AN ANALOG OF $ACTH_{4-10}$ — A LONG-ACTION STIMULATOR OF LEARNING

M. A. Ponomareva-Stepnaya, V. N. Nezavibat'ko,
L. V. Antonova, L. A. Andreeva, L. Yu. Alfeeva,
V. N. Potaman, A. A. Kamenskii,
and I. P. Ashmarin

UDC 615.357.814.3:
577.175.325].015.
154.012.1

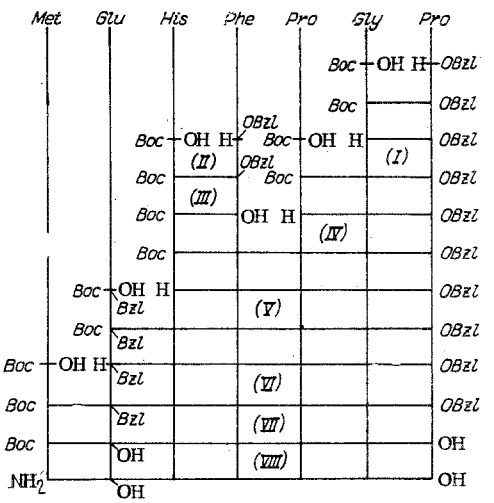
One of the promising approaches to the creation of new drugs is the synthesis and investigation of peptide regulators of a number of functions of the central nervous system. In particular, fragments of ACTH ($ACTH_{4-10}$, $ACTH_{4-7}$), devoid of hormonal activity, affect processes of learning in animals and man [3, 14]. It has been shown that they act on the central nervous sytem in extremely small doses (10-100 μg/kg) and are essentially harmless. An important shortcoming of these compounds is their brief action, possibly on account of the rapid degradation by enzymes [12], which hinders their use as drugs. In view of this, it is extremely important to prolong the action of the preparation.

A large number of analogs of the $ACTH_{4-10}$ fragment have been synthesized, and on the basis of investigations of structure versus function it has been concluded that an increase in behavioral activity is correlated with an increase in metabolic stability [10, 15]. The effect was basically achieved by introducing D-isomers of amino acids, more resistant to the action of peptidases, into the polypeptide chain [4].

At the same time it is known that portions of the polypeptide chain containing pyronine residues are less subject to decomposition by peptidases. To obtain compounds with increased metabolic stability we synthesized a series of analogs of $ACTH_{4-10}$, into which proline-glycine sequences were introduced from the C-end. The analog $ACTH_{4-7}$-Pro-Gly-Pro [15] (methionyl-glutamyl-histidyl-phenylalanyl-prolyl-glycyl-proline) proved the most effective.

The synthesis of the peptides was carried out according to the scheme presented, using 1-hydroxybenzotriazole (BT) and N,N'-dicyclohexylcarbodiimide (DCHC) [8, 11].

Scheme 1



Scheme of synthesis of the investigated peptides.

As the starting materials we used N^{α}-tert-butyloxycarbonyl (Boc) amino acids from Reanal (Hungary) and Serva (Republic of Germany). The carboxyl group of proline was protected by a benzyl ester [13]. Purification of the intermediate products was carried out by reprecipitation from the corresponding solvents. The individuality of all the synthesized com-

Institute of Molecular Genetics, Academy of Sciences of the USSR, M. V. Lomonosov Moscow University. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 18, No. 7, pp. 790-795, July, 1984. Original article submitted October 18, 1983.

0091-150X/84/1807-0445$08.50

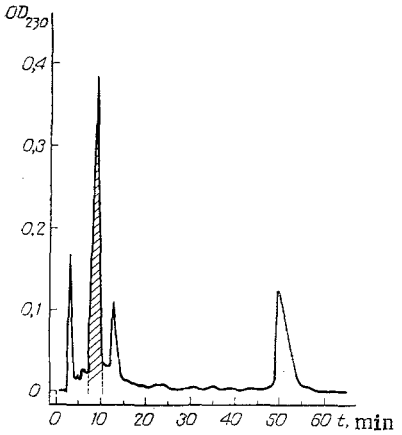


Fig. 1

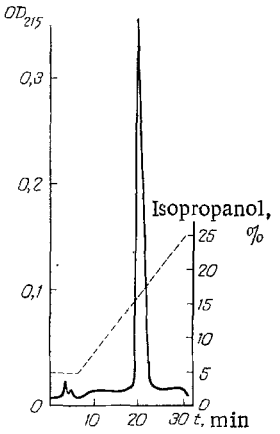


Fig. 2

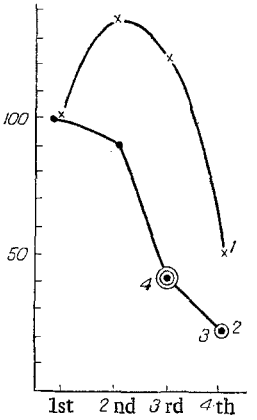


Fig. 3

Fig. 1. Isocratic preparative isolation of the peptide Met-Glu-His-Phe-Pro-Gly-Pro. Zorbax ODS column (21.2 × 250 mm), eluent 0.01 M CH_3COONH_4 pH 4.0 + 7.5% isopropanol, rate of flow 10 ml/min, t 50°C. The shading marks a collective peak corresponding to the peptide sought (15 mg), homogeneous according to thin-layer chromatography and used for analytical separation. Here and in Fig. 2, along the x axis: time (in min); along y axis: optical density (OD).

Fig. 2. Analytical chromatography of the peptide Met-Glu-His-Phe-Pro-Gly-Pro (0.2 mg). Zorbax ODS column (4.6 × 250 mm), linear sucrose concentration gradient in 0.01 M CH_3COONH_4, pH 4.0, rate of flow 1 ml/min, t 50°C.

Fig. 3. Acceleration of the learning of rats under the action of $ACTH_{4-10}$. 1) Control; 2) administration of $ACTH_{4-10}$ in a dose of 0.015 mg/kg 30 min before learning; 3) $P < 0.05$; 4) $P < 0.01$. Here and in Figs. 4 and 5, along x axis: days of learning; along y axis: CHNR (in percent of the first day of learning).

pounds was monitored by chromatography in a thin layer on Silufol UV-254 plates (Czechoslovakia). In this case various chromatographic systems are selected in such a way that the reaction products differed maximally in mobility from the starting materials, as well as possible side products.

A 50% solution of trifluoroacetic acid (TFA) in methylene chloride was used to remove the Boc-protection from the intermediate products. Benzyl esters are eliminated by transfer hydrogenation using cyclohexene with boiling in absolute ethanol medium over the catalyst palladium black [6, 7, 9]. The Boc-protection is removed from the end products by treatment with a solution of hydrogen chloride in acetic acid, and the peptide hydrochlorides obtained are desalted on Amberlist resin from Serva (Federal Republic of Germany) (A-21, acetate form) for exchange of the hydrochloride for the acetate.

If the product is not chromatographically individual, purification is performed by high-efficiency liquid chromatography on a model 830 instrument from Du Pont de Nemours (USA) (Fig. 1). The peptide obtained is investigated according to the method of teaching the animals a food-getting habit in a T-shaped maze, and also according to the "open-field" method and on an Aminex instrument from LKB (Sweden) comparing with native $ACTH_{4-10}$.

EXPERIMENTAL CHEMISTRY

Peptide synthesis was monitored on Silufol plates, spraying with ninhydrin solution in the following solvent systems: N-butanol—acetic acid—water, 5:1:2 (A); chloroform—methanol (14:1) (B); N-butanol—acetic acid—pyridine—water (30:6:20:24) (C); chloroform—methanol—ammonia, 10:5:1 (D); isobutanol—acetone—ammonia 12%, 5:3:2 (E); isopropanol—formic acid—water, 20:1:5 (F). All the solvents were rendered absolute in the following way. Acid hydrolysis of the end after stripping off of the protective groups was performed under standard condi-

tions (6 N HCl, 100°C, 48 h), and the hydrolysis products were investigated by two-dimensional chromatography ("fingerprint") in the solvent system E and F, as well as on a Hitachi LA-3B amino acid analyzer (Japan). All the amino acids have the L-configuration. The melting points were not corrected. The specific optical rotation was measured on a digital polarimeter from Perkin-Elmer, model 141 (USA).

Equipment from Du Pont de Nemours (USA) was used for reversed-phase chromatography of the peptide: Zorbax ODS column (4.6 × 250 mm); model 830 chromatograph; model 838 gradient device, spectrophotometric detector (Figs. 1 and 2).

Boc-Pro-Gly-ProOBzl (I). To a solution of 675 mg (3.14 mmoles) Boc-Pro in 10 ml tetrahydrofuran (THF) we added 475 mg (3.14 mmoles) BT, cooled to 0°C, and added 710 mg (3.45 mmoles) DCHC in 5 ml THF. After mixing for 1 h, a solution (3.14 mmoles) of the trifluoroacetate of the benzyl ester of Gly-Pro and 0.44 ml (3.14 mmoles) of triethylamine (TEA) in 10 ml THF was added in portions (over 2 h) to the reaction mixture at room temperature.

After mixing for 2 days at room temperature, the precipitate of N,N'-dicyclohexylurea (DCU) was filtered off, the filtrate evaporated under vacuum, and the residue dissolved in ethyl acetate (EA). The solution was washed successively with water, with 0.1 N HCl solution, with water, with 5% $NaHCO_3$ solution, with water, and dried over magnesium sulfate. The filtrate was evaporated under vacuum. The oil remaining was ground with absolute ether, whereupon a precipitate gradually crystallized, was filtered off, and dried under vacuum. Yield 750 mg I (53%); mp 120-2°C, R_f 0.65 (A), 0.74 (B).

Boc-His-Phe-OBzl (II) was synthesized analogously to I (with the exception of washing the ethyl acetate solution with 0.1 N HCl) from 10 mmoles of Boc-His and 10 mmoles of the p-toluenesulfonate of the benzyl ester of phenylalanine with an 88% yield. mp 164-6°C, R_f 0.43 (A), 0.836 (B).

Boc-His-Phe (III). A. A 350-mg (0.75 mmoles) portion of II was dissolved in 5 ml of methanol, 0.5 ml of acetic acid was added, along with the catalyst Pd-black, and hydrogenation was conducted at room temperature for 10 h. After the catalyst was filtered off, the solution was evaporated under vacuum and the residue ground with absolute ether. The precipitate was filtered off and dried under vacuum. Yield 270 mg III (76%); mp 173-4°C, R_f 0.35 (A).

B. A 300-mg (0.606 mmoles) portion of II was boiled in 10 ml of absolute ethanol and 3 ml of cyclohexene with an addition of 65 mg Pd-black for 1 h. Then the catalyst was filtered off and washed repeatedly with absolute ethanol. The filtrate was evaporated to dryness under vacuum, then the oil remaining was ground with absolute ether and again evaporated under vacuum. Grinding with ether produced a precipitate, which was filtered off and dried in the vacuum desiccator. Yield 226 mg III (93%), mp 172-4°C, R_f 0.35 (A). (The catalyst should not be dried out completely but should be accurately decanted by washing with the ethanol remaining in the flask. Then the catalyst is covered with absolute ethanol, which permits its reuse in a similar experiment.)

Boc-His-Phe-Pro-Gly-Pro-OBzl (IV). A 500-mg portion of I was dissolved in 3 ml methylene chloride, 3 ml TFA was added, and it was left for 40 min at room temperature. The solution was evaporated under vacuum at the temperature 25-30°C, absolute ether added, the gradually separating precipitate washed several times by decantation with ether and dried under vacuum over NaOH. Yield quantitative. R_f 0.01 (A), 0.085 (B). The peptide IV was synthesized from equimolar amounts of III and TFA-Pro-Gly-Pro-OBzL analogously to II with a yield of 70%. mp 160-2°C, R_f 0.64 (A), 0.08 (B).

Boc-γ-Bzl-Glu-His-Phe-Pro-Gly-Pro-OBzl (V). Synthesized from equimolar amounts of Boc-γ-Bzl-Glu and TFA-His-Phe-Pro-Gly-Pro-OBzl analogously to II with a yield of 75%. mp 168-70°C, R_f 0.38 (A), 0.815 (C).

Boc-Met-γ-Bzl-Glu-His-Phe-Pro-Gly-Pro-OBzl (VI). A 475-mg portion of Boc-Met-DCHC (1.1 mmoles) was suspended in 50 ml of ether, 20 ml of 0.2 N H_2SO_4 was added, and the mixture was shaken thorougly in a separatory funnel. The ether layer was removed and washed with water to pH 7.0, then dried over $MgSO_4$ and evaporated. The oil remaining (230 mg, or 0.92 mmole) was dissolved in 8 ml of THF, 125 mg (0.92 mmole) BT and 210 mg DCHC were added, and the mixture mixed for 1 h with cooling.

Then a solution (0.92 mmole) of TFA-γ-Bzl-Glu-His-Phe-Pro-Gly-Pro-OBzl and 1.3 ml (0.92 mmole) TEA in 10 ml of THF was added in 4 portions over 2 h. After mixing for three days the reaction mixture was treated as in the production of I.

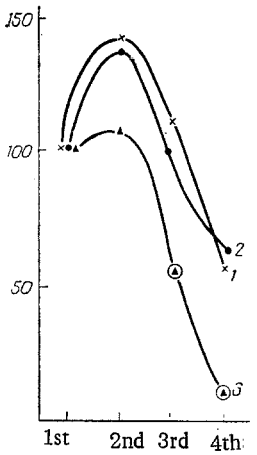


Fig. 4

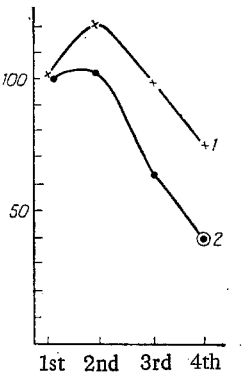


Fig. 5

Fig. 4. Acceleration of the learning of rats under the action of $ACTH_{4-7}$-Pro-Gly-Pro. 1) Control; 2) administration of $ACTH_{4-10}$ in a dose of 0.015 mg/kg 1 h before learning; 3) administration of $ACTH_{4-7}$ in a dose of 0.015 mg/kg 1 h before learning.

Fig. 5. Influence of $ACTH_{4-7}$-Pro-Gly-Pro on learning in rats. 1) Control; 2) administration of $ACTH_{4-7}$ in a dose of 0.05 mg/kg 20 h before learning.

The peptide VI obtained was reprecipitated twice from MeOH and ethyl acetate with ether.

Yield 585 mg VI (58%). mp 98-100°C; R_f 0.36 (A), 0.76 (C); $[\alpha]_D^{23} = -50.0°$ [c 0.12; acetic acid].

Boc-Met-Glu-His-Phe-Pro-Gly-Pro (VII). A 75-mg (0.062 mmole) portion of VI was boiled in 1 ml of absolute ethanol and 1 ml 1,4-cyclohexadiene in the presence of 15 mg Pd-black for 2 h.

The catalyst was filtered off and washed repeatedly with ethanol. The filtrate was evaporated under vacuum; the oil remaining was dissolved in 0.5 ml of methanol and the product precipitated by the addition of absolute ether. Yield 48 mg VII (76%), R_f 0.26 (A), 0.96 (D).

H-Met-Glu-His-Phe-Pro-Gly-Pro (VIII). A 48-mg portion of VII was dissolved in 0.5 ml of 1 N HCl in acetic acid and exposed at room temperature for 30 min. When absolute ether was added, a precipitate formed; it was washed several times with absolute ether by decantation, then dissolved in 2 ml of absolute MeOH and precipiated with absolute ether. After drying under vacuum over P_2O_5 and NaOH, we obtained 25 mg VIII, which was dissolved in 0.3 ml of water and desalted on a column with Amberlist A-21 (AcO^- form), eluting with 20 ml of H_2O. Then the aqueous solution was evaporated under vacuum and the product remaining evaporated several times with methanol and precipitated from absolute MeOH with ether. Yield 20 mg VIII (57%). R_f 0.09 (A), 0.29 (C), $[\alpha]_D^{23} = 67.55°$ (c 0.34; AcOH).

Amino acid analysis: Met 0.93 (1.0), Glu 1.00 (1.0), His 0.96 (1.0), Phe 1.00 (1.00); Pro 1.80 (2.0), Gly 1.10 (1.0).

EXPERIMENTAL BIOLOGY

Biological testing of the action of the peptides was performed according to the method of teaching the animals a food-getting habit of running to the reinforcement site (henceforth food habit) in a T-shaped maze. Noninbred male white mice weighing 150-250 g were used in the experiments. Each experiment was reproduced twice. In one experiment 10 experimental (which received the peptide) and 10 control animals (which received distilled

water) were used. The preparations were injected intraperitoneally. The rats were placed in the maze for two days for extinction of the orientative response (for 1 h per day). During these two days the animals were fed only in the maze; the food (bread) was distributed uniformly over the entire maze. During the following four days learning was conducted: Each animal was placed in the maze for 3 min five times in a row on each day of training. Bread, the location of which in one of the sections was always constant, was used as the lure. The time from the moment of placement until the animal left the starting chamber (latent period); the time required by the animal for entering the required section of the maze and taking the lure (reaction time); the number of errors, i.e., the number of entrances into the opposite section of the maze; and the number of cases when the animal did not cope with the experimental problem — did not find the lure in a 3 min stay in the maze (number of unperformed responses — NUR) were recorded in the experiments. The data obtained in each series of experiments were averaged and subjected to statistical treatment. The significance of the differences between averages was estimated using the Mann—Whitney—Wilcoxon criterion [2].

RESULTS AND DISCUSSION

The main effect of $ACTH_{4-10}$ under our experimental conditions was manifested in a more rapid decrease in the NUR during training in the animals of the experimental group in comparison with the control. The decrease in the NUR during training of the animals reflects the process of establishment of an adequate response to the given situation, which is actually the essence of the learning process. Consequently, the change in the NUR can be considered as an index of learning, and the more rapid decrease in this index under the influence of $ACTH_{4-10}$ means that this peptide stimulates the animals to learn the food habit. It was shown that stimulation of the process of establishment of a food habit by $ACTH_{4-10}$ is closely dependent on the time of administration [1] and is manifested in the case of intraperitoneal injection of this peptide in a dose of 0.015 mg/kg no earlier than 30 min before learning (Fig. 3).

The administration of such a peptide 1 h before the learning session did not change the rate of acquisition of the habit in the experimental animals in comparison with the controls. The $ACTH_{4-10}$ analog produced — $ACTH_{4-7}$-Pro-Gly-Pro — accelerated the development of the food habit in a T-shaped maze among the experimental animals when it was administered in a dose of 0.015 mg/kg 1 h before learning, in contrast to $ACTH_{4-10}$ (Fig. 4). This peptide also gives the same effect when it is administered 20 h before the training session, but when the active dose is increased to 0.05 mg/kg (Fig. 5), i.e., the addition of the amino acid sequence Pro-Gly-Pro from the C-end to $ACTH_{4-7}$ prolongs the stimulating influence of the natural fragment to the development of a food habit in a T-shaped maze. Supplementary tests showed that the analog of $ACTH_{4-10}$ does not significatly change the level of the orientative response and the emotional state of the animals in the "open-field" test and their motor activity measured on a Animex instrument. Moreover, no influence of the investigated peptide in a dose of 0.05 mg/kg was noted on the heartbeat rate, respiration rate, and body temperature of immobilized animals, which is evidence of high specificity of the action of the peptide on processes associated with learning.

Thus, the proposed approach to reducing the rate of degradation of peptides is extremely promising and will permit the production of new analogs effective for a long time.

LITERATURE CITED

1. L. V. Antonova, Influence of Fragments of ACTH and Their Analogs on Learning and Certain Physiological Reactions of White Rats. Author's Abstract of Candidate's Dissertation [in Russian], Moscow (1981).
2. I. P. Ashmarin, N. N. Vasil'ev, and V. A. Ambrosov, Rapid Methods of Statistical Treatment and Planning of Experiments [in Russian], Second Edition, Leningrad (1976).
3. V. I. Medvedev, V. D. Bakharev, and V. N. Nezavibat'ko, in: Eighth All-Union Conference on the Electrophysiology of the Central Nervous System [in Russian], Erevan (1980), p. 361.
4. US Patent No. 3850904 (1974).
5. M. A. Ponomareva-Stepnaya, V. N. Nezavibat'ko, I. P. Ashmarin, et al., USSR No 939440 (1981).
6. P. W. Baut and F. A. Haak, Rec. Trav. Chim. Pays Bas., 67, 85 (1948).

7. H. Boer and P. M. Duinber, ibid., 77, 346 (1958).
8. Eösuka Munokata, J. Synth. Org. Chem., 31, 85 (1973).
9. A. M. Felix, E. O. Heimer, T. S. Sambros, et al., J. Org. Chem., 43, 4194 (1978).
10. J. Kelder and H. Greven, J. Toy. Netherl. Chem. Soc., 98, 168 (1979).
11. W. König and R. Geiger, Chem. Ber., 103, 788 (1970).
12. N. Marks, in: Peptides in Neurobiology, London (1977), p. 221.
13. G. Li and C. H. Ramachandran, J. Org. Chem., 28, 173 (1963).
14. H. Van Rieszen, H. Rigter, and D. De Wied, Behare Biol., 20, 311-324 (1977).
15. A. Witter, H. Greven, and D. De Wied, J. Pharmacol. Exp. Ther., 193, 853 (1975).

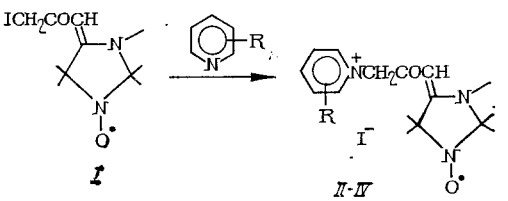
SPIN-LABELED ANALOGS OF ACETYLCHOLINESTERASE

V. A. Reznikov, T. I. Reznikova, and L. B. Volodarskii

UDC 615.31:547.781].012

It is well known that alkylpyridinium aldoximes [5] and compounds with structures analogous to the structure of isonitrosoacetone [4] are effective acetylcholinesterase reactivators when it is inhibited by organophosphorus compounds. Since nitroxyl radicals can be detected by EPR spectroscopy at extremely low concentrations (10^{-9}-10^{-7} M) [2], analogs of acetylcholinesterase reactivators that contain a nitroxyl radical center could prove to be useful for the study of the mechanism of the action of such compounds on inactivated acetylcholinesterase.

We have previously shown [1] that a pyridinium salt (II) is formed in the reaction of a nitroxyl radical (I) with pyridine. This reaction was used for the synthesis of spin-labeled pyridinium aldoxime salts. In the reaction of I with 2-oximinomethylpyridine one might have expected the formation of products of alkylation at both the oxime group and at the pyridine nitrogen atom; however, the reaction gives only one compound, the spectral characteristics of which are similar to those of pyridinium salt II [1], on the basis of which the 2-oximinomethyl-1-[3-(2,2,3,5,5-pentamethyl-1-oxylimidazolidin-4-ylidene)-2-oxopropyl]-pyridinium iodide (III) structure was assigned to it. Under similar conditions the reaction of I with 4-oximinomethylpyridine leads to 4-oximinomethyl-1-[3-(2,2,3,5,5-pentamethyl-1-oxylimidazolidin-4-ylidene)-2-oxopropyl]pyridinium iodide (IV).



II: R = H; III: R = *o*-CH=NOH; IV: R = P-CH=NOH.

In order to obtain a spin-labeled analog of isonitrosoacetone we treated pyridinium salt II with p-nitroso-N,N-dimethylaniline under the conditions of the Kröhnke reaction, as a result of which N-[4-(2-oxo-1-propyliden-3-ylidene)-2,2,3,5,5-pentamethylimidazolidinyl-1-oxyl]-p-N,N-dimethylaminophenylamine N-oxide (V) is formed. Treatment of V with hydroxylamine hydrochloride leads to the formation of VI, which has an EPR spectrum in the form of the characteristic (for nitroxyl radicals) triplet with hyperfine interaction constant α_N = 14.0 Oe. Absorption bands at 1610 and 1550 cm^{-1}, which are characteristic for an enamino ketone grouping [3], are observed in the IR spectrum of this compound; absorption with λ_{max} 343 nm (log ε = 4.26) is observed in the UV spectrum. On the basis of the spectral characteristics and the results of elementary analysis we assigned the 2,2,3,5,5-pentamethyl-4-(3-oximino-2-oxopropylidene)imidazolidine-1-oxyl structure — a structural analog of isonitrosoacetone — to VI.

Novosibirsk Institute of Organic Chemistry, Siberian Branch, Academy of Sciences of the USSR. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 18, No. 7, pp. 795-798, July, 1984. Original article submitted August 9, 1983.

 0091-150X/84/1807-0450$08.50