SYNTHESIS OF [4-HISTIDINE]OXYTOCIN

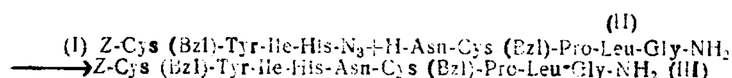
M. A. Samartsev, T. K. Lozhkina,
and V. F. Martynov

UDC 547.964.4

In a study of the analogs of oxytocin modified in position 4, we turned our attention to [4.histidine]oxytocin as a possible natural mutant of oxytocin. The question of mutations in the field of neurohypophyseal hormones has been discussed in the literature in connection with the problem of phylogenesis, i.e., the evolutionary change in the chemical structure of the hormone molecule. Each such change must be connected with one point mutation — the replacement of only one "letter" in the triplet codon responsible for the inclusion of an amino acid in the peptide chain. If mutations led to a mutant analog retaining a fairly high activity, the altered hormone could prove to be viable and be retained in the progeny. In particular, all replacements of amino acids in known natural neurohypophyseal hormones apart from the replacement of the glutamine in position 4 by serine correspond to the principle of one point mutation [1]. The question of the phylogenetic origin of the serine analogs has not yet been answered satisfactorily [1, 2].

For the [4-histidine]oxytocin that we have synthesized, a uterotonic activity of the order 25 IU/mg has been found. Table 1 gives all the oxytocin analogs that can be obtained as the result of a point mutation of the codon corresponding to histamine together with the codons for the inclusion of the corresponding amino acids and the values of their uterotonic activity. It can be seen that the histidine-containing analog possesses the greatest uterotonic activity, its magnitude being sufficient for its retention and discovery in nature. Consequently, it is not excluded that [4-histidine]oxytocin, may be found in living beings. Such a possibility is confirmed by the fact that the hormone glumitocin, or [4-serine-8-glutamine]oxytocin, which has been isolated from the hypophysis of some species of cartilaginous fish, possesses an even greater uterotonic activity, 10.0 ± 0.8 IU/mg [3].

For the synthesis of a protected nonapeptide with the sequence of [4-histidine]oxytocin we used the azide condensation of two fragments—the azide of a tetrapeptide of sequence 1-4 (I) and the amide of the pentapeptide of sequence 5-9 (II):



This division into fragments enabled histidine to be introduced into the synthesis without protection in the imidazole ring.

The synthesis of the protected pentapeptide amide of sequence 5-9 of oxytocin was performed by methods described previously [8, 9] with the exception that, in the last stage to introduce benzyloxycarbonylasparagine we used the o-nitrophenyl ester, which has recently been used successfully for the synthesis of fragments of cytochrome c [10]. This activated ester, unlike the p-nitrophenyl ester, is obtained with high yield (70-80%) and is easily purified.

The tetrapeptide of the sequence 1-4 was obtained from the methyl ester of histidine by the successive growth of the peptide chain with the aid of activated esters of benzyloxycarbonylamino acids. The benzyloxycarbonyl protection was eliminated by the action of hydrogen bromide in acetic acid. The methyl ester of the protected tetrapeptide was converted into the hydrazide by the action of hydrazine in dimethylformamide.

Leningrad State University. Translated from Khimiya Prirodnykh Khimii, No. 4, pp. 498-502, July-August, 1975. Original article submitted May 12, 1974.

© 1976 Plenum Publishing Corporation, 227 West 17th Street, New York, N.Y. 10011. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, microfilming, recording or otherwise, without written permission of the publisher. A copy of this article is available from the publisher for $15.00.

TABLE 1. Uterotonic Activity of Oxytocin Analogs

Amino acid in position 4	Coding triplets	Uterotonic activity of the analog, IU/mg	Literature
Gln	CAA, CAG	450 ± 30	—
His	CAC, CAU	25 ± 9	—
Leu	CUA, CUG	13 ± 1	4
Lys	AAA, AAG	6	5
Glu	GAA, GAG	1,5	6
Pro	CCA, CCG	0,007	7
Arg	CGA, CGG	?	

The protective groups were removed from the protected nonpeptide by the action of sodium in liquid ammonia, and then the disulfide bond was closed by the action of atmospheric oxygen in dilute aqueous solution at pH 6.5. To purify the product we used gel filtration on Sephadex G-15, first in 25% acetic acid and then twice in 2% acetic acid. The narrow zone from the middle peak after the third purification was taken for the determination of biological activity.

The testing of uterotonic activity was performed by Z. P. Auna in the Institute of Organic Synthesis of the Academy of Sciences of the Latvian SSR on isolated rat uterus under standard conditions. A mean value of the activity of 25.0 IU/mg with confidence limits of 15.8-34.3 IU/mg was obtained. The activity was calculated to unit weight of the substance corresponding to the molecular formula of the hormone; the amount of substance in the freeze-dried material was determined from the results of analysis for nitrogen, as has been recently adopted in the literature. In addition to the uterotonic activity, we also investigated the pressor activity of the analog which proved to be very low, as for the majority of compounds modified in position 4. Our preparation exhibited a pressor effect only in doses greater than 0.5 mg/kg weight.

EXPERIMENTAL METHOD

All the amino acids used in the synthesis apart from glycine had the L configuration. The purity of the substances obtained was checked chromatographically on micro plates with a nonfixed layer of silica gel or by paper ionophoresis at a voltage of 1200 V. The analyses of the protected peptides corresponded to the calculated figures.

o-Nitrophenyl Ester of Benzyloxycarbonylasparagine. With stirring and cooling to 0°C, a solution of 10.3 g of dicyclohexylcarbodiimide in 25 ml of dioxane was added to a solution of 14.6 g of benzyloxycarbonylasparagine and 8.3 g of o-nitrophenyl in 60 ml of dimethylformamide (DMFA). After stirring at 0°C for 2 h and at 20°C for 4-6 h, 2 ml of acetic acid were added, the mixture was stirred for another 15 min, and the dicyclohexylurea was filtered off and was washed with 30 ml of dioxane. The filtrate was treated with 80 ml of water and after the beginning of crystallization another 120 ml of water were gradually added. The precipitate was immediately filtered off and was washed with 5% $NaHCO_3$ solution, water, 1 N HCl, water again, and repeatedly with ether. This gave 15.25 g (79%) of a product with mp 149-150°C. After reprecipitation from DMFA with water, mp 150.5-151.5°C, $[\alpha]_D^{20}$ − 29.7° (c 2; dioxane). Composition $C_{18}H_{17}N_3O_7$. On recrystallization from a hot mixture of ethanol and ethyl acetate, the melting point rose to 154-154.5° but, according to chromatography, the product had partially decomposed with the formation of benzyloxycarbonylasparagine.

Amide of Benzyloxycarbonylasparaginyl-S-benzylcysteinyloprolylleucylglycine (IV). The hydrobromide of S-benzylcysteinylprolylleucylglycinamide, obtained by treating 3.9 g of the corresponding benzyloxycarbonyl tetrapeptide [8] in acetic acid with hydrogen bromide, was dissolved in ethanol and the solution was stirred with IRA-410 ion-exchange resin in the OH^- form until the reaction for the bromide ion was negative. After the separation of the resin, the solution was evaporated and the residue was dissolved in 10 ml of DMFA, and then 2.8 g of the o-nitrophenyl ester of benzyloxycarbonylasparagine was added. After two days, the product was precipitated with water and it was washed on the filter with 5% $NaHCO_3$, water, 1 N HCl, water, ether, and ethyl acetate, and was dried in vauum over P_2O_5. This gave 4.25 g (92%) of the pentapeptide with mp 208-210°C, $[\alpha]_D^{20}$ −52° (1.4; DMFA). According to the literature [8], mp 213-214°C, $[\alpha]_D^{21}$ −51.5° (c 1; DMFA).

Methyl Ester of Benzyloxycarbonylisoleucylhistidine (V). The dihydrochloride of histidine methyl ester (2 g) was treated with an excess of a solution of ammonia in chloroform, and the ammonium chloride was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in a mixture of 12 ml of chloroform and 12 ml of acetonitrile, and 3.1 g of the p-nitrophenyl ester of benzyloxycarbonylisoleucine was added. After a day, the solvent was distilled off, the residue was dissolved in 6 ml of methanol, and 80 ml of absolute ether was added. On standing overnight at 0°C, a gel-like precipitate deposited which, after filtration and repeated washing with ether, was dried over P_2O_5. This gave 2.0 g

of substance (60%) with mp 158-168°C. After reprecipitation from methanol ether, the yield was 1.2 g (36%), mp 175-177°C; a second reprecipitation gave a product with mp 176-178°C. Composition $C_{21}H_{28}N_4O_5$.

A similar result was obtained by synthesis using the pentachlorophenyl ester of benzyloxycarbonylisoleucine.

Methyl Ester of Dibenzyloxycarbonyltyrosylisoleucylhistidine (VI). The dipeptide (V) (0.88 g) was treated with a solution of hydrogen bromide in acetic acid (3.2 ml), and after an hour the hydrobromide was precipitated with ether, filtered off, and dried over P_2O_5 and KOH, and it was then reprecipitated from methanol with ether. The oil that had separated out was disssolved in 5 ml of DMFA, and 1.14 g of the p-nitrophenyl ester of triethylamine were added. After a day, the tripeptide formed was precipitated with water, filtered off, and washed with water, ether, 5% $NaHCO_3$, water, 2% acetic acid, ethyl acetate, acetone, and ether. This gave 0.60 g (42%) of a product with mp 170-171°C. Composition $C_{38}H_{43}N_5O_9$.

Hydrazide of Benzyloxycarbonyl-S-benzycysteinyltyrosylisoleucylhistidine (VII). The tripeptide (VI) (0.49 g) was treated with a solution of hydrogen bromide in acetic acid (2.5 ml), and after an hour the hydrobromide of the tripeptide was precipitated with ether, filtered off, washed with ether, dissolved in DMFA, and neutralized with solid sodium bicarbonate. The excess of bicarbonate was filtered off and the filtrate was treated with 0.37 g of the p-nitrophenyl ester of benzyloxycarbonyl-S-benzylcysteine and 0.1 ml of triethylamine. After two days, the methyl ester of the tetrapeptide was precipitated with water, washed with water and ether, dried, and again washed with ether. This gave 0.51 g of a product in the form of an amorphous powder with mp 166-173°C. Amino-acid analysis (hydrolysis in the presence of thioglycolic acid): His 0.98, Ile 1.00, Tyr 1.13; the cysteine was not determined.

For conversion into the hydrazide, 0.47 g of the methyl ester of the tetrapeptide was dissolved in 10 ml of DMFA, 1.2 ml of anhydrous hydrazine was added, and the mixture was kept at 20°C for two days and at 80°C for 15 min. The hydrazide was precipitated from the cool solution with water and it was washed with water and ether and dried in vacuum over sulfuric acid. This gave 0.34 g (62%) of the hydrazide; after reprecipitation from DMFA with water, mp 216-218°C. Composition $C_{39}H_{48}H_8O_7$.

Amide of Benzyloxycarbonyl-S-benzylcysteinyltyrosylisoleucylhistidylasparaginyl-S-benzylcysteinylprolylleucylglycine (III). The protected pentapeptide amide (IV) (0.48 g) was treated with hydrogen bromide in acetic acid, and the hydrobromide of the pentapeptide amide (II) was isolated and was freed from bromide anions as described in the synthesis of (IV).

To obtain the azide (I), 0.3 g of the hydrazide (VII) was suspended in a mixture of 11 ml of acetic acid, 1 ml of 1 N hydrochloric acid, and 0.6 ml of 20% sodium-chloride solution at 35°C. The suspension was cooled to −10°C and, with stirring, 0.2 ml of a 1 M solution of sodium nitrite was added. After 15 min, the azide was precipitated by the addition of 100 ml of 20% sodium-chloride solution (0°C), and the precipitate was filtered off and washed with water and, without drying, it was dissolved in 20 ml of DMFA. The solution was dried with sodium sulfate for 15 min, the desiccant was filtered off, and the solution was immediately added to the pentapeptide (II) obtained above. The reaction mixture was stirred at −10°C for 1 h and was left at −5°C for 3 days and at 20°C for another day. Then it was diluted with water, and the precipitate was filtered off and was washed with 5% $NaHCO_3$, water, 0.5 M citric acid, water, and ether. After reprecipitation from DMFA with water and drying, 0.24 g (60%) of product was obtained, and this was used immediately in the following stage.

[4-Histidine] oxytocin. A solution of 0.24 g of the protected nonapeptide (III) in 200 ml of liquid ammonia which had been freshly redistilled over sodium was treated, with stirring, with small portions of sodium until a blue coloration remaining for a minute appeared. The resideal coloration was eliminated by the addition of a small crystal of ammonium acetate. The ammonia was allowed to evaporate completely in 3 h, the residue was dissolved in 2 liters of distilled water, the pH was brought to 6.5 by the addition of acetic acid, and the solution was stirred vigorously in an open flask for two days. After filtration the solution was evaporated in vacuum at 30-35°C to a volume of 120 ml and was then lyophilized. To free it from salts, the lyophilizate was subjected to gel filtration on a column (90 × 1.5 cm) of Sephadex G-15 in 25% acetic acid. The peptide peaks were identified automatically directly

at the outlet from the column from the absorption of the solution at 280 nm. The solution corresponding to the main peptide peak was collected, evaporated to a volume of 100 ml, and lyophilized. The material so obtained was subjected to gel filtration again on the same column in 2% acetic acid, by which means it was possible to separate the bulk of the dimeric product [11] and also the tetrapeptide fragment present in the mixture. After the lyophilization of the central part of the main peak, 94 mg of substance were obtained.

For final purification, 47 mg of the lyophilizate was rechromatographed under the same conditions and a narrow fraction corresponding to the central part of the peptide peak was collected. After evaporation and lyophilization, 27 mg of an electrophoretically homogenous product was obtained. From the results of elementary analysis, the amount of main substance in the lyophilizate was 66%.

The hydrolysis of the substance for amino-acid analysis was performed in the presence of phenol and also by the standard method without phenol (figures in parentheses). The following ratios of the amino acids in the hydrolyzate were obtained: His 1.00 (0.82), Asp 1.00 (0.95), Pro 0.97 (0.94). Gly 1.05 (1.05), Cys (1.47), Ile 1.00 (1.00), Leu 1.02 (1.05), Tyr 1.22 (0.44). The amino-acid analysis was performed by A. O. Smirnov.

SUMMARY

A new synthetic analog of oxytocin — [4-histidine] oxytocin — has been obtained and its uterotonic activity has been determined.

LITERATURE CITED

1. J. Rudinger, O. V. Kesarev, K. Poduska, B. Pickering, R. Dyball, D. Ferguson, and W. Ward, Experientia, 25, 680 (1969).
2. M. Manning, E. Coy, and W. Sawyer, Biochemistry, 9, 3925 (1970).
3. W. Sawyer, M. Manning, E. Heinicke, and A. Perks, Gen. Comp. Endocrinology, 12, 387 (1969).
4. V. Hruby, G. Flouret, and V. du Vigneaud, J. Amer. Chem. Soc., 244, 3890 (1969).
5. O. A. Kaurov, V. F. Martynov, and Yu. D. Mikhailov, Zh. Obshch. Khim., 41, 1413 (1971).
6. I. Photaki and V. du Vigneaud, J. Amer. Chem. Soc., 87, 908 (1965).
7. W. Sawyer, T. Wuu, J. Baxter, and M. Manning, Endocrinolgy, 85, 385 (1969).
8. M. Bodanszky and V. du Vigneaud, J. Amer. Chem. Soc., 81, 5688 (1959).
9. Zh. D. Bespalova, T. K. Lozhkina, M. A. Samartsev, and I. M. Shchenkova, Khim. Prirodn. Soedin., 808 (1973).
10. L. Moroder, G. Borin, F. Marchiori, and E. Scoffone, Biopolymers, 12, 521, 701 (1973).
11. D. Yamashiro, D. Hope, and V. du Vigneaud, J. Amer. Chem. Soc., 90, 3857 (1958); M. Manning, T. Wuu, and J. Baxter, J. Chromatogr., 38, 396 (1968).