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

Synthesis of Succinyl[1]-isoleucyl[5]-angiotensin II and N-(Poly-O-acetyl-seryl)-isoleucyl[5]-angiotensin II[1]

BY KIKUO ARAKAWA,[2] ROBERT R. SMEBY AND F. MERLIN BUMPUS

RECEIVED SEPTEMBER 11, 1961

The synthesis of succinyl[1]-isoleucyl[5]-angiotensin II (desamino-angiotensin II), N-(poly-O-acetyl-L-seryl)-angiotensin II and N-(poly-O-acetyl-L-seryl)-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine are presented. From the pressor and myotropic activities exhibited by these peptides, the following conclusions on the relationship of structure to biological activity in angiotensin II were reached: (a) the N-terminal amino group is not essential for biological activity but it influences the degree of response; (b) an important function of the N-terminal amino acid and the adjacent amino acid is to stabilize the conformation of the peptide but this is not their sole function; (c) the peptide probably acts on the cell membrane to cause a contractile response.

Introduction

Isoleucyl[5]-angiotensin II, L-aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine is the most potent substance known causing elevation of blood pressure.[3] This octapeptide exhibits high myotropic activity. Recent studies on the biological activity of various synthetic analogues and homologues of angiotensin II[3,4] have indicated that the C-terminal amino acid must be L-phenylalanine with proline the next amino acid and tyrosine in position 4 for the peptide to exhibit biological activity.

Paiva and Paiva[5] have concluded from studies on the photo-oxidation of angiotensin II that histidine is essential for biological activity. In contrast to the structural specificity exhibited by these amino acids, the two amino acids aspartic acid and arginine can be greatly modified without significantly modifying biological activity.[3,4,6] The hexapeptide, in which these two amino acids have been removed, L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine, still retains significant pressor activity. This hexapeptide has approximately the same pressor activity as noradrenaline (arterenol) on a weight basis. Removal of valine from this hexapeptide, leaving a pentapeptide, completely destroys the biological activity. The lack of structural specificity in these two N-terminal amino acids would suggest that they function partially to stabilize the conformation of the peptide.[7] Deodhar[8] has observed that blocking the N-terminal amino group of angiotensin II with a *p*-nitrobenzoyl or *p*-aminobenzoyl group decreased the biological activity 50 to 60%. However, these blocked peptides were not purified or isolated before assay. To study the importance of this amino group in more detail, the octapeptide in which it has been removed, succinyl[1]-isoleucyl[5]-angiotensin II (desamino-angiotensin II) has been prepared. In addition, the N-terminal amino group of both the hexa- and octapeptides has been blocked using poly-O-acetyl-serine.

Synthesis

Desamino-angiotensin II was prepared by condensing succinic anhydride with nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine-*p*-nitrobenzyl ester dihydrobromide prepared previously.[9] The resulting blocked succinyl-heptapeptide was hydrogenated using palladium black as the catalyst to yield the desired peptide.

Since we were unable to prepare poly-L-serine directly from L-serine-N-carboxy-anhydride (NCA) by the method reported,[10] O-acetyl-L-serine NCA was polymerized in phosphate buffer with either angiotensin II or the hexapeptide L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine serving as the initiator. The resulting products had poly-O-acetyl-serine attached to the N-terminus. The degree of polymerization was estimated from the histidine content of an acid hydrolysate of the polymer. It was estimated that the polymer with the hexapeptide contained 110 serine residues per molecule while the polymer with angiotensin contained 220 serine residues per molecule.

O-Acetyl-L-serine hydrochloride was prepared using the procedure of Sheehan, Goodman and Hess.[11] The crystalline product, however, was shown by paper chromatography to contain a small amount of serine which could not be removed by recrystallization from ethanol. This product was converted to the free base and crystallized from aqueous ethanol to remove the serine. The resulting O-acetyl-L-serine gave a specific rotation higher than previously reported.[11]

Experimental[12]

Succinyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine-*p*-nitro-benzyl Ester.—To 0.63 g. (0.495 mmole) of nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine-*p*-nitrobenzyl ester dihydrobromide[9] dissolved in 2.5 ml. of dimethylformamide containing 0.236 ml. (0.99 mmole) tributylamine was added with stirring over a 15 min. period at 20°, 0.099 g. (0.99 mmole) of succinic anhydride dissolved in 1 ml. tetrahydrofuran. After 1 hr., no heptapep-

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

(2) Research Fellow of the Frank E. Bunts Educational Institute.

(3) I. H. Page and F. M. Bumpus, *Physiol. Rev.*, **41**, 331 (1961).

(4) F. M. Bumpus, P. A. Khairallah, K. Arakawa, I. H. Page and R. R. Smeby, *Biochim. Biophys. Acta*, **46**, 38 (1961).

(5) A. C. M. Paiva and T. B. Paiva, *ibid.*, **48**, 412 (1961).

(6) R. Schwyzer, *Helv. Chim. Acta*, **44**, 667 (1961).

(7) R. R. Smeby, K. Arakawa, F. M. Bumpus and M. M. Marsh, *Biochim. Biophys. Acta*, in press.

(8) S. D. Deodhar, *J. Exp. Med.*, **111**, 419 (1960).

(9) K. Arakawa and F. M. Bumpus, *J. Am. Chem. Soc.*, **83**, 728 (1961).

(10) G. D. Fasman and E. R. Blout, *ibid.*, **82**, 2262 (1960).

(11) J. C. Sheehan, M. Goodman and G. P. Hess, *ibid.*, **78**, 1367 (1956).

(12) All melting points were taken on a Kofler hot-stage and were 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} or acetonitrile:water (6:1) abbreviated as R_{fAW}.

TABLE I

BIOLOGICAL ACTIVITIES OF THE ANGIOTENSIN II ANALOGUES PREPARED

Peptide tested	Rat pressor activity[a] Units/μmole	Rat pressor activity[a] Per cent. of angiotensin II	Rat uterus myotropic activity[a] Units/μmole	Rat uterus myotropic activity[a] Per cent of angiotensin II	Ratio of myotropic activity to pressor activity
Desamino-angiotensin II	8,040	60.4	48,046	50.9	5.97
N-Poly-(O-acetyl-L-seryl)-hexapeptide	1,434	10.8	15,000	15.9	10.4
Hexapeptide	134	1	2,470	2.6	18.4
N-Poly-(O-acetyl-L-seryl)-angiotensin II	5,460	41	38,217	40.5	7.0
Angiotensin II	13,300	100	94,430	100	7.1

[a] The methods of assay are given in ref. 4.

tide could be observed in the reaction mixture by paper chromatography. After 2 hr., 150 ml. of a 2:1 mixture of ethyl acetate and ether was added. The white precipitate was collected by filtration and washed with the same solvent mixture to yield 0.52 g. (86.4%) of the protected desaminoangiotensin II as an amorphous product. For analysis 0.26 g. of this product was dissolved in methanol and precipitated with ethyl acetate. This precipitate was dissolved in warm methanol, allowed to precipitate by cooling and dried *in vacuo* at 78° for 15 hr.

Anal. Calcd. for $C_{57}H_{74}O_{16}N_{14}$: C, 56.52; H, 6.16; N, 16.19. Found: C, 56.46; H, 6.13; N, 16.11, $[\alpha]^{20}D$ − 27.48° (c, 0.5, dimethylformamide); m.p. 197–205° (dec); R_{fBAW}, 0.92.

Succinyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine.—The protected desaminoangiotensin II (0.3 g.) was dissolved in 10 ml. of methanol containing 0.1 ml. of glacial acetic acid and 1 ml. of water. The peptide was reduced by passing a stream of hydrogen through this solution for 10 hr. using palladium black as the hydrogenation catalyst. The catalyst was then removed by filtration and the solvent evaporated *in vacuo*. A paper chromatogram of this crude product revealed 4 spots: R_{fBAW} 0.92 (Pauly positive), R_{fBAW} 0.60 (Pauly positive), R_{fBAW} 0.55 (ninhydrin and Pauly positive), and R_{fBAW} 0.44 (ninhydrin and Pauly positive). This crude reduction mixture (0.16 g.) was chromatographed on a 2.5 × 60 cm. cellulose column prepared from 100 g. of cellulose (Whatman) using *n*-butanol:acetic acid:water (4:1:5) as the developing solvent. Fractions of 10 ml. were collected and fractions 55 to 59, representing the second peak from the column, yielded 80 mg. of a homogeneous product (R_{fBAW} 0.60). This was twice precipitated from 0.5 ml. of water and 9 ml. of ethanol. The sample was dried *in vacuo* at 110° for 15 hr. for analysis.

Anal. Calcd. for $C_{50}H_{70}N_{12}O_{12}\cdot 1^1/_2\ H_2O$: C, 56.74; H, 6.95; N, 15.88. Found: C, 56.74; H, 6.97; N, 16.13, $[\alpha]^{20}D$ − 75.0° (*c*, 0.5%, 1 *N* HCl); m.p. 215–224° dec.

O-Acetyl-L-serine.—Dry hydrogen chloride was bubbled through a suspension of 8.0 g. (76 mmoles) of powdered L-serine in 800 ml. of anhydrous acetic acid at 0° until saturation (about 1 hr.). The mixture was then allowed to stir overnight (16 hr.) at room temperature during which time a clear solution resulted. After evaporation to dryness, the above procedure was repeated. The product was crystallized from absolute ethanol by addition of ether to the point of incipient turbidity. This product, recrystallized once in the same manner, showed two spots on paper chromatography (R_{fBAW}: 0.21, 0.10 and R_{fAW}: 0.53, 0.20). It was then converted to the free base by dissolving in 180 ml. of absolute ethanol followed by the addition of 1.8 ml. (76 mmoles) of tri-*n*-butylamine in 10 ml. of absolute ethanol. The resulting precipitate was collected by filtration, washed with cold ethanol and crystallized from water–ethanol. It was recrystallized from 50% ethanol to yield 8.57 g. (77%) O-acetyl-L-serine; m.p. 168–169° (lit. 167–168° dec.[11]); $[\alpha]^{17}D$ + 22.44° (*c*, 1 in 0.1 *N* HCl) lit. + 9.15°[11] (*c*, 1.78 in 0.1 *N* HCl); $[\alpha]^{17}D$ + 9.24 (*c*, 1 in H_2O).

Anal. Calcd. for $C_5H_9O_4N$; C, 40.82; H, 6.17; N, 9.52. Found: C, 40.99; H, 6.30; N, 9.48.

O-Acetyl-L-serine-N-carboxyanhydride.—This was prepared from O-acetyl-L-serine as described previously.[10]

N-Poly-(O-acetyl-L-seryl)-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine.—To 34.2 mg. of L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine dihydrobromide dihydrate[7] in 3 ml. of water was added 70 ml. of cold phosphate buffer (*p*H 7.5, ionic strength 0.3). To this solution at 0° was added dropwise over a period of 15 min. 1.5 g. of freshly prepared O-acetyl-L-serine NCA in 5 ml. of dry dioxane. The mixture was stirred for 24 hr. at room temperature, transferred to a cellophane bag and dialyzed against changes of distilled water for 4 days. The solution remaining in the bag was dried by lyophilizing and then further dried over P_2O_5 *in vacuo*. The product was insoluble in dimethylformamide, dioxane, dichloroacetic acid, trifluoroacetic acid, chloroform and 10% urea solution. The polymer contained 0.478 μmoles of histidine per 6.92 mg. for an average molecular weight of 14,500.

N-Poly-(O-acetyl-L-seryl)-L-aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine. N-(Poly-O-acetyl-L-seryl)-angiotensin II.—To 36.2 mg. of isoleucyl[5]-angiotensin II monoacetate suspended in 3 ml. of water, acetic acid was added dropwise until the solution cleared. The *p*H was adjusted to about 7 with 0.8 *N* ammonium hydroxide and 70 ml. of phosphate buffer (*p*H 7.5, ionic strength 0.3) and 10 ml. of dioxane were added. The polymer was then prepared from 15 g. of O-acetyl-L-serine NCA as described above yielding 0.37 g. of product. From the histidine content (0.214 μmoles per 6.08 mg. polymer) the average molecular weight was calculated to be 28,500.

Discussion

The pressor and myotropic activities of the isoleucyl[5]-angiotensin II analogs prepared are presented in Table I. Removal of the N-terminal amino group of angiotensin II reduced the pressor activity 40% and the myotropic activity by 50%. Blocking this N-terminal amino group with poly-O-acetyl-L-serine reduced both activities by 60%.

These results indicate that while the N-terminal amino group of angiotensin II is not essential for the biological responses tested, it influences the extent of the response. If the sole function of aspartic acid, the N-terminal amino acid of angiotensin II, was to stabilize the conformation of the peptide,[7] desaminoangiotensin II would be expected to possess the same biological potency as angiotensin II. Since this is not observed, the N-terminus of this peptide must play an additional role in effecting a biological response. The nature of this function is at present unknown.

From the model recently suggested to represent the conformation of angiotensin II,[7] it can be predicted that extension of the chain of the hexapeptide with additional amino acid residues at the N-terminus would stabilize the conformation of the C-terminal end of the peptide. Since this portion of angiotensin II contains two groups essential to biological activity, stabilization of its conformation should fix the position of these groups relative to others on the peptide chain. Destruction of the conformation of angiotensin II is known to decrease its myotropic activity[4,7] so that increasing conformation stability of the hexapeptide would be

expected to increase biological activity. The extension of the peptide chain increases the biological potency of the hexapeptide by about 10 fold (see Table I). Thus, the contribution aspartic acid and arginine make to the stability of the conformation of angiotensin is important to its biological activity.

The rate of the myotropic and pressor responses and the duration of these responses to N-(poly-O-acetyl-L-seryl)-angiotensin II were identical to those produced by angiotensin II. Since the large polymer would probably not pass into a cell readily, this indicates these peptides are causing a myotropic response by acting on the cell membrane.

[CONTRIBUTION FROM THE ORGANIC CHEMICAL RESEARCH SECTION, LEDERLE LABORATORIES, A DIVISION OF AMERICAN CYANAMID CO., PEARL RIVER, N. Y.]

The 6-Deoxytetracyclines. III. Electrophilic and Nucleophilic Substitution[1]

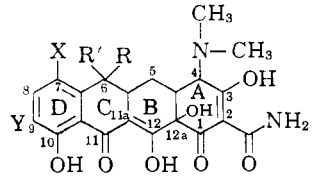
BY JOSEPH J. HLAVKA, A. SCHNELLER, H. KRAZINSKI AND J. H. BOOTHE

RECEIVED OCTOBER 26, 1961

A series of electrophilic and nucleophilic substitutions upon the aromatic D ring of 6-deoxytetracycline and 6-demethyl-6-deoxytetracycline are described. The positions of entering groups are assigned on the basis of a novel method of tritium replacement. The relationship between structure and antibacterial activity is discussed.

The tetracycline molecule, I, has always presented a special problem to the organic chemist interested in the study of structure–activity relationships. The difficulty has been to devise chemical pathways which would bring about the necessary transformation yet preserve the rather complicated and sensitive B, C, D ring chromophore. The lability of the 6-hydroxyl group to both acid[2] and base[3] degradation prevented any substantial progress in this field. Moreover, the ease of epimerization[4] at the carbon atom at position 4 added to the problem of chemical instability under many reaction conditions.

The stability of the recently isolated 6-deoxytetracycline[5] (II) and 6-demethyl-6-deoxytetracycline[5] (III) permitted, for the first time, an ingress to this problem of chemical modification with retention of biological activity by a series of electrophilic substitutions under strongly acid conditions.[1] We now wish to report on an extension of this study using both electrophilic halogenation and nucleophilic displacement in the aromatic D ring.



I, R = CH_3, R′ = OH, X = Y = H
II, R = CH_3, R′ = X = Y = H
III, R = R′ = X = Y = H
IV,[6] R = R′ = Y = H, X = Cl
V, R = R′ = Y = H, X = Br
VI, R = R′ = Y = H, X = I
VII, R = CH_3, R′ = Y = H, X = Br
VIII, R = CH_3, R′ = Y = H, X = I
IX, R = CH_3, R′ = X = H, Y = NH_2
X, R = R′ = Y = H, X = NH_2
XI, R = R′ = X = H, Y = NH_2

XII, R = CH_3, R′ = X = H, Y = $-\overset{+}{N}\equiv N$

XIII, R = R′ = Y = H, X = $-\overset{+}{N}\equiv N$

XIV, R = R′ = X = H, Y = $-\overset{+}{N}\equiv N$

XV, R = CH_3, R′ = X = H, Y = $-\overset{-}{N}-\overset{+}{N}\equiv N$

XVI, R = R′ = Y = H, X = $-\overset{-}{N}-\overset{+}{N}\equiv N$

XVII, R = R′ = X = H, Y = $-\overset{-}{N}-\overset{+}{N}\equiv N$

XVIII, R = CH_3, R′ = X = H, Y = $-S-\overset{S}{\overset{\|}{C}}-OC_2H_5$

XIX, R = R′ = Y = H, X = $-S-\overset{S}{\overset{\|}{C}}-OC_2H_5$

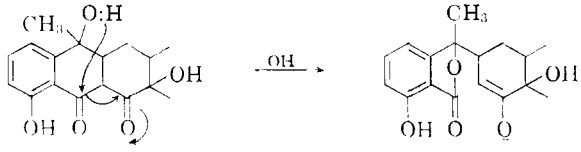
XX, R = R′ = X = H, Y = $-S-\overset{S}{\overset{\|}{C}}-OC_2H_5$

We have found that treatment of 6-demethyl-6-deoxytetracycline (III) with N-bromosuccinimide in concentrated sulfuric acid at 0° yielded a single monobromo-6-demethyl-6-deoxytetracycline, V.

(1) (a) A preliminary report of this material has been published in *J. Am. Chem. Soc.*, **82**, 1253 (1960); (b) Paper II of this series has been submitted to the *J. Med. Pharm. Chem.*; (c) *cf.* J. J. Beereboom, J. J. Ursprung, H. H. Rennhard and C. R. Stephens, *J. Am. Chem. Soc.*, **82**, 1003 (1960).

(2) C. R. Stephens, L. H. Conover, R. Pasternack, F. A. Hochstein, W. T. Moreland, P. P. Regna, F. J. Pilgrim, K. J. Brunings and R. B. Woodward, *ibid.*, **76**, 3568 (1954); C. Waller, B. L. Hutchings, C. F. Wolf, A. A. Goldman, R. Broschard and J. H. Williams, *ibid.*, **74**, 4981 (1952). Acid treatment of tetracycline results in a ready *trans* elimination of the 6-hydroxy group to yield anhydrotetracycline.

(3) *Ibid.*: Base treatment of tetracycline yields isotetracycline.



(4) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. H. Muller, R. Winterbottom and A. P. Doerschuk, *ibid.*, **79**, 2849 (1957).

(5) (a) J. R. D. McCormick, E. R. Jensen, P. A. Miller and A. P. Doerschuk, *ibid.*, **82**, 3381 (1960); (b) C. R. Stephens, *et al.*, *ibid.*, **80**, 5324 (1958).

(6) J. R. D. McCormick and E. R. Jensen, German Patent 1,082,905, June 9, 1960.