907. *Oxytocin and 4-Glycine Oxytocin* *

By A. P. FOSKER and H. D. LAW

An improved method for the synthesis of oxytocin (by the condensation of peptide intermediates) has been developed and 4-glycine oxytocin has been synthesised by the same general approach.

THE structure (I) of the posterior pituitary hormone, oxytocin, was established by synthesis by Du Vigneaud, Ressler, Swan, Roberts, Katsoyannis, and Gordon in 1953.[1,2] Many other syntheses have since been reported, mostly concerned with new methods for the preparation of the protected nonapeptide (II). The final stages have generally remained unchanged. Reduction by sodium in liquid ammonia has been used for the removal of the protecting groups and ærial oxidation at neutral pH for the formation of the disulphide bridge. A large number of analogues of oxytocin has been prepared and investigated biologically.[3]

For purity, the most satisfactory synthesis involves stepwise addition of single amino-acid residues, in the form of *N*-benzyloxycarbonyl *p*-nitrophenyl esters, to the carboxyl-protected *C*-terminal glycine residue.[4] This method has found widespread application in peptide chemistry. However, approaches which involve the condensation of the carboxyl group of one suitably protected peptide with the amino-group of another are still widely used and, in particular circumstances, can have over-riding advantages over stepwise routes, being more easily adapted to team efforts. If side reactions peculiar to certain amino-acid residues occur, or solubility problems as the length of a peptide chain increases, arise, the stepwise method becomes impracticable. Often a combination of both approaches may be used.

Ultimately to synthesise analogues of oxytocin, we further investigated the " improved synthesis of oxytocin " described in 1959 by Bodanszky and Du Vigneaud.[5] In this approach, the final stages in the formation of the protected nonapeptide (II) involve the condensation of the dipeptide, *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-tyrosine (III),

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* The suggestions of the Committee on Nomenclature which reported at the Fifth European Peptide Symposium are adopted throughout this Paper (Peptides: Proc. Fifth European Peptide Symp., Oxford, September 1962, ed. G. T. Young, Pergamon Press, Oxford, 1963).

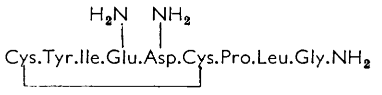
[1] V. Du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Amer. Chem. Soc.*, 1953, **75**, 4879.

[2] V. Du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *J. Amer. Chem. Soc.*, 1954, **76**, 3115.

[3] H. D. Law, Review in " Progress in Medicinal Chemistry," ed. G. P. Ellis and G. B. West, Butterworths Scientific Publications, London, 1965, vol. IV, p. 86.

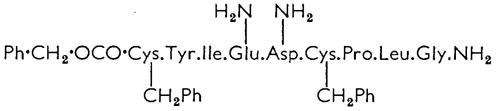
[4] M. Bodanszky and V. Du Vigneaud, *J. Amer. Chem. Soc.*, 1959, **81**, 5688.

[5] M. Bodanszky and V. Du Vigneaud, *J. Amer. Chem. Soc.*, 1959, **81**, 2504.



$$\mathrm{Cys.Tyr.Ile.}\overset{\mathrm{H_2N}}{\mathrm{Glu}}\mathrm{.}\overset{\mathrm{NH_2}}{\mathrm{Asp}}\mathrm{.Cys.Pro.Leu.Gly.NH_2}$$

(I) Oxytocin



$$\mathrm{Ph{\cdot}CH_2{\cdot}OCO{\cdot}}\underset{\mathrm{CH_2Ph}}{\mathrm{Cys}}\mathrm{.Tyr.Ile.}\overset{\mathrm{H_2N}}{\mathrm{Glu}}\mathrm{.}\overset{\mathrm{NH_2}}{\mathrm{Asp}}\mathrm{.}\underset{\mathrm{CH_2Ph}}{\mathrm{Cys}}\mathrm{.Pro.Leu.Gly.NH_2}$$

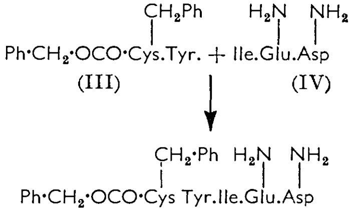
(II) *N*-Benzyloxycarbonyl-*SS′*-dibenzyloxytocin

with the tripeptide, L-isoleucyl-L-glutaminyl-L-asparagine (IV). The resulting pentapeptide (V) is subsequently condensed with the *C*-terminal tetrapeptide derivative, *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycineamide (VI), to give the required protected nonapeptide.

In the original synthesis, all intermediates (III to VI) were obtained in highly purified forms, but for the tripeptide (IV) and the tetrapeptide derivative (VI), the synthetic methods were inconvenient. Since Cash[6] has described a simplified preparation of *N*-benzyloxycarbonyl-L-prolyl-L-leucylglycineamide (VII) from which the tetrapeptide derivative can be easily obtained, it seemed desirable to devise a new synthesis of the tripeptide (IV) so that all the peptide intermediates would be accessible.

Although the dipeptide derivative (III) and the tripeptide (IV) can be coupled satisfactorily by the mixed anhydride procedure,[7] the preparation of the protected nonapeptide from the resulting pentapeptide derivative (V) and the partially protected tetrapeptide (VI) is more troublesome. We have investigated this stage further, with regard both to the separation of the products and to the use of modern coupling agents.

Finally, we have applied the general approach, as here modified, to the synthesis of 4-glycine oxytocin.



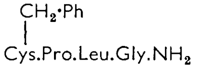
$$\mathrm{Ph{\cdot}CH_2{\cdot}OCO{\cdot}}\overset{\mathrm{CH_2Ph}}{\mathrm{Cys}}\mathrm{.Tyr.} + \mathrm{Ile.}\overset{\mathrm{H_2N}}{\mathrm{Glu}}\mathrm{.}\overset{\mathrm{NH_2}}{\mathrm{Asp}}$$

(III) (IV)

$$\downarrow$$

$$\mathrm{Ph{\cdot}CH_2{\cdot}OCO{\cdot}}\overset{\mathrm{CH_2{\cdot}Ph}}{\mathrm{Cys}}\ \mathrm{Tyr.Ile.}\overset{\mathrm{H_2N}}{\mathrm{Glu}}\mathrm{.}\overset{\mathrm{NH_2}}{\mathrm{Asp}}$$

(V)



$$\overset{\mathrm{CH_2{\cdot}Ph}}{\mathrm{Cys}}\mathrm{.Pro.Leu.Gly.NH_2}$$

(VI)

$\mathrm{Ph{\cdot}CH_2{\cdot}OCO{\cdot}Pro.Leu.Gly.NH_2}$

(VII)

Synthesis of the Tripeptide (IV).—A stepwise *N*-benzyloxycarbonyl-amino-acid *p*-nitrophenyl ester approach proved ideal. *N*-Benzyloxycarbonyl-L-asparagine was converted into the *p*-nitrobenzyl ester by the reaction of its triethylammonium salt with *p*-nitrobenzyl chloride.[8] To confirm its optical purity, the ester was hydrogenolysed and the optical rotation of the resulting L-asparagine solution measured. By the action of a solution of hydrogen bromide in acetic acid, *p*-nitrobenzyl *N*-benzyloxycarbonyl-L-asparagine was readily converted into the crystalline hydrobromide of *p*-nitrobenzyl L-asparaginate. After neutralisation by one equivalent of triethylamine, the free base was treated with *p*-nitrophenyl *N*-benzyloxycarbonyl-L-glutaminate to form the crystalline dipeptide

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[6] W. D. Cash, *J. Org. Chem.*, 1961, **26**, 2136.

[7] J. R. Vaughan, jun., and J. A. Eichler, *J. Amer. Chem. Soc.*, 1953, **75**, 5556.

[8] R. Schwyzer and P. Sieber, *Helv. Chim. Acta*, 1959, **42**, 972.

derivative, *p*-nitrobenzyl *N*-benzyloxycarbonyl-L-glutaminyl-L-asparaginate. The benzyloxycarbonyl group was removed by hydrogen bromide in acetic acid and the resulting hydrobromide neutralised with triethylamine. Reaction of the resulting free base with *p*-nitrophenyl *N*-benzyloxycarbonyl-L-isoleucinate gave the crystalline tripeptide derivative, *p*-nitrobenzyl *N*-benzyloxycarbonyl-L-isoleucyl-L-glutaminyl-L-asparaginate, in high yield. This was very insoluble in a wide variety of solvents but, by catalytic hydrogenolysis of a necessarily dilute solution in *NN*-dimethylformamide, it was readily converted into the crystalline free tripeptide.

Formation of the Nonapeptide.—Although material with higher biological activity was obtained by the use of *o*-phenylene chlorophosphite,[9] the most convenient procedure described by Bodanszky and Du Vigneaud [5] for the preparation of the protected nonapeptide employed *NN'*-dicyclohexylcarbodi-imide [10] as coupling agent. Various difficulties attended its use, but when it was taken in considerable excess of the theoretical requirement a good yield of protected nonapeptide resulted. The difficulty of separating this material from the by-product *NN'*-dicyclohexylurea was overcome by "chromatographic filtration." The crude peptide mixture was suspended in chloroform and placed on the top of a short column of silica gel. *NN'*-Dicyclohexylurea was removed from the mixture with liberal washings of chloroform. Any traces of peptide dissolved were immediately adsorbed on the silica so separation from the urea was complete. The column was afterwards washed with methanol and finally, to remove the protected nonapeptide, with *NN*-dimethylformamide.

The use of coupling agents which form water-soluble by-products seems particularly attractive for the preparation of the protected nonapeptide and we have investigated the use of two such reagents, *NN'*-carbonyldi-imidazole [11] and Reagent-K (*N*-ethyl-5-phenyl isoxazolium-3'-sulphonate).[12] Both gave products more readily purified than the product from the *NN'*-dicyclohexylcarbodi-imide reaction, but, whereas the *NN'*-carbonyldi-imidazole procedure was inferior to the di-imide one in terms of yield of active material, the Reagent-K procedure was appreciably better from all points of view (see Table).

Relative merits of various coupling agents in the preparation of the protected nonapeptide

		Avian depressor activity *		
Wt. of pentapeptide derivative (g.)	Wt. of crude product (g.)	Units/mg. of protected nonapeptide derivative	Units/mM of pentapeptide derivative	Method †
1·73	1·90 [a]	140	133,000	(i) (ref. 5)
0·62	0·816 [a]	81	92,000 [c]	(i)
0·548	0·600 [b]	50	47,200	(ii)
0·157	0·205 [b]	134	151,000	(iii)

* Activity produced in the usual way by the removal of the protecting groups from the nonapeptide derivative, followed by aerial oxidation. † (i) *NN'*-Dicyclohexylcarbodi-imide procedure. (ii) *NN'*-Carbonyldi-imidazole procedure. (iii) Reagent-K procedure.

[a] Washed with cold methanol. [b] Washed with water. [c] Some activity was invariably lost during the methanol wash.

4-Glycine Oxytocin.—Only very limited prediction is yet possible of the type and amount of biological activity which an analogue of a peptide hormone will possess and such compounds are generally synthesised speculatively, to determine the biological effects of structural modifications. Studies may be stimulated, however, by an interest in a particular biological activity, which has shown suggestive variations in related analogues. Thus, we hoped that 4-glycine oxytocin might possess a therapeutically favourable ratio of milk let-down and uterotonic activities, as suggested by the variation in the ratio of these two activities

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[9] G. W. Anderson, J. Blodinger, R. W. Young, and A. D. Welcher, *J. Amer. Chem. Soc.*, 1952, **74**, 5304; G. W. Anderson and R. W. Young, *ibid.*, p. 5307.

[10] J. C. Sheehan and G. P. Hess, *J. Amer. Chem. Soc.*, 1955, **77**, 1067.

[11] R. Paul and G. W. Anderson, *J. Amer. Chem. Soc.*, 1960, **82**, 4596.

[12] R. B. Woodward, R. A. Olofson, and H. Mayer ,*J. Amer. Chem. Soc.*, 1961, **31**, 1010.

in the series: oxytocin (450/450), 4-asparagine oxytocin (300 ± 128/108 ± 29),[13,14] 4-serine oxytocin (255 ± 45/195 ± 30),[14] 4-alanine oxytocin (240 ± 55/36 ± 6).[14]

For the synthesis of 4-glycine oxytocin, a new tripeptide, L-isoleucylglycyl-L-asparagine (VIII), was prepared by the same route as the glutaminyl tripeptide (IV), with *p*-nitrophenyl *N*-benzylocarbonylglycinate in place of *p*-nitrophenyl *N*-benzyloxycarbonyl-L-glutaminate. The reactions gave good yields and crystalline intermediates at all stages.

The partially protected pentapeptide, *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucylglycyl-L-asparagine (IX) was prepared by a mixed anhydride coupling of the dipeptide derivative (III) to the triethylammonium salt of the tripeptide (VIII). Although the pentapeptide derivative could not be crystallised, thin layer chromatography in several solvent systems failed to reveal impurities. The protected nonapeptide *N*-benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-tyrosyl-L-isoleucylglycyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycineamide (X) was prepared from the pentapeptide derivative (IX) and the tetrapeptide derivative (VI) by both the *NN'*-dicyclohexylcarbodi-imide and the Reagent-K procedures. The latter was notably superior in purity and yield.

Chromatographic filtration of the product from the *NN'*-dicyclohexylcarbodi-imide procedure gave a third compound with the expected nonapeptide and *NN'*-dicyclohexylurea. This was shown by amino-acid analysis and elemental analysis to be a deamido-nonapeptide derivative, probably *N*-benzyloxycarbonyl-*SS'*-dibenzyl-4-glycine 9-deamido-oxytocin, which could arise from a possible deamido-impurity in the tetrapeptide preparation.

The protecting groups were removed from the protected 4-glycine oxytocin by sodium in liquid ammonia and the disulphide bond was subsequently formed in the usual way by ærating a neutral aqueous solution of the dithiol. The analogue was finally purified by counter-current distribution.

In the lactating guinea-pig, 4-glycine oxytocin possessed milk ejection activity equivalent to 50 ± 25 units of oxytocin, whereas, in the isolated rat uterus, its activity was equivalent to 3·9 ± 1·8 units of oxytocin. Its avian depressor activity (fowl) was equivalent to 5·3 ± 2·5 units of oxytocin. The deamido-compound, after removal of the protecting groups and oxidation in the usual way, possessed no detectable avian depressor activity. This finding is not surprising in view of the relatively low activity of 4-glycine oxytocin and the apparent importance of the amido-groups in residues 5 and 9.[3]

[When this work had been completed, the synthesis of 4-glycine oxytocin by a different route was described.[15] The biological activity per milligram of 4-glycine oxytocin was given as 17 units, milk ejection activity in the lactating rabbit, 3 units, oxytocic activity on the isolated rat uterus, and 5 units of avian depressor activity.]

EXPERIMENTAL

The purity of most compounds was checked by thin-layer chromatography (t.l.c.) on Kieselgel, G, using the solvent systems n-butanol–acetic acid–water (62 : 12 : 26 v/v) (BAW) and s-butanol–3% ammonia (120 : 44 v/v) (BN). Spots were revealed by the chlorine–starch iodide method [16] and, where applicable, by ninhydrin. Optical rotations were measured with a Bellingham and Stanley model A polarimeter. M. p.s were measured in a Gallenkamp Electrothermal heated block apparatus and are uncorrected. Syntocinon® (Sandoz) was used to standardise the oxytocin assays. Reagent-K was purchased from K & K Laboratories Inc., Plainview, New York. Microanalyses were by H. Beiler, Organisch-Chemisches Institüt der Universität Wien.

p-*Nitrobenzyl* N-*Benzyloxycarbonyl*-L-*asparaginate*.—*N*-Benzyloxycarbonyl-L-asparagine (100·5 g., 0·378 mole), triethylamine (86 ml., 0·62 mole) and *p*-nitrobenzyl chloride (123 g.,

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[13] P. A. Jaquenoud and R. A. Boissonnas, *Helv. Chim. Acta*, 1962, **45**, 1601.

[14] S. Guttmann and R. A. Boissonnas, *Helv. Chim. Acta*, 1963, **46**, 1626.

[15] S. Drabarek, *J. Amer. Chem. Soc.*, 1964, **86**, 4477.

[16] H. N. Rydon and P. G. Smith, *Nature*, 1952, **69**, 922.

0·71 mole) in *NN*-dimethylformamide (1 l.) were heated for 5 hr. at 75°. After cooling, the solution was poured into dilute aqueous potassium hydrogen carbonate (3 l.). The precipitate was filtered off, washed with water (2 l.) and ether (2 l.) and recrystallised from hot aqueous methanol (charcoal) to yield *p*-nitrobenzyl *N*-benzyloxycarbonyl-L-asparaginate (76·2 g., 50%), m. p. 169—173°, $[\alpha]_D^{14}$ $-15{\cdot}2°$ (*c* 1·1 in DMF) {lit.,[17] m. p. 164—166°, lit.,[18] m. p. 167—168°, $[\alpha]_D^{25}$ $-63{\cdot}3°$ (*c* 0·98 in DMF)}.

Hydrogenolysis of a sample over 10% palladised charcoal in N-hydrochloric acid gave authentic L-asparagine, $[\alpha]_D^{16}$ $+40°$ (*c* 2·9 in N-HCl) {lit.,[19] $[\alpha]_D^{25}$ $+37{\cdot}8°$ (*c* 2 in N-HCl)}.

p-*Nitrobenzyl* L-*Asparaginate Hydrobromide*.—*p*-Nitrobenzyl *N*-benzyloxycarbonyl-L-asparaginate (76 g., 0·19 mole) was suspended in acetic acid (270 ml.) and treated with a solution (150 ml., 45% w/v) of hydrogen bromide in glacial acetic acid. The clear solution gradually formed a gel. After a further 2 hr. at room temperature, the gel was triturated and washed well with dry ether. The semi-solid mass was treated with ethanol and ether, filtered, and recrystallised from ethanol to give *p*-nitrobenzyl L-asparaginate hydrobromide (62·6 g., 94%), m. p. 175—178° (needles), $[\alpha]_D^{17}$ $-3{\cdot}0°$ (*c* 2 in water) (Found: C, 37·8; H, 4·05; N, 11·9. Calc. for $C_{11}H_{14}N_3O_5$,HBr: C, 37·8; H, 4·3; N, 12·0%) (lit.,[17] 77%, m. p. 170—171°).

p-*Nitrobenzyl* N-*Benzyloxycarbonyl*-L-*glutaminyl*-L-*asparaginate*.—*p*-Nitrobenzyl L-asparaginate hydrobromide (34·8 g., 0·1 mole) was dissolved in *NN*-dimethylformamide (160 ml.) and treated with triethylamine (13·9 ml., 0·1 mole). Precipitated triethylamine hydrobromide was filtered off and a solution of *p*-nitrophenyl *N*-benzyloxycarbonyl-L-glutaminate (40 g., 0·1 mole) in *NN*-dimethylformamide (120 ml.) was added to the filtrate. After 24 hr. at room temperature, the mixture was poured into dilute aqueous sodium hydrogen carbonate. The gel which formed was pressed on a filter and washed with dilute aqueous sodium hydrogen-carbonate, water, N-HCl, and water. It was finally suspended in ethanol, filtered, washed with ethanol and ether, and dried *in vacuo* to give the N-*protected dipeptide ester* (46·5 g., 88%), m. p. 212—215°, $[\alpha]_D^{17}$ $-9{\cdot}6°$ (*c* 1·5 in DMF). A small sample recrystallised from *NN*-dimethylformamide solution by the addition of water had m. p. 220—223° (Found: C, 54·4; H, 5·0; N, 13·3. $C_{24}H_{27}N_5O_9$ requires C, 54·4; H, 5·1; N, 13·2%).

p-*Nitrobenzyl* L-*Glutaminyl*-L-*asparaginate Hydrobromide*.—The benzyloxycarbonyl protecting group was removed from *p*-nitrobenzyl *N*-benzyloxycarbonyl-L-glutaminyl-L-asparaginate (46·3 g., 0·088 mole) by the use of hydrogen bromide in acetic acid as described above. The crude product was reprecipitated from ethanolic solution by ether to give the *dipeptide ester hydrobromide* (38 g., 91%), m. p. ~90° with foaming, $[\alpha]_D^{15}$ $-5{\cdot}45°$ (*c* 1·3 in water). A sample reprecipitated several times from ethanol had m. p. 120—125° (sintered 100—110°) (Found: C, 40·2; H, 4·9; N, 14·5. $C_{16}B_{21}B_5O_7$,HBr requires C, 40·3; H, 4·65; N, 14·7%).

p-*Nitrobenzyl N-Benzyloxycarbonyl*-L-*isoleucyl*-L-*glutaminyl*-L-*asparaginate*.—*p*-Nitrobenzyl L-glutaminyl-L-asparaginate was produced by neutralising a solution of the hydrobromide (24·75 g., 0·052 mole) in *NN*-dimethylformamide (180 ml.) with triethylamine (7·2 ml., 0·052 mole). Triethylamine hydrobromide was filtered off and *p*-nitrophenyl *N*-benzyloxycarbonyl-L-isoleucinate (20 g., 0·051 mole) was added to the filtrate. After 18 hr., the mixture was worked up as in the preparation of the protected dipeptide derivative to yield p-*nitrobenzyl* N-*benzyloxycarbonyl*-L-*isoleucyl*-L-*glutaminyl*-L-*asparaginate* (21 g., 63%), m. p. 247—250°, $[\alpha]_D^{15}$ $-10{\cdot}6°$ (*c* 0·4 in DMF). A sample reprecipitated from *NN*-dimethylformamide by the addition of water had m. p. 253—257° (sintered 248°) (Found: C, 56·2; H, 6·0; N, 13·1. $C_{30}H_{38}N_6O_{10}$ requires C, 56·1; H, 6·0; N, 13·1%).

L-*Isoleucyl*-L-*glutaminyl*-L-*asparagine* (IV).—*p*-Nitrobenzyl *N*-benzyloxycarbonyl-L-isoleucyl-L-glutaminyl-L-asparaginate (10 g., 0·0156 mole) was dissolved in *NN*-dimethylformamide (1·8 l.) containing concentrated hydrochloric acid (2·75 ml.) and hydrogenolysed over 10% palladised charcoal (1·1 g.) for 18 hr. The catalyst was filtered off, washed with water, and the combined filtrate and washings evaporated under reduced pressure. Water (40 ml.) was added to the residual oil and the resulting solution was basified with concentrated ammonia (4 ml.) and extracted with ether (3 × 50 ml.). The aqueous layer was concentrated under reduced pressure to 15 ml., treated with ethanol (100 ml.), and left at 0° for 1 hr. A precipitate was filtered off and washed with ethanol and ether to give the tripeptide as fine needles (5·3 g.,

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[17] E. Sondheimer and R. J. Semeraro, *J. Org. Chem.*, 1961, **26**, 1847.

[18] P. G. Katsoyannis and K. Suzuki, *J. Amer. Chem. Soc.*, 1961, **83**, 4058.

[19] J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," John Wiley and Sons, New York, 1961, vol. III, p. 1857.

83%), m. p. 214—217° (vague), $[\alpha]_D^{19}$ −34·8° (*c* 0·6 in 0·5N-$KHCO_3$). A sample recrystallised from aqueous ethanol gave the monohydrate, m. p. 245—250° (decomp.) (sintered 220—225°) (Found: C, 46·0; H, 7·4; N, 17·8. Calc. for $C_{15}H_{27}N_5O_6,H_2O$: C, 46·0; H, 7·5; N, 17·8%) {lit.,[5] m. p. 230—235° (decomp.) (sintered 225°), $[\alpha]_D^{24}$ −32·5° (*c* 1 in 0·5N $KHCO_3$)}.

N-*Benzyloxycarbonyl*-S-*benzyl*-L-*cysteinyl*-L-*tyrosyl*-L-*isoleucyl*-L-*glutaminyl*-L-*asparagine* (V).—The method was essentially that of Bodanszky and Du Vigneaud.[5] The product had m. p. 238—240°, $[\alpha]_D^{16}$ −23·9° (*c* 0·8 in DMF) {lit.,[5] m. p. 236—238°; analysis sample, m. p. 245—247° (decomp.), $[\alpha]_D^{24}$ −23·5° (*c* 1 in DMF)}.

N-*Benzyloxycarbonyl*-S-*benzyl*-L-*cysteinyl*-L-*tyrosyl*-L-*isoleucyl*-L-*glutaminyl*-L-*asparaginyl*-S-*benzyl*-L-*cysteinyl*-L-*prolyl*-L-*leucylglycineamide* (II).—(i) NN′-*Dicyclohexylcarbodi-imide procedure*.—Coupling was conducted by the method of Bodanszky and Du Vigneaud.[5] *NN*′-Dicyclohexylurea was removed from the crude protected nonapeptide either by extraction with methanol[5] or by chromatographic filtration: the crude product (from 0·7 g. of pentapeptide derivative) was suspended in chloroform (20 ml.) and placed on a column of silica gel (7 × 3 cm.). The urea was removed by copious washings of chloroform (500 ml.) followed by methanol (70 ml.). Finally, the protected nonapeptide was eluted with *NN*-dimethylformamide (200 ml.). The products isolated by these procedures were similar (~80%, based on pentapeptide derivative), m. p. 225—230°. Removal of the protecting groups by reduction with sodium–liquid ammonia followed by oxidation in the usual way gave a solution possessing avian depressor activity equivalent to 81 units/mg. of protected nonapeptide. The oxytocin isolated by countercurrent distribution of this material was fully active. T.l.c. of the crude protected nonapeptide showed the presence of an unknown compound, R_F (BAW) 0·75, as well as *NN*′-dicyclohexylurea, R_F (BAW) 0·89, and the required protected nonapeptide, R_F (BAW) 0·63. The unknown compound could not be obtained pure, but was probably the 9-deamido-protected nonapeptide. This could have been formed from traces of *S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine in the tetrapeptide amide used at the coupling stage. Evidence for the presence of this impurity was obtained during the synthesis of the corresponding protected nonapeptide of 4-glycine oxytocin (see below).

(ii) NN′-*Carbonyldi-imidazole procedure*. The pentapeptide derivative (V) (548 mg., 0·635 mmole) was dissolved in dry *NN*- dimethylformamide and the vigorously stirred solution was cooled to −15° before addition of *NN*′-carbonyldi-imidazole (114 mg. of 90% purity, 0·635 mmole). After 2½ hr. at −10° the mixture was cooled to −40° and the tetrapeptide amide (394 mg., 0·795 mmole) added. The mixture was maintained at −5° for 1 hr., then at room temperature overnight, then poured into water (300 ml.). The resulting gel was coagulated by a few drops of acetic acid and heating. The filtered gel was washed with water and dried *in vacuo* over phosphoric oxide to give the protected nonapeptide (600 mg., 70·5%), m. p. 225—230° (discoloured 150°). By the usual methods, a solution possessing avian depressor activity equivalent to 50 units/mg. of protected nonapeptide was obtained from this sample.

(iii) *Reagent-K* (N-*ethyl-5-phenyl isoxazolium-3*′-*sulphonate*) *procedure*. Reagent-K (46·1 mg., 0·182 mmole) was added to a stirred solution of the pentapeptide derivative (V) (157 mg., 0·182 mmole) in dry *NN*-dimethylformamide (3 ml.) containing triethylamine (0·025 ml.) at 0°. When the reagent had dissolved (1½ hr.), the tetrapeptide amide (100 mg., 0·210 mmole) was added and stirring was continued for a further 1 hr. at 0° and at room temperature overnight. The protected nonapeptide was obtained in a finely divided and readily filterable form (205 mg., 85%), m. p. 240—242°, $[\alpha]_D^{18}$ −44·5° (*c* 0·65 in DMF) when the mixture was poured into water. Removal of the protecting groups, followed by usual oxidation, gave a solution possessing avian depressor activity equivalent to 134 units/mg. of protected nonapeptide. Distributed over 303 transfers in the system n-butanol–ethanol–0·05% aqueous acetic acid (4 : 1 : 5 v/v), the active material migrated with *K* 0·37 (lit.,[5] 0·43) and was biologically and chromatographically indistinguishable from oxytocin.

p-*Nitrobenzyl* N-*Benzyloxycarbonylglycyl*-L-*asparaginate*.—*p*-Nitrobenzyl-L-asparaginate was treated with *p*-nitrophenyl *N*-benzyloxycarbonylglycinate in *NN*-dimethylformamide and the mixture worked up as usual to give the p-*nitrobenzyl* N-*benzyloxycarbonyl-dipeptide ester* (94%), m. p. 165—166°, $[\alpha]_D^{17}$ −9·6° (*c* 0·9 in DMF). A sample recrystallised from methanol as fine needles had m. p. 165—166° (Found: C, 54·9; H, 4·9; N, 12·3. $C_{21}H_{22}N_4O_8$ requires C, 55·0; H, 4·8; N, 12·2%).

p-*Nitrobenzylglycyl*-L-*asparaginate Hydrobromide*.—*p*-Nitrobenzyl *N*-benzyloxycarbonylglycyl-L-asparaginate was treated with hydrogen bromide in glacial acetic acid as usual. The ether-precipitated product was treated with hot ethanol, filtered, and dried at 70° *in vacuo* to give the *dipeptide ester hydrobromide* (70%), m. p. 222°, $[\alpha]_D^{17}$ $-18·2°$ (*c* 0·9 in water). A sample was crystallised from methanol for analysis (Found: C, 38·6; H, 4·4; N, 13·6. $C_{13}H_{16}N_4O_6$,HBr requires C, 38·5; H, 4·2; N, 13·8%).

p-*Nitrobenzyl* N-*Benzyloxycarbonyl*-L-*isoleucylglycyl*-L-*asparaginate*.—*p*-Nitrobenzyl glycyl-L-asparaginate was prepared from the hydrobromide and treated with *p*-nitrophenyl *N*-benzyloxycarbonyl-L-isoleucinate as usual to give the *protected tripeptide* (88%), m. p. 218—222°, $[\alpha]_D^{17}$ $-9·5°$ (*c* 0·36 in *NN*-dimethylacetamide). A sample recrystallised from *NN*-dimethylformamide solution by the addition of water had m. p. 221—223° (Found: C, 56·9; H, 5·7; N, 12·4. $C_{27}H_{33}N_5O_9$ requires C, 56·7; H, 5·8; N, 12·25%).

L-*Isoleucylglycyl*-L-*asparagine* (VIII).—*p*-Nitrobenzyl *N*-benzyloxycarbonyl-L-isoleucylglycyl-L-asparaginate (11 g., 0·019 mole) was dissolved in *NN*-dimethylformamide (1 l.) containing concentrated hydrochloric acid (3·4 ml.) and was hydrogenolysed over 10% palladised charcoal (1·1 g.). The mixture was worked up as in the preparation of (IV) to yield the *tripeptide* (4·84 g., 83%), m. p. 185—190°, $[\alpha]_D^{17}$ $+50·5°$ (*c* 0·6 in water). Repeated recrystallisation of a sample from aqueous ethanol gave the *tripeptide monohydrate*, m. p. 167—173° (Found: C, 44·9; H, 7·8; N, 17·2. $C_{12}H_{22}N_4O_5$,H_2O requires C, 45·0; H, 7·55; N, 1·7·5%).

N-*Benzyloxycarbonyl*-S-*benzyl*-L-*cysteinyl*-L-*tyrosyl*-L-*isoleucylglycyl*-L-*asparagine* (IX).—*N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteinyl-L-tyrosine (10·1 g., 0·0199 mole) was dissolved in tetrahydrofuran (200 ml.) containing triethylamine (2·75 ml., 0·0199 mole) and the stirred solution was cooled to −15° before addition of isobutyl chloroformate (2·6 ml., 0·0199 mole). When the mixture had been at −10° for 30 min., a solution of L-isoleucylglycyl-L-asparagine (6 g., 0·0199 mole) in water (30 ml.) containing triethylamine (2·75 ml.) was added. The mixture was allowed to warm to room temperature and 2½ days later, water (60 ml.) and N-HCl (60 ml.) were added. Evaporation of the tetrahydrofuran at room temperature left a gel which was pressed dry on a filter and washed with water until the washings were nearly neutral. A suspension of the dried gel [(13·6 g., 86%), m. p. 194—196°] in warm ethyl acetate (100 ml.) was filtered and the residue, after further washing with ethyl acetate, was dried *in vacuo* to give the *pentapeptide derivative* (11·3 g., 71%), m. p. 198—203°, $[\alpha]_D^{20}$ $-19·8°$ (*c* 1·1 in DMF) (Found: C, 59·1; H, 6·3; N, 10·5. $C_{39}H_{48}N_6O_{10}S$ requires C, 59·1; H, 6·10; N, 10·6%).

N-*Benzyloxycarbonyl*-S-*benzyl*-L-*cysteinyl*-L-*tyrosyl*-L-*isoleucylglycyl*-L-*asparaginyl*-S-*benzyl*-L-*cysteinyl*-L-*proly*-L-*leucylglycineamide* (X).—(i) NN′-*Dicyclohexylcarbodi-imide procedure*. The partially protected pentapeptide (2·7 g., 3·4 mmoles) was dissolved in warm *NN*-dimethylformamide (15 ml.) and the resulting solution was cooled in an ice-bath. A solution of the tetrapeptide amide (2·125 g., 4·2 mmoles) in *NN*-dimethylformamide (15 ml.) was added followed by *NN′*-dicyclohexylcarbodi-imide (2·54 g., 12·3 mmoles). After 15 min. at 0°, the mixture was allowed to warm to room temperature and left for 2½ days. The mixture was then cooled, and after the addition of glacial acetic acid (2·5 ml.), poured into ice–water (150 ml.). After 30 min. the precipitate was filtered off, washed thoroughly with water, and dried *in vacuo* over phosphoric oxide. The product (5·67 g.) was washed repeatedly with dry methanol (120 ml.) to give 1·68 g. (39%) of an off-white solid (*A*). Evaporation of the methanolic washings gave 3·85 g. of a white material (*B*).

Both (*A*) and (*B*) showed three spots on t.l.c., R_F (BAW) 0·67, 0·80, and 0·89. *NN′*-Dicyclohexyl-urea has R_F 0·89 in this system. Removal of the protecting groups from material (*A*) and oxidation as usual gave a solution possessing avian depressor activity equivalent to 1·7 units/mg. of protected nonapeptide. Material (*B*) after the same treatment gave a solution possessing 0·57 avian depressor units/mg. of protected nonapeptide.

Fraction (*A*) was further purified by chromatographic filtration on silica gel as described for the preparation of the protected nonapeptide derivative related to oxytocin. The material of R_F 0·80, was more readily eluted by methanol than material of R_F 0·67, and the latter was consequently obtained pure (494 mg., 11·5%). This was the required *protected nonapeptide*. A small sample reprecipitated from *NN*-dimethylformamide by the addition of water had m. p. 215—218° (Found: C, 59·2; H, 6·6; N, 12·0. $C_{62}H_{81}N_{11}O_{13}S_2$ requires C, 59·45; H, 6·5; N, 12·3%).

Fraction (*B*) was also filtered through silica gel and gave a further quantity of protected nonapeptide, identical to that obtained from fraction (*A*) (87 mg., 2%), m. p. 204—210°, $[\alpha]_D^{19}$ $-54·2°$ (*c* 0·5 in DMF).

The methanolic eluate from various experiments was combined and rechromatographed several times to obtain the pure material of R_F (BAW) 0·80, m. p. 128—140°. Amino-acid analysis by a t.l.c. procedure indicated that this compound had a composition very similar to that of the 4-glycine nonapeptide derivative described above and elemental analysis suggested that it was a *deamido-protected nonapeptide* (Found: C, 59·7; H, 6·8; N, 11·1. $C_{62}H_{80}N_{10}O_{14}S_2$ requires C, 59·4; H, 6·4; N, 11·2). It seems likely that this compound is the 9-deamido-analogue which could be formed from traces of deamido-impurity in the tetrapeptide amide. Hydrolysis of the terminal amide of this compound might have occurred either during the removal of the *N*-benzyloxycarbonyl group with hydrogen bromide in acetic acid solution or during the subsequent conversion of the resulting hydrobromide into the free base by anion-exchange. Removal of the protecting groups from the protected deamido-nonapeptide derivative and subsequent oxidation gave a solution with no detectable avian depressor activity.

(ii) *Reagent-K procedure.* The protected pentapeptide (1·44 g., 1·88 mmoles) was dissolved in *NN*-dimethylformamide containing triethylamine (0·26 ml., 1·88 mmoles) and the stirred solution was cooled to 0°. Reagent-K (0·476 g., 1·88 mmoles) was added followed 2 hr. later by the tetrapeptide (1·03 g., 1·16 mmoles). Stirring was continued for 1 hr. at 0° and then at room temperature overnight. The solution was poured into water (300 ml.) and the precipitate filtered off, washed with water, and dried *in vacuo* at 50—60° to give a fine powder (2·095 g., 89%), m. p. 205—215° (sintered 200°). Thin layer chromatography indicated that this material was mainly the desired protected nonapeptide together with traces of the tetrapeptide amide and supposed deamido-nonapeptide derivative. Removal of the protecting groups in the usual way followed by oxidation gave a solution possessing an avian depressor activity equivalent to 1·8 units/mg. of protected nonapeptide.

4-*Glycine Oxytocin.*—The nonapeptide derivative (X) (2·09 g.) was reduced by sodium in liquid ammonia as usual. After evaporation of the ammonia, the residue was dissolved in water (755 ml.), the pH was adjusted to 6·7 by dropwise addition of acetic acid, and air was bubbled through the solution for 3 hr. The avian depressor activity of the resulting solution was equivalent to 1·8 units/mg. of (X), *i.e.*, total activity = 3780 units. This solution was concentrated at room temperature to 100 ml. and placed in the first four tubes of a counter-current distribution apparatus (25 ml. phase) before distribution in the solvent system discussed above. The whereabouts of the material in the apparatus was determined colorimetrically by the use of the Folin–Phenol reagent [20] and biologically in terms of avian depressor activity. By the use of a recycling technique, the material was distributed over 291 transfers. Only one peak ($K = 0{\cdot}62$) which possessed biological activity was present. The assay curve was in close agreement with a theoretical curve calculated from the distribution constant.[21] Concentration under reduced pressure of the combined fractions of the active peak, followed by lyophilisation of the concentrate, gave the *free nonapeptide* (0·535 g., 34%), m. p. 207—210° (sintered 180°), $[\alpha]_D^{18}$ +21·2° ± 0·6° (*c* 0·52 in methanol). A sample precipitated from methanol by ether had m. p. 210—212° (decomp.) (Found: C, 51·1; H, 6·6; N, 16·55. $C_{40}H_{61}N_{11}O_{11}S_2$ requires C, 51·3; H, 6·6; N, 16·45%). The compound was homogeneous on thin layer chromatograms, R_F (BAW) 0·31, R_F (BN) 0·35 [cf. oxytocin R_F (BAW) 0·26, R_F (BN) 0·25] and was shown by hydrolysis followed by thin layer chromatography to possess the expected amino-acid composition.

4-Glycine oxytocin was biologically evaluated by Dr. E. W. Horton and Mr. I. H. M. Main of the Department of Physiology, St. Bartholomew's Hospital Medical College, London E.C.1. We thank Miss C. Cranwell for biological assays and Mr. W. F. Hurst for assistance in some preparations.

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