J. Biochem. 119, 441-447 (1996)

Identification of Nekoflavin as 7α-Hydroxyriboflavin

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Received for publication, September 4, 1995

Nekoflavin, which was found in cat's choroids [Matsui, K. (1965) *J. Biochem.* 57, 201-206], was identified as 7α-hydroxyriboflavin by comparing the physicochemical properties of nekoflavin acetate with those of chemically synthesized 7α-hydroxyriboflavin pentaacetate. 7α-Hydroxyriboflavin was synthesized from 4-chloro-2-methylbenzonitrile through 7-cyano-7-demethylriboflavin and 7α-aminoriboflavin, which are also new flavins.

Key words: 7α-aminoriboflavin, 7-cyano-7-demethylriboflavin, 7α-hydroxyriboflavin, nekoflavin.

The choroids of some animals contain a large amount of the free form of riboflavin (RF) (*1*). The cat's choroids also have a high level of RF, 71 μg/g fresh tissue, which is higher than that of the liver, 26 μg/g (*2*). However, the functions of the free form of RF in the choroids are not completely elucidated. Matsui found the unidentified flavin "nekoflavin" (NF) in the choroids of cat (*3*). Its function is still unknown, but the identification and the chemical synthesis of NF would improve our understanding of the functions of flavins in the eyes.

NF was assumed to be either 7α-hydroxy- (7αHOF) or 8α-hydroxyriboflavin (8αHOF), which were found in human urine (*4*), based on the molecular mass (602 Da) of its acetate and the similarity of its UV-visible absorption spectrum to that of riboflavin tetraacetate (RFa). We synthesized 8α-hydroxyriboflavin pentaacetate (8αHOFa), but its properties were not coincident with those of nekoflavin pentaacetate (NFa) (*5*). We also synthesized 7αHOF and its pentaacetate (7αHOFa), found that their properties were coincident with those of NF and NFa, respectively, and confirmed the identity of NF with 7αHOF. The details of the preparation of NFa, and of the chemical synthesis and identification are presented here. A preliminary report has been published (*6*).

EXPERIMENTAL PROCEDURES

Materials—Cat's choroids were isolated from cat's eyes 10-15 h after death, and were stocked for months at −20°C (*7*). The reagents used were of reagent grade.

Measurement—UV-visible absorption spectra were measured with a Shimadzu UV260 spectrophotometer, and ^{1}H-NMR spectra with a Hitachi R24B (60 MHz) NMR spectrometer or a JEOL GX400 or A400 (400 MHz) NMR spectrometer. ^{13}C-NMR spectra were also measured with the GX400 or A400 spectrometer. Tetramethylsilane was used as the internal standard in measuring both types of NMR spectra. pK_a was measured by spectroscopic titration. Redox potential $E^{0\prime}$ was measured by cyclic voltammetry using a Huso potentiostat HECS-972W by Dr. Ichimura.

Chromatography—For TLC, silicagel 70 plate-wako was used. The solvent systems were isoamyl alcohol-methyl ethyl ketone-acetic acid-H_2O (40:35:7:13 w/w/w/w, AMAW) for free flavins and CH_2Cl_2-methanol (20 : 1 w/w, DCM) for flavin acetates. In the AMAW system, NF showed an R_f value of 0.19-0.24, which is 0.5-0.6 times that of RF, and in DCM, NFa showed the same R_f value as that of RFa (0.2-0.4).

For silica gel column chromatography, Wakogel C-200 or C-300 and mixture of isobutanol, acetic acid, H_2O, and CH_2Cl_2 were used in the case of free flavins, and a mixture of CH_2Cl_2 and methanol in the case of flavin acetates. Eluates were monitored by TLC.

For gel filtration chromatography Sephadex G-10 (Pharmacia) and Biogel P-2 (Bio-Rad) were used, and flavins were eluted with H_2O.

Droplet Counter-current distribution Method—The apparatus was constructed with 15 glass tubes (7 mm × 150 cm) connected in series with Teflon tubes. The glass tubes were filled with the lower layer of an equilibrated mixture of 1-butanol-1-propanol-H_2O (2:1:3 v/v/v). Material dissolved in the lower layer of the mixture was pumped from the bottom of the 1st tube, and then the upper layer of the solvent mixture was also pumped from the bottom. The effluent from the last tube was divided into fractions.

Estimation of Flavins—The amount of flavin was determined by measuring the absorption at 445 nm, and was shown as absorbance units (AU); 1 AU was the amount of material contained in 1 ml of the solution, of which the absorbance at 445 nm was 1 in water.

Flavins were prepared and treated in a dark room under the light of a sodium lamp.

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Abbreviations: 7αAF, 7α-aminoriboflavin; 7αAFa, 7α-aminoriboflavin pentaacetate; 7αHOF, 7α-hydroxyriboflavin; 7αHOFa, 7α-hydroxyriboflavin pentaacetate; CNF, 7-cyano-7-demethylriboflavin; CNFa, 7-cyano-7-demethylriboflavin tetraacetate; d, doublet; dd, double doublet; δ, chemical shift in ppm; DMSO-d_6, deuterated dimethylsulfoxide; *J*, coupling constant; m, multiplet; mp, melting point; NF, nekoflavin; NFa, nekoflavin pentaacetate; PPC, partition paper chromatography; RF, riboflavin; RFa, riboflavin tetraacetate; s, singlet; t, triplet.

Isolation of NF—The stock of cat's choroids (255 g, 3,374 pieces) was extracted by the methods described previously (*7*). Flavins were partially purified by adsorption on a Florisil column. About 550 AU of flavins was recovered in the eluate, which was concentrated to 60 ml under reduced pressure. The solution was applied to a column of Sephadex G-10 (5×87 cm), and flavins were eluted with water. The eluate containing NF was collected, and evaporated to dryness under reduced pressure. The combined crude NF fraction (about 350 AU) obtained from several batches was fractionated by the droplet countercurrent method. Each 17 ml aliquot of the effluent was collected. The first flavin fraction contained most of the RF, and the second, mainly NF. The latter was divided into NF fractions of higher purity (67 AU) and of lower purity (27 AU), of which the latter was purified by retreatment.

On a column of Biogel P-2 (equilibrated with water, 1.5×82 cm) 3 ml of the sample containing 20 AU of flavin was applied, and the material was eluted with water. The NF fraction, which showed only the fluorescent spot of NF on TLC, was obtained, and it contained 87% of the applied amount. It was evaporated to dryness under reduced pressure.

About 300 AU of NF, having an absorbance equivalent to about 26 μmol of RF, was obtained from about 30,000 choroids.

Preparation of NFa—The preparation of NF (about 300 AU) was dissolved in 2 ml of anhydrous pyridine by gentle heating. The cooled solution was mixed with an equal volume of acetic anhydride, and was stirred for 4 h at room temperature. Then 5 ml of ethanol was added and the mixture was chilled with ice for 30 min. After evaporation under reduced pressure, the residue was dissolved in a mixture of 8 ml of $CHCl_3$ and 6 ml of water. The $CHCl_3$ layer was washed twice with an equal volume of water, and dried over anhydrous sodium sulfate. The $CHCl_3$ was removed, and the residue was recrystallized twice from ethanol. Yellow, fine crystals. Yield, 8 mg from about 30,000 eyeballs. Mp 196.5-198°C. Mass spectrum (field desorption), *m/z*: 602.0 (M^+) (Fig. 1a).

Synthesis of 7αHOF—4-Chloro-2-methylbenzonitrile (II): This material was prepared from *N*-acetyl-*o*-toluidine (I) by the method of Claus and Stapelberg (*8*). ^{1}H-NMR (60 MHz, DMSO-d_6), δ: 7.51 (1H, d, J=7.8 Hz, ϕ-H), 7.26 (1H, s, 3-H), 7.19 (1H, d, J=7.8 Hz, ϕ-H), 2.50 (3H, s, 2-CH_3).

4-Chloro-5-nitro-2-methylbenzonitrile (III): Concentrated H_2SO_4 (97%, 84 g) was added to chilled HNO_3 (d=1.83, 84 g) at a temperature below 10°C. The mixed acids were added in small portions to a chilled solution of II (8.4 g) in H_2SO_4 (84 g) and the mixture was stirred for 15 min at a temperature of 5-10°C, then poured into ice (1 kg) and water (0.7 kg). The precipitates were collected and washed with water. The crude product (10.4 g) was recrystallized from methanol. Yield, 7.3 g, 67%. Pale yellow, granules. Mp 85-88°C. Elem. anal. Found: C, 48.92; H, 2.48; N, 14.23%. Calcd. for $C_8H_5N_2O_2Cl$: C, 48.88; H, 2.56; N, 14.25%. ^{1}H-NMR (400 MHz, DMSO-d_6), δ: 8.66 (1H, s, ϕ-H), 7.98 (^{1}H, s, ϕ-H), 2.59 (3H, s, 2-CH_3). ^{13}C-NMR (100.5 MHz, DMSO-d_6), δ: 148.24, 145.40, 133.39, 129.88, 129.78, 115.49, 111.93 (six ϕ-C and CN), 19.82 (2-CH_3).

4-D-Ribitylamino-5-nitro-2-methylbenzonitrile (IV): D-Ribose oxime (4.0 g), which was prepared by a modification of the method of Wohl (*9*), was dissolved in anhydrous methanol (40 g), and was reduced with H_2 (50-100 atm) at 65°C for 70 min in the presence of Ni catalyst prepared from Raney's alloy (50%, 4 g). After filtration, the reaction mixture was concentrated to a thick syrup, which was redissolved in methanol (2.5 g) and mixed with anhydrous pyridine (1.0 g) and III (2.4 g). The mixture was refluxed gently for 40 min, and then evaporated under reduced pressure. The residue was dissolved in water (250 g), and extracted several times with equal volume of CH_2Cl_2 to remove unreacted III. The aqueous layer was concentrated under reduced pressure, and the formed crystals were collected, and recrystallized from methanol. Yield, 0.6 g. Yellow, fine needles. Mp 136°C. Elem. anal. Found: C, 49.97; H, 5.51; N, 13.34%. Calcd. for $C_{13}H_{17}N_3O_6$: C, 50.15; H, 5.50; N, 13.49%. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 8.74 (1H, t, J=5 Hz, 4-NH), 8.45 (1H, s, ϕ-H), 7.13 (1H, s, ϕ-H), 5.14 (1H, d, J=5.4 Hz, OH), 4.96 (1H, d, J=4.9 Hz, OH), 4.80 (1H, d, J=4.9 Hz, OH), 4.48 (1H, t, J=4.9 Hz, 5'-OH), 3.93-3.88 (1H, m, 1'-H_a), 3.64-3.36 (6H, m, 1'-H_b, 2'-, 3'-, 4'-H, 5'-H_2), 2.44 (3H, s, 2-CH_3). ^{13}C-NMR (100.4 MHz, DMSO-d_6) δ: 147.84, 146.99, 132.12, 129.05, 117.40, 115.48, 97.64 (six ϕ-C and CN), 73.03, 72.63, 68.90, 63.16, 44.60 (five ribityl-C), 20.34 (CH_3).

4-D-Ribitylamino-5-nitro-2-methylbenzonitrile tetraacetate (IVa): Compound IV (0.62 g) was dissolved in a mixture of anhydrous pyridine (8.0 g) and acetic anhydride (5.1 g), and stirred overnight. The mixture was evaporated to dryness under reduced pressure, and the residue was dissolved in ethanol and evaporated twice. The product was recrystallized from ethanol twice. Yield, 0.8 g. Yellow, fine crystals. Elem. anal. Found: C, 52.37; H, 5.14; N, 8.75%. Calcd. for $C_{21}H_{25}N_3O_{10}$: C, 52.60; H, 5.25; N, 8.76%. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.53 (1H, t, J=5.8 Hz, 4-NH), 8.46 (1H, s, ϕ-H), 6.85 (1H, s, ϕ-H), 5.40 (1H, dd, J=6.0 and 4.4 Hz, 3'-H), 5.32 (1H, dt, J=6.3 and 2.9 Hz, 2'-H), 5.27 (1H, dt, J=7.4 and 4.7 Hz, 4'-H), 4.38 (1H, dd, J=12.2 and 3.0 Hz, 1'-H_a), 4.17 (1H, dd, J=12.4 and 6.0 Hz, 1'-H_b), 3.72-3.78 (1H, m, 5'-H_a), 3.57-3.64 (1H, m,

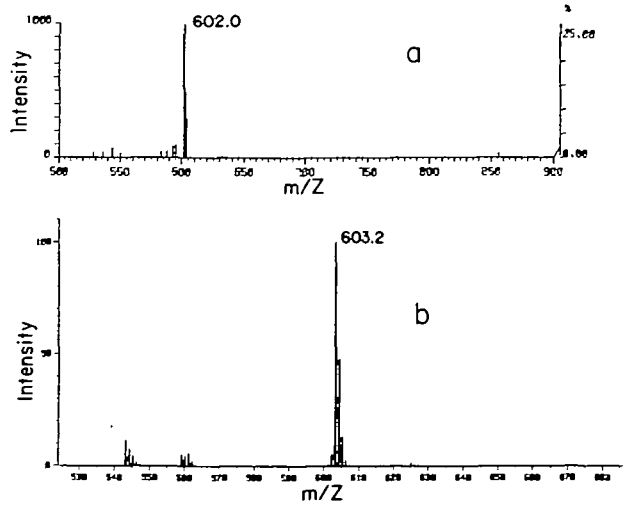


Fig. 1. **Mass spectra of NFa and 7αHOFa.** a, NFa, FD-MS; b, 7αHOFa, FAB-MS.

5′-H_b), 2.54 (3H, s, 2-CH_3), 2.15 (3H, s, CO-CH_3), 2.12 (3H, s, CO-CH_3), 2.10 (3H, s, CO-CH_3), 2.07 (3H, s, CO-CH_3). ^{13}C-NMR (100.4 MHz, $CDCl_3$) δ: 170.56, 170.09 (duplex), 169.60 (four CH_3-*C*O), 149.21, 146.58, 132.60, 130.55, 117.04, 114.43, 100.51 (six ϕ-C and CN), 70.28, 69.50, 69.41, 61.81, 42.50 (five ribityl-C), 21.16 (ϕ-CH_3), 20.89, 20.72 (duplex), 20.68 (four CO-*C*H_3).

7-Cyano-7-demethyl-D-riboflavin (CNF): Compound IV was reduced by the method of Hirashima and Manabe (*10*). The methanol solution of $FeCl_3 \cdot 6H_2O$ (1%, 25 g) was refluxed for 3 min with active charcoal (0.55 g). After dissolution of IV (3.11 g) in the above solution, hydrazine hydrate (11 g) was dropped into the boiling solution, which was then refluxed for a further 10 min. The reaction mixture was filtered, and the filtrate was evaporated to dryness. Hydrazine was removed by repeated evaporation with ethanol, and finally over H_2SO_4 *in vacuo* in a desiccator.

The white crystals (V) that remained were dissolved in acetic acid (25 g), and then boric acid (5 g) and alloxan monohydrate (2.2 g) were added. The mixture was refluxed gently for 25 min, allowed to stand overnight at room temperature, and then evaporated under reduced pressure. Boric acid was removed completely by repeated evaporation with ethanol.

The residue (3 g) was mixed with Wakogel C-200 (5 g) and a small amount of methanol in a mortar, and the mixture was air-dried. It was applied to a column of Wakogel C-200 (3.0×40 cm) with CH_2Cl_2, and developed with isobutanol (30 g)-acetic acid (20 g)-H_2O (2.5 g)-CH_2Cl_2 (200 g). The content of CH_2Cl_2 in the eluant was decreased in steps of 20 g. The eluates were monitored by TLC (solvent AMAW, R_f of CNF was about 0.5). Fractions containing CNF were collected and evaporated to dryness. Yield, 1.7 g. If necessary, the product was recrystallized from 2 M acetic acid.

Dark yellow, microcrystals. Elem. anal. Found: C, 45.67; H, 5.28; N, 15.50%. Calcd. for $C_{17}H_{17}N_5O_6 \cdot 3.33H_2O$: C, 45.64; H, 5.33; N, 15.65%. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.55 (1H, s, 3N-H), 8.60 (1H, s, ϕ-H), 8.11 (1H, s, ϕ-H), 5.10 (1H, d, J=3.7 Hz, OH), 4.91-4.82 (3H, m, 1′-H_a and two OH), 4.63 (1H, d, J=12.2 Hz, 1′-H_b), 4.50 (1H, t, J=5.2 Hz, 5′-OH), 4.24 (1H, broad, 2′-H), 3.65-3.62 (2H, m, 3′ and 4′-H), 3.50-3.44 (1H, m, 5′-H_a), 3.41-3.32 (1H, m, 5′-H_b), 2.68 (3H, s, 8-CH_3). ^{13}C-NMR (100.5 MHz, DMSO-d_6) δ: 159.25, 155.31, 151.51, 146.03, 139.03, 136.51, 136.20, 132.55, 118.94, 116.62, 109.25 (ten isoalloxazine-C, and CN), 73.39, 72.61, 68.42, 63.29, 47.23 (five ribityl-C), 20.96 (8-CH_3).

7-Cyano-7-demethyl-D-riboflavin tetraacetate (CNFa): The crude preparation of CNF (2.5 g) was acetylated as described above, and the product was recrystallized from ethanol. Yield, 1.8 g, 60%. Yellow fine granular crystals. Mp 222-225°C. Elem. anal. Found: C, 53.48; H, 4.62; N, 12.40%. Calcd. for $C_{25}H_{25}N_5O_{10} \cdot 0.33H_2O$: C, 53.48; H, 4.60; N, 12.47%. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.97 (1H, s, 3N-H), 8.49 (1H, s, ϕ-H), 7.76 (1H, s, ϕ-H), 5.61 (1H, d, J=8.8 Hz, 2′-H), 5.41 (2H, s, 3′-H, 4′-H), 5.13 (1H, broad, 1′-H_a), 4.87 (1H, broad, 1′-H_b), 4.46 (1H, d, J=11.6 Hz, 5′-H_a), 4.23 (1H, dd, J=10.4 and 3.7 Hz, 5′-H_b), 2.83 (3H, s, ϕ-CH_3), 2.28 (3H, s, CO-CH_3), 2.21 (3H, s, CO-CH_3), 2.09 (3H, s, CO-CH_3), 1.81 (3H, s, CO-CH_3). ^{13}C-NMR (100.5 MHz, $CDCl_3$) δ: 170.73, 170.38 (duplex), 170.05 (four CH_3-*C*O), 158.36, 154.16, 151.20, 148.86, 138.30, 137.77, 135.39, 133.16, 117.02, 115.82, 111.84 (ten isoalloxazine-C and CN), 70.52, 69.60, 68.94, 61.93, 45.28 (five ribityl-C), 21.87 (8-CH_3), 21.06, 20.81, 20.73, 20.04 (four CO-*C*H_3).

7α-Amino-D-riboflavin (7αAF): In an autoclave, a solution of CNFa (0.4 g) in anhydrous methanol (10 g) was reduced with hydrogen (100-50 atm) in the presence of Ni catalyst prepared from Raney's alloy (50%, 16 g) (100°C, 45 min). The reaction mixture was filtered, and the filtrate was evaporated to dryness under reduced pressure. Yield, 0.12 g. The combined residue (1.6 g) from 13 batches was mixed with silica gel (C-200, 5 g) and a small amount of methanol, and air-dried. It was applied to a dry column of Wakogel C-200 (2.5×15 cm), and developed with isobutanol (35 g)-acetic acid (2 g)-H_2O (2 g)-CH_2Cl_2 (100 g). The solvent composition was changed stepwise as follows: 34:4:4:88, 70:10:10:70, 50:10:10:25, 40:10:10:10, 40:10:10:0 in grams, and finally half a volume of methanol was added to the last mixture. The eluates were monitored by TLC (AMAW), and fractions containing the substance remaining at the origin on TLC were collected and evaporated. Yield, 0.4 g.

7α-Amino-D-riboflavin pentaacetate (7αAFa): The crude preparation of 7αAF (0.1 g) was acetylated as described above. The yield of the crude product was 0.05 g. The combined crude product (0.30 g) was dissolved in a small volume of CH_2Cl_2, applied to a column of Wakogel C-300 (2×20 cm), and developed with a mixture of CH_2Cl_2 (200 g) and methanol (1 g). The amount of methanol in the eluant was increased in steps of 1 g. The eluates were monitored by TLC (DCM), and fractions containing 7αAFa, of which the R_f (0.35-0.40) was about half that of CNFa, were collected, and evaporated. The residue was recrystallized from ethanol. Yield, 0.05 g. Yellow, fine crystals. Mp 217-218°C. Mass spectrum (fast atom bombardment), m/z: 602.2402 (MH^+). Elem. anal. Found: C, 53.68; H, 5.29; N, 11.49%. Calcd. for $C_{27}H_{31}N_5O_{11} \cdot 0.33H_2O$: C, 53.38; H, 5.25; N, 11.52%. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 9.65 (1H, s, 3N-H), 7.99 (1H, s, ϕ-H), 7.56 (1H, s, ϕ-H), 7.23 (1H, s, 7αC-NH), 5.59 (1H, m, 2′-H), 5.50-5.39 (2H, m, 3′ and 4′-H), 5.05 (1H, broad, 1′-H_a), 4.82 (1H, broad, 1′-H_b), 4.58 (2H, m, 7αC-H_2), 4.44 (1H, d, J=11.0 Hz, 5′-H_a), 4.24 (1H, dd, J=12.5 and 5.4 Hz, 5′-H_b), 2.61 (3H, s, 8-CH_3), 2.29 (3H, s, CO-CH_3), 2.22 (3H, s, CO-CH_3), 2.17 (3H, s, CO-CH_3), 2.08 (3H, s, CO-CH_3), 1.73 (3H, s, CO-CH_3). ^{1}H-NMR (400 MHz, CD_3OD) δ: 7.96 (1H, s, ϕ-H), 7.82 (1H, s, ϕ-H), 5.65 (1H, m, 2′-H), 5.52 (1H, dd, J=6.1 and 4.3 Hz, 3′-H), 5.42 (1H, dt, 9.2 and 3.1 Hz, 4′-H), 5.12 (1H, broad, 1′-H_a), 5.04 (1H, broad, 1′-H_b), 4.52 (2H, s, 7αC-H_2), 4.48 (1H, dd, J=16.2 and 3.1 Hz, 5′-H_a), 4.25 (1H, dd, J=12.2 and 6.1 Hz, 5′-H_b), 2.64 (3H, s, 8-CH_3), 2.20 (3H, s, CO-CH_3), 2.19 (3H, s, CO-CH_3), 2.08 (3H, s, CO-CH_3), 2.02 (3H, s, CO-CH_3), 1.69 (3H, s, CO-CH_3). ^{13}C-NMR (100.5 MHz, CD_3OD) δ: 173.45, 172.34, 171.83, 171.55, 171.49 (five CH_3-*C*O), 161.85, 158.05, 152.49, 148.07, 138.35, 137.98, 135.74, 133.59, 131.18, 118.47 (ten isoalloxazine-C), 71.77, 70.82, 70.70, 63.05, 46.03 (five ribityl-C), 41.42 (7-CH_2), 22.58 (8-CH_3), 21.05, 20.74, 20.61, 20.52, 20.38 (five CO-*C*H_3).

7αHOF: The chromatographed preparation of 7αAF (0.1 g) was dissolved in 1 M acetic acid (10 g), and $NaNO_2$ (0.18 g) was added to the solution. The reaction mixture was

TABLE I. **UV-Visible absorption properties of flavins.**

Flavin	Medium	Absorption maximum nm[a] ($\varepsilon \times 10^{-3}$)						
NF[b]	H_2O	222		267	368		436	
7αHOF	6 M HCl	219(22.3)		264(30.9)		393(19.9)		
	0.1 M NaOH	220(27.1)		269(36.3)	354(11.1)		443(11.1)	
	pH 6.96	222(26.8)		266(32.8)	368(9.2)		438(12.1)	
7αHOF[c]	pH 7.0			267(25.1)	369(6.9)		437(8.9)	
CNF	6 M HCl	224(18.9)		266(30.5)		390(13.6)		450s(3.0)
	pH 6.80	225(17.7)	231s(17.3)	276(35.4)	351(5.7)		427(11.1)	
RF	6 M HCl	220(21.9)		265(30.1)		394(19.7)		
	0.1 M NaOH	220(26.8)		269(32.5)	355(11.0)		448(11.0)	
	pH 6.99	222(27.8)		266(30.4)	373(9.8)		444(11.7)	

[a]s, shoulder. Data from Ref. [b]*3* and [c]*4*.

stirred for 30 min at room temperature, then 1 ml of ethanol was added and the whole was evaporated to dryness under reduced pressure. The residue was stored in a desiccator *in vacuo* at a temperature below 5°C. The combined materials from 5 batches were mixed with Wakogel C-200 (5 g) in a mortar, and transferred with a mixture of H_2O (1.5 g)-acetic acid (1.5 g)-isobutanol (22 g)-CH_2Cl_2 (15 g) to a Wakogel C-200 column (1.5×21 cm) which was packed with the aid of an isobutanol-CH_2Cl_2 (1:5 w/w) mixture. The flavins were developed with isobutanol-acetic acid-H_2O-CH_2Cl_2, of which the ratio was changed stepwise from 30:2:2:20 to 30:4:4:6 w/w/w/w. The eluates were monitored by TLC (AMAW), and fractions which contained the flavin having an R_f value of about half that of RF were collected and evaporated. The residue was washed with water to remove contaminating sodium acetate and dried. Yield, 0.07 g (12%) from 0.55 g of the preparation of 7αAF.

Yellow, fine needles. Elem. anal. Found: C, 51.20; H, 5.20; N, 13.77%. Calcd. for $C_{17}H_{20}N_4O_7 \cdot 0.35H_2O$: C, 51.21; H, 5.23; N, 14.05%. ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 11.36* (1H, s, 3N-H), 8.01 (1H, s, ϕ-H), 7.92 (1H, s, ϕ-H), 5.46* (1H, s, 2′-OH), 5.13* (1H, s, 3′-OH), 4.95 (1H, t, J=10.8 Hz, 1′-H_a), 4.88* (1H, s, 4′-OH), 4.82* (1H, d, J=5.0 Hz, 5′-OH), 4.65 (2H, s, 7α-H_2), 4.62 (1H, s, 1′-H_b), 4.50* (1H, s, 7α-OH), 4.27 (1H, m, 2′-H), 3.65 (3H, broad, 3′-H, 4′-H, 5′-H_a), 3.46 (1H, broad, 5′-H_b), 2.49 (3H, s, 8-CH_3) (*, exchangeable H with D of D_2O). The UV-visible absorption spectrum of 7αHOF at pH 6.96 is shown in Fig. 2, and the absorption properties in various media are summerized in Table I.

7αHOFa: Solid $NaNO_2$ (3.4 g) was dissolved in a solution of 7αAF (1.31 g in 130 g of 1 M acetic acid), and the solution was stirred for 1 h at room temperature. Evaporation under reduced pressure afforded a residue, which was acetylated with anhydrous pyridine (50 g) and acetic anhydride (30 g) in the same way as described for the preparation of CNFa. The crude acetate (0.59 g) was purified by chromatography.

A CH_2Cl_2 solution of the crude acetate (0.73 g) was applied to a column of Wakogel C-200 (2×20 cm, solvent CH_2Cl_2) and developed with a mixture of CH_2Cl_2 (200 g) and methanol (1.0 g). The content of methanol in the eluant was increased in steps of 0.2 g to the final ratio of 200:2.2 (w/w). The fractions containing a greenish fluorescent substance, of which the R_f value on TLC is the same as that of RFa, were collected and evaporated to dryness. The residue was recrystallized from ethanol.

Yield, 0.28 g. Orange, fine crystals. Mp, 200°C. Mass spectrum (fast atom bombardment), m/z: 603.2114 (MH^+) (Fig. 1b). Other data are given in "RESULTS AND DISCUSSION."

RESULTS AND DISCUSSION

Properties of NF and NFa—The preparation of NF was obtained as yellow, silky fine needles. The UV-visible absorption spectrum of NF in water (*3*) is shown in Fig. 2, and the wavelengths of absorption peaks in Table I. They were very similar to those of RF, though the 1st and 2nd peaks (peaks in visible and near UV region) were shifted to shorter wavelength by as much as about 6 nm. It decomposed slowly in the solution, even in the frozen state.

The preparation of NFa was obtained as yellow, fine crystals. Mp, 196.5-198°C. Molecular mass, 602.0 Da (Fig. 1a). The molecular weight of NFa, calculated from the composition $C_{27}H_{30}N_4O_{12}$ (7α-hydroxyriboflavin pentaacetate) was 602.6. The UV-visible absorption properties in $CHCl_3$ are shown in Table II. The spectrum, shown in Ref. *6*, was similar to that of RFa, but the 1st and 2nd absorption peaks were blue-shifted by as much as about 5 nm. The δ values of ^{1}H- and ^{13}C-NMR signals are shown in Tables III and IV.

Preparation of 7αHOF—7αHOF was prepared by the process shown in Scheme 1. 4-Chloro-2-methylbenzonitrile (II) was prepared by the method of Claus and Stapelberg (*8*). They also reported the preparation of III, but they did not describe its properties. As we could not prepare it by their method, we obtained it by nitrating II. Therefore, III and the following compounds are new. Their structures were confirmed by the elementary analyses and NMR data, except for V and 7αAF.

Compound IV was prepared by the reaction between III and D-ribitylamine in the presence of pyridine. The yield was not good (4±4.5%). One of the reasons for this low yield would be the low purity of the amine, which contained a variable amount of ribitol.

Compound V was prepared by the reduction of IV with hydrazine hydrate in the presence of charcoal-$FeCl_3$ (*10*). As V was expected to be easily autoxidizable and it was obtained in the form of colorless needles, it was used for preparing CNF without purification. As the acetate of V was not crystallizable, its structure was not confirmed. However, the formation of CNF is good evidence for an *o*-phenylenediamine structure of V.

CNF was prepared by condensing V with alloxan in the

presence of boric acid (*12*). The UV-visible absorption properties are summerized in Table I. The 1st and 2nd absorption peaks of CNF in the neutral medium were blue-shifted by as much as about 20 nm from those of RF. CNF (pK_a, <1 and 8.8) was more acidic than RF (pK_a, <1 and 10.2), and was labile in an alkaline medium (>pH 9). The redox potential, $E^{o'}$ (pH 7.00), of −0.108 V (*vs.* SHE, 25°C) was more positive than that of RF (−0.205 V) by as much as 0.097 V. The compound was also highly photosensitive, as reported in the case of 7-cyano-10-hydroxyethylisoalloxazine (*13*). The two absorption peaks of CNFa in $CHCl_3$ were also blue-shifted by as much as 10 nm from those of RFa (Table II).

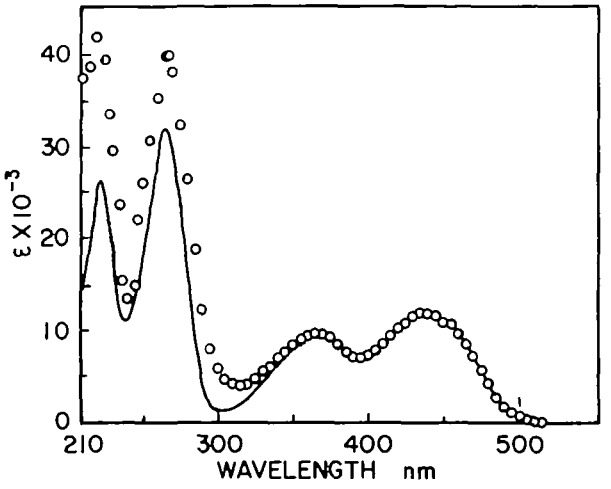


Fig. 2. **UV-visible absorption spectra of NF and 7αHOF.** ○, NF, in water, arbitrary scale; —, 7αHOF, at pH 6.96. Adapted with permission of the publisher from Ref. *1*.

7αAF was prepared by hydrogenating CNFa in the presence of an Ni catalyst. As this compound was labile even *in vacuo*, the acetate (7αAFa) was used for confirming the structure. The absorption spectrum was very similar to that of NFa, except for the shoulder at 360 nm of NFa (Table II).

7αHOF was obtained by treating 7αAF with $NaNO_2$ in acidic medium. The yield was not good, probably because of the lability of 7αAF and of the product 7αHOF.

Properties of 7αHOF—The UV-visible absorption spectrum of 7αHOF at pH 6.96 is shown in Fig. 2, and the absorption properties in various media are summerized in Table I. The spectra measured in different pH media were very similar to those of RF, except for the 1st and the 2nd peaks in neutral medium, which were blue-shifted by as much as about 5 nm from those of RF. The pK_as were 9.3 and <1. The former was smaller than that of RF (10.2) by as much as 0.9, *i.e.*, 7αHOF was more acidic than RF. The redox potential $E^{o'}$ (pH 7.00) was −0.193 V (*vs.* SHE, 25°C), and was similar to that of RF (−0.205 V). These properties were consistent with the structure, 7α-hydroxyriboflavin.

The properties of synthesized 7αHOF were compared with those of 7αHOF isolated from human urine by Ohkawa *et al.* (*4*). The wavelengths of UV-visible absorption maxima at pH 7 of urinary 7αHOF coincided with those of synthetic material within the range of error (Table I), but Ohkawa's extinction coefficients were smaller than ours. The ratios of both coefficients at the 3 peaks (1st to 3rd) were 1.31-1.36, which can be regarded as constant from the viewpoint of accuracy of measurement. Therefore, the UV-visible absorption properties of both flavins can be regarded as identical. The comparison of the

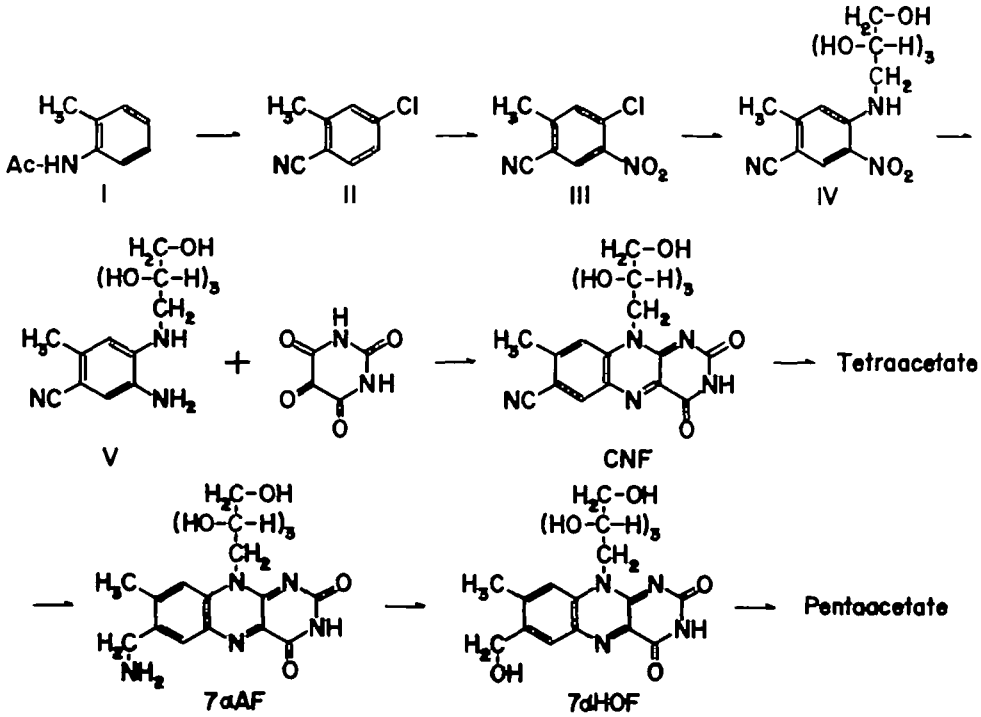
TABLE II. **UV-visible absorption properties of flavin acetates in $CHCl_3$.**

Flavin acetate	Absorption maximum nm[a] (ε ×10⁻³)						
NFa	271		344	360s	417s	442	466s
7αHOFa	271 (33.2)		344(8.2)	360s(7.3)	417s(9.5)	442(12.6)	466s(9.5)
7αAFa	271 (32.7)		346(8.4)		420s(9.1)	444(12.4)	468s(9.6)
CNFa	278s(38.0)	284(42.6)	335(3.3)		414s(5.6)	434(13.2)	458s(9.1)
RFa	270 (31.7)	284(42.6)	349(9.6)	364s(9.1)	425s(10.2)	447(13.6)	469 (11.0)

[a] s, shoulder.

TABLE III. **Comparison of ^{1}H-NMR spectra of NFa and 7αHOFa (400 MHz, $CDCl_3$).**

NFa			7αHOFa			Assignment
δ (ppm)	No. of H	J (Hz)	δ (ppm)	No. of H	J (Hz)	
8.58	1 s		8.86	1 s		3N-H
8.27	1 s		8.26	1 s		φ-H
7.62	1 s		7.62	1 s		φ-H
5.66	1 d	7.6	5.66	1 d	9.0	2′-H
5.45	1 t	4.6	5.45	1 d	3.0	3′-H
5.44-5.40	1 m		5.44-5.40	1 m		4′-H
5.29-5.21	2 m		5.29-5.21	2 m		7-CH_2
5.2	1 broad		5.2	1 broad		1′-H_a
4.89	1 broad		4.89	1 broad		1′-H_b
4.45	1 dd	12.5 2.8	4.45	1 dd	12.2 2.4	5′-H_a
4.24	1 dd	12.5 5.8	4.25	1 dd	12.3 5.5	5′-H_b
2.62	3 s		2.62	3 s		8-CH_3
2.29	3 s		2.29	3 s		CO-CH_3
2.21	3 s		2.22	3 s		CO-CH_3
2.19	3 s		2.19	3 s		CO-CH_3
2.08	3 s		2.08	3 s		CO-CH_3
1.78	3 s		1.78	3 s		CO-CH_3



Scheme 1. **Synthetic route to 7αHOF.**

TABLE IV. **Comparison of δ-values (ppm) in ^{13}C-NMR spectra of NFa and 7αHOFa (100.5 MHz, DMSO-d_6).**

NFa (ppm)	7αHOFa (ppm)	Assignment
170.61	170.61	CH_3-*C*O
170.46	170.46	〃
170.28	170.28	〃
169.92 duplex	169.92	〃
	169.82	〃
159.02	159.06	Isoalloxazine-C
154.21	154.33	〃
151.03	151.02	〃
146.32	146.29	〃
136.73	136.76	〃
134.51	134.48	〃
134.36	134.35	〃
132.39	132.39	〃
131.91	131.88	〃
116.41	116.41	〃
70.61	70.60	Ribityl-C
69.58	69.56	〃
69.07	69.07	〃
62.60	62.60	〃
61.96	61.94	〃
45.17	45.16	7-CH_2
21.05	21.05	8-CH_3
20.81 duplex	20.80 duplex	*C*H_3-CO
20.70	20.68	〃
20.35 duplex	20.35 duplex	〃

^{1}H-NMR spectra of both flavins was difficult because the solvents used in measurement were different (one in D_2O and the other in DMSO-d_6). The δ values of the two aromatic Hs coincided, but the values of 7-CH_2 and 8-CH_3 were different, 4.81:4.65 and 2.58:2.49. As the differences were small, it seems likely that the δ values of both compounds would be same in the same solvent. Therefore, the urinary 7αHOF was considered to be identical with the synthetic compound.

TABLE V. **R_f values of 7αHOF, NF, and RF on TLC and PPC.** In PPC (ascending method) Toyo 51A paper was used. The solvent BFW is a mixture of 1-butanol-90% HCO_2H-H_2O (77:10:13 v/v/v), and BAWE is 1-butanol-acetic acid-H_2O-diethyl ether (8:4:4:3 v/v/v/v). Ratio is the ratio of the R_f value of 7αHOF or NF to that of RF.

Solvent	RF	7αHOF	NF	Ratio
TLC				
AMAW	0.35	0.18		0.5
	0.31[a]		0.14[a]	0.5
BFW	0.40	0.24		0.6
	0.33[a]		0.24[a]	0.7
BAWE	0.50	0.38		0.8
	0.49[a]		0.37[a]	0.8
PPC				
5% $Na_2HPO_4 \cdot H_2O$	0.34	0.45		1.3
	0.33[b]		0.43[b]	1.3
5% H_3BO_3	0.46	0.54		1.2
	0.44[b]		0.53[b]	1.2
3.1% $Na_2B_4O_7 \cdot 10H_2O$	0.66	0.76		1.2
	0.60[b]		0.70[b]	1.2
H_2O saturated with	0.40	0.48		1.2
isoamyl alcohol	0.40[b]		0.49[b]	1.2

[a]Data from Ref. *11*. [b]Data from Ref. *3*.

Properties of 7αHOFa—Yellow, fine crystals. Mp 200°C. Molecular mass, 602.2 Da (Fig. 1b). Elem. anal. Found: C, 53.26; H, 4.96; N, 9.29%. Calcd. for $C_{27}H_{30}N_4O_{12} \cdot 0.25$ H_2O: C, 53.42; H, 5.06; N, 9.22%. The UV-visible absorption properties in $CHCl_3$ are summerized in Table II. The 1st and 2nd absorption peaks were blue-shifted by as much as 5 nm from those of RFa, as in the case of free 7αHOF. The δ values of ^{1}H- and ^{13}C-NMR spectra are shown in Tables III and IV, respectively. These properties were consistent with the structure.

Comparison of NF with 7αHOF—As the preparation of natural NF was stored in the form of the acetate, NF was compared with synthesized 7αHOF in the acetate form.

Although the melting point of NFa was lower than that of 7αHOFa by as much as about 3°C, no depression was observed in the mixed melting point test. The molecular mass of NFa, 602.0 Da, was coincident with that of 7αHOFa, 602.2 Da, and with the molecular weight of the latter, 602.6, within the range of experimental error (Fig. 1, a and b). The wavelengths of absorption peaks in $CHCl_3$ of NFa were coincident with those of 7αHOFa as shown in Table II. The absorption curves of both acetates, shown in Ref. *6*, also coincided within the range of experimental error. The ^1H- and ^{13}C-NMR spectra of NFa and 7αHOFa are shown in Ref. *6*, and the δ-values in Tables III and IV. They showed very good coincidence.

Among the recorded properties of free NF, the UV-visible absorption in water was compared with that of 7αHOF. Table I shows that the wavelengths of absorption peaks of both flavins in neutral medium are coincident within the range of error. However, the absorbance of NF in the UV region was apparently higher than that of the synthetic material (Fig. 2). This is likely to be caused by colorless impurities contained in the preparation of NF, which was difficult to purify by recrystallization because of the minute amount available at that time.

Since NF was unstable and was stored as NFa, it was necessary to clarify whether or not NF itself carries any acetyl group in its molecule. The acetyl group would affect the hydrophobicity of the molecule and appreciably change the R_f value on TLC and PPC, but not the UV-visible absorption spectrum. As exact reproduction of the conditions of chromatography was difficult, we compared the R_f values of 7αHOF and NF with that of RF, *i.e.*, the ratios of R_f values of the two flavins to that of RF were compared. Table V shows the R_f values and the ratios. The ratios of 7αHOF under various conditions of TLC and PPC coincided within the range of experimental error with those of NF, which were calculated from the reported R_f values (*3, 11*). This excludes any appreciable differences of properties, for example, of hydrophobicity between 7αHOF and NF, and eliminates the possibility that NF was acetylated 7αHOF. The report that NF was not affected by treatment with dilute HCl and NaOH (*11*) also rules out the possibility of that NF is an acetate. Based on these observations, we concluded that NF was identical with 7αHOF and carried no acetyl group.

Physiological Functions of NF—The physiological role of NF is of interest. However, it was found as a small fluorescent spot in PPC of the extract of cat's choroids, and nothing is known of its physiological function. As it was found in human urine, and also was produced by hydroxylation of RF by the microsomal electron transfer system in rat liver (*14*), it may be a metabolite of RF in cat. But important functions seem probable, because NF corresponds to several percent of the free form of flavin in cat choroids. Its function should be considered in connection with that of free RF, which is still not completely elucidated (*1*).

We are very grateful to Prof. Shoichi Kusumoto of the Faculty of Science, Osaka University for elementary analyses, Prof. Yasutsugu Shimonishi of Protein Research Institute, Osaka University for measuring molecular mass, Dr. Akio Ichimura of Osaka City University for measuring redox potential, Prof. Franz Müller of Sando Agro Ltd. for discussions and an experimental trial, and Mrs. Kei Katayama, Mrs. Masako Shirakazu, Mrs. Katsumi Matsui, Miss Yokoi, Dr. Mieko Masuoka, Dr. Kazunori Yanagiya, Mrs. Miyoko Kusaka, Miss Yoshiko Kondo, Miss Miwako Takahashi, Miss Chisa Kiyono, Miss Tamami Nagano, Miss Shihori Yamashita, Mrs. Kanako Konishi, Miss Takayo Inoue, Miss Emiko Doi, Miss Kaori Nakamura, Miss Sayuri Matsu, Miss Chihiro Setoguchi, Miss Yasuko Nishida, Miss Yasuko Matsuda, Miss Yoshiko Yoshimura, Miss Reiko Ishimizu, Miss Kumi Tanaka, Miss Hitomi Tanaka, and Miss Nao Yoshida for technical assistance.

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