Synthesis and Some Pharmacological Properties of Oxytocin and Vasopressin Analogues with Sarcosine or *N*-Methyl-L-alanine in Position 7†

Zbigniew Grzonka,‡ Bernard Lammek,‡ Franciszek Kasprzykowski,‡ Diana Gazis,§ and Irving L. Schwartz*,§

Institute of Chemistry, University of Gdansk, 80-952 Gdansk, Poland, and Department of Physiology and Biophysics and the Center for Polypeptide and Membrane Research, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029. *Received July 6, 1982*

Eight analogues of oxytocin and arginine-vasopressin were synthesized, in which the proline residue in position 7 was replaced by either sarcosine or *N*-methylalanine; some of the pharmacological properties of these analogues were evaluated. In peptides containing a β-mercaptopropionic acid residue in position 1, the additivity of the effects of deletion of the amino group in position 1 and of the above-noted replacements in position 7 on biological properties of these analogues was ascertained. All of the analogues were found to be potent in either antidiuretic or uterine activity and also selective in action. From the point of view of pharmacological properties, substitution of sarcosine in position 7 of oxytocin gave analogues with higher oxytocic and milk-ejecting activities than did the substitution of *N*-methylalanine. The opposite structure–activity relationship was observed with arginine-vasopressin, where the *N*-methylalanine-containing analogues were more potent than the sarcosine-containing analogues with respect to pressor activity and also, if not deaminated, with respect to antidiuretic activity.

Relatively few neurohypophyseal hormone analogues with changes in position 7 have been synthesized. In the oxytocin series, the naturally occurring proline residue in this position has been replaced by glycine,[1-4] alanine,[5] norvaline,[6] D-leucine,[2] D-proline,[7] hydroxyproline,[8,9] 3,4-didehydroproline,[10] thiazolidine-4-carboxylic acid,[11] and pipecolic acid.[9] In the lysine-vasopressin series, the proline residue has been replaced by glycine,[12] valine,[13] and D-proline,[12] and in the arginine-vasopressin series, the proline residue has been replaced by glycine,[14] leucine,[15] and 3,4-didehydroproline.[16] A number of analogues retained high hormonal potency, but most were of low activity or totally inactive. Isosteric substitutions in position 7, such as thiazolidine-4-carboxylic acid[11] or 3,4-didehydroproline,[10] gave oxytocin analogues with doubled oxytocic activity. These are the most potent singly substituted analogues reported to date. However, the most interesting oxytocin analogues with alterations at position 7 are those having a glycine residue substitution. These analogues have substantial oxytocic potency, but their pressor and antidiuretic activities (vasopressin-like activities) are exceedingly low. For example, [4-Thr,7-Gly]oxytocin, synthesized by Manning and associates,[4] exhibits a 640-fold increase in oxytocic/antidiuretic (O/A) selectivity relative to oxytocin.

Substitution of 3,4-didehydroproline (Δ^3-Pro) for proline in position 7 of arginine-vasopressin greatly enhances antidiuretic activity. An analogue containing two additional substitutions, [1-β-Mpa,2-Phe,7-Δ^3-Pro]arginine-vasopressin, has been reported to be 30 times as potent as the parent hormone.[16] However, analogues of lysine- and arginine-vasopressins, with a glycine residue in the place of proline at position 7, exhibit very much lower pressor and antidiuretic activities than the parent hormones.[12,14]

The results of studies of the role played by the proline residue in position 7 of neurohypophyseal hormones have led to the following generalizations: (1) isosteric substitutions (Thz, Δ^3-Pro), both in oxytocins and vasopressins yield analogues of high hormonal activity without definitely influencing selectivity of action; (2) replacement of proline by glycine in oxytocin, which results in greater conformational freedom of the C-terminal tripeptide, leads to quite different effects than does the same replacement in vasopressin; (3) other changes, such as elongation of the side chain and change in configuration of the 7-residue, result in reductions of activity.

These findings prompted us to synthesize and evaluate the biological activities of oxytocin and arginine-vasopressin analogues in which the proline residue was replaced by sarcosine or *N*-methylalanine, in the hope of increasing our understanding of the role of the proline residue in the structure and biological function of neurohypophyseal hormones.

Peptide Synthesis. The protected peptide intermediates were synthesized by solid-phase technique[17-19] and

† The symbols β-Mpa, Sar, MeAla, and Δ^3-Pro are used to indicate the β-mercaptopropionic acid, sarcosine, *N*-methylalanine, and 3,4-didehydroproline residues, respectively. All other symbols follow the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.* **1972**, *247*, 977.

‡ University of Gdansk.

§ Mount Sinai School of Medicine of the City University of New York.

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Table I. Comparison of the Biological Activity[a] of Oxytocin and Its Analogues Containing Sarcosine or *N*-Methylalanine in Position 7

X-Tyr-Ile-Gln-Asn-Cys-Y-Leu-Gly-NH_2
1 2 3 4 5 6 7 8 9

peptide	uterotonic, no Mg^{2+}	uterotonic, Mg^{2+}	milk ejectn	vasopressor	antidiuretic	O/P[e]	O/A[f]
oxytocin (OT)[b]	520 ± 12	486 ± 15	474 ± 16	4.3 ± 0.1	4.0 ± 0.8	120	130
deamino-OT[c]	684		733	1.1	15.0	122	46
[7-Sar]OT	459 ± 41	609 ± 14	564 ± 27	none	0.18 ± 0.04	∞	2 550
[1-β-Mpa,7-Sar]OT	745 ± 20	1346 ± 203	498 ± 41	none		∞	
[7-MeAla]OT	62 ± 6	184 ± 21	402 ± 30	none	0.12 ± 0.03	∞	620
[1-β-Mpa,7-MeAla]OT	98 ± 8	274 ± 14	157 ± 12	none		∞	
[7-Gly]OT[b]	93 ± 4		392 ± 13	0.01	0.0056 ± 0.0003	9300	16 600
[7-Ala]OT[d]	22 ± 1			0.4	0.08 ± 0.01	55	275

[a] Units per milligram. [b] Reference 40. [c] References 26 and 41. [d] Reference 5. [e] O/P refers to the ratio of uterotonic potency (assayed in vitro without Mg^{2+}) to pressor potency. [f] O/A refers to the ratio of uterotonic potency to antidiuretic potency.

cleaved from the resin by ammonolysis.[18] In general, the *tert*-butyloxycarbonyl group[20,21] was used for the protection of the amino group and was removed by treatment with 1.3 N HCl/AcOH. Coupling was affected by dicyclohexylcarbodiimide (DCCI)[22] or by *p*-nitrophenyl esters[23] for the asparaginyl and glutaminyl residues. The completeness of each coupling reaction was monitored by the Kaiser test.[24] Protected peptides were deblocked with sodium in liquid ammonia,[19,25] and the resulting disulfhydryl compounds were subjected to oxidative cyclization with K_3FeCN_6.[26] All analogues were purified by gel filtration on Sephadex G-15.[27]

Bioassays. Oxytocic activity was assayed on the isolated rat uterus in solutions containing either 0.5 mM Mg^{2+} or no Mg^{2+}.[28] Rat milk-ejection assays were performed as described by Bisset et al.[29] Vasopressor assays were performed on phenoxybenzamine-treated rats.[30] Antidiuretic activity was assayed according to the procedure described by Sawyer.[31]

Results and Discussion

Oxytocin Analogues. Activities of 7-Substituted Analogues. Replacement at position 7 of the proline residue by a sarcosine residue both lessens uterotonic activity (in vitro without Mg^{2+}) and increases it (in vitro with Mg^{2+}) relative to oxytocin (Table I). Replacement of proline by *N*-methylalanine lessens uterotonic activity (in vitro without Mg^{2+}) still further. Uterotonic activity in vitro in the presence of Mg^{2+} is also decreased but not nearly as much as in its absence. The ultimate in "magnesium magnification" in this series of analogues in which proline is replaced by proline "fragments" is that observed in [7-glycine]oxytocin, which has activities of 93 and 965 U/mg in the absence and presence of Mg^{2+}.

The milk-ejection activities of [7-Sar]- and [7-*N*-MeAla]oxytocin are roughly similar to that of oxytocin. In contrast, the antidiuretic activities of these two analogues are about 5% of that of oxytocin, and the pressor activities are nil.

Additivity of Substitutions in Positions 1 and 7 of Oxytocin. Removal of the amino group from the cysteine residue in position 1 of oxytocin results in a 1.5- to 2-fold increase in oxytocic activity in vitro.[32] A similar enhancement of uterine activity occurs in the [7-Sar]- and [7-*N*-MeAla]oxytocin analogues in which the N-terminal amino group is absent. This constitutes another example of the additivity of the effects of changes in positions 1 and 7 on biological activity.

Selectivity of Action. A high selectivity of action results from the relative preservation of uterotonic and milk-ejection activities and the diminution of the antidiuretic and pressor activities of 7-Sar and 7-*N*-MeAla analogues of oxytocin. The oxytocic/antidiuretic (O/A) ratio for the latter analogues is 5–20 times higher than that of oxytocin, whereas the oxytocic/pressor (O/P) ratio approaches infinity.

Conformational Differences between [7-Sar]- and [7-*N*-MeAla]oxytocin. The decrease in the oxytocic activity of the *N*-methylalanine analogue compared to the sarcosine analogue might be ascribed to differences in the conformation of the two molecules: the methyl groups attached to the neighboring N and C atoms in the *N*-methylalanine residue change the dihedral angle (ϕ) as compared with this angle in the sarcosine residue. That this change alters the conformation of the C-terminal tripeptide and the interaction of this tripeptide with the cyclic component of the hormone is suggested by circular dichroism studies of these oxytocin analogues.[34]

Arginine-vasopressin Analogues. Activities of 7-Substituted Analogues. Arginine-vasopressin analogues in which the proline residue was replaced by sarcosine or *N*-methylalanine are characterized by a sharp decrease in pressor activity (Table II). On the other hand, they

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Table II. Comparison of the Biological Activity[a] of Arginine-vasopressin and Its Analogues Containing Sarcosine or *N*-Methylalanine in Position 7

X-Tyr-Phe-Gln-Asn-Cys-Y-Arg-Gly-NH_2
1 2 3 4 5 6 7 8 9

peptide	antidiuretic	vasopressor	uterotonic		milk ejectn	A/O[e]	A/P[f]
			no Mg^{2+}	Mg^{2+}			
arginine-vasopressin (AVP)[b]	323 ± 16	369 ± 6	12 ± 0.2			27	0.9
deamino-AVP[c]	1745 ± 385	346 ± 13	29 ± 4			60	5
[7-Sar]AVP	188 ± 19	3.6 ± 0.2	9.3 ± 0.9	36 ± 3	110 ± 8	20	52
[1-β-Mpa,7-Sar]AVP	580 ± 40	0.84 ± 0.08	38.2 ± 3.7	49 ± 6	193 ± 13	15	690
[7-MeAla]AVP	343 ± 54	10.6 ± 0.4	2.8 ± 0.4	4.1 ± 0.6	35 ± 3	122	32
[1-β-Mpa,7-MeAla]AVP	113 ± 37	1.3 ± 0.1	1.7 ± 0.6	8.0 ± 2.1	35 ± 2	78	102
[7-Gly]AVP[d]		0.01	0.1				

[a] Units per milligram. [b] Reference 42. [c] Reference 43. [d] Reference 14. [e] A/O refers to the ratio of antidiuretic potency to uterotonic potency (assayed in vitro without Mg^{2+}). [f] A/P refers to the ratio of antidiuretic potency to pressor potency.

possess relatively high antidiuretic activity, the *N*-methylalanine analogue being more potent than its sarcosine counterpart.

The milk-ejection and uterine activities of [7-Sar]- and [7-*N*-MeAla]arginine-vasopressin resemble those of the corresponding oxytocin analogues in that the sarcosine analogue is more potent than the *N*-methylalanine analogue.

Additivity of Substitutions in Positions 1 and 7 of Arginine-vasopressin. The effects of deaminating residue 1 are additive to the effects of replacing proline by sarcosine in all activities measured. However, when residue 1 in [7-*N*-MeAla]arginine-vasopressin is deaminated, no such additivity is observed; on the contrary, the antidiuretic and uterine (in vitro without Mg^{2+}) activities are decreased in the deaminated analogue as compared with the amino-containing analogue.

Selectivity of Action. The antidiuretic/oxytocic (A/O) selectivity of [7-*N*-MeAla]arginine-vasopressin is three times that of the parent hormone and six times that of [7-Sar]arginine-vasopressin. Furthermore, the antidiuretic/pressor (A/P) activity ratio of these analogues is 35–700 times that of arginine-vasopressin.

With the exception of D-arginine-vasopressin analogues,[35] these [7-Sar]- and [7-*N*-MeAla]vasopressins have the highest selectivity observed yet in singly substituted arginine-vasopressin analogues. This selectivity stands in striking contrast to the selectivity of [7-Gly]arginine-vasopressin and [7-Gly]lysine-vasopressin. [7-Gly]arginine-vasopressin has 0.1 U/mg of oxytocic activity and 0.01 U/mg of pressor activity;[14] [7-Gly]lysine-vasopressin has 1.0 U/mg of antidiuretic activity and 0.15 U/mg of pressor activity[12] (these are the only activities reported for these analogues).

Comparison of 7-Substituted Oxytocin and Vasopressin Analogues. Biological results taken together with the preliminary results of circular dichroism and proton magnetic resonance spectroscopy studies suggest that the 7-proline residue plays a somewhat different role in oxytocin and in vasopressin in its effect on biofunctional conformation. With oxytocin, conformational restriction due to arrangement of the C-terminal tripeptide, -Pro-Leu-Glu-NH_2, in relation to the cyclic component is considerable, a phenomenon that might account for the diminished oxytocic activities in the series Pro > Sar > *N*-MeAla. In contrast, it is likely that in arginine-vasopressin the C-terminal tripeptide, -Pro-Arg-Gly-NH_2, possesses enhanced conformational freedom during its interaction with the receptor. Also, our results, as well as the findings recorded in the literature, show that receptors responsible for pressor activity are highly intolerant of conformational changes arising from replacement of the naturally occurring prolyl residue in position 7.

Experimental Section

Solid-phase peptide synthesis was carried out by standard procedures.[17-19] Chloromethylated resin (Bio-Rad, Bio-Beads SX-1) was esterified[36] to Boc-Gly to an incorporation of 0.51 mmol/g. Dimethylformamide (DMF) was distilled under reduced pressure; triethylamine (NEt_3) was distilled from ninhydrin. Other solvents and reagents were of analytical grade. Thin-layer chromatography was carried out on silica gel plates (Merck), and the products were detected by ninhydrin or iodine vapor. The following solvent systems were used: BAW, 1-butanol–acetic acid–water (4:1:5, v/v, upper phase); BAWP, 1-butanol–acetic acid–water–pyridine (15:3:3:10, v/v); CM, chloroform–methanol (7:3, v/v). Melting points are uncorrected. Optical rotations were determined with a Hilger–Watts polarimeter with an accuracy of 0.01°. Samples for analytical purposes were dried over P_2O_5 in vacuo for 24 h. Elemental analytical results (determined on a Carlo Erba Model 1106 analyzer) indicated by the elemental symbols were ±0.4% of theoretical values. For amino acid analysis,[37] peptides (~0.5 mg) were hydrolyzed with constant-boiling hydrochloric acid (400 μL) containing phenol (20 μL) in evacuated and sealed ampules for 18 h at 110 °C. The analyses were performed with a Beckman automatic amino acid analyzer Model 121. Sarcosine and *N*-methylalanine ratios were not calculated because of the difficulties in detection of *N*-methyl amino acids.[38] The presence of sarcosine or *N*-methylalanine in the structure of the synthesized peptides was determined by TLC of hydrolysates [silica gel; two-dimension solvent systems: (I) 1-butanol–acetic acid–water, 4:1:5, v/v, upper phase; (II) phenol–water, 3:1, v/v; ninhydrin detection]. Molecular ions of pure analogues were determined by mass spectrometry (field desorption) with a Varian MAT 711 instrument.

Boc-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Sar-Leu-Gly-resin (I). Boc-Gly-resin (3.14 g, 1.6 mmol) was subjected to seven cycles of deprotection, neutralization, and coupling. A cycle for incorporation of each amino acid residue into the growing peptide chain involved the following steps: (1) three washings with CH_2Cl_2; (2) one washing with CH_2Cl_2–EtOH (1:1); (3) three washings with EtOH; (4) one washing with EtOH–AcOH (1:1); (5) three washings with AcOH; (6) removal of the Boc group by treatment with 1.3 N HCl/AcOH (1 × 5 min, 1 × 25 min); (7) three washings with AcOH; (8) one washing with AcOH–EtOH (1:1); (9) three washings with EtOH; (10) one washing with EtOH–CH_2Cl_2 (1:1); (11) three washings with CH_2Cl_2; (12) neutralization with 10% NEt_3 in CH_2Cl_2 (1 × 5 min, 1 × 10 min); (13) three washings with CH_2Cl_2;

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(14) equilibration with 4.8 equiv of the appropriate Boc-protected amino acid in CH_2Cl_2 for 20 min, followed by the addition of DCCI (4.8 mmol) and 4 h reaction; (15) three washings with CH_2Cl_2; (16) a repeat coupling with Boc-protected amino acid and DCCI (step 14) for 16 h. *p*-Nitrophenyl esters of Boc-protected asparagine and glutamine were used for the incorporation of these amino acid residues. In these cases, in the place of steps 14–16 the following new steps were used: (14a) one washing with CH_2Cl_2–DMF, 1:1, (15a) three washings with DMF; (16a) coupling with 6.4 mmol of Boc-X-ONp (X = Asn or Gln) and 6.4 mmol of *N*-hydroxybenzotriazole in DMF for 18 h. The resin was washed three times with DMF and once with DMF–CH_2Cl_2 before the next cycle. Following glutamine incorporation, the Boc group was cleaved with trifluoroacetic acid in order to avoid the formation of pyroglutamyl residue. In the place of AcOH, CH_2Cl_2 was used as a solvent in steps 4–8. After completion of the synthesis, the protected 8-peptide-resin was washed five times each with CH_2Cl_2, EtOH, and MeOH and then dried (4.7 g, weight gain 1.56 g, 97% theory).

Z-Cys(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Sar-Leu-Gly-NH_2 (II). The foregoing 8-peptide-resin (2.35 g, ~0.8 mmol) was converted in a cycle of solid-phase peptide synthesis to the Z-9-peptide-resin (2.47 g). The resin was ammonolyzed in methanol. Following evaporation of the solvent, the product was extracted into hot DMF, precipitated with boiling water, and left overnight at room temperature. The peptide was collected by filtration, washed with water, and dried in vacuo over P_2O_5. Product was further purified by dissolving in DMF and reprecipitating with boiling EtOH: yield 962 mg (87% based on substituted Gly); mp 239–241 °C; $[\alpha]^{22}_D$ –40.4° (*c* 1.06, DMF); TLC R_f 0.73 (BAW), 0.73 (BAWP), 0.77 (CM). Anal. ($C_{70}H_{90}N_{12}O_{14}S_2$) C, H, N. Amino acid analysis: Cys(Bzl), 1.9; Tyr, 0.8; Ile, 1.0; Glu, 1.10; Asp, 1.0; Leu, 1.0; Gly, 1.0; NH_3, 2.8.

β-Mpa(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Sar-Leu-Gly-NH_2 (III). This peptide was prepared from I (2.35 g, ~0.8 mmol) and isolated in the same manner as described for II: yield 956 mg (87%); mp 241–243 °C; $[\alpha]^{23}_D$ –36.3° (*c* 0.99, DMF); TLC R_f 0.74 (BAW), 0.76 (BAWP), 0.68 (CM). Anal. ($C_{62}H_{83}N_{11}O_{12}S_2$) C, H, N.

[7-Sar]oxytocin (IV). The protected 9-peptide (146 mg, 0.105 mmol) was dissolved in 400 mL of ammonia freshly distilled from sodium and treated at the boiling point and with stirring with sodium from a stick of the metal contained in a small-bore glass tube until a light blue color persisted in the solution for 20 s. The color was discharged by the dropwise addition of dry AcOH. The solution was evaporated and the residue was taken up in 0.2% AcOH (700 mL). The pH was adjusted to 7.5 with 2 N NH_4OH, and 0.01 M $K_3Fe(CN)_6$ was added with stirring until a yellow color persisted for 30 min. The solution was stirred for an additional 10 min with AG-3-X4 resin (chloride form, 10 g), and the suspension was filtered through a bed of the resin (70 g damp weight). The resin was washed with 0.2% AcOH (100 mL), and the combined filtrate and washings were lyophilized. The material was desalted on a Sephadex G-15 column (100 × 2.5 cm) eluting with aqueous AcOH (50%) with a flow rate of 4 mL/h. The fractions corresponding to the major peak were pooled, diluted with water, and lyophilized. The resulting powder (76 mg) dissolved in 0.2 N AcOH was subjected to gel filtration on a Sephadex G-15 column (120 × 1.2 cm) in 0.2 N AcOH with a flow rate of 2.5 mL/h. [7-Sar]oxytocin (71 mg, 70%) was isolated from the fractions comprising the single symmetrical peak by lyophilization: $[\alpha]^{25}_D$ +17.3° (*c* 0.55, 1 N, AcOH); TLC R_f 0.28 (BAW), 0.45 (BAWP). M_r calcd, 981.2; found, 981. Amino acid analysis: Asp, 1.02; Glu, 1.03; Gly, 1.00; $^1/_2$-Cys, 2.02; Ile, 1.00; Leu, 1.00; Tyr, 0.97; NH_3, 2.82.

[1-β-Mpa,7-Sar]oxytocin (V). The protected 9-peptide III (149 mg, 0.12 mmol) was reduced with sodium in liquid ammonia, reoxidized to the disulfide, and purified by gel filtration as described for [7-Sar]oxytocin: yield 44.1 mg (38% based on protected 9-peptide); $[\alpha]^{22}_D$ –65.4° (*c* 0.39, 1 N, AcOH); TLC R_f 0.41 (BAW), 0.60 (BAWP). M_r calcd, 966.3; found, 966. Amino acid analysis: Asp, 0.99; Glu, 1.12; Gly, 0.97; Ile, 0.98; Leu, 1.00; Tyr, 0.98; NH_3, 2.98.

Boc-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-MeAla-Leu-Gly-resin (VI). The protected 8-peptide-resin (2.75 g, 95%) was synthesized on another portion of Boc-Gly-resin (1.96 g, 1 mmol), used for the synthesis of [7-Sar]oxytocin, by identical methods, except for the use of Boc-MeAla-OH[39] in the place of Boc-Sar-OH.

Z-Cys(Bzl)-Tyr(Bzl)-Ile-Glu-Asn-Cys(Bzl)-MeAla-Leu-Gly-NH_2 (VII). A single cycle of solid-phase peptide synthesis with Z-Cys(Bzl)-OH converted 8-peptide-resin VI (1.37 g, 0.5 mmol) to Z-9-peptide-resin (1.48 g). The material was ammonolyzed, and the product was extracted with warm DMF, precipitated by boiling water, filtered, dried, and reprecipitated by boiling EtOH from DMF solution to yield the protected 9-peptide amide (648 mg, 93% based on substituted Gly): mp 240–242 °C; $[\alpha]^{22}_D$ –51.3° (*c* 1.01, DMF); TLC R_f 0.80 (BAW), 0.79 (BAWP). Anal. ($C_{71}H_{92}N_{12}O_{14}S_2$) C, H, N. Amino acid analysis: Asp, 1.0; Glu, 1.0; Gly, 1.0; Ile, 1.0; Leu, 1.0; Tyr, 0.9; Cys(Bzl), 1.9; NH_3, 2.9.

β-Mpa(Bzl)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-MeAla-Leu-Gly-NH_2 (VIII). Boc-8-peptide-resin VI (1.37 g, 0.5 mmol) was converted to 9-peptide-resin (1.45 g) in a single cycle of deprotection, neutralization, and coupling with β-Mpa(Bzl)-OH. This material was ammonolyzed, and the amide of 9-peptide [(440 mg, overall yield 64%) mp 245–248 °C dec; $[\alpha]^{22}_D$ –40.6° (*c* 1.07, DMF); TLC R_f 0.77 (BAW), 0.87 (BAWP)] was purified by precipitation as detailed in the preparation of analogue II. Anal. ($C_{63}N_{85}N_{11}O_{12}S_2$) C, H, N.

[7-*N*-MeAla]oxytocin (IX). The protected peptide VII (180 mg, 0.128 mmol) was deblocked, reoxidized, deionized, and purified as for IV: yield 49 mg (39%); $[\alpha]^{26}_D$ +1.7° (*c* 0.33, 1 N AcOH); TLC R_f 0.29 (BAW), 0.63 (BAWP). M_r calcd, 995.2; found, 995. Amino acid analysis: Asp, 0.96; Glu, 10.02; Gly, 1.00; $^1/_2$-Cys, 2.03; Ile, 1.01; Leu, 1.00; Tyr, 0.89; NH_3, 3.10.

[1-β-Mpa,7-*N*-MeAla]oxytocin (X). Treatment of the peptide VIII (162 mg, 0.117 mmol) as detailed above for compound IV yielded analogue X (51 mg, 45%): $[\alpha]^{22}_D$ (*c* 0.5, 1 N, AcOH); TLC R_f 0.43 (BAW), 0.65 (BAWP). M_r calcd, 980.4; found, 980. Amino acid analysis: Asp, 1.00; Glu, 1.01; Gly, 1.00; $^1/_2$-Cys, 0.94; Ile, 0.99; Leu, 1.00; Tyr, 0.93; NH_3, 2.86.

Boc-Arg(Tos)-Gly-resin (XI). Boc-Gly-resin (7.84 g, 4 mmol) was subjected to one cycle of deprotection, neutralization, and coupling to yield the protected 2-peptide-resin (9.08 g, 100% of theory).

Boc-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Sar-Arg(Tos)-Gly-resin (XII). The 2-peptide-resin XI (4.54 g, 2 mmol) yielded the desired Boc-8-peptide-resin (6.39 g, 96%) in six cycles of solid-phase peptide synthesis.

Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Sar-Arg(Tos)-Gly-NH_2 (XIII). A single cycle of deprotection, neutralization, and coupling with Z-Cys(Bzl)-OH as the carboxy component converted 8-peptide-resin XII (1.60 g, 0.5 mmol) to the protected 9-peptide-resin (1.71 g, 96%). The material was ammonolyzed, and the amide of protected 9-peptide obtained was purified as detailed in the preparation of analogue II to give the chromatographically pure product (710 mg, 88%, based upon initial Gly content of the resin): mp 214–216 °C; $[\alpha]^{22}_D$ –28.3° (*c* 1, DMF); TLC R_f 0.52 (BAW), 0.65 (BAWP). Anal. ($C_{80}H_{95}N_{15}O_{16}S_3$) C, H, N. Amino acid analysis: Asp, 1.01; Glu, 1.03; Gly, 1.00; Tyr, 0.96; Phe, 1.02; Cys(Bzl), 1.95; Arg, 0.93; NH_3, 2.98.

β-Mpa(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-Sar-Arg(Tos)-Gly-NH_2 (XIV). The 8-peptide-resin XII (1.60 g, ~0.5 mmol) yielded the protected peptide-resin (1.64 g) in a single cycle of solid-phase peptide synthesis by using β-Mpa(Bzl)-OH in the coupling step. Ammonolytic cleavage of the peptide-resin and further purification as described for the synthesis of II gave the amide of protected peptide XIV (723 mg, 91%); mp 206–207 °C; $[\alpha]^{22}_D$ –26.6° (*c* 1, DMF); TLC R_f 0.47 (BAW), 0.60 (BAWP). Anal. ($C_{72}H_{88}H_{14}O_{14}S_3$) C, H, N.

[7-Sar]arginine-vasopressin (XV). The peptide derivative XIII (180 mg, 0.111 mmol) was reduced by sodium in liquid ammonia, oxidized to the disulfide, and purified by gel filtration on Sephadex G-15 as detailed above in the preparation of compound IV to yield [7-Sar]arginine-vasopressin (106 mg, 90%); $[\alpha]^{22}_D$ –8.2° (*c* 0.5, 1 N, AcOH); TLC T_f 0.04 (BAW), 0.08 (BAWP). M_r calcd, 1073.2; found 1073. Amino acid analysis: Asp, 1.00; Glu, 1.09; Gly, 1.00; $^1/_2$-Cys, 2.01; Tyr, 0.98; Phe, 0.99; Arg, 1.01; NH_3, 3.03.

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[1-β-Mpa,7-Sar]arginine-vasopressin (XVI). The protected peptide XIV (128 mg, 0.087 mmol) was deblocked, reoxidized, desalted, and purified as for IV: yield 48 mg (53%); $[\alpha]^{22}_D$ −58.7° (c 0.43, 1 N AcOH); TLC R_f 0.23 (BAW), 0.17 (BAWP). M_r calcd 1058.2; found, 1058. Amino acid analysis: Asp, 1.01; Glu, 1.08; Gly, 1.00; Tyr, 0.96; Phe, 0.98; Arg, 1.01; NH_3, 3.06. Analysis following performic acid oxidation[40] gave a Cys(O_3H)/Gly ratio of 1.02:1.00.

Boc-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-MeAla-Arg(Tos)-Gly-resin (XVII). Part of the 2-peptide-resin XI (4.54 g, 2 mmol) was converted to protected 8-peptide-resin (6.36 g, 96%) in six cycles of solid-phase peptide synthesis.

Z-Cys(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-MeAla-Arg(Tos)-Gly-NH_2 (XVIII). A single cycle of deprotection, neutralization, and coupling with Z-Cys(Bzl)-OH converted 8-peptide-resin XVII (1.59 g, 0.5 mmol) to the protected 9-peptide-resin (1.70 g). This material was ammonolyzed, and the amide of 9-peptide (701 mg, 86% based on substituted Gly) was purified by precipitation as detailed in the preparation of protected [7-Sar]oxytocin: mp 214–216 °C; $[\alpha]^{22}_D$ −35.7° (c 1, DMF); TLF R_f 0.68 (BAW), 0.74 (BAWP). Anal. ($C_{81}H_{97}N_{15}O_{16}S_3$) C, H, N. Amino acid analysis: Asp, 1.00; Glu, 1.03; Gly, 1.00; Tyr, 0.97; Phe, 0.97; Cys(Bzl), 1.97; Arg, 0.96, NH_3, 3.1.

β-Mpa(Bzl)-Tyr(Bzl)-Phe-Gln-Asn-Cys(Bzl)-MeAla-Arg(Tos)-Gly-NH_2 (XIX). Boc-8-peptide-resin XVIII (1.59 g, ~0.5 mmol) was subjected to one cycle of solid-phase peptide synthesis to yield the protected peptide-resin (1.63 g). Material was ammonolyzed and the product purified as described for the preparation of analogue II: yield of 597 mg (80% based on substituted Gly); mp 213–215 °C; $[\alpha]^{22}_D$ −32.2° (c 1, DMF); TLC R_f 0.58 (BAW), 0.48 (CM). Anal. ($C_{73}H_{90}N_{14}O_{14}S_3$) C, H, N.

[7-*N*-MeAla]arginine-vasopressin (XX). The protected 9-peptide XVIII (180 mg, 0.11 mmol) was deblocked, reoxidized, and purified as for IV: yield 97 mg (82%); $[\alpha]^{22}_D$ −13.1° (c 0.5, 1 N AcOH); TLC R_f 0.10 (BAW), 0.22 (BAWP). M_r calcd, 1087.2; found, 1087. Amino acid analysis: Asp, 1.01; Glu, 1.00; Gly, 1.00; $^1/_2$-Cys, 1.99; Tyr, 0.99; Phe, 1.02; Arg, 1.01; NH_3, 3.07.

[1-β-Mpa,7-*N*-MeAla]arginine-vasopressin (XXI). Acyl peptide XIX (190 mg, 0.128 mmol) was reduced by sodium in liquid ammonia as detailed above for IV: yield 102 mg (78%); $[\alpha]^{23}_D$ −68.8° (c 0.33, 1 N AcOH); TLC R_f 0.27 (BAW), 0.31 (BAWP). M_r calcd 1072.2; found, 1072. Amino acid analysis: Asp, 1.02; Glu, 1.04; Gly, 1.00; Tyr, 0.99; Phe, 1.01; Arg, 1.00; NH_3, 3.04. Analysis following performic acid oxidation gave a Cys(O_3H)/Gly ratio of 1.02:1.00.

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Preparation of 2-Amino-4(3*H*)-oxopyrimido[5,4-*b*][1,4]thiazines (5-Thiapterins[1]) and Their Evaluation as Cofactors for Phenylalanine Hydroxylase

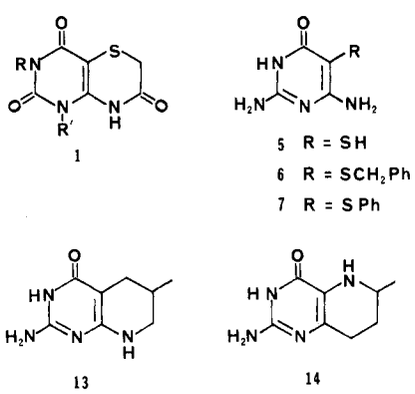
Robert N. Henrie II, Robert A. Lazarus, and Stephen J. Benkovic*

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received July 6, 1982*

Reaction of diethyl chloromalonate with β-mercapto amines, **9**, gave 1,4-thiazin-3-ones, **10**, which were alkylated exclusively at the lactam oxygen with triethyloxonium tetrafluoroborate and subsequently condensed with guanidine to give the first reported 5-thiapterins, **8**. Oxidation of **8** with *m*-chloroperoxybenzoic acid gave the *S*-oxides, **12**. Both **8** and **12** were found to be good inhibitors of rat liver phenylalanine hydroxylase competitive with 6-methyltetrahydropterin, with **8** exhibiting lower K_i's than the corresponding **12**. The 8-thiapterin **4** was a much poorer inhibitor.

L-Phenylalanine hydroxylase (phenylalanine 4-monooxygenase, EC 1.14.16.1) is a mammalian, pterin-dependent monooxygenase that catalyzes the para hydroxylation of L-phenylalanine to form L-tyrosine by using dioxygen as cosubstrate.[2] As such, PAH[3] serves as the initiator for the metabolism of phenylalanine, as well as the initiating enzyme in the biosynthesis of the catecholamines.[4] A tetrahydropterin cofactor is required for hydroxylation: L-*erythro*-tetrahydrobiopterin is the natural cofactor, although many simple tetrahydropterins

Chart I



(1) The less cumbersome name 5-thiapterin will be used in place of the systematically correct 2-amino-4(3*H*)-oxopyrimido[5,4-*b*][1,4]thiazine throughout this paper.
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