A Synthesis of Oxytocin

By Shumpei SAKAKIBARA, Yoshibumi NOBUHARA,* Yasutsugu SHIMONISHI and Reiko KIYOI

(Received July 27, 1964)

Oxytocin, which is well known as a hormone of the posterior pituitary gland, has been synthesized by many workers[1-6] in different ways since the first brilliant synthesis of the hormone by du Vigneaud et al. in 1953—1954[7]. In all cases, two protecting groups, *S*-benzyl for the cysteinyl residue and carbobenzoxy for the amino nitrogen, have been employed during the syntheses for the following reasons: (1) The *S*-benzyl group is comparatively stable during the peptide synthesis. (2) The carbobenzoxy group is, generally, the most convenient *N*-protecting group for peptide synthesis, and the selective removal of the *N*-protecting group from an *N*-carbobenzoxy-peptide derivative containing *S*-benzyl cysteine can be accomplished by using anhydrous hydrogen bromide without any complications in other parts of the peptide. (3) Both protecting groups can be removed simultaneously using sodium in liquid ammonia.

The sodium treatment of the protected peptide in liquid ammonia, however, is rather drastic, and some side-reactions may occur during the process. Moreover, it must be remembered that the removal of a protecting group from a larger peptide is, in general, more difficult than the removal of one from a smaller peptide. Actually, du Vigneaud et al. reported that the protected pre-oxytocic nona-peptide had been reduced in boiling liquid ammonia.[7] Nevertheless, the yield of active oxytocin from a large-scale sample (1.3 g.) of the protected nona-peptide was less than that from a small-scale sample (50 mg.) of the same intermediate.[5] This must be because it is difficult to remove the *S*-benzyl group with sodium in liquid ammonia. Therefore, an *S*-protecting group which is easier to remove has long been required in the preparation of such a complicated cysteine-peptide.

Only one different route to oxytocin has been reported, the method of Velluz.[3] In this the *S*-trityl group is used for the protection of the sulfhydryl group. Since, however, the *S*-trityl group is quite unstable against anhydrous hydrogen bromide, the use of the carbobenzoxy group for the protection of amino nitrogen during peptide synthesis is impossible, and the more labile *N*-trityl group is used. This limitation is fatal to the practical synthesis of the hormone, because the synthesis of *N*-trityl amino acids or peptides is, in general, much more complicated than that of carbobenzoxy derivatives.[8]

Recently, it was found by the present authors[9] that the *p*-methoxybenzyl (anisyl) group was valuable in protecting the thiol of cysteinyl residues; that is, the protecting group was stable against hydrogen bromide at room temperature, and it was easily cleaved from the final product by treatment with either sodium in liquid ammonia, or boiling trifluoroacetic acid. Moreover, it was found that the synthesis of *S*-*p*-methoxybenzyl cysteine was as easy as that of the *S*-benzyl derivative.

In the present paper, the synthesis of oxytocin using the *p*-methoxybenzyl group for the protection of cysteinyl residues will be described. For the synthesis of the hormone, the step-wise elongation method using carbobenzoxyamino acid *p*-nitrophenylesters was employed. *N*-Carbobenzoxy-*S*-*p*-methoxybenzyl-L-cysteine *p*-nitrophenylester (III) was synthesized by the dicyclohexylcarbodiimide method. Compound III was coupled with L-prolyl-L-leucylglycinamide,[10] and then the carbobenzoxy group was removed from the

* Present address: Dainippon Pharmaceutical Co., Ltd., Fukushima-ku, Osaka.

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

2) J. Rudinger, J. Honzl and M. Zaoral, *Chem. Listy*, **50**, 288 (1956); *Coll. Czech. Chem. Communs.*, **21**, 202 (1956).

3) L. Velluz, G. Amiard, J. Bartos, B. Goffinet and R. Heymès, *Bull. soc. chim. France*, **1956**, 1464.

4) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 2504 (1959).

5) M. Bodanszky and V. du Vigneaud, ibid., **81**, 5688 (1959).

6) H. C. Beyerman, J. S. Bontekoe and A. C. Koch, *Rec. trav. chim.*, **78**, 935 (1959).

7) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, ibid., **76**, 3115 (1954).

8) L. Zervas and D. M. Theodoropoulos, ibid., **78**, 1359 (1956); L. Zervas, D. Borovas and E. Gazis, ibid., **85**, 3660 (1963).

9) S. Akabori, S. Sakakibara, Y. Shimonishi and Y. Nobuhara, This Bulletin, **37**, 433 (1964).

10) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **76**, 3107 (1954).

product, *N*-carbobenzoxy-*S*-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IV), by treatment with hydrogen bromide in the normal way. A series of carbobenzoxyamino acid *p*-nitrophenylesters were then coupled one by one with the respective peptide amides after treatment with hydrogen bromide. No complications occurred with *S*-methoxybenzyl cysteine residues during the synthesis, and the final protected nona-peptide amide (IX) was obtained in a good yield.

H-CyS(MBz)-OH → Cbz-CyS(MBz)-OH →
(I) (II)

Cbz-CyS(MBz)-OPNP
(III)

III + H-Pro-Leu-Gly-NH_2 →

Cbz-CyS(MBz)-Pro-Leu-Gly-NH_2 (IV)

1) HBr/AcOH
2) Cbz-Asp(NH_2)-OPNP → Cbz-Asp(NH_2)-

CyS(MBz)-Pro-Leu-Gly-NH_2 (V)

1) HBr/AcOH
2) Cbz-Glu(NH_2)-OPNP → Cbz-Glu(NH_2)-

Asp(NH_2)-CyS(MBz)-Pro-Leu-Gly-NH_2 (VI)

1) HBr/AcOH
2) Cbz-Ileu-OPNP → Cbz-Ileu-Glu(NH_2)-

Asp(NH_2)-CyS(MBz)-Pro-Leu-Gly-NH_2 (VII)

1) HBr/AcOH
2) Cbz-Tyr(Bz)-OPNP → Cbz-Tyr(Bz)-Ileu-

Glu(NH_2)-Asp(NH_2)-CyS(MBz)-

Pro-Leu-Gly-NH_2 (VIII)

1) HBr/AcOH
2) III → Cbz-CyS(MBz)-Tyr-Ileu-

Glu(NH_2)-Asp(NH_2)-CyS(MBz)-

Pro-Leu-Gly-NH_2 (IX)

1) CF_3COOH or Na/NH_3
2) aeration → Oxytocin

MBz=*p*-Methoxybenzyl Cbz=Carbobenzoxy
PNP=*p*-Nitrophenyl

First, the protected nona-peptide (IX) was treated with boiling trifluoroacetic acid for two hours; it was confirmed that a total oxytocic activity of about 3300 units is obtained from 100 mg. of substance IX after the usual aeration procedure. Some hydrolysis of amide-bonds probably occurs during the process, and this is the main reason why the recovery of the active oxytocin is low. The trifluoroacetic acid procedure is now being improved in the laboratory. The usual procedure of sodium reduction in liquid ammonia, followed by aeration at pH 6.5, gave a high oxytocic activity, regardless of the quantity of material IX fed into the reaction (170000 units from 1 g. of substance IX, or 24000 units from 100 mg. of the same substance). This was surprising in view of the results of Bodanszky et al.,[5] who reported that 12500—15000 units were obtained from 50 mg. of a protected nona-peptide containing *S*-benzyl-cysteinyl residues, whereas 168000 units were obtained from 1.3 g. of the same material.

Experimental*

***S*-*p*-Methoxybenzyl-L-Cysteine (I).** — This compound was prepared from L-cystine (50.0 g., 0.21 mol.) and *p*-methoxybenzyl chloride (72.0 g., 0.46 mol.) according to the procedure of du Vigneaud et al. for the synthesis of *S*-benzyl derivatives. The recrystallization of the crude product (107 g.) from water gave colorless plates; wt. 78.0 g. (78%), m. p. 198—199°C (decomp.), $[\alpha]_D^{25}$ +22.6° (*c* 1.02, N NaOH).

Found: C, 54.50; H, 6.33; N, 5.89; S, 13.05. Calcd. for $C_{11}H_{15}O_3NS$: C, 54.75; H, 6.26; N, 5.81; S, 13.29%.

***N*-Carbobenzoxy-*S*-*p*-methoxybenzyl-L-cysteine (II).**—A solution of I (12.1 g., 0.05 mol.) in a cold N sodium hydroxide solution (50 ml.) was treated with carbobenzoxy chloride (10.3 g., 0.06 mol.) and 2 N sodium hydroxide (30 ml.) alternately under vigorous stirring below 0°C. The stirring was continued for an additional hour without external cooling. When usual extraction procedures were then followed, an oily material was obtained which was solidified by adding petroleum ether (60—80°C). The crude material was recrystallizable from toluene; wt. 14.2 g. (76%), m. p. 62—65°C, $[\alpha]_D^{23}$ −45.5° (*c* 1.98, acetone).

Found: C, 61.11; H, 5.81; N, 3.68; S. 8.46. Calcd. for $C_{19}H_{21}O_5NS$: C, 60.78; H, 5.64; N, 3.73; S, 8.54%.

***N*-Carbobenzoxy-*S*-*p*-methoxybenzyl-L-cysteine *p*-nitrophenylester (III).**—A solution of II (109 g., 0.29 mol.) and *p*-nitrophenol (48.4 g., 0.348 mol.) in ethyl acetate (250 ml.) was cooled to −10°C, and dicyclohexylcarbodiimide (59.7 g., 0.29 mol.) in ethyl acetate (100 ml.) was stirred in. The mixture was allowed to react for an hour at −10°C, and then for two hours more at room temperature. The precipitated materials were filtered off and washed with a small amount of ethyl acetate. The filtrate and washings were combined and concentrated to dryness in vacuo. Crude material III was collected by using a mixture of ethanol and petroleum ether (60—80°C); wt. 18.5 g., m. p. 92—97°C. Recrystallization from ethanol gave colorless fine needles; wt. 110 g. (76%), m. p. 94—97°C, $[\alpha]_D^{21}$ −39.8° (*c* 2.06, dimethylformamide).

Found: C, 60.71; H, 4.80; N, 5.78; S, 6.68. Calcd. for $C_{25}H_{24}O_7N_2S$: C, 60.48; H, 4.87; N, 5.64; S, 6.46%.

***N*-Carbobenzoxy-*S*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IV).** — A solution of III (19.8 g., 0.04 mol.) and L-prolyl-L-leucylglycinamide[10] (11.4 g., 0.04 mol.) in dimethylformamide (20 ml.) was allowed to react overnight at room

* All melting points given are uncorrected. All samples for analyses were first dried in vacuo at 110°C for 2 hr.

temperature. Ethyl acetate (60 ml.) was then added to the reaction mixture to precipitate the product as crystals, which were collected by filtration. The crude product was recrystallized from hot methanol (300 ml.) and water (300 ml.); wt. 19.8 g. (77%), m. p. 165—167°C, $[\alpha]_D^{21}$ −55.4° (*c* 2, dimethylformamide).

Found: C, 59.90; H, 6.81; N, 10.59; S, 4.91. Calcd. for $C_{32}H_{43}O_7N_5S$: C, 59.88; H, 6.75; N, 10.91; S, 5.00%.

***N*-Carbobenzoxy-L-asparaginyl-*S*-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucyl-glycinamide (V).**—A suspension of IV (15.09 g., 0.024 mol.) in acetic acid (25 ml.) was treated with 29% hydrogen bromide in acetic acid (120 g.). After one hour at room temperature, dry ether (1000 ml.) was added to the reaction mixture. The precipitated mass was separated by filtration, washed well with ether three times, and dried over sodium hydroxide. The dried material was dissolved in methanol (150 ml.), and ion exchange resin IRA-400 (in an OH cycle) was added until the bromine ions disappeared. Then the resin was filtered off and washed throughly with methanol. The filtrate and washings were combined and evaporated to dryness. The residue and *N*-carbobenzoxy-L-asparagine *p*-nitrophenylester[5] (9.09 g., 0.024 mol.) were dissolved in dimethylformamide (30 ml.), and the mixture was allowed to react at room temperature for two days. The product was then precipitated by adding ethyl acetate (100 ml.), collected by filtration, washed with ethyl acetate and with ethanol, and dried; wt. 15.4 g. (87%), m. p. 211—212°C, $[\alpha]_D^{20}$ −50.5° (*c* 1, dimethylformamide). For analysis, a part of the product was recrystallized from 80% methanol; m. p. 213—214°C, $[\alpha]_D^{21}$ −51.9° (*c* 1, dimethylformamide).

Found: C, 57.14; H, 6.47; N, 12.68; S, 4.12. Calcd. for $C_{36}H_{49}O_7N_9S$: C, 57.20; H, 6.54; N, 12.97; S, 4.24%.

***N*-Carbobenzoxy-L-glutaminyl-L-asparaginyl-*S*-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VI).**—A suspension of V (15.12 g., 0.02 mol.) in acetic acid (20 ml.) was treated with 29% hydrogen bromide in acetic acid (150 g.) for one hour at room temperature. Dry ether (1000 ml.) was added to the reaction mixture to precipitate the product, which was then separated and washed with ether three times and dried over sodium hydroxide. Then the dried mass was dissolved in methanol (200 ml.), and the bromine ions in the solution were removed by adding ion exchange resin IRA-400 (in an OH cycle). The bromine-free solution was concentrated to dryness in vacuo, and the residue was dissolved in dimethylformamide (30 ml.). *N*-Carbobenzoxy-L-glutamine *p*-nitrophenylester[5] (8.42 g., 0.021 mol.) was added to the solution, and the mixture was kept for two days at room temperature. Then the product was precipitated by adding ethyl acetate, which was collected by filtration and washed with ethyl acetate and ethanol. The yield was 13.51 g. (76%); m. p. 207—208°C, $[\alpha]_D^{21}$ −42.6° (*c* 1, dimethylformamide). For analysis, a part of the product was recrystallized from 80% methanol; m. p. 208—210°C, $[\alpha]_D^{21}$ −51.3° (*c* 1, dimethylformamide).

Found: C, 55.93; H, 6.79; N, 14.51; S, 3.43. Calcd. for $C_{41}H_{57}O_{11}N_9S$: C, 55.70; H, 6.50; N, 14.26; S, 3.63%.

***N*-Carbobenzoxy-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VII).**—A suspension of VI (9.72 g., 0.011 mol.) in 20 ml. of acetic acid was treated with 29% hydrogen bromide in acetic acid (80 g.) for one hour. The product was precipitated by adding dry ether (600 ml.), and the precipitate was washed three times with dry ether and dried. The dried product was dissolved in methanol (300 ml.), and the solution was treated with ion exchange resin IRA-400 (in an OH cycle) until bromine ions disappeared. The resin was filtered and washed well with methanol. The filtrate and washings were combined and concentrated to dryness in vacuo. The residue was dissolved in 25 ml. of dimethylformamide, and to the solution was added *N*-carbobenzoxy-L-isoleucine *p*-nitrophenylester[5] (4.63 g., 0.012 mol.). The mixture was allowed to react for two days at room temperature. Ethyl acetate (200 ml.) was added to the reaction mixture, and the precipitates were collected by filtration and washed with ethyl acetate and then with ethanol. The yield was 8.56 g. (78%); m. p. 216—217°C, $[\alpha]_D^{20}$ −43.6° (*c* 1, dimethylformamide). The product was recrystallized from 80% methanol (500 ml.); wt. 6.93 g., m. p. 217—218°C, $[\alpha]_D^{18}$ −44.2° (*c* 1, dimethylformamide).

Found: C, 56.55; H, 7.07; N, 13.89; S, 3.10. Calcd. for $C_{47}H_{68}O_{12}N_{10}S$: C, 56.61; H, 6.87; N, 14.05; S, 3.22%.

***N*-Carbobenzoxy-*O*-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (VIII).**—A suspension of VII (4.99 g., 0.005 mol.) in acetic acid (10 ml.) was treated with 29% hydrogen bromide in acetic acid (25 g.) for one hour. Dry ether (250 ml.) was added to the reaction mixture to precipitate the product, which was collected by filtration, washed with ether three times, and dried. The dried material was dissolved in methanol (500 ml.). Ion exchange resin IRA-400 (in an OH cycle) was added to the solution until bromine ions disappeared, and then the resin was filtered off. The filtrate was evaporated to dryness. The residue was dissolved in dimethylformamide (60 ml.), and *N*-carbobenzoxy-*O*-benzyl-L-tyrosine *p*-nitrophenyl ester[5] (2.63 g., 0.005 mol.) was added to the solution. After two days, ethyl acetate (300 ml.) was added to the reaction mixture to precipitate the product, which was then filtered off, washed with ethyl acetate and then with ethanol, and dried; wt. 5.33 g. (85%), m. p. 221—222°C, $[\alpha]_D^{18}$ −33.1° (*c* 1, dimethylformamide). For analysis, a part of the product was recrystallized from 80% methanol; m. p. 223—224°C, $[\alpha]_D^{18}$ −35.7° (*c* 1, dimethylformamide).

Found: C, 60.31; H, 6.54; N, 12.42; S, 2.63. Calcd. for $C_{63}H_{83}O_{14}N_{11}S$: C, 60.51; H, 6.69; N, 12.32; S, 2.57%.

***N*-Carbobenzoxy-*S*-*p*-methoxybenzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IX).**—A suspension of VIII (5.00 g.,

0.004 mol.) in acetic acid (20 ml.) was treated with 29% hydrogen bromide in acetic acid (40 g.). After one hour, dry ether (400 ml.) was added to precipitate the product, which was then separated, washed with ether, and dried. The dried mass was dissolved in dimethylformamide (40 ml.) and triethylamine (6 ml.) was carefully added to the solution. Triethylamine hydrobromide which formed was filtered off, and III (2.38 g., 0.0048 mol.) was added to the filtrate. After three days, ethyl acetate (200 ml.) was added to the reaction mixture and the precipitate which formed was collected by filtration and washed with ethyl acetate and then with ethanol. The yield was 5.16 g. (93%); m. p. 218—220°C, $[\alpha]_D^{20}$ −41.1° (*c* 1, dimethylformamide). For analysis, a part of the product was recrystallized from 80% methanol; m. p. 223—224°C, $[\alpha]_D^{18}$ −43.7° (*c* 1, dimethylformamide).

Found: C, 58.19; H, 6.68; N, 12.29; S, 4.56. Calcd. for $C_{67}H_{90}O_{16}N_{12}S_2$: C, 58.15; H, 6.56; N, 12.15; S, 4.63%.

Oxytocin.—a) A solution of IX (100 mg.) and anisole (50 mg.) in trifluoroacetic acid (30 ml.) was gently refluxed for two hr. Then the solvent was evaporated off in vacuo, and the residue was dissolved in water (100 ml.). The pH was adjusted to 6.5 with diluted aqueous ammonia, and carbon dioxide free air was bubbled through the solution until the nitroprusside-test became negative. Then the solution was acidified to pH 4 with acetic acid, and the small amount of insoluble material was filtered out. The final solution showed a total oxytocic activity of 3300 units as determined by rat-uterine contractive activity.

b) Product IX was reduced with sodium in liquid ammonia, as described by Bodanszky and du Vigneaud,[4] and the final reduced material was aerated to oxytocin as has been described before. The total oxytocic activity obtained from 100 mg. of IX was 24500 units, while that from 1 g. IX was 180000 units.

Summary

N-Carbobenzoxy-*S*-methoxybenzyl-L-cysteinyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-*p*-methoxybenzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide (IX) was synthesized by the step-wise elongation method, and highly potent oxytocin was obtained from IX by treatment not only with sodium in liquid ammonia, but also with boiling trifluoroacetic acid, followed by the usual aeration procedure. It was demonstrated during the reactions that the newly introduced *S*-*p*-methoxybenzyl group could be cleaved more easily than the usual *S*-benzyl group, and that the new *S*-protecting group was useful in the preparation of complicated cysteinyl peptides.

The authors wish to express their thanks to Professor Shiro Akabori for his encouragement and valuable advice, and to Professor Shigeru Aonuma and to Dr. Keiji Nakamura of the Dainippon Pharmaceutical Co., Ltd. for determining the oxytocic activities of the synthetic materials.

Peptide Center
Institute for Protein Research
Osaka University
Kita-ku, Osaka