5. V. I. Svergun, M. B. Smirnov, V. N. Karel'skii, E. P. Krysin, and V. P. Panov, Khim.-farm. Zh., No. 6 (1980).
6. M. A. Chlenov, E. V. Titova, L. I. Kudryashov, V. N. Karel'skii, and A. A. Antonov, Fifth All-Union Symposium on the Chemistry and Physics of Proteins and Peptides [in Russian], Moscow (1980), p. 245.
7. E. P. Krysin, V. N. Karel'skii, A. A. Antonov, and É. D. Glinka, Khim. Prir. Soedin., 482 (1978).

# SYNTHESIS OF FRAGMENT 8-12 OF THE NATURAL SEQUENCE OF ACTH

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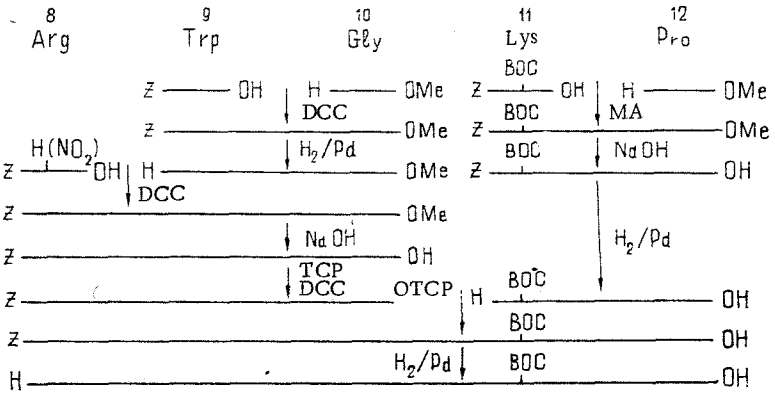
Two new variants of the synthesis of the pentapeptide 8-12 of the natural sequence of ACTH are presented. In some stages, the trimethylsilyl group was used as temporary protection of the carboxy group of the amino component. The final and intermediate compounds were obtained with good yields, were distinguished by chromatographic homogeneity, and were characterized by their angles of optical rotation, melting points, and electrophoretic behavior. Their purity was checked by TLC and by high-pressure liquid chromatography. Some physicochemical characteristics (angles of optical rotation, melting points, chromatographic mobilities) of the compounds obtained are given.

Pentapeptide 8-12 of the natural sequence of ACTH [1] is one of the widely used intermediate fragments in the synthesis of the whole moiety of the adrenocorticotropic hormone.

The total synthesis of this compound was carried out by the following scheme (BOC — tert-butoxycarbonyl; Z — benzyloxycarbonyl; TCP — trichlorophenol; MA — mixed anhydride method; DCC — dicyclohexylcarbodiimide).




We have developed a simpler (three stages shorter) scheme of synthesis of this compound in which it has been possible to avoid the use of DCC — a strong allergen. As the condensing agents we used N-ethoxycarbonyl-1,2-ethoxydihydroquinoline (EEDQ) and ethyl chloroformate. The synthesis was carried out by the scheme* on the next page.

The trityl protection was smoothly removed from the N atom of glycine by acetic acid, and the benzyloxycarbonyl and benzyl groups were eliminated by reduction with hydrogen over palladium black. In addition, we have developed another original variant of the synthesis of a similar compound which predicates the wide use in a number of stages of the trimethylsilyl

*All amino acids in the L form.

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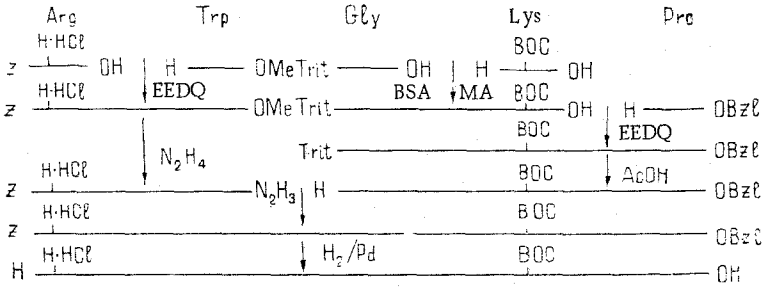
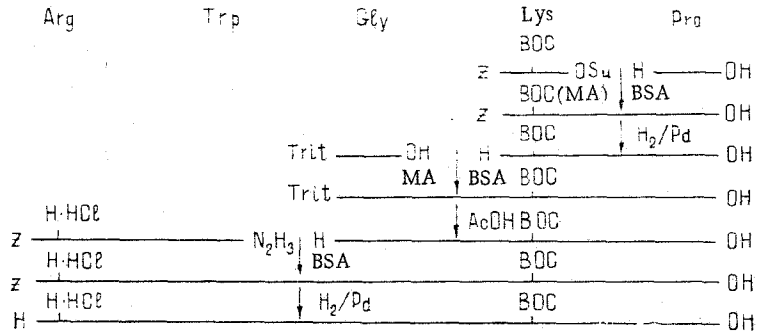



group as temporary protection of the carboxyls of amino acids and peptides: (BSA — bis(trimethylsilyl)acetamide; Su-OH — hydroxysuccinimide).




As previously [2], the silylation of amino acids and peptides was carried out under mild conditions at room temperature using bis(trimethylsilyl)acetamide as the silylating agent. The trimethylsilyl derivatives were used in the stage of peptide condensation without isolation. In the later variant, azide synthesis in the process of forming the pentapeptide also took place mostly only with the silylated derivative of the tripeptide glycyllysylproline.

In the synthesis of lysylproline we used the hydroxysuccinimide ester of $N^{\alpha}$-benzyloxycarbonyl-$N^{\varepsilon}$-tert-butoxycarbonyllysine.

In principle in this stage it is possible to use the mixed-anhydride (MA) method, but in this case the quality of the corresponding dipeptide obtained is lower.

The pentapeptide and intermediate compounds were obtained in the two variants with good yields. They were distinguished by chromatographic homogeneity and were characterized by angles of optical rotation, melting points, electrophoresis, TLC, and high-pressure liquid chromatography. Below, we give some physicochemical characteristics of the compounds synthesized.

| Compound | mp, °C | $[\alpha]_D^{20}$, deg (c 1; MeOH) |
|---|---|---|
| Z-Arg (H·HCl) TrpOMe | — | +9,5 |
| Z-Arg (H·HCl) $TrpN_2H_3$ | 95—97 | —10.0 |
| Trit GlyLyz (BOC)-OH | 72—73 | +6,5 |
| Trit-GlyLyz (BOC) ProOBzl | — | —33,5 |
| H-GlyLyz (BOC) ProOBzl | — | —42,0 |
| Z-Arg (H·HCl) TrpGlyLyz (BOC) ProOBzl | 185—190 | —35.0 |
| H-Arg (H·HCl) TrpGlyLyz (BOC) ProOH | — | —9,5 |
| Z-Lyz (BOC) ProOH | 70—72 | —38.0 |
| H-Lyz (BOC) ProOH | 126—128 | —45.0 |
| Trit-GlyLyz (BOC) ProOH | 80—82 | —27,0 |
| H-GlyLyz (BOC) ProOH·AcOH | — | —37.0 |
| Z-Arg (H·HCl) TrpGlyLyz (BOC)ProOH | 198—200 | —27,0 |

## EXPERIMENTAL

Melting points were determined in open capillaries without correction, and angles of optical rotation on a polarimeter. Chromatographic purities and mobilities were determined by the TLC method on Silufol plates in the chloroform—ethanol system, and electrophoretic homo-

geneities and mobilities by paper electrophoresis in a laboratory apparatus in the pyridine—acetic acid—water (1.2:1.0:100) system. Chromatographic analysis of the compounds by high-pressure liquid chromatography was performed on a Spectra Physics 3500 B instrument. Conditions: stationary phase Zorbax ODS.

Column 250 × 4.6 mm, mobile phase methanol—0.005 M tetrabutylammonium bromide (95:5). A LC-55 UV spectrometer was used as the detector. Detection was carried out at a wavelength of λ 220 nm. The volume of sample introduced was 10 μl (10 mg of sample in 1 ml of solution) and the rate of flow was 0.7 ml/min.

1. Preparation of Z-Arg(H·HCl)TrpOMe. With stirring, 5.4 g (26 mmole) of EEDQ was added to a solution of 7.6 g (25 mmole) of $N^{\alpha}$-benzyloxycarbonylarginine and 6.4 g (25 mmole) of the hydrochloride of the methyl ester of tryptophan in 25 ml of DMFA cooled to 0°C. After the components had dissolved completely, the reaction mixture was kept at room temperature for 10-12 h, and it was then diluted with 50 ml of methylene chloride and was washed successively with water, with 1 N hydrochloric acid, and with a 50% aqueous solution of methanol. The organic layer was evaporated and the residue was dried in vacuum to constant weight. Yield 90%; practically chromatographically homogeneous; $R_f$ 0.75 (7:3 system).

2. Preparation of Z-Arg(H·HCl)Trp$N_2H_3$. A solution of 41 g (75 mmole) of the methyl ester of $N^{\alpha}$-benzyloxycarbonylarginyltryptophan in 150 ml of methanol was treated with hydrazine hydrate, and the mixture was left at room temperature for 18-20 h. Then it was evaporated in vacuum to 2/3 of its initial volume, diluted with 100 ml of water, and washed with chloroform (3 × 25 ml). The reaction product was extracted from the salt-saturated aqueous layer with butanol, and the extract was washed successively with a saturated solution of NaCl and a saturated solution of ammonium chloride. Then it was dried by filtration through absorbent cotton in a chemical funnel and was concentrated in vacuum.

The desired product was isolated by treating the residue with an excess of chloroform. The hydrazide obtained was separated off on a glass filter, washed with chloroform, and dried in vacuum. Yield 70%; chromatographically homogeneous; $R_f$ 0.36 in the (7:3) system.

3. Preparation of Trit-GlyLys(BOC)OH. A solution of 12.8 g (40.3 mmole) of tritylglycine in 50 ml of methylene chloride was treated with 5.6 ml of triethylamine, and 4.2 ml (44 mmole) of ethyl chloroformate was added at a temperature of —20 to —25°C. The mixture was stirred at —15°C for 15 min and was added to silylated $N^{\varepsilon}$-tert-butoxycarbonyllysine, prepared from 10 g (41 mmole) of the corresponding lysine derivative, and 16 ml of BSA in 40 ml of methylene chloride cooled to —20°C [2]. The reaction mixture was stirred at —5°C for an hour and then at room temperature for 2 h. The reaction products were diluted with methylene chloride and washed successively with 1 N HCl and with water. The organic layer was dried by filtration through sodium sulfate and absorbent cotton in a chemical funnel. The solvent was driven off in vacuum. Yield 96%; chromatographically homogeneous; $R_f$ 0.60 in the (9:1) system.

4. Preparation of Trit-GlyLys(BOC)ProOBzl. By the method of paragraph 1, 8.2 g (15 mmole) of trityl-glycyl-$N^{\varepsilon}$-tert-butoxycarbonyllysine, 3.7 g (15.3 mmole) of the hydrochloride of the benzyl ester of proline, 2.2 ml of triethylamine, and 3.9 g (16 mmole) of EEDQ yielded 10.4 g (94%) of the corresponding tripeptide. Chromatographically homogeneous; $R_f$ 0.80 in the (9:1) system.

5. Preparation of H-GlyLys(BOC)ProOBzl. Water was added dropwise to a solution of 10 g of the benzyl ester of trityl-glycyl-$N^{\varepsilon}$-tert-butoxycarbonyllysylproline in 20 ml of glacial acetic acid until a permanent turbidity appeared, and the mixture was left at room temperature for 10-12 h. Then it was diluted with water, washed with ether, and neutralized with ammonia to pH 9. The oil that precipitated was extracted with methylene chloride, the organic layer was dried by passage through a layer of absorbent cotton and sodium sulfate in a chemical funnel, and the solvent was driven off in vacuum. Yield 60%; electrophoretically homogeneous, E = 100 mm, τ = 3 h, σ = 15 V/cm.

6. Preparation of Z-Arg(H·HCl)TrpGlyLys(BOC)ProOBzl. A solution of 6.3 g (11.5 mmole) of the hydrazide of $N^{\alpha}$-benzyloxycarbonylarginyltryptophan in 10 ml of DMFA cooled to —10°C was treated with 20 ml of 1 N HCl, and, with stirring, 0.85 g (12.3 mmole) of sodium nitrate was added. After 5 min, 10 ml of chloroform, 3.8 ml of triethylamine (pH 9), and then a solution of 5.8 g (12 mmole) of the benzyl ester of glycyl-$N^{\varepsilon}$-tert-butoxycarbonyllysylproline in 10 ml of chloroform cooled to —20°C were added. The organic layer was separated off, and

the aqueous layer, after being saturated with sodium chloride, was treated additionally with 15 ml of chloroform and the combined organic extract was kept at 0°C for 20 h. The reaction mixture was diluted with chloroform and was washed with 50% aqueous methanol that had been acidified with hydrochloric acid (pH 2-3) and, repeatedly, with 50% aqueous methanol. The solvent was eliminated in vacuum. Yield 72%; electrophoretically homogeneous, E = 75 ml. $\tau$ = 3 h, $\sigma$ = 15 V/cm.

7. Preparation of H-Arg(H·HCl)TrpGlyLys(BOC)ProOH. In 50 ml of ethanol, 8.2 g (8.2 mmole) of the benzyl ester of $N^{\alpha}$-benzyloxycarbonylarginyltryptophanylglycyl-$N^{\varepsilon}$-tert-butoxycarbonyllysylproline was reduced in a current of hydrogen at 50°C with vigorous shaking over palladium black (~1 g). Monitoring was carried out by the TLC method (from the total disappearance of the initial pentapeptide). Then the ethanol was evaporated off in vacuum the residue was dissolved in 50 ml of 30% aqueous methanol, and the solution was extracted with chloroform. The chloroform extract was washed with water, and the aqueous and aqueous methanol extracts were combined and evaporated in vacuum. Yield 88%; electrophoretically homogeneous, E = 90 mm, $\tau$ = 3 h, $\sigma$ = 15 V/cm.

8. Preparation of Z-Lys(BOC)ProOH. A solution of 10 g (21 mmole) of the hydroxysuccinimide ester of $N^{\alpha}$-benzyloxycarbonyl-$N^{\varepsilon}$-tert-butoxycarbonyllysine [3] in 23 ml of methylene chloride was added to the silylated protein derivative obtained from 2.53 g (22 mmole) of proline, 132 ml of BSA, and 23 ml of ethylene chloride. The mixture was stirred for 2 h and was left at room temperature for 10-12 h. Then the reaction products were diluted with methylene chloride and were washed successively with 0.5 N HCl and with water. The organic layer was dried with sodium sulfate and the solvent was driven off in vacuum. The residue was triturated with petroleum ether and the product was dried in vacuum. Yield 98%; chromatographically homogeneous, $R_f$ 0.50 in the (9:1) system.

9. Preparation H-Lys(BOC)ProOH. By reduction over palladium black in ethanol by the method of paragraph 7, 10 g (21 mmole) of $N^{\alpha}$-benzyloxycarbonyl-$N^{\varepsilon}$-tert-butoxycarbonyllysylproline yielded 6.55 g (91%) of product. Electrophoretically homogeneous, E = 89 mm, V = 520 V, I = 20 mA, $\tau$ = 2.5 h.

10. Preparation of Trit-GlyLys(BOC)ProOH. Using the MA method in methylene chloride as described in paragraph, 3, 3.17 g (10 mmole) of tritylglycine, 3.5 g (10 mmole) of $N^{\varepsilon}$-tert-butoxycarbonyllysylproline, and 5.5 ml (23 mmole) of BSA yielded 6.2 g (95%) of product. Chromatographically homogeneous, with $R_f$ 0.67 in the (8:2) system.

11. Preparation of H-GlyLys(BOC)ProOH·AcOH. A solution of 6.1 g (9.5 mmole) of tritylglycyl-$N^{\varepsilon}$-tert-butoxycarbonyllysylproline in 36 ml of glacial acetic acid was treated with 6 ml of water and was then left at room temperature for 10-12 h. The reaction mixture was diluted with 60 ml of water, the triphenyl carbinol that separated out was filtered off, and the aqueous solution was washed with methylene chloride and evaporated in vacuum. The product was dried in vacuum. Yield 68%; electrophoretically homogeneous, $\tau$ = 3 h, V = 500 V I = 10 mA, E = 84 mm.

12. Preparation of Z-Arg(H·HCl)TrpGlyLys(BOC)ProOH. To a solution of 3 g (6.5 mmole) of the acetate of glycyl-$N^{\varepsilon}$-tert butoxycarbonyllysylproline in 10 ml of DMFA was added 0.9 ml of triethylamine, and then, after 15 min, 3 ml (12.6 mmole) of BSA and, after 3 h, another 0.9 ml of triethylamine, and the reaction mixture was cooled to +5°C. In parallel, to a solution of 3.43 g (6.3 mmole) of the hydrazide of $N^{\alpha}$-benzyloxycarbonylarginyltryptophan in 6 ml of DMFA cooled to −15°C were successively added 11.2 ml of a 1 N solution of hydrogen chloride and 0.48 g (6.9 mmole) of sodium nitrite. The azide obtained was extracted with cold chloroform and, after being dried with sodium sulfate, the extract was combined with the solution of the tripeptide and the mixture was left at +5-8°C for 48 h. Then it was evaporated to a volume of 20 ml, the reaction products were diluted with 70 ml of chloroform–butanol (3:2), and this dilution was then washed with ammonia saturated with NaCl, with a solution of common salt, with 1 N hydrochloric acid, and with water. The organic layer was dried with sodium sulfate and the solvent was driven off in vacuum. The residue was dissolved in chloroform and was reprecipitated with an excess of ether. Yield 76%; chromatographically homogeneous with $R_f$ 0.47 in the (1:1) system.

## CONCLUSION

1. Two new variants of the synthesis of the peptide 8-12 of the natural sequence of ACTH have been developed.

2. In several stages of the process the trimethylsilyl group has been used as temporary protection of the carboxyl of the amino component in the process of peptide synthesis.

## LITERATURE CITED


1. US Patent No. 3,352,844, cl 260-112,5, publ. November 14, 1967; Belgian Patent No. 644,130, Chem. Abstr., 66, 38236 (1965); GFR Patent Application No. 1,543,882, publ. February 5, 1970.
2. E. P. Krysin, V. N. Karel'skii, A. A. Antonov, and É. D. Glinka, Khim. Prir. Soedin., 482 (1978).
3. G. W. Anderson, J. E. Zimmermann, F. M. Callahan, J. Am. Chem. Soc., 89, 178 (1967).


# TRANSFERASE AND HYDROLASE ACTIVITIES OF PHOSPHOLIPASE D FROM THE ROOTS OF *Aconitum arcuatum*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UDC 577.154.52



The phospholipase D from *Aconitum arcuatum* has been studied. The optimum pH values have been determined: 6.3 for the hydrolase activity and 8.0 for the transferase activity. Calcium ions activate the phospholipase D: the hydrolase activity most strongly at 8 and 30 μmole and the transferase activity at 30 μmole. The hydrolase activity of the phospholipase D is not activated by low concentrations of sodium dodecyl sulfate, while the transferase activity is activated to a considerable degree. The results obtained are compared with those on the activation of cabbage phospholipase D.


Phospholipase D is widespread in higher plants [1-3]. The isolation and properties of this enzyme have been described in [4-7]. Its most interesting feature is its manifestation of two activities: hydrolase and transferase activities [8-11]. When phospholipase D was isolated from cabbage [5] and from peanuts [6], it was established that the ratio of hydrolase and transferase activities did not change during the purification process and the two activities require the same conditions for optimum catalysis. At the present time, it is assumed that both activities belong to a single enzyme [12], although Saito et al. [13] have shown that the maximum hydrolase and transferase activities of the phospholipase D from cabbage are shown at different optimum pH values and are activated by different concentrations of $Ca^{2+}$.

We have performed the partial purification of phospholipase D from the roots of *Aconitum arcuatum* Maxim., in which one of the highest hydrolase activities has previously been detected [2] and have described its properties. We have determined the transferase and hydrolase activities of the phospholipase D in the pH range of 5.0-9.0 and have studied the influence of various concentrations of $Ca^{2+}$ and of sodium dodecyl sulfate on these activities. For comparison, we determined the two activities of cabbage phospholipase D under the same conditions. Figure 1A shows the dependence of the transferase and hydrolase activities of the aconitum and cabbage phospholipases D on the pH of the medium.

The hydrolase activities of the enzymes from the two sources are highest in the same pH region — 6.3 for the aconitum enzyme and 5.8 for the cabbage enzyme. It is known that the optimum hydrolase activities of preparations of phospholipase D from various sources are found at pH values of 5.5 to 6.0 [14, 16]. In our experiments, the transferase activities of the enzymes differed greatly according to their source: the preparation from cabbage possessed its maximum activity at pH 5.8 and that from the aconite at pH 8.0. In the paper of Yang et al. [4], the optimum pH was the same for the two activities of the cabbage phospholipase D, amounting to 5.5-6.0. According to the results of the investigations of Saito et al. [13], the optima of the two activities of the enzyme from the cabbage differed sharply: for the


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