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The Trifluoroacetate Method of Peptide Synthesis. II. An Improved Synthesis of Bradykinin

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When *p*-nitrophenyl trifluoroacetate (I), 2, 4, 5-trichlorophenyl trifluoroacetate (II) and *N*-hydroxysuccinimide trifluoroacetate (III) were subjected to Anderson's racemization test, only reagent III showed resistance against racemization. However, reagents I and II were also found to be useful for fragment-condensation with acylpeptides possessing glycine or proline at the *C*-terminus. Bradykinin was prepared using these reagents. During the synthesis, a combination of the *t*-amyloxycarbonyl *N*-protecting group and the benzyl ester was mainly used, and the alkaline hydrolysis procedure of the ester group was eliminated. Thus, colorless bradykinin with full activity was obtained in a satisfactory yield without any purification procedure.

In the first paper of this series,[1] the syntheses of various esters of trifluoroacetic acid were presented, and it was confirmed that *p*-nitrophenyl

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1) S. Sakakibara and N. Inukai, This Bulletin, **38**, 1979(1965); **37**, 1231 (1964).

trifluoroacetate (I), 2, 4, 5-trichlorophenyl trifluoroacetate (II) and *N*-hydroxysuccinimide trifluoroacetate (III) are the best reagents for preparing the respective active esters of urethan-type acyl-L-amino acids by ester-exchange reactions in pyridine. Further, it was suggested that the method should be valuable for the stepwise elongation of peptides by the active ester method without the isolation of the intermediates.

The present paper is concerned with a study of the use of reagents I, II and III not only for the stepwise synthesis, but also for so called fragment-condensation of peptides. First, reagents I, II and III were subjected to Anderson's racemization test,[2] i. e., carbobenzoxyglycyl-L-phenylalanine was treated with each reagent in pyridine until the ester-exchange reaction was complete, and then, the mixture was directly subjected to the coupling reaction with glycine ethyl ester. It was found that the ester-exchange reaction of the acylpeptide with reagent I or II was comparatively slow at room temperature, and a completely racemized product was obtained. With reagent III, however, the ester-exchange reaction was completed smoothly within one hour at room temperature, and only about 2.5% of the entire product, which was obtained in a 80% yield, was found to be racemate. Therefore, the use of reagents I and II for the fragment-condensation of peptides must be limited to the condensation of acylpeptides with glycine at their *C*-terminus. As will be described later in this paper, the *C*-terminal L-proline residue in an acylpeptide was found to be stable against racemization with reagent I, II or III. Furthermore, it has been mentioned previously[1] that amino acid residues with active hydrogen (such as histidine, serine, threonine and tyrosine) and *C*-terminal glutamine or asparagine are susceptible to these trifluoroacetate reagents in pyridine. Therefore, unless they are protected with suitable groups, acylpeptides with amino acid residues such as these should not be treated by this method. However, when these amino acid residues are in the amine components (i. e., peptide esters), this method, of course, can be used freely.

Second, the synthesis of bradykinin was carried out as an example of the application of the trifluoroacetate method using reagents I, II and III. The *t*-amyloxycarbonyl (AOC) group was mainly used as an *N*-protecting group; it had already been confirmed in this laboratory that the AOC group is useful in peptide synthesis.[3,4] The use of a methyl or ethyl ester group for protecting the carboxylic acid, the method adopted by Nicolaides and De Wald[5,6] for bradykinin synthesis, was replaced by the use of a benzyl ester group in this study, except for the preparation of hydrazide, because the removal of benzyl group by catalytic hydrogenolysis is much safer than the saponification of alkyl esters at the end of the synthesis. It was also confirmed that the AOC-group can be removed safely using trifluoroacetic acid, without any effect on the benzyl ester.[4] Moreover, it may be recalled that, although the removal of the carbobenzoxy group with anhydrous hydrogen bromide in acetic acid is accompanied by the partial *O*-acetylation of the serine residue during the procedure,[6] treatment with trifluoroacetic acid does not result in any modification of the serine residue. Boissonnas et al.[7] first prepared bradykinin using a combination of carbobenzoxy and *p*-nitrobenzyl ester groups. However, the hydrogenolysis of the *p*-nitrobenzyl group yield *p*-toluidine, and the separation of *p*-toluidine from the main product was a problem. Then, Guttmann et al.[8] improved the synthetic method by using tosylarginine as a material. The tosyl group, however, can only be removed by sodium reduction in liquid ammonia, and, after the reduction, the product had to be purified by column chromatography and/or counter current distribution.

The best route of bradykinin synthesis found so far in this study is shown in Fig. 1. The nitro-L-arginine benzyl ester was prepared from nitro-L-arginine in a good yield, using two moles of *p*-toluenesulfonic acid and excess benzyl alcohol, by the azeotropic dehydration procedure with chloroform.[9] Compound VII, AOC-L-prolyl-L-phenylalanyl-nitro-L-arginine benzyl ester, was synthesized step by step from the nitroarginine benzyl ester by the trifluoroacetate method using reagent I. In this case, it should be considered that AOC-L-phenylalanine and the AOC-L-proline *p*-nitrophenyl ester are both oily materials, while the AOC-L-phenylalanine *p*-nitrophenyl ester (V) and AOC-L-proline are easily crystallizable substances.[3] Therefore, compound V was isolated as crystals and then subjected to the reaction with the nitroarginine benzyl ester, while AOC-L-proline was coupled directly to the dipeptide ester without isolating the *p*-nitrophenyl ester. The linking of serine to the tripeptide ester was a problem, as has been mentioned above. Since the

2) G. W. Anderson and R. W. Young, *J. Am. Chem. Soc.*, **74**, 5307 (1952); G. W. Anderson, J. Blodinger and A. D. Welcher, ibid., **74**, 5409 (1952); G. W. Anderson and F. M. Callahan, ibid., **80**, 2902 (1958); G. W. Anderson and R. Paul, ibid., **82**, 4596 (1960).

3) S. Sakakibara, M. Shin, M. Fujino, Y. Shimonishi, S. Inouye and N. Inukai, This Bulletin, **38**, 1522 (1965).

4) S. Sakakibara and M. Fujino, ibid., **39**, 947 (1966).

5) E. D. Nicolaides and H. A. De Wald, *J. Org. Chem.*, **26**, 3872 (1961).

6) E. D. Nicolaides and H. A. De Wald, ibid., **28**, 1926 (1963).

7) R. A. Boissonnas, S. Guttmann and P. A. Jaquenoud, *Helv. Chim. Acta*, **43**, 1349 (1960).

8) S. Guttmann, J. Pless and R. A. Boissonnas, ibid., **45**, 170 (1962).

9) S. Akabori, S. Sakakibara and S. Shiina, This Bulletin, **31**, 784 (1958).

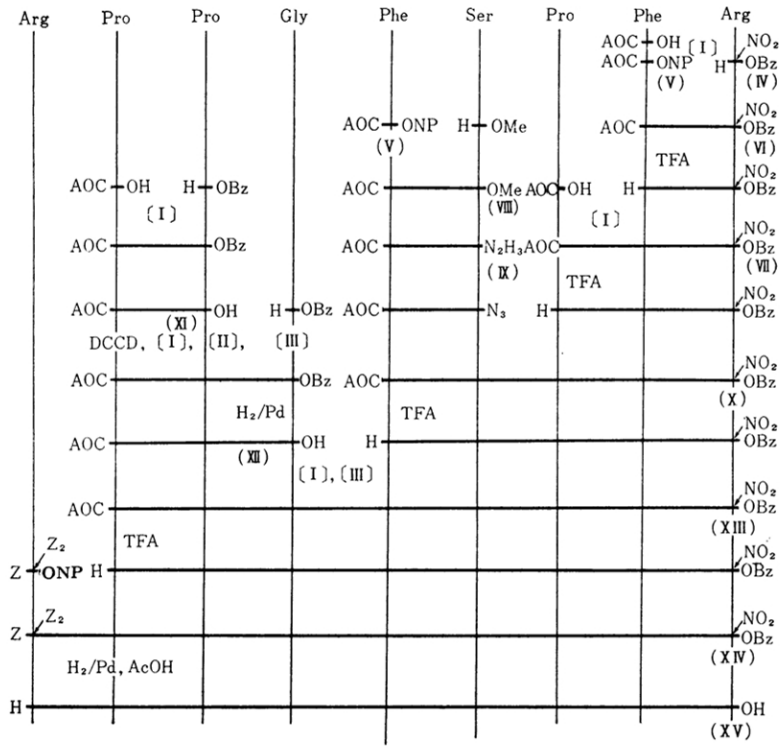

Fig. 1. Schematic diagram of the synthesis of bradykinin.

Bz=benzyl; NP=*p*-nitrophenyl; AOC=*t*-amyloxycarbonyl; TFA=trifluoroacetic acid; DCCD=dicyclohexylcarbodiimide; Z=carbobenzoxy.

application of the trifluoroacetate method for *N*, *O*-protected serine was not practical, the azide method was adopted in this case. After various attempts, it was found that the coupling of AOC-L-phenylalanyl-L-serine azide to the *C*-terminal tripeptide ester gave a much better result than that of AOC-L-serine azide. Next, AOC-L-prolyl-L-prolylglycine (XII) was prepared by coupling AOC-L-prolyl-L-proline (XI) with the glycine benzyl ester, followed by catalytic hydrogenation. A comparison was made using the reagents I, II and III in this condensation reaction; also, the dicyclohexylcarbodiimide method for preparing the same compound XII was tested. It was found that there was no difference in the optical purity of the respective products with these reagents; this result shows that the prolyl residue at the *C*-terminus of an acylpeptide is stable against racemization with the trifluoroacetate reagents I, II and III. The AOC-L-prolyl-L-prolylglycine thus formed was then satisfactorily coupled with the previously-prepared pentapeptide benzyl ester by the direct condensation method using reagent I. Finally, the tricarbobenzoxy-L-arginine *p*-nitrophenyl ester, which had been prepared by the reported method,[10] was coupled with the octapeptide,[11] and then all the protecting groups were removed simultaneously from the final product (XIV) by a single catalytic hydrogenation. The bradykinin thus obtained was completely colorless, and its biological activity was found to be slightly greater than that of a bradykinin sample prepared in the laboratory according to the method described by Nicolaides et al.[5,6]

Thus, the method of the condensation of peptides with trifluoroacetate reagents was demonstrated to be useful for the synthesis of complicated peptides.

Experimental*

Anderson's Racemization Test with Reagent III. A solution of carbobenzoxyglycyl-L-phenylalanine (0.8 g., 0.0025 mol.) in pyridine (5 ml.) was cooled to 0°C; a solution of reagent III (0.7 g., 0.0033 mol.) in pyridine (2 ml.) was then slowly added to the above solution. After the mixture had been allowed to stand at room temperature for one hour, water (0.02 ml.) was added to the reaction mixture to destroy any excess reagent. A solution of glycine ethyl ester hydrochloride (0.47 g.,

10) E. D. Nicolaides, H. A. De Wald, P. G. Shorley and H. O. J. Collier, *Nature*, **187**, 773 (1960).

11) Since one carbobenzoxy group in tricarbobenzoxyarginine was found to be susceptible to reagent I in pyridine, the use of the trifluoroacetate method in this case was disadvantageous.

* All melting points given are uncorrected. Each reaction process was checked by thin-layer chromatography.[12]

0.0034 mol.) and triethylamine (0.47 ml., 0.0034 mol.) in chloroform (5 ml.) was then stirred into the reaction mixture, and the mixture was allowed to react for 3 hr. at room temperature. The solvent was removed in vacuo, and a small volume of water was added to the residue to obtain the product as crystals; wt. 0.775 g. (78.3%); m. p. 115—117°C. The whole product was dissolved in absolute ethanol (38 ml.), and the solution was seeded with a small amount of the carbobenzoxyglycyl-DL-phenylalanylglycine ethyl ester and stored in a refrigerator. The crystals which appeared in the solution were collected by filtration at intervals and weighed. The data obtained are given below:

	Time after seeding	Crystals obtained
1)	6.5 hr.	No crystal appeared
2)	25 hr.	0.01 g., m. p. 132—133°C (DL-form)
3)	49 hr.	0.005 g., m. p. 130—133°C (DL-form)

Then the solution was concentrated to 25 ml., and stored in a refrigerator after having been seeded with L-form crystals.

4)	4.5 hr	0.315 g., m. p. 118—121°C
5)	24 hr.	0.15 g., m. p. 118—119.5°C
6)	54 hr.	0.13 g., m. p. 118—119.5°C

The optical rotation of the last three portions was $[\alpha]_D^{22}$ −12.6° (*c* 2, ethanol); lit. m. p. 118—119°C, $[\alpha]_D^{24}$ −12.3° (*c* 2, ethanol).[2] The recovery of the recrystallized whole product was 0.61 g. (79%), which includes 0.015 g. of racemate. When reagent I or II was applied in the same test, three or more hours were necessary for the completion of the ester-exchange reaction in pyridine at room temperature, and the final product was found to be entirely in the DL-form; the yield with reagent I was 81%, while the yield with reagent II was 70%.

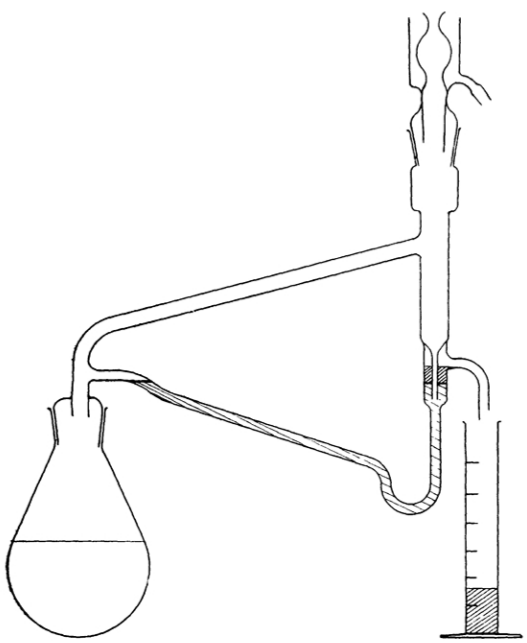

Fig. 2. An apparatus for the azeotropic dehydration with chloroform.

***g*-Nitro-L-arginine Benzyl Ester Ditosylate (IV).**—A mixture of *g*-nitro-L-arginine (11 g., 0.05 mol.), *p*-toluene sulfonic acid mono-hydrate (20 g., 0.1 mol.) and benzyl alcohol (40 ml.) in chloroform (100 ml.) was refluxed for 7—10 hr. in a dehydrating apparatus, as is illustrated in Fig. 2. After concentration to a small volume, the reaction mixture was extracted several times with petroleum ether, and the residue was crystallized by adding ether. The crude product was recrystallized from methanol-ether; wt. 29 g. (88.7%), m. p. 132—134°C, $[\alpha]_D^{24}$ +11.7° (*c* 3, pyridine).

Found: C, 49.40; H, 5.34; N, 10.61. Calcd. for $C_{27}H_{35}O_{10}N_5S_2$: C, 49.60; H, 5.41; N, 10.72%.

***t*-Amyloxycarboxyl-L-phenylalanine *p*-Nitrophenyl Ester (V).**—A solution of *t*-amyloxycarbonyl-L-phenylalanine (2.8 g., 0.01 mol.) in pyridine (5 ml.) was treated with *p*-nitrophenyl trifluoroacetate (2.7 g., 0.012 mol.) for about 2 hr. at room temperature; then water was added to the reaction mixture in order to precipitate the product as crude crystals (3.1 g., 77.5%). It was recrystallized from ethyl acetate and petroleum ether; wt. 2.6 g., yield 65%, m. p. 127—127.5°C, $[\alpha]_D^{24}$ −14.8° (*c* 1.4, ethanol); lit.[3] m. p. 128—129°C, $[\alpha]_D^{21}$ −14.4° (*c* 1.3, ethanol).

***t*-Amyloxycarbonyl-L-phenylalanyl-*g*-nitro-L-arginine Benzyl Ester (VI).**—Compound V (4 g., 0.01 mol.) was added to a solution of IV (6.5 g., 0.01 mol.) and triethylamine (2.8 ml., 0.02 mol.) in dimethylformamide (30 ml.), and the mixture was allowed to react for 60 hr. at room temperature. Then the mixture was diluted with ethyl acetate (about 200 ml.), and the solution was washed successively with water, N ammonia, 0.75 N hydrochloric acid and water, and dried over anhydrous sodium sulfate. The evaporation of the solvent left a crude product, which was recrystallized from ethyl acetate and petroleum ether; wt. 5.1 g. (ca. 90%), m. p. 120—124°C, $[\alpha]_D^{23}$ −15.8° (*c* 3, ethanol).

Found: C, 59.05; H, 6.44; N, 14.39. Calcd. for $C_{28}H_{38}O_7N_6$: C, 58.93; H, 6.71; N, 14.73%.

***t*-Amyloxycarbonyl-L-prolyl-L-phenylalanyl-*g*-nitro-L-arginine Benzyl Ester (VII).**—Compound VI (2.5 g., 0.0044 mol.) was treated with trifluoroacetic acid (5 ml.) for 30 min. at room temperature, and then the product was precipitated by adding dry ether. It was collected by filtration, washed with ether, and dried over sodium hydroxide in vacuo. *t*-Amyloxycarbonyl-L-proline (1.01 g., 0.0044 mol.) was treated with I (1.25 g., 0.0053 mol.) in pyridine (5 ml.) for about 2 hr. at room temperature, and then a solution of L-phenylalanyl-L-nitroarginine benzyl ester trifluoroacetate (2.5 g., 0.0044 mol.), prepared as described above, and triethylamine (1.36 ml., 0.0097 mol.) in dimethylformamide (55 ml.) was added to the reaction mixture. After it had stood for 2 days at room temperature, the reaction mixture was diluted with ethyl acetate. The diluted solution was washed successively with water, N ammonia, 0.75 N hydrochloric acid and water, and dried over anhydrous sodium sulfate. The dried solution was concentrated to a syrup which was solidified by trituration with ether. The product was reprecipitated from ethyl acetate and ether; wt. 2.64 g. (90.5%), m. p. 80—86°C, $[\alpha]_D^{21}$ −39.6° (*c* 1, ethanol).

Found: C, 59.86; H, 6.79; N, 14.24. Calcd. for $C_{33}H_{45}O_8N_7$: C, 59.35; H, 6.79; N, 14.68%.

***t*-Amyloxycarbonyl-L-phenylalanyl-L-serine**

Methyl Ester (VIII).—Compound V (4 g., 0.01 mol.) and L-serine methyl ester hydrochloride (1.6 g., 0.01 mol.) were dissolved in dimethylformamide (35 ml.), and then triethylamine (1.4 ml., 0.01 mol.) was added to the mixture. After 20 hr. at room temperature, the reaction mixture was diluted with ethyl acetate (300 ml.), and the solution was washed successively with water, N ammonia, 0.75 N hydrochloric acid and water, and dried over sodium sulfate. The dried solution was concentrated to a syrup, which was then solidified with petroleum ether. The product was reprecipitated as an amorphous powder from ethyl acetate-petroleum ether; wt. 3.7 g., (97.5%), m. p. 60—90°C, $[\alpha]_D^{25}$ −3.2° (*c* 1, dimethylformamide).

Found: C, 59.82; H, 7.46; N, 7.34. Calcd. for $C_{19}H_{28}O_6N_2$: C, 59.98; H, 7.42; N, 7.36%.

***t*-Amyloxycarbonyl-L-phenylalanyl-L-serine Hydrazide (IX).**—Compound VIII (4.2 g., 0.011 mol.) was dissolved in ethanol (9 ml.), and then 90% hydrazine hydrate (1.7 ml.) was added to the solution. After about 2 hr. at room temperature, the crystals (needles) which appeared were collected by filtration and recrystallized from ethanol; wt. 3.35 g. (88%), m. p. 175—177°C, $[\alpha]_D^{25}$ −4.0° (*c* 1, dimethylformamide).

Found: C, 56.44; H, 7.27; N, 14.66. Calcd. for $C_{18}H_{28}O_5N_4$: C, 56.82; H, 7.42; N, 14.73%.

***t*-Amyloxycarbonyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-*g*-nitro-L-arginine Benzyl Ester (X).**—Compound VII (2 g., 0.003 mol.) was treated with trifluoroacetic acid (4 ml.) for 30 min. at room temperature, and then the product was precipitated by adding dry ether. The precipitate was collected by filtration, washed well with ether, and dried over sodium hydroxide in vacuo; wt. 2.06 g. A solution of compound IX (1.03 g., 0.0027 mol.) in dimethylformamide (10 ml.) was cooled to −10°C; 2 N hydrochloric acid (2.73 ml., 0.0055 mol.) was stirred into the chilled solution and then a solution of sodium nitrite (0.207 g., 0.003 mol.) in water (1.5 ml.) was carefully added to the mixture at −10—−15°C. After about 15 min., a solution of previously-prepared tripeptide benzyl ester trifluoroacetate, dissolved in a mixture of dimethylformamide (3 ml.) and chloroform (8 ml.), was added to the reaction mixture, and then the pH was adjusted to 8 with triethylamine (about 0.84 ml.). Stirring was continued for 3 hr. at −10°C, and subsequently for 2 hr. at 0°C. During this period, the pH of the solution was maintained at 8 by adding triethylamine. The mixture was then stored in a refrigerator for 20 hr., after which the product was extracted thoroughly with ethyl acetate. The extracts were combined and washed well with N sodium bicarbonate, 0.75 N hydrochloric acid and water, and dried over sodium sulfate. The dried solution was concentrated to a syrup, and this was reprecipitated twice from methanol and ether as an amorphous powder; wt. 1.7 g. (70%), m. p. 100—115°C, $[\alpha]_D^{22}$ −38.2° (*c* 0.5, dimethylformamide).

Found: C, 59.85; H, 6.76; N, 14.17. Calcd. for $C_{45}H_{59}O_{11}N_9$: C, 59.92; H, 6.60; N, 13.98%.

***t*-Amyloxycarbonyl-L-prolyl-L-proline (XI).**—Reagent I (0.71 g., 0.003 mol.) was added to a solution of *t*-amyloxycarbonyl-L-proline (0.57 g., 0.0025 mol.) in dry pyridine (1.5 ml.) at room temperature. After about 2.5 hr., the excess reagent was destroyed with one small drop of water, and then a solution of L-proline benzyl ester hydrochloride (0.67 g., 0.0028 mol.) and triethylamine (0.385 ml., 0.0028 mol.) in chloroform (2.5 ml.) was added to the above reaction mixture. One more equivalent of triethylamine (0.4 ml.) was added to the mixture, which was then allowed to react for 3 days at room temperature. Then it was concentrated to a residue, which was dissolved in ethyl acetate (about 100 ml.); the solution was washed successively with N ammonia, 0.75 N hydrochloric acid and water, and dried over sodium sulfate. The dried solution was concentrated to a syrup, which was dissolved in methanol (20 ml.), and a small amount of palladium black was added to the solution. Hydrogen was bubbled through the solution for about 2 hr. with stirring, and then the catalyst was removed by filtration. The filtrate was concentrated to a syrup, and the material was crystallized by adding ether. The recrystallization of the product from ethyl acetate - petroleum ether gave prisms; wt. 0.66 g. (81%), m. p. 155—158°C, $[\alpha]_D^{21}$ −112.8° (*c* 1, ethanol).

Found: C, 58.97; H, 8.00; N, 8.53. Calcd. for $C_{16}H_{26}O_5N_2$: C, 58.88; H, 8.03; N, 8.58%.

***t*-Amyloxycarbonyl-L-prolyl-L-prolylglycine (XII).**—a) Compound XI (6.5 g., 0.02 mol.) and glycine benzyl ester tosylate (6.75 g., 0.02 mol.) were dissolved in chloroform (100 ml.), and triethylamine (2.8 ml., 0.02 mol.) was added to the mixture. After dicyclohexylcarbodiimide (4.12 g., 0.02 mol.) had been slowly added to the mixture, it was allowed to react for one day at room temperature. The urea formed was removed by filtration, and the filtrate was concentrated to a residue, which was then redissolved in ethyl acetate (about 100 ml.). This solution was washed successively with N sodium bicarbonate, N hydrochloric acid and water, and dried over anhydrous sodium sulfate. The dried solution was subjected to the hydrogenation procedure described above, and the final product was obtained as fine prisms which were dried over sulfuric acid in vacuo at room temperature; wt. 6.15 g. (80.5%), m. p. 109—112°C, $[\alpha]_D^{21}$ −132.8° (*c* 1, ethanol).[12]

Found: C, 53.70; H, 7.81; N, 10.49. Calcd. for $C_{18}H_{29}O_6N_3 \cdot H_2O$: C, 53.85; H, 7.78; N, 10.48%. The same sample was dried over phosphorous pentoxide in vacuo at 110°C for 3 hr. to remove the water of crystallization.

Found: C, 56.14; H, 7.56; N, 11.15. Calcd. for $C_{18}H_{29}O_6N_3$: C, 56.38; H, 7.62; N, 10.96%.

b) A solution of XI (0.815 g., 0.0025 mol.) in dry pyridine (1.8 ml.) was treated with reagent I, II or III at room temperature for 1—5 hr. until the ester-exchange reaction was complete; this was checked by thin-layer chromatography. Then a solution of glycine benzyl-ester tosylate (0.93 g., 0.0028 mol.) and triethylamine (0.39 ml., 0.0028 mol.) in dimethylformamide (1.5 ml.) was added to the reaction mixture, and in the cases of reagents I and II, one more equivalent of triethylamine (0.4 ml.) was also added to the reaction mixture. After about 3 or 4 days, ethyl acetate (100 ml.) was added to the reaction mixture, and the solution was washed successively with N ammonia, 0.75 N hydrochloric acid and water, and dried over sodium sulfate. After the dried solution had been concentrated to a residue, it was dissolved in methanol (about 30 ml.), and then a small amount of palladium black was added to the

solution. Hydrogen was bubbled through the solution for about 3 hr., and then the catalyst was removed by filtration. The filtrate was concentrated to a syrup, and the material was crystallized from ethyl acetate and petroleum ether. The results are listed below.

Reagent	Weight	m. p.	$[\alpha]_D^{23}$ (c 1, ethanol)[12]
I	0.84 g. (88%)	110—113°C,	−132.8°
II	0.59 g. (62%)	109—112°C,	−132.5°
III	0.82 g. (86%)	110—112°C,	−132.2°

***t*-Amyloxycarbonyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-*g*-nitro-L-arginine Benzyl Ester (XIII).**—Compound X (0.72 g., 0.8 mmol.) was dissolved in trifluoroacetic acid (2 ml.), and after about 30 min. at room temperature, the product was precipitated by adding dry ether, collected by filtration, and dried over sodium hydroxide in vacuo; wt. 0.7 g. A solution of the dried compound, XII (0.33 g., 0.86 mmol.), in pyridine (0.8 ml.) was treated with reagent I (0.3 g., 1.3 mmol.) for 2—3 hr. at room temperature. Then one small drop of water was added to the reaction mixture in order to destroy any excess reagent, and a solution of the previously-prepared pentapeptide benzylester trifluoroacetate (0.7 g.) and triethylamine (0.33 ml., 2.36 mmol.) in dimethylformamide (6 ml.) was added to the mixture. After about 24 hr. at room temperature, the reaction mixture was diluted with ethyl acetate (about 200 ml.) and the solution was washed successively with N ammonia, 0.75 N hydrochloric acid and water, and dried over anhydrous sodium sulfate. The dried solution was concentrated to a residue, which was triturated well with ether to obtain an amorphous powder. The product was reprecipitated from ethyl acetate and ether; wt. 0.69 g. (77%), m. p. 125—130°C, $[\alpha]_D^{22}$ −46.8° (c 0.5, dimethylformamide).

Found: C, 58.87; H, 6.61; N, 14.52. Calcd. for $C_{57}H_{76}O_{14}N_{12}$: C, 59.36; H, 6.64; N, 14.58%.

When reagent III was used instead of I, the yield of the product was 76%.

Tricarbobenzoxy-L-arginyl-L-prolyl-L-prolylglycyl-L-phenylalanyl-L-seryl-L-prolyl-L-phenylalanyl-*g*-nitro-L-arginine Benzyl Ester (XIV).—Compound XIII (0.2 g., 0.17 mmol.) was dissolved in trifluoroacetic acid (0.4 ml.), and after 40 min. the product was precipitated by adding dry ether. The precipitate was collected by filtration, washed well with ether, and dried over sodium hydroxide in vacuo; wt. 0.2 g. After the dried material had been dissolved in dimethylformamide (3.5 ml.), the tricarbobenzoxy-L-arginine *p*-nitrophenyl ester[10] (0.121 g., 0.175 mmol.) and triethylamine (0.0243 ml., 0.17 mmol.) were added to the solution. After one day at room temperature dry ether was added to the reaction mixture, and the precipitate which formed was washed well with ether by means of decantation. The residue was triturated with ethyl acetate, and then dissolved in chloroform. The solution was washed with water, N hydrochloric acid and water, and dried over anhydrous sodium sulfate. The dried solution was concentrated to a syrup under reduced pressure, and the remaining syrup was crystallized from ethyl acetate and ether. For purification the precipitation procedure from ethyl acetate and ether was repeated; wt. 0.17 g. (61%); m. p. 115—118°C, $[\alpha]_D^{22}$ −44.5° (c 0.5, dimethylformamide).

Found: C, 60.73; H, 6.23; N, 13.93. Calcd. for $C_{81}H_{96}O_{19}N_{16}$: C, 60.89; H, 6.06; N, 14.03%.

Bradykinin Triacetate.—Compound XIV (25 mg.) was dissolved in a mixture of acetic acid (3 ml.) and methanol (2 ml.), and a small amount of freshly-prepared palladium black was added to the solution. Hydrogen was bubbled through it for about 10 hr., until the absorption band of the solution at 267 mμ disappeared. This was measured at a concentration of 0.1 mg./5 ml. Then the catalyst was removed by filtration, and the filtrate was concentrated to a syrup under reduced pressure at room temperature. The residue was dissolved in water (15 ml.) and lyophilized. The product was completely colorless (wt. 16.7 mg.), and it was found to be homogeneous without any further purification. The homogeneity was tested by paper electrophoresis (pH 4.75, pH 3.5, pH 8.8) and by paper chromatography (BuOH : AcOH : H_2O=4 : 1 : 1, 80% pyridine, *t*-BuOH : AcOH : H_2O=2 : 1 : 1), using ninhydrin and Sakaguchi reagents respectively. The amino acid ratio in a hydrolyzate prepared with 6 N hydrochloric acid was as follows: Arg, 1.98; Pro, 2.89; Gly, 1.04; Phe, 1.96; Ser, 1.00. For analysis this material was dried at 110°C in vacuo over phosphorous pentoxide for 18 hr. About 13% of the weight was reduced during the procedure.

Found: C, 54.30; H, 7.01; N, 17.10. Calcd. for $C_{50}H_{73}O_{11}N_{15}\cdot 3CH_3COOH$: C, 54.22; H, 6.91; N, 16.94%.

The biological activity of this material was determined by the guinea-pig ileum contraction test;[13] it was thus found to be 1.4 times more than that of a sample prepared in this laboratory by the procedure of Nicolaides et al.[5,6]

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12) The concentration was calculated in a dry base.

13) T. Suzuki, Y. Mizushima, T. Sato and S. Iwanaga, *J. Biochem.* (Japan), **57**, 14 (1965).