INSULIN SECRETION-STIMULATING ACTIVITY OF CERTAIN DERIVATIVES OF THE C-TERMINAL TETRAPEPTIDE OF CHOLECYSTOKININ

N. V. Sadovnikova, V. P. Fedotov,
Yu. P. Shvachkin, A. A. Shishkina,
and L. A. Kolomeitseva

UDC 615.252.349:547.961].07

The tetrapeptide amide, possessing amino acid sequence H-Trp-Met-Asp-Phe-Nh_2 (I, CCK-4, trimafam) is a C-terminal structural fragment of gastrin, secretin, and cholecystokinin, and, as we discovered previously [2], has a pronounced stimulating effect on the secretion of insulin, glucagon, and somatostatin. According to the data of [5], the insulin-stimulating effect of tetrapeptide I is substantially more pronounced than that of other larger fragments of cholecystokinin. These investigations open up theoretically new possibilities in the treatment of diabetes mellitus using previously unknown oligopeptide compounds that activate insulin secretion in the pancreas.

In this work, in order to disclose structuro-functional interrelationships in the molecule of I, we studied the biological activity of three of its analogs (II-IV), in which the N-terminal tryptophan residue was replaced by L-ornithine (II), D-ornithine (III), or L-alanine (IV) residues, respectively. The insulin-stimulating properties of the peptides II-IV were assessed in vitro according to the influence on basal insulin secretion by a culture of islet cells of the rat pancreas and in vivo according to the ability to increase the insulin concentration in the blood after intravenous injection of the preparations. A sample of I synthesized and studied previously [1, 3] was used as the standard.

EXPERIMENTAL (CHEMICAL)

The melting points of the compounds obtained were determined on a Boecius-type instrument produced by VEB Analytik (German Democratic Republic). The optical activity was measured on a DIP-360 instrument from Tasco (Japan). Standard Silufol UV-254 plates (Czechoslovakia) were used for chromatography. The following solvent systems were used: n-butanol–acetic acid–water, 4:1:1 (A); n-butanol–acetic acid–pyridine–water, 15:3:10:12 (B); chloroform–methanol–acetic acid, 25:2:0.5 (C). Ninhydrin and iodine vapors were used to develop the chromatograms. The peptides were hydrolyzed with 6 M hydrochloric acid at 105-110°C for 24 h; the amino acid composition of the hydrolysate was determined on a Technicon TSM analyzer (USA).

Amide of N^{α}-tert-Butyloxycarbonyl-β-O-tert-butyl-L-aspartyl-L-phenylalanine (V). To a solution of 2.24 g (0.008 mmole) L-phenylalanine trifluoroacetate amide in 15 ml DMFA, chilled to −10°C, we added 1.12 ml (0.008 mole) triethylamine. The precipitate formed was filtered off, and 3.8 g (0.008 mole) of the pentafluorophenyl ester of N-tert-butyloxycarbonyl-β-O-tert-butyl-L-aspartic acid was added to the filtrate. The mixture was exposed for 1 h at 20°C; the precipitate formed was filtered off, washed with DMFA, and the combined filtrate was evaporated under vacuum. The residue was dissolved in ethyl acetate, and the solution obtained was washed successively with a 5% solution of $KHSO_4$, with water, with a 5% solution of $KHCO_3$, and again with water. The solution, dried over anhydrous sodium sulfate, was evaporated and diluted with hexane. The crystallized product was filtered off, washed with hexane and ether, and dried in a vacuum desiccator over $CaCl_2$. Yield of V 3.35 g (96%), mp 113-114°C, $[\alpha]_D^{25}$ −24.6° (c 1, MeOH). R_f 0.56 (A), 0.73 (B), 0.54 (C). Found, %: C 60.78; H 7.79; N 9.39. $C_{22}H_{33}N_3O_6$. Calculated, %: C 60.67; H 7.74; N 9.65.

Trifluoroacetate of Amide of L-Aspartyl-L-phenylalanine (VI). The protected dipeptide V (1.45 g; 0.003 mole) was dissolved in 3 ml of trifluoroacetic acid with an addition of three drops of anisole; the solution obtained was exposed for 2 h at 20°C and then evaporated under vacuum. The residue was triturated with ether, the suspension was filtered off, and

Institute of Experimental Endocrinology and Hormone Chemistry, Academy of Medical Sciences of the USSR, Moscow. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 21, No. 12, pp. 1424-1427, December, 1987. Original article submitted August 12, 1986.

 0091-150X/87/2112-0838$12.50

the substance from the filter was dried under vacuum under P_2O_5. Yield 1.1 g (84%) of compound VI, R_f 0.26 (A).

Amide of N^{α}-tert-Butyloxycarbonyl-L-methionyl-L-aspartyl-L-phenylalanine (VII). The amide (VII) was synthesized analogously to compound V from 0.905 g (0.002 mole) of the pentafluorophenyl ester of tert-butyloxycarbonylmethionine, 0.302 ml (0.002 mole) triethylamine, and 0.86 g (0.002 mole) of the trifluoroacetate VI. After evaporation of the reaction mixture under vacuum to half the volume, the residue was suspended in water, and the precipitate formed was removed by filtration, washed on the filter with ether and hexane, and dried under vacuum. Yield of VII 0.97 g (95%), mp 204°C (with dec.), $[\alpha]_D^{23}$ −32.5° (c 1, DMFA). R_f 0.69 (A), 0.76 (C).

Amide of N^{α}-tert-Butyloxycarbonyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine (IX). The amide IX was synthesized analogously to compound V from 1.98 g (0.004 mole) of the pentafluorophenyl ester of tert-butyloxycarbonyl-L-tryptophane, 0.58 ml (0.004 mole) triethylamine, and 2.2 g (0.004 mole) of the tripeptide trifluoroacetate VIII, prepared with a yield of 87% by treatment of 2.45 g (0.0047 mole) of the protected tripeptide VII with trifluoroacetic acid with anisole, as described above. After evaporation of the reaction mixture under vacuum, the residue was dissolved in ethyl acetate, and the solution obtained was washed successively with a 5% solution of $KHSO_4$, with water, with a 5% solution of $KHCO_3$, and again with water, and dried with anhydrous sodium sulfate. After removal of the solvent, the residue was dissolved in ethanol, diluted with ether, and the precipitate obtained was filtered off and dried. Yield of the tetrapeptide IX 2.58 g (88.5%), mp 204-206°C, $[\alpha]_D^{23}$ −34° (c 1, DMFA). R_f 0.51 (A), 0.66 (B), 0.65 (C), 0.48 (C).

Hydrochloride of the Amide of L-Tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine (I). Compound IX (0.53 g; 0.0007 mole) was dissolved in 10 ml of a 1 M solution of hydrogen chloride in acetic acid; the solution obtained was exposed for 1 h at 20°C and then evaporated under vacuum. The residue was dissolved in a small quantity of methanol and diluted with ether. The precipitate formed was filtered off and dried under vacuum. Yield 0.44 g (92%) of I, mp 162-163°C (with dec.), $[\alpha]_D^{25}$ −31.5° (c 1, DMFA). R_f 0.7 (A). Amino acid composition: tryptophan 0.92, methionine 0.99, aspartic acid 1.0, phenylalanine 0.91.

Amide of $N^{\alpha,\delta}$-Di-tert-butyloxycarbonyl-L-ornithyl-L-methionyl-L-aspartyl-L-phenylalanine (X). The amide X was synthesized analogously to compound V from 0.3 g (0.0006 mole) of the pentafluorophenyl ester of di-tert-butyloxycarbonyl-L-ornithine, 0.08 ml (0.0006 mole) triethylamine, and 0.32 g (0.0006 mole) of the tripeptide trifluoroacetate VIII. After evaporation of the reaction mixture, the residue was dissolved in an ethyl acetate—water mixture, 1:1. The organic layer was removed and washed with a 5% solution of $KHSO_4$, with water, with a 5% solution $KHCO_3$, and again with water. The solution, dried over anhydrous sodium sulfate, was evaporated. After recrystallization of the dry residue from a mixture of ethyl acetate and petrolium ether, we obtained 0.32 g (75%) X, mp 174-175°C, $[\alpha]_D^{22}$ −37°C (c 1, MeOH). R_f 0.41 (A), 0.68 (B), 0.46 (C). Found, %: C 54.53; H 7.43; N 11.29; S 4.48. $C_{33}H_{52}N_6O_{10}S$. Calculated, %: C 54.68; H 7.23; N 11.59; S 4.42.

Hydrochloride of the Amide of L-Ornithyl-L-methionyl-L-aspartyl-L-phenylalanine (II). Compound X (0.15 g; 0.0002 mole) was dissolved in 2 ml of a 10% solution of hydrogen chloride in dioxane; the mixture was exposed for 45 min at 20°C, the evaporated and the residue dried under vacuum. Yield of compound II 0.11 g (98%), mp 148-150°C, $[\alpha]_D^{25}$ −18.8° (c 0.5, MeOH). R_f 0.82 (A), 0.32 (B), 0.45 (C). Amino acid composition: ornithine 0.9, aspartic acid 0.98, methionine 0.8, phenylalanine 1.0.

Amide of $N^{\alpha,\delta}$-Di-tert-butyloxycarbonyl-D-ornithyl-L-methionyl-L-aspartyl-L-phenylalanine (XII). The amide XII was synthesized analogously to compound V from 0.3 g (0.0006 mole) of the pentafluorophenyl ester of di-tert-butyloxycarbonyl-D-ornithine, 0.008 ml (0.0006 mole) triethylamine, and 0.32 g (0.0006 mole) of the tripeptide trifluoroacetate VIII. After evaporation of the reaction mixture to half the volume, the residue was suspended in water, the precipitate removed by filtration, washed on the filter with ether and hexane, and dried. After recrystallization from a mixture of ethyl acetate and petrolium ether, we obtained 0.3 g (68%) of compound XI, mp 193-195°C, $[\alpha]_D^{22}$ −30° (c 1, MeOH). R_f 0.68 (B), 0.46 (C). Found, %: C 54.71; H 7.15; N 11.48; S 4.3. $C_{33}H_{52}N_6O_{10}S$. Calculated, %: C 54.88; H 7.23; N 11.59; S 4.42.

Hydrochloride of the Amide of L-Ornithyl-L-methionyl-L-aspartyl-L-phenylalanine (III). The amide hydrochloride III was synthesized analogously to the compound II from 0.15 g (0.0002 mole) of compound XI. Yield of III 0.1 g (97%), mp 139-142°C, $[\alpha]_D^{25}$ −47, (c 0.5, MeOH). R_f 0.82 (A), 0.32 (B), 0.45 (C). Amino acid composition: ornithine 0.9, aspartic acid 0.98, methionine 0.8, phenylalanine 1.0.

Amide of N^{α}-tert-Butyloxycarbonyl-L-alanyl-L-aspartyl-L-methionyl-L-phenylalanine (XII). The amide XII was synthesized analogously to compound V from 0.695 g (0.0019 mole) of the pentafluorophenyl ester of tert-butyloxycarbonyl-L-alanine, 0.27 g (0.0019 mole) triethylamine, and 1.03 g (0.0019 mole) of the tripeptide trifluoroacetate VIII. After evaporation of the reaction mixture to half the volume, the residue was suspended in water, and the precipitate removed by filtration and dried. After recrystallization from a mixture of ethyl acetate–ethanol (2:1), we obtained 0.87 g (76%) of compound XII, mp 211°C, $[\alpha]_D^{25}$ −30.3° (c 1, MeOH). R_f 0.62 (B), 0.54 (C). Found, %: C 53.42; H 6.78; N 11.7; S 5.3. $C_{26}H_{39}N_5O_8S$. Calculated, %: C 53.63; H 6.77; N 12.05; S 5.5.

Hydrochloride of the Amide of L-Alanyl-L-methionyl-L-aspartyl-L-phenylalanine Monohydrate (IV). The compound IV synthesized analogously to compound I from 0.32 g (0.0005 mole) of compound XII. Yield of compound IV 0.26 g (91%), mp 181-182°C, $[\alpha]_D^{25}$ −33 (c 1, MeOH). R_f 0.78 (A), 0.3 (B), 0.48 (C). Amino acid composition: alanine 1.05, aspartic acid 1, methionine 1.04, phenylalanine 1.

EXPERIMENTAL (BIOLOGICAL)

Experiments in vitro were conducted on a four-day culture of pancreatic islet cells of newborn rats. The technique for producing the culture was described in detail previously [1]. The cell cultures were grown on microplates for culturing (Flow) in medium 199 with an addition of 10% calf embryo serum (Serva, Federal Republic of Germany) in an atmosphere of 96% O_2 and 4% CO_2. Before the experiment the cells were washed twice with the medium to remove insulin, separated according to groups, and incubated again for 30 min in medium 199 with serum and addition of tetrapeptides to the experimental samples in concentrations of 10^{-10}, 10^{-8}, and 10^{-6} M. At the end of the incubation, the culture medium was collected from the wells and stored at −40°C until the determination of the insulin concentration in it. Figure 1a presents the results of a series of experiments on a cell culture with basal activity about 160 microunits/ml, while Fig. 1b presents the results for a cell culture with basal activity 94 microunits/ml.

The investigations in vivo were conducted on male Wistar rats with body weight 180-200 g. The tetrapeptides were dissolved in physiological saline solution in am amount of 5 μg per kg of weight and introduced into the jugular vein under light ether anesthesia. The control animals received an equal volume of physiological saline solution. The animals were decapitated 2 min after the injection. The blood serum was stored at −40°C. The insulin concentration in the culture medium and blood serum was determined by ratioimmuno assay using the RIA-insulin kits (Institute of Isotopes, Hungary) and expressed in microunits/ml.

RESULTS AND DISCUSSION

Figure 1 presents the results of the action of the tetrapeptide I and its analogs II-IV on insulin secretion in a culture of pancreatic islet cells at various peptide concentrations in the medium. The secretion of insulin under the influence of I at a concentration of 10^{-8} M (see Fig. 1a) was increased to 203 ± 21.1 microunits/ml versus 157.5 ± 9.0 microunits/ml in the control ($P = 0.5$) and continued to increase further to 275.0 ± 23.5 microunits/ml ($P < 0.05$ in comparison with the preceeding experiment). An analogous dependence of the action of I on insulin secretion is presented in Fig. 1b, where it is shown that the basal secretion of insulin in the culture was lower (94.0 ± 4.0 microunits/ml).

The insulin-stimulating effect of the peptides II and III in a culture of islet cells was manifested to the same degree as for I. The tetrapeptide IV stimulated secretion of the hormone to a substantially lesser degree. In this case a significant increase in the insulin concentration in the medium was noted only at a dose of the preparation in the medium of 10^{-8} M ($P < 0.05$).

In testing of the preparations in vivo (see Table 1), it was revealed that only the peptides II and III induce an increase in the insulin content in the blood 2 min after intravenous injection of the preparations. The peptide IV does not posses insulinogenic activity in vivo.

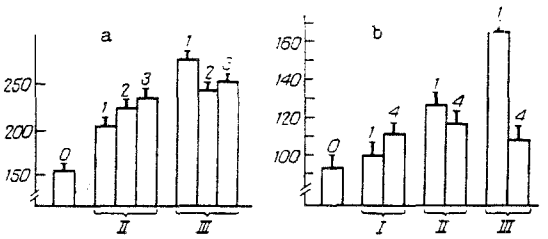


Fig. 1. Secretion of insulin under the influence of various concentrations of I and its analogs in a culture of pancreatic islet cells of newborn rats with basal activity 160 microunits/ml (a) and 94 microunits/ml (b). 0) Control; 1) peptide I; 2) peptide II; 3) peptide III; 4) peptide IV. Along the horizontal: peptide concentration: I) 10^{-10} M; II) 10^{-8} M; III) 10^{-6} M; along the vertical: insulin concentration in the medium (in microunits/ml).

TABLE 1. Change in the Insulin Content (in microunits/ml) in the Blood of Rats in Response to Intravenous Injection of the Tetrapeptide I and Its Analogs II-IV (in a dose of 5 μg/kg)

Compound	No. of animals in experiment	Insulin, microunits/ml	P
Control	8	46,7±3,8	—
I	8	62,9±6,2	<0,05
II	6	59,7±4,3	<0,05
III	7	67,7±8,1	<0,05
IV	6	48,9±7,6	>0,05

The results obtained are evidence that the replacements of tryptophan residues in the molecule of I by L- and D-ornithine residues do not lead to a loss of insulin-stimulating activity. Like I, the peptides II and III stimulate secretion in the islet cells of the pancreas, and a dependence of the effect on the dose is preserved. These peptides exhibited an equal degree of activity in vivo, providing for rapid release of insulin into the blood. When alanine was introduced into the molecule of I instead of tryptophan (peptide IV), a substantial decrease in the insulin-stimulating activity of the preparation was noted in vitro. This peptide completely lost the ability to activate insulin secretion in vivo.

The role of tryptophan in the manifestation of the biological properties of I has not been completely established. According to the data of [6], the removal of tryptophan from the molecule of I led to the formation of the tripeptide entirely inactive with respect to the endocrine function of the pancreas, while replacement of tryptophan by glycine or Boc-β-alanine led to a substantial decrease in the insulin-secreting activity of the preparations. Tetrapeptides related to I with high biological activity are also known [4]: in this case the tryptophan residue was replaced by pyruvic acid or proline. The structural modifications that we performed on the N-end of the cholecystokinin tetrapeptide are evidence that insulinogenic activity of the compound is fully preserved when the tryptophan residue is replaced by L- or D-ornithine residues. The results, together with the literature data, permit us to conclude that the tryptophan residue in the molecule of I is not essential in the manifestation of the insulin-stimulating properties of the tetrapeptide.

LITERATURE CITED

1. V. P. Fedotov, I. S. Komolov, N. V. Sadovnikova, et al., Probl. Endokrinol., No. 4, 57-60 (1978).
2. V. P. Fedotov, N. V. Sadovnikova, V. I. Gudoshnikov, et al., Byull. Éksp. Biol. Med., No. 6, 729-731 (1984).
3. Yu. P. Shvachkin and L. A. Kolomeitseva, Khim. Prir. Soedin., No. 3, 397-398 (1983).
4. N. Ahmad, S. Sharma, A. Rastogi, et al., Acta Diabretol. Lat., 21, 361-364 (1984).
5. J. Okabayashi, M. Otsuki, A. Ohki, et al., Endocrinology, 113, 2210-2215 (1983).
6. J. F. Rehfeld, Li. Larsson, N. K. Goltermann, et al., Nature, 284, 33-37 (1980).

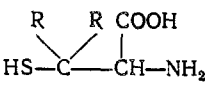
SYNTHESIS AND BIOLOGICAL ACTIVITY OF AMIDINOMERCAPTIC ACIDS AND RELATED COMPOUNDS

V. G. Granik, G. Ya. Shvarts,
S. I. Grizik, N. Z. Tugusheva, I. F. Faermark,
I. F. Faermark, E. V. Kugaevskaya,
Yu. E. Eliseeva, L. V. Pavlikhina,
V. N. Orekhovich, and M. D. Mashkovskii

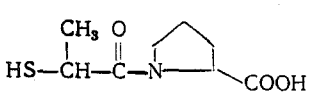
UDC 615.225.2:547.569.4].012.1

One of the new and promising areas of research on antihypertensive agents is the synthesis and study of compounds which inhibit angiotensin and dipeptidyl transferase I, dipeptidyl carboxypeptidase (DCP: EC 3.4.15.1) which catalyzes the formation of the octapeptide, angiotensin II, which has pressor properties, and which also catalyzes the inactivation of the depressor peptide bradykinin. A series of data has now been compiled on the structural features of this enzyme's active center and the requirements for making inhibitors of the enzyme [6, 11]. At the same time there is still some question about the relationship between the inhibiting and antihypertensive effects of DCP since there have been cases where no correlation between those activities was found [13].

The present work is concerned with the synthesis and investigation of the inhibiting activity of a new group of chemical compounds — the amidinomercaptic acids and related substances. A basic prerequisite to this investigation was the study made of the inhibiting action and antihypertensive activity of a series of thiol compounds, primarily L-cysteine (I) and D-penicillamine (II) [4, 5]. The amino acids (I and II), just as the known drug Captopril, contain a terminal mercapto group that is capable of bonding with Zn^{2+} at DCP's active center but do not have a water-repellent cyclic polymethylene chain that is so essential to the biological activity of III and its analogs [6].



I: R = H; II: R = CH_3.



III

In that connection, we thought it would be of interest to introduce fragments of amino acids I and II into molecules that have saturated azaheterocyclic structures. To accomplish that purpose amino acids I and II were reacted with lactim esters (IV) and lactan acetals (V). The latter are rather strong alkylating agents and are readily esterified upon reacting with acids [8]. Nevertheless, we were able to select conditions whereby esterification did not accompany the reaction and the target amidomercapto acids (VI) could be obtained at a good yield.

In addition to the hydrophobic polymethylene chain, the synthesized compounds also have a carboxyl and mercapto group as well as a C=N fragment that in principle are capable of bonding with the groups of DCP's active center.

S. Ordzhonikidze All-Union Scientific-Research Institute of Pharmaceutical Chemistry, Moscow. Translated from Khimiko-farmatsevticheskii Zhurnal, Vol. 21, No. 12, pp. 1428-1433, December, 1987. Original article submitted September 22, 1986.

 0091-150X/87/2112-0842$12.50