Flavinyl Peptides. I. Synthesis of Flavinyl-Aromatic Amino Acids (1)

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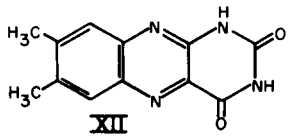
The syntheses of flavinyl peptides, in which L-tryptophan, L-tyrosine, or L-phenylalanine are attached *via* peptide linkage to the isoalloxazine system with ω-carboxyalkyl groups in position 3 or 10, are described. Lumiflavin was carboxymethylated by known methods to yield *N*-3-carboxymethyllumiflavin. Oxidation of 10-ω-hydroxyhexyl-, 10-ω-hydroxypentyl-, 10-ω-hydroxybutyl-, and 10-formylmethylflavins gave the corresponding 10-ω-carboxyalkylflavins. The 10-ω-carboxyethylflavin was obtained by condensation of 2-amino-4,5-dimethyl-*N*-ω-carboxyethylaniline with alloxan. Activations of the carboxyl group of the flavins were achieved with *N,N'*-carbonyldiimidazole and *p*-nitrophenyltrifluoroacetate to form the corresponding acyl imidazoles and *p*-nitrophenyl esters. 10-Carboxymethylflavin was hydrogenated to form 10-carboxymethyldihydroflavin and activated by carbodiimide. Reaction of the carboxy-activated flavins with the appropriate amino acid methyl esters, followed by air oxidation in the case of dihydroflavin, gave the corresponding flavinyl peptides.

Interaction of the flavin with aromatic amino acids results in a broadening of the visible flavin absorption towards the green, without the appearance of discrete new maxima, and in quenching of the flavin fluorescence. The fluorescence efficiency increases with increasing numbers of methylene groups in the flavin side chain. The nonlinear dependency of fluorescence quenching versus number of methylene groups indicates that different types of intramolecular interactions are involved.

Changes are known to occur in the absorption and fluorescence spectra of the coenzymes flavin mononucleotide and flavin-adenine dinucleotide on binding to specific apoenzymes. Associations of these coenzymes with tyrosine in flavoproteins have been reported (2,3). Flavins are also known to complex strongly with tryptophan (4).

To characterize more exactly the nature of the interactions between the flavin nucleus [7,8-dimethylisoalloxazine] and the aromatic amino acids, phenylalanine, tyrosine, and tryptophan, such as may occur in flavoproteins, flavin-aromatic amino acid peptides with varying chain length between flavin and amino acid were synthesized. The general pathway leading to flavinyl peptides is shown in Chart I.

N-10-ω-Hydroxyalkylflavins (I) [n=3,4,5] were synthesized by known methods (5). The corresponding *N*-10-ω-carboxyalkylflavins (II) were obtained in 65-75% yield by oxidations of I [n=3,4,5] with 60% nitric acid. In a similar reaction, the treatment of *N*-10-ω-hydroxyethylflavin I [n=1] with nitric acid led to a product which exhibited a blue fluorescence under ultraviolet light and had the same R_f values on thin-layer chromatograms as did authentic lumichrome (XII).

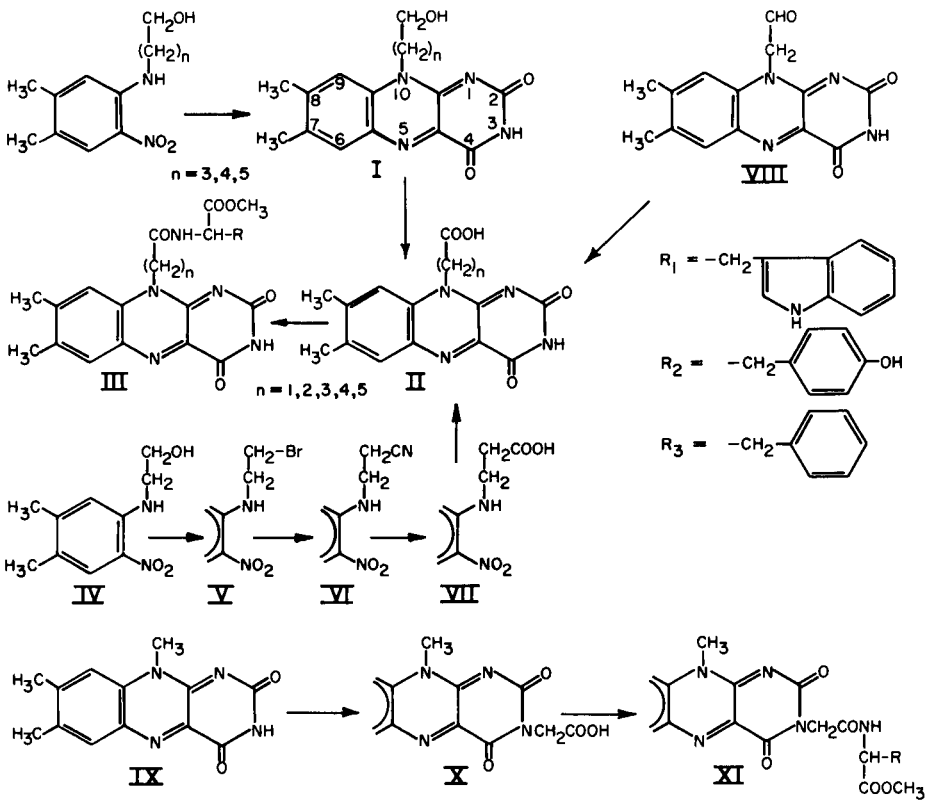


XII

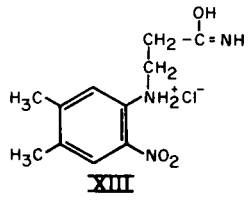
7,8-Dimethyl-10-(formylmethyl)isoalloxazine (VIII) (6) has been shown to yield lumichrome (XII) upon treatment with peroxyacetic acid (7). Since this aldehyde is an intermediate in the oxidation of I [n=1], a similar elimination of the formylmethyl group probably occurs in the present case.

Reaction of 2-nitro-4,5-dimethyl-*N*-(ω-hydroxyethyl)-aniline (IV) with triphenylphosphite-bromine (8) gave the ω-bromoethyl compound (V) in 80% yield. The corresponding *N*-ω-cyanoethylaniline (VI), obtained in 82% yield by a bromide-cyanide displacement (9), was hydrolyzed

Chart I



under alkaline conditions to the corresponding carboxylic acid (VII) in 82% yield. Acidic hydrolysis of VI led to VII in only 60% yield. In addition, a bicarbonate-insoluble compound was isolated from acid hydrolysates and characterized as 2-nitro-4,5-dimethylaniline by infrared spectroscopy and mixed melting point. A heterolytic fragmentation of the intermediate 2-nitro-4,5-dimethyl-*N*-(ω-ethylimidoester)anilinium hydrochloride (XIII) leading to cyanic acid, ethylene and 2-nitro-4,5-dimethylaniline can be considered as a possible explanation (10). Catalytic



hydrogenation of VII gave the diamino compound which was condensed with alloxan by a known method (11) to form the *N*-10-ω-carboxyethylflavin II [n=2] in 61% yield. *N*-10-Carboxymethylflavin (II) [n=1] was first synthesized by Kuhn (12) by reduction of 2-nitro-4,5-dimethyl-*N*-(carboxymethyl)aniline to the corresponding diamino compound followed by condensation with alloxan to give II [n=1] in very low yield. A more satisfactory synthesis utilizes the oxidation of the *N*-10-formylmethylflavin (VIII) with potassium permanganate in dimethylformamide to give II [n=1] in 51% yield. *N*-3-Carboxymethylflavin

TABLE I

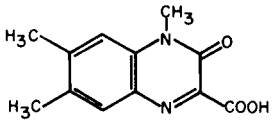
7,8-Dimethyl-10-(ω-carboxyalkyl)isoalloxazines (II)

n	% Yield	Calculated C	H	N	Found C	H	N
1	51	56.0	4.03	18.7	55.9	4.17	18.4
2	61	57.3	4.50	17.8	57.4	4.38	17.6
3	75	58.5	4.91	17.1	58.4	5.47	16.9
4	65	59.6	5.30	16.4	59.1	5.33	16.9
5	67	60.7	5.66	15.7	60.4	5.54	15.3

(X) was synthesized by alkylation of lumiflavin (IX) with the appropriate iodide in dimethylformamide-potassium

carbonate following the general method of Hemmerich (13).

Reaction of *N*-10-(II) [n=3,4,5] and *N*-3-ω-carboxyalkylflavins (X) [n=1] with *N,N'*-carbonyldiimidazole led to the corresponding acyl imidazoles which were reacted without isolation with the appropriate amino acid methyl ester hydrochlorides to give *N*-10- and *N*-3-flavinyl amino acid peptides. Yields obtained were 60-70% with III [n=4,5] and XI, but only 30-35% with impure III [n=3]. Attempts to synthesize III [n=2] under similar conditions failed. In this latter case, the reaction products on thin-layer chromatograms showed blue fluorescence under ultraviolet light. To clarify this side reaction, the carboxylic acid (II) [n=2] was activated with *N,N'*-carbonyldiimidazole and samples were taken at intervals for 48 hours and diluted with sodium phosphate buffer, *p*H 7. The change in the absorption spectrum, measured in the 300-500 mμ region, and thin-layer chromatography, indicated that the flavin degradation which occurs follows a pathway comparable with the known base-catalyzed degradation of IX (14,15) to 1,2-dihydro-1,6,7-trimethyl-2-keto-3-quinoxaline carboxylic acid (XIV). Because of the complete lack of

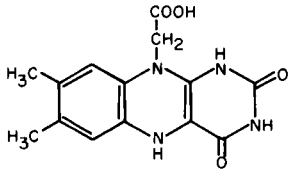


XIV

success with this method for synthesis of III [n=2], and the relatively low yield of III [n=3], other methods for peptide synthesis were attempted.

The activation of the flavin carboxylic acids II [n=2,3] was achieved by *p*-nitrophenylation with *p*-nitrophenyl trifluoroacetate (16). The isolated *p*-nitrophenyl ester of the flavin was reacted with the methyl esters of the appropriate amino acids to give the corresponding flavinyl peptides (III) [n=2,3] in 48-56% yield. *p*-Nitrophenylation of III [n=1] was not successful because of predominant dimerization (17,18) which gives a green biflavin and small amounts of ring-opened products previously mentioned. The interaction between *N*-1 of the flavin nucleus and the carbonyl function of the transient *p*-nitrophenyl ester increases markedly the electron deficiency in the flavin nucleus and activates the 8-methyl group to give 8,8'-dimerization, as studied by Ehrenberg *et al.* (18) with a similarly electron deficient 2,4-diethyllumiflavinium perchlorate.

To prevent dimerization and cleavage of the pyrimidine portion of the flavin nucleus, II [n=1] was reduced catalytically to the dihydroflavin (XV) using 10% palladium on charcoal. This compound was reacted under argon



XV

with 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate and the appropriate amino acid methyl esters to form the dihydroflavinyl amino acid peptides. Admission of air resulted in the reoxidation of the dihydroflavinyl peptides to III [n=1] in 40-59% yield.

TABLE II

Flavinyl Peptides (III) and (XI)

Compound	n	R	% Yield	Calculated C	H	N	Found C	H	N
III	1	1	40	62.4	4.83	16.8	62.4	4.94	16.5
		2	59	60.4	4.86	14.7	60.1	4.81	14.3
		3	50	62.5	5.02	15.2	62.6	5.23	15.1
	2	1	51	63.0	5.09	16.3	62.8	5.15	16.6
		2	56	61.1	5.13	14.3	61.3	5.39	14.3
	3	1	50	63.6	5.34	15.9	63.9	5.54	15.8
		2	48	61.8	5.38	13.9	61.6	5.31	14.0
	4	1	60	64.2	5.57	15.5	63.8	5.61	15.6
		2	65	62.4	5.63	13.5	62.3	5.89	13.7
	5	1	70	64.7	5.79	15.1	64.6	5.73	15.4
		2	61	63.0	5.86	13.1	62.7	5.51	13.6
XI	1	1	67	63.0	5.09	16.3	63.1	5.19	16.1
		2	66	61.1	5.13	14.3	60.6	5.23	14.8
		3	60	63.2	5.30	14.7	63.1	5.59	14.6

Absorption spectra were determined for all the flavinyl peptides. For difference spectra, the buffer blank was replaced with a solution containing the appropriate ω-carboxyalkylflavin. The considerable changes found in difference spectra of the short-chain flavinyl peptides (III) where n=1 are shown in Figure 1. The locations of the maxima (482-493 mμ and 389-393 mμ) and less significantly the minima (432-446 mμ and 352-355 mμ) are shifted to longer wavelength in the order tryptophan > tyrosine > phenylalanine. In addition, spectra of the peptides of phenylalanine and tyrosine show shoulders at 460 mμ.

The quenching of the flavin fluorescence of a homologous series of flavinyl tryptophans is shown in Figure 2. The peptides (III), where n=1,2,3, were 0.25, 0.3, 0.77% and, where n=4,5, were 2.48 and 2.74% as fluorescent as the corresponding ω-carboxyalkylflavins. The low relative fluorescence of the peptides with shorter side chains, compared with the higher fluorescence of those with longer side chains, indicates a dominant contribution of

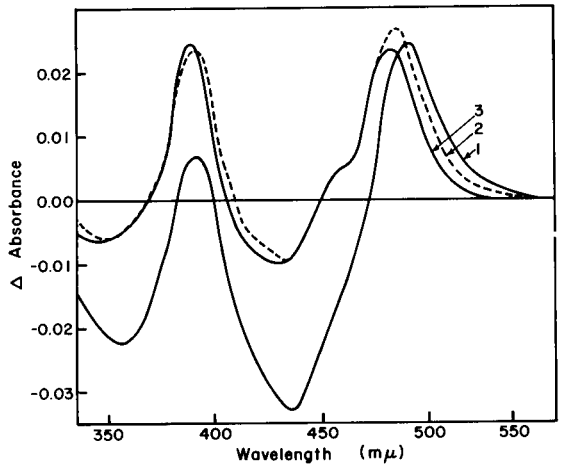


Figure 1. Difference spectra of flavinyl peptides (III) [n=1] in 5 x 10^{-2} *M* sodium phosphate, *p*H 7.0. Notations are: 1, tryptophan; 2, tyrosine; 3, phenylalanine. Sample cuvette: 2 x 10^{-5} *M* flavinyl peptide; reference cuvette: 2 x 10^{-5} *M* ω-carboxyalkylflavin.

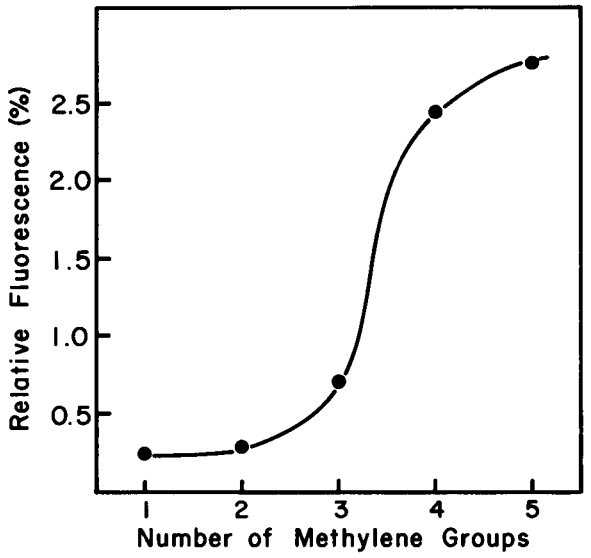


Figure 2. Intensity of fluorescence of flavinyl peptides (III) [n=1-5, R_1] measured relative to the ω-carboxyalkylflavin (II) as 100%. Flavins were 2 x 10^{-5} *M* in 5 x 10^{-2} *M* sodium phosphate, *p*H 7.0.

complex formation with the former as opposed to collisional quenching with the latter peptides. The distinction between these different mechanisms for fluoresence quenching is additionally supported by the non-linear relationship between fluorescence quenching and the number of methylene groups.

A detailed study of the absorption and fluorescence spectra of these compounds is being undertaken to investigate the forces involved in these intramolecular interactions.

EXPERIMENTAL

Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. Visible and ultraviolet spectra were determined with a Cary Model 14 recording spectrophotometer. Fluorescence measurements were made with an Aminco Bowman Spectrophotofluorimeter, using a Xenon lamp, photomultiplier tube 1P21, and slit arrangement No. 3. Stock solutions of 2 x 10^{-3} *M* flavin in dimethylformamide were appropriately diluted for all spectral measurements. Thin-layer chromatograms (TLC) were run on MN Silica Gel S-HR or MN Silica Gel S-HR/UV_{254} (Brinkmann). Spots were detected by visual examination under ultraviolet light. A mass spectrum was determined by an MS 902 mass spectrometer. Microanalyses were by Schwarzkopf Microanalytical Laboratory, Woodside, New York.

All syntheses involving isoalloxazine compounds were carried out in a darkened room.

7,8-Dimethyl-10-(ω-carboxyalkyl)isoalloxazine (II) [n=3,4,5].

ω-Hydroxyalkylflavin (I) (9 mmoles) was stirred into a solution of 60% nitric acid (35 ml.) at 5-10° and the stirring continued for three hours at room temperature. The reaction mixture was then stirred into 300 ml. of ice water and the precipitate collected on a filter and washed twice with 5 ml. of cold water. The crude flavin was dissolved in the minimal amount of 5% sodium bicarbonate. After filtration, the solution was adjusted to *p*H 1-2 with concentrated hydrochloric acid. The solid was collected on a filter and washed twice with 5 ml. of cold water. Recrystallization from concentrated hydrochloric acid-water gave II in 65-75% yield. The compounds moved as a single spot in *n*-butyl alcohol-acetic acid-water (2:1:1) on TLC.

2-Nitro-4,5-dimethyl-*N*-(ω-hydroxyethyl)aniline (IV).

A mixture of 19.6 g. (100 mmoles) of 1,2-dinitro-4,5-dimethylbenzene and 12.2 g. (200 mmoles) of 2-aminoethanol was refluxed for 18 hours in 100 ml. of *n*-amyl alcohol containing 5 ml. of pyridine. The solvent was evaporated under reduced pressure, the residue dissolved in 150 ml. of concentrated hydrochloric acid, and the mixture filtered. The filtrate was poured into 2 liters of water and the precipitate collected on a filter; yield, 19.0 g. (90%) of crude product. One recrystallization from methanol gave 17.1 g. (81%) of pure IV, m.p. 190-191°. The compound moved as a single spot in *n*-butyl alcohol-2*N* ammonium hydroxide-ethanol (3:1:1) on TLC.

2-Nitro-4,5-dimethyl-*N*-(ω-bromoethyl)aniline (V).

To 5.1 g. (16.5 mmoles) of triphenylphosphite in 50 ml. of benzene was added 2.6 g. (16.5 mmoles) of bromine with stirring. To this suspension was added 3.5 g. (16.5 mmoles) of IV; the mixture was stirred for 60 minutes at room temperature. Methanol (10 ml.) was added, and the solution was evaporated to a small volume and cooled. The resultant precipitate was collected on a filter and washed with small portions of cold methanol to give 4.0 g. of crude V. Recrystallization from benzene-methanol gave 3.6 g. (80%) of V, m.p. 88-89°. The compound moved as a single spot in benzene on TLC.

2-Nitro-4,5-dimethyl-*N*-(ω-cyanoethyl)aniline (VI).

A solution of 2.73 g. (10 mmoles) of V in 8 ml. of dimethyl-

sulfoxide was added with stirring to 0.54 g. (11 mmoles) of sodium cyanide in 10 ml. of dimethylsulfoxide at 60° over a period of 20 minutes and stirred for an additional 20 minutes at this temperature. The reaction mixture was added slowly with stirring to 500 ml. of water. The solid material was collected on a filter and washed with 10 ml. of water to give 2.1 g. (95%) of crude VII. Recrystallization from methanol gave 1.8 g. (82%) of VII, m.p. 156-157°. The compound moved as a single spot in chloroform on TLC.

2-Nitro-4,5-dimethyl-*N*-(ω-carboxyethyl)aniline (VII).

A mixture of 1.7 g. (7.75 mmoles) of VI, 30 ml. of ethanol, and 30 ml. of 4 *N* sodium hydroxide was refluxed under nitrogen for 60 minutes. The reaction mixture was added, with vigorous stirring, to 350 ml. of 1 *N* hydrochloric acid. The solid material was collected on a filter, washed with 10 ml. of water, and dried *in vacuo* to give 1.68 g. (91%) of crude VII. Recrystallization from chloroform-petroleum ether (b.p. 30-60°) gave 1.51 g. (82%) of VII, m.p. 182-184°. The compound moved as a single spot in *n*-butyl alcohol-2 *N* ammonium hydroxide-ethanol (3:1:1) on TLC.

7,8-Dimethyl-10-(ω-carboxyethyl)isoalloxazine (II) [n=2].

A suspension of 2.22 g. (9.3 mmoles) of VII and 0.1 g. of 10% palladium on charcoal in 40 ml. of 80% aqueous acetic acid was hydrogenated at room temperature and 4 atmospheres pressure until colorless. The mixture was filtered under nitrogen onto 1.94 g. (10.2 mmoles) of alloxan and 1.9 g. of boric acid and this mixture stirred for 24 hours. The precipitate formed was collected on a filter and washed with 10 ml. of cold water. The crude flavin was dissolved in a minimal amount of 5% sodium bicarbonate, treated with decolorizing carbon, and filtered. Concentrated hydrochloric acid was used to adjust the filtrate to *p*H 1-2. The precipitate formed was collected on a filter and washed four times with 5 ml. of water. The crude flavin was recrystallized from concentrated hydrochloric acid-water to give 1.8 g. (61%) of II [n=2].

7,8-Dimethyl-10-(ω-carboxymethyl)isoalloxazine (II) [n=1].

To a stirred solution of 7.2 g. (24 mmoles) of 7,8-dimethyl-10-(formylmethyl)isoalloxazine in 180 ml. of dimethylformamide at 16-20° under nitrogen was added 7.2 g. (45.5 mmoles) of potassium permanganate in five equal portions over a period of 100 minutes. The reaction mixture was added slowly with stirring to 1.2 liters of ether. The suspension was filtered and the residue washed with ether and dried *in vacuo* at room temperature. To this dark powder suspended in 200 ml. of 0.4 *N* hydrochloric acid was added 12.6 g. (100 mmoles) of solid sodium sulfite and 24.4 g. (100 mmoles) of barium chloride in 200 ml. of water. The mixture was stirred for 5 minutes and then adjusted to *p*H 8-8.5 with solid sodium bicarbonate. After the addition of 2 g. of decolorizing carbon, the solution was stirred for 5 minutes and filtered. The residue was washed with 50 ml. of saturated sodium bicarbonate followed by 50 ml. of water. The combined filtrates were extracted three times with a mixture of 225 ml. of benzyl alcohol and 75 ml. of *n*-butyl alcohol. The organic phases were washed twice with 50 ml. of saturated sodium bicarbonate. The combined aqueous solutions were extracted twice with 200 ml. of ether, adjusted to *p*H 1-2 with concentrated hydrochloric acid, and allowed to stand overnight at 4°. The precipitate was collected on a filter and washed three times with 20 ml. of cold water. Crystallization of the crude flavin from concentrated hydrochloric acid-water gave 3.6 g. (51%) of III [n=1]. The compound moved as a single spot in *n*-butyl alcohol-acetic acid-water (2:1:1) on TLC.

10-Flavinyl Amino Acid Methyl Esters (III) [n=3,4,5].

Two mmoles of II [n=3,4,5] and 0.324 g. (2 mmoles) of *N,N'*-carbonyldiimidazole in 100 ml. of dimethylformamide were stirred under nitrogen for 24 hours at room temperature. Two mmoles of the appropriate amino acid methyl ester hydrochloride were added and stirred for 24 hours. The solvent was evaporated *in vacuo* to a small volume (5 ml.) which was diluted with 200 ml. of *n*-butyl alcohol. The butanol solution was sequentially extracted with 100 ml. each of water, 0.5 *N* hydrochloric acid, water, 5% sodium bicarbonate, and water. The aqueous solutions were washed twice with 100 ml. of butanol. The combined butanol solutions were diluted with 200 ml. of chloroform, and the solution was dried with sodium sulfate and evaporated under reduced pressure to a small volume (5 ml.). The flavin was precipitated by dropwise addition of the concentrated butanol solution to rapidly stirred ether. The product was collected on a filter. One recrystallization from dimethylformamide-ether gave III in 60-70% yield. The peptides moved as a single spot in *n*-butyl alcohol-2 *N* ammonium hydroxide-ethanol (3:1:1) or chloroform-acetone (1:1) on TLC.

3-Flavinyl Amino Acid Methyl Esters (XI).

The same procedures were used as for the synthesis of III [n=3,4,5]. Yields obtained were 60-70%.

p-Nitrophenyl Ester of II [n=2,3].

A mixture of 2.3 mmoles of II [n=2,3] and 1.8 g. (4.5 mmoles) of *p*-nitrophenyl trifluoroacetate in 80 ml. of absolute pyridine was stirred at 60° under nitrogen for 20 minutes [n=2] or 90 minutes [n=3]. The reaction mixture was evaporated under reduced pressure to a small volume (15 ml.) and added to a stirred mixture of 250 ml. of ether and 250 ml. of water at 0°. The precipitate formed was collected on a filter and washed twice with 50 ml. of cold 5% sodium bicarbonate followed by 200 ml. of cold water, 200 ml. of a mixture of ether-acetone (4:1), and 100 ml. of ether. The product was dried *in vacuo* over phosphorus pentoxide for 24 hours at room temperature to yield 76% of *p*-nitrophenyl ester. This was used without further purification for the synthesis of the corresponding peptides III [n=2,3].

10-Flavinyl Amino Acid Methyl Esters (III) [n=3].

To 10 ml. of dimethylsulfoxide was added 0.225 g. (0.5 mmole) of the *p*-nitrophenyl ester of II [n=3] and 0.75 mmole of the appropriate amino acid methyl ester. The mixture was stirred at room temperature under nitrogen for 15 hours, then poured into 200 ml. of *n*-butyl alcohol. The butanol solution was extracted successively with 150 ml. each of water, 1 *N* hydrochloric acid, water, and 5% sodium bicarbonate. The aqueous solutions were washed twice with 100 ml. of butanol. The combined butanol solutions were diluted with 200 ml. of chloroform, dried with sodium sulfate, evaporated to a small volume (5 ml.), and the concentrated solution added dropwise to 100 ml. of vigorously stirred ether. The crude material was recrystallized from dimethylformamide-ether to give III [n=3] in 48-50% yield. The peptides moved as one spot in *n*-butyl alcohol-acetic acid-water (2:1:1) on TLC.

10-Flavinyl Amino Acid Methyl Esters (III) [n=2].

A mixture of 0.25 g. (0.575 mmole) of the *p*-nitrophenyl ester of II [n=2] and 0.63 mmole of the appropriate amino acid methyl ester in 10 ml. of dimethylsulfoxide was stirred at room temperature for 5 hours. Work up of the reaction mixture was as described for III [n=3], to give, after crystallization from dimethylformamide-ether, 51-56% yield of the flavin peptide III

[n=3]. The peptides showed one spot in *n*-butyl alcohol-acetic acid-water (2:1:1) on TLC.

10-Flavinyl Amino Acid Methyl Esters (III) [n=1].

A suspension of one millimole (0.3 g.) of II [n=2] and 0.1 g. of 10% palladium on charcoal in 30 ml. of dimethylformamide was hydrogenated at room temperature and 4 atmospheres pressure for 1 hour. To this mixture, under argon, was added 1.2 mmoles of 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide metho-*p*-toluenesulfonate and 1.2 mmoles of the appropriate amino acid methyl ester. The mixture was stirred for 48 hours and diluted with 70 ml. of ethyl acetate. The catalyst was removed by filtration and washed with a mixture of 100 ml. of ethyl acetate and 50 ml. of *n*-butyl alcohol. The combined filtrates were extracted twice with 200 ml. of water, followed by 200 ml. each of 0.5 *N* hydrochloric acid, water, 5% sodium bicarbonate, and water. The aqueous phases were washed twice with a mixture of 200 ml. of ethyl acetate and 50 ml. of butanol. The combined organic phases were dried over sodium sulfate and evaporated to dryness. The residue was dissolved in a minimal volume of dimethylformamide, added slowly to vigorously stirred ether (300 ml.), and the precipitated material collected on a filter. The peptides were recrystallized from dimethylformamide-water (R_1, R_3) and dimethylformamide-ether (R_2) in a yield of 40-59%. The peptides moved as a single spot in *n*-butyl alcohol-2 *N* ammonium hydroxide-ethanol (3:1:1) on TLC. A molecular weight determined by mass spectrometry for III [n=1, R_3]: calcd. 461; found 461.

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