encouragingly, this highly water soluble compound was found to be potently and cleanly sweet. The results of sensory analysis with a panel of human volunteers are given in Table I. We were pleasantly surprised to find that the sensory properties of 4 were substantially improved over stevioside.

It is clear from the sensory properties of 4 that the glucose ester moiety of 1 is unnecessary for sweet taste. It is then obvious to question the role of the sophorose sugar unit. Therefore, we elected to prepare 5, the product of glycolysis of 4. Thus, steviol (2)[11] was alkylated (K_2CO_3/DMF/1,3-propane sultone/25 °C) to give the sulfopropyl ester 5 in 34%[8] yield after ion exchange[9] and recrystallization from methanol: mp[10a] 130–133 °C; IR[10b] λ 2.90 (OH), 5.80 (C=O), 6.01 (C=CH_2), 8.45 (S=O), 9.60 (S=O) μm; NMR[10c] δ 0.82 (s, 3 H, 20-CH_3), 1.13 (s, 3 H, 18-CH_3), 4.75 (s, 1 H, 17-CH), 4.88 (s, 1 H, 17-CH). Neutralization equivalents:[10e] calcd, 463; found, 453. Ester 5 exhibited only bitter taste; no hint of a sweet taste component could be observed. Clearly the sophorose moiety present in 1, 3, and 4 and absent in 5 has some important role in the mechanism of eliciting the sweet taste of these compounds. It occurred to us, however, that the sophorose unit may have its function solely in modulation of gross molecular polarity. This would allow efficaceous receptor interaction of functionality located exclusively in the aglycon portion of 1. Thus, we chose to increase the polarity of 5 by sulfopropylation of the weakly polar hydroxyl group. Repetitive treatment of a dry Me_2SO solution of steviol[11] with portions of potassium dimsylate in Me_2SO, followed by quenching with 1,3-propane sultone, until TLC analysis [silica gel F-254; $CHCl_3/CH_3OH/H_2O$ (15:10:2)] showed complete conversion to one more polar product (R_f 0.44), resulted in a 52%[8] yield after ion exchange[9] and recrystallization (CH_3OH) of the bissulfopropyl derivative 6 of steviol as a monohydrate: mp[10a] 160–195 °C; IR[10b] λ 2.90 (H_2O), 5.84 (C=O), 6.04 (C=CH_2), 8.45 (S=O), 9.60 (S=O) μm; NMR[10c] δ 0.78 (s, 3 H, 20-CH_3), 1.12 (s, 3 H, 18-CH_3), 4.76 (s, 1 H, 17-CH), 4.81 (s, 1 H, 17-CH). Neutralization equivalents:[10e] calcd, 303; found, 297. Quite interestingly, this compound, bearing no carbohydrate functionality, although mainly bitter, exhibited significant sweet taste. Thus, we conclude that the carbohydrate portions of 1 are completely unnecessary for sweet taste and that *all* functionality involved in receptor binding is located in the aglycon. The high quality and high potency sweet taste observed for the stevioside analogue 4 suggest that it may have utility in food applications. Critical for any such usage, however, is demonstration of the absence of degradation to steviol. Thus, in vitro degradation studies, employing rat cecal bacteria, were carried out by the general method of Wingard and co-workers.[2] Stevioside was observed to degrade completely to steviol under these conditions. Analogue 4 was incubated (37 °C) at a concentration of 1.0 mg/mL with 50 mg/mL fresh rat cecal contents in Krebs–Ringer 0.25 M phosphate buffer (pH 7.4) containing 0.25 mg/mL dithiothreitol and 0.25 mg/mL α-D-glucose. TLC [silica gel F-254; $CHCl_3/CH_3OH/H_2O$ (15:10:2)] and HPLC [30 cm C-18 on μ-Bondapak; 15 min linear gradient of 10–40% CH_3CN in 0.005 M KH_2PO_4 (pH 3.45); 200 nm] analyses showed all of 4 (R_f 0.42; t_R 14.0 min) to have been consumed within 24 h to yield apparently only 5, the sulfopropyl ester of steviol (R_f 0.63; t_R 16.3 min). No steviol (R_f 0.95; t_R 31.3 min) was detected. After 3 days, the bacterial cells were sedimented by centrifugation. The sediment was extracted (THF), as was the supernatant,

(8) Crude product yields in the cases of compounds 4–6 approached quantitative; reported yields are of first crops of crystals, and the low values reflect the highly soluble nature of these compounds.

(9) The sulfoalkyl potassium salts were converted to sodium salts by passage through an ion-exchange column of Bio-Rad AG MP50 in the sodium form.

(10) The sulfoalkyl salts described herein did not exhibit well-defined melting points but rather appeared to soften and then slowly liquify. (b) IR spectra were recorded as KBr pellets at a concentration of 1%. (c) NMR spectra were recorded at 100 MHz in Me_2SO-d_6 solvent. (d) Satisfactory elemental analyses were obtained for all new compounds. (e) Neutralization equivalents were obtained by potentiometric titration vs. 0.100 N NaOH of the sulfonic acid obtained by ion exchange on Bio-Rad AG MP 50 cation-exchange resin (acid form). Values given were calculated for the sodium salts and are corrected for water content, which was determined by Karl Fischer titration.

(11) Bridel, M. M.; LaVieille, R. *Bull. Soc. Chim. Biol.* 1931, *13*, 781–796.

after lyophilization. HPLC analysis for steviol of the two THF extracts showed none to be detectable. With a detection limit of 0.05 μg, as little as 0.03 and 0.13% degradation to steviol could have been detected for the sediment and supernatant fractions, respectively.

As has been shown above, the sulfopropyl ester moiety is quite stable to the biological conditions which readily degrade the glucosyl ester of stevioside. In addition, we have found this functionality to be inert to boiling alkali and to be quite unreactive toward hot dilute sulfuric acid.[12] Clearly, in vivo formation of steviol from 4 is quite unlikely and, therefore, we expect that any toxicity related to steviol formation would be eliminated by use of sulfopropyl ester 4 rather than the biologically unstable stevioside. In addition, it should be noted that 4 exhibits ($p < 0.003$) a cleaner taste [sweet (S)/bitter (B), other (O) = 92/8] than stevioside (S/B, O = 62/38), and which appears ($p < 0.32$) to be somewhat better than that of saccharin (S/B, O = 85/15). In summary, we have synthesized a stevioside analogue which has been demonstrated by sensory panel studies to have a potent, clean sweet taste and which is expected to have improved safety for usage in food systems. Thus, we are presently pursuing the development of 4 for usage as a sweetener for all applicable food and medicinal systems.

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(12) Similar hydrolytic stability has been reported by Mosettig and Nes for analogous methyl esters; cf. Mosettig, E.; Nes, W. R. *J. Org. Chem.* **1955**, *20*, 884-899.

Articles

Synthesis and Evaluation of Some Stable Multisubstrate Adducts as Inhibitors of Catechol *O*-Methyltransferase

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A new series of methylase inhibitors has been designed in which the nucleophilic methyl acceptor is attached to the adenosine and/or homocysteine fragments of the methyl donor, *S*-adenosylmethionine, to form a "multisubstrate adduct". In the present case, catecholamine analogues attached through a phenethyl sulfide linkage to 5'-thioadenosine or homocysteine have been synthesized, together with the corresponding methylsulfonium salts. These compounds were assayed as inhibitors of catechol *O*-methyltransferase, and the adenosylsulfonium salts (4) were found to be inhibitors of the enzyme.

The methylation of several biological nucleophiles by *S*-adenosylmethionine (SAM) is catalyzed by methylases specific for each nucleophile.[1] Recently, a myriad of analogues of both SAM[2] and *S*-adenosylhomocysteine (SAH),[3,4] itself a potent product inhibitor of nearly all SAM-dependent methylases, have been reported. The 7-deaza analogue of SAH, *S*-tubercidinylhomocysteine (STH), has been shown to be a potent inhibitor of RNA methylation and biogenic amine methylation in both cell free systems[3,4] and several cell culture systems.[5-7] Whereas both SAH and STH have nearly identical K_i values against several purified methylases, STH is more effective than SAH in cell culture systems. This has been ascribed to the stability of STH to the various enzymatic reactions responsible for SAH metabolism in mammalian cells.[8] While these results are encouraging in terms of inhibition of methylation in vivo, the general lack of specificity of SAH analogues warrants the investigation of an alternative approach. An exciting new approach to the design of highly potent and specific enzyme in inhibitors is the use of "transition-state analogues",[9] whose design is based upon the chemical mechanism of enzyme action.

Catechol *O*-methyltransferase, COMT (EC 2.1.1.6), is a well-studied enzyme which methylates a relatively simple monomeric substrate, in contrast to many methyltransferases which act on macromolecular substrates.[10] Based upon kinetic and stereochemical studies of the COMT reaction and related model compound reactions,

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