(7) I. I. Geschwind, C. H. Li, and L. Barnafi, *J. Am. Chem. Soc.*, **79**, 6394 (1957).
(8) D. Yamashiro and C. H. Li, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 4945 (1974).
(9) A. Loffet, *Int. J. Peptide Protein Res.*, **3**, 297 (1971).
(10) T. Wieland, F. Flor, and C. Birr, *Justus Liebigs Ann. Chem.*, 1595 (1973).
(11) H. Hagenmaier and H. Frank, *Hoppe*-Seyler's *Z. Physiol. Chem.*, **353**, 1973 (1972).
(12) D. Yamashiro and C. H. Li, *J. Am. Chem. Soc.*, **95**, 1310 (1973).
(13) D. Yamashiro and C. H. Li, *J. Org. Chem.*, **38**, 591 (1973).
(14) D. Yamashiro and C. H. Li, *J. Org. Chem.*, **38**, 2594 (1973).
(15) D. Yamashiro, J. Blake, and C. H. Li, *J. Am. Chem. Soc.*, **94**, 2855 (1972).
(16) B. Gutte and R. B. Merrifield, *J. Am. Chem. Soc.*, **91**, 501 (1969).
(17) Preparation of synthetic βc2-MSH and βb-MSH without the initial trifluoroacetic acid treatment always gave a side product which is retarded on CMC and can be converted to the desired product either by successive lyophilizations or heat treatment of the dry powder (R. L. Noble, D. Yamashiro, and C. H. Li, *J. Am. Chem. Soc.*, in press.
(18) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).
(19) J. Lenard and A. B. Robinson, *J. Am. Chem. Soc.*, **89**, 181 (1967).
(20) R. H. Mazur and G. Plum, *Experientia*, **24**, 661 (1968).
(21) M. Ohno, S. Tsukamoto, S. Makisumi, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **45**, 2852 (1972).
(22) E. A. Peterson and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956).
(23) D. Yamashiro, *Nature (London)*, **201**, 76 (1964).
(24) K. T. Wang, J. Blake, and C. H. Li, *Int. J. Peptide Protein Res.*, **5**, 33 (1973).
(25) J. Scotchler and A. B. Robinson, *J. Org. Chem.*, **35**, 3151 (1970).
(26) A. Previero, M. A. Coletti-Previero, and J. C. Cavadore, *Biochim. Biophys. Acta*, **147**, 453 (1967).
(27) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
(28) The natural βb-MSH was further purified by partition chromatography as described herein. This step removed the methionine sulfoxide derivative of the hormone.
(29) K. Shizume, A. B. Lerner, and T. B. Fitzpatrick, *Endocrinology*, **54**, 533 (1954).
(30) J. Ramachandran, *Biochem. Biophys. Res. Commun.*, **41**, 353 (1970).

Synthesis and Some Pharmacological Properties of [1-(L-2-Hydroxy-3-mercaptopropanoic acid),4-threonine]oxytocin (Hydroxy[4-Thr]oxytocin), a Peptide with Strikingly High Oxytocic Potency, and of [1-(L-2-Hydroxy-3-mercaptopropanoic acid)]oxytocin (Hydroxy-oxytocin)

Maurice Manning,* John Lowbridge,

Department of Biochemistry, Medical College of Ohio at Toledo, Toledo, Ohio 43614

Jaya Haldar, and Wilbur H. Sawyer

Department of Pharmacology, College of Physicians and Surgeons of Columbia University, New York, New York 10032.
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[1-(L-2-Hydroxy-3-mercaptopropanoic acid),4-threonine]oxytocin (hydroxy[4-Thr]oxytocin) and [1-(L-2-hydroxy-3-mercaptopropanoic acid)]oxytocin (hydroxy-oxytocin) were synthesized by a combination of solid phase and classical methods of peptide synthesis. Protected octapeptides were synthesized by the solid-phase method and 1 + 8 couplings in solution were then employed to furnish the required key protected intermediates. Hydroxy[4-Thr]oxytocin has oxytocic potency, as measured in the rat uterus suspended in a Mg^{2+}-free solution, of about 4200 units/mg, eight times the potency of oxytocin, while its antidiuretic potency is approximately equal to that of oxytocin. It thus exhibits a significantly favorable oxytocic–antidiuretic selectivity. Hydroxy-oxytocin has an oxytocic potency of approximately 1300 units/mg, 2.5 times that of oxytocin. Threonine substitution in hydroxy-oxytocin has thus caused a significant enhancement in both oxytocic potency and selectivity. The enhancement in oxytocic potency of these two peptides relative to oxytocin and [4-Thr]oxytocin appears to correlate with their lipophilic characteristics, suggesting a significant role of lipophilicity in the interplay of oxytocin-like peptides with oxytocic receptors.

Among the many synthetic analogs of oxytocin reported to date only four are known in which a single substitution has brought about a significant enhancement of oxytocic potency relative to that of oxytocin. These substitutions and the resulting highly potent analogs are (1) replacement of the amino group at position 1 by (a) hydrogen, deamino-oxytocin[1-3] or (b) a hydroxy group, [1-(L-2-hydroxy-3-mercaptopropanoic acid)]oxytocin (hydroxy-oxytocin);[4-6] (2) substitution of threonine for glutamine at position 4, [4-Thr]oxytocin;[7,8] and (3) replacement of the sulfur atom of the cysteine residue at position 1 with a methylene group, [6-1-cystathionine]oxytocin ([carba-1]oxytocin).[9] These modifications also changed the oxytocic–pressor and oxytocic–antidiuretic ratios of the above peptides relative to those of oxytocin. The oxytocic–pressor ratio was enhanced in all peptides with the exception of hydroxy-oxytocin which exhibited a substantially diminished ratio. The antidiuretic potency of hydroxy-oxytocin has not been reported but of the three remaining peptides only [4-Thr]oxytocin exhibited diminished antidiuretic activity. Thus of these four highly potent oxytocic agents, [4-Thr]oxytocin alone possesses the added highly desirable feature of enhanced oxytocic–antidiuretic selectivity (Table I).

Combining two or more of the above four modifications in a single peptide suggested the attractive possibility of further enhancing (a) oxytocic potency and (b) oxytocic–antidiuretic selectivity. Only two such peptides had been reported prior to the present study and their pharmacological evaluation had resulted in surprising and somewhat inconsistent findings.

On the one hand, deamino[4-Thr]oxytocin,[10] which

Table I. Biological Activities (Units/mg ± Standard Errors) of Hydroxy[4-Thr]oxytocin, Oxytocin, and Some Other 1- and 4-Substituted Analogs of Oxytocin

		Rat uterus (RU)							
		No Mg^{2+}	0.5 mM Mg^{2+}	Rat mammary strip	Rat milk ejection	Rat vasopressor (P)	Rat antidiuretic (A)	RU/P	RU/A
1	Hydroxy[4-Thr]oxytocin[a]	4179 ± 222	937 ± 55	378 ± 10	808 ± 19	4.92 ± 0.09	5.3 ± 0.5	849	788
2	Hydroxy-oxytocin[a]	1275 ± 51	868 ± 101	363 ± 8	694 ± 43	14.7 ± 0.3	16.6 ± 1.3	87	77
2	Hydroxy-oxytocin[b]	1607				32		50	
3	[4-Thr]oxytocin[c]	923 ± 95	719 ± 83		543 ± 23[h]	0.43 ± 0.01	1.8 ± 0.3	2146	513
4	Deamino[4-Thr]oxytocin[c]	149 ± 21	242 ± 22		385 ± 14[h]	<0.1	0.9 ± 0.1	∞	165
5	[Carba-1]oxytocin[d]	734		177		2.95	9.3	249	79
6	Deamino[carba-1]oxytocin[e]	1899	921	581		17.5	24.1	108	79 130
7	Oxytocin[c]	520 ± 12	486 ± 15	533[g]	474 ± 16[h]	4.3 ± 0.12	4.0 ± 0.8	121	130
8	Deamino-oxytocin[f]	803 ± 36	760		541 ± 13[h]	1.44 ± 0.06	19	558	42

[a] Present communication. [b] Values reported by Hope and Walti.[4,6] [c] Values reported by Manning et al.[10] [d] Values reported by Jost et al.[9] [e] Values reported by Barth et al.[12] [f] Values reported by Ferrier et al.[3] [g] Value reported by Polacek et al.[21] [h] Rabbit.

combines modifications 1a and 2, exhibited greatly diminished oxytocic activity relative to oxytocin while on the other, deamino[carba-1]oxytocin,[11,12] which combines modifications 1a and 3, exhibited almost four times the oxytocic potency of oxytocin. In this aspect the combination of deamination and carba substitution at position 1 acted cooperatively whereas deamination at position 1 and threonine substitution at position 4 were noncooperative. Neither combination afforded significant improvement in oxytocic–antidiuretic selectivity.

In pursuing the goal of obtaining a peptide which possessed both high oxytocic potency and selectivity, it appeared worthwhile to determine whether other combinations of the above four changes would exhibit cooperativity in their effects on both (a) oxytocic potency and (b) oxytocic–antidiuretic selectivity. A highly potent and selective oxytocic agent could be of potential clinical usefulness and might also be of value in helping to elucidate the mechanisms involved in peptide receptor interactions.

We thus decided to combine modifications 1b and 2 in one molecule, i.e., hydroxy substitution at position 1 and threonine substitution at position 4. We now wish to report the synthesis and pharmacological properties of the peptide designed according to the above rationale, i.e., [1-(L-2-hydroxy-3-mercaptopropanoic acid),4-threonine]oxytocin (hydroxy[4-Thr]oxytocin).

The proposed synthetic scheme for hydroxy[4-Thr]oxytocin differed from the approach followed in the original synthesis of hydroxy-oxytocin.[5] We therefore wished to test the feasibility of this modified approach for the synthesis of hydroxy-oxytocin and thus report in this communication its synthesis and pharmacological properties. As indicated above its antidiuretic potency, essential to the comparison of hydroxy-oxytocin and hydroxy[4-Thr]oxytocin, had not previously been reported.

Peptide Synthesis. The reported synthesis of hydroxy-oxytocin[5] was via a protected precursor prepared essentially by a fragment condensation (the acylation of a protected heptapeptide, corresponding to the C-terminal sequence of oxytocin, by *S*-benzyl-L-2-hydroxy-3-mercaptopropanoyl-*O*-benzyltryosine *p*-nitrophenyl ester). It can be reasoned from this report that since the dicyclohexylcarbodiimide (DCCI) mediated acylation of the amino function of the tyrosine derivative by the propanoic acid derivative proceeded with minimal loss of optical integrity in the carboxy component, a fully stepwise strategy for chain extension, minimizing the risk of racemization in the tyrosine residue, might be employed. The coupling of *S*-benzyl-L-2-acetoxy-3-mercaptopropionic acid with the requisite octapeptide derivative was thus envisaged as the final step in the generation of protected precursors of the synthetic hormones.

S-Benzyl-DL-2-hydroxy-3-mercaptopropanoic acid was prepared, and resolved, according to the literature.[5] The acylation of the hydroxy function proved difficult, however. Acetylation with acetic anhydride was sluggish; solutions of the hydroxy acid in acetic anhydride stored for 5 weeks at room temperature contained large proportions of unreacted starting material, and warming of such reaction mixtures, or of freshly prepared solutions, resulted in a multiplicity of products. Reaction with tosyl chloride in pyridine solution gave analogous results. Such difficulty suggested the possibility that the hydroxy group need not be protected during the coupling reaction (a satisfactory coupling of the hydroxypropanoic acid derivative was reported[5]), particularly if relatively short reaction times were used. In this latter regard the efficacy of the DCCI-*N*-hydroxybenzotriazole (HOBt)[13] system was demonstrated by the synthesis of *S*-benzyl-L-2-hydroxy-3-mercaptopropanoyl-*O*-benzyltyrosine hydrazide[5] in high yield, and purity, with a much reduced coupling time. The coupling method has the added advantage of allowing the assumption of minimal racemization.[13]

The Merrifield technique[14,15] was employed for the synthesis of the *tert*-butoxycarbonyloctapeptide resin esters as for the synthesis of oxytocin[16] and [4-Thr]oxytocin.[7] These were cleaved as the required amides by ammonolysis.[7,16] The N-protecting group was removed, and these products were acylated with *S*-benzyl-L-2-hydroxy-3-mercaptopropanoic acid to yield the desired protected intermediates in good yield (Table II). Their preparation in this manner allowed the final stages of the synthesis to proceed through purified precursors, in reactions easily monitored by thin-layer chromatography (TLC), and with minimal wastage of reactants.

The protected peptides were converted to the cyclized free peptides and purified by methods previously described.[7,17] The pharmacological properties of both hydroxy-oxytocin and hydroxy[4-Thr]oxytocin were evaluated by methods previously described[18-20] and by assays on isolated strips of rat mammary gland.[21]

Results and Discussion

Oxytocic potencies of neurohypophysial hormones and analogs are usually expressed in terms of their activities in assays on the isolated rat uterus suspended in a medium containing no Mg^{2+}.[12,19] Based on such assays the oxytocic

Table II. Properties of Peptide Intermediates, X-Tyr(Bzl)-Ile-Y-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2

	X	Y	Mp, °C[a]	$[\alpha]_D$,[b] deg	R_f in solvent system A	B	C	D	Formula[c]
IV	Boc	Gln	214-215	−43	0.75	0.53	0.64	0.48	$C_{59}H_{83}N_{11}O_{14}S\cdot 2H_2O$
V	Boc	Thr(Bzl)	214-215	−28	0.80	0.62	0.69	0.58	$C_{65}H_{88}N_{10}O_{14}S\cdot H_2O$
VI	HO·CH(CH_2·S·Bzl)·CO	Gln	225-228	−38	0.67	0.51	0.63	0.48	$C_{64}H_{85}N_{11}O_{13}S_2\cdot 3H_2O$
VII	HO·CH(CH_2·S·Bzl)·CO	Thr(Bzl)	221-224	−29	0.75	0.61	0.71	0.60	$C_{70}H_{90}N_{10}O_{13}S_2\cdot 2H_2O$

[a] Melting points taken in open capillaries, with a Thomas-Hoover apparatus,,and are uncorrected. All the compounds decomposed upon melting. [b] Optical rotations measured with a Bellingham Stanley Ltd. Model A polarimeter: c 1 (DMF), T 22°. [c] Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. The compounds were analyzed for C, H, and N; results were within ± 0.4% with the exception of the analyses for N of compound VI (found N, 11.55, $C_{64}H_{85}N_{11}O_{13}S_2\cdot 3H_2O$ requires N, 11.05). The formulations as containing solvent should not be taken to imply defined solvates.

activity of hydroxy[4-Thr]oxytocin is strikingly enhanced relative to the activities of either hydroxy-oxytocin or [4-Thr]oxytocin (Table I). In exhibiting an oxytocic potency of 4200 units/mg, hydroxy[4-Thr]oxytocin is by far the most potent analog of oxytocin synthesized to date. It possesses twice the oxytocic potency of deamino-[carba-1]oxytocin, hitherto the most highly potent oxytocic agent known. Furthermore, its oxytocic potency is over three times, four times, and seven times that of hydroxy-oxytocin, [4-Thr]oxytocin, and oxytocin, respectively. Clearly the combination of the hydroxy group in position 1 and threonine substitution in position 4 has acted cooperatively in producing such an extraordinary enhancement of oxytocic potency. This is in dramatic contrast to the results obtained on combining deamination at position 1 with threonine substitution at position 4, a combination which acted noncooperatively in bringing about a drastic reduction in oxytocic potency of the resulting compound, deamino[4-Thr]oxytocin (Table I).

Oxytocin-like activities of hydroxy[4-Thr]oxytocin and hydroxy-oxytocin measured by assays other than the isolated rat uterus in the absence of Mg^{2+} are considerably weaker (Table I). These analogs appear only about twice as potent as oxytocin when assayed on the rat uterus in the presence of Mg^{2+}. Magnesium ion does not decrease the sensitivity of the rat uterus to these analogs. The fall in relative potency reflects an increase in sensitivity of the uterus to the standard. Sensitivity to hydroxy[4-Thr]-oxytocin remains about the same when Mg^{2+} is added to the medium, while that to hydroxy-oxytocin may increase only slightly. Barth et al.[12] noted that sensitivity of the rat uterus to deamino[carba-1]oxytocin was not increased by the addition of Mg^{2+} and suggested that "the interaction between the compound and the receptor is apparently so complete that it cannot be augmented by magnesium ions". Our data on hydroxy[4-Thr]oxytocin might also be interpreted in this manner. The apparent depression of relative potencies on the uterus by Mg^{2+} cannot be related specifically to the presence of the hydroxy group or the 4-threonine in hydroxy[4-Thr]oxytocin since the same phenomenon was seen in the case of deamino[carba-1]oxytocin. Thus these observations shed no light on the question of which structural features of oxytocic peptides, or their uterine receptors, may be involved in magnesium potentiation.

Milk ejection activities of hydroxy[4-Thr]oxytocin and hydroxy-oxytocin are not as high as their rat uterus activities. Among the analogs in which milk ejection assays are reported in Table I, however, the ranking of activities on milk ejection and rat uterus assays is virtually the same: hydroxy[4-Thr]oxytocin > hydroxy-oxytocin > [4-Thr]-oxytocin = deamino-oxytocin > oxytocin > deamino[4-Thr]oxytocin. Mammary gland receptors appear to differ from uterine receptors in the extent by which they are influenced by alterations in the oxytocin molecule.[21] There is also a marked difference in the potencies of the hydroxy analogs on milk ejection assays in vivo and on assays on isolated mammary strips from the same rats. The difference in activities of the hydroxy analogs, relative to oxytocin by in vivo and in vitro assays, does not seem to result from differences in metabolism or distribution of these peptides in the whole rat. The figures presented (Table I) are based on assays by close arterial injection. When hydroxy[4-Thr]oxytocin was assayed by intravenous injections its activity, relative to that of oxytocin, did not differ from that estimated from intraarterial injections in the same rat. The loss of relative sensitivity by the isolated mammary strip remains unexplained. It could reflect the absence of blood flow to the myoepithelial elements. Perhaps oxytocin gains access to myoepithelial receptors more readily than the hydroxy analogs in these circumstances. It is also possible that the properties of the oxytocin receptors are somehow altered in the isolated strips.

The oxytocic–pressor and oxytocic–antidiuretic ratios of hydroxy[4-Thr]oxytocin also present an interesting and encouraging picture. Our data on hydroxy-oxytocin (Table I) show conclusively that hydroxy substitution at position 1 enhances both pressor and antidiuretic activities. Thus hydroxy-oxytocin possesses diminished oxytocic–pressor and oxytocic–antidiuretic ratios relative to oxytocin. We had earlier shown that threonine substitution in the 4 position of oxytocin led to reductions in both pressor and antidiuretic activities. Thus [4-Thr]oxytocin possesses enhanced oxytocic–pressor and oxytocic–antidiuretic selectivities relative to oxytocin. Individually the hydroxy and threonine substitutions have opposite effects on these selectivities and in combination in hydroxy[4-Thr]oxytocin these effects cancel one another out. Thus hydroxy[4-Thr]oxytocin possesses virtually the same pressor and antidiuretic activities as oxytocin while being over seven times more potent as an oxytocic agent. Its oxytocic–antidiuretic ratio is thus enhanced sixfold over that exhibited by oxytocin. Significantly also, the oxytocic–antidiuretic selectivity of hydroxy[4-Thr]oxytocin is 1.5 times greater than [4-Thr]oxytocin and ten times that of hydroxy-oxytocin.

It is tempting to speculate on why (a) hydroxy substitution at position 1 causes enhancement of all the characteristic activities of oxytocin and (b) the combination of hydroxy substitution at position 1 and threonine substitution at position 4 act cooperatively in causing such a selective enhancement of oxytocic potency.

The conformational model proposed for oxytocin[22] locates amino acid residues 3, 4, 7, and 8 within β turns, positions not primarily involved in the intramolecular stabilization of the peptide backbone and therefore available for intermolecular interactions. Changes in these positions have been observed to modulate the characteristic profile of activities in a selective fashion.[23] Amino

Table III. Correlation between Oxytocic Activity and Chromatographic Mobility of Oxytocin and Some Highly Potent Analogs

	Peptide	Oxytocic act., units/mg	R_f in solvent system B	C
1	Oxytocin	520 ± 12	0.07	0.13
2	[4-Thr]oxytocin	923 ± 95	0.14	0.18
3	Hydroxy-oxytocin	1275 ± 51	0.17	0.24
4	Hydroxy[4-Thr]oxytocin	4179 ± 222	0.29	0.37
5	Deamino[4-Thr]oxytocin	149 ± 21	0.40	0.56

acid residues at positions 1, 2, 5, 6, and 9 are believed to be involved in intramolecular stabilization; by implication, substitutions in these positions affect the activity profile in a nonselective manner. Although the findings outlined in this report are entirely consistent with the proposed model, conformational considerations per se provide no further insights into the key topochemical features responsible for either the direction or the magnitude of the changes in oxytocic potency and selectivity observed for hydroxy-oxytocin and for hydroxy[4-Thr]oxytocin.

It may be recalled that we had earlier suggested that threonine exerted its potentiating and selective effects in [4-Thr]oxytocin by modulating the overall hydrophobic–hydrophilic balance of the molecule[8] and that the markedly reduced oxytocic potency of deamino[4-Thr]-oxytocin might be a consequence of its unusually high lipophilic character.[10] It thus appeared very worthwhile to determine whether enhanced lipophilicity might account for the unusually high oxytocic potencies of hydroxy-oxytocin and hydroxy[4-Thr]oxytocin. We thus compared the lipophilic characteristics of oxytocin, [4-Thr]oxytocin, hydroxy-oxytocin, hydroxy[4-Thr]oxytocin, and deamino[4-Thr]oxytocin (as reflected by their respective R_f values) with their oxytocic potencies (Table III) and noted an apparent correlation. Increasing lipophilic character was associated with increasing oxytocic activity for the first four members of the series followed by a marked reduction in oxytocic activity for the most highly lipophilic peptide in the series, deamino[4-Thr]oxytocin. This ordering is highly reminiscent of the parabolic relationship between biological activity and lipophilicity demonstrated by Hansch and coworkers.[24] Analysis of the data from Table III in this light did not, however, yield correlation coefficients of any significance, possibly a consequence of the small number of compounds involved and the fact that they do not constitute a congeneric series.

Further studies aimed at (a) explaining the extraordinary potentiation of oxytocic activity brought about by the combination of a hydroxy group at position 1 and a threonine at position 4 of oxytocin and (b) further enhancing the oxytocic–antidiuretic ratio with retention of oxytocic potency appear very worthwhile. Investigation of the conformational structures of both hydroxy-oxytocin and hydroxy[4-Thr]oxytocin now also seems well warranted. It will also be interesting to ascertain whether the effects of the hydroxy substitution in oxytocin extend to the vasopressins and whether hydroxy substitution, in combination with other changes, can lead to highly potent and selective analogs in this series. Studies toward these ends are now in progress.

Experimental Section

The procedure of "solid-phase" synthesis conformed to that published[7,14-16] with the exception that the chloroform washes were omitted and a pyridine hydrochloride treatment[25] followed by neutralization was included after the final coupling step. Chloromethylated resin (Bio-Rad Bio-Beads SX-1) was esterified[26] with Boc-Gly to an incorporation of 0.25 mmol g^{-1}. The amino acid derivatives used were supplied by Bachem Inc., Fox Chemical Co., or Biosynthetika. Triethylamine and *N*-methylmorpholine (NMM) were distilled from ninhydrin; trifluoroacetic acid was distilled from P_2O_5; the acetic acid used for the hydrogen chloride–acetic acid cleavage reagent and for washings bracketing the trifluoroacetic acid cleavage following glutamine incorporation[16,27] was heated under reflux with boron triacetate and distilled from the reagent. Dimethylformamide (DMF) was distilled under reduced pressure immediately prior to its use. Methanol was dried with magnesium methoxide and distilled. Other solvents and reagents were analytical grade. Thin-layer chromatography was carried out by ascending technique on silica gel (0.25 mm, Brinkman Silplate). Solvent systems were A, butan-1-ol–acetic acid–water–pyridine (15:3:3:10 v/v); B, butan-2-ol–ammonia (25% aqueous)–water (34:3:3 v/v); C, butan-1-ol–acetic acid–water (4:1:5 v/v, upper phase); D, butan-1-ol–water (3.5% in acetic acid, 1.5% in pyridine) (1:1 v/v, upper phase); E, butan-1-ol–ethanol–pyridine–0.2 M acetic acid (8:1:1:10 v/v, upper phase). The load was 10 μg. Detection was by (i) chloroplatinate reagent followed by exposure to acid and spraying with ninhydrin solution and (ii) iodine vapor. For amino acid analysis samples (0.5 mg) were hydrolyzed with constant boiling hydrochloric acid (400 μl) containing phenol (80 mg) in evacuated and sealed ampules, for 20 h at 110°. The analyses were provided by Biopolymer Corporation, Cleveland, Ohio. Ratios are referred to Gly = 1.00.

Boc-Asn-Cys(Bzl)-Pro-Leu-Gly-resin (I). Boc-Gly-resin (7.15 g, 1.80 mmol of glycine) was subjected to four cycles of deprotection, neutralization, and coupling to yield the protected pentapeptide resin (7.90 g, weight gain 0.75 g, 82% of theory).

Boc-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-resin (II). The foregoing pentapeptide resin (3.95 g) was converted to Boc-octapeptide-resin (4.28 g, weight gain 0.33 g, 90% based upon weight gain in preparation of peptide-resin I) in three cycles of deprotection, neutralization, and coupling, followed by washing with a solution of pyridine hydrochloride (0.3 M) in dichloromethane and neutralization.

Boc-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly-resin (III). The protected peptide resin I (3.93 g) was treated as above (preparation of peptide-resin II) with the substitution of Boc-Thr(Bzl) and DCCI for Boc-Gln-ONp, affording the octapeptide resin (4.32 g, weight gain 0.39 g, 95% calculated as for II).

Boc-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (IV). The peptide resin II (4.28 g) was ammonolyzed[16] and the product extracted with DMF. The solvent was evaporated in vacuo and the residue twice reprecipitated, by methanol–ether (ca. 1:1 v/v) from a decolorized (charcoal) and filtered solution in a minimum quantity of warm (60°) DMF, to yield the protected nonapeptide amide (680 mg, 64% based upon initial glycine content of the resin, Table II). Amino acid analysis gave Tyr, 0.88; Ile, 1.02; Glu, 0.99; Asp, 1.05; Cys(Bzl), 1.20; Pro, 0.88; Leu, 1.07; Gly, 1.00.

Boc-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (V). The protected octapeptide V (590 mg, 52.5% based upon initial glycine content of the resin, Table II) was derived from the peptide resin III and purified by reprecipitation, as detailed in the preparation of the glutaminyl analog IV, above. Amino acid analysis gave Tyr, 0.97; Ile, 0.96; Thr, 0.82; Asp, 0.98; Cys(Bzl), 0.96; Pro, 1.02; Leu, 1.03; Gly, 1.00.

***S*-Benzyl-L-2-hydroxy-3-mercaptopropanoyl-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (VI).** A suspension of the Boc-octapeptide amide IV (100 mg, 0.084 mmol) in cold trifluoroacetic acid (3 ml) was swirled to effect solution (ca. 40 min) and left to stand for 80 min. Toluene (5 ml) was added and the mixture evaporated. The evaporation was repeated twice further with fresh aliquots of toluene to yield a crystalline solid (R_f^A 0.64, single chloroplatinate and ninhydrin-positive spot) which was washed well with ether and dried in vacuo. This material was dissolved in DMF (0.8 ml) containing *S*-benzyl-L-2-hydroxy-3-mercaptopropanoic acid[5] (19.5 mg, 0.092 mmol), HOBt·H_2O (14.0 mg, 0.092 mmol), and NMM (8.6 mg, 0.084 mmol). The solution was chilled in ice and treated with a solution of DCCI (19.0 mg, 0.092 mmol) in DMF (0.2 ml) with vortex mixing. Such mixing was continued for 15 min, with intermittent ice cooling; then the mixture was set aside, at room temperature,

Table IV. Some Physical Properties of Hydroxy-oxytocin and Hydroxy[4-Thr]oxytocin

Peptide	$[\alpha]^{22}D$,[a] deg	R_f in solvent system C	D	E
Hydroxy-oxytocin	−100[b]	0.24 (0.80)[c]	0.15 (0.67)[c]	0.35
Hydroxy-[4-Thr]-oxytocin	−120	0.37 (0.92)[c]	0.26 (0.76)[c]	0.47

[a] c 0.1, 1 M acetic acid. [b] Lit.[5] $[\alpha]^{18}D$ −91.5 ± 2.5° (c 0.05, 1 M acetic acid). [c] Chromatography on cellulose support (Brinkman celplate, 0.10 mm).

for 3 h. The consumption of the ninhydrin-positive material and the formation of a product (R_f^A 0.67) giving no color with the reagent were demonstrated by TLC (both spots detected by the chloroplatinate reagent). The reaction mixture was chilled and diluted with cold ethyl acetate (10 ml). The gelatinous precipitate was filtered and washed exhaustively with ethyl acetate–ethanol (1:1 v/v) and ether. Reprecipitation from a filtered solution in DMF (5 ml) by ether (15 ml) and further washing with ether and drying in vacuo yielded the acylpeptide amide (95 mg, 88%, Table II). Amino acid analysis gave Tyr, 0.73; Ile, 0.99; Glu, 1.06; Asp, 1.04; Cys(Bzl), 1.05; Pro, 0.91; Leu, 0.99; Gly, 1.00.

S-Benzyl-L-2-hydroxy-3-mercaptopropanoyl-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2 (VII). The protected peptide amide V (200 mg, 0.16 mmol) was subjected to the reaction sequence described above for the preparation of the glutaminyl analog VI to yield the acyloctapeptide amide VII (175 mg, 81%, Table II). The acylation reaction was monitored by TLC (cleavage product R_f^A 0.67 detected by ninhydrin and chloroplatinate, acylation product R_f^A 0.75 detected by chloroplatinate but giving no ninhydrin color). Amino acid analysis of compound VII gave Tyr, 0.75; Ile, 1.01; Thr, 0.89; Asp, 1.09; Cys(Bzl), 1.13; Pro, 1.00; Leu, 1.04; Gly, 1.00.

[1-(L-2-Hydroxy-3-mercaptopropanoic acid)]oxytocin (Hydroxy-oxytocin) (VIII). A solution of the peptide intermediate VI (90 mg, 0.07 mmol) in sodium-dried and redistilled ammonia (ca. 300 ml) was treated at the boiling point, and with stirring, with sodium[17] from a stick of the metal contained in a small bore glass tube[1,2,7] until a light blue color persisted in the solution for 20 s. The color was discharged by the addition of dry glacial acetic acid (ca. 2 ml). The solution was evaporated (water aspirator via a splash head and two U tubes containing soda lime) and the residue taken up in aqueous acetic acid (0.2%, 750 ml) with vigorous stirring. Following the addition of aqueous ammonia (2 M, ca. 40 ml) to give a solution of pH 7, an excess of potassium ferricyanide solution (0.01 M, 10 ml) was added gradually, with stirring. The yellow solution (pH 6–7) was stirred for 10 min with anion-exchange resin (Bio-Rad AG 3-X4A, chloride form, ca. 10 g damp weight) and filtered through a bed of the resin (ca. 80 g damp weight). The bed was washed with aqueous acetic acid (0.2%, 200 ml), and the combined filtrate and washings were lyophilized. The resulting powder was desalted on Sephadex G-15 (column 110 × 2.7 cm)[10,28] eluting with aqueous acetic acid (50%) with a flow rate ca. 6 ml h^{-1}. The eluate was monitored for absorbance at 280 nm and fractionated. The fractions comprising the major peak were pooled and lyophilized, and the residue (36 mg) was further subjected to gel filtration with Sephadex G-15 (column 100 × 1.5 cm) eluting with aqueous acetic acid (0.2 M, flow rate 4 ml h^{-1}). The oxytocin analog (18 mg, 26%, Table IV) was isolated from the fractions comprising the single symmetrical peak by lyophilization and dried in vacuo over P_2O_5. Amino acid analysis gave Tyr, 0.98; Ile, 1.01; Glu, 0.98; Asp, 1.19; Cys, 0.34; Pro, 0.99; Leu, 1.00; Gly, 1.00.

[1-(L-2-Hydroxy-3-mercaptopropanoic acid),4-threonine]oxytocin (Hydroxy[4-Thr]oxytocin) (IX). The [4-Thr]oxytocin analog IX (15 mg, 25%, Table IV) was prepared from the protected precursor VII (90 mg, 0.067 mmol) as detailed above for the preparation of the oxytocin analog VIII. Amino acid analysis gave Tyr, 0.95; Ile, 0.94; Thr, 0.85; Asp, 1.17; Cys, 0.32; Pro, 0.84; Leu, 0.97; Gly, 1.00.

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References and Notes

(1) V. du Vigneaud, G. Winestock, V. V. S. Murti, D. B. Hope, and R. J. Kimbrough, *J. Biol. Chem.*, **235**, 64 (1960).
(2) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Biol. Chem.*, **237** 1563 (1962).
(3) B. M. Ferrier, D. Jarvis, and V. du Vigneaud, *J. Biol. Chem.*, **240**, 4264 (1965).
(4) D. B. Hope and M. Walti, *Biochem. J.*, **125**, 909 (1971).
(5) M. Walti and D. B. Hope, *J. Chem. Soc. C*, 1946 (1972).
(6) D. B. Hope and M. Walti, *Proc. Roy. Soc. Med.*, **67**, 12 (1974).
(7) M. Manning, E. Coy, and W. H. Sawyer, *Biochemistry*, **9**, 3925 (1970).
(8) W. H. Sawyer and M. Manning, *J. Endocrinol.*, **49**, 151 (1971).
(9) K. Jost, T. Barth, I. Krejci, L. Fruhaufova, Z. Prochazka, and F. Sorm, *Collect. Czech. Chem. Commun.*, **38**, 1073 (1973).
(10) M. Manning, E. J. Coy, and W. H. Sawyer, *Experientia*, **27**, 1372 (1971).
(11) K. Jost, *Collect. Czech. Chem. Commun.*, **36**, 218 (1971).
(12) T. Barth, I. Krejci, B. Kupkova, and K. Jost, *Eur. J. Pharmacol.*, **24**, 183 (1973).
(13) W. Konig and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
(14) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).
(15) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964).
(16) M. Manning, *J. Am. Chem. Soc.*, **90**, 1348 (1968).
(17) V. du Vigneaud, C. Ressler, J. M. Swan, P. Katsoyamnis, and C. W. Roberts, *J. Am. Chem. Soc.*, **76**, 3115 (1954).
(18) W. H. Sawyer, *Endocrinology*, **63**, 694 (1958).
(19) W. H. Sawyer in "The Pituitary Gland", Vol. 3, G. W. Harris and B. T. Donovan, Ed., Butterworths, London, 1966, p 288.
(20) G. W. Bisset, B. J. Clark, J. Haldar, M. C. Harris, G. P. Lewis, and M. Roche e Silva, Jr., *Br. J. Pharmacol. Chemother.*, **31**, 537 (1967).
(21) I. Polacek, I. Krejci, and J. Rudinger, *J. Endocrinol.*, **38**, 13 (1967).
(22) D. W. Urry and R. Walter, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 956 (1971).
(23) R. Walter, I. L. Schwartz, J. H. Darnell, and D. W. Urry, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 1355 (1971).
(24) C. Hansch and J. M. Clayton, *J. Pharm. Sci.*, **62**, 1 (1973).
(25) L. C. Dorman, *Tetrahedron Lett.*, **28**, 2319 (1969).
(26) B. F. Gisin, *Helv. Chim. Acta*, **56**, 1476 (1973).
(27) H. Takashima, R. B. Merrifield, and V. du Vigneaud, *J. Am. Chem. Soc.*, **90**, 1323 (1968).
(28) M. Manning, T. C. Wuu, and J. W. M. Baxter, *J. Chromatogr.*, **38**, 396 (1968).