tested by administering the substances orally and subcutaneously. It is known that rifamycin SV is poorly absorbed by the oral route and that the rapid elimination through the bile prevents the appearance of therapeutic blood levels when given orally.[11] For these reasons this antibiotic is used in therapy only by parenteral administration. Therefore in testing new derivatives of rifamycin B, absorption from intestine and concentration in bile were systematically investigated for each active compound. Laboratory studies on these two aspects of the pharmacology of the new rifamycins are in progress and will be reported later. A preliminary screening of the oral and subcutaneous activity of these derivatives against experimental infections was thought to give some indirect information on this aspect of the problem.

The results given in Table V lead to the following considerations. The monosubstituted amides (**3**, **8**) give very poor *in vivo* protection in agreement with the slight *in vitro* activity. With the exception of the rifamycin morpholides, the other disubstituted rifamycinamides, which show exceptionally high *in vitro* activity, have a good protective effect when administered parenterally. By the oral route the ED_{50} values are always many times higher than the s.c. ED_{50}. It is interesting to note that in the homologous series the ratio ED_{50} oral/ED_{50} s.c. is generally higher for the compounds bearing the shorter alkyl chains. For example, this ratio is >8.0 for the dimethyl-, 8.0 for the diethyl-, 5.55 for the dipropyl-, and 3.2 for the di-*n*-butylrifamycinamide. Since in a homologous series the water solubility decreases and the lipid solubility increases with the increase of the carbon chains on the nitrogen, the ratio of lipid solubility–water solubility of the different rifamycinamides can be considered of importance for gastrointestinal absorption.

As far as the *in vivo* activity of the rifamycin hydrazides is concerned, the situation is parallel to that of the rifamycinamides. With the exception of the N,N-diethylenoxy-N′-methylhydrazide (**72**), which is slightly active, all the other trisubstituted hydrazides are effective in protecting the infected animal at low doses when administered subcutaneously. By the oral route effectiveness appears at higher doses.

The acute toxicity of these derivatives can be judged from the data in Table V. In a series of homologs the toxicity increases with the molecular weight; for example, dimethylamide, 654; diethylamide, 429; dipropylamide, 175; and dibutylamide, 141. Other examples of this correlation can be observed in the series of rifamycin hydrazides. The less toxic derivatives are those containing a morpholino group, both in the series of amides (**47–49**) and in the series of hydrazides (**72**). As mentioned before these derivatives show a limited therapeutic efficacy in experimental infections.

In conclusion, comparison of the *in vitro* and *in vivo* activities and toxicity of the amides and hydrazides of rifamycin B with the corresponding data of rifamycin SV (which are included in Table III–V) indicates that a great number of the new derivatives here reported show favorable properties as potential therapeutic agents. Obviously, the comparative evaluation of these derivatives will require further pharmacological and toxicological studies. Laboratory investigations on some members of the series are in progress.

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(11) (a) G. Malfii, G. Bianchi, P. Schiatti, and G. G. Gallo, *Farmaco* (Pavia), *Ed Sci.*, **16**, 246 (1961); (b) S. Fürész and R. Scotti, *ibid.*, **16**, 262 (1961).

D-Aspartyl[1]-valyl[5]-phenylalanine[8] Amide Angiotensin II

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D-Aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine amide, an analog of bovine angiotensin II, was synthesized, and results of enzymatic studies with leucine aminopeptidase, trypsin, chymotrypsin, and carboxypeptidase support its structure. The peptide increased rat blood pressure with an average potency of 1/300th that of asparaginyl[1]-valyl[5] angiotensin II; a threefold increase in the duration of action was exhibited at dose levels which gave an equivalent absolute response.

Extensive studies of structure–activity relationships in the angiotensin series have been reviewed by Schwyzer[1] as well as by Page and Bumpus.[2] Alteration of the C-terminus of angiotensin II resulted in a striking quantitative change in biological activity, whereas modification of the N-terminus had little influence. Conversion of the C-terminal carboxyl group to a carboxamide function decreased biological activity to 1/30, and either elimination of C-terminal L-phenylalanine or its replacement by D-phenylalanine abolished activity. Substitution of the N-terminal L-aspartic acid residue by L-asparagine had no deleterious effect on activity and elimination of L-asparagine decreased activity to only 1/2. Replacement of the N-terminal amino acid by D-asparagine actually increased activity. The C-terminus of angiotensin II, therefore, appears to be of greater importance for biological activity than the N-terminus.

Recently, efforts have been directed toward the elucidation of the mode of physiological inactivation of angiotensin II. Information from experiments *in vitro* suggested that specific aminopeptidases are pri-

(1) R. Schwyzer, *Pure Appl. Chem.*, **6**, 265 (1963).
(2) I. H. Page and F. M. Bumpus, *Physiol. Rev.*, **41**, 331 (1961).

TABLE I
ACTIVITY OF ANGIOTENSIN II ANALOGS

Compound	Activity[a]
H·D-Asp-L-Arg-L-Val-L-Tyr-L-Val-L-His-L-Pro-L-Phe·OH (1)	>1 (2–3 times longer duration of action)[3b]
NH_2 | H·L-Asp-L-Arg-L-Val-L-Tyr-L-Val-L-His-L-Pro-L-Phe·NH_2 (2)	1/30[1]
H·D-Asp-L-Arg-L-Val-L-Tyr-L-Val-L-His-L-Pro-L-Phe·NH_2 (3)	1/300 (3 times longer duration of action)

[a] Activity on blood pressure relative to bovine angiotensin II in the nephrectomized rat.

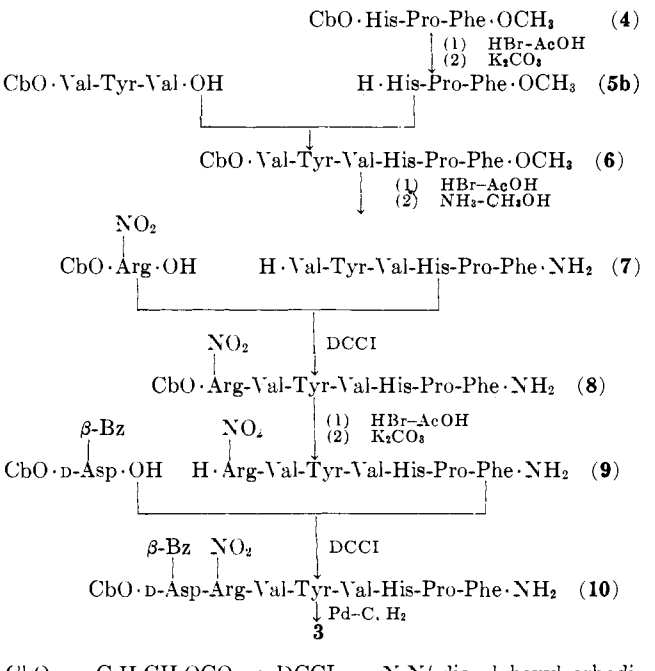
marily responsible for inactivation *in vivo*,[3] and an indication of contributions from carboxypeptidases to degradation has thus far not been obtained. These results are surprising in view of the rapid inactivation of angiotensin II *in vivo*, and especially because of the profound effect that removal of C-terminal phenylalanine displays on biological activity. Furthermore, another polypeptide hormone, bradykinin, is inactivated by carboxypeptidase,[4] a finding which suggests that carboxypeptidases are capable of playing a significant role in biological degradation.

Substrates for aminopeptidases as well as for carboxypeptidases require the L-configuration. An additional prerequisite for carboxypeptidase substrates is a free carboxyl group.[5] Compound 1, D-Asp[1]-Val[5] angiotensin II (Table I), was reported to be 50% more active than bovine angiotensin II[6] on the rat blood pressure with a two- to threefold increase in duration of action.[3b] This increase in biological activity was ascribed to the stabilizing effect of the D-aspartyl residue toward digestion of the N-terminus by aminopeptidases. Analog **2**, on the other hand, having a C-terminal carboxamide function, retained 1/30th of the activity of angiotensin II.[1] Such a substance would be expected to be resistant to carboxypeptidase degradation. Considering the biological activities of these two analogs, and the possibility that inactivation *in vivo* could take place not only at the N-terminus but simultaneously at the C-terminus, D-Asp[1]-Val[5]-Phe[8] amide angiotensin II (**3**), having both of the termini stabilized toward enzymatic degradation, could be a biologically active compound with a considerable longer duration of action than **1**.

To test this hypothesis, H·D-Asp-L-Arg-L-Val-L-Tyr-L-Val-L-His-L-Pro-L-Phe·NH_2 (**3**) was synthesized in order to determine its biological activity, specifically its duration of action *in vivo*.

The synthetic scheme employed is illustrated in Scheme I and followed essentially standard peptide procedures. The intermediate hexapeptide derivative (**6**) was prepared from two tripeptide units, carbobenzoxy-L-valyl-L-tyrosyl-L-valine[7] and L-histidyl-L-prolyl-L-phenylalanine methyl ester (**5**) utilizing 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide as the condensing agent. Crystalline **6**, which had previously been synthesized by Schwyzer, *et al.*,[7] and described as an amorphous powder, was converted to the amide (**7**) by first cleaving the carbobenzoxy group and then ammonolysis of the ester function. Stepwise addition of the remaining two amino acids, arginine and aspartic acid, elimination of the protecting groups by catalytic hydrogenation, and purification by countercurrent distribution in the system 2-butanol–0.5% acetic acid furnished D-Asp[1]-Val[5]-Phe[8] amide angiotensin II (**3**). Paper electrophoresis, paper chromatography, and quantitative amino acid determination provided support for the homogeneity of this material and its structure.

SCHEME I
SYNTHESIS OF D-ASP[1]-VAL[5]-PHE[8] AMIDE ANGIOTENSIN II



CbO = $C_6H_5CH_2OCO$—; DCCI = N,N′-dicyclohexylcarbodiimide

For further proof of structure, enzymatic degradation studies were carried out as illustrated in Scheme II; reaction products were separated by paper chromatography and compared with reference substances. Trypsin digestion furnished the hexapeptide amide (**6**), whereas incubation of (**3**) with chymotrypsin resulted in formation of the tetrapeptide valylhistidylprolylphenylalanine. A second component, believed to be D-aspartyl-L-arginyl-L-valyl-L-tyrosine, was also detected in the chymotrypsin digestion mixture. As anticipated, the octapeptide amide was stable to leucine aminopeptidase and to carboxypeptidase. The enzymatic experiments support the configurational homogeneity of the synthetic material and, together with the sequence of synthesis and the quantitative amino

(3) (a) H. Brunner and D. Regoli, *Experientia*, **18**, 504 (1962); (b) D. Regoli, B. Riniker, and H. Brunner, *Biochem. Pharmacol.*, **12**, 637 (1963); (c) P. A. Khairallah, F. M. Bumpus, I. H. Page, and R. R. Smeby, *Science*, **140**, 672 (1963).

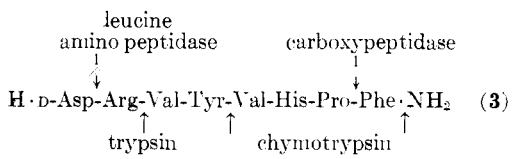
(4) E. G. Erdös and E. M. Sloane, *Biochem. Pharmacol.*, **11**, 585 (1962).

(5) H. Zuber, *Chimia* (Aarau), **14**, 405 (1960).

(6) Bovine angiotensin II has the amino acid sequence H·L-Asp-L-Arg-L-Val-L-Tyr-L-Val-L-His-L-Pro-L-Phe·OH. Asparaginyl[5]-valyl[5] angiotensin II has been reported to be as active as bovine angiotension II in nephrectomized rats; *cf.* ref. 1.

(7) R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Ritter, and H. Zuber, *Helv. Chim. Acta*, **41**, 1273, 1287 (1958).

SCHEME II
ENZYMATIC DEGRADATION OF D-ASP[1]-VAL[5]-PHE[8] AMIDE ANGIOTENSIN II



acid analysis, establish its structure as D-aspartyl[1]-valyl[5]-phenylalanine[8] amide angiotensin II.

Biological activity of **3** was evaluated *in vitro* on the isolated rat uterus and the isolated guinea pig ileum and *in vivo* through blood pressure measurements in both intact and nephrectomized phenobarbital-anesthetized rats. Weak, but definite activity, approximately 1/1000th that of asparaginyl[1]-valyl[5] angiotensin II[6] was exhibited *in vitro* at concentrations of 1–10 γ/ml. Activity shown *in vivo* was about 1/300th that of the reference compound in intact and nephrectomized rats, less than one might have expected from structure–activity considerations of **1** and **2** (Table I). This may indicate that biological activities observed in analogs in which single structural changes in different parts of the molecule have been made cannot be extrapolated to another analog in which all these changes have been made. Modifications of this sort may give rise to unpredictable subtle configurational changes, a different mode of combination with a receptor, or *in vivo* on altered transport to the receptor site, resulting in decreased or possibly increased biological activity.

Although less active than asparaginyl[1]-valyl[5] angiotensin II in absolute potency, D-aspartyl[1]-valyl[5]-phenylalanine[8] amide angiotensin II (**3**) exhibited approximately a three times longer duration of action on the blood pressure of intact and nephrectomized rats at doses which gave an equivalent absolute response. This difference in the two peptides was apparent at all dose levels in intact, and at low dose levels in nephrectomized rats. But at higher doses in nephrectomized rats, the duration of action of **3** was even more prolonged. Such a difference between intact and nephrectomized animals has also been observed by Gross and Turrian[8] after administration of moderate to high doses of angiotensin and does not seem to represent a unique feature of **3**. An explanation for this phenomenon of prolonged action at higher dose levels could be saturation of the degradative enzymes by peptide substrate.

Compound **1** was reported to have a duration of action in rats which is 2 to 3 times that of angiotensin II.[3b] The duration of response of **3** has been found to be similar to that of **1** in spite of the C-terminal amide function in **3**. If carboxypeptidases were major contributors to inactivation[9] *in vivo*, a longer duration of action of **3** relative to 1 would be expected. Since this was not the case, we conclude that our results fail to support the concept of carboxypeptidase degradation of angiotensin II, but they are in agreement with the conclusions of Regoli, *et al.*,[3a,b] and Khairallah, *et al.*,[3c] that inactivation proceeds primarily from the N-terminus.

(8) F. Gross and H. Turrian, "Polypeptides Which Affect Smooth Muscles and Blood Vessels," M. Schachter, Ed., Pergamon Press, New York, N. Y., 1960, p. 137.

(9) It has been reported recently that enzymes with chymotrypsin-like activity may participate in the degradation of angiotensin II (*cf.* ref. 3b). If the rate of hydrolysis by these enzymes occurs faster at the C-terminal amide function than at the tyrosyl[4]-valyl[5] linkage, compound **3** would be converted to compound **1**; but such a conversion is not supported by the difference in magnitude of pressor activity of compounds **1** and **3**.

Experimental

Analytical Methods.—Rotations were generally measured in 1% solutions. Melting points were taken in open capillaries and are corrected. A Misco paper electrophoresis apparatus and organic buffers containing 10% urea were used for the electrophoreses.[10] The R_f values (on Whatman paper No. 4) refer to the following paper chromatographic systems: (1) benzene–diethylamine (formamide) (9:1); (2) chloroform–diethylamine (formamide) (95:5); (3) chloroform–methanol–diethylamine–water (9:5:1:5), bottom phase; (4) 1-butanol–dioxane–2 *N* ammonia (5:1:4), top phase; (5) 2-butanol–formic acid (88%)–water (7:1:2); (6) chloroform–formic acid (88%)–ethanol (95%) (2:2:1); (7) 1-butanol–acetic acid–water (4:1:1); (8) chloroform–formamide (saturated).

Carbobenzoxy-L-histidyl-L-propyl-L-phenylalanine Methyl Ester (4).—A solution of 8.48 g. of carbobenzoxy-L-histidine hydrazide[11] in 83.7 ml. of 1 *N* hydrochloric acid was mixed with 100 ml. of ethyl acetate and cooled to 0°. An ice-cold solution of 1.93 g. of sodium nitrite in 8 ml. of water was then added, followed after 2 min. by 38 ml. of a cold 50% potassium carbonate solution. The mixture was shaken vigorously, the ethyl acetate layer was separated, and the aqueous phase was extracted with 20 ml. of ethyl acetate. The combined extracts were dried with sodium sulfate and filtered. To this solution was added at 0°, 7.7 g. of L-prolyl-L-phenylalanine methyl ester[12] in 20 ml. of ethyl acetate and the new solution was stored at 0° for 5 hr. and then at room temperature for 15 hr. A small amount of precipitate was filtered, the solution was washed with water, and dried with sodium sulfate, and the solvent was evaporated to yield 14.0 g. (91%) of **4** as a foamy residue, $[\alpha]^{25}D$ −34° (acetone), R_f (solvent 1) 0.20 (positive reaction with *p*-nitrobenzenediazonium fluoroborate spray).[13]

Trituration with hexane–ethyl acetate furnished the analytical sample, m.p. 65–70°, $[\alpha]^{25}D$ −35° (acetone), lit.[14] $[\alpha]^{26}D$ −41.5° (*c* 2.044, methanol).

Anal. Calcd. for $C_{29}H_{33}N_5O_6$: C, 63.60; H, 6.07; N, 12.79. Found: C, 63.60; H, 6.37; N, 12.59.

L-Histidyl-L-prolyl-L-phenylalanine Methyl Ester Dihydrobromide (5a).—A solution of 13.5 g. of **4** in 152 ml. of 1.2 *N* HBr in glacial acetic acid was stored at room temperature for 90 min. The mixture was poured into ether (1.5 l.), the precipitated solids were filtered, washed well with ether, and dried over phosphorus pentoxide. The hygroscopic material was dissolved in cold methanol and then precipitated with ethyl acetate to yield 11.9 g. (82%) of crystalline **5a**, m.p. 170–174°, $[\alpha]^{24}D$ −39° (H_2O).

Recrystallization from methanol–ethyl acetate gave the analytical sample, m.p. 169–171°, $[\alpha]^{24}D$ −38° (H_2O), R_f (solvent 2) 0.44 (positive reaction with *p*-nitrobenzenediazonium fluoroborate spray).

Anal. Calcd. for $C_{21}H_{27}N_5O_4 \cdot 2HBr$: C, 42.44; H, 5.43; Br, 26.89; N, 11.69. Found: C, 42.38; H, 5.39; Br, 26.89; N, 11.93.

L-Histidyl-L-prolyl-L-phenylalanine Methyl Ester (5b).—The dihydrobromide (**5a**) (6 g.) was dissolved in 15 ml. of water, 50 ml. of chloroform was added, and the mixture was cooled to 0°. The solution was transferred into a precooled separating funnel, 20 ml. of ice-cold, saturated potassium carbonate solution was added, the mixture was shaken vigorously, and the chloroform layer was separated. The water phase was extracted twice with 20 ml. of chloroform, the combined extracts were washed with saturated sodium chloride solution and with water, dried over sodium sulfate, and the solvent was evaporated to furnish 4.2 g. (100%) of **5b** as a fluffy residue.

Carbobenzoxy-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine Methyl Ester[7] (6).—Carbobenzoxy-L-valyl-L-tyrosyl-L-valine[7] (5.1 g.) was dissolved in 160 ml. of tetrahydro-

(10) L. N. Werum, H. T. Gordon, and W. Thornburg, *J. Chromatog.*, **3**, 125 (1960).

(11) R. W. Holley and E. Sondheimer, *J. Am. Chem. Soc.*, **76**, 1326 (1954).

(12) W. Ritter, B. Iselin, H. Kappeler, B. Riniker, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 614 (1957).

(13) J. H. Freeman, *Anal. Chem.*, **24**, 955 (1952).

(14) L. T. Skeggs, Jr., K. E. Lentz, J. R. Kahn, and N. P. Shumway, *J. Exptl. Med.*, **108**, 283 (1958).

furan and L-histidyl-L-prolyl-L-phenylalanine methyl ester (4.1 g.) was dissolved in 40 ml. of acetonitrile. The two solutions were cooled to 0°, combined, and 2.37 g. of 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide in 10 ml. of acetonitrile was added and the resulting solution stored at room temperature for 16 hr. The solvent was then evaporated *in vacuo*, the residue was taken up in chloroform in the presence of some methanol, and the solution was successively washed with cold 2 *N* hydrochloric acid, 0.5 *N* potassium carbonate solution, and with water. Evaporation of the solvent afforded 5.6 g. (61%) of the hexapeptide as a white powder. Trituration with ethyl acetate resulted in 3.8 g. of crystalline **6** of m.p. 152–156°, $[\alpha]^{24}D$ −55° (methanol), R_f (solvent 2) 0.85, R_f (solvent 8) 0.55 [positive reactions with $FeCl_3$–$K_3Fe(CN)_6$[15] and *p*-nitrobenzenediazonium fluoroborate sprays].

Anal. Calcd. for $C_{48}H_{60}N_8O_{10}\cdot H_2O$: C, 62.19; H, 6.74; N, 12.09. Found: C, 62.40; H, 6.81; N, 12.09.

Schwyzer, *et al.*,[7] reported m.p. *ca.* 155° and $[\alpha]D$ −57° (ethanol) for their analytical sample and m.p. 158–162°, $[\alpha]D$ 56 ± 4° (*c* 1.38, ethanol) for material purified by countercurrent distribution, which was amorphous.

L-Valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine Amide (7).—A solution of 3.1 g. of carbobenzoxy-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine methyl ester in 35 ml. of 1.2 *N* HBr in glacial acetic acid was stored at room temperature for 2 hr. After that period the solution was poured into 300 ml. of anhydrous ether and the precipitated material was filtered. The collected dihydrobromide was dissolved in 50 ml. of water, the solution was made basic (pH 7.5) with 0.5 *N* potassium carbonate solution and extracted several times with a mixture of chloroform–methanol (1:1). The combined extracts were washed with a small amount of water, dried with anhydrous sodium sulfate, and the solvent was evaporated to furnish 2.0 g. of a yellow residue. This was dissolved in 8 ml. of methanol (saturated with ammonia) and allowed to stand at room temperature for 4 days. Evaporation of the solvent yielded 1.9 g. of material, $[\alpha]^{24}D$ −48° (methanol), which was precipitated from methanol–ether to give 1.6 g. (62%) of **7**, m.p. 147–150°, $[\alpha]^{24}D$ −54° (methanol).

Countercurrent distribution in the system methanol–water–chloroform–carbon tetrachloride (8:2:5:5, 50 transfers) afforded 1.41 g. of pure hexapeptide amide (fractions 30–43 combined), m.p. 149–152°, $[\alpha]^{24}D$ −55° (methanol), R_f (solvent 2) 0.65, R_f (solvent 3) 0.6, R_f (solvent 4) 0.8, R_f (solvent 5) 0.32 [positive reactions with $FeCl_3$–$K_3Fe(CN)_6$ and *p*-nitrobenzenediazonium fluoroborate sprays].

Anal. Calcd. for $C_{39}H_{53}N_9O_7$: C, 61.64; H, 7.03; N, 16.59. Found: C, 61.61; H, 7.03; N, 16.37.

Carbobenzoxynitro-L-arginyl-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine Amide (8).—To 5.9 of **7** in 22 ml. of dimethylformamide and 51 ml. of acetonitrile at 0° was added 4.37 g. of N,N′-dicyclohexylcarbodiimide in 25 ml. of acetonitrile followed by 6.85 g. of carbobenzoxynitro-L-arginine[16] in 15 ml. of dimethylformamide. The resulting solution was stored at room temperature for 16 hr., precipitated N,N′-dicyclohexylurea (4.0 g., m.p. 228–230°) was filtered, and the filtrate was evaporated *in vacuo*. The residual oil was triturated with ethyl acetate, furnishing 11.3 g. of solid material, $[\alpha]^{23}D$ −48° (methanol). This was dissolved in a mixture of ethyl acetate–methanol, the solution was washed with dilute sodium bicarbonate solution, with water, and dried with anhydrous sodium sulfate. Evaporation of the solvent yielded a residue which was triturated with ethyl acetate to afford 7.76 g. (90%) of **8**, m.p. 151–164°, $[\alpha]^{24}D$ −57° (methanol).

A portion (3.3 g.) of this material was subjected to a 50-transfer countercurrent distribution in the system methanol–water–chloroform–carbon tetrachloride (8:2:5:5). The purified protected heptapeptide was isolated from tubes 28–41; yield, 3.0 g. Trituration with ethyl acetate furnished 2.8 g. of pure **8**, m.p. 154–160°, $[\alpha]^{24}D$ −60° (methanol), R_f (solvent 2) 0.15 [single spot with $FeCl_3$–$K_3Fe(CN)_6$ reagent].

Anal. Calcd. for $C_{53}H_{70}N_{14}O_{13}\cdot 0.5H_2O$: C, 57.65; H, 6.48; N, 17.76. Found: C, 57.64; H, 6.48; N, 17.45.

Nitro-L-arginyl-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine Amide (9).—The amide **8** (2.6 g.) was dissolved in 90 ml. of 1.2 *N* HBr in glacial acetic acid. The solution was allowed to stand at room temperature for 2 hr. and then poured into 300 ml. of anhydrous ether. The precipitated amorphous material was filtered, washed well with ether, and dried over phosphorus pentoxide to afford 2.5 g. of the dihydrobromide. This was dissolved in 40 ml. of water, the aqueous solution was cooled to 0°, washed with saturated $NaHCO_3$ solution, and then extracted several times with butanol. The combined extracts were washed with water, the solvent was evaporated (bath temperature <45°), and the resulting residue was triturated with ethyl acetate to yield **9**, 2.0 g. (97%), m.p. 154–157°, $[\alpha]^{24}D$ −52° (methanol), R_f (solvent 2) 0.6, R_f (solvent 5) 0.5 [positive reaction with $FeCl_3$–$K_3Fe(CN)_6$ spray]. The material was homogeneous by paper electrophoresis at pH 4, 6, 7.2, 8, and 9.3 [single spot with $FeCl_3$–$K_3Fe(CN)_6$ reagent].

Anal. Calcd. for $C_{45}H_{64}N_{14}O_{10}\cdot H_2O$: C, 55.21; H, 6.80; N, 20.02. Found: C, 55.16; H, 6.73; N, 19.72.

Cabrobenzoxy(β-benzyl)-D-aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine Amide (10).—To 1.4 g. of **9** in 5 ml. of dimethylformamide at 0° was added 0.605 g. of N,N′-dicyclohexylcarbodiimide in 7 ml. of acetonitrile, followed by 1.0 g. carbobenzoxy-D-aspartic acid β-benzyl ester[17] in 4 ml. of dimethylformamide. The solution was allowed to stand at room temperature for 18 hr., when 0.2 ml. of glacial acetic acid was added. After an additional standing for 30 min., the dicyclohexylurea (0.30 g.) was filtered, the filtrate was concentrated *in vacuo*, and the residue (2.4 g.) was triturated with ethyl acetate to afford 1.67 g. (90%) of crystalline **10**, m.p. 149–155°, $[\alpha]^{24}D$ −52° (methanol). For analysis a sample was dissolved in warm ethanol, chilled, and the amorphous precipitate filtered, m.p. 149–155°, $[\alpha]^{24}D$ −59° (methanol), R_f (solvent 7) 0.95 [positive reaction with $FeCl_3$–$K_3Fe(CN)_6$ reagent].

Anal. Calcd. for $C_{64}H_{81}N_{15}O_{15}\cdot 2H_2O$: C, 57.53; H, 6.41; N, 15.73. Found: C, 57.40; H, 6.31; N, 16.07.

D-Aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine Amide (3).—To 0.65 g. of **10** in 10 ml. of methanol was added 0.5 ml. of 5.3 *N* hydrochloric acid in methanol followed by 0.13 g. of 5% Pd–C catalyst. The mixture was hydrogenated at room temperaturd for 24 hr., when an additional 0.26 g. of the catalyst was added. After further hydrogenation for 48 hr., the catalyst was filtered, washed well with methanol, and the filtrate was evaporated *in vacuo* to yield 0.407 g. of a residue, $[\alpha]^{24}D$ −47° (methanol). This material was distributed in the system 2-butanol–0.5% acetic acid–water (100 transfers, phase volume 10 ml.). Electrophoresis at pH 4, 7.2, 8, and 9.3 indicated that substance in tubes 0–14 [0, 155 g., $[\alpha]^{24}D$ −57° (water)], fraction I, was essentially single spot material with ninhydrin (yellow color) and the $FeCl_3$–$K_3Fe(CN)_6$ reagent. Material in tubes 16–19 (0.086 g.) showed an additional more polar spot, which gave a gray color with the ninhydrin reagent. The latter became predominant in tubes 20–26 (0.036 g.). After a further distribution of fraction I in the same countercurrent system (200 transfers) octapeptide amide (**3**) was isolated from fractions 6–23 and lyophilized to yield 0.10 g. of a white powder, $[\alpha]^{23}D$ −54° (water), R_f (solvent 4) 0.40, R_f (solvent 5) 0.17 [single spot with $FeCl_3$–$K_3Fe(CN)_6$, *p*-nitrobenzenediazonium fluoroborate and diacetyl reagents], homogeneous by paper electrophoreses at pH 7.2 and 8.0 [single spot with ninhydrin, *p*-nitrobenzenediazonium fluoroborate, and $FeCl_3$–$K_3Fe(CN)_6$ reagents].

Quantitative amino acid determination gave the following molar ratio: Asp, 1.1; Arg, 1.0; Val, 1.8; Tyr, 0.8; His, 0.9; Pro, 1.0; Phe, 0.9.

Enzymatic Methods.—The procedures followed are essentially those described by Riniker and Schwyzer.[18] The buffer used was tris(hydroxymethyl)aminomethane adjusted with hydrochloric acid.

Carboxypeptidase.—The carboxypeptidase solution was prepared by dilution of 0.3 ml. of a 1% suspension of carboxypeptidase in water (Mann Research Laboratories) with 0.3 ml. of pH 8 buffer, careful addition of 0.2 *N* sodium hydroxide until a clear solution was obtained, and subsequent addition of 0.2 *N* hydrochloric acid to cloudiness. The pH of the solution was approximately 8, the concentration of carboxypeptidase, 0.2%.

The octapeptide (**3**, 1 mg.) was dissolved in 0.1 ml. of buffer (pH 8, containing 0.1% sodium chloride), and 0.0125 ml. of the above carboxypeptidase solution was added (enzyme–substrate,

(15) G. M. Barton, R. S. Evans, and J. A. F. Gardner, *Nature*, **170**, 249 (1952).

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

(17) Cyclo Chemical Corp., Los Angeles, Calif.

(18) B. Riniker and R. Schwyzer, *Helv. Chim. Acta*, **44**, 658 (1961).

1:40). The resulting mixture was incubated at 38° for 60 min., then acidified with dilute hydrochloric acid, and subsequently evaluated by paper chromatography on solvents 4 and 5, utilizing ninhydrin and $FeCl_3$–$K_3Fe(CN)_6$ reagents as indicators. The octapeptide remained essentially unchanged, only traces of two new spots could be observed; no phenylalanine could be detected. (The hexapeptide H·L-val-L-tyr-L-val-L-pro-L-phe·OH under identical conditions was digested quantitatively, releasing phenylalanine.)

Chymotrypsin.—The octapeptide (**3**, 1 mg.) was dissolved in 0.20 ml. of pH 8.6 buffer and 0.016 ml. of a solution of 0.25% crystalline chymotrypsin (Worthington) was added (enzyme-substrate, 1:25). After incubation for 1 hr. at 38°, the reaction mixture was acidified with dilute hydrochloric acid and then assayed by paper chromatography on solvents 4 and 5, utilizing *p*-nitrobenzenediazonium fluoroborate and $FeCl_3$-$K_3Fe(CN)_6$ reagents as indicators. The octapeptide (**3**) was digested quantitatively to the tetrapeptide L-valyl-L-histidyl-L-prolyl-L-phenylalanine, R_f (solvent 4) 0.33 and R_f (solvent 5) 0.43 (yellow reaction with *p*-nitrobenzenediazonium fluoroborate), and to another component, presumably D-aspartyl-L-arginyl-L-valyl-L-tyrosine, R_f (solvent 4) 0.15 and R_f (solvent 5) 0.11, which gave a purple spot with the same reagent and a positive reaction with the $FeCl_3$–$K_3Fe(CN)_6$ spray [L-valyl-L-histidyl-L-prolyl-L-phenylalanine amide has R_f (solvent 4) 0.74, R_f (solvent 5) 0.37].

Trypsin.—The octapeptide (**3**, 2 mg.) was dissolved in 0.20 ml. of 0.05 *N* buffer (pH 8.6), 0.016 ml. of a solution of 0.25% crystalline trypsin (Worthington, 3x crystallized) was added, and the solution was incubated at 38° for 1 hr. (enzyme–substrate, 1:50). The reaction mixture was then acidified and assayed by paper chromatography. The peptide was digested quantitatively to L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine amide, R_f (solvent 3) 0.65, R_f (solvent 4) 0.85 [positive reactions with $FeCl_3$–$K_3Fe(CN)_6$ and *p*-nitrobenzenediazonium fluoroborate sprays].

Leucine Aminopeptidase.—The enzyme (Worthington) was activated before use for 3 hr. at 40°. The activation mixture consisted of 0.20 ml. of 0.025 *M* $MnCl_2$ solution, 0.50 ml. of pH 8 buffer, 0.50 ml. of water, and 0.20 ml. of 0.5% leucine aminopeptidase solution.

The octapeptide (2 mg.) was added to 0.2 ml. of the above activated leucine aminopeptidase, the resulting solution was incubated at 38° for 24 hr. and subsequently evaluated by paper chromatography. No degradation could be detected with indicators ninhydrin and *p*-nitrobenzenediazonium fluoroborate. (The hexapeptide, L-valyl-L-tyrosyl-L-valyl-L-histidyl-L-prolyl-L-phenylalanine, under identical conditions was digested quantitatively to valine, tyrosine, and the tripeptide, L-histidyl-L-prolyl-L-phenylalanine.)

Biological Determinations.—Asparaginyl-valyl[5] angiotensin II[6,19] was used as a standard in all preparations.

Rat Uterus.—Virgin Wistar strain rats (180–200 g.) were injected intramuscularly with diethylstilbesterol, 0.1 mg./kg., 20 hr. before being sacrificed. Uterine strips were suspended in 40 ml. of DeJalon's solution maintained at 30°, and aerated with a mixture of 95% O_2 and 5% CO_2. The peptides were allowed to act for 2 min. before washing.

Guinea Pig Ileum.—Segments of terminal ileum, 4 cm. long, were excised from male Hartley strain guinea pigs (290–310 g.) and suspended in 40 ml. of Tyrode solution maintained at 38° and aerated with 95% O_2 and 5% CO_2. The peptides were allowed to act for 2 min. before washing.

Blood Pressure Measurements.—The procedures followed were essentially those described by Gross and Lichtlen.[20]

(19) Hypertensin®, Ciba. This substance produced an average increase of 50 mm. in rat blood pressure of intact phenobarbital-anesthetized rats following intravenous administration of 0.1–0.2 γ/kg.

(20) F. Gross and P. Lichtlen, *Arch. exptl. Pathol. Pharmakol.*, **233**, 323 (1958).

Synthetic Antigonadotropins. I. Triarylethylenes

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A preliminary series of triarylethanol and triarylethylene compounds was prepared and tested for hormonal and antihormonal effects. Beyond some weak estrogenic effects, the triarylethanols showed no activity. Some of the triarylethylenes proved to be very active antigonadotropins.

The Triarylethylenes.—In an attempt to make a pyridine analog of triparanol, 1-(*p*-methoxyphenyl)-1-(4-pyridyl)-2-(*p*-chlorophenyl)ethanol (I) was prepared from *p*-methoxyphenyl 4-pyridyl ketone and *p*-chlorobenzyl chloride by means of a Grignard reaction. Treatment with hydrobromic acid to hydrolyze the methoxy group also served to split out water from the aliphatic moiety to produce 1-(*p*-hydroxyphenyl)-1-(4-pyridyl)-2-(*p*-chlorophenyl)ethylene (XX). The resemblance between the latter and other compounds known to have marked estrogenic and other hormone-like effects suggested that an excursion into this area might be profitable. The present report concerns some preliminary findings resulting from this excursion.

With the single exception already noted all of the triarylethylenes prepared in this series were made by substantially the same method. The appropriate ketones and Grignard reagents reacted to give the triarylethanols which, on dehydration, gave the desired triarylethylenes.

Tables I and II list the triarylethanols and the triarylethylenes prepared in this series. Two isomeric pairs are listed in Table II, namely, XXI and XXII, and XXXIX and XL. The configurations of these compounds have not been determined.

Pharmacology.—The antigonadotropic activity was determined in intact immature male rats. The effect of oral administration of the compounds in oil, given once each day for 10 working days, on the weights of the testes and the prostate was compared to that of the controls and expressed as the per cent difference from the controls. The estrogenic activity was determined on intact immature female rats given subcutaneous injections of the compounds in oil for 3 consecutive days. The increase in uterine weight of the treated animals was compared to that of the controls and was expressed as the per cent difference from the controls.

Beyond weak estrogenic effects, the triarylethanols have so far shown no notable activity. On the other hand, some of the triarylethylenes have shown very marked antigonadotropic effects in a preliminary screen-

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