Neither leucine aminopeptidase nor chymotrypsin showed any hydrolytic action on this peptide, the peptide bond between the N-terminal phenylalanine and the penultimate amino acid being resistant to the action of both enzymes and the internal phenylalanine also being resistant to chymotrypsin.

About 0.72 mg. of the peptide was degraded by the Edman procedure through five steps, the cyclization being effected in acetic acid–HCl. The identification of the PTH amino acid obtained at each step was made by hydrolyzing a portion of the PTH amino acid in constant boiling HCl in a sealed tube at 150° for 16 hr. and chromatographing the amino acid so obtained on Whatman No. 1 paper in the system methanol–water–pyridine (80:20:4) which separates all of the amino acids found in this peptide. The amino acids identified at each step were as follows: 1, phe; 2, pro with a trace of phe; 3, asp with a trace of phe; 4, phe with a trace of ser; 5, ser with a trace of phe and lys. Lysine was identified as the residue left after the fifth step. These results indicated the sequence phe·pro·(asp or aspNH_2)·phe·ser·lys. The yield of the PTH amino acid at each step was low, however, or about one fourth of that expected.

Because of the low yield obtained above, the Edman degradation was repeated using different conditions for the cyclization step. About 1.43 mg. (1.9 μM) was degraded. The cyclization in the first step was brought about in 0.5 N HCl at 37° and through the next five steps in 2 N HCl at room temperature. The yield of the PTH in steps 1 through 6 (in μM) was 1.75, 1.5, 1.2, 1.4, 1.4 and 0.7, respectively, or from about 40–90%. The low yield obtained in the first method apparently was due to the cyclization procedure employed. The identification of the amino acid at each step was the same as that first obtained.

The average of two determinations of amide ammonia content of this peptide was 0.16 mole per mole of peptide which indicated that aspartic acid, not asparagine, was present in the peptide. This fact also was indicated by the fact that the peptide is negatively charged at pH 7, moving toward the anode when subjected to electrophoresis at that pH. Were asparagine present in the molecule, the peptide would be a strongly basic peptide and would be positively charged at pH 7. The amino acid sequence of K-0.33 may therefore be written phe·pro·asp·phe·ser·lys.

The results are summarized in Table I and Table II. The calculated recoveries reported in Table I were obtained by the formula

$$\frac{\text{mol. wt. of peptide}}{18{,}000 - \text{mol. wt. insoluble peptide}} \times 740 \text{ mg.}$$

The total weight of peptide material which had been distributed was approximately 780 mg., about 40 mg. of which had been consumed for preparations of the various distribution curves.[3] The molecular weight of the protein sub-unit is about 18,000 and the "molecular weight" of the insoluble peptide material found in the digest is about 6,000.[2] The various fractions removed from the countercurrent distribution machine usually contained only part of each component of a particular distribution coefficient. The recovery of each purified peptide must therefore be multiplied by an appropriate factor to obtain the weight of the peptide that would have been recovered had all of this component been included in the fraction taken from the machine. Thus, it was estimated that the fraction designated K-0.065 (see Fig. 1) contained about 70% of the two components (K-0.065–2 and K-0.065-7) of this distribution coefficient while about half of the material with a distribution coefficient of 0.33 was estimated to be present in the fractions designated K-0.33a and K-0.33b (see Figs. 3, 16 and 20 of ref. 2).

TABLE II

PEPTIDES ISOLATED FROM A TRYPTIC DIGEST OF TMV PROTEIN

Component	Mol. wt.	Amino acid sequence
K-0.065-2	288.3	aspNH$_2$·arg
K-0.065-7	753.7	gly·thr·ser·gly·tyr·aspNH$_2$·arg
K-0.086-Ma	601.7	thr·val·gluNH$_2$·val·arg
K-0.13	1115.2	arg·val·asp·asp·ala·thr·val·ala·ileu·arg
K-0.22-D	436.5	val·tyr·arg
K-0.33	739.8	phe·pro·asp·phe·ser·lys

It can be seen from an examination of Table I that the peptides reported in this paper were recovered in an amount that would justify the assumption that all were major products of the action of trypsin on the protein sub-unit of TMV and that none were derived from transpeptidation or from impurities.

Acknowledgments.—The authors are indebted to Mrs. Gladys Perez-Mendez and Mr. Yusei Kato for valuable technical assistance.

BERKELEY, CALIF.

[CONTRIBUTION FROM THE RESEARCH DIVISION OF THE CLEVELAND CLINIC FOUNDATION AND THE FRANK E. BUNTS EDUCATIONAL INSTITUTE]

Syntheses of an Optically Pure Tetrapeptide Contained in Angiotensin[1,2]

BY HANS SCHWARZ AND F. MERLIN BUMPUS

RECEIVED AUGUST 4, 1958

The tetrapeptide derivative cbzo-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucine methyl ester was synthesized by the three routes possible, and the products were compared for yield, optical purity and by-products. A similar comparison was made with the two intermediate tripeptides obtained each by two different routes. Mixed anhydride and carbodiimide methods were used exclusively. A mixture of diastereoisomers was obtained with either method whenever an acyl di- or higher peptide was used for condensation. Both diastereoisomers, cbzo-L-valyl-L-tyrosyl-L-isoleucine methyl ester and cbzo-L-valyl-D-tyrosyl-L-isoleucine methyl ester, were isolated in crystalline form from the reaction of cbzo-L-valyl-L-tyrosine and L-isoleucine methyl ester. The best route to synthesize a polypeptide when using either mixed anhydride or carbodiimide method seems to be the one starting with the C-terminal amino acid and adding one amino acid at a time. The products obtained in good yield are optically pure.

In our recent synthesis of the isoleucine angiotensin octapeptide[3] we were handicapped by the fact that all derivatives of peptides containing four or more amino acids were amorphous, gelatinous products. Intermediates of this nature are extremely hard to purify and, as was pointed out recently,[4,5] may contain large amounts of diastereoisomers or other by-products. They, therefore, endanger the outcome of any synthesis in which optical purity is demanded, especially of those in which the end product cannot be compared by biological activity to a naturally occurring poly-

(1) This work was supported in part by the National Heart Institute, U. S. Public Health Service, Grant No. H-96 (C7).

(2) Angiotensin is the term suggested to replace the dual name angiotonin-hypertensin: E. Braun-Menendez and I. H. Page, *Science*, **127**, 242 (1958).

(3) H. Schwarz, F. M. Bumpus and I. H. Page, THIS JOURNAL, **79**, 5697 (1957).

(4) B. F. Erlanger, W. V. Curran and N. Kokowsky, *ibid.*, **80**, 1128 (1958).

(5) K. Hoffmann, M. E. Woolner, G. Spuhler and E. T. Schwartz, *ibid.*, **80**, 1486 (1958).

peptide. To avoid later difficulties all intermediates should be analyzed enzymatically and purified until complete optical purity is obtained. There are several recent examples showing that optically pure peptide derivatives may crystallize rather easily.[4-6]

Most methods of peptide synthesis lead to racemization in varying amounts when the carboxyl carrying component is an acyl di- or higher peptide.[7] The amino acid with the free carboxyl group is racemized while the component carrying the free amino group seems to be unaffected. No racemization is observed when an acyl amino acid is employed. This has been suggested as regards the mixed anhydride method,[8,9] and, independently of us, has been found to apply to the carbodiimide method also.[5,10]

The tetrapeptide derivative cbzo-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucine methyl ester (cbzo = carbobenzyloxy) was selected as a model on which to demonstrate these points further. The above sequence is contained as amino acids 2–5 in isoleucine angiotensin,[11] and a pure derivative would serve as a very convenient intermediate in the synthesis of the hormone.

We have synthesized derivatives of all the combinations contained in the selected tetrapeptide (Fig. 1). Tripeptides and tetrapeptides were furthermore built up by all possible routes. The peptides in bold print are those built up from acyl amino acid + peptide ester and, as expected, were found to be optically pure by enzymatic hydrolysis. Peptides obtained by other routes were compared to these standards in order to detect the presence of diastereoisomers and other by-products. Carbodiimide and mixed anhydride methods were used exclusively.

Cbzo-nitro-L-arginyl-L-valine methyl ester (I) was obtained in 85% yield from cbzo-nitro-L-arginine and L-valine methyl ester with N,N'-dicyclohexylcarbodiimide as condensing agent. The yield is quite remarkable considering the usual low yield obtained from cbzo-nitro-L-arginine.[12] A simple method of converting an ester hydrochloride into the free base was used in this case. Valine methyl ester hydrochloride suspended in tetrahydrofuran was titrated with sodium methoxide using thymol blue as indicator. The solution of valine methyl ester in tetrahydrofuran–methanol obtained after filtration of the precipitated sodium chloride was used directly for the condensation. The methanol present did not decrease the yield since an equal yield was obtained using valine methyl ester extracted into ethyl acetate from a basic solution of its hydrochloride.

Formyl-L-tyrosine was condensed with L-isoleucine methyl ester by the carbodiimide method, yielding formyl-L-tyrosyl-L-isoleucine methyl ester (IV)

(6) R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, **40**, 624 (1957).
(7) H. D. Springall and H. D. Law, *Quart. Revs.*, **10**, 230 (1956).
(8) J. R. Vaughan and J. A. Eichler, THIS JOURNAL, **75**, 5556 (1953).
(9) B. F. Erlanger, H. Sachs and E. Brand, *ibid.*, **76**, 1806 (1954).
(10) G. W. Anderson and F. M. Callahan, *ibid.*, **80**, 2902 (1958).
(11) L. T. Skeggs, W. H. Marsh, J. R. Kahn and N. P. Shumway, *J. Exp. Med.*, **102**, 435 (1955).
(12) K. Hofmann, W. D. Peckham and A. Rheiner, THIS JOURNAL, **78**, 238 (1956).

Cbzo–NO_2–Arg–Val–Tyr–Ileu–Me
1 2 3 4

Dipeptides: 1 + 2, 2 + 3, 3 + 4
Tripeptides: 1−2 + 3, 1 + **2−3**, 2−3 + 4, **2 + 3**−4
Tetrapeptides: 1 + **2 − 3 − 4**, 1 − 2 + 3 − 4, 1 − 2 − 3 + 4

Fig. 1.

in 74% yield. Using O-acetyl-N-formyl-L-tyrosine the yield of dipeptide was about the same, whereas the mixed anhydride method gave only 31% of O-acetyl-N-formyl-L-tyrosyl-L-isoleucine methyl ester. It can be concluded from this that the additional step of converting N-formyl-L-tyrosine into the O-acetyl derivative is unnecessary if N,N'-dicyclohexylcarbodiimide is used as condensing agent. The removal of the formyl group by 1 *N* methanolic HCl was shown by chromatography to be both quantitative and free from side reactions. Neither L-tyrosyl-L-isoleucine methyl ester hydrochloride nor the free base could be obtained in crystalline form.

The tripeptide derivative cbzo-nitro-L-arginyl-L-valyl-L-tyrosine methyl ester (V) obtained from cbzo-nitro-L-arginine and L-valyl-L-tyrosine methyl ester using N,N'-dicyclohexylcarbodiimide as condensing agent crystallized easily in 72% yield. The alternate route starting from cbzo-nitro-L-arginyl-L-valine (II) and L-tyrosine methyl ester using the same method did not yield a crystalline product directly. By chromatography on aluminum oxide, however, crystalline tripeptide derivative V was isolated in 36% yield. In both cases the last crop of tripeptide could be isolated only after alkaline hydrolysis of the mother liquor. The tripeptide esters V obtained by the two different routes had identical melting points and rotations in two solvents. Optical purity was demonstrated on cbzo-nitro-L-arginyl-L-valyl-L-tyrosine (VI) by hydrolyzing the valyl–tyrosine bond with carboxypeptidase and both peptide bonds (after removal of the cbzo-group) by leucine aminopeptidase.

Cbzo-L-valyl-L-tyrosyl-L-isoleucine methyl ester (VII) was obtained in 69% yield from cbzo-L-valine and L-tyrosyl-L-isoleucine methyl ester. A similar peptide, cbzo-S-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucine methyl ester, had been synthesized by Boissonnas *et al.*,[13] from the mixed anhydride of cbzo-S-benzyl-L-cysteinyl-L-tyrosine and freshly distilled isoleucine methyl ester (yield 70%). We have repeated the procedure in detail using cbzo-L-valyl-L-tyrosine (III) and freshly distilled isoleucine methyl ester. A tripeptide (V) identical to the one obtained by the alternate route was isolated but in only 37% yield. The mother liquor was subjected to a fractional crystallization and furnished a second crystalline tripeptide, cbzo-L-valyl-D-tyrosyl-L-isoleucine methyl ester in 11% yield. The same two peptides, in 65 and 10% yield, respectively, were obtained when the carbodiimide method was employed, regardless of whether dimethylformamide or tetrahydrofuran was used as solvent. The two isomeric tripeptides were identical with respect to amino acid composition, microanalysis and R_f values, but different with respect to melting point, rotations in two

(13) R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud and J.-P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).

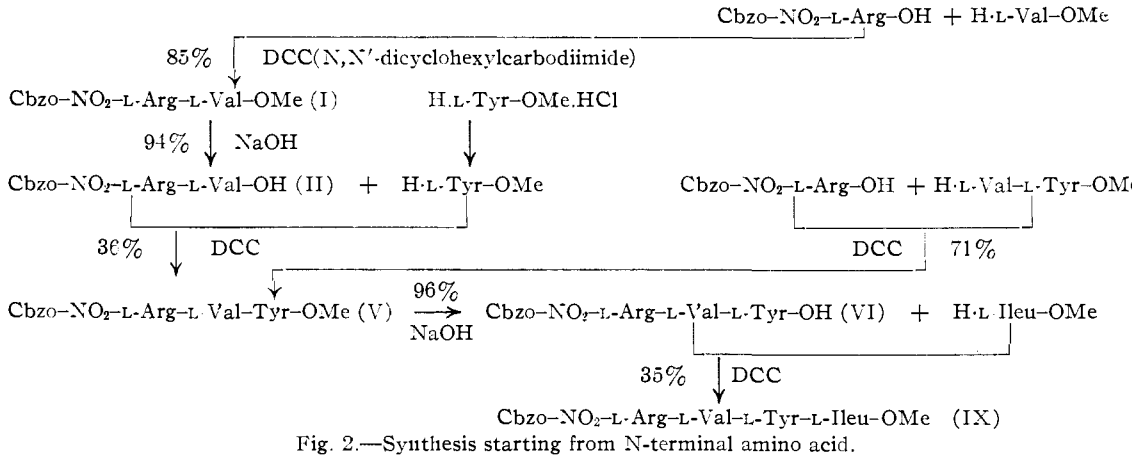

Fig. 2.—Synthesis starting from N-terminal amino acid.

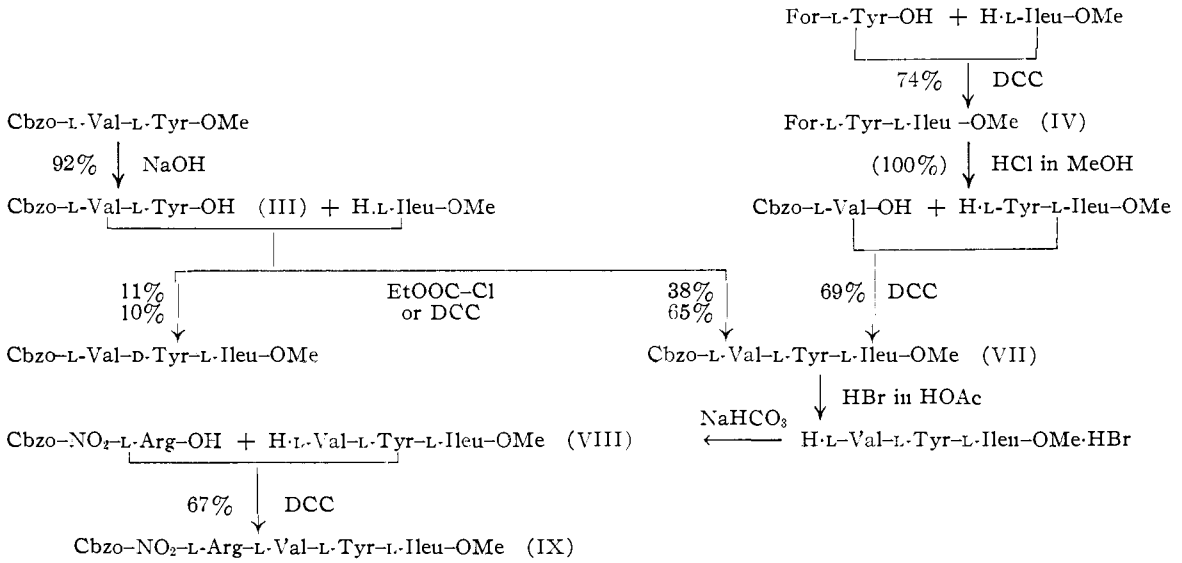

Fig. 3.—Synthesis starting from C-terminal amino acid.

solvents and solubilities. A mixture of the two did not melt below the melting point of the lower melting component. Both of them were optically pure as shown by enzymatic hydrolysis. The L-L-L-tripeptide VII was hydrolyzed completely by chymotrypsin and leucine aminopeptidase; the L-D-L-tripeptide was not at all hydrolyzed by either enzyme.

The cbzo-group was removed from tripeptide VII by HBr in glacial acetic acid. The reaction was practically quantitative (shown by paper chromatography), but recrystallization of the L-valyl-L-tyrosyl-L-isoleucine methyl ester (VIII) could only be accomplished with considerable losses due to its solubility in water.

The tetrapeptide cbzo-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucine methyl ester (IX) was synthesized by three different routes using the carbodiimide method exclusively. As was to be expected the condensation of cbzo-nitro-L-arginine with tripeptide ester VIII gave the best yield (62%) and a product that crystallized spontaneously. After two recrystallizations it was homogeneous and optically pure. The product obtained from cbzo-nitro-L-arginyl-L-valine (II) and L-tyrosyl-L-isoleucine methyl ester could not be crystallized in spite of extensive purification attempts. The amorphous, gelatinous final material still gave rise to two spots on paper chromatography. The impurity was tentatively identified as N-(cbzo-nitro - L - arginyl - L - valyl) - N,N' - dicyclohexylurea. The presence of racemized tetrapeptide (probably containing D-valine) was definitely shown in the mother liquors. From the reaction of cbzo-tripeptide (VI) with isoleucine methyl ester optically pure tetrapeptide IX was isolated in 37% yield. Again racemized tetrapeptide (containing D-tyrosine) was present in the mother liquor.

The final crystalline tetrapeptide derivative IX was analyzed enzymatically as shown in Fig. 4. Usually all protective groups (NO_2, cbzo, Me) are removed before analysis.[5] Since not all of these reactions are quantitative, the possibility remains that impurities or diastereoisomers present in the original peptide derivative are not subjected to the enzymatic analysis. Since we intended to use a derivative of our tetrapeptide, such as the hydrazide, for further synthetic work we placed great emphasis on showing the optical purity of derivative IX.

In order to render the peptide derivative water soluble, the cbzo-group was removed by HBr in

glacial acetic acid, a reaction that can be assumed not to change the optical configuration. The whole reaction product, without any purification, was then incubated with enzyme solutions. As is shown in Fig. 4 each bond was tested separately. Chymotrypsin hydrolyzed the tyrosine–isoleucine bond greater than 95%; only a trace of tetrapeptide was found when 2.5 mg. of hydrolysate was spotted on paper. Carboxypeptidase hydrolyzed the valine–tyrosine bond of the newly formed tripeptide quantitatively; no tripeptide was visible with Pauly reagent.[14] The hydrolysis with leucine aminopeptidase could again be followed by the disappearance of tetrapeptide. It was quantitative and proved the arginine–valine bond to be all L-L.

From the above results several conclusions can be drawn: 1. From all the alternatives of building up a higher peptide the one starting from the C-terminal end and adding one amino acid at a time has several advantages, such as high yields, little or no formation of diastereoisomers and pure, crystallizable products. 2. The fact that racemization occurs when employing either the mixed anhydride or the carbodiimide method with an acyl di- or higher peptide was confirmed. The presence of diastereoisomers was demonstrated in the mother liquors in two cases of carbodiimide condensations; both diastereoisomers were isolated in crystalline form when cbzo-L-valyl-L-tyrosine was condensed with L-isoleucine methyl ester by either method. This latter example may be applicable to the critical evaluation of other methods of peptide synthesis. 3. The two crystalline diastereoisomers did not show a melting point depression and, as tripeptide esters, could not be separated by paper chromatography. This exemplifies the difficulty of recognizing the presence of small amounts of isomers in a peptide derivative by methods other than enzymatic hydrolysis. The inadequacies of syntheses of large peptides from amorphous, insufficiently characterized intermediates should again be emphasized.

Experimental[15]

All peptides were shown to contain the appropriate amino acids in about equivalent amounts by hydrolysis in 6 *N* HCl at 105° for 24 hours followed by chromatography in two solvent systems. All melting points were taken on a Kofler hot-stage and are corrected. Microanalyses were done by Micro Tech Laboratories, Skokie, Ill.

Paper Chromatography of Peptide Derivatives.—Compounds containing a free amino group were chromatographed directly. Cbzo-derivatives were treated as follows: 1 to 10 mg. of peptide derivative was dissolved in 1 ml. of 2 *N* HBr in glacial acetic acid and left for 1 hour at room temperature in a tightly stoppered test-tube. The sample was then evaporated to dryness *in vacuo*, a small amount of benzene added, evaporated again, and this repeated twice more.[16] The residue was dissolved in water, and 50 to 250 γ was spotted for chromatography. Two solvent systems were employed: (a) 1-butanol–acetic acid–water 4:1:5 (BAW); (b) methyl ethyl ketone–pyridine–water 4:1:1.6 (MPW). The chromatograms were sprayed with ninhydrin and/or diazotized sulfanilic acid and/or Folin–Ciocalteu phenol reagent. All compounds, if not otherwise stated, were purified until they showed one spot only in both solvent systems.

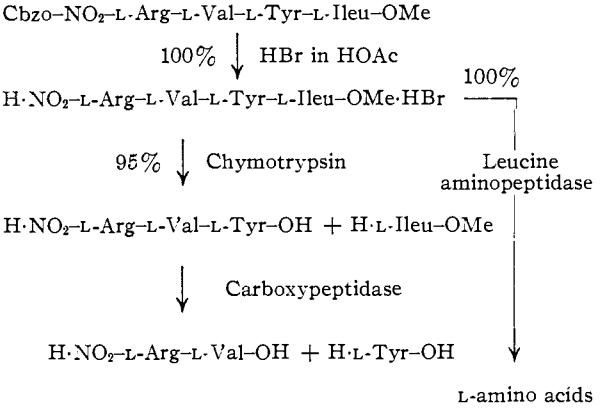

Fig. 4.—Test for optical purity.

Enzymatic Hydrolysis and Test for Optical Purity.—In order to render the peptide derivatives water soluble the cbzo-group was removed as described above prior to incubation with enzymes. Five mg. of crystalline chymotrypsin (Worthington Biochem. Corp.) was dissolved in 5 ml. of 0.01 *M* borate buffer (*p*H 8.1) which was 0.001 molar in $CaCl_2$. One ml. of this enzyme solution was added to 10 mg. of peptide or peptide ester dissolved in 3 ml. of 0.01 *M* borate buffer. Incubation was carried out at 37°. Samples of 0.1-ml. volume were removed at intervals and applied directly to paper. The chromatograms were developed in the BAW system, ascending overnight.

Carboxypeptidase was dissolved in 10% LiCl solution (1 mg./ml.), and 1 ml. of this solution was added to 10 mg. of cbzo-peptide in 3 ml. of 0.01 *M* $NaHCO_3$. The incubation was carried out at 37°, and 0.1-ml. samples were taken out and applied to paper at different time intervals. The chromatograms were developed in the BAW system, ascending overnight.

A mixture of chymotrypsin and carboxypeptidase was made by dissolving 5 mg. of chymotrypsin in 4.8 ml. of 0.01 *M* borate buffer and adding 0.2 ml. (1 mg.) of a carboxypeptidase suspension (Worthington Biochem. Corp.). One ml. of this enzyme mixture was incubated with 10 mg. of peptide substrate prepared as above.

Partially purified leucine aminopeptidase was prepared by two ammonium sulfate precipitations of an extract of an acetone powder of swine kidney.[17] This preparation following dialysis against 0.01 *M* carbonate buffer (*p*H 8.15) containing 0.001 *M* $MgCl_2$ contained 3.3% protein. Peptide (10 mg.) or peptide ester dissolved in 3 ml. of 0.001 *M* carbonate buffer at *p*H 8.1 (*p*H adjusted with Na_2CO_3 if necessary) was incubated with 0.1–0.2 ml. of enzyme solution. Aliquots were taken intermittently and spotted on paper for chromatography. The chromatograms were developed in the BAW system, ascending overnight.

L-Tyrosine Methyl Ester.[18]—L-Tyrosine methyl ester hydrochloride (1.16 g.) was dissolved in 1 ml. of water by warming. The clear solution was cooled to 0°, and 0.7 ml. of triethylamine was added. A crystalline precipitate formed slowly. After standing for about 30 min. at 0° it was collected on a filter and washed with a small amount of ice-water; yield 0.95 g. (dried over P_2O_5). One recrystallization from methanol–ether–petroleum ether gave 0.75 g. (77%), m.p. 136–137°, $[\alpha]^{22}D$ +19.8° (*c* 2.4, pyridine), $R_{f\,BAW}$ 0.61.

L-Isoleucine methyl ester hydrochloride was prepared by a modification of the method by Boissonnas, *et al.*[18] A suspension of 15 g. of L-isoleucine in 35 ml. of absolute methanol was cooled to −10° in a salt–ice-bath, and 12.3 ml. of thionyl chloride was added slowly. The mixture was kept at 40° for 3 hours after which time most of the isoleucine had dissolved. The solution was refluxed for 3 hours, and left at room temperature overnight. It was then evaporated to dryness *in vacuo*, redissolved in absolute methanol and evap-

(14) Nitro-arginyl-valine was not hydrolyzed by carboxypeptidase under these conditions; compare ref. 12.

(15) We wish to express our thanks to Jennifer King, Robert Russell, Mrs. Sally Nousek and Jefferson Jones for their valuable technical assistance.

(16) If water was used instead of benzene to remove HBr all peptides containing NO_2–Arg– gave rise to a considerable amount of ornithine peptides.

(17) D. H. Spackman, E. L. Smith and D. M. Brown, *J. Biol. Chem.*, **212**, 255 (1955).

(18) E. Fischer and W. Schrauth, *Ann.*, **354**, 34 (1907).

orated several times in order to remove excess hydrochloric acid. The final residue was dissolved in a minimum amount of cold water, overlayered with ice-cold ether, and 50% K_2CO_3 added to adjust the solution to *p*H 10. The oily isoleucine methyl ester was extracted into 3 portions of ether. The combined ether extracts were dried over Na_2SO_4 and saturated with dry HCl gas. After evaporation to dryness an oil was obtained which was dissolved in tetrahydrofuran. On addition of ether, isoleucine methyl ester hydrochloride crystallized in long needles; yield 16.85 g. (81%), m.p. 100–101°, $[\alpha]^{22}D$ +26.8° (*c* 3.0, water),[19] $R_{f\ BAW}$ 0.70.

Cbzo-nitro-L-arginyl-L-valine Methyl Ester (I).—L-Valine methyl ester hydrochloride was converted into valine methyl ester by titration of 6.09 g. (0.0362 mole) of the hydrochloride in 200 ml. of absolute tetrahydrofuran with 2 *N* $NaOCH_3$ in absolute methanol, using thymol blue as an indicator. The precipitated NaCl was filtered with the aid of some Super-cel; tetrahydrofuran was used to wash the precipitate. Cbzo-nitro-L-arginine hydrate (13.4 g., 0.0362 mole) was dissolved in the filtrate, more tetrahydrofuran being added if necessary to obtain a clear solution. After the addition of 8.95 g. of N,N′-dicyclohexylcarbodiimide (DCC) in a small volume of tetrahydrofuran, the solution was stirred at room temperature. N,N′-Dicyclohexylurea (DCU) started to crystallize after about 10 min. After 16 hours it was collected on a filter and washed with tetrahydrofuran; yield of DCU, 6.7 g. The filtrate was evaporated to an oil which was dissolved in hot ethyl acetate. Cbzo-nitro-L-arginyl-L-valine methyl ester crystallized on cooling; 14.7 g., m.p. 137–139°. The product was recrystallized from methanol–ether–petroleum ether; 13.4 g., m.p. 138–140°.

The mother liquors were combined, evaporated to dryness, taken up in ethyl acetate, and extracted with 1 *N* HCl, H_2O, 5% $KHCO_3$ and H_2O. Freshly formed dicyclohexylurea was removed by filtration. After drying over Na_2SO_4 and evaporation, an oil was obtained. Several extractions with cold tetrahydrofuran left some more DCU undissolved. From the extracts, 0.95 g. of dipeptide was obtained after several crystallizations from methanol–ether–petroleum ether; m.p. 138–140°, total yield 14.35 g., m.p. 138–140°, 85%. A sample was analyzed after 5 recrystallizations from the same solvent mixture; m.p. 139–141°, $[\alpha]^{22}D$ −17.3° (*c* 1.5, methanol), R_{fBAW} 0.55.

Anal. Calcd. for $C_{20}H_{30}N_6O_7$: C, 51.49; H, 6.48; N, 18.02. Found: C, 51.35; H, 6.65; N, 18.19.

Cbzo-nitro-L-arginyl-L-valine (II).—To 8.0 g. (0.0172 mole) of cbzo-nitro-L-arginyl-L-valine methyl ester in 25 ml. of tetrahydrofuran an equal volume of 1 *N* NaOH (1.4 equiv.) was added. The mixture was shaken for 1 hour at room temperature. The *p*H of the clear solution was then adjusted to 7, and the organic solvent removed *in vacuo*. The resulting solution was made alkaline by the addition of 20 ml. of 1 *M* K_2CO_3, and extracted at 0° with two 40-ml. portions of ethyl acetate. The extracts were discarded. Some excess ethyl acetate was removed from the aqueous solution by bubbling N_2 into it for about one hour. After some insoluble precipitate had been removed the solution was acidified with concd. HCl. The semicrystalline precipitate was filtered after standing overnight in the cold. It was recrystallized from ethanol–water without drying; yield 7.00 g., m.p. 182–184°, $[\alpha]^{22}D$ −5.3° (*c* 2.1, methanol). The mother liquor gave 0.31 g., m.p. 179–181°, total yield 7.31 g., 94%. A sample was recrystallized several times from ethanol-water, and dried for 6 hours at 78° in high vacuum; m.p. 183–185°, $[\alpha]^{22}D$ −3.6° (*c* 2.1, pyridine). The sample was not sufficiently soluble in methanol for a rotation, $R_{f\ BAW}$ 0.49, $R_{f\ MPW}$ 0.66.

Anal. Calcd. for $C_{19}H_{28}N_6O_7$: C, 50.43; H, 6.24; N, 18.58. Found: C, 50.41; H, 6.37; N, 18.36.

Cbzo-L-valyl-L-tyrosine (III).—A suspension of 4.25 g. (0.01 mole) of cbzo-L-valyl-L-tyrosine methyl ester in 50 ml. of 0.5 *N* NaOH (2.5 equiv.) was shaken for one hour at room temperature. The slightly turbid solution was extracted twice with ethyl acetate, and the extracts discarded. Excessive ethyl acetate was removed from the aqueous solution by a stream of nitrogen. On acidification with concd. HCl in the presence of seed crystals a crystalline precipitate was obtained. After cooling, the precipitate was filtered and dried over P_2O_5. It was recrystallized from ethanol–water; 3.45 g., m.p. 164–166° (loses water at 95°), $[\alpha]^{22}D$ +22.7° (*c* 5.1, pyridine). The mother liquor gave 0.68 g. of identical material; total yield 4.13 g., 92%. A sample was recrystallized three times from methanol–water and dried overnight in a desiccator over P_2O_5; m.p. 164–166°, $[\alpha]^{22}D$ +23.3° (*c* 5.0, pyridine), $[\alpha]^{22}D$ +2.5° (*c* 5.0, 0.5 *N* NaOH), $R_{f\ BAW}$ 0.71.

Anal. Calcd. for $C_{22}H_{26}N_2O_6 \cdot H_2O$: C, 61.10; H, 6.53; N, 6.48. Found: C, 61.10; H, 6.45; N, 6.48.

O-Acetyl-N-formyl-L-tyrosyl-L-isoleucine Methyl Ester.—L-Isoleucine methyl ester hydrochloride (0.91 g., 0.005 mole) in 10 ml. of absolute tetrahydrofuran was converted into the free amino ester by titration with methanolic $NaOCH_3$. The NaCl was removed by filtration, and the filtrate added to a solution of 1.26 g. of O-acetyl-N-formyl-L-tyrosine in 10 ml. of dry tetrahydrofuran. After the addition of 1.41 g. of DCC the reaction mixture was stirred for 16 hours at room temperature. Crystalline DCU was filtered off and the resulting solution evaporated *in vacuo*. The oil was dissolved in ethyl acetate and cooled to 0°. On shaking with 5 ml. of cold 1 *N* HCl a second portion of crystalline DCU was formed which again was removed by filtration. The resulting ethyl acetate solution was extracted with 1 *N* HCl (2 × 5 ml.), H_2O (2 × 5 ml.), 1 *M* $KHCO_3$ (3 × 5 ml.), H_2O (3 × 5 ml.), dried over Na_2SO_4, and evaporated *in vacuo*. The residue was dissolved in a very small volume of tetrahydrofuran. After the addition of several volumes of ether O-acetyl-N-formyl-L-tyrosyl-L-isoleucine methyl ester crystallized in long silky needles; 1.18 g., m.p. 151–153°, $[\alpha]^{22}D$ +10.3° (*c* 2.4, methanol). The mother liquor furnished another 0.07 g. of m.p. 150–152°; total yield 1.25 g., 66%. A sample was recrystallized twice from tetrahydrofuran–ether 1:1, and dried for 2 hours at 78° in high vacuum over P_2O_5; m.p. 152–153°, $[\alpha]^{22}D$ +10.7° (*c* 2.0, methanol).

Anal. Calcd. for $C_{19}H_{26}N_2O_6$: C, 60.30; H, 6.93; N, 7.40. Found: C, 60.46; H, 7.12; N, 7.40.

O-Acetyl-N-formyl-L-tyrosyl-L-isoleucine methyl ester was also synthesized by the mixed anhydride method using ethyl chlorocarbonate and tri-*n*-butylamine to form the anhydride, and tetrahydrofuran as a solvent (compare for instance cbzo-L-valyl-L-tyrosine methyl ester in ref. 2). The yield from 0.005 mole of starting materials was 0.58 g. (31%), m.p. 150–153°, $[\alpha]^{22}D$ +8.9° (*c* 2.3, methanol).

N-Formyl-L-tyrosyl-L-isoleucine Methyl Ester (IV).—A solution of 2.53 g. (0.014 mole) of L-isoleucine methyl ester hydrochloride and 3.33 ml. of tri-*n*-butylamine in 6 ml. of dimethylformamide was added to a solution of 3.66 g. (0.016 mole) of N-formyl-L-tyrosin hydrate[20] in 30 ml. of tetrahydrofuran. After cooling to 0° 3.5 g. (0.017 mole) of DCC in 5 ml. of dimethylformamide was added. The reaction mixture was stirred for several hours slowly attaining room temperature, and after 16 hours the DCU (3.09 g.) was removed by filtration. The filtrate was evaporated to dryness *in vacuo*, the residue dissolved in ethyl acetate, and this solution extracted at 0° with three 5-ml. portions each of 1 *N* HCl, water, 1 *M* $KHCO_3$, water; DCU (0.25 g.) was formed during the first extraction with acid. After drying over Na_2SO_4 the ethyl acetate solution was concentrated, treated with active charcoal, and cooled. A further 0.1 g. of DCU crystallized, and was collected. Addition of ligroin to the filtrate and cooling produced the desired N-formyl-L-tyrosyl-L-isoleucine methyl ester in clusters of white needles; 3.16 g., (67%), m.p. 142–144°, $[\alpha]^{22}D$ +5.9° (*c* 2.5, methanol).

The mother liquor was evaporated to dryness. The foamy residue (1.64 g.) was dissolved in a minimum amount of ethyl acetate–ligroin 1:1 and chromatographed on 30 g. of Florisil. Elution with the same solvent mixture gave, after about 1 g. of unidentified material, fractions that contained crystalline dipeptide. After one recrystallization from ethyl acetate–ligroin 0.30 g., m.p. 139–143°, was obtained; total yield 3.46 g., 74%. A sample was recrystallized twice from ethyl acetate–ligroin and dried for 2 hours at 78° in high vacuum over P_2O_5; m.p. 143–144°, $[\alpha]^{22}D$ + 6.7° (*c* 2.2, methanol).

Anal. Calcd. for $C_{17}H_{24}N_2O_6$: C, 60.69; H, 7.19; N, 8.33. Found: C, 60.75; H, 7.28; N, 8.42.

N-Formyl-L-tyrosyl-L-isoleucine methyl ester also was synthesized using the method, amounts and solvents as described for the O-acetyl-dipeptide; 58% was isolated directly

(19) E. L. Smith, D. H. Spackman and W. J. Polglase, *J. Biol. Chem.*, **199**, 801 (1952), reported m.p. 98–100°, $[\alpha]^{22}D$ +26.6° (*c* 2.0, water).

(20) S. G. Waley and J. Watson, *Biochem. J.*, **57**, 529 (1954).

and chromatography gave 10% of the product for a total yield of 68%.

L-Tyrosyl-L-isoleucine methyl ester was obtained by removal of the formyl group from the preceding compound, but neither the free base nor the hydrochloride or hydrobromide were isolated in crystalline form. The reaction was, therefore, tested for yield and by-products by paper chromatography: N-formyl-L-tyrosyl-L-isoleucine methyl ester (10 mg.) was dissolved in 5 ml. of 1 *N* methanolic HCl, and 50-γ aliquots were spotted on paper at different time intervals. The chromatogram was developed in the BAW system and sprayed with ninhydrin and diazotized sulfanilic acid. After 6 hours the spot due to the formyl derivative had completely disappeared. Only one spot, L-tyrosyl-L-isoleucine methyl ester, was visible with either spraying reagent. It was, therefore, concluded that the reaction was practically quantitative and free of side reactions.

N-Formyl-L-tyrosyl-L-isoleucine methyl ester was dissolved in 1 *N* methanolic HCl (1 g. per 10 ml.), and left at room temperature for 16 hours. The solution was then evaporated to an oil, and the excess HCl removed by dissolving the residue in a small amount of absolute methanol and re-evaporating for several times. The resulting foam was distributed between cold ethyl acetate and cold saturated $NaHCO_3$. The organic phase was extracted with additional bicarbonate until no more HCl was removed ($AgNO_3$). The final ethyl acetate solution of L-tyrosyl-L-isoleucine methyl ester was dried over Na_2SO_4, and used directly for condensations (R_f BAW 0.76).

Cbzo-nitro-L-arginyl-L-valyl-L-tyrosine Methyl Ester (V). A. Cbzo-amino Acid + Dipeptide Ester.—L-Valyl-L-tyrosine methyl ester hydrobromide[21] (4.50 g., 0.012 mole) was dissolved in 10 ml. of water, and, at 0°, 1.68 ml. (0.012 mole) of triethylamine added to the clear solution. The oily precipitate was extracted into three 25-ml. portions of cold ethyl acetate. The extracts were combined, dried over Na_2SO_4 and added to a solution of cbzo-nitro-L-arginine hydrate (4.45 g., 0.012 mole) in 50 ml. of tetrahydrofuran; 2.98 g. (20% excess) of DCC in 4 ml. of tetrahydrofuran was added. The reaction mixture was stirred overnight at room temperature. It was then filtered and the filtrate washed with tetrahydrofuran; precipitate (a) and filtrate (b) were worked up separately.

The precipitate (a) was triturated with hot 50% ethanol and the insoluble DCU separated by filtration. On cooling, the filtrate deposited a solid which was triturated with $KHCO_3$ solution, washed with water and dried; 5.1 g., m.p. 188–200°. It was recrystallized twice from 95% ethanol; 4.25 g., m.p. 211–214°, $[\alpha]^{22}D$ +5.0° (*c* 4.0, dimethylformamide), 56%.

The filtrate (b) was evaporated to dryness, and the residue dissolved in a very small amount of glacial acetic acid. Enough cold water was added to make the solution 5% with respect to acetic acid. It was left in the cold room overnight. The precipitate was isolated by filtration, and the filtrate discarded. The solid was then triturated with hot 50% ethanol and filtered. The insoluble part was mainly DCU and was discarded. The filtrate deposited an oil that solidified on cooling. After filtration it was triturated with cold 1 *M* $KHCO_3$, washed with water and dried. The amorphous solid was triturated with ethyl acetate and filtered; 1.50 g., m.p. 187–197°. It could not be crystallized from ethanol or methanol–ether, and was, therefore, hydrolyzed with NaOH as described below; yield of tripeptide acid after 2 recrystallizations from 50% ethanol, 1.10 g., m.p. 182–183°, $[\alpha]^{22}D$ +7.1° (*c* 3.0, pyridine); total yield of tripeptide, 71%.

B. **Cbzo-dipeptide + Amino Acid Ester.**—Cbzo-nitro-L-arginyl-L-valine (3.16 g., 0.007 mole), L-tyrosine methyl ester hydrochloride (1.62 g., 0.007 mole) and tri-*n*-butylamine (1.67 ml., 0.007 mole) were dissolved in dimethylformamide (25 ml.), and the solution cooled to 0°. A solution of 1.65 g. (0.008 mole) of DCC in 10 ml. of cold tetrahydrofuran was added. After stirring for 3 hours at 0° the reaction mixture was left overnight at room temperature. It was filtered after the addition of 10 ml. of tetrahydrofuran. The precipitate was washed with a mixture of equal parts of dimethylformamide and tetrahydrofuran: 1.08 g., 70% of DCU. The filtrate was evaporated to an oil which was triturated with absolute ether, 10% acetic acid, 1 *M* $KHCO_3$ and finally water. The oil solidified when stored overnight under water. It was filtered, washed with cold water, and dried. In order to remove some additional DCU the product was dissolved in 15 ml. of dimethylformamide and filtered: DCU 0.35 g., 23%. The filtrate was evaporated to dryness, and the residue dissolved in 95% ethanol. Crystallization of the product could not be induced by seeding with the material obtained under A. No fractionation was obtained by chromatography on Florisil. The dried product (4.5 g.) was dissolved in ethyl acetate–methanol 3:1 and chromatographed on 100 g. of aluminum oxide, Merck. The same mixture of solvents was used for elution. The first 80 ml. contained some DCU. The next fractions (120 ml.) could not be crystallized and were combined (a). All further fractions contained about 50% of cbzo-nitro-L-arginyl-L-valyl-L-tyrosine methyl ester and crystallized easily on seeding. The elution was completed with ethyl acetate–methanol 1:1 and finally methanol. All crystalline material was collected, and recrystallized once from 95% ethanol; 1.20 g., m.p. 211–214°. The mother liquors were all combined (b).

Fractions a and b were hydrolyzed separately with NaOH as described below: The yield of cbzo-nitro-L-arginyl-L-valyl-L-tyrosine was 0.37 g. and 0.1 g., respectively. The two fractions combined and recrystallized from ethanol–water gave 0.40 g., m.p. 180–185°, $[\alpha]^{22}D$ +8.5° (*c* 3.8, dimethylformamide), $[\alpha]^{22}D$ +6.8° (*c* 3.8, pyridine); total yield of tripeptide, 1.60 g., 36%.

A comparison of the two tripeptide esters obtained in A and B is

	M.p., °C.	$[\alpha]^{22}D$ dimethylformamide	$[\alpha]^{22}D$ pyridine	R_f BAW
A	211–214	+5.2° (*c* 4.2)	+0.4° (*c* 5.3)	0.64
B	211–214	+5.1 (*c* 4.1)	+0.4 (*c* 4.4)	0.64

Mixed m.p. 210–213°.

The two products were, therefore, combined, and a sample readied for analysis by two further recrystallizations (1 × 95% methanol, 1 × 95% ethanol). It was dried for 2 hours at 78° over P_2O_5 in high vacuum, m.p. 215–216°.

Anal. Calcd. for $C_{29}H_{39}N_7O_9$: C, 55.32; H, 6.24; N, 15.57. Found: C, 55.54; H, 6.41; N, 15.76, 15.63.

Cbzo-nitro-L-arginyl-L-valyl-L-tyrosine (VI).—A suspension of 2.22 g. of cbzo-nitro-L-arginyl-L-valyl-L-tyrosine methyl ester in 20 ml. of 0.5 *N* NaOH was shaken for 1 hour at room temperature. An almost clear solution resulted; 13 ml. of 1 *N* HCl was added and the precipitate was redissolved by the addition of $NaHCO_3$. The solution was extracted twice with ethyl acetate and the extracts discarded. The excess ethyl acetate was removed from the aqueous phase by bubbling nitrogen through it for about 1 hour. A small amount of precipitate was removed by filtration, and the filtrate was acidified with concd. HCl. The product was collected on a filter, washed with water and crystallized from absolute ethanol without drying; yield 2.08 g., 96%, m.p. 180–185°. A sample was recrystallized once from 95% ethanol, and once from methanol, and dried for 2 hours at 78° over P_2O_5 in high vacuum; m.p. 183–186°, $[\alpha]^{22}D$ + 9.3° (*c* 4.7, dimethylformamide), $[\alpha]^{22}D$ +8.3° (*c* 3.4, pyridine), $[\alpha]^{22}D$ +3.7 (*c* 2.9, 0.5 *N* NaOH), R_f BAW 0.57, R_f MPW 0.87.

It was observed with several preparations, that the melting point became lower and very unsharp after a short time of standing in contact with air. The dry compound was very hygroscopic, and needed an additional drying at 100° just before analysis.

Anal. Calcd. for $C_{28}H_{37}N_7O_9$: C, 54.62; H, 6.06; N, 15.93. Found: C, 54.58; H, 6.21; N, 15.93.

This tripeptide was completely hydrolyzed with carboxypeptidase; ninhydrin: tyr, val; Pauly: tyr. The hydrolysis with leucine aminopeptidase, after removal of cbzo- by HBr in glacial acetic acid, was also complete; ninhydrin: NO_2-arg, val, tyr; Pauly: tyr.

Cbzo-L-valyl-L-tyrosyl-L-isoleucine Methyl Ester (VII). A. Cbzo-amino-Acid + Dipeptide Ester.—To a solution of L-tyrosyl-L-isoleucine methyl ester in ethyl acetate (prepared from 2.0 g., 0.006 mole, of formyl-L-tyrosyl-L-isoleucine methyl ester as described above) were added 1.39 g. of cbzo-L-valine (0.0055 mole) and 1.50 g. of DCC (20% excess). The clear solution, stirred at room temperature, turned semi-solid after one hour. It was filtered after 4 hours, and the precipitate was washed with ethyl acetate and ether, and dried; 2.82 g. The filtrate was extracted with 1 *N* HCl, H_2O, 1 *M* $KHCO_3$, H_2O (0.33 g. of DCU was

(21) W. Rittel, B. Iselin, H. Kappeler, B. Riniker and R. Schwyzer, *Helv. Chim. Acta*, **40**, 614 (1957).

filtered after the first extraction with HCl) dried over Na_2SO_4, and evaporated to dryness. The residue was combined with the original precipitate, and the mixture refluxed with tetrahydrofuran. The suspension was cooled to room temperature, left for about one hour, filtered, and the precipitate washed with tetrahydrofuran. The precipitate consisted of 0.81 g. of DCU. The filtrate was concentrated to a foam *in vacuo*. The latter was dissolved in 50 ml. of boiling methanol. After cooling, 1.76 g. of cbzo-L-valyl-L-tyrosyl-L-isoleucine methyl ester, m.p. 218–219°, $[\alpha]^{22}D$ −29.9° (*c* 2.0, pyridine), was obtained. The mother liquor was evaporated to dryness, and the residue dissolved in hot tetrahydrofuran. A very small fraction of DCU was obtained on cooling. The residue from the filtrate yielded 0.29 g. of tripeptide from methanol, m.p. 214–217°, total yield 2.05 g., 69%.

B. **Cbzo-dipeptide + Amino Acid Ester, Mixed Anhydride Method.**—A solution of 17.3 g. of cbzo-L-valyl-L-tyrosine hydrate (0.04 mole) and 5.83 ml. of triethylamine in 90 ml. of tetrahydrofuran was cooled to −10°, and 3.96 ml. of ethyl chloroformate added. After 10 min. stirring at the same temperature 6.05 g. (0.042 mole) of freshly distilled L-isoleucine methyl ester in 20 ml. of tetrahydrofuran was added. The clear solution was slowly brought to room temperature and stirring was continued for three hours. The solvent was removed *in vacuo* and the solid thus obtained was heated under reflux with 225 ml. of ethyl acetate. The suspension was left at 0° overnight. Filtration gave 14.5 g. of a crystalline solid with a melting range of 165–208°. On recrystallization from methanol 2 fractions of similar purity were obtained and combined; 7.80 g. m.p. 213–216°, $[\alpha]^{22}D$ −29.7° (*c* 2.5, pyridine). The mother liquor was concentrated to a small volume and ethyl acetate added; 4.74 g. of white needles subliming between 200–240°, was obtained on cooling. They were identified as triethylamine hydrochloride. The mother liquor gave an additional 0.18 g. of the above tripeptide.

The original ethyl acetate mother liquor was extracted with 1 *N* HCl (2 ×), H_2O (2 ×), 1 *N* NH_3 (5 ×), H_2O (3 ×), and dried over Na_2SO_4. After concentration and cooling a gelatinous mass was obtained that could be crystallized by alternate heating and cooling: 2.6 g., m.p. 167–170°, unsharp. This crop and its mother liquor were then subjected to a long triangular fractional recrystallization using ethyl acetate–petroleum ether as solvent. Fractions with constant melting point and optical rotation were withdrawn and combined. Two main components were obtained. The smaller one, 0.27 g., m.p. 215–217°, $[\alpha]^{22}D$ −29.8° (pyridine), was identical with the above isolated cbzo-L-valyl-L-tyrosyl-L-isoleucine methyl ester. The second compound, 2.43 g., had m.p. 161–162°, $[\alpha]^{22}D$ −7.1° (*c* 1.8 pyridine); it gave the amino acids valine, tyrosine and isoleucine in about equal amounts on acid hydrolysis, and the microanalytical values confirmed its composition as cbzo-valyl-tyrosyl-isoleucine methyl ester. It was not hydrolyzed by either chymotrypsin or leucine aminopeptidase and was, therefore, assigned the configuration L-D-L; total yield of tripeptides: L-L-L 38%, L-D-L 11%. Due to the quite considerable losses during the fractional crystallization the above figures are probably low; the yield of crude tripeptide mixture was found to be about 14 g. or 65%.

C. **Cbzo-dipeptide + Amino Acid Ester. Carbodiimide Method.**—To a cold solution of 1.24 g. of cbzo-L-valyl-L tyrosine hydrate (0.0029 mole), 0.55 g. of L-isoleucine methyl ester hydrochloride and 0.71 ml. of tri-*n*-butylamine in 10 ml. of dimethylformamide was added 0.72 g. (0.0035 mole) of DCC in 2 ml. of the same solvent. The reaction mixture was stirred at 0° for 2 days, brought to room temperature and left overnight. An equal volume of tetrahydrofuran was added, cooled and filtered; 0.55 g., 92% of DCU. The filtrate was evaporated to dryness, and the residue triturated with ether. The insoluble fraction was crystallized from methanol; 0.84 g., m.p. 215–217°; 0.16 g. was isolated from the mother liquor to bring the total yield to 1.00 g., 65%, $[\alpha]^{22}D$ −30.0° (*c* 1.9, pyridine).

The ether extract deposited crystals on cooling; 0.30 g., m.p. 146–156°. This fraction was recrystallized from methanol–ether to give 0.16 g., 11%, m.p. 156–158°, $[\alpha]^{22}D$ −7.7° (*c* 1.8, pyridine). No melting point depression was observed on admixture with cbzo-L-valyl-D-tyrosyl-L-isoleucine methyl ester isolated under B.

An identical condensation was made by using tetrahydrofuran as solvent. The yield of L-L-L tripeptide was 59%. The ether extract could not be crystallized directly, but by chromatography on Florisil in ethyl acetate–ligroin 1:1, 10% of L-D-L tripeptide was eluted with the same solvent and ethyl acetate. No L-L-L tripeptide was eluted under the same conditions.

THE TRIPEPTIDES OBTAINED UNDER A, B AND C

	M.p., °C.	$[\alpha]^{22}D$, pyridine (*c*)	$[\alpha]^{22}D$, dimethyl-formamide (*c*)	Yield, %	R_f BAW
A (LLL)	217–219	−29.6° (2.2)	−14.6 (2.1)	69	0.85
B (LLL)	215–217	−30.6 (1.4)	−14.9 (1.9)	38	.85
C (LLL)	217–219	−30.0 (1.9)	−15.4 (2.0)	65	.85
A (LDL)				0	
B (LDL)	158–159	− 7.1 (1.8)	+11.3 (1.9)	11	.85
C (LDL)	156–158	− 7.7 (1.8)	+ 9.7 (1.8)	11	.85

The two stereoisomers do not show a melting point depression. A 1:1 mixture melts between 156–215°.

The three preparations of cbzo-L-valyl-L-tyrosyl-L-isoleucine methyl ester were combined. A sample was recrystallized twice more from methanol and dried for 3 hours at 78° over P_2O_5 in high vacuum, m.p. 215–216°.

Anal. Calcd. for $C_{29}H_{39}N_3O_7$: C, 64.30; H, 7.26; N, 7.76. Found: C, 64.28; H, 7.27; N, 7.90.

This tripeptide was completely hydrolyzed by chymotrypsin; ninhydrin: val-tyr, ileu-OMe; Pauly: val-tyr. An equally complete hydrolysis was obtained by incubation with leucine aminopeptidase; ninhydrin: val, tyr, ileu; Pauly: tyr. No trace of tripeptide was visible with either spray.

A sample of cbzo-L-valyl-D-tyrosyl-L-isoleucine methyl ester (B) was recrystallized from ethyl acetate and dried for 2 hours at 78° over P_2O_5 in high vacuum, m.p. 158–159°, $[\alpha]^{22}D$ −7.0° (*c* 1.9, pyridine). *Anal.* Found: C, 64.18; H, 7.21; N, 8.00. This tripeptide was not hydrolyzed by chymotrypsin or leucine aminopeptidase, ninhydrin: val-tyr-ileu-OMe; Pauly: same.

L-Valyl-L-tyrosyl-L-isoleucine Methyl Ester (VIII).—Cbzo-L-valyl-L-tyrosyl-L-isoleucine methyl ester (3.5 g., 0.0065 mole) was dissolved in 30 ml. of HBr 2.5 *N* in glacial acetic acid, and the solution was left for 1 hour at room temperature. The addition of 200 ml. of absolute ether produced an amorphous precipitate which, after cooling in ice, was collected on a filter and washed thoroughly with absolute ether; 3.12 g., m.p. 131–139°. It was distributed between cold ethyl acetate and cold saturated $NaHCO_3$ solution. The organic phase was extracted several times with $NaHCO_3$ until no more HBr was taken up. After two washes with small amounts of water the ethyl acetate solution was dried over Na_2SO_4, and the solvent removed *in vacuo*. The remaining solid was crystallized from acetonitrile–water; 1.50 g., 57%, m.p. 146–148°, $[\alpha]^{22}D$ −4.2° (*c* 3.4, pyridine), $[\alpha]^{22}D$ + 4.4° (*c* 2.3, 1 *N* HCl–methanol 1:1), $[\alpha]^{22}D$ −0.4° (*c* 2.5, methanol), R_f BAW 0.86.

A sample was recrystallized twice from acetonitrile–water, and dried for 2 hours at 78° over P_2O_5 in high vacuum; m.p. 147–148°. The sample proved to be very hygroscopic and made an additional drying at 100° necessary before analysis.

Anal. Calcd. for $C_{21}H_{33}N_3O_5$: C, 61.89; H, 8.16; N, 10.31. Found: C, 61.84; H, 8.20; N, 10.27.

Because of the considerable losses on crystallization and recrystallization, the above-mentioned ethyl acetate solution of L-valyl-L-tyrosyl-L-isoleucine methyl ester was used directly for peptide condensations without any attempt at crystallization.

Cbzo-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucine Methyl Ester. A. Cbzo-amino Acid + Tripeptide Ester (IX).—A solution of L-valyl-L-tyrosyl-L-isoleucine methyl ester in ethyl acetate prepared from 2.98 g. (0.0055 mole) of cbzo-L-valyl-L-tyrosyl-L-isoleucine methyl ester as described above was combined with a solution of 1.86 g. of cbzo-nitro-L-arginine hydrate in dimethylformamide. N,N′-Dicyclohexyl-carbodiimide (1.40 g., 0.007 mole) in a small volume of dimethylformamide was added, and the reaction mixture was stirred for 16 hours at room temperature. The solution was filtered, concentrated and filtered again to remove 0.83 g. (75%) of DCU. The remaining dimethylformamide was removed by evaporating several times after the addition of benzene and absolute ethanol. The final residue was dissolved in warm 67% ethanol, treated with charcoal and filtered. A voluminous precipitate was isolated by filtration

after cooling. The mother liquor was evaporated to dryness, the residue triturated with ether, and then combined with the first precipitate. The whole solid was crystallized from 95% ethanol; yield 2.17 g,. m.p. 168–182°, $[\alpha]^{22}D$ −7.1° (*c* 2.4, dimethylformamide).

The mother liquor was evaporated to dryness, dissolved in 10 ml. of hot 50% ethanol and filtered; insoluble product, 0.05 g. of DCU. The filtrate deposited an amorphous product that was isolated by filtration, and crystallized from 95% ethanol; 0.32 g., m.p. and rotation as above, total crude yield 2.49 g., 67%. Recrystallization from ethanol and then methanol gave 2.30 g., 62%, m.p. 180–182°, $[\alpha]^{22}D$ −6.2° (*c* 2.5, dimethylformamide). This tetrapeptide was hydrolyzed by chymotrypsin; ninhydrin: NO_2-arg-val-tyr, ileu·OMe; Pauly: NO_2-arg-val-tyr, very faint NO_2-arg-val-tyr-ileu·OMe (<5%), hydrolysis > 95%. Incubation with chymotrypsin + carboxypeptidase gave a similar result; ninhydrin: NO_2-arg-val, tyr, ileu·OMe; Pauly: tyr, very faint NO_2-arg-val-tyr-ileu·OMe (<5%), hydrolysis > 95%. The hydrolysis by leucine aminopeptidase was complete; ninhydrin: NO_2-arg, val, tyr, ileu, ileu·OMe; Pauly: tyr.

B. **Cbzo-dipeptide + Dipeptide Ester.**—A solution of L-tyrosyl-L-isoleucine methyl ester in ethyl acetate prepared from 3.0 g. (0.009 mole) of formyl-L-tyrosyl-L-isoleucine methyl ester as described above was added to 4.06 g. of cbzo-nitro-L-arginyl-L-valine in 20 ml. of dimethylformamide. After cooling the clear solution to 0°, 2.06 g. (10% excess) of DCC in 5 ml. of dimethylformamide was added. The reaction mixture was stirred for two hours in an ice-bath and then slowly warmed to room temperature. Stirring was continued overnight. N,N′-Dicyclohexylurea (1.68 g.) was isolated by filtration. The filtrate was evaporated to dryness and the residual sirup was almost completely dissolved in boiling ethyl acetate. On cooling to room temperature a gelatinous precipitate appeared that was collected on a filter after one day. It was reprecipitated again from ethanol, filtered, washed with ether and dried; 3.0 g., m.p. 145–150° (a). The two mother liquors were combined and worked up separately (b).

The precipitate (a) was triturated in a mortar with cold 1 *M* $KHCO_3$, filtered and washed with cold water. After drying it was dissolved in a small volume of methanol and filtered through a column consisting of 50 g. of Dowex 50, X-12 and 50 g. of Dowex 3, X-4. The effluent was evaporated to dryness; yield 2.4 g. This was dissolved in 95% ethanol and seeded with the tetrapeptide obtained under A. After several days a semi-crystalline material (crystals + gel) was isolated; yield 1.8 g., 27%, m.p. 140–180°, $[\alpha]^{22}D$ −6.2° (*c* 5.4, dimethylformamide). The product gave 2 spots by chromatography on paper, one corresponded to the tetrapeptide, the other was negative with Pauly reagent. It was assumed that the by-product was N-(cbzo-nitro-L-arginyl-L-valyl)-N,N′-dicyclohexylurea.

On incubation with chymotrypsin the tetrapeptide spot disappeared completely and gave rise to NO_2-arg-val-tyr and ileu·OMe. Incubation with leucine aminopeptidase hydrolyzed both compounds; ninhydrin: NO_2-arg, val, tyr, ileu, ileu·OMe; Pauly: tyr.

All ethyl acetate-insoluble material was removed from the filtrates (b) and the resulting solution was extracted with acid and base. The neutral components were combined with the insoluble material; yield 2.9 g. This fraction was dissolved in a minimal amount of ethyl acetate–methanol 2:1 and chromatographed on 60 g. of Al_2O_3. The same solvent mixture was used for elution. The first fraction contained 0.15 g. of DCU. The remaining fractions were combined to 4 main fractions, and were tested by paper chromatography and enzymatic hydrolysis for their possible components; fraction 2: 4 spots in BAW system, about 50% tetrapeptide, the others unidentified. Chymotrypsin + carboxypeptidase hydrolyzed only very little of the intermediate tripeptide (< 20%); ninhydrin: NO_2-arg-val, tyr, ileu·OMe, NO_2-arg-val-tyr, NO_2-arg-val-tyr-ileu·OMe; Pauly: tyr, NO_2-arg-val-tyr, NO_2-arg-val-tyr-lieu·OMe; fraction 3 and 4: 2 spots in BAW system, one corresponding to tetrapeptide (about 80%), the other not positive with Pauly's reagent, therefore, probably a derivative of nitro-arginyl-valine. Incubation with chymotrypsin + carboxypeptidase gave the same picture as fraction 2, but in fraction 4 about 50% of the tripeptide was hydrolyzed; fraction 5: 2 spots in BAW system, tetrapeptide and intermediate tripeptide are completely hydrolyzed with chymotrypsin + carboxypeptidase; ninhydrin: NO_2-arg-val, tyr, ileu·OMe; Pauly: tyr.

C. **Cbzo-tripeptide + Amino Acid Ester.**—L-Isoleucine methyl ester hydrochloride (1.09 g., 0.006 mole) in 5 ml. of dimethylformamide was converted into the free amino ester by titration with $NaOCH_3$ as described above. A solution of 3.80 g. (0.006 mole) of cbzo-nitro-L-arginyl-L-valyl-L-tyrosine in 10 ml. of dimethylformamide was added, and the mixture was cooled in an ice-bath. After the addition of 1.5 g. of DCC (20% excess) in 5 ml. of ethyl acetate the clear solution was stirred at 0° for one hour, warmed slowly to room temperature and stirred overnight. The precipitated DCU (0.94 g.) was filtered, and the filtrate evaporated to dryness. Another small crop of DCU was removed by dissolving the residue in tetrahydrofuran and adding ethyl acetate. The solvents were again evaporated *in vacuo*, and the residue was dissolved in boiling ethyl acetate. The amorphous, gelatinous precipitate obtained on cooling was filtered off and crystallized from 95% ethanol (seeded with A); a second, very small crop was obtained from the mother liquor; yield 1.30 g., m.p. 168–181°.

Ethyl acetate and ethanol mother liquors were combined, evaporated to dryness, triturated with ether, and dissolved in a small volume of ethyl acetate. The addition of ether caused a voluminous precipitation which was collected on a filter and dried; 2.62 g., m.p. 100–130°. It was dissolved in 95% ethanol, seeded and left at room temperature for several days; 0.56 g., m.p. 163–184°, total crude yield 1.85 g., 42% (mother liquor, see b).

It was observed while attempting to recrystallize this product from methanol that whereas the mother liquors crystallized easily the first crop came out amorphous. Therefore, all the mother liquors were collected and the first crop finally narrowed down to 0.1 g. of amorphous material. This was analyzed by paper chromatography and found to contain mainly tripeptide. The crystalline tetrapeptide was isolated from the collected mother liquors in several fractions that were found to be identical in melting point, rotation and behavior toward enzymes; yield 1.54 g., 35%, m.p. 178–181°, $[\alpha]^{22}D$ −6.6° (*c* 4.6, dimethylformamide). This tetrapeptide was incubated with the same enzymes as A and behaved exactly alike.

The mother liquor b was again treated with ethyl acetate–ether as described above; 1.95 g., amorphous, $[\alpha]^{22}D$ +6.7° (*c* 4.0, dimethylformamide). It contained mainly tetrapeptide, but a second spot was clearly visible in BAW; it corresponded to tripeptide, either the starting material or more likely (nitro-arginyl-valyl-tyrosyl)-N,N′-dicyclohexylurea.

Chymotrypsin hydrolyzed only about 50% of the tetrapeptide part of this material; ninhydrin: NO_2-arg-val-tyr, ileu·OMe, NO_2-arg-val-tyr-ileu·OMe; Pauly: NO_2-arg-val-tyr-ileu·OMe, NO_2-arg-val-tyr.

PHYSICAL CONSTANTS OF THE THREE TETRAPEPTIDES

	M.p., °C.	$[\alpha]^{22}D$ pyridine (*c*)	$[\alpha]^{22}D$ dimethylformamide (*c*)	Yield, %	R_f BAW
A	180–182	−27.1° (3.5)	−6.5 (4.3)	67	0.82
B	140–180	−26.0 (4.6)	−6.3 (4.6)	27	0.82, 0.53
C	178–181	−26.2 (3.3)	−6.6 (4.6)	35	0.82

A sample of A + C was recrystallized twice from methanol and dried for 2 hours at 78° over P_2O_5 in high vacuum; m.p. 179–181°, $[\alpha]^{22}D$ −27.1° (*c* 4.5, pyridine), $[\alpha]^{22}D$ −6.3° (*c* 3.9, dimethylformamide).

Anal. Calcd. for $C_{35}H_{50}N_8O_{10}\cdot H_2O$: C, 55.25; H, 6.90; N, 14.73; H_2O, 2.37. Found: C, 55.32, 55.48; H, 6.83, 6.99; N, 14.96; H_2O, 2.96. The H_2O was determined by drying a sample at 100° to constant weight: Calcd. for $C_{35}H_{50}N_8O_{10}$: C, 56.59; H, 6.79. Found: C, 56.78; H, 6.67.

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