Peptide Sweetenters. 8. Synthesis and Structure–Taste Relationship Studies of L-Aspartyl-D-alanyl Tripeptides

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Several L-aspartyl-D-alanyl tripeptides have been synthesized to investigate the structural requirements of the C-terminal amino acid needed to elicit a taste response. Following our suggestion that a rigid, hydrophobic residue is required, both α,α-dialkane and cycloalkane α-amino acid methyl esters were incorporated into the tripeptide. The L-aspartyl-D-alanine-based tripeptide derivatives of α-aminoisobutyric acid methyl ester, α,α-diethylglycine methyl ester, and α-aminocycloalkanecarboxylic acid methyl esters from three- to six-membered rings are sweet. The higher analogues of the cycloalkane series containing α-aminocycloheptanecarboxylic acid methyl ester and α-aminocyclooctanecarboxylic acid methyl ester are bitter. It is important to note that this series of tripeptides (analogous to the previously reported dipeptides) goes from sweet to bitter to tasteless as the ring size of the C-terminal amino acid increases. The relationships between effective volume of the C-terminal residue, size requirements of the sweet receptor, and taste are discussed.

We have recently reported the synthesis of L-aspartyl dipeptides with various α,α-dialkane or cycloalkane α-amino esters as the C-terminal residue.[1] The studies of the variation in the C-terminal residue are illustrative in determining the boundaries of the hydrophobic portion of the sweet (and/or bitter) receptor site. In the cycloalkane series, it was found that the dipeptides containing three- to five-membered rings are sweet, those containing six- and seven-membered rings are bitter, and that with the eight-membered ring is tasteless. In the dialkane series, the dimethyl derivative, corresponding to the cyclopropyl derivative of the cycloalkane series, is also sweet but the diethyl derivative is tasteless, although it corresponds to the sweet cyclopentyl derivative of the cycloalkane series.

The difference in these two series is attributable to the fact that the Aib residue has a strong tendency to give folded conformations,[2] similar to those of the cycloalkane amino acid residues, whereas DEG generally assumes a stiff extended conformation, with the two ethyl groups pointing upward with respect to the "ribbon" of f the main chain.[3]

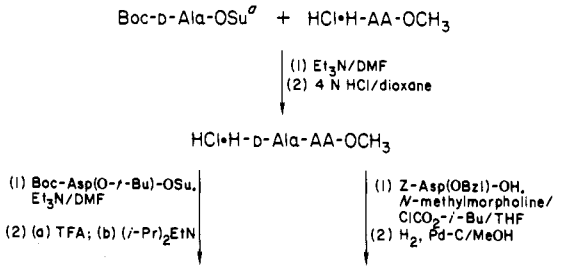
Compounds such as L-aspartyl tripeptides should further elucidate the topochemistry of the taste receptor by extending the hydrophobic portion of the molecule into a slightly different zone of the receptor. Ariyoshi[4] synthesized Asp-Gly-Gly-OMe, Asp-D-Ala-Gly-OMe and related tripeptides, all with glycine as the C-terminal amino acid; their sweet potencies however do not exceed 3 times that of sucrose. We believe the lack of hydrophobicity and the high flexibility of the C-terminal residue can account for these results. Such a belief is largely based on a view of the taste receptor similar to the model proposed by Temussi and his associates.[5] The key aspect of this model is the precise geometrical relationship between a hydophobic zone and the hydrogen-bonding groups of the receptor. We therefore undertook the synthesis of a series of L-aspartyl-D-alanyl tripeptides containing various dialyklglycine methyl esters or α-aminiocycloalkanecarboxylic acid methyl esters as the C-terminal residue. We chose D-alanine as the second residue because it should orient the C-terminal residue into the hydrophobic region of the sweet receptor, as suggested by the sweet taste of Asp-D-Ala-OBzl.[1] This series should allow us to study the effects on taste of size and hydrophobicity of conformationally constrained C-terminal amino acids.

Synthesis

The synthesis of the compounds was carried out according to Scheme I. The hydrochloride salt of the appropriate amino acid methyl ester was coupled to the *N*-(*tert*-butyloxycarbonyl)-D-alanine *N*-hydroxysuccinimide ester. This method provided pure protected dipeptide ester as oils, which were not characterized. Deprotection of the Boc group was carried out by using 4 N HCl in dioxane. The hydrochloride salt of the dipeptide esters were very hydroscopic solids. Therefore, we were not able to report accurate melting points for these compounds (see Table I).

The dipeptide esters were coupled either to *N*-(*tert*-butyloxycarbonyl)-β-*tert*-butylaspartic acid *N*-hydroxysuccinimide ester or *N*-(benzoylcarbonyl)-β-benzylaspartic acid by the mixed-anhydride method. The use of acid-labile protecting groups was mandatory in the case of the cyclopropane derivative, since opening of the cyclopropane ring has been reported during hydrogenolysis.[6] Further,

Scheme I



AA	dipeptides	tripeptides
Aib	1	9
DEG[b]	2	10
AC_3C[c]	3	11
AC_4C	4	12
AC_5C	5	13
AC_6C	6	14
AC_7C	7	15
AC_8C	8	16

[a] OSu = *N*-hydroxysuccinimide ester. [b] DEG = α,α-diethylglycine. [c] AC_nC signifies 1-aminocycloalkane-1-carboxylic acid where *n* denotes the ring size (i.e., AC_3C is 1-aminocyclopropane-1-carboxylic acid).

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Table I. HCl·H-D-Ala-AA-OCH_3

no.	AA	yield,[a] %	mp, °C	$[\alpha]^{25}D$ (c 1, CH_3OH)	R_f (B)	formula[b]
1	Aib	89	95–105	−18.2	0.09	$C_8H_{17}N_2O_3Cl·0.5H_2O$
2	DEG[c]	67	85–90	−19.1	0.24	$C_{10}H_{21}N_2O_3Cl·0.5H_2O·0.5CH_3OH$
3	AC_3C[d]	73	e	−25.6	0.08	$C_8H_{15}N_2O_3Cl·0.5H_2O$
4	AC_4C	82	95–105	−27.5	0.10	$C_9H_{17}N_2O_3Cl·0.25H_2O·0.25C_4H_8O_2$[f]
5	AC_5C	95	105–115	−28.7	0.14	$C_{10}H_{19}N_2O_3Cl·0.125H_2O·0.125C_4H_8O_2$[f]
6	AC_6C	77	115–125	−18.0	0.15	$C_{11}H_{21}N_2O_3Cl$
7	AC_7C	87	150–160	−22.4	0.16	$C_{12}H_{23}N_2O_3Cl$
8	AC_8C	90	150–160	−21.7	0.18	$C_{13}H_{25}N_2O_3Cl$

[a] All yields are for purified product. [b] All compounds analyzed for C, H, and N within ±0.4% of the calculated values. [c] DEG = α,α-diethylglycine. [d] AC_nC signifies 1-aminocycloalkane-1-carboxylic acid where n denotes the ring size (i.e., AC_3C is 1-aminocyclopropane-1-carboxylic acid). [e] Obtained as an oily material. [f] $C_4H_8O_2$ = dioxane.

Table II. Asp-D-Ala-AA-OCH_3

no.	AA	yield,[a] %	mp, °C	$[\alpha]^{25}D$ (c 1, CH_3OH)	R_f (C)	formula[b]	taste[c]
9	Aib	73	138–140	+40.6	0.58	$C_{12}H_{21}N_3O_6·0.5H_2O$	+
10	DEG[d]	82	105–115	+32.6	0.83	$C_{14}H_{25}N_3O_6·1.25H_2O$	+
11	AC_3C[e]	90	184–186	+29.3	0.35	$C_{12}H_{19}N_3O_6·0.75H_2O$	+
12	AC_4C	75	130–140	+21.3	0.38	$C_{13}H_{21}N_3O_6·CH_3OH$	+
13	AC_5C	59	130–140	+23.3	0.40	$C_{14}H_{23}N_3O_6·0.25CH_3OH$	+
14	AC_6C	84	125–130	+30.8	0.42	$C_{15}H_{25}N_3O_6·2H_2O·1.5CF_3CO_2H$	+
15	AC_7C	74	115–125	+28.1	0.44	$C_{16}H_{27}N_3O_6·0.75H_2O$	−
16	AC_8C	75	150–155	+20.7	0.50	$C_{17}H_{29}N_3O_6·H_2O·0.25CH_3OH$	−

[a] All yields are for purified product. [b] All compounds analyzed for C, H, and N within ±0.4% of the calculated values. [c] Key: +, sweet; −, bitter. The sweet potencies were about 20 times that of sucrose; higher homologues of the sweet compounds were accompanied with an unpleasant metallic taste. [d] DEG = α,α-diethylglyine. [e] AC_nC signifies 1-aminocycloalkane-1-carboxylic acid where n denotes the ring size (i.e., AC_3C is 1-amino-cyclopropane-1-carboxylic acid).

deprotection affords the tripeptide esters **9–16**. Their physical properties are listed in Table II.

Results and Discussion

We first synthesized Asp-D-Ala-Aib-OMe (**9**), which was found to be many times sweeter than sucrose or the tripeptide Asp-D-Ala-Gly-OMe synthesized by Ariyoshi.[4] This concurs with our suggestion that a higher degree of hydrophobicity and rigidity of the C-terminal residue is required for sweetness. The conformational preferences of the sequence D-Ala-Aib should be very similar to those of the achiral Aib-Aib sequence. The synthesis of Asp-Aib-Aib-OMe showed that it has a sweet potency similar to that of Asp-D-Ala-Aib-OMe. Interestingly the Asp-D-Ala-X-OMe tripeptide containing diethylglycine (DEG) (**10**) as the C-terminal residue is also sweet. This demonstrates that a large volume can be accommodated by the sweet receptor in the particular zone of interaction probed by the X residue; the DEG-containing tripeptide may be extended in contrast to the folded conformation for the other tripeptides. A direct comparison with Asp-DEG-OMe however is not possible since the orientations of the side chains are quite different.

In the homologous series of tripeptides containing α-aminocycloalkanecarboxylic acid methyl esters as the C-terminal residue, the peptides **11–14**, (i.e., containing cyclopropane through cyclohexane α-amino acids, respectively) are sweet, with potencies of about 20 times that of sucrose. As the cycloalkane ring is expanded to include the cycloheptane and cyclooctane rings, the peptides **15** and **16** are bitter. These observations though phenomenological are important in relating size to taste and sweet to bitter response.

There is evidently a shift in size requirements from the area of the sweet receptor probed by the dipeptide series to that probed by the tripeptide series, as demonstrated by the accommodation of larger cycloalkane rings in the tripeptide series. In addition, the taste elicited by the tripeptide DEG derivative is sweet, in contrast to the complete loss of taste found for the analogous dipeptide.[1]

Since the compounds in the series of L-aspartyl-D-alanyl-α-aminocycloalkanecarboxylic acid methyl esters vary only in the number of methylenes in the cycloalkane ring, this study provides further evidence that these taste responses (sweet and bitter) occur at receptors of closely related shape and electronic poperties or even at the same receptor, if a suitable triggering mechanism for the two different sensations is hypothesized.

Experimental Section

Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 141 with a 10-cm water-jacketed cell. NMR spectra were obtained from a Varian EM 360 spectrometer. All chemical shifts are reported in parts per million downfield from Me_4Si. Multiplet (m), quartet (q), triplet (t), doublet (d), or singlet (s) describe the multiplicity of resonances. IR spectra were recorded on a Perkin-Elmer 180 spectrometer. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. The analytical values are within 0.4% of the theoretical values.

Analytical TLC plates were purchased from E. Merck: silica gel 60 F-254, aluminum backed. The TLC plates were visualized with ninhydrin, Cl_2/toluidine reagent, or UV light (254 nm). The following developing systems were used: (A) EtOAc; (B) $CHCl_3$/MeOH/AcOH, 85:10:5; (C) $CHCl_3$/MeOH/AcOH, 55:30:15.

Usual workup refers to concentration of the reaction mixture under vacuum, dissolving the residue in ethyl acetate, successive washings with 1 M $NaHSO_4$, water, 5% $NaHCO_3$, and brine, and removal of the solvent under reduced pressure, after drying over $MgSO_4$.

The new compounds were qualitatively taste tested by four volunteers from our laboratories as solids and in solution. Tasting of solutions were carried out using double-blind tastings of dilute solutions in a "sip and spit" fashion. Three subjects tasted all the compounds including 8% sucrose and 0.1% aspartame solutions as standards. Each compound was initially designated as sweet, bitter, or tasteless. Relative intensities of sweet taste were obtained by comparison of the standards with 2% solutions of the unknown tastants.

General Method for the Synthesis of the Dipeptide Methyl Ester Hydrochloride 1–8. To a cold (0 °C) solution of the appropriate amino acid methyl ester hydrochloride (1 mmol) in

5 mL of DMF were successively added triethylamine (1 mmol, 0.14 ml) and *N*-(*tert*-butyloxycarbonyl)-D-alanine *N*-hydroxysuccinimide ester (0.9 mmol, 257 mg). The reaction was allowed to proceed overnight at room temperature. The usual workup afforded the protected dipeptide ester as a clear oil, homogeneous by TLC (solvent A). This material was dissolved in 5 mL of 4 N HCl/dioxane. After 30 min, the solvent was evaporated and the residue triturated with 3 × 50 mL of ether, to yield the dipeptide methyl ester hydrochloride as a very hygroscopic white solid.

Physical data of these compounds are listed in Table I.

As an example, NMR data of 1 are as follows: NMR (CD_3OD) δ 4.00 (q, 1 H, C_tH D-Ala), 3.68 (s, 3 H, OCH_3), 1.52 (d, 3 H, CH_3 D-Ala), 1.50 (s, 6 H, CH_3 Aib).

General Method for the Synthesis of the Tripeptide Methyl Esters 10, 12, 13, 15, and 16. A solution of β-benzyl *N*-(benzyloxycarbonyl)-L-aspartate (1 mmol, 357 mg) in 10 mL of THF was cooled to −15 °C. *N*-Methylmorpholine (1 mmol, 0.101 mL) and isobutyl chloroformate (1 mmol, 0.130 mL) were successively added. The mixture was stirred for 5 min at −20 °C, and a solution of **2**, **4**, **6**, or **7** (1.1 mmol) and triethylamine (1.1 mol, 0.154 mL) in 10 mL of THF was added. The reaction was allowed to proceed for 1 h at 0 °C and 2 h at room temperature. The usual workup yielded the protected tripeptide ester as a clear oil, homogeneous by TLC (solvent A).

This material was dissolved in 20 mL or methanol and hydrogenated in the presence of palladium on charcoal 10% for 4 h at room temperature and under atmospheric pressure. The catalyst was then removed by filtration, the solvent evaporated under reduced pressure, and the residue dissolved in 2 mL of methanol. Upon addition of ether, the zwitterionic tripeptide ester precipitated. It was collected by filtration, washed with ether, and dried under vacuum over phosphorus pentoxide.

Physical data of compounds **10**, **12**, **13**, **15**, and **16** are listed in Table II.

General Method for the Synthesis of the Tripeptide Methyl Esters 9, 11, and 14. To a cold (0 °C) solution of **2**, **3**, or **6** (1 mmol) in 5 mL of DMF were successively added triethylamine (1 mmol, 0.14 mL) and *N*-(*tert*-butyloxycarbonyl)-β-*tert*-butyl-L-aspartic acid *N*-hydroxysuccinimide ester (0.9 mmol, 348 mg). The mixture was stirred for 1 h at 0 °C and 2 h at room temperature. The usual workup afforded the pure, homogeneous by TLC (solvent A) protected tripeptide ester as a clear oil.

This material was dissolved in 5 mL of trifluoroacetic acid. The reaction, monitored by TLC, was complete in 45 min. The trifluoroacetaic acid was removed under reduced pressure and the residue triturated with 3 × 20 mL of ether, to leave a white solid, which was dissolved in 20 mL of ethyl acetate. Upon addition of *N*,*N*-diisopropyl-*N*-ethylamine (1 mmol, 0.172 mL) the zwitterionic tripeptide ester precipitated. It was collected by filtration, washed with ethyl acetate (3 × 5 mL), and ether (3 × 10 mL), and dried under vacuum over phosphorus pentoxide.

Physical data of compounds **9**, **11**, and **14** are listed in Table II.

As an example, NMR data of **9** are as follows: NMR (CD_3OD) δ 4.33 (q, 1 H, C_α H D-Ala), 4.10 (m, 1 H, C_α H Asp), 3.65 (s, 3 H, OCH_3), 2.67 (m, 2 H, CH_2 Asp), 1.43 (s, 6 H, CH_3 Aib), 1.32 (d, 3 H, CH_3 D-Ala).

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Registry No. 1, 97522-41-3; **2**, 97522-42-4; **3**, 97522-43-5; **4**, 97522-44-6; **5**, 97522-45-7; **6**, 97522-46-8; **7**, 97522-47-9; **8**, 97522-48-0; **9**, 97522-49-1; **10**, 97522-50-4; **11**, 97522-51-5; **12**, 97522-52-6; **13**, 97522-53-7; **14**, 97522-54-8; **15**, 97522-55-9; **16**, 97522-56-0; H-Aib-OMe·HCl, 15028-41-8; H-DEG-OMe·HCl, 92398-54-4; H-AC_3C-OMe·HCl, 72784-42-0; H-AC_4C-OMe·HCl, 92398-47-5; H-AC_5C-OMe·HCl, 60421-23-0; H-AC_6C-OMe·HCl, 37993-32-1; H-AC_7C-OMe·HCl, 92398-50-0; H-AC_8C-OMe·HCl, 92398-52-2; Boc-D-Ala-OSu, 34404-33-6; Z-Asp-(OBzl)-OH, 3479-47-8; Boc-Asp(O-*t*-Bu)-OSu, 50715-50-9.

Synthesis of the C-Terminal Octapeptide of Pig Oxyntomodulin. Lys-Arg-Asn-Lys-Asn-Asn-Ile-Ala: A Potent Inhibitor of Pentagastrin-Induced Acid Secretion

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The synthesis of Lys-Arg-Asn-Lys-Asn-Asn-Ile-Ala representing the C-terminal octapeptide of oxyntomodulin isolated from pig intestine is described. Its structure was confirmed by its 360-MHz ^1H NMR spectra. The octapeptide was tested for its ability to inhibit pentagastrin-induced acid secretion, in the anaesthetized rat, in the conscious rat with chronic gastric fistula, and in the conscious cat with gastric chronic fistula. The octapeptide inhibits pentagastrin-induced acid secretion in all three models. Compared to oxyntomodulin, the parent hormone, the synthetic peptide was approximately 150 times less potent but has the same efficacy. Biological data are presented and discussed.

A porcine intestinal peptide that is able to display glucagon-like immunoreactivity was also shown to interact with the glucagon receptors and to activate the adenylate cyclase present in liver membranes.[1,2] On the basis of these criteria, Bataille et al.[3] isolated a peptide of 37 amino acid residues (G-37) representing the whole glucagon molecule (G-29) elongated at its C-terminal end by a basic octapeptide, Lys-Arg-Asn-Lys-Asn-Asn-Ile-Ala[4] (Figure 1). Biological activities of the G-37 were quite different from those of glucagon (G-29). The affinity of G-37 for antibodies directed against the C-terminal sequence of glucagon (such as K47 or 30K) was ≃1% that of glucagon. Indeed, the C-terminal extension is likely to hinder the

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