Synthesis of [1-(L- and [1-(D-2-Hydroxy-3-mercaptopropanoic acid)-8-lysine]-vasopressin

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Analogues of [lysine]-vasopressin containing a hydroxy-group in place of the primary amino-group (both L- and D-configurations) were synthesized by coupling *p*-nitrophenyl *N*-[L-2-acetoxy-3-(benzylthio)propanoyl]-L-tyrosinate and *O*-benzyl-*N*-(D-3-benzylthio-2-hydroxypropanoyl)-L-tyrosine hydrazide with a crystalline heptapeptide. The pressor and uterotonic activities of the L-isostere were 431 and 17.9 i.u.mg^{-1}, respectively, and those of the D-diastereoisomer 0.913 and 0.015 i.u.mg^{-1}. The hormone analogues did not bind to neurophysin.

THE neurohypophysial polypeptide hormones, oxytocin and vasopressin, are found together with neurophysin, a group of hormone-binding proteins, in the neurosecretory granules of the pituitary gland.[1] Presumably the proteins retain the hormones within the granules for transport and storage within the neurones of the hypothalamo-neurohypophysial system.

Oxytocin and vasopressin satisfy two different structural requirements in their non-covalent binding to receptor material and to neurophysin. On the one hand they bind with and stimulate tissue receptors and on the other they are bound by neurophysin to form protein–hormone complexes. The binding to neurophysin is vitally dependent on the presence of a charged amino-group at position 1 of the polypeptide. The importance of the amino-group is revealed by results obtained with synthetic analogues of the hormones in which the primary amino-group was replaced by a hydrogen atom.[2] Neurophysin fails to form a complex [3] with either deamino-oxytocin [4] or deamino-[8-arginine]-vasopressin.[5] The hormonal activities of both deamino-analogues are comparable with those of the parent hormones.

To test the possibility that oxytocin is bound to neurophysin *via* hydrogen bonds, we recently synthesized [1-(L-2-hydroxy-3-mercaptopropanoic acid)]-oxytocin [6] and its D-diastereoisomer.[7] In these analogues the *N*-terminal primary amino-group was replaced by the strongly hydrogen-bonding hydroxy-group. Thin-film dialysis showed that neither of the two analogues was bound to neurophysin at pH 5.8 where the hormones are bound maximally. The pharmacological activities however, were greatly enhanced: the oxytocic activity of the L-hydroxy-analogue was three times greater than that of oxytocin. This result was surprising because the greater activity of deamino-oxytocin as compared with oxytocin had led us to believe that a substituent at the α-position in amino-acid residue 1 reduced hormonal activity. A further surprising observation was the high pressor activity of the L-hydroxy-analogue of oxytocin, *ca.* ten times more than that of the hormone itself.[8]

Although the hormonal activities (anti-diuretic) of the deamino-analogues of both vasopressins (lysine and arginine) are greater than those of the parent hormones the pressor activities are greatly reduced. The relationship between pressor activity and the basic character of the amino-acid at position 8 (Arg > Lys > His) has been recognised for some time. The amino-group at position 1 would contribute to the overall basic character of the vasopressins and hence to the pressor activities. However, the high pressor activity of the L-hydroxy-analogue of oxytocin suggested otherwise. The observation prompted us to replace the terminal amino-group in [8-lysine]-vasopressin by a hydroxy-group for a study of its pressor and other biological activities.

The recent synthesis of *p*-nitrophenyl *N*-[L-2-acetoxy-3-(benzylthio)propanoyl]-L-tyrosinate [6] and *O*-benzyl-*N*-(D-3-benzylthio-2-hydroxypropanoyl)-L-tyrosine hydrazide [7] enabled us to prepare protected ' nonapeptides ' of [8-lysine]-vasopressin by a ' dipeptide '–heptapeptide coupling. The sequence 3—9 of [8-lysine]-vasopressin was prepared as a protected heptapeptide by the stepwise *p*-nitrophenyl ester procedure.[9] Deprotection with hydrogen bromide in acetic acid and treatment with the strongly basic anion exchanger Amberlite IRA-400 gave the free heptapeptide, which was crystallized from water to remove diastereoisomerides. The ' L-hydroxy-nonapeptide ' was synthesized by condensing the 'dipeptide ' nitrophenyl ester to the heptapeptide, and the ' D-hydroxy-nonapeptide ' by coupling the ' dipeptide ' azide to the heptapeptide. The protecting groups were removed with sodium in liquid ammonia and the dithiol intermediates were oxidized to the cyclic nonapeptides either with carbon dioxide-free oxygen or with potassium ferricyanide.[10] The hormone analogues were purified by counter-current distribution until the curves of the Folin–Lowry colour values [11] and pressor activity [12] were in

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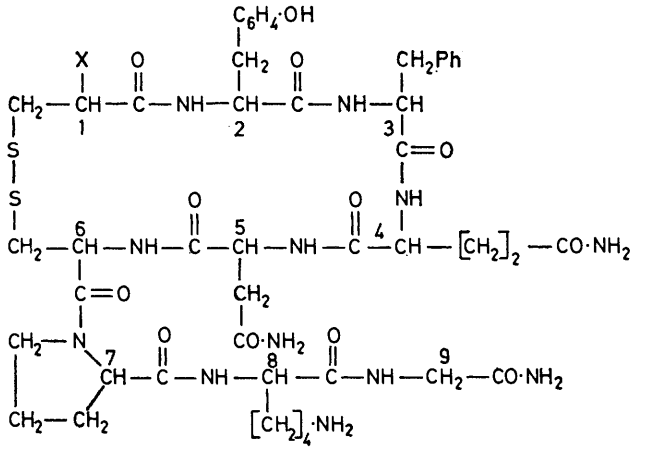
excellent agreement with the theoretical curve.[13] The diastereoisomers were isolated as lyophilized powders.



(I) X = NH_2 ([8-lysine]-vasopressin)
(II) X = H (deamino-[8-lysine]-vasopressin)
(III) X = OH {[1-(2-hydroxy-3-mercaptopropanoic acid)-8-lysine]-vasopressin}

[8-Lysine]-vasopressin itself was synthesized under comparable conditions (for biological activities see Table).

The pressor activity of deamino-[8-lysine]-vasopressin [14] is *ca.* half that of [8-lysine]-vasopressin,[14]

Biological activities

	Rat blood pressure (i.u. mg^{-1})	Rat uterus (i.u. mg^{-1})
[8-Lysine]-vasopressin	276 ± 13	4.7 ± 0.5
L-Hydroxy-deamino-[8-lysine]-vasopressin	431 ± 10.3	17.9 ± 0.6
D-Hydroxy-deamino-[8-lysine]-vasopressin	0.913 ± 0.029	0.015 ± 0.003
Deamino-[8-lysine]-vasopressin [14]	126 ± 2	12 ± 0.5
[8-Arginine]-vasopressin [15]	435 ± 45	17 ± 4

whereas introduction of a hydroxy-group to give the L-configuration increases the activity by approximately half. Insertion of the hydroxy-group to give the D-configuration reduces both pressor and oxytocic activities by more than 99.5%. It is interesting that the values for the pressor and oxytocic activities of [1-(L-2-hydroxy-3-mercaptopropanoic acid)-8-lysine]-vasopressin coincide with those of [8-arginine]-vasopressin [15] (Table).

Thin-film dialysis and salting-out experiments [2] showed that the hydroxy-analogues were not bound by bovine neurophysin I or II.

A hydroxy-analogue of vasopressin, [8-ε-hydroxy-norleucine]-vasopressin, in which the ε-amino-group of the lysine residue of [8-lysine]-vasopressin was replaced by a hydroxy-group was recently prepared in Bodanszky's laboratory by Dreyfuss.[16] The biological activities reported were considerably less than those of [8-lysine]-vasopressin. The pressor activity was 31 ± 1 i.u.mg^{-1} and the antidiuretic activity was given as *ca.* 75 i.u.mg^{-1}. These results support the view that the charged form of the amino-group in the side chain of vasopressin is required for full expression of the biological activities of the hormone.

We conclude that the *N*-terminal amino-group of [8-lysine]-vasopressin is not essential for biological activity, nor does its basic character contribute to pressor activity. The higher pressor activity of the L-hydroxy-analogue as compared with the deamino-analogue indicates that uncharged groups approximately isosteric with the amino-group can contribute to pressor activity. The hydroxy-group and to a lesser extent the amino-group contribute to the activity either sterically or by hydrogen bonding. Experiments are in progress to distinguish between these two possibilities by synthesis of an analogue in which the *N*-terminal amino-group of vasopressin is replaced by a methyl group.

EXPERIMENTAL

M.p.s were determined on a Kofler hot-stage apparatus. 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 butan-1-ol–acetic acid–water–pyridine (15 : 3 : 12 : 10).[17] Sulphur-containing substances were located with a solution of platinum(IV) iodide in acetone [18] [with one modification: the concentration of platinum(IV) chloride was increased ten-fold]. Iodine solution (0.5% in chloroform) [19] was 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 *et al.*[20] Samples were hydrolysed *in vacuo* in constant-boiling hydrochloric acid [21] for 18 h. Counter-current distribution was carried out with the solvent system butan-1-ol–propan-1-ol–0.05% acetic acid (2 : 1 : 3) in an automatic train of 200 tube-units (H.O. Post and Co., Maspeth, New York). The ion-exchange columns were prepared from Dowex AG 2-X8 (50—100 mesh) (Cl^-) (Bio-Rad Laboratories, Richmond, California), and from Amberlite IRA-400 (200—400 mesh) (OH^-) (B.D.H.). The binding of the hormone analogues to neurophysin was assessed by a modification of the thin-film dialysis pro-

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cedure,[22] and by the salting-out procedure.[2] The pressor activity was measured by the method of Dekanski [12] on male albino rats anaesthetized with urethane (1.2 g kg^{-1}, i.p.). The oxytocic activity was assayed on the isolated uterus of the rat by the method of Holton [23] with Mg^{2+}-free van Dyke–Hastings solution suggested by Munsick [24] against synthetic oxytocin, standardized against the IIIrd International Oxytocic Standard.[25] Peptide concentrations were determined by the Folin–Lowry colour reaction.[11]

L-*Phenylalanyl*-L-*glutaminyl*-L-*asparaginyl*-S-*benzyl*-L-*cysteinyl*-L-*prolyl*-N$^{\epsilon}$-*tosyl*-L-*lysylglycinamide*.— *N*-Benzyloxycarbonyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide [9] (2.0 g) was suspended in glacial acetic acid (10 ml) and treated with 45% hydrogen bromide in glacial acetic acid (12 ml) for 1½ h at room temperature. The hydrobromide salt was precipitated with dry ether (220 ml) and washed with ether (3 × 80 ml) by decantation. The solid was dried *in vacuo* (conc. H_2SO_4 and solid NaOH), dissolved in water (75 ml), and passed down a column (1.5 × 11.5 cm) of Amberlite IRA-400. The peptide precipitated in the column and was washed out as a suspension with water (200 ml) at 50 °C. The effluent was evaporated to dryness under reduced pressure at 50 °C and the residue (1.3 g, 73% yield) was recrystallized from the minimum amount of boiling water and dried *in vacuo* (P_2O_5). The *product* (1.0 g, 77% recovery) had m.p. 185—186° (sintering at 177°), $[\alpha]_D^{25.5}$ −44.5° (*c* 1 in Me_2N·CHO), R_F 0.69 (Found: C, 54.9; H, 6.4; N, 15.2; S, 6.0. $C_{48}H_{65}N_{11}O_{11}S_2,0.5H_2O$ requires C, 55.1; H, 6.4; N, 14.8; S, 6.1%).

L-2-*Acetoxy*-3-(*benzylthio*)*propanoyl*-L-*tyrosyl*-L-*phenylalanyl*-L-*glutaminyl*-L-*asparaginyl*-S-*benzyl*-L-*cysteinyl*-L-*prolyl*-N$^{\epsilon}$-*tosyl*-L-*lysylglycinamide*.—The foregoing peptide (400 mg) was dissolved in dimethylformamide (6 ml) and *p*-nitrophenyl *N*-[L-2-acetoxy-3-(benzylthio)propanoyl]-L-tyrosinate [6] (219 mg) was added (10% excess). The solution was left at room temperature for 4 days. Ethyl acetate (40 ml) was added and the precipitate was filtered off. It was washed with ethyl acetate (10 ml), ethyl acetate–ether (1 : 1; 5 ml), and ethanol–ether (1 : 1; 3 ml), and dried *in vacuo* (P_2O_5). The *peptide* (468 mg, 90% yield) had m.p. 183—184.5°, $[\alpha]_D^{24}$ −38.9° (*c* 0.5 in Me_2N·CHO), R_F 0.75 (Found: C, 56.0; H, 6.1; N, 11.7; S, 6.2. $C_{69}H_{86}N_{12}O_{14}S_3,4H_2O$ requires C, 56.2; H, 6.4; N, 11.4; S, 6.5%). Amino-acid analysis gave the following molar ratios (Pro taken as 1): Asp, 1.0; Glu, 1.1; Pro, 1.0; Gly, 1.0; Tyr, 0.9; Phe, 1.0; Cys(Bzl), 1.0; Lys, 1.0; NH_3, 2.9.

D-2-*Hydroxy*-3-(*benzylthio*)*propanoyl*-O-*benzyl*-L-*tyrosyl*-L-*phenylalanyl*-L-*glutaminyl*-L-*asparaginyl*-S-*benzyl*-L-*cysteinyl*-L-*prolyl*-N$^{\epsilon}$-*tosyl*-L-*lysylglycinamide*.— *O*-Benzyl-*N*-(D-3-benzylthio-2-hydroxypropanoyl)-L-tyrosine hydrazide [7] (160 mg) was mixed with peroxide-free dioxan (2.8 ml) and tetrahydrofuran (1.4 ml). Hydrochloric acid (5N; 0.48 ml) was added and the clear solution was cooled to −20 °C. Aqueous sodium nitrite (40 mg in 0.2 ml) was added slowly with stirring and after 15 min it was diluted with precooled ethyl acetate (15 ml). The solution was washed with a saturated solution (15 ml) of sodium hydrogen carbonate in 16.8% sodium chloride [26] at −20 °C. The aqueous phase was removed with a syringe and the remaining organic phase was dried ($MgSO_4$) and filtered directly into a solution of L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide (361 mg) in dimethylformamide (20 ml) at −20 °C. The mixture was left for 24 h at 0 °C and evaporated to dryness under reduced pressure at 60 °C. The residue was triturated with cold hydrochloric acid (0.2N; 2 × 12.5 ml), collected on the filter, and washed with water (2 × 7.5 ml), ethyl acetate–ether (1 : 1; 3 ml), and ether (5 ml). After drying *in vacuo* (P_2O_5), the solid (211 mg, 43% yield) was purified by reprecipitation from dimethylformamide (10 ml) with ethyl acetate–ether (1 : 1; 55 ml). The precipitate was washed on the filter pad with ether (2 × 5 ml) and dried *in vacuo* (P_2O_5). The *peptide* (131 mg, 62% recovery) had m.p. 190—191°, $[\alpha]_D^{23}$ −25.8° (*c* 0.5 in Me_2N·CHO), R_F 0.79 (Found: C, 58.0; H, 6.3; N, 11.0; S, 6.5. $C_{74}H_{90}N_{12}O_{16}S_3,H_2O$ requires C, 58.5; H, 6.1; N, 11.1; S, 6.3%). Amino-acid analysis showed the following molar ratios (Asp taken as 1): Asp, 1.0; Glu, 1.1; Pro, 1.1; Gly, 1.2; Tyr, 0.9; Phe, 1.0; Cys(Bzl), 0.9; Lys, 1.0; NH_3, 3.3.

[1-(L-2-*Hydroxy*-3-*mercaptopropanoic acid*)-8-*lysine*]-*vasopressin*.— L-2-Acetoxy-3-(benzylthio)propanoyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide (400 mg) was dissolved in anhydrous liquid ammonia (300 ml) and methanol (40 ml) was added with stirring. The ammonia was allowed to evaporate off overnight. The residual solution was evaporated to dryness under reduced pressure at 40 °C, and the residue redissolved in liquid ammonia (300 ml) and reduced with sodium by the stick method [27] until a blue colour persisted for 10 s. Glacial acetic acid (0.5 ml) was added and the ammonia was evaporated off at 30 °C. The last traces were removed with a rapid stream of nitrogen. The residue was dissolved in water (500 ml) and carbon dioxide-free oxygen was passed through until the Ellman test [28] was negative. The solution was evaporated under reduced pressure at 40 °C, and the residue taken up in the lower phase (40 ml) of the system butan-1-ol–propan-1-ol–0.05% acetic acid (2 : 1 : 3) and divided between the first four tubes of the counter-current distribution train. After 425 transfers one main peak (*K* 0.31) was separated from two minor Folin-positive peaks (*K* 0.02 and 0.13). The major band (tubes 80—160) was not in complete agreement with the theoretical curve. The contents of these tubes were therefore pooled, evaporated to a volume of *ca.* 25 ml, filtered, and lyophilized. The amorphous solid (155 mg, 53% yield) was again subjected to counter-current distribution in the same solvent system. The Folin–Lowry tests after 360 transfers defined one main peak (*K* 0.31), well separated from a faster travelling, very shallow band. The curves of the Folin–Lowry colour values and pressor activity were in excellent agreement with the calculated curve for a substance with a partition coefficient of 0.31. The contents of tubes 70—100 containing the [1-(L-2-hydroxy-3-mercaptopropanoic acid)-8-lysine]-vasopressin were pooled and freed from organic solvents under reduced pressure at 30 °C. The remaining

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aqueous solution was filtered and lyophilized. The resulting white, fluffy powder (93 mg, 60% recovery) had $[\alpha]_D^{23}$ −79.8° (*c* 0.05 in N-AcOH), R_F 0.57 (Found: C, 52.0; H, 6.0; N, 15.5; S, 5.8. $C_{46}H_{64}N_{12}O_{13}S_2$ requires C, 52.3; H, 6.1; N, 15.9; S, 6.1%). Amino-acid analysis gave the following molar ratios (Pro taken as 1): Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; Tyr, 1.0; Phe, 1.0; Lys, 1.1; NH_3, 2.7. Cystine (0.4) and the mixed disulphide of L-cysteine and L-2-hydroxy-3-mercaptopropanoic acid [29] (0.5) together account for the half-cystine residue in the hormone analogue. The pressor activity (rat blood pressure) was 431 ± 10.3 i.u.mg^{-1} [standard error of mean (SEM) (n = 5)] and the oxytocic activity (isolated uterus of the rat) was 17.9 ± 0.6 i.u.mg.$^{-1}$ [SEM (n = 5)].

[1-(D-*Hydroxy*-3-*mercaptopropanoic acid*)-8-*lysine*]-*vasopressin*.— D-2-Hydroxy-3-(benzylthio)propanoyl-*O*-benzyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-N^{ϵ}-tosyl-L-lysylglycinamide (86 mg) in liquid ammonia (350 ml) was deprotected as above. The residue after removal of the ammonia was dissolved in water (500 ml) and the dithiol intermediate was oxidized with 0.02M-potassium ferricyanide [10] under nitrogen [30] to the cyclic 'nonapeptide'. This was subjected to counter-current distribution in butan-1-ol–propan-1-ol–0.05% acetic acid (2 : 1 : 3). After 600 transfers a separation into five peaks with K values of 0.013 (medium), 0.14 (medium), 0.21 (large), 0.32 (small), and 0.38 (small) was accomplished as detected by the Folin–Lowry colour reaction. Determination of the pressor activity indicated that the Folin-positive main peak (tubes 85—140) had a low activity and was contaminated with material of much greater biological activity. The contents of tubes 90—130 were collected and the peptide material (28 mg, 48% yield) was isolated by lyophilization. The non-crystalline solid was further purified by counter-current distribution in the same system. Measurement of Folin–Lowry colour values and pressor activities after a total of 1430 transfers showed the presence of one major component (K 0.21) with minute biological activity and a small peptide peak (K 0.29) associated with an appreciable amount of pressor activity. The Folin curve of the main peak was in good agreement with the theoretical curve. The material of the peak with K_{1430} 0.21 was recovered by lyophilization. The white, fluffy product (15.6 mg, 60% recovery) had $[\alpha]_D^{23}$ −102.8° (*c* 0.05 in N-AcOH), R_F 0.57. Amino-acid analysis showed the following molar ratios (Pro taken as 1); Asp, 1.0; Glu, 1.0; Pro, 1.0; Gly, 1.0; Tyr, 0.9; Phe, 1.1; Lys, 1.0; NH_3, 3.0. In addition, cystine (0.5) and the mixed disulphide of L-cysteine and D-2-hydroxy-3-mercaptopropanoic acid [29] (0.4) were present. These two sulphur compounds fully account for the half-cystine residue. Cystine would be expected from an acid-catalysed disulphide interchange.[31] The pressor and oxytocic activities were 0.913 ± 0.029 [SEM (n = 5)] and 0.015 ± 0.003 i.u.mg^{-1} [SEM (n = 5)], respectively. Isolation of the material from peak K 0.29 in the usual way yielded an amorphous product, crude [1-(L-2-hydroxy-3-mercaptopropanoic acid)-8-lysine]-vasopressin (3.7 mg). The amino-acid analysis was identical with that of the D-diastereoisomer except for cystine (0.5) and the mixed disulphide of L-cysteine and L-2-hydroxy-3-mercaptopropanoic acid [29] (0.5). A pressor activity of 263 ± 16 i.u.mg^{-1} [SEM (n = 4)] was found, corresponding to a total of 973 i.u., *i.e.* 2.3 mg of L-diastereoisomer.

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