[33] Synthesis of N^6-(2-Aminoethyl)-FAD, N^6-(6-Carboxyhexyl)-FAD, and Related Compounds

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Introduction

X-ray diffraction studies of the three-dimensional structure of crystalline complexes of flavin adenine dinucleotide (FAD)-dependent enzymes with its coenzyme in many cases reveal an externally oriented adenine moiety of the FAD embedded in the active site.[1] Hence the C-6-bound amino group of the adenine nucleus is the preferred target of chemical modification for any functionalization of the FAD molecule in which the redox activity is to be maintained.

An established strategy for introducing reactive carboxyl or primary amino groups at this position for NAD or NADP includes some crucial steps.[2] The first step is alkylation of the N-1 position of the adenine ring by nucleophilic displacement to give, under aqueous conditions, an alkyl-bound reactive group leading to an N^1-functionalized coenzyme analog. A few highly electrophilic reagents are available for such alkylation reactions, such as iodoacetic acid, propiolactone, or 3,4-epoxybutyric acid for a carboxylic group and ethyleneimine for the primary amino group. N^1-Functionalized adenine-containing coenzymes are, in the long term, chemically unstable due to spontaneous rearrangement to the N^6-functionalized derivative. The next step is a base-catalyzed Dimroth rearrangement under aqueous conditions for conversion to this chemically stable N^6-functionalized derivative. The reaction conditions needed for an acceptable fast quantitative conversion are normally extreme (e.g., pH 10.5–11.0, 70°, 2- to 3-hr reaction time). Under such conditions, undesirable side reactions of FAD are likely, for instance cleavage of the alkaline-susceptible C–N-10 bond between the ribitol and isoalloxazine moieties. Bückmann found that as an exception N^1-(2-aminoethyl)-FAD can still be relatively quickly rearranged to N^6-(2-aminoethyl)-FAD under mild aqueous conditions (e.g., pH 6.0–7.0, 50°, 6- to 7-hr reaction time for 100%

[1] F. S. Mathews, *Curr. Opin. Struct. Biol.* **1,** 954 (1991).
[2] A. F. Bückmann and G. Carrea, *Adv. Biochem. Eng.* **39,** 97 (1989).

0076-6879/97 $25

conversion).[3,4] Under these mild conditions, only about 10% rearrangement can be achieved within 7 hr in the case of N^1-(carboxylalkyl)-modified coenzyme derivatives. Presumably, the primary amino group of the N^1-attached 2-aminoethyl moiety eases the intramolecular Dimroth rearrangement required for base-catalyzed ring opening between the alkylated N-1 and the C-2 position of the adenine. Simultaneously, tricyclic 1,N^6-ethanoadenine-FAD is formed from N^1-(2-aminoethyl)-FAD by a parallel reaction. This tricyclization reaction under mild aqueous conditions is new in heterocyclic organic chemistry. Surprisingly, the presence of arsenate or organic/inorganic phosphate strongly catalyzes this tricyclization reaction. The parallel Dimroth rearrangement and tricyclization have been studied in detail using the model compound N^1-(2-aminoethyl)-adenosine.[5]

Usually the straightforward approach of starting out from native coenzymes for synthesizing N^6-functionalized adenine-containing coenzyme analogs has the disadvantage that the spacer length of the alkyl chain cannot be varied at will by CH_2 increments because of the limited availability of alkylating reagents. However, a coupling strategy for the synthesis of FAD from FMN and AMP by Cramer and Neunhofer[6] has been adapted by Stocker *et al.*[7] for the synthesis of N^6-functionalized FAD derivatives with terminal carboxylic groups on a more variable spacer. FMN, purified from a commercial preparation by a new method based on reversed-phase chromatography, is covalently attached to the heptyl ester of N^6-(carboxyalkyl)-AMP by diphosphate coupling. The protected carboxyl group of the resulting heptyl ester of N^6-(carboxyalkyl)-FAD is enzymatically deprotected by lipase to give N^6-(carboxyalkyl)-FAD. The protection of the carboxyl group is required, as a free carboxyl group interferes considerably by cross-reactivity with the diphosphate coupling.

The synthesis according to an established strategy under mild conditions, purification, and characterization of N^1-(2-aminoethyl)-FAD, tricyclic 1,N^6-ethanoadenine-FAD, and N^6-(2-aminoethyl)-FAD from native FAD are described in detail here. Similarly the synthesis, purification, and characterization of N^6-(carboxyalkyl)-FAD and intermediate compounds, as exemplified for N^6-(6-carboxyhexyl)-FAD and its reactive succinimido ester, are also described. The role of N^6-(2-aminoethyl)-FAD and N^6-(6-carboxy-

[3] A. F. Bückmann, H. Erdmann, M. Pietzsch, J. M. Hall, and J. V. Bannister, *in* "Flavins and Flavoproteins" (K. Yagi, ed.), p. 597. de Gruyter, Berlin, 1994.
[4] A. F. Bückmann, Eur. Patent 0,247,537 (1991); U.S. Patent 5,399,681 (1995).
[5] A. F. Bückmann, V. Wray, and H. C. van der Plas, *Heterocycles* **41,** 1399 (1995).
[6] F. Cramer and H. Neunhofer, *Chem. Ber.* **95,** 1612 (1961).
[7] A. Stocker, H. J. Hecht, and A. F. Bückmann, *Eur. J. Biochem.* **238,** 519 (1996).

hexyl)-FAD as starting compounds for further derivatizations and applications is considered.

Representative Synthetic Procedures

N^6-(2-Aminoethyl)-FAD and Related FAD Derivatives

General. The structural assignments are based on ultraviolet–visible (UV–Vis) spectrophotometry, ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and negative ion electrospray ionization–mass spectrometry (ESI–MS). Ultraviolet spectra are recorded in 0.1 *M* sodium phosphate buffer, pH 7.0, using 11,300 M^{-1} cm^{-1} as molar absorption coefficient at 450 nm for concentration determinations. Thin-layer chromatography is carried out on silica gel 60-F_{254} plates (0.2 mm; Merck, Rahway, NJ) with a fluorescence marker in isobutyric acid–25% aqueous NH_3–H_2O (66 : 33 : 1, v/v/v), pH 3.7, to follow the progress of the conversion of FAD by reaction with ethyleneimine and the Dimroth rearrangement of N^1-(2-aminoethyl)-FAD to N^6-(2-aminoethyl)-FAD and the parallel conversion to tricyclic 1,N^6-ethanoadenine-FAD. Quantitative determination of the composition of the reaction mixtures is performed at 450 nm by high-speed scanning after thin-layer chromatography.

N^6-(2-Aminoethyl)-FAD. Ethyleneimine (2 ml, 40 mmol; Serva, Heidelberg, Germany) is added slowly to an aqueous solution of FAD (sodium salt, 11 g, 12.7 mmol; Serva) in 15 ml, maintaining the pH at 3.5 by simultaneous manual addition of $HClO_4$ (70%, w/w). The reaction solution is gently stirred in the dark at 30° for 76 hr. During this period, the pH is maintained at pH 3.5 by periodic addition of $HClO_4$ (70%, w/w). After diluting the reaction solution with water to 60 ml, the crude product is precipitated in cold ethanol (600 ml) to remove unreacted ethyleneimine. The precipitated crude product is recovered by centrifugation and redissolved in water (60 ml). This precipitation procedure is repeated three times. The final precipitate containing FAD (2.5 mmol, 19.7%, R_f 0.37), N^1-(2-aminoethyl)-FAD (8.5 mmol, 67%, R_f 0.29), and by-products, presumably mainly N^1-(2-aminoethylaminoethyl)-FAD (1.68 mmol, 13.3%, R_f 0.10) is dissolved in water (900 ml). After adjusting the pH to 6.3 with LiOH (1 *M*), the solution is incubated at 40° until N^1-(2-aminoethyl)-FAD conversion is complete.

After reducing the volume to 20 ml by rotary evaporation, the reaction mixture is lyophilized. The final dry reaction mixture (14 g) contains FAD (2.28 mmol, 18.8%), N^6-(2-aminoethyl)-FAD (6.69 mmol, 55%, R_f 0.35), tricyclic 1,N^6-ethanoadenine-FAD (1.82 mmol, 15%, R_f 0.26) and by-products, mainly N^6-(2-aminoethylaminoethyl)-FAD (1.37 mmol, 11%, R_f 0.15). Lyophilized reaction mixture (600 mg), dissolved in distilled water (2 ml),

pH 7.0 (adjusted with 1 *N* NaOH), is loaded on a LiChroprep RP-18 reversed-phase column (Lobar, 310-25, 40–63 μm; Merck), integrated into a fast protein liquid chromatography (FPLC) system (Pharmacia, Piscataway, NJ) and equilibrated against water at room temperature. After gradient elution [3000 ml, 0–30% (v/v) methanol in water; flow rate, 7 ml/min] and lyophilization of the pooled fractions (controlled by thin-layer chromatography), 100 mg of pure N^6-(2-aminoethyl)-FAD (0.115 mmol) is obtained in 22% overall yield (λ_{max} at 267 nm; calculated for $C_{26}H_{39}O_{15}N_{10}P_2$ 828, found ESI–MS $[M - H]^-$ 827).

1,N^6-Ethanoadenine-FAD. Lyophilized crude 1,N^6-ethanoadenine-FAD, collected from two gradient runs for the purification of N^6-(2-aminoethyl)-FAD (80 mg) and dissolved in water (6 ml), is applied on a LiChroprep RP-18 reversed-phase column (Lobar, 310-25, 40–63 μm; Merck), integrated into an FPLC system (Pharmacia) and equilibrated against water at room temperature. After gradient elution [2000 ml, 0–20% (v/v) methanol in water; flow rate, 7 ml/min] and lyophilization of the pooled fractions, 16 mg of pure 1,N^6-ethanoadenine-FAD (0.019 mmol) is obtained in 9.5% overall yield (λ_{max} at 262 nm; calculated for $C_{29}H_{34}O_{15}N_9P_2Na_2$ 854, found ESI–MS $[M - H]^-$ 853).

N^1-(2-Aminoethyl)-FAD. Lyophilized reaction mixture containing FAD (0.192 mmol), N^1-(2-aminoethyl)-FAD (0.354 mmol), and by-products (0.054 mmol), dissolved in water (20 ml) and adjusted to pH 5.0 with LiOH (1 *M*), is applied to a Bio-Rex 70 (Bio-Rad, Richmond, CA) cation-exchanger column (50–100 mesh, 1.5 × 60 cm), equilibrated against water at 4°, pH 3.5, adjusted with 0.1 *N* HCl. Elution under these conditions gives FAD within 250 ml. Subsequent elution with LiCl (0.5 *M*, pH 3.5) affords a fraction with pure N^1-(2-aminoethyl)-FAD in 1 liter. After concentration to 10 ml by rotary evaporation, repeated precipitation in ethanol (three 250-ml volumes per charge) at 4°, and lyophilization, salt-free pure N^1-(2-aminoethyl)-FAD (188 mg, 0.215 mmol) is obtained in 36% overall yield (λ_{max} at 261 nm; calculated for $C_{29}H_{39}O_{15}N_{10}P_2$ 828, found ESI–MS $[M - H]^-$ 827). [The development of a convenient reversed-phase procedure for the purification of N^1-(2-aminoethyl)-FAD from the reaction mixture was not successful owing to the need for at least two rechromatography steps, leading to a much lower yield compared to that of the cation-exchange procedure.]

All purified FAD analogs are pure on the basis of the results of thin-layer chromatography (one fluorescent spot excited at 366 nm), have typical FAD absorption maxima at 377 and 450 nm, and characteristic NMR spectra (Tables I and II[7a]).

[7a] A. F. Bückmann and V. Wray, *Biotechnol. Appl. Biochem.* **15,** 303 (1992).

TABLE I
^{1}H CHEMICAL SHIFTS OF FAD, N^1-(2-AMINOETHYL)-FAD, N^6-(2-AMINOETHYL)-FAD, AND 1,N^6-ETHANOADENINE-FAD[a]

Assignment	FAD	N^1-(2-Amino-ethyl)-FAD[b]	N^6-(2-Amino-ethyl)-FAD	1,N^6-Ethanoadenine-FAD
Flavin moiety				
F6	7.51	7.49	7.53	7.53
F7-Me	2.28[c]	2.28	2.28	2.28
F8-Me	2.36	2.43	2.39	2.41
F9	7.52	7.68	7.57	7.69
F1′A	4.89	4.84	4.90	4.91
F1′B	4.40	4.38	4.36	4.58
F2′	4.35	4.38	4.31	4.26
F3′	3.92	3.96	3.91	3.91
F4′	4.03	4.17	4.03	3.97
F5′A	4.29	4.30	4.22	4.10
F5′B	4.11	4.10	4.06	4.04
Adenine moiety				
A2	7.85	8.59	7.94	8.55
A8	8.29	8.66	8.27	8.63
A1′	5.85	6.11	5.79	6.06
A2′	4.54	4.64	4.48	4.71
A3′	4.48	4.57	4.42	4.47
A4′	4.36	4.36	4.31	4.25
A5′AB	4.28	4.26	4.22	4.11
CH_2(1)		4.69	3.62	4.81
CH_2(2)		3.57	3.21	4.21

[a] At pD 7 in D_2O.
[b] At pD 4.0.
[c] All ^{1}H signals of FAD were referenced to the methyl signal of the sodium salt of 3-(trimethylsilyl)propanesulfonic acid and subsequently the highest field signal in the derivative spectra, belonging to F7-Me, was corrected to the value in FAD (2.28 ppm).

Dimroth rearrangement of N^1-(2-aminoethyl)-FAD to N^6-(2-aminoethyl)-FAD leads to a typical shift of λ_{max} from 261 to 267 nm. An absorption maximum at 262 nm for 1,N^6-ethanoadenine-FAD indicates alkylation of the N-1 position of the adenine moiety. The reaction scheme for the synthesis of N^6-(2-aminoethyl)-FAD, 1,N^6-ethanoadenine-FAD, and intermediate N^1-(2-aminoethyl)-FAD is depicted in Fig. 1.

N^6-(2-Aminoethyl)-FAD as Starting Compound for Further Derivatization

N^6-(2-Aminoethyl)-FAD has been reacted with *N*-(2-methylferrocene)-caproic acid, activated in the presence of water-soluble 1-ethyl-3-(3-dimeth-

TABLE II

^{13}C CHEMICAL SHIFTS OF FAD, N^1-(2-AMINOETHYL)-FAD, N^6-(2-AMINOETHYL)-FAD, AND 1,N^6-ETHANOADENINE-FAD[a]

Assignment	FAD	N^1-(2-Amino-ethyl)-FAD[b]	N^6-(2-Amino-ethyl)-FAD	1,N^6-Ethanoadenine-FAD
F2[c] s	160.26	160.11	160.38	160.38
F4[c] s	163.71	163.27	163.84	163.71
F6 d	133.00	132.83	132.97	132.99
F7 s	141.78	141.99	141.89	142.04
F8 s	153.18	152.94	153.34	153.06[c]
F9 d	119.21	119.61	119.34	119.01
F11 s	134.04	134.47	134.10	134.35
F12 s	136.51	136.49	136.57	136.59
F13[c] s	137.09	137.02	137.11	137.09
F14[c] s	152.59	152.49	152.64	152.93
F1′ t	50.01	50.02	49.97	50.18[d]
F2′ d	72.03	71.84	71.93	71.88
F3′ d	75.21	75.74	75.23	75.12
F4′ d	73.92[e]	74.14[e]	73.93[e]	73.79[e]
F5′ t	70.39[e]	70.08[e]	70.29[e]	69.68[e]
F8-Me q	23.39	23.44	23.43	23.44
F7-Me q	21.27[f]	21.27	21.27	21.27
A2 d	155.06	150.40d	155.00	147.54
A4 s	150.85	148.95	150.58	152.57
A5 s	120.46	121.47	121.11	119.13
A6 s	157.42	153.40s	156.62	153.35[c]
A8 d	141.88	145.84d	142.05	146.41
A1′ d	89.84	91.71	89.73	91.15
A2′ d	77.55	77.94	77.54	77.82
A3′ d	72.77	72.20	72.75	72.95
A4′ d	86.23[e]	86.40	86.32	87.06[e]
A5′ t	67.82[e]	67.23[e]	67.84[e]	67.46[e]
CH_2(1) t		50.81	41.76	51.64[d]
CH_2(2) t		39.33	40.95	50.69[d]

[a] In D_2O at pD 7. The assignments for FAD were unambiguously established from the 2D COSY (^{1}H assignments) and inverse 2D HMQC and HMBC spectra.

[b] At pD 4.0.

[c] These assignments can be interchanged.

[d] The assignment of these signals follows from COSY and HMQC spectra, and comparison with the corresponding NAD derivatives.[7a]

[e] These signals appear as doublets from coupling to phosphorus.

[f] All ^{13}C signals of FAD were referenced to the methyl signal of the sodium salt of 3-(trimethylsilyl)propanesulfonic acid and subsequently the highest field signal in the derivative spectra, belonging to F7-Me, was corrected to the value in FAD (21.27 ppm).

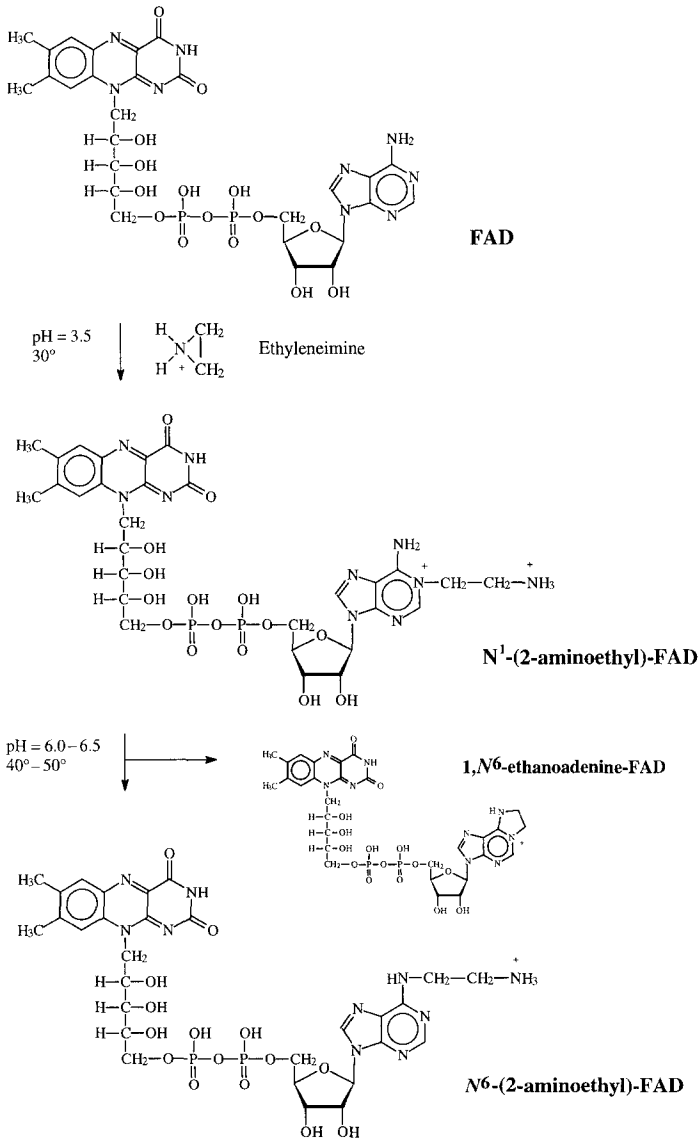


FIG. 1. Reaction scheme for the synthesis of N^6-(2-aminoethyl)-FAD, tricyclic 1,N^6-ethanoadenine-FAD, and intermediate N^1-(2-aminoethyl)-FAD under mild aqueous conditions.

ylaminopropyl)carbodiimide for the synthesis of the ferrocene-modified FAD analog N^6-[*N*-(2-methylferrocene)caproylamidoethyl]-FAD. This redox relay–FAD conjugate has been reconstituted with apoglucose oxidase from *Aspergillus niger* and apo-D-amino-acid oxidase from pig liver, leading to a hologlucose oxidase and holo-D-amino-acid oxidase with one specifically site-oriented electron-mediating ferrocene moiety per enzyme subunit. The semisynthetic electroglucose oxidase and electro-D-amino-acid oxidase exhibited electrical communication via an electrode similar to that of native FAD-containing glucose oxidase and D-amino-acid oxidase in the presence of an excess of dissolved ferrocenecarboxylic acid.[8] Compared to glucose oxidase, randomly modified with several unspecifically positioned ferrocene molecules on the surface according to Badia *et al.*,[9] it was calculated that the new ferrocene-FAD glucose oxidase conjugate showed even superior electrical communication properties. The site-specific reconstitution to semisynthetic electroflavoenzymes leads to a novel general method for establishing electrical communication through a structurally defined path between flavoenzymes and electrode surfaces.

Similarly, N^6-(2-aminoethyl)-FAD has been covalently coupled to a short-spacered thio derivative of pyrroloquinoline quinone (PQQ), strongly adsorbed as a monolayer on the gold surface of an electrode. Reconstitution with apoglucose oxidase resulted in an electrode-bound glucose oxidase monolayer with a surface density close to the theoretical limit and containing integrated PQQ as electron relay for electronic communication between glucose oxidase and the electrode. This relayed reconstituted glucose oxidase monolayer provides an unprecedentedly high glucose electrooxidation rate with a current density reaching the theoretical limit for a maximally packed enzyme layer without serious interference from molecular oxygen or ascorbate in the physiologically relevant glucose concentration range of 2–50 m*M*.[10]

Nitrospiropyran-functionalized FAD has been synthesized by coupling of *N*-hydroxysuccinimide-activated photochromic 1-(β-carboxyethyl)-3,3-dimethyl-6′-nitrospiro[indoline-2,2′-2*H*-benzospiran] to N^6-(2-aminoethyl)-FAD. A photoswitchable glucose oxidase was prepared by reconstitution of apoglucose oxidase with the photochromic FAD derivative that exhibits reversible photoisomerizable properties between the nitrospiropyran–FAD-reconstituted glucose oxidase (switched off at 360 nm $< \lambda <$

[8] A. Riklin, E. Katz, I. Willner, A. Stocker, and A. F. Bückmann, *Nature (London)* **376,** 672 (1995).

[9] A. Badia, R. Carlini, A. Fernandez, F. Batteglini, S. R. Mikkelsen, and A. M. English, *J. Am. Chem. Soc.* **115,** 7053 (1993).

[10] I. Willner, V. Heleg, R. Blonder, E. Katz, G. Tao, A. F. Bückmann, and A. Heller, *J. Am. Chem. Soc.* **118,** 10321 (1996).

400 nm with low electron transport activity reduced to <10%) and the nitromerocyanine–FAD-reconstituted glucose oxidase (switched on at $\lambda >$ 475 nm with high electron transport activity). The off-and-on switching effect is a consequence of structural changes in the enzyme protein owing to a conformational difference between the nitrospiropyran and nitromerocyanine moieties of the incorporated photochromic FAD derivative. Presumably, the penetration path of the diffusional electron-relay ferrocenecarboxylic acid into the nitrospiropyran–FAD-reconstituted glucose oxidase is perturbed. Both photoisomeric forms of reconstituted glucose oxidase, assembled as a monolayer on a gold electrode, reveal different electrical communication with electrode surfaces and photoregulated bioelectrocatalytic activities for the electrochemical oxidation of glucose in the presence of ferrocenecarboxylic acid as electron transfer mediator.[11] In principle, the resulting novel enzyme electrode acts as an active interface for the cyclic amperometric transduction of recorded optical signals.

N^6-(6-Carboxyhexyl)-FAD and Related Compounds

General. The structural assignments are based on UV-Vis spectrophotometry, ^{1}H NMR data, and negative ion fast atom bombardment (FAB)–MS with glycerol as matrix. Spectrophotometric measurements of nucleotide and flavin compounds are carried out in sodium phosphate buffer (0.1 *M*), pH 7.0, using molar absorption coefficients of 12,500 M^{-1} cm^{-1} at 445 nm for FMN, 11,300 M^{-1} cm^{-1} at 450 nm for FAD and FAD analogs, and 8400 M^{-1} cm^{-1} at 263 nm for 6-chloroadenine-AMP. The progress of the conversions and the purity of all nucleotides and flavin compounds synthesized were checked by high-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC). HPTLC is carried out on silica gel 60 F_{254} HPTLC plates (Merck) in triethylammonium bicarbonate buffer (1 *M*)–ethanol [3:7 (v/v), pH 7.5]. Analytical HPLC is performed in ammonium formate (0.1 *M*), pH 3.7, containing 20% (v/v) methanol, at 35° at a flow rate of 1.0 ml/min on a Nucleosil (120-5 C_{18}, 4 × 125 mm; Macherey-Nagel, Dueren, Germany) reversed-phase column.

Purification of 5'-FMN from Commercial FMN. Crude FMN (520 mg, 1.0 mmol; Fluka, Buchs, Switzerland) is dissolved in 8 ml of water and loaded on a LiChroprep NH_2 column (Lobar B, 310-25, 40–63 μm; Merck) equilibrated against triethylammonium acetate buffer (1 m*M*), pH 5.8. A linear gradient is applied from equilibration buffer to triethylammonium acetate (1 *M*), pH 5.8 (1250 ml at a flow rate of 6 ml/min). Two fractions

[11] I. Willner, R. Blonder, E. Katz, A. Stocker, and A. F. Bückmann, *J. Am. Chem. Soc.* **118,** 5310 (1996).

are collected containing a mixture of 3′-FMN and 5′-FMN and pure 4′-FMN, respectively. 5′-FMN is separated from 3′-FMN by loading the first fraction on a LiChroprep RP-18 reversed-phase column (Lobar B, 310-25, 40–63 μm; Merck) and applying a linear gradient from water to methanol (1600 ml at a flow rate of 6 ml/min). After collection of the fractions with pure 5′-FMN, removal of the solvent by rotary evaporation, and redissolving in water (25 ml), lyophilization leads to 5′-FMN (235 mg, 0.42 mmol), 99% pure by HPLC in 45% yield. After dissolving in water (100 ml) and passage through an AG-50W-X4 column (pyridinium resin, 2.5 × 15 cm; Bio-Rad), pure 5′-FMN can be converted to its tri-*n*-octylammonium salt by adding tri-*n*-octylamine (153 mg, 0.43 mmol) to the eluate. After removal of the solvent by rotary evaporation, the viscous residue is dried by repeated addition and evaporation of anhydrous pyridine (three 10-ml washes) followed by addition and evaporation of dry dimethylformamide (three 10-ml washes).

6-Chloroadenine-AMP. A solution of freshly distilled phosphoryl chloride (4.5 ml, 49 mmol) and water (0.3 ml) in triethyl phosphate (15 ml) is added at −17° to a stirred solution of 6-chloro-9-β-D-ribofuranosylpurine (8.4 g, 30 mmol; Pharma Waldhof, Düsseldorf, Germany) in triethyl phosphate (150 ml). The addition is repeated three times at intervals of 30 min and the reaction mixture is left overnight at 4°. The mixture is then titrated by adding NaOH (4 *M*) at 4° to pH 6.0 until no pH change is observed within 10 min. After extraction of triethyl phosphate with diethyl ether (three 500-ml washes) and subsequent lyophilization, the residue (48.5 g) contains 6-chloroadenine-AMP and inorganic salts. This is divided into three charges of ca. 16 g and each charge, dissolved in 40 ml of ethanol–water (1:9, v/v), is loaded on an Amberlite XAD-16 column (5 × 36 cm; Serva) equilibrated against the same solvent. Elution with this solvent at a flow rate of 5 ml/min and subsequent removal of the solvent from the pooled fractions give a salt-free product of 6-chloroadenine-AMP (6.4 g, 16.8 mmol) as a white powder in 60% yield and 98% purity by HPLC. The product is identical with commercial 6-chloroadenine-AMP by HPTLC, HPLC, and UV-Vis spectrophotometry.

N^6-(6-Carboxyhexyl)-AMP Heptyl Ester. 6-Chloroadenine-AMP (0.56 g, 1.47 mmol) is added at room temperature to 6-aminocaproic acid heptyl ester (7.0 g, 30.5 mmol), prepared from 6-aminocaproic acid and 1-heptanol by azeotropic esterification in the presence of *p*-toluenesulfonic acid as described by Braun *et al.*[12] The mixture is heated to 80° and incubated at this temperature overnight with stirring. 6-Chloroadenine-AMP dissolves completely within 1 hr under these conditions and after 4 hr almost complete

[12] P. Braun, H. Waldmann, W. Vogt, and H. Kunz, *Liebigs Ann. Chem.* 165 (1991).

conversion is observed as monitored by HPLC. The reaction mixture, dissolved in 150 ml of 2-propanol–water (1 : 1, v/v) at pH 3.7 (adjusted with 1 *N* HCl), is passed through a Bio-Rex 70 cation-exchange column (hydrogen resin, 2.5 × 30 cm; Bio-Rad), equilibrated against the same solvent as eluent. Removal of the solvent from the pooled fractions by rotary evaporation gives the product, N^6-(6-carboxyhexyl)-FAD heptyl ester (0.79 g, 1.4 mmol), as an oily residue in 95% yield, 97% pure by HPLC. ^{1}H NMR (CD_3OD) δ/ppm = 0.92 (t, 3H, H7‴), 1.30–1.80 (m, rest of N^6 side-chain protons), 2.38 (t, 2H, H5″), 3.61 (bm, 2H, H1″), 4.08 (t, 2H, H1‴), 4.14 (m, 2H, A5′AB), 4.27 (m, 1H, A4′), 4.44 (q, 1H, A3′), 4.72 (t, 1H, A2′), 6.12 (d, 1H, A1′), 8.26 (s, 1H, A2), 8.51 (s, 1H, A8). Calculated for $C_{23}H_{38}N_5O_9P$ 559, found FAB–MS $[M - H]^-$ 558.

N^6-(6-Carboxyhexyl)-FAD Heptyl Ester. N^6-(6-carboxyhexyl)-AMP heptyl ester is converted to its tri-*n*-octylammonium salt by adding tri-*n*-octylamine (520 mg, 1.47 mmol) in 20 ml of dimethylformamide–dioxane (1 : 1, v/v) and subsequently removing the solvent by rotary evaporation. Diphenyl phosphochloridate (0.39 ml, 1.9 mmol) and tri-*n*-butylamine (0.83 ml, 3.5 mmol) are added to the tri-*n*-octylammonium salt of N^6-(6-carboxyhexyl)-AMP heptyl ester (1.28 g, 1.4 mmol) dissolved in a mixture of dimethylformamide (1.5 ml) and dioxane (10.5 ml) with vigorous shaking. The mixture is left at room temperature for 2.5 hr and the solvent is removed by rotary evaporation. After shaking the residue in dry diethyl ether (50 ml) and removing the solvent, the precipitated gum is dissolved in dioxane (3 ml) and the last traces of diethyl ether are removed by evaporation. A solution of the tri-*n*-octylammonium salt of 5′-FMN (2 mmol) in a mixture of dimethylformamide (3 ml) and pyridine (7 ml) is added to the residue with vigorous shaking and incubated at room temperature overnight. After removal of the solvent, the residue is dissolved in 100 ml of water–2-propanol (1 : 1, v/v) and the solution is passed through an AG-50W-X4 cation-exchange column (pyridinium resin, 2.5 × 15 cm; Bio-Rad), equilibrated against the same solvent as the eluent. The residue from the pooled fractions, after evaporation, is dissolved in a mixture of water (36 ml) and pyridine (4 ml) and applied to a LiChroprep RP-18 reversed-phase column (Lobar B, 310-25, 40–63 μm; Merck) integrated into an FPLC system (Pharmacia) and equilibrated against ammonium formate buffer (0.1 *M*), pH 3.7, containing methanol (10%, v/v). After elution with two column volumes of this solvent, the residue is purified by gradient elution (equilibration buffer to methanol, 1.5 liters, 6 ml/min). Removal of the solvent from the pooled fractions gives the pure product, N^6-(6-carboxyhexyl)-FAD heptyl ester (0.7 g, 0.7 mmol) in 48% yield, 97% pure by HPLC. ^{1}H NMR (dimethyl sulfoxide) δ/ppm = 0.82 (t, 3H, H7‴), 1.1–1.6 (m, rest of N^6 side-chain protons), 2.25 (m, 2H, H5″), 2.31 (s, 3H, F7-Me), 2.37 (s,

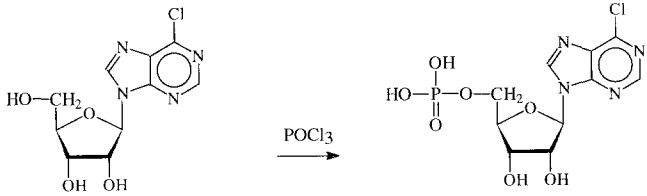
3H, F8-Me), 3.3–5.0 (m, rest of sugar protons, and H1″ and H-1‴ of N^6 side chain), 5.91 (d, 1H, A1′), 7.77 (s, 1H, F6), 7.88 (s, 1H, F9), 8.16 (s, 1H, A2), 8.44 (s, 1H, A8). Calculated for $C_{40}H_{56}N_9O_{17}P_2$ 997, found FAB–MS $[M - H]^-$ 996.

N^6-(6-Carboxyhexyl)-FAD. Commercial lipase Lipomod 224 (0.4 g; Biocatalysts, Mid Glamorgan, U.K.) is added to a clear solution of N^6-(6-carboxyhexyl)-FAD heptyl ester (0.43 g, 0.43 mmol) in sodium phosphate buffer (0.25 *M*, 400 ml), pH 7.0. After stirring the mixture gently for 4 hr at 35°, total enzymatic deprotection of the carboxyhexyl group is achieved as shown by HPTLC: R_f for N^6-(6-carboxyhexyl)-FAD heptyl ester) is 0.55; R_f for N^6-(6-carboxyhexyl)-FAD is 0.40. After concentration, filtration, and lyophilization, the crude residue is dissolved in water and applied to a LiChroprep RP-18 reversed-phase column (Lobar B, 310-25, 40–63 μm; Merck), equilibrated against ammonium formate (0.1 *M*), pH 3.7, and eluted with two column volumes of equilibration buffer. The residue is purified by gradient elution (equilibration buffer to methanol, 4.0 liters, 6 ml/min) to give, after removal of the solvent from the pooled fractions, a pure product, N^6-(6-carboxyhexyl)-FAD (0.243 g, 0.27 mmol) in 63% yield, 97% pure by HPLC. ^{1}H NMR (D_2O) δ/ppm = 1.16 (m, 2H, H-3″), 1.25 (m, 2H, H-4″), 1.42 (m, 2H, H2″), 2.12 (t, 2H, H-5″), 2.23 (s, 3H, F7-Me), 2.32 (s, 3H, F8-Me), 2.88 (m, 2H, H1″), 3.90–4.80 (m, rest of sugar protons), 5.78 (d, 1H, A1′), 7.48 (s, 1H, F6), 7.75 (s, 1H, F9), 8.23 (s, 1H, A2), 8.41 (s, 1H, A8). Calculated for $C_{33}H_{43}N_9O_{17}P_2$ 899, found FAB–MS $[M - H]^-$ 898. A typical absorption maximum at 267 nm indicates the alkylated N^6 state of the adenine moiety.

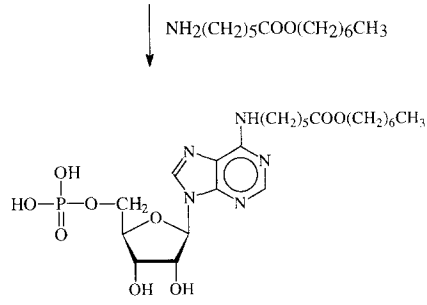
N^6-(6-Carboxyhexyl)-FAD Succinimido Ester. Diisopropylethylamine (15 μl, 87.6 μmol) and *O*-(*N*-succinimidyl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (16 mg, 52.5 μmol) in dimethylformamide (1.8 ml) is added to N^6-(6-carboxyhexyl)-FAD (16 mg, 17.8 μmol) dissolved in water (200 μl). The same two reagents are added again in the same quantities after 1 hr to drive the activation of the carboxyhexyl group to completion (control by HPTLC). Repeated extraction of the reaction mixture with diethyl ether (four 3-ml washes) and subsequent removal of the solvent by rotary evaporation yield an orange-colored gum containing N^6-(6-carboxyhexyl)-FAD succinimido ester in quantitative yield and 100% pure by HPTLC (R_f, 0.50). The activated form of N^6-(6-carboxyhexyl)-FAD is always freshly prepared before use, as it is relatively unstable (slow decomposition, even when stored at −20°).

The reaction pathway for the synthesis of N^6-(6-carboxyhexyl)-FAD, its activated form, and intermediate compounds is depicted in Fig. 2.

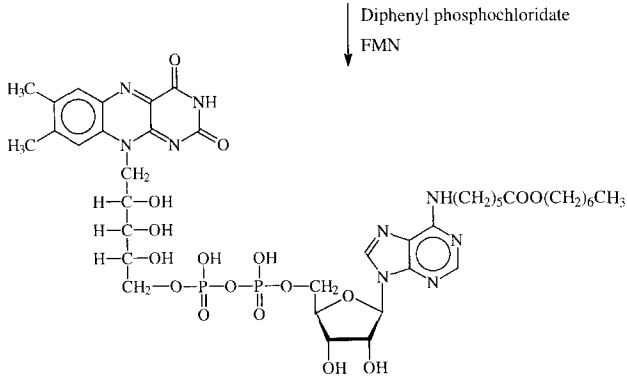
It is anticipated that other N^6-(carboxyalkyl)-FAD derivatives with different spacer lengths can be synthesized analogously without difficulty.



6-chloroadenosine **6-chloroadenine-AMP**

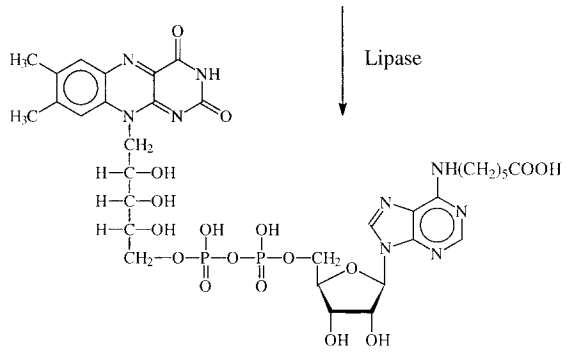


N^6-(6-carboxyhexyl)-AMP-heptyl ester

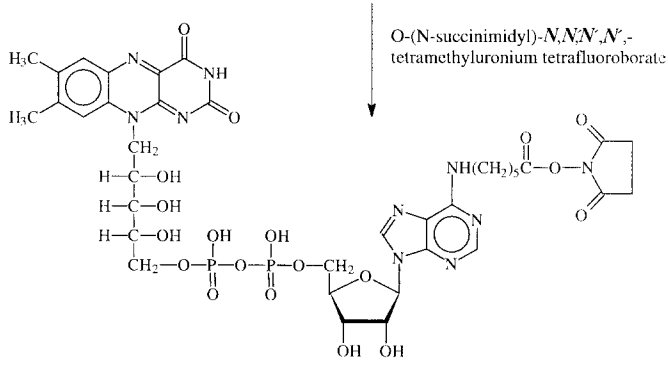


N^6-(6-carboxyhexyl)-FAD-heptyl ester

FIG. 2. Reaction scheme for the synthesis of N^6-(6-carboxyhexyl)-FAD, its activated succinimido ester, and intermediate compounds.



N^6-(6-carboxyhexyl)-FAD



N^6-(6-carboxyhexyl)-FAD succinimido ester

FIG. 2. (*continued*)

Application of N^6-(6-Carboxyhexyl)-FAD Succinimido Ester. A procedure for the covalent attachment of N^6-(6-carboxyhexyl)-FAD succinimido ester to apo-D-amino-acid oxidase from pig kidney has been developed, leading to semisynthetic N^6-(6-carbamoylhexyl)-FAD-D-amino-acid oxidase with one covalently bound FAD analog per subunit coupled to one defined lysine (Lys-163), as demonstrated by UV–Vis spectrophotometry, ESI–MS, and peptide mapping.[7] Presumably, the N^6-(6-carbamoylhexyl)-FAD moiety of this new semisynthetic D-amino-acid oxidase has a structurally similar position as a prosthetic group compared to noncovalently bound FAD in the native holoenzyme, as both enzyme forms have similar K_m and V_{max} values. There is no difference regarding their pH optimum, but their

isoelectric points differ owing to the loss of one free lysine side chain in the case of the semisynthetic D-amino-acid oxidase.

The semisynthetic D-amino-acid oxidase is a considerably more stable enzyme than the native holoenzyme, shows mesothermostability, and withstands inactivation on dilution.[7] Presumably, these phenomena are a consequence of the absence of the unstable apoform of D-amino-acid oxidase. The improvement in enzyme stability, through the use of semisynthetic flavoenzymes, may lead to more exploitable flavoenzymes for biotechnological purposes, such as biochemical analysis and biosynthesis.

Convenient and reproducible crystallization conditions have been found for the semisynthetic D-amino-acid oxidase.[7] Crystals, obtained within 3 weeks, diffract to at least 0.3-nm resolution and have two monomers in the asymmetric unit, which indicates a two-subunit quaternary structure. The absence of unstable apo-D-amino-acid oxidase is likely the cause of these favorable crystallization conditions. Hence synthesis of such semisynthetic flavoenzymes may afford a new approach to the crystallization and structural analysis of flavoenzymes.

Acknowledgments

We thank Dr. K.-D. Klöppel for the MS data. This work was partly supported by a grant from the Biotechnology programme BRIDGE 9BIOT-CT91-0279) and the Scientific Cooperation Programme with Mediterranean Countries (CI1-CT92-0024) of the European Commission.

[34] Biosynthesis of Riboflavin: 3,4-Dihydroxy-2-butanone-4-phosphate Synthase

By G. RICHTER, C. KRIEGER, R. VOLK, K. KIS, H. RITZ, E. GÖTZE, and A. BACHER

The riboflavin precursor, 6,7-dimethyl-8-ribityllumazine (**2**), is formed by condensation of 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione (**1**) with 3,4-dihydroxy-2-butanone 4-phosphate (**3**) (Fig. 1, structures **1**–**5**).[1,2] The structure of the carbohydrate **3** was established relatively re-

[1] A. Bacher, *in* "Chemistry and Biochemistry of Flavoproteins" (F. Müller, ed.), p. 215. CRC Press, Boca Raton, Florida, 1991.

[2] A. Bacher, S. Eberhardt, and G. Richter, *in* "*Escherichia coli* and *Salmonella*" (F. C. Neidhardt, ed.), p. 657. American Society for Microbiology, Washington, DC, 1996.

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