The ether solution was dried over anhydrous Na_2SO_4 and then evaporated to dryness at room temperature under a stream of nitrogen; yield 650 mg. of a colorless oil (theory 677 mg.). Calcd. for dipalmitolein: hydrogen number,[16] 282; sapn. equiv., 282.5. Found: hydrogen number, 282; sapn. equiv., 282; $[\alpha]^{25}D$ −2.3° (*c* 12.0 in $CHCl_3$).

Hydrogenation of this unsaturated compound gave as the only product, D-1,2-dipalmitin, m.p. 67–68° (reported value[17] 67–67.5°). Alkaline hydrolysis of 350 mg. of the unsaturated diglyceride, followed by acidification and extraction with diethyl ether yielded 300 mg. of palmitoleic acid (theory 316 mg.). Calcd. for palmitoleic acid: neut. equiv., 254.0; hydrogen number, 254.0. Found: neut. equiv., 255.0; hydrogen number, 253.5. Fifty milligrams of the acid was converted to the amide[10]; yield 42 mg., m.p. 68.5–69.5°. Admixture with pure palmitoleamide[10] did not alter the melting point.

Hydroxylation of 50 mg. of the unsaturated fatty acid by the procedure of Swern, *et al.*,[18] gave 40 mg. of *cis*-dihydroxypalmitic acid, m.p. 85–86° (reported value[10] 85–86°).

The infrared pattern of the free unsaturated fatty acid was identical with that reported for palmitoleic acid.[13]

D-1,2-Dipalmitin.—Under exactly the same conditions as described above for the preparation of the unsaturated diglyceride, the saturated diglyceride may be obtained from (dipalmitoyl)-L-α-lecithin. In a typical run, 625 mg. of dipalmitin was obtained from 1.00 g. of saturated lecithin. The residue from the evaporation of the ether-soluble fraction (see above) was crystallized three times from $CHCl_3$–petroleum ether (1:10); yield 590 mg., m.p. 67.5–68° (reported value[17] 67–67.5°).

Anal.[19] Calcd. for $C_{35}H_{68}O_5$ (568): C, 73.8; H, 12.05. Found: C, 73.75; H, 12.13; $[\alpha]^{25}D$ −2.3° (*c* 11.7 in $CHCl_3$), reported value[17] $[\alpha]^{25}D$ −2.3°, in $CHCl_3$.

p-Nitrobenzoyl derivative, m.p. 60–61° (reported value[17] 60–60.5°). Hydrolysis of 100 mg. of this diglyceride under the same conditions as for the unsaturated diglyceride gave 75 mg. of fatty acid (theory 80 mg.). After recrystallization three times from 60% ethanol, 60 mg. of acid, m.p. 60–61°, was obtained which when admixed with an authentic sample of palmitic acid had m.p. 60–61°; neut. equiv. 256.0 (theory for palmitic acid 256.0).

Phosphorylcholine.—The combined original aqueous fraction and the washings were concentrated to dryness and the calcium salt of phosphorylcholine was isolated in 90% yields, essentially by the procedure described by MacFarlane and Knight.[2]

Anal. Calcd. for $C_5H_{13}O_4NPClCa5H_2O$ (348): P, 8.91; N, 4.02. Found: P, 8.87; N, 4.10.

Addenda.—After the completion of this manuscript, a communication by C. Long and M. F. Maguire appeared in *Biochem. J.*, **55**, xv (1953), wherein they report that *Cl. welchii* type A α-toxin acts as expected on ovolecithin but exhibited no activity toward saturated lecithins in an aqueous medium.

(16) Hydrogen number = wt. of sample in mg. × 2.4/ml. of hydrogen absorbed (S.T.P.).

(17) J. C. Sowden and H. O. L. Fischer, THIS JOURNAL, **63**, 3244 (1941).

(18) D. Swern, G. N. Billen, T. W. Findley and J. T. Scanlan, *ibid.*, **67**, 1786 (1945).

(19) Analyzed by the Elek Micro Analytical Laboratories, 4763 W. Adams Blvd., Los Angeles, Calif.

SEATTLE, WASHINGTON

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY]

The Synthesis of Peptides Related to Gramicidin S

BY BERNARD F. ERLANGER,[1] HOWARD SACHS AND ERWIN BRAND[2]

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The synthesis of a crystalline decapeptide containing the same sequence and configurations of amino acids as exist in gramicidin S is described. The synthetic methods were chosen so as to preclude the possibility of the formation of mixtures of diastereoisomers.

We are reporting the synthesis of a decapeptide having the sequence and configurations of amino acids believed to exist in gramicidin S. Consden, Gordon, Martin and Synge[3] have determined the sequence of the five different amino acid residues of gramicidin S to be[4]: -Val-Orn-Leu-Phe-Pro- (L-L-L-D-L). It has been established by Battersby and Craig[5] that the molecular weight of gramicidin S is that of a decapeptide. Thus the sequence must appear twice. Sanger[6] has shown that the only free amino group is the δ-amino group of ornithine. This result and X-ray analysis[7] support the hypothesis that gramicidin S is a cyclic decapeptide.

Harris and Work[8] have synthesized three pentapeptide derivatives having the sequence of gramicidin S: *p*-Tos·Val-Orn-Leu-Phe-Pro·OMe·HCl (L-L-L-D-L)[9]; *p*-Tos·Val-Orn-Leu-Phe-Pro·NH_2·HCl (L-L-L-D-L); H·Val-Orn-Leu-Phe-Pro·OMe·2HCl (L-L-L-D-L). The last peptide could not be crystallized and no analysis or physical data were reported. The synthetic techniques used were those developed by Bergmann and his collaborators.[10]

Recently, Schumann and Boissonas[11] reported the synthesis of H·Val-Z·Orn-Leu-Phe-Pro·OH-(L-L-L-D-L), an intermediate which could be useful for the synthesis of the decapeptide. They used, exclusively, the mixed anhydride method of peptide synthesis devised by Boissonnas[12] and by Vaughan and Osato.[13] They employed phthaloyl[14–16] as a protecting group.

It has been found by Vaughan[17] and by the au-

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(2) Deceased July 11, 1953.

(3) R. Consden, A. Gordon, A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **41**, 596 (1947).

(4) For key to abbreviations, see footnotes to Table I and B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951).

(5) A. R. Battersby and L. C. Craig, *ibid.*, **73**, 1887 (1951).

(6) F. Sanger, *Biochem. J.*, **40**, 261 (1946).

(7) D. C. Hodgkin, *Cold Spring Harbor Symposia*, **14**, 65 (1949).

(8) J. I. Harris and T. S. Work, *Biochem. J.*, **46**, 196, 582 (1950).

(9) *p*-Tos = *p*-toluenesulfonyl.

(10) For references *cf.* J. S. Fruton, *Adv. Prot. Chem.*, **5**, 1 (1949).

(11) I. Schumann and R. A. Boissonnas, *Helv. Chim. Acta*, **35**, 2237 (1952).

(12) R. A. Boissonnas, *ibid.*, **35**, 874 (1951).

(13) J. R. Vaughan and R. L. Osato, THIS JOURNAL, **73**, 5553 (1951); **74**, 676 (1952).

(14) J. C. Sheehan and V. S. Frank, *ibid.*, **71**, 1856 (1949).

(15) F. E. King and D. A. A. Kidd, *J. Chem. Soc.*, 3315 (1949).

(16) F. E. King, R. S. Jackson and D. A. A. Kidd, *ibid.*, 243 (1951).

(17) J. R. Vaughan, THIS JOURNAL, **74**, 6137 (1952).

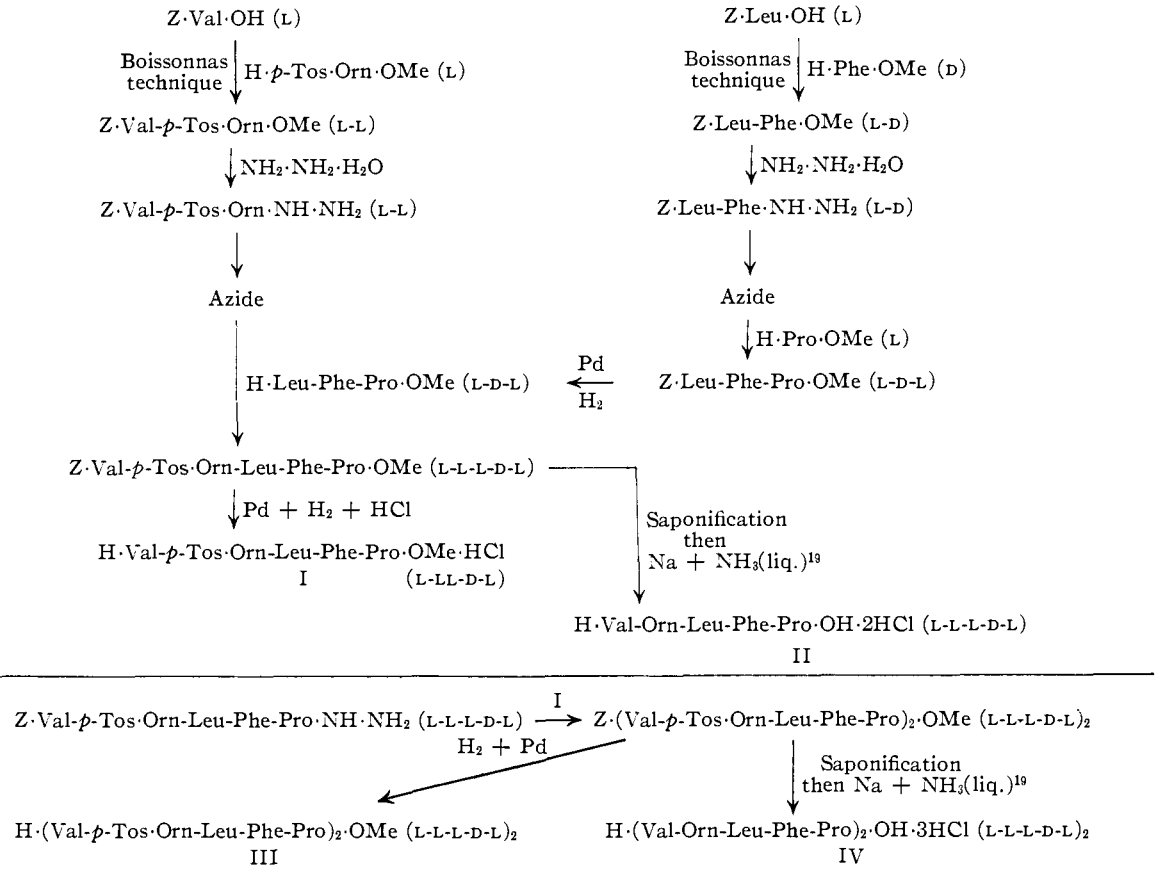

Fig. 1.

thors[18] that the mixed anhydride method, when used to synthesize tripeptides and higher peptides in the manner reported by Boissonnas, leads to mixtures of diastereoisomers. This is probably why Schumann and Boissonnas were able to crystallize only one of the higher intermediates. It is unfortunate that no optical rotations are reported by these authors, especially in the case of H·Leu-Phe-Pro·OMe·HCl (L-D-L) which we, like Harris and Work,[8] have found to be crystalline and which Schumann and Boissonnas report as an "extremely hygroscopic" solid.

We have synthesized the following penta- and decapeptides and derivatives by a combination of methods which we believe ensures the optical homogeneity of the products:

I, H·Val-*p*-Tos·Orn-Leu-Phe-Pro·OMe·HCl (L-L-L-D-L)
II, H·Val-Orn-Leu-Phe-Pro·OH·2HCl (L-L-L-D-L)
III, H·(Val-*p*-Tos·Orn-Leu-Phe-Pro·)$_2$·OMe (L-L-L-D-L)$_2$
IV, H·(Val-Orn-Leu-Phe-Pro)$_2$·OH·3HCl (L-L-L-D-L)$_2$

Compounds I, II and III were obtained in crystalline form. Compound IV when studied under the microscope is a mixture of needles in combination with amorphous material. It has an ash of about 1.5%, undoubtedly sodium chloride.

Dipeptide derivatives were prepared by the method of Boissonnas.[12] Further steps utilized the Bergmann azide technique.[10] In addition, we have utilized the *p*-toluenesulfonyl group in such a manner as to enable us to produce higher peptides and have succeeded in removing it by the action of sodium in liquid ammonia.[19] For the general scheme, see Fig. 1.

In the case of Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·NH·NH$_2$ (L-L-L-D-L) difficulty was encountered in getting a sharp melting point despite the obviously crystalline appearance of the compound and its satisfactory analysis. The isopropylidene derivative, however, had a sharp melting point. Recovery of the hydrazide from this derivative yielded a product with no better melting point. No explanation for this can be advanced.

Antibacterial activity of these open chain compounds is being studied and will be reported in a subsequent paper, along with comparative data on the cyclic gramicidin S.[19a]

Experimental[20]

Starting Materials.—The specific rotations of the amino acids used are as follows: L-leucine +16.5° (6 *N* HCl), D-phenylalanine[21] +35.1° (H_2O), L-proline −83.1° (H_2O), L-valine +27.1° (6 *N* HCl), L-ornithine, +30.8° (6 *N* HCl).

1. **δ-*p*-Toluenesulfonyl-L-ornithine.**—10.1 g. (0.06 mole) of L-ornithine monohydrochloride was dissolved in 30 ml. of water. The solution was brought to boiling and 7 g. (0.07 mole) of $CuCO_3$·$Cu(OH)_2$ was added carefully over a period

(18) B. F. Erlanger, H. Sachs and E. Brand, unpublished experiments.

(19) *Cf.* V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937); also D. W. Wooley, *ibid.*, **172**, 71 (1948).

(19a) NOTE ADDED IN PROOF.—The decapeptide H·(Val-Orn-Leu-Phe-Pro)$_2$·OH·3HCl (L-L-L-D-L)$_2$ has now been crystallized as platelets from 0.1 *N* HCl.

(20) All melting points are corrected. Analytical data for compounds 5 through 20 are in Tables I and II.

(21) Resolved according to method of H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 475 (1951).

TABLE I

CARBOBENZYLOXY DERIVATIVES

No.	Compound[a]	Molecular formula	Mol. wt.	M.p., °C. (cor.)	Nitrogen, % Calcd.	Found	Carbon, % Calcd.	Found	Hydrogen, % Calcd.	Found	Neut. equiv.[b] Calcd.	Found
	Dipeptide derivatives											
5	Z·Val-*p*-Tos·Orn·OH (L-L)	$C_{25}H_{33}O_7N_3S$	519.6	150	8.1	8.1					520	531
6	Z·Val-*p*-Tos·Orn·OMe (L-L)	$C_{26}H_{35}O_7N_3S$	533.6	145	7.9	7.9						
7	Z·Val-*p*-Tos·Orn·$NHNH_2$ (L-L)	$C_{25}H_{35}O_6N_5S$	533.6	213	13.1	13.0						
8	Z·Leu·Phe·OH (L-D)[24]	$C_{23}H_{28}O_5N_2$	412.5	76	6.8	6.8					413	420
9	Z·Leu-Phe-OMe (L-D)	$C_{24}H_{30}O_5N_2$	426.5	112[c]	6.6	6.5						
10	Z·Leu-Phe·$NHNH_2$ (L-D)	$C_{23}H_{30}O_4N_4$	426.5	166[d]	13.1	13.1						
	Tripeptide derivatives											
11	Z·Leu-Phe-Pro·OMe (L-D-L)	$C_{29}H_{37}O_6N_3$	523.6		Amorphous[e]							
	Pentapeptide derivatives											
12	Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·OMe (L-L-L-D-L)	$C_{46}H_{62}O_{10}N_6S$	891.1	184	9.4	9.4						
13	Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·OH (L-L-L-D-L)	$C_{45}H_{60}O_{10}N_6S$	877.0	157	9.6	9.7					890	877
14	Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·$NHNH_2$ (L-L-L-D-L)	$C_{45}H_{62}O_9N_8S$	891.1	[f]	12.6	12.5	60.7	60.4	7.0	7.1		
	Decapeptide derivatives											
15	Z·(Val-*p*-Tos·Orn-Leu-Phe-Pro)$_2$·OMe (L-L-L-D-L)$_2$	$C_{83}H_{114}O_{17}N_{12}S_2$	1616.0	209–211	10.4	10.4	61.7	61.6	7.1	6.8		
16	Z·(Val-*p*-Tos·Orn-Leu-Phe Pro)$_2$·OH (L-L-L-D-L)$_2$	$C_{82}H_{111}O_{17}N_{12}S_2$	1601.9	223–224	10.5	10.3	61.5	61.0	7.0	7.2	1601	1632

[a] The following abbreviations are used (*cf.* B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951)): Z, carbobenzyloxy, $C_6H_5CH_2OCO$; *p*-Tos, *p*-toluenesulfonyl, $C_7H_7SO_2$; Leu, $NH(CHC_4H_9)CO$; Phe, $NH(CHCH_2C_6H_5)CO$; Pro, $N(CHC_3H_6)CO$; Val, $NH(CHC_3H_7)CO$; Orn, $NH(CHC_3H_6NH_2)CO$; Me, CH_3; configuration follows compound in parentheses; *e.g.*, carbobenzyloxy-L-leucyl-D-phenylalanyl-L-proline methyl ester; Z·Leu-Phe-Pro·OMe (L-DL): carbobenzyloxy-L-valyl δ-*p*-toluenesulfonyl-L-ornithine: Z·Val-*p*-Tos·Orn·OH (L-L). [b] E. Ellenbogen and E. Brand, Am. Chem. Soc., Phila. Meeting, April, 1950, Abstracts p. 55-C. [c] Harris and Work (ref. 8) report 110–112°. [d] Harris and Work (ref. 8) report 169. [e] Harris and Work report an amorphous glass-like material. [f] See Compound 14 in Experimental section.

TABLE II

PEPTIDE DERIVATIVES

No.	Compound[a]	Molecular formula	Mol. wt.	Nitrogen, % Calcd.	Found	Amino N, % Calcd.	Found	Carbon, % Calcd.	Found	Hydrogen, % Calcd.	Found
17	H·Val-*p*-Tos·Orn-Leu-Phe-Pro-OMe·HCl (L-L-L-D-L)[b]	$C_{38}H_{57}O_8N_6SCl$	793.4	10.6	10.6	1.8	1.8				
18	H·(Val-*p*-Tos·Orn-Leu-Phe-Pro)O_2·Me (L-L-L-D-L)$_2$	$C_{75}H_{108}O_{15}N_{12}S_2$	1481.8	11.3	11.4			60.8	60.9	6.8	7.1
19	H·Val-Orn-Leu-Phe-Pro·OH·2HCl (L-L-L-D-L)	$C_{30}H_{50}O_6N_6Cl_2$	661.7	12.7	12.7	4.2	4.4[c]	54.4	54.0	7.6	7.7
20	H·(Val-Orn-Leu-Phe-Pro)$_2$·OH·3HCl (L-L-L-D-L)$_2$	$C_{60}H_{97}O_{11}N_{12}Cl_3$	1268.8	13.3	13.2	3.3	3.0[c]	56.8	55.6	7.6	7.7

[a] See Table I, footnote *a* [b] Calcd.: Cl, 4.5. Found: Cl, 4.4. [c] Run for 20 minutes.

of 15 minutes. The blue solution was allowed to cool over a period of one hour, with stirring.

It was then filtered into a 500-ml. 3-neck flask equipped with stirrer and cooled to 20°; 7.75 g. (0.04 mole) of *p*-toluenesulfonyl chloride in 30 ml. of ether and 65 ml. of 2 *N* NaOH were added and vigorous stirring started. After 40 minutes another 7.75 g. (0.04 mole) of *p*-toluenesulfonyl chloride in 35 ml. of ether and 15 ml. of 2 *N* NaOH was added. Stirring was continued for another three hours. The light blue copper compound was filtered off and washed with alcohol and ether; yield 14 g. (75%). It was dissolved in 75 ml. of 2 *N* HCl (green solution) and H_2S passed in for 2 hours. Supercel was added and the copper sulfide filtered off. Pyridine was added to the filtrate until the *p*H was about 5–6, whereupon crystallization occurred; yield 11 g. (67%). After recrystallization from 300 ml. of boiling water and charcoal, the yield was 9 g. (55%) (1.5 g. of material can be recovered from the mother liquor); m.p. 212° dec., $[\alpha]^{23}D$ +20.8° (2% in 6 *N* HCl).

Anal. Calcd. for $C_{12}H_{18}O_4N_2S$ (286.3): N, 9.8; NH_2–N, 4.9. Found: N, 9.7; NH_2–N, 4.9.

2. δ-*p*-Toluenesulfonyl-L-ornithine Methyl Ester Hydrochloride.—11.4 g. (0.04 mole) of δ-*p*-toluenesulfonyl-L-ornithine was dissolved in 250 ml. of anhydrous methanol and the solution saturated with dry HCl gas at ice–salt temperature. The saturated solution was allowed to stand one hour at that temperature after which the methanol was removed *in vacuo*. The resulting oil was redissolved in methanol and retreated with gaseous HCl in the same way. After distillation the oil was placed in an evacuated desiccator over P_2O_5 and NaOH pellets overnight. The result was a glass-like substance which could not be crystallized but was used as such.

3. H·Phe·OMe·HCl (D).—Twenty-five grams (0.15 mole) of D-phenylalanine was dissolved in 500 ml. of anhydrous methanol, and dry HCl gas was passed into the solution until refluxing began and then for another ten minutes. The methanol was taken off *in vacuo*, the residue redissolved in methanol and retreated with HCl. The methanol was removed *in vacuo* and the crystalline product taken up in 50 ml. of hot methanol. Ether was added until slightly turbid. After standing overnight in the ice-box the crystals

were collected and washed with anhydrous ether; yield 27.4 g. (88%), $[\alpha]^{25}D$ +3.9° (2.67% in water) (calcd. as free ester).

Anal. Calcd. for $C_{10}H_{14}O_2NCl$ (215.68): N, 6.5; NH_2–N, 6.5; neut. equiv., 216. Found: N, 6.4; NH_2–N, 6.5; neut. equiv., 216.

4. H·Pro·OMe·HCl (L).—11.5 g. (0.1 mole) of L-proline was dissolved in 125 ml. of anhydrous methanol and cooled in an ice–salt-bath. Gaseous HCl was passed into the solution until the saturation point, after which the methanol was removed *in vacuo*. The saturation and distillation procedures were repeated and the oil was allowed to stand in a desiccator over P_2O_5 and NaOH overnight. The oil crystallized. The crystals were washed thoroughly with ether; yield 10.5 g. (64%), $[\alpha]^{24}D$ −40.1° (0.544% in water) (calcd. as free ester).

Anal. Calcd. for $C_6H_{12}O_2NCl$ (165.7): N, 8.5; HCl, 22.0; neut. equiv., 166. Found: N, 8.4; HCl, 21.9; neut. equiv., 161.

5. Z·Val-*p*-Tos·Orn·OH (L-L).—8.3 ml. (0.036 mole) of tri-*n*-butylamine was added to 9.4 g. (0.036 mole) of carbobenzoxy-L-valine[22] in 65 ml. of dioxane. The solution was cooled to 10° and 3.22 ml. (0.036 mole) of ethyl chlorocarbonate was added. The mixture was allowed to stand at 10° for 30 minutes. Then a 10° solution of 10.4 g. (0.036 mole) of δ-*p*-toluenesulfonyl-L-ornithine (compd. 1) in 18.2 ml. of 2 *N* NaOH was added. There was a vigorous evolution of CO_2. It was allowed to stand overnight in the ice-box, 150 ml. of H_2O and 10 ml. of 2 *N* NaOH were added. The mixture was extracted twice with ether and the clear water layer acidified. The oil which separated out was taken up in ether, dried over sodium sulfate and the ether removed *in vacuo*. The oil was solidified by trituration with ether; yield 9.7 g. (52%). It was recrystallized from ethyl acetate–petroleum ether; yield 9.2 g. (48%), m.p. 150°.

6. Z·Val-*p*-Tos·Orn·OMe (L-L). **(a) Using Diazomethane.**—9.2 g. (0.018 mole) of Z·Val-*p*-Tos·Orn·OH (L-L) (compd. 5) was dissolved in 75 ml. of ethyl acetate. A solution of diazomethane in ether was added dropwise until orange color persists. Then a drop of acetic acid was added and the solvents removed *in vacuo*. The residue was taken up in 15 cc. of ethyl acetate and ether added until turbid. The ester crystallized as needles; yield was 8.2 g. (86%), m.p. 145°. **(b)** 9.6 g. (0.038 mole) of carbobenzoxy-L-valine[22] was dissolved in 50 ml. of dioxane and cooled to 10°. Then 9.3 ml. (0.04 mole) of tri-*n*-butylamine was added and followed by 3.52 ml. (0.04 mole) of ethyl chlorocarbonate. The solution was kept at 10° for 30 minutes. Then a solution of 12.9 g. (0.039 mole) of δ-*p*-toluenesulfonyl-L-ornithine methyl ester hydrochloride (compd. 2) and 8.9 ml. (0.039 mole) of tri-*n*-butylamine in dioxane (10°) was added over a period of 2 minutes, with swirling. The reaction mixture evolved CO_2 and was put in the ice-box overnight. It was washed with dilute HCl, water, 2% sodium bicarbonate and water, then dried over anhydrous sodium sulfate and taken down to an oil *in vacuo*. Addition of ethyl acetate and then ether yielded 16 g. (78%) of needles, m.p. 145°.

7. Z·Val-*p*-Tos·Orn·NH·NH$_2$ (L-L).—16 g. (0.03 mole) of Z·Val-*p*-Tos·Orn·OMe (L-L) (compd. 6) was dissolved in 100 ml. of anhydrous methanol. Four grams (0.08 mole) of hydrazine hydrate was added, the solution refluxed for 45 minutes and then allowed to stand overnight at room temperature. It was cooled in an ice-bath and the crystals of the hydrazide filtered off and washed with cold ethanol; yield 14 g. (88%), m.p. 211°. This material was satisfactory for the next reaction. However, it can be recrystallized from 350 ml. of hot methanol; yield 12.6 g. (80%), m.p. 213°.

8. Z·Leu-Phe·OH (L-D).—14.5 g. (0.055 mole) of carbobenzoxy-L-leucine[23] and 13.2 ml. (0.055 mole) of tri-*n*-butylamine were dissolved in 50 ml. of dioxane and the solution cooled to 10°. 5.24 ml. (0.055 mole) of ethyl chlorocarbonate was added and the solution kept at 10° for 30 minutes. Then a 10° solution of 10.0 g. (0.061 mole) of D-phenylalanine in 30.3 ml. of 2 *N* NaOH and 15 ml. dioxane was added with swirling. There occurred a vigorous CO_2 evolution. The solution was allowed to stand in the ice-box overnight, 150 ml. of H_2O and 30 ml. of 2 *N* NaOH were added and it was then extracted clear with ether. The water layer was acidified with 2 *N* HCl and the resultant oil taken up in ether. The ether layer was washed with water, dried over anhydrous sodium sulfate, and taken down in vacuum to an oil. The oil was dissolved in methanol and made very faintly turbid with water. On standing at room temperature for several days the product crystallized out as needles; yield 14 g. (62%), m.p. 63–65°. This product was satisfactory for the next step. Repeated crystallization brought the melting point to 76°.[24]

9. Z·Leu-Phe·OMe (L-D).—This compound was prepared in the same way as Z·Val-*p*-Tos·Orn·OMe (L-L) (compd. 6). The materials and quantities were 13.5 g. (0.05 mole) of Z·Leu·OH (L), 24.4 ml. (0.10 mole) of tri-*n*-butylamine, 4.75 ml. (0.05 mole) of ethyl chlorocarbonate and 9.0 g. (0.05 mole) of D-phenylalanine methyl ester hydrochloride (compd. 3). The yield of pure product (needles) was 12.5 g. (70%); crystallized from ethyl acetate–petroleum ether, m.p. 112°.

It was also prepared by reaction of diazomethane on compd. 8 in quantitative yield (see prepn. of compd. 6).

10. Z·Leu-Phe·NH·NH$_2$ (L-D).—Nine grams (0.021 mole) of Z·Leu-Phe·OMe (L-D) (compd. 9) was dissolved in a solution of 2.5 g. (0.05 mole) of hydrazine hydrate and 50 ml. of anhydrous methanol. The solution was refluxed for one hour and then allowed to stand overnight at room temperature. Most of the methanol was taken off *in vacuo*, and water was added to precipitate the hydrazide; yield 9 g. (100%). After recrystallization from ethanol–water, m.p. 166°, the crude material was pure enough for the next step.

11. Z·Leu-Phe-Pro·OMe (L-D-L).—11.0 g. (0.026 mole) of Z·Leu-Phe-NH·NH$_2$ (L-D) (compd. 10) was dissolved in a solution containing 88 ml. of glacial acetic acid, 18.9 ml. of 5 *N* HCl and 300 ml. of H_2O. The solution was cooled to 0°, after which 1.89 g. (0.027 mole) of sodium nitrite dissolved in a small amount of water was added in one portion. The precipitated azide was extracted with ice-cold ethyl acetate, washed with ice-cold water, dilute sodium bicarbonate and water and then added to an ethyl acetate solution (cold) of proline methyl ester (previously prepared from 6.6 g. (0.038 mole) of the hydrochloride (compd. 4)). It was kept in the ice-box for 48 hours, washed with dilute HCl, water, dilute sodium bicarbonate and water, dried over anhydrous sodium sulfate and taken down to an oil *in vacuo*. All attempts to crystallize this oil failed. It was finally dried to a glass-like powder[8] and used as such.

12. Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·OMe (L-L-L-D-L).—13.8 g. (0.026 mole) of Z·Val-*p*-Tos·Orn·NH·NH$_2$ (L-L) (compd. 7) was dissolved in 110 ml. of glacial acetic acid, 55 ml. of HCl and 450 ml. of water. The solution was cooled to −5° and 2.1 g. (0.026 mole) of sodium nitrite in a small amount of water added. The azide precipitate was extracted with cold ethyl acetate, washed with cold water, dilute sodium bicarbonate and water, dried over sodium sulfate and then added to a cold ethyl acetate solution of H·Leu-Phe-Pro·OMe (L-D-L) (previously prepared from 11 g. (0.026 mole) of the hydrochloride).[25] After standing in the ice-box for 48 hours, the precipitate was filtered off and washed with ether; yield was 7.3 g. (31.5%); m.p. 160–163°. The filtrate was washed with dilute hydrochloric acid, water, dilute sodium bicarbonate and water. The solvent was removed *in vacuo*, the oil taken up in a small quantity of ethyl acetate and precipitated as a solid with petroleum ether; yield 12.5 g. (54%), m.p. 149–154°. This was combined with the first crude product (total 19.8 g. (85.5%)) and recrystallized twice from 65% ethanol; yield 14.5 g. (63%), m.p. 184°, $[\alpha]^{23}D$ −52.3° (0.936% in glacial acetic acid), $[\alpha]^{25}D$ −54.0° (0.595% in anhydrous methanol).

13. Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·OH (L-L-L-D-L). 2.4 g. (2.7 mmoles) of Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·OMe (L-L-L-D-L) (compd. 12) was dissolved in 50 ml. of anhydrous methanol; 5.4 ml. of *N* NaOH was added and kept at 37°. After 2 hours, another 2 ml. of *N* NaOH was added, followed

(22) R. L. M. Synge, *Biochem. J.*, **42**, 99 (1948).

(23) M. Bergmann and L. Zervas, *Ber.*, **65**, 1192 (1932).

(24) Schumann and Boissonnas (ref. 11) report no crystals or melting point.

(25) J. I. Harris and T. S. Work, *Biochem. J.*, **46**, 196 (1950), reported $[\alpha]^{20}D$ −38.9 (2% in methanol); m.p. 240° for the hydrochloride. We found $[\alpha]^{28}D$ −40.6° in same solvent; m.p. 242–243°. Schumann and Boissonnas[11] report an extremely hygroscopic solid; they report no rotation or m.p.

in one hour by 1 ml. of *N* NaOH (total is 8.4 mmoles). After a total of 4 hours at 37° the addition of a drop of this solution to a large excess of water resulted in a clear solution. The batch was poured into 250 ml. of water and acidified with dilute hydrochloric acid. On standing, the product crystallized out as needles; yield 2.2 g. After recrystallization from acetone–water the yield was 1.5 g. (63%), m.p. 157°. Another 350 mg. was recovered pure from the mother liquor; $[\alpha]^{25}D$ −53.9° (1.002% in glacial acetic acid).

14. **Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·NH·NH$_2$** (L-L-L-D-L). —3.1 g. (3.36 mmoles) of the methyl ester (compd. 12) was dissolved in a solution of 10 ml. of anhydrous methanol and 3 g. (60 mmoles) of hydrazine hydrate, by gentle warming. The solution stood at room temperature for 2 hours and then in the ice-box for 36 hours. The crystalline solid was filtered off and washed with 70% methanol to get rid of excess hydrazine; yield 2.3 g. (75%), m.p. 160–168°. Another 600 mg. was recovered from the mother liquor by addition of water; m.p. 145–170°. Repeated recrystallization of these products from a variety of solvents would not sharpen or raise the melting point. The combined crops were converted into the isopropylidene derivative by boiling for five minutes in 10 ml. of acetone and then allowing to cool. The derivative was recrystallized from ethyl acetate as needles; yield 2.8 g., m.p. 198°.

Anal. Calcd. for $C_{48}H_{66}O_9N_8S$ (933.1): N, 12.08. Found: N, 12.20.

The hydrazide was recovered by dissolving in 15 ml. of anhydrous ethanol, adding excess of ethanol saturated with HCl gas and precipitating with ether. The melting point was still not sharp, being about 145–160°, depending on the rate of heating; yield 2.5 g. (67%), $[\alpha]^{26}D$ −75.11° (0.442% in methanol).

15. **Z·(Val-*p*-Tos·Orn-Leu-Phe-Pro)$_2$·OMe** (L-L-L-D-L)$_2$. —2.7 g. (3.22 mmoles) of compd. 14 was dissolved in 20 ml. of glacial acetic acid, 10 ml. of *N* HCl and 15 ml. of water. It was converted to the azide with 0.22 g. (3.25 mmoles) of $NaNO_2$ in a manner analogous to the procedure for the preparation of Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·OMe (L-L-L-D-L) (compd. 12). The azide was extracted, washed and dried as described above and coupled with H·Val-*p*-Tos·Orn-Leu-Phe-Pro·OMe prepared from 3.0 g. (3.8 mmoles) of the hydrochloride (compd. 17). After standing in the ice-box for 48 hours, the solution was taken down to a volume of about 20 ml. *in vacuo*, the bath temperature never rising above 40°. The precipitate was filtered off; yield 1.0 g. (19%), m.p. 204–208°. The mother liquor was washed with dilute hydrochloric acid, water and dilute bicarbonate, dried and taken down to a semi-solid. Petroleum ether was added and the yellowish material was filtered off; yield 3.0 g. (57%), m.p. 186–194°.

The first crop was recrystallized twice and the mother liquor crop was recrystallized three times from 70% ethanol to yield a total of 2.3 g. (44%) of material, m.p. 209–211°, $[\alpha]^{27}D$ −111.11° (1.089% in anhydrous methanol).

16. **Z·(Val-*p*-Tos-Orn-Leu-Phe-Pro)$_2$·OH** (L-L-L-D-L)$_2$.— 1.2 g. (0.74 mmole) of the carbobenzoxy decapeptide ester (compd. 15) was dissolved in 15 ml. of anhydrous methanol by slight warming. Then, after cooling to room temperature, 1.2 ml. of 2 *N* NaOH was added. The solution was kept at 37° until one drop added to water produced no turbidity (5 hours). A slight precipitate was filtered off, 100 ml. of water was added and 5 ml. of *N* HCl. A precipitate formed; yield 1.12 g. (92%), m.p. 197–213°. Recrystallization three times from 65% ethanol yielded 590 mg. (45%) of product, m.p. 223–224°, $[\alpha]^{27}D$ −103.4° (0.716% in anhydrous methanol).

17. **H·Val-*p*-Tos·Orn-Leu-Phe-Pro·OMe·HCl** (L-L-L-D-L).—8.9 g. (0.01 mole) Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·OMe (compd. 12) was hydrogenated in methanol with 15 ml. of *N* HCl using palladium black as catalyst. When no more CO_2 was evolved, the palladium black was filtered off and the solvent taken off *in vacuo*. The residue was kept in a desiccator over P_2O_5 for 24 hours. The white powder was taken up in absolute ethanol and anhydrous ether added till slightly turbid. After standing several days in the cold, the crystalline product (needles) was filtered off; yield 7.2 g. (91%). It was dried to constant weight at 78° for analysis; $[\alpha]^{25}D$ −48.7° (0.511% in 0.01 *N* HCl).

18. **H·(Val-*p*-Tos·Orn-Leu-Phe-Pro)$_2$·OMe** (L-L-L-D-L)$_2$. —200 mg. (0.13 mmole) of Z·(Val-*p*-Tos·Orn-Leu-Phe-Pro)$_2$·OMe (L-L-L-D-L)$_2$ (compd. 15) was dissolved in 3 ml. of methanol and hydrogenated in the presence of palladium black until no more CO_2 was evolved. The palladium was filtered off and the solution blown down to a small volume. Water was added, resulting in the precipitation of 137 mg. (75%) of a white powder. It was dissolved in 10 ml. of warm 60% EtOH. On standing at room temperature rosettes of needles crystallized out; yield 100 mg. (54%). Another 20 mg. was recovered from the mother liquor; $[\alpha]^{27}D$ −113.7° (0.219% in anhydrous ethanol), m.p. 220–222° (browning).

19. **H·Val-Orn-Leu-Phe-Pro·OH·2HCl** (L-L-L-D-L).— 2.1 g. (2.4 mmole) of Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·OH (L-L-L-D-L) (compd. 13) was suspended in 85° ml. of liquid ammonia. Small quantities of sodium were added with stirring. The compound dissolved and sodium was added until a deep blue color persisted for 30 seconds; 550 mg. (24 mmoles) was required. A very small amount of NH_4Cl was added to remove the blue color and then the NH_3 was allowed to evaporate off. The flask was placed in a desiccator over sulfuric acid overnight. The yellow powder was taken up in 5 ml. of absolute ethanol previously saturated with HCl gas and precipitated with anhydrous ether. The mixture of peptide and salt was dissolved in 25 ml. of anhydrous ethanol, filtered and taken down *in vacuo* to 10 ml. after which more salt was filtered off. Addition of large excess of ether yielded 1.2 g. (76%) of crude peptide. It was dissolved in 10 ml. of water, decolorized with charcoal, 50 ml. of ethanol was added and distilled off *in vacuo*. Repeated additions of absolute ethanol were carried out to remove water. After four such additions and distillations the residue was taken up in 20 ml. of absolute ethanol and anhydrous ether added until turbid. The peptide appeared as long needles and in a short time the solution became a gel; yield was 900 mg. (55%). Recrystallized from alcohol–ether. For analysis the compound was dried to constant weight at 78°. On paper chromatograms the compound gave a single spot with both butanol–water and collidine–water. On hydrolysis and developing with collidine–water[8] there were five spots corresponding to the five amino acids; $[\alpha]^{25.7}$ −62.38° (0.495% in 0.5 *N* HCl).

20. **H·(Val-Orn-Leu-Phe-Pro)$_2$·OH·3HCl** (L-L-L-D-L)$_2$. —575 mg. (0.36 mmole) of the carbobenzoxy decapeptide (compd. 16) was suspended in about 50 ml. of liquid NH_3. Small quantities of sodium were added with stirring until a blue color appeared which persisted for 30 seconds; 150 mg. (6.5 mmoles) was required. A speck of NH_4Cl was added and the ammonia was allowed to evaporate off. The resulting powder was kept overnight in an evacuated desiccator over sulfuric acid.

It was dissolved in 10 ml. of absolute ethanol previously saturated with HCl gas, filtered with the aid of Analytical Grade Celite, which was previously washed with ethanol. It was then blown down to about 1.5 ml. and ether added; yield was 426 mg. It was dissolved in 15 ml. of absolute ethanol, filtered and ether added. The product came down as microscopic needles; yield 311 mg. (68%); m.p. 229° with browning.

It was redissolved in 10 cc. of absolute ethanol and 1 drop of ethanolic HCl, clarified with charcoal, evaporated in a current of air to 2.5 cc. and recrystallized by the slow addition of anhydrous ether; yield was 275 mg. (60%); m.p. 233° with browning, $[\alpha]^{25.7}D$ −89.3° (0.310% in 0.5 *N* HCl).

Product was not completely crystalline; amorphous material could be seen under the microscope. Also about 1.5% ash was present—undoubtedly sodium chloride. Analytical data are not corrected for ash.

Hydrolysis with 6 *N* HCl and paper chromatography[8] revealed 5 spots corresponding to the five amino acids.

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