SYNTHESIS OF A FRAGMENT OF BOVINE FIBRINOPEPTIDE B WITH THE AMINO ACID SEQUENCE 12-21

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UDC 547.964.4+577.17.07

Bovine fibrinopeptide B, formed from fibrinogen by the action of thrombin on it [1], possesses a pressor action [2]. Fibrinopeptide B differs from other vasoactive peptides such as angiotensin by a weaker but prolonged effect. Thus, in rats fibrinopeptide B in doses ten times larger ($4 \cdot 10^{-4}$ μmole) than angiotensin II possessed approximately 33% of the activity of the latter and acted for more than 15 min.

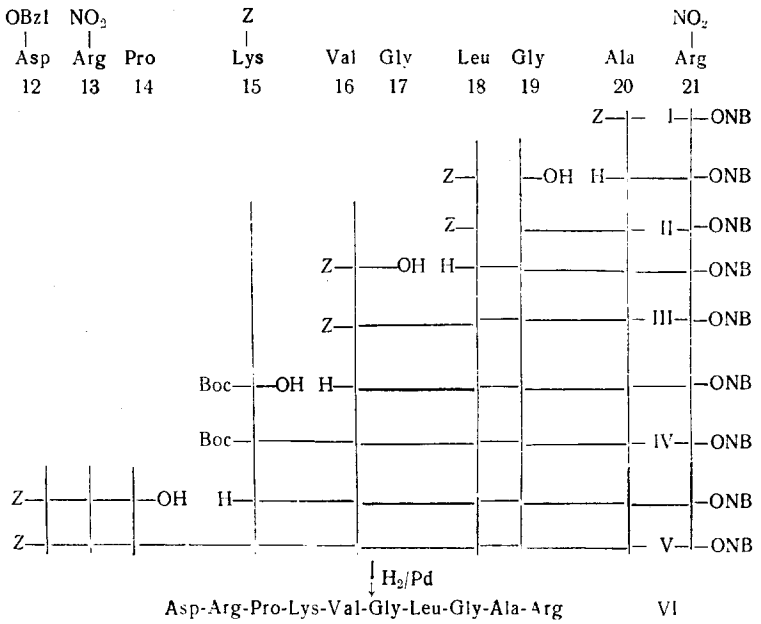
The primary structure of fibrinopeptide B contains a fragment with the amino acid sequence $Asp^{12}-Arg^{13}-Pro^{14}-$, the structural elements of which are frequently found in other low-molecular-weight peptide hormones ("common" fragments). It has been shown previously that peptides containing a common fragment have the same structural organization [3] (in all cases, this fragment is located immediately adjacent or close to the "active center" – the smallest amino acid sequence still possessing the specific action of the natural hormone) and that the "common" fragments make a considerable contribution in the formation of the secondary signal at the level of the receptor – the removal or addition of the common fragment is connected with a change in the specific biological action of the hormones averaging three orders of magnitude. In view of this, the synthesis of the presumed specific active center of fibrinopeptide B and the investigation of the potentiation of its biological activity after the addition of the common fragment are matters of interest. A priori, on the basis of a model of the structural-functional organization of several groups of peptides [4], it may be assumed that the specific activity of fibrinopeptide B is determined mainly by the C end of the heptapeptide with the amino acid sequence 15-21 located adjacent to the common fragment (12-14). The N-terminal amino acid sequence of the peptide mainly contains polar acidic amino acids. In this respect, the molecule of fibrinopeptide B is somewhat similar to that of gastrin [5]. The C-terminal hexa-, hepta-, and nonapeptides of fibrinopeptide B have been described by Suzuki [6]. The structural elements of the latter resemble bradykinin in some respects, but the nonapeptide, just like the heptapeptide, possesses only one-thousandth of the activity of bradykinin, and the hexapeptide is inactive [6].

In the present communication are described the results of the synthesis and biological testing of fragments of fibrinopeptide B with the amino acid sequences 15-21 (presumed active center) and 12-21 (common fragment with the added active center). The synthesis of the heptapeptide 15-21 was performed by the mixed-anhydride method (see scheme). After the addition of the tripeptide 12-14 [7] by the dicyclohexylcarbodimide method with the addition of N-hydroxysuccinimide, a protected decapeptide was obtained. The protective groups were eliminated by catalytic hydrogenolysis, and the free decapeptide was purified on a column of carboxymethylcellulose in a linear concentration gradient of ammonium acetate solution.

The pressor activity of the fragments synthesized was investigated on intact rats under urethane anesthesia. As compared with (1-asparagine, 5-valine)angiotensin II, the decapeptide (VI) in 100-times larger doses ($1.5 \cdot 10^{-4}$ g/kg) possessed a 25% activity, and the heptapeptide (VII) had 10% of the activity of the decapeptide in the same test. No prolonged action, as compared with (1-asparagine, 5-valine)angiotensin II was observed in either case. Evidently the N-terminal part of the peptide is responsible for this effect.

Thus, in this case the addition of the common fragment potentiates the action of the heptapeptide by one order of magnitude. The poor potentiating effect can apparently be explained by the fact that after the

Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 768-773, November-December, 1973. Original article submitted September 25, 1972.



Scheme of the synthesis of the fragment of bovine fibrinopeptide B with the amino acid sequence 12-21. Notes. Z – Benzyloxycarbonyl; Boc – t-butoxycarbonyl; ONB – p-nitrobenzyl; OBzl – β-benzyl.

addition of the common fragment, the structure of the whole hormone is not produced. A similar phenomenon has been observed in the case of eledoisin on passing from the hexapeptide$^{6-11}$ to the nonapeptide$^{3-11}$ [8]. When the addition of the common fragment leads to the structure of the whole hormone, the activity increases more considerably. Thus, the activity rises 10^4-fold in the case of angiotensin II (fragment$^{4-8}$ as compared with the hormone$^{1-8}$) [9], [8-arginine]vasopressin (fragment$^{1-6}$, hormone$^{1-9}$) [10], and bradykinin (fragment$^{5-9}$, kinin$^{1-9}$) [11], etc.

At the present time it is not possible to make pharmacological tests of fibrinopeptide B and its fragments under similar conditions. However, the available information shows that the decapeptide possesses approximately 10% of the activity of whole fibrinopeptide B, which consists of 21 amino acids.

EXPERIMENTAL

The experiments were performed with amino acids of the L series with the exception of glycine. The melting points were determined in open capillaries and the optical rotations on a Perkin-Elmer 141 spectropolarimeter at 20°C. The peptides obtained were characterized by thin-layer chromatography on Silufol plates in the chloroform–methanol (9:1) system (1), by paper chromatography (Filtrak FN-15) in the butan-1-ol–acetic acid–water 5:1:2 (2) and the sec-butanol–3% aqueous ammonia (8:2) (3) systems, and by paper electrophoresis (Filtrak FN-16) in 1 N acetic acid solution at 20 V/cm for 30 min. In the isolation of the protected peptides, solutions of them in organic solvents were washed successively with 0.5 N hydrochloric acid, water, 1 N bicarbonate solution, and water again. The results of the analyses of all the compounds corresponded to the calculated figures.

p-Nitrobenzyl Ester of Benzyloxycarbonylalanylnitroarginine (I). To a solution of 4.9 g (22 mmoles) of benzyloxycarbonylalanine in 15 ml of chloroform were added 3.0 ml (22 mmoles) of triethylamine and, at −15°C, 2.8 ml (22 moles) of n-butyl chloroformate. The mixture was stirred at −5°C for 20 min, and a solution of 9.6 g (22 moles) of the hydrobromide of the p-nitrobenzyl ester of nitroarginine [12] in 20 ml of chloroform was added at such a rate that the temperature of the reaction mixture did not rise above −5°C. After being stirred for another 4 h at the same temperature, the chloroform solution was washed and was dried with sodium sulfate, and the solvent was evaporated off in vacuum. After crystallization from ethanol with the addition of ether, the yield of the dipeptide (I) was 8.5 g (69%), mp 153-154°C, $[\alpha]_D^{20}$ −16.6° (c 1.0; acetic acid), R_f 0.37 (1), $C_{24}H_{29}N_7O_9$.

p-Nitrobenzyl Ester of Benzyloxycarbonylleucylglycylalanylnitroarginine, Chloroform Solvate (II). To a solution of 5.0 g (9.0 mmoles) of the protected dipeptide (I) in 10 ml of acetic acid was added 30 ml of a 3.8 N solution of hydrogen bromide in glacial acetic acid. The mixture was left at room temperature

for 1 h, and then 300 ml of ether was added. The oil that precipitated was triturated with ether and was kept in a vacuum over potassium hydroxide. The solidified residue, containing 3.5 meq/g of hydrogen bromide (Volhard determination), was dissolved in 15 ml of dimethylformamide, the solution was brought to a weakly alkaline reaction with triethylamine, and at −10°C it was added to a solution of the mixed anhydride obtained from 2.9 g (9.0 mmoles) of benzyloxycarbonylleucylglycine [13], 1.24 ml of triethylamine, and 1.14 ml of n-butyl chloroformate. The mixture was kept at 0°C for 24 h, the precipitate was filtered off, and the solvent was evaporated off in vacuum. The residue was triturated with petroleum ether and was precipitated from chloroform with carbon tetrachloride. The yield of the tetrapeptide (II) was 4.2 g (55%), mp 80°C (decomp.), $[\alpha]_D^{20}$ −21.0° (c 1.0; ethanol), R_f 0.25 (1), $C_{32}H_{43}N_9O_{11} \cdot CHCl_3$.

p-Nitrobenzyl Ester of Benzyloxycarbonylvalylglycylleucylglycylalanylnitroarginine, Chloroform Solvate (III). To a solution of 2.5 g (3.0 mmoles) of the protected tetrapeptide (II) in 5 ml of acetic acid was added 16 ml of a 3.8 N solution of hydrogen bromide in glacial acetic acid. The mixture was left at room temperature for 1 h, and then 300 ml of ether was added. The residual oil was triturated with ether and kept in vacuum over potassium hydroxide. The solidifying residue was dissolved in 7 ml of dimethylformamide and the solution was made feebly alkaline with triethylamine and was added at −10°C to a solution of the mixed anhydride prepared from 0.92 g (3.0 mmoles) of benzoxycarbonylvalylglycine [14], 0.41 ml of triethylamine and 0.38 ml of n-butyl chloroformate. The mixture was kept at 0°C for 24 h, the precipitate of triethylamine hydrobromide was filtered off, and the solvent was evaporated in vacuum. The residue was dissolved in ethyl acetate, the solution was washed and was dried with sodium sulfate, the ethyl acetate was evaporated off to small volume, and the petroleum ether was added. After reprecipitation of the resulting precipitate from chloroform with carbon tetrachloride, the yield of the hexapeptide (III) was 1.6 g (53%), mp 110°C (decomp.), $[\alpha]_D^{20}$ −9.4° (c 1.0; dimethylformamide), R_f 0.20 (1), $C_{39}H_{55}N_{11}O_{13} \cdot CHCl_3$.

p-Nitrobenzyl Ester of t-Butoxycarbonyl-ε-benzyloxycarbonyllysylvalylglycylleucylglycylalanylnitroarginine (IV). To a solution of 1.0 g (1.0 mmole) of the protected hexapeptide (III) in 2.5 ml of acetic acid was added 7.5 ml of a 3.8 N solution of hydrogen bromide in glacial acetic acid. The mixture was left at room temperature for 1 h, and then 100 ml of ether was added and the oil that separated out was triturated with ether and was kept in vacuum over potassium hydroxide. The solidifying residue was dissolved in 4 ml of dimethylformamide, and the resulting solution was brought to a weakly alkaline reaction with triethylamine, and 0.46 g (1.2 mmole) of t-butyloxycarbonyl-ε-benzyloxycarbonyllysine, 0.28 g (2.4 mmoles) of N-hydroxysuccinimide [15], and, at −5°C, 0.23 g (1.1 mmole) of dicyclohexylcarbodiimide were added. The mixture was kept at 0°C for 18 h and at room temperature for 6 h. The precipitate was filtered off, the filtrate was evaporated in vacuum to small volume, the residue was dissolved in 10 ml of ethyl acetate, the solution was washed (with acid at 0°C) and was dried with sodium sulfate, the ethyl acetate was evaporated off, and the residue was triturated with petroleum ether. After reprecipitation from methanol with ether, the yield of the heptapeptide (IV) was 0.78 g (70%), mp 160-165°C (decomp.), $[\alpha]_D^{20}$ −27.8° (c 0.9; ethanol), R_f 0.23 (1), $C_{50}H_{75}N_{13}O_{16}$. Literature data: mp 175°C [6].

p-Nitrobenzyl Ester of Benzyloxycarbonyl(β-COO-benzylaspartyl)nitroarginylprolyl(ε-benzyloxycarbonyllysyl)valylglycylleucylglycylalanylnitroarginine (V). A solution of 0.50 g (0.45 mmole) of the protected heptapeptide (IV) in 1.5 ml of acetic acid was treated with 10 ml of a 1 N solution of hydrogen chloride in glacial acetic acid. The mixture was left at room temperature for 1 h, and 100 ml of ether was added. The precipitate that deposited was filtered off, washed with ether, and kept in vacuum over potassium hydroxide. The residue was dissolved in 4 ml of dimethylformamide, the solution was brought to an alkaline reaction with triethylamine, and 0.36 g (0.55 mmole) of benzyloxycarbonyl(β-COO-benzylaspartyl)-nitroarginylproline [7], 0.10 g (0.9 mmole) of N-hydroxysuccinimide, and, at −5°C, 0.10 g (0.49 mmole) of dicyclohexylcarbodiimide were added. The mixture was kept at 0°C for 48 h and at room temperature for 12 h. The precipitate was filtered off, and the filtrate was evaporated in vacuum (40°C). The residue was dissolved in 6 ml of a mixture of butan-1-ol and chloroform (1 : 1), the solution was washed, dried with sodium sulfate, and evaporated in vacuum, and the residue was triturated with ether. After reprecipitation from ethanol with ether, the yield of protected decapeptide (V) was 0.40 g (54%), mp 180°C (decomp.), $[\alpha]_D^{20}$ −21.4° (c 0.4; dimethylformamide), R_f 0.44 (1), $C_{75}H_{102}N_{20}O_{23}$.

Aspartylarginylprolyllysylvalylglycylleucylglycylalanylarginine, Diacetate (VI). A solution of 248 mg (0.15 mmole) of the protected decapeptide (V) in 10 ml of a mixture of methanol, acetic acid, and water (6 : 1 : 1) was hydrogenated in the presence of palladium black for 50 h. After the catalyst had been filtered off, the solution was evaporated to dryness in vacuum and was kept over potassium hydroxide. The residue was purified on a column (2 × 20 cm) of carboxymethylcellulose in a concentration gradient of am-

monium acetate (300 ml of a 0.25 M solution of ammonium acetate in the reservoir and 300 ml of water in the mixer, pH 6.5). The fraction issuing at a concentration of ammonium acetate solution of about 0.21 M and containing all the expected amino acids, as shown by the hydrolysis of a sample, was evaporated in vacuum and freeze-dried to constant weight. The yield of the decapeptide (VI) was 62 mg, mp above 150°C, $[\alpha]_D^{20}$ −42.0° (c 0.5; water), R_f 0.11 (2), 0.12 (3), E_{Arg} 0.88 (ninhydrin, Sakaguchi, positive spot). Amino acid analysis: alanine 0.96, aspartic acid 0.85, arginine 2.10, valine 0.92, glycine 1.76, leucine 0.95, lysine 1.12, proline 0.77.

Lysylvalylglycylleucylglycylalanylarginine, Diacetate (VII). A mixture of 123 mg (0.11 mmole) of the protected heptapeptide (IV) and 5 ml of a 1 N solution of hydrogen chloride in glacial acetic acid was evaporated in vacuum and the residue was hydrolyzed and purified in a similar manner to compound (VI). The fraction issuing from the column at a concentration of ammonium acetate of about 0.15 M and containing all the expected amino acids, as shown by the hydrolysis of a sample, was evaporated in vacuum and freeze-dried to constant weight. The yield of the heptapeptide (VII) was 36 mg, decomp. pt. above 165°C, $[\alpha]_D^{20}$ −25.5° (c 0.5; water), R_f 0.10 (2), 0.07 (3), E_{Arg} 0.91 (ninhydrin, Sakaguchi, positive spot). Amino acid analysis: alanine 0.93, arginine 1.04; valine 0.94, glycine 1.98, leucine 1.06, lysine 1.09. Literature data: $[\alpha]_D^{27}$ −26.9° (c 0.5; water) [6].

SUMMARY

1. The assumed specifically active fragment of bovine fibrinopeptide B with the amino acid sequence 15-21 and the fragment 12-21 containing the tripeptide residue Asp^{12}-Arg^{13}-Pro^{14} have been synthesized.

2. Their pressor activities on rats have been determined, and it has been shown that the addition of the tripeptide raises the pressor activity of the heptapeptide by one order of magnitude.

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