5,7-Diamino-3-phenyl-v-triazolo(d)pyrimidine (XIV, R = C_6H_5).—Fifteen grams of the 4-anilino-2,5,6-triaminopyrimidine bisulfite (XII) was dissolved in 300 ml. of boiling water. To this solution was added 50 ml. of glacial acetic acid, and the solution was stirred while 10 g. of sodium nitrite in 100 ml. of water was slowly added. The solution was then heated on the steam-bath for 1 hr. and cooled. The resulting precipitate was filtered and washed with water. For analysis the compound was recrystallized from N,N-dimethylformamide to yield 4.5 g. of 5,7-diamino-3-phenyl-v-triazolo(d)pyrimidine (XIV, R = C_6H_5), m.p. >300°. This product was insoluble in aqueous potassium hydroxide solution.

Anal. Calcd. for $C_{10}H_9N_7$: C, 52.5; H, 3.6; N, 43.1. Found: C, 52.8; H, 3.9; N, 43.5.

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Synthesis of Peptides Related to Gramicidin S. III.[1] The Decapeptide Containing L-Lysine Residues in Place of L-Ornithine[2]

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The synthesis of a decapeptide analog of gramicidin S is described. It differs from the latter in being acyclic and containing two L-lysine residues instead of two L-ornithines. It was obtained in crystalline form as were fourteen of the fifteen polypeptide intermediates, adsorption chromatography being utilized for the purification of the higher intermediates.

This paper reports further progress in the synthesis of decapeptide analogs of gramicidin S. The polypeptide described in this paper is the decapeptide H·Val-Lys-Leu-Phe-Pro-Val-Lys-Leu-Phe-Pro·OH·3HCl(L-L-L-D-L)$_2$[3](I).

The structure of gramicidin S and the decapeptides synthesized to date are shown in Fig. 1; gramicidin S is a cyclic decapeptide containing a repeated sequence of five amino acid residues. Two of the component amino acid residues, L-ornithine and D-phenylalanine, are not frequently encountered in naturally occurring polypeptides and appear rarely, if at all, in proteins. It is the purpose of this program to ascertain which parts of the chemical structure of gramicidin S are responsible for its antibiotic activity. This objective is being pursued by means of the synthesis and antibacterial assay of various decapeptide analogs.

It was reported earlier[4] that decapeptide (II)[1] possessed antibacterial activity, although it was less potent than gramicidin S. It was proposed that its lower activity might be the result of its greater susceptibility to bacterial hydrolytic enzymes because of its acyclic structure. The suggestion was made that the cyclic structure of gramicidin S, though not necessary for its antimicrobial activity, prevents destruction of the peptide by the microörganism. Antibacterial studies of decapeptides I, II and III and several to be prepared will test this hypothesis and perhaps establish the chemical structure responsible for the bactericidal properties of gramicidin S.

It should be noted here that Schwyzer and Sieber[5] have recently synthesized gramicidin S, utilizing a pentapeptide intermediate described in paper I[1] of this series.

The synthetic methods used to prepare the decapeptide I are described in Fig. 2. As emphasized in previous papers, choice of synthetic techniques was governed by the necessity of preventing diastereoisomer formation. For this reason, the azide route was employed in all cases where acylated peptides served as intermediates. Fourteen of the fifteen compounds were obtained in crystalline form, a positive demonstration of the efficacy of the azide method for the preparation of complex polypeptides.

Val-Orn-Leu-Phe-Pro-Val-Orn-Leu-Phe-Pro
(L-L-L-D-L)$_2$ Gramicidin S

H·Val-Lys-Leu-Phe-Pro-Val-Lys-Leu-Phe-Pro·OH
(L-L-L-D-L)$_2$ (I)

H·Val-Orn-Leu-Phe·Pro-Val-Orn-Leu-Phe-Pro·OH
(L-L-L-D-L) (II)

H·Val-Orn-Leu-Tyr-Pro-Val-Orn-Leu-Tyr-Pro·OH
(L-L-L-D-L) (III)

Fig. 1.—Synthetic peptides.

As in the preparation of decapeptide III (ref. 1, paper II), it was necessary to perform the synthesis by the reaction of a tetrapeptide, Z·Val-*p*-Tos·Lys-Leu-Phe·NH·NH$_2$(L-L-L-D) with a hexapeptide, H·Pro-Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe(L-L-L-L-D-L). Decapeptide II (ref. 1, paper I) was synthesized by the reaction of two pentapeptide derivatives, but this scheme was not feasible here because Z·Val-*p*-Tos·Lys-Leu-Phe-Pro·NH·NH$_2$(L-L-L-D-L) could not be obtained in pure crystalline form.

The pentapeptide, H·Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe·HCl (L-L-L-D-L)(compd. 11) was found to crystallize in two forms, as needles and as rhombohedra, depending upon the quantity of methanol in the recrystallizing solvent.

(1) Paper I: B. F. Erlanger, H. Sachs and E. Brand, THIS JOURNAL, **76**, 1806 (1954); paper II: B. F. Erlanger, W. V. Curran and N. Kokowsky, *ibid.*, **80**, 1128 (1958).

(2) This research is supported by the Office of Naval Research under contract N-onr-266(44). A preliminary account appears in the Abstracts of the 133rd American Chemical Society meeting, San Francisco, Calif., April, 1958, p. 27-C.

(3) For an explanation of the abbreviations, see papers I and II (ref. 1). Briefly: Z, carbobenzyloxy, $C_6H_5CH_2OCO$; *p*-Tos, *p*-toluenesulfonyl, $C_7H_7SO_2$; Leu, leucyl, NH(CHC$_4$H$_9$)CO; Val, valyl, NH-(CHC$_3$H$_7$)CO; etc. The configurations of the amino acid residues appear in parentheses after the name of the compound.

(4) B. F. Erlanger and L. Goode, *Nature*, **174**, 840 (1954).

(5) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).

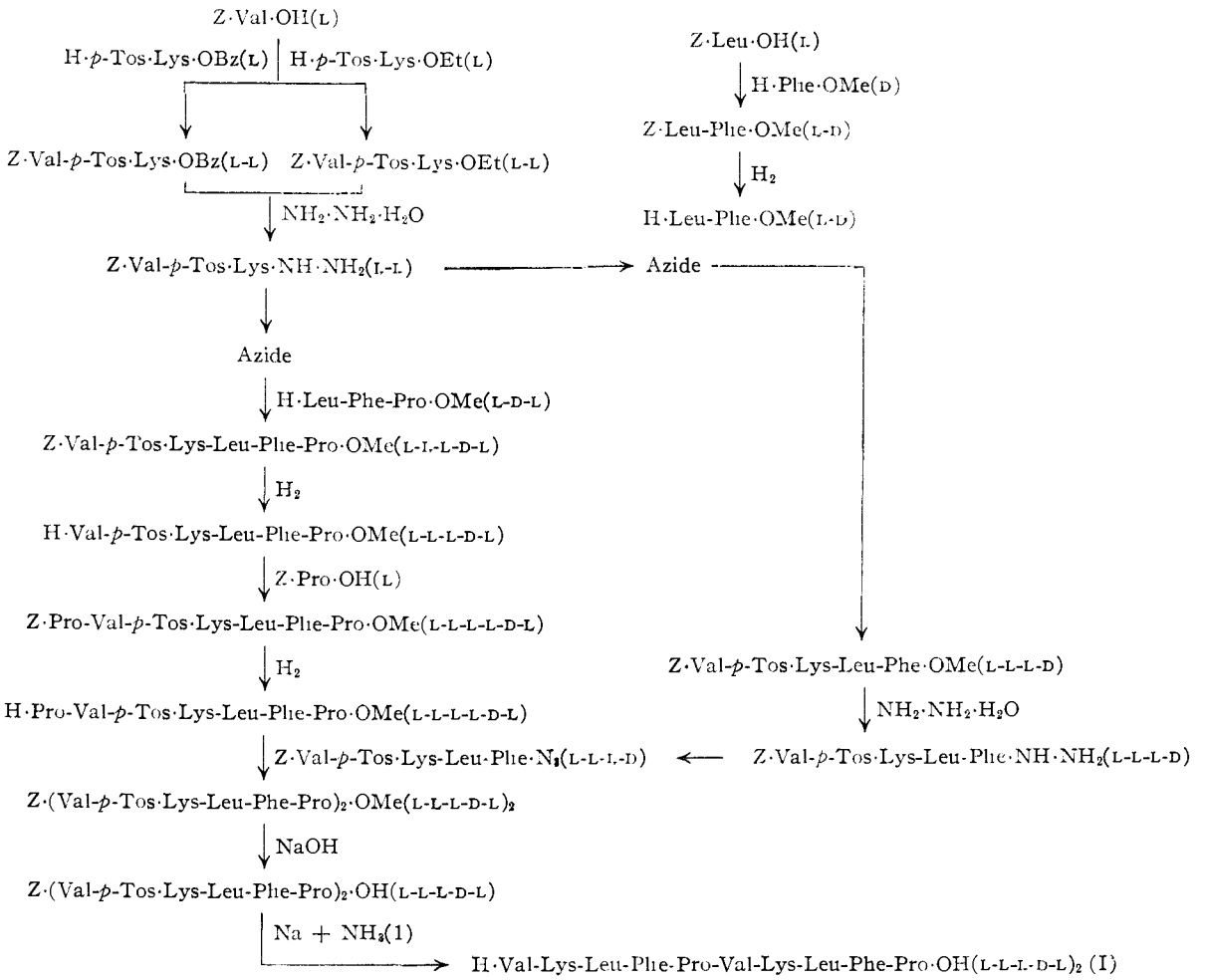

Fig. 2.

Adsorption chromatography on Florisil was utilized for the purification of the decapeptide Z·(Val-*p*-Tos·Lys-Leu-Phe-Pro)$_2$·OMe(L-L-L-D-L)$_2$ (compd. 14) and its saponified product (compd. 15). The latter was converted to the final product by reduction by sodium in liquid ammonia.[6] Excess sodium metal was decomposed by the addition of solid NH_4Cl. After removal of the liquid ammonia the residue was dissolved in water, made acid with hydrochloric acid and then passed through a column of IR100A in the acid form. The decapeptide passed through free of cations and evaporation of the eluate resulted in a crystalline product. Recrystallization could be effected by dissolving it (with warming) in water and then subsequent evaporation of the solvent.

The decapeptide was characterized by hydrolysis and by dinitrophenylation studies.

Experimental[7]

1. ε-*p*-Toluenesulfonyl-L-lysine.—Copper hydroxide was prepared by mixing a cold solution of 10.2 g. (0.041 mole) of copper sulfate pentahydrate with 41 ml. (0.082 mole) of cold 2 *N* sodium hydroxide solution. The gelatinous precipitate was collected by centrifugation, washed several times with cold water and added to a solution of 12.3 g. (0.067 mole) of L-lysine monohydrochloride in 34 ml. (0.068 mole) of 2 *N* sodium hydroxide. To this solution was added, with vigorous stirring, 17.2 g. (0.090 mole) of *p*-toluenesulfonyl chloride in 100 ml. of ether followed by a dropwise addition of 40 ml. (0.080 mole) of 2 *N* sodium hydroxide over a period of 45 minutes. Stirring was continued for an additional 3 hr., after which the chelate was filtered and washed with water, alcohol and ether. The light blue product was dissolved in 100 ml. of 2 *N* hydrochloric acid and treated with hydrogen sulfide to precipitate the copper as the sulfide. The copper sulfide was removed by filtration, using celite and the filtrate adjusted to *p*H 6 with pyridine to precipitate ε-*p*-toluenesulfonyl-L-lysine, which was collected after standing overnight in the refrigerator; yield 14.0 g. (70%). Recrystallization from 5% acetic acid gave 11.9 g. (59%), m.p. 234–237° dec.[8] $[\alpha]^{25}D$ +16.4° (2% in 6 *N* hydrochloric acid). Calcd. for $C_{13}H_{20}O_4N_2S$ (300.4): N, 9.4. Found: N, 9.5.

2. ε-*p*-Toluenesulfonyl-L-lysine Benzyl Ester Hydrochloride.—5.0 g. (0.0167 mole) of ε-*p*-toluenesulfonyl-L-lysine (compd. 1) was dissolved in 25 ml. of benzyl alcohol containing 7.5 g. of polyphosphoric acid.[9] The solution was stirred for 4 hr. in an oil-bath at 95°. After cooling, 165 ml. of anhydrous ether was added and dry hydrogen chloride passed in until the solution was saturated. The precipitated ester hydrochloride, after standing in the refrigerator overnight, was filtered and washed with dry ether; yield 6.48 g. (90.8%), m.p. 172–174°. The crude product was recrystallized from methanol–ether to give 5.3 g. (75%), $[\alpha]^{25}D$ −7.5 (1% in 0.1 *N* HCl) (calcd. as free base). *Anal.*

(6) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

(7) Compounds are numbered to correspond with numbering in Table I.

(8) R. Roeske, F. H. C. Stewart, R. J. Stedman and V. du Vigneaud, THIS JOURNAL, **78**, 5883 (1956), report m.p. 237–238° dec., $[\alpha]^{21}D$ +13.6 (*c* 3, 2 *N* HCl).

(9) *Cf.* B. F. Erlanger and R. M. Hall, *ibid.*, **76**, 5781 (1954).

TABLE I

PEPTIDE DERIVATIVES

No.	Compound	Mol. formula	Mol. wt.	M.p., °C.	Nitrogen, % Calcd.	Nitrogen, % Found	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
4	Z·Val-*p*-Tos·Lys·OBz(L-L)	$C_{33}H_{42}O_7N_4S$	624.8	124–125.5	6.9	7.0				
5	Z·Val-*p*-Tos·Lys·OEt(L-L)	$C_{28}H_{38}O_7N_4S$	561.7	139–140	7.5	7.4				
6	Z·Val-*p*-Tos·Lys·NH·NH_2(L-L)	$C_{26}H_{37}O_6N_5S$	457.6	211–211.5	12.9	12.9				
7	Z·Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe (L-L-L-D-L)	$C_{47}H_{64}O_{11}N_6S$	905.1	219–221.5	9.3	9.2	62.3	62.4	7.1	7.2
8	H·Leu-Phe·OMe·HCl(L-D)	$C_{16}H_{25}O_3N_2Cl$	328.8	217–219	8.5	8.4				
9	Z·Val-*p*-Tos·Lys-Leu-Phe·OMe(L-L-L-D)	$C_{42}H_{57}O_9N_5S$	808.0	218–220	8.5	8.7	62.5	62.3	7.1	7.0
10	Z·Val-*p*-Tos·Lys-Leu-Phe·NH·NH_2(L-L-L-D)	$C_{41}H_{57}O_8N_7S$	808.0	235–238 (dec.)	12.1	12.1	61.0	61.1	7.1	7.0
11	H·Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe·HCl (L-L-L-D-L)	$C_{39}H_{59}O_8N_6SCl$	807.4	155–157	10.4	10.4	58.0	57.9	7.4	7.7
12	Z·Pro-Val-*p*-Tos·Lys-Leu-Phe-1-Pro·OMe (L-L-L-L-D-L)	$C_{52}H_{71}O_{11}N_7S$	1002.2	157–159	9.8	10.0	62.3	62.0	7.1	7.1
13	H·Pro-Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe·HCl (L-L-L-L-D-L)	$C_{44}H_{68}O_7N_7SCl$	904.5	167–169.5	10.8	10.7	58.4	57.9	7.5	7.4
14	Z(Val-*p*-Tos·Lys-Leu-Phe-1-Pro)$_2$·OMe (L-L-L-D-L)$_2$	$C_{86}H_{118}O_{17}N_{12}S_2$	1644.0	194–196	10.4	10.6	62.0	61.5	7.1	7.2

Calcd. for $C_{20}H_{27}O_4N_2SCl$ (427.0): N, 6.7. Found: N, 6.6.

3. **ε-*p*-Toluenesulfonyl-L-lysine Ethyl Ester Hydrochloride.**—18.7 g. (0.0623 mole) of ε-*p*-toluenesulfonyl-L-lysine (compd. 1) was suspended in 650 ml. of absolute ethanol and cooled in an ice-salt bath. Anhydrous hydrogen chloride was passed in until solution was complete (20–30 minutes). The flask was stoppered and stored in the cold for 2 hr., after which the solvent was removed *in vacuo*, the resulting oil dissolved in 600 ml. of absolute ethanol and retreated with hydrogen chloride. The solution, after being at 4° for 48 hr. was concentrated *in vacuo* to yield a crystalline solid. Recrystallization from ethanol–ether gave 20.4 g. (90%), m.p. 136–137.5°, $[\alpha]^{22}D$ +10.1 (2% in 6 *N* hydrochloric acid). Calcd. for $C_{15}H_{25}O_4N_2SCl$ (364.9): N, 7.8. Found: N, 7.8.

4. **Z·Val-*p*-Tos·Lys·OBz(L-L).**—10.3 g. (0.041 mole) of carbobenzoxy-L-valine[10] was dissolved in 50 ml. of dioxane containing 9.75 ml. (0.41 mole) of tri-*n*-butylamine and the solution cooled to 10°; 3.9 ml. (0.041 mole) of ethyl chlorocarbonate was added slowly with swirling, after which the reaction mixture was allowed to stand at 12° for 30 minutes. Then a precooled solution of 17.5 g. (0.041 mole) of ε-*p*-toluenesulfonyl-L-lysine benzyl ester hydrochloride (compd. 2) and 9.75 ml. (0.041 mole) of tri-*n*-butylamine in 50 ml. of dioxane was added with stirring. The stirring was continued for 1 hr., the solution left overnight at room temperature and then poured into three volumes of water. The precipitated dipeptide derivative was extracted with ethyl acetate, the ethyl acetate solution washed with dilute hydrochloric acid, water, dilute sodium bicarbonate and water, and then dried over magnesium sulfate. The compound, obtained as an oil by removal of the solvent *in vacuo*, was crystallized from ethyl acetate–petroleum ether; yield 18.8 g. (73.5%), $[\alpha]^{20}D$ −27.1 (1% in methanol).

5. **Z·Val-*p*-Tos·Lys·OEt(L-L).**—This compound was prepared in the same manner as the corresponding benzyl ester (compd. 4), except that isobutyl chlorocarbonate was used in the preparation of the mixed anhydride. The yield from 20 g. (0.0548 mole) of the ε-*p*-toluenesulfonyl-L-lysine ethyl ester hydrochloride (compd. 3) was 24 g. (84%), $[\alpha]^{20}D$ −22.6 (1% in methanol).

6. **Z·Val-*p*-Tos·Lys·NH·NH_2(L-L).**—18.5 g. (0.03 mole) of Z·Val-*p*-Tos·Lys·OBz (compd. 4) was dissolved in 100 ml. of methanol, 3.7 g. (0.074 mole) of hydrazine hydrate added and the solution refluxed for 1 hr. The hydrazide crystallized out on cooling and after standing overnight in the refrigerator, it was filtered and washed with cold methanol; yield 14.3 g. (88%), $[\alpha]^{20}D$ −18.5 (1% in glacial acetic acid).

This compound was also prepared from the ethyl ester (compd. 5) by the same procedure. 12.0 g. (0.0213 mole) of the ethyl ester gave 10.6 g. (92%) of the hydrazide, m.p. 210–213°.

7. **Z·Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe(L-L-L-D-L).**—9.1 g. (0.0166 mole) of Z·Val-*p*-Tos·Lys·NH·NH_2(L-L) (compd. 6) was dissolved in a solution containing 86 ml. of glacial acetic acid, 19 ml. of 2 *N* hydrochloric acid and 250 ml. of water. After cooling to 0°, 1.2 g. (0.0174 mole) of sodium nitrite was added. The precipitated azide was extracted into cold ethyl acetate and washed with cold water, dilute sodium bicarbonate and again with water. The solution was dried over sodium sulfate in the cold and added to a cold ethyl acetate solution of H·Leu-Phe-Pro·OMe(L-D-L)[11] previously prepared from 7.1 g. (0.0166 mole) of the hydrochloride. After standing 18 hr. in the refrigerator and then 24 hr. at room temperature, the precipitate was filtered off and dried *in vacuo* to give 11.9 g. (79%) of a product melting at 207–211°. Recrystallization from aqueous ethanol gave 11.4 g. (76%), m.p. 219–221.5°, $[\alpha]^{25}D$ −54.5 (0.5% in methanol).

8. **H·Leu-Phe·OMe·HCl(L-D).**—5.0 g. (0.0107 mole) of Z·Leu-Phe·OMe(L-D) (ref. 1, paper I) was dissolved in 150 ml. of ethanol containing 5.4 ml. (0.0108 mole) of 2 *N* hydrochloric acid and hydrogenated over activated palladium until carbon dioxide evolution ceased. The palladium was filtered off and the solvents removed *in vacuo* to yield an oil which crystallized as needles from methanol–ether; yield 2.7 g. (77%), m.p. 217–219° dec., $[\alpha]^{23}D$ −43.8 (2% in methanol).

9. **Z·Val-*p*-Tos·Lys-Leu-Phe·OMe(L-L-L-D).**—9.6 g. (0.0175 mole) of Z·Val-*p*-Tos·Lys·NH·NH_2(L-L) (compd. 6) was dissolved in 80 ml. of 1 *N* hydrochloric acid and 295 ml. of water. After cooling to 0°, 1.25 g. (0.0180 mole) of sodium nitrite was added and the precipitated azide extracted, washed and dried in the same manner as described in the preparation of Z·Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe(L-L-L-D-L) (compd. 7). To the azide solution was added a cold ethyl acetate solution of H·Leu-Phe·OMe(L-D) prepared from 5.75 g. (0.0175 mole) of the hydrochloride (compd. 8). The solution was allowed to stand overnight in the refrigerator, followed by 4 hr. at room temperature, after which it was cooled again and the product recovered by filtration. The yield was 11.1 g. (81%), $[\alpha]^{20}D$ −32.0 (1% in methanol).

10. **Z·Val-*p*-Tos·Lys-Leu-Phe·NH·NH_2(L-L-L-D).**—11.0 g. (0.0136 mole) of Z·Val-*p*-Tos·Lys-Leu-Phe·OMe(L-L-L-D). (compd. 9) was suspended in 165 ml. of absolute alcohol containing 4.5 ml. (0.090 mole) of hydrazine hydrate. The mixture was refluxed for 7 hr. Most of the ester dissolves after *one hour* and *hydrazide* then starts to precipitate. After standing overnight in the refrigerator, the precipitate was collected and washed with cold ethanol and ether; yield 8.79 g. (80%), m.p. 220–224°, $[\alpha]^{25}D$ −31.6° (1% in glacial acetic acid). It was recrystallized from dimethylformamide–water.

11. **H·Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe·HCl(L-L-L-D-L).**—8.24 g. (9.1 mmoles) of Z·Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe(L-L-L-D-L) (compd. 7) was dissolved in 70 ml. of 80% acetic acid containing 9.1 ml. (9.1 mmoles) of 1 *N* hydrochloric acid. After addition of activated palladium, hydrogen was passed through the solution until carbon dioxide evolution ceased. The catalyst was filtered off and the solvents removed *in vacuo*. The resulting glass-like solid was crystallized by slow cooling of a solution containing 100 ml. of ethyl acetate and 5 ml. of methanol; yield 5.2 g. (70%), $[\alpha]^{25}D$ −46.6° (0.5% in methanol).

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

(11) J. I. Harris and T. S. Work, *ibid.*, **46**, 196 (1950); also ref. 1, paper I.

The product was found to be capable of crystallizing as needles or rhombohedra, depending, apparently, upon the quantity of methanol in the solvent.

12. **Z·Pro-Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe**(L-L-L-L-D-L).—1.96 g. (7.87 mmoles) of Z·Pro·OH(L)[12] was dissolved in 15 ml. of tetrahydrofuran containing 1.8 ml. (7.55 mmoles) of tri-*n*-butylamine. The solution was cooled to 0° and 0.99 ml. (7.55 mmoles) of isobutyl chlorocarbonate was added slowly with swirling. The solution was allowed to stand for 1 hr. at 0°. During this time the free ester was prepared by dissolving 5.3 g. (6.56 mmoles) of H·Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe·HCl (compd. 11) in a small amount of water and adding two equivalents of sodium bicarbonate, causing the precipitation of the free ester, which was extracted into ethyl acetate. After washing with water several times, the ethyl acetate solution was evaporated *in vacuo* and the residue dried by twice dissolving in 25 ml. of dry benzene and removing the solvent *in vacuo*. It was then dissolved in 50 ml. of tetrahydrofuran, cooled to 0° and added to the mixed anhydride. After standing overnight in the refrigerator the solution was concentrated *in vacuo* and added to 5 volumes of water. The precipitated peptide derivative was extracted with ethyl acetate, which was then washed with dilute hydrochloric acid, dilute sodium bicarbonate and water. The solution was dried over magnesium sulfate and the ethyl acetate replaced by acetone. Water was added to the point of turbidity and after three days at room temperature, the compound crystallized as needles; yield 5.09 g., m.p. 149–156°. The product was recrystallized from acetone–water to give 3.9 g. (60%), m.p. 157–159°, $[\alpha]^{25}D$ −76.3 (0.5% in methanol).

13. **H·Pro-Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe·HCl**(L-L-L-L-D-L).—4.8 g. (4.79 mmoles) of Z·Pro-Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe(L-L-L-L-D-L) (compd. 12) was dissolved in 150 ml. of methanol and 5.5 ml. (5.5 mmoles) of 1 *N* hydrochloric acid and hydrogenated over activated palladium until one drop of the solution added to several ml. of water produced no turbidity. After filtering the palladium, the solvents were removed *in vacuo*. The oily ester hydrochloride was repeatedly dissolved in absolute ethanol which was evaporated *in vacuo*. Finally, the product was taken up in methanol and ether added until the solution became slightly cloudy. The compound crystallized after standing overnight at room temperature. Recrystallization from the same solvents gave 2.71 g. (63%), $[\alpha]^{23}D$ −78.5 (0.5% in 0.01 *N* hydrochloric acid).

14. **Z·(Val-*p*-Tos·Lys-Leu-Phe-Pro)$_2$OMe**(L-L-L-D-L)$_2$.—2.25 g. (2.76 mmoles) of Z·Val-*p*-Tos·Lys-Leu-Phe·NH·NH$_2$(L-L-L-D) (compd. 10) was dissolved in 1.6 ml. of 4 *N* hydrochloric acid, 28 ml. of glacial acetic acid and 10 ml. of water. The solution was cooled to −2°, and 0.23 g. (3.31 mmoles) of sodium nitrite dissolved in 10 ml. of water was added dropwise with efficient stirring. After addition of the sodium nitrite the solution was stirred for several minutes, and 60 ml. of cold water was added to ensure complete precipitation of the azide. The mixture was extracted with cold ethyl acetate washed with cold water, dilute sodium bicarbonate and water and then dried over magnesium sulfate in the cold. To this was added a cold dry solution of H·Pro-Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe(L-L-L-L-D-L) prepared from 2.5 g. (2.76 mmoles) of the hydrochloride (compd. 13) by the same procedure described for the preparation of Z·Pro-Val-*p*-Tos·Lys-Leu-Phe-Pro·OMe(L-L-L-L-D-L) (compd. 12). After mixing the solution was allowed to stand at room temperature overnight. The solution was then kept at room temperature for 24 hr. and the precipitate collected after chilling in the refrigerator for 4 hr.; yield 5.22 g. (60%), m.p. 163–179° dec. (sinters at 151°). Repeated precipitation by the addition of petroleum ether to an ethyl acetate solution resulted in 3.9 g. of amorphous material with a wide melting range.

1.52 g. of the above material was applied as a chloroform solution to a column of 90 g. of Florisil (60–100 mesh) which was wet with chloroform. After a preliminary elution with 1100 ml. of 0.5% methanol in chloroform and 300 ml. of 1% methanol in chloroform, fractions were eluted with 700 ml. of 1% methanol in chloroform and 1600 ml. of 1.5% methanol in chloroform. These fractions were combined and taken to dryness to yield about 500 mg. of material, m.p. 179–185°, $[\alpha]^{20}D$ −85 (2% in methanol).

This material was rechromatographed on 30 g. of Florisil. Preliminary elution with 450 ml. of 0.5% methanol in chloroform yielded about 10 mg. of a colored resinous product. Then elution was carried out using 425 ml. of 1% methanol in chloroform, 980 ml. of 1.5% methanol in chloroform and 560 ml. of 2% methanol in chloroform, yielding a combined product with m.p. 188–192°. This product was crystallized from 70% ethanol, to yield 325 mg., m.p. 194–196°, $[\alpha]^{25}D$ −110 (2% in methanol).

15. **Z·(Val-*p*-Tos·Lys-Leu-Phe-Pro)$_2$·OH**(L-L-L-D-L)$_2$.—A solution of 300 mg. (0.18 mmole) of Z·(Val-*p*-Tos·Lys-Leu-Phe-Pro)$_2$·OMe(L-L-L-D-L) (compd. 14) in 4 ml. of methanol containing 0.4 ml. of 1.85 *N* NaOH was allowed to stand 3.5 hr. at 37°. The solution was then acidified and concentrated *in vacuo* to an oil which was dissolved in ethyl acetate. The ethyl acetate solution was washed with water, dried over anhydrous sodium sulfate and concentrated *in vacuo* to a glass-like solid; yield 210 mg.

One hundred and fifty mg. of this material was chromatographed on 9 g. of Florisil (60–100 mesh), and the fraction eluted by 25% methanol in chloroform was collected and evaporated to dryness; yield 96 mg.; neut. equiv., calcd. 1629; found, 1685; $[\alpha]^{25}D$ −96.5 (0.3% in methanol).

16. **H·Val-Lys-Leu-Phe-Pro-Val-Lys-Leu-Phe-Pro·OH·3HCl**(L-L-L-D-L)$_2$.—49.8 mg. (0.03 mmole) of Z·(Val-*p*-Tos·Lys-Leu-Phe-Pro)$_2$·OH(L-L-L-D-L)$_2$ (compd. 15) was reduced in liq. NH_3 by the addition of 30 mg. of sodium. (This was the quantity of sodium required to effect a permanent blue solution). A small quantity of NH_4Cl was added to decompose excess sodium and the NH_3 was allowed to evaporate off. After being in the desiccator over phosphorus pentoxide for 24 hr. the residue was dissolved in 3 ml. of water plus 3 drops of 2 *N* HCl and passed through a column of 2 g. of IR 100 A in the acid form. NH_4^+ and Na^+ were picked up by the column while the peptide passed through. The column was washed with an additional 5 ml. of water and all washings were combined and taken to dryness in a desiccator in the presence of P_2O_5. The residue was redissolved in 1 ml. of water (slight warming was necessary) and allowed to evaporate slowly in a desiccator over P_2O_5. Crystals appeared. They were separated from the supernatant after being chilled for several days in the refrigerator; yield 20 mg. (60%).

The compound was characterized as follows.

(a) A fraction of a milligram was deposited on a sheet of Parafilm. One drop of 6 *N* HCl was added and the compound brought into solution by mixing with a stirring rod drawn to a fine tip. The drop of solution was drawn up into a melting point capillary which was then sealed at both ends and kept at 110° for 18 hr. The contents of the capillary was deposited as a drop on a watch glass and dried *in vacuo* in a desiccator over P_2O_5, then redissolved in water and transferred to Whatman #1 filter paper and chromatographed, using butanol–acetic acid–water, 4:1:5, as the developing solvent. The amino acids were then developed with 0.4% ninhydrin in acetone and determined quantitatively by the method of Giri.[13] Leucine, phenylalanine, valine, proline and lysine were found in approximately equimolar quantities.

(b) Two mg. of decapeptide was dinitrophenylated by the method of Sanger.[14] Subsequent hydrolysis in 6 *N* HCl was followed by extraction with ether. The ether phase was evaporated to dryness and chromatographed on Whatman #1 paper, using benzene: 1% acetic acid, 1:1[15] as the developing solvent. The resulting DNP amino acid had the same R_f as authentic DNP-valine (R_f 0.6).

Chromatography of the aqueous fraction (after evaporation to dryness and redissolving the residue in water) on paper, using butanol–acetic acid–water 4:1:5, revealed the presence of ε-DNP-lysine. Treatment with ninhydrin and quantitative analysis as above[13] showed leucine, phenylalanine, valine and proline in ratios of approximately 1:1:0.5:1 (±10%). Lysine was absent.

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