SYNTHESIS AND INVESTIGATION OF THE MYOTROPIC ACTIVITY OF SOME FRAGMENTS OF [5-VALINE]ANGIOTENSIN

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To investigate the mechanism of the action of peptide hormones and kinins, it is important to study their functional organization, i.e., to isolate those features of the structure of the molecule that are responsible for the "recognition" and binding of the compound to the receptor and for the formation of a secondary signal [1, 2].

The present paper describes the synthesis of a number of fragments of the tissue hormone angiotensin [compound (I), Table 1], one of the strongest natural pressor compounds, and gives the results of an investigation of their myotropic activity.

We synthesized the fragments of angiotensin by the methods of classical peptide chemistry; to form the peptide bonds we used mainly the dicyclohexylcarbodiimide method (Scheme 1). The protective groups were removed from the intermediates by alkaline hydrolysis (alkyl ester groups), by hydrogen bromide in acetic acid (benzyloxycarbonyl groups), or by catalytic hydrogenation (benzyloxycarbonyl groups, the nitro group of arginine, and aryl ester groups). The free peptides (III-V) were purified on columns of carboxymethylcellulose.

The myotropic activity of the fragments of angiotensin synthesized was investigated by recording cumulative "concentration–effect" curves by the method of van Rossum and Ariëns [3] on the isolated ascending great colon of the rat, which is the most sensitive and specific organ for testing angiotensin under in vitro conditions. All the compounds synthesized, in concentrations of 10^{-10}-10^{-9} M, caused the contraction of the smooth musculature of the colon [6]. The parallel nature of the cumulative concentration–effect curves of these compounds permits the assumption that they act on the same receptor system (for the values of the parameters α and pD_2, see Table 1). It is extremely important to note the nature of the change of such curves for angiotensin (I) and its fragments; namely the gradual increase in the sensitizing

TABLE 1. Parameters Characterizing the Interaction of Angiotensin I and Its Fragments (II-IX) with the Receptors of the Smooth Musculature of the Ascending Great Colon of the Rat (method of van Rossum and Ariëns [3])

Compound	Primary structure	Internal activity α with confidence limits (P=0.05)	Index of specific affinity pD_2
I	Asn-Arg-Val-Tyr-Val-His-Pro-Phe	1,00	9,70
II	Asn-Arg	0,76±0,10	8,34
III	Asn-Arg-Val	0,55±0,12	9,26
IV	Asn-Arg-Val-Tyr	0,53±0,09	9,31
V	Asn-Arg-Val-Tyr-Val-His	0,35±0,07	9,11
VI	Val-Tyr-Val-His	0,72±0,18	9,98
VII	Val-Tyr-Val-His-Pro-Phe [4]	0,74±0,13	8,24
VIII	Val-His-Pro-Phe [4]	0,59±0,18	9,21
IX	Pro-Phe	0,55±0,14	8,74

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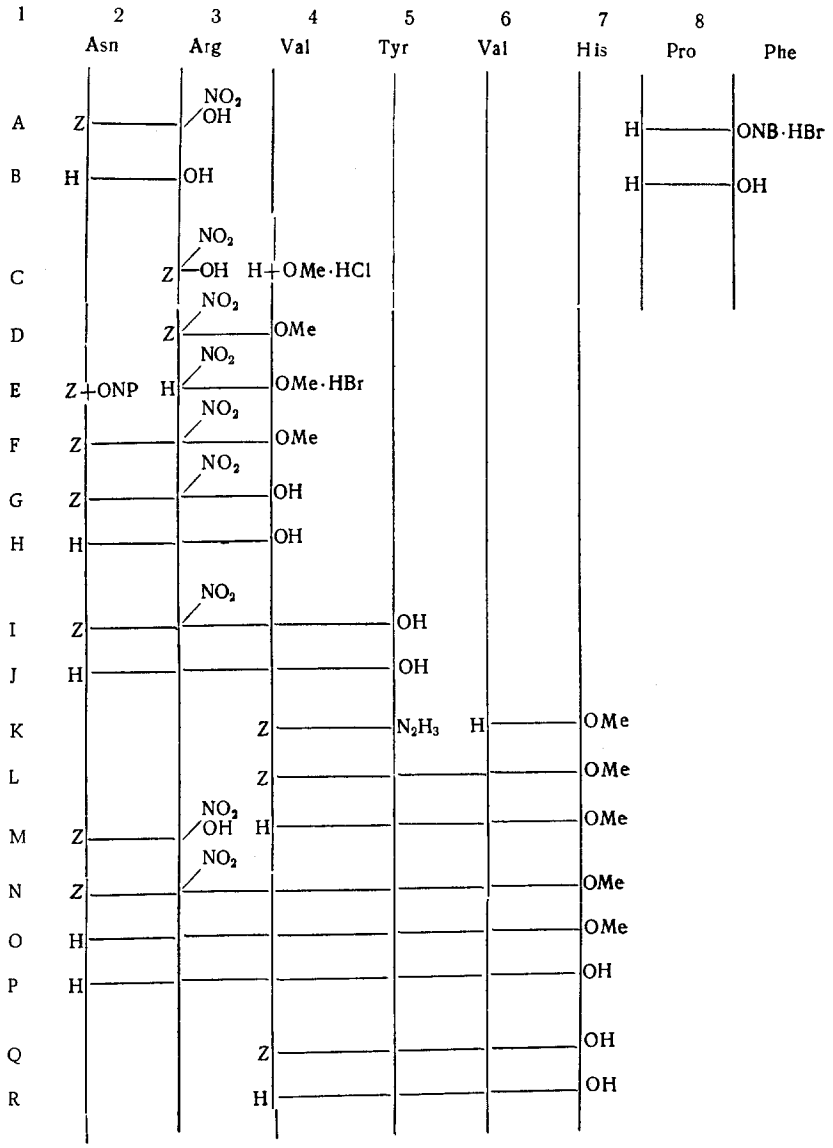
and potentiating properties of the fragments in the direction of the N-end of the angiotensin molecule and, conversely, the increase in the antagonistic properties in the direction of the C-end of the molecule. For example, on a background of the N-terminal fragments (compounds III-IV) in concentrations of 10^{-7}-10^{-6} M the sensitization and potentiation of the myotropic action of angiotensin are observed. At the same time, the C-terminal fragments of angiotensin (VIII, IX) in the same concentrations act as antagonists of the hormone, the strongest antagonist being the C-terminal dipeptide (IX).

Scheme 1



These results clearly show the different functional values of the individual parts of the molecule of the hormone in the formation of the secondary signal and support the hypothesis put forward previously according to which the bulk of the information necessary for complex formation of the hormone with the receptor is coded in the C-terminal amino acids and the information ensuring the formation of the secondary signal in the N-terminal acids. This is also confirmed by the results of a comparison of the parameters characterizing the internal activities of the two terminal fragments: the numerical value of the internal activity α of the N-terminal peptide (II) ($\alpha = 0.76 \pm 0.10$) exceeds the corresponding value for the C-terminal dipeptide (IX) ($\alpha = 0.55 \pm 0.14$).

The common fragment of angiotensin includes the three N-terminal amino acids Asp-Arg-Val. As can be seen from Table 1, the addition of valine to the terminal dipeptide (II) lowers the internal activity [compound (III)], but simultaneously increases the affinity for the receptor. The C- and N-terminal tetrapeptides (IV and VIII) differ only slightly in activity, but in their presence the nature of the cumulative concentration–effect curves of angiotensin changes in different ways.

The interpretation of the results obtained is complicated to some extent by the absence of information on the conformation of both angiotensin and its fragments. The conformation of an individual fragment and of the same fragment in the structure of the hormone molecule may differ considerably. The smallest differences in this respect are apparently possessed by the central tetrapeptide of angiotensin [compound (VI)], the affinity of which for the receptor is comparable with that for angiotensin itself. This compound also has the third-largest internal activity ($\alpha = 0.72 \pm 0.18$). It may be assumed that the conformation of the tetrapeptide (VI) in the angiotensin molecule differs little from the conformation of the individual fragment. Apparently, in the determination and stabilization of the conformation of this tetrapeptide a large role is played by the two valine residues with voluminous and branched hydrophobic side chains at the C^{β} atom. The addition to the tetrapeptide (VI) of the N-terminal dipeptide Asn-Arg leads to a fall in the internal activity (compound V), obviously because of changes in spatial structure.

EXPERIMENTAL

The work was performed with amino acids of the L configuration. The solutions were evaporated in a rotary vacuum evaporator at a residual pressure of 12-15 mm. The melting (decomposition) points were determined in open capillaries without correction; the specific optical rotations were measured on a digital polarimeter (Perkin-Elmer model 141). Filtrak FN-3 paper and the two-phase system butan-1-ol–acetic acid–water (4:1:5) were used for descending chromatography. The electrophoretic mobilities E_{His} were determined as the ratios of the distances migrated by the substance to the distance migrated by histidine on Filtrak FN-16 paper in 1 N acetic acid (pH 2.4; voltage 15 V/cm; time 1.5 h). The chromatograms and electrophoregrams were revealed with ninhydrin and Pauli's reagent [7] or the Reindel–Hoppe reagent [8]. Samples for elementary analysis were dried over phosphorus pentoxide at a residual pressure of 1-2 mm. To determine the amino acid compositions, the terminal products were hydrolyzed with 6 N hydrochloric acid in sealed galss tubes at 105°C for 20-24 h; the hydrolyzates were analyzed on a Bio-Cal BC-200 amino acid analyzer.

Asparaginylarginine (B 1-2). In solution in 40 ml of a mixture of methanol, acetic acid, and water (6:1:1), 2.50 g (5.3 mmoles) of benzyloxycarbonylasparaginylnitroarginine (A 1-2) [9] was hydrogenated over palladium black [10] for 14 h. After the elimination of the catalyst, the solution was evaporated and the residue was dissolved in distilled water and freeze-dried. The yield of asparaginylarginine (B 1-2) was 1.85 g (95%), composition $C_{12}H_{24}N_6O_6 \cdot CH_3COOH \cdot H_2O$ mp 134°C (decomp.), $[\alpha]_D^0 + 8°$ (c 1; water), R_f 0.16, E_{His} 1.00.

Hydrobromide of the Methyl Ester of Nitroarginylvaline (E 2-3). A suspension of 8.40 g (50 mmoles) of the hydrochloride of the methyl ester of valine (C 3) [11] in 40 ml of ethyl acetate was treated with 6.90 ml (50 mmoles) of triethylamine, and the mixture was stirred for 4 h. The precipitate of triethylammonium chloride that deposited was separated off and the filtrate was treated with a solution of 17.7 g (50 mmoles) of benzyloxycarbonylnitroarginine (C 2) [12] in 60 ml of dioxane and then, at 0°C with stirring, a solution of 10.3 g (50 mmoles) of dicyclohexylcarbodiimide in 30 ml of ethyl acetate was added and the resulting mixture was kept at 0°C for 15 h. The crystals of dicyclohexylurea that deposited were filtered off, and the filtrate was evaporated to dryness. The residue was dissolved in 150 ml of ethyl acetate and the solution was washed with 1 N hydrochloric acid, distilled water, 1 N sodium carbonate solution, and distilled water again and was dried over anhydrous sodium sulfate and evaporated to dryness. The methyl ester of benzyloxycarbonylnitroarginylvaline (D 2-3) so obtained was dissolved in 35 ml of glacial acetic acid and the solution was treated with 60 ml of a 34% solution of hydrogen bromide in glacial acetic acid and was kept at room temperature for 1 h. The product was precipitated with 1000 ml of dry ether, filtered off with suction, washed with dry ether (3 × 100 ml), and dried in vacuum over phosphorus pentoxide and potassium hydroxide. After reprecipitation from ethanol with ether, the yield of the hydrobromide of the methyl ester of nitroarginylvaline (E 2-3) was 15.60 g (76%; composition $C_{12}H_{25}N_6O_5 \cdot HBr$, mp 176°C (decomp.), $[\alpha]_D^{20} + 6°$ (c 1; water), R_f 0.67.

Methyl Ester of Benzyloxycarbonylasparaginylnitroarginylvaline (F 1-3). Dry ammonia was passed for 10 min through a solution of 8.06 g (20 mmoles) of the hydrobromide of the methyl ester of nitroarginylvaline (E 2-3) in 30 ml of chloroform at 0°C. The precipitate of ammonium chloride that deposited was separated off, and the solution was partially evaporated and filtered again. The filtrate was treated with a solution of 7.95 g (20 mmoles) of the p-nitrophenyl ester of benzyloxycarbonylasparagine (E 1) [13] in 15 ml of dimethylformamide and 1.40 g of imidazole and was kept at room temperature for 48 h. The reaction product was precipitated with ethyl acetate and was triturated successively with ether, 1 N hydro-

chloric acid, distilled water, and ether, and was dried in vacuum over phosphorus pentoxide and potassium hydroxide. The yield of the methyl ester of benzyloxycarbonylasparaginylnitroarginylvaline (F 1-3) was 5.10 g (45.3%), composition $C_{24}H_{36}N_8O_9$, mp 114°C, $[\alpha]_D^{20}-8°$ (c 1; methanol), R_f 0.88.

Asparaginylarginylvaline (H 1-3). To a solution of 5.00 g (8.6 mmoles) of the methyl ester of benzyloxycarbonylasparaginylnitroarginylvaline (F 1-3) in 50 ml of methanol was added 2.5 ml of a 20% solution of sodium hydroxide (1.3 mmoles), and the mixture was stirred for 1 h. The methanol was evaporated off in vacuum, the residue was dissolved in 50 ml of distilled water, and the solution was washed three times with ethyl acetate, acidified with 1 N hydrochloric acid to pH 1, and extracted with ethyl acetate (5 × 50 ml). The ethyl acetate extract was dried over anhydrous sodium sulfate and evaporated to dryness. The benzyloxycarbonylasparaginylnitroarginylvaline (G 1-3) obtained was dissolved in 40 ml of methanol–acetic acid–water (6:1:1) and hydrogenated over palladium black for 18 h. After the separation of the catalyst, the solution was evaporated to dryness and the product was purified on carboxymethylcellulose and freeze-dried. The yield of asparaginylarginylvaline (H 1-3) was 1.10 g (27%); composition $C_{17}H_{37}N_7O_9 \cdot CH_3COOH \cdot 2H_2O$, mp 114°C (decomp.), $[\alpha]_D^{20}-13°$ (c 1, water), R_f 0.30, E_{His} 0.91.

Asparaginylarginylvalyltyrosine (J 1-4). In solution in 8 ml of methanol–acetic acid–water (6:1:1), 0.66 g (0.9 mmole) of benzyloxycarbonylasparaginylnitroarginylvalyltyrosine (I 1-4) [9] was hydrogenated over palladium black for 18 h. The catalyst was filtered off and the filtrate was evaporated to dryness. The product was purified on carboxymethylcellulose and was freeze-dried. The yield of asparaginylarginylvalyltyrosine (J 1-4) was 0.33 g (66%), mp 205-210°C (decomp.), $[\alpha]_D^{22}-37°$ (c 1; water), R_f 0.27, E_{His} 0.79. Amino-acid composition: aspartic acid 0.95, arginine 0.98, valine 1.08, tyrosine 1.00.

Methyl Ester of Benzyloxycarbonylvalyltyrosylvalylhistidine (L 3-6). With stirring, 1.5 ml of tert-butyl nitrite was added to a solution of 4.60 g (10.7 mmoles) of the hydrazide of benzyloxycarbonylvalyltyrosine (K 3-4) [14] in 5.0 ml of dimethylformamide and 13.3 ml of a 1.25 N solution (16.6 mmoles) of hydrogen chloride in tetrahydrofuran cooled to −25°C in such a way that the temperature of the solution did not rise above −10°C. Stirring was continued at the same temperature for 10 min, and then the solution was diluted with 25 ml of cooled acetonitrile and the excess of hydrogen chloride was neutralized by the addition of 2.3 ml (16.6 mmoles) of triethylamine. To the reaction mixture was added a cooled solution of 10.7 mmoles of the methyl ester of valylhistidine (K 5-6) in 30 ml of acetonitrile (the amino component was prepared from 4.32 g (10.7 mmoles) of the methyl ester of benzyloxycarbonylvalylhistidine by the method of Schwyzer et al. [15]) and the mixture was kept at 0°C for 18 h. The gel-like product was filtered off with suction, carefully washed with water, and dried in vacuum over phosphorus pentoxide. The yield of the methyl ester of benzyloxycarbonylvalyltyrosylvalylhistidine (L 3-6) was 4.00 g (56%), mp 228-229°C (decomp.), R_f 0.88; E_{His} 0.64 (mp 228-230°C [15]).

Methyl Ester of Valyltyrosylvalylhistidine (M 3-6). The hydrogenation of 7.00 g (10.5 mmoles) of the methyl ester of benzyloxycarbonylvalyltyrosylvalylhistidine (L 3-6) was performed in 30 ml of dimethylformamide and 5 ml of acetic acid over palladium black for 8 h. The catalyst was filtered off, and the filtrate was evaporated at a residual pressure of 1-2 mm. The resulting diacetate of the methyl ester of valyltyrosylvalylhistidine (diacetate of M 3-6) crystallized on the addition of ethyl acetate (mp 209-211°C, decomp.); it was dissolved in a mixture of 40 ml of water and 5 ml of acetic acid and the mixture was filtered. To isolate the base (M 3-6), with stirring at 0°C a 50% solution of potassium carbonate was added to the filtrate to pH 8-9. The white precipitate that deposited was filtered off with suction and was washed three times with ice water and dried in a vacuum over phosphorus pentoxide. The yield of the methyl ester of valyltyrosylvalylhistidine (M 3-6) was 4.20 g (75%), composition $C_{36}H_{38}N_6O_6$, mp 214-219°C (decomp.), R_f 0.60, E_{His} 0.88 (mp 190°C [16]).

Methyl Ester of Benzyloxycarbonylasparaginylnitroarginylvalyltyrosylvalylhistidine (N 1-6). At −30°C with stirring, a solution of 0.42 g (2 mmoles) of dicyclohexylcarbodiimide in 5 ml of dimethylformamide was added to a solution of 0.94 g (2 mmoles) of benzyloxycarbonylasparaginylnitroarginine (A 1-2) [9], 1.06 g (2 mmoles) of the methyl ester of valyltyrosylvalylhistidine (M 3-6), and 0.46 g (4 mmoles) of N-hydroxysuccinimide in 30 ml of dimethylformamide, and the mixture was kept at 0°C for 24 h. The crystals of dicyclohexylurea that had deposited were filtered off, and the product was precipitated by the addition of 100 ml of water. The precipitate was filtered off with suction and was washed with water, 10% sodium hydrogen carbonate solution, water again, 0.1 N hydrochloric acid, and then three times with water again and was dried in vacuum over phosphorus pentoxide. The yield of the methyl ester of benzyloxycarbonylasparaginylnitroarginylvalyltyrosylvalylhistidine (N 1-6) was 1.20 g (61%), mp 227-232°C (decomp.), R_f 0.91 (mp 170-188°C [16]).

Methyl Ester of Asparaginylarginylvalyltyrosylvalylhistidine (O 1-6). In solution in 30 ml of dimethylformamide–acetic acid–methanol–water (1:2:2:1), 1.20 g (1.1 mmole) of the methyl ester of benzyloxycarbonylasparaginylnitroarginylvalyltyrosylvalylhistidine (N 1-6) was hydrogenated over palladium black for 8 h. The catalyst was separated off, and the filtrate was evaporated to dryness at a residual pressure of 1-2 mm. The product was purified by partition chromatography on columns of Sephadex G-25 in the two-phase system butan-1-ol–water–acetic acid (4:5:1) and on columns of carboxymethylcellulose, and was freeze-dried. The yield of the methyl ester of asparaginylarginylvalyltyrosylvalylhistidine (O 1-6) was 0.30 g (38%), mp 236-240°C (decomp.), $[\alpha]_D^{20}$ −46° (c 1.1; water); R_f 0.16, E_{His} 0.92.

Asparaginylarginylvalyltyrosylvalylhistidine (P 1-6). A solution of 0.08 g (0.1 mmole) of the methyl ester of asparaginylarginylvalyltyrosylvalylhistidine (O 1-6) in a mixture of 5 ml of distilled water and 0.2 ml (0.2 mmole) of a 1 N solution of sodium hydroxide was stirred at room temperature for 30 min. Then the solution was acidified to pH 3 with acetic acid and was evaporated to dryness. After purification on a column of carboxymethylcellulose and freeze-drying, the yield of asparaginylarginylvalyltyrosylvalylhistidine (P 1-6) was 0.05 g (65%), mp 220-240°C (decomp.), $[\alpha]_D^{20}$ −28° (c 1.1; water), R_f 0.15, E_{His} 0.80. Amino acid composition: aspartic acid 1.06, arginine 0.95, valine 2.13, tyrosine 1.01, histidine 1.16.

Benzyloxycarbonylvalyltyrosylvalylhistidine (Q 3-6). A suspension of 3.00 g (4.5 mmoles) of the methyl ester of benzyloxycarbonylvalyltyrosylvalylhistidine (L 3-6) in 20 ml of methanol was treated with 6.0 ml of a 1 N solution (6 mmoles) of sodium hydroxide, and the mixture was stirred at room temperature for 30 min. Then dioxane was added dropwise until the suspended matter had dissolved completely, and stirring was continued for another 1.5 h. The solution was diluted with 20 ml of water, acidified with 6 ml of 1 N hydrochloric acid, and partially evaporated. The precipitate that deposited was filtered off with suction, washed with water, and dried in vacuum over phosphorus pentoxide. The yield of benzyloxycarbonylvalyltyrosylvalylhistidine (Q 3-6) was 2.70 g (99%), mp 231-233°C (decomp.), (mp 236-238°C, decomp. [15]).

Valyltyrosylvalylhistidine (R 3-6). In suspension in 20 ml of methanol–acetic acid–water (6:1:1), 2.00 g (3.3 mmoles) of benzyloxycarbonylvalyltyrosylvalylhistidine (Q 3-6) was hydrogenated over palladium black for 6 h. The catalyst was filtered off, and the filtrate was evaporated to dryness. The residue was dissolved in 30 ml of water and the solution was washed with n-butanol (2 × 15 ml) and was then freeze-dried and finally dried in vacuum over phosphorus pentoxide and potassium hydroxide. The yield of valyltyrosylvalylhistidine (R 3-6) was 1.50 g (87%), mp 225-229°C (decomp.), $[\alpha]_D^{24}$ −6° (c 2.45; water), R_f 0.38; E_{His} 0.74 (mp 227-229°C, decomp.) $[\alpha]_D^{24}$ −6 ± 2° (c 2.45; water) [15]. Amino acid composition: valine 1.98, tyrosine 1.05, histidine 1.00.

Prolylphenylalanine (B 7-8). A suspension of 2.78 g (5.8 mmoles) of the hydrobromide of the p-nitrobenzyl ester of prolylphenylalanine (A 7-8) [4] in 30 ml of dry ethyl acetate was treated with 0.80 ml (5.8 mmoles) of triethylamine, and the mixture was stirred for 10 min. The crystals of triethylammonium bromide that deposited were separated off, and the filtrate was acidified with 5 ml of acetic acid and evaporated to dryness. The oily residue was dissolved in a mixture of 15 ml of methanol and 15 ml of acetic acid and was hydrogenated over palladium black for 5 h. The catalyst was filtered off and the filtrate was evaporated to dryness. The product obtained was reprecipitated from acetic acid with methanol. The yield of prolylphenylalanine (B 7-8) was 1.38 g (91%), mp 238-240°C (decomp.), $[\alpha]_D^{20}$ −42° (c 1; 6 N hydrochloric acid), R_f 0.60 [mp 244-246°C (decomp.); $[\alpha]_D^{24}$ −41.7° (c 1.7; 6 N hydrochloric acid) [14].

SUMMARY

1. A number of fragments of angiotensin have been synthesized: asparaginylarginine, asparaginylarginylvaline, asparaginylarginylvalyltyrosine, asparaginylarginylvalyltyrosylvalylhistidine, valyltyrosylvalylhistidine, and prolylphenylalanine.

2. The fragments of the hormone differ both in their affinity for the receptor and in their internal activity and capacity for antagonizing or potentiating the action of angiotensin, which shows the existence of a definite functional organization of the molecule of the hormone.

3. The C-terminal amino acid residues of angiotensin include the bulk of the information necessary for the formation of a complex with the receptor, and the N-terminal acids that for the formation of the secondary signal.

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