THE PREPARATION OF L-ARGINYL DIPEPTIDES OF ASPARAGINE, GLUTAMINE, AND SOME BASIC AMINO ACIDS[1]

CASIMIR BERSE, LUCIEN PICHÉ, AND AKIRA UCHIYAMA

ABSTRACT

The synthesis of the following dipeptides is described: L-arginyl-L-asparagine acetate, L-arginyl-L-glutamine acetate, L-arginyl-L-lysine diacetate, and L-arginyl-L-ornithine diacetate. The two former were prepared by coupling a mixed anhydride of α-carbobenzoxy-ω-nitro-L-arginine directly with L-asparagine or with L-glutamine; catalytic hydrogenolysis of the resulting intermediates gave the dipeptides. The two others were obtained by combining α-carbobenzoxy-L-arginine with ω-carbobenzoxy-L-lysine methyl ester hydrochloride or with ω-carbobenzoxy-L-ornithine methyl ester hydrochloride in the presence of N,N′-dicyclohexylcarbodiimide; saponification and hydrogenation was necessary to uncover the resulting dipeptides.

Arginine is a universal constituent of proteins and is found in most of the physiologically active polypeptides. Its introduction in synthetic peptides has presented unusual difficulties caused by the strongly basic character of the guanidino group (1). The arginyl peptides, in which the carboxyl group of arginine is involved, were not prepared until three groups of workers reported initial success almost at the same time in 1953 (2, 3, 4). Although a variety of these compounds have now been synthetized by appropriate methods (5–8), the arginyl dipeptides containing basic amino acids have not hitherto been described; L-arginyl-L-arginine is the only exception (9).

Two methods were adapted to the combination of arginine with L-asparagine and L-glutamine or with L-lysine and L-ornithine respectively. In the first of these, α-carbobenzoxy-ω-nitro-L-arginine (6, 10) was converted to a mixed anhydride (11) by the action of ethyl chloroformate in the presence of an equimolar amount of tri-*n*-butylamine; it was then condensed directly with L-asparagine or with L-glutamine in alkaline solution. Catalytic hydrogenolysis of the resulting intermediates removed the carbobenzoxyl and nitro groups and resulted in the respective dipeptides; these were crystallized as the acetates.

The synthesis of L-arginyl-L-lysine and of L-arginyl-L-ornithine was attempted by the same procedure: the mixed anhydride of ethyl carbonate and α-carbobenzoxy-ω-nitro-L-arginine was condensed with ω-carbobenzoxy-L-lysine methyl ester hydrochloride and with ω-carbobenzoxy-L-ornithine methyl ester hydrochloride. Saponification of the resulting covered dipeptide esters was successful but the carboxylic compounds resisted hydrogenolysis in acidic, neutral, or basic media.

An alternate procedure based on the previous experience of Boissonnas *et al.* (15) was adopted. In this procedure the guanido group of α-carbobenzoxy-L-arginine is protected by a proton (2, 3) while condensation is carried out with N,N′-dicyclohexylcarbodiimide (12). Use of the proton to cover the guanido group avoided the necessity for hydrogenolysis of the nitro group and in contrast to previous experience of the authors (7) gave satisfactory yields of the protected dipeptides. The esters obtained by condensation of α-carbobenzoxy-L-arginine with ω-carbobenzoxy-L-lysine methyl ester or with ω-carbobenzoxy-L-ornithine methyl ester were saponified in the usual manner. Hydrogenation in 10% acetic acid over palladium catalyst converted the carboxylic intermediates into arginyl dipeptides; these were isolated as the diacetates.

[1]*Manuscript received in original form February 1, 1960, and, as revised, July 11, 1960.*
Contribution from the Department of Chemistry, Université de Montréal, Montreal, Que.

EXPERIMENTAL

*α-Carbobenzoxy-ω-nitro-*L*-arginyl-*L*-asparagine*

Ethyl chloroformate (2.44 ml, 20 mmoles) was added to a solution of α-carbobenzoxy-ω-nitro-L-arginine (7.06 g, 20 mmoles) (5, 6, 10) and tri-*n*-butylamine (2.8 ml, 20 mmoles) in dry dioxane (50 ml). After 10 minutes, L-asparagine (2.64 g, 20 mmoles) in 0.4 *N* sodium hydroxide (50 ml) was added to the mixture at 10°. Carbon dioxide gas was given off vigorously. The mixture was shaken for 15 minutes at 10°, and then it was acidified to Congo red with *N* hydrochloric acid. Dioxane was evaporated *in vacuo* in the water bath, water was decanted, and the resulting oily product was triturated with cold acetonitrile until crystallization occurred. The compound was recrystallized from a mixture of N,N-dimethylformamide and acetonitrile. Yield 4.6 g (48%), m.p. 201°, $[\alpha]_D^{25}$ +6.2 (*c*, 4.17 in N,N-dimethylformamide). Anal. Calc. for $C_{18}H_{25}O_8N_7$: C, 46.25; H, 5.39; N, 20.98. Found: C, 46.27; H, 5.46; N, 20.88.

L-*Arginyl-*L*-asparagine Acetate*

α-Carbobenzoxy-ω-nitro-L-arginyl-L-asparagine (2.12 g, 4.53 mmoles) was dissolved in 0.1 *N* sodium hydroxide (4.65 ml). Palladium catalyst was added and the mixture was shaken with hydrogen for 5 hours. The catalyst was filtered off; pH of the solution was 8.6; it was brought to 5–6 with glacial acetic acid, then the solvent was evaporated until dryness *in vacuo* at 30–40°. The resulting product was washed with absolute ethanol and treated with N,N-dimethylformamide. On standing, the residue crystallized. The compound was recrystallized from aqueous methanol. Yield 1.2 g (75%), m.p. 260° (decomp.), $[\alpha]_D^{25}$ +11.1° (*c*, 0.83 in water). Anal. Calc. for $C_{12}H_{24}O_6N_6$: C, 41.34; H, 6.95; N, 24.13. Found: C, 41.32; H, 7.07; N, 24.16.

*α-Carbobenzoxy-ω-nitro-*L*-arginyl-*L*-glutamine*

This compound was prepared from α-carbobenzoxy-ω-nitro-L-arginine (3.53 g, 10 mmoles) and L-glutamine (1.46 g, 10 mmoles) in the manner described for α-carbobenzoxy-ω-nitro-L-arginyl-L-asparagine; the product was crystallized several times from a mixture of a small amount of absolute ethanol and methylene chloride. Yield 2.8 g (45%), m.p. 168°, $[\alpha]_D^{25}$ −0.5° (*c*, 3.8 in N,N-dimethylformamide). Anal. Calc. for $C_{19}H_{27}O_8N_7$ (1/2 H_2O): C, 46.65; H, 5.72; N, 19.99. Found: C, 46.72; H, 5.91; N, 19.97.

L-*Arginyl-*L*-glutamine Acetate*

α-Carbobenzoxy-ω-nitro-L-arginyl-L-glutamine (2 g, 4.16 mmoles) was dissolved in a mixture of water (2 ml), methanol (2 ml), and glacial acetic acid (20 ml). The compound was hydrogenated for 12 hours at room temperature and at atmospheric pressure over 1 g of 10% palladium on carbon. The catalyst was filtered off and the solvent was evaporated *in vacuo* at 40–50°. The residue was crystallized from methanol–acetone. Yield 1.0 g (66%), m.p. 160°, $[\alpha]_D^{25}$ +19.2° (*c*, 3.87 in water). Anal. Calc. for $C_{13}H_{26}O_6N_6$ (1/2 H_2O): C, 42.16; H, 7.35; N, 22.65. Found: C, 42.34; H, 7.11; N, 22.67.

*α-Carbobenzoxy-ω-nitro-*L*-arginyl-ω-carbobenzoxy-*L*-lysine Methyl Ester*

α-Carbobenzoxy-ω-nitro-L-arginine (3.53 g, 10 mmoles) was dissolved in dry dioxane (100 ml) and tri-*n*-butylamine (2.4 ml, 10 mmoles) was added. The solution was cooled to 10° C; ethyl chloroformate (0.96 ml, 7.8 mmoles) was added with stirring and the solution was kept at 11–12° for 15 minutes. The product was then added to 150 ml of N,N-dimethylformamide containing 3.31 g (10 mmoles) of ω-carbobenzoxy-L-lysine methyl ester hydrochloride (13, 14). The mixture was shaken for 2 hours at room temperature and the solvent was evaporated at 40–50°. The residue was dissolved in ethyl acetate,

and the solution was washed successively with *N* hydrochloric acid, water, *N* sodium bicarbonate and water, dried over sodium sulphate, and evaporated to dryness *in vacuo*; it was then treated with isoamyl alcohol – methylene chloride (1:50). The resulting crystals were washed with ether and recrystallized from N,N-dimethylformamide–water. Yield 3.9 g (62%), m.p. 104°, $[\alpha]_D^{25}$ −2.5° (*c*, 4.64 in N,N-dimethylformamide). Anal. Calc. for $C_{29}H_{39}O_9N_7$: C, 55.35; H, 6.25; N, 15.58. Found: C, 55.28; H, 6.32; N, 15.39.

*α-Carbobenzoxy-ω-nitro-*L*-arginyl-ω-carbobenzoxy-*L*-lysine*

α-Carbobenzoxy-ω-nitro-L-arginyl-ω-carbobenzoxy-L-lysine methyl ester (6.35 g, 10.01 mmoles) was dissolved in methanol (20 ml) and *N* sodium hydroxide (20 ml, 20 mmoles) was added. The mixture was shaken for 3 hours; the alkaline solution was washed with ethyl acetate, then acidified to Congo red with *N* hydrochloric acid; the oily residue crystallized on standing. It was recrystallized from hot water or aqueous methanol. Yield 4.7 g (77%), m.p. 159°, $[\alpha]_D^{25}$ +3.8° (*c*, 1.44 in N,N-dimethylformamide). Anal. Calc. for $C_{28}H_{37}O_9N_7$: C, 54.80; H, 6.07; N, 15.93. Found: C, 54.55; H, 6.19; N, 15.74.

*α-Carbobenzoxy-*L*-arginyl-ω-carbobenzoxy-*L*-lysine Methyl Ester Hydrochloride*

α-Carbobenzoxy-L-arginine (3.08 g, 10 mmoles) (15, 17) and ω-carbobenzoxy-L-lysine methyl ester hydrochloride (3.65 g, 11 mmoles) were suspended in N,N-dimethylformamide (10 ml) containing diethyl phosphite (2 ml) and the mixture was stirred for 2 hours (15). To this solution was added N,N-dicyclohexylcarbodiimide (3.10 g, 15 mmoles). The mixture was stirred for 2 days at room temperature, then it was cooled to −10°; N,N-dicyclohexylurea precipitated and was filtered off. The urea was washed with a small amount of N,N-dimethylformamide which was combined with the filtrate. The solvent was removed *in vacuo*. The residue was triturated several times with anhydrous ether; it was then dissolved in methylene chloride (200 ml). The solution was washed with *N* ammonium hydroxide, water, *N* hydrochloric acid and water, and it was dried over sodium sulphate; the solvent was removed *in vacuo*. The crude product thus obtained was crystallized from methylene chloride – ether. It was recrystallized from the same solvent and again recrystallized from ethanol–ether. Yield 4.05 g (65%), m.p. 131°, $[\alpha]_D^{25}$ +9.9° (*c*, 5.69 in N,N-dimethylformamide). Anal. Calc. for $C_{29}H_{41}O_7N_6Cl$: C, 56.00; H, 6.66; N, 13.62. Found: C, 57.40; H, 7.01; N, 13.88.*

*α-Carbobenzoxy-*L*-arginyl-ω-carbobenzoxy-*L*-lysine Hydrochloride*

Sodium hydroxide (4 *N*, 2 ml, 8 mmoles) was added to α-carbobenzoxy-L-arginyl-ω-carbobenzoxy-L-lysine methyl ester hydrochloride (2.6 g, 4.2 mmoles) dissolved in methanol. The mixture was shaken at room temperature for 2 hours. The solution was diluted with water (2 ml), acidified with concentrated hydrochloric acid under cooling. Methanol was evaporated *in vacuo*. The product precipitated as an oil and crystallized on standing. It was recrystallized from hot water. Yield 1.53 g (60%), m.p. 205° $[\alpha]_D^{25}$ +27.8° (*c*, 1.82 in N,N-dimethylformamide). Anal. Calc. for $C_{28}H_{39}O_7N_6Cl$: C, 55.50; H, 6.50; N, 13.83. Found: C, 56.19; H, 7.12; N, 13.83.*

L-*Arginyl-*L*-lysine Diacetate*

(*a*) A number of attempts towards the hydrogenation of α-carbobenzoxy-ω-nitro-L-arginyl-ω-carbobenzoxy-L-lysine in methanol containing acetic acid or ammonium hydroxide or in neutral methanol, over palladium catalyst in the usual manner, were unsuccessful.

**In view of the poor agreement of the analytical data the other properties of the compound are subject to question. However, the intermediate at this stage of purity was suitable for the synthesis of* L*-arginyl-*L*-lysine diacetate.*

(*b*) α-Carbobenzoxy-L-arginyl-ω-carbobenzoxy-L-lysine hydrochloride (1 g, 1.64 mmoles) was dissolved in methanol (20 ml) containing 10% by volume of glacial acetic acid. The compound was hydrogenated for 3 hours at room temperature and atmospheric pressure over palladium catalyst. The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residue was crystallized from methanol–acetone. Yield 0.41 g (58%), m.p. 167°, $[\alpha]_D^{25}$ +5.1 (*c*, 3.41 in water). Anal. Calc. for $C_{16}H_{33}O_7N_6$: C, 45.48; H, 8.11; N, 19.99. Found: C, 45.59; H, 8.24; N, 20.61.

α-Carbobenzoxy-ω-nitro-L-arginyl-ω-carbobenzoxy-L-ornithine Methyl Ester

This compound was prepared from α-carbobenzoxy-ω-nitro-L-arginine (3.53 g, 10 mmoles) and ω-carbobenzoxy-L-ornithine methyl ester hydrochloride (3.46 g, 10 mmoles) (16) in the manner described for α-carbobenzoxy-nitro-L-arginyl-ω-carbobenzoxy-L-lysine methyl ester hydrochloride. The product was recrystallized from methanol – methylene chloride (1:100 by volume). Yield 3.53 g (58%), m.p. 88°, $[\alpha]_D^{25}$ −16.2° (*c*, 1.0 in N,N-dimethylformamide). Anal. Calc. for $C_{28}H_{37}O_9N_7$: C, 54.70; H, 6.06; N, 15.91. Found: C, 54.35; H, 6.20; N, 15.85.

α-Carbobenzoxy-ω-nitro-L-arginyl-ω-carbobenzoxy-L-ornithine

α-Carbobenzoxy-ω-nitro-L-arginyl-ω-carbobenzoxy-L-ornithine methyl ester (7.00 g, 14 mmoles) was dissolved in methanol (20 ml) and *N* sodium hydroxide (20 ml, 20 mmoles) was added. The mixture was shaken for 3 hours. The product was isolated in the usual manner and recrystallized from hot water. Yield 4.9 g (72%), m.p. 185°, $[\alpha]_D^{25}$ −12.7° (*c*, 0.77 in N,N-dimethylformamide). Anal. Calc. for $C_{27}H_{35}O_9N_7$: C, 54.00; H, 5.86; N, 16.31. Found: C, 53.81; H, 5.84; N, 16.25.

α-Carbobenzoxy-L-arginyl-ω-carbobenzoxy-L-ornithine Methyl Ester Hydrochloride

This compound was prepared from α-carbobenzoxy-L-arginine (3.08 g, 10 mmoles) and ω-carbobenzoxy-L-ornithine methyl ester hydrochloride (3.80 g, 11 mmoles) in the manner described for α-carbobenzoxy-L-arginyl-ω-carbobenzoxy-L-lysine methyl ester hydrochloride. The crude product was precipitated from ethanol–ether and was recrystallized from methylene chloride – ether. Yield 3.73 g (62%), m.p. 122°, $[\alpha]_D^{25}$ +4.6 (*c*, 4.78 in N,N-dimethylformamide). Anal. Calc. for $C_{28}H_{39}O_7N_6Cl$: C, 55.30; H, 6.48; N, 13.85. Found: C, 55.51; H, 6.60; N, 13.88.

α-Carbobenzoxy-L-arginyl-ω-carbobenzoxy-L-ornithine Hydrochloride

α-Carbobenzoxy-L-arginyl-ω-carbobenzoxy-L-ornithine methyl ester hydrochloride (1.00 g, 1.75 mmoles) was saponified in the manner described for α-carbobenzoxy-L-arginyl-ω-carbobenzoxy-L-lysine hydrochloride. The product was recrystallized from hot water. Yield 0.61 g (58%), m.p. 103–106°, $[\alpha]_D^{25}$ +3.5° (*c*, 1.72 in N,N-dimethylformamide). Anal. Calc. for $C_{27}H_{37}O_7N_6Cl$: C, 54.70; H, 6.29; N, 14.17. Found: C, 54.48; H, 7.03; N, 13.80.

L-Arginyl-L-ornithine Diacetate

α-Carbobenzoxy-L-arginyl-ω-carbobenzoxy-L-ornithine hydrochloride (1.00 g, 1.68 mmoles) was dissolved in methanol (10 ml) containing 10% by volume of glacial acetic acid. The compound was hydrogenated for 3 hours at room temperature and atmospheric pressure over the palladium catalyst. The final product was obtained in the manner described for L-arginyl-L-lysine diacetate. Yield 0.53 g (77%), hygroscopic. The crude product (0.52) in 5% acetic acid (50 ml) was applied to the top of the curtain of the continuous flow paper electrophoresis cell (Spinco Model CP) and was carried downward by acetic acid solution (5%, 16 liters). Sample feed rate was 0.8 ml/hour. The acetic

acid solution was maintained at 1° C and the curtain temperature was 10° C. Power supply was 30 milliamperes and 500 volts. The curtain came to equilibrium after 2 hours. Collection of the sample solution was started 1/2 hour after the first fraction reached the drip point. Thirty-two (32) test tubes were used to collect the solution. Duration of a typical run was 65 hours. The curtain was then dried and developed with ninhydrin.

The sample solution in test tubes No. 27 to 32 was combined and the solvent was evaporated *in vacuo* at 40–50° C. The residue was precipitated from methanol–ether. Yield 0.36 g (53.9%), $[\alpha]_D^{25}$ +9.6 (*c*, 2.9 in water). Anal. Calc. for $C_{15}H_{22}O_7N_6$: C, 44.11; H, 7.90; N, 20.60. Found: C, 44.14; H, 8.05; N, 20.49.

ACKNOWLEDGMENTS

This research was supported by a grant of the National Research Council of Canada. One of us (A. U.) held a Canadian Industries Limited Fellowship during part of the period of work. The authors wish to express their appreciation of this support.

REFERENCES

1. J. S. FRUTON. Advances in Protein Chem. **51** (1949).
2. G. W. ANDERSON. J. Am. Chem. Soc. **75**, 6081 (1953).
3. D. T. GISH and F. H. CARPENTER. J. Am. Chem. Soc. **75**, 5872 (1953).
4. K. HOFMANN, W. D. PECKHAM, and A. RHEINER. J. Am. Chem. Soc. **75**, 6083 (1953).
5. H. O. VAN ORDEN and E. L. SMITH. J. Biol. Chem. **208**, 751 (1954).
6. K. HOFMANN, W. D. PECKHAM, and A. RHEINER. J. Am. Chem. Soc. **78**, 238 (1956).
7. C. BERSE and L. PICHÉ. J. Org. Chem. **21**, 808 (1956).
8. N. IZUMIYA and S. MAKISUMI. J. Chem. Soc. Japan, **78**, 1768 (1957).
9. L. ZERVAS, T. OTANI, M. WINITZ, and J. P. GREENSTEIN. Arch. Biochem. Biophys. **75**, 290 (1958).
10. M. BERGMANN, L. ZERVAS, and H. RINKE. Z. physiol. Chem. **224**, 40 (1934).
11. R. A. BOISSONNAS. Helv. Chim. Acta, **34**, 874 (1951).
12. J. C. SHEENAN and G. P. HESS. J. Am. Chem. Soc. **77**, 1067 (1955).
J. C. SHEENAN, M. GOODMAN, and G. P. HESS. **78**, 1367 (1956).
13. A. NEUBERG and F. SANGER. Biochem. J. **37**, 515 (1943).
14. M. BERGMANN, L. ZERVAS, and F. WILLIAM. J. Biol. Chem. **111**, 249 (1935).
15. R. A. BOISSONNAS, ST. GUTTMANN, R. L. HUGUENIN, P. A. JAQUENOUD, and ED. SANDRIN. Helv. Chim. Acta, **41**, 1867 (1958).
16. R. L. M. SYNGE. Biochem. J. **42**, 100 (1948).
17. M. BERGMANN and L. ZERVAS. Ber. **65**, 1199 (1932).