(3×10^{-4} *M*) in aqueous acetonitrile (1%). After 45 minutes the dienone intensity at 260 mμ was measured. Aliquots were removed and assayed with ninhydrin against a glycine standard.[18] The results are recorded in Table I. Control experiments showed that ninhydrin values were unaffected by the presence of dienone-lactone, even after boiling.

TABLE VIII

*p*H	Buffer	Final conc., *M*	*p*H	Buffer	Final conc., *M*
1.9	HCl	0.016	5.2	Acetate	0.08
2.3	Phosphate	.04	5.9	Acetate	.08
3.5	Acetate	.08	6.8	Phosphate	.04
4.7	Acetate	.08	8.6	Bicarbonate	.016

Similar methods were used to obtain the data summarized in Tables II, IV, VI, VII and Figs. 3a, 3b and 4. Where necessary, dilute potassium carbonate was used to neutralize acidic solutions prior to ninhydrin assay.

Dibromophloretylglycine, when reacted with 1 equivalent of NBS or bromine in 4% methanol–water over the *p*H range 2.2–8.4, exhibited a similar range of results.

Stability of Dienone-lactone (II) in Sodium Bicarbonate Solution.—Solutions of II (10^{-4} *M*) in aqueous sodium bicarbonate (1% acetonitrile) were examined by spectrophotometric assay at 260 mμ. The decrease in intensity with time is recorded in Fig. 1 at several concentrations of bicarbonate. No difference in rate of destruction was observed in the presence of glycine (10^{-4} *M*) or succinimide (3×10^{-4} *M*).

Effect of *p*H on NBS Oxidation of Leucine.—To solutions of leucine in sulfuric acid or buffer (Table VII) was added 4 equivalents of NBS in acetonitrile (final concentrations-amino acid 0.01 *M* and NBS 0.04 *M*, 1% acetonitrile) and the disappearance of positive halogen followed by thiosulfate titration. The results are recorded in Fig. 2.

Competition Between Tyrosine and Tryptophan Cleavage.—To reaction mixtures containing phloretylglycine and indolepropionyl-DL-phenylalanine (each at 2.5×10^{-3} *M* in 50% acetic acid–6% acetonitrile–water) was added either 1.5, 2.25 or 3 equivalents of NBS (per mole of either peptide). After 5 minutes at room temperature, the solutions were concentrated to dryness *in vacuo*, the residues dissolved in water and the solutions reconcentrated and the residues finally dissolved in 0.5 ml. of 50% ethanol.

Aliquots were spotted on Whatman #1 paper and the chromatograms run in the descending technique using either methyl ethyl ketone–propionic acid–water (15:5:6) or methanol–benzene–*n*-butanol–water (1:1:1:1) with concentrated ammonia added to 1% of total volume. The chromatograms were dried in air and developed with a spray of 0.25% ninhydrin in acetone containing 5% acetic acid.

By visual estimation increasing amounts of phenylalanine were observed. Glycine appeared only after 3 moles of NBS has been added and only in trace amounts. In a control experiment which omitted the tryptophyl analog a considerable amount of glycine was released.

Cleavage of Valyl-Hypertensin.—Solutions of hypertensin (2×10^{-4} *M*) in 50% acetic acid were treated with 3, 4 and 5 equivalents of NBS and with 4 equivalents in water alone. After 10 minutes, the solutions were concentrated to dryness and treated with fluorodinitrobenzene in bicarbonate solution.[19] The resulting mixtures of DNP-peptides were hydrolyzed at 105–110° for 14 hr. Dinitrophenol was removed by sublimation and the residual materials separated by two-dimensional chromatography with DNP-valine as a standard.[19] Actual recoveries of DNP-valine were determined by elution of the spots and spectrophotometric assay. Results are summarized in Table IX.

TABLE IX

RECOVERIES OF DNP-VALINE FROM HYPERTENSIN CLEAVAGE

NBS, moles	Solvent	DNP-valine, %
3	50% acetic	5
4	50% acetic	7
5	50% acetic	15
4	Water	2

(18) S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 907 (1954). Glycine was identified (chromatographically) as its dinitrophenyl derivative.

(19) H. Fraenkel-Conrat, J. Ieuan Harris and A. L. Levy, "Methods in Biochemical Analysis," Vol. II, Interscience Publishers, Inc., New York, N. Y., 1955, p. 359.

[CONTRIBUTION FROM THE RESEARCH DIVISION OF THE CLEVELAND CLINIC FOUNDATION AND THE FRANK E. BUNTS EDUCATIONAL INSTITUTE, CLEVELAND, OHIO]

An Improved Synthesis of Isoleucine[5] Angiotensin Octapeptide[1]

BY KIKUO ARAKAWA[2] AND F. MERLIN BUMPUS

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Both high yield and high purity have been achieved in the synthesis of angiotensin octapeptide. The product was also more active pharmacologically than those previously synthesized. This great increase in biological activity probably is due to an increased optical purity of the peptide. The synthesis was started from the C-terminal end by adding amino acids one at a time except for valyl-tyrosine. An excess of the carboxyl entering amino acids insured high yields. As a C-terminal blocking group, *p*-nitro-benzyl ester proved more stable to hydrogen bromide cleavage than the benzyl ester. Despite this, cleavage occasionally occurs which introduces difficulties if the hydrobromide salt of the resulting peptide is hard to crystallize.

Introduction

Angiotensin I (a decapeptide), the product of the action of renin on renin substrate, has been isolated in highly purified form and its structure postulated in two laboratories.[3,4] This decapeptide is converted to angiotensin II (an octapeptide) by a plasma enzyme by removal of dipeptide L-histidyl-L-leucine from the C-terminus.[5] The octapeptide was first synthesized in our Laboratory.[6] The asparaginyl[1] analog of angiotensin II was simultaneously reported[7] and has similar physiological properties to angiotensin II. Several angiotensin-peptide syntheses have been reported more recently.[8]

(1) This work was supported in part by the National Heart Institute, U. S. Public Health Service, Grant No. H-96.

(2) Research Fellow of the Frank E. Bunts Educational Institute.

(3) D. F. Elliott and W. S. Peart, *Biochem. J.*, **65**, 246 (1957).

(4) L. T. Skeggs, K. E. Lentz, J. R. Kahn, N. P. Shumway and K. K. Woods, *J. Exp. Med.*, **104**, 193 (1956).

(5) L. T. Skeggs, W. H. Marsh, J. R. Kahn and N. P. Shumway, *ibid.*, **99**, 275 (1954).

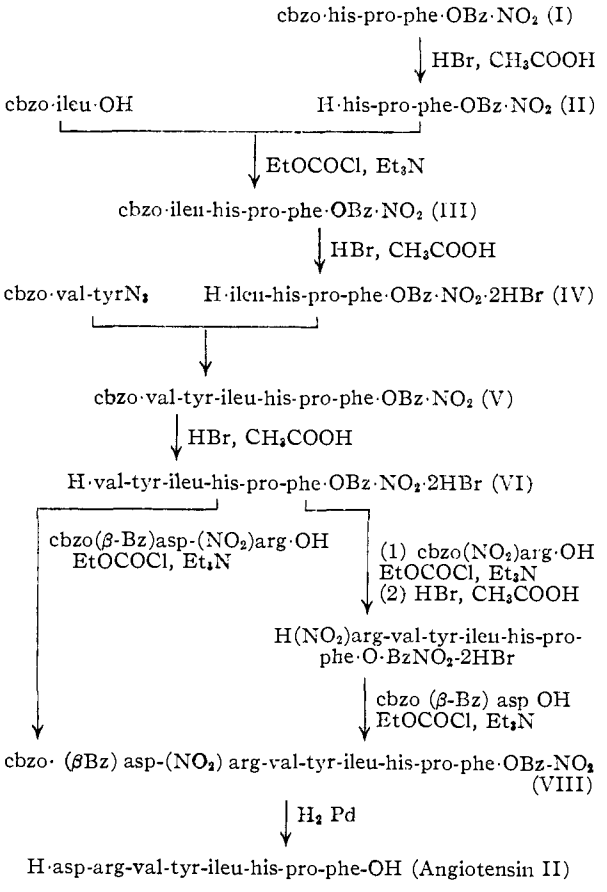
(6) F. M. Bumpus, H. Schwarz and I. H. Page, *Science*, **125**, 886 (1957); H. Schwarz, F. M. Bumpus and I. H. Page, THIS JOURNAL, **79**, 5697 (1957).

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

(8) R. Schwyzer, *Record of Chemical Progress*, **20**, 147 (1959).

Discussion

In the method employed previously in this Laboratory, the carboxyl groups of angiotensin were blocked as methyl esters. This necessitated saponification as one of the final steps, which possibly could have resulted in α to β shift of the aspartyl linkage.[9] To eliminate the possibility of this type of rearrangement, benzyl and *p*-nitro benzyl esters were used. These protecting groups can be removed quantitatively along with the nitro and carbobenzoxy protecting groups by catalytic hydrogenation as a final step in angiotensin synthesis.



It has been shown that with the use of the mixed anhydride method partial racemization occurs when the carboxyl carrying component is an acyl di- or higher peptide.[10,11] It was recently suggested that peptides should be synthesized by starting with the C-terminal amino acid ester and adding one amino acid at a time until the desired peptide is obtained.[12] Using this procedure an optically pure product could be more readily produced. The synthesis reported here is started from phenylalanine-*p*-nitrobenzyl ester, the C-terminal amino acid. The chain is built up one amino acid at a time with the exception of the addition of L-valyl-L-tyrosine which was added by the azide method. By using an excess (up to 1.5 equivalents) of the carbobenzoxy amino acid for the mixed anhydride condensation, almost quantitative yields (calculated on amine bearing component) were realized. Likewise, the use of an excess of carbobenzoxy peptide azide increased the yield to 91%.

The tripeptide, carbobenzoxy-L-histidyl-L-prolyl-L-phenylalanine-*p*-nitrobenzyl ester was prepared as previously described.[13] The carbobenzoxy group was removed with anhydrous hydrogen bromide resulting in a small loss of nitrobenzyl ester. Since this tripeptide ester dihydrobromide was easy to separate by crystallization from free peptide this ester cleavage presented no difficulties.

In subsequent steps where purification was more difficult, conditions were varied according to the method of separation of the products. Carbobenzoxyisoleucine was condensed by the mixed anhydride method with L-histidyl - L - prolyl - L - phenylalanine-*p*-nitrobenzyl ester to yield an amorphous tetrapeptide (III) in 98% yield. The tetrapeptide ester hydrobromide salt obtained by treating III with hydrogen bromide, contained traces of the carbobenzoxy peptide. By a series of solvent extractions, a crystalline salt was obtained, but upon analysis it proved to be a mixture of mono- and dihydrobromide. A pure salt (IV) could not be obtained. For this reason the amorphous salt of L - isoleucyl - L - histidyl - L - prolyl - L - phenylalanine-*p*-nitrobenzyl ester was converted directly into free base, extracted into ethyl acetate and condensed with carbobenzoxy-L-valyl-L-tyrosine azide to yield carbobenzoxy-L-valyl-L-tyrosyl-L-isoleucyl-L - histidyl - L - prolyl - L - phenylalanine - *p* - nitrobenzyl ester. The carbobenzoxy group was removed with hydrogen bromide to give a crystalline hexapeptide ester dihydrobromide. This intermediate also easily lost some hydrogen bromide during crystallization. It could easily be recrystallized from solvents containing a trace of hydrogen bromide. The high yields in the latter two steps, 91% in each, indicate that the peptide IV was pure even though it formed an unstable hydrobromide salt.

By condensing this hexapeptide with carbobenzoxy β - benzyl - L - aspartyl - L - nitro - arginine (2+6 condensation) the protected angiotensin octapeptide was obtained. An alternative method was first to form the carbobenzoxy heptapeptide ester by condensing carbobenzoxy-nitro-L-arginine with the hexapeptide. After removal of the carbobenzoxy group, this heptapeptide free base was condensed with carbobenzoxy-β-benzyl-L-aspartic acid (1+7 condensation) to yield protected angiotensin (VIII) with very similar physical properties to those obtained from the 2 + 6 condensation. The over-all yield of the protected octapeptide from (VI) hexapeptide was very nearly equivalent by both routes.

Hydrogenolysis of the protected form VIII (1 + 7 condensation) yielded angiotensin octapeptide in good yield. It was separated from minor components formed during reduction by chromatography on cellulose powder using butanol:acetic acid:water (4:1:5) solvent system. Color reactions indicated two of the minor components to be due to incom-

(9) A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955).
(10) J. R. Vaughan and J. A. Eichler, THIS JOURNAL, **75**, 5556 (1953).
(11) B. F. Erlanger, H. Sachs and E. Brand, *ibid.*, **76**, 1806 (1956).
(12) H. Schwarz and F. M. Bumpus, *ibid.*, **81**, 890 (1959).
(13) H. Schwarz and K. Arakawa, *ibid.*, **81**, 5691 (1959).

plete hydrogenation. Aspartic acid was also produced during reduction and evaporation, possibly due to acidic cleavage of peptide bond before removal of the carbobenzoxy group. We have observed that carbobenzoxy asparagine loses ammonia rapidly during heating in aqueous solution. This is not true for asparagine. This same lability should exist for acylaspartyl peptides.

Purified angiotensin (1+7) assayed 12,700 pressor units per mg. dry weight as compared to 9200 previously reported.[6] Angiotensin prepared by the (2+6) condensation was less active.

Carbobenzoxy-nitro-L-arginyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine-*p*-nitrobenzyl ester was also synthesized in good yield by the 1+7 condensation. Its biological activity will be reported elsewhere.

Experimental[14]

Carbobenzoxy-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester.—To a cold solution of 21.1 g. (82.5 mmoles) of carbobenzoxy-L-isoleucine in 130 ml. of tetrahydrofuran was added 11.55 ml. (82.5 mmoles) of triethylamine and 7.86 ml. (82.5 mmoles) of ethylchloroformate. After stirring for 15 minutes at −5°, the precipitate of triethylamine hydrochloride was removed by filtration (cold) (10.7 g., 94.5%). The filtrate was combined with a precooled solution of 38.3 g. of L-histidyl-L-prolyl-L-phenylalanine-*p*-nitrobenzyl ester dihydrobromide[11] (55 mmoles) in 150 ml. of tetrahydrofuran, 1.98 ml. of distilled water and 27.5 ml. of tributylamine (115.5 mmoles). The mixture was stirred for 15 minutes at 0° and 2 hr. at room temperature. The solvents were removed *in vacuo* under 50° and the residue was dissolved in ethyl acetate. The ethyl acetate solution was washed twice with water, three times with *N* hydrochloric acid, once with water, three times with saturated sodium bicarbonate, twice with water and once with saturated salt solution. All extractions were carried out in cold. After drying over anhydrous sodium sulfate, the solvent was removed *in vacuo*. Benzene was added to the residue and evaporated three times. The residue was kept *in vacuo* over phosphorus pentoxide for two days. Yield of amorphous solid, 42.2 g., 98.3%. R_{fBAW}: 0.91; R_{fAW}: 0.98. Small amount of the sample was precipitated from ethyl acetate–petroleum ether four times. The final precipitated oil was dried *in vacuo* to glassy foam and further dried at 64° in high vacuum for 20 hr.

The amorphous product melted at 81–85°, $(\alpha)^{22}D$ − 63.85 (*c* 1.1 in methanol).

Anal. Calcd. for $C_{41}H_{47}O_9N_7$: C, 62.98; H, 6.06; N, 12.54. Found: C, 62.56; H, 6.14; N, 12.78.

L-Isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester Dihydrobromide.—Carbobenzoxy-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-nitrobenzyl ester (42.2 g., 54 mmoles) was dissolved in 160 ml. of glacial acetic acid and treated with 190 ml. of approximately 4 *N* hydrogen bromide in glacial acetic acid for 35 minutes at room temperature. After the addition of 2 liters of absolute ether, the precipitate was collected and washed with a large volume of absolute ether. Chromatography on paper (BAW) of the product obtained after reprecipitation from absolute methanol–ethyl acetate–ether indicated approximately 20% of the unreacted carbobenzoxy peptide. Retreating the above mixture with freshly prepared 190 ml. of 4 *N* hydrogen bromide in glacial acetic acid for 90 minutes at room temperature and precipitating as described above, yielded 39.06 g. of amorphous product, 89.5% yield, R_{fBAW}: 0.90 (Pauly's *R*) 0.64 (ninhydrin and Pauly's *R*).

A second very minor spot at R_f 0.90 was evident only with diazotized sulfanilic acid. The unreacted carbobenzoxy peptide amounted to no more than 5% of total product. In other experiments of more prolonged treatment with hydrogen bromide, a notable amount of the ester was removed. The product contaminated with carbobenzoxy peptide was used for further synthesis.

For further purification 3.0 g. of the above product dissolved in 50 ml. of water was extracted 24 times (3°) with ethyl acetate. After adjustment of the *p*H of the aqueous layer to 8.5, the resulting precipitated gum was extracted into ethyl acetate. The combined ethyl acetate extracts of the basic solution were dried over anhydrous sodium sulfate and saturated with dry hydrogen bromide. The oily precipitate was triturated with ethyl acetate, ether and reprecipitated from ethanol–ether yielding 0.85 g. of amorphous product. Crystallization of this product from 95% ethanol yielded 300 mg. of needles, m.p. 134–138°, $(\alpha)^{23}D$ + 36.6 (*c* 1.0 in methanol). Analysis showed this to be a mixture of a mono- and dihydrobromide salt. Further attempts did not yield either a pure mono- or dibasic salt of the tetrapeptide.

Carbobenzoxy-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester.—To 27.5 g. (64.1 mmoles) of carbobenzoxy-L-valyl-L-tyrosine hydrazide dissolved in 500 ml. of glacial acetic acid and 100 ml. of 5 *N* hydrochloric acid was added 700 ml. of ethyl acetate and the resulting mixture cooled to 0°. To this was added an aqueous solution of 4.43 g. (64.1 mmoles) of sodium nitrite. After 5 minutes one liter of cold water was added to form two layers. Upon separation of the organic phase, the aqueous layer was further extracted three times with 400 ml. of ethyl acetate. The combined ethyl acetate was washed (cold) three times with water, three times with saturated sodium bicarbonate, twice with water and twice with saturated salt solution. The azide solution was dried over anhydrous sodium sulfate for 20 minutes.

A solution of 34.6 g. (42.7 mmoles) of tetrapeptide *p*-nitrobenzyl ester dihydrobromide in 300 ml. of water was extracted four times with equal volumes of ethyl acetate to remove carbobenzoxy-tetrapeptide *p*-nitrobenzyl ester. The *p*H of the aqueous layer was adjusted to 9.0 with 50% potassium carbonate and diluted with 500 ml. of saturated sodium chloride. This was extracted with 1000 ml. of ethyl acetate containing 100 ml. of tetrahydrofuran followed by 3 additional extractions of 400 ml. of ethyl acetate. These combined extracts were dried over anhydrous sodium sulfate and evaporated *in vacuo* to 400 ml.

To this was added the above filtered azide solution. After standing 1 hr. at 3° and 16 hr. at room temperature, the reaction mixture was extracted twice with water and once with *N* hydrochloric acid. During the acid wash a precipitate formed which was removed by filtration of both layers. The ethyl acetate layer was then extracted with water, sodium carbonate solution and water and then dried over anhydrous sodium sulfate. After removal of the solvent *in vacuo* the amorphous residue was combined with the solid obtained previously. Precipitation from methanol–ether yielded 40.5 g. (91% yield) of an amorphous product.

R_{fBAW} 0.98, ninhydrin negative, Pauly's reagent positive. One μ mole of the product was hydrolyzed with a mixture of glacial acetic acid and concentrated hydrochloric acid (1:1) for 72 hr. at 110°. The amino acid ratios were: val, 1.01 μM; tyr, 0.89 μM; ileu, 1.00 μM; his, 1.02 μM; pro, 0.92 μM; phe, 1.03 μM.

L-Valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester Dihydrobromide.—To a solution of 13.6 g. of carbobenzoxy-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine-*p*-nitrobenzyl ester in 60 ml. of glacial acetic acid was added 200 ml. of 4 *N* hydrogen bromide, in glacial acetic acid. After 30 minutes at room temperature, 3 liters of cold absolute ether was added. The resulting precipitate was collected and washed with a large volume of absolute ether. This amorphous product was crystallized from 95% ethanol to yield 12.8 g. (91.5%) of needle crystals, m.p. 192–196.5°.

For analysis, a sample was recrystallized from methanol–ethyl acetate (1:2) containing 0.5 ml. of hydrogen bromide in glacial acetic acid. Crystallization was very slow and required 1 week for completion. After drying *in vacuo* at 56° for 2 hr., the sample melted at 193–199°, R_{fAW} = 0.75, $(\alpha)^{20}D$ −36.18 (*C* 1.0 in methanol).

Anal. Calcd. for $C_{47}H_{60}O_{10}N_9 \cdot 2HBr$: C, 52.61; H, 5.83; N, 11.75; Br, 14.90. Found: C, 52.34; H, 5.84; N, 11.89; Br, 15.16.

Carbobenzoxy-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester.

(14) All melting points were taken on Kofler hot-stage and are corrected. Microanalyses were done by Micro tech Laboratories, Skokie, Ill. All samples were chromatographed in two solvent systems: (A) Butanol:acetic acid:H_2O (4:1:5), abbreviated as R_{fBAW} (B) Acetonitrile:water (3:1), abbreviated as R_{fAW}.

—To a quite cold (−5°) solution of 4.45 g. (12 mmoles) of carbobenzoxynitro-L-arginine monohydrate dissolved in 35 ml. of tetrahydrofuran and 2 ml. of dimethylformamide were added 2.86 ml. of tributylamine and 1.45 ml. of ethyl chloroformate. After stirring for 15 minutes, a solution of 10.2 g. (8 mmoles) of hexapeptide *p*-nitrobenzyl ester dihydrobromide in 13 ml. of dimethylformamide and 3.81 ml. of tributylamine was added. The reaction mixture was stirred for 15 minutes at −5° and 1 hr. at room temperature. The addition of 700 ml. of ethyl acetate to the reaction mixture caused a slight turbidity which was removed with a small amount of methanol. The organic layer was extracted with 200 ml. of water followed by three extractions of 400 ml. of *N* hydrochloric acid. After the addition of 100 ml. of methanol and 200 ml. of ethyl acetate, the resulting organic layer was extracted with water followed by three extractions with aqueous sodium bicarbonate. Sufficient methanol was added to the ethyl acetate between the extractions to prevent precipitation of the peptide. After drying over anhydrous sodium sulfate, the organic layer was evaporated *in vacuo*. Benzene was added to the residue and evaporated several times to remove all traces of moisture. After drying over phosphorus pentoxide the amorphous product melted at 184–200°; yield, 8.5 g., 85.3% $(\alpha)^{22}D$ −60.4 (*C* 2, in methanol), R_{fAW}: 0.98, R_{fBAW}: 0.89.

It was crystallized from 85% ethanol to yield 6.32 g., 63.3% yield, m.p. 202–205°. A small sample of this material was twice recrystallized from 95% ethanol to yield a product which melted at 202–206°, $(\alpha)^{22}D$ − 59.5 (*C* 2.0, in methanol), $(\alpha)^{22}D$ − 31.2 (*C* 1.0, in pyridine).

Anal. Calcd. for $C_{61}H_{76}O_{15}N_{14}$: C, 58.83; H, 6.15; N, 15.75. Found: C, 58.61; H, 6.11; N, 15.66.

Nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester Dihydrobromide.—To a solution of 6.32 g. of crystalline carbobenzoxy heptapeptide *p*-nitrobenzyl ester in 20 ml. of glacial acetic acid was added 40 ml. of freshly prepared 4 *N* hydrogen bromide in glacial acetic acid. After 40 minutes at room temperature, the product was precipitated by the addition of 5 volumes of absolute ether, collected by filtration and washed with a large volume of absolute ether. It was reprecipitated from absolute ethanol–ethyl acetate to yield 6.05 g. (4.75 mmoles) of amorphous product, 94%, R_{fAW} 0.80.

Carbobenzoxy-β-benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester (A:1+7).—To a cold solution of 2.41 g. of carbobenzoxy-β-benzyl-L-aspartic acid[15] (6.75 mmoles) in 15 ml. of tetrahydrofuran (−5°) were added 0.95 ml. of triethylamine and 0.643 ml. of ethyl chloroformate. After stirring for fifteen minutes triethylamine hydrochloride was removed by filtration in the cold. The filtrate was combined with a solution of 5.73 g. (4.5 mmoles) of the heptapeptide *p*-nitrobenzyl ester dihydrobromide dissolved in 20 ml. of dimethylformamide and 2.15 ml. of tributylamine. The reaction mixture was stirred in an ice-bath for 15 minutes, at room temperature for 2 hr., then transferred into a separatory funnel with 50 ml. of methanol and 1.7 liters of ethyl acetate. It was extracted with 200 ml. of water, four times with 500 ml. of *N* hydrochloric acid, 200 ml. of water, four times with 500 ml. of saturated sodium bicarbonate and finally four times with 300 ml. volumes of water. The aqueous extractions caused a precipitation due to removal of methanol and dimethylformamide from the ethyl acetate layer. This was overcome by the addition of 30 ml. of dimethylformamide, 50 ml. of methanol and 250 ml. of ethyl acetate following the acid washes and 50 ml. of methanol and 150 ml. of ethyl acetate after the basic washes. After evaporation *in vacuo* the residue was dissolved in methanol and decolorized with charcoal. It was precipitated from methanol–ether to yield 4.97 g. of an amorphous product, 76% yield. For analysis it was precipitated from 95% ethanol and from methanol–dioxane–ethyl acetate and finally dried at 110° for 20 hr. *in vacuo*. The amorphous product melted at 180–184°; $(\alpha)^{22}D$ −29.5 (*c* 1.1, in pyridine).

Anal. Calcd. for $C_{72}H_{87}O_{18}N_{15}\cdot 2H_2O$: C, 58.17; H, 6.17; N, 14.13. Found: C, 58.42; H, 6.16; N, 14.31.

(15) A. Berger and E. Katchalski, THIS JOURNAL, **73**, 4084 (1951).

L-Aspartyl-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine Monoacetate.—To a solution of 2.176 g. (1.5 mmoles) of carbobenzoxy-β-benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-nitrobenzyl ester (1+7) in the warm mixture of 60 ml. of methanol, 20 ml. of glacial acetic acid was added 1.5 g. of palladium black suspended in 15 ml. of water. Hydrogen was bubbled through this mixture with vigorous shaking for 30 hr. Another 10 ml. of methanol and 15 ml. of water were added during the hydrogenation. After removal of the catalyst by filtration the solvents were removed *in vacuo*. Methanol and benzene were added to the residue and evaporated at reduced pressure. This was repeated to remove the last traces of acetic acid. The yield of amorphous product after drying over P_2O_5 was 1.93 g. The chromatogram of 500 μg. of this material gave the results: R_{fBAW}: 0.14 (ninhydrin positive), 0.29 (Pauly, ninhydrin, Sakaguchi positive), 0.40 (Pauly, ninhydrin and Sakaguchi positive), 0.81 (Pauly positive) and 0.96 (Pauly positive). The intensity of the spot (Pauly) at R_f 0.29 was 3 to 4 times that at R_f 0.40. The three remaining spots were extremely faint in intensity.

Samples of the reduced peptide were chromatographed on a column 3.6 × 60 cm. containing 200 g. cellulose (Whatman) powder using butanol: acetic acid:water (4:1:5) as the developing solvent. The column was packed by preparing a suspension of the cellulose in the organic phase. Washing of the packed column was continued until no more color was eluted.

A solution of 570 mg. of the reduced sample dissolved in 15 ml. of the organic phase was added to the column. It was then eluted with 2100 ml. of the same solvent. There was considerable overlapping of peaks which necessitated several passes through the column to obtain optimum purity. Chromatographically homogeneous samples as shown by paper chromatograms of serial samples were combined. The first 1300 ml. of eluent from the column was discarded; the next 240 ml. which contained 310 mg. was recycled through the same column. The third fraction (465 ml.) contained 200 mg. of peptide which was shown to be homogeneous by paper chromatography, using both acetonitrile: water and the butanol: acetic acid:water systems described before.

A combined fraction of 860 mg. (corresponding to R_{fBAW} 0.29) after treatment with charcoal and drying in high vacuum over phosphorus pentoxide and sodium hydroxide melted at 230–247° with decomposition $(\alpha)^{21}D$ − 65.87 (*c* 0.8, in *N* hydrochloric acid).

In preparation for analysis a sample was twice reprecipitated by dissolving in 50% hot ethanol and cooling and dried at 110° in high vacuum for 24 hours.

Anal. Calcd. for $C_{50}H_{71}O_{12}N_{13}\cdot C_2H_4O_2$: C, 56.46; H, 6.83; N, 16.46, Found: C, 55.98; H, 6.55; N, 16.74, 16.20. $(\alpha)^{20}D$ − 66.98 (*c* 0.4, in *N* hydrochloric acid).

Pressor activity as determined both in rat and dog's blood pressure was 12000 units per mg.[16]

Carbobenzoxy-β-benzyl-L-aspartyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitrobenzyl Ester (B:2+6).—To a solution of 0.56 g. (1 mmole) of carbobenzoxy-β-benzyl-L-aspartyl-nitro-L-arginine[17] dissolved in 10 ml. of tetrahydrofuran and cooled to −5° were added 0.238 ml. of tributylamine and 0.095 ml. of ethyl chloroformate. The solution was stirred for 15 minutes in an ice–salt bath and then combined with a solution of 0.858 g. (0.8 mmole) of L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-nitrobenzyl ester dihydrobromide dissolved in a mixture of 5 ml. of dimethylformamide and 10 ml. of ethyl acetate containing 0.418 ml. of tributylamine.

The reaction mixture was stirred at −5° for 20 minutes and at room temperature for 3 hr. The solvents were removed *in vacuo* at 50° and the oily residue dissolved in warm 95% ethanol. The amorphous precipitate obtained upon cooling was reprecipitated by dissolving in 95% hot ethanol and cooling to yield 0.43 g. of material which was chromatographically homogeneous. The filtrates were combined, evaporated to dryness and triturated with ethyl acetate. The residue was dissolved in warm methanol, a small amount

(16) A. A. Green and F. M. Bumpus, *J. Biol. Chem.*, **210**, 281 (1954).

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

of glacial acetic acid added and finally precipitated with water. This product was combined with the solid product obtained above to yield 0.63 g. (54.3% yield). A sample was reprecipitated twice from 95% ethanol and dried in high vacuum at 78° for 4 hr. This amorphous product melted at 188.5–191°, $(\alpha)^{23}D$ −28.5 (*c* 1.0, in pyridine).

Anal. Calcd. for $C_{72}H_{87}O_{18}N_{18}\cdot 2H_2O$: C, 58.17: H, 6.17: N, 14.13. Found: C, 58.06: H, 5.90: N, 13.98.

Carbobenzoxy-nitro-L-arginyl-nitro-L-arginyl-L-valyl-L-tyrosyl-L-isoleucyl-L-histidyl-L-prolyl-L-phenylalanine *p*-Nitro-benzyl Ester (1+7).—To a cold solution of 0.557 g. (1.5 mmoles) of carbobenzoxy-nitro-L-arginine monohydrate dissolved in 5 ml. of tetrahydrofuran was added 0.357 ml. of tributylamine and 0.143 ml. of ethyl chloroformate. After 15 minutes at this temperature, it was combined with the solution of 1.27 g. (1.0 mmoles) of the heptapeptide *p*-nitrobenzyl ester dihydrobromide dissolved in 5 ml. of dimethylformamide containing 0.5 ml. of tributylamine. After stirring for 15 minutes at −5° and 2 hr. at room temperature, the reaction mixture was transferred to a separatory funnel with 200 ml. of tetrahydrofuran and 400 ml. of ethyl acetate. The organic layer was extracted once with water, twice with *N* hydrochloric acid, once with water, twice with saturated sodium bicarbonate and twice with water. The precipitate which formed during each extraction was redissolved by addition of dimethylformamide and tetrahydrofuran. Sufficient ethyl acetate was added to maintain the two layers. The organic layer was dried over anhydrous sodium sulfate and evaporated *in vacuo*. Precipitation from methanol–ether yielded 0.71 g. (49.1% yield) of an amorphous product which melted at 170–180°. Chromatography in two solvent systems indicated that this product was homogeneous: R_{fAW} 0.95, R_{fBAW} 0.90.

In preparation for analysis a sample was twice reprecipitated from 95% ethanol and once from methanol–dioxane–ethyl acetate. After drying *in vacuo* at 110° for 24 hr., the product melted at 173–180°, $(\alpha)^{22}D$ −34.45 (*c* 2.0 in pyridine).

Anal. Calcd. for $C_{87}H_{87}O_{18}N_{19}$: C, 55.63; H, 6.06; N, 18.40. Found: C, 55.38; H, 6.30; N, 18.15.

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES AND THE NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE, BETHESDA, MARYLAND]

Oxidation of Kynuramine Derivatives by Monoamine Oxidase and the Enzymatic Conversion of Dihydronorkynuramine to Indigo

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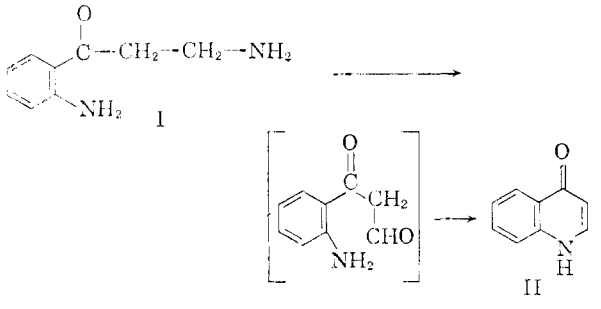
Norkynuramine (VI), dihydronorkynuramine (VIIa) and dihydrokynuramine, synthesized by standard methods, have been found to be oxidized by monoamine oxidase at rates of 20–40% compared with kynuramine, as determined manometrically. Only dihydronorkynuramine (VIIa) in this oxidation with monoamine oxidase gave rise to a colored product, namely indigo, which makes possible a simple qualitative visual test for the detection of that enzyme.

It has been shown in a preceding study[2] that kynuramine (I) is a good substrate for monoamine (but not diamine) oxidase and that its enzymatic oxidation and transformation to 4-hydroxyquinoline (γ-carbostyryl, II) may be conveniently followed spectrophotometrically. This method has been developed into a new rapid assay for monoamine oxidase. These enzymatic studies have now been extended to dihydrokynuramine, norkynuramine (VI), dihydronorkynuramine (VII) and its amino acid analog, *erythro-o*-amino-DL-phenylserine, substrates which were synthesized by standard methods (III-VIII).



Materials[3]

ω-Bromo-*o*-acetaminoacetophenone (III).—To a hot solution (70°) of 25 g. of *o*-acetaminoacetophenone (m.p. 76.5°)[4] in 170 ml. of glacial acetic acid was added dropwise and with stirring a solution of 22.6 g. of bromine in 140 ml. of glacial acetic acid. The reaction mixture was irradiated with light from a 125-watt ultraviolet lamp. The yellow crystalline addition product which formed initially dissolved after 10–20 min., during which the color of bromine was discharged. The temperature had to be kept at 65 ± 5°. In experiments where the temperature was raised above 75°, colorless crystals, m.p. 169–170° (dec.), presumably the dibromo compound, were formed. After 2 hr. the reaction mixture was evaporated *in vacuo*, the residue taken up in chloroform, washed with water and the dried chloroform extract was again evaporated to dryness. The residue was crystallized from benzene–hexane to yield pale brown needles, m.p. 124–125.5° (reported 126–127°).[5] The yield was 27.1 g. or 74% of theory.

ω-Phthalimido-*o*-acetaminoacetophenone V.—To a solution of 10.0 g. of ω-bromo-*o*-acetaminoacetophenone (III) in 30 ml. of dimethylformamide[6] was added 8.3 g. of potassium phthalimide over a period of 15 min. with stirring. A slightly exothermic reaction occurred. The mixture was then warmed to 70° and maintained there for 30 min. After cooling 200 ml. of chloroform was added to the reaction mixture, the organic phase was washed with 150 ml. of water, 100 ml. of 0.5 *N* sodium hydroxide and with three portions of 50 ml. of water. The chloroform extract was dried (Na_2SO_4) and evaporated *in vacuo*. The residue was triturated with ethyl acetate to give a crystalline powder, m.p. 212–216° (sintering at 205°). The yield was 5.36 g. or 43% of theory. Recrystallization from ethyl acetate gave almost colorless prisms, m.p. 222–224°.

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(3) All melting points are uncorrected. We are indebted to Mr. H. G. McCann and associates of the Analytical Services Unit of this Laboratory for the analytical data.

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