the floral aroma/taste of a beer with Tettnanger (high ratio).

An attempt was made (Table V) to quantify the amount of floral hop aroma/taste one could expect in a finished beer brewed with the same amount of various hops. By the formula explained in the table, each of the flavor components is weighted according to its sensory threshold concentration in beer. In the case of geranyl isobutyrate, the flavor potential is calculated assuming that one-third of that present in the hop is converted to geraniol in the beer. These contributions are added and multiplied by the yield of oil from the hops to give the column denoted as "floral index". We believe this index gives a fairly accurate measure of the amount of floral aroma/taste one can expect in a beer brewed with a standard amount of one of these hops.

The column on the extreme right of Table V is the floral index weighted to a standard amount of α-acids to give an estimation of the relative amount of floral hop aroma/taste one could expect in beers brewed to a standard bitterness by using the various hops. This index may be of more use to a brewer than the floral index. Using the floral index divided by % α-acids column, a brewer can estimate the relative intensity of the overall floral aroma/taste of a beer to a standard bitterness as a function of the hop variety used. A beer brewed with a hop variety with a high value in this column would have a more intense floral aroma/taste than a beer brewed to the same bitterness level with a hop with a lower value. Of course, subtle changes in the floral aroma/taste profile will occur as the linalool/geraniol–geranyl isobutyrate ratio changes in the beers.

ACKNOWLEDGMENT

We thank Donald Griffin for assistance in mass spectrometry and Wanda Gore for assistance in preparing the sensory evaluation samples. We also thank Dr. D. Pierce, Statistics, OSU, for assistance in the statistical evaluation of the data.

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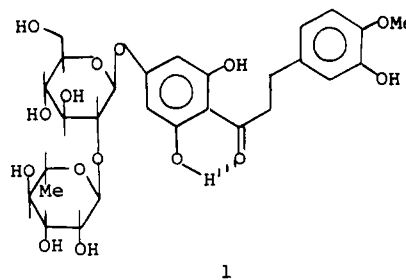
Dihydrochalcone Sweeteners. Synthesis and Sensory Evaluation of a Homoserine–Dihydrochalcone Conjugate with Low Aftertaste, Sucrose-like Organoleptic Properties

Grant E. DuBois,* Guy A. Crosby, Janice F. Lee, Rebecca A. Stephenson, and Patricia C. Wang

Neohesperidin dihydrochalcone (NHDHC; 1), known since 1963 as a potently sweet compound (340 ± 60 times the sweetness of sucrose), is plagued by a non-sucrose-like, lingering, sweet aftertaste. An analogue, 4, involving substitution of the neohesperidosyl moiety of 1 with homoserine ether functionality, is demonstrated to be potently sweet (400 ± 30) and to be the *first* sweet dihydrochalcone to have diminished aftertaste. Three additional amino acid–dihydrochalcone conjugates, 6, 7, and 8, were synthesized and evaluated. Differences in sensory properties were rationalized by changes in hydrophilic–hydrophobic balance as quantitated by the chromatographic parameter k'.

Eighteen years have now elapsed since the original report (Horowitz and Gentili, 1963) on the potent sweetness of the grapefruit-derived flavanoid neohesperidin dihydrochalcone (NHDHC; 1). The pleasant taste of 1 is unfortunately beset with one severe handicap, a long-lingering menthol–licorice-like sweetness, quite unlike that of sucrose, the consumers standard. Within the last 8 years, we have been intensively studying the molecular topography of 1, attempting to design and synthesize analogues which retain NHDHC's potent sweetness but



lack its non-sucrose-like aftertaste (DuBois et al., 1977a,b 1981). We report here the synthesis and sensory evaluation of the *first* NHDHC analogue with significantly decreased aftertaste. In addition, we report the synthesis and sensory

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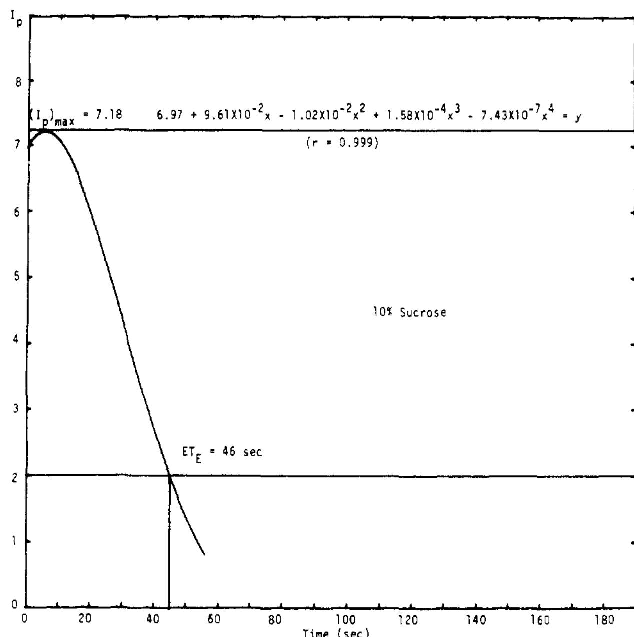


Figure 1. TI curve for 10% sucrose.

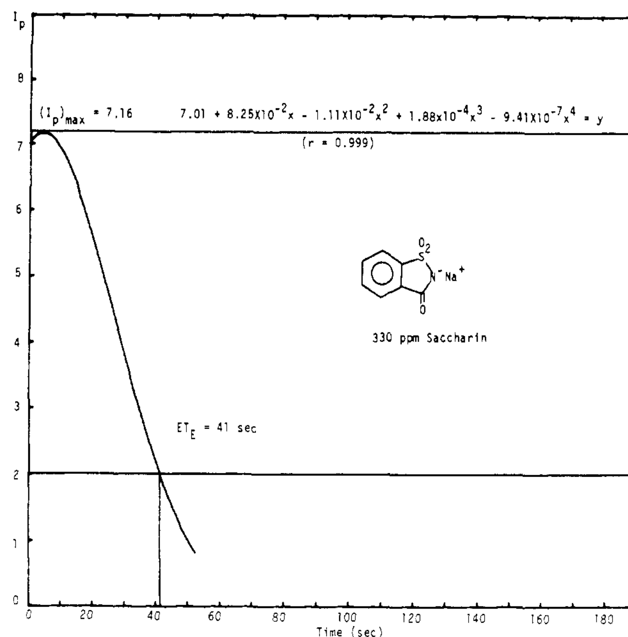


Figure 2. TI curve for 330 ppm of saccharin.

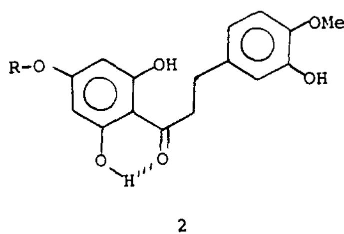
evaluation of three structurally related dihydrochalcone (DHC)–amino acid conjugates which exhibit surprisingly different sensory properties.

SENSORY EVALUATION

The four experimental compounds, which are described below, were evaluated by a human sensory panel. The same criteria, regarding purity and absence of toxicity, which were applied in our earlier work (DuBois et al., 1981) were applied to these materials. Since none of the four synthetic DHCs showed any toxicity, they were subjected to sensory analysis by a trained panel of judges. Panelists were required to carry out magnitude estimation [vs. 0.25 M (8.55%) sucrose] and taste quality (percent sweet, sour, salty, bitter and other) evaluations in one sensory session. From this analysis comparative taste potency data, calculated on both weight (P_w) and molar (P_m) bases, and taste quality data were obtained. One compound, found to have both high taste potency and quality, was subjected to time–intensity (TI) analysis. This method provides a measure of the *time* required for *extinction* of sample taste (ET). All of the sensory methods which were employed for the present study are described in complete detail in our earlier work (DuBois et al., 1981); the parameters I_w and I_m are equivalent to P_w and P_m, respectively, in the present work.

RESULTS AND DISCUSSION

Since beginning our work in the DHC sweetener area, we have prepared some 21 compounds of the general structure **2** (DuBois et al., 1977a,b, 1981). Substituent R



has been varied so as to charge **2** as monoanionic [R = $(CH_2)_nCOONa$, $(CH_2)_nSO_3K$, $(CH_2)_2NHSO_3K$, and $(CH_2)_3PO(OH)(OK)$], dianionic [R = $CH(COONa)_2$, $CH(COONa)CH_2CH_2COONa$, and $(CH_2)_2CH[(CH_2)_3SO_3K]SO_3K$], and cationic [R = $(CH_2)_nNH_3Cl$] species. In general, it was found that the monoanionic dihydrochalcones were potently and cleanly sweet, the dianionic compounds, tasteless, and the cationic dihydrochalcones, bittersweet. Unfortunately, all of the cleanly and potently sweet monoanionic DHCs were plagued by the same lingering sweet aftertaste problem observed for NHDHC. To further assess the effects of charge nature on taste character, we decided to prepare a zwitterionic DHC. First prepared was the homoserine–DHC conjugate **4**, synthesized from hesperetin (**3**) in 60% overall yield as illustrated in Scheme I. In common with most α-amino acids with large alkyl substitutents, **4** exhibited poor water solubility (45.3 mg/L

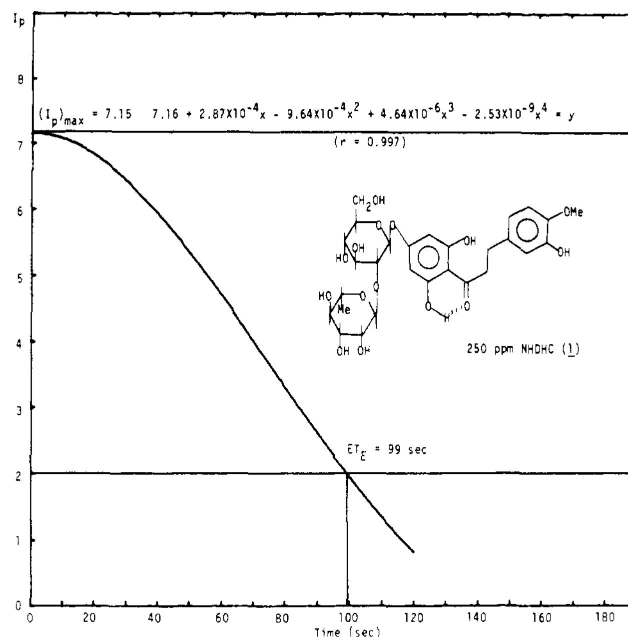


Figure 3. TI curve for 250 ppm of NHDHC.

Table I. Sensory Analysis of NHDHC and Analogues[a]

compd	judgments	concn, ppm	I_p	P_w	P_m	taste character: sweet	sour	salty	bitter	other	sweet: bitter
1[b]	12	240	0.94 (0.17)	340 (60)	600 (110)	77 (14)	0 (0)	0 (0)	8 (5)	15 (10)	90:10
4 (HCl)	16	212	1.0 (0.1)	400 (30)	480 (40)	85 (6)	2 (1)	0 (0)	5 (4)	8 (4)	94:6
6	12	248	0.60 (0.07)	210 (20)	240 (30)	82 (12)	0 (0)	1 (2)	14 (11)	3 (3)	85:15
7 (HCl)	1	1000	0	0	0						
8 (HCl)	4	1000	0.8 (0.3)	70 (30)	110 (50)	60 (8)	20 (24)	0 (0)	13 (15)	7 (10)	82:18

[a] Sensory data are reported as follows: mean value ($2S_m$); statistical calculations were carried out as described by Gordon and Ford (1972). [b] NHDHC.

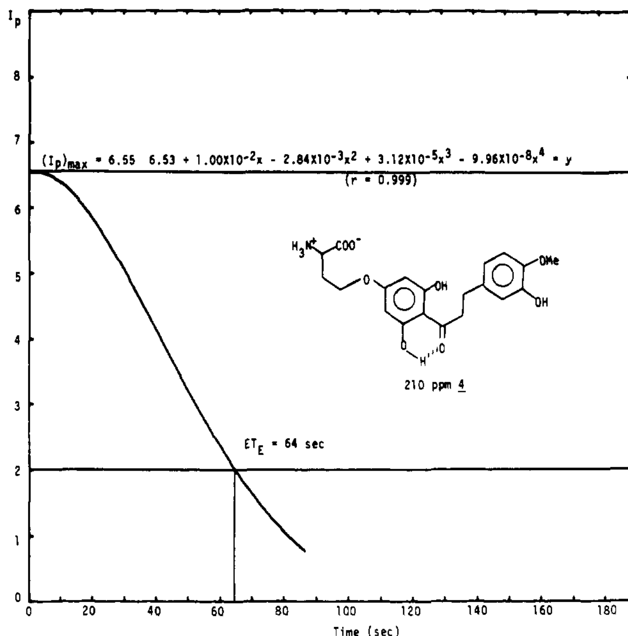


Figure 4. TI curve for 250 ppm of NHDHC analogue 4.

Table II. Normalized Taste Extinction Times (ET_N) for Sucrose, Saccharin, NHDHC, and NHDHC Analogue 4 As Determined by the TI Technique

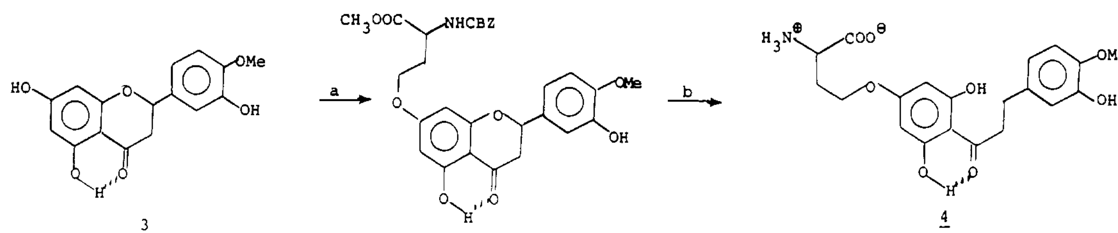
compd	ET_N,[a] s
sucrose	31 (7)
saccharin	26 (7)
NHDHC	85 (11)
4	59 (14)

[a] Normalized extinction times, defined as the times required for the tastes of solutions having "moderate" maximum perceived intensities [$(I_p)_{max} = 6.0$] to decay to a level of "faint" ($I_p = 2.0$), are reported as ET_N ($2S_m$).

at 23 °C). It was soon learned, however, that highly crystalline hydrochloric, sulfuric, and phosphoric acid salts of 4 could be obtained in excellent yield by simple crystallization from the corresponding dilute aqueous mineral acid. These salts dissolved readily in warm water to give solutions which were usually stable, after cooling, for hours. The results of sensory analysis of the hydrochloride salt of 4 are summarized in Table I. We were quite encouraged to note that DHC 4 exhibited a taste having character and potency at least comparable to, if not better than those of NHDHC (1). In addition, it was the general consensus of the panelists that 4 exhibited much less aftertaste than 1. For this reason, we attempted to quantitate the relative aftertastes of 1 and 4 by TI sensory analysis as mentioned above. NHDHC, saccharin, and sucrose were evaluated with 4 in a single session so as the best possible comparison of *times* required to *extinction* of taste would be obtained. *Experimental* extinction times (ET_E) were *normalized* to provide ET_N values which reflect the times required for tastes having a common maximum perceived intensity of "moderate" [$(I_p)_{max} = 6.0$] to decay to a perceived intensity of "faint" ($I_p = 2.0$). The TI curves for sucrose, saccharin, 1, and 4 are illustrated in Figures 1–4, respectively. The normalized extinction times for these four compounds are given in Table II.

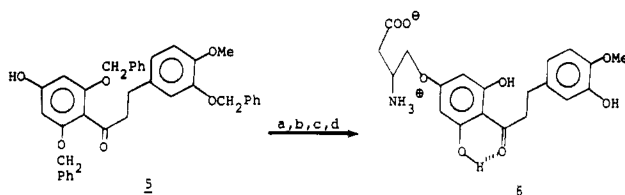
The data of Table II show a significant decrease in sweet taste linger between NHDHC (85 s) and analogue 4 (59 s). This is the first example of a dihydrochalcone rigorously demonstrated to have significantly less aftertaste

Scheme I[a]



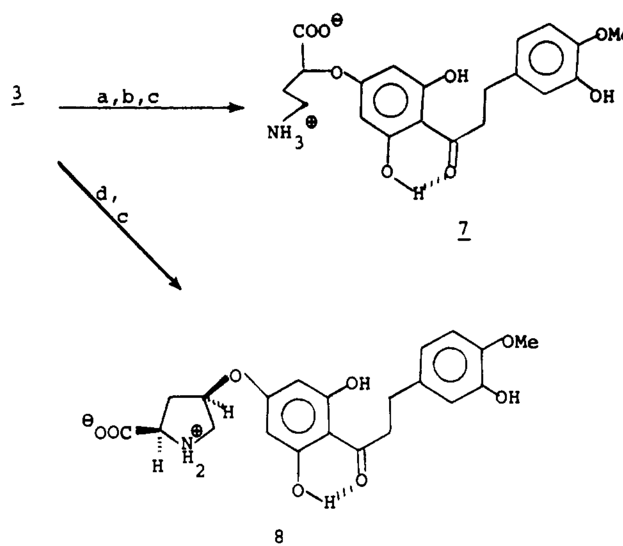
[a] a = K_2CO_3-DMF-$BrCH_2CH_2CH(NHCBZ)COOMe$; b = H_2-Pd/C-10% KOH.

Scheme II[a]



[a] a = K_2CO_3-DMF-$BrCH_2CH=CHCOOCH_3$; b = NH_3-THF; c = KOH-H_2O-THF; d = H_2-Pd/C-THF-H_2O.

Scheme III[a]



[a] a = K_2CO_3-DMF-$BrCH_2CH_2CHBrCOOMe$; b = NaN_3-DMF; c = H_2-Pd/C-10% KOH; d = K_2CO_3-DMF-1-carbobenzoxy-2(*R*)-carbomethoxy-4(*S*)-[(methylsulfonyl)oxy]pyrrolidine.

than NHDHC. Although the aftertaste associated with 4 was not observed to be as low as that of saccharin (ET_N = 26 s) or sucrose (ET_N = 31 s), the improvement was enough that 4 was judged to be significantly better than NHDHC in food systems such as coffee, tea, and carbonated beverages. Although the ET_N obtained for NHDHC in this study is significantly higher than previously obtained (DuBois et al., 1981), the ET_N values for 4 and NHDHC obtained from the same study show them to be significantly different.

The promising sensory results on DHC 4 led to our examination of other zwitterionic DHCs in the hope that a compound exhibiting both low ET_N and improved solubility relative to those of 4 could be prepared. It is generally true (Greenstein and Winitz, 1961) that β-amino acids have solubility greater than their α-amino isomers. The difference is likely due largely to an increase in ionization. For this reason, **6**, the β-amino acid isomer of 4, was prepared, as is illustrated in Scheme II. Encouragingly, a solubility increase of 5.3 times (solubility at 23 °C = 240 mg/L) was observed. Solubility was great enough to allow sensory evaluation of **6** as the free amino acid, the results of which are summarized in Table I. Although β-amino acid **6** exhibited a high quality taste (sweet:bitter = 85:15), we were disappointed to find that the potency was only approximately half that of 4. In addition, even though the perceived sample intensity was only 60% that of the reference, a very high proportion of the panelists reported a notable lingering sweet aftertaste. Thus, the diminished aftertaste property observed for α-amino acid 4 may not be a property common to zwitterionic DHCs but rather a property specific to 4 for reasons unknown at this point.

In an effort to further explore zwitterionic DHCs, amino acid conjugates **7** and **8** were also prepared. They were synthesized from the common starting material, hesperetin (**3**), as is illustrated in Scheme III. It was hoped that the high taste quality and diminshed aftertaste properties of 4 would be retained and solubility improved. Neither **7** nor **8** exhibited significant water solubility and were therefore converted to hydrochloride salts which dissolved readily in warm water to give solutions of the stability required for panel investigation. Quite surprisingly, **7**, a γ-amino acid isomer of the potently sweet 4, was found to be totally tasteless. Also surprising was the observation that hydroxy proline–DHC conjugate **8** exhibited less than 20% of the potency of 4. DHC **8** may be viewed as an analogue of α-amino acid 4 where flexibility has been restricted by methylene bridging of the amino nitrogen and C-1 side chain carbon atoms. Thus it appears that the potent, diminished aftertaste sweetness of 4 is somehow specifically due to the homoserine functionality of 4.

The differences in taste potencies between 4 (400×), **6** (210×), **7** (0×), and **8** (70×) were quite unanticipated. We (DuBois et al., 1981) and others (Esaki et al., 1975; Kamiya et al., 1976, 1978) have prepared a large number of potently sweet NHDHC analogues with quite diverse C-4 functionality. Why then are such great differences in potency observed for the above amino acids which appear to have very similar C-4 substituents? Molecular hydrophilic–hydrophobic balance provides a likely rationalization of this apparent anomaly. In recent years, many differences in various types of biological activity have been successfully correlated with changes in nonspecific hydrophobic effects. In some of the early work of this type, such changes were quantitated by comparison of 1-octanol–water partition coefficients (Hansch and Fujita, 1964). These parameters have been shown to be essential in correlating the potency of nitroaniline sweeteners (Deutsch and Hansch, 1966). More recently, the activity of a wide variety of tastants has been shown to correlate well only with these hydrophobic constants (Greenberg, 1980). Within the last several years, the octanol–water partition coefficient has been largely replaced by the chromatographic parameter k' as a quan-

Table III. Comparative Molecular Polarities of Amino Acid–DHC Analogues 4, 6, 7, and 8 As Quantitated by k' [a]

compd	k'
4	11.0
5	9.6
7	5.8
8	10.0

[a] k' values were calculated by the formula $k' = (t_R - t_0)/t_0$ where t_R is the compound retention time in minutes and t_0 the time for elution of the column void volume.

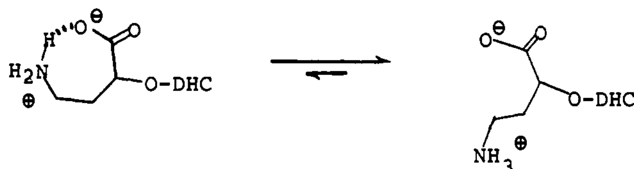


Figure 5. Preferred conformation of NHDHC analogue 7.

titator of hydrophobicity (Tanaka and Thornton, 1977). k' can be considered to be a ratio of the *time* a compound spends interacting with a chromatographic *stationary phase* to the *time* it spends in the *mobile phase*. Reverse-phase high-performance liquid chromatography (HPLC) provides the method of choice for rapid measurement of k' values within a series of compounds. In this system, k' provides a measure of the tendency of a compound to participate in *nonspecific* hydrophobic interactions with a nonpolar stationary phase. k' values thus provide a convenient index for comparison of relative hydrophobicity within a series of compounds. It should be recognized that low k' values are expected for hydrophilic congeners which interact weakly with the hydrophobic stationary phase and that k' will increase within a series as hydrophobic character increases. The k' values which were detd. for the four amino acid–DHC conjugates are given in Table III.

Inspection of the data in Table III leads immediately to some very interesting observations. First, 7, the γ-amino acid isomer of 4 which was found to be tasteless, is noted to be much more polar ($k' = 5.8$) than the other three compounds ($k' = 9.6$–11.0). Quite likely, this increased hydrophilicity prevents partitioning onto a receptor which presumably has significant hydrophobic character. It seems reasonable that the charge separation in 7, which is capable of intramolecular hydrogen bonding only through the energetically disfavored seven-membered ring, as illustrated in Figure 5, causes 7 to behave as a doubly charged, and thus highly polar, compound. The α-amino acids 4 and 8 and the β-amino acid 6 are capable of intramolecular hydrogen bonding through lower energy five- and six-membered rings, respectively, and this is reflected in decreased hydrophilicities as quantitated by higher k' values.

It is also interesting to note that compounds 6 and 8, both having sweetness potencies significantly less than 4, also both exhibit k' values less than that of 4. It thus appears that sweetness potency is quite dependent on hydrophobicity and can be successfully correlated with the relative and nonspecific interactions of the compounds with the hydrophobic stationary phase of a standard reverse-phase HPLC column.

In summary, the differences in potency between the four amino acid–DHCs can be explained quite simply on a polarity basis. The decrease in aftertaste of homoserine–DHC 4 over NHDHC remains unexplained, however. It has been speculated (Boudreau et al., 1979) that receptors for dihydrochalcone sweeteners exist, in the tongue posterior, that are distinct from receptors which interact with sucrose, saccharin, and other sweeteners, located in the tongue anterior. Further, these posterior receptors are said to produce a sweetness of long duration. We have further speculated (DuBois et al., 1981) that dihydrochalcones stimulate both anterior and posterior receptor systems since actual tasting of NHDHC and analogues results in a sweetness quickly perceived at the front of the tongue but which develops slowly in the back and then decays over a lengthy period of time. Sensory evaluation of the homoserine–DHC conjugate 4 results in rapid sensation at the tongue anterior but with little effect in the tongue posterior. Thus, for reasons not clear at this junction, DHC 4 is capable of efficacious interaction with tongue anterior receptors but only poorly with posterior receptors. The reduced aftertaste of DHC 4 may thus be best understood in terms of reduced interaction with tongue posterior sweet taste receptors.

EXPERIMENTAL SECTION

Synthetic Procedures. All organic starting materials and reactants were obtained from Aldrich Chemical Co. except for hesperetin, which was obtained from Sigma Chemical Co. All inorganic reagents were obtained from J. T. Baker Chemical Co. except for 5% palladium on carbon (Pd/C) hydrogenation catalyst (Engelhard Minerals & Chemicals Corp.) and ammonia, anhydrous HBr, and anhydrous HCl which were obtained from Matheson Gas Corp. Solvents used were reagent grade and obtained from either J. T. Baker Chemical Co. or Fisher Scientific Co. The following reaction solvents and reagents were additionally purified by distillation from the respective drying agents (in parentheses) and stored over activated (400 °C; 3 h) molecular sieves, 3A (J. T. Baker Chemical Co.): DMF (CaH_2), Et_3N (P_2O_5), and CH_2Cl_2 (P_2O_5).

Melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer Model 137 infrared spectrometer, ultraviolet–visible (UV–vis) spectra on a Cary Model 119 spectrometer, and proton magnetic resonance spectra (NMR) on a Varian T-60A spectrometer and are reported as parts per million (ppm, δ) relative to tetramethylsilane. Combustion analyses were performed by the Microanalytical Laboratory, Stanford University, Stanford, CA.

Analytical thin-layer chromatography (TLC) was carried out on prelayered silica gel F-254 plates (E. Merck, Darmstadt, Germany), visualizing with either UV light or iodine staining. Preparative thick-layer chromatography was carried out on 20 × 20 cm plates of 2-mm thickness prepared on silica gel PF-254 (E. Merck). Column chromatography was carried out on 60–200-mesh silica gel powder (J. T. Baker Chemical Co.).

High-pressure liquid chromatography (HPLC) was performed on a Waters Associates Instrument equipped with a Model 660 solvent programmer and two Model 6000A pumps. analytical work was carried out on a μBondapak C-18 reverse-phase column (30 cm × 3.9 mm i.d.) eluting with a linear program (15 min; 2 mL/min) of 10–100% CH_3OH in 0.03 M KH_2PO_4 buffer. The detector employed was a Schoeffels Model SF 770 spectroflow monitor equipped with a Model GM 770 monochromator. Capacity factor (k') determinations were carried out on the same column as above, eluting with 40% CH_3OH in 0.03 M KH_2PO_4 and monitoring at 286 nm. Capacity factors were calculated from the relationship $k' = (t_R - t_0)/t_0$ where t_R is the compound retention time and t_0 is the time for elution of the column void volume.

Unless otherwise indicated, all reactions were carried out under an inert atmosphere of argon at ambient temperature with vigorous stirring, magnetic for homogeneous and overhead for nonhomogeneous reactions, and all reagents employed were anhydrous. Standard workup of reactions employing H_2O-immiscible solvents (e.g., Et_2O, CH_2Cl_2, toluene, etc.) involved addition of H_2O, neutralization with aqueous acid or base, extraction of the mixture with an appropriate organic solvent (Et_2O, $CHCl_3$, EtOAc, etc.), drying the combined extracts over $MgSO_4$, and concentration in vacuo. Standard workup of reactions employing DMF solvent involved dilution with H_2O, neutralization, extraction of the mixture with an appropriate solvent (Et_2O, EtOAc, etc.), washing the combined extracts with H_2O (6×), drying over $MgSO_4$ and concentration in vacuo. Hydrogenation reactions were carried out on a Parr hydrogenation apparatus. Standard workup involved filtration through Celite and neutralization with 10% HCl, if the solvent was an aqueous base, followed by product isolation either by filtration or, if an oil, by extraction with EtOAc. If the solvent was organic, it was simply removed in vacuo to yield the product.

The amino acids or mineral acid salts thereof were assayed with regard to purity by a combination of (1) proton titration, (2) chloride titration, (3) HPLC, and (4) Karl Fischer analyses. Titrations were carried out with a Brinkmann Metrohm Herisau Potentiograph E576.

2,3′,6-Trihydroxy-4-(3-amino-3-carboxypropoxy)-4′-methoxydihydrochalcone (4). As has been previously described (Nollet et al., 1969), anhydrous HBr was bubbled vigorously for 3 h, with a gas dispersion tube, into a suspension of 3.64 g (20 mmol) of α-amino-γ-butyrolactone hydrobromide in 180 mL of glacial acetic acid while stirring at ambient temperature. After standing overnight, the reaction mixture was concentrated in vacuo to yield 5.20 g (99%) of 2-amino-4-bromobutanoic acid hydrobromide (**9**) as a white solid. A solution of 5.20 g of carboxylic acid **9** in 25 mL of absolute methanol was saturated with anhydrous HBr and refluxed 72 h. The resultant reaction mixture was concentrated in vacuo to yield methyl 2-amino-4-bromobutanoate hydrobromide (**10**) as an impure viscous oil. The crude amine hydrobromide **10** was dissolved with stirring in 25 mL of water and the resultant solution was cooled to 0 °C. In one portion, 3.70 g (44 mmol) of $NaHCO_3$ was added, followed immediately by 4.10 g (24 mmol) of benzyl chloroformate. The reaction mixture was allowed to warm to ambient temperature over 30 min after which Et_2O extraction and standard workup yielded methyl 2-[(benzyloxycarbonyl)amido]-4-bromobutanoate (**11**) as an oily solid. Recrystallization from hexane–Et_2O yielded 1.96 g of colorless clusters: mp 87–89 °C; IR (KBr) 2.95 (N-H), 5.83 (ester and carbamate C═O) μm; NMR ($CDCl_3$) δ 1.99–2.56 (m, 2 H, BrC—CH_2), 3.40 (t, 2 H, J = 7 Hz, $BrCH_2$), 3.76 (s, 3 H, OCH_3), 4.24–4.69 (m, 1 H, CH-N), 5.10 (s, 2 H, $PhCH_2$), 5.30 (s, 1 H, N-H), 7.36 (s, 5 H, Ph-H) ppm.

A mixture of 3.02 g (10.0 mmol) of hesperetin, 1.52 g (11.0 mmol) of K_2CO_3, and 3.30 g (10.0 mmol) of **11** in 50 mL of DMF was reacted at ambient temperature for 72 h. Standard workup followed by column chromatography over 500 g of silica gel ($CHCl_3$–CH_3OH) yielded 3.68 g (67%) of 3′,5-dihydroxy-4′-methoxy-7-[3-(carbomethoxy)-3-[(benzyloxycarbonyl)amido]propoxy]flavanone (**12**) as a viscous oil: IR (film) 2.96 (O—H, N—H), 5.81 (ester, carbamate C═O), 6.09 (ketone C═O) μm. The flavanone **12** (3.68 g; 6.7 mmol) was dissolved in 100 mL of 10% KOH after which 2.1 g of 5% Pd/C was added and the resultant reaction mixture was hydrogenated at 30 psi. After 15 h, the alkaline reaction mixture was filtered through Celite and acidified to pH 6 with 10% HCl. After the mixture was allowed to stand overnight, filtration yielded 2.44 g (90%) of **4** as tiny light tan clusters: mp 182–184 °C dec; IR (KBr) 2.90–4.20 (O—H, N—H), 6.36 (C═O), 6.42 (C═O) μm; UV (CH_3OH) 226 nm (ϵ 14700), 284 (ϵ 14500); NMR (CD_3OD) δ 2.04–2.55 (m, 2 H, $OCCH_2CN$), 2.51–2.98 (m, 4 H, $ArCOCH_2CH_2Ar'$), 3.82 (s, 3 H, $Ar'OCH_3$), 3.90–4.36 (m, 3 H, OCH_2CCHN), 5.98 (s, 2 H, Ar aromatic H), 6.60–6.93 (m, 3 H, Ar′ aromatic H). Anal. ($C_{20}H_{23}NO_8 \cdot 1.25H_2O$) C, H.

Amino acid **4** (27.0 g, 0.0666 mol) was dissolved with heating in 1400 mL of 1 N HCl. After being cooled to ambient temperature, the crystallization solution was cooled briefly in an ice bath. The crystals were then filtered and allowed to air dry for 3 days to yield 30.0 g of the hydrochloride salt as a fine nonhygroscopic powder. Proton titration indicated this product to be 84.3% DHC 4, while chloride titration indicated 7.1% HCl to be present. Karl Fischer analysis showed the presence of 10.6% water. Combination of proton and chloride titration results indicated the product to be 94.2% amino acid hydrochloride and 5.8% free amino acid.

2,3′,6-Trihydroxy-4-(2-amino-3-carboxypropoxy)-**4′-methoxydihydrochalcone (6).** A mixture of 2.30 g (4.00 mmol) of 2,3′,6-(tribenzyloxy)-4-hydroxy-4′-methoxydihydrochalcone (**5**), 0.83 g (6.00 mmol) of anhydrous K_2CO_3, and 1.43 g (8.00 mmol) of methyl 4-bromo-2-butenoate in 20 mL of DMF was reacted at 0 °C for 40 h after which standard workup yielded 3.66 g of crude 2,3′,6-(tribenzyloxy)-4-(3-carbomethoxy-2-propenoxy)-4′-methoxydihydrochalcone (**13**) as an oily solid. Without purification, the crude **13** was dissolved in 30 mL of dry THF in a Fisher-Porter pressure bottle. While the mixture was cooled to −78 °C, the reaction vessel was charged with 30 mL of anhydrous NH_3 after which the reaction mixture was allowed to warm and was then stirred at ambient temperature for 20 h. The excess NH_3 was then allowed to evaporate after which the THF was removed in vacuo and the residue was chromatographed over 100 g of silica gel ($CHCl_3$–CH_3OH) to yield 1.83 g (66%) of 2,3′,6-(tribenzyloxy)-4-(3-carbomethoxy-3-aminopropoxy)-4′-methoxydihydrochalcone (**14**) as a TLC-pure [$CHCl_3$–CH_3OH (98:2); R_f = 0.18] light yellow solid: IR ($CHCl_3$) 3.19 (N—H), 5.79 (ester C═O), 5.93 (ketone C═O) μm; NMR ($CDCl_3$) δ 1.74 (br m, 2 H, NH_2), 2.48 (t, J = 6 Hz, 2 H, $ArCOCCH_2Ar'$), 2.94 (t, J = 6 Hz, 2 H, $ArCOCH_2CAr'$), 2.97 (m, 2 H, CH_2COO), 3.64 (s, 3 H, $COOCH_3$), 3.75 (s, 3 H, $Ar'OCH_3$), 3.86 (m, 1 H, CHN), 4.97 (s, 6 H, OCH_2Ph), 6.20 (s, 2 H, Ar aromatic H), 6.56–6.88 (m, 3 H, Ar′ aromatic H), 7.33 (s, 15 H, Ph aromatic H).

Twenty milliliters of 10% KOH was added to a solution of 1.83 g (2.65 mmol) of ester **14** in 40 mL of THF while stirring at 0 °C. The resultant solution was allowed to warm to room temperature over 21 h after which the THF was removed in vacuo. The aqueous solution thus obtained was then cooled and neutralized with 10% HCl to yield a voluminous white precipitate. Filtration yielded 1.62 g (90%) of 2,3′,6-(tribenzyloxy)-4-(2-amino-3-carboxypropoxy)-4′-methoxydihydrochalcone (**15**) as a white powder which appeared pure by TLC [toluene–dioxane–AcOH (4.5:4.5:0.8); R_f = 0.23].

Tribenzyl ether **15** (1.62 g, 2.39 mmol) was hydrogenated over 1.0 g of 5% Pd/C at 3 atm for 6 h as a solution in 160 mL of THF–CH_3CH_2OH–H_2O (10:5:1). Standard workup followed by recrystallization (CH_3CH_2OH–H_2O) yielded 341 mg (35%) of **6** as tiny light tan clusters: mp 193–195 °C dec; IR (KBr) 3.0–4.4 (O—H), 6.17 (ketone and car-

boxylate anion C═O) μm; UV (CH_3OH) 226 nm (ε 20 500), 283 (ε 18 600); NMR (Me_2SO-d_6) δ 2.46 (m, 2 H, CH_2COO), 2.83 (t, 2 H, J = 7 Hz, $ArCOCCH_2Ar'$), 3.36 (t, 2 H, J = 7 Hz, $ArCOCH_2CAr'$), 3.80 (s, 3 H, OCH_3), 3.91 (m, 2 H, $ArOCH_2$), 4.20 (m, 1 H, CHN), 6.07 (s, 2 H, Ar aromatic H), 6.56–7.00 (m, 3 H, Ar' aromatic H). Anal. ($C_{20}H_{23}NO_8·H_2O$) C, H.

2,3',6-Trihydroxy-4-(1-carboxy-3-aminopropoxy)-4'-methoxydihydrochalcone (7). A mixture of 3.02 g (10.0 mmol) of hesperetin, 1.52 g (11.0 mmol) of K_2CO_3, and 5.20 g (20.0 mmol) of methyl 2,4-dibromobutyrate in 50 mL of DMF was reacted at ambient temperature for 72 h. Standard workup followed by column chromatography over 500 g of silica gel ($CHCl_3$–CH_3OH) yielded 2.30 g (48%) of 3',5-dihydroxy-4'-methoxy-7-(1-carbomethoxy-3-bromopropoxy)flavanone (**16**) as a colorless oil which solidified on standing. Recrystallization from $CHCl_3$–hexane yielded an analytical sample: mp 130–134 °C; IR (KBr) 2.90 (O—H), 5.74 (ester C═O), 6.14 (ketone C═O) μm; UV (CH_3OH) 284 nm (ε 21 900), 324 (ε 3820); NMR ($CDCl_3$) δ 2.44 (q, 2 H, J = 6 Hz, Br—C—CH_2), 2.73–3.26 (m, 2 H, $ArCOCH_2$), 3.56 (t, 2 H, J = 6 Hz, $BrCH_2$), 3.78 (s, 3 H, $Ar'OCH_3$), 3.90 (s, 3 H, $COOCH_3$), 4.91 (t, 1 H, J = 6 Hz, ArOCHCOO), 5.33 (AB q, 1 H, J = 11 Hz, J = 5 Hz, ArCOCCHAr'), 5.79 (s, 1 H, Ar'OH), 6.06 (s, 2 H, Ar aromatic H), 6.80–7.14 (m, 3 H, Ar' aromatic H), 11.93 (s, 1 H, ArOH). Anal. ($C_{21}H_{21}BrO_8$) C, H.

To a solution of 722 mg (1.50 mmol) of flavanone **16** in 15 mL of dry DMF was added 107 mg (1.65 mmol) of NaN_3. The resultant reaction mixture was stirred vigorously at ambient temperature for 15 h after which standard workup yielded 665 mg (100%) of 3',5-dihydroxy-4'-methoxy-7-(1-carbomethoxy-3-azidopropoxy)flavanone (**17**) as a light yellow oil. Analytical HPLC (70% MeOH in 0.03 M KH_2PO_4; 286 nm) indicated the product (RT = 4.6 min) to be pure, uncontaminated by the starting material (RT = 5.6 min).

Flavanone **17** was dissolved in 75 mL of 10% KOH after which 300 mg of 5% Pd/C was added and the resultant reaction mixture was hydrogenated at 30 psi. After 6 h, the alkaline mixture was filtered through Celite, acidified to pH 5.1, and concentrated to dryness. Trituration with water followed by filtration yielded 567 mg (93%) of dihydrochalcone **6** as a light tan powder. An analytical sample of **6** was obtained by dissolving the crude product with warming in 30 mL of 1 N HCl. When the mixture was cooled, the crystallized hydrochloride salt was filtered and dried. The finely powdered salt was then dissolved in hot (80 °C) water, after which the solution was filtered and allowed to cool slowly, thus yielding **6** as long needle-like crystals: mp 222–224 °C dec; IR (KBr) 2.7–5.2 (O—H), 6.25 (ketone C═O, carboxylate anion C═O) μm; UV (CH_3OH–H_2O, 1:1) 221 nm (ε 22 900), 287 (ε 23 400); NMR (Me_2SO-d_6) δ 2.10–3.60 (m, 8 H, $ArCOCH_2CH_2Ar'$, NCH_2CH_2), 3.77 (s, 3 H, $Ar'OCH_3$), 4.64 (m, 1 H, ArOCHCOO), 6.06 (s, 2 H, Ar aromatic H), 6.46–6.96 (m, 3 H, Ar' aromatic H). Anal. ($C_{20}H_{23}NO_8·0.25H_2O$) C, H, N.

2,3',6-Trihydroxy-4-[2(*R*)-carboxypyrrolidinyl-4-(*R*)-oxy]-4-methoxydihydrochalcone (8). Twenty-five milliliters of absolute CH_3OH was added to 2.30 g (20 mmol) of L-(−)-proline after which the resultant mixture was saturated with anhydrous HCl and refluxed for 72 h. The reaction mixture was then concentrated in vacuo to yield L-(−)-proline, methyl ester, hydrochloride (**18**) as a white solid. The crude amine hydrochloride **18** was dissolved in 25 mL of H_2O to yield a homogeneous solution which was cooled to 0 °C after which 3.70 g (44 mmol) of $NaHCO_3$ was added in one portion followed immediately by 4.10 g (24 mmol) of benzyl chloroformate. After 30 min at 0 °C, the reaction mixture was allowed to warm to ambient temperature over 2 h after which Et_2O extraction and standard workup, followed by precipitation ($CHCl_3$ → hexane), yielded 5.47 g (98%) of TLC-pure [silica gel F-254; hexane–EtOAc (1:1); R_f = 0.12] 1-carbobenzoxy-2-(*R*)-carbomethoxy-4(*S*)-hydroxypyrrolidine (**19**) as a viscous oil. To an ice-cold solution of 4.19 g (15 mmol) of alcohol **19** and 3.33 g (33 mmol) of dry triethylamine in 110 mL of dry CH_2Cl_2 was added dropwise over 30 min 3.45 g (30 mmol) of methane sulfonyl chloride. After 2.5 h at 0 °C, standard aqueous workup with Et_2O followed by high vacuum removal (0.025 mmHg for 24 h at 20 °C) of all volatile materials yielded 5.36 g (100%) of 1-carbobenzoxy-2(*R*)-carbomethoxy-4(*S*)-[(methylsulfonyl)oxy]-pyrrolidine (**20**) as a light yellow oil: IR ($CHCl_3$) 5.71 (ester C═O), 5.85 (carbamate C═O), 7.41 (S═O), 8.53 (S═O) μm; NMR ($CDCl_3$) δ 2.04–2.70 (m, 2 H, NC(COO)CH_2), 2.96 (s, 3 H, CH_3SO_2), 3.43–3.96 (m, 5 H, $COOCH_3$, NCH_2), 4.49 (t, 1 H, J = 7 Hz, NCHCOO), 5.14 (s, 2 H, $PhCH_2$), 5.00–5.43 (m, 1 H, SO_2OCH), 7.34 (s, 5 H, PhH). Anal. ($C_{15}H_{19}NO_7S$) C, H, N, S.

A mixture of 1.16 g (3.85 mmol) of hesperetin, 0.53 g (3.85 mmol) of anhydrous K_2CO_3, and 1.25 g (3.5 mmol) of mesylate **20** in 10 mL of DMF was reacted at 60 °C for 6 days. Standard workup followed by preparative TLC on silica gel (hexane–EtOAc) yielded 0.25 g (13%) of 3',5-dihydroxy-4'-methoxy-7-[1-carbobenzoxy-2(*R*)-carbomethoxypyrrolidinyl-4(*R*)-oxy]flavanone (**21**) as a viscous oil: IR ($CHCl_3$) 2.85 (O—H), 5.70 (ester C═O), 5.85 (carbamate C═O), 6.10 ketone C═O) μm; NMR ($CDCl_3$) δ 2.21–2.67 (m, 2 H, NC(COO)CH_2), 2.70–2.96 (m, 2 H, $ArCOCH_2$), 3.44–3.96 (m, 6 H, $COOCH_3$, ArOCHCN, NCH_2), 3.84 (s, 3 H, $Ar'OCH_3$), 4.37–4.80 (m, 1 H, NCHCOO), 5.17 (s, 2 H, $PhCH_2$), 5.10–5.50 (m, 1 H, ArOCHAr'), 5.94 (s, 2 H, ArH), 6.50 (s, 1 H, Ar'OH) 6.77–7.15 (m, 3 H, Ar'H), 7.34 (s, 5 H, PhH), 11.93 (s, 1 H, ArOH).

The flavanone **21** (0.25 g, 0.44 mmol) was dissolved in 25 mL of 5% KOH after which 0.10 g of 5% Pd/C was added and the resultant reaction mixture was hydrogenated at 38 psi for 16 h. The reaction mixture was then filtered through Celite and acidified to pH 5.0 with 10% HCl. After the mixture was allowed to stand overnight, the precipitate was filtered to give 0.13 g (70%) of **8** as a light tan solid. An analytical sample was obtained by dissolving 98 mg of crude **8** with warming in 15 mL of 1 N HCl. When the mixture was cooled, the crystalline hydrochloride salt was filtered and dried. The finely powdered salt was dissolved in 50 mL of warm (65 °C) water, after which the solution was filtered and allowed to cool slowly, thus yielding 35 mg of **8** as light tan clusters: mp 168–172 °C dec; IR (KBr) 2.65–4.6 (N—H, O—H), 6.15 (ketone C═O), 6.28 (carboxylate C═O) μm; UV (CH_3OH) 225 nm (ε 24 700), 286 (ε 24 400); NMR (Me_2SO-d_6) δ 2.44–2.70 [m, 6 H, $ArCOCH_2CH_2Ar'$, NC(COO)CH_2], 3.40–3.80 (m, 3 H, NCH_2CHO), 3.80 (s, 3 H, $Ar'OCH_3$), 5.96 (s, 1 H, Ar aromatic H), 6.60–6.84 (m, 3 H, Ar' aromatic H). Anal. ($C_{21}H_{23}NO_8·0.75H_2O$) C, H, N.

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Characterization of Meat and Bone Meal Flavor Volatiles

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The vacuum distillate of meat and bone meal (MBM), a pet food ingredient, has been analyzed by WCOT glass capillary gas–liquid chromatography–mass spectrometry. A total of 64 components were identified in this volatile oil. The major volatile identified was 1-hexanal (43% relative concentration). Other major volatiles identified in decreasing order of relative concentration are 1-heptanal, 1-octanal, 3-octen-2-one, 1-nonanal, 1-pentanal, 3,5-octadien-2-one (two isomers), 1-octen-3-ol, and 2-(*n*-pentyl)furan. Other important flavor compounds identified include *trans*-2-alkenals, *trans,trans*-2,4-alkadienals, and several pyrazines. These compounds contributed to the notes characteristic of MBM which may play a role in the acceptance of MBM-containing pet foods by pet and pet owner.

Meat and bone meal (MBM) is a relatively inexpensive protein source that is used extensively in animal feed (meals for poultry and pigs) and pet foods. The rendering industry prepares MBM from viscera, bone scraps, and fallen and unsound animals from the livestock dressing processing. The processing and utilization of MBM have been described by Herbert et al. (1974). The application of meat and bone meal as a food protein concentrate for human use has been described by Nash and Mathews (1971).

The composition of MBM has been studied by Doty (1969) and Nash and Mathews (1971). These authors found MBM to contain 54–59% protein, 10–12% crude fat, 28–29% ash, and 3–6% moisture. Amino acid composition studies indicated that glutamic acid and glycine were the most abundant amino acids. The least abundant amino acids were the sulfur-containing amino acids cystine and methionine.

Few studies exist in the literature that describe the flavor chemistry of meat byproducts which are utilized in pet foods. A recent study by Greenberg (1981) described the flavor volatiles found in poultry byproduct meal (PBPM), a meat meal used in dog and cat foods. The lack of literature reports on meat byproduct volatiles is in contrast to the enormous volume of literature on the flavor chemistry of prime meats used primarily in human consumption. The flavor chemistry of prime meats has been reviewed recently by Wasserman (1979) and Shibamoto (1980).

It is the objective of this study to identify key flavor components in meat and bone meal (MBM). A discussion of how these MBM voltailes may be formed and how they differ from volatiles of prime meats and meat byproducts such as PBPM will also be presented.

EXPERIMENTAL SECTION

Materials. Meat and bone meal was obtained from a major supplier, Badger By-Products Co., Milwaukee, WI. The meal was refrigerated and, within 1 week after purchase, subjected to flavor isolation. The MBM was used "as is" during the isolation procedures.

Authentic chemical reference compounds were obtained from commercial sources (e.g., Aldrich Chemical Co. and Alpha Chemical Co.).

Isolation of the Volatile Flavor: Vacuum Degassing. The volatile flavor components were isolated from a total of 28 lb of meat and bone meal by using a vacuum degassing technique.

Each isolation involved degassing 1600 g of meat and bone meal at room temperature with a pressure of 0.02 mmHg and collecting the volatiles in a series of coiled traps such as those described by Chang et al. (1977) which were immersed in dry ice–2-propanol. At the end of 6 h the volatiles were taken up in redistilled diethyl ether. The ether was dried over anhydrous sodium sulfate, concentrated to 0.5 mL by using a Kuderna-Danish concentrator, and concentrated to a final volume of 0.3 mL under a nitrogen stream.

Capillary GLC–Mass Spectral (GLC–MS) Analysis.

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