with GC retention times longer than those of *N*-nitrosodibutylamine, were observed in most samples.

Second, all samples that were examined were found to contain *N*-nitrosodimethylamine after treatment with acidic sodium nitrite. The identity of NDMA in shrimp sauce was confirmed by GC–MS; the retention times and the mass spectra were indistinguishable for authentic NDMA and for the compound formed upon nitrosation of shrimp sauce. The major ions in the mass spectrum of an NDMA standard occurred at m/z 74 (100%), 43 (46%), 42 (98%), 40 (12%), and 30 (27%); the mass spectrum of the shrimp sauce nitrosation product had major ions at m/z 74 (100%), 43 (43%), 42 (93%), 40 (7%), and 30 (26%). The levels of NDMA were higher in shrimp sauce than in anchovy sauce, and kimchi that had been prepared with shrimp sauce contained higher levels than kimchi that had been prepared with anchovy sauce. This ranking is consistent with the relative levels of trimethylamine oxide, trimethylamine, dimethylamine, and betaine that have been reported for shrimp sauce, anchovy sauce, and kimchi (Chung and Lee, 1976; Pyeon et al., 1976). The NDMA concentrations in fresh Chinese cabbage with added shrimp sauce were very near those that would be expected from the amount of shrimp sauce that had been added to the preparation, i.e., about 3000 μg/kg NDMA was detected in a nitrosated mixture of about 100 g of Chinese cabbage and 25 g of shrimp sauce while about 13000 μg/kg NDMA was detected in nitrosated shrimp sauce (Table II). The concentrations of NDMA found in nitrosated kimchi (34–83 μg/kg) are essentially the same as those (54–63 μg/kg) reported earlier (Kim et al., 1984) and are much lower than the levels found after nitrosation of a mixture of fresh Chinese cabbage and shrimp sauce, implying that kimchi contains a nitrosation inhibitor, e.g., ascorbic acid (Kim et al., 1974). This idea is supported by the observation (Table II) that the addition of a 2-fold molar excess of ascorbic acid (over nitrite) to shrimp sauce prior to nitrosation almost completely suppressed the formation of NDMA.

Registry No. NDMA, 62-75-9; ascorbic acid, 50-81-7; nitrite, 14797-65-0.

LITERATURE CITED

Chung, S. Y.; Lee, E. H. *Bull. Korean Fish. Soc.* **1976**, *9*, 79–110.
Doerr, R. C.; Fiddler, W. *J. Chromatogr.* **1977**, *140*, 284–287.
Fine, D. H.; Rufeh, E.; Lieb, D.; Rounbehler, D. P. *Anal. Chem.* **1975**, *47*, 1188–1111.
Groenen, P. J.; Luten, J. B.; Dhont, J. H.; de Cock-Bethbeder, M. W.; Prins, L. A.; Vreeken, J. W. In "*N*-Nitroso Compounds: Occurrence and Biological Effects"; Bartsch, H.; O'Neill, I. K.; Castegnaro, M.; Okada, M., Eds.; International Agency for Research on Cancer, Lyon, 1982; IARC. Scientific Publication No. 41, pp 99–112.
Harvery, D. C.; Fazio, T.; Howard, J. W. In "Environmental Aspects of *N*-Nitroso Compounds"; International Agency for Research on Cancer, Lyon, 1978; IARC Scientific Publication No. 19, pp 1–52.
Hotchkiss, J. H.; Libbey, J. F.; Barbour, J. F.; Scanlan, R. A. In "*N*-Nitroso Compounds: Anaysis, Formation and Occurrence"; Walker, E. A.; Griciute, L.; Castegnaro, M.; Borzsony, M.; International Agency for Research on Cancer, Lyon, 1980; IARC Scientific Publicaion No. 31, pp 361–373.
Kim, S. H.; Kawabata, T.; Endo, T.; Ishibashi, T.; Matsui, M.; Lee, E. H. *Bull. Jpn. Soc. Sci. Fish.* **1984**, in press.
Marquardt, H.; Rufino, F.; Weisburger, J. H. *Food Cosmet. Toxicol.* **1977**, *15*, 97–100.
Mirvish, S. S. *Toxicol. Appl. Pharmacol.* **1975**, 325–351.
Ohshima, H.; Bartsch, H. *Cancer Res.* **1981**, *41*, 3658–3662.
Pyeon, J. H.; Jeoung, B. Y.; Hwang, K. S. *Bull. Korean Fish. Soc.* **1976**, *9*, 223–231.
Reed, P. I.; Haines, K.; Smith, P. L. R.; House, F. R.; Walters, C. L. *Lancet* **1981**, 550–552.
Ruddell,W. S. J.; Bone, E. S.; Hill, M. J.; Blendis, L. M.; Walters, C. L. *Lancet* **1976**, 1037–1039.
Sander, J. *Arch. Hyg. Bakteriol.* **1967**, *151*, 22–28.
Schlag, P.; Ulrich, H.; Merkle, P.; Bockler, R.; Peter, M.; Herfarth, C. *Lancet* 1980, 727–729.
Tannenbaum, S. R. *Lancet* 1983, 629–631.
Tannenbaum, S. R.; Moran, D.; Rand, W.; Cuello, C.; Correa, P. *JNCI, J. Natl. Cancer Inst.* **1979**, *62*, 9–12.

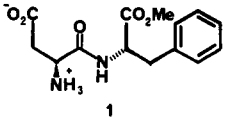
Received for review April 19, 1984. Accepted September 20, 1984. This work was supported by the Korea Science and Engineering Foundation and by U.S. Public Health Service Grant 1-PO1-CA26731-04 awarded by the National Cancer Institute, Department of Health and Human Services.

Design of New Artificial Sweeteners Based on Aspartic Acid Amides

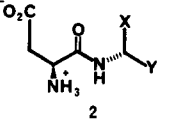
George P. Rizzi

The syntheses and taste properties of several new aspartic acid amide sweeteners are described. Infrared and circular dichroism data were used to demonstrate differences in molecular conformations due to hydrogen bonding.

Since the discovery (Mazur et al., 1969) that certain α-amides of L-aspartic acid tasted sweet, much research has been done to establish molecular requirements for the sweet taste (Pavlova et al., 1981). Despite the dozens of sweet compounds synthesized, only one, α-L-aspartyl-L-phenylalanine methyl ester (Aspartame), **1**, entered commercial development and recently gained FDA approval for use in foods (Beck, 1978).



1



2

Compounds like **1** have attracted much attention because of their potent sweet taste, which when diluted is more similar to sucrose than other artificial sweeteners. Also, these compounds are completely free of bitter aft-

The Procter & Gamble Co., Miami Valley Laboratories, Cincinnati, Ohio 45247.

0021-8561/85/1433-0019$01.50/0

Table I. *N*-(α-L-Aspartyl)-2-amino-1-alkanols or -alkenols, 4

compd	R	C-2 stereochemistry	mp, °C	$[\alpha]_D$, deg (c)[a]	method of synthesis	rel sweetness (ref sucrose concn, %)
4a	i-C_3H_7	*S*	197–204.5	+8.3 (1.07)	A	0
4b	i-C_4H_9	*S*	229–231	−3.1 (1.05)	C	0
4c	i-C_5H_{11}	*S*	229.5–231	+13.9 (1.21)	C	25× (7.6)
4d	i-C_5H_{11}	*R*	191–192.5	+20.5 (1.20)	C	0
4e[b]	i-C_5H_{11}	*S*	179–180	+19.3 (1.02)	C	14× (2.7)
4f	n-C_5H_{11}	*S*	229.5–230	+7.5 (1.03)	C	25× (7.6)
4g	n-C_5H_{11}	*R*	199.5–200	+20.6 (0.73)	C	0
4h[c]	i-$C_3H_7CH{=}CH$	*S*			A	32× (4.7)
4i	i-$C_3H_7CH{=}CH$	*S*	230–231	+19.6 (0.57)	A	5× (2.0)
4j	$(CH_3)_2C{=}CHCH_2$	*S*	219–220.5	+11.2 (1.86)[d]	D	23× (4.4)

[a] Solvent 0.5 N HCl unless otherwise specified; temperature 22–25 °C; concentration in %, w/v. [b] *O*-Methyl ether derivative. [c] Z:E ratio was 5:95 by HPLC analysis. [d] Acetic acid solvent.

Table II. *N*-(α-L-Aspartyl)-3-amino-2-alkanols or -alkenols, 5

compd	R	stereochemistry C-2	stereochemistry C-3	mp, °C	$[\alpha]_D$, deg (c)[a]	method of synthesis	rel sweetness (ref sucrose concn, %)
5a	i-C_5H_{11}	*S*	*S*	197.5–200	+4.8 (1.04)	B	60× (8.5)
5b	i-C_5H_{11}	*R*	*S*	182–184	+11.6 (2.21)	B	5× (2.4)
5c	i-C_5H_{11}	*S*	*R*	216–217	+31.3 (1.31)	B, C	0
5d	i-C_5H_{11}	*R*	*R*	190.5–192	+29.2 (0.72)	B	0
5e	n-C_5H_{11}	*S*	*S*	205–206	−1.4 (1.84)	B	35× (5.3)
5f[b]	i-$C_3H_7CH{=}CH$	*S*	*S*	186–187		D	29× (2.8)
5g[c]	i-C_5H_{11}	*R*	*S*	196–198	−18.6 (1.00)[d]	B	0

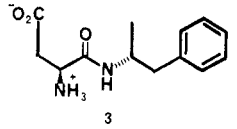
[a] Solvent 0.5 N HCl unless otherwise specified; temperature was 22–25 °C; concentration (c) in %, w/v. [b] HPLC indicated a Z:E isomer ratio of 1:3. [c] *O*-Methyl ether derivative. [d] Methanol.

ertaste and lingering sweetness that often accompany the taste of potently sweet substances.

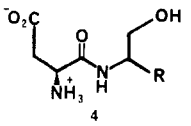
If one considers artificial sweeteners from a design standpoint, additional factors must be included besides potency and quality of sweetness. We envision the ideal product as one that is toxicologically safe, hydrolytically stable, water soluble, and cheap to manufacture, as well as being potently sweet. This report describes part of our effort to develop novel aspartic acid amide sweeteners keeping in mind the criteria listed above.

Basic Design Considerations. The sweet taste of aspartic acid amides depends greatly on molecular structure and stereochemistry at chiral carbon atoms. Many sweet amides have the general structure **2** in which X is a small group like carbomethoxy or methyl and Y is a larger, usually hydrophobic group.

For stability reasons we avoided amides where X was an ester group, since compounds like **1** are prone to chemical change and loss of sweetness, especially at neutral pH (Beck, 1978). Simple amides analogous to **1** without an ester group, i.e., **3**, are also potently sweet (Mazur, 1973)



3



4

and predictably more stable to the mild hydrolytic conditions associated with food preparation and storage. Unfortunately, enzyme-mediated hydrolysis of **3** in the gut could lead to formation of amphetamine, a well-known CNS-active agent. Thus, for toxicological reasons the use of α-L-aspartyl-2-aminoalkanes is limited by the biological activity of amines formed on hydrolysis. In a related study it was shown that vasopressor and myocardial stimulant activity of 1,5-dimethylhexylamine were lessened by substitution of a hydroxyl group on the alkyl chain (Marsh and Herring, 1951). Thus, we concluded that sweeteners based on aspartic acid amides of ethanolamine derivatives could have the advantage of increased hydrolytic stability and reduced toxicity (Brand, 1982). At the onset of our work, others had demonstrated that side-chain hydroxylation of **3** led to diminished potency, **4** (R = benzyl; 1X; Mazur et al., 1970). However, we found that replacing the benzyl group by isopentyl, i.e., **4c** (Table I) led to a product with moderate potency, 25×.

In an attempt to synthesize molecules with hydrolytic stability greater than **4**, we prepared the methyl homologues, **5** (Rizzi, 1983a,b), Table II. Within this series, compound **5a** exhibited the greatest potency (60×).

MATERIALS AND SYNTHETIC METHODS

N-(α-L-Aspartyl) amino alcohols were prepared by four conventional peptide-forming reactions (Bodanszky et al., 1976) starting with method A, employing methyl α-L-aspartate (Kovacs et al., 1963), method B, employing *N*-*t*-Boc-L-aspartic acid, α-*p*-nitrophenyl ester, β-benzyl ester (Bachem Corp.), method C, employing *N*-Cbz-α-L-aspartic acid β-benzyl ester (Bachem Corp.) in the form of a mixed carboxylic–carbonic anhydride (Anderson et al., 1966), or method D, employing *N*-trifluoroacetyl-L-aspartic acid anhydride (Lapidus and Sweeney, 1973) and appropriately substituted amino alcohols. Optically pure amino alcohols and their *O*-methyl ethers were obtained commercially, e.g., valinol, leucinol, and phenylalaninol (Aldrich Chemical Co.) or synthesized by standard techniques of organic chemistry (Brand, 1982; Rizzi, 1983a,b). Compounds **7a** and **7b** were previously described (Mazur et al., 1970). Compound **8** was prepared by method B using 4-methylpentylamine obtained via *d*-carvone-catalyzed decarboxylation (Rizzi, 1976) of homoleucine (Brand, 1982). Common reagents and solvents were of analytical grade and purity. Palladium-on-charcoal catalyst (5%) was purchased from Matheson Coleman and Bell.

Product structures were confirmed by proton nuclear magnetic resonance (NMR), infrared spectroscopy (IR), optical rotation, and combustion analysis. The 60-MHz NMR spectra were obtained in deuterated solvents on a Varian Associates T-60 spectrometer. NMR data are expressed in the format chemical shift (δ) in ppm downfield

from tetramethylsilane (Me_4Si) reference, with multiplicity designated by s = singlet, d = doublet, t = triplet, and m = complex multiplet, followed by spin–spin coupling constants (J), peak area, and structural assignment.

IR spectra were recorded in KBr disks on a Perkin-Elmer Model 298 spectrophotometer. Optical rotations were measured on a Rudolf Autopol polarimeter. Circular dichroism data were obtained on aqueous solutions by using a Jasco J-500C instrument calibrated with *d*-camphor-10-sulfonic acid. Melting points were obtained in open capillaries and were not corrected. Microcombustion analyses were performed by Galbraith Laboratories, Knoxville, TN.

Product purity was confirmed by thin-layer chromatography (TLC) or by high-performance liquid chromatography (HPLC). TLC was done on 0.25-mm precoated silica gel plates (F-254, E. Merck) by using 4:1:1 butanol–acetic acid–water (v/v) or methyl *tert*-butyl ether solvents. Spots were visualized with ninhydrin spray or by ultraviolet light (UV). Analytical HPLC was done on a 15 × 0.46 cm Supelcosil LC-18 column by using 10:90 acetonitrile–water solvent buffered at pH 6.0 with 0.02 M phosphate and 0.025 M sodium perchlorate. The flow rate was ca. 1.5 mL/min through a UV detector set at 220 nm.

Preparative-scale HPLC was used to isolate geometric isomers of the unsaturated amide, **4h,i**. Separation was performed on a 10-μm LiChrosorb RP-18 column (30 × 0.4 cm. i.d.) by using 12:88 acetonitrile–water solvent buffered at pH 3.0 with 0.02 M phosphate and 0.32 M sodium perchlorate at a flow rate of 1.5 mL/min. Products were isolated from pooled fractions following removal of buffer components by passage through a short column of Bio-Rad AG 50W-X8 resin (80–100 mesh, H^+ form). Examples of synthetic methods A–D are given below.

***N*-(α-L-Aspartyl)-(2*S*)-2-amino-3-methyl-1-butanol, 4a (Method A).** A mixture containing methyl α-L-aspartate (0.71 g, 0.0048 mol), L-valinol (0.56 g, 0.0054 mol), and methanol (1.0 mL) was stirred and heated at reflux for 0.5 h. An additional 0.5 mL of methanol was added to facilitate stirring over a total reaction time of 2.25 h. Tetrahydrofuran (THF) (15 mL) was added to complete the precipitation of product that was filtered, recrystallized from hot water (8 mL), and vacuum-dried to give 0.413 g of glistening plates: mp 197–204.5 °C dec (39% yield); NMR (D_2O/DCl, external Me_4Si reference) δ 0.82 [d, J = 6 Hz, 6 H, $(CH_3)_2CH$], 1.77 (m, 7 Hz, 1 H, $(CH_3)_2CH$), 3.00 (d, 7 Hz, 2 H, CH_2CO_2H), 3.55 (br s, 3 H, CH_2OH and $CHCH_2OH$), and 4.30 (t, 6 Hz, 1 H, $CHNH_3^+$). Anal. Calcd for $C_9H_{18}N_2O_4$: C, 49.53; H, 8.31; N, 12.84. Found: C, 49.52; H, 8.38; N, 12.76.

***N*-(α-L-Aspartyl)-(3*S*,2*R*)-3-amino-2-methoxy-6-methylheptane, 5g (Method B).** A solution containing 0.203 g (0.00128 mol) of (3*S*,2*R*)-3-amino-2-methoxy-6-methylheptane in 5 mL of dry THF was treated with *N*-(*tert*-butoxycarbonyl)-L-aspartic acid, α-*p*-nitrophenyl ester, β-benzyl ester (0.577 g, 0.0013 mol) and stirred for 18 h at 25 °C. Following vacuum removal of THF, ether was added, and the resulting solution was exhaustively extracted with 5% aqueous sodium carbonate to remove *p*-nitrophenol. The colorless ether solution was dried (Na_2SO_4) and evaporated to give 0.646 g of crude coupled product. Recrystallization from toluene followed by ethyl acetate–hexane gave pure, diprotected **5g**: mp 120.5–121.5 °C; $[\alpha]^{25}_D$ −16.95° (*c* 0.767, $CHCl_3$). Anal. Calcd for $C_{25}H_{40}N_2O_6$: C, 64.63; H, 8.68; N, 6.03. Found: C, 64.72; H, 8.55; N, 5.92.

Deprotection of **5g** was done in two steps. A solution of recrystallized diprotected **5g** from above, in acetic acid (15 mL), was treated with 0.2 g of 5% Pd–charcoal catalyst and subjected to hydrogenation at 21 °C and 1 atm of pressure. After 2 h when gas absorption ceased, thin-layer chromatography (TLC) indicated debenzylation was complete. Following filtration of the catalyst HOAc was evaporated to afford 0.531 g of *N*-*t*-Boc-**5g** as a pale brown oil. For *t*-Boc cleavage the monoprotected **5g** (0.531 g) was treated with 5 mL of 70:30 v/v trifluoroacetic acid–water and heated 30 min at 85–90 °C in an open flask. After removal of excess acid under vacuum, the residue was dissolved in 95:5 v/v HOAc–water (10 mL) and transferred to a 7 cm × 20 mL column of Bio-rad AG50W-X8 ion exchange resin (50–100 mesh, H^+ form). The column was eluted with 100 mL of 5:95 HOAc–water, water (50 mL), and 1 N ammonium hydroxide (200 mL). Evaporation of the latter two eluants and recrystallization of the residue from methanol–ethyl acetate gave 0.125 g of colorless **5g**: mp 196–198 °C dec; NMR (d_4-MeOH, Me_4Si) δ 0.92 [d, 6 Hz, 6 H, $(CH_3)_2CH$] and 1.13 (d, 6 Hz, 3 H, CH_3CH–O) above a broad envelope of unresolved peaks at 0.67–1.90 ($CHCH_2CH_2$, total area 14 H), 2.65 (d, 8 Hz, 2 H, $CH_2CO_2^-$), 3.38 (s, 3 H, CH_3O), and 3.57–4.30 (group of unresolved peaks, 3 H, CH^+NH_3, $CHOCH_3$, and CHNCO). Anal. Calcd for $C_{13}H_{26}N_2O_4 \cdot H_2O$: C, 53.40; H, 9.65; N, 9.58. Found: C, 53.91; H, 9.57; N, 9.76.

***N*-(α-L-Aspartyl)-(2*S*)-2-amino-4-methyl-1-pentanol, 4b (Method C).** A solution containing 2.10 g (0.00588 mol) of *N*-carbobenzoxy-L-aspartic acid β-benzyl ester in dry THF (50 mL) was stirred in an argon atmosphere at −15 °C (dry ice–ethanol bath) and treated with *N*-methylmorpholine (0.656 mL, 0.00588 mol). The cold mixture was treated dropwise during 3 min with 0.80 mL (0.0061 mol) of isobutyl chloroformate, and stirring was continued for 10 min at ca. −20 °C to complete the formation of the mixed anhydride. A solution of L-leucinol (0.706 g, 0.0060 mol) in 1.5 mL of THF was added dropwise (2–3 min) to the cold anhydride, and after 15 min at −15 °C the mixture was allowed to slowly warm to 25 °C. THF was evaporated, and a solution of the residue in ethyl acetate was washed with 0.1 N HCl, water, and saturated aqueous sodium bicarbonate and finally dried over anhydrous magnesium sulfate. Evaporation of ethyl acetate gave *N*-Cbz-**4b** β-benzyl ester, which was recrystallized from benzene–hexane to obtain 2.30 g of colorless solid: mp 94–95 °C; $[\alpha]^{25}_D$ −23.92° (*c* 1.02, MeOH). Anal. Calcd for $C_{25}H_{32}N_2O_6$: C, 65.77; H, 7.07 N, 6.14. Found: C, 65.93; H, 7.31; N, 6.05.

Deblocking of doubly protected **4b** was accomplished in one step by hydrogenolysis. A solution of *N*-Cbz-**4b** benzyl ester (1.70 g) in acetic acid (25 mL) was treated with 0.55 g 5% Pd–charcoal catalyst and agitated under hydrogen gas at 50 psig at 25 °C for 2 h. Filtration of the catalyst followed by vacuum evaporation of solvent and recrystallization from aqueous ethanol gave 0.743 g of **4b**, mp 229–231 °C dec, as shiny platelets: NMR (HOAc-d_4, Me_4Si) δ 0.95 [d, 6 Hz, 6 H, $(CH_3)_2CH$] and 1.45 (d, 7 Hz, $CHCH_2CH$) above an unresolved envelope of peaks 1.18–1.85 [$CH(CH_3)_2$, total integral 3 H], 3.05 (d, 6 Hz, 2 H, CH_2CO_2H), 3.52 (br s, 2 H, CH_2O), 3.82–4.32 (unresolved m, 1 H, CH–NHCO), and 4.50 (t, 6 Hz, 1 H, CH–NH_3^+). Anal. Calcd for $C_{10}H_{20}N_2O_4$: C, 51.70; H, 8.68; N, 12.06. Found: C, 51.66; H, 8.63; N, 11.82.

***N*-(α-L-Aspartyl)-(2*S*)-2-amino-5-methyl-4-hexen-1-ol, 4j (Method D).** Under anhydrous conditions a solution of (2*S*)-2-amino-5-methyl-4-hexen-1-ol (0.822 g, 0.0063 mol) in 4 mL of THF was added dropwise to a stirred slurry of *N*-(trifluoroacetyl)-L-aspartic acid anhydride (2.11 g, 0.010 mol) in 20 mL of THF at 0 °C. After

1 h at 0 °C the mixture was allowed to slowly reach ca. 25 °C, and THF was removed under vacuum. The residue was treated with 20 mL of 7.4 N ammonium hydroxide and heated at 85–90 °C for 5 min to cleave the trifluoroacetamide. Evaporation to dryness in vacuum gave 0.43 g of solid, which was recrystallized from hot water to yield 0.219 g of **4j**: mp 219–220.5 °C dec; NMR (HOAc-d_4, Me_4Si) δ 1.63 (s, 3 H, CH_3), 1.72 (d, 1 Hz, 3 H, CH_3), 2.25 (t, 2 H, CH_2CH=), 3.05 (d, 6 Hz, 2 H, $CH_2CO_2^-$), 3.72 (br s, 2 H, CH_2O), 3.97 (m, 5 Hz, 1 H, CH–NHCO), 4.50 (t, 6 Hz, 1 H, $CHNH_3^+$), and 5.13 (t, 6 Hz, 1 H, CH_2CH=). Anal. Calcd for $C_{11}H_{20}N_2O_4$: C, 54.08; H, 8.25; N, 11.47. Found: C, 54.14; H, 8.39; N, 11.46.

Sensory Method. The sweet taste of aspartic acid amides was measured by comparing the taste of their aqueous solutions with sucrose.

Five to ten male subjects were picked at random from a group of ca. twenty persons who had previously been selected on the basis of proven tasting acuity, i.e., persons who could easily recognize the four basic tastes (sweet, sour, bitter, and salty) and who were adept at quantifying their physiological responses numerically. Subjects were asked to taste and expectorate 10 mL of aqueous solutions containing test compounds. Unknowns were compared with five standard sucrose solutions containing 0.04 M (1.4%) to 0.35 M (12%) sucrose. Standards were letter coded A–E with concentration increasing alphabetically. Letters were designated on a ballot by using a closed linear scale. Subjects indicated levels of sweet taste by marking the scale at points they considered equal in sweet taste among the standards. A graduated numeric scale was later superimposed on the lettered scale to obtain numerical data that were averaged. Reference sucrose concentrations were determined by referring to a plot of standard sucrose concentrations vs. the numeric scale. The potency of sweet taste reported in Tables I and II is the ratio of reference sucrose concentration to the actual concentration of novel sweetener.

The panel's ability to quantitate sweet taste was checked periodically with sucrose solutions of known strengths. With five panelists, the precision for determining reference sucrose concentrations and hence potency of sweet taste was ca. ±10%.

Because potency is known to vary inversely with sucrose reference concentration (Homler, 1984) exact comparisons of potencies are possible only if the same reference is used. We attempted to evaluate novel sweeteners at practical sucrose levels (5–10%) insofar as compound solubilities permitted.

Novel sweeteners were subjected to an acute oral toxicity test in rats including necropsy and examination of tissue sections prior to taste testing in humans. None of the test compounds showed toxic effects when dosed to rats at 100× the maximum human exposure.

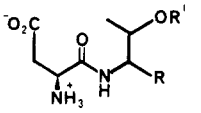
A sweet taste quality very similar to that of sucrose was observed for all of the novel aspartic acid amides.

RESULTS AND DISCUSSION

Substitution of a small isoalkane group for the R group in **4** led to a series of compounds with varying potency, Table I. Optimum potency was obtained with the isopentyl derivative, **4c**, 25×. Lower homologues **4a** and **4b** although water soluble were completely tasteless. Hydrocarbon chains containing more than five carbon atoms led to water-insoluble compounds that were not useful as sweeteners. Minor structural change in R going from **4c,d** to straight chain analogues **4f,g** had little effect on potency, but stereochemical configuration at C-2 had to be *S* for the compound to exhibit sweet taste. C-2 *R*-configured compounds **4d** and **4g** were completely tasteless. The hydroxyl group in **4c** undoubtedly plays an important role in potency since a more potently sweet compound (100×) is obtained by replacing the hydroxyl by hydrogen (Mazur et al., 1970). O-Methylation of **4c** leads to a partial loss of sweet taste, which was surprising in view of its close structural similarity to the methyl ester **2** [X = COOMe, Y = $(CH_3)_2CHCH_2CH_2$], reported to be 160× (Mazur et al., 1973).

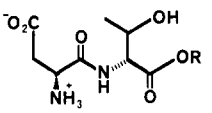
The presence of an olefinic bond in **4** also affects sweetness potency. Disubstituted olefins **4h** and **4i** with a double bond at C_3–C_4 were observed to be significantly different from each other. Amide **4i** with a cis olefinic bond is nearly tasteless compared to 32× observed for the trans isomer **4h**. The lack of sweetness in **4i** is probably related to the shorter overall Y-group length resulting from geometric restraint of the cis bond. A similar effect of Y-group length on sweet taste was already seen by comparing the tastes of **4a–c**, and the phenomenon has been well documented by earlier workers (Ariyoshi, 1976). A molecule containing a trisubstituted olefinic bond at C_4–C_5, **4j**, is indistinguishable from its saturated counterpart, **4c**, by taste potency or sweetness quality.

In a separate study we sought to improve the hydrolytic stability of **4** by adding a methyl substituent at C-1 (Rizzi, 1983a,b). The resulting compounds, **5**, Table II, are



5a–5f (R'=H)

5g (R'=CH_3)



6

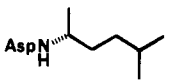
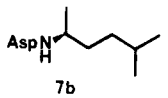
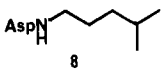
structurally similar to the α-L-aspartyl-D-threonine esters **6**, previously shown to be potently sweet (Ariyoshi et al., 1974). Structures with two chiral carbon atoms in the amino alcohol moiety, e.g., **5** can exist in four diastereomeric forms, and for R = i-C_5H_{11} we obtained **5a–d**. Sweet taste potency was highly dependent on the absolute configurations at C-2 and C-3. The most potent isomer (60×) proved to be the *S,S* (threo) compound, **5a**. Erythro isomer **5b** [C-2 (*R*), C-3 (*S*)] is also sweet, but potency was greatly reduced (5×). Both isomers with the *R* configuration at C-3, **5c** and **5d**, are completely tasteless. Our taste results on **5c** and **5d** closely parallel the Japanese experience with **6** in that they observed no sweet taste for amides of L-threonine or L-allothreonine esters.

Small structural changes in the Y group of **2** did not lead to increased potency of sweetness. Straight chain analogue **5e** had reduced potency compared to **5a**. Also, introduction of a double bond, **5f**, led to significantly less sweet taste potency (29×).

It was reported that hydroxyl groups in compounds like **6** are involved in intramolecular hydrogen bonding and that this phenomenon was related to increasing sweet taste potency (Ariyoshi, 1979) by rendering the X group more hydrophobic. In the following discussion we describe our use of infrared and circular dichroism spectroscopy to probe the effects of hydrogen bonding on conformations of **4** and **5** and the possible ramifications of these effects upon sweet taste potency.

Results of Infrared Spectral Analysis. The degree of intramolecular hydrogen bonding (IHB) and its possible relationship to sweet taste in *N*-(α-L-aspartyl) amino alcohols was investigated by infrared (IR) spectroscopy. The position of IR bands for NH and C=O functions served to indicate the degree of IHB between hydroxy-substituted X groups and the peptide bond, Table III. The parent

Table III. Infrared Data for Aspartic Acid Amides[a]

compd	IR absorption bands, cm^{-1}: amide (NH stretch)	amide I (C=O)
4c	3260	1668
4d	3290	1655
4e	3280	1660
4f	3260	1670
4g	3280	1655
5a	3270	1665
5b	3240	1670
5c	3300	1650
5d	3310	1660
5g	3300	1663
 **7a**	3300	1660
 **7b**	3300	1650
 **8**	3300	1655

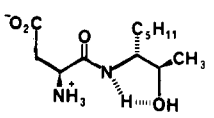
[a] Asp = α-L-aspartyl.

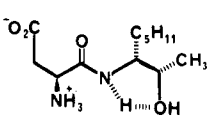
amide **8**, which has no substituent adjacent to the amide nitrogen, showed strong absorptions at 3300 cm^{-1} (amide NH) and 1655 cm^{-1} (amide I, C=O) characteristic of a peptide bond with only intermolecular hydrogen bonding (Bellamy, 1966). Similar behavior was observed for methyl derivatives **7a** and **7b**. The presence of a hydroxymethyl group led to IHB, evidenced by a shift of NH absorption to lower frequencies (3260–3290 cm^{-1}) in compounds **4c**, **4d**, **4f**, and **4g**.

Some compounds with IHB, e.g., **4c**, also exhibited higher frequency amide carbonyl absorptions (1655–1670 cm^{-1}) relative to **8**. This behavior was consistent with greater carbon–oxygen double bond character and less involvement of the carbonyl oxygen in intermolecular hydrogen bonding.

When spectra of diastereomeric pairs **4c**, **4d** and **4f**, **4g** were compared, it became apparent that sweet isomers **4c** and **4f** were more prone to IHB than tasteless compounds **4d** and **4g**. Apparently the degree of IHB was dependent on the stereochemical configuration of the carbon atom attached to the amide nitrogen (C-2 for amides in Table I). Sweet-tasting hydroxymethyl amides with the *S* configuration at C-2 exhibited greater IHB, evidenced by a lower NH stretching frequency than tasteless isomers with the *R* configuration.

A similar trend was observed for amides **5a–d**, whose amino alcohol moieties contained two chiral carbons. Sweet compounds **5a** and **5b** with the *S* configuration at


5a


5b

C-3 (Table II) showed greater evidence of IHB via their NH absorptions at 3270 and 3240 cm^{-1} than C-3 *R* configured diastereomers **5c** and **5d**. In compounds **5a** and **5b** the difference in sweet taste potency was apparently due to the absolute configuration of the C-2 methyl groups in structures held conformationally rigid by IHB.

Although the IR data provided evidence for IHB effects in hydroxyalkyl amides, the importance of the effect on sweet taste was still uncertain.

Table IV. Circular Dichroism Data for Aspartic Acid Amides

compd	concn[a]	λ_m[b]	$[\theta]$[c]	$\Delta\epsilon$[d]
4c	0.60	201	+9300	+2.83
4d	0.60	196	+7100	+2.15
4e	3.30	207	+9670	+2.93
5a	0.60	204	+7790	+2.36
5b	0.60	204	+8290	+2.51
5c	0.60	191	+12200	+3.68
		220	−460	−0.14
5d	0.60	190	+9790	+2.97
5g	0.60	210	+10920	+3.31
7a	1.63	204	+5440	+1.65
7b	0.79	200	+2900	+0.88
8	1.23	205	+4350	+1.32

[a] $\times 10^{-3}$ g/cm^3 in water. [b] Wavelength at CD maxima/minima, nm (±2 nm). [c] Molecular ellipticity, deg cm^2/dmol at λ_m. [d] Differential dichroic absorption at λ_m, L/(mol cm).

O-Methylation was used to assess the importance of IHB to sweetness in hydroxyalkyl amides **4c** and **5b**. It was assumed a priori that methylation of the hydroxyl groups would lead to reduced IHB and result in reduced potency of sweet taste. O-Methylation is known to lead to less IHB in the case of salicylic acid (Gould, 1959). Methylation of **4c** gave the methyl ether **4e**, which had reduced potency, 14×, and showed IR evidence of slightly less IHB than **4c**.

The methyl ether **5g** derived from **5b** was completely tasteless, and its IR spectrum showed no evidence for IHB. The results of methylation provided indirect evidence that IHB might be important for sweet taste in compounds **4c** and **5b**. Our conclusion must be considered tentative because the increased size of the X group due to methylation may in itself have contributed to loss of potency.

Circular Dichroism Studies. Circular dichroism (CD) measurements can be used to determine conformations and configurations of organic molecules in solution (Crabbé, 1965). The CD of the peptide bond has been extensively studied in proteins; however, simple chiral amides have received less attention. Pertinent CD data for 11 aspartic acid amides are given in Table IV.

All compounds exhibited strong positive Cotton effects with broad maxima at 190–210 nm (λ_m) probably resulting from $\pi \rightarrow \pi^*$ electronic transitions of the amide chromophor. A weak negative Cotton effect was observed for **5c** at 220 nm, apparently due to a lower intensity $n \rightarrow \pi^*$ transition. Molecular ellipticity, $[\Theta]$, is sensitive to changes in configuration within the amino alcohol moiety. Diastereoisomeric pairs **4c**, **4d** and **7a**, **7b** with a single chiral carbon adjacent to amide nitrogen were readily distinguished by 31 and 88% differences in $[\Theta]$ values, respectively.

L, *S* isomer **4c** and its *O*-methyl ether **4e** gave nearly the same value of $[\Theta]$, suggesting that they had similar conformations in solution. This result paralleled our IR spectral analysis of **4c** and **4e**, which had already shown that certain conformations could be preferred due to IHB.

CD parameters are also sensitive to structural changes in amides with two chiral centers in the amino alcohol moiety, **5a–d**. Sweet amides **5a** and **5b** with L, *S*, *S* and L, *S*, *R* stereoconfigurations, respectively, have similar values of λ_m and $[\Theta]$. Nonsweet diastereomers **5c** (L, *R*, *S*) and **5d** (L, *R*, *R*) gave parameter values that differed from each other and from values obtained from **5a** and **5b**. Taken together, these data suggested that **5a** and **5b** had similar conformations in solution, possibly as a consequence of hydrogen bonding. IHB is known to occur in aqueous solution, and the effect has been quantitated for certain dibasic acids (Hammond, 1956).

In contrast to the behavior of **4c**, O-methylation of sweet amide **5b** led to a tasteless product, **5g**. The CD parameters of **5g** differed significantly from those of the parent compound and reflected an obvious conformational difference in solution. The absence of IHB in **5g** is probably the reason for enhanced conformational mobility.

ACKNOWLEDGMENT

I thank Sanford Kirksey, Jr., for analytical support, especially in connection with HPLC method development and preparative separations, and Allen J. Fehl for obtaining the circular dichroism data. I also recognize the fine technical assistance of Mary Bruns, Richard S. Echler, and Dennis Toepker.

LITERATURE CITED

Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1966**, *88*, 1338.
Ariyoshi, Y. *Agric. Biol. Chem.* **1976**, *40*, 983.
Ariyoshi, Y. *ACS Symp. Ser.* **1979**, *No. 115*, 133–148.
Ariyoshi, Y.; Yasuda, N.; Yamitani, T. *Bull. Chem. Soc. Jpn.* **1974**, *47*, 326.
Beck, C. I. in "Low Calorie and Special Dietary Foods"; Dwivedi, B. K., Ed.; CRC Press: West Palm Beach, FL, 1978; pp 59–114.
Bellamy, L. J. "The Infra-red Spectra of Complex Molecules", 2nd ed.; Wiley: New York, 1966; p 203.
Bodanszky, M.; Klausner, Y. S.; Ondetti, M. A. In "Peptide Synthesis", 2nd ed.; Wiley: New York, 1976.
Brand, L. M. U.S. Patent 4 338 346, 1982.
Crabbé, P. "Optical Rotatory Dispersion and Circular Dichroism In Organic Chemistry"; Holden-Day: San Francisco, CA, 1965.
Gould, E. S. "Mechanism and Structure In Organic Chemistry"; Holt, Rinehart and Winston: New York, 1959; p 209.
Hammond, G. S. In "Steric Effects In Organic Chemistry"; Newman, M. S., Ed.; Wiley: New York, 1956; pp 426–436.
Homler, B. E. In "Aspartame, Physiology and Biochemistry"; Stegink, L. D.; Filer, L. J., Jr., Eds.; Marcel Dekker: New York, 1984; Chapter 11.
Kovacs, J.; Kovacs, H. N.; Ballina, R. *J. Am. Chem. Soc.* **1963**, *85*, 1839.
Lapidus, M.; Sweeney, M. *J. Med. Chem.* **1973**, *16*, 163.
Marsh, E. F.; Herring, D. A. *J. Pharmacol. Exp. Ther.* **1951**, *102*.
Mazur, R. H. In "Sweeteners"; Inglett, G. E., Ed.; AVI Publishing Co.: Westport, CT, 1973; pp 159–163.
Mazur, R. H.; Goldkamp, A. H.; James, P. A.; Schlatter, J. M. *J. Med. Chem.* **1970**, *13*, 1217.
Mazur, R. H.; Reuter, J. A.; Swiatek, K. A.; Schlatter, J. M. *J. Med. Chem.* **1973**, *16*, 1284.
Mazur, R. H.; Schlatter, J. M.; Goldkamp, A. H. *J. Am. Chem. Soc.* **1969**, *91*, 2684.
Pavlova, L. A.; Komarova, T. V.; Davidovich, Yu. A.; Rogozhin, S. V. *Russ. Chem. Rev. (Engl. Transl.)* **1981**, *50*, 316.
Rizzi, G. P. *ACS Symp. Ser.* **1976**, *No. 26*, 122–132.
Rizzi, G. P. U.S. Patent 4 423 029, 1983a.
Rizzi, G. P., unpublished synthetic work, 1983b.

Received for review July 2, 1984. Accepted October 17, 1984.

Polychlorinated Biphenyls: Congener-Specific Analysis of a Commercial Mixture and a Human Milk Extract

Stephen Safe,* Lorna Safe, and Michael Mullin

On the basis of the relative retention times and response factors of all 209 synthetic polychlorinated biphenyls (PCBs), this paper reports the first congener-specific analysis of a commercial PCB preparation, Aroclor 1260, and the PCB composition of a human milk extract. The analysis indicates that Aroclor 1260 contains nearly 80 different PCB congeners with the major components identified as 2,2',3,4',5',6-, 2,2',4,4',5,5'-, 2,2',3,4,5,5'-, and 2,2',3,4,4',5'-hexachlorobiphenyl and 2,2',3,3',4,4',5-, 2,2',3,3',4,5,6'-, 2,2',3,4,4',5,5'-, and 2,2',3,4',5,5',6-heptachlorobiphenyl. In contrast, the major PCB components of the human milk fraction were the 2,4,4'-tri-, 2,4,4',5-tetra-, 2,2',4,4',5-penta-, 2,3',4,4',5-penta-, 2,2',3,4,4',5'-hexa-, 2,2',4,4',5,5'-hexa-, 2,2',3,3',4,4',5-hepta-, and 2,2',3,4,4',5,5'-heptachlorobiphenyls. The significance of congener-specific PCB analysis is discussed in terms of the structure–activity effects on PCB persistence, bioaccumulation, and toxicity.

Polychlorinated biphenyls (PCBs) are highly stable industrial chemical products that are synthesized by the direct chlorination of biphenyl. Commercial PCBs are distinguished by their stability and resistance to breakdown by acids, bases, oxidation, and reduction, their miscibility with numerous organic solvents, their nonflammability, and their excellent electrical insulation properties. Because of these highly desirable physical properties PCBs have enjoyed widespread use as industrial fluids, flame retardents, diluents, hydraulic fluids, and dielectric fluids for capacitors and transformers. Due to their widespread use, careless disposal practices, and environmental stability, PCBs have been widely identified in diverse environmental matrices including fish, wildlife, and domestic animals, rivers, lakes, and oceans and their underlying sediments, aquatic and marine flora, air, rain, and snow (Risebrough et al., 1968; Fishbein, 1972; Buckley, 1982; Ballschmiter et al., 1981; Wasserman et al., 1979). It was also apparent from several analytical studies that PCBs preferentially bioaccumulate in the food chain and residues are routinely detected in human adipose tissue, blood and human milk (Wasserman et al., 1979; Cordle et al., 1978; Holdrinet et al., 1977; Safe, 1982). Thus, the chemical stability of PCBs is paralleled by their environmental stability and potential for environmental transport, and it is evident from analytical surveys that PCBs are the

Department of Physiology and Pharmacology, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843 (S.S. and L.S.), Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1 (S.S. and L.S.), and Large Lakes Research Station, ERL—Duluth at Grosse Ile, U.S. Environmental Protection Agency, Grosse Ile, Michigan 48138 (M.M.).

0021-8561/85/1433-0024$01.50/0