Synthesis of a Peptide Lactone Related to Vernamycin B_α

Miguel A. Ondetti and Patsy L. Thomas

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The synthesis of a peptide lactone, namely N-(3-hydroxypicolinyl)-L-threonyl-D-α-aminobutyryl-L-prolyl-N-methyl-L-phenylalanyl-L-prolyl-L-phenylglycine threonine hydroxyl lactone, is described. This structure incorporates some of the most significant features of the antibiotic vernamycin Bα. The synthetic analog, like the natural compound, enhances the antibacterial activity of vernamycin A.

In the past few years, the structures of a number of new antibiotics have been reported in the literature with the common feature of a lactone that is formed from the carboxyl function of an amino acid and the hydroxyl group of an hydroxyamino acid. Moreover, in most of the cases, the amino function of the latter is acylated by a heterocyclic acid which constitutes the chromophoric group of the molecule. The remaining portion of the lactone ring is usually formed by a peptidic chain, in which some of the building blocks are "unnatural" amino acids, that is, amino acids which do not commonly occur in proteins. Examples of this class of compounds are the antibiotics actinomycins,[1] etamycin,[2] echinomycin,[3] vernamycins B,[4] staphylomycin S,[5] etc. The structure of vernamycin B_α (Figure 1) exemplifies the characteristic features of this group of natural products for which the name peptide lactones has been suggested.[6]

Some of the members of this group, *e.g.*, ostreogrycins B, mikamycin B, vernamycins B, and staphylomycin S, are accompanied in the fermentation liquors by a second group of antibiotics of quite unrelated structure.[7] However, the biological activity of the substances belonging to this second group is considerably enhanced by addition of components of the former.[8]

Synthetic approaches in the field of peptide lactone antibiotics have been, so far, limited to the actinomycins, probably because of their remarkable cytotoxic properties. On the other hand, a considerable amount of attention has been devoted by Shemyakin and his collaborators[9] to a closely related group of compounds in which the hydroxyl group of the lactone function is contributed by an hydroxy acid (depsipeptides or peptolides).[10] The studies on vernamycin[4] suggested the possibility that the synthesis of compounds related to the peptide lactones would throw some light on the problem of the structural requirements for the remarkable synergistic activity mentioned above. Preliminary investigations showed that this activity was closely connected with the peptidic lactone character.[5] Therefore, the synthesis of a peptide lactone was planned in which the amino acids forming the peptide chain were selected among those known to be present in the natural compounds. Other structural features considered valuable enough to be retained in the synthetic analog were the chromophoric heterocyclic acid, the D configuration of one of the building blocks of the peptide chain, and the presence of at least one N-methyl amino acid. In brief, the synthetic approach described in this paper aimed at a compound with the structure depicted in Figure 2.

Two alternatives are possible for the synthesis of this type of cyclic compound: (a) closing of the ring by formation of an ester bond, or (b) cyclization by formation of an amide bond. Of these two methods, the second seemed the most appropriate one, because of the number and variety of procedures available for the formation of peptide bonds. Indeed, this is the pathway most commonly used in the synthesis of depsipeptides, and this was the approach that led to the first synthesis of actinomycin C_3 reported by Brockmann and Lackner.[11] A disadvantage of approach a is evident when the synthesis of I is considered. Cyclization with formation of the ester bond involves activation of the carboxyl function of a peptide having C-terminal phenylglycine which could cause considerable racemization[12] and, therefore, substantial decrease in the yields of the desired product. When the synthesis here described was near completion, Brockmann and Lackner reported three new syntheses of actinomycins[13] in which approach a was followed. Lactonization was achieved by heating the hydroxy acid at 55° for 1 hr. with a mixture of acetyl chloride–acetylimidazole[14] in tetrahydrofuran. However, in the case of compound I, the use of this reagent would have led to considerable racemization of the phenylglycine residue, particularly because of the presence of imidazole derivatives in the reaction mixture.[15]

(1) H. Brockmann, G. Bohnsack, B. Franck, H. Gröne, H. Muxfeldt, and C. Süling, *Angew. Chem.*, **68**, 70 (1956).

(2) J. C. Sheehan, H. G. Zachau, and W. B. Lawson, *J. Am. Chem. Soc.*, **80**, 3349 (1958); R. B. Arnold, A. W. Johnson, and A. B. Mauger, *J. Chem. Soc.*, 4466 (1958)

(3) W. Keller-Schierlein, M. Lj. Mihailović, and V. Prelog, *Helv. Chim. Acta*, **42**, 305 (1959).

(4) M. Bodanszky and M. A. Ondetti, "Antimicrobial Agents and Chemotherapy—1963," American Society for Microbiology, Ann Arbor, Mich., 1964, pp. 360–365. Vernamycin B_α was shown to be identical with mikamycin B and ostreogrycin B; *cf.* K. Watanabe, *J. Antibiot.*, **A14**, 14 (1961); F. W. Eastwood, B. K. Snell, and A. Todd, *J. Chem. Soc.*, 2286 (1960).

(5) H. Vanderhaeghe and G. Parmentier, *J. Am. Chem. Soc.*, **82**, 4414 (1960).

(6) E. Schröder and K. Lübke, *Experientia*, **19**, 57 (1963).

(7) Lord A. Todd, International Symposium on Organic Chemistry of Natural Products, Brussels, 1962; *cf.* also ref. 4, 5, and K. Okabe, *J. Antibiot.*, **A12** 86 (1959).

(8) K. Watanabe, *ibid.*, **A13**, 62 (1960), *cf.* also ref. 4 and 5.

(9) A. A. Kiryushkin, Yu. A. Ovchinnikov, and M. M. Shemyakin, *Tetrahedron Letters*, **No. 2**, 143 (1965), and preceding papers by the same group.

(10) For reviews of this field see M. M. Shemyakin, *Angew. Chem.*, **72**, 342 (1960), and ref. 6.

(11) H. Brockmann and H. Lackner, *Naturwiss.*, **47**, 230 (1960); **48**, 555 (1961).

(12) P. A. Levene and R. E. Steiger, *J. Biol. Chem.*, **86**, 703 (1930).

(13) H. Brockmann and H. Lackner, *Naturwiss.*, **51**, 384 (1964), **51**, 407 (1964); **51**, 435 (1964).

(14) H. A. Staab, *Angew. Chem.*, **74**, 407 (1962).

(15) F. Weygand, A. Prox, L. Schmidhammer, and W. König, *ibid.*, **75**, 282 (1963).

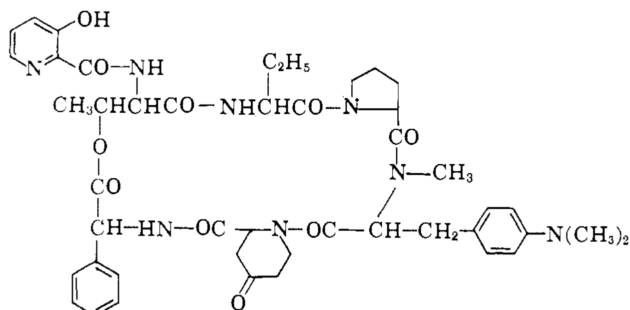

Figure 1.

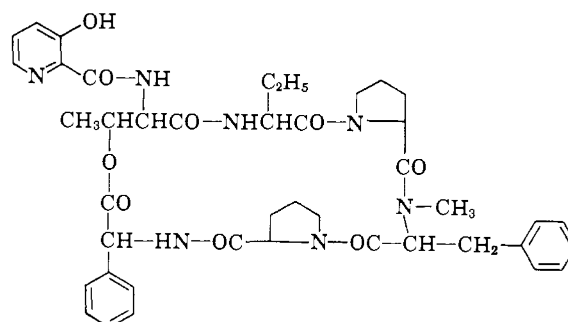

Figure 2.

On the basis of the aforementioned considerations, method b was finally followed, and the amide bond between proline and phenylglycine was selected for the cyclization step because of the well-known stability of proline toward racemization. For this approach, it was necessary to synthesize a chain (Figure 3) that would include the desired ester bond between phenylglycine and threonine. This circumstance limited the selection of carboxyl-protecting groups of the C-terminal amino acid of the chain (proline) to those that could be removed without affecting the already formed ester involving the hydroxyl function of threonine. A *t*-butyl ester was employed for this purpose because of its facile removal under acidic conditions. *t*-Butyloxycarbonyl protection was selected for the amino function of phenylglycine because it could be eliminated simultaneously with the above-mentioned *t*-butyl ester prior to the cyclization step without affecting the temporary protection of the amino group of threonine. The well-known benzyloxycarbonyl protection was employed for this last purpose to avoid or minimize the danger of racemization in the introduction of the threonine moiety. This protecting group could be removed by hydrogenolysis in order to introduce the 3-hydroxypicolinic acid residue. This step could be performed before or after cyclization, and eventually, the former possibility was used in the present synthesis which is schematically described in Figure 3.

The preparation of the protected tetrapeptide VIII was attempted by two different approaches: by stepwise addition of amino acid residues starting from proline *t*-butyl ester and by coupling two suitably protected dipeptides (IV and VI). Even though this last procedure appeared the most direct one, a considerable number of complications were found because of the formation of side products. When dicyclohexylcarbodiimide[16] was used for the coupling of IV and VI, besides the desired tetrapeptide VIII, a substituted urea, N-(benzyloxycarbonyl-L-α-aminobutyryl-L-prolyl)-N,N′-dicyclohexylurea, was also obtained. If the acylation of IV was conducted with a mixed anhydride of VI and isobutyl chloroformate,[17] mainly N-isobutyloxycarbonyl-N-methyl-L-phenylalanyl-L-proline *t*-butyl ester was isolated and only a small amount of the desired tetrapeptide. Finally, the stepwise procedure was selected for the preparation of large amounts of the tetrapeptide VIII. Unfortunately, probably because of the simultaneous presence of two proline residues and a *t*-butyl ester, all the intermediates were very soluble in the usual organic solvents which caused considerable difficulties in the purification procedures. Countercurrent distribution was found to be an invaluable help, not only because it permitted separations and purifications very difficult otherwise, but also because it allowed the handling of relatively large batches (*ca.* 16 g.) without undue complications.

The preparation of a suitably protected "ester portion" of the molecule, namely, O-(N-*t*-butyloxycarbonyl-L-phenylglycyl)-N-benzyloxycarbonyl-L-threonine (X), was attempted in a separate stage of the synthesis because of the difficulties foreseen in the esterification of the secondary alcoholic hydroxyl of threonine. Several carboxyl-activating methods were tested, *e.g.*, mixed carboxylic anhydride,[18] mixed carboxylic–carbonic anhydride,[19] benzenesulfonyl chloride,[20] and N,N′-carbonyldiimidazole.[21] Only the mixed carboxylic–carbonic anhydride and the N,N′-carbonyldiimidazole methods afforded fairly good yields, particularly when a large excess of benzyloxycarbonyl-L-threonine was employed. However, a closer scrutiny of the products obtained by these two methods showed that partial racemization of the phenylglycine moiety had occurred in the material prepared with N,N′-carbonyldiimidazole, and therefore this last procedure was abandoned. The "ester portion" X was coupled by the dicyclohexylcarbodiimide method to the tetrapeptide *t*-butyl ester obtained after removal of the protecting group from VIII. The introduction of the 3-hydroxypicolinic acid moiety was achieved by means of the crystalline nitrophenyl ester of the 3-benzyl derivative of this acid. A short treatment with anhydrous trifluoroacetic acid was sufficient to remove both the amino- and the carboxyl-protecting groups from the open-chain intermediate XIII, and the trifluoroacetate thus obtained was converted with an anion exchange resin to the zwitterion XIV.

Cyclization of XIV was originally attempted with dicyclohexylcarbodiimide in anhydrous tetrahydrofuran[22] using an excess of diimide. The crude product consisted mainly of the substituted ureide of the open

(16) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
(17) J. R. Vaughan and R. L. Osato, *ibid.*, **74**, 676 (1952); T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).
(18) E. D. Nicolaides, R. D. Westland, and E. L. Wittke, *J. Am. Chem. Soc.*, **76**, 2887 (1954).
(19) L. Velluz, G. Amiard, and R. Heymes, *Bull. soc. chim. France*, 1283 (1955).
(20) M. M. Shemyakin, E. I. Vinogradova, M. Yu. Feigina, N. A. Aldanova, N. A. Oladkina, and L. A. Shchukina, *Dokl. Akad. Nauk SSSR*, **140**, 387 (1961).
(21) H. A. Staab, *Angew. Chem.*, **71**, 194 (1959).
(22) H. Brockmann and H. Bujard, *Naturwiss.*, **49**, 515 (1962).

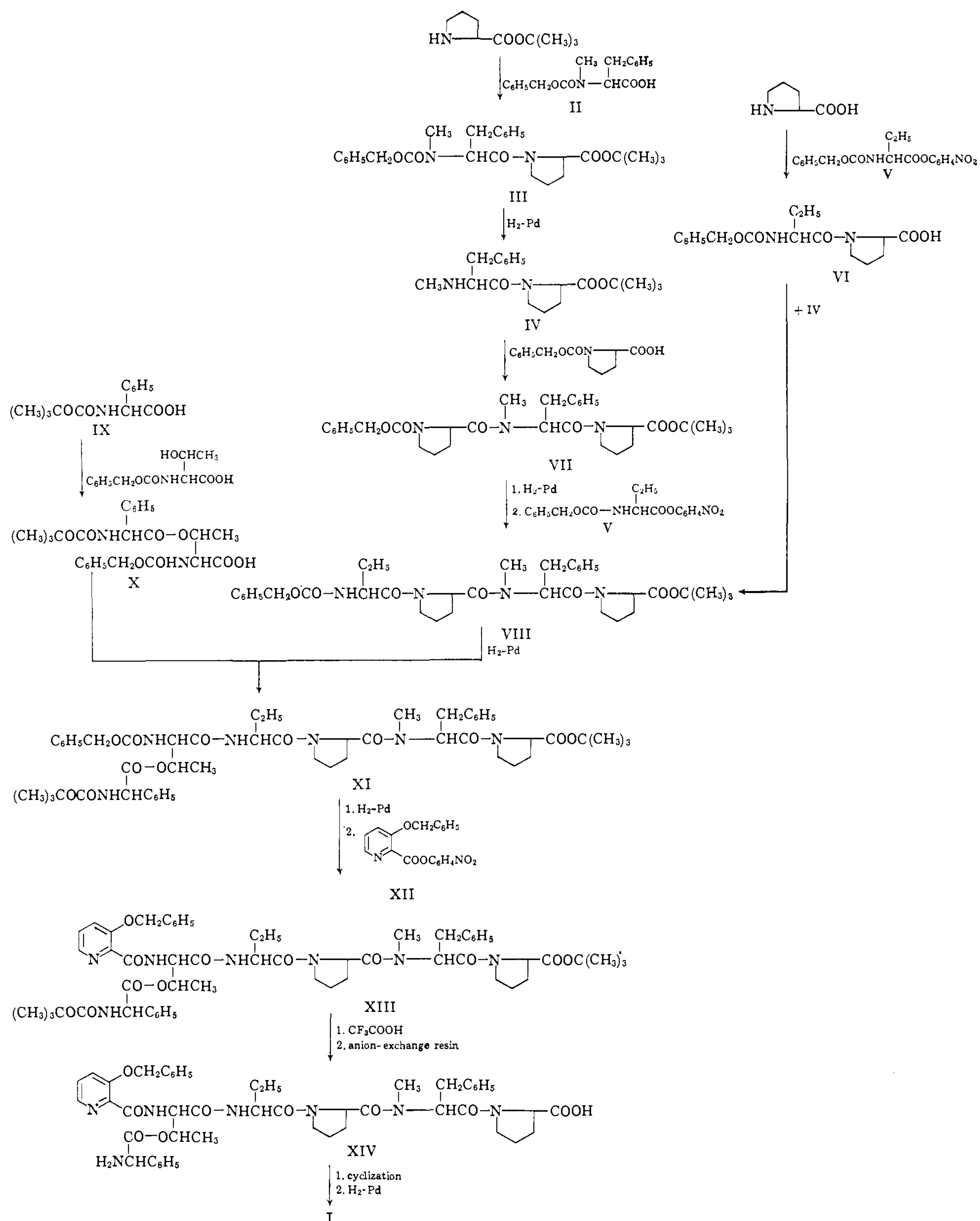

Figure 3.

chain, which was very difficult to separate from the small amount of the desired cyclic product. To facilitate this separation, a polar diimide was employed,[23] namely, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide. The substituted acylurea formed with this reagent could be easily separated from the cyclic product due to its polar character, but the yield of the desired compound I was still very low. A considerable improvement in the yield of cyclized product was achieved by replacing tetrahydrofuran with dichloromethane, a solvent which seems to reduce the

(23) J. C. Sheehan and D. N. McGregor, *J. Am. Chem. Soc.*, **84**, 3000 (1962); K. D. Kopple and D. E. Nitecki, *ibid.*, **83**, 4103 (1961); J. C. Sheehan and J. J. Hlavka, *J. Org. Chem.*, **21**, 439 (1956).

tendency of the active intermediate to rearrange to the substituted ureide.[24] From all the other coupling reagents tested, only N,N′-carbonyldiimidazole[25] and N-ethyl-5-phenylisoxazolium 3′-sulfonate[26] permitted the isolation of cyclized material, even though the yields were much lower than those provided by the diimide method. After removal of the benzyl group from the 3-hydroxypicolinyl moiety by catalytic hydrogenolysis, a short countercurrent distribution of the crude reaction mixture led to the isolation of I in homogeneous form and in comparatively good yield (34%). Crystallization was eventually achieved from methanol with almost quantitative recovery. The analytical data of this material were in full agreement with the results expected for I.

No attempts were made to establish by enzymatic degradation the optical purity of the intermediates and the final product of this synthesis for the following reasons. Cyclic peptides are generally resistant to enzymatic attack, and most of the peptide bonds of the linear intermediates of the present synthesis are not cleaved by the enzymes commonly used to ascertain the optical purity of synthetic peptides. However, as it was pointed out above, in all the peptide bond-forming steps, protecting groups and subunits were selected in such a way that, according to the present knowledge of peptide synthesis, the possibility of racemization should be excluded.

Unlike vernamycin B_α, compound I does not inhibit *per se* the growth of *Staphylococcus aureus*. However, it is active against *Bacillus subtilis* with a minimum inhibitory concentration of 4.8 μg./ml. The peculiar enhancement of the activity of vernamycin A is also shown by compound I, *e.g.*, a mixture of 20% of vernamycin A and 80% of compound I shows, against *S. aureus*, eight times the activity calculated for vernamycin A alone.

Experimental Section

Melting points were taken in capillary tubes and are not corrected. Countercurrent distributions were run in a 500-tube, all glass, automatic Craig apparatus (H. O. Post Scientific Instrument Co., Inc., New York, N. Y.) with capacity for 10 ml. of each phase. The distributions were followed by determination of weight curves. When fractionation of large amounts was required, the material was applied to a bank of tubes at the beginning of the machine in such a way that a load of no more than 1 g. per tube was obtained. The homogeneity of intermediates was checked by thin layer chromatography on silica gel using mixtures of chloroform–methanol (95:5) for protected peptides and of *n*-butyl alcohol–acetic acid–water (4:1:1) for the unprotected intermediates. Amino acid analyses, performed by Mr. F. Russo-Alesi using a Technicon amino acid Auto Analyzer (Technicon Chromatography Corp., Chauncey, New York), and microanalyses by Mr. J. F. Alicino and his co-workers are gratefully acknowledged.

*Benzyloxycarbonyl-N-methyl-*L*-phenylalanine (II).* N-Methyl-L-phenylalanine[27] (10.74 g., 60 mmoles) was dissolved in a mixture of 0.9 *N* lithium hydroxide (69.6 ml.) and tetrahydrofuran (114 ml.). Benzyloxycarbonyl chloride (15 ml.) and 0.9 *N* lithium hydroxide (120 ml.) were added in ten portions over 20 min., keeping the temperature near 10° with an ice bath. By the final addition, the mixture was milky. Stirring in the ice bath was continued for a total of 5 hr., and then the reaction mixture was extracted with ether (150 ml.). A dense, white precipitate formed (the lithium salt of the acid) which was washed again with ether. The aqueous suspension was acidified to pH 1–3 with 5 *N* hydrochloric acid and extracted with ether (four 100-ml. portions). The combined ether extracts were washed with water, dried over magnesium sulfate, and concentrated to dryness *in vacuo*. The oily residue was crystallized from ether–hexane (1:2), yield 15.3 g. (81%), m.p. 70–72°. The mother liquor gave a second crop, 0.5 g., m.p. 68–70°. The two crops were joined and recrystallized from ethyl acetate–hexane, yield 11.7 g. (62%), m.p. 70–71°. A second crop weighed 1.1 g., m.p. 69–71° (total yield 68%), $[\alpha]^{21}D$ −71.2° (*c* 2.5, ethyl acetate) (lit.[28] m.p. 69.5–70.5°, $[\alpha]^{26}D$ −70.3° (*c* 2.5, ethyl acetate)).

Anal. Calcd. for $C_{18}H_{19}NO_4$: C, 69.01; H, 6.07; N, 4.47. Found: C, 69.03; H, 6.08; N, 4.42.

A dicylohexylammonium salt was prepared in ethyl acetate–hexane, m.p. 116–118°.

Anal. Calcd. for $C_{30}H_{42}N_2O_4$: C, 72.85; H, 8.56; N, 5.66. Found: C, 72.78; H, 8.64; N, 5.84.

*t-Butyl Benzyloxycarbonyl-N-methyl-*L*-phenylalanyl-*L*-prolinate (III).* Benzyloxycarbonyl-N-methyl-L-phenylalanine (12.50 g., 40 mmoles) and *t*-butyl prolinate[29] (6.85 g., 40 mmoles) were dissolved in ethyl acetate (95 ml.) with stirring and cooling in an ice bath. Dicyclohexylcarbodiimide (8.24 g., 40 mmoles) was added and, after 2 hr. in the ice bath, 3.5 hr. at room temperature, and 0.5 hr. in the ice bath, the precipitate of dicyclohexylurea (7.35 g., 82%) was filtered off and washed with ethyl acetate (100 ml.). The filtrate was extracted with 5% citric acid (50 ml.), water (50 ml.), saturated sodium bicarbonate solution (50 ml.), and water. It was dried over magnesium sulfate and concentrated to dryness *in vacuo*. The oily residue weighed 18.6 g. (100%). This product was used without further treatment in the preparation of the tripeptide.

For purposes of characterization, after removal of the benzyloxycarbonyl group, the oxalate and hydrochloride of the free base *t*-butyl N-methyl-L-phenylalanyl-L-prolinate (IV) were prepared by adding anhydrous oxalic acid or hydrochloric acid to an ether solution of the free base. The oxalate has m.p. (145) 147–149°, $[\alpha]^{31}D$ −45.5° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{21}H_{30}N_2O_7$: C, 59.70; H, 7.16; N, 6.63. Found: C, 59.70; H, 7.23; N, 6.92.

The hydrochloride has m.p. 224–225° dec., $[\alpha]^{19}D$ −64° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{19}H_{29}ClN_2O_3$: C, 61.87; H, 7.87; Cl, 9.63; N, 7.60. Found: C, 61.47; H, 7.62; Cl, 10.06; N, 7.20.

(24) J. C. Sheehan, M. Goodman, and G. P. Hess, *J. Am. Chem. Soc.*, **78**, 1367 (1956).
(25) R. Paul and G. W. Anderson, *ibid.*, **82**, 4597 (1960).
(26) R. B. Woodward, R. A. Olofson, and H. Mayer, *ibid.*, **83**, 1010 (1961).
(27) P. Quitt, J. Hellerbach, and K. Vogler, *Helv. Chim. Acta*, **46**, 327 (1963).
(28) M. Goodman and K. C. Stueben, *J. Org. Chem.*, **27**, 3409 (1962).
(29) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).

Benzyloxycarbonyl-D-*α*-*aminobutyric Acid.* D-α-Aminobutyric acid (California Corp. for Biochemical Research, Los Angeles) (20.60 g., 0.2 mole) was dissolved in 5 *N* sodium hydroxide (40-ml.) with stirring and cooling in an ice bath. Benzyloxycarbonyl chloride (38 ml.) and 5 *N* sodium hydroxide (50 ml.) were added in five portions over 30 min., keeping the pH strongly alkaline. After an additional 30 min., the mixture was washed with ether (three 40-ml. portions) to remove excess acid chloride, then acidified to congo red end point with 5 *N* hydrochloric acid. The crystalline precipitate was filtered and washed with water, yield 45.82 g. (97%), m.p. 78–79°, $[\alpha]^{21}\text{D}$ +9.1° (*c* 2.8, absolute ethanol), $[\alpha]^{22}\text{D}$ +14.4° (*c* 4, 1 *N* sodium hydroxide) (lit.[30] m.p. 76–78°, $[\alpha]^{19}\text{D}$ +14.4° (*c* 4, 1 *N* sodium hydroxide)).

Anal. Calcd. for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 60.94; H, 6.32; N, 6.10.

p-Nitrophenyl Benzyloxycarbonyl-D-*α*-*aminobutyrate* (*V*). This compound was obtained following the general procedure described for the preparation of nitrophenyl esters.[31] Diisopropyl ether was used for the crystallization of the product, yield 77%, m.p. 60–61°, $[\alpha]^{24}\text{D}$ +34.4° (*c* 2, dimethylformamide).

Anal. Calcd. for $C_{18}H_{18}N_2O_6$: C, 60.33; H, 5.06; N, 7.82. Found: C, 60.23; H, 5.26; N, 7.84.

Benzyloxycarbonyl-D-*α*-*aminobutyryl*-L-*proline* (*VI*). A solution of *p*-nitrophenyl benzyloxycarbonyl-D-α-aminobutyrate (7.16 g., 20 mmoles) in tetrahydrofuran (50 ml.) was added to a solution of L-proline (2.96 g., 24 mmoles) in water (50 ml.). The pH was maintained at 9.0 by the controlled addition of 4 *N* sodium hydroxide. When the reaction was completed (after 2 hr., no more uptake of sodium hydroxide was observed), the clear, yellow solution was acidified to pH 6.8–7.0 with 5 *N* hydrochloric acid, concentrated *in vacuo* to remove tetrahydrofuran, and finally saturated with solid sodium bicarbonate. This solution was washed several times with ethyl acetate until it was nearly colorless. The ethyl acetate extracts were twice re-extracted with saturated sodium bicarbonate solution. The aqueous phases were acidified to pH 2–3 with concentrated hydrochloric acid and extracted five times with ethyl acetate. The combined ethyl acetate extracts were washed with water, dried over magnesium sulfate, and concentrated to dryness *in vacuo*. The oily residue was crystallized from ether in the form of a dicyclohexylammonium salt, yield 7.60 g. (74%), m.p. 112–114°, $[\alpha]^{21}\text{D}$ −28.3° (*c* 2, 95% ethanol).

Anal. Calcd. for $C_{29}H_{45}N_3O_5$: C, 67.50; H, 8.80; N, 8.15. Found: C, 67.37; H, 9.02; N, 8.10.

For the conversion to the free acid VI, the dicyclohexylammonium salt (3.1 g.) was dissolved in a mixture of water (6 ml.) and ethyl acetate (15 ml.). After acidification with 6 *N* HCl (3 ml.) the precipitate of dicyclohexylammonium hydrochloride was filtered off and the aqueous phase was extracted three times more with 30-ml. portions of ethyl acetate. The combined ethyl acetate extracts were dried over magnesium sulfate and concentrated to dryness *in vacuo*, yield 2.0 g. (100%).

(30) H. Uchino, *Nippon Kagaku Zasshi*, **81**, 1851 (1960); *Chem. Abstr.*, **56**, 2509*a* (1962).
(31) "Biochemical Preparations," Vol. 9, M. J. Coon, Ed., John Wiley and Sons, Inc., New York, N. Y., 1962, p. 110.

t-Butyl Benzyloxycarbonyl-L-*prolyl-N-methyl*-L-*phenylalanyl*-L-*prolinate* (*VII*). *t*-Butyl benzyloxycarbonyl-N-methyl-L-phenylalanyl-L-prolinate (18.7 g., 40 mmoles) was dissolved in a mixture of absolute ethanol (100 ml.) and acetic acid (100 ml.). To this solution, 10% palladium on charcoal (2.0 g.) was added and the mixture was stirred for 4.5 hr. in an atmosphere of hydrogen. The catalyst was filtered off, and the filtrate was concentrated to near dryness *in vacuo* and reconcentrated twice more after dilution with benzene. The oily residue was dissolved in ethyl acetate (200 ml.) and washed with a saturated solution of sodium bicarbonate (50 ml.) containing enough excess solid bicarbonate so that the wash remained alkaline. The ethyl acetate was then washed with water (30 ml.) and the bicarbonate and water washes were pooled and extracted with ethyl acetate (two 40-ml. portions). The combined ethyl acetate extracts were washed with water (25 ml.), dried over magnesium sulfate, and concentrated to dryness *in vacuo*. The oil weighed 11.7 g. (35.2 mmole, 88%).

This oil and benzyloxycarbonyl-L-proline (8.33 g., 33.4 mmole) were dissolved in ethyl acetate (70 ml.) and the solution was stirred in an ice bath. Dicyclohexylcarbodiimide (6.90 g., 33.4 mmoles) was added and the mixture was stirred for 2 hr. in the ice bath, and 4 hr. at room temperature and kept overnight at 5°. The precipitate of dicyclohexylurea (6.18 g., 82%) was filtered off, and the filtrate, after dilution with ethyl acetate (300 ml.), was washed with 5% citric acid (50 ml.), water (50 ml.), saturated sodium bicarbonate solution (50 ml.), water (two 50-ml. portions), and saturated sodium chloride solution (20 ml.), dried over magnesium sulfate, and concentrated to dryness *in vacuo*. The oily residue (17.7 g., 94%) behaved as a homogeneous compound on thin layer chromatograms. Attempts to prepare a derivative of the free base after removal of the benzyloxycarbonyl group failed. This product was used without further treatment in the preparation of the tetrapeptide VIII.

t-Butyl Benzyloxycarbonyl-D-*α*-*aminobutyryl*-L-*prolyl-N-methyl*-L-*phenylalanyl*-L-*prolinate* (*VIII*). *A. t*-Butyl benzyloxycarbonyl-L-prolyl-N-methyl-L-phenylalanyl-L-prolinate (17.7 g., 31.4 mmoles) was dissolved in a mixture of absolute ethanol (100 ml.) and acetic acid (100 ml.). To the resulting solution, 10% palladium on charcoal (2.0 g.) was added and the mixture was stirred in an atmosphere of hydrogen for 4.5 hr. The catalyst was filtered off, and the filtrate was concentrated to dryness *in vacuo* and reconcentrated twice after dilution with benzene.

The oily residue and *p*-nitrophenyl benzyloxycarbonyl-D-α-aminobutyrate (10.8 g., 30.1 mmoles) were dissolved in pyridine (30 ml.) and allowed to react at room temperature for 3 days. The reaction mixture was then concentrated *in vacuo* and the residue was dissolved in ethyl acetate (250 ml.), washed with 2 *N* hydrochloric acid (50 ml.), water (50 ml.), 1 *N* sodium carbonate (twenty 30-ml. portions), and water (four 50-ml. portions). After drying over magnesium sulfate, the solvent was removed *in vacuo*. The oily residue was dissolved in benzene, filtered from a small insoluble portion, and freeze-dried, yielding 18.6 g. This crude product was distributed in the solvent system chloroform–hexane–methanol–water (5:15:16:4)

for 500 transfers. Only one major peak was observed ($K = 0.69$), and the contents of the corresponding tubes were pooled, concentrated *in vacuo*, and freeze-dried from benzene, yield 13.3 g. (68%), $[\alpha]^{32}D$ $-110°$ (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{36}H_{48}N_4O_7$: C, 66.64; H, 7.46; N, 8.64. Found: C, 66.34; H, 7.62; N, 8.54.

Quantitative amino acid analysis showed the presence of α-aminobutyric acid and proline in a ratio of 1.0:2.0. N-Methylphenylalanine was determined by n.m.r. spectroscopy in $CDCl_3$. The area of the peak corresponding to the N-methyl protons (τ 6.85) was equal to that of the C-methyl triplet centered at τ 9.11, corresponding to the α-aminobutyric acid moiety.

B. Benzyloxycarbonyl-D-α-aminobutyryl-L-proline (VI, 582 mg., 1.75 mmoles, isolated from 920 mg. of the dicyclohexylammonium salt) and *t*-butyl N-methyl-L-phenylalanyl-L-prolinate (IV, 648 mg., 1.95 mmoles) were dissolved in ethyl acetate (8 ml.) and cooled in an ice bath. Dicyclohexylcarbodiimide (360 mg., 1.75 mmoles) was added, and the mixture was stirred in the ice bath for 3 hr. and then at room temperature overnight. The dicyclohexylurea (270 mg., 69%) was filtered off and the filtrate was washed with 5% citric acid, water, saturated sodium bicarbonate solution, and water. After drying over magnesium sulfate, the solvent was removed *in vacuo*, and the residue was freeze-dried from benzene. This residue (1.0 g.) was distributed in the solvent system chloroform–hexane–methanol–water (5:15:16:4) for 100 transfers. Two peaks were observed, the larger with $K = 0.69$ and the smaller with $K = 3.0$. The peak $K = 0.69$ was the desired tetrapeptide. The contents of the tubes in this peak were pooled, concentrated to dryness *in vacuo*, and freeze-dried from benzene, yield 595 mg. (53%), $[\alpha]^{25}D$ $-104°$ (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{36}H_{48}N_4O_7$: C, 66.64; H, 7.46; N, 8.64. Found: C, 66.48; H, 7.41; N, 8.78.

The contents of the tubes in the peak $K = 3.0$ were also pooled, concentrated to dryness, and freeze-dried from benzene; 140 mg. Paper chromatograms of an acid hydrolysate of this material showed spots corresponding to α-aminobutyric acid, proline, and dicyclohexylamine. These data and the analysis of the infrared spectrum support the conclusion that this material is N-(benzyloxycarbonyl-D-α-aminobutyryl-L-prolyl)-N,N′-dicyclohexylurea.

C. Benzyloxycarbonyl-D-α-aminobutyryl-L-proline (VI, 2.01 g., 6 mmoles, isolated from 3.18 g. of the dicyclohexylammonium salt) and triethylamine (0.83 ml., 6 mmoles) were dissolved in tetrahydrofuran (7 ml.) and the solution was cooled in an ice–salt bath. Isobutyl chloroformate (0.79 ml., 6 mmoles) was added, followed, after 5 min. stirring in the cold bath, by a solution of N-methyl-L-phenylalanyl-L-proline *t*-butyl ester (IV, 1.76 g., 5.3 mmoles) in tetrahydrofuran (5 ml.). The reaction mixture was stirred for another 2 hr. in the cold bath and 3.5 hr. at room temperature. After dilution with ethyl acetate (100 ml.), the suspension was washed with 5% citric acid (25 ml.), water (25 ml.), saturated $NaHCO_3$ solution (25 ml.), and finally water (three 25-ml. portions). The organic layer was dried over magnesium sulfate and the solvent was removed *in vacuo*. Upon dilution of the oily residue with ether, a crystalline precipitate was observed which was filtered and washed with ether. It weighed 450 mg., m.p. 103–104°. The ether filtrate was concentrated to dryness and the residue was distributed in the solvent system chloroform–hexane–methanol–water (5:15:16:4) for 100 transfers. Three major peaks ($K = 0.05$, 0.69, and 4.0) and two minor ones ($K = 0.33$ and 1.5) were observed. The peak with $K = 0.69$ gave the desired tetrapeptide, 590 mg. (17%), $[\alpha]^{25}D$ $-107°$ (*c* 2, dimethylformamide).

The peak with $K = 4.0$ gave 400 mg. of a crystalline material, m.p. 104–105°, $[\alpha]^{32}D$ $-101°$ (*c* 1, dimethylformamide), identical with that isolated from the crude reaction mixture. Paper chromatography of an acid hydrolysate of this product showed only spots corresponding to N-methylphenylalanine and proline. The infrared spectrum showed three carbonyl bands (5.78, 5.90, and 6.10 μ), no NH band, no amide II band, and a *t*-butyl ester band at 8.7 μ. On the basis of this evidence, the crystalline material was identified as isobutyloxycarbonyl-N-methyl-L-phenylalanyl-L-proline *t*-butyl ester.

Anal. Calcd. for $C_{24}H_{36}N_2O_5$: C, 66.64; H, 8.39; N, 6.48. Found: C, 66.74; H, 8.01; N, 6.83.

The other three peaks of this countercurrent distribution were not investigated.

t-Butyloxycarbonyl-L-phenylglycine (IX). *t*-Butyl carbazate[32] (50 g., 0.38 mole) was dissolved in acetic acid (44 ml.) and water (65 ml.) with vigorous stirring and cooling in an ice–salt bath. A solution of sodium nitrite (28.7 g.) in water (80 ml.) was added gradually over a period of 1 hr., keeping the inside temperature between 3 and 11°. After the addition was complete, stirring was continued for 1 hr. longer. Water (65 ml.) was then added, the yellow *t*-butyloxycarbonyl azide layer was separated, and the aqueous layer was extracted with ether (four 25-ml. portions). The combined azide layer and ether extracts were washed with water (three 25-ml. portions) and 1 *M* sodium bicarbonate (three 20-ml. portions). This azide solution was then added to a mixture of L-phenylglycine (30 g., 0.2 mole), magnesium oxide (15.2 g., 0.4 mole), water (300 ml.), and dioxane (500 ml.). The resulting suspension was stirred at 45–50° for *ca.* 24 hr. The mixture was then concentrated *in vacuo* to remove dioxane, diluted with ethyl acetate (600 ml.) and water (600 ml.), and filtered to remove magnesium oxide. The filtrate aqueous layer was extracted with ethyl acetate (250 ml.), acidified with solid citric acid (congo red), and extracted with ethyl acetate (four 400-ml. portions). The ethyl acetate extract was washed with water (four 250-ml. portions), dried over magnesium sulfate, and concentrated to dryness *in vacuo*. The oily residue was crystallized from hexane, yield 37.15 g. (74%), m.p. 90–93°. This material was recrystallized from hot hexane, yield 33.30 g. (66%), m.p. 90.5–92.5°, $[\alpha]^{27}D$ $+139°$ (*c* 2, 95% ethanol).

Anal. Calcd. for $C_{13}H_{17}NO_4$: C, 62.14; H, 6.82; N, 5.57. Found: C, 62.16; H, 6.85; N, 5.75.

O-(t-Butyloxycarbonyl-L-phenylglycyl)-N-benzyloxycarbonyl-L-threonine (X). *A.* *t*-Butyloxycarbonyl-L-phenylglycine (2.5 g., 10 mmoles) and N,N′-carbonyldiimidazole[21] (1.65 g., 10 mmoles) were dissolved in dry tetrahydrofuran (10 ml.). The solution was kept at

(32) L. A. Carpino, *J. Am. Chem. Soc.*, **82**, 2725 (1960); R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

room temperature for 20 min. and then added with another solution of N-benzyloxycarbonyl-L-threonine[33] (5.06 g., 20 mmoles) and triethylamine (2.8 g., 20 mmoles) in dry tetrahydrofuran (15 ml.). The reaction mixture was stored overnight at room temperature and then diluted with ethyl acetate (200 ml.) and washed with 1 *N* hydrochloric acid (50 ml.) and water (six 50-ml. portions). The organic layer was dried over magnesium sulfate and the solvent was removed *in vacuo*. The residue was distributed in the system chloroform–cyclohexane–methanol–water (5:5:8:2) for 200 transfers. Three main peaks were observed with *K* values 0.70, 1.8, and 5.7. The products isolated from the tubes corresponding to the peaks *K* = 1.8 and 5.7 were shown to be *t*-butyloxycarbonyl-L-phenylglycine and N-benzyloxycarbonyl-L-threonine, respectively. The material with *K* = 0.70, which was shown to be the desired product, was isolated in the form of a crystalline dicyclohexylammonium salt from acetone–hexane, yield 3.4 g. (52%), m.p. 142–144° with softening at 140°, $[\alpha]^{30}D$ +25.5° (*c* 1, dimethylformamide). The infrared spectrum shows three carbonyl (5.76, 5.85, and 5.89 μ) and a carboxylate band (6.13 μ). Quantitative amino acid analysis showed only the two amino acids, phenylglycine and threonine in the ratio, 1.0:1.0.

Anal. Calcd. for $C_{37}H_{53}N_3O_8$: C, 66.57; H, 7.95; N, 6.30. Found: C, 66.70; H, 7.98; N, 6.35.

B. *t*-Butyloxycarbonyl-L-phenylglycine (2.6 g., 10 mmoles) and triethylamine (1.35 ml., 10 mmoles) were dissolved in dry tetrahydrofuran (17.5 ml.), and the solution was cooled in an ice–salt bath. Isobutylchloroformate (1.3 ml., 10 mmoles) was added, followed, after 5 min. stirring in the cold bath, by a solution of N-benzyloxycarbonyl-L-threonine (5 g., 20 mmoles) and triethylamine (4 ml., 30 mmoles) in dry tetrahydrofuran (20 ml.). The reaction mixture was stirred at room temperature for about 20 hr. and then processed as described in the preceding paragraph. The crude reaction product was distributed for 250 transfers in the aforementioned system. In addition to the peaks corresponding to the desired product, *t*-butyloxycarbonyl-L-phenylglycine and N-benzyloxycarbonyl-L-threonine, three minor peaks were observed, but not further investigated. The desired product was isolated in the form of a dicyclohexylammonium salt, yield 3.5 g. (54%), m.p. 145–147° softening at 143°, $[\alpha]^{28}D$ +35.1° (*c* 1, dimethylformamide). The infrared spectrum of this material and the one obtained in paragraph A are identical.

Anal. Calcd. for $C_{37}H_{53}N_3O_8$: C, 66.57; H, 7.95; N, 6.30. Found: C, 66.46; H, 7.97; N, 6.38.

t-Butyl O-(t-Butyloxycarbonyl-L-phenylglycyl)-N-benzyloxycarbonyl-L-threonyl-D-α-aminobutyryl-L-prolyl-N-methyl-L-phenylalanyl-L-prolinate (XI). *t*-Butyl-N-benzyloxycarbonyl-D-α-aminobutyryl-L-prolyl-N-methyl-L-phenylalanyl-L-prolinate (VIII, 10.5 g., 16 mmoles) was dissolved in absolute ethanol (75 ml.) and acetic acid (75 ml.). To the resulting solution, 10% palladium on charcoal (1.05 g.) was added, and the mixture was stirred in a hydrogen atmosphere for 4.5 hr. The catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo* and reconcentrated twice after dilution with benzene. The oily residue was dissolved in ethyl acetate (260 ml.) and washed with saturated sodium bicarbonate (45 ml.) containing enough excess solid bicarbonate so that the wash remained alkaline. After washing the ethyl acetate layer with water (45 ml.), the aqueous washes were pooled and re-extracted with ethyl acetate (two 40-ml. portions). The ethyl acetate portions were pooled and washed with water (30 ml.). The organic layer was dried over magnesium sulfate and concentrated to dryness *in vacuo*. The residue was dried to constant weight; 6.9 g. (13.5 mmoles).

This residue and O-(*t*-butyloxycarbonyl-L-phenylglycyl)-N-benzyloxycarbonyl-L-threonine (X, 7.3 g., 15 mmoles, isolated from 10.0 g. of the dicyclohexylammonium salt, as described for VI) were dissolved in ethyl acetate (60 ml.), cooled in an ice bath, and dicyclohexylcarbodiimide (2.8 g.) was added. After 2 hr. of stirring in the ice bath, 4 hr. at room temperature, and 0.5 hr. in the ice bath, the precipitate of dicyclohexylurea was filtered off and washed with ethyl acetate (100 ml.). The filtrate was diluted with more ethyl acetate (100 ml.) and washed with 5% citric acid (50 ml.), water (50 ml.), a saturated solution of sodium bicarbonate (50 ml.), and water (three 50-ml. portions). After drying over magnesium sulfate, the solvent was removed *in vacuo*. The residue (*ca.* 14 g.) was distributed in the solvent system chloroform–hexane–methanol–water (5:15:16:4) for 500 transfers. Only one major peak was observed (*K* = 1.05), and the contents of the corresponding tubes were pooled, concentrated to dryness *in vacuo*, and freeze-dried from benzene, yield 9.2 g. (62%), $[\alpha]^{32}D$ −36.8° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{53}H_{70}N_6O_{12}$: C, 64.77; H, 7.13; N, 8.55. Found: C, 64.59; H, 7.23; N, 8.57.

Quantitative amino acid analysis showed the presence of phenylglycine, threonine, α-aminobutyric acid, and proline in the ratio 1.0:1.0:1.0:2.0.

p-Nitrophenyl 3-Benzyloxypicolinate (XII). 3-Benzyloxypicolinic acid hydrochloride sesquihydrate[34] (22.0 g., 75 mmoles) was suspended in a mixture of ethyl acetate (320 ml.) and triethylamine (11.2 ml.) and stirred for 1 hr. at room temperature. The crystals of triethylamine hydrochloride were filtered off and washed with ethyl acetate (two 30-ml. portions). *p*-Nitrophenol (11.12 g.) and dicyclohexylcarbodiimide (16.48 g.) were added to the filtrate and the mixture was stirred for 2 hr. at room temperature and 0.5 hr. in an ice bath. The precipitate of dicyclohexylurea (9.60 g., 53%) was filtered off and the filtrate was concentrated to dryness *in vacuo*. The crystalline residue was suspended in ether, filtered, and washed with ether, yield 14.38 g. (51%), m.p. 120–121° dec.

Anal. Calcd. for $C_{19}H_{14}N_2O_5$: C, 65.14; H, 4.03; N, 8.00. Found: C, 65.24; H, 4.06; N, 8.14.

t-Butyl O-(t-Butyloxycarbonyl-L-phenylglycyl)-N-(3-benzyloxypicolinyl)-L-threonyl-D-α-aminobutyryl-L-prolyl-N-methyl-L-phenylalanyl-L-prolinate (XIII). *t*-Butyl O-(*t*-butyloxycarbonyl-L-phenylglycyl)-N-benzyloxycarbonyl-L-threonyl-D-α-aminobutyryl-L-prolyl-N-methyl-L-phenylalanyl-L-prolinate (XI, 3.0 g., 3.06 mmoles) was dissolved in a mixture of absolute ethanol (30 ml.) and acetic acid (30 ml.) and hydrogenated in the

(33) E. Wünsch and J. Jentsch, *Ber.*, 97, 2490 (1964).

(34) J. T. Sheehan, in preparation.

presence of 10% palladium on charcoal (300 mg.) for 4.5 hr. The catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo* and freeze-dried from benzene. The residue was dissolved in dry pyridine (9 ml.) and *p*-nitrophenyl 3-benzyloxypicolinate (XII, 1.6 g., 4.5 mmoles) was added. After standing at room temperature for 3 days, the reaction mixture was concentrated to dryness *in vacuo* and freeze-dried from benzene. The residue was distributed in the solvent system chloroform–hexane–methanol–water (4:6:8:2) for 250 transfers. The contents of the tubes in the peak with K = 1.94, which corresponded to the desired intermediate XIII, as demonstrated by the analytical data given below, were pooled and concentrated to dryness *in vacuo*, yield 1.55 g. (50%). The ultraviolet spectrum in ethanol showed a maximum at 291 mμ (ϵ 5200), $[\alpha]^{32}$D −38.2° (*c* 1, dimethylformamide).

Anal. Calcd. for $C_{58}H_{73}N_7O_{12}$: C, 65.70; H, 6.94; N, 9.25; mol. wt., 1060. Found: C, 65.59; H, 6.95; N, 9.09; neut. equiv. (perchloric acid in acetic acid), 1060.

Quantitative amino acid analysis showed the presence of threonine, phenylglycine, α-aminobutyric acid, and proline in the ratio 0.9:1.0:1.0:2.0.

O-(L-Phenylglycyl)-N-(3-benzyloxypicolinyl)-L-threonyl-D-α-aminobutyryl-L-prolyl-N-methyl-L-phenylalanyl-L-proline (XIV). *t*-Butyl O-(*t*-butyloxycarbonyl-L-phenylglycyl)-N-(3-benzyloxypicolinyl)-L-threonyl-D-α-aminobutyryl-L-prolyl-N-methyl-L-phenylalanyl-L-prolinate (1.2 g., 1.1 mmoles) was dissolved in anhydrous trifluoroacetic acid (7.5 ml.), and the solution was held at room temperature for 15 min. Ether (250 ml.) was added, and after 0.5 hr. at room temperature the white precipitate which formed was filtered, washed with ether, and dried *in vacuo* over potassium hydroxide. This dried product was dissolved in methanol (60 ml.) and stirred with Amberlyst XN-1003 (Rohm and Haas, Philadelphia, Pa.) (6.6 g., previously washed with methanol) for 3 hr. at room temperature. The resin was filtered off, the filtrate was concentrated to dryness *in vacuo*, and the residue was freeze-dried from dioxane, yield 870 mg. (90%), $[\alpha]^{24}$D −44.5° (*c* 1, dimethylformamide). For analysis a sample was dried *in vacuo* at 110°.

Anal. Calcd. for $C_{49}H_{57}N_7O_{10}$: C, 65.18; H, 6.36; N, 10.86; mol. wt., 903. Found: C, 65.33; H, 6.40; N, 10.48; neut. equiv. (sodium hydroxide in ethanol), 915.

N-(3-Hydroxypicolinyl)-L-threonyl-D-α-aminobutyryl-L-prolyl-N-methyl-L-phenylalanyl-L-prolyl-L-phenylglycine Threonine Hydroxyl Lactone (I). O-(L-Phenylglycyl)-N-(3-benzyloxypicolinyl)-L-threonyl-D-α-aminobutyryl-L-prolyl-N-methyl-L-phenylalanyl-L-proline (2.8 g., 3.1 mmoles) and cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate[23] (7 g., 16 mmoles) were dissolved in dichloromethane (4 l.). After 3 days standing at room temperature, 50% aqueous acetic acid (40 ml.) was added and the mixture was stirred for 2 more hr. After this period the solvent was removed *in vacuo*, and the residue was dissolved in ethyl acetate. The resulting solution was extracted with a saturated solution of $NaHCO_3$ (50 ml.) and then with water (50 ml.). The organic layer was dried over magnesium sulfate and the solvent was evaporated *in vacuo*. The residue was dissolved in absolute ethanol (100 ml.) and after addition of 10% palladium on charcoal (300 mg.) the mixture was stirred in an atmosphere of hydrogen for 4.5 hr. The catalyst was filtered off and the filtrate was concentrated to dryness *in vacuo*. The material thus obtained and the products from two more similar batches were pooled (7.3 g.) and distributed in the system chloroform–hexane–methanol–water (4:6:8:2) for 250 transfers. Two peaks were observed with K = 0.04 and 1.1. The former was a mixture of polar side products, *e.g.*, polymers, substituted acylurea, etc. The peak with K = 1.1, which showed an experimental curve in good agreement with the calculated curve, corresponded to the desired cyclic product, as demonstrated by the analytical data given below. The contents of the corresponding tubes were pooled and concentrated to dryness to give 2.55 g. (34%). This material crystallized from methanol in elongated prisms, yield 2.25 g. (31%), m.p. 163–165° sintering from 145°. For analysis a sample was dried *in vacuo* at 110° without change in the melting point, $[\alpha]^{26}$D −61.2° (*c* 1, in absolute ethanol), λ_{max} in ethanol 304 mμ (ϵ 7560) and in 0.1 *N* sodium hydroxide 332 mμ (ϵ 8350). The infrared spectrum shows a lactone carbonyl band at 5.70 μ.

Anal. Calcd. for $C_{42}H_{49}N_7O_9$: C, 63.37; H, 6.20; N, 12.32; NCH_3, 1.9; mol. wt., 796. Found: C, 63.16; H, 6.41; N, 12.08; NCH_3, 3.2; mol. wt., (Rast) 785; neut. equiv. (sodium hydroxide in ethanol), 806; neut. equiv. (perchloric acid in acetic acid), 804.

Quantitative amino acid analysis showed the presence of threonine, phenylglycine, α-aminobutyric acid, and proline in the ratio 1.0:1.0:1.0:2.1.

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