Anal. Calcd. for $C_{13}H_{18}O_8N_2S$: C, 43.09; H, 5.01; N, 7.73; S, 8.85. Found: C, 43.30; H, 5.26; N, 7.51; S, 8.54.

1-(2′-*O*-Mesyl-β-D-xylofuranosyl)-uracil (XVIII).—The mesyl-isopropylidene derivative (XVII, 51 mg.) was added to 50 ml. of 90% ethanol containing 5 drops of concentrated hydrochloric acid. The clear solution was refluxed for 15 minutes, then concentrated to a light acidic sirup to which benzene was added repeatedly and evaporated to obtain a glass. 2′-*O*-Mesyl-xylosyluracil could not be crystallized. It was demonstrated by paper electrophoresis (*p*H 6, borate buffer)[18] that XVII was absent and that only XVIII was present as one ultraviolet absorbing spot. The yields were shown to be quantitative by the amount of sodium hydroxide consumed in a subsequent reaction; XVIII was converted *exclusively* to 1-β-D-lyxofuranosyluracil.

2,2′-Anhydro-1-(β-D-lyxofuranosyl)-uracil (XIX).—A glass containing 0.0009 mole of XVIII obtained from the deacetonation of XVII was placed in 12 ml. of water. Sodium hydroxide (0.02 *N*) was added dropwise to the stirred solution until the consumption of alkali (35 ml.) ceased (phenolphthalein indicator). The neutral solution was concentrated to dryness and 40 ml. of ethanol was added. The insoluble sodium mesylate was filtered and discarded. The filtrate was again evaporated to about 10 ml. when crystallization of prisms occurred. An additional crop was obtained from the mother liquor giving a total yield of 35%. Recrystallization of XIX from 95% ethanol afforded a pure sample, m.p. 252.5–253° dec.

Anal. Calcd. for $C_9H_{10}O_5N_2$: C, 47.79; H, 4.46; N, 12.39. Found: C, 47.98; H, 4.46; N, 12.70.

Paper electrophoresis (0.1 *M* sodium borate[18]) revealed in the mother liquor another component which migrated similarly to the 2′,3′-epoxide of 1-β-D-lyxosyluracil,[24] and which, upon heating for 10 minutes in dilute alkali, was unaltered.

1-β-D-Lyxofuranosyluracil (XX).—An aqueous solution (10 ml.) of the 2′-*O*-mesyl-xylosyluracil (XVIII, 0.00014 mole) was refluxed for 3 hours. The course of the reaction was followed chromatographically and the end-point was determined when only one ultraviolet-absorbing spot, migrating identically with authentic 1-β-D-lyxofuranosyluracil,[8] remained. Exactly one molecular equivalent of methylsulfonic acid was liberated, as shown by titration with 0.01 *N* sodium hydroxide (methyl red). The neutral solution was taken to dryness *in vacuo*, ethanol added and warmed. Insoluble sodium mesylate was filtered and the filtrate concentrated to about 2 ml., whereupon crystallization occurred. The crude material (26 mg.) melted at 193–195°. Recrystallization from absolute ethanol afforded pure compound, m.p. 200–202°. A mixture of XX and an authentic sample[8] did not depress the melting point.

1-β-D-Lyxofuranosyluracil was also prepared from the anhydronucleoside intermediate XIX. Treatment of XIX with warm, dilute alkali for 10 minutes followed by paper electrophoresis of the reaction solution revealed only one spot (borate buffer, *p*H 6)[18] corresponding to that for XX.

2,3′-Anhydro-1-(2′,5′-di-*O*-benzoyl-β-D-xylosyl)-uracil (XXII). Method A.—Benzoyl chloride (0.48 ml.) was added slowly to a stirred solution of 3′-*O*-mesyluridine (X, 0.64 g.) in 30 ml. of dry pyridine. The reaction mixture was kept at 50–55° for 20 hours, after which it was poured into a stirred ice–water mixture. The product was extracted with chloroform, washed with 2 *N* sulfuric acid, then with saturated bicarbonate solution and finally with water. 1-(2′,5′-Di-*O*-benzoyl-3′-*O*-mesyl-β-D-ribosyl)-uracil (XXI) was not obtained in crystalline form. A dried glass (1.1 g.) of XXI was treated with 1.5 g. of sodium benzoate in 20 ml. of DMF for 1 hour at 110° (internal temperature). The mixture was poured into approximately 500 ml. of water, stirred and cooled overnight. The solids were collected on a Celite pad and washed thoroughly with water. The residue was dissolved in chloroform, dried over sodium sulfate and concentrated to a sirup *in vacuo*. Two crystallizations from ethanol affored a 10% yield of XXII, m.p. 251–252° with resolidification to colorless needles which melted at 270–272° dec. Further recrystallizations did not alter the melting point. The ultraviolet spectral data for XXII are given in Table I (ratio of 230/260 mμ = 5.40). The infrared spectrum (KBr disk) of XXII is shown in Fig. 6.

Anal. Calcd. for $C_{23}H_{18}O_7N_2$: C, 63.65; H, 4.17. Found: C, 63.57; H, 4.29.

Method B.—2,3′-Anhydro-1-(β-*D*-xylofuranosyl)-uracil (0.1 g.) was suspended in 4 ml. of dry pyridine and treated with 0.15 ml. of benzoyl chloride. The stirred mixture was allowed to remain at 40° overnight. Fine needle crystals separated and were collected on a filter (80 mg.) and washed well with ethanol. Recrystallization from ethanol gave pure material, with identical melting point, ultraviolet and infrared spectral properties as the sample obtained by method A.

2,2′-Anhydro-1-(3′,5′-di-*O*-benzoyl-β-D-arabinosyl)-uracil (XXIV).—A sample of XXII was placed in a tube and immersed in a bath. The bath temperature was raised cautiously to 252° whereupon the solid melted and resolidified into needles. The bath temperature was maintained at 252° for approximately 3 minutes. The product was recrystallized from absolute ethanol to give pure XXIV, m.p. 270–272° (without pre-melting). The pure material gave no melting-point depression when admixed with an authentic sample of XXIV.[15] The ultraviolet absorption spectrum also agreed with that previously listed[15] (found 230/260 mμ = 4.52, reported 4.59). The infrared spectrum of XXIV (see Fig. 6) is significantly different from that exhibited by XXII (KBr disk). The infrared spectrum of XXIV was similar to that obtained with an authentic sample of XXIV prepared previously by another route.[15]

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA]

Synthesis of a Heptapeptide Sequence Derived from Bovine Insulin[1]

BY JAMES E. SHIELDS[2] AND FREDERICK H. CARPENTER

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The synthesis of glycyl-phenylalanyl-phenylalanyl-tyrosyl-threonyl-prolyl-lysine (all L), a heptapeptide sequence from bovine insulin, is described. The synthetic material appeared identical with the natural heptapeptide released by the action of trypsin on bovine insulin. The result represents a synthetic confirmation of this part of the insulin structure. A unique feature of the synthesis involved the use of the *p*-nitrobenzyloxycarbonyl group to cover the ε-amino function of the lysine during the preferential removal of a carbobenzoxy group from the α-amino function of lysine-containing peptides. Experiences in the preparation of *p*-nitrobenzyl esters of peptides and their use in peptide synthesis are described.

Since the proposal of the complete amino acid sequence of bovine insulin by Sanger and co-workers in 1955,[3] a number of synthetic studies on various parts of the amino acid sequence have appeared.[4] However, to date, none of these synthetic

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(2) National Science Foundation predoctoral fellow, 1956–1959.

(3) A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai, *Biochem. J.*, **60**, 541 (1955).

(4) D. Chillemi, L. Scarso and E. Scoffone, *Gazzetta chim. itali.*, **87**, 1356 (1957); *C. A.*, **52**, 14540 (1958); J. A. Maclaren, W. E. Savige and J. M. Swan, *Australian J. Chem.*, **11**, 345 (1958); G. F. Holland

peptides have been compared with products obtained by cleavage of the insulin molecule. Although confirmation of the proposed structure of the whole insulin molecule by synthetic methods may be beyond the scope of present techniques, still it should be possible to confirm the sequence in parts of the chain by the synthetic approach. Of interest in this connection is the observation that trypsin catalyzes the hydrolysis of insulin at the COOH-terminal end of the B-chain (phenylalanyl-chain) to liberate desocatepeptide-insulin, alanine and the heptapeptide, glycyl-phenylalanyl-phenylalanyl-tyrosyl-threonyl-prolyl-lysine.[5,6]

This reaction has recently been studied in detail by Nicol and Smith[7] at Cambridge and by Young and Carpenter[8] of this Laboratory. In connection with studies now in progress on the properties of the heptapeptide, a good supply of this compound was needed. Rather than isolate the heptapeptide from insulin, we decided to undertakes its synthesis since this would not only give adequate amounts of the material for study but might also lead to a synthetic confirmation of this part of the insulin structure. The present paper describes the synthesis of the heptapeptide.

In the synthesis of the heptapeptide, which is outlined in Fig. 1, we selected a route involving the coupling of a derivative of the tetrapeptide, glycyl-phenylalanyl-phenylalanyl-tyrosine, with the tripeptide, threonyl-prolyl-lysine. This route was chosen from the point of view of costs of starting material (especially threonine) and also because the synthesis of the tetrapeptide appeared to be a straight-forward problem whereas the synthesis of threonyl-prolyl-lysine promised (and in fact turned out) to be a somewhat more difficult task. The tetrapeptide was prepared by coupling derivatives of the two dipeptides, glycyl-phenylalanine and phenylalanyl-tyrosine. The derivative of the tripeptide, threonyl-prolyl-lysine, was built up by sequential addition at the amino end, starting with lysine. In order to minimize the possibility of racemization, the azide procedure was used wherever two peptides were connected.[9] Coupling of amino acid derivatives was accomplished by carboxyl activation through the use of either the acid chloride, *p*-nitrophenyl ester[10] or di*cyclo*hexylcarbodiimide.[11]

The *p*-nitrobenzyloxycarbonyl-group[12] (abbreviated as "PNBC") was used to protect the amino function in a number of steps. Berse, Boucher and Piche[13c] have reported that the PNBC groups are more readily removed by hydrogenolysis than the carbobenzoxy group. On the other hand, in the cleavage of the PNBC-group by hydrogen bromide in acetic acid, Gish and du Vigneaud[13b] used somewhat more vigorous conditions than those normally used for the removal of the carbobenzoxy group.[14] The marked stability of the PNBC-group to acid cleavage was noted in the present work where it was necessary to heat PNBC-phenylalanyl-tyrosine methyl ester (IV) for 2 hr. at 60° in 4 *N* hydrogen bromide in acetic acid in order to obtain a good cleavage of the group. This difference in stability to acid cleavage between the carbobenzoxy group and the PNBC-group was exploited in the synthesis of the tripeptide. The ε-amino group of lysine was covered by the PNBC group while the carbobenzoxy group was preferentially cleaved from carbobenzoxy-prolyl-N$^{\epsilon}$-PNBC-lysine (VIII) and carbobenzoxy-threonyl-prolyl-N$^{\epsilon}$-PNBC-lysine *p*-nitrobenzyl ester (XI). After the latter compound had been coupled with the tetrapeptide, all of the protecting groups were removed by palladium catalyzed hydrogenolysis. This procedure avoided the use of a sodium and liquid ammonia reduction which would have been required if the *p*-toluenesulfonyl group had been used to protect the N$^{\epsilon}$-amino function of lysine. Although the use of the PNBC-group to cover the ε-nitrogen of lysine during peptide synthesis was successful in the present instance, it cannot be recommended as a general procedure where a carbobenzoxy group must be removed preferentially. The difference in the rate of acid cleavage between the PNBC and carbobenzoxy group was not as great as could be desired in that some of the PNBC group was also

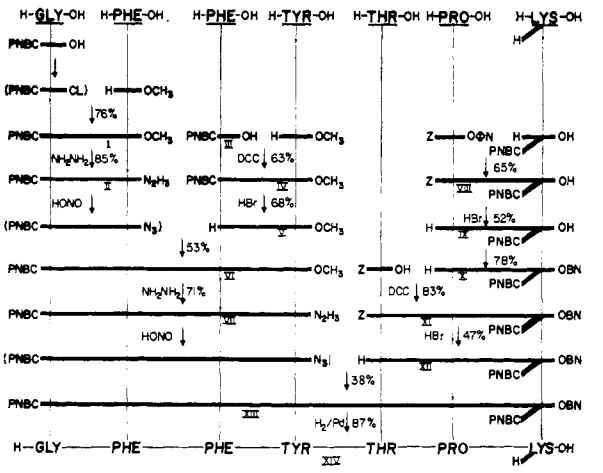

Fig. 1.—Schematic diagram of the synthesis of the heptapeptide. Yields are the average obtained in several runs. PNBC = *p*-nitrobenzyloxycarbonyl-; Z = carbobenzoxy-; -φN = *p*-nitrophenyl-; -BN = *p*-nitrobenzyl-; DCC = di*cyclo*hexylcarbodiimide.

and L. A. Cohen, *J. Am. Chem. Soc.*, **80**, 3765 (1958); G. L. Tritsch and D. W. Woolley, *ibid.*, **82**, 2787 (1960); H. Zahn and N. H. La France, *Ann.*, **630**, 26, 37 (1960); P. G. Katsoyannis, *J. Polymer Sci.*, **49**, 51 (1961).

(5) All the amino acids discussed in this paper are of the L-configuration.

(6) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951); J. I. Harris and C. H. Li, *J. Am. Chem. Soc.*, **74**, 2945 (1952).

(7) D. S. H. W. Nicol and L. F. Smith, *Biochem. J.*, **64**, 17 P (1956); D. S. H. W. Nicol, *ibid.*, **75**, 395 (1960).

(8) J. D. Young and F. H. Carpenter, *J. Biol. Chem.*, **236**, 743, (1961).

(9) M. Goodman and G. W. Kenner, *Advances in Protein Chem.*, **12**, 465 (1957); K. Hofmann, T. A. Thompson, H. Yajima, E. T. Schwartz and H. Inouye, *J. Am. Chem. Soc.* **82**, 3715 (1960).

(10) M. Bodanszky, *Nature*, **175**, 685 (1955); B. Iselin. W. Rittel, P. Sieber and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).

(11) J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955); H. G. Khorana, *Chemistry and Industry*, 1087 (1955).

(12) F. H. Carpenter and D. T. Gish, *J. Am. Chem. Soc.*, **74**, 3818 (1952).

(13) (a) D. T. Gish and F. H. Carpenter, *ibid.*, **75**, 950 (1953); (b) D. T. Gish and V. du Vigneaud, *ibid.*, **79**, 3579 (1957); (c) C. Berse, R. Boucher and L. Piche, *J. Org. Chem.*, **22**, 805 (1957).

(14) D. Ben-Ishai and A. Berger, *ibid.*, **17**, 1564 (1952).

cleaved during the removal of the carbobenzoxy group. In the present synthesis the procedure was successful because the various products of hydrogen bromide cleavage could be readily separated from one another, but this may not always be the case. The possibility still remains to be explored that because of its enhanced acid stability the PNBC group may be used advantageously to cover the ε-nitrogen of lysine (or other similar groups) while acid labile groups such as formyl,[15] *t*-butyloxycarbonyl[16] and trityl[17] are employed to temporarily block other amino functions.

In one step of the synthesis of the tripeptide, carbobenzoxy-proline *p*-nitrophenyl ester was coupled with N^{ϵ}-PNBC-lysine using dimethylformamide as a solvent in the presence of triethylamine. This involves the coupling of an active ester with the α-amino group of an amino acid bearing a free α-carboxyl group. The procedure offers advantages in ease of purification which are not found when an active ester is used to form a neutral peptide derivative. In such cases it may prove to be difficult to separate the products from any unreacted starting materials. We encountered this difficulty when carbobenzoxy-proline *p*-nitrophenyl ester was coupled with N^{ϵ}-PNBC-lysine *p*-nitrobenzyl ester. However, when the coupling was performed with the unesterified compound (N^{ϵ}-PNBC-lysine), one of the reactants is a neutral compound and the other is an amphoteric compound while the product is an acidic compound which can be separated from unreacted starting materials by simple extraction procedures.

In order to avoid an alkaline saponification step at the end, we desired to cover the terminal carboxyl with a group that could be removed by hydrogenolysis. Benzyl esters have frequently been used in such circumstances.[18] However, benzyl esters are prone to cleavage with the hydrogen bromide–acetic acid treatment used to remove the carbobenzoxy group in intermediates peptides.[14,19] From the point of view of acid stability the *p*-nitrobenzyl esters are preferred.[20] In connection with the present investigation, an improved synthesis of the *p*-nitrobenzyl esters of amino acids was devised. This procedure, which has already been reported,[21] involves the formation of the ester by an azeotropic distillation procedure. In the synthesis of the heptapeptide, this azeotropic method was applied with success in the esterification of a *peptide*. Thus, prolyl-N^{ϵ}-PNBC-lysine was converted in 89% yield to its *p*-nitrobenzyl ester. The ready formation of these *p*-nitrobenzyl esters under mild conditions should make them more attractive for use in peptide syntheses.

In the synthesis of the heptapeptide the lowest yield was encountered in the azide coupling of the protected tetrapeptide VIII with the tripeptide XII. Only a 35 to 45% yield of the crystalline protected heptapeptide was obtained from this reaction. The hydrogenolysis of the protected heptapeptide XIII proceeded smoothly, and the heptapeptide was isolated in crystalline form as its acetate. The synthetic product was indistinguishable in paper chromatography from a sample of the natural material prepared by Young and Carpenter[8] by tryptic cleavage of insulin.

Experimental

Melting points are uncorrected. Elementary analyses were performed at the Microchemical Laboratory, Chemistry Department, University of California, Berkeley. Amino acid analyses were performed on samples that had been hydrolyzed *in vacuo* for 24 hours at 110° in constant boiling hydrochloric acid. The method of Spackman, Stein and Moore[22] was used with a Beckman/Spinco Model 120 Amino Acid Analyzer.[23] Results are reported as the ratio of the various components to glycine, assuming a value of unity for this amino acid. The small amounts of ammonia formed from the decomposition of threonine and tyrosine during the hydrolysis procedure are also reported.

***p*-Nitrobenzyloxycarbonyl-glycyl-L-phenylalanine Methyl Ester (I).**—PNBC-Glycyl chloride,[12] prepared from 5.68 g. (0.022 mole) of PNBC-glycine, was reacted with methyl L-phenylalanate, prepared by the method of Fischer and Fourneau[24] from 6 g. (0.028 mole) of L-phenylalanine methyl ester hydrochloride which had been prepared by the thionyl chloride method.[25] The acyl chloride in ether was added to the solution of ester in 50 ml. of ether. Triethylamine (4.1 ml., 0.03 mole) was added to the reaction mixture. A voluminous white precipitate formed at once. The reaction mixture was allowed to stand at room temperature for 0.5 hr. at which time, the solids were filtered and taken up in ethyl acetate and 1 *N* hydrochloric acid. The ethyl acetate solution was washed in succession with 1 *N* hydrochloric acid, water, 1 *N* sodium bicarbonate and water and dried over anhydrous magnesium sulfate. Evaporation of the solvent left an oily residue which gradually crystallized; wt. 7.37 g., 78%, m.p. 109–111°. Yields varied from 69 to 81%. A sample was recrystallized from ethanol for analysis; m.p. 109–112°, $[\alpha]^{23}D$ +57.8° (*c* 2, chloroform).

Anal. Calcd. for $C_{20}H_{21}N_3O_7$: C, 57.84; H, 5.10; N, 10.12. Found: C, 58.18; H, 5.16; N, 10.31.

***p*-Nitrobenzyloxycarbonyl-glycyl-L-phenylalanine Hydrazide (II).**—To a solution of 5 g. (0.011 mole) of PNBC-glycyl-L-phenylalanine methyl ester (I) in 50 ml. of warm ethanol was added 1.0 ml. (0.03 mole) of 95% hydrazine. After the reaction mixture had remained overnight at room temperature, long needles of the hydrazide formed; yield 4.08 g., 81%, m.p. 148–153°. A second and a third crop were obtained from the mother liquors; combined wt. 0.62 g., 12%. The three crops were combined and recrystallized from 150 ml. of hot 95% ethanol to give 4.52 g., 90%, m.p. 152–155°. Yields varied from 63 to 90% with an average of 85%. A sample was recrystallized from ethanol and dried at 100° *in vacuo* over phosphoric anhydride for analysis, m.p. 164–166°, $[\alpha]^{24}D$ +5.3° (*c* 4, acetic acid).

Anal. Calcd. for $C_{19}H_{21}N_5O_6$: C, 54.93; H, 5.10; N, 16.86. Found: C, 55.27; H, 5.22; N, 16.64.

***p*-Nitrobenzyloxycarbonyl-L-phenylalanine (III).**—This compound was prepared according to the procedure of Gish and Carpenter[13a] for the DL-compound, starting with 8.25 g. (0.05 mole) of the L-isomer. In purifying the product by

(15) J. C. Sheehan and D. D. H. Yang, *J. Am. Chem. Soc.*, **80**, 1154 (1958).

(16) F. C. McKay and N. F. Albertson, *ibid.*, **79**, 4686 (1957); G. W. Anderson and A. C. McGregor, *ibid.*, **79**, 6180 (1957).

(17) A. Hillmann-Elies, G. Hillmann and H. Jatzkewitz, *Z. Naturforschung*, **8b**, 445 (1953); L. Zervas and D. M. Theodoropoulos, *J. Am. Chem. Soc.*, **78**, 1359 (1956).

(18) M. Bergmann, L. Zervas and L. Salzmann, *Ber.*, **66**, 1288 (1933); M. Bergmann and J. S. Fruton, *J. Biol. Chem.*, **117**, 189 (1937).

(19) D. Ben-Ishai, *J. Org. Chem.*, **19**, 62 (1954).

(20) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **42**, 972 (1959); H. Schwarz and K. Arakawa, *J. Am. Chem. Soc.*, **81**, 5691 (1959).

(21) J. E. Shields, W. H. McGregor and F. H. Carpenter, *J. Org. Chem.*, **26**, 1491 (1961).

(22) D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

(23) We wish to thank Mr. A. Hirotoshi Nishikawa for performing the amino acid analyses.

(24) E. Fischer and E. Fourneau, *Ber.*, **34**, 2868 (1901).

(25) R. A. Boissonnas, St. Guttmann, P. A. Jaquenoud and J. P. Waller, *Helv. Chim. Acta*, **39**, 1421 (1956).

extractions between ethyl acetate and aqueous bicarbonate, three phases formed. The middle one contained much of the ethyl acetate, some water and all of the product. This phase was separated and evaporated *in vacuo*. The oily residue was dissolved in 50 ml. of water, acidified with 1 *N* hydrochloric acid and extracted with ethyl acetate. The ethyl acetate solution was washed with 1 *N* hydrochloric acid and water, then extracted with 1 *N* sodium bicarbonate solution. The middle and lower phases were washed with ethyl acetate and then acidified. A white solid precipitated; yield 15 g., 88%.

A sample was recrystallized from hot 95% ethanol (2.0 g., from 20 ml.). The material was dried *in vacuo* over phosphorus pentoxide at room temperature; yield 0.82 g., 41% recovery, m.p. 149.5–151.5°, $[\alpha]^{24}D$ −27.9° (*c* 3, 1 *N* potassium hydroxide).

Anal. Calcd. for $C_{17}H_{16}N_2O_6$: C, 59.30; H, 4.68; N, 8.14. Found: C, 58.68; H, 4.77; N, 8.00.

p-Nitrobenzyloxycarbonyl-L-phenylalanyl-L-tyrosine Methyl Ester (IV).—Compound III (2.36 g., 6.8 mmoles) was dissolved in distilled acetonitrile by heating. L-Tyrosine methyl ester hydrochloride, prepared by the thionyl chloride method,[25] (1.44 g., 7 mmoles) was suspended in 100 ml. of acetonitrile and treated with 0.97 ml. (7.5 mmoles) of triethylamine. Di*cyclo*hexylcarbodiimide (1.44 g., 7 mmoles) was dissolved in *ca.* 5 ml. of acetonitrile, and the three solutions were cooled in an ice-bath and mixed. The reaction mixture was stirred overnight at + 3°. After filtration to remove di*cyclo*hexylurea, the solution was evaporated *in vacuo*, and the yellow residue was dissolved in ethyl acetate. The ethyl acetate solution was washed with water, 1 *N* hydrochloric acid, water, 1 *N* sodium bicarbonate and water. After the solution had been dried over anhydrous magnesium sulfate and filtered, the solvent was removed *in vacuo* and the residue was crystallized from 17 ml. of hot 95% ethanol; wt. 2.91 g., 82%, m.p. 161–163°. Preparations of this peptide on a scale 5 times this large gave products in yields of 60–70%, m.p. 160–161°. Samples were dried *in vacuo* at 78° for analysis, $[\alpha]^{23}D$ + 41.3° (*c* 2, chloroform).

Anal. Calcd. for $C_{27}H_{27}N_3O_8$: C, 62.19; H, 5.03; N, 8.06. Found: C, 62.15; H, 5.23; N, 7.86.

L-Phenylalanyl-L-tyrosine Methyl Ester Hydrobromide (V).—PNBC-L-Phenylalanyl-L-tyrosine methyl ester (IV) (3.40 g., 6.5 mmoles) was heated in 23 ml. of 35% by weight hydrogen bromide in glacial acetic acid at 65° for 2 hr. After concentration of the reaction mixture *in vacuo*, the residual oil was refluxed in 30 ml. of 1 *N* methanolic hydrogen bromide for 15 min. The alcohol was removed *in vacuo*, and the oil was crystallized by repeated precipitations from methanol with ether; wt. 1.44 g., 52%, m.p. 201–203°, $[\alpha]^{23}D$ +9.7° (*c* 2, methanol). A second crop was obtained from the mother liquors; wt. 0.60 g., 12%. Yields varied widely, 31 to 95%, with an average of 68%. A sample with m.p. 197–200° was submitted for analysis.

Anal. Calcd. for $C_{19}H_{23}N_2O_4Br$: N, 6.62; Br, 18.88. Found: N, 6.80; Br, 19.02.

p-Nitrobenzyloxycarbonyl-glycyl-L-phenylalanyl-L-phenylanyl-L-tyrosine Methyl Ester (VI).—All reagents were precooled and all operations were performed in the cold room at +3°. PNBC-Glycyl-L-phenylalanine hydrazide (II) (2.13 g., 5 mmoles) was suspended in 50 ml. of water and 18 ml. of 1 *N* hydrochloric acid. The mixture was cooled in an ice-bath and treated with 0.42 g. (6 mmoles) of sodium nitrite dissolved in 2 ml. of water. After allowing the reaction to proceed for a few minutes, the azide was extracted from the mixture with ethyl acetate, which was washed with 1 *N* sodium bicarbonate and water. The ethyl acetate solution was dried with anhydrous magnesium sulfate and filtered. L-Phenylalanyl-L-tyrosine methyl ester hydrobromide (V) (2.13 g., 5 mmoles) was dissolved in 50 ml. of redistilled tetrahydrofuran. Triethylamine (0.83 ml., 6 mmoles) was added, and the solution of the ester was mixed with the azide. The mixture was kept at +3° overnight and then concentrated *in vacuo*. The residue was taken up in 75 ml. of ethyl acetate and the solution was washed with water, 1 *N* hydrochloric acid, water, 1 *N* sodium bicarbonate and water and then dried over anhydrous magnesium sulfate. The solvent was evaporated *in vacuo*, the residue was dissolved in 40 ml. of hot ethanol, and water was added to incipient opalescence. A granular white gel separated and was dried *in vacuo* over phosphorus pentoxide; wt. 2.02 g., 55%, m.p. 133–140°. Yields varied from 41 to 67%. A sample was recrystallized twice from ethanol–water; m.p. 141.5–143°, $[\alpha]^{25}D$ −20.8° (*c* 3, acetonitrile).

Anal. Calcd. for $C_{38}H_{39}N_5O_{10}$: C, 62.89; H, 5.42; N, 9.65. Found: C, 62.61; H, 5.77; N, 10.19.

p-Nitrobenzyloxycarbonyl-glycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosine Hydrazide (VII).—The protected tetrapeptide methyl ester VI (4.00 g., 5.5 mmoles) was dissolved in 100 ml. of hot ethanol. Hydrazine (2.0 ml., 0.06 mole) was added, and the reaction mixture was allowed to stand at 37° for two weeks. In later runs it was found that a reaction time of one week or less sufficed. The gel which formed was then broken up, filtered and dissolved in 300–400 ml. of hot ethanol. On cooling, the gel re-formed and was filtered. The product was dried *in vacuo* over phosphorus pentoxide; wt. 2.50 g., 67%, m.p. 216–219°. Yields varied from 55 to 89%. A sample (0.205 g.) was crystallized from 15 ml. of 95% ethanol and 3 ml. of water; wt. 0.137 g., m.p. 222–224°.

Anal. Calcd. for $C_{37}H_{39}N_7O_9$: C, 61.24; H, 5.42; N, 13.51. Found: C, 60.67; H, 5.26; N, 13.21.

Carbobenzoxy-L-prolyl-N$^{\epsilon}$-(*p*-nitrobenzyloxycarbonyl)-L-lysine (VIII).—N$^{\epsilon}$-PNBC-L-Lysine[21] (1.01 g., 3.1 mmoles) and carbobenzoxy-L-proline *p*-nitrophenyl ester[26] (1.27 g., 3.4 mmoles) were suspended in 25 ml. of distilled dimethylformamide and 2 ml. of triethylamine. The reaction mixture was stirred at 37° overnight, giving a clear yellow solution. The solvent was concentrated slightly *in vacuo* and diluted with several volumes of water containing 6 meq. of potassium carbonate. After the aqueous solution had been washed with ethyl acetate, it was acidified with hydrochloric acid and was cooled. An oil separated and was dissolved in ethanol. Water was added to the solution, and the product crystallized. The crystalline product was dried *in vacuo* over phosphorus pentoxide; wt. 1.33 g., 77%, m.p. 139–41°, $[\alpha]^{24}D$ −29.1° (*c* 2, 0.85 *N* potassium hydroxide). Yields varied from 48 to 77%.

Anal. Calcd. for $C_{27}H_{32}N_4O_9$: C, 58.26; H, 5.80; N, 10.07. Found: C, 57.78; H, 5.71; N, 10.02.

L-Prolyl-N$^{\epsilon}$-(*p*-nitrobenzyloxycarbonyl)-L-lysine (IX).—Carbobenzoxy-L-prolyl-N$^{\epsilon}$-PNBC-L-lysine (VIII) (14.3 g., 0.026 mole) was treated with 70 ml. of *ca.* 2 *N* hydrogen bromide in acetic acid at room temperature for 5 min. The reaction mixture was then poured into 700 ml. of ether. A white solid precipitated and was washed by decantation with fresh ether. The solid was taken up in water, and the solution was adjusted to *p*H 2 to 3 with hydrochloric acid. Some unreacted starting material was filtered off, and the filtrate was neutralized to *p*H 5 to 6 with sodium hydroxide solution. A white precipitate formed and was filtered, and the wet filter-cake was boiled up with 150–200 ml. of water to convert it to a crystalline form; wt. 5.51 g., 51%, m.p. 213–214°, $[\alpha]^{24}D$ −65.4° (*c* 2, 1.5 *N* hydrochloric acid). Yields varied from 42 to 70%. A sample was dried *in vacuo* at 100° for 12 hours for analysis.

Anal. Calcd. for $C_{19}H_{26}N_4O_7$: C, 54.03; H, 6.21; N, 13.27. Found: C, 54.35; H, 6.34; N, 13.34.

L-Prolyl-N$^{\epsilon}$-(*p*-nitrobenzyloxycarbonyl)-L-lysine *p*-Nitrobenzyl Ester Benzenesulfonate (X).—L-Prolyl-N$^{\epsilon}$-PNBC-L-lysine (IX) (2.54 g., 6 mmoles) was suspended in 60 ml. of carbon tetrachloride with 15 g. of *p*-nitrobenzyl alcohol and 1.5 g. (8.5 mmoles) of benzenesulfonic acid monohydrate, and the peptide was esterified according to the procedure for esterifying amino acids, described elswehere.[21] The reaction mixture was refluxed for 2 to 3 days; the reflux condensate passed through a bed of anhydrous calcium sulfate before returning to the pot. After the reaction mixture had cooled, the solution was decanted from the solid, which was dissolved in ethanol and precipitated with ether. The insoluble oil was redissolved in ethanol and a crystalline solid was forced out of solution by the slow addition of about one volume of ether. The product was filtered, washed with ethanolic ether (*ca.* 1:1) and dried *in vacuo* over phosphorus pentoxide; wt. 3.09 g., 71%, m.p. 126–127°, $[\alpha]^{24}D$ −19.2° (*c* 2, dimethylformamide). When the reaction was refluxed 24 hr., the yield was 51%. As described above, the yield varied from 69 to 89%.

(26) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

Anal. Calcd. for $C_{32}H_{37}N_5O_{12}S$: C, 53.70; H, 5.21; N, 9.79; S, 4.48. Found: C, 53.80; H, 5.54; N, 9.93; S, 4.78.

Carbobenzoxy-L-threonyl-L-prolyl-N$^{\epsilon}$-(*p*-nitrobenzyloxycarbonyl)-L-lysine *p*-Nitrobenzyl Ester (XI).—L-Prolyl-N$^{\epsilon}$-PNBC-L-lysine *p*-nitrobenzyl ester benzene-sulfonate (X) (3.61 g., 5 mmoles) and carbobenzoxy-L-threonine[27] (1.65 g., 6.5 mmoles) were dissolved in 50 ml. of distilled methylene chloride. The solution was cooled in an ice-ethanol bath. Di*cyclo*hexylcarbodiimide (1.34 g., 6.5 mmoles) was dissolved in 10 ml. of methylene chloride and added to the reaction mixture. Triethylamine (0.7 ml., 5 mmoles) was added and the reaction mixture was allowed to stand at room temperature overnight. The solvent was evaporated *in vacuo*, and the residue was taken up in ethyl acetate and 1 *N* hydrochloric acid. The mixture was filtered to remove di*cyclo*hexylurea. The ethyl acetate solution was washed with 1 *N* hydrochloric acid and water, then cooled to 5° and washed with cold, freshly prepared 1 *N* potassium bicarbonate and cold water and then dried with anhydrous magnesium sulfate. The solvent was evaporated *in vacuo* to give an amorphous foam; wt. 3.75 g., 93%. Yields varied from 63 to 94%.

Anal. Calcd. for $C_{38}H_{44}N_6O_{13}$: C, 57.57; H, 5.59; N, 10.60. Found: C, 57.95; H, 5.73; N, 10.55.

L-Threonyl-L-prolyl-N$^{\epsilon}$-(*p*-nitrobenzyloxycarbonyl)-L-lysine *p*-Nitrobenzyl Ester Hydrobromide (XII).—The protected tripeptide XI (3.00 g., 3.78 mmoles) was dissolved in 15 ml. of glacial acetic acid. To this was added 35% hydrogen bromide in glacial acetic acid (15 ml.). After 5 min. at room temperature, the reaction mixture was poured into 500 ml. of ether cooled in Dry Ice. The flocculent white precipitate was washed with fresh ether four times by decantation. It was filtered quickly and dried *in vacuo* over phosphorus pentoxide and potassium hydroxide.

The crude solid was dissolved in 50 ml. of the lower phase from equilibration of *n*-butyl alcohol and 1% acetic acid. A two-transfer counter-current distribution was performed manually using 50-ml. phases. The upper phases from tubes 1 and 2 were combined and yielded 2.0 g. of oil on evaporation. This material was dissolved in 50 ml. of water and washed twice with 50-ml. portions of ethyl acetate. The aqueous solution was evaporated and the residual oil was dried *in vacuo* over phosphorus pentoxide; wt. 1.50 g., 53%. Yields varied from 30 to 57%; ave. 47%.

Anal. Calcd. for $C_{30}H_{33}N_6O_{11}Br$: C, 48.72; H, 5.31; N, 11.36; Br, 10.81. Found: C, 48.21; H, 5.91; N, 11.18; Br, 11.15.

***p*-Nitrobenzyloxycarbonyl-glycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-N$^{\epsilon}$-(*p*-nitrobenzyloxycarbonyl)-L-lysine *p*-Nitrobenzyl Ester (XIII).**—The tetrapeptide hydrazide VII (1.44 g., 1.98 mmoles) was dissolved by warming in 75 ml. of 50% aqueous acetic acid containing 7.5 meq. of hydrochloric acid. The solution was cooled to 0° in an ice–ethanol bath and treated in the cold with 150 mg. (2.2 mmoles) of sodium nitrite. After two minutes, water (200 ml.) and ethyl acetate (150 ml.) were added to the reaction mixture. (All solutions and reagents had been pre-cooled to about 0° before use; all operations were performed in the cold room at +3°.) The ethyl acetate solution was washed with cold water, followed by 2 *N* potassium bicarbonate until all effervescence ceased, then with water again. The ethyl acetate solution was dried with anhydrous magnesium sulfate and filtered.

The tripeptide ester derivative XII (1.42 g., 1.91 mmoles) was dissolved in 30 ml. of tetrahydrofuran. Triethylamine (0.3 ml., 2.2 mmoles) was added, and this solution was added to the solution of tetrapeptide azide. The reaction mixture was allowed to stand overnight in a refrigerator. The protected heptapeptide XIII crystallized out of the reaction mixture. After decantation of the solution, the crystalline solid was washed with fresh ethyl acetate and then with water and finally dried *in vacuo* over phosphorus pentoxide; wt. 1.22 g., 47%, m.p. 116–19°, $[\alpha]^{29}D$ −26.5° (*c* 2.5, dimethylformamide).

The ethyl acetate mother liquor was washed with 1 *N* hydrochloric acid, water, 1 *N* potassium bicarbonate and water. It was dried with anhydrous magnesium sulfate, filtered and evaporated *in vacuo*. The residual oil was triturated with 11 ml. of ethanol–ethyl acetate (1:10), whereupon it solidified. The solid was recrystallized from ethanol–ethyl acetate; wt. 0.108 g., 4%, m.p. 121–132°.

A sample, 0.202 g., was recrystallized from 1.0 ml. of hot ethyl acetate–ethanol (1:1) and dried *in vacuo* at 78° overnight for analysis; wt. 0.159 g., m.p. 122–124°.

Anal. Calcd. for $C_{67}H_{73}N_{11}O_{20}$: C, 59.50; H, 5.44; N, 11.39. Found: C, 59.82; H, 5.75; N, 10.88.

Amino acid analysis gave the following ratios to glycine (1.00): phenylalanine (2.13): tyrosine (0.98): threonine (0.96): proline (1.08): lysine (1.10): ammonia (0.09).

Glycyl-L-phenylalanyl-L-phenylalanyl-L-tyrosyl-L-threonyl-L-prolyl-L-lysine Acetate (XIV).—The protected heptapeptide XIII (0.997 g., 0.72 mmole) was suspended in 80 ml. of 95% ethanol and 6 ml. of acetic acid. Palladium oxide[28] (0.20 g.) was added, and the system was flushed with nitrogen. Hydrogen was passed through the reaction mixture at atmospheric pressure with magnetic stirring. After 3 hr., the catalyst had coagulated; fresh catalyst (0.05 g.) was added, and hydrogen was passed through the reaction mixture with stirring for 3 more hr., at which time a small amount of water was added to bring the gel-like free peptide into solution. Hydrogenation was continued for another 2–3 hr. The catalyst was filtered off and the filtrate was evaporated *in vacuo*. The residual oil was triturated with acetone (*ca.* 30 ml.), which treatment caused it to become solid. The product was dried *in vacuo* over phosphorus pentoxide; wt. 0.666 g., m.p. 185–188°. The material was dissolved in hot 85% aqueous ethanol (8 ml.). A white solid separated on cooling; wt. 0.552 g., 87%, m.p. 185–188°, $[\alpha]^{27}D$ −51.0° (*c* 1, 0.02 *N* hydrochloric acid). For analysis, a sample was dried *in vacuo* at 100° for 12 hr.

Anal. Calcd. for $C_{44}H_{58}N_8O_{10}\cdot CH_3COOH$: C, 60.12; H, 6.58; N, 12.21. Found: C, 59.7; H, 6.9; N, 11.9.

Amino acid analysis of this material gave the following ratios to glycine (1.00): phenylalanine (2.03): tyrosine (0.94): threonine (0.88): proline (1.04): lysine (1.04): ammonia (0.15).

The synthetic product was compared with an authentic sample of the heptapeptide prepared by Young and Carpenter[8] by tryptic digestion of bovine insulin. The two materials were found to be indistinguishable by paper chromatography on Whatman No. 1 in five solvent systems: *n*-butanol: acetic acid:water (4:1:1),[29] R_f 0.33; *n*-butanol:acetic acid:water:pyridine (30:6:24:20),[30] R_f 0.61; phenol:-water (160:40),[31] R_f 0.91; ethanol:water:0.9 *N* ammonia (180:10:10),[31] R_f 0.40; *t*-butanol:water:methyl ethyl ketone:diethylamine (80:80:40:8),[32] R_f 0.55.

The infrared spectrum of the synthetic material showed a number of incompletely resolved bands in the 3200, 1650 and 1550 cm.$^{-1}$ regions and very few peaks below 1200 cm.$^{-1}$. The absorption peaks (p) and shoulders (s) of the synthetic material, 0.25% in potassium bromide pellet, were as follows: 3450(s), 3250(p), 3050(s), 2910(s), 1690(s), 1675(s), 1660(s), 1645(s), 1630(p), 1560(s), 1550(p), 1540(p), 1520(s), 1500(s), 1460(s), 1450(p), 1400(p), 1380(p), 1277(s), 1245(p) 920(p), 833(p), 750(p), 696(p) cm.$^{-1}$. The natural heptapeptide had not been isolated in sufficient quantities to prepare it in crystalline form.[8] However, a lyophilized powder of the natural material exhibited an infrared spectrum identical with the synthetic material except for an ill-defined, broad absorption between 1140–1000 cm.$^{-1}$ which was present in the natural material but absent in the synthetic sample. This discrepancy can probably be attributed to a small amount of impurity in the lyophilized sample or to a difference in physical state of the two samples.

(27) R. B. Merrifield, *J. Biol. Chem.*, **232**, 43 (1958).

(28) R. L. Shriner and R. Adams, *J. Am. Chem. Soc.*, **46**, 1683 (1924).

(29) L. J. Reed, *J. Biol. Chem.*, **183**, 451 (1950).

(30) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

(31) I. Smith, "Chromatographic Techniques," Interscience Publishers, Inc., New York, N. Y., 1958, p. 60.

(32) R. R. Redfield, *Biochem. Biophys. Acta*, **10**, 344 (1953).