The Structure-Taste Relationships of the Dipeptide Esters Composed of L-Aspartic Acid and β-Hydroxy Amino Acids

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In order to evaluate the contribution of a hydroxyl group to the degree of sweetness of aspartyl dipeptides, we have synthesized a number of α-L-aspartyl dipeptides, such as the esters of α-L-aspartyl-L-hydroxynorleucine (**16** and **17**), -*O*-acyl-L-serine (**18**—**21**), -D-serine (**22**—**28**), -D-threonine (**30**—**36**), and -D-allothreonine (**37**—**43**); their sweetness potencies were compared with those of the corresponding dipeptides without a hydroxyl group. It has been found that a hydroxyl group makes a major contribution to the sweetness of the α-L-aspartyl dipeptide esters. The introduction of a hydroxyl group into the L–L peptides gave compounds with reduced potency. However, no such regularity was observed in the L–D peptides; the esters of α-L-aspartyl-D-serine (**22**—**28**) and -D-threonine (**30**—**36**) were sweeter than the corresponding esters of α-L-aspartyl-D-alanine and -D-α-amino-*n*-butyric acid respectively, while the esters of α-L-aspartyl-D-allothreonine (**37**—**43**) were less sweet than the corresponding esters of α-L-aspartyl-D-α-amino-*n*-butyric acid. The enzymatic resolution of *N*-acetyl-*erythro*- and -*threo*-β-hydroxy-DL-norleucine is also described.

The pronounced sucrose-like sweetness of α-L-aspartyl-L-phenylalanine methyl ester (APM) was reported by Mazur *et al.*[1] At that time, L-aspartic acid seemed to be necessary for sweetness, but the phenylalanine part could be modified considerably.[1,2] A number of sweet dipeptide esters related to APM have been synthesized by several groups,[3] according to the generalization made by Mazur *et al.*[1,2] Recently, however, Briggs and Murley described that the aspartic acid part could be replaced by amino malonic acid without losing its sweetness.[4] Furthermore, Lapidus and Sweeney reported that an *N*-protected APM, *N*-trifluoroacetyl-L-aspartyl-L-phenylalanine methyl ester, was 120 times as sweet as sucrose,[5] though the free amino group of L-aspartic acid had been considered to be necessary for sweetness.[1]

APM appears attractive as a low-calorie sweetener, but it is somewhat unstable in an aqueous solution, in which APM spontaneously cyclizes to cyclo-L-aspartyl-L-phenylalanyl, which lacks sweetness. In order to obtain more stable sweeteners than APM and, in particular, to draw on for more definite structure-taste relationships of aspartyl dipeptides, we synthesized a number of aspartyl dipeptide derivatives, including D-amino acids and L- and D-hydroxy amino acids, and tasted them.

During the replacement of the phenylalanine part of APM by other amino acids, it was found that α-L-aspartyl-L-norvaline methyl ester (**1**) was somewhat sweet, about 4 times sucrose, while α-L-aspartyl-L-α-amino-*n*-butyric acid methyl ester (**2**) was devoid of sweetness. Mazur *et al.* reported that the methyl esters of α-L-aspartyl-L-valine, -L-leucine, and -L-isoleucine were bitter.[1] The results show that, in the cases of the aspartyl dipeptides with the L–L configuration, L-norvaline seems to be the smallest amino acid to give a sweet derivative; they also show that the substitution of a methyl group for a hydrogen on the β- or γ-carbon of the amino acid results in a bitter taste (Table 2). A similar relationship was found to hold for the methyl esters of α-L-aspartyl-*O*-acyl-L-threonine, -*O*-acyl-L-allothreonine, and -D-alanylsarcosine (*vide post*). α-L-Aspartyl-β-alanine methyl ester (**6**) and -β-alanine *i*-propyl ester (**7**) lacking an asymmetric carbon were sweet, about equal to sucrose and 7 times sucrose respectively. α-L-Aspartyl-DL-β-amino-*n*-butyric acid methyl ester (**4**) and -γ-amino-*n*-butyric acid methyl ester (**5**) were similar in potency to sucrose. α-L-Aspartyl-L-lysine methyl ester (**8**) was bitter, while α-L-aspartyl-N^{ε}-acetyl-L-lysine methyl ester (**9**) as well as α-L-aspartyl-N^{δ}-acetyl-L-ornithine methyl ester (**10**) was sweet, about equal to sucrose. α-L-Aspartyl-DL-(α-methyl)phenylalanine methyl ester (**11**) and -DL-(α-methyl)glutamic acid dimethyl ester (**12**) were sweet, 5 times sucrose and 8 times sucrose respectively. The results show that the α-hydrogen is not necessary for sweetness, but can be replaced by a methyl group without losing sweetness. α-L-Aspartyl-D-alanylglycine methyl ester (**13**), a tripeptide, was 3 times as sweet as sucrose, and the replacement of the glycine by sarcosine resulted in a bitter taste. They are reasonable results if the D-alanylglycine methyl ester is considered as approximately equivalent to an L-2-amino-alkane such as L-2-amino-octane,[2] and if the replacement of the glycine by sarcosine is also considered as approximately equivalent to the introduction of a methyl group into the γ-position of alkyl amines (*e.g.*, L-1,3-dimethylbutylamine[2]) or amino acids (*e.g.*, L-Leu-OMe in Table 2). Therefore, the former is sweet and the latter is bitter. No compound sweeter than APM was found. The results are summarized in Table 1.

At the present time, the phenomenon of a sweet taste seems best explained by the hydrogen bond between receptor site of the "sweet-sensitive protein" and the sweet unit of the chemical compound. Therefore, we are interested in the contribution of a hydroxyl group to the degree of sweetness, since there has been no systematic and thorough study of the evaluation of a hydroxyl group, though the introduction of hetero-atoms, including a hydroxyl group, resulted in the diminution of the potency.[1,2] In order to evaluate the contribution of a hydroxyl group, we intended to introduce a hydroxyl group into the β-position of the norleucine of α-L-aspartyl-L-norleucine methyl ester (**15**) with a sweetness potency of 45 times sucrose. When *erythro*- and *threo*-β-hydroxy-L-norleucine were substituted for the norleucine, the sweetness fell off

Table 1. Taste of aspartyl peptides (α-L-Asp-X)

	X[a]	Taste[b]
1	L-Nva-OMe	4
2	L-Abu-OMe	tasteless
3	D-Abu-OMe	16
4	DL-β-Abu-OMe	+
5	γ-Abu-OMe	+
6	β-Ala-OMe	+
7	β-Ala-OPr^i	7
8	L-Lys-OMe	bitter
9	L-Lys(Ac)-OMe	+
10	L-Orn(Ac)-OMe	+
11	DL-(α-Me)Phe-OMe	5
12	DL-(α-Me)Glu-$(OMe)_2$	8
13	D-Ala-Gly-OMe	3
14	D-Ala-Sar-OMe	bitter

a) Abbreviations according to IUPAC-IUB Commission on Biochemical Nomenclature, *Arch. Biochim. Biophys.*, **150**, 1 (1972). b) Number times sweeter than sucrose. In addition, + = 1—2 times sucrose (5% sucrose level).

rapidly; α-L-aspartyl-*erythro*-β-hydroxy-L-norleucine methyl ester (**16**) was 18 times as sweet as sucrose, and the corresponding *threo*-isomer (**17**) was 7 times sucrose (Table 3). It seems to be true that the introduction of a hydroxyl group into a dipeptide with the L–L configuration results in a diminution in the potency.

Next, we synthesized the esters of α-L-aspartyl-*O*-acyl-L-serine (**18**—**21**). α-L-Aspartyl-*O*-acetyl-L-serine methyl ester (**18**), as well as the *O*-*n*-propionyl (**19**), *O*-*n*-butyroyl (**20**), and *O*-*i*-butyroyl (**21**) analogs, was sweet (Table 3). From an examination of a model, this is a reasonable result if the *O*-acylated part is considered as approximately equivalent to the *n*-butyl part of norleucine of **15** (Table 3). The replacement of the methyl ester group by the ethyl ester gave compounds with reduced potency; α-L-aspartyl-*O*-acetyl-L-serine ethyl ester was slightly sweet, about equal to sucrose, the *O*-*n*-butyroyl analog was 10 times as sweet as sucrose, and the *O*-*i*-butyroyl analog was 3 times sucrose. The replacement of the serine by L-threonine or by L-allothreonine resulted in bitter compounds; the methyl esters of α-L-aspartyl-*O*-acyl-L-threonine and -*O*-acyl-L-allothreonine (acyl: *n*-butyroyl and *i*-butyroyl) were bitter (Table 2). These results support the idea that the substitution of a methyl group for a hydrogen on the β-carbon of amino acids may result in a bitter substance (Table 2 and see also Ref. 1).

In order to find out more information about hydroxyl function, α-L-aspartyl-D-serine esters (**22**—**28**) were synthesized. α-L-Aspartyl-D-serine methyl ester (**22**), as well as the ethyl (**23**), *n*-propyl (**24**), *i*-propyl (**25**), *n*-butyl (**26**), *i*-butyl (**27**), and cyclohexyl (**28**) esters, was found to have an intensely sweet taste (Table 3). It was surprising to us that α-L-aspartyl-D-serine esters, in spite of having a hydroxyl group, were sweeter than α-L-aspartyl-D-alanine esters[3g] without a hydroxyl group; *e.g.*, α-L-aspartyl-D-serine *n*-propyl ester (**24**) was 320 times as sweet as sucrose, while the corresponding α-L-aspartyl-D-alanine *n*-propyl ester (**29**) was 125 times sucrose. The increased sweetness of the peptides

Table 2. Taste of α-L-aspartyl peptides and amides (α-L-Asp-X)

X: Symbol[a]	Structure	Taste[b]
L-Nva-OMe (**1**)	$\begin{matrix}COOCH_3\\\vert\\HNCHCH_2CH_2CH_3\end{matrix}$	4
L-Leu-OMe	$\begin{matrix}COOCH_3\\\vert\\HNCHCH_2CHCH_3\\\quad\vert\\\quad CH_3\end{matrix}$	bitter[2]
L-Ile-OMe	$\begin{matrix}COOCH_3\\\vert\\HNCHCHCH_2CH_3\\\quad\vert\\\quad CH_3\end{matrix}$	bitter[2]
D-Ala-Gly-OMe (**13**)	$\begin{matrix}CH_3\\\vert\\HNCHCONHCH_2COOCH_3\end{matrix}$	3
D-Ala-Sar-OMe (**14**)	$\begin{matrix}CH_3\quad CH_3\\\vert\quad\vert\\HNCHCONCH_2COOCH_3\end{matrix}$	bitter
L-2-Amino-octane	$\begin{matrix}CH_3\\\vert\\NHCHCH_2CH_2CH_2CH_2CH_2CH_3\end{matrix}$	10[2]
L-Ser-OMe (**21**)	$\begin{matrix}COPr^i\quad COOCH_3\\\vert\quad\vert\\HNCHCH_2OCOCH(CH_3)_2\end{matrix}$	50
L-Thr-OMe	$\begin{matrix}COPr^i\quad COOCH_3\\\vert\quad\vert\\HNCHCHOCOCH(CH_3)_2\\\quad\vert\\\quad CH_3\end{matrix}$	bitter
D-Ser-OPr^n (**24**)	$\begin{matrix}CH_2OH\\\vert\\HNCHCOOCH_2CH_2CH_3\end{matrix}$	320
D-Ala-OPr^n (**29**)	$\begin{matrix}CH_3\\\vert\\HNCHCOOCH_2CH_2CH_3\end{matrix}$	125
L-Amphetamine	$\begin{matrix}CH_3\\\vert\\HNCHCH_2C_6H_5\end{matrix}$	50[2]
L-1-Hydroxymethyl-2-phenylethyl-amine	$\begin{matrix}CH_2OH\\\vert\\HNCHCH_2C_6H_5\end{matrix}$	1—2[2]
D-Thr-OPr^n (**32**)	$\begin{matrix}CH_3\\\vert\\H\text{-}C\text{-}OH\\\vert\\HN\text{-}C\text{-}H\\\vert\\COOCH_2CH_2CH_3\end{matrix}$	150
D-aThr-OPr^n (**39**)	$\begin{matrix}CH_3\\\vert\\HO\text{-}C\text{-}H\\\vert\\HN\text{-}C\text{-}H\\\vert\\COOCH_2CH_2CH_3\end{matrix}$	40
D-Abu-OPr^n (**44**)	$\begin{matrix}CH_3\\\vert\\CH_2\\\vert\\HNCHCOOCH_2CH_2CH_3\end{matrix}$	95

a) See Table 1 for abbreviations. b) Number times sweeter than sucrose (5% sucrose level).

(**22**—**28**) would not have been predicted from the previous results, since the replacement of the amino acids or amines by those with a hydroxyl group always resulted in the diminution of the potency;[1,2] *e.g.*, α-L-aspartyl-L-amphetamine is 50 times as sweet as sucrose, while α-L-aspartyl-1-hydroxymethyl-2-phenylethylamine is only 1—2 times sucrose in sweetness[2] (Table 2).

This finding led us to synthesize the esters of α-L-aspartyl-D-threonine (**30**—**36**) and -D-allothreonine (**37**—**43**). It was found that they all have an intensely sweet taste (Table 3). It is very interesting to note that the esters of α-L-aspartyl-D-threonine (**30**—**36**) were sweeter than the esters of α-L-aspartyl-D-α-amino-*n*-butyric acid,[3g] which lacks a hyrdoxyl group of the D-threonine, as in the case of the esters of α-L-aspartyl-D-serine and -D-alanine. The replacement of the D-threonine by D-allothreonine resulted in a significant diminution of the potency; *e.g.*, α-L-aspartyl-D-α-amino-*n*-butyric acid *n*-propyl ester (**44**) was 95 times as sweet as sucrose, α-L-aspartyl-D-threonine *n*-propyl ester (**32**) was 150 times sucrose, and α-L-aspartyl-D-allothreonine *n*-propyl ester (**39**) was 40 times sucrose. With an increase in the size of the ester, the potency increased peaking at the chain length of three carbons, such as the *n*-propyl and *i*-butyl esters (Table 3). It is also of interest to note that the configurational change in the hydroxyl group results in a change in the potency, as is shown in Table 3 (**16** and **17**, and **30**—**36** and **37**—**43**). In the cases of the peptides with the L–D configuration, the *threo*-isomers were sweeter than the corresponding *erythro*-isomers, in contrast to the cases of the L–L peptides. In the cases of the L–L peptides, the *erythro*-isomers were sweeter than the corresponding *threo*-isomers, as has been described in the case of α-L-aspartyl-β-hydroxy-L-norleucine methyl ester (**16** and **17**). The results (Table 3) indicate that the introduc-

Table 3. Sweetness values of aspartyl dipeptide esters

	Compound[a]	Mp (°C)	Sweetness value[b]	Formula	Calcd %			Found %		
					C	H	N	C	H	N
15	α-L-Asp-L-Nle-OMe	148—149	45	$C_{11}H_{20}O_5N_2\cdot 1/2H_2O$	49.06	7.86	10.40	49.59	7.60	10.50
16	α-L-Asp-L-*e*-HyNle-OMe	172—173	18	$C_{11}H_{20}O_6N_2O\cdot H_2O$	44.89	7.54	9.52	45.08	7.61	9.51
17	α-L-Asp-L-*t*-HyNle-OMe	167—168	7	$C_{11}H_{20}O_6N_2O\cdot H_2O$	44.89	7.54	9.52	44.76	7.61	9.66
18	α-L-Asp-L-Ser-OMe $COCH_3$	88—89.5	10	$C_{10}H_{16}O_7N_2\cdot 1/2H_2O$	42.10	6.01	9.82	41.72	5.82	9.76
19	α-L-Asp-L-Ser-OMe $COCH_2CH_3$	86—87	40	$C_{11}H_{18}O_7N_2\cdot 1/2H_2O$	44.14	6.40	9.36	44.24	6.32	9.24
20	α-L-Asp-L-Ser-OMe $COCH_2CH_2CH_3$	102—103	30	$C_{12}H_{20}O_7N_2\cdot 1/2H_2O$	46.00	6.76	8.94	45.72	6.59	8.81
21	α-L-Asp-L-Ser-OMe $COCH(CH_3)_2$	116—116.5	50	$C_{12}H_{20}O_7N_2\cdot 1/2H_2O$	46.00	6.76	8.94	45.96	6.62	9.07
22	α-L-Asp-D-Ser-OMe	183—184	45	$C_8H_{14}O_6N_2$	41.02	6.03	11.96	40.74	5.96	11.86
23	α-L-Asp-D-Ser-OEt	193—194	115	$C_9H_{16}O_6N_2$	43.54	6.50	11.29	43.46	6.41	11.26
24	α-L-Asp-D-Ser-OPr^n	194—195	320	$C_{10}H_{18}O_6N_2$	45.79	6.92	10.68	45.42	6.83	10.68
25	α-L-Asp-D-Ser-OPr^i	198—199	120	$C_{10}H_{18}O_6N_2$	45.79	6.92	10.68	45.59	6.78	10.66
26	α-L-Asp-D-Ser-OBu^n	192—193	70	$C_{11}H_{20}O_6N_2$	47.82	7.30	10.14	47.56	7.21	10.05
27	α-L-Asp-D-Ser-OBu^i	188—189	200	$C_{11}H_{20}O_6N_2$	47.82	7.30	10.14	47.63	7.22	10.30
28	α-L-Asp-D-Ser-OC_6H_{11}	198—199	60	$C_{13}H_{22}O_6N_2\cdot 1/2H_2O$	50.15	7.45	9.00	50.10	7.13	8.97
29	α-L-Asp-D-Ala-OPr^n	143—144	125	$C_{10}H_{18}O_5N_2\cdot H_2O$	45.44	7.63	10.60	45.39	7.38	10.66
30	α-L-Asp-D-Thr-OMe	158—159	25	$C_9H_{16}O_6N_2\cdot H_2O$	40.60	6.81	10.52	40.45	6.63	10.14
31	α-L-Asp-D-Thr-OEt	160—161	110	$C_{10}H_{18}O_6N_2\cdot 3/2H_2O$	41.51	7.32	9.68	41.34	6.79	9.62
32	α-L-Asp-D-Thr-OPr^n	155—156	150	$C_{11}H_{20}O_6N_2$	47.82	7.30	10.14	47.96	7.25	10.33
33	α-L-Asp-D-Thr-OPr^i	84—85	105	$C_{11}H_{20}O_6N_2\cdot 3/2H_2O$	43.56	7.64	9.24	43.42	7.65	9.29
34	α-L-Asp-D-Thr-OBu^n	82—83	30	$C_{12}H_{22}O_6N_2\cdot H_2O$	46.74	7.85	9.09	46.50	7.43	9.05
35	α-L-Asp-D-Thr-OBu^i	142—143	110	$C_{12}H_{22}O_6N_2$	49.64	7.64	9.65	49.89	7.42	9.58
36	α-L-Asp-D-Thr-OC_6H_{11}	180—181	30	$C_{14}H_{24}O_6N_2\cdot 1/2H_2O$	51.68	7.75	8.61	51.31	7.58	8.60
37	α-L-Asp-D-aThr-OMe	181—182	7	$C_9H_{16}O_6N_2$	43.54	6.50	11.29	43.37	6.30	11.40
38	α-L-Asp-D-aThr-OEt	181—182	6	$C_{10}H_{18}O_6N_2$	45.79	6.92	10.68	45.91	6.70	10.77
39	α-L-Asp-D-aThr-OPr^n	176—177	40	$C_{11}H_{20}O_6N_2$	47.82	7.30	10.14	47.86	7.12	9.98
40	α-L-Asp-D-aThr-OPr^i	182—183	10	$C_{11}H_{20}O_6N_2$	47.82	7.30	10.14	47.91	7.03	10.26
41	α-L-Asp-D-aThr-OBu^n	173—174	20	$C_{12}H_{22}O_6N_2$	49.64	7.64	9.65	49.51	7.35	9.66
42	α-L-Asp-D-aThr-OBu^i	183—184	27	$C_{12}H_{22}O_6N_2$	49.64	7.64	9.65	49.65	7.44	9.61
43	α-L-Asp-D-aThr-OC_6H_{11}	198—199	22	$C_{14}H_{24}O_6N_2$	53.15	7.65	8.86	53.18	7.36	8.89
44	α-L-Asp-D-Abu-OPr^n	85—86.5	95	$C_{11}H_{20}O_5N_2\cdot H_2O$	47.47	7.97	10.07	47.26	7.74	9.97

a) See Table 1 for abbreviations. b) Number times sweeter than sucrose (5% sucrose level).

tion of a hydroxyl group into the L–D peptides does not always result in a diminution of their potencies, but sometimes increase their potencies, in contrast to the cases of the L–L peptides. In the cases of the L–L peptides, the potencies always decreased when a hydroxyl group was introduced, as has been described above and as has been reported by Mazur *et al.*[1,2] The contribution of a hydroxyl group to the L–D peptides seems to differ significantly from that to the L–L peptides. These results would appear to hold considerable promise for clarifying the interaction between the receptor site and the sweet unit. More detailed studies concerning this problem are now in progress.

Experimental

All the melting points are uncorrected. The optical rotations were measured with a visual polarimeter. All the compounds reported here were homogeneous on thin-layer chromatography on silica. Spots were detected first by the ninhydrin method and then by the starch-iodide method, as modified by one of us.[6] The acylated NH groups, if any, can be detected at the 0.1—0.25 μg level after chromatography by the latter method.

Starting Materials. L- and D-α-Amino-*n*-butyric acid, L-norvaline, L-norleucine, D-serine, and D-allothreonine were obtained without any difficulty *via* the L-directed asymmetric hydrolytic action of the mold acylase on their *N*-acetyl racemates by the usual procedure.[7] L-α-Amino-*n*-butyric acid: $[\alpha]_D^{21}$ +20.5° (*c* 2, 5 M HCl). lit,[7] $[\alpha]_D^{25}$ +20.6° (*c* 2, 5 M HCl). D-α-Amino-*n*-butyric acid: $[\alpha]_D^{21}$ −20.5° (*c* 2, 5 M HCl). lit,[7] $[\alpha]_D^{25}$ −20.7° (c 2, 5 M HCl). L-Norvaline: $[\alpha]_D^{26}$ +24.3° (*c* 2, 5 M HCl). lit,[7] $[\alpha]_D^{25}$ +24.9° (*c* 2, 5 M HCl). L-Norleucine: $[\alpha]_D^{23}$ +24.0° (*c* 1, 6 M HCl). lit,[8] $[\alpha]_D^{23}$ +23.3° (*c* 4.15, 6 M HCl). D-Serine: $[\alpha]_D^{19}$ −15.8° (*c* 2, 6 M HCl). lit,[7] $[\alpha]_D^{25}$ −15.0° (*c* 2, 5 M HCl). D-Allothreonine: $[\alpha]_D^{26}$ −9.6° (*c* 4, water). lit,[7] $[\alpha]_D^{25}$ −9.6° (*c* 4, water). D-Threonine, which had been resolved by the preferential crystallization, was left over from a previous investigation.[9] $[\alpha]_D^{29}$ +28.7° (*c* 2, water). lit,[7] $[\alpha]_D^{25}$ +28.5° (c 2, water). *threo-* and *erythro-β*-Hydroxy-L-norleucine: β-Hydroxynorleucine was resolved *via* the asymmetric synthetic action of papain on *N*-benzoyl-β-methoxynorleucine by Adams and Niemann.[10] We will now describe a more convenient method of resolving the two diastereoisomeric racemates of β-hydroxynorleucine *via* the L-directed asymmetric hydrolytic action of a mold acylase on their *N*-acetyl derivatives. β-Hydroxynorleucine was prepared by the condensation of copper glycinate with *n*-butyraldehyde according to the method described by Mix[11] and was then separated into the two diastereoisomeric racemates through their tetrachlorophthalic acid salts according to the method previously reported by one of the present authors.[12] *N*-Acetyl-*threo-* and *-erythro-β*-hydroxy-DL-norleucine were obtained by the usual acetylation of *threo-* and *erythro-β*-hydroxy-DL-norleucine with acetic anhydride. *N*-Acetyl-*threo-β*-hydroxy-DL-norleucine: yield, 82%; mp 149—150.5 °C (decomp.). Found: C, 50.86; H, 8.08; N, 7.40%. Calcd for $C_8H_{15}O_4N$: C, 50.78; H, 7.99; N, 7.40%. *N*-Acetyl-*erythro-β*-hydroxy-DL-norleucine: yield, 82%; mp 137—138 °C. Found: C, 50.97; H, 8.02; N, 7.35%. The optical resolution of *N*-acetyl-*threo-* and *-erythro-β*-hydroxy-DL-norleucine was not easily accomplished because the rate of the hydrolysis was found to be considerably lower than those observed with other amino acids, such as norvaline and serine.

Incubation with Enzyme. A solution of *N*-acetyl-*threo-β*-hydroxy-DL-norleucine (18.4 g) in water (1800 ml) was adjusted to pH 7.0 with an aqueous sodium hydroxide solution. To the solution, there were then added cobaltous chloride hexahydrate (0.43 g) and the mold acylase (0.36 g).[13] The solution was incubated at 38 °C for 8 days. During the incubation, an additional acylase (0.18 g) was added every other day to the solution.

Isolation of threo-*β-Hydroxy-*D*-norleucine.* In order to separate the free L-amino acid and *N*-acetyl-D-amino acid, the solution was passed through a column (3×50 cm) of Diaion SK 1B[14] (H-form) and the column was washed with water. The fractions containing the *N*-acetyl-D-amino acid were combined and concentrated *in vacuo* to dryness. The residue was dissolved in water and decolorized with charcoal. The solution was concentrated *in vacuo* until the crystals appeared. After storage in a refrigerator overnight, the crystals of *N*-acetyl-*threo-β*-hydroxy-D-norleucine thus formed were collected by filtration; yield, 8.3 g (89%); mp 136—138 °C. Found: C, 50.85; H, 7.95; N, 7.40%.

A solution of *N*-acetyl-*threo-β*-hydroxy-D-norleucine (8.3 g) in 3 M hydrochloric acid (83 ml) was heated under reflux for 6 hr and then concentrated *in vacuo* to dryness. The residue was dissolved in water, and the solution was adjusted to pH 6.0 with an aqueous sodium hydroxide solution and poured onto a column (3×50 cm) of Diaion SK 1B (H-form). The column was washed with water, and then the D-amino acid was eluted with 1.5 M ammonium hydroxide. The eluate was concentrated *in vacuo* to a small volume, and then a sufficient amount of ethanol was added to the concentrate to give 5.7 g (88%) of crystals (*threo-β*-hydroxy-D-norleucine), which were subsequently recrystallized from water-ethanol; yield, 4.8 g (74%); mp 222—224 °C (decomp.); $[\alpha]_D^{25}$ −4.8° (*c* 2, water) and −18.5° (*c* 2, 5 M HCl); $[M]_D^{25}$ −7.0° (*c* 2, water) and −27.2° (*c* 2, 5 M HCl). lit,[10] mp 185—188 °C (decomp.); $[\alpha]_D^{25}$ −4.6° (*c* 3.50, water) and −18.5° (*c* 2.28, 6.03 M HCl). Found: C, 49.40; H, 8.79; N, 9.44%. Calcd for $C_6H_{13}O_3N$: C, 48.96; H, 8.90; N, 9.52%.

Isolation of threo-*β-Hydroxy-*L*-norleucine.* The column, adsorbing the free L-amino acid, was washed with water (200 ml) to remove completely the trace amount of the *N*-acetyl-D-amino acid from the column. The L-amino acid was eluted with 1.5 M ammonium hydroxide. The eluate was concentrated *in vacuo* to a small volume, and then a sufficient amount of ethanol was added to the concentrate to give 5.8 g (89%) of the L-amino acid, which was subsequently recrystallized from water-ethanol; yield, 5.0 g (70%); mp 222—223 °C (decomp.); $[\alpha]_D^{25}$ +4.5° (*c* 2, water) and +18.5° (*c* 2, 5 M HCl); $[M]_D^{25}$ +6.6° (*c* 2, water) and +27.7° (*c* 2, 5 M HCl). lit,[10] mp 184—188 °C (decomp.); $[\alpha]_D^{25}$ +4.6° (*c* 3.46, water) and +18.6° (*c* 2.42, 6.07 M HCl). lit,[15] $[M]_D^{25}$ 6.8° (*c* 2, water) and +27.4° (*c* 2, 5 M HCl). Found: C, 48.80; H, 8.63; N, 9.40%.

The resolution of the racemic *erythro*-isomer was carried out by the manner described in the resolution of the racemic *threo*-isomer, except that the *N*-acetyl-D-amino acid was obtained as an oil; it was subjected to acid hydrolysis without further purification.

erythro-β-Hydroxy-D-norleucine: yield, 76%; mp 241—242 °C (decomp.); $[\alpha]_D^{25}$ +2.3° (*c* 2, water) and −28.3° (*c* 2, 5 M HCl); $[M]_D^{25}$ +3.4° (*c* 2, water) and −41.6° (*c* 2, 5 M HCl). lit,[10] mp 198—202 °C(decomp.); $[\alpha]_D^{26}$ +2.0° (*c* 8.81, water) and $[\alpha]_D^{25}$ −27.4° (*c* 2.63, 6.07 M HCl). Found: C, 48.99; H, 8.72; N, 9.57%.

erythro-β-Hydroxy-L-norleucine: yield, 89%; mp 240.5—241.5 °C (decomp.); $[\alpha]_D^{25}$ −2.3° (*c* 2, water) and +28.3°

(*c* 2, 5 M HCl); $[M]_D^{25}$ −3.4° (*c* 2, water) and +41.6° (*c* 2, 5 M HCl). lit,[10] mp 203—205 °C (decomp.); $[\alpha]_D^{22}$ −2.0° (*c* 5.87, water) and $[\alpha]_D^{25}$ +27.1° (*c* 2.47, 6.07 M HCl). lit,[15] $[M]_D^{25}$ −2.9° (*c* 2, water) and +39.9° (*c* 2, 5 M HCl). Found: C, 48.96; H, 8.63; N, 9.45%.

Hydrochlorides of Amino Acid Esters. In a typical procedure (D-serine *i*-propyl ester hydrochloride), into a suspension of D-serine (10.5 g) in *i*-propyl alcohol (200 ml) was bubbled dry hydrogen chloride gas without external cooling. After the crystals had gone into solution, the reaction mixture was boiled under reflux for 5 hr and then concentrated *in vacuo*. Benzene (100 ml) was added to the residue; the solution was then evaporated *in vacuo* to remove the trace amount of water, and then the esterification procedure was repeated. The reaction mixture was concentrated *in vacuo* to give the crude ester, which was recrystallized from *i*-propyl alcohol–ether; yield, 13.7 g (75%); mp 145—146 °C.

The other amino acids were esterified with an appropriate alcohol by the same procedure, and the resulting hydrochlorides of amino acid esters were recrystallized from the appropriate alcohol and ether. When *n*-butanol, *i*-butyl alcohol and cyclohexanol were employed, the reaction temperature was maintained at 100 °C.

*α-L-Aspartyl-O-acyl-L-serine Esters (**18**—**21**).* In a typical procedure (**21** in Table 3), *β*-benzyl-*N*-carbobenzoxy-L-aspartyl-L-serine methyl ester[16] (4.6 g) was dissolved in pyridine (50 ml) and cooled in an ice bath, and then *i*-butyric anhydride (3.2 g) was stirred in. After stirring for 3 hr at room temperature, the reaction mixture was stirred into 500 ml of water containing ice flakes. The crystals thus formed were collected by filtration and washed with 0.7 M hydrochloric acid and then water; yield, 5.1 g (98%). Recrystallization from ethyl acetate–petroleum ether gave 4.7 g (91%) of *β*-benzyl-*N*-carbobenzoxy-L-aspartyl-*O*-*i*-butyroyl-L-serine methyl ester; mp 95—96 °C. Found: C, 61.58; H, 6.16; N, 5.31%. Calcd for $C_{27}H_{32}O_9N_2$: C, 61.35; H, 6.10; N, 5.30%.

A solution of the dipeptide derivative (4.0 g) in 90% methanol (50 ml) was hydrogenated in the presence of 5% palladium on charcoal (0.4 g) for 5 hr. After the removal of the catalyst by filtration, the filtrate was concentrated *in vacuo* to give a crystalline residue. Recrystallization from aqueous methanol gave 1.3 g (54%) of α-L-aspartyl-*O*-*i*-butyroyl-L-serine methyl ester.

The results are shown in Table 3.

*The Esters of α-L-Aspartyl-*erythro- *and* -threo-*β-hydroxy-*L-*norleucine (**16** and **17**), -D-serine (**22**—**28**), -D-threonine (**30**—**36**), and -D-allothreonine (**37**—**43**).* In a typical procedure (**25** in Table 3), *β*-benzyl-*N*-carbobenzoxy-L-aspartate[17] (7.2 g) was dissolved in chloroform (50 ml) and cooled in an ice bath, and then *N*,*N*′-dicyclohexylcarbodiimide (4.3 g) was added. After 20 mins' stirring in the ice bath, a solution of *i*-propyl D-serinate hydrochloride (4.0 g) and triethylamine (2.3 g) in chloroform (50 ml) was added to the reaction mixture. After stirring for 6 hr at room temperature, a few drops of acetic acid were added to the reaction mixture. After the removal of *N*,*N*′-dicyclohexylurea by filtration, the filtrate was concentrated *in vacuo*. The residue was dissolved in ethyl acetate and washed successively with 1 M hydrochloric acid, a 5% sodium bicarbonate solution, and water, and finally dried over sodium sulfate. After filtration, the filtrate was concentrated *in vacuo* to give 7.6 g (78%) of *β*-benzyl-*N*-carbobenzoxy-L-aspartyl-D-serine *i*-propyl ester; mp 80.5—81.5 °C. Found: C, 61.35; H, 6.16; N, 5.65%. Calcd for $C_{25}H_{30}O_8N_2$: C, 61.71; H, 6.22; N, 5.76%.

A solution of the protected dipeptide derivative (5.0 g) in 75% acetic acid (100 ml) was hydrogenated in the presence of 5% palladium on charcoal (0.5 g) at room temperature for 5 hr. After the removal of the catalyst by filtration, the filtrate was concentrated *in vacuo* to give crystals, which were subsequently recrystallized from aqueous methanol; yield, 2.2 g (82%). Recrystallization from water gave 1.5 g of α-L-aspartyl-D-serine *i*-propyl ester.

The results are shown in Table 3.

Sweetness Evaluation. The sweetness values of the dipeptide derivatives were organoleptically determined by panel evaluation, with ten to fifteen people per panel, in our Foodstuff Development Laboratory. In a series of tests with aqueous solutions, samples of the peptides and samples of various levels of sucrose (3—8%) were compared for relative sweetness. The tests were carried out at concentrations higher than threshold in order to provide a comparison at a practical level. In a typical comparison test, an aqueous solution of α-L-aspartyl-D-serine *n*-propyl ester containing 0.02% was rated in between a 6.08 and a 7.00% sucrose solution, putting it in the 305—350 sweetness range. Therefore, the compound was assumed about 320 times as sweet as sucrose.

The results are shown in Tables 1, 2, and 3.

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