similar. In the counter-current distribution (500 transfers) $K = 0.16$ was observed; the hormone analog has an R_f value of 0.15 in the solvent systems described by XII; $[\alpha]^{20}D$ −20° (*c*, 1, N acetic acid). Amino acid analysis gives the molar ratios (glutamic acid = 1.0): aspartic acid 1.09, proline 0.92, glycine 0.97, cystine 1.2, isoleucine 0.98, tyrosine 0.90, ammonia 3.8, citrulline 0.77, and ornithine 0.26.

In the assay on the isolated rat uterus the oxytocic potency of XVI is about 500 u./mg., while about 250 u./mg. was exhibited in the avain depressor test. The pressor activity in rat was found to be about 7 u./mg. In the amphibian bladder test XII is only slightly more active than oxytocin and much less active than arginine vasotocin.

Acknowledgments.—The authors wish to thank Drs. B. Rubin and J. Piala for carrying on the bioassays. They are indebted also to Mr. J. Alicino and his co-workers for the microanalytical work reported herein and to Mrs. Nina J. Williams for the amino acid analyses.

[CONTRIBUTION FROM THE RESEARCH DIVISION OF THE CLEVELAND CLINIC FOUNDATION, CLEVELAND 6, OHIO]

Synthesis of Alanyl⁴-isoleucyl⁵-angiotensin II[1]

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RECEIVED MARCH 15, 1962

The octapeptide alanyl⁴-isoleucyl⁵-angiotensin II has been synthesized to study further the significance of the aromatic ring in position 4 of the natural peptide. This synthetic peptide possesses about 3% of the pressor activity of isoleucyl⁵-angiotensin II.

Recent studies on analogs and homologs of angiotensin II, the pressor octapeptide L-aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine, have shown the two amino acids with aromatic rings are important to biological activity.[3,4,5] Removal of the aromatic ring of phenylalanine greatly reduces pressor activity.[3] The peptide without the phenolic hydroxyl group of tyrosine, phenylalanyl⁴-angiotensin, has only 2 to 10%[4,6] of the activity of the parent octapeptide. The two aromatic side groups are positioned very close to each other in a conformation recently suggested for angiotensin II.[7] When the position of the two groups relative to each other is changed by the substitution of alanine for proline in position 7,[8] pressor activity is greatly reduced. To study further the importance of the aromatic ring of tyrosine for biological activity, we have synthesized alanyl⁴-isoleucyl⁵-angiotensin II, the octapeptide without this ring in position four.

Experimental[9]

Carbobenzoxy-L-valyl-L-alanine Methyl Ester (I).—To 25.1 g. (100 mmoles) of carbobenzoxy-L-valine in 53 ml. of tetrahydrofuran was added 23.8 ml. of tri-*n*-butylamine. The solution was cooled to −10° and then 9.45 ml. of ethyl chloroformate was added. After stirring for 20 minutes at −10°, 14.0 g. (100 mmoles) of alanine methyl ester hydrochloride and 24.0 ml. of tri-*n*-butylamine in 100 ml. of tetrahydrofuran were added. The mixture was stirred at room temperature for 2 hours and then 200 ml. of water and 200 ml. of ethyl acetate were added. The two liquid phases were separated and the aqueous phase was again extracted with 50 ml. of ethyl acetate. The combined extract then was washed twice with 50 ml. of *N* hydrochloric acid and once with 50 ml. of distilled water. Then 350 ml. of ethyl acetate and 50 ml. of methanol were added to redissolve a precipitate which formed in the organic phase. The organic phase was further washed once with 50 ml. of distilled water, twice with 50 ml. of saturated sodium bicarbonate, and twice with 50 ml. of distilled water. This entire extraction procedure was conducted at 4°. The organic solution was dried over sodium sulfate and the solvent removed *in vacuo*. The residue was dissolved in a minimum of ethyl acetate; then pet. ether (b.p. 30–60°) was added to the point of incipient turbidity. The peptide crystallized on standing to yield 23.3 g. of product, m.p. 162°. An additional 2 g. of product was obtained from the mother liquor (yield 75%). Upon recrystallization as above, a product melting at 162.5–163° was obtained; $[\alpha]^{22}D$ −49.45° (*c*, 1.65, in methanol). *Anal.* Calcd. for $C_{17}H_{24}N_2O_5$: C, 60.70; H, 7.19; N, 8.33. Found: C, 60.70; H, 7.13; N, 8.16.

Carbobenzoxy-L-valyl-L-alanine Hydrazide (II).—To 15 g. of carbobenzoxy-L-valyl-L-alanine methyl ester in 375 ml. of methanol was added 15 ml. of 85% hydrazine hydrate and the solution was heated under reflux for two hours. Upon cooling to room temperature, 1100 ml. of water was added and the solution was allowed to stand at 4° overnight. The crystalline product was collected by filtration and recrystallized from methanol by the addition of water to yield 13.9 g. (92.5%) of peptide melting at 219–222°; $[\alpha]^{22}D$ −57.67° (*c*, 0.86, in methanol). *Anal.* Calcd. for $C_{16}H_{24}N_4O_4$: C, 57.30; H, 7.19; N, 16.66. Found: C, 57.78; H, 7.25; N, 16.90.

Carbobenzoxy-L-valyl-L-alanyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine-*p*-nitrobenzyl Ester (III).—To 10.95 g. (14 mmoles) of carbobenzoxy-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-nitrobenzyl ester[10] in 35 ml. of anhydrous acetic acid was added 30 ml. of freshly prepared 4.2 *N* hydrogen bromide in anhydrous acetic acid. After 50 minutes at room temperature, 900 ml. of anhydrous ether was added to precipitate the peptide ester dihydrobromide. The precipitate was collected by filtration, washed with a large volume of anhydrous ether and dried over sodium hydroxide and phosphorus pentoxide. This material on paper chromatography showed two spots; R_{fBAW} 0.70 and 0.46 (both ninhydrin and Pauly positive), so it was converted to the free base as described below.

(1) This work was supported in part by the National Heart Institute U. S. Public Health Service, Grant H-96.

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(9) All melting points were taken on a Kofler hot-stage and are corrected. Microanalyses were done by Micro-Tech Laboratories, Skokie, Ill. Paper chromatograms were developed using butanol:acetic acid:water (4:1:5) abbreviated as R_{fBAW}; acetonitrile:methanol (1:1) abbreviated as R_{fAM}; acetonitrile:1-butanol (1:1) abbreviated as R_{fAB}; dimethylformamide:methanol (1:3) abbreviated as R_{fDM}. The conditions used for paper electrophoresis were: solvent, 95 ml. of acetic acid and 36 ml. of formic acid diluted to 2 liters with distilled water; *p*H 2.1; voltage, 14.8 volts per cm. of paper. Electrophoretic mobilities are reported as the ratio: distance the peptide moved/distance glutamic acid moved and abbreviated as E_G.

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The dried peptide ester dihydrobromide was dissolved in 30 ml. of water and extracted twice with 35 ml. of ethyl acetate. The pH of the cold (4°) aqueous solution then was adjusted to 9 with cold (4°) 50% potassium carbonate solution. The alkaline solution was extracted five times with 200 ml. of ethyl acetate at 4°. The combined ethyl acetate extracts were washed with 100 ml. of 50% potassium carbonate solution, twice with saturated sodium chloride solution and dried over anhydrous sodium sulfate. After removal of the sodium sulfate, the volume of the solution was reduced to 40 ml. *in vacuo*. The tetrapeptide ester prepared in this manner gave one spot on paper chromatography and was used without further treatment.

To a cold (0°) solution of 7.16 g. (21 mmoles) of carbobenzoxy-L-valyl-L-alanine hydrazide in 80 ml. of glacial acetic acid and 30 ml. of 5 *N* hydrochloric acid was added 1.45 g. (21 mmoles) of sodium nitrite in 10 ml. of cold water. After five minutes, 300 ml. of cold (0°) 25% potassium carbonate solution was added and the mixture was extracted four times with 175 ml. of cold (−3°) ethyl acetate. The combined ethyl acetate extracts were washed twice with 50 ml. of cold (0°) 50% potassium carbonate solution, twice with 100 ml. of cold water, once with 60 ml. of saturated sodium chloride and dried by shaking for 10 minutes with anhydrous sodium sulfate. This azide solution then was filtered into the cold solution of tetrapeptide ester prepared as described above. The mixture was stirred for two and one-half hours at 0° and overnight at room temperature. The reaction mixture was washed twice with 40 ml. of 1 *N* hydrochloric acid, twice with 80 ml. of water, twice with 50 ml. of 1 *M* potassium carbonate, twice with 80 ml. of water and dried over anhydrous sodium sulfate all at 4°. After removal of the sodium sulfate the solution was evaporated to dryness *in vacuo*. The solid material was dissolved in 400 ml. of methanol and the product precipitated by the addition of ether; yield 9.95 g. (74.7%); m.p. 150–160°; R_{fAM} 0.80. The material was redissolved in methanol and precipitated by the addition of ether; yield 8.45 g. (63.5%); m.p. 158–161°. After hydrolysis of a small portion of this sample for 50 hours at 110° in 6 *N* hydrochloric acid, amino acid analyses (in μ mole) obtained using the method of Spackman, Stein and Moore[11]; proline 1.0, isoleucine 0.95, alanine 0.86, phenylalanine 0.94 and valine 0.86. For further analysis a small sample was precipitated twice from methanol with ether and the product dried at 110° overnight (16 hours); m.p. 163–166°; $[\alpha]^{22}D$ −40.10° (*c*, 1.0, in dimethylformamide); R_{fAM} 0.88; R_{fAB} 0.90. *Anal.* Calcd. for $C_{40}H_{61}N_9O_{11}$: C, 61.80; H, 6.45; N, 13.24. Found: C, 61.54; H, 6.73; N, 13.14.

L-Valyl-L-alanyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester Dihydrobromide (IV).—To 8.1 g. (8.27 mmole) of carbobenzoxy hexapeptide ester (III) in 30 ml. of anhydrous acetic acid was added 30 ml. of freshly prepared 4.8 *N* hydrogen bromide in anhydrous acetic acid. After 57 minutes at room temperature the product was precipitated by the addition of 500 ml. of anhydrous ether, collected by filtration, washed with anhydrous ether and dried over sodium hydroxide and phosphorus pentoxide *in vacuo*. The dried product was dissolved in methanol–tetrahydrofuran (1:1) and precipitated with ether; yield 8.0 g. This product yielded two spots on paper chromatography. It was reprecipitated twice as above; yield 7.6 g. (91%); m.p. 171–174°. A small sample of this product was further reprecipitated twice as above and dried at 110° for 10 hours *in vacuo* for analysis; m.p. 168–172°; $[\alpha]^{22}D$ −38.20° (*c*, 1, in acetic acid); R_{fBAW} 0.68; E_G 1.17. *Anal.* Calcd. for $C_{41}H_{57}N_9O_9Br_2 \cdot H_2O$: C, 49.35; H, 5.96; N, 12.63; Br, 16.02. Found: C, 49.38; H, 5.94; N, 12.58; Br, 15.88.

Carbobenzoxy-nitro-L-arginyl-L-valyl-L-alanyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester (V).—To a cold (−13°) solution of 1.86 g. (5 mmoles) of carbobenzoxy-nitro-L-arginine monohydrate and 1.2 ml. (5 mmoles) of tri-*n*-butylamine in 10 ml. of anhydrous dimethylformamide was added 0.47 ml. (5 mmoles) of ethyl chloroformate. After stirring for 15 minutes at −10°, a cold (−10°) solution of 4.9 g. (5 mmoles) of hexapeptide ester dihydrobromide (IV) and 2.88 ml. (12 mmoles) of tri-*n*-butylamine in 23 ml. of anhydrous dimethylformamide was added. After 30 minutes at −10°, a second portion of the mixed anhydride prepared as described above from 1.86 g. (5 mmoles) of carbobenzoxy-nitro-L-arginine monohydrate was added to the reaction mixture. After standing thirty minutes at −10° and overnight (about 16 hours) at room temperature, 800 ml. of ethyl acetate was added to the reaction mixture. The resulting hazy solution was washed twice with water, once with 60 ml. of 1 *N* hydrochloric acid, once with 60 ml. of 0.5 *N* hydrochloric acid, twice with water, three times with 50 ml. of 1 *M* potassium carbonate, and five times with water. The final clear solution was dried over anhydrous sodium sulfate, the volume was reduced *in vacuo* and the product precipitated by the addition of ether. The precipitate was collected by filtration, redissolved in methanol and precipitated with ether. This precipitation procedure was repeated and the final product dried over phosphorus pentoxide; yield 3.61 g. (62.7%); m.p. 209–213°; R_{fDM} 0.81. A small sample of this product was precipitated two more times from methanol with ether, once from methanol with water, and once from methanol with ethyl acetate. This sample was dried overnight at 140° *in vacuo;* m.p. 203–206°; $[\alpha]^{22}D$ −30.50° (*c*, 1.0, in dimethylformamide). *Anal.* Calcd. for $C_{56}H_{72}N_{14}O_{14}$: C, 57.28; H, 6.29; N, 17.01. Found: C, 57.20; H, 6.57; N, 16.77.

Nitro-L-arginyl-L-valyl-L-alanyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester (VII).—To 1.15 g. (1.0 mmole) of carbobenzoxyheptapeptide ester (V), which had been dried overnight at 110° *in vacuo*, in 10 ml. of anhydrous acetic acid was added 10 ml. of freshly prepared 4 *N* hydrogen bromide in anhydrous acetic acid. After 45 minutes at room temperature, 200 ml. of anhydrous ether was added, the resulting precipitate collected by filtration and washed with a large volume of anhydrous ether. This product, after drying overnight over sodium hydroxide and phosphorus pentoxide *in vacuo*, gave three spots on paper chromatography; R_{fBAW} 0.66, 0.73 (both ninhydrin and Pauly positive) and 0.95 (Pauly positive). This material was dissolved in 5 ml. of cold water. The solution was extracted once with 20 ml. of ethyl acetate, cooled to 0°, and the pH was adjusted to 9 with saturated sodium carbonate. The precipitate was collected by filtration, washed with a large volume of cold water, redissolved in methanol and precipitated with ether. This product was dried over phosphorus pentoxide *in vacuo;* yield 0.71 g. (70%), m.p. 145–149°. A portion of this product was dissolved in methanol, treated with charcoal (Darco G-60), and precipitated with ether. Then precipitation from methanol with ethyl acetate was carried out three times. This product still gave two spots on paper chromatography (R_{fBAW} 0.67 and 0.73). The minor contaminant (R_{fBAW} 0.67) finally was removed by repeating the aqueous precipitation procedure given above, and precipitation twice from methanol with ether. The final product was dried for 20 hours at 110° over P_2O_5 *in vacuo;* m.p. 149–158°; $[\alpha]^{22}D$ −101.20° (*c*, 1.0, in dimethylformamide); R_{fBAW} 0.74; R_{fAW} 0.73; E_G 1.04. *Anal.* Calcd. for $C_{47}H_{66}N_{14}O_{12}$: C, 55.39; H, 6.53; N, 19.24. Found: C, 55.20; H, 6.64; N, 18.91.

Carbobenzoxy-β-benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-L-alanyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester (VIII).—To a cold (−13°) solution of 0.72 g. (2 mmoles) of carbobenzoxy-β-benzyl-L-aspartic acid[12] and 0.46 ml. (2 mmoles) of tri-*n*-butylamine in 6 ml. of anhydrous dimethylformamide was added 0.192 ml. (2 mmoles) of ethyl chloroformate. The mixture was stirred at −13° for 15 minutes and then a solution of 0.82 g. (0.82 mmole) of the heptapeptide ester (VII) in 10 ml. of anhydrous dimethylformamide was added. The reaction mixture was stirred for 40 minutes at −10°, then three and one-half hours at room temperature and concentrated under reduced pressure to about one-half the original volume. This solution was cooled to 4° and 4 ml. of 1 *N* hydrochloric acid and 30 ml. of cold water were added. The precipitate was removed by filtration, washed carefully with water and air dried. The product then was dissolved in 10 ml. of dimethylformamide, cooled to 0°, and precipitated by the addition of 5 ml. of saturated sodium carbonate solution and 50 ml. of water. After standing overnight at 0°, the precipitate was collected by filtration, washed with water and dissolved in dimethylformamide. The peptide was precipitated with ether and dried *in vacuo* over phosphorus

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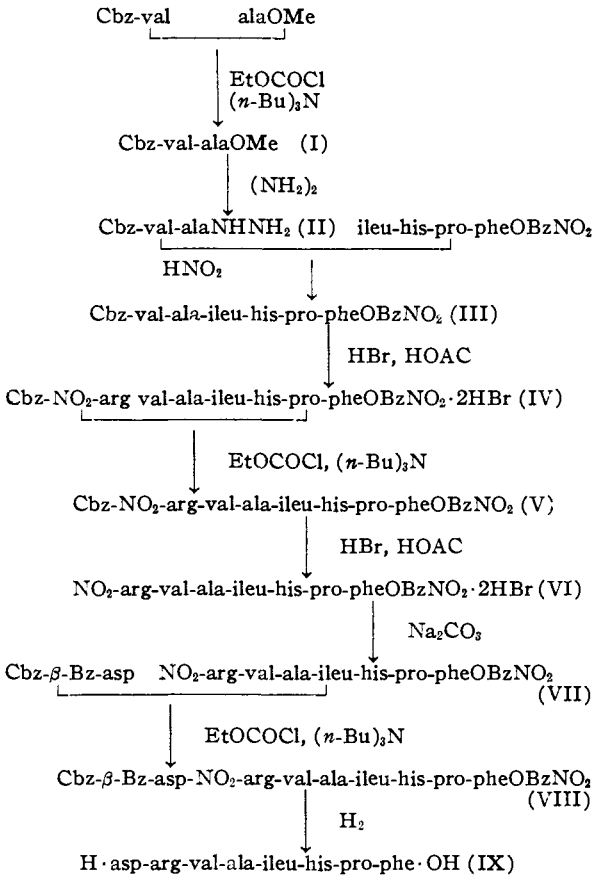

Fig. 1.—Outline of the synthesis of alanyl[4]-isoleucyl[5]-angiotensin II.

pentoxide; yield 1.0 g. (90.9%); m.p. 184–188°. A portion of this sample was precipitated twice from hot methanol by cooling the solution, once from methanol with ether and dried 10 hours at 110° *in vacuo;* m.p. 195–200°; $[\alpha]^{24}D$ −37.00° (*c*, 1.0, in acetic acid). *Anal.* Calcd. for $C_{66}H_{83}N_{15}O_{17}$: C, 58.35; H, 6.16; N, 15.47. Found: C, 58.23; H, 6.31; N, 15.33.

L-Aspartyl-L-arginyl-L-valyl-L-alanyl-L-isoleucyl-L-histidyl - L - prolyl - L - phenylalanine Monoacetate. Alanyl[4]-isoleucyl[5]-angiotensin II (IX).—To a solution of 1.13 g. (0.9 mmole) of blocked octapeptide (VIII) in 25 ml. of acetic acid and 9 ml. of methanol was added a suspension of 0.3 g. of palladium black in 3 ml. of water. Hydrogen gas, which was passed through 50% aqueous methanol, was bubbled through the mixture for 57 hours. The catalyst was removed by filtration, the filtrate concentrated *in vacuo* to 10 ml. and the peptide precipitated by the addition of 180 ml. of acetone and collected by filtration. The product was precipitated three times from water with acetone and dried over phosphorus pentoxide *in vacuo*. After precipitation the peptide gave two spots on paper chromatography (R_{fBAW} 0.49 and 0.55) and three spots on paper electrophoresis. The product was washed twice by suspending it in hot anhydrous methanol; yield 0.71 g. (84.1%); m.p. 230–245° (dec.). This material gave only one spot on paper chromatography but a trace of contaminating peptide was detected on paper electrophoresis. The peptide was purified further by precipitating it twice from water with acetone. The final product was dried for 18 hours at 110° over phosphorus pentoxide *in vacuo;* m.p. 230–260° (dec.); $[\alpha]^{25}D$ −61.34° (*c*, 1.0, in 0.1 *N* acetic acid); R_{fBAW} 0.41; E_G 1.37. *Anal.* Calcd. for $C_{46}H_{71}N_{13}O_{13}$: C, 54.48; H, 7.06; N, 17.96. Found: C, 54.64; H, 6.97; N, 18.16.

This peptide was found to possess 410 units of activity per mg. in the rat blood pressure assay.[5] After hydrolysis of the peptide[13] with swine kidney leucine aminopeptidase[14] the expected eight amino acids were detected by paper chromatography and the spot due to peptide could not be detected.

Discussion

The synthesis of alanyl[4]-isoleucyl[5]-angiotensin II is outlined in Fig. 1. The C-terminal tetrapeptide as the *p*-nitrobenzyl ester was prepared as described previously.[10] The protected dipeptide (II) was added to this tetrapeptide using the azide method to give the protected hexapeptide (III) in 64% yield. After removal of the carbobenzoxy group from this hexapeptide, it was condensed with carbobenzoxy-nitro-L-arginine by the mixed-anhydride procedure to give the protected heptapeptide (V) in 63% yield. The mixed-anhydride procedure was again used to add carbobenzoxy-β-benzyl-L-aspartic acid to the heptapeptide to give the fully protected octapeptide (VIII) in 91% yield. All blocking groups were removed by hydrogenation to give alanyl[4]-isoleucyl[5]-angiotensin II (IX) in 84% yield. Final purification of the octapeptide was achieved by several precipitation steps. Alanyl[4]-isoleucyl[5]-angiotensin II is considerably more soluble in aqueous solutions, such as 0.1 *M* acetic acid, than isoleucyl[5]-alanyl[7]-angiotensin II[8] and is also more soluble than isoleucyl[5]-angiotensin II. The reason for the increased solubility upon removal of the phenolic ring is not known.

Alanyl[4]-isoleucyl[5]-angiotensin II was found to possess about 3% of the pressor activity of isoleucyl[5]-angiotensin II. Since this is approximately the same pressor activity previously found for phenylalanyl[4]-valyl[5]-angiotensin II,[4,6] removal of this aromatic ring does not decrease pressor activity further than removal of the –OH group alone.

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