SYNTHESIS AND STUDY OF THE BIOLOGICAL ACTIVITY OF FRAGMENTS OF SALMON CALCITONIN II

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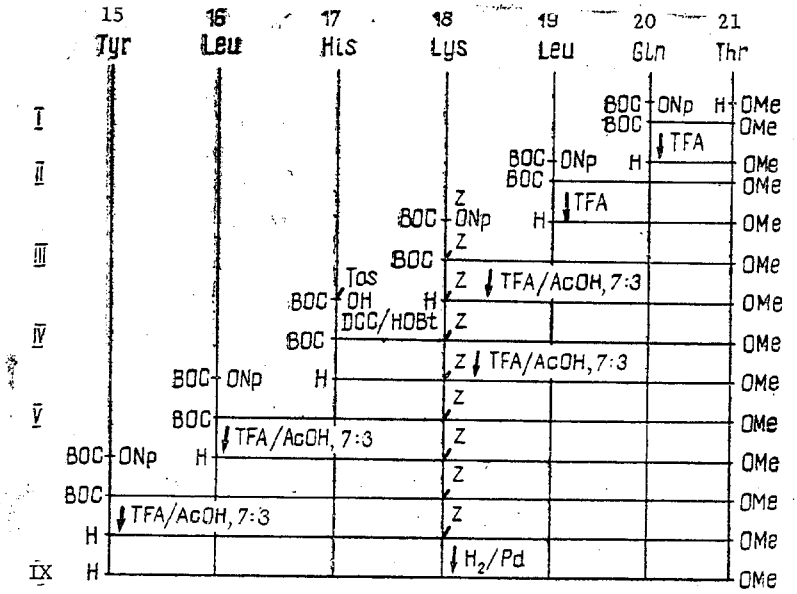
A number of fragments of salmon calcitonin II possessing analgesic activity of the nonopioid type have been synthesized.

The results of numerous investigations show an extremely broad spectrum of the biological activity of salmon calcitonin II (SCTII). Thus, there are reports of the existence in the hormone, in addition to its main (hypocalcemic) activity, of analgesic, anorexogenic, and other types of activity [1-3]. Since the use of calcitonin as a therapeutic agent requires a contraction of the spectrum of the biological activity of the hormone, we set ourselves the task of finding the minimum structures responsible for particular types of activity. The present work was devoted to determining the minimum peptide fragments of the hormone responsible for its analgesic activity.

We have previously reported the synthesis of salmon calcitonin II and its fragments [4-6]. The preliminary screening performed showed the presence of a high epileptogenic activity in the case of the 16-21-OMe peptide and some other sections of the sequence of the hormone [6]. At the same time, it was shown that the threshold epileptogenic dose (TED) of the 16-21 OMe fragments was only 1.3 ± 1.0 μmole/kg [6]. The TED leu-enkephalin, chosen as standard, is 47 ± 3 μmole/kg.

We assumed that the epileptogenic test can be used for finding peptide fragments interacting with definite types of receptors [7], i.e., that these fragments may possess, in addition to an epileptogenic action, other types of activity also due to a central mechanism. To elucidate the possibility of using the epileptogenic test for finding peptides with a central mechanism of their action and with the aim of localizing the section of the sequence responsible for the appearance of the analgesic action, we have carried out the synthesis of fragments from the 11-21 sequence of SCTII.



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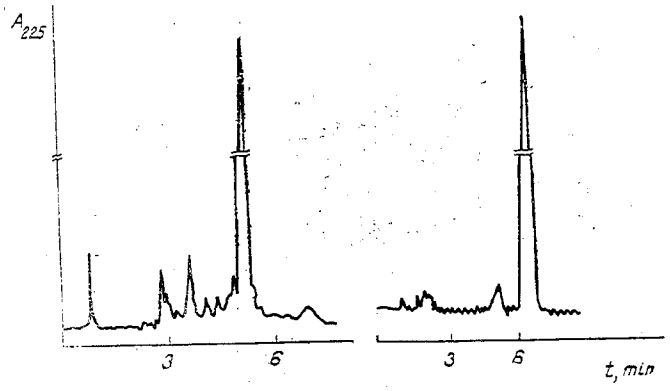
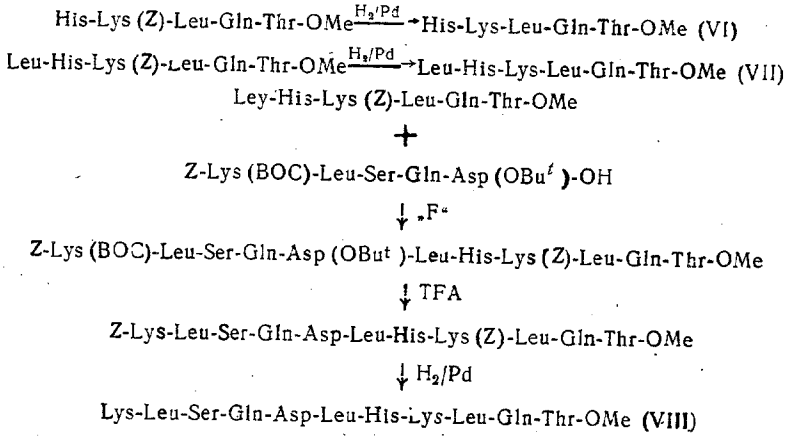


Fig. 1. HPLC of the peptides Z-Leu-His-Lys (BOC)-Leu-Gln-Thr-Ome [5] (on the left) and of peptide (V) (on the right); MeOH/0.01 N TFA (70:30).



Scheme of the synthesis of fragments of SCTII

The corresponding peptides were obtained by the methods of classical peptide synthesis in solution by a scheme forming a modification of one that we have published previously [5]. The changes introduced permitted a substantial simplification of the production and purification of the substances. In the case of the 17-21-OMe and 16-21-OMe fragments, the successive growth of the peptide chain was carried out by the activated: ester method, while the histidine residue was attached by the carbodiimide method with the addition of 1-hydroxybenzotriazole (HOBt). The 11-21-OMe fragment was obtained by fragment condensation by a 5 + 6 scheme using complex F. For blocking the α-amino groups we used tert-butoxycarbonyl protection and for the imidazole grouping of histidine tosyl protection, the HOBt being eliminated simultaneously with the formation of the peptide bond. The use of the new scheme permitted the yield to be increased by 20% at the stage of obtaining BOC-Lys(Z)-Leu-Gln-Thr-OMe (see [5], and also Table 3), while the use of the protected His in the successive growth of the peptide chain led to a substantial increase in the purity of the intermediate compounds, as can be seen from Fig. 1.

The results of the biological tests of the peptides synthesized and of the formalin test [10] showed that the 16-21-OMe peptide led to a substantial difference from the control when administered in doses smaller than those of the other preparations (Table 1). Both a lengthening and a shortening of the amino acid sequence led to a fall in activity (the 11-21-OMe and 17-21-OMe peptides). As a result of the replacement of a methyl ester group by a free carboxy group (the 16-21 peptide, Table 2), the analgesic activity likewise decreased. It must be mentioned that when the 17-21-OMe and 16-21-OMe peptides were administered in large doses (1 μg/rat) complete absence of the pain reaction was observed in a large proportion of the experimental animals.

At the same time, in the test of the electrostimulation of the tooth pulp in cats* [8] a high analgesic activity was found both for the 11-15 peptide, the synthesis of which we

* The 11-15 and 16-21 peptides were investigated in the tooth pulp electrostimulation tests by B. T. Moroz of the Department of Pharmacology of the 1st Leningrad Medical Institute (St. Petersburg).

TABLE 1. Biological Activities of the Calcitonin Peptides in the Formalin Tests [10]*

Peptide	Dose, μg/rat**	Number of animals in the series	Beginning of the appearance of the pain reaction, s	Duration of the pain reaction, s
Control	0	14	22,5±3,62	395,3±29,78
11—15	1	6	10,8±2,57 $\alpha<0,1$	554,2±44,1 $\alpha<0,01$
16—21 OMe	0,01	7	30,0±5,45 $\alpha<0,1$	237,1±42,06 $\alpha<0,01$
Tyr-16—21 OMe	1	5	63,0±15,99 $\alpha<0,05$	309,0±68,06
11—21 OMe	0,1	8	76,3±16,87 $\alpha<0,05$	252,5±27,98 $\alpha<0,01$
17—21 OMe	1	4	108,8±24,0 $\alpha<0,05$	411,3±67,65

* Formalin was administered to the rats in a concentration of 1.2%.
**The minimum doses at which the results of the experiment differed significantly from the control are given.

have described previously [4] and for the 16-21-OMe peptide, the antinociceptive effect of the preparations being retained on the administration of naloxone, which shows the nonopioid nature of their action. The existence of nonopioid analgesic activity in the 16-21-OMe peptide was also confirmed in the hotplate test [9]. The 11-15 peptide was inactive in this test.

It must be mentioned that the heptapeptide Tyr-16-21-OMe exhibited a pronounced analgesic action, although in a larger dose. This permits this substance to be used for the introduction of an isotopic label and for the study of the hormone-receptor interaction.

Thus, optimization has been carried out of the scheme of synthesizing a number of peptides of the 16-21 sequence, the existence of a considerable analgesic activity of the nonopioid type in the 16-21-OMe fragment of STII has been established and a definite correlation has been shown between the results of the epileptogenic test and the test of analgesia.

EXPERIMENTAL

In this work we used amino acids and their derivatives produced by the firm Reanal (Hungary).

Melting points of the compounds obtained were determined on a Boëtius instrument from the firm Nagema (Germany).

Amino acid analysis was conducted on Biotronik LC-2000 (Germany) and T 339 M (Czechoslovakia) amino acid analyzers.* For amino acid analysis, the peptides were hydrolyzed with 6 N hydrochloric acid at 110°C for 20 h.

The optical rotations of the peptides were determined on a Bellingham and Stanley Pepol-60 spectropolarimeter (United Kingdom) at 22°C.

The individuality of the compounds obtained was checked with the aid of thin-layer chromatography, electrophoresis, and high-performance liquid chromatography (HPLC).

Thin-layer chromatography was conducted on Silufol plates from the firm Kavalier (Czechoslovakia) and also on plates from the German firm Merck. The solvent systems used were: A) benzene—ethyl acetate—ethanol (3:1:1); B) n-butanol—acetic acid—water (4:1:1); C) sec-butanol—12 N ammonia—water (24:1:5); D) ethyl acetate—pyridine—acetic acid—water (60:20:6:11); E) ethyl acetate—pyridine—acetic acid—water (45:20:6:11); F) isopropanol—pyridine—acetic acid—water (10:5:4:4); G) t-ButOH—AcOH—H_2O (4:1:1); and H) n-ButOH—AcOH—Py—H_2O (15:3:10:12).

The electrophoresis of the peptides was performed on a Laboratorium Felszeresek instrument (Hungary) in 2% acetic acid on Filtrak FN-12 paper with a potential difference at the electrodes of 1500 V. Electrophoretic mobilities were determined relative to glycine (E_{Gly}).

High-performance liquid chromatography was conducted on a Du Pont chromatograph (USA) in the methanol—0.01 M trifluoroacetic acid system using Du Pont Zorbax C_8, 5 μm (4.6 × 250 mm) columns. Detection was carried out spectrophotometrically at a wavelength of 225 nm.

* The amino acid analyses of the peptide synthesized were carried out by V. V. Doshechkina.

TABLE 2. Comparison of the Analgesic Activities of the 16-21 and 16-21OMe Peptides in the Formalin Test

Peptide	Dose, μg/rat	Number of animals in the series	Beginning of the appearance of the pain reastion, s	Duration of the pain reaction, s
Control	0	14	4,5±1,68	362,6±33,55
16—21	1	13	21,7±5,16 α<0,01	247,2±18,88 α<0,01
16—21 OMe	0,01	12	18,3±5,05 α<0,02	185,5±29,23 α<0,001 α<0,1*

* Significant difference for the 16-21 and 16-21OMe peptides.

TABLE 3. Yields and Constants of the Intermediate Compounds of the Fragments of Salmon Calcitonin II

Compound	Formula	Yield, %	mp, °C	$[\alpha]_D^{23}$ (c 1; MeOH)
I	$C_{15}H_{10}N_2O_2$	56	116—117	—34
II	$C_{20}H_{38}N_3O_6$	65	179—183	—40
III	$C_{34}H_{56}N_6O_{10}$	78	169—175	—38
IV	$C_{40}H_{63}N_9O_{11}$	62	179—181	—32
V	$C_{45}H_{74}N_{10}O_{12}$	67	202—205	—34

The characteristics of the intermediate compounds are given in Table 3.

The amino acid and elementary analysis of the compounds synthesized corresponded to the calculated values.

The biological tests — namely, the hotplate test and the formalin test — were performed in the Institute of Experimental Medicine of the Russian Academy of Sciences under the direction of A. M. Cotin.

Methyl Ester of tert-Butoxycarbonylglutaminylthreonine (I). A solution of 8.94 g (0.062 mole) of HCl·H-Thr-OMe in dimethylformamide (DMF) was brought to pH 8 with N-methylmorpholine, and then 22.17 g (0.074 mole) of BOC—Gln—ONp was added and the mixture was stirred for a day, after which the DMF was evaporated off and the residue was triturated with ether; it was then dissolved in ethyl acetate and the solution was washed with water. The aqueous extracts were combined and evaporated, and the product so obtained was purified by column chromatography on silica gel L 100/160 (Chemapol, Czechoslovakia) in a 40 × 400 mm column, the column being washed successively with the solvents systems benzene—ethyl acetate—ethanol (3:1:1) and (1:1:1). R_f 0.37 (A), 0.40 (B), 0.72 (C) (Silufol).

Methyl Ester of tert-Butoxycarbonylleucylglutaminylthreonine (II). A solution of 12.15 g (0.034 mole) of BOC—Gln—Thr—OMe in trifluoroacetic acid (TFA) was stirred for 30 min, and the product was precipitated with ether, filtered off, and dried in a desiccator for 1 h. E_{Gly} = 1.98. The TFA·H—Gln—Thr—OMe was dissolved in DMF, the pH was brought to 8 with N-methylmorpholine, and 14.22 g (0.040 mole) of BOC—Leu—ONp was added and the mixture was stirred for a day. The DMF was evaporated off, and the residue was triturated with ether and dissolved in water-saturated butanol, after which the solution was washed with water, the butanol was evaporated off, and the residue was recrystallized from ethyl acetate. R_f 0.40 (A), 0.71 (B), 0.75 (C) (Silufol).

Ethyl N^{α}-tert-Butoxycarbonyl-N^{ε}-benzyloxycarbonyllysolleucylglutaminylthreonine (III). A solution of 1.45 g (0.003 mole) of BOC—Leu—Gln—Thr—OMe in TFA was stirred for 30 min, and the product was precipitated with ether, filtered off, and dried in the desiccator for 1 h. E_{Gly} = 1.51. The TFA·H—Leu—Gln—Thr—OMe was dissolved in DMF, the solution was brought to pH 8 with N-methylmorpholine, 2.26 g (0.0045 mole) of N^{α}-BOC-N^{ε}-Z—Lys—ONp was added, the mixture was stirred for a day, the DMF was evaporated off, the residue triturated with ether and dissolved in water-saturated butanol, the solution was washed with water, the butanol was evaporated off, and the product was recrystallized from isopropanol—ether. R_f 0.65 (A), 0.84 (B), p.75 (C) (Silufol).

Methyl Ester of tert-Butoxycarbonylhistidyl-N^{ε}-benzyloxycarbonyllysylleucylglutaminyl-threonine (IV). A solution of 2.4 g (0.003 mole) of N^{α}-BOC-N^{ε}Z—Lys—Leu—Gln—Thr—OMe in TFA—AcOH (7:3) was stirred for 30 min, and then the acid was evaporated off in vacuum

with benzene at 30°C and the residue was dried in a desiccator for 1 h. E_{Gly} = 1.05. The TFA·H–Lys(Z)–Leu–Gln–Thr–OMe was dissolved in DMF, the pH was brought to 8 with N-methylmorpholine, 2.0 g (0.005 mole) of BOC-His(Tos)-OH was added, the mixture was cooled to 0°C, and then a solution of 0.7 g (0.005 mole) of hydroxybenzotriazole (HOBt) in DMF and 1.05 g (0.005 mole) of dicyclohexylcarbodiimide (DCC) in DMF were added. The reaction mixture was stirred at 0°C for 1 h and was left in the refrigerator for a day, the urea was filtered off, the DMF was evaporated off, the residue was triturated with ether and was then dissolved in water-saturated butanol, the solution was washed with water, with 5% $NaHCO_3$ and with water again, the butanol was evaporated off and the residue was boiled with ethyl acetate. The product was deposited on a 30 × 150 column containing silica gel L 40/100. The column was washed with the benzene–ethyl acetate–ethanol (1:1:1) system and the substance was eluted with the ethyl acetate–pyridine–acetic acid–water (60:20:6:11) system. R_f 0.29 (B), 0.64 (C), 0.60 (D) (Silufol).

Methyl Ester of Histidyllysylleucylglutaminylthreonine (VI). A solution of 300 mg (0.34 mmole) of BOC-His-Lys(Z)Leu-Gln-Thr-OMe in TFA–AcOH (7:3) was stirred for 30 min, and the acid was evaporated off in vacuum with benzene at 30°C and the residue was dried in a desiccator; E_{Gly} = 1.6. The substance obtained was puridied by column chromatography on Merck silica gel 60 in the ethyl acetate–pyridine–acetic acid–water (45:20:6:11) system in a 13 × 330 column. The product was hydrogenated over a palladium catalyst in aqueous methanol, and final purification was performed by gel filtration on a Sephadex G-15, 20 × 1000 column, in 0.2 N AcOH. Then the preparation was lyophilized. R_f 0.59 (F) Merck. According to HPLC, the purity of the product was more than 96%.

Methyl Ester of tert-Butoxycarbonylleucylhistidyl-N^{ε}-benzyloxycarbonyllysylleucylglutaminylthreonine (V). A solution of 1.097 g (0.48 mmole) of BOC-His-Lys(Z)-Leu-Gln-Thr-OMe in TFA–AcOH (7:3) was stirred for 30 min, the acid was evaporated off in vacuum at 30°C, and the product was dried in a desiccator for 1 h. The TFA·H-His-Lys(Z)-Leu-Gln-Thr-OMe was dissolved in DFM, the pH of the solution was brought to 8 with N-methylmorpholine, 0.263 g (0.75 mmole) of BOC-Leu-ONp was added, the mixture was stirred for a day, the DMF was evaporated off, the residue was triturated with ether and dissolved in water-saturated n-butanol, the solution was washed with water, with 5% $NaHCO_3$, and with water, the butanol was evaporated off, and the product so obtained was boiled with ethyl acetate. Gel chromatography was carried out on Sephadex L-20 in methanol. R_f 0.35 (B), 0.71 (C), 0.74 (D) (Silufol). E_{Gly} = 0.84.

Methyl Ester of Leucylhistidyllysylleucylglutaminylthreonine (VII). A solution of 130 mg (0.137 mmole) of BOC-Leu-His-Lys(Z)-Leu-Gln-Thr-OMe in TFA–AcOH (7:3) was stirred for 30 min, the acid was evaporated off, and the residue was dried in a desiccator for 1 h. The product so obtained was purified on a 13 × 330 column of silica gel L 40/100 in the n-butanol–pyridine–acetic acid–water (45:20:6:11) system. R_f 0.46 (E), E_{Gly} = 1.45. The substance was dissolved in a 4.4% solution of formic acid in ethanol, the catalyst was added, and the mixture was stirred in an atmosphere of nitrogen for 1 h. The catalyst was filtered off, and the solvent was evaporated off. Final purification was carried out by gel filtration on Sephadex G-15 in 0.2 N AcOH. The product was lyophilized. R_f 0.83 (F), 0.25 (C) Merck), E_{Gly} = 2.19. According to HPLC, the purity of the product was 98%.

Methyl Ester of Lysylleucylserylglutaminylaspartylleucylhistidyllysylleucylglutaminylthreonine (VIII). A solution of 253 mg (0.266 mmole) of BOC-Leu-His-Lys(Z)-Leu-Gln-Thr-OMe in TFA–AcOH (7:3) was stirred for 30 min, the acid was evaporated off, and the residue was dried in a desiccator. The product obtained was dissolved in DMF, and after the pH had been brought to 8 with N-methylmorphyline, 195 mg (0.222 mmole) of Z-Lys(BOC)-Leu-Ser-Gln-Asp(OBut)-OH [4] and 184.79 mg (0.244 mmole) of complex F were added. The reaction mixture was stirred for a day, and then another 84.1 g (0.111 mmole) of complex F was added and the mixture was stirred for another 2 days. The urea was filtered off, the solvent was distilled off in vacuum, the residue was triturated with ether and was dissolved in water-saturated butanol, the solution was washed, the butanol was evaporated off, and product obtained was chromatographed on a 20 × 1000 column of Sephadex LH-20 in DMF, the fraction containing the desired product being treated with trifluoroacetic acid by the standard procedure. E_{Gly} = 1.1. R_f 0.25 (C) (Merck). The product obtained was hydrogenated in aqueous ethanol over a palladium catalyst. E_{Gly} = 2.14. R_f 0.26 (H). Final purification was carried out with the aid of reversed-phase HPLC. According to HPLC, the purity of the product was 95%. The peptide was lyophilized.

Methyl Ester of Tyrosylleucylhistidyllysylleucylglutaminylthreonine (IX). A solution of 241 mg (0.254 mmole) of BOC-Leu-His-Lys(Z)-Leu-Gln-Thr-OMe in TFA–AcOH (7:3) was stirred for 30 min, the acid was evaporated off, and the product was purified on a 10 × 400 column, with Merck silica gel 60 in the ethyl acetate–pyridine–acetic acid–water (45:20:6:11) system. The TFA·H-Leu-His-Lys(Z)-Leu-Gln-Thr-OMe was dissolved in DMF and, after the pH had been brought to 8 with N-methylmorpholine, 189 mg (0.500 mmole) of BOC-Tyr-ONSu was added. The reaction mixture was stirred for a day, the DMF was evaporated off, the residue was triturated with ether and dissolved in water-saturated n-butanol, the solution was washed with water, the butanol was evaporated off, and the product obtained was boiled in ethyl acetate. The residue was dissolved in TFA–AcOH (7:3), the solution was stirred for 30 min, the acid was evaporated off, and the peptide was purified on a 12 × 215 column with sulfoethyl-Sephadex C-25 in a gradient (0.005-1 M) of pyridine–acetate buffer. E_{Gly} = 1.57. R_f 0.55 (B), 0.48 (C). The benzyloxycarbonyl group was eliminated by hydrogenation in aqueous methanol over palladium black. The product obtained was purified by gel filtration on a 20 × 1000 column of Sephadex G-15 in 0.2 N AcOH, and the peptide was lyophilized. The purity of the product was more than 95% (according to HPLC). E_{Gly} = 2.2; R_f 0.16 (B), 0.92 (F).

Leucylhistidyllysylleucylglutaminylthreonine (X). This peptide was synthesized by the solid-phase method on a Pab-polymer using a Neosystem Laboratoire NPS-4000 synthesizer by a standard program. After preparative purification by the HPLC method, the purity of the product was more than 95%. The peptide was lyophilized.

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