

SYNTHESIS AND PROPERTIES OF [2-[3,5-³H₂]-TYROSINE, 4-GLUTAMIC ACID]DEAMINO-1-CARBA-OXYTOCIN*

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The title compound (specific activity 11.1–32.7 Ci (0.41–1.22 TBq)/mmol) was prepared by iodination and subsequent catalytic replacement of iodine by tritium. The analogue which was unstable in the form of a lyophilizate was purified by reversed phase liquid chromatography. Using the N,N'-dicyclohexylcarbodiimide method, the pure analogue was converted into N-hydroxybenzotriazolyl ester, an irreversible oxytocin inhibitor. However, attempts to label specifically the uterotonic receptor, present in the enriched rat myometrium fraction, were hitherto unsuccessful.

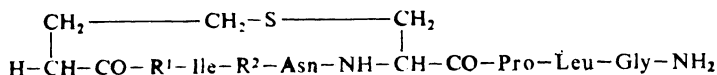
Specific irreversible inhibitors represent a useful tool for studying mechanism of hormone interaction with receptor. A series of compounds, expected to inhibit irreversibly the uterotonic activity of oxytocin, has been synthesized^{1–5}. However, in most cases the desired effect was not achieved, although similar modifications of other hormones, tested on other target tissues, led to the desired irreversible inhibitors.

In our Laboratory we prepared and studied^{6,7} a series of compounds inhibiting irreversibly the uterotonic effect of oxytocin. Most of the synthesized compounds had their own uterotonic activity and therefore their inhibition parameters could not be determined by comparison with competitive inhibitors. We assumed that an interaction with uterotonic receptor is accompanied with its stimulation and formation of a chemical bond between the activated γ -carboxyl of the glutamic acid in position 4 and the possibly present amino group in the receptor protein. In order to decide whether this bond is sufficiently specific it was necessary to prepare a labelled irreversible inhibitor whose fate after possible bonding with the receptor protein could be followed. We decided therefore to prepare a tritium-labelled analogue because alternative introduction of a bulky iodine atom into the molecule could

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considerably change the character of the analogue-receptor interaction. An introduction of radioactive sulfur or carbon atoms would require an almost complete synthesis of the labelled analogue. On the other hand, the tritium-labelled analogue should have a very high specific activity since the emitted radiation energy is low. The irreversible inhibitors, prepared in our Laboratory^{6,7} can be synthesized by one- or two-step synthesis from the already described⁸ [4-glutamic acid]deamino-1-carba-oxytocin (*I*). We prepared the labelled compound *via* the analogue, containing iodinated tyrosine; this method had been elaborated for labelling of neurohypophyseal hormones^{9,10}. We checked the method first using a mixture of tyrosine and S-(γ -methoxycarbonylpropyl)cysteine, or deamino-1-carba-oxytocin (*II*) alone. The fact that the iodination gives absolutely no sulfoxide derivative is very important. Particularly in the case of 1-carba-oxytocin analogues, any sulfoxide present could considerably reduce the affinity of the analogue to the uterotonic receptor¹¹, since catalytic reduction of deamino-1-carba-oxytocin sulfoxide was found to afford, instead of the sulfide, a mixture of hitherto unidentified products. The compound *I* was iodinated in good yield and simple gel filtration gave the pure product *III*, homogeneous according to thin-layer chromatography and reversed-phase liquid chromatography. On oxidation with periodate, compound *III* was converted into a mixture of diastereoisomeric sulfoxides, separable by liquid chromatography. The analogue *III* is relatively stable: even storage for three years at -17°C did not affect its properties.

We have used a catalytic hydrogenation procedure¹⁰ affording a relatively high yield of the desired analogue *IV* which was purified by gel filtration or reversed-phase high pressure liquid chromatography. The yield of the pure analogue *IV* depends on how long the crude tritiation product is left in the form of evaporation residue or lyophilizate. If the product is purified immediately after the catalytic tritiation, yields up to 67% of pure *IV* can be obtained. The highest specific radioactivity of the analogue *IV* was 32.7 Ci (1.22 TBq)/mmol; the compound was homogeneous according to thin-layer chromatography as well as reversed-phase liquid chromatography. Its quality remained practically unchanged on storing in aqueous-methanolic solution at -17°C for two months. On the other hand, as a lyophilizate the com-



- I*, $\text{R}^1 = \text{Tyr}$, $\text{R}^2 = \text{Glu}$
II, $\text{R}^1 = \text{Tyr}$, $\text{R}^2 = \text{Gln}$
III, $\text{R}^1 = \text{Tyr}(3,5\text{-I}_2)$, $\text{R}^2 = \text{Glu}$
IV, $\text{R}^1 = \text{Tyr}(3,5\text{-}^3\text{H}_2)$, $\text{R}^2 = \text{Glu}$
V, $\text{R}^1 = \text{Tyr}$, $\text{R}^2 = \text{Glu}(\text{O---C}_6\text{H}_4\text{N}_3)$
VI, $\text{R}^1 = \text{Tyr}(3,5\text{-}^3\text{H}_2)$, $\text{R}^2 = \text{Glu}(\text{O---C}_6\text{H}_4\text{N}_3)$

pound underwent rapid autoradiolysis and after twenty days it contained only 29% of the analogue *IV*.

When the radioactive analogue was used in the form of lyophilizate (*e.g.* for transport), its purification by liquid chromatography was necessary immediately before preparation of the irreversible inhibitor. In the thus-obtained solution the analogue concentration was determined by UV spectrum and the solution was taken down *in vacuo*. The residue was employed for the preparation of the inhibitor which was immediately used in the incubation with membrane myometrium fractions.

Of the irreversible inhibitors, prepared by us previously⁶, we selected the N-hydroxybenzotriazole ester which is the most potent inhibitor and which can be easily prepared and purified. In the model experiments with the analogue *I* we found optimal conditions for its preparation and purification. The reaction was followed by liquid chromatography which was used also for evaluation of quality of the prepared active ester (after conversion into compound *II* by treatment with ammonia). The ester was purified by precipitation with ether whose efficiency, checked with compound *I*, exceeded 90%. In the preparation of the radioactive analogue we omitted the filtration of the reaction mixture since it removes (and only incompletely) N,N'-dicyclohexylurea which very likely does not influence the bonding to the membrane fractions.

The rat myometrium fraction, containing oxytocin receptors, was prepared as described previously¹². After incubation with the labelled active ester *VI*, this analogue was separated by centrifugation or filtration through membranes of various type. Neither case proved the specific character of binding with the receptor protein, mainly because of the very high radioactive background retained on the membrane filters or centrifuge tubes. After solubilization of the incubated membrane myometrium fraction, followed by SDS (sodium dodecyl sulphate) gel electrophoresis and autoradiography we did not observe a band corresponding to covalently bound peptide.

EXPERIMENTAL

The analytical samples were dried *in vacuo* (150 Pa) over phosphorus pentoxide at room temperature. Thin-layer chromatography was carried out on silica gel plates (Silufol, Kavalier, Czechoslovakia) in the systems 2-butanol-98% formic acid-water (75 : 13.5 : 11.5) (S1), 2-butanol-25% aqueous ammonia-water (85 : 7.5 : 7.5) (S2), 1-butanol-acetic acid-water (4 : 1 : 1) (S3), and 1-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 6) (S4). Compounds were detected by chlorination method, by scanning with Hewlett-Packard Scanner (Hewlett-Packard, U.S.A.) or by autoradiography on DuPont Cronex Safety Film. Solvents were evaporated on a rotatory evaporator at room temperature at 150 Pa. Samples for amino acid analysis were hydrolyzed with 6M-HCl at 105°C for 20 h and analyzed on type 6020 analyzer (Development Workshops, Czechoslovak Academy of Sciences). The liquid chromatography was carried out on an SP-8700 instrument equipped with an SP-8400 detector and SP-4100 integrator (Spectra-Physics, U.S.A.) or on a Beckmann 110 A instrument (Beckmann, U.S.A.).

[2-3,5-Diiodotyrosine, 4-Glutamic Acid]deamino-1-carba-oxytocin (*III*)

A solution of the analogue *I* (50 mg) in 50% aqueous methanol (4 ml) was made alkaline with 26% ammonia (0.26 ml) and a 2% solution of iodine in methanol (1.3 ml) was added. After standing for 10 min at room temperature, the mixture was adjusted to pH 6 with acetic acid (1.8 ml), diluted with 3M acetic acid (2 ml) and applied on a column of Bio-Gel P-4 (2.5 × 100 cm). Elution with 3M acetic acid and freeze-drying afforded 56 mg (89%) of product, pure according to TLC and HPLC. R_F (values for *I* in parentheses): 0.35 (0.26) in S1, 0.05 (0.04) in S2, 0.40 (0.31) in S3, 0.61 (0.57) in S4; k' = 2.87 (0.79) on Separon SI-C-18 (25 × 0.46 cm) in methanol-0.1% trifluoroacetic acid (62.5 : 37.5). Oxidation with sodium periodate gave the diastereoisomeric sulfoxides of k' = 1.97 and 2.45. Amino acid analysis: Asp 0.99, Glu 0.95, Pro 0.93, Gly 1.00, Ile 0.99, Leu 1.06, Tyr 1.05, Cys(C₃H₆COOH) 1.01. For C₄₄H₆₄N₁₀I₂O₁₃S (1 227) calculated: 43.07% C, 5.26% H, 11.42% N, 20.69% I; found: 43.24% C, 5.47% H, 11.63% N, 22.56% I. UV spectrum (H₂O): 287 nm (log ϵ = 3.54), 295 nm (log ϵ = 3.53), upon addition of NaOH: 314 nm (log ϵ = 3.83).

Model Hydrogenation of Sulfoxide of Compound *II*

Deamino-1-carba-oxytocin sulfoxide (3.5 mg) was hydrogenated under the same conditions as described for the preparation of *IV*. Gel filtration through a column of Bio-Gel P-4 (100 × 1 cm) in 3M acetic acid, followed by freeze-drying, afforded 2 mg of product which did not contain any deamino-1-carba-oxytocin (thin-layer chromatography with an authentic sample) but consisted of compounds of higher R_F (in all systems).

Model Iodination

S-(γ -Methoxycarbonylpropyl)cysteine (10 mg) and tyrosine (10 mg) were dissolved in a mixture of water (2.8 ml) and methanol (2.8 ml). The solution was made alkaline with 26% ammonia (280 μ l) and 2% solution of iodine in methanol (1.45 ml) was added. After standing for 10 min at room temperature, the mixture was taken down and the residue analyzed by thin-layer chromatography and on amino acid analyzer. The product did not contain any S-(γ -methoxycarbonylpropyl)cysteine sulfoxide or tyrosine.

Preparation of Active Ester *V* resp. *VI*

a) *p*-Nitrophenol (1.7 mg), N-hydroxybenzotriazole (1.8 mg) and dicyclohexylcarbodiimide (2.7 mg) were added to a solution of the analogue *I* (0.66 mg) in dimethylformamide (33 μ l). The mixture was shaken at room temperature and 1 μ l samples were withdrawn and analyzed by liquid chromatography (25 × 0.46 cm Zorbax ODS column; gradient elution during 15 min with 30–50% methanol and 0.05M ammonium acetate buffer pH 7; 1.5 ml/min; the analogue *I* was eluted in 7.6 min). When the peak of the compound *I* disappeared (2 h), the mixture was filtered through a stainless net (4 mm diameter; mesh size 3 μ m). The product was repeatedly precipitated with ether and centrifuged. The obtained material was dissolved in dimethylformamide (30 μ l), 10% aqueous ammonia (10 μ l) was added and the solution was analyzed by HPLC in the above-mentioned system. The yield of the whole reaction sequence, calculated from the amount of the formed compound *II*, ranged from 18% to 68% and depended strongly on the quality of the dimethylformamide employed. The efficiency of the precipitation with ether was determined as follows: the compound *I* (0.16 mg) was dissolved in methanol (2 ml) and after measuring the UV spectrum and rinsing the cell with methanol (0.5 ml) the analogue was precipitated with diethyl ether (9 ml; no visible precipitate appeared). After centrifugation in a clinical

centrifuge for 15 min, the sediment was suspended in ether, the suspension centrifuged for 10 min and the sediment dissolved in methanol (2 ml). According to UV spectrum, the solution contained 92% of the starting amount of *I*. Similarly, it was found that this double centrifugation removed at least 98% of *p*-nitrophenol and *N*-hydroxybenzotriazole.

b) A solution of the compound *IV* in aqueous methanol after purification by liquid chromatography (0.35 mg, as calculated from the UV spectrum) was taken down at 20°C *in vacuo* (oil pump). The residue was dried for 40 min *in vacuo*, dissolved in dimethylformamide (40 µl) and transferred into a centrifuge tube, containing *p*-nitrophenol (0.8 mg), *N*-hydroxybenzotriazole (1.2 mg) and dicyclohexylcarbodiimide (1.8 mg). The mixture was set aside at room temperature for 3 h, mixed with ether (10 ml) and centrifuged for 15 min. After resuspending in ether and centrifugation, the sediment was dried *in vacuo*, dissolved in ethanol (90 µl), diluted with water to the desired concentration and immediately incubated with the myometrium fraction. For verification of the product character, this solution was also incubated with ammonia and analyzed by TLC.

[2-[3,5-³H₂]-Tyrosine,4-Glutamic Acid]deamino-1-carba-oxytocin (*IV*)

Palladium oxide was reduced with hydrogen in dioxane (0.5 ml). Dioxane and the arising water were removed by freeze-drying, a solution of the diiodo peptide in dimethylacetamide (0.5 ml) was added and the mixture was stirred in ³H₂ atmosphere at room temperature and 80 kPa for 1 h. The mixture was freeze-dried and the product was dissolved in 6% acetic acid (0.5 ml). The catalyst was removed by centrifugation, washed with 6% acetic acid (0.5 ml) and the combined solutions were freeze-dried. The remaining labile ³H was removed by repeated freeze-drying with 6% acetic acid (1 ml). The residue was dissolved in methanol–water (1 : 2) to obtain concentration 20 mg/ml and this solution (100 µl) was applied on a column of Separon SI-C-18 (25 × 0.46 cm). The main peak was eluted with a mixture of methanol and 0.05% aqueous trifluoroacetic acid (1 : 1). Then the mobile phase was replaced by methanol and after 10 min the column was regenerated for another injection of the crude mixture solution. Concentration of the desired compound in the combined fractions was determined by absorption at 278 nm ($\epsilon = 1\,382$ for [4-glutamic acid]deamino-1-carba-oxytocin) and the radioactivity by scintilla-

TABLE I
Experimental details for particular tritiations

Experiment	<i>I</i>	<i>II</i>	<i>III</i>	<i>IV</i>
PdO, mg	34	23.8	35	28
Compound <i>III</i> , mg	4.5	3.5	7.7	10
³ H ₂ (% of theoretical specific radioactivity)	35	44	94	75
Crude product, mCi	57	41	270	216
Pure product, mCi	12.2	10.1	54.7	139
Specific radioactivity, Ci/mmol	11	12.7	32.7	26.7
Yield, %	31.0	29.4	28.1	67.5

tion of an aliquot. The solution was then taken down *in vacuo*, divided into ampoules and freeze-dried. The identity of the product was verified by comparison with the compound *I* (UV spectrum, TLC in four systems and HPLC). The detailed conditions and results of four preparations are given in Table I.

REFERENCES

1. Walter R., Schwartz I. L., Hechter O., Douša T., Hoffman P. L.: *Endocrinology* **91**, 39 (1972).
2. Rich D. H., Geselchen P. D., Tong A., Cheung A., Buchner C. K.: *J. Med. Chem.* **18**, 1004 (1975).
3. Krojidl M., Barth T., Bláha K., Jošt K.: *This Journal* **41**, 1954 (1976).
4. Rudinger J. in the book: *Drug Design* (E. J. Ariëns, Ed.), Vol. 2, p. 319. Academic Press, New York 1971.
5. Pliška V., Marbach P.: *Eur. J. Pharmacol.* **49**, 213 (1978).
6. Lebl M., Bojanovska V., Barth T., Jošt K.: *This Journal* **44**, 2573 (1979).
7. Bojanovska V.: *Thesis*. Czechoslovak Academy of Sciences, Prague 1979.
8. Lebl M., Jošt K.: *This Journal* **43**, 523 (1978).
9. Flouret G., Terada S., Yang F., Nakagawa S. H., Nakahara T., Hechter O.: *Biochemistry* **16**, 2119 (1977).
10. Morgat J. L., Hung L. T., Cardinaud R., Fromageot P., Bockaert J., Imbert M., Morel F.: *J. Label. Compounds* **6**, 276 (1970).
11. Lebl M., Barth T., Jošt K.: *This Journal* **43**, 1538 (1978).
12. Crankshaw D. J., Branda L. A., Matlib M. A., Daniel E. E.: *Eur. J. Biochem.* **86**, 481 (1978).

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