Synthesis and Antibacterial Properties of Some Tripeptides[1]

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The antibacterial activity of tripeptides of the hydrophobic amino acids, valine, isoleucine, alloisoleucine, leucine, cycloleucine, and phenylalanine, was found chiefly in peptides of the composition L-valyl-L-valyl-D-amino acid. Several synthetic paths for the free peptides were evaluated. A rearrangement of some carbobenzoxy tripeptide esters to disubstituted ureas took place during saponification. Tagged L-valyl-L-valyl-D-valine was prepared from Z-L-valine-C^{14} and L-valyl-D-valine benzyl ester with carbodiimide coupling and hydrogenolysis. This sequence is generally applicable for the synthesis of radioactive peptides. The synthesis and properties of nine tripeptides and various intermediates are given.

In studies of structure of small peptides and their antibacterial activity,[2] it was established that only L-valyl-L-valyl-D-valine of the eight stereoisomers of trivaline inhibited the growth of *Pediococcus cerevisiae* and *Lactobacillus plantarum*. Substitution of any valine residue by leucine substantially destroyed inhibitory activity for *L. plantarum*.[3] Of the seven leucine homologs, only valyl-valyl-leucine possessed significant activity for *P. cerevisiae*. Substitution of the D-valine residue by several amines also led to loss of activity. However, N^4-(valyl-valyl)-sulfanilamide was highly effective against these organisms. Its mode of action was quite different from sulfanilamide itself.[4]

At present, we report the synthesis of tripeptides of isoleucine, alloisoleucine, cycloleucine, valine, and phenylalanine together with the relation of structure to antibacterial activity. Shown in Table I are the comparative antibacterial properties of several peptides of the L-L-D configuration. Effective peptidic inhibitors of *P. cerevisiae* are the valyl-valyl-D-amino acids, where the D-amino acid may be valine, leucine, isoleucine, or alloisoleucine among others. These peptides also inhibit *L. plantarum* at about 10^{-4} *M*, but at 10^{-3} or 10^{-2} *M* the latter three peptides stimulate growth. Of the other L-L-D tripeptides, only isoleucyl-valyl-valine was an effective inhibitor of growth. Other substitution of the N-terminal valyl-valine portion of either trivaline or val-val-sulfa[5] led to loss of inhibitory ability. The tetrapeptide val-val-val-val (L-L-L-D and L-D-L-D) was 0.05 as active as the tripeptide for *L. plantarum* and about 0.2 as active for *P. cerevisiae*. Of several peptide antibiotics (Table II), only Actinomycin D was much more inhibitory than the active L-L-D tripeptides for *P. cerevisiae*. For *L. plantarum*, the peptide antibiotics were considerably more active than trivaline and of the same order of magnitude as val-val-sulfa. It appears that the structure of tripeptides which inhibit growth is highly specific. It is noteworthy that the isoleucine tripeptide was substantially inactive.

The marked structural specificity for inhibitory properties resembles the specificity of carbohydrate structure and uptake in *Escherichia coli*,[6] in which the presence of specific uptake mechanisms or permeases was postulated. From other evidence,[7] we have suggested that L-valyl-L-valyl-D-valine binds or modifies sites necessary for amino acid uptake. The present findings of specific structure support this view.

TABLE I
RELATIVE INHIBITORY ACTIVITY OF L-L-D TRIPEPTIDES

	P. cerevisiae	*L. plantarum*
Val-val-val	1.0[a]	1.0[b]
Val-val-ileu	0.7	1.0–0.01 S[c,d]
Val-val-aleu	.7	1.0–0.05 S[c,d]
Val-val-leu	.8[e]	0.7 S[e]
Val-val-cyc	.01	S
Ileu-ileu-ileu	.02	S
Ileu-ileu-val	.05	S
Ileu-val-val	.4	0.3–0.01 S[e]
Leu-leu-leu	.06	S
Leu-leu-val	.05	S
Leu-val-val	.1	S
Phe-phe-phe	N	S
Met-met-met	.03	S
N^4-(val-val)-sulfa (L,L)	2.0[f]	100.0[f]
N^1-(cyc-cyc)-sulfa	N	0.1

[a] Concentration at which growth is reduced 50% (ID_{50}) was 0.5×10^{-4} *M*; lit.[2b] 10^{-4} *M*. [b] $ID_{50} = 0.6 \times 10^{-4}$ *M*. [c] These compounds inhibited well at low concentrations of inhibitor, 0.5×10^{-4} *M*, but at 10^{-3} and 10^{-2} *M* became less effective and stimulated growth. [d] N = no effect, S = stimulation. [e] See ref. 3. [f] See ref. 4.

TABLE II
RELATIVE INHIBITORY ACTIVITY[a,b]

	P. cerevisiae	*L. plantarum*
L-Val-L-val-D-val	1.0	1.0
Val-lys-leu-D-phe-pro | | pro-D-phe-leu-lys-val (Lysine analog of Gramicidin S)	2.0	20.0
Gramicidin D	N	500.0
Gly-phe-leu-gly-phe-leu (-D,L,-D,L)	<0.02	<0.04
(-D,L,-L,L)	<0.02	<0.04
(-G,G,-L,G)	<0.02	<0.04
Penicillin G	2–10	>100.0
Actinomycin D	>100	>100.0
Actinomycin P_2	<0.1	<0.1

[a] Weight basis. [b] Other noninhibitory peptides were leu-phe-pro-OMe (L-D-L), val-tyr-val-his-pro-phe (L-D-L-L-L-L), his-leu-leu-val-tyr-ser-OMe (all L), pro-phe-his-OMe (all L), α-hydroxy-isovaleryl-val-leu (L-D-D, D-L-L, D-D-D, all tested separately), and valyl-O-glycocoll benzyl ester.

(1) This work was supported by Grants CY-4318, CY-3609, and E-1883 of the National Institutes of Health.

(2) (a) S. Shankman, S. Higa, and V. Gold, *J. Am. Chem. Soc.*, **82**, 990 (1960); (b) S. Shankman, V. Gold, and S. Higa, *Texas Rept. Biol. Med.*, **19**, 358 (1960).

(3) S. Shankman and S. Higa, *J. Pharm. Sci.*, **51**, 137 (1962).

(4) S. Shankman, S. Makineni, and V. Gold, *Arch. Biochem. Biophys.*, **100**, 431 (1963).

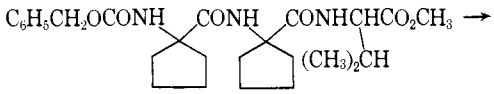
(5) Sulfa = sulfanilamide; Z = carbobenzoxy; Aleu = alloisoleucine; cyc = cycloleucine; OBz = benzyl ester; and OMe = methyl ester. Otherwise, standard abbreviations have been used.

(6) G. N. Cohen and J. Monod, *Bacteriol. Rev.*, **21**, 169 (1957).

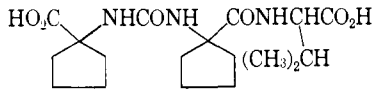
(7) S. Shankman, V. Gold, S. Higa, and R. Squires, *Biochem. Biophys. Res. Commun.*, **9**, 25 (1962).

The carbobenzoxy tripeptide methyl esters containing isoleucine, alloisoleucine, valine, phenylalanine, and cycloleucine (1-aminocyclopentanecarboxylic acid) were readily obtained by mixed anhydride[8] or carbodiimide coupling.[9] These peptide esters saponified very slowly for the most part as compared to the dipeptides.[10] Generally they did not react with hypochlorite–KI–starch to give a test for the peptide bond. Since these properties are characteristic of peptides of the amino acids which have a 3 to 6 carbon R group it is apparent that the hydrocarbon character of the R group decreases the reactivity of the Z-tripeptide esters towards aqueous reactions.

In addition to a decreased rate of saponification, determined by isolation of carbobenzoxy tripeptides, the carbobenzoxy tripeptide esters could not be recovered in high yield or good purity from saponification trials. A saponification experiment at 60° for 24 hr. on a model tripeptide, carbobenzoxycycloleucyl-cycloleucyl-D-valine methyl ester (I) gave as principal product II, N^1-(cyclopentyl-1-carboxylic acid)-N^2-(cyclopentyl-1-carboxyl-valine)-urea. Racemization of the C-terminal-D-valine did not occur.



I



II

A corresponding rearrangement of carbobenzoxy dipeptides containing a C-terminal glycine ethyl ester has been reported by Maclaren[11] and discussed by Anderson.[12] It may be noted that α-hydrogen is not involved. Strangely enough, carbobenzoxy-valyl-valyl-cycloleucine methyl ester was readily saponifiable in tetrahydrofuran and aqueous base. Two other tripeptides containing N-terminal carbobenzoxy-valyl-valine could also be obtained by saponification, although with more difficulty. Carbobenzoxy-phenylalanyl-phenylalanyl-phenylalanine was similarly obtained in low yield.

The benzyl ester of the C-terminal dipeptide was useful in obtaining free tripeptides, especially in microsynthesis of radioactive tripeptides or in those cases where the carbobenzoxy dipeptide benzyl ester could be cleaved preferentially by a short-period reaction with hydrogen bromide in glacial acetic acid to give a dipeptide benzyl ester hydrobromide. In some cases, carbobenzoxy and benzyl groups were removed simultaneously. The dipeptides were re-esterified with benzyl alcohol and *p*-toluenesulfonic acid to give high yields of the dipeptide benzyl ester tosylates. The sodium salt of the C-terminal dipeptide moiety was useful in other cases. However, the Z-amino acid and Z-tripeptide had similar solubilities. Although the presence of Z-amino acid resulting from decomposition of the mixed anhydride was not detectable by ninhydrin or hypochlorite–KI–starch sprays, the free tripeptide prepared in this manner at times contained the free N-terminal amino acid, limiting the utility of this procedure.

Early in this study, several carbobenzoxy tripeptide methyl esters were made by the 2 + 1 sequence now known to be prone to racemization on the C-terminal amino acid of the dipeptide.[3,12] The N-terminal dipeptide moiety was carbobenzoxy-L-valyl-L-valine. L-Valyl-L-valyl-D-isoleucine of satisfactory optical purity was obtained, whereas the L-valyl-L-valyl-D-phenylalanine methyl ester was significantly racemized. These data suggest that the entering amine plays a significant role in the stereochemistry of the resultant peptide, which we have observed previously.[4] These and other esters have proved useful in a study of the binding of the L-valyl-L-valyl-D-valine by *P. cerevisiae*.[7]

Experimental

A. Amino Acid Benzyl Ester Tosylates.—D-Isoleucine benzyl ester tosylate and other benzyl ester tosylates were prepared from D-amino acids, containing less than 0.3% of L-amino acid, by the azeotropic distillation procedure,[13] with yields from 85–96%, after 20 hr. reflux.

III. D-Isoleucine Benzyl Ester Tosylate.—m.p. 149°, lit.[13] m.p. 153–154° for L.

IV. D-Valine Benzyl Ester Tosylate.—m.p. 159°, lit.[13] m.p. 158–160° for L.

V. D-Alloisoleucine Benzyl Ester Tosylate.—m.p. 164–165°, lit.[13] m.p. 162–164° for D.

VI. Phenylalanine Benzyl Ester Tosylate.—m.p. 144.5–145°, lit.[13] m.p. 170.5–171.5° for L.

They were chromatographically homogeneous in pyridine–isoamyl alcohol–water 35:35:30 [PIW]. Employing bacterimetric assay after hydrolysis, less than 0.3% of L-ileu was found in D-aleu ester, and less than 0.3% L-val in D-val ester. In contrast, the D-phenylalanine had racemized about 90%.

B. Carbobenzoxy Dipeptide Benzyl Esters by Mixed Anhydride.[8]—Twenty mmoles each of carbobenzoxyamino acid and triethylamine were dissolved in methylene chloride and the solution cooled to −10°. Twenty mmoles of ethyl chlorocarbonate was added with stirring and anhydride formation occurred. To the solution at −10° was added 21 mmoles of amino acid benzyl ester tosylate in methylene chloride which had been neutralized with triethylamine. The temperature of the reaction mixture was allowed to rise slowly to room temperature and remain there for 5 hr. After washing with water, dilute HCl, water, 5% K_2CO_3, and water, the methylene chloride solution was dried with sodium sulfate. The solvent was removed on the rotary evaporator and the residue was dried *in vacuo*. Crude yields were from 50 to 90%. Other compounds were similarly prepared. The compounds were recrystallized from ethyl acetate–water (90:10), hot ethanol, or chloroform–petroleum ether one or more times. The following compounds were prepared in this manner.

VII. Z-L-ileu-D-ileu-OBz, crude yield 93%, m.p. 131–131.5°, $[\alpha]_D$ +5.5° [*c* 2 in DMSO (dimethyl sulfoxide)]. *Anal.* Calcd. for $C_{27}H_{36}N_2O_5$: C, 69.1; H, 7.72; N, 5.99. Found: C, 69.0; H, 7.53; N, 6.25.

VIII. Z-L-val-D-ileu-OBz, crude yield 47%, m.p. 141–141.5°, $[\alpha]_D$ +11.9° (*c* 2, DMSO). *Anal.* Calcd. for $C_{26}H_{34}N_2O_5$: C, 68.8; H, 7.55; N, 6.16. Found: C, 69.0; H, 7.29; N, 6.20.

IX. Z-L-ileu-D-val-OBz, crude yield 78%, m.p. 136–137°, $[\alpha]_D$ +7.6° (*c* 2, DMSO). *Anal.* Calcd. for $C_{26}H_{34}N_2O_5$: C, 68.8; H, 7.55; N, 6.16. Found: C, 68.8; H, 7.32; N, 6.16.

X. Z-L-val-D-val-OBz, crude yield 78%, m.p. 145–146°, $[\alpha]_D$ −6.4° (*c* 2, chloroform). *Anal.* Calcd. for $C_{25}H_{32}N_2O_5$: C, 68.2; N, 7.31; N, 6.25. Found: C, 68.7; H, 6.85; N, 6.35.

XI. Z-L-val-D-aleu-OBz, crude yield 78%, m.p. 131–132°, $[\alpha]_D$ +10.0° (*c* 2, DMSO). *Anal.* Calcd. for $C_{26}H_{34}N_2O_5$: C, 68.8; H, 7.55; N, 6.16. Found: C, 69.0; H, 7.51; N, 6.16. Reaction solvent $CHCl_3$.

(8) J. R. Vaughan and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).
(9) J. C. Sheehan and J. J. Hlavka, *J. Org. Chem.*, **21**, 439 (1956).
(10) S. Shankman, S. Higa, F. Lew, and M. E. Roberts, *J. Med. Pharm. Chem.*, **5**, 42 (1962).
(11) J. A. Maclaren, *Australian J. Chem.*, **11**, 360 (1958).
(12) G. W. Anderson, *Ann. N. Y. Acad. Sci.*, **88**, Art. 3, p. 676 (1960).
(13) (a) J. D. Cifrera and R. B. B. Nicholls, *Chem. Ind.* (London), 16 (1955); (b) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 2, John Wiley and Sons, Inc., New York, N. Y., 1961, p. 941.

C. Dipeptide Benzyl Ester Hydrobromides. XII. L-Val-D-val-OBz·HBr.—Z-L-val-D-val-OBz (1 g.) was dissolved in about 4 ml. of 4 *N* HBr in glacial HOAc and let stand 20 min. at room temperature. Two hundred ml. of ethyl ether was added. Crystals appeared at 1 hr. Crystallization was allowed to proceed in the cold; yield 88%, m.p. 183–185°, $[\alpha]_D$ +55.9° (*c* 2, water).

Anal. Calcd. for $C_{17}H_{27}BrN_2O_3$: C, 52.6; H, 7.00; N, 7.22. Found: C, 52.2; H, 7.17; N, 7.33.

It was chromatographically homogeneous in butanol–water–acetic acid 90:30:10 [BAW] and in PIW.

XIII. L-Ileu-D-val-OBz·HBr was prepared similarly, allowing the reaction to proceed until the evolution of gas was complete. An oil was obtained which was triturated with 1:1 ethyl ether–isopropyl ether. Crystals formed, m.p. 187.5–188°, yield 55%, $[\alpha]_D$ +53.8° (*c* 2, water), chromatographically homogeneous.

Anal. Calcd. for $C_{18}H_{29}BrN_2O_3$: C, 53.9; H, 7.29; N, 6.95. Found: C, 53.3; H, 7.21; N, 6.94.

The L-val-D-aleu-OBz was obtained by reaction with HBr in dioxane. After removal of dioxane and excess HBr, the compound was precipitated by acetone and ether. It was homogeneous on chromatography with detection by ninhydrin and NaOCl–KI–starch but had a second component visible on the ultraviolet screen; probably unreacted material. Carbobenzoxy-L-val-phe-OBz reacted with HBr in glacial acetic acid until gas evolution ceased, giving an impure mixture of ester and free peptide identified chromatographically. Since removal of Z and benzyl was essentially quantitative on hydrogenolysis with Pd/H_2 and the re-esterification also took place in 95% yield, the use of HBr in glacial acetic acid or dioxane was not pursued further.

D. Dipeptide Hydrochlorides.—Ten mmoles of Z-dipeptide benzyl ester was dissolved in methanol. Twelve mmoles of aqueous HCl and 10% by weight of catalyst (either 5 or 10% palladium on charcoal) was added. Hydrogen was passed in at atmospheric pressure until no further evolution of CO_2 occurred. The catalyst was filtered and the methanol removed by evaporation *in vacuo*. After flushing with acetone, precipitation occurred. The compounds were freed of moisture and excess HCl by repeated flushing with acetone and finally ethyl ether. Yields were 90–95%. The peptide hydrochlorides were chromatographically homogeneous in the BAW and PIW systems. The following compounds were prepared in this manner.

XIV. L-Val-D-ileu·HCl, m.p. 259°, $[\alpha]_D$ +36.5 (*c* 3, DMSO). *Anal.* Calcd. for $C_{11}H_{23}ClN_2O_3$: C, 49.4; H, 8.72; N, 10.5. Found: C, 49.7; H, 8.84; N, 10.23.

XV. L-Val-D-aleu·HCl, m.p. 246.5–247.5°; $[\alpha]_D$ +38.9° (*c* 3, water). *Anal.* Calcd. for $C_{11}H_{23}ClN_2O_3$: C, 49.4; H, 8.72; N, 10.5. Found: C, 49.6; H, 8.59; N, 10.5.

XVI. L-Ileu-D-ileu·HCl, m.p. 263.5°; $[\alpha]_D$ +39.9° (*c* 3, water). *Anal.* Calcd. for $C_{12}H_{25}ClN_2O_3$: C, 51.4; H, 9.02; N, 10.0. Found: C, 51.4; H, 8.86; N, 9.9.

L-Val-D-val·HCl, m.p. 251–252°, $[\alpha]_D$ +39.6° (*c* 3, DMSO); $[\alpha]_D$ +50.8° (*c* 1, water); lit.[14] $[\alpha]_D$ +51.1° in water.

E. Dipeptide Benzyl Ester Tosylates from Dipeptides.—Yields were 86–98% for the following compounds prepared as in A. They were recrystallized from methanol–ether when necessary. They were chromatographically pure in the PIW system.

XVII. L-Val-D-aleu-OBz Tosylate, m.p. 226–228°, $[\alpha]_D$ +23.6° (*c* 3, DMSO). *Anal.* Calcd. for $C_{32}H_{36}N_2O_6S$: N, 5.80. Found: N, 5.70.

XVIII. L-Val-D-val-OBz Tosylate, m.p. 246.5–248°, $[\alpha]_D$ +32.7° (*c* 2, methanol). *Anal.* Calcd. for $C_{24}H_{34}N_2O_6S$: C, 60.3; H, 7.18; N, 5.85. Found: C, 60.6; H, 7.45; N, 5.52.

XIX. L-Ileu-D-ileu-OBz Tosylate, m.p. 242–242.5°, $[\alpha]_D$ +28.1° (*c* 2, methanol). *Anal.* Calcd. for $C_{26}H_{38}N_2O_6S$: C, 61.7; H, 7.52; N, 5.54. Found: C, 61.4; H, 7.48; N, 5.2.

F. Z-Tripeptides *via* Sodium Salt Procedure.—Into 1.04 g. (4.1 mmoles) of Z-L-val and 0.404 g. of triethylamine in dioxane cooled to 0° was stirred 0.43 g. of ethyl chlorocarbonate. After 20 min. at 0°, 1.1 g. (4.1 mmoles) of L-val-D-ileu·HCl in cold 50:50 dioxane–water containing 8.8 mmoles of NaOH was added with stirring. The mixture was allowed to reach room temperature slowly and stood overnight. After evaporation to dryness and suspension in water, the acidic suspension was neutralized with 2*N* NaOH. The aqueous mixture was extracted with ether, charcoal treated, filtered, and acidified with HCl to pH 2. The carbobenzoxy tripeptides precipitated out. They were recrystallized from hot acetone–petroleum ether. Yields for this and other homologs were about 50%. The following compounds were prepared in this way.

XX. Z-Val-val-ileu (L,L,D), m.p. 224–225°, $[\alpha]_D$ −6.7° (*c* 2, DMSO). *Anal.* Calcd. for $C_{24}H_{37}N_3O_6$: C, 62.2; H, 8.05; N, 9.08. Found: C, 62.2; H, 8.03; N, 9.25.

XXI. Z-Val-val-aleu (L,L,D), m.p. 222–224°, $[\alpha]_D$ −11.4° (*c* 2, DMSO). *Anal.* Calcd. for $C_{24}H_{37}N_3O_6$: C, 62.2; H, 8.05; N, 9.08. Found: C, 62.3; H, 7.92; N, 9.08.

XXII. Z-Ileu-val-val (L,L,D); this compound was recrystallized from acetone, then chloroform–petroleum ether, m.p. 224–225°, $[\alpha]_D$ −9.3° (*c* 2, DMSO). *Anal.* Calcd. for $C_{24}H_{37}N_3O_6$: C, 62.2; H, 8.05; N, 9.08. Found: C, 61.7; H, 7.45; N, 8.78.

These compounds were not examined for chromatographic purity since the Z-amino acid, the most likely impurity, is not detectable by ninhydrin.

Z-Val-val-val (L,L,D) similarly prepared in 85% yield and recrystallized from ethanol–water contained valine which was demonstrated by chromatography after hydrogenolysis. When a second preparation of Z-val-val-ileu also contained Z-valine, similarly detected, the method was replaced by the benzyl ester procedure given in H.

G. Tripeptide from Z-Tripeptides.—Compounds XX, XXI, XXII were submitted to hydrogenolysis as in D. Yields were about 90%. When not white, they were treated with charcoal. Recrystallization was from methanol–isopropyl ether or water–ethanol. They were chromatographically homogeneous in both systems after recrystallization. For ileu-val-val, HCl was omitted during hydrogenolysis.

XXIII. Val-val-ileu·HCl (L,L,D), m.p. 175°, solidifies 225° with decomposition, melts 238–240°, $[\alpha]_D$ −3.2° (*c* 2, water). *Anal.* Calcd. for $C_{16}H_{31}N_3O_4$·HCl: C, 52.5; H, 8.76; N, 11.5; neut. equiv. 366; L-val, 64.0%. Found: C, 52.9; H, 8.81; N, 11.8; neut. equiv. 368; L-val, 57.6%.

XXIV. Val-val-aleu·HCl·H_2O (L,L,D), m.p. 176–178°, $[\alpha]_D$ −5.4° (*c* 2, water); $[\alpha]_D$ +7.8° (*c* 3, DMSO). *Anal.* Calcd. for $C_{16}H_{31}N_3O_4$·HCl·H_2O: C, 50.2; H, 8.95; N, 11.0; neut. equiv. 384; L-val, 61.0%. Found: C, 49.8; H, 8.51; N, 11.2; neut. equiv. 372; L-val, 65.4%.

XXV. Ileu-val-val·H_2O (L,L,D), m.p. 222–223°, $[\alpha]_D$ −5.1° (*c* 2, water). *Anal.* Calcd. for $C_{16}H_{31}N_3O_4$·H_2O: C, 55.4; H, 9.58; N, 12.1; neut. equiv. 347; L-val, 33.8%; L-ileu, 37.8%. Found: C, 56.0; H, 9.47; N, 11.8; neut. equiv. 371; L-val, 32.8%; L-ileu, 36.1%.

H. Z-Tripeptide Benzyl Esters.—These were made as in B from carbobenzoxy-L-amino acid and dipeptide benzyl ester tosylates. The compounds were recrystallized from acetone–ether, hot ethanol, or 70% ethanol.

XXVI. (a) Z-Ileu-val-val-OBz (L,L,D), yield 77%, m.p. 194.5–195°, $[\alpha]_D$ −2.1° (*c* 2, DMSO). *Anal.* Calcd. for $C_{31}H_{43}N_3O_6$: C, 66.4; H, 7.8; N, 7.58. Found: C, 67.3; H, 8.04; N, 7.48.

XXVI. (b) This was also made employing a water-soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide metho-*p*-toluenesulfonate. The urea precipitated from reaction mixture. Yield 66% of lower purity material, m.p. 186.5–187.5°, N = 7.25.

XXVII. Z-Val-val-val-OBz (L,L,D), yield 85%, m.p. 186.5–187.0°, $[\alpha]_D$ +18.2° (*c* 2, chloroform). *Anal.* Calcd. for $C_{30}H_{41}N_3O_6$: C, 66.7; H, 7.68; N, 7.78. Found: C, 67.1; H, 7.86; N, 7.8.

XXVIII. Z-Ileu-ileu-val-OBz (L,L,D), yield 71%, m.p. 195.5–196.5°, $[\alpha]_D$ −3.8° (*c* 2, DMSO). *Anal.* Calcd. for $C_{32}H_{45}N_3O_6$: C, 67.6; H, 8.00; N, 7.40. Found: C, 67.6; H, 8.02; N, 7.47.

XXIX. Z-Ileu-ileu-ileu-OBz (L,L,D) prepared as in XXVI (b). Yield 66%, m.p. 186.5–187.5°, $[\alpha]_D$ −4.0° (*c* 2, DMSO). *Anal.* Calcd. for $C_{33}H_{47}N_3O_6$: C, 68.2; H, 8.18; N, 7.22. Found: C, 68.5; H, 8.51; N, 7.29.

I. Tripeptides from Z-Tripeptide Benzyl Esters.—Hydrogenolysis as in D was used for the following compounds. Yields were 85–90%. On evaporation of the methanolic HCl solutions of free tripeptides, esters were frequently found, as determined by chromatography. Re-esterification of the tripeptide takes place readily during room temperature evaporation of the methanolic HCl solutions. It was avoided by addition of water before concentration. Hydrogen bromide in glacial acetic acid did not remove the carbobenzoxy group quantitatively in a single trial, although CO_2 evolution had ceased. The matter was not pursued further.

XXX. Ileu-ileu-ileu·HCl (L,L,D), m.p. 234–235°, $[\alpha]_D$ +0.3 ± 0.3° (*c* 2, water).

(14) S. Shankman and Y. Sebyo, *J. Am. Chem. Soc.*, **80**, 1164 (1958).

Anal. Calcd. for $C_{18}H_{35}N_3O_4 \cdot HCl$: C, 55.0; H, 9.14; N, 10.7; neut. equiv., 394; L-ileu, 66.6%. Found: C, 55.6; H, 9.13; N, 10.45; neut. equiv., 393; L-ileu, 66.8%.

XXXI. Ileu-ileu-val·HCl (L,L,D), m.p. 226–227°, $[\alpha]_D$ +4.6° (*c* 2, DMSO).

Anal. Calcd. for $C_{17}H_{33}N_3O_4 \cdot HCl$: C, 53.8; H, 9.05: N, 11.05; neut. equiv. 380; L-ileu, 69.0%; L-val, 0.0%. Found: C, 53.1; H, 8.81; N, 11.1; neut. equiv. 381; L-ileu, 63.3%; L-val, 0.4%.

The amino acid values have not been corrected for racemization during hydrolysis.

J. Phenylalanine Tripeptides and Intermediates. XXXII.—Z-Phe-phe-OMe (L,D) was prepared from Z-L-phe and D-phe methyl ester by the mixed anhydride as in B with chloroform as solvent. Yield 76%, m.p. 141–143.5°, $[\alpha]_D$ +21.8° (*c* 2, DMSO).

Anal. Calcd. for $C_{27}H_{28}N_2O_5$: C, 70.4; H, 6.13; N, 6.08. Found: C, 70.4; H, 5.98; N, 6.10.

The *p*-nitrophenyl ester procedure gave similar yields but low melting material. Dicyclohexyl carbodiimide coupling gave material melting at 145–146.5°.

XXXIII. The dipeptide XXXII was hydrogenated as in D and recrystallized from methanol–ether to give L-phe-D-phe-OMe·HCl. Yield 93%, m.p. 182–184.5°, $[\alpha]_D$ −41.0 (*c* 2, $CHCl_3$). *Anal.* Calcd. for $C_{19}H_{22}N_2O_3 \cdot HCl$: C, 63.0; H, 6.39; N, 7.77; neut. equiv. 361. Found: C, 62.7; H, 6.19; N, 7.48; neut. equiv. 361.

XXXIV. Z-Phe-phe-phe-OMe (L,L,D).—This tripeptide was prepared from Z-phe and L-phe-D-phe-OMe by mixed anhydride in $CHCl_3$. It was recrystallized from tetrahydrofuran and petroleum ether. Yield 76%, m.p. 199–200°, $[\alpha]_D$ +3.0 (*c* 2, DMSO). *Anal.* Calcd. for $C_{36}H_{37}N_3O_6$: C, 71.2; H, 6.16; N, 6.91. Found: C, 71.1; H, 6.49; N, 6.92.

XXXV. Z-Phe-phe-phe (L,L,D).—XXXIV (1.5 g., 2.5 mmoles) in 200 ml. of tetrahydrofuran and 2.7 mmoles of aqueous *N* NaOH were allowed to react for 16 hr. at room temperature. The residue, after solvent removal, was extracted into water, then $CHCl_3$. The aqueous phase was acidified to congo red with HCl and XXXV precipitated out. Crude yield 40%, m.p. 200–205°. Recrystallization from methanol gave crystals. Final yield 20%, m.p. 209–211.5°, $[\alpha]_D$ −17° ± 20% (*c* 0.18, DMSO). *Anal.* Calcd. for $C_{35}H_{35}N_3O_6$: C, 70.8; H, 5.95; N, 7.08. Found: C, 70.6; H, 6.17; N, 7.01.

Recovered unsaponified from $CHCl_3$ was 0.45 g. or 30%.

XXXVI. Phe-phe-phe·HCl·2H₂O (L,L,D).—(a) XXXV (0.28 g.) was hydrogenated as in D. After catalyst removal and solvent concentration, XXXVI was precipitated from solution with ether in the cold. Yield 60%, m.p. 198–201°, $[\alpha]_D$ −12.9° (*c* 2, *N* HCl). *Anal.* Calcd. for $C_{27}H_{29}N_3O_4 \cdot HCl \cdot 2H_2O$: C, 60.8; H, 6.28; N, 7.90; neut. equiv. 532; L-phe, 62.0%. Found: C, 62.5; H, 6.08; N, 7.65; neut. equiv. 540; L-phe, 61.1%.

The L-phenylalanine was determined after 8 days hydrolysis in 4 *N* HCl at 116°, sealed, corrected for racemization on hydrolysis.

The compound had 0.4% sodium by flame photometry. The neutral equivalent, determined as in Q, was corrected for the sodium. XXXIII and XXXVI were chromatographically homogeneous in the BAW system.

(b) Phe-phe-phe-OMe·HCl prepared by hydrogenolysis of XXXV was saponified in the manner used for valine tripeptide methyl esters.[14] It was suspended in 0.5 *N* KOH and kept at 37° for 2 hr. The unreacted material was removed by filtration, and upon acidification to pH 5.2, a gel precipitated. It was filtered and dried, m.p. 244–245°. Chromatography gave material with one ninhydrin spot and two NaOCl–KI–starch spots. The slower starch spot also gave a strong ultraviolet spot. Three products, then, resulted from saponification of the tripeptide ester, namely free tripeptide, and two possible polymers of unknown length. These products were separated on IRC-50 resin by 0.1 *N* HCl but were not further identified.

K. Peptides of Cycloleucine. N⁴-(Z-Cyc-cyc)-sulfanilamide. XXXVII.—Z-Cyc-cyc (0.025 mmole), m.p. 186–188°, reported 184–185°,[10] in 100 ml. of cold (−5°) tetrahydrofuran were coupled *via* the anhydride as in B. XXXVII precipitated in part. The entire mass was taken to dryness (crude yield 93%), and washed as in B employing trituration. The wide melting solid was only slightly soluble in chloroform, toluene, ether, ethyl acetate, or acetonitrile. It was washed with warm acetone. The acetone insolubles were recrystallized from methanol–ether. Yield 54%, m.p. 248–251°, needles. *Anal.* Calcd. for $C_{26}H_{32}N_4O_6S$: C, 59.0; H, 6.06; N, 10.6, S, 6.06. Found: C, 59.4; H, 6.41; N, 10.4; S, 6.35.

XXXVIII. N⁴-(Cyc-cyc)-sulfanilamide.—Compound XXXVII was suspended in methanol and hydrogenated as in D, without HCl. On hydrogenolysis, solution occurred. On evaporation of methanol, needles precipitated, yield 60%. They were recrystallized from methanol–ether, m.p. 250–252°.

Anal. Calcd. for $C_{18}H_{26}N_4O_4S$: C, 54.8; H, 6.64; N, 14.2; S, 8.13; neut. equiv. 394; Sulfa, 43.8%. Found: C, 54.7; H, 6.96; N, 14.3; S, 8.07; neut. equiv. 399; Sulfa, 41.8%.

Sulfanilamide was determined by the Bratton-Marshall procedure.[15]

XXXIX. Z-Cyc-cyc-D-val-OMe.—This compound was prepared *via* the mixed anhydride from Z-cyc-cyc as in B, with chloroform as solvent. It was precipitated from a small volume with petroleum ether and recrystallized from methanol–water, yield 61%; m.p. 152–153°; $[\alpha]_D$ +23.7° (*c* 2, DMSO).

Anal. Calcd. for $C_{26}H_{37}N_3O_6$: C, 64.0; H, 7.66; N, 8.62. Found: C, 64.3; H, 7.29; N, 8.47.

XL. Cyc-cyc-D-val-OMe·HCl.—XXXIX was hydrogenated as in D, with additional catalyst added after gas evolution had ceased. After solvent removal, some ninhydrin-positive material was found. The residue was dissolved in carbonate buffer and extracted with ether. The aqueous phase was re-extracted with ethyl acetate. The residues from the ether and ethyl acetate extracts were both chromatographically homogeneous by the peptide bond test and free of ninhydrin-positive material. The amino group of cycloleucine does not give a ninhydrin test. After conversion to the hydrochloride by aqueous HCl, the compound was recrystallized from methanol–ether, m.p. 166.5–167.5°, $[\alpha]_D$ +27.1° (*c* 2, water).

Anal. Calcd. for $C_{18}H_{31}N_3O_4 \cdot HCl$: C, 55.4; H, 8.3; N, 10.8; neut. equiv. 390. Found: C, 54.9; H, 8.63; N, 10.7; neut. equiv. 393.

XLI. Z-Val-val-cyc-OMe (L,L,-).—L-Val-cyc-OMe was prepared from Z-L-val-cyc-OMe[10] by hydrogenolysis as in D, without HCl. Some diketopiperazine val-cyc was obtained, which was insoluble in ether. The desired dipeptide ester was soluble in ether. The tripeptide XLI was prepared in 78% yield by coupling Z-L-val with L-val-cyc-OMe as in B. It was recrystallized from chloroform and petroleum ether, m.p. 180–182°; $[\alpha]_D$ −44.1° (*c* 2, methanol).

Anal. Calcd. for $C_{25}H_{37}N_3O_6$: C, 63.0; H, 7.82; N, 8.84; L-val, 48.1%. Found: C, 63.4; H, 8.00; N, 8.80; L-val, 46.9%.

XLII. L-Val-L-val-cyc-HOAc·2H₂O.—Saponification of XLI in methanol for 16 hr. gave a low yield of Z-tripeptide. Saponification in tetrahydrofuran gave the Z-tripeptide in 85% yield, m.p. 110–112°. The Z-tripeptide was hydrogenated in acidic methanol as in D and gave material with two ninhydrin-positive spots on chromatography. Since recrystallization did not eliminate one spot, multiple full-sheet chromatography was used to separate the compounds. After elution, HCl was removed by passing the peptide hydrochloride through IR-45 resin. It was converted to the acetate and recrystallized from methanol–ether. The compound stood for several months prior to analysis. It analyzed as a dihydrate; m.p. 125° dec., $[\alpha]_D$ −35.4° (*c* 2, glacial acetic acid).

Anal. Calcd. for $C_{16}H_{29}N_3O_4 \cdot HOAc \cdot 2H_2O$: C, 51.0; H, 8.79; N, 9.94; neut. equiv. 423; L-val, 55.4%. Found: C, 50.5; H, 8.40; N, 10.1; neut. equiv. 416; L-val, 55.0%.

M. Carbobenzoxy Tripeptide Methyl Esters from Z-Dipeptides.—The Z-dipeptides, Z-L-val-L-val, Z-L-ileu-L-ileu, or Z-L-ileu-L-aleu were coupled as in B with a D-amino acid ester. The Z-dipeptides were prepared by saponification of the Z-L-L-dipeptide methyl esters which proceeded readily. The D-amino acid methyl esters were prepared and used as hydrochlorides. Several of the tripeptide esters were recrystallized repeatedly from hot methanol, methanol–water, ethyl acetate–hexane, or chloroform–petroleum ether. Melting points on XLIII to XLV are not corrected.

XLIII. Z-Ileu-ileu-ileu-OMe (L,L,D), m.p. 198–200°, $[\alpha]_D$ −30.2° (*c* 2, $CHCl_3$). *Anal.* Calcd. for $C_{27}H_{43}N_3O_6$: N, 8.32; L-ileu, 51.9%. Found: N, 8.14; L-ileu, 45.7%.

XLIV. Z-Ileu-aleu-aleu-OMe (L,L,D), m.p. 186.5–189°, $[\alpha]_D$ −21.8° (*c* 2, $CHCl_3$). *Anal.* Calcd. for $C_{27}H_{43}N_3O_6$: N, 8.32; L-ileu, 26.0%. Found: N, 8.18; L-ileu, 24.4%.

(15) Methods of the Assoc. of Official Ag. Chemists, 7th Ed., p. 619, Washington, D. C. (1950).

XLV. Z-Ileu-ileu-aleu-OMe (L,L,D), m.p. 182.5–184.5°, $[\alpha]_D$ −30.6° (*c* 2, $CHCl_3$). *Anal.* Calcd. for $C_{27}H_{43}N_3O_6$: N, 8.32; L-ileu, 51.9%. Found: N, 8.15; L-ileu, 47.0%.

XLVI. Z-Val-val-ileu-OMe (L-L,D), m.p. 200–201°, $[\alpha]_D$ −32.8° (*c* 2, $CHCl_3$). *Anal.* Calcd. for $C_{25}H_{39}N_3O_6$: C, 62.8; H, 8.22; N, 8.80; L-val, 49.0%. Found: C, 62.7; H, 7.96; N, 8.70; L-val, 44.1%.

XLVII. Z-Val-val-aleu-OMe (L,L,D), m.p. 188–189°, $[\alpha]_D$ −34.3° (*c* 2, $CHCl_3$). *Anal.* Calcd. for $C_{25}H_{39}N_3O_6$: C, 62.8; 8.22; N, 8.80; L-val, 49.0%. Found: C, 62.9; H, 8.23; N, 8.61; L-val, 44.7%.

XLVIII. Z-Val-val-phe-OMe (L,L,D + L,D,D), m.p. 210–212°, $[\alpha]_D$ +7.7° (*c* 2, DMSO). *Anal.* Calcd. for $C_{28}H_{37}N_3O_6$: C, 65.8; H, 7.30; N, 8.21. Found: C, 65.6; H, 7.35; N, 8.28.

Racemization may occur[3,12] in this sequence of coupling. However, L-amino acid assays on the peptide esters given below obtained after hydrogenolysis would indicate that racemization did not occur or was of minor consequence, except for Z-val-val-phe-OMe.

N. Tripeptide Methyl Esters from Z-Tripeptide Methyl Esters.—These were prepared as in D. They were recrystallized from methanol–isopropyl ether, or acetone–ether.

XLIX. Ileu-ileu-ileu-OMe·HCl (L,L,D), m.p. 219–221°, $[\alpha]_D$ +2.1° (*c* 2, H_2O). *Anal.* Calcd. for $C_{19}H_{38}ClN_3O_4$: C, 55.9; H, 9.4; N, 10.35; neut. equiv. 408; L-ileu, 64.2%. Found: C, 55.5; H, 9.17; N, 10.2; neut. equiv. 406; L-ileu, 68.3%.

L. Ileu-aleu-aleu-OMe·HCl (L,L,D), m.p. 231–232°. *Anal.* Calcd. for $C_{19}H_{38}ClN_3O_4$: C, 55.9; H, 9.4; N, 10.35; neut. equiv. 408; L-ileu, 32.1%. Found: C, 55.5; H, 9.3; N, 10.2; neut. equiv. 406; L-ileu, 31.7%.

LI. Ileu-ileu-aleu-OMe (L,L,D), m.p. 197–199°. *Anal.* Calcd. for $C_{19}H_{38}ClN_3O_4$: C, 55.9; H, 9.4; N, 10.35; neut. equiv. 408; L-ileu, 64.2%. Found: C, 54.8; H, 9.4; N, 10.15; neut. equiv. 405; L-ileu, 60.0%.

LII. Val-val-phe-OMe·HCl (L,L,D + L,D,D), m.p. 218.5–220.5°; $[\alpha]_D$ +19.8° (*c* 2, H_2O). *Anal.* Calcd. for $C_{19}H_{32}ClN_3O_4$: C, 58.2; H, 7.80; N, 10.15; neut. equiv. 415; L-val, 56.8%. Found: C, 57.8; H, 7.3; N, 9.90; neut. equiv. 394; L-val, 44.0%.

Racemization of the central valine[3,12] may be the cause of low valine assay.

O. Rearrangement on Saponification.—On saponification in methanol at 37° for 2 hr. or room temperature for 16 hr., several carbobenzoxy tripeptide methyl esters gave yields of 10–20% or no isolatable yield of Z-tripeptide. These included Z-val-val-phe-OMe, Z-phe-phe-phe-OMe, Z-val-val-ileu-OMe among others. Better yields were obtained with tetrahydrofuran as solvent. Recycling of recovered product was only possible once, since recovered material was not identical with starting material. The question of base racemization of the C-terminal amino acid which would give L-L-L tripeptide rather than L-L-D was of consequence, since L-L-L tripeptides reverse the antibacterial activity of L-L-D trivaline for *L. plantarum* at 1:100 and for *P. cerevisiae* at 1:1.[25]

Employing Z-cyc-cyc-D-val-OMe as a model compound, the rate of saponification and the extent of possible racemization were studied. Five tenths mmole of the tripeptide in 30 ml. of methanol containing 0.55 mmole of KOH and 2 ml. of H_2O, was kept closed for 24 hr. at 20, 40, and 60°. The base consumed was a measure of carboxyl formation. Assuming one carboxyl liberated per mole of base, the amounts saponified were 11, 60, and 105%, respectively.

A larger scale run with 1–4 mmoles of the tripeptide gave an isolation 5% yield at 20°, m.p. 181–186°, with recovered ester of m.p. 150–151.5°. The 40° run gave 27% yield of material with an 80° range of melting point. The 60° trial gave a 35% yield of material melting over a 70° range. Purification of the 60° saponificate was accomplished by recrystallization from methanol–petroleum ether with charcoaling. The isolated compound melted at 149–154°. The compound gave a single spot by silica thin-layer chromatography and Morin detection[16]; $[\alpha]_D$ −1.1° (*c* 2, methanol).

Anal. Calcd. for **II**, $C_{18}H_{29}N_3O_6$: C, 56.5; H, 7.60; N, 11.0. Found: C, 56.7; H, 7.45; N, 10.35.

Calcd. for Z-cyc-cyc-val, $C_{25}H_{35}N_3O_6$: C, 63.0; H, 7.42; N, 8.80.

The compound did not have a free amino group by perchloric acid tritration. By tritration of carboxyl group,[17] it had an equiv. wt. of approximately 255. The infrared spectrum was compared to Z-val-val-OMe. It had no ester band at 5.75 μ and no benzyl bands at 13μ or higher. Both infrared and analytic data for the rearrangement product fit a substituted urea.

The entire amount of each of the 0.5 mmole saponificates above was acidified, then taken to dryness, treated with HBr in dioxane to remove Z, again taken to dryness, suspended in 1 ml. water, ether extracted, again dried, then hydrolyzed with 4 *N* HCl for 40 hr. at 110°, and sealed. L-Val determined bacterimetrically was 0.3, 0.3, and 0.6% in the 20, 40, and 60° preparations. If all of the valine were racemized, the expected L-val would be 12.0%. A sample of D-val similarly treated but omitting the saponification step contained 1.5% L-val. D-Val in the C-terminal position is not significantly racemized under these conditions. The absence of racemization on saponification suggests that oxazolone formation as in the mechanism of Goodman[18] did not occur here.

P. Synthesis of Radioactive Val-val-val (L,L,D) Labeled in the N-Terminal Amino Acid.—Fifty microcuries of L-val weighed about 0.6 mg. Carbobenzoxylation trials on cold valine with 0.5, 1, and 5 mg. of valine gave 90–95% yields at 0.5 and 1 mg. when a 10-fold excess of ethereal carbobenzoxy chloride and bicarbonate was employed. Yields decreased sharply without excess. The extent of carbobenzoxylation was determined by ninhydrin estimation of the unreacted valine.

The radioactive valine was carbobenzoxylated, excess carbobenzoxy chloride removed by ether extraction, and after acidification the Z-val-C^{14} was extracted into ethyl ether. It was allowed to react with 23 mg. of L-val-D-val benzyl ester tosylate, 5 mg. of triethylamine, and 9.9 mg. of the carbodiimide (XXVIb)[19] in 1 ml. of methylene chloride. After 24 hr., the mixture was washed with water, 0.1 *N* HCl, and water. The solvent was evaporated and the residue dissolved in methanol and hydrogenolyzed with 1 mg. of 10% Pd on charcoal as catalyst. The radioactive val-val-val was isolated by paper chromatography with the BAW system. Cold authentic material was used to establish the position of the tripeptide. The principal product was the radioactive tripeptide. In some runs, there was some evidence in the radioactive chromatograms of a valyl-urea spot and tripeptide benzyl ester spot, as well as tripeptide. Of 5×10^6 c.p.m. as Z-val-C^{14} + val-C^{14}, present after carbobenzoxylation, 2×10^6 c.p.m. were extracted into ether after acidification. This gave 1.7×10^6 c.p.m. as protected tripeptide in methylene chloride after washing. Some runs did not give product. Mixed anhydride has been used to obtain radioactive glycine peptides.[18] The presence of traces of water could seriously reduce yields by an anhydride procedure. A tripeptide was not obtained in a single trial of the mixed anhydride procedure.

Q. Other Procedures.—L-Amino acids were determined bacterimetrically. For L-ileu, if L-aleu could be present, *L. mesenteroides* or *P. cerevisiae* were employed. For these cultures, D-ileu and D-aleu were inactive and L-aleu had about 1/100 of the growth-promoting activity of L-ileu. L-Aleu could be determined by *L. plantarum* with pyridoxal in the medium if L-ileu was absent. L-Val was determined with *L. plantarum*. For L-phe, *P. cerevisiae* or *L. mesenteroides* were used. D-Amino acids were the highest purity grades and assayed less than 0.3% L-amino acid by bacterimetric assay. The isodecines were resolved at the National Institutes of Health, under the direction of Dr. M. Winitz.

Hydrolysis of peptides was conducted at 110°, in sealed tubes, using 4 *N* HCl and varied times of hydrolysis, 2–8 days. Values were extrapolated to zero time when either substantial destruction of L-amino acid or production of L-amino acid from C-terminal D-amino acid occurred on hydrolysis. Appropriate mixtures were hydrolyzed and assayed as controls for destruction or racemization occurring on hydrolysis. The bacterimetric assay was particularly useful to establish that the peptide ileu-ileu-ileu (L,L,D) was indeed optically active but had an optical rotation within the limit of error of the instrument used.

Rotations were determined on the Keston photo-electric polarimetric attachment to the Beckman DU spectrophotometer. Neutral equivalent was determined by titration of amino group with perchloric acid in acetic acid, with mercuric acetate used to remove HCl. Corrected melting points were determined with a

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(17) A. Patchornick and S. E. Rogozinski, *Anal. Chem.*, **31**, 985 (1959).

(18) M. Goodman and K. C. Stueben, *J. Org. Chem.*, **27**, 3109 (1962).

(19) E. M. Levine and S. Simmonds, *J. Biol. Chem.*, **235**, 2902 (1960).

TABLE III

INHIBITORY ACTIVITY OF OTHER PEPTIDES

ID_{50}[a] *L. plantarum* Inhibitory dipeptides (all L)	*P. cerevisiae*	Noninhibitory peptides (all L)		Noninhibitory amino acids
Try-try 0.01,	≪0.02	Cyc-pro	Leu-cyc	Ornithine
Try-gly .025,	≪0.02	Cyc-ser	Cyc-cyc	Citrulline
Try-tyr .06,	≪0.02	Ser-cyc	Ala-cyc	ε-Aminocaproic acid
Gly-try .05,	0.03	Val-cyc	Phe-cyc	Diaminopropionic acid
Leu-try .02,	.02	Arg-glu	Glu-arg	3-Aminotyrosine
Arg-cyc .06,	.1	Arg-asp	Arg-ala	S-Ethylcysteine
His-cyc .1,	.1	Pro-arg	Ileu-aleu	Homocysteic acid
			Ileu-ileu	
Tyr-phe N,[b]	.06	N-Formyl-phenylalanyl-melphelan·OEt		α-Methylglutamic acid
Lys-gly Inhibits		Val-gly-melphelan-OEt·2HCl		Melphelan (L-sarcolysin)
Arg-val N,	.08	Cys-bis-(melphelan-OEt)·2HCl		*ortho*-DL-Merphelan
Arg-leu N,	.08	Ala-melphelan-OEt picrate		Medphelan
Meth-cyc Variable		Val-melphelan		N,N,Di-(2-chloroethyl)-*p*-aminophenylbutyric acid

[a] ID_{50} is the concentration in mg./ml. at which growth is reduced 50%. [b] N, noninhibitory.

Thomas-Hoover melting point apparatus, calibrated against U.S.P. reference standards.

Butanol–water–acetic acid, 90:30:10, and pyridine–isoamyl alcohol–water, 35:35:30, were used routinely for paper chromatography with detection by ninhydrin, NaOCl–KI–starch, or ultraviolet for Z-peptides. Peptides with an N-terminal cycloleucine do not give a ninhydrin spot. Several blocked tripeptides did not give spots on the NaOCl–KI–starch peptide bond test. In these systems, protected peptide esters move close to the solvent front. Changes in optical configuration do not give R_f changes sufficient to detect.

R. Free Peptides by Other Procedures.—Hydrolysis at 37 and 100° of ileu-ileu-ileu-methyl ester hydrochloride by dilute HCl to remove the methyl group was used as an approach to the desired peptides. Hydrolysis of both ester and peptide bonds occurred, determined chromatographically. Base saponification of the tripeptide ester as used for valine[14] gave some polymeric products. Hydrolysis of ester moiety of L-val-L-val-D-phe methyl ester with crystalline chymotrypsin[20] was very slow. The peptide bonds cleaved in this time. The method could not be used for preparation of free L-L-D peptides.

S. Inhibitory Activity of Other Peptides.—Other peptides were evaluated[2b] to determine other possible types of inhibitory peptides. These are listed in Table III. The inhibition indices for *L. plantarum* and *P. cerevisiae* are given in that order after each peptide.

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A New Series of Substances which Block the Adrenergic β-Receptors

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A series of phenylethanolamines and phenylpropanolamines with chlorine or alkyl substitution in the benzene nucleus has been synthesized which block adrenergic β-receptors in mammals. A clear relationship between structure and activity allowed us to synthesize potent β-receptor blockers with or without intrinsic sympathomimetic activity.

To explain the effects of catecholamines [norepinephrine (Ia), epinephrine (Ib), and isoproterenol (Ic)] on different organs, Ahlquist[1] proposed that responses to catecholamines might be elicited by stimulation of two different receptors. Relaxation of smooth muscle resulting in vasodilatation and bronchodilatation, the positive inotropic and chronotropic effect on the heart, as well as metabolic effects (*e.g.*, glycogenolysis) are due to stimulation of β-receptors, while other effects

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