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Synthesis and Antipepsin Activities of Peptides having a Valine or Valylvaline Moiety at the N-Terminus[1)]

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A series of peptides having a valine or valylvaline moiety at the N-terminus, **1**—**13**, was prepared and their antipepsin activities were tested. All of the peptides possessing an N-acyl–Val–Val moiety, except one, showed some inhibitory activity against pepsin, while compounds lacking N-acyl–Val–Val showed no inhibition even at a concentration of 50 μg/ml.

Compound **12**, a pepstatin analog, in which a tyrosine residue is present instead of AHMHA of pepstatins, was the most potent inhibitor among the synthetic peptides, but its activity was markedly lower than that of pepstatin A. Compounds **10**, **12**, and **13** showed neither agonistic nor antagonistic effects on pepstatin A, suggesting major differences in binding abilities of the synthetic peptides and of pepstatin A to the enzyme. The importance of the AHMHA residue of pepstatins in relation to the inhibitory activity is discussed.

Keywords——antipepsin activity; pepstatin analogs; N-acylvalylvaline sequence; oligopeptides; 4-amino-3-hydroxy-butyric acid

Pepstatins, which are produced by Actinomycetes species, are pentapeptides with a valylvaline sequence at the N-terminus, and are specific inhibitors of acid proteases and the renin-angiotensin system.[3)]

Recently Aoyagi *et al.*[4)] showed that the partial sequence Val-Val-AHMHA is a minimum requirement for pepsin-inhibiting activities, and Shigezane *et al.*[5)] reported that Leu–Val–Tyr or Leu–Val–Phe is necessary for antirenin activity. These results suggested to us that peptides with hydrophobic amino acids at the N-terminus might be effective inhibitors of acid proteases.

Here we report the synthesis and the antipepsin activities of thirteen oligopeptides possessing a valine or valylvaline moiety at the N-terminus, **1**—**13** (Table I). Of these, the N-acylpentapeptides, **12** and **13**, differ from pepstatin G[4)] only in that they contain Tyr and aminohydroxy acid (AHB), respectively, instead of the AHMHA moiety, and **12** is N-acylated with Z instead of Oct.

1) The amino acid residues except for glycine, 4-amino-3-hydroxy-butyric acid (AHB), and 4-amino-3-hydroxy-6-methylheptanoic acid (AHMHA) are of the L-configuration, unless otherwise stated. The abbreviations used to denote amino acids, peptides, and their derivatives are those recommended in *J. Biol. Chem.*, **247**, 977 (1972). Other abbreviations used are: ONBzl=*p*-nitrobenzyl ester; OSu=N-hydroxysuccinimide ester, Oct=octanoyl; DCC=dicyclohexylcarbodiimide; THF=tetrahydrofuran; TFA=trifluoroacetic acid; NMM=N-methylmorpholine; BCC=isobutyloxycarbonyl chloride.

2) Location: *a) Kowakae, Higashi-Osaka, 577, Japan; b) Yoshikawa, Matsumoto, 399-65, Japan; c) Kanagawa-machi, Kanazawa, 920, Japan.*

3) *a)* H. Umezawa, T. Aoyagi, H. Morishima, M. Matsuzaki, M. Hamada, and T. Takeuchi, *J. Antibiotics*, **23**, 259 (1970); *b)* H. Morishima, T. Takita, T. Aoyagi, T. Takeuchi, and H. Umezawa, *ibid.*, **23**, 263 (1970); *c)* T. Miyano, M. Tomiyasu, H. Iizuka, S. Tomisaka, T. Takita, T. Aoyagi, and H. Umezawa, *ibid.*, **25**, 490 (1972).

4) T. Aoyagi, H. Morishima, R. Nishizawa, S. Kunimoto, T. Takeuchi, and H. Umezawa, *J. Antibiotics*, **25**, 687 (1972).

5) A. Ide, K. Shigezane, S. Shigezane, T. Mizoguchi, and S. Saito, *Yakugaku Zasshi*, **90**, 850 (1970); K. Shigezane, M. Muraki, T. Morikawa, and T. Mizoguchi, *ibid.*, **91**, 987 (1971).

Z–Val–OH was condensed with H–Val–OMe by the mixed anhydride method followed by saponification to afford Z–Val–Val–OH (**1**). Similarly, the diastereomer (**2**) and Z–Val–Leu–OH (**3**) and its diastereomer (**4**) were obtained by saponification of the corresponding dipeptide methyl esters. Z–Val–Ser–OH (**5**) and Z–Val–Thr–OH (**6**) were prepared by coupling Z–Val–OH with serine and threonine, respectively, *via* the mixed anhydride. Z–Val–Val–OMe, after removal of the Z group by catalytic hydrogenation, was condensed with Z–Val–OH and the resulting Z–Val–Val–Val–OMe was converted in the usual manner to the free acid Z–Val–Val–Val–OH (**7**). **5** and **6** were hydrogenolyzed over Pd and the resulting free dipeptides were coupled to Z–Val–OH by the mixed anhydride procedure to give Z–Val–Val–Ser–OH (**8**) and Z–Val–Val–Thr–OH (**9**), respectively.

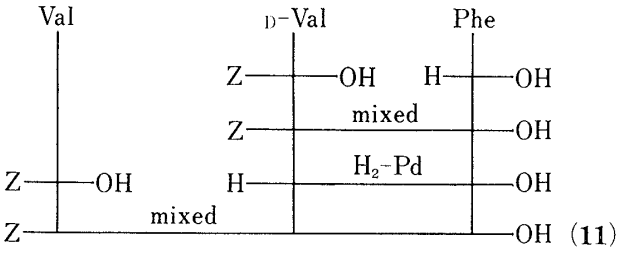

Fig. 1. Synthetic Scheme for **11**

H–Val–Phe–OMe, obtained after hydrogenation of Z–Val–Phe–OMe, was condensed with Z–Val–OH *via* its mixed anhydride to give Z–Val–Val–Phe–OMe, which was converted to Z–Val–Val–Phe–OH (**10**) in the usual manner. Z–Val–D-Val–Phe–OH (**11**) was prepared sequentially by the mixed anhydride procedure starting from phenylalanine, as shown in Fig. 1. An attempt to prepare **11** by a route similar to that used for its diastereomer **10** was unsuccessful, since the condensation of Z–Val–D-Val–OH and H–Phe–OMe using either DCC/HOSu or the azide procedure failed to afford Z–Val–D-Val–Phe–OMe in pure form.

The two pepstatin analogs **12** and **13** were prepared by connecting valine and two dipeptide subunits containing hydroxyamino acids according to the schemes illustrated in Figs. 2 and 3, respectively. Z–Ala–OH and H–Tyr–OEt were coupled by the mixed anhydride procedure to give Z–Ala–Tyr–OEt. This method was also applied to couple Z–Val–OH and H–Tyr–OEt, affording Z–Val–Tyr–OEt, which, after conversion to the corresponding hydrazide, was condensed with H–Ala–Tyr–OEt (derived from Z–Ala–Tyr–OEt) by the azide procedure.[6] The resulting protected tetrapeptide Z–Val–Tyr–Ala–Tyr–OEt was hydrogenated

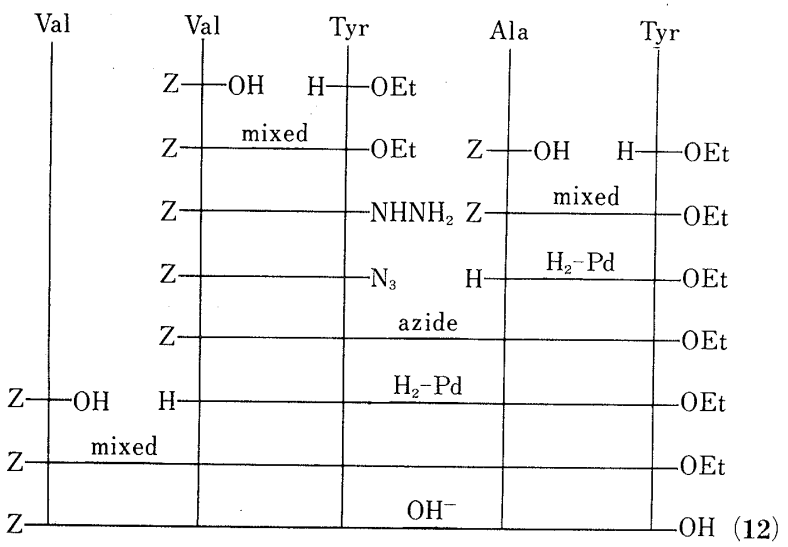

Fig. 2. Synthetic Scheme for **12**

6) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).

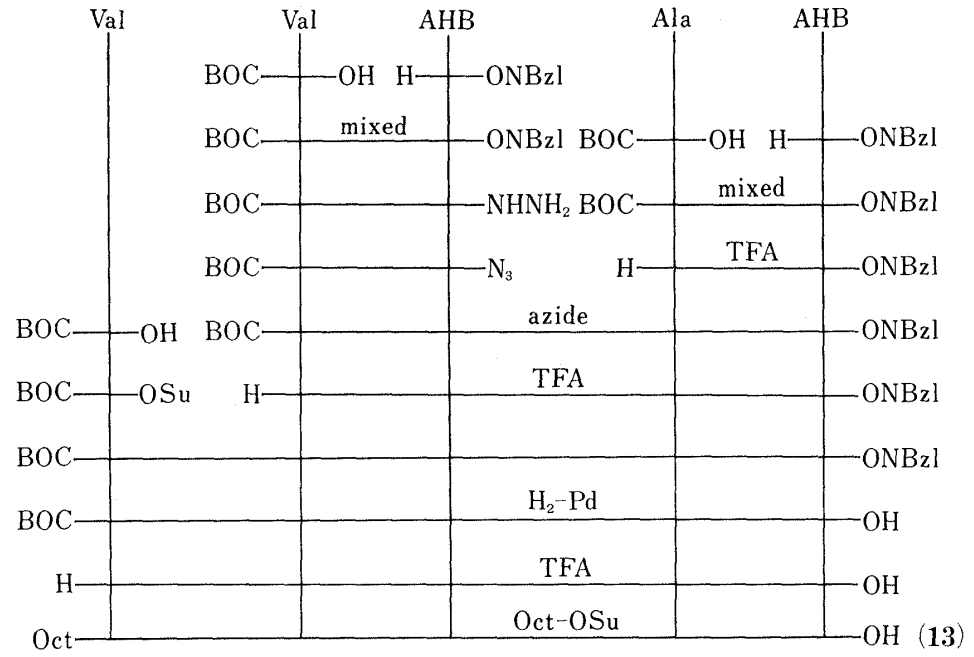

Fig. 3. Synthetic Scheme for **13**

and coupled with Z–Val–OH by the mixed anhydride procedure. The product was purified by silica gel column chromatography and saponified to give **12**. Preparation of the intermediate tetrapeptide ester BOC–Val–AHB–Ala–AHB–ONBzl for the synthesis of **13** was achieved in the manner described for Z–Val–Tyr–Ala–Tyr–OEt, except that BOC and ONBzl groups were employed for the protection of the amino and carboxyl groups, respectively.

Fortunately, esterification of AHB with the ONBzl group gave a good crystalline derivative and at the final step of synthesis this protecting group could be easily removed under mild conditions. The usual treatment of BOC–Val–AHB–Ala–AHB–ONBzl with TFA in the presence of anisole followed by reaction with BOC–Val–OSu afforded BOC–Val–Val–AHB–Ala–AHB–ONBzl. This, after similar treatment with TFA, was acylated with Oct–OSu to give Oct–Val–Val–AHB–Ala–AHB–OMBzl, which was finally de-*p*-nitrobenzylated by catalytic hydrogenation. The resulting **13** was obtained in analytically pure form after gel filtration on Sephadex LH 20.

The acid protease-inhibitory activities of the synthesized peptides, determined according to a slight modification of the method described by Aoyagi *et al.*,[7] are listed in Table I. All of the peptides with an R–Val–Val moiety at the N-terminus, except for **8**, showed more or less inhibitory activities on proteolysis by pepsin. However, compounds lacking the Val–Val moiety at the N-terminus, **2**—**6**, and **11**, showed no inhibition at a concentration of 50 μg/ml. This result suggests that the Val–Val moiety in the N-terminal region may play an important role in the binding of the peptides with pepsin. Compounds **10** and **12** showed the most potent inhibition. Unexpectedly, **13** was less active than **10** and **12**. The inhibitory activities of these three peptides towards pepsin are compared in Fig. 4. The 20% inhibition concentration obtained for each compound was as follows: **10**, 48 μg/ml; **12**, 28 μg/ml; **13**, 98 μg/ml. Pepstatin A gave 60% inhibition of pepsin even at a concentration of 0.32 μg/ml. It is clear that these compounds are less potent inhibitors than pepstatin A. The main difference in the structure of **13** compared to pepstatins lies in the two AHB residues, which lack a side chain in the C^r position, unlike the AHMHA residue in pepstatins. Therefore the unusual amino acid with a bulky side chain (AHMHA) in the latter peptide molecule appears to be an important structural requirement for antipepsin activity. This

7) T. Aoyagi, S. Kunimoto, H. Morishima, T. Takeuchi, and H. Umezawa, *J. Antibiotics*, **24**, 687 (1971).

Table I. Physical Data and Antipepsin Activities of the Peptide Derivatives Synthesized (1—13)

R-Val-X_n-OH
1—12: R=Z[a]
13: R=Oct

Compd. No.	X_n	Recryst. solv.	Yield %	mp °C	$[\alpha]_D$ (c, solv., t°)	Antipepsin activity, %[b]
1[c]	Val	MeOH–H_2O	65	139.5—140	−22 (1, MeOH, 20)	16
2[d]	D-Val	MeOH–H_2O	58	184—185	−20 (1, 1N KOH, 16)	0
3	Leu	AcOEt–ether	68	130.5—133	−30 (1, MeOH, 16)	0
4[e]	D-Leu	EtOH–H_2O	65	136—139	+ 4 (1, MeOH, 16)	0
5	Ser	MeOH–AcOEt	44	182—183	− 7 (1, MeOH, 16)	—[f]
6	Thr	MeOH–AcOEt	62	168—169	+ 4 (1, $CHCl_3$/MeOH =15:1, 16)	—[f]
7	Val–Val	EtOH–H_2O	48	218—218.5	−44 (1, $CHCl_3$, 20)	9
8	Val-Ser	MeOH–H_2O	35	210—211.5[g]	−19 (1, $CHCl_3$/MeOH =15:1, 16)	0
9	Val-Thr	EtOH–ether–petr. ether	40	197—198[g]	−37 (1.37, MeOH, 16)	12
10	Val-Phe	MeOH–H_2O	51	214—215	−32 (1, MeOH, 26)	23
11	D-Val-Phe	$CHCl_3$–ether–petr. ether	30	217—218	− 9 (1, AcOH, 26)	0
12	Val–Tyr–Ala–Tyr	MeOH–H_2O	37	248	−35 (1, MeOH, 27)	28
13[h]	Val–AHB–Ala–AHB	MeOH–H_2O	36	228—233		10

a) Matsushita *et al.* [Y. Matsushita, H. Tone, S. Hori, A. Takamatsu, H. Morishima, T. Aoyagi, T. Takeuchi, and H. Umezawa, *J. Antibiotics*, **28**, 1016 (1975)] showed that the nature of the N-acyl groups of pepstatins has no marked effect on the antipepsin activity.
b) The percent inhibition was determined at a concentration of 50 μg/ml for all compounds.
c) lit.[12] mp 139.5—140°, $[\alpha]_D^{20}$ −36.6° (c=3.2, 1N KOH); lit.[8] mp 132—134°, $[\alpha]_D$ +7.7° (acetone)
d) Prepared by the saponification of Z–Val–D-Val–OMe; lit.[12] mp 184—185°, $[\alpha]_D^{25}$ −21.3° (c=1.5, 1N KOH).
e) Prepared by the method described in a previous paper [K. Okada, Y. Kurosawa, and M. Hiramoto, *Chem. Pharm. Bull.* (Tokyo), **22**, 2136 (1974)].
f) The incubation mixture showed slightly higher proteolytic activity than the control.
g) Decomp.
h) The AHB residue is racemic.

conclusion is in accord with the observation of Aoyagi *et al.*[4] that the AHMHA moiety in pepstatins is important for binding with pepsin. Unlike **1** and **10**, their diastereomers **2** and **11** showed no inhibition; this confirms a stereospecificity in the action of pepsin inhibitors.

Next, the agonistic or antagonistic effects of the three peptides **10**, **12**, and **13** on pepstatin A were examined by adding the peptides (25 μg/ml each) to 0.32 μg/ml of pepstatin A and measuring the antipepsin activity of the resulting solution. None of compounds **10**, **12**, and **13** showed a significant effect, as can be seen in Table II. This again suggests a marked difference in the enzyme-binding abilities of the synthetic peptides and pepstatin A.

Table II. Effects of Compounds **10**, **12**, and **13** on the Inhibition of Pepsin by Pepstatin A[a)]

Compd.	% inhibition
—	60
10	80
12	72
13	56

a) The incubation mixture consisted of 1 ml of 0.6% casein solution, 0.8 ml of 0.02 M KCl–HCl buffer (pH 2.0) containing 0.64 μg of pepstatin A, 0.1 ml of AcOH with or without a test compound (50 μg), and 0.1 ml of enzyme solution (porcine pepsin 1.2 mg). The enzymic activity was determined as described in "Experimental".

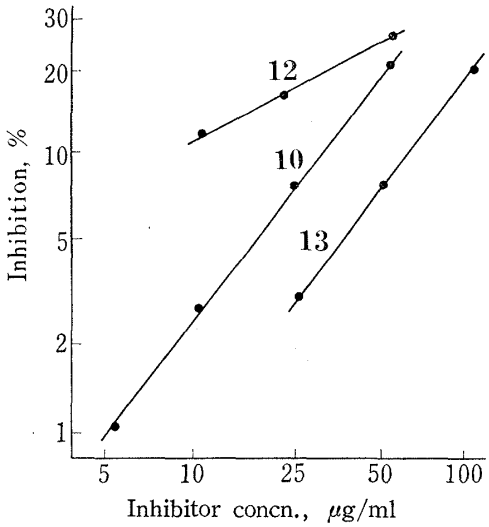

Fig. 4. Inhibition of Pepsin by Compounds **10**, **12**, and **13**

The reaction mixtures consisted of 0.01M KCl–HCl buffer, pH 2.0, containing 0.30% casein, and 30 μg/ml enzyme.

Experimental

The melting points are uncorrected. Rotations were determined on a Jasco DIP-SL polarimeter. Thin-layer chromatography was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the follwoing solvent systems; Rf_1 $CHCl_3$–MeOH–AcOH (95:5:3), Rf_2 $CHCl_3$–MeOH–pyridine (95:5:3), Rf_3 *n*-BuOH–AcOH–H_2O (4:1:5). D-Valine and D-leucine were purchased from Tanabe Amino Acid Research Foundation, Osaka. Pepstatin A was kindly provided by Dr. T. Aoyagi, Institute of Microbial Chemistry, Tokyo.

The yields and physical constants of the products (**1**—**13**) are listed in Table I.

Preparation of Z-Peptide Methyl Ester (General Method)——BCC (4 mmol) was added to a stirred solution of Z-amino acid (4 mmol) and Et_3N or NMM (used for the preparation of peptides larger than tetrapeptide) (4 mmol) in dry THF (6 ml) at −10°, and after 10 min a solution of amino acid methyl ester or peptide methyl ester [prepared from the hydrochloride (4 mmol) and Et_3N (4 mmol)] in dry $CHCl_3$ (16 ml) was added dropwise. The mixture was stirred at 0° for 1 hr and then at room temperature for 2 hr.

The solvent was removed *in vacuo*, and the residue was dissolved in AcOEt (150 ml). The solution was washed with 1 N HCl, 5% $NaHCO_3$ and H_2O, dried over $MgSO_4$ and concentrated *in vacuo*. Trituration of the residue with ether or petr. ether gave a crystalline product which was recrystallized from an appropriate solvent.

Z-Val-Val-OMe——This was prepared according to the general method and recrystallized from AcOEt–ether–petr. ether; yield 80%, mp 105.5—106°, $[\alpha]_D^{20}$ −34° (*c*=1, MeOH), Rf_1 0.48; Rf_2 0.66. *Anal.* Calcd. for $C_{19}H_{28}N_5O_5$: C, 62.62; H, 7.74; N, 7.64. Found: C, 62.91; H, 7.67; N, 7.54. (lit.[8]) prepared by the DCC plus 1,2,3-benzotriazine procedure, mp 107—109°).

Z-Val-D-Val-OMe——This was prepared according to the general method and recrystallized from $CHCl_3$–ether–petr. ether; yield 71%, mp 161—162°, $[\alpha]_D^{12}$ −18° (*c*=1, $CHCl_3$), Rf_1 0.07; Rf_2 0.78. *Anal.* Calcd. for $C_{19}H_{28}N_2O_5$: C, 62.62; H, 7.74; N, 7.64. Found: C, 62.94; H, 7.24; N, 7.61.

Z-Val-Leu-OMe——Z–Val–OSu (1.1 g) was added to a solution of H–Leu–OMe·HCl (0.56 g) in dioxane (4 ml) and Et_3N (0.45 ml). The mixture was stirred at room temperature for 24 hr, then, after adding N,N-dimethyl-1,3-propanediamine[9] (0.03 ml), for a further 30 min. Removal of the solvent gave a crystalline residue which was recrystallized from $CHCl_3$–AcOEt–petr. ether; yield 1.0 g (86%), mp 100—101°, $[\alpha]_D^{16}$ −42° (*c*=1, MeOH), Rf_1 0.45; Rf_2 0.59. *Anal.* Calcd. for $C_{20}H_{30}N_2O_5$: C, 63.47; H, 7.99; N, 7.40. Found: C, 63.56; H, 7.77; N, 7.62.

Z-Val-Val-OH (**1**)——A solution of Z–Val–Val–OMe (0.82 g) in MeOH (11 ml) was treated with 1 N NaOH (3.4 ml) at room temperature for 3 hr and then MeOH was removed by evaporation. Acidification of the aqueous solution to pH 1 gave the desired compound.

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9) M. Low and L. Kisfaludy, *Acta Chim. Hung.*, **44**, 61 (1965).

Z-Val-Phe-OMe——This was prepared according to the general method and recrystallized from AcOEt–petr. ether; yield 80%, mp 138—139°, $[\alpha]_D^{25}$ +37° (c=1, $CHCl_3$); $[\alpha]_D^{25}$ −4.6° (c=1, DMF), Rf_1 0.69; Rf_2 0.82 (lit.[10,11] prepared by the DCC method, mp 139—140°, $[\alpha]_D$ −5° in DMF; mp 132—134°, $[\alpha]_D$ +14° in dioxane).

Z-Val-Leu-OH (3)——Z–Val–Leu–OMe was saponified as described for **1**; Rf_1 0.45; Rf_2 0.19. *Anal.* Calcd. for $C_{19}H_{28}N_2O_5$: C, 62.62; H, 7.74; N, 7.69. Found: C, 62.44; H, 7.55; N, 7.76.

Z-Val-D-Leu-OH (4)——Z–Val-D-Leu–OMe[12] was saponified as described for **1**; Rf_1 0.49; Rf_2 0.19. *Anal.* Calcd. for $C_{19}H_{28}N_2O_5 \cdot 1/2H_2O$: C, 61.11; H, 7.83; N, 7.50. Found: C, 61.69; H, 7.59; N, 7.61.

Z-Val-Ser-OH (5)——BCC (1.04 ml) was added to a stirred solution of Z–Val–OH (2 g) in dry THF (16 ml) and Et_3N (1.12 ml) at −10°, and the mixture was stirred for 5 min. Et_3N (1.4 ml) and serine (1.05 g) in H_2O (5 ml) were added and the mixture was stirred for 20 min. The solvent was evaporated off *in vacuo* and the residue was acidified with 1 N HCl, then extracted with AcOEt (50×2 ml). The organic layer was washed with H_2O–NaCl, dried over Na_2SO_4 and evaporated down. Treatment of the residue with ether afforded the desired compound. *Anal.* Calcd. for $C_{17}H_{21}N_2O_6 \cdot 1/2H_2O$: C, 56.97; H, 6.19; N, 7.82. Found: C, 56.75; H, 6.50; N, 7.82.

Z-Val-Thr-OH (6)——This compound was prepared from Z–Val–OH (1.0 g) and threonine (0.6 g) as described for **5**. *Anal.* Calcd. for $C_{18}H_{23}N_2O_6 \cdot 1/2H_2O$: C, 58.00; H, 6.50; N, 7.52. Found: C, 57.71; H, 6.86; N, 7.54.

Z-D-Val-Phe-OH——Z-D-Val–OH (1.0 g) was condensed with phenylalanine (0.66 g) in the manner described for **5**, yielding the desired compound, which was recrystallized from AcOEt–ether–petr. ether; yield 0.27 g (22%), mp 134—135°, $[\alpha]_D^{16}$ +12° (c=1, MeOH), Rf_1 0.44, Rf_2 0.25. *Anal.* Calcd. for $C_{22}H_{26}N_2O_5 \cdot H_2O$: C, 63.44; H, 6.78; N, 6.73. Found: C, 63.53; H, 6.68; N, 6.84.

Z-Val-Val-Val-OMe——Z–Val–OH (1.35 g) was coupled to H–Val–Val–OMe·AcOH (1.4 g; prepared by catalytic hydrogenation of Z–Val–Val–OMe) according to the general procedure. The product was recrystallized from $CHCl_3$–ether; yield 1.41 g (68%), mp 218—218.5°, $[\alpha]_D^{16}$ −35° (c=1, $CHCl_3$), Rf_1 0.53, Rf_2 0.83. *Anal.* Calcd. for $C_{26}H_{37}N_3O_6 \cdot H_2O$: C, 61.76; H, 7.73; N, 8.31. Found: C, 62.07; H, 7.90; N, 8.80.

Z-Val-Val-Val-OH (7)——A solution of Z–Val–Val–Val–OMe (0.39 g) in MeOH (10 ml) and DMF (0.5 ml) was treated with 1 N NaOH at 40° for 5 hr. The reaction mixture was acidified with 1 N HCl to give a gelatinous material which was dissolved in dioxane–MeOH–H_2O (25 : 25 : 3) and applied to a Dowex 50×4 column (H^+; 3.5×4 cm). The column was eluted with the same solvent. The fractions containing the desired compound were collected and the solvent was evaporated off *in vacuo* to give **7** as a solid mass; Rf_2 0.11. *Anal.* Calcd. for $C_{25}H_{35}N_3O_6 \cdot 1/2H_2O$: C, 62.22; H, 7.52; N, 8.71. Found: C, 61.71; H, 7.89; N, 8.92.

Z-Val-Val-Ser-OH (8)——Z–Val–Ser–OH (1.0 g) in 70% MeOH (70 ml) was hydrogenated over a Pd catalyst for 2 hr in the usual manner. After removal of the solvent, the residue was dissolved in H_2O (4 ml) containing Et_3N (0.44 ml). A mixed anhydride prepared from Z–Val–OH (0.93 g) with Et_3N (0.49 ml) and BCC (0.46 ml) in dry THF (7 ml) was added and the mixture was stirred for 30 min at room temperature. The organic solvent was evaporated off *in vacuo* and the residue was acidified (pH 1) with 1 N HCl. Trituration of the resulting oil with ether furnished **8** as a solid mass. *Anal.* Calcd. for $C_{21}H_{31}N_3O_7 \cdot H_2O$: C, 55.37; H, 7.30; N, 9.23. Found: C, 55.92; H, 6.94; N, 9.44.

Z-Val-Val-Thr-OH (9)——Z–Val–OH (0.53 g) was condensed with H–Val–Thr–OH [obtained by the hydrogenolysis of **6** (0.62 g)] in the usual manner. *Anal.* Calcd. for $C_{22}H_{33}N_3O_7 \cdot 1/2H_2O$: C, 57.37; H, 7.44; N, 9.12. Found: C, 56.97; H, 7.21; N, 9.45.

Z-Val-Val-Phe-OMe——Z–Val–Phe–OMe (1.0 g) in a mixture of MeOH (30 ml), DMF (5 ml), and conc. HCl (0.21 ml) was hydrogenated over 5% Pd–C for 3 hr. After filtration, the filtrate was evaporated down *in vacuo* and the residue was treated with ether to yield H–Val–Phe–OMe·HCl (mp 193—196°)[13] as fine crystals. This compound was coupled with Z–Val–OH (0.59 g) according to the general method. The product was recrystallized from $CHCl_3$–petr. ether; yield 0.87 g (73%), mp 214—215°, $[\alpha]_D^{26}$ +2° (c=1, DMF), Rf_1 0.43, Rf_2 0.74. *Anal.* Calcd. for $C_{28}H_{37}N_3O_6 \cdot 1/2H_2O$: C, 64.60; H, 7.35; N, 8.07. Found: C, 64.62; H, 7.29; N, 8.21.

Z-Val-Val-Phe-OH (10)——A solution of Z–Val–Val–Phe–OMe (0.38 g) in DMF (3 ml) was treated with 1 N NaOH (3 ml) at room temperature for 24 hr. After removal of the solvent, the residue was dissolved in H_2O (10 ml) and acidified with 1 N HCl to afford a crystalline material. *Anal.* Calcd. for $C_{27}H_{35}N_3O_6 \cdot 1/2H_2O$: C, 64.01; H, 7.16; N, 8.29. Found: C, 63.97; H, 7.09; N, 7.97.

Z-Val-D-Val-Phe-OH (11)——Z-D-Val–Phe–OH (0.20 g) was decarbobenzoxylated and condensed with Z–Val–OH (0.25 g) as described for **8** to give **11** (0.10 g). *Anal.* Calcd. for $C_{27}H_{35}N_3O_6$: C, 65.17; H, 7.09; N, 8.45. Found: C, 65.06; H, 7.01; N, 8.62.

Z-Val-Tyr-Ala-Tyr-OEt——Z–Ala–Tyr–OEt[14] (1.0 g) in a mixture of MeOH (30 ml), DMF (3 ml), and

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conc. HCl (0.22 ml) was hydrogenated over a Pd catalyst for 3 hr. After removal of the catalyst and the solvent, the residue was dissolved in DMF (5 ml) and Et_3N (1.55 ml).

Isoamylnitrite (1.3 ml) and 4 N HCl (1.85 ml) were added to a cooled solution of Z–Val–Tyr–$NHNH_2$[15] (0.85 g) in DMF (4.5 ml), and the mixture was stirred at −25° for 5 min, until the hydrazine test[16] became negative. The solution was neutralized with Et_3N (1.2 ml) and mixed under cooling with the above solution of H–Ala–Tyr–OEt. The mixture was stirred at 4° for 48 hr, then poured into 0.01 N HCl (100 ml). The separated oil was extracted with AcOEt (50×2 ml) and the extract was washed with 1 N HCl, H_2O, 5% $NaHCO_3$, and H_2O, dried over $MgSO_4$, and then evaporated down. The residue was recrystallized from MeOH–H_2O; yield 0.94 g (70%), mp 235—236°, $[\alpha]_D^{16}$ −28° (c=1, MeOH), Rf_1 0.45. *Anal.* Calcd. for $C_{36}H_{43}N_4O_9 \cdot 1/2H_2O$: C, 63.14; H, 6.48; N, 8.18. Found: C, 63.24; H, 6.64; N, 7.80.

Z-Val-Val-Tyr-Ala-Tyr-OEt——Z–Val–Tyr–Ala–Tyr–OEt (0.91 g) in a mixture of MeOH (70 ml), DMF (1 ml), and AcOH (0.5 ml) was hydrogenated over a 5% Pd catalyst for 3 hr. Removal of the catalyst and the solvent, and trituration of the residue with ether gave a powder; yield 0.60 g (79%). This was coupled with Z–Val–OH (0.25 g) according to the general method. The separated solid product was collected by filtration, washed with ether, dissolved in $CHCl_3$–MeOH–AcOH (95 : 5 : 3), and chromatographed on silica gel (42×19 cm). The fractions containing the compound of Rf_1 0.33 were combined and the solvent was evaporated off. The resulting residue was recrystallized from MeOH–$CHCl_3$–ether; yield 0.41 g (56%), mp 225°, Rf_2 0.25. *Anal.* Calcd. for $C_{41}H_{52}N_5O_{10} \cdot H_2O$: C, 62.18; H, 6.74; N, 8.84. Found: C, 62.30; H, 6.78; N, 8.81.

Z-Val-Val-Tyr-Ala-Tyr-OH (12)——Z–Val–Val–Tyr–Ala–Tyr–OEt (0.14 g) in a mixture of EtOH (30 ml) and DMF (1 ml) was treated with 5% KOH (1 ml) for 2 hr. The mixture was acidified with 1 N HCl and the resulting precipitate was collected and washed with H_2O; Rf_1 0.10. *Anal.* Calcd. for $C_{39}H_{48}N_5O_{10} \cdot H_2O$: C, 61.24; H, 6.59; N, 9.16. Found: C, 60.77; H, 6.85; N, 9.50.

H-AHB-ONBzl·TosOH——AHB (5 g), *p*-nitrobenzyl alcohol (9 g) and *p*-toluenesulfonic acid (12 g) were refluxed in CCl_4 (70 ml) for 24 hr. The resulting precipitate was collected, washed with ether, and recrystallized from MeOH; yield 14.7 g (79%), mp 164—167°. *Anal.* Calcd. for $C_{11}H_{14}N_5O_5 \cdot C_7H_8O_3S$: C, 50.70; H, 5.20; N, 6.57. Found: C, 51.22; H, 5.24; N, 6.43.

BOC-Ala-AHB-ONBzl——BOC–Ala–OSu (2.16 g), Et_3N (1.33 ml) and H–AHB–ONBzl·TosOH (4.0 g) in DMF (20 ml) were stirred at room temperature for 48 hr and then poured into AcOEt (70 ml). The solution was washed with 1 N HCl, 5% $NaHCO_3$, and H_2O, dried over $MgSO_4$, and evaporated down. The solid residue was recrystallized from AcOEt–petr. ether; yield 2.47 g (80%), mp 108—111°. *Anal.* Calcd. for $C_9H_{27}N_3O_8$: C, 53.64; H, 6.40; N, 9.88. Found: C, 53.53; H, 6.52; N, 9.70.

BOC-Val-AHB-ONBzl——This compound was prepared from BOC–Val–OSu and H–AHB–ONBzl·TosOH in the manner described for BOC–Ala–AHB–ONBzl; yield 87%, mp 78—80°. *Anal.* Calcd. for $C_{21}H_{31}N_3O_8$: C, 55.62; H, 6.89; N, 9.26. Found: C, 55.81; H, 6.74; N, 8.92.

BOC-Val-AHB-$NHNH_2$——Hydrazine hydrate (80%, 0.65 ml) was added to a solution of BOC–Val–AHB–ONBzl (0.60 g) in MeOH (2.5 ml), and the solid formed on standing for three days was recrystallized from MeOH–AcOEt–ether; yield 0.35 g (79%), mp 141—142°. *Anal.* Calcd. for $C_{14}H_{28}N_4O_5$: C, 50.58; H, 8.49; N, 16.86. Found: C, 50.26; H, 8.49; N, 16.38.

BOC-Val-AHB-Ala-AHB-ONBzl——TFA (4.5 ml) was added to a cooled solution of BOC–Ala–AHB–ONBzl (0.63 g) in $CHCl_3$ (7 ml), and the reaction mixture was stirred for 1 hr at room temperature. Concentration *in vacuo* gave H–Ala–AHB–ONBzl·TFA as an oil which was washed with dry ether, dried and dissolved in DMF (2 ml). A mixture of Et_3N (0.28 ml) and BOC–Val–AHB–N_3 (prepared from 0.6 g of the corresponding hydrazide with 1.7 ml of 4 N HCl-dioxande, 0.28 ml of isoamylnitrite and 0.98 ml of Et_3N) in DMF (4 ml) was added to this ice-cooled solution and the mixture was stirred at −20° for 30 min, then at 4° for 24 hr. EtOAc (40 ml) was added and the mixture was washed with 1 N HCl, 4% $NaHCO_3$, and H_2O, then dried over $MgSO_4$. Removal of the solvent *in vacuo* gave a solid which was recrystallized from MeOH–ether–petr. ether; yield 0.45 g (48%), mp 118—120°, Rf_1 0.30. *Anal.* Calcd. for $C_{28}H_{43}N_5O_{11}$: C, 53.75; H, 6.92; N, 11.93. Found: C, 53.66; H, 7.19; N, 11.45.

BOC-Val-Val-AHB-Ala-AHB-ONBzl——TFA (2.4 ml) was added to an ice-cooled suspension of BOC–Val–AHB–Ala–AHB–ONBzl (0.4 g) in $CHCl_3$ (4 ml), and the mixture was stirred at room temperature for 1 hr. Concentration of the mixture *in vacuo* and trituration of the residue with dry ether gave H–Val–AHB–Ala–AHB–ONBzl·TFA as a powder. This was dissolved in DMF (2 ml), and Et_3N (0.11 ml) and BOC–Val–OSu (0.33 g) were added. The mixture was stirred at room temperature for 3 hr, then poured into H_2O (200 ml). The resulting precipitate was collected by filtration and recrystallized from MeOH–ether; yield 0.38 g (76%), mp 171—175°, Rf_1 0.46; Rf_2 0.31. *Anal.* Calcd. for $C_{33}H_{52}N_6O_{12}$: C, 54.69; H, 7.23; N, 11.59. Found: C, 54.77; H, 7.31; N, 11.23.

Oct-Val-Val-AHB-Ala-AHB-ONBzl——TFA (1 ml) was added to a suspension of BOC–Val–Val–AHB–Ala–AHB–ONBzl (0.23 g) in $CHCl_3$ (2.5 ml) at −5°. The mixture was stirred at room temperature for 1 hr, then evaporated down *in vacuo*. The residue was washed with ether, dissolved in DMF (1.5 ml) and Et_3N

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(0.56 ml), and allowed to react with Oct-OSu[17] (0.12 g). After stirring at room temperature for 30 min, the resulting precipitate was collected by filtration and washed with ether and MeOH; yield 0.17 g (66%), mp 237—241°, Rf_1 0.18. *Anal.* Calcd. for $C_{36}H_{58}N_6O_{11}$: C, 57.58; H, 7.78; N, 11.19. Found: C, 57.75; H, 7.76; N, 11.37.

Oct-Val-Val-AHB-Ala-AHB-OH (**13**)——A solution of Oct–Val–Val–AHB–Ala–AHB–ONBzl (0.17 g) in MeOH (30 ml) containing AcOH (1.5 ml) was hydrogenated over a Pd catalyst for 1.5 hr. The mixture was filtered, and the filtrate was concentrated to dryness. Gel filtration of the residue in MeOH on a column (2.6×34 cm) of Sephadex LH-20 gave **13**, which was recrystallized from MeOH–H_2O; Rf_1 0.20; Rf_3 0.78. *Anal.* Calcd. for $C_{29}H_{53}N_5O_9 \cdot H_2O$: C, 54.95; H, 8.75; N, 11.05. Found: C, 54.45; H, 8.49; N, 11.45.

Assay for Inhibitory Activity Against Pepsin——Milk casein (Hammarsten) and porcine pepsin (3200 units) were purchased from Merck Co. and Sigma Chemical Co., respectively.

One ml of 0.6% casein solution in 0.75% lactic acid, 0.8 ml of 0.02 M KCl–HCl buffer (pH 2.0), and 0.1 ml of AcOH with or without a test sample were mixed and incubated at 37° for 3 min, then 0.1 ml of the enzyme solution (600 μg/ml) was added and the mixture was further incubated at 37° for 10 min. After this time, 3 ml of 5% trichloroacetic acid was added and the mixture was stored for 30 min in a refrigerator, then filtered. The extinction at 280 nm of the filtrate was measured.

The percent inhibition was calculated as follows: % inhibition$=100\times(A-B)/A$; where A is the absorbance in the absence of test sample and B is that with the test sample.

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