The Deamino Derivatives of [4-Threonine]-Oxytocin and [4-Threonine]-Mesotocin; Analogs Possessing a Surprising Spectrum of Diminished Pharmacological Activities[1]

During the course of an investigation on the molecular phylogeny of the neurohyphophysical hormones [4-threonine]-oxytocin was synthesized and pharmacologically evaluated[2]. This analog was found to possess most unique properties. It exhibits enhanced oxytocin-like activities and diminished vasopressin-like activities. Subsequent studies have shown that [4-threonine]-mesotocin exhibits a similar spectrum of activities[3]. It had previously been shown that removal of the amino group from oxytocin[4] and other 4-substituted analogs of oxytocin[5] gave analogs which are strikingly more potent than the parent compound in each case. It thus seemed of particular interest to determine whether removal of the amino group from [4-threonine]- oxytocin and [4-threonine]-mesotocin would bring about a similar further enhancement of their biological activities. These two deamino analogs IIa and IIb (Figure) have been synthesized via their respective protected β-mercaptopropionyl octapeptide intermediates Ia an Ib by use of the solid phase method as described for the synthesis of oxytocin[6] and of [4-threonine]-oxytocin[2]. Purification of the two deamino analogs was accomplished in each case by gel filtration on Sephadex G-15[7]. They have been pharmacologically evaluated by methods previously described[8]. The results obtained are presented in Table I.

Bzl-S-$(CH_2)_2$-CO-Tyr(Bzl)-Ile-Thr(Bz)-Asn-Cys(Bzl)-Pro-X-Gly-NH_2

Ia Protected β-mercaptopropionyl octapeptide of [Deamino, 4-threonine]-oxytocin: X = Leu

Ib Protected β-mercaptopropionyl octapeptide of [Deamino, 4-threonine]-mesotocin: X = Ile

1 2 3 4 5 6 7 8 9
S$(CH_2)_2$-CO-Tyr-Ile-Thr-Asn-Cys-Pro-X-Gly-NH_2
|_____|

IIa [Deamino, 4-threonine]-oxytocin: X = Leu
IIb [Deamino, 4-threonine]-mesotocin: X = Ile

Fig. 1. The Deamino Derivatives of [4-threonine]-oxytocin, [4-threonine]-mesotocin, and their protected octapeptide intermediates.

The protected β-mercaptopropionyl octapeptide intermediate Ia was synthesized in a stepwise fashion starting with 5.0 g of BOC-glycyl-resin (purchased from Schwarz Bioresearch, Inc.) containing 2.4 mmole of glycine. The procedure outlined in the earlier communication[2b] was followed to introduce each new residue into the growing peptide chain. Eight cycles of deprotection, neutralization and coupling were carried out on successive days with the following amino acid derivatives[9]: BOC-L-leucine, BOC-L-proline, BOC-S-benzyl-L-cysteine, BOC-L-asparagine, BOC-*O*-benzyl-L-threonine, BOC-L-isoleucine, BOC-*O*-benzyl-L-tyrosine, S-benzyl-β-mercaptopropionic acid[4] being incorporated in the final step. All coupling reactions to form peptide bonds were mediated by dicyclohexylcarbodiimide[10] in methylene chloride, except in the case of BOC-L-asparagine, and S-benzyl-β-mercaptopropionic acid, which were allowed to react as the nitrophenylester derivatives[11,4] in redistilled dimethylformamide (DMF).

At the conclusion of the synthesis, the protected peptide-resin was washed out of the reaction vessel with ethanol, DMF, and methanol, collected on a filter, and dried in vacuo, weight 7.55 g. The weight gain of 2.55 g (2.25 mmole), at this stage, indicated a 92% incorporation of protected peptide based on the initial BOC-glycine content (2.4 mmole) in the resin.

Ammonolytic cleavage of the protected peptide resin (3.0 g) was carried out as described earlier[2,6]. Following the bubbling with ammonia, the peptide precipated from solution during the overnight storage at room temperature. Due to the unusual insolubility of this protected peptide, it was necessary to use warm (70 °C) DMF (6 × 20 ml) for the extraction from the resin. The resin was further washed with methanol (2 × 20 ml). Removal of the solvents in vacuo on a rotary evaporator followed by trituration with ethanol and ether[2,6] gave the required protected peptide amide intermediate Ia as a white amorphous powder: weight 980 mg (0.73 mmole), mp 239–241 °, $[\alpha]_D^{21}$ °– 41.8 ° (c, 1.04 hexamethylphosphoramide). Anal. Calcd. for $C_{70}H_{90}N_{10}O_{12}S_2$: C, 63.30; H, 6.83; N, 10.55. Found: C, 63.29; H, 6.93; N, 10.34.

—The yield of the purified protected peptide amide Ia from the ammonolytic cleavage and trituration was 82.5% of the amount expected, based on the weight gain on the resin. The yield based on the amount of glycine originally esterified to the resin was 77.5%. Amino acid analysis[12] gave: Asp, 1.04; Pro, 1.06; Gly, 1.00; Ile, 0.97; Tyr, 0.82; Bzl-Cys, 0.93; Thr, 0.90; Leu, 1.03; and NH_3, 2.07. When subjected to thin layer chromatography in the solvent system butanol: acetic acid water 4:1:5 as described earlier[2b], the 1-β-mercaptopropionyl protected octapeptide amide

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Ia gave a single spot, Rf 0.81. Under the same conditions the protected nonapeptide of [4-threonine]-oxytocin had an Rf value of 0.75.

The β-mercaptopropionyl protected octapeptide Ib was prepared in a similar fashion with the use of an automated machine (purchased from Schwarz Bioresearch, Inc.), starting with 5.10 g of the BOC-glycyl resin containing 2.55 mmole of glycine. BOC-L-isoleucine was used in place of BOC-L-leucine in the first incorporation step with all subsequent steps being identical to those used for the synthesis of Ia. The weight of protected peptide resin was 7.65 g. The weight gain of 2.55 g (2.25 mmole) represents an 88.5% incorporation of the protected peptide based on the initial glycine content (2.55 mmole) of the resin.

Ammonolytic cleavage and extraction of the protected peptide resin (2.5 g) was carried out as described for Ia with warm DMF again being required to extract the relatively insoluble protected peptide. The solvents were evaporated in vacuo and the insoluble precipitate was dried in vacuo over P_2O_5 to give Ib as a white amorphous powder: weight 789 mg (0.59 mmole) mp 254–255° $[\alpha]_D^{22}$°–41.2° (c, 1, hexamethylphosphoramide). Anal. Calcd. for $C_{70}H_{90}N_{10}O_{12}S_2$: C, 63.30; H, 6.83; N, 10.55. Found: C, 63.09; H, 6.83; N, 10.34.

Amino acid analysis gave: Asp, 0.96; Gly, 1.00; Bzl-Cys, 0.89; Ile, 1.90; Tyr, 0.72; Pro, 1.00; Thr, 0.95; NH_3, 1.95. When subjected to thin layer chromatography as described earlier[2b], the β-mercaptopropionyl protected octapeptide amide Ib gave a single spot with an Rf value of 0.81; the protected nonapeptide of [4-threonine]-mesotocin had an Rf value of 0.75 under the same conditions. The yield of the protected peptide amide from the cleavage was 79% of the amount expected based on the increase in weight of the resin. The overall yield based on the amount of glycine originally esterified to the resin was 70%.

Debenzylation of Ia (250 mg, 0.188 mmole) and Ib (250 mg, 0.188 mmole), with sodium and liquid ammonia[13] followed by oxidation with potassium ferricyanide[4] to give IIa and IIb respectively was carried out as described for the synthesis of (4-threonine-oxytocin[2]. 24 ml of 0.011 *M* $K_3[Fe(CN_6)]$ was required in each case to complete the oxidative cyclization of the disulphydryl intermediate. The crude products thus obtained were purified separately by gel filtration on Sephadex G-15 in a two step procedure[7]. This procedure requires the use of a) 50% acetic acid for elution in the first step and b) 0.2 *N* acetic acid for elution in the second step. The 50% acetic acid elution was carried out using a larger column than that previously described[7]. The column size for these purifications was 110 × 2.7 cm. The column was eluted in each case with 50% acetic acid at a rate of 30 ml/h and 120 fractions of 5.0 ml each were collected. A plot of the UV absorbance values at 280 nm of the various fractions showed the presence of the usual 2 peaks corresponding to dimer material and the required peptide in each instance.

In both cases the lyophilized peak 2 material from this step was found to be very insoluble in 0.2 *N* acetic acid, the solvent required for the second purification step, and formed an intractable gum. The insolubility encountered at this stage led to appreciable losses of the desired peptides IIa and IIb. The overall yields, based on the initial glycine incorporation on the resin were 28.1 mg (12.0%) for IIa and 23.1 mg (8.5%) for IIb. Accordingly, these values were lower than those previously obtained by identical methods for similar peptides[2,6,14]. The free peptides IIa and IIb showed satisfactory amino acid analysis. Single spots were obtained with the platinum reagent[15] when separate aliquots of IIa and IIb were examined by thin layer chromatography in comparison with (4-threonine]-oxytocin and [4-threonine]-mesotocin respectively by methods previously described[2b]. The Rf values and optical rotations are given in Table II. It will be noted that the Rf values of both IIa and IIb are much higher than those of [4-threonine]-oxytocin and [4-threonine]-mesotocin respectively. Single components in the direction of the cathode were observed with the same detecting reagent when paper electrophoresis of aliquots (50 μg) of each peptide in 2 pyridine acetate buffers of pH 3.5 and 6.5 were carried out.

Table I. Pharmacological activities (in USP or IU/mg ± S. E.) of [deamino, 4-threonine]-oxytocin (IIa), and [deamino, 4-threonine]-mesotocin (IIb) compared with those of [4-threonine]-oxytocin, [4-threonine]-mesotocin, oxytocin, mesotocin and deamino-oxytocin

Assay	*Deamino 4-Thr-Oxytocin[a]	*4-Thr-Oxytocin	*Oxytocin[b]	*Deamino-[c] Oxytocin	Deamino-4-Thr-mesotocin[a]	4-Thr-Mesotocin[d]	Mesotocin[e]
Rat uterus, no Mg^{++}	149 ± 21	923 ± 95	520 ± 12	803 ± 36	128 ± 28	520 ± 28	382 ± 14
Rat uterus, 0.5 m*M* Mg^{++}	245 ± 22	719 ± 83	486 ± 15	760[f]	276 ± 62	565 ± 23	478 ± 10
Fowl vasodepressor	781 ± 136	1480 ± 28	554 ± 22	975 ± 24	1113 ± 30	1545 ± 59	830 ± 24
Rabbit milk-ejection	385 ± 14	543 ± 23	474 ± 16	541 ± 13	251 ± 13	519 ± 37	298 ± 23
Rat antidiuretic	0.9 ± 0.1	1.8 ± 0.3	4.0 ± 0.8	19	2.1 ± 0.4	2.6 ± 0.2	6.1 ± 0.4
Rat vasopressor	<0.1	0.43 ± 0.01	4.3 ± 0.12	1.44 ± 0.06	<0.5	1.08 ± 0.03	6.4 ± 0.2

*Represents essentially identical values from 2 independent duplicate syntheses. [a] Present communication. [b] Values from M. MANNING and W. H. SAWYER, unpublished data for synthetic oxytocin, reported in Ref.[6]. [c] Values reported in Ref.[4c]). [d] Values reported in Ref.[3]. [e] Assays performed on a sample of synthetic mesotocin supplied by Dr. J. RUDINGER; J. RUDINGER, O. V. KESAREV, K. PODUSKA, B. T. PICKERING, R. E. J. DYBALL, D. R. FERGUSON and W. R. WARD, Experientia 25, 680 (1969). [f] Value reported in R. A. MUNSICK and S. C. JERONIMUS, Endocrinology 76, 90 (1956).

It can be seen by examination of the data presented in Table I that the removal of the amino group from both [4-threonine]-oxytocin and [4-threonine]-mesotocin has

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Table II. Physical properties of [deamino, 4-threonine]-oxytocin (IIa) and [deamino, 4-threonine]-mesotocin (IIb) compared with those of [4-threonine]-oxytocin, [4-threonine]-mesotocin, oxytocin, mesotocin, and deamino-oxytocin

Physical properties	Deamino-4-Thr-oxytocin[a]	4-Thr-oxytocin[b]	Deamino-4-Thr-Mesotocin[a]	4-Thr Mesotocin[c]	Mesotocin[d]	Deamino-Oxytocin[e]	Oxytocin[f]
Rf[g]	0.56	0.42	0.57	0.39	0.33	0.78[h]	0.34 (0.59)[h]
$[\alpha]_D^{T°}$[k]	−85.7°	−10.4°	−83.4°	−8,2°	−31.8°	−107°	−24.0°

[a] Present Communication. [b] See Ref. [2b]. [c] Values from M. MANNING and W. H. SAWYER, unpublished data. [d] Value reported by RUDINGER et al. (1969) see Footnote[e] Table I. [e] See Ref.[4b]. [f] See Ref.[6]. [g] Thin layer chromatography in the upper phase of the solvent system Butanol: acetic acid: water: 4:1:5 as described in Ref.[2b]. [h] Values reported in Ref.[4b]; are for decending chromatography on Whatman No. 1 Paper in the same solvent system as in [g]. [k] In 1 *N* acetic acid (C = 0.50 for all except mesotocin); T° = 22°, 24°, 24°, 24–21°, 22.5°, respectively.

resulted in a general marked reduction in potency of all of the characteristic pharmacological activities of both of these highly active analogs. This unexpected pattern of diminished activities is in striking contrast to that obtained by removal of the amino group from oxytocin. The resulting compound, deamino-oxytocin possesses a marked enhancement of all activities except the rat vasopressor. In the case of IIa and IIb it is interesting to note that the most drastic reduction in both instances is in the rat uterus activity. On the other hand the fowl vasodepressor activity of both analogs was not diminished nearly to the same extent. In fact, the fowl vasodepressor potency of IIb is higher than that of deamino-oxytocin. It is of further interest to note that whereas both IIa and IIb exhibit a fowl vasodepressor activity which is more potent in both instances than that possessed by oxytocin and mesotocin respectively, yet all of the other characteristic activities of both IIa and IIb are lower than those of oxytocin and mesotocin respectively.

These results illustrate a rather curious anomaly from the structure-function point of view. On the one hand, the substitution of glutamine by threonine in the 4-positions of oxytocin and mesotocin has resulted in analogs which are much more potent in the characteristic assay systems than the parent peptide in each case: yet, on the other hand, the identical substitution in deamino oxytocin and deamino mesotocin has led to a surprising diminishment of these same activities.

In searching for clues which might lead to a possible explanation for these unexpected findings it is tempting to speculate that the unusual solubility characteristics of both IIa and IIb might somehow be involved. As indicated above, both IIa and IIb were found to be much less soluble in aqueous acetic acid than any oxytocin analogs hitherto encountered in these laboratories. Also the higher Rf values of IIa and IIb (Table II) as compared to those of [4-threonine]-oxytocin and [4-threonine]-mesotocin indicate that each of the deamino derivatives is generally much more lipophilic than the parent compound in each case. It is thus possible that the overall diminishment of activities may in some way be related to these very pronounced differences in solubilities.

The findings outlined here represent the first reported instance in which the removal of the amino group from an analog of oxytocin, which has been modified in only a single position, has resulted in a diminishment rather than in an enhancement of the characteristic oxytocin-like activities as well as the antidiuretic activity. In a planned extension of these studies, it is hoped that knowledge of the pharmacological and physical characteristics of the deamino derivatives of a) synthetic 4-threonine analogs of the other neurohypophysial hormones and b) synthetic oxytocin analogs with other hydroxy-amino acids in the 4-position, may help to illuminate and possible further clarify the surprising findings reported here.

Zusammenfassung. Synthese (Merrifield-Methode) und pharmakologische Eigenschaften der Desamino-Derivate von (4-Threonin)-Oxytocin und (4-Threonin)-Mesoxytocin werden beschrieben.

M. MANNING, E. J. COY and W. H. SAWYER

Department of Biochemistry, Medical College of Ohio at Toledo, P.O. Box 6190, Toledo, (Ohio 43614, USA) and Department of Pharmacology, College of Physicians and Surgeons, Columbia University New York, (New York, USA), 3 May 1971.

PRO EXPERIMENTIS

Redaktionelle Vorbemerkung. Die nachstehende Arbeit bringt unseres Erachtens methodologische Anregungen (z.B. verbesserte Schätzungen für die Parameter der Schätzgleichung), welche geeignet erscheinen, die Qualität der statistischen Auswertung von Versuchsergebnissen zu heben. H.M.

Ein Beispiel zur Anwendung mehrfacher linearer Regression in der Biochemie

R. STRASSER und A. MISEREZ[1] beschreiben die Ergebnisse von Untersuchungen über das Verhalten von Polysacchariden während der Mikroelektrophorese. Sie geben in ihrer Arbeit Werte für die Wanderstrecke einiger Polysaccharide als Funktion der Zeit und der Stromstärke an.

Die Wanderstrecke ist eine lineare Funktion der Zeit, wobei die Wandergeschwindigkeit wiederum linear zunimmt als Funktion der Stromstärke. STRASSER und MISEREZ geben eine Formel für die Wanderstrecke

$$w_{(t)} = n_{(I)} \cdot t \qquad (1)$$

und für die Geschwindigkeit

$$n_{(I)} = m \cdot I + q. \qquad (2)$$

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