Agric. Biol. Chem., **52** (3), 819~827, 1988

A Mechanism for Bitter Taste Sensibility in Peptides†

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Received October 27, 1987

To estimate the steric distance between the bitter taste determinant sites in peptides, some cyclic dipeptides, amino acid anilides, amino acid cyclohexylamides, and benzoyl amino acids were synthesized and their tastes were evaluated. The diketopiperazine ring of cyclic dipeptides acted as a bitter taste determinant site due to its hydrophobicity. The steric distance between 2 sites was estimated as 4.1 Å from the molecule models of cyclic dipeptides composed of typical amino acids in the bitter peptides. Due to the hypothesis of two bitter taste determinant sites, which bind with the bitter taste receptor *via* a "binding unit" and a "stimulating unit," a mechanism for the bitterness in peptides was postulated.

In our previous reports on this topic,[1~4] the mechanism for bitter taste sensibility in peptides has been extensively studied. Numerous bitter peptides and their diverse structures were noted, and the mechanism for bitter taste in peptides was based on a much simplified principle. Two determinant sites participated in the mechanism for the bitter taste of peptides, the primary one a hydrophobic group, and for the secondary site, another hydrophobic group or a bulky basic group such as a guanidino group or an α-amino group. It was demonstrated that the adjacency of these two sites, in the steric conformation of peptides, was essential for the bitter taste.

Finally we attempted to measure the steric distance between the two bitter taste determinant sites in peptides. In a series of bitter peptides, in which there were comparatively rigid molecular conformations, distances between the two bitter taste determinant sites were measured. Cyclic dipeptides, which had been reported to be a bitter component in cocoa[5] or aged sake,[11] were tested for an authentic bitter peptide of a rigid molecular conformation. Minamiura *et al.*[6] reported that the structure of BPII, a bitter peptide isolated from casein hydrolysate, was cyclo(-Trp-Leu-Trp-Leu-), while Shiba *et al.*[7] demonstrated the structure of BPII to be cyclo(-Trp-Leu-) in his report concerning the taste of cyclic peptides. Matoba *et al.*[12] also synthesized some cyclic dipeptides and reported their bitterness.

In this study, we synthesized numerous cyclic dipeptides taking account of the preceding results on the taste of the variety of peptide, and compared their taste with the corresponding linear dipeptides. Amino acid anilides, amino acid cyclohexylamides, and benzoyl amino acids, which are more flexible in confor-

† Studies on Flavored Peptides. Part V.

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The abbreviations recommended by the IUPAC-IUB Commission of Biochemical Nomenclature [*J. Biol. Chem.*, **247**, 977 (1972)] were used. Amino acids except for glycine are of the L-configuration unless otherwise noted.

mation than cyclic dipeptides, were also synthesized and tested. The mechanism for bitter taste sensibility in peptides was discussed in detail.

MATERIALS AND METHODS

Synthesis of peptides and their derivatives.

General procedure. The melting points which are shown were uncorrected. Thin layer chromatography (TLC) was done on Merck Silicagel G with the solvent systems, Rf^1, 1-butanol–acetic acid–pyridine–water (4 : 1 : 1 : 2, v/v) or Rf^2, chloroform–methanol (5 : 1, v/v). The peptides retaining free amino groups were detected by spraying ninhydrin, while 25% hydrogen bromide was sprayed previously in the case of protected amino groups. Cyclic dipeptides were detected on TLC by spraying 10% sulfuric acid because they were ninhydrin negative. $[\alpha]_D$ was measured with a Union PM-101 polarimeter. Before the analysis and sensory tests, all the peptides were dessicated over phosphorus pentaoxide at 66°C and 2 mmHg for 4 hr.

Synthesis of cyclic dipeptides. Cyclic dipeptides were synthesized from linear dipeptide esters by Fischers' method (Method A)[8] and Niteckis' method[9] (Method B).

a) Method A, for preparation of cyclo(-Arg-Phe-). Two mmol of Z-Arg(NO_2)-Phe-OMe was dissolved in acetic acid saturated with HBr at 0°C. After standing for 30 min, the solution was triturated with ether. The resultant precipitate was dissolved in methanol containing dry ammonia at 0°C. The solution was placed in a glass-stoppered bottle and kept at room temperature for 12 hr. The reaction mixture was then evaporated *in vacuo*. The crystalline residue was filtered with the aid of methanol. The resultant cyclo(-Arg(NO_2)-Phe-) was suspended in a mixture of methanol and acetic acid (1 : 1, v/v), and hydrogenated with palladium black as catalyst for 22 hr. After the catalyst was removed, the filtrate was evaporated. The oily residue was crystallized from ether.

b) Method B, for preparation of cyclo(-Asp-Gly-). Two mmol of Boc-Asp(OBzl)-Gly-OMe was dissolved in 50 ml of formic acid (99%), and the solution was left at room temperature for 2 hr. The excess formic acid was evaporated *in vacuo* (<30°C) and the residual oily substance was then dissolved in the mixture of 100 ml of *sec*-buthylalcohol and 20 ml of toluene. The solution was refluxed for 5 hr. After being concentrated and chilled to 0°C, the precipitate was collected by filtration and was recrystallized from methanol. Crystalline cyclo(-Asp(OBzl)-Gly-) was then suspended in a mixture of methanol and acetic acid (1 : 1, v/v) and hydrogenated with palladium black as catalyst. After the catalyst was removed by filtration, the filtrate was evaporated and the crystalline product was harvested from the oily residue by adding ether. Yield and analytical data of synthesized cyclic dipeptides are summarized in Table I.

Synthesis of amino acid anilides and amino acid cyclohexylamides. Five mmol of benzyloxycarbonyl amino acids and 5 mmol of aniline or cyclohexylamine were conjugated by the conventional mixed anhydride method. The resultant benzyloxycarbonyl amino acid anilides or cyclohexylamides were hydrogenated in the presence of palladium black in methanol containing hydrogen chloride. The products were obtained as hydrochloride salts.

Synthesis of benzoyl amino acids. Amino acids were acylated with benzoyl chloride in alkaline solution.

Yields and analytical data of the synthesized compounds are in Table II.

Sensory test. For all the synthesized compounds, the feature and intensity of taste, as measured by the threshold concentration (TH. V), was evaluated by 4 to 5 panels. The relative bitterness of the peptides, against the standard threshold concentration for caffeine (1 mM), was used as an index for bitterness. The taste of benzoyl amino acids was evaluated without neutralization, because a bitter taste was easily distinguishable from a sour taste. The procedure for the sensory test was described in our previous report[1] in detail.

RESULTS AND DISCUSSION

Taste of cyclic dipeptides

The tastes for 23 synthesized cyclic dipeptides and corresponding linear dipeptides are shown in Table III. The tastes of cyclic and linear dipeptides were compared. Regarding the peptides containing neutral amino acids, all the cyclic and linear dipeptides of glycine and alanine were tasteless. The cyclic dipeptides composed of valine and glycine were bitter, while linear peptides had no bitterness or only slight bitterness. All the cyclic and linear dipeptides containing leucine, isoleucine, or phenylalanine, a typical hydrophobic amino acid, were considerably bitter, but the bitter taste of cyclic dipeptides was more intense than linear dipeptides. With dipeptides composed of a hydrophobic amino acid and glycine, the difference in bitterness between cyclic and linear dipeptides was more distinct. It was noted that the bitterness in terms of TH.V of cyclic dipeptides containing leucine, isoleucine, or phenylalanine, a typical hydrophobic amino acid, varied only limitedly and inconsistently with the number of residues of hydrophobic amino acid in their molecules.

TABLE I. YIELD AND ANALYTICAL DATA OF CYCLIC DIPEPTIDES

Compound	Method	Yield (%)	mp (°C)	$[\alpha]_D^{23}$/° (Solvent)	Formula	Found (Calcd.) (%) C	H	N	Rf^1	Rf^2
Cyclo(Gly-Gly)	A	62	>290	—	$C_4H_6O_2N_2$	41.93 (42.10)	5.35 (5.30)	24.96 (24.55)	0.79	0.32
Cyclo(Ala-Ala)	A	52	272~275	−24 (cl, H_2O)	$C_6H_{10}O_2N_2$	51.10 (50.70)	7.01 (7.04)	19.85 (19.72)	0.82	0.61
Cyclo(Val-Val)	A	36	286~288	−31 (cl, H_2O)	$C_{10}H_{18}O_2N_2$	60.27 (60.61)	9.11 (9.09)	14.54 (14.14)	0.81	0.61
Cyclo(Leu-Leu)	A	63	272~274	−24 (cl, H_2O)	$C_{12}H_{22}O_2N_2$	62.92 (63.68)	9.94 (9.80)	12.35 (12.38)	0.97	0.83
Cyclo(Ile-Ile)	A	41	265~266	−8 (cl, H_2O)	$C_{12}H_{22}O_2N_2$	63.21 (63.68)	9.75 (9.80)	12.34 (12.38)	0.91	0.83
Cyclo(Phe-Phe)	A	58	>290	−102 (cl, AcOH)	$C_{18}H_{18}O_2N_2$	73.24 (73.45)	6.24 (6.16)	9.56 (9.53)	0.89	0.73
Cyclo(Orn(Z)-Gly)	A	69	218~220	+15 (cl, DMF)	$C_{15}H_{19}O_4N_3$	58.96 (59.02)	6.21 (6.23)	14.01 (13.77)	0.81	0.66
Cyclo(Orn-Gly)·HCl		99	Hygroscopic	+12 (cl, H_2O)	$C_7H_{14}O_2N_3Cl$				0.40	0.00
Cyclo(Lys(Z)-Gly)	A	72	207~210	+16 (cl, DMF)	$C_{16}H_{21}O_4N_3$	60.12 (60.17)	6.70 (6.63)	13.07 (13.16)	0.82	0.79
Cyclo(Lys-Gly)·HCl		99	230~235	+13 (cl, H_2O)	$C_8H_{16}O_2N_3Cl$	42.07 (43.38)	7.33 (7.22)	18.15 (18.96)	0.44	0.00
Cyclo(Arg(NO_2)-Gly)	A	73	220~223	+14 (cl, DMF)	$C_8H_{14}O_4N_6$	37.05 (37.21)	5.56 (5.46)	32.26 (32.55)	0.74	0.56
Cyclo(Arg-Gly)·HCl		94	217~220	+12 (cl, H_2O)	$C_8H_{16}O_2N_5Cl$	37.91 (38.48)	5.95 (6.01)	28.21 (28.06)	0.56	0.00
Cyclo(Orn(Z)-Orn(Z))	A	63	220	−24 (cl, DMF)	$C_{26}H_{32}O_6H_4$	62.78 (62.89)	6.61 (6.50)	11.32 (11.28)	0.91	0.68
Cyclo(Orn-Orn)·2AcOH	A	98	Hygroscopic						0.35	0.00
Cyclo(Lys(Z)-Lys(Z))	A	68	214~216	+4 (cl, DMF)	$C_{28}H_{36}O_6N_4$	63.83 (64.10)	7.08 (6.92)	10.72 (10.68)	0.83	0.54
Cyclo(Lys-Lys)·2AcOH		96	115~116	−46 (cl, H_2O)	$C_{16}H_{32}O_6N_4$	38.19 (38.30)	6.42 (6.38)	15.01 (14.89)	0.54	0.00
Cyclo(Arg(NO_2)-Arg(NO_2))	A	74	228	−15 (cl, DMF)	$C_{12}H_{22}O_6N_{10}$	35.53 (35.82)	5.66 (5.51)	34.51 (34.81)	0.75	0.54
Cyclo(Arg-Arg)·2AcOH	A	82	252~256	−13 (cl, H_2O)	$C_{16}H_{32}O_6N_8$	43.57 (44.47)	7.15 (7.40)	25.32 (25.91)	0.50	0.00
Cyclo(Asp(OBzl)-Gly)	B	45	207~210	−8 (cl, DMF)	$C_{13}H_{14}O_4N_2$	60.71 (59.53)	5.99 (5.38)	10.21 (10.68)	0.84	0.51
Cyclo(Asp-Gly)		97	205	+46 (cl, H_2O)	$C_5H_8O_4N_2 \cdot 2/3\,H_2O$	38.96 (39.13)	4.37 (4.35)	15.16 (15.22)	0.46	0.23
Cyclo(Glu(OBzl)-Gly)	B	37	197~199	−6 (cl, DMF)	$C_{14}H_{16}O_4N_2$	39.66 (40.86)	5.43 (5.84)	10.80 (10.14)	0.84	0.42
Cyclo(Glu-Gly)		98	197	+6 (cl, H_2O)	$C_7H_{10}O_4N_2 \cdot 1/2\,H_2O$	43.15 (43.08)	5.08 (5.13)	14.21 (14.36)	0.45	0.21
Cyclo(Ala-Gly)	A	46	244~246	−5 (cl, H_2O)	$C_5H_8O_2N_2$	46.75 (46.87)	6.40 (6.29)	21.90 (21.87)	0.78	0.69
Cyclo(Val-Gly)	A	22	208~210	−207 (cl, H_2O)	$C_7H_{12}O_2N_2$	58.30 (58.83)	7.87 (7.74)	18.06 (19.74)	0.77	0.68
Cyclo(Leu-Gly)	A	22	243~245	+21 (cl, H_2O)	$C_8H_{14}O_2N_2$	56.21 (56.45)	8.30 (8.29)	16.32 (16.46)	0.85	0.62
Cyclo(Ile-Gly)	A	35	245~250	+11 (cl, H_2O)	$C_8H_{14}O_2N_2$	56.15 (56.45)	8.43 (8.29)	16.31 (16.46)	0.84	0.63

TABLE I. (continued)

Compound	Method	Yield (%)	mp (°C)	$[\alpha]_D^{23}$/° (Solvent)	Formula	Found (Calcd.) (%) C	H	N	Rf^1	Rf^2
Cyclo(Phe-Gly)	A	31	268~270	+101 (cl, H_2O)	$C_{11}H_{12}O_2N_2$	64.39 (64.69)	5.86 (5.92)	13.64 (13.72)	0.90	0.73
Cyclo(Pro-Pro)	A	72	212~216	−41 (cl, H_2O)	$C_{10}H_{14}O_2N_2$	61.54 (61.83)	7.32 (7.27)	14.33 (14.42)	0.75	0.31
Cyclo(Arg(NO_2)-Phe)	A	16	217~220	−34 (cl, DMF)	$C_{15}H_{20}O_4N_6$	53.72 (53.89)	5.91 (6.00)	21.23 (20.96)	0.88	0.33
Cyclo(Arg-Phe)·AcOH		83	217	−10 (cl, H_2O)	$C_{15}H_{21}O_2N_5$·AcOH·1/2H_2O	47.95 (48.39)	5.60 (5.65)	18.65 (18.82)	0.65	0.01
Cyclo(Arg(NO_2)-Pro)	A	34	202~208	−84 (cl, DMF)	$C_{11}H_{18}O_4N_6$	44.59 (44.24)	6.29 (6.08)	28.06 (28.18)	0.81	0.45
Cyclo(Arg-Pro)·HCl		59	245~250	−72 (cl, H_2O)	$C_{11}H_{19}O_2N_5$·HCl	45.32 (45.33)	7.10 (6.87)	23.98 (24.04)	0.69	0.00
Cyclo(His-Phe)	B	65	230	−144 (cl, DMF)	$C_{15}H_{16}O_2N_5$	58.41 (58.63)	5.31 (5.21)	29.02 (28.80)	0.65	0.31

The TH.V was within a narrow range irrespective of the presence of glycine residues in the dipeptides.

Only cyclo(-Lys-Gly-) had a slight bitterness in the peptides containing lysine or ornithine. A bitter taste was sensible in both the cyclic and linear peptides containing arginine, and the bitterness of the cyclic peptides was more intense.

Bitter taste was not observed in all the cyclic and linear peptides composed of acidic amino acids.

The taste of proline peptides was notable. Cyclo(-Pro-Pro-) was completely tasteless, but linear Pro-Pro was very bitter. The comparison of the linear and cyclic dipeptides composed of arginine, proline, and phenylalanine, which were identified as essential components of a typical bitter peptide, demonstrated that the bitterness of cyclic dipeptides greatly exceeded the bitterness of linear dipeptides. The TH.V values of the cyclic dipeptides, described above, remained at the same level, 0.45 mM, irrespective of the amino acid sequence and composition. It was therefore evident that the bitter taste intensity of cyclic dipeptides almost exceeded the bitterness of the corresponding linear dipetides. The bitter taste of cyclic dipeptides was not much varied irrespective of the structural changes of amino acid components in terms of hydrophobic amino acids, arginine, proline, and phenylalanine.

It was concluded that the diketopiperazine ring acts as a bitter taste determinant site due to its hydrophobicity. This aspect of the diketopiperazine ring seemed to resemble the case of the imino ring in proline residues. From this point of view, it was conceived that cyclo(-Pro-Pro-) was tasteless. The conformational molecular model of cyclo(-Pro-Pro-) demonstrated that two imino rings and the diketopiperazine ring disposed on nearly a single plane, and they were unable to approach in the steric conformation which was essential for bitterness. On the contrary, the two imino rings in the linear Pro-Pro peptide, were able to form the adjacent conformation. Shiba[7] and Matoba[12] discussed the relationship between the bitter taste and chemical structure of cyclic dipeptides. However, they did not give an account of the role of the diketopiperazine ring as a bitter taste determinant site.

To estimate the distance between the two bitter taste determinant sites of the peptides that bind with the bitter taste receptor, topological models of cyclic dipeptides, composed of arginine, proline, phenylalanine, and histidine, which were typical amino acids in bitter peptides, were measured. The results are shown in Table IV. Guanidino groups in ar-

TABLE II. YIELDS AND ANALYTICAL DATA OF SYNTHETIC COMPOUNDS

Compound*	Yield (%)	mp (°C)	Formula	Found (Calcd.) (%)			*Rf*
				C	H	N	
Z-Gly-NH-R_1	84	144~145	$C_{16}H_{16}O_3N_2$	67.73 (67.59)	5.48 (5.67)	9.67 (9.85)	0.98
Z-β-Ala-NH-R_1	60	137~138	$C_{17}H_{18}O_3N_2$	68.76 (68.44)	5.86 (6.08)	9.26 (9.39)	0.98
Z-γ-Abu-NH-R_1	69	122	$C_{18}H_{20}O_3N_2$	69.42 (69.21)	6.50 (6.45)	8.84 (8.97)	0.98
Z-δ-Ava-NH-R_1	87	125~126	$C_{19}H_{22}O_3N_2$	70.02 (69.92)	6.72 (6.79)	8.33 (8.53)	0.95
Z-ε-Aca-NH-R_1	84	133~134	$C_{20}H_{24}O_3N_2$	70.51 (70.56)	7.33 (7.11)	8.09 (8.23)	0.98
Z-Gly-NH-R_2	64	108~110	$C_{16}H_{22}O_3N_2$	66.43 (66.18)	7.48 (7.64)	9.37 (9.65)	0.98
Z-β-Ala-NH-R_2	80	155~156	$C_{17}H_{24}O_3N_2$	67.31 (67.08)	7.85 (7.95)	9.09 (9.20)	0.98
Z-γ-Abu-NH-R_2	82	128	$C_{18}H_{26}O_3N_2$	68.16 (67.90)	8.13 (8.23)	8.61 (8.80)	0.98
Z-δ-Ava-NH-R_2	95	132~134	$C_{19}H_{28}O_3N_2$	68.77 (68.64)	8.24 (8.49)	8.60 (8.43)	0.96
Z-ε-Aca-NH-R_2	93	122~124	$C_{20}H_{30}O_3N_2$	69.55 (69.33)	8.41 (8.53)	8.24 (8.09)	0.97
H-Gly-NH-R_1·HCl	87	200	$C_8H_{11}ON_2Cl$	51.59 (51.48)	5.86 (5.94)	14.90 (15.01)	0.86
H-β-Ala-NH-R_1·HCl	88	199~200	$C_9H_{13}ON_2Cl$	53.98 (53.86)	6.50 (6.53)	14.11 (13.96)	0.86
H-γ-Abu-NH-R_1·HCl	95	142~144	$C_{10}H_{15}ON_2Cl$	56.20 (55.94)	6.93 (7.04)	12.87 (13.05)	0.89
H-δ-Ava-NH-R_1·HCl	83	169~170	$C_{11}H_{17}ON_2Cl$	57.63 (57.76)	7.64 (7.49)	12.36 (12.25)	0.81
H-ε-Aca-NH-R_1·HCl	93	172~173	$C_{12}H_{19}ON_2Cl$	59.62 (59.37)	7.81 (7.89)	11.33 (11.54)	0.81
H-Gly-NH-R_2·HCl	87	201~203	$C_8H_{17}ON_2Cl$	50.19 (49.87)	8.62 (8.89)	14.31 (14.54)	0.86
H-β-Ala-NH-R_2·HCl	94	185~186	$C_9H_{19}ON_2Cl$	52.40 (52.29)	9.41 (9.27)	13.28 (13.55)	0.83
H-γ-Abu-NH-R_2·HCl	92	114~115	$C_{10}H_{21}ON_2Cl$	54.78 (54.41)	9.38 (9.59)	12.67 (12.69)	0.89
H-δ-Ava-NH-R_2·HCl	89	190~192	$C_{11}H_{23}ON_2Cl$	56.20 (56.27)	10.15 (9.88)	11.62 (11.93)	0.80
H-ε-Aca-NH-R_2·HCl	87	142~144	$C_{12}H_{25}ON_2Cl$	58.21 (57.93)	10.02 (10.13)	11.04 (11.26)	0.79
Bz-Gly-OH	56	187	$C_9H_9O_3N$	60.46 (60.33)	5.10 (5.06)	7.80 (7.82)	0.74
Bz-β-Ala-OH	64	118~119	$C_{10}H_{11}O_3N$	62.24 (62.16)	5.80 (5.74)	7.18 (7.25)	0.88
Bz-γ-Abu-OH	71	78~80	$C_{11}H_{13}O_3N$	63.94 (63.75)	6.21 (6.32)	6.55 (6.76)	0.86
Bz-δ-Ava-OH	80	92~93	$C_{12}H_{15}O_3N$	65.30 (65.14)	6.69 (6.83)	6.30 (6.33)	0.87
Bz-ε-Aca-OH	88	79~80	$C_{13}H_{17}O_3N$	66.54 (66.36)	7.36 (7.28)	5.79 (5.95)	0.90

* R_1 = phenyl; R_2 = cyclohexyl.

TABLE III. TASTE OF CYCLIC AND LINEAR DIPEPTIDES

Cyclic cipeptides	Taste	TH.V. (mM)	R_{caf}	Linear dipeptides	Taste	TH.V (mM)	R_{caf}
Cyclo(-Gly-Gly-)	Flat	—	—	Gly-Gly	Flat	—	—
Cyclo(-Ala-Ala-)	Flat	—	—	Ala-Ala	Flat	—	—
Cyclo(-Ala-Gly-)	Flat	—	—	Ala-Gly	Flat	—	—
				Gly-Ala	Flat	—	—
Cyclo(-Val-Val-)	Bitter	5	0.2	Val-Val	*Umami**		—
Cyclo(-Val-Gly-)	Bitter	5	0.2	Val-Gly	*Umami*		—
				Gly-Val	Bitter	4.5	0.22
Cyclo(-Leu-Leu-)	Bitter	1.2	0.83	Leu-Leu	Bitter	2.5	0.4
Cyclo(-Leu-Gly-)	Bitter	2	0.5	Leu-Gly	Bitter	20	0.05
				Gly-Leu	Bitter	25	0.04
Cyclo(-Ile-Ile-)	Bitter	0.6	1.67	Ile-Ile	Bitter	1.5	0.67
Cyclo(-Ile-Gly-)	Bitter	1.2	0.83	Ile-Gly	Bitter	4.5	0.22
				Gly-Ile	Bitter	2.3	0.44
Cyclo(-Phe-Phe-)	Insoluble	—	—	Phe-Phe	Bitter	1.2	0.83
Cyclo(-Phe-Gly-)	Bitter	1.0	1.0	Phe-Gly	Bitter	6	0.17
				Gly-Phe	Bitter	1.2	0.83
Cyclo(-Orn-Orn-)	*Umami*		—	Orn-Orn	Flat	—	—
Cyclo(-Orn-Gly-)	*Umami*		—	Orn-Gly	*Umami*		—
				Gly-Orn	*Umami*		—
Cyclo(-Lys-Lys-)	*Umami*		—	Lys-Lys	Flat	—	—
Cyclo(-Lys-Gly-)	Bitter	5	0.2	Lys-Gly	*Umami*		—
				Gly-Lys	*Umami*		—
Cyclo(-Arg-Arg-)	Bitter	0.75	1.33	Arg-Arg	Bitter	9.5	0.1
Cyclo(-Arg-Gly-)	Bitter	5	0.2	Arg-Gly	Bitter	9.5	0.1
				Gly-Arg	Bitter	75	0.01
Cyclo(-Asp-Gly-)	Sour		—	Asp-Gly	Sour		—
				Gly-Asp	Sour		—
Cyclo(-Glu-Gly-)	Sour		—	Glu-Gly	Sour		—
Cyclo(-Pro-Pro-)	Flat	—	—	Pro-Pro	Bitter	4.5	0.22
Cyclo(-Arg-Phe-)	Bitter	0.45	2.22	Arg-Phe	Bitter	2.3	0.43
Cyclo(-Arg-Pro-)	Bitter	0.45	2.22	Arg-Pro	Bitter	0.8	1.25
				Pro-Arg	Bitter	3	0.33
Cyclo(-His-Phe-)	Bitter	0.5	2				

* Umami is a relish taste like MSG.

ginine, phenyl groups in phenylalanine, imino rings in proline, imidazole groups in histidine, and diketopiperazine rings had already been recognized as bitter taste determinant sites in bitter peptides. The distance between the centers of the molecular models of each group was measured. As shown in Table IV, the distances between the respective bitter taste determinant sites were approximately equal, despite their different structures. The average (4.1Å) was assessed to be the distance between the bitter taste determinant sites in peptides. This result was compatible with our preceding study concerning natural bitter peptides and many synthetic model peptides.[13] Small changes in this distance probably affect the intensity of the bitterness. The bitterness of the compounds in terms of TH.V was not altered by introducing multiple pairs of bitter taste determinant sites in a cyclic dipeptide molecule. It was evident that only a piar of sites reacted with a bitter taste receptor. The bitter taste receptors evidently recognized the bitter peptides on the molecular level.

To clarify the range of allowance of molecular recognition in bitter taste receptors for the bitter substances, we examined the tastes of a group of compounds with higher flexibility in molecular conformation than cyclic dipeptides, the benzoyl amino acids, amino acid

TABLE IV. DISTANCE BETWEEN ACTIVE SITES FOR BITTERNESS

Compounds	TH.V (mM)	Sites*	Distance (Å)
Cyclo(-Gly-Gly-)	—	D	—
Cyclo(-Pro-Pro-)	—	D-I	(Plane)
Cyclo(-Arg-Phe-)	0.45	A-P	4.2
		D-A	4.0
		D-P	4.2
Cyclo(-Arg-Pro-)	0.45	A-D	4.4
		D-I	(Plane)
Cyclo(-His-Phe-)	0.5	H-P	3.9
		D-H	4.0
		D-P	4.2
		Average	4.1

* A, guanidino group in arginine; P, phenyl group in phenylalanine; H, imidazole group in histidine; D, diketopiperazine ring; I, imino ring in proline.

TABLE V. TASTE OF AMINO ACID ANILIDES, AMINO ACID CYCLOHEXYLAMIDES AND BENZOYL AMINO ACIDS

Compound	Taste	TH.V (mM)
Benzoyl-NH-CH_2-COOH	Sour	3.0
Benzoyl-NH-$(CH_2)_2$-COOH	Sour	3.0
Benzoyl-NH-$(CH_2)_3$-COOH	Sour	3.0
Benzoyl-NH-$(CH_2)_4$-COOH	Sour	3.0
Benzoyl-NH-$(CH_2)_5$-COOH	Sour	3.0
H-Gly-NH-Phenyl	Bitter	8.4
H-β-Ala-NH-Phenyl	Bitter	4.2
H-γ-Abu-NH-Phenyl	Bitter	2.0
H-δ-Ava-NH-Phenyl	Bitter	1.5
H-ε-Aca-NH-Phenyl	Bitter	0.8
H-Gly-NH-Cyclohexyl	Bitter	8.4
H-β-Ala-NH-Cyclohexyl	Bitter	5.2
H-γ-Abu-NH-Cyclohexyl	Bitter	3.1
H-δ-Ava-NH-Cyclohexyl	Bitter	1.5
H-ε-Aca-NH-Cyclohexyl	Bitter	0.8
Gly-Phe	Bitter	1.2
Gly-Gly-Phe	Bitter	1.5

anilides and cyclohexylamides. These compounds are composed of linear aliphatic carbon chains which linked hydrophobic benzoyl, phenyl or cyclohexyl groups at one terminus. Anilides and cyclohexylamides linked amino groups at the opposite terminus. The tastes of these compounds are listed in Table V.

According to the increase of the chain length of the benzoyl amino acids, which has the hydrophobic group in the molecule, the hydrophobic character appears at the part of the aliphatic carbon chain, thereby a bitter taste could be expected. However, all the synthesized benzoyl amino acids had only a sour taste. The aliphatic carbon chain was not active as a bitter taste determinant site. This results also suggested that the carboxyl group were not significant as bitter taste determinant sites in peptides. Here the existence of two bitter taste determinant sites was suggested again. This is compatible with the observation that Asp-Gly and Glu-Glu were not bitter.

Amino acid anilides and cyclohexylamides were the same in taste. The enhancement of the bitter taste according to the elongation of chain length was observed. The derivative of ε-amino capronic acid was 10 times as bitter as the glycine derivative. According to the thermodynamic theory on the stable state, the distance between the hydrophobic group and amino group in these molecule increases as the chain length is elongated. The increase in the distance between the determining sites for bitterness was presumed to cause the lesser bitterness. However the experimental results were contrary to this presumption. The stepwise enhancement of bitter taste in accordance with the increase of the chain length seemed to depend on the conformational flexibility of the carbon chain of these compounds. Gly-Phe (TH.V 1.2 mM) or Gly-Gly-Phe (TH.V 1.5 mM), which were comparatively less flexible, gave rise to an almost equal bitterness. These results indicated the high affinity or selectivity of the bitter taste receptor for the bitter taste determinant sites.

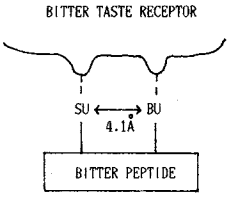
Mechanism for bitter taste sensibility in peptides

According to the experimental results in our previous[1~4] and present studies, the mechanism for bitter taste sensibility is summarized in a simplified scheme. It is well known that a hydrophobic amino acid residue is essential for bitterness. However, as already mentioned in this series of studies, the overall hydrophobicity of the peptide did not directly

correlate with the bitterness. The hydrophobic group of the peptide offered a binding site with the bitter taste receptor. The results concerning the taste of leucine and phenylalanine diastereomers, in which the steric conformation was not required for the bitter taste, clarified that the number of binding sites in the bitter peptides for bitter taste receptors should be fewer than three. From the results about benzoyl amino acids, cyclo(-Asp-Gly-) and cyclo(-Glu-Gly-), it was conceivable that the bitterness was not ensured with only a single hydrophobic group. The carboxyl group was inert as a bitter taste determinant site. Then it was concluded that 2 sites were involved in the bitter taste in peptides and the adjacent situation of these sites in the steric conformation was essential for it. It was observed in cyclo(-Pro-Pro-), that the peptide possessed more than 2 bitter taste determinant sites, but the bitter taste was not sensible unless they were disposed with a suitable configuration.

The hypothesis that a specific steric configuration of these two sites was required for the bitterness was also compatible with experimental results showing that arginine and proline were less significant for the bitterness in peptides, but they had strong bitterness when they constituted Arg-Pro.

The distance between the two bitter taste determinant sites was measured by devising molecular models of cyclic dipeptides, composed of phenylalanine, arginine, proline, and histidine, which were typical components in bitter peptides. The distance between the two sites was estimated to be 4.1Å. A primary site, which was to determine the bitterness of the peptide, was the bulky hydrophobic group composed of at least a 3-carbon skeleton. The imino ring in proline or the diketopiperazine ring also played the role of this hydrophobic group. For a secondary determinant site for bitterness, a bulky basic group including an α-amino group was functional. The hydrophobic group also operated for a secondary site to determine the bitter taste. From this view, the primary and the secondary bitter taste determinant sites were termed the "binding unit" (BU) and the "stimulating unit" (SU), respectively. The experimental results of this study were quite compatible with a hypothesis by Okai,[8] one of the authors, in 1976 on the mechanism of bitter taste sensibility in peptides.



BU: binding unit (hydrophobic group).
SU: stimulating unit (hydrophobic or basic group).

Scheme of Binding of Bitter Peptide with Bitter Taste Receptor.

The intensity of bitterness is assumed to be dependent on the features of the binding and stimulating units and on the distance between the units. This mechanism will be elucidated owing to the systematic analysis of the relationship between the chemical structure and bitter taste intensity of the peptides extensively. The investigation on molecular dimensions of bitter peptides which the bitter taste receptor can recognize is in progress.

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