# *N,N'*-Carbonyldiimidazole in Peptide Synthesis. III.[1] A Synthesis of Isoleucine-5 Angiotensin II Amide-1

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The versatility of *N,N'*-carbonyldiimidazole as a peptide forming reagent was tested by synthesizing isoleucine-5 angiotensin II amide-1. The yields and purities of the products compare favorably with the literature except for the formation of the valyl-tyrosine bond.

The application of *N,N'*-carbonyldiimidazole to the synthesis of di- and tri-peptides has been described previously.[1] To become truly useful to the peptide chemist, however, the reagent must be capable of synthesizing larger molecules. A peptide that has been well studied and one which would be a good test for the reagent is equine angiotensin II amide-1. This compound was synthesized by Schwyzer and co-workers.[2] the Schwyzer synthesis[2] except, of course, using *N,N'*-carbonyldiimidazole as the peptide-forming reagent. The synthetic scheme is outlined in Fig. 1. A comparison of our yields to those of the literature is made in Table I for those reactions involving *N,N'*-carbonyldiimidazole. It should be noted that some of the compounds compared are similar but not identical. This is considered a valid comparison since the differences are only

TABLE I

PEPTIDES MADE USING *N,N'*-CARBONYLDIIMIDAZOLE: A COMPARISON TO THE LITERATURE

| Compound | % Yield[a] | M.P. | $[\alpha]_D$[b] |
|---|---|---|---|
| Z·asp($NH_2$)-arg($NO_2$)·OMe[c] (B 1–2) | 54 | 180–183 | +5.4 |
| *Z·asp($NH_2$)-arg($NO_2$)·OMe*[c,d] | 30[e] | 170–173 | +5 |
| B·val-tyr·OEt[c] (B 3–4) | 50 | 139.5–141 | −20 |
| *Z·val-tyr-·OMe*[c] | 81[f,g] | 144–147 | +54 |
| B·ileu-his·OMe[c] (B 5–6) | 81 | 168.5–170 | −34.4 |
| *Z·ileu-his·OMe*[c] | 78[f] | 160–172 | −34 |
| B·pro-phe·OMe[c] (B 7–8) | 72 | 74–76 | −53 |
| *Z·pro-phe·OMe* | ..[h] | 75–77 | −39 |
| Z·asp($NH_2$)-arg($NO_2$)-val-tyr·OEt (D 1–4) | 55 | 224–225 | −4.6 |
| *Z·asp($NH_2$)-arg($NO_2$)-val-tyr·OMe* | 33[f] | 202–206 | −4 |
| B·ileu-his-pro-phe·OMe (D 5–8) | 64 | 92–100 | −62.5 |
| *Z·ileu-his-pro-phe·OMe* | 68[i] | 105–110 | −56 |
| Z·asp($NH_2$)-arg($NO_2$)-val-tyr-ileu-his-pro-phe·OMe (F 1–8) | 45 | 221–222.5 | −37 |
| *Z·asp($NH_2$)-arg($NO_2$)-val-tyr-ileu-his-pro-phe·OMe* | 22 | 190–205 | −29 |

[a] Only purified yields are compared since crude yields have very little significance. [b] All rotations were run in the same solvent at the same concentration as the literature. [c] Crystalline. [d] Literature compounds are italicized. The values are taken from ref. 2 except where otherwise stated. [e] Diethylchlorophosphite method. [f] Dicyclohexylcarbodiimide method. [g] Also ref. 5. Z-val-tyr·OEt, 64%, m.p. 155–157°, $[\alpha]^{25}D$ +56°. [h] Mixed anhydride method. Ref. 9b. Ref. 2 obtained an oil. [i] Azide method.

Variations and analogs of angiotensin II amide-1 have also been made.[3-11] We decided to follow minor changes in the protecting groups. Of the four A→B reactions the first dipeptide, methyl carbobenzoxy-L-asparaginylnitro-L-argininate (B 1–2), was made in better yield and with a higher melting point than the literature[2] material. Ethyl *t*-butyloxycarbonyl-L-valyl-L-tyrosinate (B 3–4) presented considerable difficulty, and for a large scale preparation it was necessary to make ethyl car-

(1)(a) G. W. Anderson and R. Paul, *J. Am. Chem. Soc.*, **80**, 4423 (1958); (b) R. Paul and G. W. Anderson, *ibid.*, **82**, 4596 (1960).

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

(3) H. Schwarz, F. M. Bumpus, and I. H. Page, *J. Am. Chem. Soc.*, **79**, 5697 (1957).

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

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

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

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

(8)(a) R. Schwyzer, *Helv. Chim. Acta*, **44**, 667 (1961); (b) B. Riniker and R. Schwyzer, *ibid.*, **44**, 674, 677, 685 (1961).

(9)(a) R. Schwyzer and H. Turrian in "Vitamins and Hormones," Vol. 18, Academic Press, New York, N. Y., 1960, pp. 237–284; (b) R. Schwyzer and H. Turrian, *ibid.*, p. 268; (c) R. Schwyzer and H. Turrian, *ibid.*, p. 273.

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

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

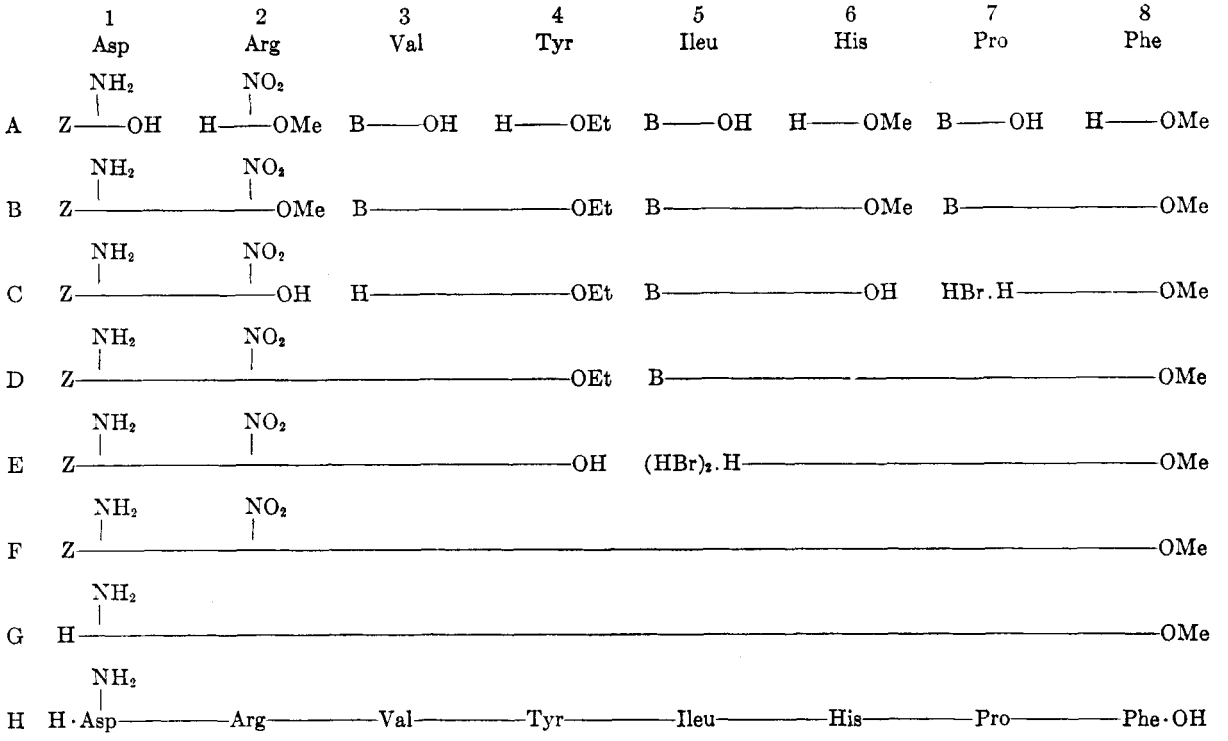


Fig. 1.—Scheme for the Synthesis of Angiotensin II Amide-1

TABLE II

KNOWN PEPTIDES MADE BY THE REMOVAL OF A PROTECTING GROUP

| Compound | Yield, % | M.P. | $[\alpha]_D$[a] | Literature Yield, % | Literature M.P. | Literature $[\alpha]_D$ |
|---|---|---|---|---|---|---|
| Z·asp($NH_2$)-arg($NO_2$)·OH·$2H_2O$ (C 1–2) | 81 | 107–110 | +7.4 | 71[b] | 98–101[f] | +10 |
| HBr·H·pro-phe·OMe (C 7–8) | 92 | 177–178.5 | −36.9 | 78[c,d] | 169.5–170.5 | −37 |
| Z·asp($NH_2$)-arg($NO_2$)-val-tyr·OH·$^1/_2H_2O$[e] (E 1–4) | 24 | 188–189.5 | −17.9 | 70[b,c] | 165–170 | 0 |
| $(HBr)_2$·H·ileu-his-pro-phe·OMe·$H_2O$[e] (E 5–8) | 93 | 159–168 | −4.8 | 92[b,c] | 130–140 | +18 |

[a] Rotations were run in the same solvent and at the same concentration as the reference. [b] Ref. 2. [c] Crude yield. [d] Ref. 25. [e] Amorphous. [f] Ref. 9b, m.p. 136–141°, $[\alpha]_D$ +7°.

bobenzoxy L-valyl-L-tyrosinate by the mixed anhydride method according to the procedure of Skeggs.[5] The yields of the other two dipeptides, B 5–6 and B 7–8, were satisfactory, and B 7–8 was found to be crystalline. The carbobenzoxy analog of B 7–8 was reported to be an oil,[2] which later crystallized.[9b]

The conversions B→C involving only the removal of protecting groups were routine. It was found that ethyl L-valyl-L-tyrosinate (C 3–4) was stable as the free base, undoubtedly due to the acidity of the phenolic group. Only C 5–6 was amorphous.

The next preparation, D 1–4, gave a crystalline product in significantly higher yield and melting point than that reported.[2] The second tetrapeptide, D 5–8, was obtained as an amorphous material in approximately the same yield as the literature.[2] The saponification of D 1–4 to E 1–4 proved to be a difficult operation, possibly due to the presence of the nitroarginine.[12] The yield was a third that of the literature[2] while the physical properties of the product were quite different (see Table II). It was noted that a sodium salt or complex of carbobenzoxy-L-asparaginylnitro-L-arginyl-L-valyl-L-tyrosine (E 1–4) was insoluble in water at room temperature. No attempt was made to identify it other than a sodium flame test and conversion to the free acid. This phenomenon has been described for carbobenzoxyphenylalanine.[13]

The compound E 5–8 was also quite different from the same compound described in the literature. Since the sign of rotation was the opposite of the literature compound, our ester was saponified and the free peptide digested with leucine amino-

(12) R. Paul, G. W. Anderson, and F. M. Callahan, *J. Org. Chem.*, **26**, 3347 (1961).

(13) W. Grassmann and E. Wünsch, *Ber.*, **91**, 462 (1958); M. Goodman and K. C. Stueben, *J. Org. Chem.*, **24**, 112 (1959).

peptidase.[14] Complete digestion was found showing the compound was all L. From the analysis cited in the literature[2] it is probable the authors have a hydrated peptide like ours, although they do not indicate it as such. The formation of F 1–8 went fairly well. The protected octapeptide was converted to the free octapeptide H 1–8 essentially by Schwyzer's[2] procedure, *i.e.*, removal of protecting groups, extraction, and countercurrent distribution. The angiotensin II amide-1 (H 1–8) thus produced was chromatographically pure. It had 20 times the pressor activity of epinephrine in rats. Leucine aminopeptidase completely hydrolyzed the peptide indicating it had the all L configuration.[14] A quantitative amino acid analysis of an acid hydrolysate was in close agreement with the theoretical value. A sample of H 1–8 was submitted to a second countercurrent distribution of 356 transfers in a different system. Only one peak was found. On paper chromatography of a concentrate of this peak, a sharper spot than before was seen, but no other changes appeared.

*N,N'*-Carbonyldiimidazole thus seems to be useful for the synthesis of larger peptides. In this study the yields are usually as good as or better than those reported with other reagents. The poor result of the reagent in forming ethyl *t*-butyloxycarbonyl-L-valyl-L-tyrosinate (B 3–4) is being investigated. The occurrence of dimorphic forms of B 3–4 is a complicating factor. Recently Ramachandran[15] has reported the isolation of an *O*-substituted product in the coupling of *p*-nitrophenyl carbobenzoxy-L-valinate and methyl L-tyrosinate. There is some evidence of small amounts of *O*-substituted by-product in our case.

In the amino acid analysis of angiotensin II amide-1 the presence of 1% leucine and 1% alloisoleucine was detected. Our synthesis involved extensive purification at each step. It may be assumed, therefore, that the starting isoleucine[16] contained higher percentages of these contaminants. It is interesting to note how much does remain after eight steps. Schwyzer[8a] has reported 7% alloisoleucine in commercial isoleucine. Another interesting point is that the isoleucine compounds are all amorphous except the dipeptide B 5–6 which is crystalline. Since that compound must be contaminated with isoleucine and alloisoleucine also, crystallinity obviously does not prove the purity in this particular case. On the other hand, the impurities are one factor in explaining the amorphous state of subsequent compounds.

(14) E. Smith and R. L. Hill in "The Enzymes," P. D. Boyer, H. Lardy, and K. Myrbäck, eds., Vol. 4, 2nd ed., Academic Press, New York, N. Y., 1960, pp. 37–62.

(15) J. Ramachandran, Abstracts of Papers Presented at Chicago, Ill., American Chemical Society Meeting, September 3–8, 1961, p. 49C. Schwyzer[4] ran the same reaction obtaining Z·val-tyr·OEt, m.p. 145–147, $[\alpha]^{33}D$ +34° (*c* 4, chloroform). Compare to Table I, footnote *g*.

(16) The isoleucine used was manufactured by Mann Research Laboratories and is the "allo-isoleucine free."

It also was concluded from this work that treatment of peptides with base gave poor yields and impure products. As has been shown before these steps are probably best avoided if possible.

It has been observed, both in this synthesis and in many cases in the literature, that frequently peptides are hydrated. This situation prevails especially when large peptides are involved. In these cases the carbon-hydrogen analyses are oftimes not sensitive enough to determine the exact amount of hydration. If an amino- or carboxy-protected peptide or amino acid is hydrated with an undetermined amount of water it presents a problem in the use of *N,N'*-carbonyldiimidazole since an excess of the reagent gives ureas with the amine portion. We have solved this problem in several cases by treating the acid portion with an excess of reagent, cooling the solution of the acyl imidazole to −5°, adding a small amount of water to destroy the excess reagent and then adding the amine.

## Experimental

All solvents used for *N,N'*-carbonyldimidazole reactions were pre-dried. Melting points were taken on a standardized Fisher-Johns block. Crystallinity was determined under an American Optical Co. polarizing microscope.

**Methyl Carbobenzoxy-L-asparaginylnitro-L-argininate (B 1–2).**—A solution of 26.6 g. (0.10 mole) of carbobenzoxy-L-asparagine[17] in 100 ml. of dimethylformamide was treated with 16.3 g. (0.10 mole, 100% purity) of *N,N'*-carbonyldiimidazole[1] at −20° for 6 hr. Meanwhile, 27.0 g. (0.10 mole) of methyl nitro-L-argininate hydrochloride[18] was dissolved in 100 ml. of hot methanol, cooled to room temperature and combined with a solution of 2.30 g. (0.10 g.-atom) of sodium in methanol. Ether was added and the precipitated salt filtered off. The filtrate was concentrated under vacuum, dissolved in 50 ml. of chloroform, and reconcentrated. The residue, after being held under high vacuum for 2 hr., was dissolved in 25 ml. of dimethylformamide and added to the reaction mixture from above. After a few minutes at −20°, the reaction mixture was permitted to warm to room temperature slowly and then to stand overnight. The solvent was removed at 60–70°/1 mm. and the residual oil heated with 1 l. of water. Cooling gave a solid. The supernatant was made more basic with sodium bicarbonate and the solid collected. It was triturated with *N* hydrochloric acid, washed with water, and dried to give 34.2 g. (71%) of material, m.p. 179–183°. Recrystallization from 1.5 l. of water gave 25.9 g. (54%) of a crystalline dipeptide, m.p. 180–183°, $[\alpha]^{25}D$ +5.4° (*c* 2, acetic acid); lit.,[2] m.p. 170–173°, $[\alpha]^{24}D$ +5° (*c* 2, acetic acid); yield, 30%.

*Anal.* Calcd. for $C_{19}H_{27}N_7O_8$: C, 47.40; H, 5.66; N, 20.37. Found: C, 47.75; H, 5.99; N, 20.22.

**Carbobenzoxy-L-asparaginylnitro-L-arginine (C 1–2).**—Methyl carbobenzoxy-L-asparaginylnitro-L-argininate (24.21 g., 0.0504 mole) was suspended in 55.0 ml. of *N* sodium hydroxide. The resulting slurry was shaken in a Narda ultrasonic generator, series 400, at 40 kc. for 30 min. When the vibration stopped the mixture solidified. It was diluted with water and unchanged starting material filtered of (6.3 g., m.p. 182.5–184°). Acidification of the filtrate gave on cooling 16.2 g. of material, m.p. 104–128°. Repetition of the reaction on the recovered material gave an additional 5.1 g., m.p. 110–125°. The combined products

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

(18) K. Hofmann, W. D. Peckham, and A. Rheiner, *J. Am. Chem. Soc.*, **83**, 728 (1961).

were recrystallized from 1.5 l. of acetonitrile, then from 500 ml. of water giving 19.6 g. (81%) of crystalline, free acid dihydrate, m.p. 107–110°; neut. equiv. calcd., 503, found, 500. A sample was dried at 100°/1 mm. for several hours. It became amorphous, m.p. 109–113°; neut. equiv. calcd., 467, found 466. $[\alpha]^{25}D$ +7.4° (*c* 2, methanol). Lit., m.p. 98–101°,[2] $[\alpha]^{22}D$ +10 ± 4° (*c* 1, methanol); yield, 71%; and m.p. 136–141°,[9b] $[\alpha]D$ +7°.

*Anal.* Calcd. for $C_{18}H_{25}N_7O_8$: C, 46.25; H, 5.39; N, 20.97. Found: C, 46.42; H, 5.58; N, 20.85.

**Ethyl *t*-Butyloxycarbonyl-L-valyl-L-tyrosinate (B 3–4).**—To a solution of 1.63 g. (0.0075 mole) of *t*-butyloxycarbonyl-L-valine[19] in 5 ml. of tetrahydrofuran was added 1.42 g. (0.0075 mole, 86% purity) of *N,N'*-carbonyldiimidazole. The solution was permitted to stand for 30 min. after the effervescence stopped. Then 1.88 g. (0.0090 mole) of ethyl L-tyrosinate[20] was added. After 24 hr. the solvent was removed under vacuum and the residue taken up in 50 ml. of ether. The ethereal solution was washed with 30 ml. of *N* sulfuric acid, 25 ml. of saturated aqueous sodium bicarbonate solution, and 25 ml. of water. Drying the organic layer over anhydrous sodium sulfate and concentrating it under an air stream gave 3.59 g. of part-solid, part-gummy material. This was boiled with 65 ml. of carbon tetrachloride; an insoluble fraction A, 0.36 g., m.p. 136–139°, was collected. Cooling the carbon tetrachloride solution gave 1.87 g. of a second fraction B, m.p. 134–135.5°. Concentration of the filtrate to dryness gave an oil which solidified on boiling with petroleum ether to a third fraction C, 0.47 g., m.p. 62–68° (foam).

Fractions A and B (72% yield) were combined and recrystallized from ethyl acetate–petroleum ether giving 1.53 g. (50%) of crystalline dipeptide, m.p. 139.5–141°, $[\alpha]^{25}D$ −20.0 ± 1.25° (*c* 2, ethanol).

*Anal.* Calcd. for $C_{21}H_{32}N_2O_6$: C, 61.74; H, 7.90; N, 6.86. Found: C, 61.98; H, 8.23; N, 6.98.

In six other runs the yields varied from 4 to 54%. In several cases fraction A melted at 151–152°, $[\alpha]^{25}D$ −18.8° (*c* 2, ethanol). Infrared spectra, run in solution, were identical for A and B. A mixed melting point of equal parts A and B merely ground together melted at 151–152°. The evidence proves that these are dimorphic forms of B 3–4.

Fraction C has not been obtained in pure form so far. Preliminary studies of the ultraviolet spectrum in neutral and basic solution suggest *O*-acylation of the tyrosine.[15] This matter is under investigation.

**Ethyl L-Valyl-L-tyrosinate (C 3–4).**—To 4.08 g. (0.01 mole) of ethyl *t*-butyloxycarbonyl-L-valyl-L-tyrosinate was added 10 ml. of a saturated solution of hydrogen bromide in acetic acid. After the effervescence had stopped and a solution had formed, the tan liquid was poured into 750 ml. of ether. An oil formed from which the supernatant was decanted. Methylene chloride (150 ml.) was added and ammonia bubbled through until the oil dissolved. A precipitate of ammonium bromide was filtered off. The addition of petroleum ether gave 2.50 g. of free base. An additional 0.19 g., m.p. 110–112.5° was recovered from the mother liquors. Recrystallization from methylene chloride–petroleum ether gave 2.68 g. (87%) of crystalline product m.p. 113.5–114.5°.

*Anal.* Calcd. for $C_{16}H_{24}N_2O_4$: C, 62.31; H, 7.85; N, 9.09. Found: C, 62.35; H, 8.06; N, 9.13.

**Alternate Method.**—Ethyl carbobenzoxy-L-valyl-L-tyrosinate[4,5] (4.43 g., 0.01 mole) was dissolved in 75 ml. of ethanol and 10 ml. of *N* hydrochloric acid and 0.5 g. of 10% palladium-on-carbon added. Hydrogen was passed through the solution at atmospheric pressure until the carbon dioxide evolution stopped (1 hr.) and then an additional 3 hr. The catalyst was filtered off and the filtrate concentrated under vacuum. The residual gum was suspended in 100 ml. of methylene chloride and anhydrous ammonia bubbled through until the gum disappeared. A little anhydrous sodium sulfate was added to aid crystallization of the ammonium chloride and all the salts were filtered off. The addition of petroleum ether to the filtrate caused the dipeptide ester to crystallize giving 2.72 g. (88%), m.p. 114–115.5°, $[\alpha]^{25}D$ +22.4° (*c* 2, *N* hydrochloric acid).

**Ethyl Carbobenzoxy-L-asparaginylnitro-L-arginyl-L-tyrosinate (D 1–4).**—A solution of 10.9 g. (0.0233 mole) of anhydrous carbobenzoxy L-asparaginylnitro-L-arginine in 50 ml. of dimethylformamide was cooled to −15° and 3.98 g. (0.0233 mole, 94.5% pure) of *N,N'*-carbonyldiimidazole was added with stirring. After 45 min. the reagent had dissolved and 7.18 g. (0.0233 mole) of ethyl L-valyl-L-tyrosinate was added. The solution was permitted to warm to room temperature slowly and to stand overnight. It was then concentrated under high vacuum with warming. Trituration of the residue with 5% aqueous sodium bicarbonate, *N* hydrochloric acid, and water gave an amorphous solid. A hot solution of the solid in 2.5 l. of 2-propanol was cooled to give 10.9 g. of a gel, m.p. 197–203°. This was crystallized from 210 ml. of hot acetic acid to give 10.4 g. (55%) of tetrapeptide, m.p. 224–225° when placed on the block at 195° with a temperature rise of 2°/min. $[\alpha]^{25}D$ −4.6 ± 2.5° (*c* 1, dimethylformamide).

*Anal.* Calcd. for $C_{34}H_{47}N_9O_{11}$: C, 53.89; H, 6.25; N, 16.64. Found: C, 53.64; H, 6.62; N, 16.34.

**Carbobenzoxy-L-asparaginylnitro-L-arginyl-L-valyl-L-tyrosine (E 1–4).**—Using the method of Schwyzer[2] a 24% yield of tetrapeptide acid was obtained m.p. 188–189.5°, $[\alpha]^{25}D$ −17.9° (*c* 0.56, methanol); lit.,[2] yield 70%, m.p. 165–170°, $[\alpha]^{23}D$ 0 ± 4° (*c* 4, methanol). A sample for analysis was dried under vacuum at 100° for 3 min. and at room temperature for several hours; neut. equiv. calcd. for hemihydrate, 739, found, 757 ± 38.

*Anal.* Calcd. for $C_{23}H_{43}N_9O_{11}\cdot{}^1/_2H_2O$: C, 52.03; H, 6.00; N, 17.06; $H_2O$, 1.22. Found: C, 52.01; H, 6.23; N, 17.04; $H_2O$, 1.69.

**Methyl *t*-Butyloxycarbonyl-L-isoleucyl-L-histidinate (B 5–6).**—Using the Fischer method[21] two solutions were prepared, one of 2.3 g. (0.10 g.-atom) of sodium in methanol and another of 12.1 g. (0.05 mole) of methyl L-histidinate dihydrochloride[22] in 125 ml. of hot methanol. The hydrochloride solution was quickly chilled and the basic solution added before crystallization occurred. After filtering off 4.8 g. of salt, the filtrate was concentrated under vacuum. The residue was taken up in chloroform, 3.1 g. of brown material filtered off, and the filtrate taken to dryness under vacuum leaving an oil.

A solution of 9.60 g. (0.04 mole) of *t*-butyloxycarbonyl-L-isoleucine[19] hemihydrate in 40 ml. of dry tetrahydrofuran was treated with 11.04 g. (0.06 mole, 88% purity) of *N,N'*-carbonyldiimidazole. After 30 min. the methyl L-histidinate was added and the solution warmed slightly. It was permitted to stand overnight whereupon the solvent was removed under vacuum. The residue was treated with water causing the product to crystallize; yield 15.5 g., m.p. 162–165°. Recrystallization from 40% methanol–water gave 12.3 g. (81%) of long needles, m.p. 168.5–170°, $[\alpha]^{25}D$ −34.4° (*c* 1, methanol–*N* hydrochloric acid, 1:1).

*Anal.* Calcd. for $C_{18}H_{30}N_4O_5$: C, 56.53; H, 7.91; N, 14.65. Found: C, 56.48; H, 8.10; N, 14.57.

***t*-Butyloxycarbonyl-L-isoleucyl-L-histidine (C 5–6).**—An 18.77-g. (0.0492 mole) sample of methyl *t*-butyloxycarbonyl-L-isoleucyl-L-histidinate was dissolved in 75 ml. of methanol and 55 ml. of *N* sodium hydroxide was added. After 30 min. the solution was poured through an Amberlite IRC-50 (acid form) column of 105 ml. volume, at 8 ml./min. The column was washed with 800 ml. of water. The effluent was concentrated under vacuum. The residual material was taken up in tetrahydrofuran (50 ml.) and

(19) G. W. Anderson and A. C. McGregor, *J. Am. Chem. Soc.*, **79**, 6180 (1957).

(20) E. Fischer, *Ber.*, **34**, 433 (1901).

(21) E. Fischer and L. H. Cone, *Ann.*, **363**, 107 (1908); R. B. Merrifield and D. W. Wooley, *J. Am. Chem. Soc.*, **78**, 4646 (1956).

(22) V. du Vigneaud and O. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

poured into 1 l. of anhydrous ether. Trituration of the resulting gum gave an amorphous solid, weighing 14.92 g. (79% yield of monohydrate[23]) m.p. 137–145°; $[\alpha]^{25}D$ −11.5 ± 1.2° (*c* 2, water). A sample was dried at 100°/0.5 mm. for analysis; neut. equiv. calcd., 377, found, 369.

*Anal.* Calcd. for $C_{17}H_{28}N_4O_5 \cdot {}^1/_2H_2O$: C, 54.09, H, 7.74; N, 14.85. Found: C, 54.30; H, 7.67; N, 14.91.

**Methyl *t*-Butyloxycarbonyl-L-propyl-L-phenylalaninate (B 7–8).**—*t*-Butyloxycarbonyl-L-proline[19] (10.75 g., 0.05 mole) was dissolved in 50 ml. of tetrahydrofuran and 8.9 g. (0.05 mole, 91% pure) *N,N'*-carbonyldiimidazole was added. After the effervescence subsided, the solution was stirred for 1 hr. Then 8.95 g. (0.05 mole) of freshly distilled methyl L-phenylalaninate[24] was added. After the solution had remained at room temperature overnight, the solvent was removed under vacuum. The oily residue was taken up in 300 ml. of ether and washed with aqueous sodium bicarbonate solution, *N* hydrochloric acid, and water. The organic layer was dried over anhydrous sodium sulfate and then concentrated under high vacuum. The residue crystallized, giving 18.28 g. (98%), m.p. 72–75°. Recrystallization from isopropyl ether gave 13.46 g. (72%) of dipeptide derivative, m.p. 74–76°, $[\alpha]^{25}D$ −53° (*c* 1, chloroform).

*Anal.* Calcd. for $C_{20}H_{28}N_2O_5$: C, 63.81; H, 7.50; N 7.44. Found: C, 63.96; H, 7.75; N, 7.65.

**Methyl L-Propyl-L-phenylalaninate Hydrobromide (C 7–8).**—To a saturated solution of hydrogen bromide in 60 ml. of acetic acid was added 14.6 g. (0.0388 mole) of methyl *t*-butyloxycarbonyl L-propyl-L-phenylalaninate. The foaming was over in about 1 min., and the tan solution was permitted to stand for 2 or 3 min. more. Ether (900 ml.) was added and the solution cooled. A 12.8-g. (92%) yield of crystalline hydrobromide, m.p. 178–180°, was obtained. Recrystallization from methanol–ether gave 12.75 g. (92%) of peptide, m.p. 177–178.5°, $[\alpha]^{25}D$ −36.9° (*c* 3, water); lit.,[25] m.p. 169.5–170.5°, $[\alpha]^{19}D$ −37 ± 1° (*c* 2, water).

*Anal.* Calcd. for $C_{15}H_{21}N_2O_3Br$: C, 50.42; H, 5.92; N, 7.84; Br, 22.37. Found: C, 50.27; H, 6.07; N, 7.81; Br, 22.40.

**Methyl *t*-Butyloxycarbonyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalaninate (D 5–8).**—A weighed flask containing 16.80 g. (0.0435 mole) of *t*-butyloxycarbonyl-L-isoleucyl-L-histidine monohydrate was heated on a steam cone at 0.5 mm. After 45 min. the powder stopped spraying around and 0.50 g. of water (theory 0.40 g. for 0.5 mole) had been lost. The hemihydrate thus formed was dissolved with warming in 40 ml. of dimethylformamide. The solution was cooled to −40° and 10.58 g. (0.065 mole) of *N,N'*-carbonyldiimidazole was added. The mixture was kept between −20° and −15° for 4 hr. With the aid of stirring, the reagent dissolved during the first hour.

During this time 15.5 g. (0.0435 mole) of methyl L-prolyl-L-phenylalaninate hydrobromide was dissolved in 750 ml. of methylene chloride and ammonia was bubbled through the solution for 10–15 min. The ammonium bromide was filtered off and the filtrate concentrated under vacuum. The crystalline residue was taken up in 25 ml. of tetrahydrofuran and added to the reaction solution at −40°. The solution was slowly allowed to warm to room temperature and then to stand overnight. The solvent was removed at 60°/0.5 mm., leaving a tan gum. This was taken up in chloroform and washed with water. The organic layer was dried over anhydrous sodium sulfate and concentrated under an air stream. The oil thus obtained was reprecipitated with trituration from carbon tetrachloride–petroleum ether to give 23.0 g. (82%) of a violet amorphous solid, m.p. 56–95° (foaming). The solid was dissolved in 300 ml. of chloroform and placed on a column containing 400 g. of alumina. The alumina had been acid washed and dried at 130° for 2 days. The column was eluted with 1.25 l. each of chloroform, chloroform–0.5% methanol, chloroform–1% methanol, then 0.5 l. each of 1.5 and 2%, and finally 250 ml. each of 3, 5, and 100% methanol. Twenty-three fractions of 250 ml. were taken. On evaporation to dryness fractions 6–23 were combined and reprecipitated from carbon tetrachloride–petroleum ether to give 17.8 g. (64%) of amorphous, slightly pink tetrapeptide hemihydrate, m.p. 92–100°, $[\alpha]^{25}D$ −62.5 ± 2.4° (*c* 1, methanol).

*Anal.* Calcd. for $C_{32}H_{46}N_6O_7 \cdot {}^1/_2H_2O$: C, 60.45; H, 7.45; N, 13.22. Found: C, 60.11; H, 7.50; N, 13.45.

**Methyl L-Isoleucyl-L-histidyl-L-prolyl-L-phenylalaninate Dihydrobromide (E 5–8).**—A 17.8-g. (0.028 mole) sample of methyl *t*-butyloxycarbonyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalaninate hemihydrate was treated for 0.5 hr. with 75 ml. of acetic acid saturated with hydrogen bromide. Ether (1 l.) was added and the resulting gum triturated until solid. It was collected giving 20.5 g., m.p. 156–165°. Reprecipitation from methanol–ether followed by trituration gave an amorphous solid, weight 18.0 g. (93%), m.p. 159–168° (foaming), $[\alpha]^{25}D$ −4.8 ± 2.4° (*c* 1, methanol). The literature reports[2] m.p. 130–140°, $[\alpha]^{22}D$ +18 ± 4° (*c* 1, methanol). A sample for analysis was dried at 100°/0.1 mm.

*Anal.* Calcd. for $C_{27}H_{40}N_6O_5Br_2 \cdot H_2O$: C, 45.90; H, 6.00; N, 11.90; Br, 22.62. Found: C, 46.11; H, 6.06; N, 12.16; Br, 22.77.

**Methyl Carbobenzoxy-L-asparaginylnitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalaninate (F 1–8).**—Carbobenzoxy-L-asparaginylnitro-L-arginyl-L-valyl-L-tyrosine hemihydrate (2.217 g., 3.00 mmoles) was taken up in 15 ml. of dimethylformamide, cooled to −15 to 10° and treated with 0.773 g. (4.50 mmoles, 94.5% pure) of *N,N'*-carbonyldiimidazole. The temperature was maintained while the mixture stood for 2.5 hr. Meanwhile, 3.579 g. (5.00 mmoles) of methyl L-isoleucyl-L-histidyl-L-prolyl-L-phenylalaninate dihydrobromide hydrate was dissolved in 20 ml. of water and washed with 20 ml. of ethyl acetate. The organic layer was discarded. The aqueous layer was made basic with 20 ml. of saturated aqueous sodium carbonate solution and extracted with four 30-ml. portions of chloroform. The organic layer was washed with 20 ml. of saturated aqueous sodium carbonate solution and 20 ml. of saturated aqueous sodium sulfate solution. It was dried over anhydrous sodium sulfate and concentrated under high vacuum for several hours. The amine was then dissolved in 10 ml. of dimethylformamide and added to the reaction solution. The mixture was permitted to warm to room temperature slowly, then to stand overnight. Removal of the solvent at 60°/1 mm. and trituration of the residue with water gave on drying 4.68 g. (3.71 theory) of material, m.p. 160–172°. Some of the dimethylformamide apparently had not been removed. The product was taken up in hot methanol and cooled to give a gel. This process was repeated a second time giving a gel again. The product was boiled with chloroform and the insoluble part collected from the hot solution. It was washed with ether and dried to give 1.672 g. (45%), m.p. 221–222.5° when placed on the block at 215° with a temperature increment of 1°/min., $[\alpha]^{25}D$ −37° (*c* 0.675, dimethylformamide); lit.,[2] yield 22%, m.p. 190–205°, $[\alpha]^{23}D$ −29 ± 4° (*c* 0.52, dimethylformamide). A sample for analysis was dried at 65°/1 mm.

*Anal.* Calcd. for $C_{59}H_{79}N_{15}O_{15} \cdot 2H_2O$: C, 55.62; H, 6.57; N, 16.49; $H_2O$, 2.89; $OCH_3$, 1.18. Found: C, 55.99; H, 6.65; N, 16.63; $H_2O$, 3.34; $OCH_3$, 1.64.

**Angiotensin II Amide-1 (H 1–8).**—The protected octapeptide F 1–8 (1.04 g., 0.82 mmole) was reduced and saponified by the method of Schwyzer.[2] The disappearance of the nitro group was followed in the ultraviolet spectrum at 270 mμ. The crude angiotensin was purified by a 193 transfer countercurrent distribution in the system *n*-butyl

(23) Calculated from weight loss on drying.

(24) Prepared by treating a methylene chloride suspension of methyl L-phenylalaninate hydrochloride [R. A. Boissonnas, St. Guttmann, P. A. Jaquenoud, and J. O. Waller, *Helv. Chim. Acta*, **39**, 1421 (1956)] with ammonia. The product was purified by distillation, b.p. 82–83°/0.4 mm., $n^{20}D$ 1.5198.

(25) E. Bricas and C. Nicot-Gutton, *Bull. soc. chim. France*, 466 (1960).

alcohol–ethanol–0.01 *M* ammonium acetate 4:1:5. The product was located by spot testing every fifth tube with Pauly reagent. This was further checked by examining the ultraviolet absorption of every fifteenth tube at 280 mμ (tyrosine absorption). A single peak was found by both methods at tubes 113–132. Concentration to a small volume, followed by lyophilization gave 104 mg. (11% based on fully protected octapeptide) of an amorphous white powder. The product gave a single spot $R_f$ 0.49 and $\frac{R_f \text{ ang.}}{R_f \text{ H·pro·OH}} = 1.36$ in *n*-butyl alcohol–acetic acid–water (BAW) 4:1:5 using ninhydrin or Pauly reagent. In a separate study the above countercurrent system gave a 25% recovery of angiotensin II amide-1 from a pure sample placed in it.

In an attempt to prove further the purity of the compound, it was distributed for 356 transfers in the system 2-butanol–water, 1:1. Every fifth tube was spot tested with Pauly reagent. Only one Pauly positive peak (tubes 108–113) was found. An 18% recovery of material was obtained. This gave on paper chromatography (BAW) a single Pauly-reagent, ninhydrin- and Sakaguchi-reagent positive spot, $R_f$ 0.48, and in 2-butanol–3% ammonia 3:1, one Pauly positive spot $R_f$ 0.51. In an attempt to locate the rest of the material the contents of the tubes before and after the product tubes were concentrated and examined chromatographically. Only traces of Pauly reagent positive spots were found. This leads to the speculation that the extensive decomposition that occurred involved in some fashion the imidazole ring of the histidine.[26] The once distributed material was tested for pressor activity in rats[27] and found to be 20 times as active as epinephrine.

A quantitative amino acid analysis[28] on an acid hydrolysate gave asp—1.00, arg (plus orn of 0.04)—0.94, val—1.05, tyr—0.83, ileu—0.97, alloilei—0.01, leu—0.01, his—0.94, pro—1.00, phe—1.03, $NH_3$—1.31. The excess ammonia may be accounted for by the decomposition of arginine and tyrosine[29] ($2 \times 0.04 + 0.17 = 0.25$). The alloisoleucine and leucine must have been present in the starting isoleucine.[16]

Leucine aminopeptidase[30] completely hydrolyzed the octapeptide indicating that all the amino acids were in the L-configuration. The material had $[\alpha]^{25}D$ $-57 \pm 8°$ (*c* 0.29, water), lit.,[2] $[\alpha]^{22}D$ $-44 \pm 6°$ (*c* 0.56, water), and more recently[9c] $[\alpha]D$ $-59.3°$ (water) as a monoacetate monohydrate which we assume ours is.

**Ethyl Carbobenzoxyglycyl-L-tyrosinate.**—*N,N'*-Carbonyldiimidazole (2.45 g., 0.013 mole; 86% purity) was added to a solution of 2.09 g. (0.01 mole) of carbobenzoxyglycine[31] in dry tetrahydrofuran. After standing at room temperature for 30 min., the soluion was cooled to −5° in a salt-ice bath and 0.5 ml. of water (0.028 mole) was added. Several minutes later 3.14 g. (0.015 mole) of ethyl L-tyrosinate[20] was added and the solution slowly warmed to room temperature, where it remained overnight. The solvent was removed by an air stream and the residue triturated with *N* hydrochloric acid, water, 5% aqueous sodium bicarbonate, and water. It was cooled to 0°. Crystallization occurred in 1 hr., and the product was collected and dried to give 4.27 g., m.p. 111–113°. Recrystallization from 60% aqueous ethanol gave 3.04 g. (76%) m.p. 126.5–127°, compared with a 95% yield, m.p. 127–128°, by the anhydrous procedure with an equivalent of reagent.[1b]

Other experiments of a similar nature gave:

| | —Dry Reaction[1b]— | | —Water Added— | |
|---|---|---|---|---|
| Compound | % Yield | M.P. | % Yield | M.P. |
| Z·gly-phe·OEt(DL) | 83 | 90–91° | 74 | 91–92° |
| Z·ala-gly·OEt (L) | 65 | 98–99° | 72 | 100.5–101.5° |
| B·phe-gly·OEt(L) | 78 | 89–91.5° | 74 | 88–90° |

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(26) Riniker[8b] describes a solvolytic cleavage of asparagine from Val[5] angiotensin amide-1. This is probably not pertinent here since the imidazole ring would not be involved.

(27) We wish to thank Dr. J. R. Cummings and Mr. H. Falk of our Experimental Pharmacology Section for carrying out this assay.

(28) We thank Prof. M. Brenner and Dr. R. Weber of the Organic Chemistry Institute, University of Basel, for the amino acid analysis.

(29) It is interesting to note that tyrosine decomposition in the presence of aspartic acid during acid hydrolysis confirms an observation made by R. G. Shepherd, P. H. Bell, *et al.*, *J. Am. Chem. Soc.*, **78**, 5067 (1956), ref. 39.

(30) We are indebted to Prof. K. Hofmann of the University of Pittsburgh for a sample of leucine aminopeptidase. A recent report by Guttmann[16] of poor optical specificity with leucine aminopeptidase caused us to check our sample. It hydrolyzed glycyl-L-leucine and glycyl-D-leucine. D-Leucylglycine gave no detectable hydrolysis and D-leucyl-L-tyrosine only a trace. Thus our use of leucine aminopeptidase as a diagnostic tool for determining the all L configuration of our synthetic angiotensin is valid. The four dipeptides mentioned were obtained from Mann Research Laboratories.

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