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Solid-Phase Synthesis and Some Pharmacological Properties of 4-Threonine Analogs of Vasopressins and Vasotocin and of Arginine-vasopressin and Arginine-vasotocin†

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[4-Threonine]arginine-vasopressin I, [4-threonine]lysine-vasopressin II, [4-threonine]arginine-vasotocin III, arginine-vasopressin IV, and arginine-vasotocin V were synthesized using the solid-phase method. The deblocked and reoxidized peptides were purified by sequential gel filtration on Sephadex G-15 in 50% AcOH and 0.2 *N* AcOH. By comparison with the parent 4-glutamine-containing peptide in each case I–III exhibited: (a) increases in rat uterus activity of 34, 28, and 58% and in fowl vasodepressor activity of 200, 400, and 69%, respectively; (b) decreases in rat vasopressor activity of 71, 80, and 21%. With respect to the antidiuretic activity, I and II gave decreases of 28 and 45% whereas III had an increase of 21%. The substitution of threonine for glutamine therefore has brought about a selective increase of the antidiuretic to pressor ratio in all three analogs. The potencies of IV and V were in general agreement with those in the literature and were used for comparisons with those of I and III.

Substitution of threonine for glutamine in position 4 of the neutral neurohypophysial principles, oxytocin and mesotocin, gave rise to analogs possessing quite remarkable properties. On the one hand, oxytocin-like activities were markedly enhanced and, on the other, vasopressin-like activities were decreased.[1b-3] These findings immediately raised the question: how would a similar threonine/glutamine interchange affect the characteristic activities of the basic neurohypophysial peptides, arginine-vasopressin, lysine-vasopressin, and arginine-vasotocin? This study was therefore carried out (a) to provide an immediate answer to this question, (b) to determine whether or not the effects produced might fit into a predictable pattern, and (c) to give some further insight into the role of the amino acid in position 4 in determining the biological characteristics of the basic neurohypophysial peptides. In addition to reporting on the synthesis and pharmacological properties of these 4-threonine analogs, we present here also independent syntheses and pharmacological properties of both arginine-vasopressin and arginine-vasotocin. Both of these compounds had been synthesized by a number of investigators using a variety of classical methods of peptide synthesis.[4] However, no reports on the synthesis of either compound by the solid-phase method[5,6] had appeared when these syntheses were first undertaken. Following completion of the present synthesis of arginine-vasopressin, a solid-phase synthesis of a preparation possessing very high antidiuretic and pressor activities was reported.[7] However, our approach is sufficiently distinct to justify its inclusion in the present report. It should be noted also that syntheses of both arginine-vasopressin and arginine-vasotocin by the solid-phase method have very recently been reported elsewhere,[8] but no details of either the syntheses or of the characteristic pharmacological properties were given. All of the required protected nonapeptide intermediates were synthesized using the Merrifield method[5,6] as adapted for the synthesis of of oxytocin,[9] [8-phenylalanine]oxytocin,[10] and [4-threonine]oxytocin[2] by either manual or automated methods, and the final purified compounds were obtained by previously described methods[11-14] as outlined in the Experimental Section. Measurements of the pharmacological potencies were carried out as previously described.[15]

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Results and Discussion

The data presented in Table I show that substitution of threonine for glutamine in the 4 position of all three basic neurohypophysial peptides increased oxytocic and fowl vasodepressor activities. These changes closely parallel in a qualitative but not in a quantitative sense those observed when threonine is substituted for glutamine in oxytocin and mesotocin. Thus, the [4-threonine] analogs of arginine-vasopressin, lysine-vasopressin, and arginine-vasotocin exhibited enhancements in rat uterus activity of 34, 28, and 58%, respectively, whereas the corresponding value for [4-threonine]oxytocin was 80%. All three [4-threonine]-substituted analogs of the basic neurohypophysial peptides demonstrated substantially increased potencies in fowl vasodepressor activity of 200, 400, and 69% when compared with

Table I. Biological Activities of the Vasopressins and Vasotocin and Their 4-Threonine Analogs

No.	Analogs	Biological activities (units/mg ± S.E. of the assays)					Ratio, antidiuretic / vasopressor
		Rat uterus		Fowl depressor	Antidiuretic	Vasopressor	
		No Mg^{2+}	0.5 m*M* Mg^{2+}				
2	Arginine-vasopressin (AVP)	13.9 ± 0.5	25.5 ± 0.6	105 ± 5	323 ± 16	369 ± 6	0.9
6	Thr^4-AVP	18.6 ± 0.4	65 ± 3	321 ± 15	231 ± 29	104 ± 2	2.2
	Lysine-vasopressin[a] (LVP)	10.1 ± 0.3	22.1 ± 0.9	52 ± 6	284 ± 39	(270 ± 15)	1.1
10	Thr^4-LVP	12.9 ± 0.4	25.1 ± 0.8	267 ± 5	155 ± 17	49 ± 2	3.2
4	Arginine-vasotocin (AVT)	127 ± 9	194 ± 15	493 ± 17	231 ± 30	160 ± 4	1.4
8	Thr^4-AVT	201 ± 10	260 ± 21	831 ± 27	279 ± 25	106 ± 25	2.6

[a]Assays on a solution of Sandoz synthetic lysine-vasopressin. The absolute pressor activity was assumed to be that reported in B. Berde and R. A. Boissonnas in "Neurohypophysial Hormones and Similar Polypeptides," B. Berde, Ed., Springer-Verlag, Berlin, 1968, p 802. All other assays were done by the authors and, except where indicated, the values shown represent units per milligram of lyophilized peptides synthesized by the authors.

Table II. Pharmacological Potencies of a Series of Analogs of Lysine-vasopressin (LVP) with Modifications at Postion 4

Analogs	Side chain in 4 position	Biological activities (units/mg ± S.E. of the assays)				Ratio, antidiuretic / vasopressor
		Rat uterus (no Mg^{2+})	Fowl depressor	Antidiuretic	Vasopressor	
4-α-Aminobutyric acid LVP[a]	$-CH(CH_3)H$	1.54 ± 0.10	13.1 ± 0.7	707 ± 107	10.2 ± 0.6	70
LVP[b]	$-CH(H)CH_2 \cdot CONH_2$	10.1 ± 0.3	52 ± 6	284 ± 39	(270 ± 15)	1.1
Thr^4-LVP[b]	$-CH(CH_3)OH$	12.9 ± 0.4	267 ± 5	155 ± 17	49 ± 2	3.2
Ser^4-LVP[c]	-CH(H)OH	0.9 ± 0.2	10 ± 0.7	70 ± 11	3.3 ± 0.5	21.1
Ala^4-AVP[c]	-CH(H)H	0.5 ± 0.05	2.2 ± 0.1	30 ± 0.3	1.6 ± 0.3	18.7
Asn^4-LVP[c]	$-CH(H) \cdot CONH_2$	3 ± 0.4	23 ± 1	25 ± 3	56 ± 12	0.45

[a]See ref 16. [b]See Table I. [c]R. A. Boissonnas, St. Guttmann, R. L. Hugenin, P-A. Jaquenoud, and E. D. Sandrin, *Helv. Chim. Acta,* 46, 2347 (1963).

their respective [4-glutamine] counterparts. This activity of [4-threonine]oxytocin was increased 200%. The effects on antidiuretic and vasopressor activities reveal an interesting selectivity. While both activities are diminished in all three analogs (except for [4-threonine]vasotocin in which there is a slight enhancement of antidiuretic activity), the degrees of diminishment are not the same. The loss of vasopressor activity is more pronounced, resulting, in all three cases, in an increase in the ratio of antidiuretic to pressor activities. It may be recalled that [4-threonine]oxytocin was found to possess reductions of 55 and 90% in antidiuretic and vasopressor potencies, respectively. These findings recall a similar but more enhanced selective increase in antidiuretic: vasopressor activity ratio observed when the lipophilic amino acid, α-aminobutyric acid, was substituted for glutamine in arginine-vasopressin, lysine-vasopressin, and their deamino analogs.[16,17]

It is tempting to suggest that an analogous though much less pronounced lipophilic effect may be responsible for the selectivity observed here. We have previously found[18] that removal of the amino group from [4-threonine]-substituted analogs of both oxytocin and mesotocin resulted in analogs which were much more lipophilic than the respective parent compounds in each case. Synthesis of the deamino derivatives of the 4-threonine analogs reported here was an obvious route toward further exploring the effects of lipophilicity upon the antidiuretic:pressor selectivity. Thus, these deamino derivatives have been synthesized and are being pharmacologically evaluted. The results obtained will be reported in a following paper.

A comparison of the properties of [4-threonine]lysine-vasopressin with those of ther 4-substituted analogs of lysine-vasopressin (LVP) (Table II) sheds further light on the role of the amino acid at position 4 in contributing to the specific biological properties of these peptides. As was observed for the series of 4-substituted oxytocin analogs,[2] the 4-threonine derivative is again unique by virtue of its enhancement of both rat uterus and fowl vasodepressor activities over the corresponding values for LVP. The data on the antidiuretic activities of these 4-substituted lysine-vasopressins provide further clues to the nature of the significant factors which govern and regulate the selective antidiuretic properties of these basic peptides. These clues can point out other possible worthwhile approaches to further exploring this most intriguing phenomenon. The peptides are listed in the order of decreasing antidiuretic potency, starting with the highly potent [4-α-aminobutyric acid] analog. All of the analogs listed differ only in the groups or atoms attached to the β-carbon atom of their respective side chains at position 4. The wide differences in their antidiuretic potencies must therefore be related to relatively minor structural alterations on a very small area of the total molecule. Comparisons of the side-chain structural features of these analogs point to the vital role of lipophilic and steric-size factors in mediating the antidiuretic response. The importance of the lipophilic factor can be illustrated by comparing [4-α-aminobutyric acid]-LVP and [4-alanine]-LVP with respect to the probable relationship between the differences in lipophilic character of the α-aminobutyric acid and alanine side chains and the dramatic differences in the antidiuretic activity of each analog. α-Aminobutyric acid has a methyl group attached to its β carbon, whereas this position is occupied by a hydrogen atom in alanine. The 25-fold enhancement in antidiuretic activity of [4-α-aminobutyric acid]-LVP as compared to [4-alanine]-LVP must in some way be directly related to the increased lipophilicity of the α-aminobutyric acid side chain as compared to that of alanine. That this enhancement of antidiuretic activity is not merely the result of a steric-size effect can be attested to by the low antidiuretic activity of [4-asparagine]-LVP, an analog having a carboxamide grouping attached to the β carbon at position 4. However, the steric-size factor at position 4 undoubtedly contributes to the elaboration of high antidiuretic potency. Thus, [4-alanine]-LVP is clearly more lipophilic than [4-serine]-LVP but the side chain at position 4 in the latter is

Table III. Protected Nonapeptides of Arginine-vasopressin (1), Arginine-vasotocin (3), [4-Threonine]arginine-vasopressin (5), [4-Threonine]arginine-vasotocin (7), and [4-Threonine]lysine-vasopressin (9)

Bzl Bzl Bzl
Z–Cys–Tyr–(X)–(X)–Asn–Cys–Pro–(X)–Gly–NH_2
1 2 3 4 5 6 7 8 9

No.	Amino acids in position 3	4	8	Formula[a]	Mp, °C[b]	$[\alpha]^T_D$[c]	Yield in resin,[d] %	Yield on ammonolysis,[d] %
1	Phe	Gln	Arg(Tos)	$C_{82}H_{97}N_{15}O_{16}S_3$	223–224	−33.4	66.2	61.7[e]
3	Ile	Gln	Arg(Tos)	$C_{79}H_{99}N_{15}O_{16}S_3$	230–231	−33.5	49	24
5	Phe	Thr(Bzl)	Arg(Tos)	$C_{88}H_{102}N_{14}O_{16}S_3 \cdot 2H_2O$	217–232	−18.8	98	86.3
7	Ile	Thr(Bzl)	Arg(Tos)	$C_{86}H_{104}N_{14}O_{16}S_3$	231–232	−23.3	98	80.0
9	Phe	Thr(Bzl)	Lys(Tos)	$C_{88}H_{102}N_{12}O_{16}S_2$	215–216	−20.4	100	53.3

[a]Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. The analytical results were within ±0.4% of the theoretical values; all compounds were analyzed for C, H, and N. [b]Melting points were taken in an open capillary in a Thomas-Hoover melting point apparatus and are uncorrected. [c]In DMF [c 1.0, except for 3 (0.5); T = 22, 22, 23, 19, and 24°]. [d]Yields are based on the initial glycine incorporation in the resin. [e]This value compares very favorably with the 28% yield obtained at this stage by J. Meienhofer, *et al.*[7]

larger than that in the former. The twofold enhancement in antidiuretic potency of the serine-containing peptide over the alanine-containing peptide must, in this case, be attributable to the predominance of the steric-size effect over the lipophilic character effect. The question as to which factor, steric size or lipophilicity, is of greater importance for the elaboration of maximal antidiuretic potency is indeed an intriguing one. The answer may be ascertained by comparing both the side-chain structural features and the antidiuretic properties of [4-α-aminobutyric acid]-LVP with those of [4-threonine]-LVP. The side chain of α-aminobutyric acid possesses more lipophilic character while the side chain of threonine possess more steric size. [4-α-Aminobutyric acid]-LVP is over four times as active as [4-threonine]-LVP in the antidiuretic assay system. It is therefore reasonable to conclude that, for the optimalization of antidiuretic potency in LVP analogs, the lipophilic factor is more effective than the steric-size factor at position 4. Increasing the lipophilic character of the amino acid residue in the four position should help to define the limits of the lipophilic factor, while at the same time providing clues to the optimal steric-size characteristics of the side chain at position 4.

In this regard, the synthesis and pharmacological study of a series of lysine-vasopressin analogs possessing valine, norvaline, leucine, norleucine, and isoleucine at position 4 should be of value in clarifying this intriguing question and may thereby lead to the design of analogs, with even greater antidiuretic selectivity, of potential clinical value.

Experimental Section‡

The required protected nonapeptide intermediates **1**, **3**, **5**, 7, and 9 were all synthesized by the Merrified method[5,6] using previously described methods[2,9,10] with the variations and modifications indicated below.

Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (1). Boc-glycyl resin (2.34 g, 0.875 mmol of glycine) was treated in an 8-cycle procedure as described for oxytocin[9] and [8-phenylalanine]oxytocin,[10] except that Boc-Arg(Tos) (in DMF) and Boc-Phe were used in the first and sixth incorporation steps, respectively, to give the protected nonapeptide resin, weight 3.23 g. Ammonolytic cleavage of the protected nonapeptide resin (2.45 g) was carried out as described for oxytocin,[9] the protected peptide was extracted with DMF and MeOH, and the solvents were removed *in vacuo*. The semisolid residue was dissolved in DMF (20 ml) and precipitated by the addition of H_2O (1 l.) to give 1 as an amorphous white powder, weight 625 mg (Table III). Amino acid analysis[19] gave: Asp, 1.01; Glu, 1.00; Phe, 1.11; Gly, 1.00; Bzl-Cys, 1.89; Arg, 0.99; Tyr, 0.82; Pro, 1.00; NH_3, 2.95.

Arginine-vasopressin (2). The protected nonapeptide **1** (150 mg) was deblocked by the sodium–liquid ammonia procedure[20] as used in the original syntheses of oxytocin.[11,12] Reoxidation in aqueous solution at pH 6.5 was effected with the use of potassium ferricyanide.[13] The lyophilized product was purified by gel filtration on Sephadex G-15 in a two-step procedure using 50% AcOH and 0.2 *N* AcOH, respectively, for elution in each step.[10,14] Arginine-vasopressin obtained as a fluffy white powder, weight 35.0 mg, was shown to be homogeneous§ by thin-layer chromatography and paper electrophoresis at different pH's as described for [8-phenylalanine]oxytocin[10] (Table IV). Amino acid analysis[19] gave: Asp, 1.00; Glu, 1.05; Gly, 1.00; Pro, 1.03; Cys, 1.92; Phe, 1.03; Tyr, 0.94; Arg, 0.99; NH_3, 2.90.

Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (3). Boc-glycyl resin (4.20 g, 1.60 mmol of glycine) was treated by the 8-cycle procedure with the use of an automated machine (purchased from Schwartz Bioresearch, Inc.) as described for the synthesis of [8-phenylalanine]oxytocin,[10] except that Boc-Arg(Tos) was used in the first incorporation step to give the protected nonapeptide resin, weight 5.38 g. Ammonolytic cleavage[9,10] of the protected nonapeptide resin (2.5 g) gave **3** as a white amorphous powder, which was purified by trituration with MeOH (20 ml), weight 300 mg (Table III). Amino acid analysis[19] gave: Asp, 1.01; Glu, 1.07; Pro, 0.96; Gly, 1.00; Ile, 0.92; Tyr, 0.80; Bzl-Cys, 1.90; Arg, 0.90; NH_3, 3.20.

Arginine-vasotocin (4). The protected nonapeptide **3** (150 mg) was deblocked, reoxidized,[11-13] deionized, lyophilized, and purified as outlined for 2 to give arginine-vasotocin, weight 35.0 mg. It was shown to be homogeneous by thin-layer chromatography and paper electrophoresis[10] (Table IV). Amino acid analysis[19] gave: Asp, 1.05; Glu, 1.00; Pro, 1.06; Gly, 1.10; Cys, 1.98; Ile, 0.98; Tyr, 0.93; Arg, 0.95; and NH_3, 3.11.

Z-Cys(Bzl)-Tyr(Bzl)-Phe-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (5). The protected nonapeptide resin was prepared from Boc-glycyl resin (2.75 g, 1.01 mmol of glycine) by the method used for the synthesis of [4-threonine]oxytocin[2] except that Boc-Arg(Tos) and Boc-Phe were used respectively in the first and sixth incorporation steps to give the protected nonapeptide resin, weight 4.5 g. Ammonolytic cleavage[2,9] of this nonapeptide resin (2.55 g) yielded **5**, which was purified by trituration with MeOH (2 × 20 ml) to give a white amorphous powder, weight 835 mg (Table III). Amino acid analysis[19] gave: Asp, 0.98; Thr, 0.91; Gly, 1.00; Bzl-Cys, 1.92; Phe, 0.97; Tyr, 0.76; Pro, 0.92; Arg, 0.96; NH_3, 1.93.

[4-Threonine]arginine-vasopressin (6). The protected nonapeptide **5** (250 mg) was deblocked,[13,20] reoxidized,[11-13] and purified[14] as for 2, weight 50 mg (Table IV). It was shown to be homogeneous by the usual methods.[10] Amino acid analysis[19] gave: Asp, 1.02; Thr, 0.94; Gly, 1.00; Pro, 0.95; Cys, 1.99; Phe, 0.97; Tyr, 0.91; Arg, 0.92; NH_3, 1.95.

Z-Cys(Bzl)-Tyr(Bzl)-Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (7). Boc-glycyl resin (2.90 g, 1.1 mmol of glycine) was

‡Amino acid analyses were performed on a Beckman/Spinco amino acid analyzer Model 121C by the method of Spackman, *et al.*[19] All optical rotations were measured using a Bellingham Stanley Ltd. Model A polarimeter, Type PI.

§When the preparation of arginine-vasopression reported earlier[7] was purified as described here, it was found to contain an impurity as revealed by paper electrophoresis. This impurity was removed by further purification by ion-exchange chromatography on IRC-50. The absence of this impurity in our Sephadex purified preparation indicates that differences in the potencies of their IRC-50 purified preparation and ours cannot be accounted for on the basis that this impurity might be present in ours.

Table IV. Arginine-vasopressin (2), Arginine-vasotocin (4), [4-Threonine]arginine-vasopressin (6), [4-Threonine]arginine-vasotocin (8), and [4-Threonine]lysine-vasopressin (10)

Cys–Tyr–(X)–(X)–Asn–Cys–Pro–(X)–Gly–NH_2

1 2 3 4 5 6 7 8 9

No.	Amino acids in positions 3	4	8	$[\alpha]^T D^a$	$R_f{}^b$	Yield from protected nonapeptide, %	Yield[c] overall, %
2	Phe	Gln	Arg	−19.0	0.18	35.0	21.5
4	Ile	Gln	Arg	−9.6	0.11	36.3	8.8
6	Phe	Thr	Arg	−19.6	0.20	39.0	35.0
8	Ile	Thr	Arg	+3.8	0.19	43.3	35.0
10	Phe	Thr	Lys	−19.0	0.13	38.0	20.3

[a] In 1 *N* AcOH (*c* 0.5, 0.55, 0.51, 0.53, and 0.51); *T* = 27, 18, 26, 20, and 23°, respectively. [b] Samples run on silica gel H plates in the upper phase of the solvent system *n*-BuOH–AcOH–H_2O (4 : 1 : 5 v/v, ascending). Electrophoresis in two pyridine acetate buffers of pH 3.5 and 6.5 gave only one spot with the platinum and ninhydrin reagents in all cases.[10] [c] Based on the initial glycine incorporation on the resin.

treated by the 8-cycle procedure used for the synthesis of [4-threonine]oxytocin[2] with Boc-Arg(Tos) being incorporated in the first step in place of Boc-Leu to give the protected nonapeptide resin, weight 4.66 g. Ammonolysis of the protected nonapeptide resin[2,9] (1.71 g) gave 7 as an amorphous powder, which was purified as for **5**, weight 503 mg (Table III). Amino acid analysis[19] gave: Asp, 1.05; Gly, 1.00; Thr, 0.94; Bzl-Cys, 1.85; Tyr, 0.94; Pro, 0.85; Arg, 0.91; Ile, 0.96; NH_3, 2.10.

[4-Threonine]arginine-vasotocin (8). Reduction,[13,20] reoxidation,[11-13] and purification[14] of 7 (150 mg) gave 8 as a white fluffy powder, weight 42 mg, shown to be homogeneous by thin-layer chromatography and by electrophoresis at two different pH values[10] (Table IV). Amino acid analysis[19] gave: Asp, 1.06; Thr, 0.94; Gly, 1.00; Pro, 1.00; Cys, 1.95; Ile, 0.96; Tyr, 0.97; Arg, 0.94; NH_3, 2.15.

Z-Cys(Bzl)-Tyr(Bzl)-Phe-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Lys(Tos)-Gly-NH_2 (9). Boc-glycyl resin (4.95 g, 1.83 mmol of glycine) was treated by the 8-cycle procedure as described for the synthesis of [4-threonine]oxytocin[2] except that Boc-Lys(Tos) and Boc-Phe were used in the first and sixth incorporation steps, respectively, to give the protected nonapeptide resin, weight 7.64 g. Ammonolytic cleavage[2,9] of the protected nonapeptide resin (2.85 g) gave 9 as a white amorphous powder after trituration with MeOH, weight 610 mg (Table III). Amino acid analysis[19] gave: Asp, 1.19; Thr, 1.10; Pro, 0.98; Gly, 1.00; Phe, 1.19; Tyr, 0.68; Bzl-Cys, 1.90; Lys, 0.88; NH_3, 2.20.

[4-Threonine]lysine-vasopressin (10). The protected nonapeptide 9 (150 mg) was reduced,[13,20] reoxidized,[11-13] deionized, and purified[14] as for 2, weight 35 mg. It was shown to be homogeneous by thin-layer chromatography and paper electrophoresis[9] (Table IV). Amino acid analysis[19] gave: Asp, 1.00; Thr, 0.96; Pro, 0.98; Gly, 1.00; Cys, 1.19; Phe, 1.00; Tyr, 0.88; Lys, 1.15; NH_3, 2.24.

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