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

Synthesis of Peptides Related to Gramicidin S. II.[1] The Decapeptide Containing D-Tyrosine Residues in Place of D-Phenylalanine[2]

BY BERNARD F. ERLANGER, WILLIAM V. CURRAN AND NICHOLAS KOKOWSKY

RECEIVED SEPTEMBER 30, 1957

The synthesis of a decapeptide analog of gramicidin S is described. It differs from the latter in being acyclic and containing two D-tyrosine residues instead of two D-phenylananines.

As part of a study of the antibacterial activity of synthetic decapeptides related to gramicidin S,[3]

H·Val-Orn-Leu-Tyr-Pro-Val-Orn-Leu-Tyr-Pro·OH·3HCl
(L-L-L-D-L)$_2$(I)[4]

was synthesized. This decapeptide differs from gramicidin S in two respects: (a) it is acyclic and (b) it contains D-tyrosine residues in place of the D-phenylanaine residues of gramicidin S. The crystalline decapeptide described in paper I of this series,[1] H·Val-Orn-Leu-Phe-Pro-Val-Orn-Leu-Phe-Pro·OH·3HCl (L-L-L-D-L)$_2$ (II) differs from gramicidin S only with respect to its acyclic nature. These interrelationships are illustrated in Fig. 1.

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

H·Val-Orn-Leu-Tyr-Pro-Val-Orn-Leu-Tyr-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)$_2$ II

Fig. 1.—Synthetic decapeptides.

The methods used to synthesize decapeptide I were chosen with the purpose of avoiding the formation of mixtures of diastereoisomers. Thus the azide route was employed when acylated dipeptides (or acylated derivatives of higher peptides) served as intermediates.[5] An outline of the methods and techniques used appears in Fig. 2.

The over-all synthetic scheme differs from the one used to synthesize decapeptide II with respect to the polypeptide derivatives which were linked to produce the desired decapeptide. Decapeptide II was formed from two pentapeptide derivatives: Z·Val-*p*-Tos·Orn-Leu-Phe-Pro·NH·NH$_2$ (L-L-L-D-L) and H·Val-*p*-Tos·Orn-Leu-Phe-Pro·OMe (L-L-L-D-L). On the other hand, decapeptide I was the product of the reaction between a tetrapeptide derivative and a hexapeptide derivative: Z·Val-*p*-Tos·Orn-Leu-Tyr·NH·NH$_2$ (L-L-L-D) and H·Pro-Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe (L-L-L-L-D-L). This modification was necessary because the pentapeptide hydrazide, Z·Val-*p*-Tos·Orn-Leu-Tyr·Pro·NH·NH$_2$ (L-L-L-D-L), could not be prepared in a sufficiently pure state.

The condensation of the tetra- and hexapeptide derivatives yielded a product which could not be crystallized despite many attempts using a number of solvent systems. This is not an uncommon experience when high molecular weight polypeptides are being synthesized. However, since we had been fortunate enough to have obtained crystalline, sharp melting decapeptide derivatives as intermediates for the synthesis of II,[1] it was decided to investigate the possibility that the product was impure. Consequently, an attempt was made to purify the amorphous preparation by means of adsorption chromatography on alumina and Florisil. A product was obtained which analyzed correctly and could be crystallized. Since this purified material comprised only about 30% by weight of the total crude product, it is quite apparent that utilization of the latter, without further purification, for subsequent synthetic steps would not have been a feasible procedure. It is suggested that the use of adsorption chromatography for the purification of peptide intermediates could have aided in the crystallization of a number of those derivatives previously obtained (by this and other laboratories) as amorphous solids. Our experience, in addition, emphasizes the risks inherent in the utilization of non-crystallizable intermediates in peptide synthesis.

Adsorption chromatography also was used to purify the saponified decapeptide derivative. The free decapeptide was prepared from the latter by removal of the carbobenzyloxy and *p*-toluenesulfonyl groups with sodium and liquid ammonia.[6] It was separated from inorganic salts by isolation of the picrate derivative and subsequent decomposition of the latter by acid. Characterization was by dinitrophenylation and hydrolysis studies.

Assay for antibacterial activity will be carried out when the synthesis of several other decapeptides is completed.

Experimental[7]

1. **H·Tyr·OBz·HCl (D).**—This compound was prepared by the method of Erlanger and Hall.[8] The yield from 14 g. (0.077 mole) of D-tyrosine was 14 g. (60%), m.p. 200°, $[\alpha]^{25}D$ +20.8 (0.5% in 0.1 *N* hydrochloric acid, calcd. as free base). Reference 8 reports m.p. 205°, $[\alpha]^{25}D$ −23.3 for the L-isomer.

2. **Z·Leu-Tyr·OEt (L-D).**—18.8 g. (0.071 mole) of Z·Leu·OH (L)[9] was dissolved in 100 ml. of tetrahydrofuran contain-

(1) For paper I, see: B. F. Erlanger, H. Sachs and E. Brand, THIS JOURNAL, **76**, 1806 (1954).

(2) This work is supported by the Office of Naval Research under contract N-onr-266(44). A preliminary account appears in the Abstracts of the 131st American Chemical Society meeting, Miami, Fla., April, 1957, p. 18-C.

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

(4) For explanation of the abbreviations, see B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 3508 (1951), and paper I of this series (ref. 1). Briefly: Z, carbobenzyloxy, $C_6H_5CH_2OCO$; *p*-Tos, *p*-toluenesulfonyl, $C_7H_7SO_2$; Leu, NH·(CHC$_4$H$_9$)CO; Val, NH·(CHC$_3$H$_7$)CO; etc. The configurations of the amino acid residues appear in parentheses after the name of the compound.

(5) For a discussion of diastereoisomer formation, see H. D. Springall and H. D. Law, *Quart. Rev.*, **10**, 230 (1956).

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

(7) The compounds are numbered to correspond with the numbering in Table I.

(8) B. F. Erlanger and R. M. Hall, THIS JOURNAL, **76**, 5781 (1954).

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

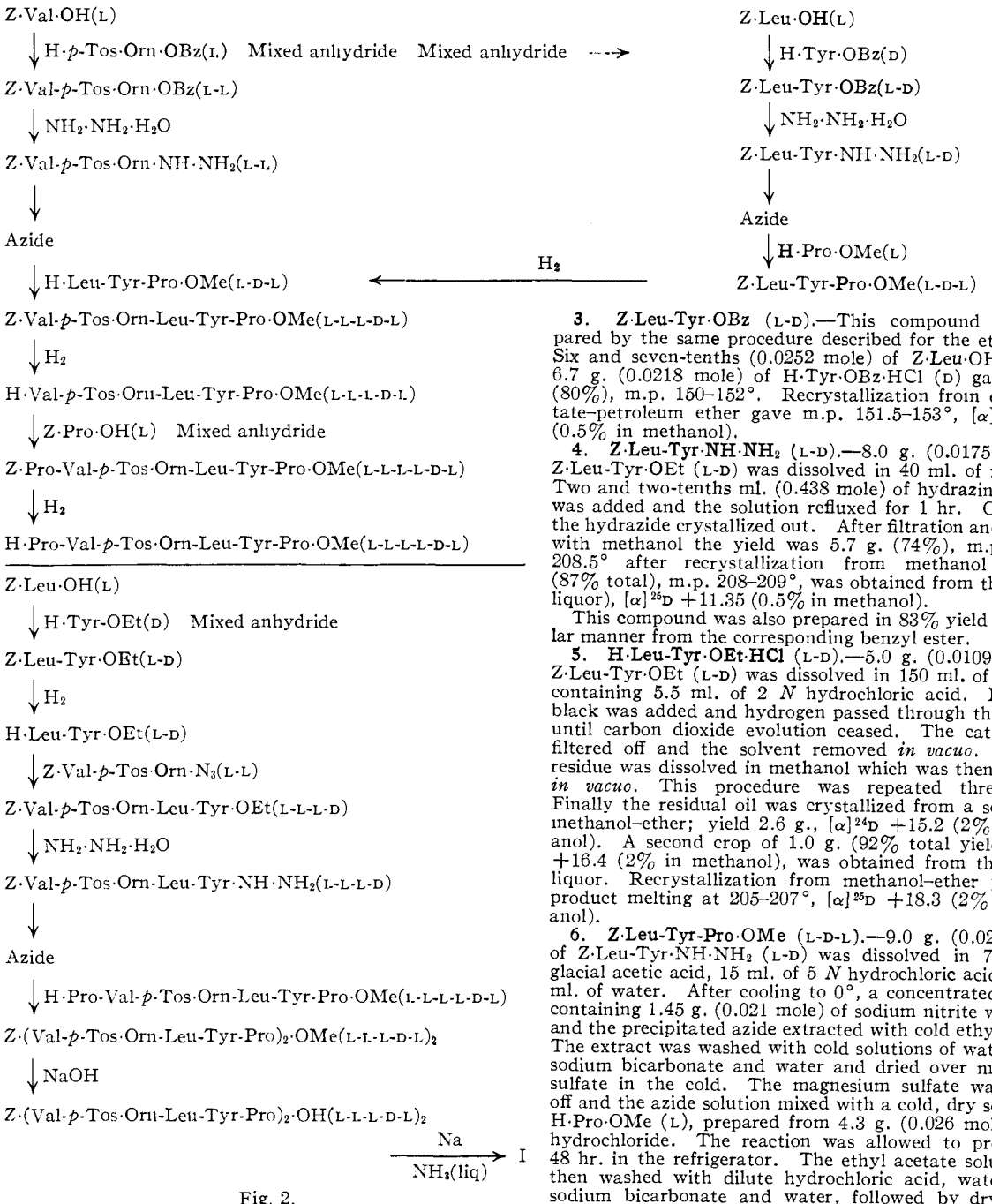

Fig. 2.

ing 17 ml. (0.071 mole) of tri-*n*-butylamine and the solution cooled to 0°. Nine and two-tenths ml. (0.071 mole) of isobutyl chlorocarbonate was added slowly with swirling, and the solution was allowed to stand for 30 minutes at 0°. To this mixture was added a cold solution of 14.5 g. (0.0592 mole) of H·Tyr·OEt·HCl(D) and 14.2 ml. (0.059 mole) of tri-*n*-butylamine in 100 ml. of tetrahydrofuran. The reaction mixture stood at room temperature overnight after which the solvent was removed *in vacuo* and three volumes of water added. The oily dipeptide derivative was extracted into ethyl acetate and washed with dilute hydrochloric acid, water, dilute sodium bicarbonate and water. The ethyl acetate solution was dried over magnesium sulfate, filtered, concentrated *in vacuo* and the residue crystallized from ethyl acetate–petroleum ether; yield 20.5 g. (78%), m.p. 122–123.5° (a second crop of 1.3 g. (82.5% total), m.p. 120–122° was obtained from the mother liquor.) $[\alpha]^{25}D$ +29.7 (1% in methanol).

3. Z·Leu-Tyr·OBz (L-D).—This compound was prepared by the same procedure described for the ethyl ester. Six and seven-tenths (0.0252 mole) of Z·Leu·OH (L) and 6.7 g. (0.0218 mole) of H·Tyr·OBz·HCl (D) gave 9.1 g. (80%), m.p. 150–152°. Recrystallization from ethyl acetate–petroleum ether gave m.p. 151.5–153°, $[\alpha]^{25}D$ +5.1 (0.5% in methanol).

4. Z·Leu-Tyr·NH·NH$_2$ (L-D).—8.0 g. (0.0175 mole) of Z·Leu-Tyr·OEt (L-D) was dissolved in 40 ml. of methanol. Two and two-tenths ml. (0.438 mole) of hydrazine hydrate was added and the solution refluxed for 1 hr. On cooling the hydrazide crystallized out. After filtration and washing with methanol the yield was 5.7 g. (74%), m.p. 207.5–208.5° after recrystallization from methanol (1.0 g. (87% total), m.p. 208–209°, was obtained from the mother liquor), $[\alpha]^{25}D$ +11.35 (0.5% in methanol).

This compound was also prepared in 83% yield in a similar manner from the corresponding benzyl ester.

5. H·Leu-Tyr·OEt·HCl (L-D).—5.0 g. (0.0109 mole) of Z·Leu-Tyr·OEt (L-D) was dissolved in 150 ml. of methanol containing 5.5 ml. of 2 *N* hydrochloric acid. Palladium black was added and hydrogen passed through the solution until carbon dioxide evolution ceased. The catalyst was filtered off and the solvent removed *in vacuo*. The oily residue was dissolved in methanol which was then removed *in vacuo*. This procedure was repeated three times. Finally the residual oil was crystallized from a solution of methanol–ether; yield 2.6 g., $[\alpha]^{24}D$ +15.2 (2% in methanol). A second crop of 1.0 g. (92% total yield), $[\alpha]^{25}D$ +16.4 (2% in methanol), was obtained from the mother liquor. Recrystallization from methanol–ether yielded a product melting at 205–207°, $[\alpha]^{25}D$ +18.3 (2% in methanol).

6. Z·Leu-Tyr-Pro·OMe (L-D-L).—9.0 g. (0.0203 mole) of Z·Leu-Tyr·NH·NH$_2$ (L-D) was dissolved in 73 ml. of glacial acetic acid, 15 ml. of 5 *N* hydrochloric acid and 230 ml. of water. After cooling to 0°, a concentrated solution containing 1.45 g. (0.021 mole) of sodium nitrite was added and the precipitated azide extracted with cold ethyl acetate. The extract was washed with cold solutions of water, dilute sodium bicarbonate and water and dried over magnesium sulfate in the cold. The magnesium sulfate was filtered off and the azide solution mixed with a cold, dry solution of H·Pro·OMe (L), prepared from 4.3 g. (0.026 mole) of the hydrochloride. The reaction was allowed to proceed for 48 hr. in the refrigerator. The ethyl acetate solution was then washed with dilute hydrochloric acid, water, dilute sodium bicarbonate and water, followed by drying over anhydrous magnesium sulfate. The latter was removed by filtration and the filtrate concentrated *in vacuo*, resulting in an oil which could not be crystallized.

7. H·Leu-Tyr-Pro·OMe·HCl (L-D-L).—The oily carbobenzoxy tripeptide ester prepared above (compound 6) was hydrogenated in methanol in the presence of excess hydrochloric acid, using palladium black as catalyst until no carbon dioxide could be detected in the effluent gas. It was worked up in the same manner as described for compound 5, crystallizing from ethanol–ether; yield 2.8 g., m.p. 239–241° dec., $[\alpha]^{21}D$ −51.0 (2% in methanol). One and four tenths g. was obtained from the mother liquor with m.p. 236–238° dec., $[\alpha]^{26}D$ −51.8 (2% in methanol).

8. Z·Val-p-Tos·Orn-Leu-Tyr·OEt (L-L-L-D).—Ten and eight-tenths g. (0.0202 mole) of Z·Val-*p*-Tos·Orn·NH·NH$_2$ (L-L)[1] was dissolved in 87 ml. of glacial acetic acid, 42 ml. of 1 *N* hydrochloric acid and 345 ml. of water. The solution was cooled to 0° and converted to the azide with 1.41 g. (0.0204 mole) of sodium nitrite. The azide was ex-

TABLE I

No.	Compound	Mol. formula	Mol. wt.	M.p., °C.	Nitrogen, % Calcd.	Nitrogen, % Found	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
2	Z·Leu-Tyr·OEt (L-D)	$C_{24}H_{32}O_6N_2$	456.5	122–123.5	6.1	6.2				
3	Z·Leu-Tyr·OBz (L-D)	$C_{30}H_{34}O_6N_2$	518.6	151.5–153	5.4	5.2				
4	Z·Leu-Tyr·NH·NH_2 (L-D)	$C_{23}H_{30}O_5N_4$	442.5	207.5–208.5	12.7	12.8				
5	H·Leu-Tyr·OEt·HCl (L-D)	$C_{17}H_{27}O_4N_2Cl$	358.9	205–207	7.8	7.9				
6	Z·Leu-Tyr-Pro·OMe (L-D-L)	$C_{29}H_{37}O_7N_3$	539.6	109–110	7.6	7.8				
7	H·Leu-Tyr-Pro·OMe·HCl (L-D-L)	$C_{21}H_{32}O_5N_3Cl$	442.0	239–241	9.6	9.6				
8	Z·Val-*p*-Tos·Orn-Leu-Tyr·OEt (L-L-L-D)	$C_{42}H_{57}O_{10}N_5S$	834.0	182–185.5	8.5	8.8	61.2	60.9	7.0	7.0
9	Z·Val-*p*-Tos·Orn-Leu-Tyr·NH·NH_2 (L-L-L-D)	$C_{40}H_{55}O_9N_7S$	810.0	236–239	12.1	11.8	59.3	59.5	6.9	6.6
10	Z·Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe (L-L-L-D-L)	$C_{46}H_{62}O_{11}N_6S$	907.1	154–156	9.3	9.1	60.9	60.7	6.9	6.7
11	H·Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe·HCl (L-L-L-D-L)	$C_{38}H_{57}O_9N_6SCl$	809.4	.....	10.4	10.3	55.4	55.2	7.1	6.8
12	Z·Pro·Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe (L-L-L-L-D-L)	$C_{51}H_{69}O_{12}N_7S$	1004.2	199–201	9.8	9.6	61.0	60.6	6.9	7.0
13	H·Pro-Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe·HCl (L-L-L-L-D-L)	$C_{43}H_{64}O_{10}N_7SCl$	906.5	210–213	10.8	10.7	56.9	57.0	7.1	6.9
14	Z·(Val-*p*-Tos·Orn-Leu-Tyr-Pro)$_2$·OMe (L-L-L-D-L)$_2$	$C_{88}H_{114}O_{19}N_{12}S_2$	1647.8	218–220	10.2	10.2	60.4	60.4	6.9	7.4
15	Z·(Val-*p*-Tos·Orn-Leu-Tyr-Pro)$_2$·OH (L-L-L-D-L)$_2$	$C_{82}H_{112}O_{18}N_{12}S_2$	1633.8	230–235 (dec.)	10.3	10.3	60.2	60.0	6.9	7.1

tracted, washed and dried as described for the preparation of compound 6. To the cold, dry azide solution was added a cold, dry ethyl acetate solution of H·Leu-Tyr·OEt (L-D) which previously had been prepared from 7.27 g. (0.0202 mole) of the hydrochloride. (This was accomplished by dissolving the H·Leu-Tyr·OEt·HCl in 10 ml. of water, cooling in an ice-bath and adding 2.5 g. (0.030 mole) of sodium bicarbonate. The free ester was taken up in cold ethyl acetate and dried over magnesium sulfate.) After mixing, the solution stood overnight in the cold, at room temperature for 4 hr., and then was chilled again and filtered; yield 10.7 g. (65%), m.p. 182–185.5°, $[\alpha]^{25}D$ −17.0 (1% in methanol).

9. **Z·Val-*p*-Tos·Orn-Leu-Tyr·NH·NH_2** (L-L-L-D).—Nine and eight-tenths grams (0.0119 mole) of Z·Val-*p*-Tos·Orn-Leu-Tyr·OEt (L-L-L-D) was dissolved in 98 ml. of methanol containing 1.79 ml. (0.0357 mole) of hydrazine hydrate and refluxed for 1 hr. After standing overnight in the cold, the crystalline mass (needles) was filtered off and washed with methanol followed by water. The compound was dried over phosphorus pentoxide *in vacuo;* yield 7.5 g. (78%), softens at 208°, m.p. 232–235°. One and fourteen one-hundredths g. (90% total yield) softens 232°, m.p. 236–239°, was obtained from the mother liquor; recrystallized from methanol, $[\alpha]^{25}D$ −27.6 (1% in solution of methanol–0.1 *N* HCl–acetic acid, 3:1:1).

10. **Z·Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe** (L-L-L-D-L).—Eight and eight-tenths g. (0.0165 mole) of Z·Val-*p*-Tos·Orn·NH·NH_2 (L-L) was dissolved in 83 ml. of glacial acetic acid, 145 ml. of water and 19 ml. of 2 *N* hydrochloric acid. The azide, prepared by addition of a concentrated solution of 1.12 g. (0.017 mole) of sodium nitrite to the cold solution, was extracted, washed and dried as described in the preparation of compound 6. Seven and three-tenths g. (0.0165 mole) of H·Leu-Tyr-Pro·OMe·HCl (L-D-L) was converted to the free ester using 2.8 g. (0.032 mole) of sodium bicarbonate as described for the preparation of compound 8. The ethyl acetate solution was added to the cold, dried solution of the azide and the reaction mixture allowed to stand overnight in the cold, followed by 48 hr. at room temperature. It was washed with dilute hydrochloric acid, water, dilute sodium bicarbonate and water and then dried over magnesium sulfate. The ethyl acetate was taken off *in vacuo* and the compound crystallized from aqueous isopropyl alcohol. After recrystallizing from the same solvent, a total of 6.0 g. (40%) was obtained; m.p. 154–156°, $[\alpha]^{25}D$ −56.0 (2% in methanol).

11. **H·Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe·HCl** (L-L-L-D-L).—6.0 g. (6.62 mmoles) of the carbobenzyloxy derivative (compound 10) was dissolved in 150 ml. of methanol containing 6 ml. of 1 *N* hydrochloric acid and hydrogenated over palladium black until evolution of CO_2 ceased. After filtering off the catalyst the methanol was removed *in vacuo* and the residue taken up in methanol and again taken to dryness. After repeating this three times the compound was obtained as a glass powder; yield 5.0 g. (94%), $[\alpha]^{26}D$ −52.1 (0.01% in 0.01 *N* hydrochloric acid).

12. **Z·Pro-Val-*p*-Tos·Orn-Leu-Tyr·Pro·OMe** (L-L-L-L-D-L).—One and ninety-two hundredths g. (7.73 mmoles) of Z·Pro·OH (L)[10] was dissolved in 30 ml. of tetrahydrofuran containing 1.85 ml. (7.73 mmoles) of tri-*n*-butylamine and converted to the mixed anhydride using 1.0 ml. (7.73 mmoles) of isobutyl chlorocarbonate by the procedure described for compound 2. Five and two tenths g. (6.45 mmoles) of H·Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe·HCl (L-L-L-D-L) was dissolved in 40 ml. of water and 1.3 g. (15.4 mmoles) of sodium bicarbonate added. The precipitated free ester was extracted with ethyl acetate. After drying over magnesium sulfate the ethyl acetate was removed *in vacuo* and replaced by tetrahydrofuran, cooled to 0° and added to the cold mixed anhydride. The reaction mixture was left in the cold overnight, then at room temperature for 4 hr. Most of the solvent was removed *in vacuo* and 5 volumes of water added. The oily peptide derivative was extracted into ethyl acetate and washed and dried as described for compound 2. After removing the ethyl acetate the product was crystallized from aqueous ethanol; yield 4.6 g. (72%), m.p. 197.5–200°. A second recrystallization raised the melting point to 199–201°; $[\alpha]^{20}D$ −84.0 (0.5% in methanol).

13. **H·Pro-Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe·HCl** (L-L-L-L-D-L).—Four and six tenths g. (4.57 mmoles) of Z·Pro-Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe (L-L-L-L-D-L) was dissolved in 100 ml. of methanol containing 4.5 ml. of *N* hydrochloric acid and decarbobenzoylated in the same manner as described for compound 11. It crystallized from methanol–ethyl acetate; yield 2.9 g. (71%), m.p. 210–213°, $[\alpha]^{26}D$ −83.0 (0.5% in 0.01 *N* hydrochloric acid). Sixty-one hundredths g. (86% total), m.p. 206–213°, $[\alpha]^{25}D$ −83.0 (0.5% in 0.01 *N* hydrochloric acid) was obtained from the mother liquor.

14. **Z·(Val-*p*-Tos·Orn-Leu-Tyr-Pro)$_2$·OMe** (L-L-L-D-L)$_2$.—Two and ninety-one hundredths g. (3.58 mmoles) of Z·Val-*p*-Tos·Orn-Leu-Tyr·NH·NH_2 (L-L-L-D) (compound 9) was dissolved in 37 ml. of glacial acetic acid and 8 ml. of 1.23 *N* hydrochloric acid. The solution was cooled to 0°, and 0.254 g. (3.68 mmoles) of sodium nitrite, dissolved in 10 ml. of water, was added slowly with efficient stirring. After several minutes 30 ml. of cold water was added and

(10) Cf. E. Abderhalden and H. Nienberg, *Fermentforschung*, **13**, 573 (1933).

the azide extracted into ethyl acetate. This was washed and dried as in the preparation of compound 6. Three and twenty-five hundredths g. (3.58 mmoles) of H·Pro-Val-*p*-Tos·Orn-Leu-Tyr-Pro·OMe·HCl (L-L-L-L-D-L) (compound 13) was converted to the free ester with 0.60 g. (7.16 mmoles) of sodium bicarbonate by the procedure employed in the preparation of compound 8. After mixing of the azide and the ester, a small quantity of a gelatinous precipitate appeared almost immediately. The mixture was allowed to stand overnight at room temperature and then for 24 hr. in the refrigerator. The small precipitate was filtered off and discarded and the ethyl acetate solution washed as described in the preparation of compound 6. Removal of the solvent yielded 3.2 g. of a solid with a very wide melting point range. It could not be purified by crystallization and therefore adsorption chromatography was utilized.

Properties of the crude: m.p., softens at 143°, melts 156–165°; $[\alpha]^{25}D$ −84.8 (0.5% in methanol).

One and six-tenths g. of crude was dissolved in a small quantity of chloroform and applied to a column of alumina[11,12] in chloroform. The column contained 96 g. of alumina and its height to diameter ratio was about 3:1.

Fractions were collected as follows:

Fraction	Yield, mg.	Solvent	Ml.	$[\alpha]^{25}D$ (0.5% MeOH)
IA	580	0.1% MeOH–$CHCl_3$	1000	− 85.3
IB	280	.1% MeOH–$CHCl_3$	700	− 93.9
II	583	.1% MeOH–$CHCl_3$	1700	−103
III	167	.15% MeOH–$CHCl_3$	3000	− 93
IV	69	.25% MeOH–$CHCl_3$	3000	− 81.2

Fractions IB, II and III were combined and rechromatographed on Florisil (60–100 mesh). Eight hundred and twenty-five mg. was applied, as a chloroform solution, to 50 g. of Florisil in a column similar in dimensions to the one described above. Elution was carried out as above, the purified product appearing during elution with 1.2 liters of 5% MeOH–$CHCl_3$. It crystallized from methanol–water; yield 425 mg.; m.p. 218–220°; $[\alpha]^{25}D$ −112 (0.5% in methanol).

Over-all yield (resulting from several chromatographic runs), 638 mg. (10.8%).

15. Z·(Val-*p*-Tos-Orn-Leu-Tyr-Pro)$_2$OH (L-L-L-D-L)$_2$.—Three hundred and ninety mg. (0.25 mmole) of Z·(Val-*p*-Tos·Orn-Leu-Tyr-Pro)$_2$·OMe (compound 14) was dissolved in a solution of 1.3 ml. of *N* NaOH and 1.5 ml. of water. The clear solution was kept at 37° and tested periodically for unsaponified ester by adding a fraction of a drop to *M*/15 phosphate buffer, *p*H 6.8. Any turbidity indicates the presence of ester. After 5 hr., the test still showed some unsaponified ester. However, rather than expose the product to alkaline conditions for a longer period of time, the solution was brought to *p*H 7.5. At this *p*H, the unsaponified ester precipitated out of solution and was centrifuged off. The supernatant was acidified to *p*H 2 and placed in the refrigerator overnight. The suspension was then centrifuged and the pellet washed with water and dried over P_2O_5 *in vacuo;* yield 125 mg.

The unsaponified ester was retreated with base in the same manner twice, resulting in an over-all yield of 310 mg. (81%); m.p., sinters at 160°, melts 175–220; neut. equiv., Calcd.: 1633; found: 1150; $[\alpha]^{25}D$ −110 (0.5% in methanol).

The crude was dissolved in a small quantity of chloroform containing 7.5% methanol and applied to a column of 24 g. of Florisil. After the elution of an impurity by 10% methanol in chloroform, the purified product was eluted from the column by 30% methanol in chloroform; m.p. 230–235° dec., $[\alpha]^{25}D$ −113.4 (0.5% in methanol); neut. equiv., calcd.: 1633; found: 1690; yield 205 mg. (53%).

16. H·Val-Orn-Leu-Tyr-Pro-Val-Orn-Leu-Tyr-Pro·OH·3 HCl (L-L-L-D-L)$_2$.—One hundred mg. (0.061 mmole) of compound 15 was suspended in approximately 20 ml. of liquid NH_3. Small quantities of sodium were added with stirring until the blue color persisted for one minute (60 mg. was required). The reaction was run at the boiling point of liquid NH_3. A drop of acetic acid was added to dispel the blue color and the NH_3 allowed to evaporate off. After standing overnight in a desiccator over sulfuric acid, the solid residue was dissolved in water, brought to *p*H 1 with hydrochloric acid and extracted with ethyl acetate. The slightly yellow aqueous layer was treated with charcoal, filtered and allowed to evaporate to dryness in a desiccator over P_2O_5; yield 60 mg., including a considerable quantity of salt.

The crude product was dissolved in 5 ml. of water, an excess of saturated aqueous picric acid added dropwise and the mixture allowed to stand 48 hr. in the refrigerator. After centrifugation, the solid residue was dissolved in 2 *N* HCl, thoroughly extracted with ether to remove picric acid and evaporated to dryness in a desiccator over P_2O_5.

The resulting solid was retreated with picric acid as described above and after decomposition of the picrate, 5 mg. of purified product remained. It was characterized as follows: (a) acid hydrolysis, subsequent chromatography on Whatman No. 1 paper using butanol–acetic acid–water, 4:1:5 as the solvent system, development with 1% ninhydrin in ethanol and elution with 50% acetone solution showed the presence of equimolar quantities of leucine, valine, ornithine, proline and tyrosine.

(b) Dinitrophenylation by the method of Sanger[13] and chromatography according to Biserte and Osteux[14] showed valine as the N-terminal amino acid residue. In addition, spectrophotometric estimation of the delta DNP-ornithine[15] yielded two moles for every mole of DNP-valine. Estimation of the quantity of free amino acids in the acid hydrolysate showed valine, leucine and proline in ratios of approximately 1:2:2.

The product had a specific rotation of $[\alpha]D$ −143 ± 10 (*c* 0.03 in 70% ethanol). Its ultraviolet absorption curve, in 70% alcohol, possessed a maximum at 278 mμ; ϵ_{max} 2980, based upon a molecular weight of 1300. The extinction coefficient of leucyltyrosine at *p*H 7.8, λ 278 mμ, was shown by Fromageot and Schnek[16] to be *ca.* 1500. Our data, therefore, are in agreement with the presence of two tyrosine residues per molecule of decapeptide.

The above data conform with the expected properties of the decapeptide H·Val-Orn-Leu-Tyr-Pro-Val-Orn-Leu-Tyr-Pro·OH·3 HCl (L-L-L-D-L)$_2$.

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(11) Harshaw alumina (200 mesh) was refluxed with ethyl acetate for 3 hr., filtered and washed with ligroin and methanol. It was then dried for 6 hr. at 60°.

(12) We wish to thank Dr. S. Solomon and Dr. S. Lieberman for generous quantities of this material.

(13) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(14) G. Biserte and R. Osteux, *Bull. soc. chim. biol.*, **33**, 50 (1951).

(15) The aqueous phase of the ether-extracted acid hydrolysate was chromatographed on Whatman No. 1 using butanol–acetic acid–water, 4:1:1.

(16) C. Fromageot and G. Schnek, *Biochim. Biophys. Acta*, **6**, 113 (1950).