Synthetic Flavinyl Peptides Related to the Active Site of Mitochondrial Monoamine Oxidase. I. Chemical and Spectral Properties†

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ABSTRACT: Various 8α-sulfur-linked flavinyl peptides related to the flavinyl peptides isolated from mitochondrial monoamine oxidase were synthesized in high yield and purity. The peptides, protected by an acetyl-blocking group on the amino terminus, were synthesized by conventional liquid-phase techniques and coupled to a tetraacetylriboflavin derivative activated in the 8α position. In some cases, the ribityl side chains of the flavinyl peptides were selectively deacetylated. In other cases, the thioether functions were oxidized to form sulfones. These flavinyl peptides were studied by uv-visible absorption and circular dichroic spectroscopies. A close correspondence in spectroscopic and other chemical properties indicated the identity of the synthetic and naturally obtained flavinyl peptides. Differences between the tetraacetylriboflavinyl and riboflavinyl peptides indicate an interaction between the ribityl side chain and thioether function in aqueous media. Evidence was obtained for an intramolecular complex between the tyrosyl and isoalloxazine moieties in aqueous media. Substitution in the 8α position was accompanied by an impairment of the protonation of the N^1 position of the isoalloxazine ring and a lowering of the redox potential relative to the parent 8-methylflavins.

Alteration of the spectral and redox properties of flavins usually occurs upon binding to flavoproteins. Therefore, determining the nature of the interactions of flavins with the amino acid components is prerequisite to understanding the enzymic behavior resulting from the composite of such interactions.

An amino acid residue that has been found to be intimately involved in the flavin-binding site of several flavoproteins is tyrosine (Strittmatter, 1961; Tu and McCormick, 1973; Watenpaugh et al., 1972; Anderson et al., 1972). Flavins have been shown to complex intermolecularly with phenols, including tyrosine, with varying tenacity (Weber, 1950; Harbury and Foley, 1958; Yagi and Matsuoka, 1956; Draper and Ingraham, 1970; Radda, 1966; Fleischman and Tollin, 1965a-c). Flavin-tyrosine interactions also have been investigated with model compounds in which the distance between interacting components was restricted by alkyl bridges (Föry et al., 1968, 1970; MacKenzie et al., 1969; Wu et al., 1970; Wu and McCormick, 1971a,b). Such studies surveyed the types of bonding that are involved in these complexes and some of the resultant changes in the flavin chemistry.

Another type of flavin-amino acid interaction is found in the various covalently appended flavin coenzymes. In these flavoenzymes, FAD is covalently bound via the flavin 8α position. It has been shown that attachment is to a nitrogen in position 3 of a histidyl residue of the protein in succinate dehydrogenase (Salach et al., 1972; Walker et al., 1972) and D-6-hydroxynicotine oxidase (Brühmüller et al., 1972; Möhler et al., 1972). A sulfur atom is involved as a thiohemiacetal linkage in cytochrome c_{552} (Walker et al., 1974; Kenney et al., 1974a) and as a thioether linkage in monoamine oxidase (Kearney et al., 1971; Walker et al., 1971). Some of these flavinyl peptides have been isolated, and some effects of these 8α-substituted amino acids on the properties of the flavins have been reviewed (Singer and Edmondson, 1974; Singer and Kenney, 1974). Further, aromatic amino acyl residues, especially tyrosyl residues, have been found in close proximity to the covalently bound flavin in several of these flavoproteins (Kenney et al., 1974a,b; Kearney et al., 1971; Walker et al., 1971, 1974; Möhler et al., 1972; Brühmüller et al., 1972).

Kearney and coworkers (1971) isolated a flavinyl pentapeptide from monoamine oxidase, 8α-(*S*-Ser-Gly-Gly-Cys-Tyr)-riboflavin 5'-phosphate. This flavinyl peptide, with the covalently bound cysteinyl residue and vicinal tyrosyl residue, provides a natural example where both the 8α-thioether linkage and phenolic function affect the properties of the flavin. Similar flavinyl peptide derivatives, as summarized in Table I, have now been synthesized and the nature of these interactions and their effects on the properties of flavins examined.

Materials and Methods

Amino acid derivatives were from commercial sources and recrystallized before use. Tetrahydrofuran was distilled from $LiAlH_4$ according to Fieser and Fieser (1967); purification of dioxane and dimethylformamide, and preparation of dioxane-4 N HCl, were done according to Stewart and Young (1969). Melting points were determined on a Fisher-

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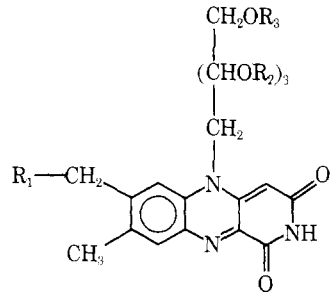
Table I: Abbreviations and Symbols for Synthetic and Natural Flavins.



Compound				
No.	Abbreviation	R_1	R_2	R_3
Riboflavin	RF	H	H	H
FMN	FMN	H	H	PO_3^{2-}
XVIII	Ac_4RF	H	$CO-CH_3$	$CO-CH_3$
XIX	Bromoflavin	Br	$CO-CH_3$	$CO-CH_3$
XXIII	AcCysAc$_4$RF	*N*-Ac-Cys	$CO-CH_3$	$CO-CH_3$
XXIV	AcCysRF	*N*-Ac-Cys	H	H
XXV	AcCys(O_2)Ac$_4$RF	*N*-Ac-Cys(O_2)	$CO-CH_3$	$CO-CH_3$
XX	AcCysTyrAc$_4$RF	*N*-Ac-Cys-Tyr	$CO-CH_3$	$CO-CH_3$
XXI	AcCysTyrRF	*N*-Ac-Cys-Tyr	H	H
XXII	AcCys(O_2)TyrAc$_4$RF	*N*-Ac-Cys(O_2)-Tyr	$CO-CH_3$	$CO-CH_3$
XXVI	AcGlyCysTyrAc$_4$RF	*N*-Ac-Gly-Cys-Tyr	$CO-CH_3$	$CO-CH_3$
XXVII	AcGlyCys(O_2)TyrAc$_4$RF	*N*-Ac-Gly-Cys(O_2)-Tyr	$CO-CH_3$	$CO-CH_3$
XXVIII	AcCysTyr(*O*-Me)Ac$_4$RF	*N*-Ac-Cys-Tyr(*O*-Me)	$CO-CH_3$	$CO-CH_3$

Johns melting point apparatus and are uncorrected.

The pK_a of the N^1 position was determined essentially according to the method of McCormick (1974) by diluting flavin stock solutions into solutions of varying HCl concentration (final flavin concentrations were 4×10^{-5} M) and immediately measuring A_{390}. Deacetylation of the ribityl side chain and hydrolysis of the 8α-substituent were determined to be negligible in the time required.

The oxidation-reduction potentials were determined by anaerobic titration with sodium dithionite in the presence of the internal standards, indigo disulfonate or anthraquinone 2,6-disulfonate, according to Edmondson and Singer (1973).

All syntheses involving flavins were carried out in a darkened room; all syntheses involving thioethers were performed with deoxygenated, peroxide-free, anhydrous solvents. Solvents were deoxygenated by bubbling through nitrogen gas (which had been deoxygenated with a vanadous sulfate solution (Meites and Meites, 1948) and dried with sulfuric acid) for 24 h. Thin-layer chromatograms were run on silica gel N-HR (Brinkmann) and compounds detected by iodine vapor or ultraviolet (uv) light. Solvent systems, referred to by subscripts to R_f values, were prepared in v/v ratios as follows: 1, chloroform–methanol (19:1); 2, chloroform–methanol–pyridine (18:1:5); 3, chloroform–methanol–acetic acid (18:1:1); 4, chloroform–methanol–acetic acid (18:3:1); 5, 1-butanol–acetic acid–water (4:1:1); 6, chloroform–methanol–ammonia (18:1:1); 7, chloroform–acetic acid (19:1); and 8, benzene–acetic acid–water (11:11:2, upper phase). All peptides were characterized by melting points, thin-layer chromatography, infrared (ir), and proton magnetic resonance (^{1}H NMR) spectra, and some by microanalysis. Many peptides were prepared by more than one synthetic route to further confirm their identities.

Infrared spectra were determined with either a Model 137B infracord or a Model 521 IR spectrophotometer (Perkin-Elmer). Ultraviolet and visible spectra were obtained with either a Cary Model 15 spectrophotometer or a Jasco ORD/UV/CD-5 optical rotatory dispersion (ORD) recorder. Circular dichroic (CD) spectra were determined in 1-cm, cylindrical, thermojacketed cells on the Jasco ORD/UV/CD-5. The temperature of the CD cell was regulated by a Haake Model F constant-temperature circulator. Proton magnetic resonance spectra were obtained with a Varian Model A60-A spectrometer and are given in parts per million (δ) from dimethyl sulfoxide (Me_2SO: δ 2.5) as internal standard. Only the blocking groups are listed below.

The syntheses of AcCysTyrAc$_4$RF, AcCysTyrRF, and AcCys(O_2)TyrAc$_4$RF are included below as representative of the synthesis of all the compounds listed in Table I. A description of the syntheses of all the compounds made for this investigation is included as supplementary material.

Ethyl N-Acetyl-S-benzyl-L-cysteinyl-L-tyrosinate (II). *N*-Acetyl-*S*-benzyl-L-cysteine (I; 2.5 g, 10 mmol) and 1.3 ml of *N*-methylmorpholine (10 mmol) were dissolved in 20 ml of tetrahydrofuran and cooled to -10 to -15 °C with stirring. Isobutyl chloroformate (1.3 ml, 10 mmol) was added, and the solution was stirred for 75 s. Ethyl L-tyrosinate (2.1 g, 10 mmol) was dissolved in 20 ml of dimethylformamide, precooled in the same bath, and added to the mixed-anhydride solution. The reaction mixture was stirred for 4 h at room temperature. After concentrating the reaction mixture in vacuo at 35 °C, the resultant oil was dissolved in 200 ml of ethyl acetate, filtered, and extracted successively with 5% $NaHCO_3$, water, 1 N HCl, water, and saturated NaCl. The organic phase was dried over anhydrous Na_2SO_4 and concentrated. Crystallization of product occurred upon the addition of hexane for 3.5 g of IX (7.9 mmol, 79% yield); R_{f_1} 0.35; NMR (Me_2SO-d_6) δ 1.8 (s, 3) *N*-Ac; 1.05 (t, 3), 3.92 (q, 2) ethyl ester; 7.32 (s, 5) *S*-Bzl.

N-Acetyl-S-benzyl-L-cysteinyl-L-tyrosine (III). II (3.4 g, 7.5 mmol) was dissolved in 32 ml of warm methanol, cooled to room temperature, and treated with 5.3 ml of 4 N NaOH. The mixture was left at room temperature for 75

min with occasional stirring, acidified to the Congo red end point with 1 N HCl, and diluted with water until no further turbidity was detected. The product was filtered, dried in vacuo over P_2O_5, and recrystallized from ethanol–water for 2.23 g of III (5.4 mmol, 72% yield; R_{f_4} 0.75; NMR (Me_2SO-d_6) δ 1.82 (s, 3) *N*-Ac; 7.29 (s, 5) *S*-Bzl; no ethyl ester.

N-Acetyl-L-cysteinyl-L-tyrosine (*V*). III (1.0 g, 2.4 mmol) was dissolved in 1 l. of liquid ammonia (distilled over sodium under strictly anhydrous conditions), and sodium was added in small pieces until the blue color persisted for 75 s. The blue color was quenched with $(NH_4)_2SO_4$ and the ammonia removed under aspirator vacuum. The mixture was further dried in vacuo over P_2O_5, dissolved in 50 ml of 0.5 N H_2SO_4, and treated with 3.6 ml of mercuric sulfate (Hopkin's reagent; Heidelberger and Kendall, 1929). The precipitate was collected by filtration, washed five times with water, and dried in vacuo over P_2O_5 to yield mercuric di(*N*-acetyl-L-cysteinyl-L-tyrosine) (IV).

IV (1.3 g) was suspended in 55 ml of deoxygenated water, saturated with H_2S until the white precipitate disappeared, and then gassed for an additional 30 min. The black HgS was removed by filtration and washed with deoxygenated water. The aqueous filtrate and washings were combined and concentrated in vacuo until precipitation commenced. The suspension was cooled and filtered to obtain some crystals, and the filtrate was concentrated to yield a second crop. The crystals were dried in vacuo over P_2O_5 to yield 0.43 mg of V (1.3 mmol, 55% yield); R_{f_4} 0.39, R_{f_5} 0.88; NMR (Me_2SO-d_6) δ 1.83 (s, 3); no ethyl ester; no *S*-Bzl.

Methyl N-tert-butyloxycarbonyl-S-benzyl-L-cysteinyl-L-tyrosinate (*VI*): mp 116–117 °C; R_{f_1} 0.57; NMR ($CDCl_3$) δ 1.39 (s, 9) *N*-Boc; 3.57 (s, 3) methyl ester; 7.32 (s, 5) *S*-Bzl.

Methyl S-benzyl-L-cysteinyl-L-tyrosinate (*VII*): R_{f_2} 0.81; NMR (Me_2SO-d_6) δ 3.54 (s, 3) methyl ester; 7.32 (s, 5) *S*-Bzl; no *N*-Boc.

p-Nitrophenol N-acetylglycinate (*VIII*): mp 130–131 °C; NMR (Me_2SO-d_6) δ 1.89 (s, 3) *N*-Ac; 7.44 (d, 2), 8.36 (d, 2) ONp ester.

Methyl N-acetylglycyl-S-benzyl-L-cysteinyl-L-tyrosinate (*IX*): mp 161.5–163.5 °C; R_{f_1} 0.18; NMR (Me_2SO-d_6) δ 1.86 (s, 3) *N*-Ac; 3.55 (s, 3) methyl ester; 7.32 (s, 5) *S*-Bzl.

N-Acetylglycyl-S-benzyl-L-cysteinyl-L-tyrosinate (*X*): mp 187–189 °C; R_{f_4} 0.45; NMR (Me_2SO-d_6) δ 1.86 (s, 3) *N*-Ac; 7.34 (s, 5) *S*-Bzl, no methyl ester.

Mercuric di(N-acetylglycyl-L-cysteinyl-L-tyrosine) (*XI*).

N-Acetylglycyl-L-cysteinyl-L-tyrosine (*XII*): R_{f_4} 0.25, R_{f_5} 0.85; NMR (Me_2SO-d_6) δ 1.86 (s, 3) *N*-Ac; no methyl ester, no *S*-Bzl.

Methyl O-methyl-L-tyrosinate hydrochloride (*XIII*): mp 184.5–186 °C; R_{f_6} 0.84; NMR (Me_2SO-d_6) δ 3.67 (s, 3) methyl ester; 3.75 (s, 3) *O*-Me.

Methyl N-acetyl-S-benzyl-L-cysteinyl-O-methyl-L-tyrosinate (*XIV*): mp 154–155 °C; R_{f_1} 0.75, R_{f_7} 0.53; NMR (Me_2SO-d_6) δ 1.82 (s, 3) *N*-Ac; 3.52 (s, 3) methyl ester; 3.71 (s, 3) *O*-Me; 7.32 (s, 5) *S*-Bzl, no methyl ester.

N-Acetyl-S-benzyl-L-cysteinyl-O-methyl-L-tyrosine (*XV*): mp 180–183 °C; R_{f_3} 0.54; NMR (Me_2SO-d_6) δ 1.82 (s, 3) *N*-Ac; 3.73 (s, 3) *O*-Me; 7.33 (s, 5) *S*-Bzl, no methyl ester, no *S*-Bzl.

Mercuric di(N-acetyl-L-cysteinyl-O-methyl-L-tyrosine) (*XVI*).

N-Acetyl-L-cysteinyl-O-methyl-L-tyrosine (*XVII*): R_{f_4} 0.6, R_{f_5} 0.73; NMR (Me_2SO-d_6) δ 1.82 (s, 3) *N*-Ac; 3.73 (s, 3) *O*-Me.

2′,3′,4′,5′-Tetra-O-acetylriboflavin (*XVIII*): mp 250–252 °C; R_{f_3} 0.51.

8α-Bromo-2′,3′,4′,5′-tetra-O-acetylriboflavin (*XIX*). XVIII (0.9 g, 1.7 mmol) was dissolved in 8.8 ml of dioxane and heated to reflux with stirring. Dibenzoyl peroxide (5 mg dissolved in a few drops of dioxane) was added, and 0.93 g of dioxane dibromide (3.7 mmol) in 6 ml of dioxane was added dropwise over 8 min. The reaction mixture was refluxed 20 min after the addition of dioxane dibromide was completed, cooled to room temperature, and evaporated to dryness in vacuo at 30 °C. The oily residue was dissolved in 25 ml of chloroform and washed successively with 0.05 M potassium phosphate buffer (pH 7) and water. The solution was dried over Na_2SO_4, concentrated to dryness in vacuo, dissolved in 8 ml of chloroform, dropped into 18 ml of ether, and cooled to −15 °C to give 0.81 g of XIX (1.3 mmol); R_{f_8} 0.64 (>95% XIX, no detectable 8α-dibromotetraacetylriboflavin as judged by NMR). This is essentially a modification of the synthesis of Walker et al. (1972).

8α-S-(N-Acetyl-L-cysteinyl-L-tyrosyl)-2′,3′,4′,5′-tetra-O-acetylriboflavin (*XX*). V (0.40 g, 1.23 mmol) and XIX (0.78 g, 1.25 mmol) were dissolved in 12 ml of dimethylformamide, and the solution was made anaerobic by bubbling through deoxygenated N_2 gas. Triethylamine (0.31 ml, 2.2 mmol) was added dropwise over 5 min and the reaction left at room temperature under N_2 for 50 min. The reaction mixture was dropped into 300 ml of cold ether; the product was removed by filtration, washed with ether, and dried in vacuo. The product was dissolved in 15 ml of dimethylformamide and stirred into 300 ml of 0.05 M potassium phosphate buffer (pH 6, deoxygenated). The precipitate was filtered and washed twice with 25-ml portions of deoxygenated water. The combined aqueous filtrates were extracted with chloroform, acidified to pH 1.5 with 1 N HCl, and again extracted with chloroform. The combined chloroform extracts from the acidified solution were dried over Na_2SO_4, concentrated to about 20 ml, and stirred into cold ether. The product was filtered and dried in vacuo for 0.45 g (0.52 mmol, 43% yield); R_{f_4} 0.46.

8α-S-(N-Acetyl-L-cysteinyl-L-tyrosyl)riboflavin (*XXI*). XX (0.19 g, 0.2 mmol) was suspended in 50 ml of deoxygenated 2 N HCl and heated to 50 °C under N_2 for 2 h. The solution was cooled to room temperature, adjusted to pH 4–5 with deoxygenated, saturated $NaHCO_3$ solution (deoxygenated by bubbling CO_2 through the solution), and saturated with $(NH_4)_2SO_4$. The flavin was extracted into two 5-ml portions of liquified phenol (92% in water), and the combined phenolic extracts were stirred into 100 ml of cold ether, which had been recently treated with neutral alumina. The ether was decanted, leaving an oily precipitate. The ether layer was extracted three times with 5-ml portions of water, and the aqueous extracts were used to dissolve the oily precipitate. The aqueous solutions were combined and extracted with 5- then 2.5-ml portions of liquified phenol. The combined phenolic extracts were stirred into 100 ml of cold ether. The ether was decanted and extracted three times with 5-ml portions of water. The aqueous extracts were used to dissolve the oily precipitate, combined, extracted with ether, treated with activated charcoal, filtered, and lyophilized for 55 mg (0.09 mmol, 40% yield); R_{f_5} 0.34.

Table II: Absorption Maxima and Molar Extinction Coefficients for Synthetic and Natural Flavins.

Flavin	Absorption maxima, nm (Extinction Coefficient × 10^4 l. mol^{-1} cm^{-1})			
	Neutral[a]		"Cationic"[b]	
	Thioether	Sulfone	Thioether	Sulfone
$AcCysAc_4RF$	448 (1.2), 365 (0.8), 270 (2.9), 223 (3.1)	448 (1.2), 352 (1.0), 271 (3.2), 223 (3.6)	396 (1.4), 268 (2.8), 220 (3.3)	400 (sh), 380 (1.6), 269 (3.1), 222 (3.0)
$AcCysTyrAc_4RF$	448 (1.2), 365 (0.8), 270 (2.9), 223 (4.2)	448 (1.2), 352 (1.0), 271 (3.4), 223 (4.7)	396 (1.5), 268 (2.9), 221 (2.7)	400 (sh), 384 (1.7), 271 (3.5), 224 (4.2)
$AcCysAc_4RF$ + AcTyrEt	448 (1.2), 365 (0.8), 268 (2.9), 223 (3.8)			
$AcCysTyr(O\text{-Me})Ac_4RF$	448 (1.2), 366 (0.8), 271 (3.2), 224 (4.7)		395 (1.6), 271 (3.1), 225 (3.8)	
$AcGlyCysTyrAc_4RF$	448 (1.2), 359 (0.86), 271 (3.1), 222 (3.6)	448 (1.2), 352 (1.0), 271 (3.8), 223 (4.7)	388 (1.4), 268 (2.9), 221 (3.2)	400 (sh), 384 (1.6), 271 (3.5), 224 (4.2)
AcCysRF	448 (1.2), 368 (0.87), 269 (3.1), 224 (3.8)		394 (1.7), 268 (3.4), 221 (3.5)	
AcCysTyrRF	448 (1.2), 366 (0.85), 269 (3.3), 222 (4.0)		394 (1.6), 269 (3.1), 222 (2.7)	
CysRF[c]	448 (1.2), 367 (0.86), 270 (?)	448 (1.2), 354 (1.0), 270 (?)	395 (1.5), 268 (?)	400 (sh), 375 (?), 268 (?)
Naturally obtained flavinyl pentapeptide[c]	448 (1.2), 367 (0.86), 270 (?)	448 (1.2), 354 (1.0), 270 (?)	395 (1.5), 268 (?)	400 (sh), 375 (?), 268 (?)
Ac_4RF	445 (1.2), 374 (1.0), 268 (5.1), 224 (3.3)		396 (2.1), 268 (5.3), 224 (2.8)	

[a] Neutral spectra were 4×10^{-5} M flavin in 0.05 M potassium phosphate buffer (pH 7). [b] Cationic spectra were 4×10^{-5} M flavin in 8 N HCl. [c] Taken from Walker et al. (1971). Cationic spectra were taken in 6 N HCl.

8α-S-(N-Acetyl-L-cysteinyl-L-tyrosyl)-2′,3′,4′,5′-tetra-O-acetylriboflavin S,S-Dioxide (XXII). XX (0.09 g, 1 mmol) and 0.1 ml of acetic anhydride were dissolved in 1 ml of glacial acetic acid. The solution was treated with 1 ml of 25% hydrogen peroxide–acetic acid (1:10, v/v; peroxyacetic acid, 40 mmol) and left overnight at room temperature. The mixture was diluted with 250 ml of ether and filtered, and the precipitate was dried in vacuo for 0.079 g (0.87 mmol, 84% yield); R_{f_4} 0.23.

8α-S-(N-Acetyl-L-cysteinyl)-2′,3′,4′,5′-tetra-O-acetylriboflavin (XXIII): R_{f_4} 0.29.

8α-S-(N-Acetyl-L-cysteinyl)riboflavin (XXIV): R_{f_5} 0.23.

8α-S-(N-Acetyl-L-cysteinyl)-2′,3′,4′,5′-tetra-O-acetylriboflavin S,S-dioxide (XXV): R_{f_4} 0.18.

8α-S-(N-Acetylglycyl-L-cysteinyl-L-tyrosyl)-2′,3′,4′,5′-tetra-O-acetylriboflavin (XXVI): R_{f_4} 0.71.

8α-S-(N-Acetylglycyl-L-cysteinyl-L-tyrosyl)-2′,3′,4′,5′-tetra-O-acetylriboflavin S,S-dioxide (XXVII): R_{f_4} 0.16.

8α-S-(N-Acetylglycyl-L-cysteinyl-O-methyl-L-tyrosyl)-2′,3′,4′,5′-tetra-O-acetylriboflavin (XXVIII): R_{f_4} 0.69.

Results and Discussion

The present procedure for the synthesis of 8α-bromoflavin is essentially a modification of the earlier procedure of Walker et al. (1972). As this compound is used for further syntheses with little purification, a purer product than that reported earlier was desired.

8α-(S-Cysteinyl)-2′,3′,4′,5′-tetra-O-acetylriboflavin was synthesized first by Ghisla and Hemmerich (1971) and later by Falk et al. (1972). The properties of a somewhat purer form of the synthetic compound were compared to those of the naturally obtained flavinyl pentapeptide of monoamine oxidase (Kearney et al., 1971; Walker et al., 1971). The earlier syntheses (Ghisla and Hemmerich, 1971) did not report yield, and, in our hands, the yields were unsatisfactory (Falk et al., 1972). The thioether moiety of the product was found to be constantly decomposing, both hydrolytically and oxidatively, during even the mildest purification procedures; it was only somewhat stable as a solid under nitrogen. The incorporation of the *N*-acetyl-blocking group on the amino terminus of the amino acid or peptide moiety imparts an unusual stability for reasons not completely understood. The blocked amino function permits the use of higher concentrations of reactants because of increased solubility, and permits base catalysis, which increases the rate and yield of the reaction of the amino acid of peptide with the bromoflavin and decreases the formation of side products. Standard purification and storage procedures become feasible; consequently, the *N*-acetyl peptidyl flavins were synthesized in the present work for up to 50% yields and 99% purities (free of flavin-like contaminants, as judged by thin-layer chromatography). Further, the N-blocked flavinyl peptides are better models for the milieu of the active site of the enzyme.

The spectral properties of the N-blocked flavinyl peptides are essentially identical with the properties of free amino flavinyl peptides, either synthetic (Ghisla and Hemmerich, 1971) or purified from natural sources (Kearney et al., 1971).

The present synthetic flavinyl peptides, $AcCysAc_4RF$, $AcCysTyrAc_4RF$, $AcCysTyr(O\text{-Me})Ac_4RF$, $AcGlyCysTyrAc_4RF$, AcCysRF, and AcCysTyrRF, all give tests that are positive with chloroplatinate and negative with iodine azide, nitroprusside, and cyanide nitroprusside (except with excess reagent and long incubation times). After oxidation by peroxyacetic acid, the synthetic compounds, viz., $AcCys(O_2)Ac_4RF$, $AcCys(O_2)TyrAc_4RF$, and $AcGlyCys(O_2)Ac_4RF$, were negative to all of the above tests. This behavior is identical with that observed by Walker et al. (1971) for both the naturally obtained flavinyl pentapeptide and the synthetic cysteinyl riboflavin, and is consistent with a highly reactive and oxidizable thioether linkage.

Visible–Uv Absorption Spectra. The neutral and cationic absorption maxima and molar extinction coefficients of the

synthetic *N*-acetyl flavinyl peptides and naturally obtained flavinyl pentapeptide are compiled in Table II. Walker et al. (1971) reported that substitution in the 8α position by a thioether brings about only a slight bathochromic shift in the 445-nm band but causes hypochromicity and a hypsochromic shift of the near-uv band, viz., λ_{max} 445 and 374 nm, with ϵ 1.2 and 1.0×10^4, respectively, for Ac_4RF; λ_{max} 448 and 367 nm, with ϵ 1.2 and 0.86×10^4, respectively, for the flavinyl pentapeptide. Upon oxidation of the 8α-thioether to a sulfone, the near-uv absorption band undergoes a further hypsochromic shift and a hyperchromicity effect, viz., λ_{max} 367 nm, with ϵ 0.86×10^4, for the flavinyl pentapeptide thioether; λ_{max} 354 nm, with ϵ 1.0×10^4, for the corresponding sulfone. All of these trends are apparent in Table II for the flavinyl peptides synthesized in the present investigation.

The "cationic" spectra of the flavinyl pentapeptide reported by Walker et al. (1971) exhibit a hypochromicity of the long-wavelength absorption band relative to Ac_4RF. After oxidation of the thioether to a sulfone, the cationic spectrum evidences a shoulder at 400 nm and shifts hypso- and hyperchromically. Again, this behavior is paralleled by the synthetic tetraacetylated flavinyl peptides.

The spectral similarity of the isolated flavinyl pentapeptide and the synthetic riboflavinyl (deacetylated) derivatives is even more striking. A comparison of $AcCysTyrAc_4RF$ with an equimolar mixture of $AcCysAc_4RF$ and AcTyrEt reveals no apparent hypo- or hyperchromicity of the 270-nm absorption band, while the 223-nm band of the deacetylated flavinyl dipeptide is slightly hyperchromic. This hyperchromism may be due to a weak dispersion-force interaction between the closely spaced transitions of the flavinyl and tyrosyl moieties and would indicate a "head-to-tail" alignment of the two transitions (Urry, 1973). The lack of a strong hyper- or hypochromism in the uv absorption spectrum of $AcCysTyrAc_4RF$ can be explained by either an ineffective alignment or too weak an interaction between the two chromophores, or both.

The difference absorption spectra in Figure 1 reveal the broadening at long wavelengths and the hypsochromic shift of the near-uv absorption band exhibited by all of the 8α-sulfur-linked flavinyl peptides. Minor differences are apparent among the 8α-thioether-substituted Ac_4RF derivatives, which largely disappear after deacetylation of the ribityl side chain. Previously, the acetyl-blocking groups on the ribityl side chain, electronically insulated from the chromophoric isoalloxazine ring system by aliphatic carbon atoms, had been thought not to affect the absorption spectrum. The variance between the flavinyl peptide-Ac_4RF and -RF spectra may be due to dissimilar conformation of the molecules in solution brought about by a ternary interaction of the peptide, isoalloxazine ring system, and ribityl side chain moieties.

pK_a of N^1 of the Isoalloxazine Ring. Acidification of typical flavins leads first to protonation of the N^1 position of the isoalloxazine ring and results in both spectral and fluorescence changes (Penzer and Radda, 1967; McCormick, 1974). In the visible region, the absorption spectra change from the double-banded, neutral flavoquinone spectra to the single-banded, cationic spectra, with a λ_{max} near 390 nm (cf. Table II). Protonation can be measured, then, by recording the increase in the absorbance at this wavelength. The pK_a values are determined by the inflection (midpoint) from plots of A vs. the Hammett acidity function (H_0). H_0 for various HCl concentrations were obtained from the data

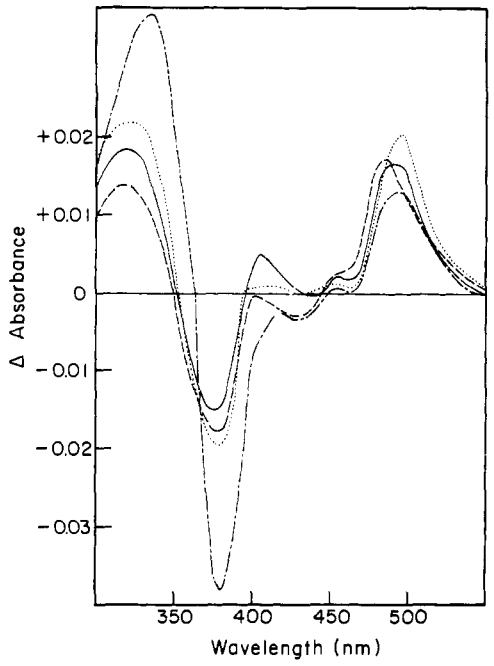


FIGURE 1: Difference absorption spectra of tetraacetylated flavinyl peptides vs. Ac_4RF. Flavins were 10^{-5} M in 0.05 M potassium phosphate buffer (pH 7). Compounds are: AcGlyCysTyrAc$_4$RF (···); AcCysTyrAc$_4$RF (- - -); AcCysAc$_4$RF (—); and AcCys(O$_2$)TyrAc$_4$RF (-·-).

Table III: pK_a Values for Flavins.

Flavin	pK_a	
	Thioether	Sulfone
Ac_4RF	−1.3 ± 0.1	
$AcCysAc_4RF$	−1.7 ± 0.1	−2.2 ± 0.1
$AcCysTyrAc_4RF$	−1.8 ± 0.1	−2.2 ± 0.1
$AcGlyCysTyrAc_4RF$	−1.8 ± 0.1	
RF or FMN	−0.3 ± 0.1	
AcCysRF	−0.7 ± 0.1	
AcCysTyrRF	−0.7 ± 0.1	

of Paul and Long (1957). The pK_a values in Table III are in essential agreement with those obtained by McCormick (1974), and earlier by Michaelis et al. (1936), for riboflavin.

Acetylation of the ribityl chain has been shown to increase the acidity of N^1 (McCormick, 1974). This is confirmed by the present data, as the pK_a values for all acetylated compounds tested are more negative by one pK_a unit than the corresponding free ribityl derivatives. Substitution by a thioether in the 8α position additionally leads to an increase in acidity of N^1 due to the electron-withdrawing properties of the sulfur. Oxidation of the thioether to a sulfone leads to an expected further increase in acidity. The vicinal tyrosyl residue in some of the flavinyl peptides has no significant effect on the pK_a of N^1. Because the sulfone derivatives are only partly protonated in 6 N HCl (H_0 −2.1), absorption spectra of these compounds in this solvent represent mixtures of neutral and cationic species. This is the reason for the shoulder at 410 nm in so-called cationic absorption spectra previously reported for such compounds (Walker et al., 1971). Although the 8α-sulfonyl-Ac_4RF derivatives are incompletely protonated, even in 8 N HCl, sufficiently rapid decomposition of the flavinyl peptides occurs

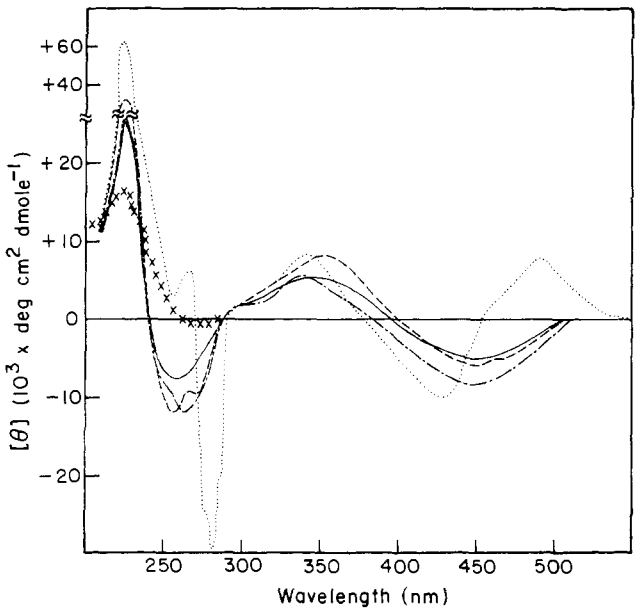


FIGURE 2: CD spectra of tetraacetylated flavinyl peptides in aqueous solution. Above 300 nm, flavins were 2×10^{-4} M, and below 300 nm, 4×10^{-5} M in 0.05 M potassium phosphate buffer (pH 7). Compounds are: AcGlyCysTyrAc$_4$RF (···); AcCysAc$_4$RF (—); AcCysTyrAc$_4$RF (- - -); AcCys(O$_2$)TyrAc$_4$RF (-·-); and AcTyrEt (×××).

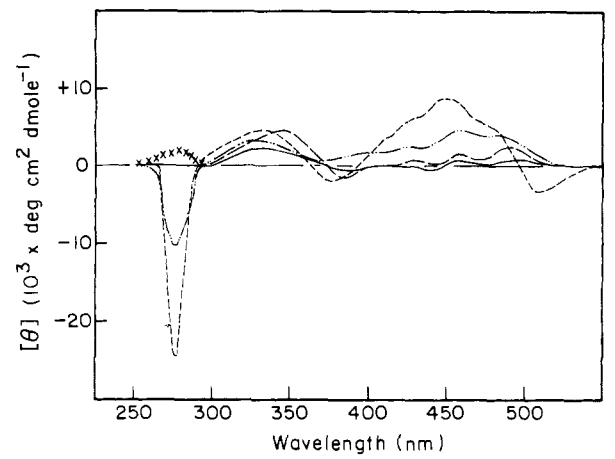


FIGURE 3: CD spectra of tetraacetylated flavinyl peptides in chloroform. Above 300 nm, flavins were 2×10^{-4} M, and below 300 nm, 4×10^{-5} M. Compounds are: AcCysTyrAc$_4$RF (- - -) AcCysTyr(*O*-Me)Ac$_4$RF (-·-); Ac$_4$RF (- -); AcCysAc$_4$RF (—); and AcTyrEt (×××).

with higher HCl concentrations to prevent the accumulation of reliable spectral data.

Oxidation-Reduction Potentials. Oxidation-reduction potentials were calculated by the method of Edmondson and Singer (1973) from a plot of E_h vs. log [oxidized flavin]/[reduced flavin]. Only titrations that corresponded closely to a theoretical two-electron reduction were accepted. RF and Ac$_4$RF yielded $E_{m,7}$ values of −0.199 and −0.179 V, respectively. $E_{m,7}$ values were −0.174 and −0.154 for AcCysRF and AcCysAc$_4$RF, respectively. The $E_{m,7}$ values for AcCysTyrAc$_4$RF, AcCysTyr(*O*-Me)Ac$_4$RF, and AcCysTyrRF did not differ significantly from those of the corresponding 8α-AcCys derivatives. AcCys(O$_2$)Ac$_4$RF decomposed upon reduction by dithionite, as was previously observed for the corresponding analogue with an unblocked amino terminus (Edmondson and Singer, 1973).

It is apparent that 8α-thioether substitution is accompanied by a rise in the oxidation-reduction potential of approximately 0.025 V, and there is no additional shift induced by the vicinal tyrosyl residue. Acetylation of the ribityl side chain induced a rise in the oxidation-reduction potential of approximately 0.02 V for all of the analogues tested. This points out the large effect on the oxidation-reduction potential of flavins that can be caused by merely sterically protecting the N^1 position of the isoalloxazine ring within a binding site of a flavoprotein.

Circular Dichroic Spectra. The circular dichroism observed with flavins is due to perturbation of the environment about the optically inactive chromophoric group, the isoalloxazine ring, by either the optically active (but nonabsorbing in the visible and near-uv regions) ribityl side chain (Tollin, 1968) and/or other compounds capable of complex formation with, or otherwise constrained near, the isoalloxazine ring (Miles and Urry, 1968; Kenney et al., 1974). The long-wavelength extremum has been attributed to "through space" interactions between all of the chiral centers of the ribityl side chain with the chromophore (Scola-Nagelschneider and Hemmerich, 1972; Harders et al., 1974), whereas the near-uv extremum may be due to "through chain" interactions with the 2′-chiral center only.

No such assignments have been made for the uv Cotton effects. Reciprocal relationships, such as those exhibited in the uv region of the CD spectra of FAD, have been attributed to a coupling of transitions of closely spaced frequency of the two chromophores (isoalloxazine and adenine), which are juxtaposed in the most-favored conformation in solution (Miles and Urry, 1968). Similar perturbation in conjunction with additional tyrosine-dependent perturbations of the isoalloxazine ring are reflected in the CD spectra of the various proteinase-liberated peptides of cytochrome c_{552} (Kenney et al., 1974).

The CD spectra of synthetic 8α-substituted flavinyl peptides are reported in Figures 2 and 3. The CD spectrum of AcCysAc$_4$RF is quite similar to that reported for Ac$_4$RF (Edmondson and Tollin, 1971). The small but significant differences between AcCysAc$_4$RF and AcCysTyrAc$_4$RF may be due to an interaction between the isoalloxazine and tyrosyl moieties. The control CD spectrum (not shown) of an equimolar mixture of AcCysAc$_4$RF and AcTyrEt is exactly equal to the sum of the CD spectra of the two components, which substantiates the additional interaction between the two moieties in AcCysTyrAc$_4$RF and, by extension, in AcCys(O$_2$)TyrAc$_4$RF and AcGlyCysTyrAc$_4$RF. Deacetylation of the ribityl side chain of Ac$_4$RF decreases the observed ellipticity (Edmondson and Tollin, 1971); the same trend is apparent for the flavinyl peptides in the present study. Interestingly, while AcCysRF and AcCysTyrRF exhibit the greatest differences between the longest wavelength Cotton effects, and no apparent differences between the 350-nm Cotton effects, the opposite is true for the corresponding tetraacetylated derivatives. This is another indication of additional interactions from the free ribityl hydroxyl functions. The small differences between the CD spectra of AcCysTyrAc$_4$RF and AcCys(O$_2$)TyrAc$_4$RF may indicate minor conformational changes. The CD spectrum of AcGlyCysTyrAc$_4$RF, wherein the longest wavelength Cotton effect is split into positive and negative extrema and the uv region exhibits more fine structure and new extrema, suggests that additional interactions, such as may occur in the milieu of the monoamine oxidase flavin-binding site,

may alter the electronic properties of the flavin.

A comparison of the CD spectra of AcCysAc$_4$RF, AcTyrEt, and AcCysTyrAc$_4$RF in chloroform (Figure 3) also reveals an interaction between the tyrosine and isoalloxazine chromophores. Not surprisingly, in this aprotic and relatively nonpolar solvent, hydrogen bonding of the phenolic hydroxyl function to the isoalloxazine ring undoubtedly plays a role in stabilizing this interaction, since the CD spectra of AcCysTyr(*O*-Me)Ac$_4$RF shows significantly lower ellipticity in all respects than AcCysTyrAc$_4$RF.

Acknowledgment

We acknowledge the competent assistance of Mr. Peter C. Preusch in the determination of oxidation–reduction potentials.

Supplementary Material Available

Experimental material describing syntheses of all compounds made for this investigation (12 pages). Ordering information is given on any current masthead page.

References

Anderson, R. D., Apgar, P. A., Burnett, R. M., Darling, G. D., LeQuesne, M. E., Mayhew, S. G., and Ludwig, M. L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3189.

Brühmüller, M., Möhler, H., and Decker, K. (1972), *Eur. J. Biochem. 29,* 143.

Draper, R. D., and Ingraham, L. L. (1970), *Arch. Biochem. Biophys. 139,* 265.

Edmondson, D. E., and Singer, T. P. (1973), *J. Biol. Chem. 248,* 8144.

Edmondson, D. E., and Tollin, G. (1971), *Biochemistry 10,* 113.

Falk, M. C., Johnson, P. G., and McCormick, D. B. (1972), Abstracts, 164th National Meeting of the American Chemical Society, New York, N.Y., No. Biol-250.

Fieser, L. F., and Fieser, M. (1967), Reagents for Organic Synthesis, Vol. 1, New York, N.Y., Wiley.

Fleischman, D. E., and Tollin, G. (1965a), *Biochim. Biophys. Acta 94,* 248.

Fleischman, D. E., and Tollin, G. (1965b), *Proc. Natl. Acad. Sci. U.S.A. 53,* 38.

Fleischman, D. E., and Tollin, G. (1965c), *Proc. Natl. Acad. Sci. U.S.A. 53,* 237.

Föry, W., MacKenzie, R. E., and McCormick, D. B. (1968), *J. Heterocycl. Chem. 5,* 625.

Föry, W., MacKenzie, R. E., Wu, F. Y.-H., and McCormick, D. B. (1970), *Biochemistry 9,* 515.

Ghisla, S., and Hemmerich, P. (1971), *FEBS Lett. 16,* 229.

Harbury, H. A., and Foley, K. A. (1958), *Proc. Natl. Acad. Sci. U.S.A. 44,* 662.

Harders, H., Förster, S., Voelter, W., and Bacher, A. (1974), *Biochemistry 13,* 3360.

Heidelberger, M., and Kendall, F. E. (1929), *J. Biol. Chem. 84,* 639.

Kearney, E. B., Salach, J. I., Walker, W. H., Seng, R. L., Kenney, W. C., Zeszotek, E., and Singer, T. P. (1971), *Eur. J. Biochem. 24,* 321.

Kenney, W. C., Edmondson, D. E., and Singer, T. P. (1974a), *Eur. J. Biochem. 48,* 449.

Kenney, W. C., Edmondson, D. E., Singer, T. P., Steenkamp, D. J., and Schabort, J. C. (1974b), *FEBS Lett. 41,* 111.

Law, H. D., and duVigneaud, V. (1960), *J. Am. Chem. Soc. 82,* 4579.

MacKenzie, R. E., Föry, W., and McCormick, D. B. (1969), *Biochemistry 8,* 1839.

McCormick, D. B. (1970), *J. Heterocycl. Chem. 7,* 447.

McCormick, D. B. (1974), *J. Heterocycl. Chem. 11,* 969.

Meites, L., and Meites, T. (1948), *Anal. Chem. 20,* 984.

Michaelis, L., Schubert, M., and Smythe, C. V. (1936), *J. Biol. Chem. 116,* 587.

Miles, D. W., and Urry, D. W. (1968), *Biochemistry 7,* 2791.

Möhler, H., Brühmüller, M., and Decker, K. (1972), *Eur. J. Biochem. 29,* 152.

Paul, M. A., and Long, F. A. (1957), *Chem. Rev. 57,* 1.

Penzer, G. R., and Radda, G. K. (1967), *Q. Rev., Chem. Soc. 21,* 43.

Radda, G. K. (1966), *Biochim. Biophys. Acta 112,* 448.

Salach, J., Walker, W. H., Singer, T. P., Ehrenberg, A., Hemmerich, P., Ghisla, S., and Hartmann, U. (1972), *Eur. J. Biochem. 26,* 267.

Scola-Nagelschneider, G., and Hemmerich, P. (1972), *Z. Naturforsch. B. 27,* 1044.

Singer, T. P., and Edmondson, D. E. (1974), *FEBS Lett. 42,* 1.

Singer, T. P., and Kenney, W. C. (1974), *Vitam. Horm. (N.Y.) 32,* 1.

Stewart, J. M., and Young, J. D. (1969), Solid Phase Peptide Synthesis, San Francisco, Calif., W. H. Freeman.

Strittmatter, P. (1961), *J. Biol. Chem. 236,* 2329.

Tollin, G. (1968), *Biochemistry 7,* 720.

Tu, S.-C., and McCormick, D. B. (1973), *J. Biol. Chem. 248,* 6339.

Urry, D. W. (1973), *Res. Dev. 24,* 28.

Walker, W. H., Kearney, E. B., Seng, R. L., and Singer, T. P. (1971), *Eur. J. Biochem. 24,* 328.

Walker, W. H., Kenney, W. C., Edmondson, D. E., Singer, T. P., Cronin, J. R., and Hendriks, R. (1974), *Eur. J. Biochem. 48,* 439.

Walker, W. H., Singer, T. P., Ghisla, S., and Hemmerich, P. (1972), *Eur. J. Biochem. 26,* 279.

Watenpaugh, K. D., Sieker, L. C., Jensen, L. H., LeGall, J., and Dubourdieu, M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3185.

Weber, G. (1950), *Biochem. J. 47,* 114.

Wu, F. Y.-H., MacKenzie, R. E., and McCormick, D. B. (1970), *Biochemistry 9,* 2219.

Wu, F. Y.-H., and McCormick, D. B. (1971a), *Biochim. Biophys, Acta 229,* 440.

Wu, F. Y.-H., and McCormick, D. B. (1971b), *Biochim. Biophys. Acta 236,* 479.

Yagi, K., and Matsuoka, Y. (1956), *Biochem. Z. 228,* 138.

Zahn, H., and Otten, H. G. (1962), *Justus Liebigs Ann. Chem. 653,* 139.