Analysis of structural features responsible for the sweetness of the sesquiterpene, hernandulcin [1, 2]

C. M. Compadre, R. A. Hussain, R. L. Lopez de Compadre, J. M. Pezzuto and A. D. Kinghorn

Department of Medicinal Chemistry and Pharmacognosy, and Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago (Illinois 60612, USA)
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Summary. The relationship between sweetness and structure was studied for several analogues of the intensely sweet sesquiterpene, hernandulcin. These derivatives were prepared synthetically, and were subjected to spectroscopic and conformational analysis. With the exception of the parent substance, none of the derivatives tested proved to be sweet. Evidence gathered in this study suggests that hernandulcin binds to its putative receptor through a three-point interaction, involving the C-1 carbonyl and C-1′ hydroxyl groups, and the double bond between C-4′ and C-5′. In the course of a preliminary safety assessment, the 3-desmethyl derivative of hernandulcin was found to be mutagenic toward *Salmonella typhimurium* strain TM677.
Key words. Hernandulcin; intense sweetener; synthetic analogues; taste attributes; conformational analysis; structure-activity relationships; safety assessment.

There is a great interest in the development of non-cariogenic, non-toxic, non-caloric and inexpensive sugar substitutes [3, 4]. However, the search for novel sweeteners is somewhat impaired by the lack of uniform criteria regarding the structural features necessary for a proper fit of sweet compounds with their receptor site(s) [5–7].

We have recently reported the isolation of the sweet bisabolane sesquiterpene, (+)-hernandulcin (**1**) [6-(1,5-dimethyl-1-hydroxy-hex-4-enyl)-3-methylcyclohexen-2-one], from a herb grown in Mexico, *Lippia dulcis* Trev. [8, 9]. Mori and Kato have determined that the absolute configuration of naturally occurring hernandulcin is (*6S, 1′S*) [10]. This compound is the prototype of a new class of intense sweeteners, and is an interesting candidate for the further study of sweetness-structure relationships, because of its intense sweetness, structural simplicity, and the availability of a facile synthesis. Thus, in the present investigation, the taste characteristics of six synthetic analogues were compared with those of hernandulcin (**1**). A conformational analysis was performed for each substance, in order to study further the steric and spatial requirements for the sweet response of this compound class.

Materials and methods. (*S**, *R**)-(±)-Epihernandulcin (**2**) was prepared as a minor reaction product in the synthesis of (±)-hernandulcin (**1**), as described previously [8, 9]. Compounds **3**–**8** were prepared by directed-aldol condensations between appropriate starting ketones, following the procedure described for the synthesis of hernandulcin [8, 9]. All of these compounds were generated as a mixture of two racemic products, with the exception of compound **3**. With reference to the chromatographic (TLC, HPLC) behavior of hernandulcin (**1**) and epihernandulcin (**2**), the more abundant and less polar racemate in each case was considered to be the product with the same stereochemistry as (±)-hernandulcin (*R**, *R**). All of the compounds were characterized by IR, UV, MS and ^{1}H- and ^{13}C- NMR spectroscopy (tables 1 and 2). The ^{13}C-NMR chemicals shifts of compounds **3**–**8** (table 2) are in very good agreement with the stereochemistry proposed [8, 9].

The mutagenic potential of the test compounds was evaluated utilizing *Salmonella typhimurium* strain TM677, according to a procedure previously reported [11], both in the presence and absence of a metabolic activating system. The compounds were evaluated at concentrations ranging from 0.1 to 5.0 mg/ml. Compound **7** produced a dose-related mutagenic response (table 3), and was therefore not submitted to further toxicity or organoleptic evaluations. Acute toxicity studies were carried out using male Swiss-Webster mice, according to a previously described procedure [9]. Test compounds were administered by oral intubation at 1 g/kg b. wt. All compounds (with the exception of compound **7**) were determined as non-mutagenic and non-toxic at the dose-levels used.

The sensory attributes of the test compounds deemed to be safe in preliminary studies were evaluated by a panel of three volunteers, at concentrations of 1, 10 and 100 mg/ml in distilled water. Samples were presented at random (in duplicate), and the participants tasted each sample for about 20 s, before expectorating and rinsing the mouth with distilled water. The results of this evaluation are presented in table 1.

Table 1. Organoleptic attributes and spectroscopic data of hernandulcin (**1**) and related compounds (**2**–**8**)

Compound	Taste	HPLC R_t, min[a]	UV λ_{max} (log ε)[b]	IR ν_{max} (OH,C = O)[c]	HRMS[d]
1	Sweet	3.34	236 (4.23)	3365, 1644	236.1800
2	Bitter	4.28	237 (4.18)	3375, 1644	236.1777
3	Bitter	4.79	237 (4.17)	3360, 1645	150.1035[e]
4	Bitter	3.20	237 (4.10)	3350, 1650	224.1770
5	Bitter	3.12	237 (4.15)	3350, 1654	220.1835[e]
6	Neutral	2.99	213 (3.27)	3368, 1715	220.1813[e]
7	Not tasted[f]	3.80	236 (4.11)	3358, 1647	222.1626
8	Bitter	3.55	229 (4.07)	3355, 1679	222.1634

[a] Column used, Bondapak C-18 (3.9 mm × 30 cm; Waters Associates, Milford, Massachusetts); detection, uv, 216 nm; for other conditions, see Compadre et al. [9]. [b] Run in methanol. [c] Neat film. [d] Varian MAT 112S spectrometer at 70 eV in the EI mode, experimental elemental compositions of $[M]^{+\cdot}$ ions within 20 ppm of calculated values. [e] Elemental composition of $[M\text{-}H_2O]^{+\cdot}$ ion. [f] Not tasted because compound exhibited a mutagenic response.

Table 2. ^{13}C-NMR data for compounds **3**–**8**[a]

Carbon	Compound 3	4	5	6	7	8
1	203.45s	204.25s	204.78s	213.23s	204.48s	213.57s
2	127.22d	127.53d	127.49d	53.88t	130.73d	130.91d
3	163.87s	163.59s	163.61s	29.28d	151.09d	179.50s
4	31.25t	31.33t	31.26t	34.61t	26.25t[b]	37.04t
5	25.36t	25.05t	24.99t	25.35t	25.74t[b]	53.74d
6	54.70d	52.29d	52.10d	31.78d	53.39d	19.55q
7	24.10q	24.42q	23.70q	22.10q		
1′	72.42s	71.73s	74.06s	71.05s	73.95s	73.80s
2′	24.64q	40.31t	40.48t	41.16t	40.02t	41.14t
3′	28.27q	22.54t	20.53t	21.22t	21.57t	22.10t
4′		22.74t	39.44t	122.45d	124.42d	124.36d
5′		23.79t	27.96d	132.82s	131.61s	131.60s
6′		14.12q	22.52q[b]	25.66q	25.19q	25.77q
7′		24.07q	22.71q[b]	17.65q	17.68q	17.40q
8′			24.06q	22.54q	23.65q	21.87q

[a] Spectra were recorded in $CDCl_3$ on a Varian XL-300 spectrometer at 75.4 MHz, using Me_4Si as internal standard. Values are in ppm. [b] Assignments are exchangeable in each column.

Table 3. Evaluation of the mutagenic activity of 3-desmethyl-hernandulcin (**7**)[a]

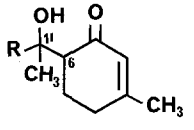
Concentration (mg/ml)	Mutant fraction ($\times 10^5$) without S-9[b]	Mutant fraction ($\times 10^5$) with S-9[b]
0	7.0	6.6
0.31	55.2	24.6
0.63	70.3	48.5
1.25	58.4	49.6
2.5	74.8	49.3
5.0	55.4	63.1

[a] This compound was evaluated with *Salmonella typhimurium* strain TM677 in the presence and absence of a metabolic activator, as indicated in the 'Materials and methods' section.
[b] Significant mutagenic responses ($p < 0.05$) observed at 0.31 mg/ml and all higher concentrations.

Conformational analysis of the molecules studied was performed by structure optimization, using steric, electrostatic and hydrogen bond energy terms, derived from molecular mechanics (MMF) and partial atomic charge (CNDO/2) calculations, with the use of CHEMLAB-II software[9, 12].

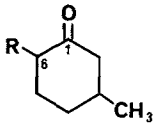
Discussion and conclusions. Shallenberger and Acree[13] have suggested that a characteristic of nearly all known sweet substances is the presence of proton donor (AH) and proton acceptor (B) entities separated by a distance of 2.4–4.0 Å. AH and B act, respectively, as an acid and a base, and the whole AH,B unit combines with a reciprocal AH,B moiety in the sweet receptor, forming a doubly hydrogen-bonded complex[13]. In addition, to explain some of the stereochemical requirements of the sweet receptor, the same group postulated the presence of a 'spatial barrier', located at a distance of about 3–4 Å from the AH,B unit[14]. Hernandulcin (**1**) seems to fit Shallenberger's model very closely, since the C-1 carbonyl and C-1′ hydroxyl groups are located about 2.6 Å apart, and reduction and acetylation, respectively, of these putative AH and B entities produces derivatives that are not sweet[9]. Futhermore, Shallenberger's 'barrier' could be used to explain the observation that, of the four possible stereoisomers, (+) and (−)-hernandulcin (**1**) and (+) and (−)-epihernandulcin (**2**), only the naturally occurring (6*S*,1′*S*)-(+)-hernandulcin (**1**) is sweet[10]. The computer matching of compounds (*S*,*S*)-(**1**) and (*S*,*R*)-(**2**), in their predicted preferred conformation (fig. 1 a) shows that the major difference between the molecules is the spatial orientation of the moieties comprised by carbon atoms 2′–7′ and the methyl group 8′. Thus, it could be hypothesized that, in the case of the non-sweet compound **2**, the bulkier fragment C-3′ through C-7′ is barring the molecule from fitting the receptor site.

In an alternative model, it has been suggested[15, 16] that intensely sweet-tasting compounds contain a third binding

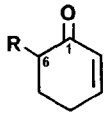


R

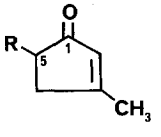
$(CH_3)_2C=CHCH_2CH_2$-	**1**	(6*R**, 1′*R**)
$(CH_3)_2C=CHCH_2CH_2$-	**2**	(6*R**, 1′*S**)
CH_3-	**3**	(6*R**, 1′*R**)
$CH_3(CH_2)_4$-	**4**	(6*R**, 1′*R**)
$(CH_3)_2CH(CH_2)_3$-	**5**	(6*R**, 1′*R**)



6 (6*R**, 1′*R**)



7 (6*R**, 1′*R**)



8 (5*R**, 1′*R**)

R = $(CH_3)_2C=CHCH_2CH_2C(CH_3)(OH)-$

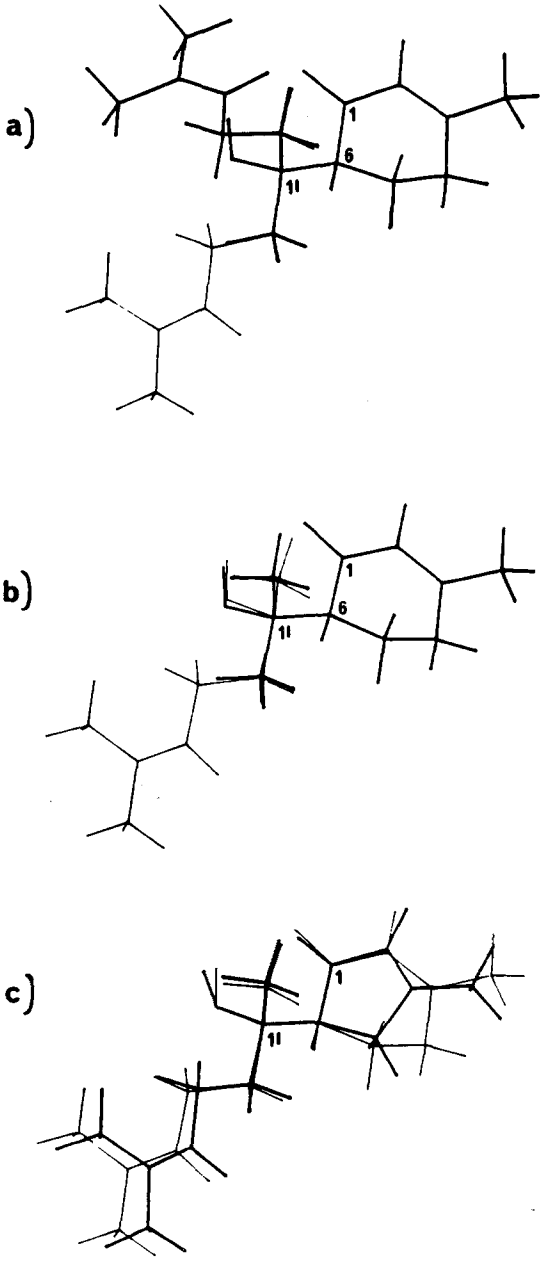


Figure 1. Computer superimposition of the preferred conformations of hernandulcin (**1**) (light) with compounds **2** (a), **3** (b), and **8** (c). Compounds **2**, **3** and **8** are shown in bold. For clarity, oxygenated functional groups and double bonds are omitted.

moiety, X, that interacts with the receptor through dispersion or hydrophobic forces. Support for such a model was obtained in the present study, since compounds **3**–**5** proved to be devoid of sweetness when tasted. The almost perfect match found when optimized structures of compounds **1** and **3** are superimposed (fig. 1 b), suggests that if the interaction with the 'spatial barrier' were the limiting factor for a proper fit with the receptor, compound **3** should be sweet. Thus, the lack of sweetness of compound **3** clearly points to the presence of an additional binding site, X. Since compounds **4** and **5** were not sweet, it is likely that the double bond between C-4′ and C-5′ is this additional binding site.

The lack of sweetness of compound **8** is more difficult to explain but as shown in figure 1 c, this compound can not adopt the relatively planar conformation exhibited by compound **1**. A conformational difference from **1** may also explain the fact that compound **6** was devoid of sweetness at the concentrations tasted.

It may be seen from table 3 that compound **7**, the 3-desmethyl-derivative of hernandulcin (**1**), was found to have substantial mutagenic activity. Compounds **1**–**6** and **8** were not active in this capacity (data not shown). Although we have not attempted to characterize the mechanism of the mutagenic process mediated by compound **7**, it is likely to involve the Michael reaction, similar to other α,β-unsaturated carbonylic compounds [17, 18]. This suggestion is supported by comparison of compound **7** with compounds **1**–**5** (which are not mutagenic and have a methyl group attached to the β-carbon atom) and the fact that compound **7** was a direct-acting mutagen (since the S-9 fraction derived from Aroclor 1254-pretreated rats was not required for activity). Further, it is interesting to note that since the related compound, 2-cyclohexen-1-one, has been reported to demonstrate only weak mutagenic activity [17], the aliphatic substituent affixed to C-6 of compound **7** may also contribute to its rather intense mutagenic response.

An interesting property of many of the known sweet molecules is the fact that minor structural or stereochemical changes may lead to the production of bitter-tasting compounds. This observation has led some workers to propose that the sweet and bitter receptors are relatively flat cavities in which the AH-B moieties can be interchanged, by a C_2 axis [6, 19]. Alternatively, it has been suggested that both sweet and bitter compounds interact with the same receptor, with sweet compounds binding through an AH,B-X system and bitter compounds producing only a partial interaction, e.g., AH-X [20]. Therefore, the fact that small structural variations of **1** furnished bitter compounds such as **4** and **5** seems to support the latter point of view. It can be argued that these compounds indeed interact with the AH,B sites on the receptor, but, as they lack the X moiety, the resultant partial interaction produces a bitter response.

Thus, evidence acquired during the course of this investigation indicates that hernandulcin (**1**) is highly specific in its structural and steric requirements, since in addition to its AH,B and X structural units, it also must adopt a relatively planar conformation in order to evoke the sweet response.

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Address reprint requests to either C. M. Compadre at Seaver Chemistry Laboratory, Pomona College, Claremont, California 91711, USA, or to A. D. Kinghorn at the University of Illinois at Chicago.

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