SYNTHESIS AND PHARMACOLOGICAL ACTIONS OF N-MALEOYLGLYCYL-[2-O-METHYLTYROSINE]OXYTOCIN*

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Received December 9th, 1975

N-Maleoylglycyl-[2-O-methyltyrosine]oxytocin was obtained by reacting N-maleoylglycine with [2-O-methyltyrosine]oxytocin. The substance behaved as a reversible competitive inhibitor of the uterotonic activity of oxytocin, as determined on the rat uterus *in vitro*.

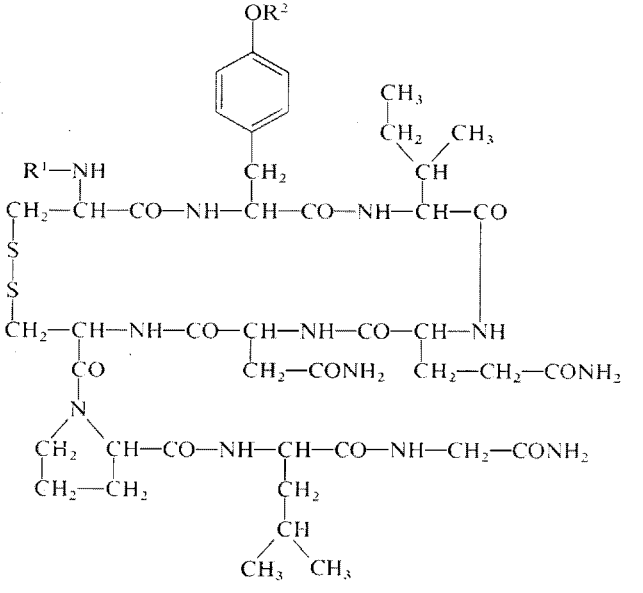
Previous work[1] has been concerned with the preparation and studies of the inhibitory properties of analogues of oxytocin (*Ia*). The type of structural change which should result in an irreversible inhibitor (such substances being of possible use for isolation of receptors from biological tissues) can be characterised as follows: the hydroxyl group of the tyrosine residue** should be substituted in order to increase steric bulk (the same effect can be obtained by introducing halogen atoms into other positions of the benzene nucleus[3,4]) and the amino group of the hemicystine in position 1 should be substituted by a group capable of forming a covalent bond with a reactive group of the receptor. N-Bromoacetyl-[2-O-methyltyrosine]oxytocin[1] (*Id*) behaved, however, only as a reversible inhibitor of the uterotonic activity of oxytocin. In the present work we describe the synthesis and properties of N-maleoylglycyl-[2-O-methyltyrosine]oxytocin (N-2,5-dihydro-2,5-dioxopyrrol-1-ylacetyl-[2-O-methyltyrosine]oxytocin, *Ic*). The maleimide residue should have the property, although not exclusively[5-7], of addition reaction with a free thiol group.

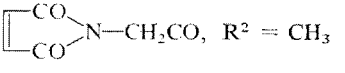
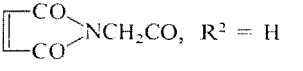
Analogue *Ic* was prepared by acylation of [2-O-methyltyrosine]oxytocin (*Ib*) by 2,5-dihydro-2,5-dioxopyrrol-1-ylacetic acid (*IIb*) in the presence of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline[8]. Substance *IIb* was obtained from the corresponding tert-butyl ester by hydrolysis with trifluoroacetic acid. Ester *IIa* was prepared by reaction of the tert-butyl ester of glycine with maleic anhydride in acetic acid in the presence of sodium acetate and acetic anhydride, by a procedure described for the preparation of N-arylmaleimides[9-11]. Other methods for preparation of this type

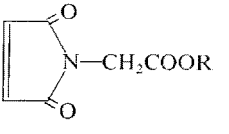
* Part CXXXIII in the series Amino Acids and Peptides; Part CXXXII: This Journal *41*, 1805 (1976).

** All amino acids in the present work are in L-configuration. The nomenclature and symbols are according to published suggestions[2].

of compound were published after the present synthetic work was completed[12,13]. Analogue *Ic* was purified by countercurrent distribution and gel filtration and characterised in the usual manner. The stability of the maleiimide group under conditions utilised for determining uterotonic activity (in buffered medium according to Munsick[14]) was demonstrated for substance *IIb* by electrophoretic analysis.



Ia, $R^1 = R^2 = H$
Ib, $R^1 = H$, $R^2 = CH_3$
Ic, $R^1 =$  , $R^2 = CH_3$
Id, $R^1 = BrCH_2CO$, $R^2 = CH_3$
Ie, $R^1 =$  , $R^2 = H$



IIa, $R = (CH_3)_3C$, *IIb*, $R = H$

Analogue *Ic* behaved as a reversible competitive inhibitor of the uterotonic activity of oxytocin as determined on isolated rat uterus: $pA_2 = 7{\cdot}27$ (7·19–7·42), $pD_2 =$ = 8·66–9·28. Up to dosages of $1 \cdot 10^{-3}$ mg there was no evidence of an intrinsic uterotonic activity. In tests on anaesthetised rats the substance showed an antidiuretic activity of about 0·001 I.U./mg. In tests on the mammary gland of lactating rats the

substance was inactive up to dosages of $2 . 10^{-3}$ mg; in a ratio of 1 : 500 there was no inhibitory action to the galactogogic activity of oxytocin.

It would appear from these results that in the present case as well we did not succeed in producing an irreversible inhibitor. As opposed to the recently published analogue[13] *Ie*, there was evidence of a reversible inhibitory activity; differences in the properties of both analogues can be attributed to structural differences in the side-chain of the amino acid residue in position 2 (free or substituted hydroxyl group). Even though it has been shown both for oxytocin[15-17] and vasopressin[18] that the mechanism of action is not based upon a disulphide–sulphydryl interaction, it would nevertheless appear to be possible that the sulphydryl groups of receptors in biological tissue play some role in the interaction of the hormone with the receptor, *e.g.* an effect on cleavage of the disulphidic hormone (oxytocin[19], vasopressin[20]) during its binding to the receptor. The fact that not even analogue *Ic* or analogue *Id* showed irreversible inhibitory properties to the uterotonic action of oxytocin demonstrates that the reactive functional groups of the analogues, which should be able to form covalent bonds with thiol groups (or other reactive groups) of the receptor, did not come into reactive contact.

EXPERIMENTAL

Melting points were determined on a Kofler block and were corrected. Samples for elemental analysis were dried over P_2O_5 for 24—48 h at room temperature at a pressure of 1 Torr. Thin-layer chromatography was carried out on silica gel plates (Kieselgel G, Merck) in systems 2-butanol–90% formic acid–water 75 : 13·5 : 11·5 (S1), benzene–ethyl acetate 10 : 1 (S2) and 1-butanol–acetic acid–water 4 : 1 : 1 (S3). Electrophoresis was carried out on Whatman 3MM paper for 1 h at a potential drop of 20 V/cm in buffers: 1M acetic acid (pH 2·4) and pyridine–acetic acid (pH 5·7). Detection was by means of an aqueous solution of potassium permanganate. Where values of R_F and E are presented, pure substances were involved.

Tert-butyl Ester of 2,5-Dihydro-2,5-dioxopyrrol-1-ylacetic Acid (*IIa*)

To a solution of the hydrochloride of the tert-butyl ester of glycine[21] (6·6 g) in acetic acid (120 ml) we added maleic anhydride (3·5 g) and, after heating to 40°C, sodium acetate (20 g). The temperature was increased to 80°C and after addition of acetic anhydride (200 ml) the temperature was maintained at 80°C for 2 h. After cooling to 50°C the mixture was evaporated and the remainder was again evaporated with water several times. After dissolving in water, the product was extracted into ether, the etheric solution was filtered with activated charcoal and then evaporated. The remnant was dissolved in benzene and chromatographed on a column of silica gel (25 × 2·5 cm; benzene with 10% ether). The product was crystallised from light petroleum; the yield was 2·0 g (24%) of a product with m.p. 79—80°C. For $C_{10}H_{13}NO_4$ (211·2) calculated: 56·87% C, 6·20% H, 6·63% N; found: 57·26% C, 6·25% H, 6·73% N. UV spectrum (ethanol): λ_{max} 221 nm (log ε 3·89), λ_{min} 253 nm (log ε 2·33); IR spectrum (nujol): 1725, 1712 (maleimide), 1743, 1235, 1158 cm^{-1} (ester).

Stability in buffer at pH 7·65 (Munsick[14]): To a solution of ester *IIa* (3·4 mg) in tert-butanol (0·1 ml) we added buffer (0·3 ml) and at various time intervals samples were removed for paper

electrophoresis. The quantity of side-products, migrating to the anode, was less than 5% after 10 min, 6—8% after 1 h and 20% after 5 h.

2,5-Dihydro-2,5-dioxopyrrol-1-ylacetic Acid (*IIb*)

A solution of ester *IIa* (0·21 g) in trifluoroacetic acid (1·5 ml) was left for 1 h at room temperature, evaporated and the remainder was dissolved in 1M acetic acid and then freeze-dried. The lyophilisate was triturated several times with ether, filtered and washed with ether; the yield was 0·12 g (66%) of a product with m.p. 111—113·5°C. Mass spectrum: molecular peak 155. The literature presents a m.p. of 105—106°C (ref.[12]) and 113—113·5°C (ref.[13]).

N-Maleoylglycyl-[2-O-methyltyrosine]oxytocin (*Ic*)

To a solution of [2-O-methyltyrosine]oxytocin[22] (*Ib*, 50 mg) in dimethylformamide (0·5 ml) we added substance *IIb* (7·75 mg) and 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline[8] (13·5 mg). After 16 h at room temperature, the product was precipitated with ether (47 mg) and purified by countercurrent distribution in the system 2-butanol–0·05% acetic acid (in the all-glass instrument manufactured by Quickfit & Quartz Ltd., Stone, Staffordshire, England) in an atmosphere of nitrogen. 60 shifts of the upper phase were carried out. The peptide material was localised using the Folin-Ciocalteau reaction (samples removed from every other tube); a peak with a partition coefficient of 3·3 was evaporated and lyophilised (41·5 mg). Gel filtration on a column (160 × 2·5 cm) of Bio-Gel P-4 (Bio-Rad Laboratories, Richmond, Ca., USA) in 3M acetic acid (localisation by OD_{280}) gave a yield of 17·8 mg (30%) of product; R_F 0·35 (S1), 0·46 (S3); $[\alpha]_D$ −7·2° (*c* 0·15, 3M acetic acid). Amino acid analysis (150 h hydrolysis at 105°C in 6M-HCl at 1 Torr): Asp 1·16, Glu 1·16, Pro 1·08, Gly 1·75, Cys 0·91, Ile 1·00, Leu 1·16, Tyr 1·00. For $C_{50}H_{71}N_{13}O_{15}S_2 \cdot 5\,H_2O$ (1248) calculated: 48·12% C, 6·54% H, 14·59% N; found: 48·12% C, 6·31% H, 13·82% N.

Pharmacological Methods

The assay on the isolated rat uterus was carried out on strips of uterus of adult virgin Wistar strain rats, under the influence of oestrogens. The strips were placed into medium[14] and bubbled through with 95% O_2—5% CO_2 at 30°C. Isometric contractions were recorded using a magneto-electric transducer[23]; oxytocin was used as the standard. The inhibitory effects of the analogue were measured by the shift of the log dose–response curve and expressed as an inhibition constant according to ref.[24]. Milk-ejecting activity was determined on lactating female rats (5—14 days after delivery) as described in ref.[25]. For determination of the antidiuretic activity we used ethanol anaesthetised rats[26] with a water load maintained at 6—8% of body weight.

We would like to thank Dr J. Smolíková and Dr A. Trka for spectral measurements. Further thanks should be given to Mrs H. Kovářová and Mrs J. Kellerová for technical assistance, to H. Farkašová for carrying out the amino acid analyses and to Mrs Z. Ledvinová for measuring optical rotation. The elemental analyses were carried out in the analytical laboratories of this Institute.

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Translated by J. H. Cort.