A Crown Ether Flavin Mimic: Synthesis and Properties of a Flavin Bearing a Crown Ring as a Recognition Site[1]

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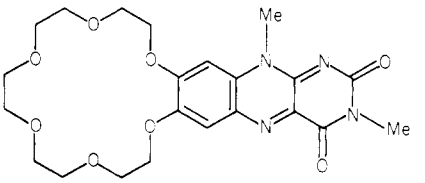
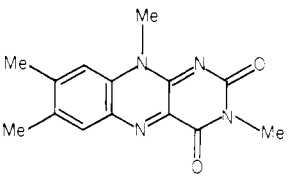
Abstract: A crown ether flavin mimic (3,10-dimethyl-1′,4′,7′,10′,13′,16′-hexaoxacyclooctadec-2′-eno[2′,3′-*i*]isoalloxazine, crFl) which has within a molecule both flavin as a catalytic site and crown ring as a recognition site was synthesized. The absorption band and the fluorescence intensity of crFl decreased with increasing alkali metal concentrations. From plots of OD/OD_0 (or I/I_0) vs. metal ion concentrations were determined the association constants (K), which were in the order $K^+ > Rb^+ > Cs^+ \approx Na^+$. The fluorescence was efficiently quenched by tryptamine hydrochloride ($I/I_0 = 0.224$ at 10^{-2} M), suggesting the pseudointramolecular fluorescence quenching due to recognition of the ammonium group by the crown ring. The fluorescence study using a series of benzimidazole derivatives with $2\text{-}(CH_2)_nNH_3^+$ showed that the maximum fluorescence quenching occurs at $n = 3$. The fluorescence intensity in the presence of these additives increased with increasing K^+ concentration, indicating the competitive binding of K^+ and additives to the crown ring. The second-order rate constants (k_2) for the reaction with 1-benzyl-1,4-dihydronicotinamide were enhanced by 1.4- to 2.4-fold by added alkali metal and ammonium cations. Furthermore, the reaction with N^3-dodecyl-1-(*p*-(ammoniomethyl)benzyl)-1,4-dihydronicotinamide proceeded according to Michaelis–Menten kinetics, and a rate enhancement of 29-fold was attained. This is also due to binding of the ammonium group to the crown ring. These results show that crFl is capable of mimicking several important properties of flavoenzymes owing to the attached crown ring as a recognition site.

Coenzymes are prosthetic groups in enzymes and catalyze the enzyme-mediated reactions in the active sites. The catalytic actions have attracted much attention of bioorganic chemists because some of them are capable of catalyzing the reactions even in the absence of apoenzymes.[2-4] Thus, the investigations on the coenzyme catalyses in the model system have provided many clues to elucidate the enzymatic reaction mechanisms reasonably.[2-6] In contrast to holoenzymes having both a catalytic site and a recognition site, however, coenzymes themselves consist only of a catalytic site. One may thus expect that the coenzymes bearing an intramolecular recognition site would behave as more attractive enzyme model systems.[7] The ability of crown ethers, as well as that of cyclodextrins,[8,9] to associate with a variety of charged and uncharged substrates bears resemblance to early reaction steps in enzyme-mediated reactions. Therefore, a crown ether family may be a useful candidate for the recognition sites. In fact, there have been several crown ether mimics of prosthetic groups reported, but to the best of our knowledge the precedents are rather limited: sulfhydryl coenzyme models,[10,11] NADH models,[12,13] and heme porphyrins.[14-16] Here we report the first example of a crown ether mimic of flavin coenzymes, 3,10-dimethyl-1′,4′,7′,10′,13′,16′-hexaoxacyclooctadec-2′-eno[2′,3′-*i*]isoalloxazine (crFl). One can expect for the crown ether cavity to recognize not only spherical metal cations but also ammonium cations and others through hydrogen bonding, and the complexation would induce a change in the flavin reactivities. Furthermore, absorption and fluorescence spectra of the isoalloxazine would be affected by bound guest species. In order to differentiate the contribution of the crown ring in crFl, we used 3-methyllumiflavin (LFl) as a reference compound.



CrFl



LFl

Experimental Section

Materials. crFl was synthesized according to Scheme I.

4′-(Tosylamino)benzo-18-crown-6 (**1**) was prepared from 4′-aminobenzo-18-crown-6 and tosyl chloride in the presence of excess triethylamine: mp 81–84 °C; yield 83.8%.

1 (15.0 g, 31 mmol) was dissolved in 380 mL of water containing 5.4 g of NaOH, and dimethyl sulfate (3.91 g, 31 mmol) was added dropwise to the stirred solution. The reaction was continued at 40 °C for 5 h. The progress of the reaction was followed by a TLC method. In order to complete the reaction, NaOH and dimethyl sulfate (one-third of the above amounts) were added after 0.5, 1.0, and 2.5 h. After cooling, the oily layer that separated was recovered by decantation. The aqueous layer was extracted with *n*-butyl alcohol, the *n*-butyl alcohol layer being combined with the oil. Concentration of the solution gave brown crystals. The crystals were dissolved once in hot NaOH solution and treated with active charcoal. After filtration, the solution was neutralized by dilute HCl. We thus obtained 4′-(*N*-methyl-*N*-tosylamino)benzo-18-crown-6 (**2**) as an almost colorless oil: yield 48.4%; single spot on TLC; IR (neat) ν_{SO_2} 1150, 1350 cm^{-1}, $\nu_{C\text{-}O\text{-}C}$ 1070 cm^{-1}.

2 (7.54 g, 15 mmol) was nitrated in chloroform (50 mL)–acetic acid (45 mL) at room temperature by 18.8 mL of 70% nitric acid. The small amount of the reaction mixture was withdrawn, neutralized with Na_2CO_3, extracted with chloroform, and subjected to TLC analysis to follow the progress of the reaction. It took about 24 h to complete the

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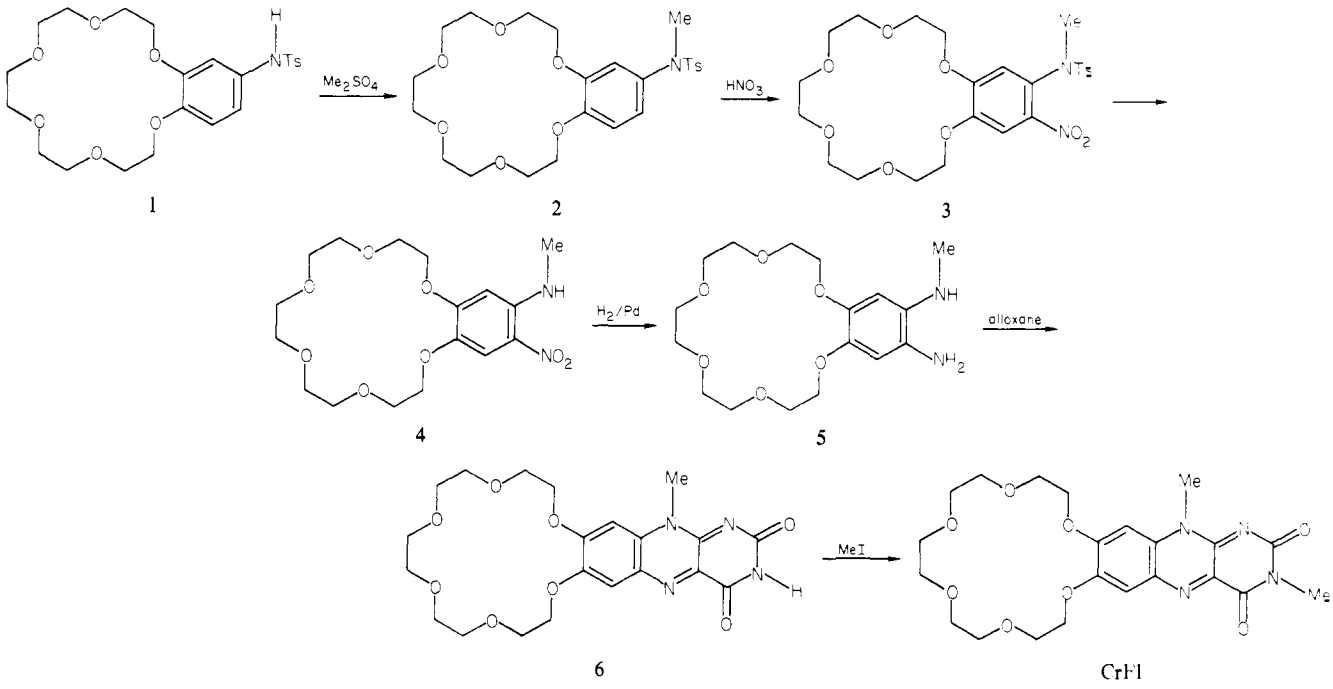
Scheme I



reaction. The reaction mixture was poured into ice water and extracted with chloroform. The chloroform solution was washed with water and aqueous Na_2CO_3 solution and evaporated to dryness. We thus obtained 4′-(*N*-methyl-*N*-tosylamino)-5′-nitrobenzo-18-crown-6 (**3**): mp 143–146 °C; yield 86.7%; IR (KBr disk) ν_{NO_2} 1340, 1520 cm^{-1}, ν_{SO_2} 1150, 1350 cm^{-1}, ν_{C-O-C} 1100 cm^{-1}; NMR ($CDCl_3$) δ 2.44 (3 H, s, CH_3 in Ts), 3.24 (3 H, s, NCH_3) 3.63–4.31 (20 H, m, crown protons), 6.64 (1 H, s, 3′-H), 7.30 (2 H, d, H ortho to methyl), 7.47 (1 H, s, 6′-H), 7.63 (2 H, d, H meta to methyl). Anal. ($C_{24}H_{32}N_2SO_{10}$) C, H, N.

3 (6.42 g, 11.9 mmol) was hydrolyzed to 4′-methylamino)-5′-nitrobenzo-18-crown-6 (**4**) in 50 mL of 40% H_2SO_4 at 80–100 °C for 1 h. After neutralization with Na_2CO_3, the solution was extracted with chloroform. The chloroform solution was treated with active charcoal and then evaporated to dryness. The solid residue (**4**) (4.19 g, 90.8% yield) was dissolved in 90 mL of methanol and reduced by hydrogen in the presence of 0.39 g of Pd on carbon. The reaction mixture was filtrated in a nitrogen stream. The filtrate containing 4′-(methylamino)-5′-aminobenzo-18-crown-6 (**5**), which was sensitive to air oxidation, was used directly for the following reaction. The completion of the reaction was confirmed by TLC.

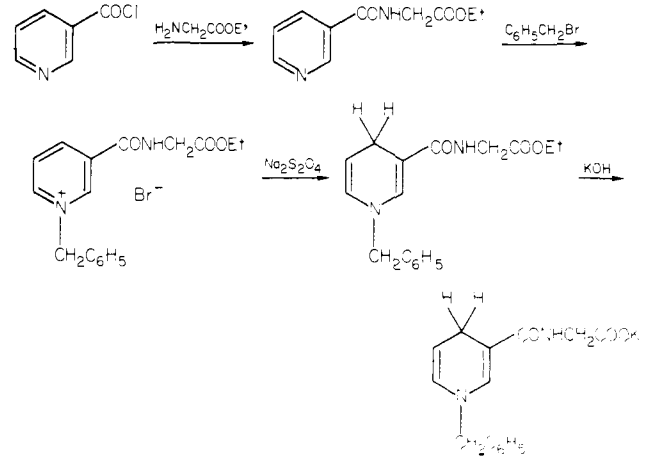
To 20 mL of a methanol solution containing 1.73 g (10.8 mmol) of alloxane was added 1 g of concentrated H_2SO_4. The methanol solution obtained from catalytic hydrogenation of **4** was mixed with the alloxane solution, and the mixture was refluxed for 1.5 h under a nitrogen stream. After cooling, the orange precipitate was collected by filtration and recrystallized from acetic acid: mp 323–328 °C; yield of 10-methyl-1′,4′,7′,10′,13′,16′-hexaoxacyclooctadec-2′-eno[2′,3′-*i*]isoalloxazine (**6**) from **3**, 28.0%.

6 (0.26 g, 0.56 mmol) was dissolved in 250 mL of *N*,*N*-dimethylformamide (DMF) containing 0.78 g (5.6 mmol) of powdered K_2CO_3 and methylated with methyl iodide (0.80 g, 5.6 mmol) at 30–40 °C. After 6 h, the additional amount of methyl iodide (1.45 g, 10.2 mmol) was added. TLC analysis showed that the reaction was over after 12 h. The reaction mixture was concentrated in vacuo, the residue being dissolved in chloroform. The chloroform layer was washed with 0.1 N NaOH and water and evaporated to dryness. The residue (crFl) was recrystallized from ethanol–isopropyl ether: mp 280–284 °C; yield 89.7%; IR (KBr disk) $\nu_{C=O}$ 1650 cm^{-1}, ν_{C-O-C} 1110 cm^{-1}; mass spectrum, *m/e* 476 (M^+). Anal. ($C_{22}H_{28}N_4O_8$) C, H, N. The NMR spectrum could not be measured because of its poor solubility.

Among NADH model compounds, preparations of 1-benzyl-1,4-dihydronicotinamide (BzlNAH) and N^3-dodecyl-1-benzyl-1,4-dihydronicotinamide (DodBzlNAH) were described previously.[17,18]

The potassium salt of N^3-carboxymethyl-1-benzyl-1,4-dihydronicotinamide (BzlNAHCOOK) was synthesized as shown in Scheme II.

Scheme II



BzlNAHCOOK

Nicotinyl chloride (5.1 g, 36 mmol) in 10 mL of DMF and triethylamine (7.6 g, 75 mmol) were added dropwise from different dropping funnels to 50 mL of a dehydrated DMF solution containing glycine ethyl ester hydrochloride (5.0 g, 36 mmol). The reaction mixture was stirred and kept at 0 °C. After 2 h triethylamine hydrochloride was removed by filtration and the filtrate was concentrated in vacuo. The residue was dissolved in chloroform and washed with water. Concentration of the chloroform solution gave an oily product (N^3-((ethoxycarbonyl)-methyl)nicotinamide) which showed a single spot on TLC: yield 92%; IR (neat) ν_{NH} 3100 cm^{-1}, $\nu_{C=O}$ 1760, 1670 cm^{-1}. The product was used for the next reaction without further purification.

The oily product (5.0 g, 24 mmol) was heated with benzyl bromide (30 g, 180 mmol) in 40 mL of benzene for 12 h. After cooling the separated oily layer was recovered by decantation. The product solidified in a refrigerator after one day. The solid (N^3-((ethoxycarbonyl)-methyl)-1-benzylnicotinamide bromide) was recrystallized from *n*-butyl alcohol: mp 151–153 °C; yield 91%. Anal. ($C_{17}H_{19}N_2O_3Br$) C, H, N.

The nicotinamide salt was reduced to N^3-((ethoxycarbonyl)-methyl)-1-benzyl-1,4-dihydronicotinamide by $Na_2S_2O_4$ according to the method of Kim and Chaykin.[19] The product was oil: yield 98%; one spot on TLC; NMR (Me_2SO-d_6) δ 1.18 (3 H, t, CH_3), 3.00 (2 H, d, 4-CH_2), 3.76 (2 H, d, N^3CH_2), 4.04 (2 H, q, OCH_2), 4.32 (2 H, s, N^1CH_2), 4.64 (1 H, m, 5-H), 5.92 (1 H, d, 6-H), 6.96 (1 H, s, 2-H), 7.3 (5 H, s, benzene protons).

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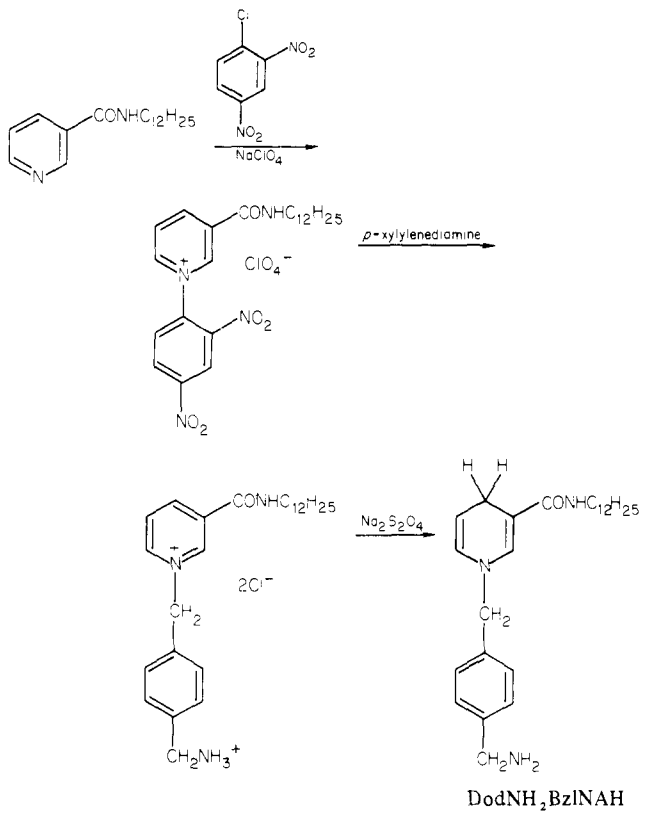
Scheme III



DodNH$_2$BzlNAH

N^3-((Ethoxycarbonyl)methyl)-1-benzyl-1,4-dihydronicotinamide (0.50 g, 1.66 mmol) in 1 mL of methanol was mixed with 10 mL of 1 N KOH solution and hydrolyzed at room temperature under a nitrogen stream. After 2 h the solution was neutralized by CO_2 gas and concentrated to dryness in vacuo. The residue was taken in anhydrous methanol and the solution was evaporated to dryness. This operation was repeated five times. We finally obtained a slightly yellow solid (BzlNAHCOOK): mp ca. 35 °C; yield 70%; one spot on TLC; NMR (D_2O) δ 3.76 (2 H, s, N^3CH_2), 3.02 (2 H, d, 4-H), 4.21 (2 H, s, N^1CH_2), 4.72 (1 H, m, 5-H), 5.75 (1 H, d, 6-H), 7.02 (1 H, s, 2-H), 7.26 (5 H, s, benzene protons). Anal. ($C_{15}H_{15}N_2O_3K$) C, H, N.

N^3-Dodecyl-1-(*p*-(aminomethyl)benzyl)-1,4-dihydronicotinamide (DodNH$_2$BzlNAH) was synthesized according to Scheme III and protonated to DodNH$_3^+$BzlNAH in situ. The ^3N-dodecyl group was attached in order to facilitate the extraction of the 1,4-dihydro compound from the aqueous layer into the organic layer after reduction with $Na_2S_2O_4$. We also synthesized 1-(*p*-(aminomethyl)benzyl)-1,4-dihydronicotinamide, but after reduction with $Na_2S_2O_4$ the 1,4-dihydro compound could not be extracted into the chloroform layer. The isolation from the aqueous solution was also difficult.

N^3-Dodecylnicotinamide[18] (20 g, 9.9 mmol) and 2,4-dinitrochlorobenzene (3.0 g, 10 mmol) were heated at 70 °C for 1 day. The mixture was poured into water, the precipitate being removed by filtration. From the aqueous filtrate N^3-dodecyl-1-(2,4-dinitrophenyl)nicotinamide perchlorate was precipitated by the addition of sodium perchlorate: mp 98–99 °C; yield 64%; IR (KBr disk) $\nu_{C=O}$ 1660 cm^{-1}, ν_{NO_2} 1540, 1340 cm^{-1}.

The perchlorate salt (1.50 g, 2.69 mmol) in 30 mL of methanol was added slowly to 20 mL of a stirred methanol solution containing *p*-xylylenediamine (0.92 g, 6.75 mmol). The reaction was continued at room temperature for 40 min. The slightly yellow crystals (2,4-dinitroaniline hydrochloride) were precipitated by the addition of 3 mL of concentrated HCl. The filtrate was concentrated to 15 mL and kept in a refrigerator. It gave the precipitate of *p*-xylylenediamine dihydrochloride. The precipitate was removed by filtration, the filtrate being poured into 35 mL of diethyl ether. After keeping one day in a refrigerator the formed precipitate (N^3-dodecyl-1-(*p*-(ammoniomethyl)benzyl)nicotinamide dichloride) was recovered by filtration: mp 233–238 °C; yield 25%; IR (KBr disk) $\nu_{C=O}$ 1640 cm^{-1}; NMR (Me_2SO-d_6) δ 0.84 (3 H, t, CH_3), 1.21 (20 H, m, $(CH_2)_{10}$), 3.40 (2 H, t, N^3CH_2), 4.00 (2 H, s, *p*-CH_2N^+), 5.96 (2 H, s, N^1CH_2), 7.60 (4 H, d, benzene protons), 8.22 (1 H, q, 5-H), 9.00 (1 H, d, 4-H), 9.35 (1 H, d, 6-H), 9.78 (1 H, s, 2-H). Elemental analysis and TLC showed that the product contains a trace amount of *p*-xylylenediamine dihydrochloride which cannot be removed by purification. We thus decided to remove the dihydrochloride after reduction to DodNH$_2$BzlNAH.

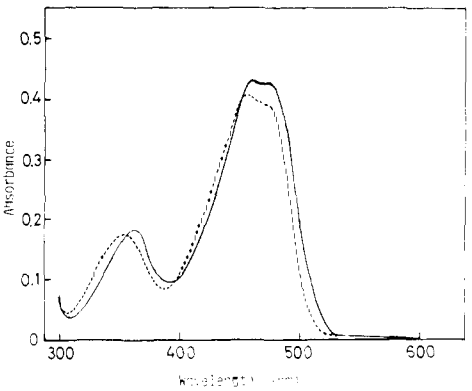


Figure 1. Absorption spectra of crFl (1.85×10^{-5} M) at 30 °C in methanol: (—) crFl; (- -) crFl + K^+ (1.75×10^{-4} M).

The nicotinamide dichloride was reduced to DodNH$_2$BzlNAH by $Na_2S_2O_4$ in a manner similar to that used for the preparation of DodBzlNAH.[18] The product was oil which gave a single spot on TLC: NMR (Me_2SO-d_6) δ 0.85 (3 H, t, CH_3), 1.24 (20 H, m, $(CH_2)_{10}$), 3.20 (2 H, t, N^3CH_2), 3.00 (2 H, d, 4-H), 3.65 (2 H, s, *p*-CH_2N), 4.25 (2 H, s, N^1CH_2), 4.55 (1 H, m, 5-H), 5.90 (1 H, d, 6-H), 6.92 (1 H, s, 2-H), 7.21 (4 H, d, benzene protons).

Benzimidazole derivatives were prepared from *o*-phenylenediamine and carboxylic acids with the terminal amino group according to the method of Revankar et al.[20] The products were identified by IR and elemental analysis. 2-(Aminomethyl)benzimidazole hydrochloride mp 227–229 °C; 2-(2-aminoethyl)benzimidazole hydrochloride, mp 193–196 °C; 2-(3-aminopropyl)benzimidazole hydrochloride, strongly hygroscopic oil (dihydrochloride, mp 276–279 °C); 2-(5-aminopentyl)benzimidazole hydrochloride, mp 180–186 °C; 2-(7-aminoheptyl)benzimidazole hydrochloride, mp 220–224 °C; 2-(10-aminodecyl)benzimidazole hydrochloride, mp 150–152 °C.

Kinetic Measurements. The kinetic measurements for oxidation of NADH model compounds by flavins were carried out at 30 °C aerobically under the recycle conditions by following the decrease in the absorption maximum of NADH model compounds.[17] In aqueous solutions the decrease obeyed the first-order rate equation for up to three half-lives. In methanolic solutions containing 12.2–30 vol % water the reaction was so slow that the rate constants were estimated from the initial slopes. The further details of the reaction conditions are recorded in captions and footnotes to each table and figure. When the acid-catalyzed decomposition of NADH model compounds, which was much slower than the net redox reaction, took place concomitantly, the contribution was offset by the rate in the absence of flavin.

Spectral Measurements. Absorption spectra of flavins were taken at 30 °C on a Shimadzu UV-3000 (dual-wavelength spectrophotometer) equipped with a thermostated cell holder. The spectrophotometer is useful to determine the small absorbance difference induced by added alkali metal cations. Fluorescence spectra were taken at 30 °C on a Hitachi 650-10S spectrophotometer equipped with a thermostated cell holder. The further details are described in captions and footnotes.

Results and Discussion

Influence of Added Metal Cations on Absorption and Fluorescence Spectra. The absorption spectrum of crFl in methanol (λ_{max} 363 and 460 nm (ϵ_{max} 9650 and 23 300, respectively)) was affected, although slightly, by added alkali metal chlorides ($\sim 1.75 \times 10^{-4}$ M). In particular, the relatively large decrease in the absorption maxima was observed on the addition of K^+ (Figure 1). Since no spectral change was observed for LFl at [MCl] < 10^{-3} M, the spectral change is due to the metal–crown interaction. According to theoretical calculations of the isoalloxazine skeleton, the lowest singlet state (around 450 nm) corresponds to the polarization along the long axis and the second transition at around 360 nm is polarized along the axis between the short and the long molecular axes.[21] The orientation of the permanent dipole moment in

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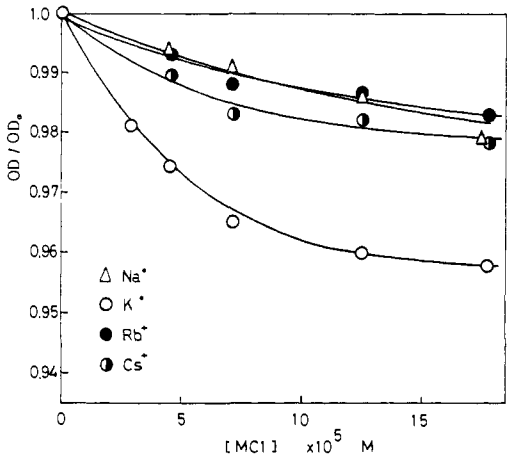

Figure 2. Influence of added alkali metal cations on the optical density of the absorption maximum (460 nm) of crFl (1.85×10^{-5} M) at 30 °C in methanol.

Table I. Association Constants (K) and Ratio of Quantum Yield (Φ_c/Φ_0) at 30 °C in Methanol

		low [M⁺] region		high [M⁺] region	
metal	K, M^{-1} [a]	K, M^{-1} [b]	Φ_c/Φ_0	K, M^{-1} [b]	Φ_c/Φ_0
Na^+	5700	3000	0.91	3500	1.0
K^+	17000	8000	0.97	2300	0.99
Rb^+	8200	4500	0.92	2100	0.98
Cs^+	6600	2900	0.88	2200	0.95

[a] From absorption spectrum. [b] From fluorescence spectrum.

isoalloxazine is almost parallel to the direction of the lowest transition dipole moment.[21] One may thus expect that the binding of alkali metal cations to the crown ring would offset the transition from the benzene moiety to the uracil moiety to some extent, leading to the decrease in the molar extinctions. We also measured the absorption spectra of crFl in the presence of $AgNO_3$, $Pb(OCOCH_3)_2$, and NH_4Cl which are known to have a large affinity with crown ethers,[22] but the spectral change was negligibly small at $[M^+(\text{or } M^{2+})] < 2 \times 10^{-4}$ M.

In Figure 2, the decrease in the absorption maximum at 460 nm (OD/OD_0) was plotted against metal concentrations. Except for K^+, the magnitude of the decrease was rather small. We determined the OD/OD_0 using a dual-wavelength spectrophotometer.[23] The association constants (K) were estimated from the plots according to eq 1, where ϵ_c is the molar extinction

$$\frac{1 - (OD/OD_0)}{[M^+]} = K\frac{OD}{OD_0} - \frac{\epsilon_c K[crFl]}{OD_0} \qquad (1)$$

coefficient of the metal–crFl complex. Plots of the data $[1 - (OD-OD_0)]/[M^+]$ vs. OD/OD_0 gave good straight lines with a correlation coefficient better than 0.98. We thus estimated the slope (K) from least-squares computation. The results (Table I) show that the affinity for alkali metal cations is in the order $K^+ > Rb^+ > Cs^+ \approx Na^+$. The K values are approximately comparable or somewhat smaller than those reported for 18-crown-6 and its homologues.[22,24,25]

There are a few examples of alkali metal cations bound to crown rings that affect the photochemical quantum yield of intramolecular chromophores.[26-28] For example, Sousa and Larson[27,28]

Table II. Influence of Alkali Metal Cations and Other Additives on the Relative Fluorescence Intensity (I/I_0)[a]

additive (mM)	I/I_0 for crFl	I/I_0 for LFl
NaCl (0.893)	0.981	1.00
KCl (0.893)	0.891	1.00
RbCl (0.893)	0.896	1.00
CsCl (0.898)	0.932	1.00
NH_4Cl (1.00)	1.00	1.02
$AgNO_3$ (1.00)	0.764	0.782
$Rb(OCOCH_3)_2$ (1.00)	0.983	0.998
$HgCl_2$ (5.00)	0.959	0.977
L-phenylalanine methyl ester·HCl (10.0)	0.981	0.980
L-tyrosine ethyl ester·HCl (10.0)	0.665	0.658
histamine·HCl (5.00)	0.971	0.964
tryptamine·HCl (10.0)	0.224	0.739

[a] 30 °C; methanol(absolute); [flavin] = 1.00×10^{-7} M; excitation wavelength 370 nm; emission wavelength 529 nm for crFl and 519 nm for LFl.

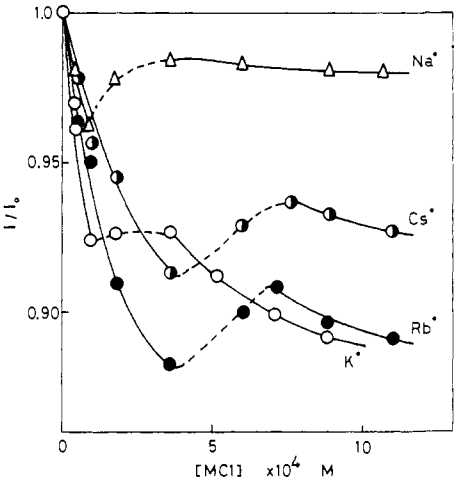

Figure 3. Influence of added alkali metal cations on the fluorescence intensity of crFl (1.00×10^{-7} M) at 30 °C in methanol. Excitation, 370 nm; emission, 529 nm.

reported that complexation of alkali metal chloride salts with a crown ring fused with naphthalene at the 2,3-positions causes a decrease in fluorescence quantum yield, whereas complexation with a crown ring fused with naphthalene at the 1,8-positions causes a perceptible increase in fluorescence quantum yield. The difference was accounted for by the difference in the geometrical orientation of perturbers. We have found that the fluorescence intensity of crFl (excitation 370 nm, emission 529 nm) decreases on the addition of alkali metal chloride salts, indicating the situation is similar to that of 2,3-substituted naphthocrown ether. No change in the fluorescence intensity was observed for LFl in the presence of comparable amounts of alkali metal cations (excitation 370 nm, emission 519 nm: Table II). Therefore, the decrease in crFl was attributed to the crown–metal interaction.

Strangely, the plots of I/I_0 against metal concentrations (Figure 3) showed a biphasic dependence; that is, I/I_0 decreases efficiently at low metal concentrations and after experiencing a transition the decrease in I/I_0 becomes small and less sensitive to metal concentrations. The larger the size of the metal ion, the higher is the metal concentration where the transition occurs. Such transitions were not found for the fluorescence of naphtho crown ethers,[27] so that the isoalloxazine ring must be responsible for the peculiar biphasic dependence. Being different from benzo crown and naphtho crown ethers, the solubility of crFl in methanol is very poor ($\sim 5 \times 10^{-4}$ M). Probably, this is due to the association tendency of the isoalloxazine ring. In fact, it is known that flavin

(22) Christensen, J. J.; Eatough, D. J.; Izatt, R. M. *Chem. Rev.* **1974**, *74*, 351.
(23) The association constants were recalculated on the basis of OD/OD_0 or I/I_0 values obtained averaging 3–4 determinations. The plot of OD/OD_0 vs. [KCl] gave an abnormal decrease at [KCl] = 1×10^{-5} M which is due to the change in the spectrum pattern. This change was neglected in the calculation.
(24) Weber, E.; Vögtle, F. In "Host Guest Complex Chemistry I"; Vögtle, Ed.; Springer-Verlag: Berlin, 1981; p 20.
(25) Ungaro, R.; Haj. B. E.; Smid, J. *J. Am. Chem. Soc.* **1976**, *98*, 5198.
(26) Hautala, R. R.; Hastings, R. H. *J. Am. Chem. Soc.* **1978**, *100*, 648.
(27) Sousa, L. R.; Larson, J. M. *J. Am. Chem. Soc.* **1977**, *99*, 307.
(28) Larson, J. M.; Sousa, L. R. *J. Am. Chem. Soc.* **1978**, *100*, 1943.

molecules readily form stacked dimeric or polymeric aggregates,[21] which sometimes lead to a decrease in the fluorescence intensity due to concentration quenching.[29,30]

It is believed that 18-crown-6 and its homologues usually form a 1:1 complex with Na^+ and K^+.[22,24,25] Recently, Ishizu et al.[31,32] proposed on the basis of an ESR spin–spin interaction that the formation of sandwich-type complex depends on the structure of the crown substituents. For example, a Tanol derivative of benzo-15-crown-5 forms, as expected, a 1:1 complex with Na^+, whereas a verdazyl derivative of benzo-15-crown-5 gives a strong spin–spin interaction, suggesting the formation of a 1:2 cation/crown complex. They considered that the sandwitch-type complex in the verdazyl derivative is facilitated by stacked face-to-face aggregation of two verdazyl groups. It is likely, therefore, that two isoalloxazine rings of crFl form a similar stacked aggregation in the presence of (especially large) alkali metal cations. At present, we presume that a 1:2 metal/crFl complex is formed below the transition concentration while the formation of a 1:1 metal/crFl complex becomes favorable with increasing metal concentrations.

We analyzed the data in Figure 3 by the Mataga–Tsuno equation (eq 2),[33] where Φ_0 and Φ_c are the quantum yields of

$$\frac{(I_0/I) - 1}{[M^+]} = K - \frac{\Phi_c}{\Phi_c}\frac{\epsilon_c}{\epsilon_0}K\frac{I_0}{I} \qquad (2)$$

fluorescence of crFl and complex, and ϵ_0 and ϵ_c are the molar extiction coefficients of crFl and complex at the wavelength of the exciting light, respectively. The ϵ_c/ϵ_0 values at 370 nm are 0.90, 0.84, 0.86, and 0.94 for Na^+, K^+, Rb^+, and Cs^+, respectively. From plots of $((I_0/I) - 1)/[M^+]$ vs. I_0/I we obtained two independent linear lines for low and high metal concentration regions. We determined the K from the intercepts and calculated the Φ_c/Φ_0 from the slopes. The results are summarized in Table I. Since we assume the formation of the 1:1 complexes for all data, it is inevitable that the accuracy is somewhat inferior. Examination of Table I reveals the following: (i) The order of the metal affinity determined from the data at low concentration region is what is expected for 18-crown-6 ($K^+ > Rb^+ > Na^+ \approx Cs^+$). (ii) The K values are about half of those determined by an absorption spectroscopic method. (iii) The K values determined by the data at high concentration region are very small except for Na^+. (iv) Except for Na^+ at the low concentration region the Φ_c/Φ_0 values decrease with an increase in the metal ion size.

It is known that the association constants of the monobenzo-15-crown-5 series are reduced by introducing electron-withdrawing substituents at the 4′-position and a good Hammett correlation with a negative slope (−0.45) is seen between log K and $\sigma_p^- + \sigma_m$.[25] For example, the association constant of 4′-nitrobenzo-15-crown-5 for Na^+ (365 M^{-1} in acetone) is smaller by about one order of magnitude than that of benzo-15-crown-5 (3440 M^{-1} in acetone).[25] The isoalloxazine moiety of crFl can be regarded as an electron-withdrawing "substituent" because the C-8 methyl group of flavin usually shows acidity as strong as that of *p*-nitrotoluene.[34] Therefore, the somewhat smaller association constants of crFl relative to benzo-18-crown-6 are primarily associated with the electronic effect. Meanwhile, Larson and Sousa[27,28] showed with their "rope-skipping" crown ethers[35] that the fluorescence quantum yield is most effectively quenched by Cs^+ and next by Rb^+ because of a heavy-atom effect operating for Cs^+ and perhaps for Rb^+. As shown in Table I, the Φ_c/Φ_0 values decrease in the presence of heavy-metal cations such as Cs^+ and Rb^+. However, the change

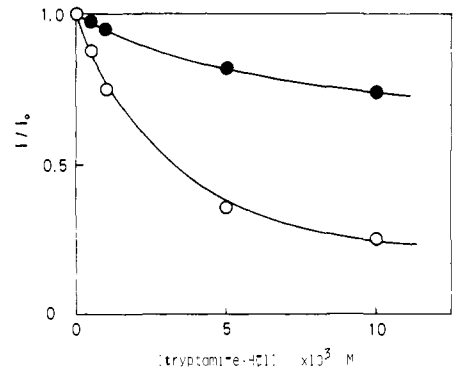

Figure 4. Influence of tryptamine hydrochloride on the fluorescence intensity of crFl (○) and LFl (●) at 30 °C in methanol. Wavelengths of excitation and emission are 370 and 529 nm for crFl and 370 and 519 nm for LFl ([flavin] = 1.00×10^{-7} M).

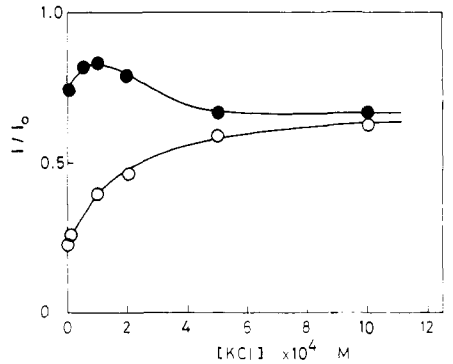

Figure 5. Competitive binding of tryptamine hydrochloride and K^+ to crFl at 30 °C in methanol: [flavin] = 1.00×10^{-7} M; [tryptamine hydrochloride] = 1.00×10^{-2} M; (○) crFl; (●) LFl.

Scheme IV

$$\text{tryptamine-NH}_3^+ + \text{crFl} \rightleftharpoons \underset{(\text{high } I/I_0)}{\text{tryptamine-NH}_3^+\cdots\text{crFl}} \overset{-K^+}{\underset{K^+}{\rightleftharpoons}} \underset{(\text{low } I/I_0)}{K^+\cdots\text{crFl}} + \underset{(\text{medium } I/I_0)}{\text{tryptamine-NH}_3^+}$$

is not so drastic as observed for "rope-skipping" crown ethers.[28] In "rope-skipping" crown ethers metal cations are held on the face of the naphthalene π system, whereas in crFl metal cations are held at the end of the isoalloxazine π system. Thus the fluorescence behaviors are largely governed, as pointed out by them,[27,28] by the geometrical orientation between metal and chromophore.

Pseudointramolecular Fluorescence Quenching through Recognition of Ammonium Groups. Flavins are known to form association complexes with a large variety of substances of biological relevance. Charge transfer character has been postulated for most of these complexes with the flavins acting as acceptors.[36-40] In particular, the flavin–amino acid interaction is of special importance in connection with that in the active sites of flavoproteins. The association constants are conveniently estimated by a fluorescence quenching method.[37,38] Visser et al.[41] and Johnson and McCormick[42] observed efficient intramolecular fluorescence

(29) Shinkai, S.; Harada, A.; Ishikawa, Y.; Manabe, O. *J. Chem. Soc., Perkin Trans. 2* **1982**, 125.
(30) Shinkai, S.; Harada, A.; Manabe, O.; Yoneda, F. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 928.
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(36) Slifkin, M. A. In "Charge Transfer Interactions of Biomolecules": Academic Press: London, 1971.
(37) Slifkin, M. A. *Nature (London)* **1963**, *197*, 275.
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(41) Visser, A. J. W. G.; Li, T. M.; Drickamer, H. G.; Weber, G. *Biochemistry* **1977**, *16*, 4883.
(42) Johnson, P. G.; McCormick, D. B. *Biochemistry* **1973**, *12*, 3359.

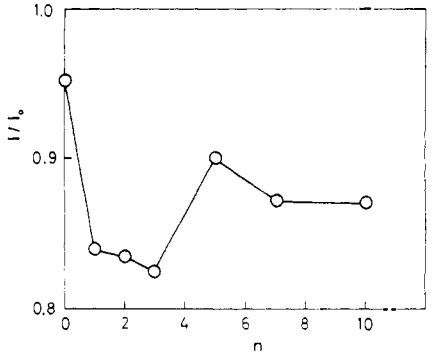
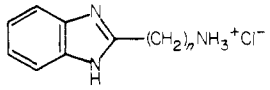


Figure 6. Relative fluorescence intensity of crFl (1.00×10^{-7} M) in the presence of BI-$(CH_2)_n$-NH_3^+ (2.00×10^{-3} M) at 30 °C in methanol (n = 0 is benzimidazole).

quenching for flavins bearing intramolecular tryptophan or histidine which is separated by polymethylene spacers. We considered that if the ammonium group of amino acids is "complexed" by the crown ring of crFl, it would lead to efficient pseudointramolecular fluorescence quenching. We tested several amino acid ammonium salts which are known to quench the fluorescence of flavins.[37-39] The results in Table II show that the fluorescence of crFl was in fact quenched by hydrochloride salts of L-phenylalanine methyl ester, L-tryosine ethyl ester, and histamine, but the decreases at [quencher] = $(0.5 - 10) \times 10^{-3}$ M were quite comparable with those observed for LFl. The result implies that recognition of the ammonium groups does not contribute to subsequent pseudointramolecular fluorescence quenching. In contrast, we found a marked difference in the fluorescence decrease in the presence of tryptamine hydrochloride (Table II and Figure 4): I/I_0 for crFl decreased three times more effectively than that for LFl. In order to further confirm the contribution of the crown ring we measured I/I_0 in the presence of constant tryptamine hydrochloride (1.00×10^{-2} M) as a function of K^+ concentration (Figure 5). In contrast to the almost constant I/I_0 value (0.77 ± 0.09) for LFl, the I/I_0 value for crFl increased with increasing K^+ concentration, and two values became almost equal at high K^+ concentration. The phenomenon can be explained by the Scheme IV, which is similar to competitive inhibition in enzyme catalysis.

The foregoing results suggest that there exist some prerequisites to the efficient pseudointramolecular fluorescence quenching. When the histidine derivatives were linked to the N(10) position of flavin as $-(CH_2)_n$CONHCH(COOR)CH_2–imidazole, the most efficient fluorescence quenching was observed for the chain length with six atoms (i.e., n = 2) separating the flavin and imidazole.[42] It is undoubted that the ammonium groups of the quenchers form the complexes with the crown ring of crFl. It is likely, therefore, that even though the ammonium groups are bound to the crown ring of crFl, the chain length with only two carbon atoms may be too short for chromophores to interact with the isoalloxazine intramolecularly. The efficient intramolecular interaction with tryptamine hydrochloride is ascribed either to the large molecular size of tryptophan or to the a priori strong association ability of tryptophan with flavin.

We tried the quantitative estimation of the spacer length by using a series of benzimidazole (BI) derivatives having different polymethylene chains at the 2-position. The data (Figure 6) are



compared at [BI-$(CH_2)_n$-NH_3^+] = 2.00 mM where the I/I_0 values are almost saturated. Clearly, the most efficient quenching occured at n = 3. Since the molecular size of BI is as large as that of tryptophan, n = 3 may be enough for the pseudointramolecular energy transfer. On the other hand, the ammonium salts of amino acids and histamine have the spacers with only two carbon atoms and the chromophore sizes are smaller than tryptophane and BI. One may conclude, therefore, that the spacers are too short to interact efficiently with the isoalloxazine.

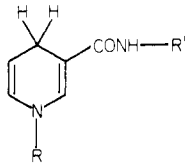
Table III. Second-Order Rate Constants (k_2) for the Reaction of crFl and LFl with 1-Benzyl-1,4-dihydronicotinamide (BzlNAH)[a]

entry	medium	metal ion (M) present in medium	flavin	k_2, M^{-1} s^{-1}
1	water	K^+ (0.005)	LFl	22.3
2	water	K^+ (1.00)	LFl	22.9
3	water	K^+ (0.005)	crFl	1.91
4	water	K^+ (1.00)	crFl	3.48
5	water-MeOH[b]	none	LFl	0.749
6	water-MeOH[b]	K^+ (0.20)	LFl	0.691
7	water-MeOH[b]	none	crFl	0.096
8	water-MeOH[b]	K^+ (0.20)	crFl	0.202
9	water-MeOH[b]	NH_4^+ (0.20)	crFl	0.230
10	water-MeOH[b]	Na^+ (0.20)	crFl	0.134
11	water-MeOH[b]	Rb^+ (0.20)	crFl	0.211
12	water-MeOH[b]	Cs^+ (0.20)	crFl	0.200

[a] 30 °C. Buffer: KOH–boric acid (pH 8.9) for entries 1–4 and *N*-ethylmorpholine (0.030 M)–HCl (0.015 M) for entries 5–12. Chloride salts were used to adjust the ion concentrations. [b] Water–MeOH (3:7 v/v).

Redox Reactions with NADH Model Compounds. It is interesting to know whether the reaction between crFl and NADH model compounds is amenable to "complexation" with NADH model compounds. The reaction is a typical intercoenzyme reaction and first-order in flavin and NADH model compounds[17] unless an abnormally high concentration (~0.1 M) of NADH model compounds is used.[43] According to Gascoigne and Radda,[44] the logarithm of the second-order rate constants (k_2) is linearly correlated with the polarographic half-wave potential of flavins. Thus, one may easily estimate the shift of the redox potential from the rate constant.

We determined the k_2 values for the following NADH model compounds in the absence and the presence of alkali metal cations. Since the dihydropyridine structure is very sensitive to acidic protons, $DodNH_2BzlNAH$ was synthesized and protonated to $DodNH_3^+BzlNAH$ in situ in the reaction mixture. Since the pK_a



BzlNAH, R = $C_6H_5CH_2$; R' = H
BzlNAHCOOK, R = $C_6H_5CH_2$; R' = $KOOCCH_2$
DodBzlNAH, R = $C_6H_5CH_2$; R' = $CH_3(CH_2)_{11}$
DodNH_3^+BzlNAH, R = *p*-$NH_3^+CH_2C_6H_4CH_2$; R' = $CH_3(CH_2)_{11}$

of *N*-ethylmorpholine hydrochloride (7.70) used as a buffer is much lower than that of benzylamine (9.34), the amino group of $DodNH_2BzlNAH$ must be protonated in the presence of excess *N*-ethylmorpholine hydrochloride. The dodecyl group at the N^3 position was introduced for the sake of the synthetic simplicity. The kinetic results are summarized in Tables III and IV.

Entries 1–4 in Table III show that (i) in aqueous solution the rate constant for crFl is smaller by one order of magnitude than that for LFl because of the electron-donating nature of the 7,8-ether groups and (ii) the rate constants for LFl are scarcely affected by K^+ concentration, whereas those for crFl are enhanced by 1.8-fold in the presence of 1.0 M K^+ ion. The small but perceptible rate increase in the presence of K^+ is attributed to the complexation of K^+ with the crown ring which would suppress the electron-donating ability of the ether groups in crFl. The similar trend was observed more clearly in a water–methanol (3:7 v/v) mixed solvent (entries 5–12 in Table III). The rate constants

(43) Blankenhorn, G. *Biochemistry* **1975**, *14*, 3172.
(44) Gascoigne, I. M.; Radda, G. K. *Biochim. Biophys. Acta* **1967**, *131*, 498.

Table IV. Second-Order Rate Constants (k_2) for the Functionalized NADH Model Compounds[a]

entry	medium	metal ion (M) present in medium	NADH model	flavin	k_2, M^{-1} s^{-1}
1	water	K^+ (0.005)	BzlNAHCOO$^-$	LFl	24.6
2	water	K^+ (1.00)	BzlNAHCOO$^-$	LFl	26.0
3	water	K^+ (0.005)	BzlNAHCOO$^-$	crFl	2.38
4	water	K^+ (1.00)	BzlNAHCOO$^-$	crFl	4.22
5	water-MeOH[b]	K^+ (0.005)	BzlNAH	crFl	0.072
6	water-MeOH[b]	K^+ (0.005)	BzlNAHCOO$^-$	crFl	0.247
7	water-MeOH[b]	K^+ (0.005)	BzlNAH	LFl	0.345
8	water-MeOH[b]	K^+ (0.005)	BzlNAHCOO$^-$	LFl	0.810
9	water-MeOH[b]	none	DodBzlNAH	crFl	0.0126
10	water-MeOH[b]	none	DodNH_3^+BzlNAH	crFl	0.361[c]

[a] 30 °C. Buffer: KOH–boric acid (pH 8.9) for entries 1–4 and *N*-ethylmorpholine (0.030 M)–HCl (0.015 M) for entries 5–10. KCl was used to adjust the K^+ concentration. [b] Water-MeOH (12.2:87.8 v/v). [c] k_{cat}/K_m.

for LFl was again unaffected by K^+ concentration, whereas those for crFl were enhanced by 1.4- to 2.4-fold by the addition of 0.20 mol of alkali metal and ammonium cations. Although the rate constant for Na^+ was a little smaller than others, alkali metal and ammonium cations gave similar enhanced rate constants.

Bruice et al.[40] found that plots of log k_2 for both NADH and 1-propyl-1,4-dihydronicotinamide vs. log of the association constants (determined by fluorescence quenching) of flavins for tryptophan are approximately linear. The findings mean that kinetically important preequilibrium complex formation would take place between flavins and NADH (and its model compounds). Blankenhorn[43,45] considered that the reaction proceeds though a face-to-face orientation. As demonstrated in the foregoing chapter, chromophores which have an intramolecular recognizable function (e.g., ammonium group) can efficiently quench the fluorescence of crFl. One may thus expect that NADH model compounds having such a recognizable group would be efficiently oxidized by crFl. BzlNAHCOOK is one of the candidates, because the interaction of the COO^- group with K^+ bound to the crown ring may facilitate the face-to-face orientation between the dihydropyridine ring and isoalloxazine. Entries 5–8 in Table IV show, however, that this is not the case. In aqueous methanol the rate constant for the reaction of BzlNAHCOOK and crFl is 3.4 times greater than that for the reaction of BzlNAH and crFl, but a similar rate increase (2.3-fold) was also observed for the reaction with LFl. The results merely mean that in aqueous methanol BzlNAHCOOK is 2–3 times more reactive than BzlNAH. On the other hand, the significant reactivity difference was not seen in aqueous solution (see entries 1–4 in Tables III and IV). There is a similar precedent found by Hadju and Sigman[46] that the negatively charged intramolecular carboxylate in an NADH model compound stabilizes partial positive charge which develops on the nicotinamide moiety in the transition state. Thus, the rate increase was observed in less polar solvents but not in water. Probably, the 3-carboxylate group in BzlNAHCOOK is able to stabilize the positively charged transition state.

Instead, we used DodNH_3^+BzlNAH which has an ammonium group as a recognizable function. In order to compare the kinetic results with the fluorescence data in Figure 6 we attempted the synthesis of an NADH model with 1-$(CH_2)_nNH_3^+$, but the isolation and purification were extremely difficult. We also synthesized 1-(*p*-(aminomethyl)benzyl)-1,4-dihydronicotinamide but could not isolate the dihydropyridine (obtained by $Na_2S_2O_4$ reduction in aqueous solution). We thus synthesized DodNH_3^+-BzlNAH with a dodecyl group in the N^3 position. As shown in Figure 7, the reaction of DodBzlNAH and crFl, followed by the decrease in the absorption band of DodBzlNAH (350 nm), showed a first-order dependence on the concentration of DodBzlNAH. On the other hand, the plot of v_{obsd} vs. DodNH_3^+BzlNAH concentration gave a saturation tendency. Assuming that a 1:1 complex is formed between crFl and DodNH_3^+BzlNAH, the double reciprocal plot of v_{obsd} vs. DodNH_3^+BzlNAH concentration

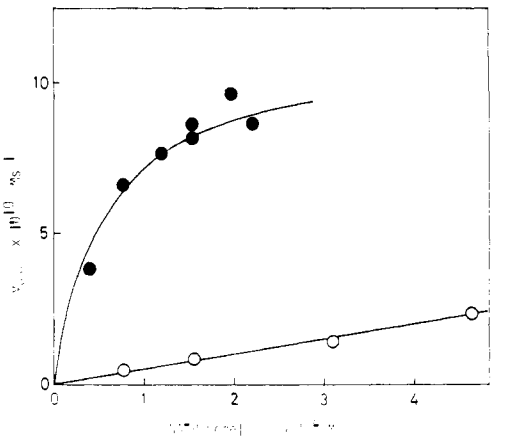
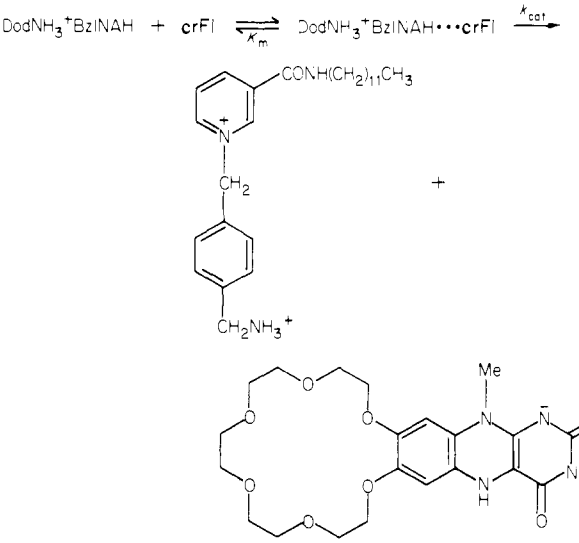


Figure 7. Reaction of crFl with DodBzlNAH (○) and DodNH_3^+-BzlNAH (●) at 30 °C in methanol (12.2 vol % water). [crFl] = 3.83 × 10^{-5} M, [*N*-ethylmorpholine] = 0.030 M; [HCl] = 0.015 M.

Scheme V



(Lineweaver–Burk plot[47]) provided a good straight line (r = 0.986). The result shows that the reaction apparently proceeds according to the Michaelis–Menten kinetics (Scheme V). Thus, k_{cat} and K_m were determined by the least-squares method from the slope and the intercept: k_{cat} = 3.65 × 10^{-5} s^{-1} and K_m = 1.01 × 10^{-4} M. Examination of CPK models suggests that when the NH_3^+ of DodNH_3^+BzlNAH is bound into the 18-crown-6 of crFl, the dihydropyridine moiety of DodNH_3^+BzlNAH exactly overlaps with the central pyrazine ring of crFl. The kinetically estimated association constant ($K = 1/K_m = 10^4$ M^{-1}) is in accord with that

(45) Blankenhorn, G. *Eur. J. Biochem.* **1975**, *50*, 351.
(46) (a) Hadju, J.; Sigman, D. S. *J. Am. Chem. Soc.* **1975**, *97*, 3524; (b) *Biochemistry* **1977**, *13*, 2841.
(47) Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *56*, 658.

expected for the ammonium group.[22,24,25] When comparing the k_{cat}/K_m (= 0.361 M^{-1} s^{-1}) of DodNH_3^+BzlNAH with k_2 (= 1.26 × 10^{-2} M^{-1} s^{-1}) of DodBzlNAH, the rate increment of 29-fold was attained through the recognition of the ammonium group.

Conclusion

As a crown ether flavin mimic, crFl, has brought forth several novel phenomena: (i) a change in the absorption and fluorescence spectra by added metal cations, (ii) efficient intramolecular fluorescence quenching by chromophores having the ammonium group, (iii) a change in the reactivity by added metal cations, and (iv) Michaelis-Menten type saturation kinetics in the reaction with DodNH_3^+BzlNAH. These phenomena imitate well several biological concepts important in enzyme chemistry: for example, allosteric effectors, competitive inhibition, recognition of metal cations, intracomplex reactions, etc. The close imitation can be achieved because crFl has within a molecule flavin as a catalytic site and crown ether as a recognition site, which are the minimum constituents required for the enzyme model system. One may say, therefore, that in a sense crFl is a well-constructed miniature of flavoenzymes. Since a crown ether family has a wide variety of association abilities, including the asymmetric recognition, we believe that modification of the crown moiety would lead to the further development of novel flavin chemistry.

Acknowledgment. We thank Dr. K. Kano (Doshisha University) for helpful discussions. This research was supported by a grant from the Ministry of Education of Japan.

Registry No. 1, 86996-27-2; **2**, 86996-28-3; **3**, 86996-29-4; **4**, 86996-30-7; **5**, 86996-31-8; **6**, 86996-32-9; CrFl, 86996-33-0; CrFl (Na^+ complex), 88729-62-8; CrFl (K^+ complex), 88729-63-9; CrFl (Rb^+ complex), 88729-64-0; CrFl (Cs^+ complex), 88729-65-1; BzlNAHCOOK, 86996-34-1; DodNH_2BzlNAH, 88704-70-5; LFl, 18636-32-3; BzlNAH, 952-92-1; DodBzlNAH, 83239-12-7; DodNHBzlNAH·HCl, 88704-77-2; H_2NCH_2COOEt, 459-73-4; NaCl, 7647-14-5; KCl, 7447-40-7; RbCl, 7791-11-9; CsCl, 7647-17-8; NH_4Cl, 12125-02-9; $AgNO_3$, 7761-88-8; $Pb(OCOCH_3)_2$, 301-04-2; $HgCl_2$, 7487-94-7; Na^+, 17341-25-2; Rb^+, 22537-38-8; Cs^+, 18459-37-5; K^+, 24203-36-9; NH_4^+, 14798-03-9; 4'-aminobenzene-18-crown-6, 68941-06-0; alloxane, 50-71-5; nicotinyl chloride, 10400-19-8; glycine ethyl ester hydrochloride, 623-33-6; N^3-((ethoxycarbonyl)methyl)nicotinamide, 54466-74-9; N^3-((ethoxycarbonyl)methyl)-1-benzylnicotinamide bromide, 88704-66-9; N^3-((ethoxycarbonyl)methyl)-1-benzyl-1,4-dihydronicotinamide, 88704-67-0; N^3-dodecylnicotinamide, 81475-38-9; 2,4-dinitrochlorobenzene, 97-00-7; N^3-dodecyl-1-(2,4-dinitrophenyl)nicotinamide perchlorate, 88704-69-2; *p*-xylylenediamine, 539-48-0; N^3-dodecyl-1-(*p*-(ammoniomethyl)-benzyl)nicotinamide dichloride, 88704-71-6; 2-(aminomethyl)benzimidazole hydrochloride, 7757-21-3; 2-(2-aminoethyl)benzimidazole hydrochloride, 88704-72-7; 2-(3-aminopropyl)benzimidazole hydrochloride, 88704-73-8; 2-(3-aminopropyl)benzimidazole dihydrochloride, 88765-77-9; 2-(5-aminopentyl)benzimidazole hydrochloride, 88704-74-9; 2-(7-aminoheptyl)benzimidazole hydrochloride, 88704-75-0; 2-(10-aminodecyl)benzimidazole hydrochloride, 88704-76-1; L-phenylalanine methyl ester hydrochloride, 7524-50-7; L-tyrosine ethyl ester hydrochloride, 4089-07-0; histamine hydrochloride, 23758-34-1; tryptamine hydrochloride, 14733-29-0; 2-aminobenzimidazole hydrochloride, 26893-41-4.

Total Synthesis of 3-(5-Tetrazolyl)carbapenems

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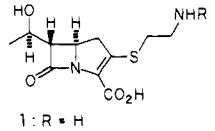
Abstract: The synthesis of the title compounds, β-lactam antibiotics, is described. A new tetrazole protecting group, (((*p*-nitrobenzyl)carbonyl)oxy)methyl, was successfully applied. Substitution of carboxyl by tetrazole on the carbapenem nucleus results in stability to a degradative renal enzyme.

Synthetic modifications of carbapenem natural products, represented by thienamycin (**1**), have yielded many potent new antibiotics.[1] In particular, formimidoylation of thienamycin has resulted in a derivative, MK0787 (**2**) selected for clinical studies.[2]



1: R = H
2: R = CH=NH

In considering other sites on the carbapenem nucleus for analogue studies, we were struck by the paucity of literature precedent for carboxylic acid substitution.[3] Herein we describe our work on 3-(5-tetrazolyl)carbapenems[4] which was directed toward renal dehydropeptidase, the major degradative enzyme for carbapenems in vivo.[5]

Our synthetic plan was initially designed to incorporate the efficient chemistry developed at Merck.[6] Utilizing a chiral intermediate **3**,[7] the tetrazolyl moiety was to be appended by the magnesium-mediated homologation procedure[6,8] used to prepare the corresponding keto esters. The success of subsequent transformations was then dependent upon the chemically equivalent behavior of the protected tetrazole and ester functional groups.

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