Synthesis of [1-(D-2-Hydroxy-3-mercaptopropanoic acid)]oxytocin, a Diastereoisomer of the Hydroxy-isostere of Oxytocin

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[1-(D-2-Hydroxy-3-mercaptopropanoic acid)]oxytocin was synthesized by azide coupling of *O*-benzyl-*N*-(D-3-benzylthio-2-hydroxypropanoyl)-L-tyrosine hydrazide with a heptapeptide. The hormone analogue had an uterotonic activity of 27·3 i.u. mg^{-1} and did not bind to neurophysin.

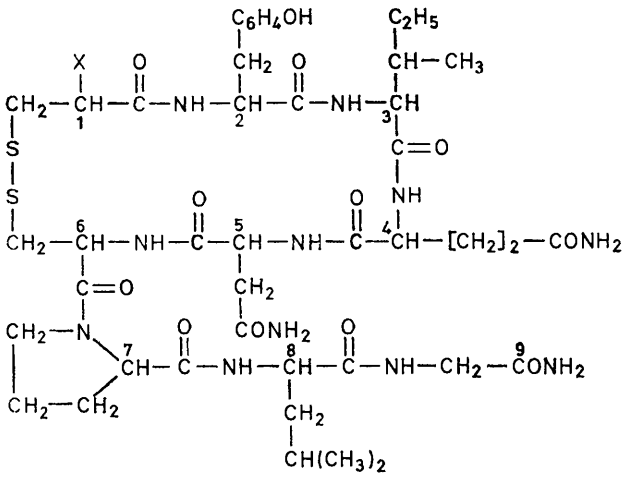
We recently reported the synthesis of an isostere of the posterior pituitary hormone oxytocin (1) in which the *N*-terminal primary amino-group was replaced by a hydroxy-group.[1,2] This isostere [L-(3)] contained the α-hydroxy-analogue[3] of L-cysteine at position 1. It was not bound by the protein neurophysin indicating that a net positive charge is vital for the formation of a protein–hormone complex.[1] The results of previous work were compatible with the possibility that a hydrogen bond was responsible for binding. The pharmacological activity of this analogue was very high (three times more potent than oxytoxin itself on the isolated rat uterus). Thus an *N*-terminal amino-group is essential for the binding of the hormone to neurophysin, a protein that carries the hormone from the site of synthesis in the brain to the pituitary gland, but not for hormonal activity.

The p*K* of the amino-group in oxytocin is approximately 6·2, consequently less than 10% of the molecules would be charged at a physiological pH of 7·4. It seems reasonable to assume therefore that the pharmacologically active form of oxytocin is the unchanged molecule. If charged molecules of oxytocin are pharmacologically inactive this would account in part for the lower activity of the hormone. The local conditions of pH and dielectric constant in the neighbourhood of the receptor would determine the importance of this effect. It is also possible that the difference in activity between the hormone and its hydroxy-isostere could be accounted for enzymically.

Some time ago it was shown that the diastereoisomer of oxytocin, [1-hemi-D-cystine]oxytocin,[4-6] possessed

[1] D. B. Hope and M. Wälti, *Biochem. J.*, 1971, **125**, 909.
[2] M. Wälti and D. B. Hope, *J.C.S. Perkin I*, 1972, 1946.
[3] D. B. Hope and M. Wälti, *J. Chem. Soc.* (*C*), 1970, 2475.
[4] K. Jošt, J. Rudinger, and F. Šorm, *Coll. Czech. Chem. Comm.*, 1963, **28**, 2021.
[5] D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Amer. Chem. Soc.*, 1963, **85**, 3686.
[6] D. Yamashiro, D. Gillessen, and V. du Vigneaud, *J. Amer. Chem. Soc.*, 1966, **88**, 1310.

less than 1% of the oxytocic activity of the hormone. This suggested that an amino-group in the unnatural configuration interfered with the access of the peptide



(1) X = NH_2 (oxytocin)
(2) X = H (deamino-oxytocin)
(3) X = OH [1-(2-hydroxy-3-mercaptopropanoic acid)-oxytocin]

to the receptor site. The amino-group might interfere sterically or by formation of an electrostatic or hydrogen bond with polar groups in or close to the receptor. We therefore decided to synthesize the diastereoisomer [D-(3)] of the hydroxy-isostere [L-(3)], differing from the latter only in the configuration of the hydroxy-group.

The synthesis was accomplished by coupling of *O*-benzyl-*N*-(D-3-benzylthio-2-hydroxypropanoyl)-L-tyrosine with the (3—9)-heptapeptide, prepared from *N*-benzyloxycarbonyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide by deprotection with hydrogen bromide in acetic acid. After treatment with Amberlite IRA-400 (OH^-) resin the heptapeptide was recrystallized from water to remove diastereoisomerides.[7] Sequence 1—2 was synthesized by a dicyclohexylcarbodi-imide coupling of D-2-acetoxy-3-(benzylthio)propanoic acid with methyl *O*-benzyl-L-tyrosinate. The resulting semi-solid methyl ester was converted *in situ* into the crystalline hydrazide. Fragments 1—2 and 3—9 were coupled by the azide procedure to give D-3-benzylthio-2-hydroxypropanoyl-*O*-benzyl-L-tyrosyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide. The protecting groups were removed with sodium in liquid ammonia. The dithiol intermediate was oxidized with potassium ferricyanide[7] under nitrogen[8] to the cyclic 'nonapeptide,' which was subjected to counter-current distribution[9] in n-butanol–toluene–0·05% acetic acid.[10] Measurement of Folin–Lowry colour values and oxytocic activities (rat uterus) after 600 transfers showed the presence of one major component (*K* 0·27) with low activity. The main peak slightly overlapped a small peptide peak (*K* 0·38) associated with much greater biological activity. The activity present in the main peak may represent a slight contamination with L-(3) from the peak with *K* 0·38. Further distribution was required to determine whether D-(3) was biologically active. Complete separation was achieved after 800 transfers, and the diastereoisomers were isolated as lyophilized powders.

The cyclization of the linear dithiol intermediate of oxytocin always results to a certain extent in the formation of dimers.[8] It was surprising therefore to find no dimeric [1-(2-hydroxy-3-mercaptopropanoic acid)]oxytocin (3) in either of the two diastereoisomer preparations.

The effect of the configuration of the α-hydroxy-group on the specific rotation of the hormone analogues in *N*-acetic acid is remarkable: L-(3) oxytocin is laevorotatory ($-91{\cdot}5 \pm 2{\cdot}5°$) whereas D-(3) is dextrorotatory ($+116 \pm 3°$).

The oxytoic activity of deamino-oxytocin[11] is almost twice that of oxytocin itself. The hormonal activity is further enhanced by the introduction of a hydroxy-group in the L-configuration but not in the D-configuration. Comparison of the oxytocic activities (Table)

Oxytocic activities

	Rat uterus (i.u. mg^{-1})
Oxytocin (1) [2]	523 ± 8
[1-(Hemi-D-cystine)]oxytocin [5,6]	1·9 ± 0·1
[1-(L-2-Hydroxy-3-mercaptopropanoic acid)]-oxytocin [L-(3)]	1607 ± 20
[1-(D-2-Hydroxy-3-mercaptopropanoic acid)]oxytocin] [D-(3)]	27·3 ± 1·2
Deamino-oxytocin (2) [11]	750

shows that the configurational effect of the hydroxy-group is similar both qualitatively and quantitatively to that of an amino-group inserted into deamino-oxytocin in the unnatural configuration.

EXPERIMENTAL

M.p.s were determined on a Kofler block; optical rotations were measured on a Bellingham and Stanley Polarimeter (model A, type P3) with a 2 dm tube. Chromatographic data refer to t.l.c. on Kieselgel G with n-butanol–acetic acid–water–pyridine (15 : 3 : 12 : 10 v/v)[12] or to descending paper chromatography (p.c.) on Whatman No. 1 paper with n-butanol–acetic acid–water.[13] Sulphur-containing substances were located with a solution of platinum(IV) iodide in acetone[14] [with one modification: the concentration of platinum(IV) chloride was increased ten-fold]. Iodine solution (0·5% in chloroform)[15] was

[7] D. B. Hope and V. du Vigneaud, *J. Biol. Chem.*, 1962, **237**, 3146.
[8] M. Wälti and D. B. Hope, *Experientia*, 1973, **39**, 389.
[9] L. C. Craig, W. Hausmann, E. H. Ahrens, jun., and E. J. Harfenist, *Analyt. Chem.*, 1951, **23**, 1236.
[10] D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Biol. Chem.*, 1962, **237**, 1563.
[11] D. Jarvis and V. du Vigneaud, *Science*, 1964, **143**, 545.
[12] E. Schillinger, O. Loge, E. Schröder, E. Klieger, and K. Lübke, *European J. Biochem.*, 1972, **27**, 473.
[13] S. M. Partridge, *Biochem. J.*, 1948, **42**, 238.
[14] G. Toennies and J. Kolb, *Analyt. Chem.*, 1951, **23**, 823.
[15] D. Waldi, in 'Thin-Layer Chromatography,' ed. E. Stahl, Springer, Berlin, 1965, p. 493.

used as a general reagent for detecting organic compounds. Elemental analyses were carried out by A. Bernhardt, Elbach, Germany. Amino-acid analyses were performed with an automatic analyser (Evans Electroselenium Ltd.) by the method of Spackman, Stein, and Moore.[16] Samples were hydrolysed *in vacuo* in constant-boiling hydrochloric acid [17] for 18 h. Counter-current distribution was carried out in an automatic train of 200 tube-units (H.O. Post and Co., Maspeth, New York). Oxytocic activity was assayed on the isolated rat uterus [18] with Mg^{2+}-free van Dyke–Hastings solution suggested by Munsick [19] against synthetic oxytocin, standardized against the IIIrd International Oxytocic Standard.[20] Binding to bovine neurophysin-II was assessed by a modification of the thin-film dialysis [21] procedure in the ' alternate ' cell.[22] Peptide concentrations were determined by the Folin–Lowry colour reaction.[23]

O-*Benzyl*-N-(D-3-*benzylthio*-2-*hydroxypropanoyl*)-L-*tyrosine Hydrazide*.—Methyl *O*-benzyl-L-tyrosinate hydrochloride [24] (1182 mg) was suspended in peroxide-free dioxan (50 ml) and neutralized with triethylamine (0·51 ml). D-2-Acetoxy-3-benzylthiopropanoic acid monohydrate [2] (1000 mg) was then added, followed by *NN'*-dicyclohexylcarbodi-imide (758 mg) at −10°. After stirring for 30 min the mixture was left at room temperature for 3 days. The urea was filtered off and washed with dioxan (2 × 5 ml) and the combined filtrate and washings were evaporated to dryness under reduced pressure at 60°. The semi-solid methyl *O*-benzyl-*N*-(D-2-acetoxy-3-benzylthiopropanoyl)-L-tyrosinate was dissolved in methanol (10 ml) and refluxed with hydrazine hydrate (99—100%; 3 ml) for 10 min. The hydrazide was precipitated with ice–water (50 ml), filtered off, washed with ice–water (3 × 10 ml), dried, recrystallized from the minimum of aqueous methanol, and dried *in vacuo* (conc. H_2SO_4). The *product* (1025 mg, 73%) had m.p. 155—157°, $[\alpha]_D^{22}$ +23·1 ± 1·2° (*c* 0·5 in MeOH), $[\alpha]_D^{22}$ +12·5 ± 0·3° (*c* 1 in $Me_2N{\cdot}CHO$), R_F 0·80 (t.l.c.) (Found: C, 65·0; H, 6·0; N, 8·8; S, 6·6. $C_{26}H_{29}N_3O_4S$ requires C, 65·1; H, 6·1; N, 8·8; S, 6·7%).

D-3-*Benzylthio*-2-*hydroxypropanoyl*-O-*benzyl*-L-*tyrosyl*-L-*isoleucyl*-L-*glutaminyl*-L-*asparaginyl*-S-*benzyl*-L-*cysteinyl*-L-*prolyl*-L-*leucylglycinamide*.— *O*-Benzyl-*N*-(D-3-benzylthio-2-hydroxypropanoyl)-L-tyrosine hydrazide (238 mg) was mixed with peroxide-free dioxan (2·8 ml) and tetrahydrofuran (1·4 ml). Hydrochloric acid (5N; 0·48 ml) was then added and the resulting solution was treated with aqueous sodium nitrite (40 mg in 0·2 ml) at −20°. After stirring for 15 min it was diluted with precooled ethyl acetate (15 ml) and the solution was washed with a saturated solution of sodium hydrogen carbonate in 16·8% sodium chloride [25] at −20°. The aqueous phase was removed with a syringe and the organic phase was briefly dried ($MgSO_4$). It was then filtered directly into a solution of L-isoleucyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-leucylglycinamide [7] (380 mg) in dimethylformamide (20 ml) at −20° and the mixture was set aside at 0° for 24 h. It was evaporated to dryness under reduced pressure at 60°. The residue was triturated with cold 0·2N-hydrochloric acid (25 ml), and washed on the filter pad with water (15 ml), ethyl acetate–ether (1 : 1; 3 ml), and ether (5 ml) alone. It was then dried (P_2O_5), reprecipitated from dimethylformamide (8 ml) with water (45 ml), washed with ethanol–ether (1 : 2; 6 ml), and dried *in vacuo* (P_2O_5). The *peptide* (215 mg, 37%) had m.p. 228—229·5°, $[\alpha]_D^{20}$ −29·0 ± 0·3° (*c* 1 in $Me_2N{\cdot}CHO$), R_F 0·76 (t.l.c.) (Found: C, 59·8; H, 6·7; N, 12·2; S, 4·9. $C_{64}H_{85}N_{11}O_{13}S_2$ requires C, 60·0; H, 6·7; N, 12·0; S, 5·0%). Amino-acid analysis of an acid hydrolysate gave the following molar ratios (Pro taken as 1): Cys(Bzl), 1·0; NH_3, 3·1; Asp, 1·1; Glu, 1·0; Pro, 1·0; Gly, 1·1; Ile, 0·9; Leu, 1·0; Tyr, 0·9.

[1-(D-2-*Hydroxy*-3-*mercaptopropanoic acid*)]*oxytocin* [D-(3)].—The foregoing peptide (187 mg) was dissolved in anhydrous liquid ammonia (400 ml) and reduced with sodium by the stick method [26] until a blue colour persisted for 10 s. Glacial acetic acid (1 ml) was added and the ammonia was evaporated off at 30—40° with a rapid stream of oxygen-free nitrogen. The residue was dissolved in deaerated water (500 ml) and oxidized at pH 6·8 in a nitrogen atmosphere [8] with 0·02M-potassium ferricyanide (13·2 ml). The excess of ferri- and ferro-cyanide ions was removed on a column (2 × 3 cm) of Dowex 2 × 8 (200—400 mesh; Cl^- form) resin. The effluent was concentrated in a flash evaporator at 30° to *ca.* 15 ml, then diluted to 40 ml with the lower phase of the system n-butanol–toluene–0·05% acetic acid,[10] and divided between the first four tubes of a 200-tube counter-current distribution train. After 600 transfers a separation into four peaks with *K* values of 0·02 (medium), 0·19 (small), 0·27 (large), and 0·38 (small, partially separated from the main peak) was accomplished as detected by the Folin–Lowry colour reaction.[23] Determination of the oxytocic activity showed that the main peak (*K* 0·27) was weakly active whereas the small peak (*K* 0·38) contained an appreciable amount of oxytocic activity. Complete separation was effected after 800 transfers, showing a medium-sized peak (*K* 0·02), two minor peaks (0·17 and 0·19), and one main peak (0·27). The curve was in agreement with the calculated curve for a substance with a partition coefficient of 0·27. The material of the peak with K_{600} 0·38 was recovered in the overflow. The contents of tubes 140—200 containing D-(3) were pooled, evaporated in a flash evaporator at 30° to a volume of *ca.* 10 ml, and diluted to 60 ml with water. The solution was filtered and lyophilized. The resulting white fluffy *powder* (65·8 mg, 45%) had $[\alpha]_D^{22\cdot5}$ +116 ± 3° (*c* 0·05 in N-AcOH). The moisture content of the lyophilized powder was 5·0% (P_2O_5; 80° at 2 mmHg) (Found: C, 51·5; H, 6·7; N, 15·4; S, 6·2. $C_{45}H_{65}N_{11}O_{13}S_2$ requires C, 51·2; H, 6·5; N, 15·3; S, 6·4%). Amino-acid analysis of an acid hydrolysate gave the following molar ratios (Tyr taken as 1): NH_3, 2·7; Asp, 1·0; Glu, 1·0; Pro, 1·0; Gly, 1·0; Ile, 0·9; Leu, 1·0; Tyr, 1·0. Cystine (0·3) and the mixed disulphide of L-cysteine and D-2-hydroxy-

[16] D. H. Spackman, W. H. Stein, and S. Moore, *Analyt. Chem.*, 1958, **30**, 1190.
[17] A. M. Crestfield, S. Moore, and W. H. Stein, *J. Biol. Chem.*, 1963, **238**, 622.
[18] P. Holton, *Brit. J. Pharmacol. Chemother.*, 1948, **3**, 328.
[19] R. A. Munsick, *Endocrinol.*, 1960, **66**, 451.
[20] D. R. Bangham and M. W. Mussett, *Bull. World Health Org.*, 1958, **19**, 325.
[21] M. D. Hollenberg and D. B. Hope, *Biochem. J.*, 1967, **104**, 122.
[22] L. C. Craig and W. Konigsberg, *J. Phys. Chem.*, 1961, **65**, 166.
[23] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.
[24] E. Wünsch, G. Fries, and A. Zwick, *Chem. Ber.*, 1958, **91**, 542.
[25] K. Jošt, V. G. Debabov, H. Nesvadba, and J. Rudinger, *Coll. Czech. Chem. Comm.*, 1964, **29**, 419.
[26] V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyannis, *J. Amer. Chem. Soc.*, 1954, **76**, 3115.

3-mercaptopropanoic acid [27] (0·4) together account for the half-cystine residue in the hormone analogue. The presence of cystine would be expected from an acid-catalysed disulphide interchange.[28] The oxytocic activity of D-(3) was 27·3 ± 1·2 ($n = 4$) i.u. mg^{-1}. The overflow collected during the counter-current transfers 600—800 was concentrated to 10 ml at 40°, diluted to 50 ml with water, filtered, and lyophilized. The amorphous material, crude L-(3) (14·7 mg) had $[\alpha]_D^{22.5}$ −32 ± 4° (*c* 0·05 in N-AcOH), R_F 0·7 (p.c.). With the exception of cystine (0·3) and the mixed disulphide of L-cysteine and L-2-hydroxy-3-mercaptopropanoic acid [27] (0·4) the amino-acid analysis was identical with that of D-(3). An oxytocic activity (rat uterus) of 556 ± 15 ($n = 3$) i.u. mg^{-1} was found, corresponding to a total of 8173 i.u., *i.e.* 5·1 mg of L-(3).

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[27] M. Wälti and D. B. Hope, *J. Chem. Soc.* (*C*), 1971, 2326.
[28] A. P. Ryle and F. Sanger, *Biochem. J.*, 1955, **60**, 535.