Studies on Polypeptides. XLI. The Synthesis of [5-Valine,6-β-(pyrazolyl-3)-alanine]-angiotensin II, a Potent Hypertensive Peptide

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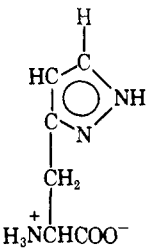
Abstract: A synthesis is described of the octapeptide aspartylarginylvalyltyrosylvalyl-β-(pyrazolyl-3)-alanylprolylphenylalanine ([Val[5], Pyr(3)Ala[6]]-angiotensin II). This peptide is an analog of [Val[5]]-angiotensin II in which histidine[6] is replaced by β-(pyrazolyl-3)-alanine. The observation that this peptide exhibits potent hypertensive and myotropic activities eliminates the characteristic acid–base properties of histidine as essential for these pharmacological properties.

Since its first synthesis[5] many analogs of [Val[5]]-angiotensin II have been prepared and evaluated pharmacologically.[6] Based on these investigations it has been generally concluded that six of the eight amino acid residues constituting this peptide are essential for biological activity. These are tyrosine-4, histidine-6, proline-7, and phenylalanine-8. In addition, the C-terminal carboxyl group must be unsubstituted. The assumption that histidine-6 was essential for biological activity was based on the following experimental results. Paiva and Paiva[7] photolyzed [Asn[1],Val[5]]-angiotensin II ("angiotensinamide") in an atmosphere of oxygen in the presence of methylene blue and observed that the decrease in biological activity (pressor, oxytocic, and myotropic) paralleled the destruction of the imidazole portion of the histidine residue. Schröder[8] replaced histidine in [Asn[1],Val[5]]-angiotensin II by phenylalanine and found that this substitution lowered the pressor activity to approximately 1% that of the parent compound. An analog of [Asn[1],-Val[5]]-angiotensin II in which histidine was replaced by lysine[9] exhibited only 0.1% of the activity of angiotensinamide.

β-(Pyrazolyl-3)-alanine (I),[3,10] a synthetic amino acid, which does not appear to occur in nature, offers interesting possibilities for evaluation of the importance



β-(pyrazolyl-3)-alanine (I)

of the acid–base properties of the imidazole portion of histidine in biologically active peptides. β-(Pyrazolyl-3)-alanine is isosteric with histidine but the pK of the pyrazole ring (approximately 2.2) differs markedly from that of the imidazole ring (approximately 6.0).

In view of the results of Paiva and Paiva,[7] it was not unreasonable to expect that the acid–base characteristics of the histidine residue of angiotensin II may be intimately involved in its biological function. This reasoning prompted the present investigation, *i.e.*, the synthesis of [5-valine,6-β-(pyrazolyl-3)-alanine]-angiotensin II (III) and its pharmacological evaluation. In this peptide the histidine residue of [Val[5]]-angiotensin II (II) is replaced by β-(pyrazolyl-3)-alanine (I).

H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-OH
[Val[5]]-angiotensin II (II)

H-Asp-Arg-Val-Tyr-Val-Pyr(3)-Ala-Pro-Phe-OH
[Val[5],Pyr(3)Ala[6]]-angiotensin II (III)

Preparative Aspects and Biological Activity

The synthesis of III (Scheme I) followed a route which was developed by Guttmann[11] for the preparation of [Val[5]]-angiotensin I.

The crystalline acetate salt of benzyloxycarbonyl-aspartylarginylvalyltyrosine hydrazide (X) was prepared by the stepwise procedure. Valyltyrosine *t*-butoxycarbonylhydrazide (IV)[11] was acylated with a mixed anhydride of N$^{\alpha}$-benzyloxycarbonylnitroarginine (V)[12] and the resulting protected tripeptide *t*-butoxycarbonylhydrazide (VI) was hydrogenated. The ensuing arginylvalyltyrosine *t*-butoxycarbonylhydrazide (VII) was then acylated with α-N-hydroxysuccinimido-

(1) The authors wish to express their appreciation to the U. S. Public Health Service for generous support of this investigation.

(2) The amino acid residues in the various peptides and peptide derivatives are of the L variety. The following abbreviations are used: Boc = *t*-butoxycarbonyl; Z = benzyloxycarbonyl; ONP = *p*-nitrophenolate; ONHS = N-hydroxysuccinimido; Pyr(3)Ala = β-(pyrazolyl-3)-alanine; Narg = nitroarginine; O-*t*-Bu = *t*-butyl ester; TEA = triethylamine; TFA = trifluoroacetic acid; THF = tetrahydrofuran; CMC = carboxymethylcellulose; AG-1X2 = anion-exchange resin (Bio-Rad); AP-M = aminopeptidase M [G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964)].

(3) See K. Hofmann, R. Andreatta, and H. Bohn, *J. Amer. Chem. Soc.*, **90**, 6207 (1968), for paper XL in this series.

(4) A preliminary communication describing some of the results presented in this study has appeared: K. Hofmann, R. Andreatta, J. P. Buckley, W. E. Hageman, and A. P. Shapiro, *ibid.*, **90**, 1654 (1968).

(5) R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, and H. Zuber, *Helv. Chim. Acta*, 41, 1287 (1958).

(6) For a recent survey see E. Schröder and K. Lübke, "The Peptides," Vol. II, Academic Press, New York, N. Y., 1966, p 52.

(7) A. C. M. Paiva and T. B. Paiva, *Biochim. Biophys. Acta*, 48, 412 (1961).

(8) E. Schröder, *Ann. Chem.*, **680**, 142 (1964).

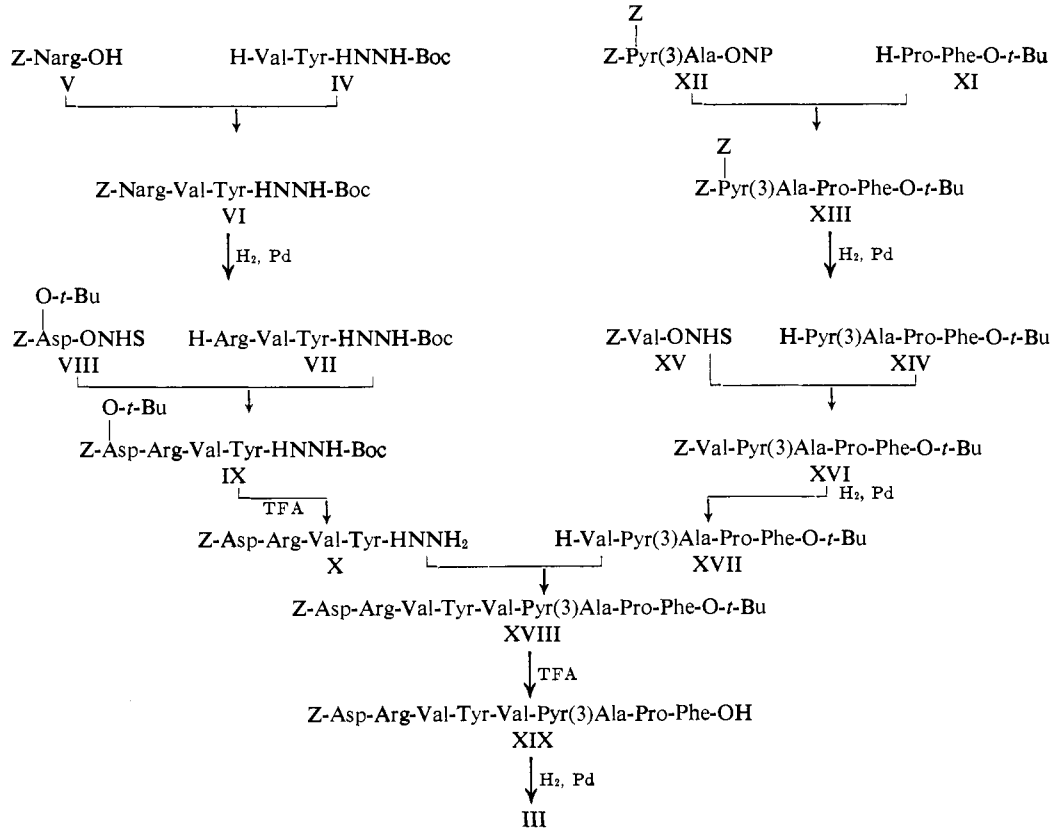
(9) E. Schröder and R. Hempel, *ibid.*, **684**, 243 (1965).

(10) K. Hofmann and H. Bohn, *J. Amer. Chem. Soc.*, **88**, 5914 (1966).

(11) St. Guttmann, *Helv. Chim. Acta*, **44**, 721 (1961).

(12) K. Hofmann, W. D. Peckham, and A. Rheiner, *J. Amer. Chem. Soc.*, **78**, 238 (1956).

Scheme I



β-*t*-butyl benzyloxycarbonylaspartate (VIII)[13] and the reaction product IX partially deblocked with trifluoroacetic acid. Trifluoroacetate ions were exchanged for acetate ions on Amberlite IRA-400 and the hydrazide acetate X was obtained in crystalline form following chromatography on carboxymethylcellulose.

For the synthesis of *t*-butyl valyl-β-(pyrazolyl-3)-alanylprolylphenylalaninate (XVII), *t*-butyl prolylphenylalaninate (XI)[14] was coupled with *p*-nitrophenyl N^{α},N^{pyr}-dibenzyloxycarbonyl-β-(pyrazolyl-3)-alaninate (XII)[3] and the reaction product XIII decarbobenzoxylated by hydrogenolysis. The resulting tripeptide *t*-butyl ester XIV was then acylated with N-hydroxysuccinimido benzyloxycarbonylvalinate (XV).[15] Hydrogenolysis converted the reaction product XVI into *t*-butyl valyl-β-(pyrazolyl-3)-alanylprolylphenylalaninate (XVII). The protected octapeptide *t*-butyl ester XVIII was prepared *via* the Rudinger modification of the azide procedure[16] from the hydrazide X and the tetrapeptide *t*-butyl ester XVII. Exposure of XVIII to trifluoroacetic acid removed the *t*-butyl ester group with formation of XIX which was purified by chromatography on the ion exchanger AG-IX2. Hydrogenolysis of the homogeneous benzyloxycarbonyl-octapeptide XIX gave the desired compound III. Some preparations of III were slightly contaminated with a material which migrated lower than the desired octapeptide on thin layer chromatograms. Countercurrent distribution in the solvent system 1-butanol–10% acetic acid removed this contaminant. Acid hydrolysates of the octapeptide III showed the expected amino acid composition and the peptide was completely digestible with aminopeptidase M with a 93% average recovery of amino acids.

Pharmacological evaluation[17] characterized [5-valine,-6-β-(pyrazolyl-3)-alanine]-angiotensin II as a highly potent pressor and myotropic agent.

The pressor activity of the pyrazole analog was determined in two rat preparations against [Asn^1,Val^5]-angiotensin II (angiotensinamide, Ciba). In the pithed rat the pyrazole analog exhibited 78.9 ± 1.33%, in the nephrectomized rat pretreated with "pentolinium tartrate" 56.6 ± 2.6% the potency of the reference standard. In the dog, the pressor activity of III ranged from 53 to 57% that of the standard. Determination of the myotropic activity on the isolated guinea pig ileum showed III to possess 23.8% of the potency of angiotensinamide (Ciba).[18]

Experimental Section[19]

N^{α}-Benzyloxycarbonylnitroarginylvalyltyrosine *t*-Butoxycarbonylhydrazide (VI). N^{α}-Benzyloxycarbonylnitroarginine (V) (2.23 g)

(13) K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *J. Amer. Chem. Soc.*, **87**, 631 (1965).
(14) E. Schröder, *Ann. Chem.*, **680**, 132 (1964).
(15) G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Amer. Chem. Soc.*, **86**, 1839 (1964).
(16) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

(17) We wish to express our appreciation to Dr. Joseph P. Buckley and Mr. William E. Hageman of the Department of Pharmacology, University of Pittsburgh School of Pharmacy, and to Dr. Alvin P. Shapiro of the Department of Medicine, University of Pittsburgh School of Medicine, for these results which will be published in detail elsewhere.
(18) In a preliminary communication,[4] we reported 52 ± 0.98%; however, careful reevaluation of the data gave the lower figure.
(19) Melting points are uncorrected. Rotations were determined with a Zeiss precision polarimeter. Measurements were carried out with a mercury lamp at 546 and 576 mμ and extrapolated to the 589-mμ sodium line. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. The amino acid composition of acid and enzymic hydrolysates was determined with a

was dissolved in sodium-dried dioxane (60 ml) by slight warming, and TEA (1.51 ml) was added. The solution was cooled at 11–12°, ethyl chloroformate (0.51 ml) was added with stirring over a period of 5 min, and the mixture was kept at 11–12° for 15 min. This mixture was then added to a precooled solution of valyltyrosine *t*-butoxycarbonylhydrazide (IV)[11] (2.49 g) in dioxane (30 ml). The mixture was stirred for 45 min at 11° and for 1 hr at room temperature, and the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate (200 ml); the solution was washed successively with 1 *N* hydrochloric acid, water, 1 *N* sodium carbonate, and water, dried over sodium sulfate, and evaporated. Precipitation from EtOH–ethyl acetate with benzene yielded a white powder: 2.98 g (65%); mp 139–141° dec; $[\alpha]^{26}D$ −31.9° (*c* 3.86, MeOH); R_f^{I} 0.79; R_f^{III} 0.80; single chlorine- and hydrazide-positive spot.

Anal. Calcd for $C_{33}H_{47}O_{10}N_9$: C, 54.3; H, 6.5; N, 17.3. Found: C, 54.3; H, 6.7; N, 17.0.

Arginylvalyltyrosine *t*-Butoxycarbonylhydrazide Diacetate (VII). N^{α}-Benzyloxycarbonylnitroarginylvalyltyrosine *t*-butoxycarbonylhydrazide (VI) (2.73 g) was hydrogenated over palladium for 20 hr in a mixture of MeOH (50 ml) and 10% acetic acid (20 ml). The catalyst was removed by filtration, the filtrate was evaporated to dryness, and the residual oil was evaporated four times with MeOH. Finally the material was lyophilized from water. Precipitation from hot MeOH (10 ml) with ethyl acetate (100 ml) gave a white amorphous powder: 2.36 g (94%); mp 98–102° dec; $[\alpha]^{28}D$ −16.1° (*c* 4.23, MeOH); R_f^{I} 0.18; R_f^{III} 0.63; single chlorine-, ninhydrin-, and hydrazide-positive spot; amino acid ratios in acid hydrolysate: $Arg_{0.97}Val_{0.98}Tyr_{1.05}$; amino acid ratios in AP-M digest: $Arg_{0.94}Val_{1.03}Tyr_{1.03}$.

Anal. Calcd for $C_{25}H_{42}O_6N_8 \cdot 2AcOH$: C, 51.9; H, 7.5; O, 23.9; N, 16.7. Found: C, 51.9; H, 7.6; O, 23.8; N, 16.6.

Guttmann[11] reports the following data for the free base: mp 175° dec; $[\alpha]^{23}D$ −19.0 ± 1.0° (*c* 1.2, MeOH); $[\alpha]^{23}D$ −25.0 ± 0.5° (*c* 1.0, DMF).

Benzyloxycarbonyl-β-*t*-butylaspartylarginylvalyltyrosine *t*-Butoxycarbonylhydrazide Monoacetate Dihydrate (IX). Arginylvalyltyrosine *t*-butoxycarbonylhydrazide diacetate (VII) (2.18 g) and α-N-hydroxysuccinimido-β-*t*-butyl benzyloxycarbonylaspartate (VIII)[13] (1.51 g) were dissolved in a mixture of DMF (35 ml) and THF (35 ml). The solution was cooled at 0°, TEA (0.56 ml) was added, and the mixture was kept at 0° for 1 hr and at room temperature for 24 hr. The solvents were evaporated, the residue was distributed between water and ethyl acetate containing 5% 1-butanol, and the organic layer was washed with half-saturated sodium bicarbonate and saturated sodium chloride. Evaporation of the solvent gave an oil which solidified on addition of petroleum ether. The compound was precipitated from EtOH with petroleum ether: 2.60 g (84%); mp 135–140° dec; R_f^{I} 0.80; R_f^{III} 0.83; R_f^{IV} 0.82; single chlorine- and hydrazide-positive spot; $[\alpha]^{23}D$ −28.2° (*c* 4.73, MeOH).

Anal. Calcd for $C_{41}H_{61}O_{11}N_9 \cdot AcOH \cdot 2H_2O$: C, 54.2; H, 7.3; O, 25.2; N, 13.2. Found: C, 53.4; H, 7.3; O, 24.8; N, 13.4.

Benzyloxycarbonylaspartylarginylvalyltyrosinehydrazide Acetate (X). Benzyloxycarbonyl-β-*t*-butylaspartylarginylvalyltyrosine *t*-butoxycarbonylhydrazide monoacetate dihydrate (IX) (2.26 g) was dissolved in TFA (20 ml) and the solution kept at room temperature for 30 min. The solvent was evaporated, the residue was dissolved in water (25 ml), and the solution was lyophilized. The residue dissolved in EtOH (20 ml) and water (30 ml) was passed through a column of Amberlite IRA-400 (acetate cycle) which was washed with EtOH–water (2:3) until the eluate reacted hydrazide negative. Hydrazide-positive fractions were pooled; the solvent was evaporated, and the residue was dissolved in water–MeOH (1:1) (50 ml). The solution was passed through a CMC column (2.0 × 11 cm) which was washed with MeOH–water (1:1) to elute the desired product. Hydrazide-positive fractions were pooled; the bulk of the solvents was removed *in vacuo*, and the aqueous residue was placed in a refrigerator. The solid was collected and dried: 1.52 g (84%); mp 201–204° dec; R_f^{I} 0.50; R_f^{II} 0.63; R_f^{III} 0.72; R_f^{I} 0.88; R_f^{3} 5.3 × His; single chlorine-, Sakaguchi-, and hydrazide-positive spot. A sample for analysis was recrystallized from water: colorless needles; mp 205–208° dec; $[\alpha]^{25}D$ −22.1° (*c* 3.77, 90% AcOH).

Anal. Calcd for $C_{32}H_{45}O_9N_9 \cdot AcOH$: C, 53.7; H, 6.5; O, 23.2; N, 16.6. Found: C, 53.9; H, 6.5; O, 22.9; N, 16.6.

Aspartylarginylvalyltyrosinehydrazide Acetate. A sample of X (110 mg) was hydrogenated over palladium in MeOH–water–acetic acid (40:10:2) (20 ml). After removal of the catalyst, the bulk of the solvents was evaporated and the residue lyophilized from water to give a white fluffy powder: 88 mg; R_f^{I} 0.25; R_f^{III} 0.41; R_f^{I} 0.53; R_f^{3} 2.70 × His; single chlorine-, ninhydrin-, and hydrazide-positive spot; amino acid ratios in AP-M digest: $Asp_{1.03}Arg_{0.94}Val_{1.03}Tyr_{1.00}$.

***t*-Butyl Benzyloxycarbonylprolylphenylalaninate.** *t*-Butyl phenylalaninate hydrochloride[20] (2.58 g) was suspended in a solution of N-hydroxysuccinimido benzyloxycarbonylprolinate[15] (3.46 g) in THF (100 ml) and the mixture cooled at 0°. TEA (2.8 ml) was added, and the reaction mixture stirred for 1 hr at 0° and at room temperature for 12 hr. The solvent was removed *in vacuo*, the residue was distributed between ethyl acetate (300 ml) and water (150 ml), and the organic phase was washed with two 100-ml portions each of water, 10% acetic acid, and saturated sodium chloride. Drying over anhydrous sodium sulfate and evaporation of the solvent gave a yellow oil, which was dissolved in ether–petroleum ether (bp 30–60°) 1:1 (100 ml). This solution was passed through a column of silica gel G (3 × 25 cm), and the desired product was eluted with ether; a yellow impurity remained on the column. Evaporation of the ether extracts yielded a colorless oil which was used for the next step without further purification; 3.84 g (85%). A sample crystallized on standing and was recrystallized from ether–cyclohexane–petroleum ether to give colorless prisms: mp 74–75°; $[\alpha]^{26}D$ −22.1° (*c* 4.76, benzene); R_f^{I} 0.84; R_f^{III} 0.85; single chlorine-positive spot; amino acid ratios in acid hydrolysate: $Pro_{1.06}Phe_{0.97}$.

Anal. Calcd for $C_{26}H_{32}O_5N_2$: C, 69.0; H, 7.1; N, 6.2. Found: C, 69.3; H, 7.3; N, 6.4.

Schröder[14] prepared the same compound in 87% yield from *t*-butyl phenylalaninate and a mixed anhydride of benzyloxycarbonylproline, mp 70–71°; $[\alpha]^{23}D$ −34.0° (*c* 1.0, $CHCl_3$).

***t*-Butyl N^{α},N^{pyr}-dibenzyloxycarbonyl-β-(pyrazolyl-3)-alanylprolylphenylalaninate (XIII).** *t*-Butyl benzyloxycarbonylprolylphenylalaninate (3.0 g) was hydrogenated over palladium in ethyl acetate (50 ml) until carbon dioxide evolution had ceased (approximately 4 hr). The catalyst was removed by filtration and the filtrate was evaporated. The resulting oily *t*-butyl prolylphenylalaninate (XI)[21] was immediately dissolved in THF (35 ml) and *p*-nitrophenyl N^{α},N^{pyr}-dibenzyloxycarbonyl-β-(pyrazolyl-3)-alaninate (XII)[3] (3.61 g) was added. The solution was cooled in an ice bath, TEA (1.2 ml) was added while stirring, and the reaction mixture was kept at 0° for 1 hr and at 5° for 24 hr. The solvent was evaporated, the residue was dissolved in ethyl acetate, and the solution was washed with water, 1 *N* sodium carbonate, 5% acetic acid, and saturated sodium chloride. Evaporation of the sodium sulfate dried organic phase gave a yellow oil which was dissolved in chloroform and the solution was passed through a column of silica gel G which retained colored impurities. The solvent was evaporated to give a colorless oil: 4.49 g (94%); R_f^{I} 0.78; R_f^{III} 0.74; single chlorine-positive spot; $[\alpha]^{27}D$ −34.7° (*c* 1.25, $CHCl_3$); amino acid ratios in acid hydrolysate: $Pyr(3)Ala_{0.96}Pro_{1.02}Phe_{1.02}$.

Anal. Calcd for $C_{40}H_{43}O_8N_5$: C, 66.4; H, 6.3; N, 9.7. Found: C, 66.6; H, 6.4; N, 9.6.

***t*-Butyl Benzyloxycarbonylvalyl-β-(pyrazolyl-3)-alanylprolylphenylalaninate (XVI).** *t*-Butyl N^{α},N^{pyr}-dibenzyloxycarbonyl-β-(pyrazolyl-3)-alanylprolylphenylalaninate (XIII) (4.5 g) was hydrogenated over palladium in MeOH (40 ml) and 10% acetic acid (10 ml) until evolution of carbon dioxide ceased (approximately 6 hr). The catalyst was removed by filtration and the filtrate evaporated to dryness. The oily *t*-butyl β-(pyrazolyl-3)-alanylprolylphenylalaninate acetate (XIV) (2.75 g) was dissolved in THF (50 ml) and N-hydroxysuccinimido benzyloxycarbonylvalinate (XV)[15] (1.87 g) was added. The solution was cooled at 0°, TEA (2 ml) was added dropwise with stirring, and the mixture was kept at ice-bath temperature for 1 hr and at room temperature for

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Beckman-Spinco Model 120 amino acid analyzer according to the method of S. Moore, D. H. Spackman, and W. H. Stein, *Anal. Chem.*, **30**, 1185 (1958). Paper chromatograms were performed on Whatman No. 1 filter paper by the descending technique with the following solvent systems: R_f^{1}, 1-butanol–acetic acid–water, 4:1:5 (upper phase); R_f^{3}, 1-butanol–pyridine–acetic acid–water, 45:30:9:36. Thin layer chromatograms were performed with the following solvent systems: R_f^{1}, 1-butanol–acetic acid–water, 60:20:20; R_f^{II}, dioxane–water, 9:1; R_f^{III}, 1-butanol–pyridine–acetic acid–water, 30:20:6:24; R_f^{IV}, chloroform; AP-M digests were performed as described in [K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Amer. Chem. Soc.*, **88**, 3633 (1966)].

(20) R. Roeske, *J. Org. Chem.*, **28**, 1251 (1963).

(21) Schröder obtained this compound in the form of crystals melting at 65–66°.[14]

24 hr. The solvent was evaporated, the residue was dissolved in ethyl acetate (100 ml), and the solution was washed successively with water, 10% acetic acid, and saturated sodium chloride. The organic phase was dried and the solvent removed to give a yellow oil which was dissolved in ether (25 ml). The solution was applied to a column of silica gel G (2.5 × 12 cm), which was eluted with ether, chloroform, and ethyl acetate. The ethyl acetate eluates which contained the desired material were pooled, the solvent was removed, and petroleum ether was added to the residue. The solid was collected and reprecipitated from ether with petroleum ether; 2.10 g (49%); mp 65–68°; $[\alpha]^{23}D$ −18.5° (*c* 2.40, benzene); R_f^{I} 0.84; R_f^{II} 0.74; R_f^{III} 0.83; R_f^{IV} 0.87; single chlorine-positive spot; amino acid ratios in acid hydrolysate: $Val_{1.03}Pyr(3)Ala_{1.08}Pro_{0.95}Phe_{0.93}$.

Anal. Calcd for $C_{37}H_{48}O_7N_6$: C, 64.5; H, 7.0; O, 16.3; N, 12.2. Found: C, 64.5; H, 7.3; O, 16.3; N, 12.3.

***t*-Butyl Valyl-β-(pyrazolyl-3)-alanylprolylphenylalaninate Hemihydrate (XVII).** *t*-Butyl benzyloxycarbonylvalyl-β-(pyrazolyl-3)-alanylprolylphenylalaninate (XVI) (1.88 g) was hydrogenated over palladium in a mixture of water (15 ml), MeOH (20 ml), and acetic acid (1.5 ml). The catalyst was removed by filtration, the filtrate was concentrated *in vacuo*, and the residue lyophilized (1.54 g). A sample (770 mg) in water (10 ml) was applied to a CMC column (2.4 × 21 cm) which was eluted successively with water (300 ml) and 4% acetic acid (200 ml). Individual fractions, 10-ml each, were collected. Ninhydrin-positive fractions of the acetic acid eluates were pooled; the bulk of the solvent was removed, and the residue was lyophilized to give a white fluffy powder: 684 mg; $[\alpha]^{26}D$ −52.8° (*c* 1.35, water); R_f^{I} 0.76; R_f^{III} 0.69; single chlorine- and ninhydrin-positive spot; amino acid ratios in acid hydrolysate: $Val_{0.98}Pyr(3)Ala_{0.88}Pro_{1.13}Phe_{0.98}$; amino acid ratios in AP-M digest: $Val_{0.99}Pyr(3)Ala_{0.90}Pro_{1.27}Phe_{0.84}$.

Anal. Calcd for $C_{29}H_{42}O_5N_6 \cdot 0.5H_2O$: C, 61.8; H, 7.7; O, 15.8; N, 14.9. Found: C, 62.2; H, 7.5; O, 15.6; N, 14.7.

Benzyloxycarbonylaspartylarginylvalyltyrosylvalyl-β-(pyrazolyl-3)-alanylprolylphenylalanine Trihydrate (XIX). A solution of *t*-butyl nitrite in DMF (12% v/v) (1.3 ml) was added to a solution cooled at −30° of X (780 mg) in DMF (10 ml) containing 6.9 *N* hydrogen chloride in dioxane (0.65 ml). The mixture was stirred at −30° for 30 min, then cooled at −60°, and 10% (v/v) TEA in DMF (7.0 ml) was added dropwise. To this solution was added dropwise with stirring a solution of XVII (320 mg) in DMF (3 ml) containing 10% (v/v) TEA in DMF (1.5 ml). The solution was stirred for 1 hr at −20° and for 24 hr at 4° (pH maintained at 8.0–8.5 by addition of TEA), then the solvents were evaporated. The residue was distributed between 1-butanol and 2% acetic acid (six funnels, 50-ml upper and 50-ml lower phase); the organic layers were combined, and the solvent was evaporated. The residue was lyophilized from 90% aqueous dioxane to give crude XVIII (1.1 g). This material was combined with 340 mg of the same material from a previous run and dissolved in 90% TFA (15 ml). The solution was kept at room temperature for 40 min, the solvent was evaporated, and the residue was lyophilized from 90% aqueous dioxane. The ensuing slightly yellow solid was dissolved in water–MeOH–2-propanol (1:1:1) (50 ml), and the solution was applied to a 3.0 × 19 cm column of AG-1X2 (acetate cycle) which was eluted successively with water–MeOH–2-propanol (1:1:1) (250 ml), 0.05 *M* acetic acid–MeOH–2-propanol (1:1:1) (500 ml), 0.1 *M* acetic acid–MeOH–2-propanol (500 ml) (1:1:1), and 0.15 *M* acetic acid–MeOH–2-propanol (250 ml) (1:1:1). Individual fractions, 10 ml each, were collected at a flow rate of 3 ml/min. The chlorine test and thin layer chromatography served to locate the desired material in the 0.1 and 0.15 *M* acetic acid eluates. Fractions containing homogeneous material were pooled; the bulk of the solvents was removed and the residue lyophilized from 90% dioxane; fluffy white powder; 688 mg; $[\alpha]^{27}D$ −40.9° (*c* 2.97, 80% aqueous dioxane); R_f^{I} 0.65; R_f^{III} 0.68; R_f^{1} 0.85; R_f^{3} 4.0 × His; single chlorine-, ninhydrin-, and Sakaguchi-positive spot.

Anal. Calcd for $C_{57}H_{75}O_{14}N_{13} \cdot 3H_2O$: C, 56.1; H, 6.7; O, 22.3; N, 14.9. Found: C, 56.0; H, 6.7; O, 22.1; N, 14.6.

[Val⁵,Pyr(3)Ala⁶]-angiotensin II (III). Benzyloxycarbonylaspartylarginylvalyltyrosylvalyl-β-(pyrazolyl-3)-alanylprolylphenylalanine trihydrate (XIX) (89 mg) was hydrogenated over palladium in water–MeOH–2-propanol (1:1:1) (20 ml). The catalyst was removed by filtration, the filtrate was concentrated to a small volume, and precipitated material was dissolved by addition of dioxane. Lyophilization gave a white fluffy solid: 78 mg; R_f^{I} 0.37; R_f^{III} 0.55; R_f^{1} 0.49; R_f^{3} 3.2 × His; single chlorine-, ninhydrin-, and Sakaguchi-positive spot; $[\alpha]^{27}D$ −47.5° (*c* 0.29, 20% aqueous dioxane); amino acid ratios in acid hydrolysate: $Asp_{1.2}Arg_{1.01}Val_{2.02}Tyr_{0.84}{}^{22}Pyr(3)Ala_{0.84}Pro_{1.05}Phe_{0.94}$; amino acid analysis in AP-M digest: $Asp_{1.04}Arg_{1.04}Val_{1.95}Tyr_{1.04}Pyr(3)Ala_{0.99}Pro_{0.99}Phe_{0.94}$. Some batches of the octapeptide contained an impurity which migrated lower than the main component on thin layer chromatograms: R_f^{I} 0.33; R_f^{III} 0.46. This contaminant was readily removed by countercurrent distribution. A typical experiment was performed as follows. The lyophilisate obtained from hydrogenation of XIX (286 mg) was dissolved in 1-butanol equilibrated with 10% acetic acid (30 ml); the solution was added to the first three tubes of a countercurrent machine, and was distributed between 1-butanol and 10% acetic acid (10-ml upper, 10-ml lower phase, 400 transfers). Thin layer chromatography of samples from the various tubes using the chlorine test located the homogeneous peptide in tubes 105–160. The contents of these tubes were pooled, and the solution was concentrated to a small volume and lyophilized: 223 mg; R_f^{I} 0.37; R_f^{III} 0.55; single chlorine- and Sakaguchi-positive spot; $[\alpha]^{25}D$ −32.4° (*c* 1.33, 90% acetic acid); amino acid ratios in acid hydrolysate: $Asp_{1.02}Arg_{1.06}Val_{2.03}Tyr_{0.66}{}^{22}Pyr(3)Ala_{1.00}Pro_{1.26}Phe_{0.96}$; amino acid ratios in AP-M digest: $Asp_{0.97}Arg_{1.03}Val_{1.91}Tyr_{0.94}Pyr(3)Ala_{0.97}Pro_{1.22}Phe_{0.94}$.

Discussion

Histidine plays a key role in biology since it constitutes an important structural element of the active site of several enzymes, and the acid–base properties of the imidazole ring appear to be connected intimately with catalytic function.[23] Since histidine and β-(pyrazolyl-3)-alanine are isosteric amino acids which differ markedly in the dissociation behavior of their ring systems, we explored the effect of histidine–β-(pyrazolyl-3)-alanine exchanges on the biological activity of certain peptides. Substitution by β-(pyrazolyl-3)-alanine of the catalytically essential His^{12} in S-$peptide_{1-14}$ afforded [$Pyr(3)Ala^{12}$]-S-$peptide_{1-14}$. This peptide dissociates the enzymically fully active S-peptide–S-protein complex (ribonuclease $S^{24,25}$) with formation of an enzymically inactive [$Pyr(3)Ala^{12}$]-S-$peptide_{1-14}$–S-protein complex. The shape of the inhibition curve demonstrated equally strong binding to S-protein of both peptides. This experiment led to the conclusion that the histidine–β-(pyrazolyl-3)-alanine substitution did not alter the conformation of S-$peptide_{1-14}$, that imidazole and pyrazole provide equally effective binding sites, and that the inability of the analog to activate S-protein was unquestionably the result of the acidity of the pyrazole ring. Based on these results it would appear that the replacement of histidine by β-(pyrazolyl-3)-alanine offers an important tool for exploring the importance of the acid–base properties of the imidazole portion of histidine for biological function of peptides.

The high degree of pharmacological activity of [Val^5,$Pyr(3)Ala^6$]-angiotensin II, which is reported in this investigation, eliminates histidine dissociation as an essential element for biological function. However, the azole ring appears to contribute to the activity of

(22) These values are uncorrected. Low recoveries of tyrosine from acid hydrolysates of angiotensin analogs have been reported previously: M. C. Khosla, R. R. Smeby, and F. M. Bumpus, *Biochemistry*, **6**, 754 (1967).

(23) For reviews see F. H. Westheimer, *Advan. Enzymol.*, **24**, 441, (1962), and M. L. Bender and F. J. Kézdy, *Ann. Rev. Biochem.*, **34**, 49 (1965).

(24) F. M. Finn and K. Hofmann, *J. Amer. Chem. Soc.*, **89**, 5298 (1967).

(25) F. M. Richards, *Proc. Nat. Acad. Sci. U. S.*, 44, 162 (1958); ribonuclease S, subtilisin modified beef ribonuclease A; S-$peptide_{1-20}$, the eicosapeptide obtained from ribonuclease S; S-protein, the protein component obtained from ribonuclease S; S-$peptide_{1-14}$, a synthetic peptide corresponding to the N-terminal amino acid sequence of S-$peptide_{1-20}$ which is equally as effective in activating S-protein as is S-$peptide_{1-20}$ [K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Amer. Chem. Soc.*, **88**, 3633 (1966)].

angiotensin since its replacement by a phenyl ring ([Val^5,Phe^6]-angiotensinamide[8]) or the destruction by photolysis (Paiva and Paiva[7]) of the imidazole portion of the histidine residue results in an almost complete loss of potency. The pharmacological nonequivalence of [Val^5]-angiotensin II and [Val^5,Pyr(3)Ala^6]-angiotensin II may be attributable to many factors. These may include different stability to tissue enzymes (angiotensinases), weaker binding to the receptor, and possibly others. Insight into the molecular events which underlie the biological function of angiotensin, particularly information regarding the chemical nature of the cell receptors, will be necessary before these aspects of the problem can be understood.

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Chemical Transformation of 4-Thiouracil Nucleosides to Uracil and Cytosine Counterparts[1]

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Abstract: Periodate oxidation at pH 7 and 35° of low concentrations of 4-thiouracil nucleosides with suitably protected pentose moieties yields the corresponding 2-oxypyrimidine-4-sulfonate nucleosides. Subsequent H^+ or OH^- catalyzed hydrolysis yields uracil nucleosides, while ammonolysis with NH_3 or methylamine yields cytosine and N^4-methylcytosine nucleosides, respectively. It is suggested that the mild conditions required for these reactions are suitable for specific modification of 4-thiouridylate residues in transfer RNA.

Recent attention has been drawn to the presence of 4-thiouridylate in tRNA[3] of *E. coli*.[4] Apart from the special chemical properties of greater acidity, ease of oxidation, and disulfide bond formation,[4,5] this residue would appear to have structural and hydrogen-bonding characteristics like those of uridylate.[6] Thus, the reason for its occurrence in tRNA presents an intriguing problem. A preliminary study[7] showed that susceptibility of 4-thiouridylate in tRNA to oxidation by sodium periodate depended on the conformational state of the tRNA, and that oxidation was accompanied by a decrease in the capacity of such modified tRNA's to be enzymatically deacylated. A quantitative interpretation of the results, however, was precluded by the absence of information regarding the chemical transformations undergone by the sensitive residue. This prompted an investigation of the periodate oxidation of appropriate 4-thiouridine model compounds. The present report describes reactions in which suitably protected nucleoside derivatives of 4-thiouracil are converted by periodate oxidation and subsequent nucleophilic attack to the corresponding uracil, cytosine, or N^4-methylcytosine derivatives. The conditions employed appear suitable for similar conversion of the residue in a tRNA molecule.

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(2) U. S. Public Health Service Predoctoral Fellow, 1964–1968.

(3) tRNA = transfer ribonucleic acid.

(4) M. N. Lipsett, *J. Biol. Chem.*, **240**, 3975 (1965).

(5) (a) M. N. Lipsett, *ibid.*, **242**, 4067 (1967); (b) M. N. Lipsett and B. P. Doctor, *ibid.*, **242**, 4072 (1967).

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(7) E. Ziff and J. R. Fresco, *Federation Proc.*, **26**, 871 (1967).

Results

Periodate Oxidation of 4-Thiouracil Nucleosides. Starting compounds for this study were either 2′-deoxy-4-thiouridine (Ia) or 2′,3′-O-isopropylidene-4-thiouridine (Ib)—see Chart I. In tRNA the ribose of 4-thiouridylate is protected from periodate oxidation by phosphate ester linkage at the 3′ position; the sugar moieties of these model compounds are similarly not reactive.

The oxidation of Ia (5×10^{-5} *M*) by sodium periodate (0.01 *M*) in aqueous solution was observed at 330 mμ (near its λ_{max}, *cf.* Figure 1a) to proceed with pseudo-first-order kinetics ($t_{1/2}$ = 15 sec at 35°, pH 7.0). The ultraviolet absorption spectrum of the final reaction mixture revealed a nucleoside product (II) with λ_{max} near 316 mμ, and greatly diminished absorption at 330 mμ. Upon addition of acid, base, or amines, a reaction of this product (II) was observed, as evidenced by irreversible loss of the absorption maximum at 316 mμ. A compound with such spectral and reaction properties (IIa or b) (Figure 1) was isolated when such oxidation mixtures were fractionated by sodium chloride gradient elution from Dowex 1 (Cl^-) columns. Attempts to isolate II free from sodium chloride were impeded by its instability. However, IIb was partially desalted by ethanol extraction of the lyophilized product.

Purified IIb was shown to contain sulfur by the nitroferricyanide, sodium azide–iodine, and lead acetate paper tests after sodium fusion. Upon acid hydrolysis or ammonolysis of IIb (see below), the sulfur was liberated as sulfite ion. The observation that synthesis of II proceeds readily at low concentrations of I (see below) indicated that II is not a dimeric compound such as a disulfide or thiosulfate. Thus, the liberation of sulfite suggested that II is the salt of a sulfinic or sul-