THE DIMER OF [2-O-METHYLTYROSINE]OXYTOCIN: PREPARATION AND PROPERTIES*

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Received February 7th, 1975

The dimeric form of [2-O-methyltyrosine]oxytocin was isolated as a side product in the synthesis of [2-O-methyltyrosine]oxytocin using counter-current distribution and free-flow electrophoresis. The dimeric character was demonstrated by indirect methods; it was not possible to determine whether the fraction consisted of parallel, antiparallel or a mixture of the two dimeric forms. In comparison with [2-O-methyltyrosine]oxytocin some of the pharmacological activities of the dimer show qualitative differences particularly in its action on the uterus. The chiroptical properties of the monomeric and dimeric forms were compared.

Oxidative closure of the disulphide bond in the final synthetic stages of the preparation of neurohypophyseal hormones and most of their analogues** yields both monomeric and dimeric forms of the final product. In the case of oxytocin and vasopressin, the dimers have been isolated and studied[2-5]. In the present work we describe the preparation and some of the properties of the dimeric form of [2-O-methyltyrosine]oxytocin.

EXPERIMENTAL

Materials

Synthetic oxytocin and [8-lysine]vasopressin were the commercial products of SPOFA, Prague. [2-O-Methyltyrosine]oxytoxin (*I*) and its fully protected linear precursor *II* were prepared as described elsewhere[6,7].

Purification and Isolation Methods

The all-glass, fully automatic Steady State Distribution Machine produced by Quickfit & Quartz, Ltd., Stafordshire, England, with possibilities of transfers of both upper and lower phases, was

* Part CXXVIII in the series Amino Acids and Peptides; Part CXXVII: This Journal *40*, 905 (1975).

** All amino acids are in the L-configuration. Nomenclature and symbols are in accordance with published suggestions[1].

used for counter-current distribution. The solvent system was 2-butanol–0·05% acetic acid (1 : 1) and the peptide material was located by the Folin-Ciocalteau reaction. Continuous free-flow electrophoresis was carried out on an instrument described elsewhere[8,9] under normal conditions for this instrument[9,10] (6% acetic acid; 3 kV across the electrodes). For gel filtration we used Bio-gel P-4 (95 × 1 cm; 3M acetic acid, flow rate 4 ml/h), Sephadex G-15 (60 × 1·8 cm; 50% acetic acid, flow rate 34 ml/h), and Sephadex G-25 (140 × 2·4 cm; 0·2M acetic acid, flow rate 7 ml/h). The peptide material was detected by continual measurement of optical density at 284 nm.

Analytical Methods and Characterisation

Unless otherwise stated, paper electrophoresis was carried out on Whatman 3 MM sheets for 60 min at a potential drop of 20 V/cm in a buffer of 1M acetic acid (pH 2·4) and pyridine–acetic acid (pH 5·7). Ninhydrin was used for detection. Thin-layer chromatography on silica gel was carried out on Merck plates in systems chloroform–methanol–acetic acid–water (60 : 30 : 5 : 5) (S_1) and 2-butanol–99% formic acid–water (75 : 12·3 : 12·7) (S_2). Detection was by means of ninhydrin and chlorination[11].

Samples for amino-acid analysis were hydrolysed for 20 h at 105°C in 6M-HCl (in ampoules sealed at 1 Torr) and analyses were carried out on an automatic analyser (Developmental Workshops, Czechoslovak Academy of Sciences, type 6020). Optical rotation was determined on a Perkin-Elmer 141 polarimeter.

Circular dichroic spectra were measured with a Roussel-Jouan CD 185 instrument in quartz cells with an optical path of 0·01 to 2·0 cm at a temperature of about 24°C. Solutions were made in 0·02M phosphate buffer (pH 7·1) and in hexafluoroacetone trihydrate (Hynes Chemical Research Co.) at a concentration of about 0·4 mg/ml. The spectral data are presented in values of molar ellipticity ($[\Theta]$, deg cm^2 dmol^{-1}) calculated on the basis of the molecular weight of the monomeric unit [2-O-methyltyrosine]oxytocin, and the data are not corrected for the refraction index of the solvent. Reduction of the disulphide bonds with dithiothreitol was carried out according to Hase and Walter[12]. Partial substitution of free amino groups by potassium 4-chloro-3,5-dinitrobenzenesulphonate was carried out as reported elsewhere[13] and evaluation of the result was by paper electrophoresis[14] (10 V/cm, 2 h, buffer: formic acid–acetic acid–water 3 : 3 : 4).

Pharmacological Methods

Isolated rat uterus assay was carried out on uterine strips of adult virgin Wistar rats, placed in Van Dyke–Hastings solution[15] which was bubbled through with a mixture of 95% O_2 — 5% CO_2 at 30°C. The concentration of Ca^{2+} in the bath was 0·5 mM. Isometric contractions were recorded using a magneto-electric transducer[16] and a 4-point dose-response curve[17] was determined or comparisons of threshold doses were made. Inhibitory effects were measured from the shift of the log dose–response curve and expressed as an inhibition constant as described elsewhere[18]. For the rat pressor assay despinalised Wistar strain males[19] were used. To test for an inhibitory action, the preparation was administered just before administration of the standard. Milk-ejecting activity was measured on lactating rats, 5—14 days following delivery in an arrangement described elsewhere[20]. Antidiuretic activity was estimated on ethanol anaesthetised rats[21], with a maintained water load of 8—10% of body weight.

Isolation of *III*

p-Toluenesulphonyl-S-benzylcysteinyl-O-methyltyrosyl-isoleucyl-glutaminyl-asparaginyl-S-benzylcysteinyl-prolyl-leucyl-glycinamide[6] (*II* 4·2 g), was deprotected by sodium in liquid ammonia

and oxidized by aeration. The reaction mixture was separated by means of counter-current distribution; after 260 upper and 60 lower phase transfers 2 peaks were found, one corresponding to compound *I* ($K = 0{\cdot}5$) and the second ($K = 0{\cdot}2$) which was a mixture of peptides and inorganic salts. The latter were removed using the ion-exchanger Ostion KM (35 × 3 cm) to give a substance (1·2 g) which, according to amino acid analysis and paper electrophoresis was a mixture of two peptides, compound *III* and the amide of prolyl-leucyl-glycine. Part of this mixture (500 mg) was separated by continuous free-flow electrophoresis to give a yield of 50 mg of electrophoretically and chromatographically pure substance. Under comparable conditions analytical values for compound *I* were $E^{Gly}_{2.4}$ 0·54, $E^{His}_{5.7}$ 0·35; R_F 0·85 (S1), R_F 0·15 (S_2) and for compound *III* $E^{Gly}_{2.4}$ 0·76, $E^{His}_{5.7}$ 0·56; R_F 0·7 (S_1), R_F 0·1 (S_2). Amino-acid analysis: Asp 1·02, Cys 1·78, Glu 1·02, Gly 0·96, Ile 1·05, Leu 0·99, Pro 0·96, Tyr 0·60, Tyr(Me) 0·40; $[\alpha]_D^{20}$ −68·4° (*c* 0·2, 1M acetic acid). For $C_{88}H_{134}N_{24}O_{24}S_4.2\ CH_3CO_2H.3\ H_2O$ (2215) calculated: 49·88% C, 6·74% H, 15·18% N; found: 49·62% C, 6·52% H, 15·22% N.

Determination of Dimeric Structure

On filtration on Bio-gel P-4, compound *III* had an elution volume of 40—44 ml, monomeric form *I* 50—54 ml. Partial substitution of the amino groups by potassium 4-chloro-3,5-dinitrobenzenesulphonate showed on paper electrophoresis that compound *III* gave two spots; compound *I* under the same conditions showed only a single spot. Reduction by dithiothreitol and isolation on Sephadex G-15 gave a single product which after oxidation by aeration and gel filtration (Bio-gel P-4) yielded compound *I* (elution volume 50—54 ml) and compound *III* (41—43 ml) in a ratio of 4 : 1. Both products were isolated and identified by paper electrophoresis and biological properties. Gel filtration on Sephadex G-25, carried out under practically the same conditions as used for separation of both dimers (parallel *IIIa* and antiparallel *IIIb*) of oxytocin[2] showed a single symmetrical peak (V_e = 537 ml) with no signs of separation. Localisation of peptide material was by continuous recording of optical density at 284 nm and individual fractions were further evaluated by the Folin-Ciocalteau reaction. The fraction corresponding to the peak was divided into 2 halves at the maximum point and CD spectra were measured from each. Both halves gave exactly the same dichroic curves.

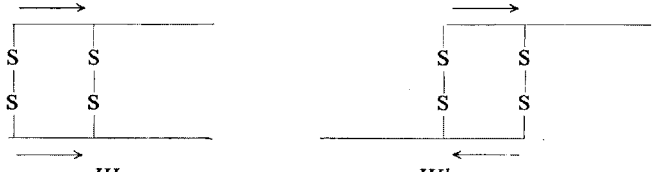
RESULTS AND DISCUSSION

In the synthesis of cystine-containing cyclopeptides where the disulphide bond forms the heterodetic joining element of the ring, oxidation of the sulphydryl groups is usually the last synthetic step. This reaction yields high-molecular-weight polymers, which can be diminished in amount by a sufficient dilution of the reaction mixture, and monomeric and dimeric products. This reaction has been studied in detail[22] on model substances of type *IV*. It was shown that the ratio of monomer to dimer depends on the number of amino-acid residues between the two cysteines. For example with $n = 4$, *i.e.* compound *IV* with a ring size approximately the same as that in neurohypophyseal hormones, the monomeric form prevails. In substances with $n = 1-3$, an antiparallel dimer was the main product; the investigators[22] have reported the view that the probability of formation of parallel dimers is quite small. However, the stability of cyclic peptides containing a disulphide bridge is also dependent on the amino acids which make up the basic peptide backbone chain[23,24].

Cys-Tyr(Me)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2
I

Tos-Cys(Bzl)-Tyr(Me)-Ile-Gln-Asn-Cys(Bzl)-Pro-Leu-Gly-NH_2
II

(Cys-Tyr(Me)-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2)$_2$
III



IIIa *IIIb*

Cys-$(Gly)_n$-Cys
IV

The situation with dimers of neurohypophyseal hormones is not completely clear. Dimeric forms are common products, both during synthesis (with oxidative closure of the disulphide bridge) and with exposure of the monomer to an alkaline environment[2,4,25]. However, it is extremely difficult to provide clear evidence whether the product has a parallel (*IIIa*) or an antiparallel (*IIIb*) arrangement. The dimeric form of oxytocin[2] was isolated by partition chromatography on Sephadex and by gel filtration it was subdivided into two fractions with different optical activities (α and β dimer). Later, the parallel dimer was synthesized[3] and it was shown that it was identical with the so-called α dimer, because on gel filtration of a mixture of α and parallel dimer, only a single symmetrical peak resulted. The final experiment which would clearly confirm this finding, gel filtration of a mixture of β and parallel dimer which should yield two peaks, was not described. Schally and coworkers[4,5] isolated the dimeric form of [8-lysine]vasopressin and by electrophoretic analysis of chymotryptic fragments they showed that this compound had an antiparallel arrangement. From the reported work[5] it is not, however, clear whether they used only isolated or also synthetic[26] dimer for their experiments with chymotryptic cleaving. Such data would be very important in order to decide whether the origin of one or two dimers is determined by primary structure or whether trace impurities in the isolated substance can influence (in the case of vasopressin) the preferred formation of an antiparallel arrangement. Detailed study of the biological effects of the vasopressin dimer showed[27] that in synthetic and isolated dimer there was not only a quantitative but also a qualitative difference in activities (the natural but not the synthetic dimer produced an increase in the release of ^{131}I from thyroid tissue of mice[27]).

In [2-O-methyltyrosine]oxytocin[7,28] (*I*) we found a number of interesting pharmacological effects[19,29–31], and clinically this analogue is of use because of its decreased vasoconstrictor activity in the uterus with reference to oxytocin, so that use of the analogue in inducing labour does not result in increased plasma levels of lactic and pyruvic acids[32,33]. In the synthesis of this analogue, the dimeric form is a side-product, and is of interest not only for its biological activity but also because apparently it can be used to prepare analogue *I*. Dimer *III* was isolated as a side-product in the synthesis of [2-O-methyltyrosine]oxytocin using counter-current distribution, removal of inorganic salts by ion exchange, and isolation from a mixture of peptides using preparative continuous free-flow electrophoresis. With compound *III* we were not able to determine molecular weight by a direct method but there are a number of indirect lines of evidence for its dimeric nature: on gel filtration it has a higher mobility than analogue *I*, with partial substitution of free amino groups it gives two

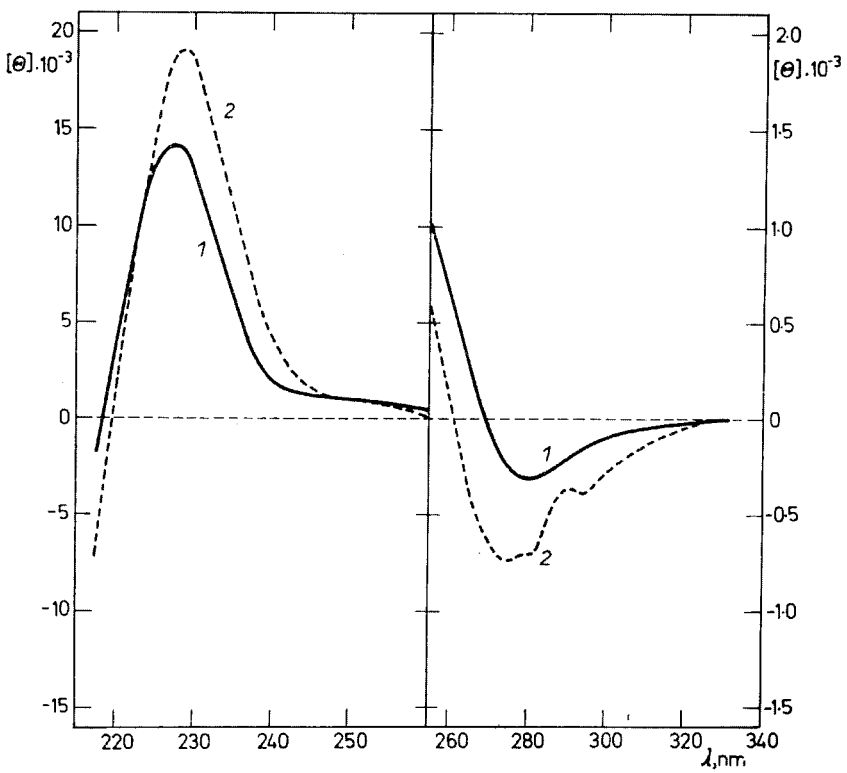


FIG. 1

Circular Dichroism of the Monomer (*1*) and Dimer (*2*) of [2-O-Methyltyrosine]oxytocin in 0·02M Phosphate Buffer, pH 7·1

products (as opposed to *I* which gives only one product) and finally its reduction by dithiothreitol and subsequent oxidation gives a mixture of compounds *I* and *III*. We were not able to demonstrate whether this isolated product was a mixture of dimeric forms *IIIa* and *IIIb* or only one of them. On the basis of the fact that practically the same conditions of gel filtration which were used to separate both dimeric forms of oxytocin[2] produced only a single substance (continual measurements of optical density in the UV region and the same CD spectrum of the rising and falling portions of the peak) it can be argued that just as in the case of vasopressin, here as well the product was only a single dimeric form. Recognition of whether this proposed single dimeric form is parallel or antiparallel is not the subject of the present report.

The CD measurements of the [2-O-methyltyrosine]oxytocin monomer and dimer are shown in Figs 1 and 2. In neutral aqueous solution the spectra of both substances are very similar (Fig. 1). Both have the same dichroic bands of the same sign and similar intensity. This is also true for the short-wave region of the spectrum which is not shown in the figures. Despite this general similarity, one can observe in Fig. 1

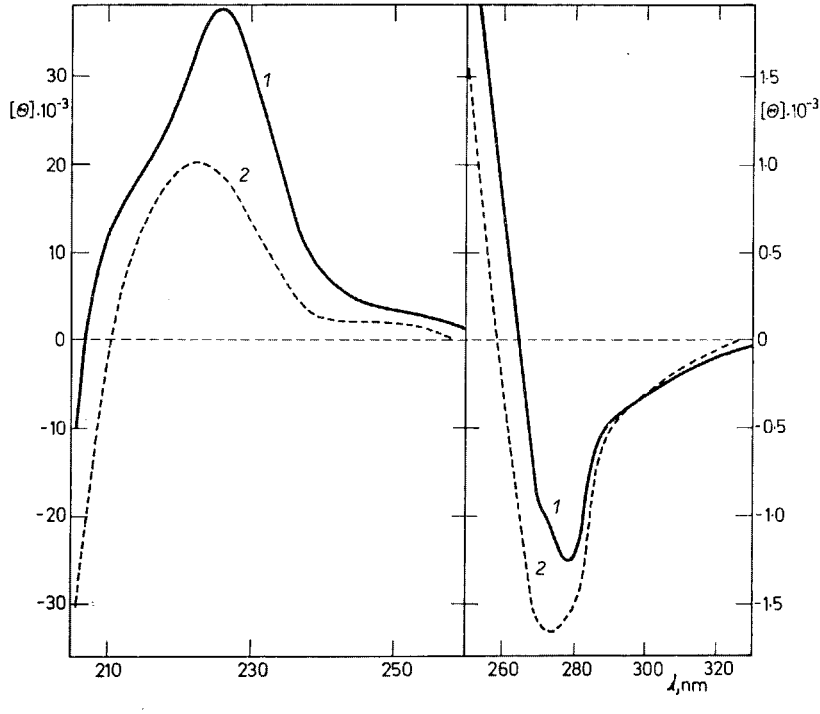


FIG. 2

Circular Dichroism of the Monomer (*1*) and Dimer (*2*) of [2-O-Methyltyrosine]oxytocin in Hexafluoroacetone Trihydrate

some differences which are characteristic for the two compounds. The most significant differences are in the two bands which are associated with the presence of the tyrosine side-chain[34]. The negative band at 280 nm and the positive complex band at 225 nm are significantly more intensive in the dimer. The spectral changes produced by transfer of the compounds into hexafluoroacetone (where one can assume a limitation of intramolecular interactions which stabilise conformation) also show marked differences in the tyrosine bands (Fig. 2). As one might expect, the intensity of these bands increases[34]. This effect is much greater in the monomer than in the dimer. In the region of bands which can be attributed to the disulphide group, there were no significant differences under the same conditions. The CD spectra of [2-O-methyltyrosine]oxytocin monomer and dimer suggest that the conformation of both compounds are basically similar but that the conformational freedom of the tyrosine side-chain is relatively decreased in the dimer.

The biological activities of the dimer *III* are clearly different from those of [2-O-methyltyrosine]oxytocin (*I*). In the antidiuretic assay compound *III* has a low activity which is somewhat protracted (0·24 ± 0·07 I.U./mg) and is about twice the peak intensity of analogue *I*. Both compounds behaved in a similar manner in the pressor assay. Compound *III* up to doses of 0·1 mg had no intrinsic activity on the rat. Such doses did, however, inhibit the pressor activity of [8-lysine]vasopressin. In a dose of $2 . 10^{-2}$ mg the effect of the latter hormone was decreased by 25% (with a dosage of [8-lysine]vasopressin of $2 . 10^{-5}$ mg); at a dose of $1 . 10^{-1}$ mg inhibition was complete. On the rat uterus *in vitro*, dimer *III* had no intrinsic activity up to a concentration of 0·017 mg/ml (as opposed to analogue *I*) but inhibited the agonist activity of both oxytocin (pA_2 6·53) as well as, at higher concentrations, substance *I*. Milk-ejecting activity was quite low but in contrast with the monomer form, very prolonged. Pharmacological observations of the oxytocin dimer[35] have shown that as opposed to compound *III*, activities in all assay systems are about two orders lower than with oxytocin but qualitatively the same. The activities of the dimer of [8-lysine]vasopressin[27] were different for different isolated and synthetic preparations. In pressor and antidiuretic assays the latter dimers had about 10 I.U./mg and in the case of antidiuresis the duration of the effect was shorter, and in the pressure assay longer than the duration of the effects of the standard. In the present dimer *III* we found in the uterotonic assay qualitative differences in comparison with monomer *I*. This result is in agreement with the view[27] that the observed biological effects are produced directly by the dimer, so that despite a doubling of the molecular weight the conformation of the dimer must be sufficiently similar to that of the monomer that it be acceptable for receptor binding and activation. In other assays, particularly those carried out under *in vivo* conditions (where protracted effects were found) the possibility cannot be excluded that the dimer serves as a "hormonogen", *i.e.* that by a transsulphidation reaction the monomer form can be formed from it and produce some of the observed biological effects.

We would like to thank Mrs H. Farkašová for carrying out the amino-acid analyses, Mrs Z. Ledvinová for measuring optical rotation and Mrs H. Kovářová and Mrs J. Kellerová for assistance with the pharmacological assays. Elemental analyses were carried out in the analytical department of this Institute.

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Translated by J. H. Cort.