Schormüller, J., Kochman, H. J., *Z. Lebensm.-Unters. Forsch.* **141**, 1 (1969).
Schultz, T. H., Flath, R. H., Black, D. R., Guadagni, D. G., Schultz, W. G., Teranishi, R., *J. Food Sci.* **32**, 279 (1967).
Tressl, R., Drawert, F., Heimann, W., *Z. Naturforsch. B* **24**, 1201 (1969).
Viane, R., Bricout, J., Marion, J. P., Müggler-Chavan, F., Reymond, D., Egli, R. H., *Helv. Chim. Acta* **52**, 887 (1969).
Winter, M., Willhalm, B., *Helv. Chim. Acta* **47**, 1215 (1964).

Received for review November 29, 1976. Accepted February 7, 1977.

Dihydrochalcone Sweeteners. Synthesis and Sensory Evaluation of Sulfonate Derivatives

Grant E. DuBois,* Guy A. Crosby, Rebecca A. Stephenson, and Robert E. Wingard, Jr.*

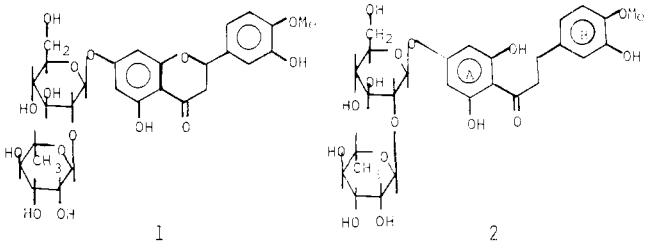
Fifteen sulfonate analogues of hesperetin dihydrochalcone (DHC), the aglycone portion of the intensely sweet glycosidic flavonoid neohesperidin DHC, were prepared and subjected to sensory analysis. Three distinct synthetic routes, the most general of which involves the regioselective alkylation of hesperetin at the 7 position followed by alkaline hydrogenation, were developed for the preparation of these materials. The simple linear 4-*O*-sulfoalkyl-DHC derivatives exhibited taste properties comparable to neohesperidin DHC. These sulfonates were found, however, to exhibit a slow taste onset followed by a lingering aftertaste as appears typical for DHC sweeteners. Taste–timing properties are discussed from the viewpoint of modern sensory theory, and a model, speculating on the various aspects of the DHC molecule responsible for the observed taste, has been developed.

Several years ago Horowitz and Gentili reported that the peels of oranges, lemons, and grapefruit contained a number of flavonoid derivatives which could, by way of a simple chemical modification, be converted into a new class of sweet compounds (1963, 1969). They found, for example, that the flavanone rhamnoglucoside, neohesperidin (1), the predominant bitter principle of the Seville orange rind, readily afforded the intensely sweet dihydrochalcone (DHC) derivative 2 upon alkaline hydrogenation. Similar results were reported for other flavanones which are conjugated with a β-neohesperidose sugar residue through the 7 phenolic hydroxyl position.

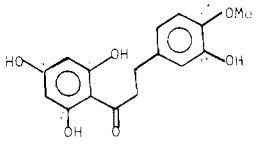
Neohesperidin DHC (2) has been indicated to be an attractive sweetener from a safety viewpoint (Booth and Robbins, 1968; Booth et al., 1973; Gumbmann et al., 1975) and it has been shown that the material can be prepared in a reasonably economic fashion on an industrial scale (Robertson et al., 1974). On the other hand, a serious problem is derived from the fact that the intense sweetness of DHC 2 is rather slow in onset and lingers considerably. These poor taste–timing characteristics render the sweetener unsuitable for use in most food products (Inglett et al., 1969).

It has been known for some time that hesperetin dihydrochalcone (3), which is the poorly soluble aglycone portion of 2, is sweet (Horowitz, 1964; Rizzi and Neely, 1973). We recently reported that water-soluble derivatives of 3 could be prepared by attaching carboxyalkyl chains to the hydroxyl group at position 4 (DuBois et al., 1977a). These compounds, although intensely sweet, were found to suffer from the same poor taste–timing characteristics which affected DHC 2. Additionally, these carboxylate-derived sweeteners were found to have limited solubility in the pH range of beverage systems.

We report here the preparation and taste properties of 15 sulfonated hesperetin dihydrochalcone derivatives.



1 2



3

These compounds were prepared with the expectation that the ionic sulfonate group would lead to high water solubility throughout the useful pH range and might, as the result of the increase in hydrophilic character, provide DHC sweeteners with improved taste–timing characteristics.

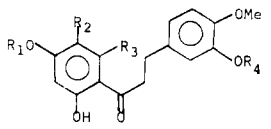
MATERIALS AND METHODS

Sensory Evaluation Procedure. All new compounds were given a preliminary taste evaluation by sampling a dilute aqueous solution. Compounds of further interest were submitted to a panel of six trained judges for sensory analysis. The panel evaluated the overall taste intensity of aqueous solutions of the materials and characterized the basic tastes present using standard psychophysical procedures (Acton et al., 1970).

The panel members were trained in the recognition of the basic tastes of sweet (as sucrose), sour (as citric acid), salty (as NaCl), and bitter (as quinine sulfate), as well as in the technique of magnitude estimation which consists of ranking the total intensity of a test solution relative to a sucrose standard. The tasting procedure consisted of giving panel members samples in coded beakers along with

Chemical Synthesis Laboratories, Dynapol, Palo Alto, California 94304.

Table I. Sulfoalkyl-, Sulfo-, and Sulfatodihydrochalcones Prepared



Compd	R_1	R_2	R_3	R_4
4	CH_2SO_3M	H	OH	H
5	$(CH_2)_2SO_3M$	H	OH	H
6	$(CH_2)_3SO_3M$	H	OH	H
7	$(CH_2)_4SO_3M$	H	OH	H
8	$(CH_2)_2CH(COOM_1)SO_3M_2$	H	OH	H
9	$(CH_2)_2CH(CHOHPh)SO_3M$	H	OH	H
10	$(CH_2)_2CH[(CH_2)_3SO_3M]SO_3M$	H	OH	H
11	$(CH_2)_3SO_3M$	H	H	H
12	$(CH_2)_3SO_3M$	H	OCH_3	H
13	$(CH_2)_3SO_3M$	H	OCH_2Ph	H
14	$(CH_2)_3SO_3M$	H	$O(CH_2)_3SO_3M$	H
15	H	H	$O(CH_2)_3SO_3M$	H
16	H	H	OH	SO_3M
17	H	SO_3M	OH	H
18	H	CH_2SO_3M	OH	H

an identified reference sample (0.25 M sucrose). Charcoal filtered, distilled water for rinsing and French bread sticks to reduce taste carry-over were supplied. At each test session, the judges also received the reference unidentified as a check on response variability.

A modified magnitude estimation procedure was employed for scoring. Each panel member tasted the test solution and judged its intensity relative to the reference. The panelist was then asked to characterize the flavor by indicating the degree of sweetness, sourness, saltiness, bitterness, or any other detected taste such that the total equaled 100%. Each panelist was also asked to describe the presence or absence, and if present the quality, of any aftertaste.

All samples were evaluated by each panelist on two occasions in sessions held 4 h apart. The results of the 2 judgements for the 6 panelists, totaling 12 determinations, were averaged. Compounds which exhibited considerable sweetness were evaluated in further sessions resulting in additional determinations.

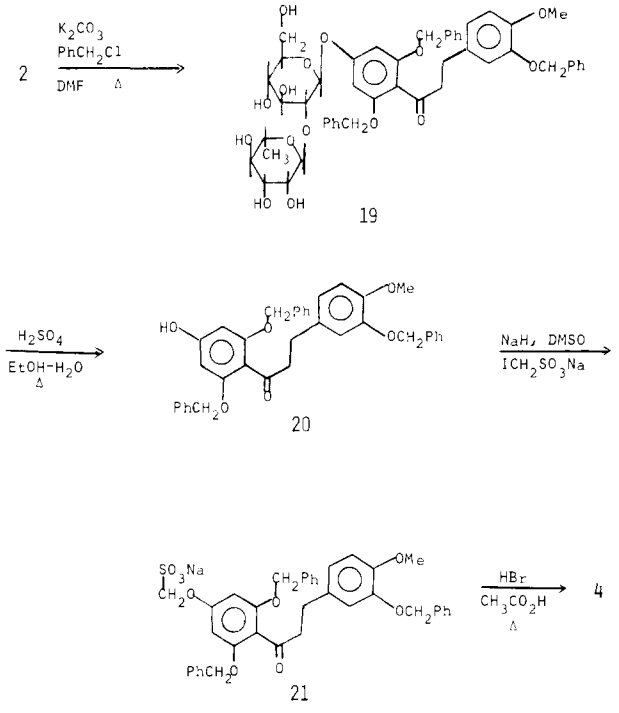
The biological safety testing procedures presently employed require satisfactory scientific evidence as to the safety of the samples prepared as a prerequisite to taste evaluation in humans. Samples are screened for mutagenicity with five *Salmonella typhimurium* tester strains with and without microsomal activation (Ames et al., 1975; Brown and Brown, 1976) to confirm the absence of carcinogenic reactants in the final product. The samples also undergo limited scale single oral dose toxicity testing in mice. Results of these tests are reviewed by an institutional medical review board prior to approval for sensory evaluation. Voluntary consent of all panelists is obtained in writing.

RESULTS AND DISCUSSION

Synthetic Considerations. The 15 sulfonated hesperetin DHC derivatives prepared are listed in Table I. Three distinct synthetic routes were developed for the preparation of these materials. Schemes II and III depict the most generally applicable of these which involves the regioselective alkylation of hesperetin at the 7 position followed by alkaline hydrogenation. The elements affecting this reaction have recently been discussed (DuBois et al., 1977b).

Synthesis of the lowest homologue of the series, 4-*O*-sulfomethyl hesperetin DHC (**4**), began with the conversion of neohesperidin DHC (**2**) into tribenzylated intermediate

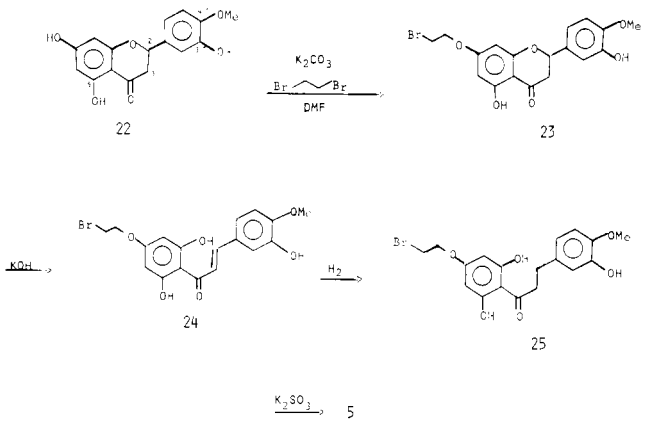
Scheme I



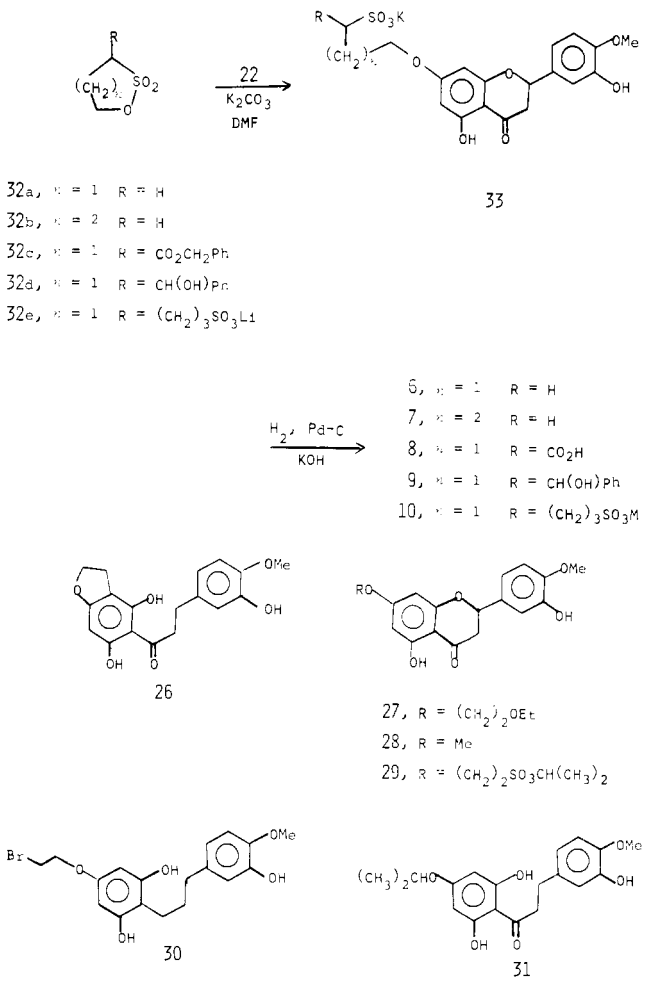
20 (Scheme I). Small amounts of glycoside O-benzylation and A-ring carbon alkylation occurred during the first step, but this caused no serious difficulty. Significant amounts of C-2 O-debenzylation product were formed during the acid hydrolysis of glycoside **19**. Optimum yields of **20** were obtained by using dilute sulfuric acid in ethanol–water at reflux. Increased amounts of debenzylation were noted when acids possessing more nucleophilic counterions (e.g., HCl) were employed, but no improvement was seen for acids having less nucleophilic counterions (e.g., $HClO_4$). Good yields of pure **20** were obtained after removal of the C-2 O-debenzylation by-product through chromatography on basic alumina.

The remaining steps consisted of alkylation with sodium iodomethanesulfonate and removal of the blocking groups. In accordance with previous reports (Lauer and Langkammerer, 1935; Barber et al., 1953), iodomethanesulfonate was found to be an extremely sluggish alkylating agent under normal conditions. Reaction of this reagent

Scheme II



Scheme III



with the sodium phenolate of **20** in Me_2SO proceeded only 1–2% after 3 days at 65 °C. Further heating (120–130 °C) resulted in the complete consumption of **20** to produce **21** and an unidentified side product in a ratio of 8:1 (HPLC analysis). Sulfomethyl-DHC **4** was obtained in the final step by debenzylation with HBr in aqueous acetic acid solution.

An indirect method of preparing 4-*O*-sulfoalkyl hesperetin DHC derivatives is illustrated in Scheme II for the case of sulfoethyl-DHC **5**. The alkylation of the flavanone hesperetin (**22**) with 1,2-dibromoethane resulted in clean, regioselective formation of the 7-*O*-bromoethyl product. No significant amount of the 3′,7-di-O-alkylation product, which was found to be a major contaminant in product mixtures obtained on the alkylation of **22** with α-halo esters (DuBois et al., 1977b), was observed. The reaction of bromoethyl flavanone **23** with aqueous K_2SO_3 produced a complex mixture of sulfonates. The alkaline sulfite medium undoubtedly caused the formation of chalcone **24** which underwent conjugate sulfite addition and other side reactions. Attempts to combine the alkaline flavanone ring opening (**23** → **24**) with hydrogenation (**24** → **25**) resulted only in the internally cyclized product **26**. The direct conversion of **23** to **25** by catalytic hydrogenolysis of the benzylic C-2–O bond also failed. The hydrogenation of **23** (60 psi of H_2, 5% Pd on C) in absolute ethanol resulted in no reaction until a catalytic amount of $HClO_4$ was added, at which point bromide solvolysis occurred to produce **27**. When the reduction was carried out in acetic acid containing $HClO_4$, slow conversion to **30** was found. No intermediate formation of **25** could be detected.

The conversion of flavanone **23** into bromoethylchalcone **24** was found to occur smoothly in 5% aqueous KOH (2 h, 25 °C). The crude product was contaminated only by a small amount of starting material. The hydrogenation of **24** quantitatively afforded bromoethyl-DHC **25** which was then treated with aqueous K_2SO_3 to produce sulfoethyl-DHC **5**.

A number of more direct approaches to the sulfoethyl-DHC were investigated. Sodium 2-bromoethanesulfonate has been reported to alkylate simple phenols (Beringer and Falk, 1959). The attempted alkylation of hesperetin with this reagent was unsuccessful, however, because the elevated temperatures required for this reaction resulted in decomposition. Alkyl vinylsulfonates have been reported to be reactive sulfoethylating agents for phenols (Werner and Distler, 1961). The reaction of hesperetin with methyl vinylsulfonate produced only O-methylation product **28**. The use of isopropyl vinylsulfonate resulted in the formation of desired adduct **29**. However, when this material was hydrogenated in aqueous base only *O*-isopropyl-DHC **31** was obtained. The transformation of **29** into **31** probably involves base-catalyzed desulfoethylation followed by an irreversible isopropylation of the hesperetin produced.

A simple, versatile, and efficient method for the preparation of 4-O-sulfoalkylation derivatives of hesperetin DHC is presented in Scheme III. This route is based on the regioselective alkylation of hesperetin (**22**) with cyclic sulfonate esters (sultones). 1,3-Propane sultone (**32a**) and 1,4-butane sultone (**32b**) were employed directly for the preparations of sulfopropylated DHC **6** and sulfobutylated DHC **7**, respectively. Durst and du Manoir reported the preparation of a variety of substituted sultones from **32a** and **32b** by generation of their α-lithio carbanions (*n*-butyllithium, −78 °C) followed by treatment with alkylating and acylating agents (1969). The treatment of **32a** in this manner with benzylchloroformate, benzaldehyde, and additional 1,3-propane sultone provided, respectively, sultones **32c**, **32d**, and **32e**. The incorporation of these alkylating agents into Scheme III provided substituted sulfopropylated hesperetin DHC derivatives **8**, **9**, and **10**.

1,3-Propane sultone and its substituted derivatives reacted quantitatively with hesperetin at ambient temperature. 1,4-Butane sultone proved less reactive and required heating at 55 °C in order to obtain a complete reaction. Small amounts (<10%) of 3′,7-di-O-alkylation products were produced in these reactions (HPLC analysis), but recrystallization of the crude hydrogenation products normally provided pure sulfoalkylated DHC derivatives.

The difference between the acidities of the three phenolic hydroxyls of sulfopropylated DHC **6** was used to advantage for the preparation of two 2,4-di-O-alkylated

Table II. Sensory Evaluation of Compounds

Compd	Judgments	Concn, ppm	Perceived intensity[c]	Calcd intensity: Wt basis	Calcd intensity: Molar basis	Flavor judgment: Sweet	Sour	Salty	Bitter	Other[d]	Aftertaste[e]
4 (M = Na)	32	250	1.27	432	534	84	1	3	10	2	28
5 (M = K)	12	228	1.88	705	929	83	2	3	7	5	75[f]
5 (M = $Ca_{0.5}$)	12	250	1.63	557	703	84	0	2	7	7	58[f]
6 (M = Na)	12	250	1.45	496	650	80	0	3	9	8	42
6 (M = K)	60	250	1.13	386	525	77	4	5	9	5	65
6 (M = $Ca_{0.5}$)	108	250	1.46	499	650	83	0	3	8	6	78
6 (M = $Mg_{0.5}$)	12	250	1.52	520	665	82	1	3	10	4	50
6 (M = $Zn_{0.5}$)	12	250	1.42	486	650	80	0	1	10	9	50
7 (M = K)	12	250	0.66	226	316	50	0	8	19	23	50[g]
7 (M = $Ca_{0.5}$)	12	250	0.63	215	289	68	0	8	11	13	25[g]
8 (M_1 = H; M_2 = K)	12	250	0.45	154	229	72	9	6	13	0	42[g]
8 (M_1 = M_2 = K)	12	250	0.36	123	197	50	7	8	35	0	67[g]
9 (M = K)	2[a]	1 130				~50			~50		Strong lingering sweetness
10 (M = K	1[b]	1 000		0	0						
11 (M = K)	32	250	0.52	178	233	81	2	2	15	0	31[g]
12 (M = K)	12	255	0.73	245	342	57	0	0	25	18	67
13 (M = K)	12	235	0.66	240	389	28	2	0	38	32	92
14 (M = K)	1[b]	2 000		0	0						
15 (M = K)	1[b]	1 000		0	0						
16 (M = Na)	1[b]	1 000		0	0						
17 (M = Na)	6	400	0.62	133	157	7	2	2	70	19	100 (bitter)
18 (M = K)	1[b]	10 500							100		
2	42	250	1.79	612	1097	80	4	4	12	0	76[f]
3	10	90	0.70	665	592	84	0	2	11	3	60
38	27	90	0.53	504	566	82	0	4	7	7	30[g]
39	12	250	0.90	308	386	74	9	5	11	1	67
Sodium cyclamate	24	1 250	0.53	36	21	75	5	13	4	3	17
Sodium saccharin	17	60	0.32	456	274	75	3	6	12	4	77 metallic-bitter)[g]
Sucrose	221	85 500	1.00	1.0	1.0	97	1	1	1	0	4

[a] Determined by two judges to be intensely and unpleasantly bittersweet and not subjected to detailed panel analysis. [b] Compounds judged tasteless in a preliminary analysis were not analyzed by the sensory panel. [c] Times the 0.25 M (8.55%) sucrose reference. [d] The balance of flavor was made up by tastes described as medicinal, phenolic, and most commonly menthol and licorice-like. [e] Percentage of judgments where presence of unpleasant lingering aftertaste was reported; aftertastes reported were sweet except where noted. [f] Percentage somewhat high due to a high perceived intensity. [g] Percentage somewhat low due to a low perceived intensity.

derivatives. The potentiometric titration (Metrohm Herisau Potentiograph E 576, Brinkmann Instruments) of **6** with tetrabutylammonium hydroxide in aqueous Me_2SO allowed calculation of pK_a values of 10.7 and 13.1 for the hydroxyl groups at positions 6 and 3′, respectively (the hydroxyl group at position 2 is too weakly acidic to titrate under these conditions). These values provide the rationale for the regioselectivity observed in the alkylation of **6**. Disubstituted derivatives **13** and **14** were prepared from the sulfopropylated DHC by treatment with benzyl chloride and 1,3-propane sultone, respectively, in the presence of 1 equiv of base.

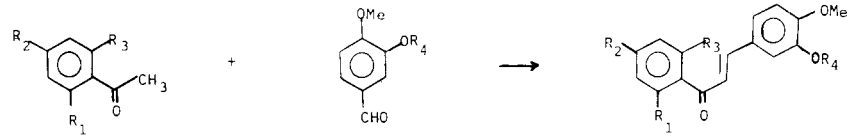
Scheme IV depicts a synthetic route to sulfoalkylated DHC derivatives which begins with nonflavonoid starting materials. This approach was employed to prepare DHC **11** from **37a** via alkylation with 1,3-propane sultone (**32a**). As anticipated, only C-4 O-alkylation took place. Alkylation of the previously reported (Honohan et al., 1976) chalcone **36b** with **32a**, followed by hydrogenation, afforded 2-O-sulfopropylated DHC **15** in good yield. The methylation and hydrogenation of this same chalcone provided **37b**, which on treatment with **32a** under the usual conditions gave 2-*O*-methyl-4-*O*-sulfopropyl hesperetin DHC **12**.

The reaction of phenols with sulfamic acid is reported to provide phenol *O*-sulfate esters (Hofmann and Biesalski, 1912). Hesperetin was treated with this reagent in pyridine and the resulting flavanone sulfate hydrogenated with the expectation that the 4-*O*-sulfate DHC ester would be obtained. The only product obtained, however, proved to be 3′-*O*-sulfate ester **16**. The reason for this unusual selectivity is not clear.

Hydroxylated aromatic compounds are known to react with the *p*-dioxane–sulfur trioxide complex to produce aromatic sulfonic acids (Walsh and Davenport, 1958). The reaction with hesperetin gave the expected sulfonated flavanone, which upon reduction in the standard manner afforded nuclear sulfonated DHC **17**. Phenolics are also known to efficiently react with aqueous sodium bisulfite–formaldehyde to yield C-sulfomethylation products (Suter et al., 1945). Accordingly, the reaction of hesperetin DHC (**3**) under these conditions provided fair yields of C-sulfomethyl DHC **18** along with a lesser amount of a disulfomethylated product.

Sensory Evaluation. The results of the structure–taste evaluations are presented in Table II. Relative taste intensity values were calculated, both on a weight and a molar basis, assuming nonhydrated materials, from perceived intensities relative to a 0.25 M (8.55%) sucrose standard. The later values are considered more useful for the comparison of relative saporific power. Sensory data obtained in the present study on sucrose, saccharin, cyclamate, and neohesperidin DHC are included for purposes of comparison. Also included are previously

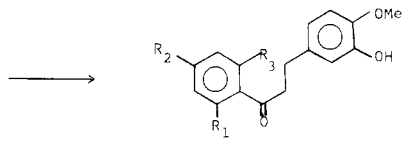
Scheme IV



34a, $R_1 = R_2 = OH$; $R_3 = H$
34b, $R_1 = R_2 = OCH_2Ph$; $R_3 = OH$

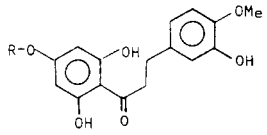
35a, $R_4 = H$
35b, $R_4 = CH_2Ph$

36a, $R_1 = R_2 = OH$; $R_3 = R_4 = H$
36b, $R_1 = R_2 = OCH_2Ph$; $R_3 = OH$; $R_4 = CH_2Ph$
36c, $R_1 = R_2 = OCH_2Ph$; $R_3 = O(CH_2)_3SO_3M$; $R_4 = CH_2Ph$
36d, $R_1 = R_2 = OCH_2Ph$; $R_3 = OMe$; $R_4 = CH_2Ph$



37a, $R_1 = R_2 = OH$; $R_3 = H$
37b, $R_1 = R_2 = OH$; $R_3 = OMe$

reported data on hesperetin DHC (3) and carboxyalkyl DHC salts 38 and 39 (DuBois et al., 1977a).



38, R = CH_2CO_2Na
39, R = $(CH_2)_3CO_2K$
40, R = SO_3Na

All data were determined relative to a 0.25 M sucrose standard and are not intended to provide absolute values for either taste intensities or flavor percentages. In addition, it is recognized that the relative taste intensities and flavor percentages will change when compared with varying concentrations of sucrose and the presence of other taste qualities. Nonetheless, these results are extremely helpful in tracking changes in taste intensity and taste quality as a function of specific structural modifications. It is noteworthy that the taste intensities determined for cyclamate (36×), saccharin (456×), and neohesperidin DHC (612×) compare well with the literature values of 41× (Benson and Spillane, 1976), 330× (Inglett et al., 1969), and 950× (Inglett et al., 1969), respectively. The differences observed are due mainly to differences in the methods of measurement. The many factors affecting this type of sensory evaluation have recently been reviewed (Spencer, 1971).

Evaluation of the taste quality and the relative intensity of individual compounds is a difficult task, even though one is using experienced judges who have received special training. Pure substances rarely exhibit a single taste quality (i.e., sweet, salty, etc.) and usually possess many flavor characteristics. In our analyses, samples were prescreened in order to eliminate materials that are completely bitter or have no perceivable sweetness, and also to allow for the adjustment of test compound concentration such that perceived intensities would approximate that of the sucrose reference. This procedure minimizes the testing of compounds with extremely unpleasant taste qualities, allows for a more reasonable comparison with the sucrose reference, and also minimizes taste carryover. However, it is still possible to encounter situations where interpretation of sensory data is rendered difficult by taste carryover. In one series of tests, sucrose

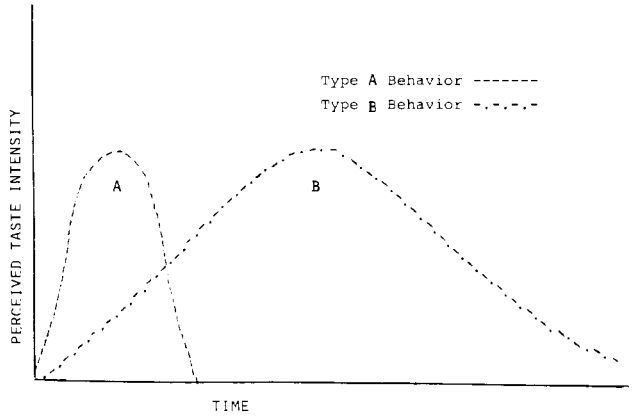


Figure 1. Perceived taste intensity vs. time for type A sweetener (rapid taste onset and cutoff) compared to a type B sweetener (slow taste onset, lingering aftertaste).

(30 800 ppm) was observed to be only 58% sweet with the remaining taste quality assigned to bitterness (24%) and the other categories. This 30 800-ppm sucrose solution is well above the accepted taste threshold concentration of 3400 ppm for sucrose (Amerine et al., 1965). Taken alone, these data are inconsistent with all other results and reflect the complexity of evaluation of the test materials.

The sweetness of neohesperidin DHC (2), as well as the sulfo and sulfoalkyl analogues prepared in this study, is characterized as slow in onset followed by a lingering sweet aftertaste. These taste properties result in unique problems when such materials are evaluated relative to sucrose which exhibits both a rapid taste onset and cutoff. These taste–timing effects are illustrated in Figure 1 by a plot of perceived intensity vs. time. Curve A illustrates the behavior of sucrose, cyclamate, and saccharin, while curve B approximates the relative behavior of most DHC derivatives. For our sensory studies panelists were trained to report maximum perceived taste intensities (i.e., maximum amplitudes of curves A or B) without regard to time. This process is more of a problem with type B compounds since it is more difficult to decide at what point one experiences the maxima in perceived taste intensity.

The lingering sweetness of DHC derivatives was found to affect subsequent evaluations by the panelists. It was observed that intensity values for nonlingering sweeteners were lower if they were tasted after, rather than before, a DHC sample. For this reason, most compounds were

presented to judges in a balanced order so that the effect of taste carryover was minimized for all samples evaluated in a single session. In special cases, compounds with known strong lingering taste properties were reserved as the final sample in a taste session.

The sulfonates prepared in this study were found to have excellent water solubility throughout the pH range. The 4-*O*-sulfopropyl-DHC **6** (M = K), which is representative, was determined by UV measurements to have a solubility of 20.0 g/L (0.043 M) at neutral pH and ambient temperature. The ready dissolution of these salts in water distinguishes them from neohesperidin DHC which after prolonged stirring and heating achieves a solubility of 1.22 g/L (0.002 M) at 25 °C (Nutrilite Products, Inc., 1970). As shown in Table II, intense, pleasant, sweet taste properties were observed for the simple 4-*O*-sulfoalkyl derivatives, especially **4–6**, which compare favorably with neohesperidin DHC (**2**). Although the taste intensity, taste quality, and solubility properties of these three materials are promising, the less than desirable taste–time profiles exhibited detract from their potential general use in food systems. This was determined by evaluating a limited number of the sweeteners in coffee, tea, and cola and comparing with sucrose and saccharin sweetened products.

Sensory panelists were trained to report the observation of any notable aftertastes and especially the presence of lingering sweetness. Unfortunately, the aftertaste results given in Table II do not accurately reflect the relative amounts of lingering sweet aftertaste since this phenomenon appears to be intimately related to the perceived intensity of the sample. Only when comparing equiintense samples is it possible to estimate relative lingering behaviors. Samples of greater perceived intensity will always appear to exhibit more of a prolonged sweet aftertaste.

A qualitative comparison of approximately equiintense solutions of derivatives **4–7** suggested a significant increase in lingering sweet aftertaste with the length of the sulfoalkyl chain (i.e., sulfomethyl-DHC **4** appeared to linger less than sulfoethyl-DHC **5** which lingered less than sulfopropyl-DHC **6**, and which lingered less than sulfobutyl-DHC **7**). Qualitatively, both the delay in onset and the lingering tastes of **4–7** appear to be less notable than observed for neohesperidin DHC. The apparent decrease in sweetness linger on proceeding from **7** to **4** is accompanied by an increase in taste intensity while proceeding in the same direction (with the exception of **4**, vide infra). A regular increase in lipophilic character seems the only apparent change in the series **4–7**. In the case of the *m*-nitroaniline class of sweeteners, an increase in lipophilic character results in an increase in taste intensity. Deutsch and Hansch explained this effect as being the result of increased partitioning from the aqueous saliva phase onto the presumably more lipophilic receptor phase (1966). An opposite effect is observed in the case of **4–7**. It appears that as the lipophilicity increases in the series, the molecular partitioning into all manner of oral lipophilic material also increases so that only after increasingly longer periods of time does the concentration of glucophore at the receptor reach an equilibrium concentration sufficient to elicit a response. This hypothesis is supported by the fact that when very concentrated DHC solutions are sampled the delayed onset characteristic is not observed. In this extreme, all of the oral lipophilic phase, including the less readily available receptor sites, may rapidly be saturated with glucophore thus resulting in rapid taste onset.

If the above hypothesis is correct, equimolar solutions of derivatives **4–7** will exhibit perceived intensity vs. time curves (Figure 2) which are expected to show increased perceived intensities, as measured by the maxima of the curves, in proceeding from **7** to **4**. The total magnitudes of the taste responses, as measured by the integrals of the curves, may be essentially identical for **4–7**, but the actual measured taste intensities, as determined from curve maxima, result in the conclusion that taste intensity increases in the order of **7–4**. The above hypothesis agrees well with the data with the exception that sulfomethyl-DHC **4** is observed to be a less intense sweetener than sulfoethyl-DHC **5**. It seems reasonable, however, that the optimum hydrophilic–lipophilic balance is reached in the case of sulfoethyl derivative **5**. Thus, increasing the hydrophilic character even more, as is the case for **4**, results in a relative decrease in the amount of partitioning of glucophore from saliva onto the lipophilic receptor and therefore a decreased perceived intensity. The slowness in onset and lingering aftertaste of **4** would be expected to be reduced from **5**, as is actually observed.

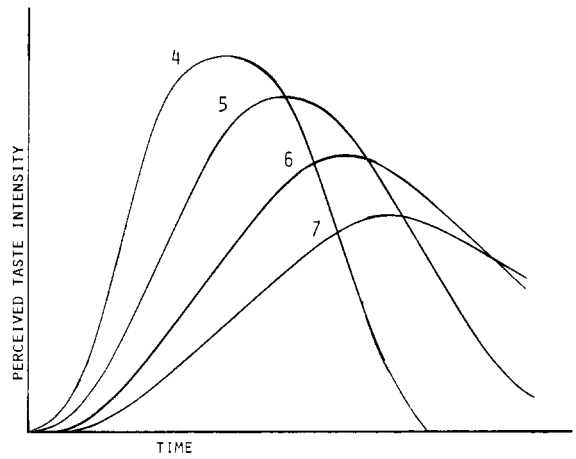


Figure 2. Hypothetic perceived intensity vs. time curves for sulfoalkyl DHC derivatives **4–7**.

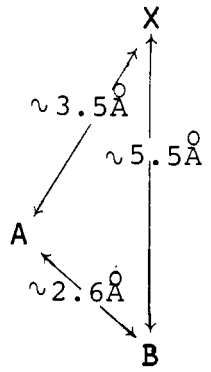


Figure 3. Hypothetical interspatial relationship of three binding sites required for intense sweetness (Kier, 1976).

A further regular decrease in lipophilic character in the series **7–4** would result in phenol *O*-sulfate ester **40**. To determine if further improved taste–timing properties could be observed, the synthesis of **40** was attempted. Unfortunately, sulfation of hesperetin led only to the 3′-*O*-sulfate which upon reduction yielded the hydrolytically unstable DHC 3′-*O*-sulfate **16**. This compound was observed to be tasteless as would be predicted from the work of Horowitz and Gentili on analogues of neohesperidin DHC where the B ring was found to be quite sensitive to structural change (1971, 1974).

Significant increases in the hydrophilic character of sulfopropyl-DHC **6** result either in loss of taste intensity or loss of taste entirely. When the moderately hydrophilic

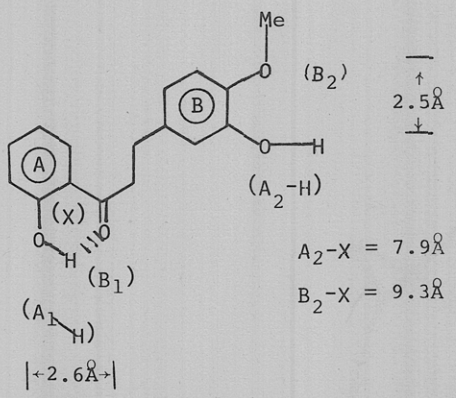


Figure 4. Postulated essential binding sites for DHC sweeteners.

carboxyl functionality is added to the sulfopropyl side chain (DHC **8**), the molar taste intensity decreases significantly (525× → 229×). Substitution of more potent hydrophilic groups such as sulfopropyl (DHC **10**) results in elimination of taste. These results may be understood in terms of decreased partitioning from the aqueous saliva onto the lipophilic receptor sites.

Increases in the lipophilic character of sulfopropyl-DHC **6** via alkyl substitution resulted not only in an increased lingering aftertaste, but also in changes in the taste profile. It was found that even the small hydrophobic methyl substituent (DHC **12**) increased the bitter flavor from 9 (**6**, M = K) to 25%. Further increases in lipophilic character, as in **9**–**13**, resulted in the taste becoming predominantly bitter.

An alternative explanation for the slow taste onset of DHC sweeteners is that some chemical modification of the molecule must occur within the oral cavity before the active glucophore is produced. This could be as simple as complexation with some metal ion present in low concentration in the saliva. For this reason, various salts of DHC derivatives **5**–**7** were synthesized and evaluated. Qualitatively, no decrease in taste onset could be detected. It was found, however, that the bivalent metal salts of **6** are superior to the potassium salt from an intensity standpoint.

One additional explanation for the lingering taste of DHC sweeteners involves a strong and slowly reversible binding to the receptor site. Phenolic compounds are known to strongly bind to protein (Seikel, 1962). The lingering taste characteristics might then, assuming the binding site is proteinaceous as is currently accepted (Dastoli and Price, 1966; Beidler, 1967; Hiji et al., 1968; Price and DeSimone, 1977), be caused by strong hydrogen bonding. If this premise were correct, a decrease in the phenolic character of **6** would be expected to reduce aftertaste. Although any one or a combination of the three phenolic hydroxyls at positions 2, 3′, or 6 could be involved, the 6-hydroxyl would seem most likely as it is the most acidic. The 6-norhydroxyl analogue **11** was prepared and

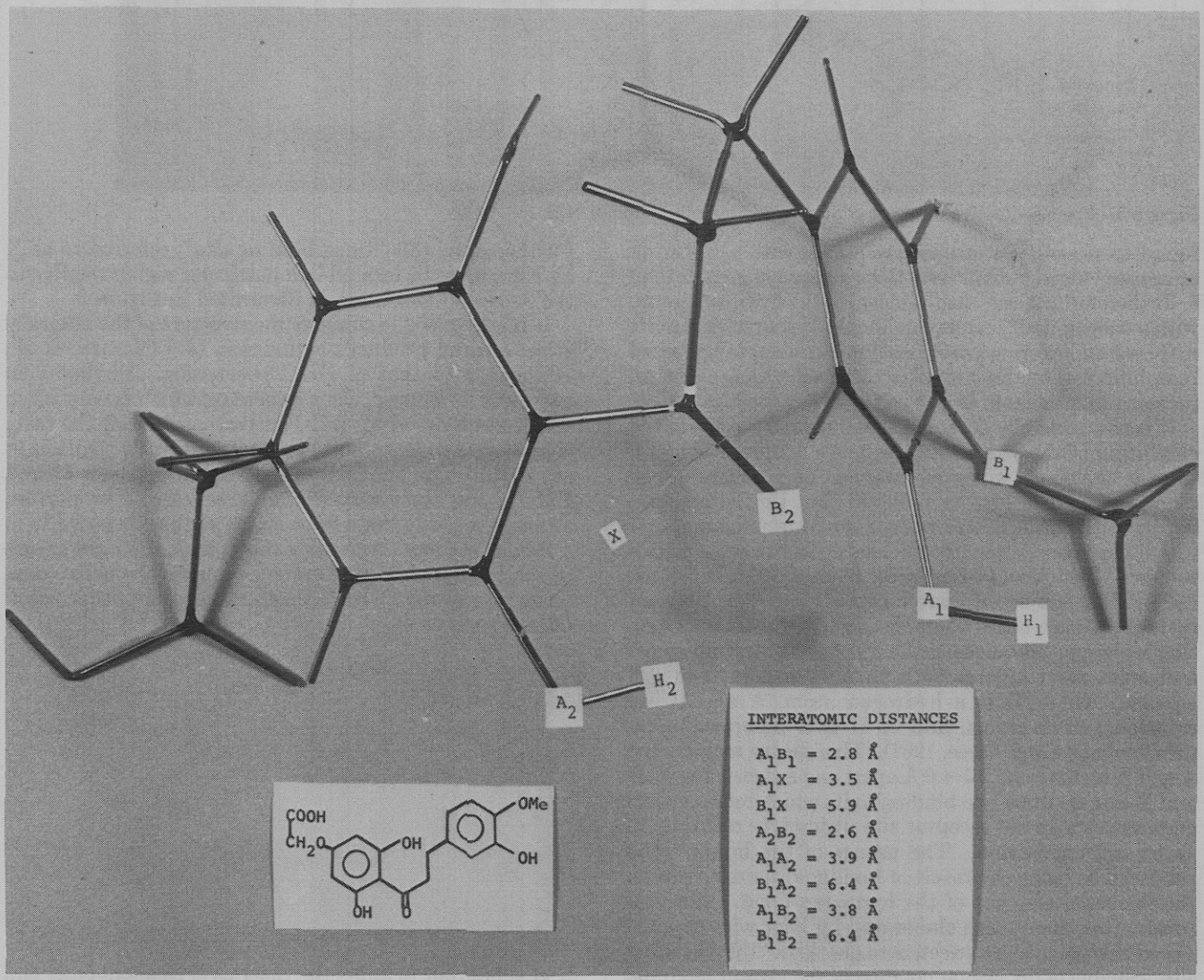


Figure 5. Proposed active or sweet conformation for the DHC molecule illustrated for the case of **38**.

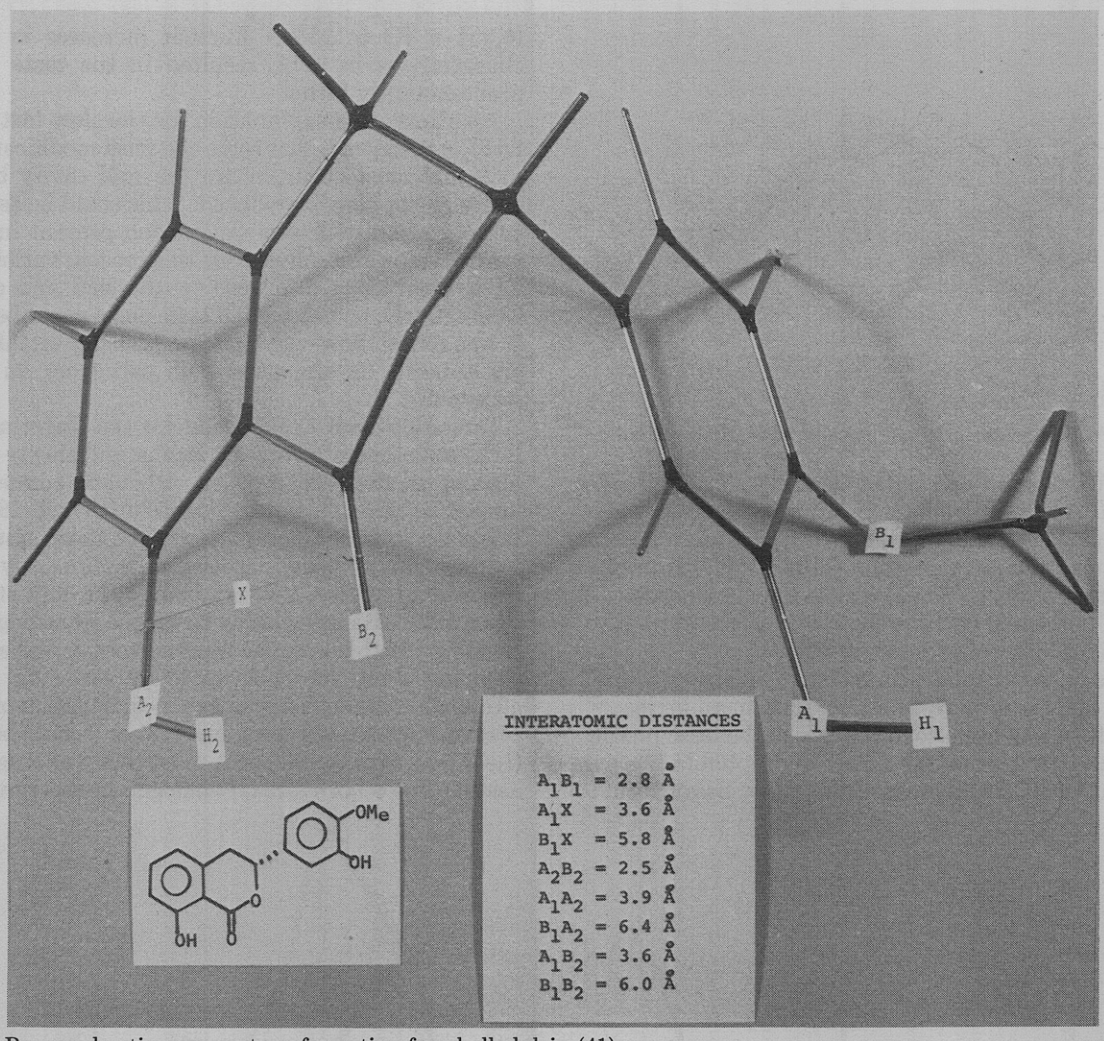
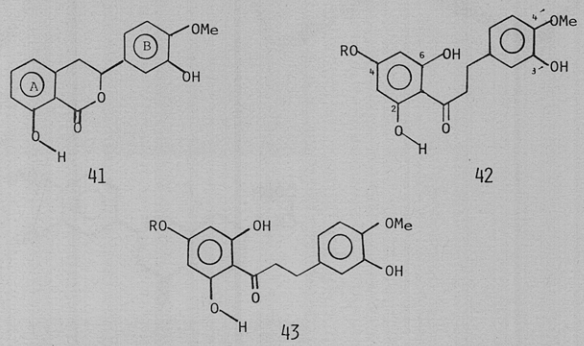


Figure 6. Proposed active or sweet conformation for phyllodulcin (41).

found to possess an intense sweetness with a lingering aftertaste judged qualitatively to be somewhat greater than **6**. It therefore appears that removal of the hydroxyl group, with a concomitant increase in lipophilic character, results in increased lingering sweetness due, possibly, to increased lipophilic interactions similar to those which cause an increase in aftertaste in the series **4**–**7**.

It is now generally accepted that a primary event in the elicitation of the sweet taste response is interaction of a tastant molecule with the exterior of a receptor cell membrane. It is further believed that this interaction occurs with some proteinaceous membrane component which has been frequently referred to as "sweet sensitive protein" (Dastoli and Price, 1966; Beidler, 1967; Hiji et al., 1968). The subject of taste receptors has recently been reviewed (Price and DeSimone, 1977). Some time ago, Shallenberger advanced a theory stating that all sweet molecules must possess both an A–H element and a B element, where H is a hydrogen atom, A and B are electronegative atoms, and A–H is a polarized bond (Shallenberger and Acree, 1967). This theory requires the A and B atoms to be 2.4–4.0 Å apart and suggests the A–H and B centers to be two binding units which interact with counterparts at the receptor site ultimately resulting in sweet taste sensation. The nature of the binding was proposed as being the result of London dispersion forces, the principal element of the hydrogen bond. More recently, this theory was elaborated by Kier who hypothesized that all *intense* sweeteners possess a "third binding site" capable of dispersion bonding of the tastant molecule to the receptor (Kier, 1972, 1976; Kier and Holtje, 1974). Furthermore, this "third binding site", referred to as X by Kier, must be located in a particular region relative to the A–H and B centers as illustrated in Figure 3.

It is interesting to compare the structure of the intensely sweet natural product phyllodulcin (**41**) (Yamato et al., 1972a,b) with that of DHC sweeteners. Phyllodulcin possesses an intense, clean sweetness which is very much like that observed for DHC derivatives in that the taste is both slow in onset and lingers significantly (DuBois et al., 1975). The similarity in structure between **41** and DHC's, and their very similar organoleptic properties, strongly suggests them to be acting at the receptor site in a similar manner. Assuming this is true, the rigid nature of the phyllodulcin ring system requires the active conformation of the DHC to be **42** rather than other possibilities such as **43**.



It is relevant to apply the theories of Shallenberger and Kier to the DHC molecule. Since the 6-OH is unnecessary

for sweetness (cf. **11**), and since a multitude of structural variations can be tolerated in the 4-OR moiety without major changes in taste character (cf. **2–8**, **38**, and **39**), it seems very unlikely that the critical A–H, B, and X binding sites are located in these parts of the DHC molecule. It follows that the essential binding sites for DHC sweeteners must be contained within the formula illustrated in Figure 4. Inspection of this structure suggests two potential A–H/B binding units which both satisfy the spatial requirements described by Shallenberger. If we consider the A-ring A–H/B binding unit to be formally equivalent to Kier's hypothesized X dispersion binding site, measurements with Drieding models show this center to be too distant from the B-ring A–H/B binding sites for the case of a planar DHC molecule as illustrated in Figure 4. If, however, the B ring is rotated so as to move into a plane nearly perpendicular to the A-ring binding unit, the B-ring A–H/B system now becomes spatially related to the X system as required by Kier's model. The result of this manipulation is the "bent" conformation illustrated in Figure 5 which we propose to be the conformation of the DHC molecule active in elicitation of sweet taste.

It seems likely that a similar "bent" conformation is the active or sweet conformation in the case of phyllodulcin as well. This requires the B ring of phyllodulcin to be pseudoaxial with respect to the lactone ring as illustrated in Figure 6.

The hypothesis that the active conformation of DHC sweeteners is nonplanar is not strictly new. This possibility was alluded to in a comparison of the structural aspects of glycosidic sweeteners in which neohesperidin DHC was speculated as having an active "U-shaped" conformation where the legs of the "U" were said to be the hydrophilic B-ring substituents on one side with the disaccharide on the other (Inglett, 1974).

It appears that three binding sites are required in DHC sweeteners. The B-ring center can be thought of as the A–H/B binding sites described by Shallenberger and the A-ring center as the X dispersion binding site discussed by Kier. It should be pointed out that the reverse, where the A-ring center is considered to be an A–H/B binding unit and the B-ring center an X dispersion binding unit, could be considered with equal validity. Alternatively, both A-ring and B-ring units may be considered as A–H/B binding centers. In any case, two binding centers, which must work in concert, appear to be necessary for the sweet taste response to occur.

Since the A ring of the DHC appears to be involved in some sort of binding with the receptor, it is expected that structural modification in this region may result in gross changes in taste properties. Thus, the absence of sweet taste in the 2-*O*-sulfopropyl (**15**), 2,4-disulfopropyl (**14**), 3-sulfo (**17**), and 3-sulfomethyl (**18**) compounds can be understood in terms of interference with the A-ring binding center to the extent that interaction with the sweet taste receptor does not occur.

The proposed "bent" active conformation for phyllodulcin suggests that only one of its enantiomers may be capable of interaction with the receptor since the sweet taste receptor is generally believed to have asymmetric character (Kier, 1976). We are currently investigating the effects of such asymmetry on taste as a probe for information on the topography of the sweet taste receptor.

ACKNOWLEDGMENT

The authors thank N. M. Weinshenker of Dynapol and H. Stone of Tragon Corporation for helpful criticism and stimulating discussions. We would like to thank the Biological Sciences and Food Sciences Departments of Dynapol for their help. In particular, the skillful assistance of T. Seitz, R. Phillips, J. Brown, B. Ryerson, and M. Swartz is gratefully acknowledged. The authors also thank R. M. Horowitz for providing numerous samples.

Supplementary Material Available: Synthetic procedures and physical and spectroscopic properties for dihydrochalcones **4–18** (21 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

Acton, E. M., Leaffer, M. A., Oliver, S. M., Stone, H., *J. Agric. Food Chem.* **18**, 1061 (1970).
Amerine, M. A., Pangborn, R. M., Roessler, E. B., "Principles of Sensory Evaluation of Food", Academic Press, New York, N.Y., 1965, p 88.
Ames, B. N., McCann, J., Yamasaki, E., *Mutation Res.* **31**, 347 (1975).
Barber, H. J., Cottrell, H. J., Green, M. B., *J. Appl. Chem.* **3**, 253 (1953).
Beidler, L. M., in "Olfaction and Taste", Vol. II, Hayashi, T., Ed., Pergamon Press, Elmsford, N.Y., 1967, pp 509–534.
Benson, G. A., Spillane, W. J., *J. Med. Chem.* **19**, 869 (1976).
Beringer, F. M., Falk, R. A., *J. Am. Chem. Soc.* **81**, 2997 (1959).
Booth, A. N., Robbins, D. J., Agricultural Research Service Publication CA 74-18, Western Regional Research Laboratory, U.S. Department of Agriculture, Albany, Calif., 1968.
Booth, A. N., Robbins, D. J., Gumbmann, M. R., Gould, D. H., presented at the Citrus Research Conference, Pasadena, Calif., Dec 4, 1973.
Brown, J. P., Brown, R. J., *Mutation Res.* **40**, 203 (1976).
Dastoli, F., Price, S., *Science* **154**, 905 (1966).
Deutsch, E. W., Hansch, C., *Nature (London)* **211**, 75 (1966).
Doak, S. M. A., Simpson, B. J. E., Hunt, P. F., Stevenson, D. E., *Toxicology* **6**, 139 (1976).
Druckrey, H., Kruse, H., Preussmann, R., *Naturwissenschaften* **55**, 449 (1968).
DuBois, G. E., Crosby, G. A., Saffron, P., *Science* **195**, 397 (1977a).
DuBois, G. E., Crosby, G. A., Saffron, P., *Synth. Commun.* **7**, 44 (1977b).
DuBois, G. E., Crosby, G. A., Stephenson, R. A., Wingard, R. E., unpublished results, 1975.
Durst, T., du Manoir, J., *Can. J. Chem.* **47**, 1230 (1969).
Gilbert, E. E., "Sulfonation and Related Reactions", Interscience, New York, N.Y., 1965, pp 10–11.
Gumbmann, M. R., Gould, D. H., Robbins, D. J., Booth, A. N., presented at the Citrus Research Conference, Pasadena, Calif., Dec 16, 1975.
Helberger, J. H., Sproviero, J. F., *Justus Liebigs Ann. Chem.* **666**, 67 (1963).
Hiji, Y., Kobayashi, N., Sato, M., *Kumamoto Med. J.* **21**, 137 (1968).
Hofmann, K. A., Biesalski, E., *Chem. Ber.* **45**, 1394 (1912).
Honohan, T., Hale, R. L., Brown, J. P., Wingard, R. E., *J. Agric. Food Chem.* **24**, 906 (1976).
Horowitz, R. M., in "Biochemistry of Phenolic Compounds", Harborne, J. B., Ed., Academic Press, New York, N.Y., 1964, p 561.
Horowitz, R. M., Gentili, B., U.S. Patent 3 087 821 (1963); *Chem. Abstr.* **59**, 11650c (1963).
Horowitz, R. M., Gentili, B., *J. Agric. Food Chem.* **17**, 696 (1969).
Horowitz, R. M., Gentili, B., in "Sweetness and Sweeteners", Birch, G. G., Green, L. F., Coulson, C. B., Ed., Applied Science, London, 1971, pp 69–80.
Horowitz, R. M., Gentili, B., in "Symposium: Sweeteners", Inglett, G. E., Ed., Ari Publishing Co., Westport, Conn., 1974, Chapter 16.
Inglett, G. E., in "Symposium: Sweeteners", Inglett, G. E., Ed., Ari Publishing Co., Westport, Conn., 1974, Chapter 20.
Inglett, G. E., Krbechek, L., Dowling, B., Wagner, R., *J. Food Sci.* **34**, 101 (1969).
Kier, L. B., *J. Pharm. Sci.* **61**, 1394 (1972).
Kier, L. B., in "Structure-Activity Relationships in Chemoreception", Benz, G., Ed., Information Retrieval, London, 1976.
Kier, L. B., Holtje, H-D., *J. Pharm. Sci.* **63**, 1722 (1974).

Lauer, W. M., Langkammerer, C. M., *J. Am. Chem. Soc.* **57**, 2360 (1935).
Nutrilite Products, Inc., Technical Bulletin "FLAV-O-LAST #1 (Neohesperidin Dihydrochalcone)", Buena Park, Calif., 1970.
Price, S., DeSimone, J. A., *Chem. Senses Flavor*, submitted for publication (1977).
Rizzi, G. P., Neely, J. S., U.S. Patent 3743716 (1973); *Chem. Abstr.* **77**, 86777 (1972).
Robertson, G. H., Clark, J. P., Lundin, R., *Ind. Eng. Chem., Prod. Res. Dev.* **13**(2), 125 (1974).
Shallenberger, R. S., Acree, T. E., *Nature (London)* **216**, 480 (1967).
Seikel, M. K., in "The Chemistry of Flavonoid Compounds", Geisman, T. A., Ed., Pergamon Press, Elmsford, N.Y., 1962, p 42.
Spencer, H. W., in "Sweetness and Sweeteners", Birch, G. G., Green, L. F., Coulson, C. B., Ed., Applied Science, London, 1971, pp 112–129.
Suter, C. M., Bair, R. K., Bordwell, F. G., *J. Org. Chem.* **10**, 470 (1945).
Ulland, B., Finkelstein, M., Weisburger, E. K., Rice, J. M., Weisburger, J. H., *Nature (London)* **230**, 460 (1971).
Walsh, J. A., Davenport, D. A., presented at the 134th National Meeting of the American Chemical Society, Chicago, Ill., Sept 7–12, 1958.
Werner, H., Distler, H., German Patent 1098202 (1961); *Chem. Abstr.* **55**, 24106 (1961).
Yamato, M., Hashigaki, K., Kuwano, Y., Koyama, T., *Yakugaku Zasshi* **92**, 535 (1972a).
Yamato, M., Kitamura, T., Hashigaki, K., Kuwano, Y., Yoshida, N., Koyama, T., *Yakugaku Zasshi* **92**, 367 (1972b).

Received for review December 15, 1976. Accepted March 11, 1977.

Flavor of Enzyme-Solubilized Fish Protein Concentrate Fractions

Patricio Hevia and Harold S. Olcott*

The soluble portion of fish protein concentrate (FPC) hydrolysates, obtained by treatment with the proteolytic enzymes bromelain, ficin, or Pronase, were fractionated by size and charge. All fractions were tasted. Bitterness and glutamic acid taste (acid-like) were the main contributors to off-flavors in the hydrolysates. Pronase hydrolysates were less bitter than those from bromelain or ficin. Pronase hydrolysates had a higher proportion of low molecular weight peptides (mol wt ~300) than those obtained with bromelain or ficin. The last two exhibited a higher proportion of peptides in the 800 molecular weight region where bitterness was also predominantly found. Paper electrophoresis showed that the bitter fractions had more basic peptides while the acid-like fractions had more acidic peptides. A basic bitter mixture isolated from the 800 molecular weight region of a ficin hydrolysate contained glycine, isoleucine, leucine, phenylalanine, and valine as N-terminal residues. Further separations of this bitter fraction suggested the presence of a basic bitter tripeptide or peptides with the following structures: N-terminus, leucine or glycine; middle, asparagine; C-terminus, lysine.

Proteolytic enzyme hydrolysis of fish protein concentrate (FPC) improves the solubility of the product (Cheftel et al., 1971; Hevia et al., 1976). However, the formation of an undesirable bitter taste is a consistent side effect of the treatment (Cheftel et al., 1971; Fujimaki et al; 1973, 1974; Noguchi et al., 1975). The degree of bitterness in the soluble product differs with the enzyme used. The bacterial protease Pronase has been reported to produce less bitterness than ficin or bromelain (Cheftel et al., 1971; Hevia et al., 1976). In the present study, soluble fish protein concentrates obtained by treatment with Pronase, ficin, and bromelain were compared with regard to molecular weight, charge, and taste of the resultant mixture of peptides. The purpose was to determine the nature of the bitter components.

EXPERIMENTAL MATERIAL AND METHODS

Fish protein concentrate hydrolysates containing equal amounts of soluble products, formed in equal time periods, were obtained by incubation of solvent-extracted FPC with bromelain (25 mg/g of FPC), ficin (40 mg/g of FPC), or Pronase (10 mg/g of FPC) for 1 and 7 h as previously described (Hevia et al., 1976).

The molecular weight distribution of the different hydrolysates was determined by gel filtration through a column of Bio-Gel P-2 polyacrylamide gel (100–200 mesh, Bio-Rad). A total gel bed of 450 mL was packed into a K 26/100 Sephadex column (100 × 2.6 (i.d.) cm). Two hundred milligrams of each of the solubilized fractions was dissolved in 2.5 mL of 10% acetic acid, added to the column, and eluted with 10% acetic acid (0.5 mL/min). Ten-milliliter fractions were collected and freeze-dried. The void volume of the column was determined by the elution of bovine serum albumin (OD at 280 nm). The elution patterns of the hydrolysates or of calibration standards (oxytocin, oxidized glutathione, reduced glutathione, tetraglycine, and glycine) were monitored manually by reaction with ninhydrin as described by Hirs (1967).

The charge distribution of the peptides in the whole hydrolysates and in their Bio-Gel P-2 fractions was determined by paper electrophoresis, conducted in a horizontal Savant high-voltage electrophoresis apparatus provided with a cooling plate on the bottom. The sample was applied to the center of the paper (Whatman No. 3) and covered with a siliconized glass plate. The buffer (10% pyridine adjusted to pH 6.5 with glacial acetic acid) was allowed to reach the application point by capillarity.

Institute of Marine Resources, Department of Food Science and Technology, University of California, Davis, California 95616.