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ANALOGS OF OXYTOCIN WITH L- AND D-PENTAFLUOROPHENYLALANINES IN THE SECOND POSITION

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On the basis of the results of a study of the biological activity of oxytocin analogs, we have previously put forward the hypothesis that the side chain of tyrosine, which is present in the second position of the hormone molecule, takes a direct part in hormone-receptor interaction, abandoning its original position in the receptor on stimulation of the chain of events leading to the biological effect [1], and therefore noncovalent interactions between the tyrosine and the receptor have an optimum nature. If the modification leads to a decrease in the intensity of noncovalent interactions, the affinity of the hormones for the receptor decreases correspondingly and, consequently, so does its biological activity. An increase in the intensity of interaction prevents the subsequent migration of the side chain, thus decreasing the capacity of the hormone-receptor complex formed for stimulating the biological effect. Consequently, such a modification may lead either to partial agonists or to antagonists of the natural hormone.

As can be seen from Table 1, a change in configuration of the modifying amino acid from L to D frequently leads to oxytocin antagonists. However, where the loss of biological activity is caused by an intensification of noncovalent interactions, the analogs with D-(amino acid)s either have a greater activity than the compound with the corresponding L-(amino acid)s (see compound (V)), or show more effective antagonism than [2-D-Phe]-oxytocin (compare compounds (IV) and VI)). If, however, the loss of biological activity is caused by a decrease in the affinity of the hormone for the receptor, the value of pA_2 for compounds with D-(amino acid)s is less than for [2-D-Phe]oxytocin (see compound (III)). The analog with phenylalanine in the second position of the oxytocin molecule occupies a boundary position between the groups of compounds under consideration.

It can also be seen from Table 1 that the interpretation of the results of a study of the dependence of the biological activity of the series of oxytocin analogs under consideration as a function of their structure is complicated by the fact that the structural modifications performed change the nature of the forces responsible for hormone-receptor interaction in an ambiguous manner. Any structural modification causes a change in some forms of noncovalent interactions and always to some degree or other changes the intensity of donor-acceptor interaction. And it is just this factor which apparently plays an important role in the stimulation of the biological effect.

In order to elucidate the influence of donor-acceptor interaction in the more or less pure form, it appeared to us to be of interest to synthesize analogs of oxytocin with a L- or D-pentafluorophenylalanine in position 2 and to compare their biological activities with those of [2-L- and -D-Phe]oxytocins.

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TABLE 1. Uterotonic Activity of Some Oxytocin Analogs Modified in Position 2

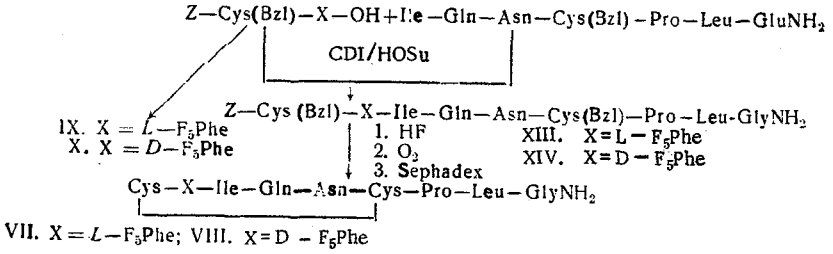
Analogs	Amino acid in position 2	Nature of the noncovalent interactions causing an increase in hormone-receptor interaction	Factors adversely affecting hormone-receptor interaction	Uterotonic activity (L-isomer), IU/mg	Uterotonic activity (D-isomer),* IU/mg
I	Tyr	Oxytocin – the natural hormone	Oxytocin – the natural hormone	450 [2]	34 [2]†
II	Phe	‡	Absence of electrostatic interactions, absence of H bonds	30 [2]	pA_2=6,00 [1]
III	Leu	—	Absence of electrostatic interactions, H bonds, and donor-acceptor interaction	1,4 [2]	pA_2=5,23 [1]
IV	Tyr (NO_2)	Electrostatic, donor – acceptor	Steric effect	1,1 [3,4]	pA_2=6,28 [3]
V	Phe (OEt)	Hydrophobic, donor – acceptor	Steric effect, absence of electrostatic interaction, change in the nature of the H bonds	0,15 [2]†	2,59 [1]
VI	Trp	Hydrophobic, donor – acceptor	Steric effect, absence of electrostatic interaction, change in the nature of the H bonds	0,24 [1]	pA_2=6,87 [1]
VII	F_5Phe	Donor – acceptor	Absence of electrostatic interaction and H bonds	0,01	pA_2=6,27

For antagonists: *) value of $pA_2 = -\log C$, where C is the concentration of antagonists producing a twofold decrease in the uterotonic activity of oxytocin; †) under nonstandard conditions show antagonism to the natural hormone; ‡) small changes in hydrophobicity and donor–acceptor properties.

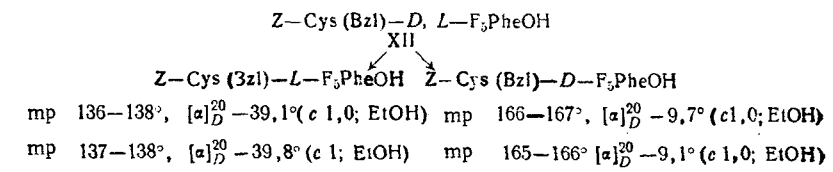
In actual fact, when hydrogen is replaced by fluorine the steric characteristics of the benzene ring do not greatly change (van der Waals radii 0.9 and 1.25 Å, respectively) nor does its hydrophobicity (logarithm of the partition coefficient in the water–octanol system 2.22 for hexafluorobenzene and 2.13 for benzene [6]). Basically what changes is the capacity for donor-acceptor interaction (for example, hexafluorobenzene can form charge-transfer complexes [7]).

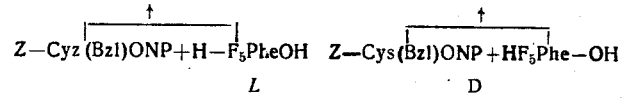
Pharmacological trials of the [2-L-F_5Phe]oxytocin (VII) that we synthesized showed that this compound has a uterotonic activity almost three orders of magnitude smaller than that of compound (II). Such a fall in activity cannot be explained by a change in the conformation of the analog, since compounds (VII) and (II) have similar CD spectra [8]. Consequently, the replacement of hydrogen by fluorine in the benzene ring of phenylalanine is strongly reflected on the hormone-receptor interaction solely as the result of the change in the capacity for donor–acceptor interactions, this sharp decrease in activity being apparently connected not with the loss of the affinity of the analog for the receptor but with difficulties in the subsequent transition, since the other analog that we synthesized – [2-D-F_5Phe]oxytocin (VIII) – inhibits the uterotonic action of oxytocin to a far greater degree than [2-D-Phe]oxytosin.

The oxytocin analogs were synthesized by the following Scheme:



The dipeptides (IX) and (X) required for this synthesis were obtained by the fractional crystallization of dipeptide (XII) the synthesis of which has been described previously [9]. To determine the configurations of compounds (IX) and (X) they were synthesized independently from the optically active pentafluorophenylalanines:





We first obtained D-pentafluorophenylalanine by separating the diastereomeric salts of acylated D,L-pentafluorophenylalanines with natural alkaloids by crystallization. Various natural alkaloids – brucine, cinchonine, and quinine – were tested, and also optically active α-phenylethylamine. Successful resolution was achieved by using brucine in aqueous ethanolic solution as the base. As a result of the decomposition of the salt we obtained optically active benzoylpentafluorophenylalanine with a specific rotation of $[\alpha]_D^{20}+65.0°$ (c 0.5; ethanol). After the elimination of the benzoyl group and the purification of the amino acid by means of KU-2 cation-exchange resin in the H^+ form and recrystallization from aqueous ethanol, optically active pentafluorophenylalanine was isolated with specific rotations of $[\alpha]_D^{20}-22.1°$ (c 1.02; H_2O) and $[\alpha]_D^{20}-16.5°$ (c 1.1; 5N. HCl).

According to Schwyzer et al. [10], L-pentafluorophenylalanine can be synthesized by the stereospecific hydrolysis of the N-trifluoroacetyl derivative of pentafluorophenylalanine by acylase I. By this method we isolated optically active pentafluorophenylalanine with a specific rotation of $[\alpha]_D^{20}+22.4°$ (c 0.5; H_2O). Since asymmetric hydrolysis with amylase always gives free amino acids of the L configuration, the antipode that we obtained with the aid of brucine had the D configuration.

It is interesting that L-pentafluorophenylalanine has a positive shift on the angle of optical rotation when both acetic and hydrochloric acids are added to its aqueous solution:

$$\Delta\alpha = [\alpha]_{HCl} - [\alpha]_{H_2O} \cong +6°.$$

EXPERIMENTAL

Thin-layer chromatography was carried out on Kieselgel Merck silica gel in the following systems: 1) 3% ammonia–sec-butanol (1:3); 2) methanol–chloroform (4:9); 3) butan-1-ol–acetic acid–water (4:1:1); 4) nitromethane–dioxane (1:1); and 5) methanol–toluene (1:4). Electrophoresis was performed on "Whatman filter paper No. 3" in 2% acetic acid followed by treatment with chlorine and benzidine. The substances were analyzed for their fluorine contents by the method of Shishikin and Mamaev [11]. The analyses of all the compounds corresponded to the calculated figures. Amino-acid analysis was carried out on a "Hitachi" amino-acid analyzer.

Benzoyl-D-pentafluorophenylalanine. A suspension of 6.196 g of benzoylpentafluorophenylalanine [12] in 200 ml of a mixture of ethanol and water (2:3) was treated with 8.3 g of brucine. The solution was heated, and to complete to dissolution of the solid matter another 215 ml of the mixture was added. The reaction mixture was carefully cooled and was left to stand at room temperature. After three weeks, the crystals that had deposited were filtered off. Two recrystallizations from aqueous ethanol gave 5 g (37%) of a salt with mp 143-144°C and $[\alpha]_D^{20}-5.0°$ (c 0.508; ethanol). The salt was decomposed with alkali. The brucine that deposited (2.1 g) was filtered off and the filtrate was acidified with 20% HCl. The precipitate of benzoyl derivative (2.5 g) with mp 186-187°C was recrystallized from aqueous ethanol. The melting point after recrystallization was 187°C, $[\alpha]_D^{20}+63°$ (c 0.502; ethanol).

The benzoylpentafluorophenylalanine obtained from the mother solution from the separation process by decomposing the more soluble salt had mp 189-200°C and $[\alpha]_D^{20}-18°$ (c 0.5; ethanol).

D-Pentafluorophenylalanine. Benzoylpentafluoroalanine (0.9 g) was boiled in a mixture of 12 ml of 40% and 6 ml of glacial acetic acid for 5 h and the mixture was evaporated to dryness under reduced pressure. The residue was treated with 10 ml of water, the mixture was heated to the boil, and after cooling, the benzoic acid that had deposited was filtered off. An aqueous solution of pentafluorophenylalanine hydrobromide was passed through a column (1.5 × 80 cm) of KU-2 cation-exchange resin in the K^+ form. The column was washed with water until the reaction for Br^- ion was negative. Then the amino acid was eluted with a 2 N aqueous solution of ammonia until the ninhydrin reaction was negative. The ammoniacal solution was evaporated under reduced pressure at 40°C to dryness. The residue was dried at 100°C in vacuum. This gave 0.52 g of D-pentafluorophenylalanine with mp 257-258°C; $[\alpha]_D^{20}-22.1°$ (c 1.02; water), $[\alpha]_D^{20}-16.5°$ (c 1.1; 5 N. HCl), $[\alpha]_D^{20}-12.2°$ (c 0.496; acetic acid).

Separation of S-Benzyl-N-benzyloxycarbonylcysteinyl-D,L-pentafluorophenylalanine into Disastereomers by Fractional Recrystallization. Compound (XII) [9] with mp 116-118°C (1.5g) was recrystallized from 11 ml of absolute ethanol. This gave 645 mg of a colorless amorphous powder with mp 152-153°C, three recrystalliza-

tions of which from absolute ethanol led to a product with a constant melting point (166-167°C) and a specific rotation of $[\alpha]_D^{20}$ −9.7 (c 1.0; ethanol). R_{f_5} 0.51, R_{f_2} 0.93.

The mother solution from the first recrystallization was evaporated and the 740 mg of substance so obtained was recrystallized four times from aqueous acetone to constant specific rotation. The dipeptide obtained (420 mg) had mp 138°C and $[\alpha]_D^{20}$ −9.7° (c 1.0; ethanol). R_{f_5} 0.52, R_{f_2} 0.91.

S-Benzyl-N-benzyloxycarbonylcysteinyl-D-pentafluorophenylalanine (X). S-Benzyl-N-benzyloxycarbonylcysteinyl-D-pentafluorophenylalanine was synthesized by a method described previously [9] starting from D-pentafluorophenylalanine obtained by the decomposition of the brucine salt.

The yield of product with mp 165-166°C and $[\alpha]_D^{20}$ −9.1° (c 1.0; ethanol) was 86%.

By a similar method, starting from L-pentafluorophenylalanine, obtained by the hydrolysis of the N-trifluoroacetyl derivative with acylase I, S-benzyl-N-benzyloxycarbonylcysteinyl-L-pentafluorophenylalanine (IX) with mp 137-138°C and $[\alpha]_D^{20}$ −39.8° (c 1.0; ethanol) was obtained with t yield of 81%.

Amide of N-Benzyloxycarbonyl-S-benzylcysteinyl-L-pentafluorophenylalanyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine (XIII). With stirring, 192 mg of N-benzyloxycarbonyl-S-benzylcysteinyl-L-pentafluorophenylalanine and 42 mg of N-hydroxysuccinimide and then, at −10°C, 72 mg of N,N'-dicyclohexylcarbodiimide were added to 280 mg of the amide of L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-leucylglycine −13] in 2 ml of dimethylformamide. The reaction mixture was stirred for an hour at −2 to −8°C and was left overnight at 0°C. Then two drops of glacial acetic acid was added and the mixture was stirred for an hour. The N,N'-dicyclohexylurea was filtered off and the filtrate was acidified with 1 N hydrochloric acid. The precipitate that deposited was filtered off and was washed with 1 N hydrochloric acid, 5% sodium bicarbonate solution, water, hot ethyl acetate, and ethanol.

This gave 320 mg of protected nonapeptide, which was recrystallized from 80% acetic acid, mp 229-232°C, R_{f_5} 0.02, R_{f_3} 0.75.

[2-L-Pentafluorophenylalanine]oxytocin (VII). The protected nonapeptide (300 mg) was treated with anhydrous HF with the addition of anisole [14]. After drying in vacuum over caustic soda, the oily residue was transferred to a flask with 2 liters of water, the pH of the solution was brought to 6.5 with ammonia, and it was stirred in the air for 1.5 days. Then the solution was evaporated at 25°C and the residue was lyophilized.

The lyophilizate gave two electrophoretically mobile spots with $E_{fGly} = 0.45$ and 0.94.

The lyophilizate (168 mg) was dissolved in 2 ml of 25% acetic acid and was passed through a column of Sephadex G-15 (2 × 70 cm) in 25% acetic acid. The product was eluted with 25% acetic acid, 1.3-ml samples being collected. The substance from tubes 95-112 was repurified on Sephadex G-15 in 5% acetic acid. The substance from tubes 90 to 117 was electrophoretically individual, $E_{fGly} = 0.2$.

The yield of [2-L-pentafluorophenylalanine]oxytocin was 81 mg. Amino-acid analysis: F_5Phe-1.06, Cys-1.56, Ile-0.98, Leu-1.12, Asn-1.0, Gln-1.04, Pro-0.97, Gly-1.1.

Amide of S-Benzyl-N-benzyloxycarbonyl-L-cysteinyl-D-pentafluorophenylalanyl-L-isoleucyl-L-glutaminyl-L-asparaginyl-S-benzyl-L-cyteinyl-L-prolyl-L-leucylglycine (XIV). Using a method analogous to that for the production of compound (XIII) starting from 294 mg of S-benzyl-N-benzyloxycarbonylcysteinyl-D-pentafluorophenylalanine we obtained 325 mg of protected nonapeptide with mp 228°C. R_{f_3} 0.73.

[2-D-Pentafluorophenylalanine]oxytocin (VIII). The removal of the protective groups, oxidation, and the purification of the analog were performed similarly to the case of [2-L-pentafluorophenylalanine]oxytocin.

This gave 41 mg of [2-D-pentafluorophenylalanine]oxytocin. Amino-acid analysis; F_5Phe−1.12, Cys−1.61, Ile −1.08, Leu −1.02, Asn −1.0, Gln −1.01, Pro −0.92, Gly − 0.99.

SUMMARY

1. The separation of pentafluorophenylalanine into optical antipodes has been effected.

2. Two new analogs of oxytocin – [2-L-pentafluorophenylalanine]oxytocin and [2-D-pentafluorophenylalanine]oxytocin – have been synthesized and their uterotonic activities have been studied.

3. It has been shown that donor–acceptor interaction plays a fundamental role in the stimulation of the biological effect.

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PURIFICATION OF CARBOXYLIC PROTEINASES ON AMINOSILOCHROME

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Carboxylic proteinases, especially the pepsins of various animals and rennin or chymosin find wide practical use, but to obtain specimens of these proteinases with a high degree of purity in preparative amounts remains a difficult task.

Anion-exchange resins mainly based on cellulose are usually used for the chromatographic fractionation of proteins with a low isoelectric point. Sorbents based on inorganic materials (silica – Silochrome) have a number of advantages over organic matrices. The inorganic matrix of Silochrome with amino groups immobilized on the surface of the support does not contract or swell in solvents and is stable to the action of acids and organic solvents. The macroporous support (250-700 Å) has no denaturing action on the labile molecule of an enzyme. The particle size of Silochrome (0.25-0.5 mm) ensures a high rate of flow of the solution. Such an ion-exchange material can be used repeatedly. We have shown the possibility of obtaining analytical amounts of porcine pepsin by chromatography on a column of Aminosilochrome C-80 [1]. The present communication is devoted to a description of the preparative separation and purification of a number of carboxylic proteinases: the separation of calf pepsin and chymosin and the purification of bovine and porcine pepsins on Aminosilochrome.

The presence of a large number of carboxy groups on the surface of an enzyme when the number of residues of basic amino acids is low shifts the isoelectric points of the carboxylic proteinases into the acid region (pI of pepsin atout 2.0) or the weakly acid region (pI of the chymosins about 4.5). Knowing the values of pI enabled us to select the conditions [1] for the sorption and desorption of proteinases on aminated Silochrome. Sorption of the enzymes was carried out at pH 5.5. At this pH the possibility of autolysis is reduced to a minimum and at the same time the enzymes possess a charge opposite in sign to that of the anion-exchange material. The conversion of the enzymes into the isoelectric state ensures the suppression of these ionic interactions and their desorption.

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