Z-γ-*tert*-Bu-Glu-*O*-*tert*-Bu-Tyr-Ala-Gly Pentachlorophenyl Ester (4).—To a soln of 9.0 g (0.0129 mole) of the fully blocked tetrapeptide **3** in 150 ml of MeOH was added 13.0 ml of 1 *N* NaOH and the soln was stirred for 90 min and then concd under reduced pressure. The residue was flooded with H_2O, acidified with 10% citric acid soln, and extd into EtOAc. The EtOAc soln was dried (Na_2SO_4) and concd under reduced pressure to give the tetrapeptide free acid as a solid; yield 8.0 g (91%). To this material in 200 ml of CH_2Cl_2 was added 3.1 g (0.0165 mole) of pentachlorophenol and 5.0 g (0.0118 mole) of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. The mixt was shaken for 48 hr at room temp. The solvent was removed *in vacuo* and the residue was washed with H_2O and crystd from MeOH to yield 5.1 g (47%): mp 187–188°, $[\alpha]^{27}D$ −12.9° (*c* 2.75, DMF). *Anal.* ($C_{41}H_{47}Cl_5N_4O_{10}$) C, H, N.

γ-*tert*-Bu-Glu-*O*-*tert*-Bu-Tyr-Ala-Gly Pentachlorophenyl Ester·HCl (5).—A fine suspension of 4.5 g (0.0048 mole) of the tetrapeptide active ester **4** and 0.5 g of 10% Pd/C in 150 ml of MeOH was treated with 0.173 g (0.0048 mole) of dry HCl in MeOH, and the suspension was hydrogenated for 2 hr. The reaction mixt was filtered and the filtrate was concd. The residue was crystd from MeOH–Et_2O to give 3.8 g (95%): mp 172°, $[\alpha]^{27}D$ +2.5° (*c* 4.2, DMF). *Anal.* ($C_{33}H_{42}Cl_5N_4O_8$) C, H, N.

Poly(Glu-Tyr-Ala-Gly)Gly-*1*-^{14}C Et Ester (1).—To a soln of 1.1 mg of glycine-*1*-^{14}C Et ester HCl (specific activity 3.15 mCi/mmole) and 1.82 g (0.018 mole) of Et_3N in 5 ml of DMSO was slowly added a soln of 3.0 g (0.00359 mole) of the polymerizing unit **5** in 25.0 ml of DMSO. The transfer vessels were washed with 5.5 ml of DMSO which was added to the reaction mixt giving a final concn of 100 nmoles/l. The reaction mixt was shaken for 6 days and then centrifuged to yield the product which was washed with three 35-ml portions of H_2O, three 35-ml portions of MeOH, and three 35-ml portions of Et_2O and dried to give 0.95 g (49.5%) of the blocked polymer. The protected polypeptide was dissolved in 50 ml of 90% F_3CCO_2H and stirred for 50 min, and then concd under reduced pressure to yield the crude polypeptide 1. This material was washed with Et_2O, suspended in 20 ml of H_2O, and dissolved by the addition of 1 *N* NaOH to pH 7.5. The soln was dialyzed against distd H_2O for 2 days, then lyophilized to yield the Na salt of the polypeptide. This material was converted into its free acid by acidification with 1 *N* HCl to pH 2.5. The pptd polypeptide was collected by centrifugation and then lyophilized to yield 0.50 g (32%); radio assay, 33.3 nCi/mg indicates 73.7% incorporation of the radioactive label. *Anal.* ($C_{19}H_{24}N_4O_7 \cdot 1.5\ H_2O$) C, H, N.

Molecular Weight Determination.—Calibrated columns of Sephadex G-100 (2.5 × 38.5 cm) and of Corning CPG 10-240 glass granules (2.0 × 28 cm) were employed for the molecular weight determination. Using 0.1 *M* NaCl–0.05 *M* KH_2PO_4 corrected to pH 8.0 as eluent, 4 mg of poly(Gly-Tyr-Ala-Gly)Gly-*1*-^{14}C Et ester was passed through each of these columns. The polypeptide was eluted from each column in a vol equiv to that corresponding to a mol wt of at least 1 × 10^5.

Immunochemical Results.—Two rabbits were treated at weekly intervals with 500 μg of poly(Glu-Tyr-Ala-Gly)Gly-*1*-^{14}C Et ester **1**. The first 2 weeks they were injected intradermally using complete Freunds adjuvant as suspension medium and the 3rd week they were injected sc. The injection on the 4th week was done iv using buffered saline. Bleedings were conducted on the following week and the serum from each animal was not found to give a precipitin reaction with up to 10,000 μg of the polymer **1**.

Inhibition Studies.—To 1-ml aliquots of rabbit antisera to poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester was added incremental amounts of up to 7000 μg of the polypeptide **1**. To each tube was added the equiv point amount of the antigen (30 μg) and the tubes were then incubated at 37°. Each tube showed a precipitin reaction. After standing at 4° for 48 hr, the ppts were collected, washed twice with H_2O, and collected by centrifugation. The amount of protein pptd was estimated by the absorbancy at 280 mμ of a 0.1 *N* NaOH soln of the ppts. It was found that the precipitin reaction between poly(Tyr-Glu-Ala-Gly)Gly-*1*-^{14}C Et ester and its antisera was 50% inhibited by the addition of 6000 μg of poly(Glu-Tyr-Ala-Gly)Gly-*1*-^{14}C Et ester 1.

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Synthesis of [5-Valine,7-*N*-methylalanine]-angiotensin II, a Hypertensive Peptide[1-3]

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A synthesis of the octapeptide H-Asp-Arg-Val-Tyr-Val-His-Mal-Phe-OH ([Val5,Mal7]-angiotensin II) by the solid phase method is described. This peptide is an analog of [Val5]-angiotensin II, in which 7-proline is replaced by Mal. The observation that this peptide exhibits marked hypertensive and myotropic activities indicates that the proline residue is not essential for these pharmacological properties; instead the presence of an N-methylated amino acid in position 7 may be sufficient.

To date, more than 100 analogs[4] of angiotensin have been reported[5] in addition to the naturally occurring [Val5]- and [Ile5]-angiotensins I and II. Many of these analogs have been prepared and their biological activity evaluated for the purpose of elucidating the correlation between structural features and biological function. On the basis of these investigations, it has been concluded[5] that 4 of the 8 amino acids constituting these peptides are essential for biological activity. These are 4-Tyr, 6-His, 7-Pro, and 8-Phe. In addition, it has been asserted[5] that the C-terminal CO_2H must not be substituted. In a recent investigation,[6] it was shown that the imidazole portion of 6-His is not required for biological function. The conclusion that 7-Pro is essential for biological activity followed from an investigation of Page and Bumpus,[7] who found that the replacement of 7-Pro in [Ile5]-angiotensin II by Ala was accompanied by a total loss of hypertensive activity. Replacement of Pro in angiotensin has been the subject of

(1) This work was supported by research grants from the National Science Foundation (GB-7571X) and from the National Heart Institute, National Institutes of Health, U. S. Public Health Service (HE-01662).

(2) All amino acid residues are of the L variety. The following abbreviations are used: Boc = *t*-butoxycarbonyl; Narg = nitroarginine; Mal = *N*-methylalanine; TEA = Et_3N; TFA = F_3CCO_2H; AP-M = aminopeptidase *M*.[3]

(3) G. Pfleiderer, P. G. Celliers, M. Stanulovic, E. D. Wachsmuth, H. Determann, and G. Braunitzer, *Biochem. Z.*, **340**, 552 (1964).

(4) These include analogs with substituted functional groups, with modified sequences, and with shorter or extended chain length.

(5) For a survey of the literature up to Oct 1965, see E. Schröder and K. Lübke, *Peptides*, **2**, 52 (1966).

(6) K. Hofmann, R. Andreatta, J. P. Buckley, W. E. Hageman, and A. P. Shapiro, *J. Amer. Chem. Soc.*, **90**, 1654 (1968); R. Andreatta and K. Hofmann, *ibid.*, **90**, 7334 (1968).

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

only two other studies. Its exchange for hydroxyproline reduces the biological activity to 7%,[8] and its replacement (expansion of ring) by L-pipecolic acid lowered the hypertensive activity to 1% of that of the parent compound,[9] indicating again that the Pro is required for biological activity. It may be that the importance of the Pro side chain lies in the ability of the rigid pyrrolidine ring to bring the functionally essential amino acids 4-Tyr, 6-His, and 8-Phe into proper relative positions, as has been suggested,[9,10] when the peptide reacts with its receptor; the isolated peptide seems to be devoid of a unique structure.[11]

The aim of the present study is to determine the importance of the proline ring for biological activity. For this purpose, [Val^5,Mal^7]-angiotensin II was synthesized, and its biological activity was determined. In this peptide, the Pro residue of [Val^5]-angiotensin II is replaced by the acyclic amino acid *N*-methylalanine: H-Asp-Arg-Val-Tyr-Val-His-Pro-Phe-OH ([Val^5]-angiotensin II); H-Asp-Arg-Val-Tyr-Val-His-Mal-Phe-OH ([Val^5,Mal^7]-angiotensin II). Like in Pro, but in contrast to Ala, the N of Mal (a secondary amino acid) cannot participate in H bonding when this residue is in the interior of a polypeptide chain.

Preparative Aspects and Biological Activity.—Tosyl-L-alanine served as the starting material for the preparation of Mal. Methylation with MeI–NaOH according to Fischer and Lipschitz[12] gave Ts-Mal. Removal of the Ts group was effected by treatment with AcOH saturated with HBr at room temp in the presence of phenol.[13] This mild procedure, which gives high yields, was chosen over the more severe one applied by Shemyakin, *et al.*,[14] in similar work. The resulting Mal·HBr was converted into the free amino acid with the aid of an anion-exchange resin.

The pathway employed for the synthesis of [Val^5,Mal^7]-angiotensin II was basically the same as that described by Marshall and Merrifield[15] for the synthesis of [Ile^5]-angiotensin II. Boc-Phe was esterified to chloromethylated poly(styrene-*co*-2% divinylbenzene). The stepwise synthesis was carried through 7 cycles to give the fully protected octapeptide-resin ester, Boc-β-benzylaspartylnitroarginylvalyl-*O*-benzyltyrosylvalyl-N^{im}-benzylhistidyl-*N*-methylalanylphenylalanyl resin. The cycle for each amino acid consisted of removal of the Boc group by 1 *N* HCl in AcOH, neutralization of the resulting hydrochloride with TEA in DMF, and then coupling of the next Boc-amino acid to the free base, using DCI as the condensing agent. An aq solution of $Et_3NH^+Cl^-$, collected after each neutralization step, was titrated for Cl^- by the Volhard method. The results provided a measure for the reaction rates in each cycle.

The data of Table I indicate that almost complete reaction has taken place at every stage but the His one. In a preliminary run with the usual coupling time of 4 hr for N^{im}-benzylhistidine, this amino acid was incorporated only to approximately 60%. Extension of the reaction time to 16 hr greatly improved the rate of its incorporation. The difficulties encountered in the coupling of Boc-N^{im}-benzylhistidine to the Mal-Phe-resin ester must be due entirely to the inherent low susceptibility of the amino group of the Mal-Phe resin ester towards acylation, since Boc-N^{im}-benzylhistidine has been used widely in solid phase synthesis, and no mention is found in the literature which might suggest that the difficulties are due to the histidine, even with proline as the component to be acylated, as in previously prepared angiotensin and angiotensin analogs.[9,15] The fact that yields obtained in coupling reactions between acylmethylalanine *p*-nitrophenyl ester and Mal are considerably lower than those obtained from reactions, under identical conditions, between acylproline *p*-nitrophenyl esters and Pro[16] supports this view.

TABLE I

Cl^- CONTENT (= NH_2 CONTENT) AFTER EACH CYCLE[a]

Cycle	Amino acid	Cl^-, mmoles
8	β-BzAsp	
7	Narg	5.30
6	Val	5.36
5	*O*-BzTyr	5.42
4	Val	5.38
3	N^{im}-BzHis	5.48
2	Mal	3.00
1	Phe	3.05

[a] For total amt of resin used (3.0 g).

The protected octapeptide was cleaved from the resin by bubbling HBr through a suspension of the peptide resin in TFA. This treatment also removed the N-terminal Boc group and the benzyl groups of both the Asp and Tyr residues. The remaining protecting groups, the NO_2 of Arg and the *im*-benzyl group of His, were finally removed by catalytic hydrogenation over freshly prepared Pd catalyst. The course of the hydrogenation was monitored by tlc. The crude [Val^5,Mal^7]-angiotensin II preparation contained one major component and 2 contaminants which were separated on Sephadex G25.

Homogeneity of the purified product was determined by chromatography on paper and tl. Acid hydrolysates of the octapeptide showed the expected amino acid composition. Digestion with AP-M gave Asp, Arg, Val, Tyr, and Phe in the expected amounts and ratios, together with the dipeptide His-Mal which is totally resistant towards this enzyme under the conditions used. Complete liberation of the C-terminal Phe could be explained by the possible presence of a carboxypeptidase in the commercial AP-M preparation used.

Pharmacological evaluation[17] showed that [Val^5,Mal^7]-angiotensin II is a potent hypertensive and myotropic agent. The hypertensive activity of the Mal analog was determined in 2 rat preparations against

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(9) N. C. Chaturvedi, W. K. Park, R. R. Smeby, and F. M. Bumpus, *J. Med. Chem.*, **13**, 177 (1970).
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(12) E. Fischer and W. Lipschitz, *Chem. Ber.*, **48**, 360 (1915).
(13) D. I. Weisblat, B. J. Magerlain, and D. R. Myers, *J. Amer. Chem. Soc.*, **75**, 3630 (1953). See also R. H. Andreatta, V. Nair, A. V. Robertson, and W. R. J. Simpson, *Aust. J. Chem.*, **20**, 1493 (1967).
(14) M. M. Shemyakin, Yu. A. Ovchinnikov, V. T. Ivanov, and A. A. Kiryushkin, *Tetrahedron*, **19**, 581 (1963).
(15) G. R. Marshall and R. B. Merrifield, *Biochemistry*, **4**, 2394 (1965).
(16) R. H. Andreatta and H. A. Scheraga, unpublished results.
(17) We wish to express our appreciation to Dr. Therezinha B. Paiva of the Escola Paulista de Medicina, Sao Paulo, Brazil, for these results.

[Asn1,Val5]-angiotensin II[18] (angiotensinamide, Ciba). In the pithed rat the Mal analog exhibited 22 ± 2%, and in the nephrectomized rat pretreated with "pentolinium tartrate" 16 ± 1% of the potency of the reference standard. Determination of the myotropic activity on the isolated guinea pig ileum showed it to possess 4.3 ± 0.1% of the potency of angiotensinamide (Ciba). Hypertensive activities of angiotensin II and analogs modified in position 7 are compared in Table II.

TABLE II

BIOLOGICAL ACTIVITIES OF ANGIOTENSIN AND ANALOGS

Peptide[a]	Residue in position 7	Pressor activity[b]
[Val5]- and [Ile5]-angiotensin II	Pro	100
[Ile5,Hyp7]-angiotensin II	Hydroxyproline	7
[Ile5,Pip7]-angiotensin II	Pipecolic acid	1
[Ile5,Ala7]-angiotensin II	Ala	0
[Val5,Mal7]-angiotensin II	Mal	22

[a] See text for lit refs to the various peptides. [b] In per cent of standard [Asn1,Val5]-angiotensin II.

Experimental Section

A. Materials.—All solvents used were spectral grade. DMF was dried (BaO) and distd. The Boc-amino acids were prepd according to the procedure of Schwyzer, *et al.*[19] Purity was checked by tlc. Boc-N^{im}-benzylhistidine was purchased from Mann Research Laboratories. The prepn contained approximately 10% of a ninhydrin-positive impurity (slower moving on tlc) which was removed in the following manner: 2.0 g of the material was treated with boiling dioxane (100 ml), and some insol material was removed by filtration. The filtrate was concd *in vacuo* to approximately 50 ml, and the desired product crystd by the addition of hexane; white crystals: mp 180–182°; single Cl-positive, ninhydrin-negative spot on tlc. The ion-exchange resin AG1-X2 was purchased from Bio-Rad Laboratories, Richmond, Calif. Sephadex G25 superfine was from Pharmacia, Uppsala, Sweden. Cross-linked chloromethylated polystyrene and DCI were from Mann Research Laboratories. Aminopeptidase M was obtained from Röhm and Haas GmbH, 61 Darmstadt, West Germany (distributor: Henley and Co., Inc., New York, N. Y. 10017).

B. General Procedures.—Melting points are uncorrected. The values reported for $[\alpha]_D$ were determined on a Cary 60 spectropolarimeter. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Prior to analyses the compounds were dried *in vacuo* over P_2O_5 at 60°. The amino acid composition of acid and enzymic hydrolysates was determined with a Technicon amino acid autoanalyzer. Paper chromatography was performed on Whatman No. 1 filter paper by the descending technique with the following systems: R_f^1, *n*-BuOH-AcOH-H_2O, 4:1:5 (upper phase); R_f^2, *n*-BuOH-pyridine-AcOH-H_2O, 45:30:9:36. Using Merck's silica gel G plates, tlc was performed with the following solvent systems: R_f^I, *n*-BuOH-AcOH-H_2O, 60:20:20; R_f^{II}, *n*-BuOH-AcOH-H_2O, 40:30:30; R_f^{III}, *n*-BuOH-pyridine-AcOH-H_2O, 30:20:6:24. Acid hydrolysis was carried out in constant boiling HCl, contg 10% by vol of a 0.1 *M* soln of phenol, at 105° for 24 hr. AP-M digestion was performed as described by Hofmann, *et al.*[20]

C. Synthetic Procedures. Ts-*N*-methylalanine.—Methylation of Ts-alanine[21] with MeI according to Fischer and Lipschitz[12] gave Ts-*N*-Mal in 94% yield; mp 121–122° (after 2 recrystns from $CHCl_3$-hexane); $[\alpha]_D^{20}$ −8.8° (*c* 2.0, EtOH).[22]

***N*-Methylalanine·HBr.**—A soln of Ts-*N*-Mal (5.15 g) and phenol (3.76 g) in glacial AcOH satd with HBr (60 ml) was allowed to stand at room temp overnight. The mixt was introduced into dry Et_2O (400 ml) yielding an oil which crystd upon standing. After 1 hr at 4° the crystals were collected and washed with Et_2O. Recrystn from MeOH-Et_2O gave white, slightly hygroscopic needles; 3.10 g (86%); mp 146–148°; $[\alpha]_D^{20}$ +5.8° (*c* 2.0, EtOH). *Anal.* ($C_4H_{10}BrNO_4$) C, H, Br, N.

***N*-Methylalanine.**[23]—A soln of *N*-Mal·HBr (2.76 g) in H_2O (15 ml) was passed through a column with anion-exchange resin AG1-X2 (acetate cycle) (dimensions, 2.0 × 10 cm). The column was eluted with water until ninhydrin neg. The solvent was evapd *in vacuo*, and the solid residue was recrystd from abs EtOH and dried *in vacuo* at 60° over P_2O_5; yield, 1.30 g (88%); $[\alpha]_D^{20}$ +4.8° (*c* 2.0, H_2O).

Boc-*N*-Mal.—*N*-Methylalanine was converted into its Boc derivative by the method of Schwyzer, *et al.*,[19] in 87% yield; white needles from Et_2O-hexane, mp 91–92°; $[\alpha]_D^{20}$ −31.8° (*c* 2.0, EtOAc). *Anal.* ($C_9H_{17}NO_4$) C, H, N. Olsen[24] obtained the same product by basic saponification of methyl Boc-*N*-methyl-L-alaninate; mp 89–91°.

Boc-Phe Resin.—A soln of 5.31 g (20 mmoles) of Boc-Phe and 2.8 ml (20 mmoles) of TEA in 100 ml of EtOAc was added to 10 g of chloromethylated poly(styrene-*co*-2.0% divinylbenzene) which contained 2.07 mmoles of Cl/g. The reaction mixt was stirred at reflux temp for 24 hr. The esterified resin was collected and washed with EtOAc, MeOH, H_2O, and MeOH, and dried *in vacuo;* yield, 12.63 g. A sample was hydrolyzed in a sealed tube with 12 *N* HCl-dioxane (1:1) for 24 hr at 105°. The amino acid content of the hydrolysate was determined spectrophotometrically using the ninhydrin test as described by Yemm and Cocking, modified by Rosen.[25] The substituted resin was found to contain 1.018 mmoles of Phe/g.

Boc-β-benzylaspartylnitroarginylvalyl-*O*-benzyltyrosylvalyl-N^{im}-benzylhistidyl-*N*-methylalanylphenylalanyl Resin.—A portion of the Boc-Phe resin (3.0 g, contg a total of 3.05 mmoles of Boc-Phe) was introduced into a solid phase reaction vessel, and the following steps were used to introduce each new amino acid residue: (1) 3 washings with 40-ml portions of glacial AcOH; (2) cleavage of the Boc group by treatment with 1 *N* HCl in glacial AcOH (40 ml) for 30 min at room temp; (3) 3 washings with 40-ml portions of glacial AcOH; (4) 3 washings with 40-ml portions of abs EtOH; (5) 3 washings with 40-ml portions of DMF; (6) neutralization of the hydrochloride with 6 ml of TEA in 40 ml of DMF for 10 min; (7) 3 washings with 40-ml portions of DMF; (8) 3 washings with 40-ml portions of CH_2Cl_2, (9) addn of 10 mmoles of the appropriate Boc-amino acid in 20 ml of CH_2Cl_2 and mixing for 10 min; (10) addn of 10 mmoles of DCI in 20 ml of CH_2Cl_2, followed by a reaction period of 4 hr at room temp; (11) 3 washings with 40-ml portions of CH_2Cl_2; (12) 3 washings with 40-ml portions of abs EtOH.

For coupling of the Boc derivatives of N^{im}-benzylhistidine and nitroarginine step 8 was deleted, and DMF was used as the solvent in place of CH_2Cl_2 in steps 9–11. The reaction period for coupling of the Boc-N^{im}-benzylhistidine was increased to 16 hr.

The fully protected polypeptide resin was washed 3 times with 40-ml portions of DMF, followed by 3 washings with 40-ml portions of abs EtOH, and drying *in vacuo* over KOH pellets; yield, 6.20 g (theor yield: 6.57 g, 3.05 mmoles of peptide resin).

Following neutralization of the hydrochloride, the combined TEA soln (step 6) and DMF washings (step 7) of each cycle were evapd to dryness, the residue was dissolved in dil HNO_3, and the Cl^- content of the soln was detd by the Volhard method.[26,27]

(18) Proposed as an international standard by the Department of Biological Standards, National Institute for Medical Research, England.

(19) R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959).

(20) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *J. Amer. Chem. Soc.*, **88**, 3633 (1966).

(21) E. W. McChesney and W. K. Swann, Jr., *ibid.*, **59**, 1116 (1937).

(22) Fischer and Lipschitz[12] give mp 121.5–122.5°, $[\alpha]_D^{20}$ −6.6° (*c* 2.0, EtOH).

(23) Fischer and Lipschitz[12] obtained this product by treatment of Ts-Mal with concd HCl at 100° and neutralization of the resulting hydrochloride with PbO. They report $[\alpha]_D^{20}$ +5.5° (*c* 1.5, H_2O).

(24) R. K. Olsen, *J. Org. Chem.*, **35**, 1912 (1970).

(25) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955); H. Rosen, *Arch. Biochem. Biophys.*, **67**, 10 (1957); see also J. P. Greenstein and M. Winitz, Chemistry of the Amino Acids, Vol. 2, Wiley, New York, N. Y., 1961, p 1309.

(26) S. Visser, J. Roeloffs, K. E. T. Kerling, and E. Havinga, *Recl. Trav. Chim. Pays-Bas*, **87**, 559 (1968).

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[Val^5,Mal^7]-angiotensin II.—The protected octapeptide polymer was suspended in 40 ml of anhyd TFA, and a slow stream of Br_2-free HBr was passed through the fritted disk of the reaction vessel into the suspension at room temp, with exclusion of H_2O. After 10 min the suspension was filtered, and the resin was washed 3 times with 20-ml portions of TFA. The filtrates were evapd to dryness under reduced pressure at 25°. The product was redissolved in TFA (30 ml), and the soln was reevapd. Et_2O (250 ml) was added to the syrupy residue, and the ppt was collected, washed with Et_2O, and dried *in vacuo* over KOH pellets; yellow powder, 1.42 g. This crude, partially protected octapeptide was dissolved in MeOH–AcOH–H_2O (5:1:1) (200 ml) and hydrogenated at ordinary pressure over freshly prepared Pd black (1 g). After 24 hr an additional 1 g of catalyst was added, and the hydrogenation was contd for another 24 hr. The catalyst was filtered off with the aid of Celite, and the filtrate was concd to dryness *in vacuo*. The residue was dissolved in H_2O (30 ml), small amounts of insol material were removed by filtration, and the soln was passed through a column with anion-exchange resin AG1-X2 (acetate cycle) (dimensions: 2.0 × 10 cm). The column was eluted with H_2O until ninhydrin neg. The bulk of the solvent was removed *in vacuo* below 40°, and the remainder was lyophilized. After drying *in vacuo* over P_2O_5, 1.041 g of a crude [Val^5,Mal^7]-angiotensin II preparation was obtained; it showed two major Cl-pos impurities on tlc, one moving faster, the other slower than the main component. Amino acid ratios of this crude material in acid hydrolysate are: Asp, 1.09; Arg, 0.99; Val, 2.12; Tyr, 1.04; His, 0.80; Phe, 0.96.[28]

This lyophilized powder was dissolved in 4.5 ml of 0.2 *N* AcOH and subjected, in 3 approximately equal portions, to gel filtration on a Sephadex G25 (superfine) column (dimensions: 2.1 × 165 cm) that had been equilibrated with 0.2 *N* AcOH, and 150 fractions of 4.0 ml each were collected. A plot of the absorbances at 280 nm of the various fractions showed 1 major peak with the maximum at fraction 104 (with 2 minor peaks at fractions 120 and 132). Tlc (systems I and II) of samples showing an absorbance of more than 10% of that with maximum absorbance, using the Cl_2 test, located practically homogeneous peptide in fractions 99–106, with fractions 90–98 and 107–115 containing detectable amounts of the desired peptide besides slower- (fractions 90–99) and faster- (fractions 107–115) moving contaminants. A typical chromatogram, with 379 mg of the crude peptide applied, yielded 178 mg in the central portion. Rechromatography of fractions 99–106 in the same way gave a plot with a single symmetrical peak (maximum at fraction 106), and the homogeneous fractions from this second chromatogram (96–113) were pooled and lyophilized to give a white powder; yield, 141 mg, total yield 405 mg; $[\alpha]^{20}D$ −48.1° (*c* 0.6, H_2O); R_f^{I} 0.30; R_f^{II} 0.55; R_f^{III} 0.35; R_f^{1} 0.27; R_f^{2} 0.36 (2.1 × His); single Cl-, Sakaguchi-, and ninhydrin-pos spot; amino acid ratios in acid hydrolysate: Asp, 1.03; Arg, 1.06; Val, 2.00; Tyr, 0.99; His, 0.97; Phe, 0.95;[29] amino acid ratios in AP-M digest: Asp, 0.95; Arg, 1.05; Val, 2.05; Tyr, 1.00; Phe, 0.95.[30] *Anal.* ($C_{50}H_{73}N_{13}O_{11}\cdot CH_3COOH\cdot 2H_2O$) C, H, N.

(28) This result is in agreement with the values of the Volhard titrations which also indicate incomplete incorporation of His.

Discussion

The marked degree of pharmacological activity exhibited by [Val^5,Mal^7]-angiotensin II, which is reported in this investigation, constitutes evidence that Pro in position 7 of angiotensin is not essential for biological function. Our own results, together with those of previous investigations (compare Table II), lead us to conclude that an opened pyrrolidine ring of 7-Pro can also lead to pharmacological activity provided that the chemical nature of the *imide* N is not altered, as it is, *e.g.*, in the case of the biologically inactive 7-Ala analog; *i.e.*, all that seems to be required in position 7 for biological activity is an N-methylated amino acid. While N-methylation can block a potential H bonding site, alter the allowed conformations of the nearby backbone, or increase the likelihood of occurrence of cis peptide bonds, among other things, it is not possible, at this time, to determine which of these effects is responsible for the observed biological behavior.

While [Val^5]- and [Val^5,Mal^7]-angiotensin II are far from pharmacologically equivalent, consideration of Mal as a tool for exploring the importance of proline for biological function of certain peptides is nevertheless suggested.

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(29) Mal, present but not determined; the ninhydrin color yield of Mal (max 440 nm) is only approximately one-third that of Pro at its maximum (440 nm). Its presence was further proven by tlc of the acidic hydrolysate (system I) in which Mal was perfectly sepd from the slower moving Arg, Asp, and His, and the faster moving Val, Tyr, and Phe.

(30) AP-M does not hydrolyze the His–Mal bond. This residual dipeptide is eluted just before Arg, at a position distinctly different from both Mal and His.