acid, 1.0; proline, 1.1; glycine, 0.9; cystine, 1.0; isoleucine, 1.0; leucine, 2.0; and ammonia, 3.0.

N-Benzyloxycarbonylvalylisoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide.—A solution of 1.67 g of crystalline isoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide[24] in 20 ml of DMF was stirred at room temperature with 0.75 g of *p*-nitrophenyl N-benzyloxycarbonylvalinate[25] for 20 hr, 300 ml of EtOAc was added, and the slurry was cooled to −20°. The precipitate was filtered off and washed with 75 ml of EtOAc, two 75-ml portions of EtOH, and 75 ml of EtOAc. The powder was dried *in vacuo* to give 2.04 g of white powder, mp 252–253°, $[\alpha]^{16}D$ −53.9° (*c* 0.40, DMF). *Anal.* ($C_{51}H_{75}N_{11}O_{12}S$) C, H, N.

N-Benzyloxycarbonyl-S-benzylcysteinylvalylisoleucylglutaminylasparaginyl-S-benzylcysteinylprolylleucylglycinamide.—A solution of 0.85 g of the preceding protected octapeptide in 10 ml of AcOH was converted to the free octapeptide in the usual manner. The product was dissolved in 15 ml of DMF, 0.38 g of *p*-nitrophenyl N-benzyloxycarbonyl-S-benzylcysteinate[4] was added, the mixture was stirred for 20 hr at room temperature, and 150 ml of EtOAc was added. The slurry was cooled and the precipitate was filtered off, washed with EtOAc (50 ml), two 50-ml portions of EtOH, and 50 ml of EtOAc and dried *in vacuo* to give 0.85 g of white powder, mp 261.5–262.5°, $[\alpha]^{25}D$ −46.9° (*c* 0.49, DMF). *Anal.* ($C_{61}H_{86}N_{12}O_{13}S_2$) C, H, N.

[2-Valine]-oxytocin.—The preceding protected nonapeptide (252 mg) was converted to [2-valine]-oxytocin, and the resulting preparation was subjected to partition chromatography on a 2.85 × 54 cm column of Sephadex G-25 according to the procedures used for [2-leucine]-oxytocin. One hundred 9.6-ml fractions from the partition chromatography were collected, the fractions corresponding to the principal peak (R_f 0.24) (determined by plotting of the Folin–Lowry color values) were pooled, and 300 ml of H_2O was added. The mixture was concentrated to about 40 ml and the solution was lyophilized to give a pale yellow hygroscopic glass which was dissolved in 10 ml of 0.2 *N* AcOH and subjected to gel filtration on Sephadex G-25. One hundred 5-ml fractions were collected and the eluates corresponding to the major peak (fractions 48–57) were pooled, concentrated to 40 ml, and lyophilized to give 196 mg of a hygroscopic powder, which was again subjected to partition chromatography as before and worked up in the usual way to give 119 mg of a white powder. Subjection of this material to gel filtration as before afforded 104 mg of [2-valine]-oxytocin as a white powder, $[\alpha]^{25}D$ −33.5° (*c* 0.51, 1 *N* AcOH). *Anal.* ($C_{30}H_{66}N_{12}O_{11}S_2 \cdot C_2H_4O_2$) H, N; C: calcd, 49.0; found, 48.5.

The analog was hydrolyzed in 6 *N* HCl at 110° for 22 hr and the following molar ratios of amino acids and ammonia were found with glutamic acid taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.0; glycine, 1.0; cystine, 1.0; valine, 0.72; isoleucine, 0.72; leucine, 1.0; and ammonia, 2.7. A 45-hr hydrolysis under the same conditions gave the following results with glutamic acid taken as 1.0: aspartic acid, 1.0; glutamic acid, 1.0; proline, 1.1; glycine, 1.0; cystine, 1.05; valine, 0.9; isoleucine, 0.9; leucine, 1.0; and NH_3, 3.0. The prolonged hydrolysis was necessitated by the difficulty in the hydrolysis of a valyl-isoleucine peptide bond.

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(24) D. B. Hope and V. du Vigneaud, *J. Biol. Chem.*, **237**, 3146 (1962).

(25) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).

Angiotensin II Analogs. I. Synthesis and Biological Evaluation of [Gly[1],Gly[2],Ile[5]]-angiotensin II, [Ac-Gly[1],Gly[2],Ile[5]]-angiotensin II, and [Gly[1],Gly[2],Ile[5],His(Bzl)[6]]-angiotensin II[1]

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[Gly[1],Gly[2],Ile[5]]-angiotensin II has been synthesized by fragment condensation and also in better yield and purity by solid-phase synthesis. This peptide showed 16–20% of the pressor activity of [Asn[1],Val[5]]-angiotensin II in the rat and the dog, while its N-acetyl derivative showed an activity of 0.4%. These results show that only a single basic group is necessary in the N-terminal dipeptide for good pressor potency. This essential basic group may be correlated with either the terminal amino group or the guanido group of arginine in angiotensin II. The low activity of the acetylated peptide shows that extension of the peptide backbone from six to eight amino acids does not alone contribute measurably to the potency of natural angiotensin II. The synthetic intermediate [Gly[1],Gly[2],Ile[5],His(Bzl)[6]]-angiotensin II showed a pressor activity of 0.3% indicating the importance of the free imidazole ring.

The octapeptide tissue hormone angiotensin II, Asp-Arg-Val-Tyr-Ile- (or Val-) His-Pro-Phe,[2] has been the subject of numerous studies on the relationship between chemical structure and biological activity because of its possible relationship to hypertension. Most of this work has been reviewed by Law[3] and by Schröder and Lübke.[4] The portion of the molecule which has been most thoroughly studied is the N-terminal dipeptide, Asp-Arg-. The analogs described by Schwyzer,[5] Khosla, *et al.*,[6] and Arakawa, *et al.*,[7] listed in Table I show that none of the functional groups in this portion of the molecule is essential for significant pressor activ-

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(2) It is assumed in this paper that the bovine [Val[5]]- and equine [Ile[5]]-angiotensins have equivalent pressor activity.

(3) H. D. Law, *Progr. Med. Chem.*, **4**, 86 (1965).

(4) E. Schröder and K. Lübke, "The Peptides," Vol. 2, Academic Press Inc., New York, N. Y., 1966, Chapter 1.

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

(6) M. C. Khosla, R. R. Smeby, and F. M. Bumpus, *Biochemistry*, **6**, 754 (1968).

(7) K. Arakawa, R. R. Smeby and F. M. Bumpus, *J. Amer. Chem. Soc.*, **84**, 1424 (1962).

TABLE I
RELATIVE PRESSOR ACTIVITIES OF ANGIOTENSIN II ANALOGS IN THE RAT

Peptide	Pressor act., %
Asp-Arg-Val-Tyr-Val-His-Pro-Phe	100
Asn-Arg-Val-Tyr-Val-His-Pro-Phe	100[a]
Gly-Arg-Val-Tyr-Val-His-Pro-Phe	50[a]
Ile-Arg-Val-Tyr-Ile-His-Pro-Phe	40[b]
Desamino-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	60[c]
Arg-Val-Tyr-Val-His-Pro-Phe	50[a]
Asn-Arg(NO_2)-Val-Tyr-Val-His-Pro-Phe	50[a]
Asp-Orn-Val-Tyr-Val-His-Pro-Phe	20[a]
Val-Tyr-Val-His-Pro-Phe	0.1[a]

[a] See ref 5. [b] See ref 6. [c] See ref 7.

ity although each contributes to the intensity of action. These examples show that the carboxyl or amino groups of aspartic acid can be omitted entirely and the guanido group of arginine may be nitrated or replaced by a simple amino group without seriously reducing the pressor potency. However, the only analog reported in which arginine is replaced by an amino acid which does not have a side chain which may mimic one of the functional features of the guanido group (positive charge, hydrogen bonding) is [Asn[1],Val[2],Val[5]]-angiotensin II.[8] This analog has a pressor activity of 5% but it is not possible to say whether this is due solely to the loss of the functional group, an increase in hydrophobic character, or to the steric effect of β-branching of the valine side chain on the peptide backbone. Therefore, the functional importance of the arginine side chain requires further definition.

Despite the large number of analogs which have been synthesized and tested, it is still not clear exactly how the two N-terminal amino acids, aspartic acid and arginine, magnify the activity of the hexapeptide, Val-Tyr-Ile-His-Pro-Phe, from its low level (reported variously as <0.1–1%[5,7]) to the high level of the natural peptides. There are three obvious possibilities: (a) extension of the peptide backbone to eight residues stabilizes an "active" conformation; (b) the side chain functional groups themselves are responsible, perhaps by strengthening binding to a receptor or by regulating the partitioning or transport of the peptide; (c) a combination of both a and b. The data available in the literature are not sufficient to permit a choice between these possibilities. Arakawa, *et al.*,[7] suggested stabilization of conformation based upon the activities of N-Poly-[Ser(Ac)]-Val-Tyr-Ile-His-Pro-Phe (11%) and N-Poly-[Ser(Ac)]-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (41% of angiotensin II). Smeby, *et al.*,[9] have proposed a strained α-helix in which the amide bonds of aspartic acid and arginine are involved in the helix-stabilizing intramolecular hydrogen bonds. In order to help clarify the roles of these two amino acids we have synthesized [Gly[1],Gly[2],Ile[5]]-angiotensin II as a test of the importance of the aspartic acid and arginine side chains. [Ac-Gly[1],Gly[2],Ile[5]]-angiotensin II was prepared to test the contribution of the peptide backbone without a contribution from the terminal ammonium ion. The synthetic intermediate in which the imidazole nitrogen of histidine is protected by a benzyl group was also purified and evaluated as a test of the importance of the free imidazole ring.

Chemistry. A conventional fragment condensation was used in the initial synthesis of [Gly[1],Gly[2],Ile[5]]-angiotensin II. Purification by preparative electrophoresis of the product obtained by catalytic hydrogenation of the protected octapeptide gave a product containing only 36% of the expected amount of tyrosine. Since Z-Val-Tyr-N_3 was used in preparing the intermediate hexapeptide, the low tyrosine value was probably due to the presence of aza-1-homotyrosine resulting from a Curtius rearrangement of the azide to the isocyanate. This problem was encountered by Riniker[10] and it led him to prepare [Asn[1],aza-1-homo-Tyr[4],-Val[5]]-angiotensin II. Since Riniker's peptide had only 30% of the activity of angiotensin II, this preparation of [Gly[1],Gly[2],Ile[5]]-angiotensin II was unsuitable for accurate evaluation of the contribution of the two glycine residues to the biological activity. Rather than attempt to further purify the limited amount of material available, the pure analog was prepared in good yield by a modification of Merrifield's solid-phase method.[11]

In our modified procedure, the polymer was washed with triethylammonium acetate in AcOH after each coupling step to remove ionically bound Boc-amino acid which would otherwise interfere with the subsequent analysis. A 5–10-mg sample of polymer was removed, treated with HBr in trifluoroacetic acid for 30 min, and filtered, and the filtrate was concentrated then analyzed by paper electrophoresis. Due to differences in mobility, ninhydrin color, and specific color reactions it was possible to detect small amounts of unacylated amino component at each step, thus permitting the acylation to be repeated if necessary. Since acetylation was also carried out prior to deprotection to block trace amounts of unacylated amino components, reacylation was not necessary unless more than 5% of unacylated amine was present. In this peptide, reacylation was not necessary although traces of amino component were detectable at various stages. In related peptides repeated acylations have been required. The use of acetylation to block traces of amino components was originally used by Merrifield[11a] but it is not a part of the solid-phase method as now generally used. It has been found to be very useful in the present preparation of angiotensin analogs since the acetylated fragments, rather than free peptides, were much easier to remove from the product. The presence of N-acetylated peptide fragments, however, made it necessary to acetylate the purified peptide rather than to prepare the acetylated peptide, [Ac-Gly[1],Gly[2],Ile[5]]-angiotensin II, on the polymer. The incorporation of the acetylation step made mandatory protection of the hydroxyl group of tyrosine and the imidazole ring of histidine, whereas these groups were not protected in the conventional synthesis. Another variation from Merrifield's procedure was the incorporation of the readily available carbobenzyloxyglycylglycine in a single step. The carbobenzyloxy group rather than the usual *t*-butyloxycarbonyl group could be used since it was the last coupling. Either group would be removed at the same time that the peptide is cleaved from the polymer by HBr.

Cleavage from the polymer yielded the [Gly[1],Gly[2],-

(8) E. Schröder, *Justus Liebigs Ann. Chem.*, **680**, 132 (1964).

(9) R. R. Smeby, K. Arakawa, F. M. Bumpus, and M. M. Marsh, *Biochim. Biophys. Acta*, **58**, 550 (1962).

(10) B. Riniker, *Metabolism*, **13**, 1247 (1964).

(11) (a) R. B. Merrifield, *J. Amer. Chem. Soc.*, **85**, 2149 (1963); (b) R. B. Merrifield, *Biochemistry*, **3**, 1385 (1964); (c) G. R. Marshall and R. B. Merrifield, *ibid.*, **4**, 2394 (1965).

Ile[5],His(Bzl)[6]]-angiotensin II analog. A portion of this was hydrogenated to the free octapeptide. The peptides were purified by chromatography on sulfoethylcellulose. The desired fraction was lyophilized to remove most of the volatile buffer salts (ammonium acetate) then precipitated from solution with picric acid. The insoluble picrate salt was washed thoroughly with water to remove traces of salts and carbohydrates, then the picrate was exchanged for acetate with an anion-exchange resin.

Bioassay.—The compounds were tested for pressor activity in nephrectomized, pentolinium-treated male rats anesthetized with pentobarbital.[12] The same technique was used in the dog except that the animal was maintained on a respirator to give a more stable base line and samples were introduced through a cannula in the femoral vein. This dog preparation gave a much more reproducible response than did the rat. The compounds were compared with [Asn[1],Val[5]]-angiotensin II.[13] The results are listed in Table II.

TABLE II
RELATIVE PRESSOR ACTIVITIES OF ANGIOTENSIN II ANALOGS

Peptide	Pressor act., % Rat	Dog
Asn-Arg-Val-Tyr-Val-His-Pro-Phe[a]	100	100
Gly-Gly-Val-Tyr-Ile-His-Pro-Phe	16–20	16
Ac-Gly-Gly-Val-Tyr-Ile-His-Pro-Phe	...	0.4
Gly-Gly-Val-Tyr-Ile-His(Bzl)-Pro-Phe	0.3	...

[a] Hypertensin®.

Structure-Activity Relationships.—The significant enhancement in activity shown by Gly-Gly-Val-Tyr-Ile-His-Pro-Phe (16%) over the hexapeptide, Val-Tyr-Ile-His-Pro-Phe (<1%) appears at first to support a role of conformational stabilization of the peptide backbone by the N-terminal dipeptide, since the side-chain residues of aspartic acid and arginine are absent. However, this stabilization would be expected to take place primarily through hydrogen-bonding interactions of the amide linkages, a property retained by Ac-Gly-Gly-Val-Tyr-Ile-His-Pro-Phe, which showed activity of only 0.4%. It may therefore be concluded that, in the absence of the functional groups, the peptide linkages of the two N-terminal amino acid residues do not contribute significantly to biological activity by stabilization of a favorable backbone conformation. The feature present in Gly-Gly-Val-Tyr-Ile-His-Pro-Phe, and absent in its N-acetyl derivative, is the N-terminal amino group. This appears therefore to be the primary feature of the Gly-Gly- residue which activates the hexapeptide, Val-Tyr-Ile-His-Pro-Phe; however, the corresponding desamino compound should be evaluated to confirm this conclusion. This relationship does not exclude a possible important functional role for the basic side chain of arginine. As shown in Figure 1, the N-terminal amino group in Gly-Gly-Val-Tyr-Ile-His-Pro-Phe may function in place of either the terminal amino group, or the guanido group of angiotensin II, since the distance from the valine nitrogen is approximately the same in each case. Therefore, both the guanido group of the arginine side chain and the N-terminal amino group may be important for activity and may be interacting with either two receptors, or competing for a common receptor. Evidence for a primarily hydrogen-donating role in a hydrogen-bonding association for the arginine side chain has been presented by Havinga and Schattenkerk.[14] No such detailed study is available on functional variations of the N-terminal amino group. At present, it may only be concluded that a single basic group separated by five atoms from the valine nitrogen of Val-Tyr-Ile-His-Pro-Phe is sufficient to significantly enhance the pressor activity of the hexapeptide, and that such a function may be correlated with either the terminal amino group or basic side chain of arginine.

(12) R. Boucher, R. Veyrat, J. de Champlain, and J. Genest, *Can. Med. Assoc. J.*, **90**, 194 (1964).

(13) A generous gift of Ciba Pharmaceutical Company, Summit, N. J.

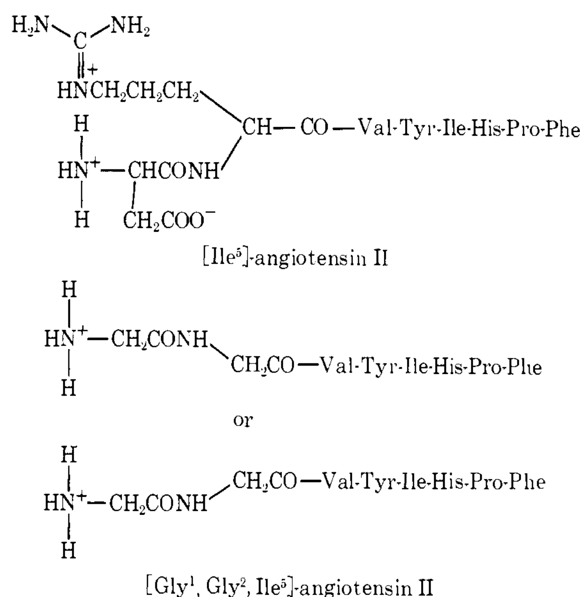

Figure 1.—Comparison of the terminal amino group in [Gly[1], Gly[2],Ile[5]]-angiotensin II with the guanido and amino groups in angiotensin II.

The low activity of Gly-Gly-Val-Tyr-Ile-His(Bzl)-Pro-Phe relative to the corresponding deprotected octapeptide suggests that the free hydrogen on the imidazole nitrogen is necessary for activity. Further analogs, however, should be studied to eliminate the possibility that the bulky benzyl group is exerting an unfavorable steric or hydrophobic effect.

Experimental Section[15]

Gly-Gly-Val-Tyr-Ile-His-Pro-Phe. Fragment Condensation.—Val-Tyr-Ile-His-Pro-Phe-OBzl(NO_2)·2HBr was prepared as described by Arakawa and Bumpus.[16] Intermediates had physical properties as described,[16,17] except that Z-His-Pro-Phe-OBzl(NO_2) prepared as described by Schwartz and Arakawa[17] in a noncrystalline state was obtained by trituration with cold EtOAc as a white powder, mp 139–140°, $[\alpha]^{26}D$ −42.8° (*c* 1,

(14) E. Havinga and C. Schattenkerk, *Tetrahedron, Suppl.*, **8**, 313 (1966).

(15) Melting points were measured in a Thomas–Hoover Uni-Melt apparatus and are uncorrected. Amino acid analyses were done on a Spinco 120B analyzer. Microanalyses were performed by the Microanalytical Department, University of California, Berkeley, Calif. Where analyses are indicated only by symbols of the elements, analytical results obtained were within ±0.4% of the theoretical values. Rotations were measured with a Rudolph photoelectric polarimeter. Silica Gel G (E. Merck) was used for tlc. The following solvent systems were used: I, 1-BuOH–HOAc–H_2O (4:1:5) (upper phase); II, 1-BuOH–HOAc–H_2O (3:1:1); III, BuOH–HOAc–pyridine–H_2O (15:3:10:12); IV, MeCOEt–HOAc–H_2O (14:6:5). Except where indicated, electrophoresis was carried out on Whatman No. 1 paper at 5000 V using HOAc–HCOOH buffer, pH 1.85, in a Savant apparatus. E_H indicates the electrophoretic mobility relative to histidine = 1.00.

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

(17) H. Schwartz and K. Arakawa, *ibid.*, **81**, 5691 (1959).

EtOAc), $R_{f\text{I}}$ 0.48, Pauly +, ninhydrin −. *Anal.* ($C_{35}H_{36}O_8N_6$) C, H, N. Ile-His-Pro-Phe-OBzl(NO_2)·2HBr, reported[16] previously as a mixed mono- and dihydrobromide, showed an opposite rotational sign from that reported: mp 140° dec, $[\alpha]^{27}D$ −26.5° (*c* 1, MeOH); lit.[16] mp 134–138°, $[\alpha]^{23}D$ +36.6 (*c* 1, MeOH).

Z-Gly-Gly[18] (93 mg, 0.35 mmole) and Et_3N (0.05 ml, 0.35 mmole) were dissolved in a mixture of 0.7 ml of THF and 0.2 ml of DMF. The solution was cooled to −5° and 0.033 ml (0.35 mmole) of ethyl chloroformate was added. This solution was kept at −5° for 10 min then added to a cold solution of 188 mg (0.175 mmole) of Val-Tyr-Ile-His-Pro-Phe-OBzl(NO_2)·2HBr and 0.080 ml (0.35 mmole) of tributylamine in 0.7 ml of DMF. This mixture was stirred at 0° for 2 hr and at room temperature overnight. The solvent was removed with a rotary evaporator and the residue was dissolved in EtOAc–MeOH (1:1) and washed with H_2O (50 ml), 1 *N* HCl (four 50-ml portions), H_2O (50 ml), saturated $NaHCO_3$ (four 50-ml portions), H_2O (two 50-ml portions), and saturated NaCl (50 ml). The EtOAc solution was dried ($MgSO_4$) and the solvent was removed *in vacuo* to yield 50 mg (25%) of an amorphous yellow solid, Z-Gly-Gly-Val-Tyr-Ile-His-Pro-Phe-OBzl(NO_2), tlc (one spot) $R_{f\text{I}}$ 0.43, Pauly +, ninhydrin −.

The protected octapeptide (25 mg) was dissolved in a mixture of 30 ml of MeOH, 3 ml of H_2O, and 15 ml of HOAc. The cloudy solution was clarified by filtration, 20 mg of 10% Pd–C was added, N_2 was bubbled through the suspension for a few minutes, then H_2 was bubbled through the vigorously stirred suspension, the reaction being followed by electrophoresis. After 15 min a large spot corresponding to the free acid appeared at E_H 0.3 (pH 3.5), Pauly +, ninhydrin −. The fully protected peptide did not migrate because of its insolubility at pH 3.5 and at pH 6.5. After 90 min the starting material and the spot at E_H 0.3 had disappeared. The product was at E_H 0.50 (pH 3.5) and at E_H 0.62 (pH 6.5), $R_{f\text{II}}$ 0.15, ninhydrin and Pauly +. There were numerous minor spots present. The mixture was filtered and the filtrate was evaporated *in vacuo* at 40°. The residue was lyophilized from 100 ml of H_2O giving 12.6 mg (65%) of a fluffy white powder. This was purified by preparative electrophoresis on Whatman No. 3MM paper at pH 3.5. The main fraction at E_H 0.51 was cut out, eluted with H_2O, and lyophilized to give 5.7 mg of a white powder. Electrophoresis at pH 6.5 showed a large spot at E_H 0.54 and a faint spot at E_H 0.17, both ninhydrin and Pauly +. A sample hydrolyzed for 24 hr at 110° in 5.5 *N* HCl in an evacuated, sealed tube showed amino acid ratios: Gly 2.07, Val 1.08, Tyr 0.36, Ile 0.94, His 0.92, Pro 1.03, Phe 1.00.

Solid-Phase Synthesis. Boc-Phe-polymer.—Chloromethylated copolystyrene–2% divinylbenzene[11] (100 g, 80 mequiv, of Cl) was suspended in 200 ml of EtOH containing 22.2 g (84 mmoles) of Boc-L-phenylalanine.[19] Et_3N (8.5 g, 84 mmoles) was added and the suspension was refluxed for 48 hr. The esterified resin was filtered off, washed with EtOH, and dried *in vacuo*. Amino acid analysis showed that the product contained 0.20 mmole of phenylalanine/g.

Z-Gly-Gly-Val-Tyr(Bzl)-Ile-His(Bzl)-Pro-Phe-polymer.—Boc-Phe-polymer (2.5 g, 0.5 mmole) was placed in the reaction vessel.[11] The resin was washed for 2 min each with three 30-ml portions of HOAc, then it was rocked for 30 min with 30 ml of 1.2 *N* HCl in HOAc to remove the Boc group. It was then washed with HOAc (three 30-ml portions), absolute EtOH (three 30-ml portions), and DMF (three 30-ml portions). The HCl was removed by rocking for 10 min with 30 ml of 10% Et_3N in DMF. The resin was washed with DMF (three 30-ml portions) and with CH_2Cl_2 (three 30-ml portions), then it was rocked with 2.0 mmoles of Boc-amino acid in 25 ml of CH_2Cl_2. After 10 min, a solution of 413 mg (2.0 mmoles) of DCCI (purified by sublimation) in 5 ml of CH_2Cl_2 was added and the mixture rocked for 2 hr. Excess reagents were removed by washing with CH_2Cl_2 (three 30-ml portions). A 10-mg sample was removed and washed with 5 ml of 10% Et_3N in HOAc to remove ionically bound Boc-amino acid. This sample was washed with HOAc (three 5-ml portions), then it was suspended in CF_3COOH. Anhydrous HBr was bubbled through the suspension for 30 min to liberate the peptide. The suspension was filtered, the filtrate was evaporated, and the liberated peptide was checked by paper electrophoresis to verify that the coupling reaction had exceeded 95%. The resin was rocked with Ac_2O (1 ml) and Et_3N (1 ml) in 30 ml of CH_2Cl_2 for 1 hr to acetylate unreacted amino groups. The resin was then washed with CH_2Cl_2 (two 30-ml portions). It was rocked for 10 min with 5 ml of EtOH in 25 ml of CH_2Cl_2, then was finally washed with EtOH (two 30-ml portions). This series of operations was carried out with Boc-L-proline, Boc-*im*-benzyl-L-histidine, Boc-L-isoleucine, Boc-O-benzyl-L-tyrosine, and Boc-L-valine. The same series was used with Z-Gly-Gly but because of its poor solubility in CH_2Cl_2 the coupling was carried out in purified[20] DMF using 3.0 mmoles to compensate for possible N-acylurea formation.

Gly-Gly-Val-Tyr-Ile-His(Bzl)-Pro-Phe.—The protected peptide resin was washed with HOAc (three 30-ml portions) and CF_3COOH (two 30-ml portions) and suspended in CF_3COOH (30 ml) and anisole (5 ml). Dry HBr was passed through towers of tetralin and $CaCl_2$ then bubbled through the fritted disk of the reaction vessel for 1 hr. The polymer was filtered off and washed with CF_3COOH (three 20-ml portions). The filtrate and washes were evaporated at 25° *in vacuo* and the residue was washed with anhydrous Et_2O (three 50-ml portions) then lyophilized from HOAc yielding 500 mg of an off-white powder. A 95-mg portion was dissolved in 8 ml of 1 *M* HOAc and centrifuged and the clear supernatant was applied to a 1.5 × 100 cm column of sulfoethylcellulose, NH_4^+ form, packed in 1 *M* HOAc (Cellex-SE, Bio-Rad Laboratories, Richmond, Calif.). The column was operated at 40°, and was eluted at 28 ml/hr with 1 *M* HOAc for 15 hr (420 ml). A linear gradient of NH_4OAc in 1 *M* HOAc was then started using a concentration change of 10^{-3} *M*/ml. The effluent was monitored at 280 mμ and 5-ml fractions were collected. The main peak was between 950 and 1020 ml. The contents of the tubes were pooled and evaporated at 40° to a sticky residue. The remaining H_2O and NH_4OAc was removed at 30° under high vacuum. The residue was dissolved in 98% HOAc and centrifuged, and the supernatant was lyophilized yielding 60 mg of an off-white powder. The powder was dissolved in 5 ml of H_2O and centrifuged and 2 ml of saturated aqueous picric acid was added to the supernatant. The precipitated picrate salt was washed with H_2O (three 5-ml portions), then suspended in 3 ml of 1 *M* HOAc. Two milliliters (sedimented volume) of AG 1-X2 (acetate) ion-exchange resin (Bio-Rad) was added and the mixture was stirred until all of the peptide picrate had decomposed. The almost colorless supernatant was passed through a 0.4 × 8 cm column of the same resin to remove last traces of picric acid. The resin was washed thoroughly with 1 *M* HOAc. The colorless solution was evaporated *in vacuo* at 40° and the residue was lyophilized from 98% HOAc giving 34 mg of white powder. A sample hydrolyzed for 72 hr at 110° in 5.5 *N* HCl had the following amino acid ratios: Gly 2.17, Val 1.03, Tyr 0.95, Ile 0.92, His 0.00, Pro 0.97, Phe 0.96. A 40-hr aminopeptidase-M[21,22] digest had Gly 1.98, Val 1.04, Tyr 1.00, Ile 0.90, His 0.00, Pro 0.02, Phe 0.95. Electrophoresis at pH 1.85 showed one spot, E_H 0.50; tlc showed one spot, $R_{f\text{III}}$ 0.82, and two spots, $R_{f\text{II}}$ 0.57 (very strong) and $R_{f\text{II}}$ 0.68 (faint). Spots were detected with ninhydrin, Pauly reagent, chlorination,[23] and 1-nitroso-2-naphthol.[24]

Gly-Gly-Val-Tyr-Ile-His-Pro-Phe.—To the benzylated peptide (400 mg) dissolved in 40 ml of 50% MeOH and 1 ml of HOAc was added 400 mg of 10% Pd–C. The mixture was stirred under 3 atm of H_2 for 90 hr at room temperature. The catalyst was filtered off (filter aid) and washed rapidly with 95% HOAc. The filtrate was evaporated *in vacuo* at 40° and the residue was lyophilized from 98% HOAc giving 300 mg of white powder. One milligram was hydrolyzed in 5.5 *N* HCl at 110° for 24 hr. No

(18) M. Bergmann and L. Zervas, *Chem. Ber.*, **65**, 1192 (1932).

(19) Boc-amino acids were prepared according to the methods of R. Schwyzer, P. Sieber, and H. Kappeler, *Helv. Chim. Acta*, **42**, 2622 (1959), or were purchased from Fox Chemical Co., Los Angeles, Calif. They were purified where necessary until homogeneous on tlc in *i*-Pr_2O–$CHCl_3$–HOAc (6:3:1) and in xylene–pyridine–HOAc (100:15:5).

(20) A. B. Thomas and E. G. Rochow, *J. Amer. Chem. Soc.*, **79**, 1843 (1957).

(21) K. Hofmann, F. M. Finn, M. Limetti, J. Montibeller, and G. Zanetti, *ibid.*, **88**, 3633 (1966).

(22) It was not possible to establish the steric purity of the histidine or proline residues with the samples of aminopeptidase-M used. Degradation of Hypertensin® (Ciba) under the same conditions gave similar results (Asn −, Arg 0.80, Val 2.08, Tyr 1.00, His 0.48, Pro 0.51, Phe 1.15). Further incubation caused an increase in free proline but a decrease in free histidine.

(23) D. E. Nitecki and J. W. Goodman, *Biochemistry*, **5**, 665 (1966).

(24) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. III, John Wiley and Sons, Inc., New York, N. Y., 1961, p 2353.

im-benzylhistidine was detectable in the hydrolysate by electrophoresis at pH 3.5.

This product (100 mg) was purified in the same way as described for the benzyl derivative. The flow rate was 28 ml/hr. The gradient was started after 18.5 hr (520 ml) and the main peak was eluted between 980 and 1040 ml. The combined fractions were lyophilized to give 65 mg. After purification *via* the picrate salt there was 45 mg of white powder. A sample hydrolyzed in 5.5 *N* HCl at 110° for 72 hr had the following amino acid composition: Gly 2.03, Val 1.03, Tyr 0.95, Ile 0.97, His 0.93, Pro 1.05, Phe 1.00. A sample hydrolyzed for 40 hr with aminopeptidase-M[21] had Gly 2.00, Val 1.10, Tyr 1.00, Ile 0.85, His 0.33, Pro 0.41, Phe 0.94. Electrophoresis at pH 1.85 showed one spot, E_H 0.57; tlc showed one spot, R_{fII} 0.48, R_{fIII} 0.72; positive reaction with ninhydrin, Pauly reagent, chlorination,[23] and 1-nitroso-2-naphthol.[24]

Ac-Gly-Gly-Val-Tyr-Ile-His-Pro-Phe.—To 15 mg of purified Gly-Gly-Val-Tyr-Ile-His-Pro-Phe in 2 ml of DMF was added 0.05 ml of Ac_2O and 0.05 ml of Et_3N. The solution was stirred for 2 hr at room temperature. A small amount of insoluble material was removed by centrifugation then 20 ml of Et_2O was added to the supernatant giving a voluminous white precipitate. The precipitated peptide was washed with Et_2O (two 20-ml portions) then dried *in vacuo* over KOH pellets at room temperature yielding 10 mg of an off white powder. Electrophoresis at pH 1.85 showed one spot, E_H 0.30, ninhydrin −, Pauly +. There was no detectable free peptide at E_H 0.57 under conditions where 1% could have been detected. A 5-mg portion of the peptide was dissolved in 0.2 ml of 0.1 *M* NaOH and kept at room temperature for 30 min to saponify any acetyl groups on tyrosine. The solution was neutralized with 0.2 ml of 0.1 *M* HCl giving a gelatinous precipitate. Normal saline containing 0.1% polyvinylpyrrolidone (20 ml) was added to prevent adsorption on glass. This solution was used directly for biological assay.

Synthesis and Microbiological Properties of Dipeptides Containing Cyclopentaneglycine and β-2-Thienylalanine[1a]

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Dipeptides synthesized included L-cyclopentaneglycyl-β-2-thienyl-L-alanine, β-2-thienyl-L-alanyl-L-cyclopentaneglycine, L-phenylalanyl-L-isoleucine, and glycyl-L-cyclopentaneglycine. Cyclopentaneglycine and β-2-thienylalanine may be viewed as structural analogs of isoleucine and phenylalanine, respectively. The effect of the dipeptides on the growth of three organisms, *Escherichia coli*, *Leuconostoc mesenteroides*, and *Lactobacillus arabinosus*, was studied. Under the test conditions, the peptides had greater growth-stimulating or growth-inhibiting effects than equivalent amounts of the corresponding free amino acids.

Peptides often display unique growth-stimulating effects in microorganisms.[2] Other peptides, containing amino acid analogs such as β-2-thienylalanine, may be more effective growth inhibitors than the free amino acid analog.[3] Where an organism was inhibited by a mixture of β-2-thienylalanine and one of its peptides, the inhibition was more effectively nullified by phenylalanine peptides than by free phenylalanine.[4]

Kihara and Snell[5] described the "double inhibition" of *Leuconostoc mesenteroides* by high levels of L-alanine and L-leucine. This "double inhibition" was reversed by the addition of a mixture of glycine and L-isoleucine and, more effectively, by a single dipeptide, glycyl-L-isoleucine. This concept of "double inhibition" suggested to us the preparation of a dipeptide containing two amino acid analogs in order to test whether such peptides would be more effective growth inhibitors than the constituent free amino acid analogs. The amino acid analogs chosen were cyclopentaneglycine[6] and β-2-thienylalanine,[7] structural analogs of isoleucine and phenylalanine, respectively.

This report describes the synthesis and microbiological properties of L-cyclopentaneglycyl-β-2-thienyl-L-alanine, β-2-thienyl-L-alanyl-L-cyclopentaneglycine, glycyl-L-cyclopentaneglycine, and L-phenylalanyl-L-isoleucine.

Experimental Section[8]

Peptide synthesis employed standard coupling and deprotection procedures.[9] The following compounds used in the present work were prepared earlier in this laboratory: L-cyclopentaneglycine, carbobenzoxy-β-2-thienyl-L-alanine, carbobenzoxy-L-phenylalanine, carbobenzoxy-L-isoleucine, β-2-thienyl-L-alanine methyl ester hydrochloride, L-phenylalanine methyl ester hydrochloride, L-isoleucine methyl ester hydrochloride, carbobenzoxyglycine, glycyl-L-isoleucine, glycyl-L-phenylalanine, glycyl-β-2-thienyl-DL-alanine, and L-isoleucyl-L-phenylalanine.

Carbobenzoxy-L-cyclopentaneglycine (I).—To a solution of 4.45 g of L-cyclopentaneglycine (31 mmoles) in an equivalent amount of 2 *N* NaOH were slowly added, with stirring at 0°, 6.39 g (36 mmoles) of carbobenzoxy chloride and 18.5 ml (37 mmoles) of 2 *N* NaOH, maintaining a pH of approximately 8. The product obtained upon acidifying the reaction mixture was purified by dissolving in ethyl acetate, extracting into 0.5 *M* $KHCO_3$, and reprecipitating with HCl; yield 6.8 g (85%), mp

(1) (a) Presented in part at the 48th annual meeting of the Federation of American Societies of Experimental Biology, Chicago, Ill., April 1964. This investigation was supported by U. S. Public Health Service Grant No. AI03710 from the National Institute of Allergy and Infectious Diseases. (b) Part of this work was taken from the M.S. thesis of Jim T. Hill, University of Tennessee, June 1964. (c) To whom requests for reprints should be sent: Department of Chemistry, Abilene Christian College, Abilene, Texas 79601.

(2) S. Shankman, S. Higa, H. A. Florsheim, Y. Schvo, and V. Gold, *Arch. Biochem. Biophys.*, **86**, 204 (1960).

(3) F. W. Dunn, J. M. Ravel, and W. Shive, *J. Biol. Chem.*, **219**, 809 (1956).

(4) F. W. Dunn, J. Humphreys, and W. Shive, *Arch. Biochem. Biophys.*, **71**, 475 (1957).

(5) H. Kihara and E. E. Snell, *J. Biol. Chem.*, **235**, 415 (1960).

(6) (a) W. M. Harding and W. Shive, *ibid.*, **206**, 401 (1954); (b) J. T. Hill and F. W. Dunn, *J. Org. Chem.*, **30**, 1321 (1965).

(7) V. DuVigneaud, H. McKennis, Jr., S. Simmonds, K. Dittmer, and G. Brown, *J. Biol. Chem.*, **159**, 385 (1945).

(8) All melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. The elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. Where analyses are indicated only by the symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values. The authors gratefully acknowledge the technical assistance of Miss Sansern Tangchai and Miss Pranee Limdhamarose in carrying out the microbiological assays.

(9) (a) M. Bergman and L. Zervas, *Ber.*, **65B**, 1192 (1932); (b) J. R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951); (c) D. Ben-Ishai, *J. Org. Chem.*, **19**, 62 (1954).