C-4 and C-5 Adducts of Cofactor PQQ (Pyrroloquinolinequinone). Model Studies Directed toward the Action of Quinoprotein Methanol Dehydrogenase†

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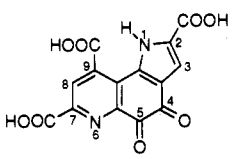
Received June 9, 1993®

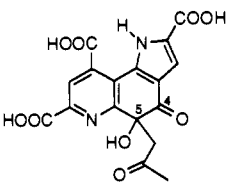
Abstract: Methanol addition to the trimethyl ester of cofactor PQQ (PQQTME) was investigated in detail to obtain information on the action of quinoprotein methanol dehydrogenase. The hemiacetal-type adduct was easily isolated from a methanol solution of PQQTME. The crystal structure of the adduct was determined by X-ray diffraction for the first time, showing that methanol addition occurred at the 5-position (C-5) of the quinone as in the case of the acetone adduct formation. On the other hand, treatment of PQQTME in methanol under acidic conditions gave the dimethyl acetal derivative as a major product for which the addition position of methanol was determined to be C-4 by X-ray crystallographic analysis. Studies of the adduct formation reactions with methanol using a series of PQQ model compounds and the molecular orbital calculations provided a clear-cut explanation for the difference in positions between the hemiacetal formation and the acetal formation. Because the C-5 hemiacetal was not very stable, it readily reverted to the quinone in solution, while the C-4 acetal was reduced to the quinol derivative when treated with base. The spectral characteristics and biological significance (particularly in the enzymatic alcohol oxidation mechanism) of the C-4 and C-5 adducts of cofactor PQQ are discussed.

Introduction

PQQ (**1**) is a novel cofactor that was first isolated and identified from methanol dehydrogenase of methylotrophic bacteria in 1979.[1] Since then, much effort has been devoted to finding several kinds of quinione-containing enzymes (referred to as *quinoproteins*) from a variety of organisms of both baterial and mammalian origins.[2] In addition to the enzymological importance, the growth-stimulating activity for microorganisms,[3] the pharmaceutical activities,[4-6] and the nutritional importance[7] of PQQ itself have also been revealed to indicate that PQQ serves versatile functions in several living systems. Therefore, recent attention has been focused on the chemistry of cofactor PQQ in order to clarify its biological functions at a molecular level.



1 (PQQ)



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One of the most intriguing aspects of the chemical properties of PQQ is the high reactivity toward nucleophiles.[8] Acetone easily adds to the quinone carbonyl carbon of PQQ to form the aldol-type adduct **2** under weakly alkaline conditions. In fact, PQQ was first isolated and crystallized as the acetone adduct from methanol dehydrogenase of methylotrophic bacteria.[1] Addition of water and alcohols to the quinone function of PQQ was also studied spectrophotometrically in solution.[9] Covalent addition of hexanol to PQQ was applied to develop the detection method for PQQ (the so-called hexanol extraction procedure).[10] The addition position of water and alcohols has always been considered to be C-5,[9,10] but no direct evidence has been reportd to date.

It has been proposed that nucleophilic addition to the quinone is a key step in the redox reactions of PQQ with biologically important substances such as amines,[11,12] amino acids,[11a,13,14]

† This paper is respectfully dedicated to Professor Teddy G. Traylor for his friendship, inspiration, and leadership as a scholar and teacher.

• Abstract published in *Advance ACS Abstracts,* October 1, 1993.

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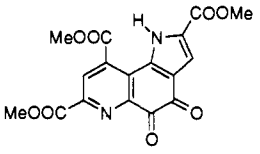
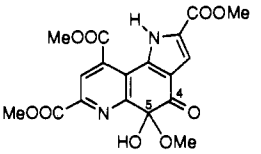
hydrazine derivatives,[15-17] and thiols.[18] Several types of PQQ adducts were isolated and characterized in those model reactions to support such ionic mechanisms. The addition position of the substrates is still ambiguous, however. Inactivation of quinoproteins by hydrazine derivatives[19] and by cyclopropanol[20,21] has been attributed to the irreversible adduct formation of the active-site cofactor with those inhibitors. Furthermore, the high reactivity of PQQ toward amino acids or peptides makes it difficult to identify free PQQ in biological fluids.[22] In spite of such importance of the adduct formation reactions, only two crystal structures of the PQQ adducts have been reported.[1,23]

In this paper, we report the structural and chemical characterizations of the C-4 and C-5 adducts of cofactor PQQ. Since methanol dehydrogenase is one of the most important quinoproteins, methanol addition was mainly investigated. Little was known about the mechanistic details of the alcohol oxidation by quinoprotein alcohol dehydrogenases. In the present study, the trimethyl ester of cofactor PQQ (PQQTME, **3**) was employed



3 (PQQTME)



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as a model compound to make the product analysis much easier. Since active sites of enzymes are generally considered to consist of a hydrophobic environment and PQQ is known to be tightly bound at the active site of the enzymes, it might be more advantageous to use the ester derivative in organic media than to use free PQQ in aqueous solutions.

Results

Hemiacetal Formation. Methanol addition to quinone **3** was first investigated by UV–vis spectroscopy in solution (eq 1). Figure

$$3 + \mathrm{MeOH} \overset{K_{add}}{\rightleftarrows} \mathbf{4} \qquad (1)$$

$$\frac{1}{\Delta A} = \frac{1}{(\epsilon_3 - \epsilon_4)[\mathbf{3}]_0 K_{add}[\mathrm{MeOH}]} + \frac{1}{(\epsilon_3 - \epsilon_4)[\mathbf{3}]_0} \qquad (2)$$

1 shows the spectral change in the titration of **3** by methanol at 30 °C in CH_3CN. A remarkable increase in the absorption at 371 nm, and decreases of the shoulders at around 275 and 440 nm were observed with isosbestic points at 252, 354, and 413 nm. The equilibrium constant $K_{add} = [\mathbf{4}]/[\mathbf{3}][\mathrm{MeOH}]$ was calculated to be 0.63 M^{-1} by eq 2, where ΔA is the absorption change ($A - A_0$) at methanol concentrations from 0.24 to 1.18 M, ϵ_3 and ϵ_4 are molar absorption coefficients of **3** and **4**, and $[\mathbf{3}]_0$ is the initial concentration of the quinone, respectively. A double-reciprocal plot of ΔA_{371} versus [MeOH] gave a straight line with an intercept, as shown in Figure 2, from which the K_{add} was calculated.

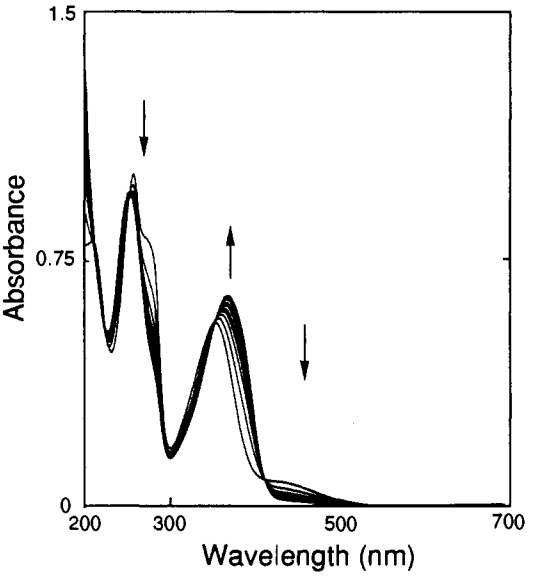

Figure 1. Spectral changes in the titration of **3** (4.84×10^{-4} M) with methanol in CH_3CN. In order to obtain large spectral changes, methanol was added in large excess (0–12 M). All spectra are of solutions contained in a 1 mm path length UV cell and are normalized to that of the initial concentration of **3**.

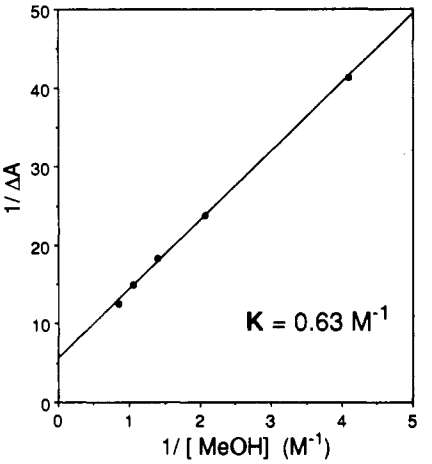

Figure 2. Plot of $1/\Delta A_{371}$ vs 1/[MeOH] for the titration of **3** (4.84×10^{-4} M) with methanol in CH_3CN. Data were collected at methanol concentrations from 0.24 to 1.18 M.

Pale yellow single crystals of adduct **4** suitable for X-ray analysis were easily formed in a methanol solution of **3** by allowing it to stand for a few days at room temperature. Because the crystals were not very stable against dryness, the X-ray measurement was carried out by sealing them in a glass capillary tube containing the mother solution. As clearly shown in Figure 3, methanol adduct **4** is the C-5 *hemiacetal* derivative of PQQTME, as has already been suggested by Dekker *et al.*[9] According to the reported X-ray structure of **1**,[24] the two heterocyclic rings (pyridine and pyrrole) of PQQ are almost coplanar. In hemiacetal **4**, on the other hand, the two heterocyclic rings deviate slightly from coplanarity; the dihedral angle of the two heterocyclic rings is 9.9°. It is interesting to note that the hemiacetal is stabilized by the intermolecular hydrogen-bond network among the ester carbonyl at the 7-position, the hydroxyl group of the crystallization

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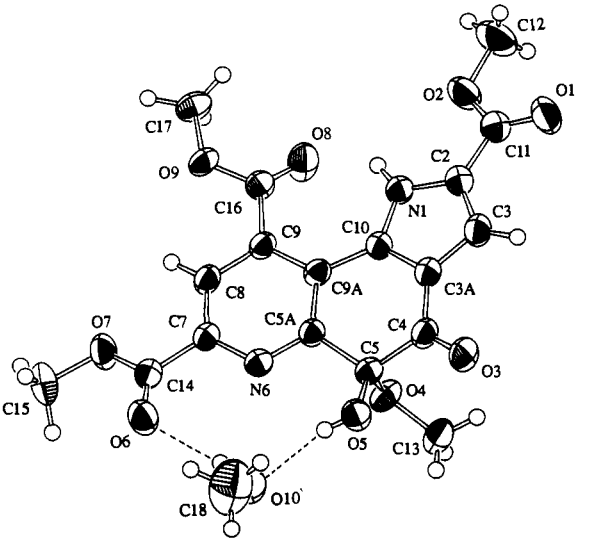
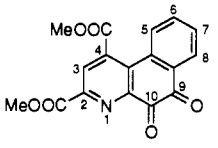


Figure 3. ORTEP drawing of hemiacetal **4**. Intermolecular hydrogen bonds are indicated by dashed lines.



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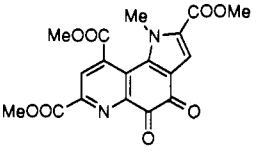

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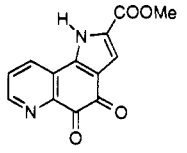

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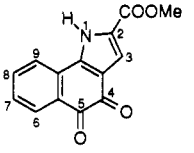

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solvent, and the hemiacetal function, as indicated by dotted lines in Figure 3 [O(5)- - -O(10) = 2.772(4) Å and O(6)- - -O(10) = 2.896(4) Å]. The dihedral angles between the methyl ester groups at the 2-, 7-, and 9-positions and the aromatic planes are 8.5, 15.2, and 25.8°, respectively. The larger deviation of the 9-ester group from the plane is due to the steric hindrance around the 1-position together with the intramolecular hydrogen bond between the ester carbonyl and H-1 [O(8)- - -H(1) = 2.642(3) Å]. The intermolecular hydrogen bond between the ester carbonyl and methanol may be an important factor to retain the geometry of the 7-ester group.

Methanol addition was further investigated using other model compounds (**5–8**) heretofore synthesized by us.[17] The equilibrium constants for methanol addition to these quinones were determined spectrophotometrically in similar manners and are listed in Table I. Since the ^{1}H NMR study of the acetone adducts of these quinone compouns indicated that the adition positions are C-5 in the case of **6–8** and C-10 in the case of **5**,[17] it is assumed that hemiacetal formation took place at the same positions, C-5 in the case of **6–8** and C-10 in the case of **5**. From the order of the equilibrium constants in Table I, it is easily recognized that the pyridine nucleus and the ester groups on it considerably enhance methanol addition, but the pyrrole ring shows an opposite effect. A similar tendency of the reactivity, **8** ≪ **7** < **3** < **6** < **5**, was observed in the acetone adduct formation.[17]

Acetal Formation. When quinone **3** was treated in refluxing methanol under acidic conditions (in the presence of a catalytic amount of *p*-toluenesulfonic acid) for a few hours, a mixture of products, **9** and **10**, and the starting material, **3**, was obtained in

Table I. Equilibrium Constants K_{add} for Addition of Methanol to the Quinones at 30 °C in CH_3CN

quinone	K_{add}, M^{-1}	quinone	K_{add}, M^{-1}
5	3.38	**7**	0.13
6	1.04	**8**	0.036
3	0.63		

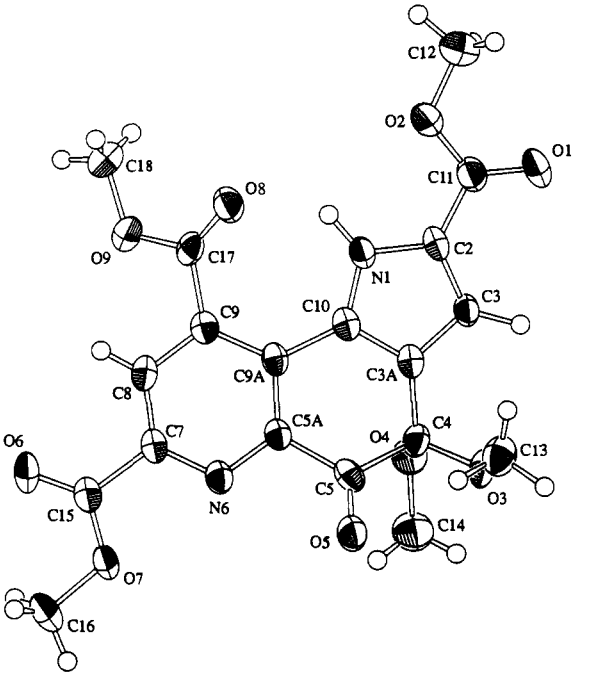
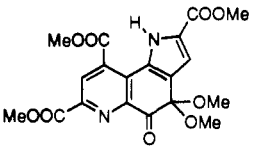


Figure 4. ORTEP drawing of acetal **9**.



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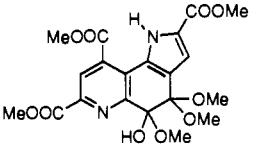

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a 33:45:22 ratio after workup with CH_2Cl_2–0.1 M K_2CO_3. Quinone **3** may be regenerated from hemiacetal **4** by hydrolysis during the workup treatment. The ^{1}H NMR spectrum of the mixture suggested that the products are a dimethyl acetal derivative of the quinone and a dimethyl acetal with another methoxyl group (probably as a hemiacetal function), respectively. In order to obtain the acetal derivative selectively, we applied Corey's method.[25] Treatment of **3** with an excess of methyl orthoformate and a trace of PTS in refluxing methanol for a few hours gave the acetal derivative whose ^{1}H NMR spectrum was completely identical to that of product **9**.

Pale yellow single crystals of dimethyl acetal **9** suitable for X-ray analyhsis were also obtained by recrystallization from benzene. Figure 4 shows the crystal structure of **9**. Unexpectedly, acetal formation took place at the 4-position of the quinone. So far, acetal formation has always been considered to occur at C-5,[10,25] but such a conclusion proved to be false. The reason for this difference in the addition positions (C-4 vs C-5) is discussed later. In the case of acetal **9**, the molecule is slightly distorted in the same degree; the dihedral angle of the two heterocyclic rings is about 9.6°, but the 7-ester group is free from the intermolecular hydrogen bond with methanol, as observed in **4**, and is nearly coplanar with the pyridine ring. Considering the above mentioned results, product **10** was assigned to the C-4 acetal with a hemiacetal function at C-5.

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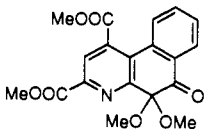
Table II. NOEs Detected on the Aromatic Protons of the Dimethyl Acetal Derivatives with Irradiation on the Methoxyl Group of the Acetal Function[a]

acetal	proton	NOE, %	acetal	proton	NOE, %
9	H-3	12	**12**	H-3	1
11	H-8	4	**13**	H-3	14

[a] Measured in $CDCl_3$.

NOE measurements were useful tools for estimation of the addition position of methanol. In acetal **9**, a large NOE (12%) was detected between the methoxyl groups of the acetal function and H-3, but only a small effect (ca. 2%) was observed on H-3 of hemiacetal **4** when the methoxy group of the hemiacetal function was irradiated. Dimethyl acetal derivatives of model compounds **5**, **6**, and **8** were also synthesized in a similar manner. NOEs detected betrween each methoxyl group of the acetal functions and the aromatic protons are listed in Table II. Judging from the results, it was concluded that acetal formation took place at the quinone carbonyl carbon closer to the pyridine ring in the case of **5** and **6** but at the opposite site in the case of **8**, affording acetals **11–13**, respectively.



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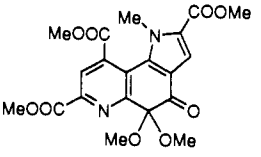

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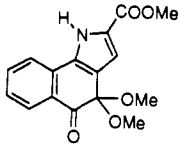

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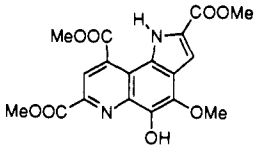
Because hemiacetal **4** itself is not very stable in CH_3CN, it reverted to the original quinone within a few minutes, even under neutral conditions. Addition of a base such as triethylamine or an acid such as PTS in the solution accelerated the reversion to the quinone. On the other hand, treatment of acetal **9** with excess triethylamine (100 equiv) in CH_3CN gave reduced compound **14**



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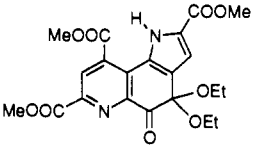

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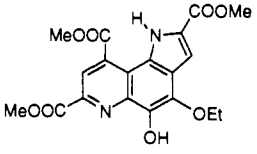


16

in a 29% isolated yield. The position of the methoxyl group that remained in **14** was confirmed to be C-4 by a large NOE (6%) detected between the methoxyl group and H-3, excluding occurrence of any rearrangements in the course of the reaction. Thus, reduced compound **14** is considered to be formed by oxidative elimination of the methanol moiety in the acetal function, although the attempt to detect formaldehyde, the oxidation product of methanol, failed. In order to confirm the formation of aldehyde, the same reaction was carried out using diethyl acetal **15**, which was prepared from **3** by a similar manner (see

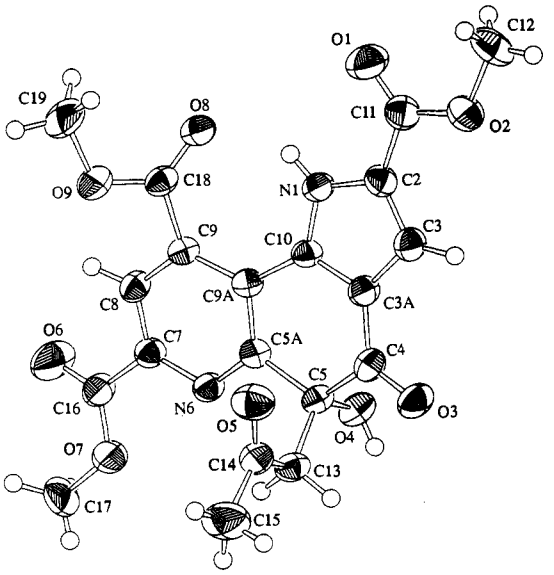
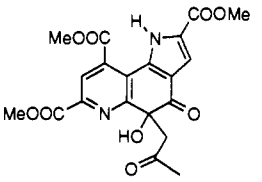


Figure 5. ORTEP drawing of acetone adduct **17**.

Experimental Section). In this case, a relative amount of acetaldehyde (58%) was obtained as the (2,4-dinitrophenyl)hydrazone together with the corresponding reduced compound **16** (45%).

Acetone adduct formation was already investigated under weakly alkaline conditions.[12c,17] ^1H NMR studies of the acetone adduct suggested that the addition position was C-5. In the present study, single crystals of acetone adduct **17** were successfully



17

isolated and its crystal structure was determined by X-ray diffraction, as shown in Figure 5. The molecular geometry of **17** is comparable to that of **2**.[1] Deviation of the molecule from planarity is similar to those of **4** and **9**; the dihedral angle of the two heterocyclic rings is about 10.7°.

Discussion

The hemiacetal derivative of cofactor PQQ was isolated and characterized for the first time. The crystal structure of the methanol adduct clearly indicated that the addition position is C-5. This has already been suggested by the results of an NMR study in solution.[9] Considering the electron-withdrawing nature of the pyridine nucleus having two carboxyl groups, this tendency to add at the C-5 position in adduct formation is very reasonable, and this was the case in the adduct formation reactions with acetone and (2,4-dinitrophenyl)hydrazine.[1,23] Studies of the hemiacetal formation (Table I) and the acetone adduct formation,[17] using a series of model compounds, further made it clear that the pyridine nucleus and the ester groups on it considerably enhance adduct formation. In general, hydration or alcohol addition to carbonyl compounds is largely enhanced when the carbonyl compounds have a highly electron-withdrawing substituent.[26] The pyrrole ring, on the other hand, showed an opposite effect, which can be attributed to its electron-releasing nature. A small difference in K_{add} between **3** and **6** may reflect the increase in steric hindrance around the 1-position due to replacement of

(26) Carey, F. A.; Sundberg, R. J. *Advanced Organic Chemistry*, 3rd ed.; Plenum Press: New York and London, 1990; Part A, Chapter 8.

Table III. Heats of Formation of Hemiacetals 4 and 18[a]

hemiacetal	heat of formation, kcal/mol		
	MNDO	AM1	PM3
4	-294.20	-275.41	-295.79
18	-291.39	-273.22	-294.78

[a] Calculated by MOPAC, version 6.1.

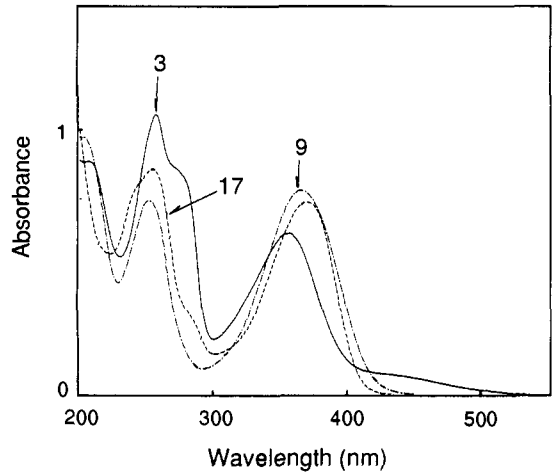


Figure 6. UV-vis spectra of quinone 3, acetal 9, and acetone adduct 17 in CH_3CN (4.0×10^{-5} M).

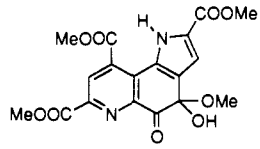
Table IV. Spectral Data for 3, 4, 9, and 17

quinone	^{1}H NMR, δ[a]			^{13}C NMR, ppm[b]		UV-vis λ_{max}, nm[c]
	H-1	H-3	H-8	C-4	C-5	
3	12.96	7.48	8.89	173.5	177.2	258, 356, 440
4	12.65	7.44	8.75			254, 371
17	12.75	7.43	8.67	167.3	74.8	256, 370
9	12.22	7.13	8.71	96.8	193.1	253, 364

[a] In $CDCl_3$. [b] In DMSO-d_6. [c] In CH_3CN.

H-1 with the methyl group. Such steric hindrance could be diminished to some extent by adduct formation.

Surprisingly, quinone 3 was mainly converted into the C-4 acetal derivatives 9 and 10 in the reaction with methanol under acidic conditions. In order to discuss the mechanism, we performed semiempirical molecular orbital calculations on hemiacetals 4 and 18. The calculated values of the heats of formation



18

listed in Table III clearly indicate that the C-5 hemiacetal is more stable than the C-4 adduct by about a few kcal/mol (1–3 kcal/mol, depending on the method used). Since hemiacetal 4 is readily converted into the original quinone in solution (*vide ante*), the *hemiacetal formation step* is considered to be completely reversible. Under such circumstances, the reaction can be regarded as thermodynamically controlled.[26] Thus, it is reasonable that hemiacetal 4 is formed as a solo isolable product under neutral conditions (eq 1). In the presence of acid, elimination of water from the protonated intermediate $18H^+$ may proceed much faster than that from protonated hemiacetal $4H^+$, because the generated carbocationic intermediate can be stabilized by simultaneous release of the pyrrole proton (H-1) ($18H^+$ to 19; Scheme I). Attack by the second molecule of the solvent gives C-4 acetal 9. There is no such stabilizing effect on the carbocationic intermediate derived from C-5 hemiacetal 4. Once C-4 acetal 9 is formed, it cannot revert to C-4 hemiacetal 18 in the presence of excess methanol ([MeOH] ≫ [H_2O]). Therefore, C-4 acetal 9 is gradually accumulated under acidic conditions. This mechanism is supported by the results of acetal formation reactions of other quinones. C-4 acetal 13 was also obtained in the case of 8, which has the same indole-4,5-quinone skeleton in the molecule, but the acetal formation occurred at the opposite site of the quinone, C-10 an C-5, in the cases of 5 and 6, respectively. Acetals 11 and 12 were derived from the corresponding C-10 and C-5 hemiacetals, which must be more stable than the corresponding C-9 and C-4 adducts, as discussed above.

Some spectral characteristics of the C-4 and C-5 adducts of cofactor PQQ are worth noting. The UV-vis spectra of the C-4 adduct 17 and C-5 acetal 9 in acetonitrile are shown in Figure 6 together with that of quinone 3. Basically, there is not much difference in shape and λ_{max} between the C-4 and C-5 adducts. Upon adduct formation, the absorption band at around 356 nm shifts toward a longer wavelength by about 10–15 nm and the quinonoid n–π^* transition at 400–500 nm and a shoulder around 270 nm disappear.

In the ^{1}H NMR spectra (Table IV), the aromatic proton H-8 shows a greater upfield shift than H-3 when they are converted into the C-5 adducts ($\Delta\delta_{H\text{-}3}$ = 0.04 and 0.05 and $\Delta\delta_{H\text{-}8}$ = 0.14 and 0.22 for 4 and 17, respectively), but in the case of C-4 adduct 9, H-3 shows a greater upfield shift than H-8 ($\Delta\delta_{H\text{-}3}$ = 0.35, $\Delta\delta_{H\text{-}8}$ = 0.18). These results could be attributed to the disappearance of the mesomeric effect of the C-5 carbonyl on the pyridine ring and that of the C-4 carbonyl on the pyrrole nucleus, respectively. The larger upfield shift of H-1 of 9 as compared with those of 4 and 17 may reflect the disappearance of the conjugation between the C-4 carbonyl group and the pyrrole nucleus.

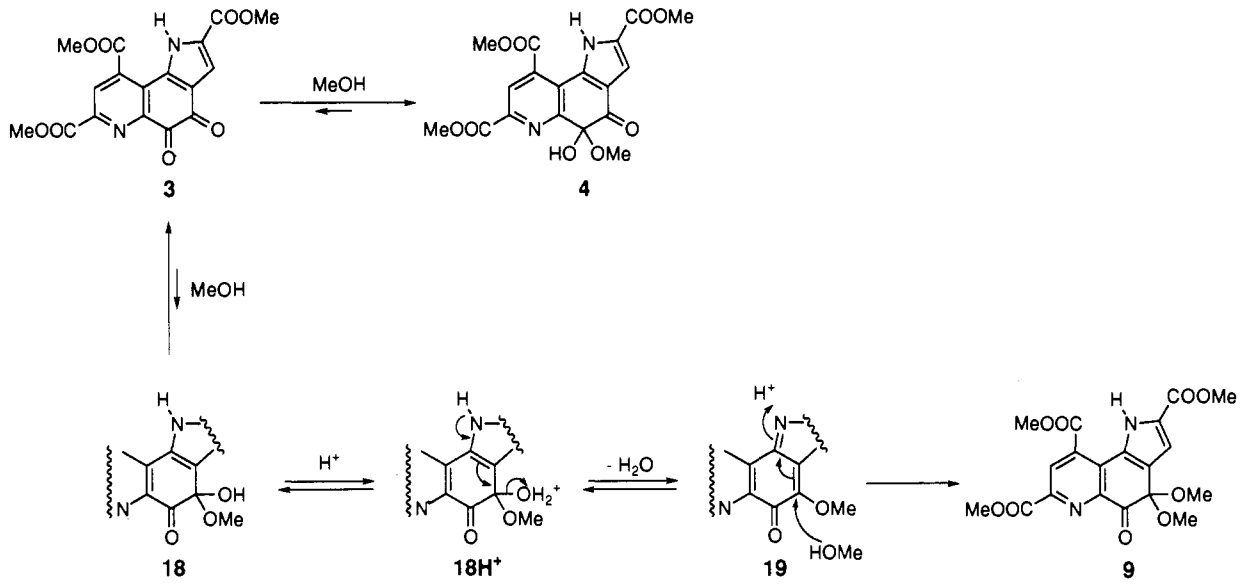
Little was known about the mechanistic details of the enzymatic reaction of quinoprotein alcohol dehydrogenases. Frank *et al.* proposed that oxidation of alcohols by PQQ in enzymatic systems proceeds via an addition–elimination mechanism through the C-5 hemiacetal intermediate (Scheme II).[27] This mechanism seems to be plausible, because quinone 3 was easily converted into hemiacetal 4 by treatment with methanol under neutral conditions. Oxidation of amines by PQQ has also been considered to proceed via a similar addition–elimination mechanism through a C-5 carbinolamine-type intermediate.[11,12] However, hemiacetal 4 itself is not very stable in solution, and it reverts to the original quinone immediately when treated with acid or base. If the enzyme operated according to Frank's mechanism, it should have a special device to stabilize the hemiacetal intermediate.

There is another possible ionic mechanism, where the C-4 acetal derivative is a key intermediate (Scheme III). In the reaction with alcohols under the acidic conditions, quinone 3 was predominantly converted into the C-4 acetals 9 and 15. This corresponds to the formation of the C-4 acetal intermediate in Scheme III. Treatment of 9 and 15 with a base such as triethylamine gave reduced products 14 and 16, respectively, suggesting the possible occurrence of the base-catalyzed oxidative elimination of alcohol from the acetal intermediate. In fact, aldehyde was detected together with the reduced compound in the reaction with diethyl acetal 15. Recently, Xia and co-workers reported the three-dimensional structure of quinoprotein methanol dehydrogenase at 2.6-Å resolution.[28] According to the reported X-ray structure of the enzyme-active site, there are two hydroxyl groups (Ser-175 and Thr-159) near the quinone function of PQQ (within a few angstroms). Thus, one of the hydroxyl groups can act as a nucleophile to form the hemiacetal intermediate, and the other one can be a general-acid catalyst to facilitate the

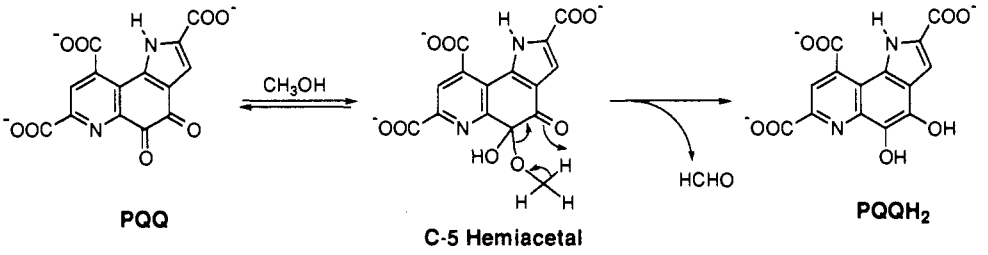
(27) Frank, J.; Dijkstra, M.; Duine, J. A.; Balny, C. *Eur. J. Biochem.* **1988**, *174*, 331.

(28) Xia, Z.-x.; Dai, W.-w.; Xiong, J.-p.; Hao, Z.-p.; Davidson, V. L.; White, S.; Mathews, F. S. *J. Biol. Chem.* **1992**, *267*, 22289.

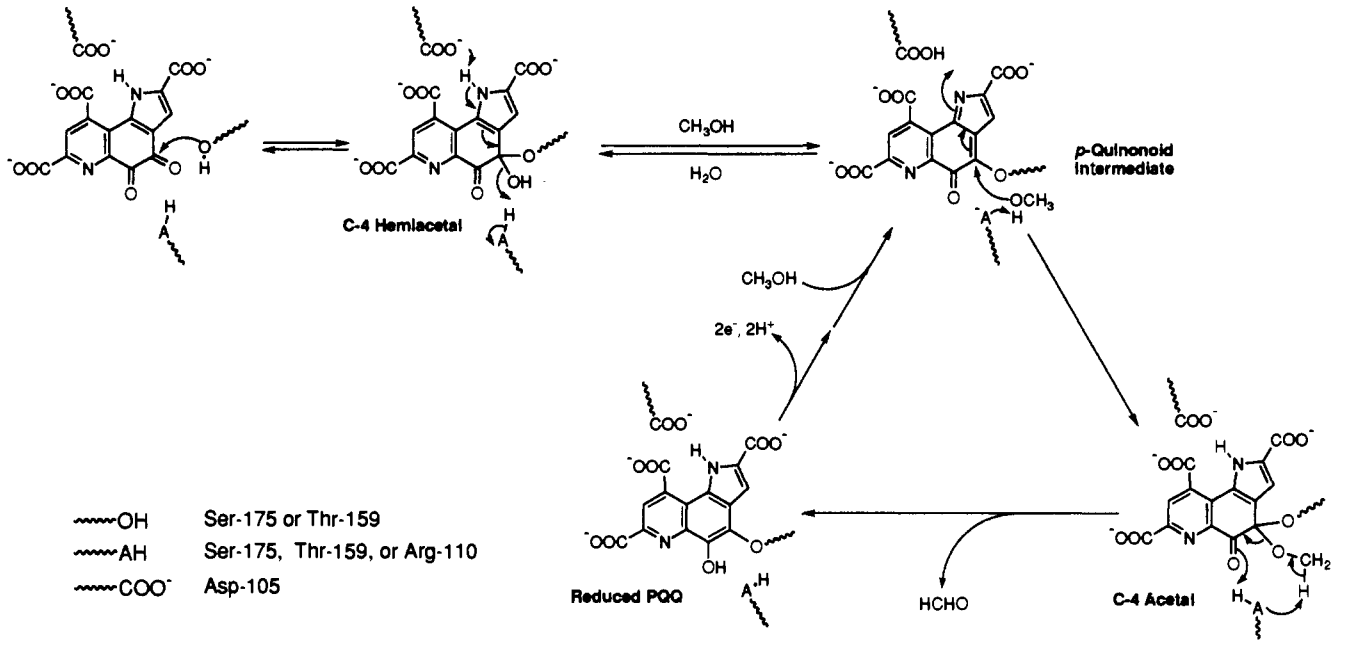
Scheme I



Scheme II



Scheme III



dehydration from the C-4 hemiacetal intermediate. Alternatively, the side chain of Arg-110, which extends into the active-site region, can be a general-acid catalyst. Furthermore, there is a carboxylate group of Asp-105 near the pyrrole ring of PQQ that can act as a general-base catalyst to accept the pyrrole proton (H-1), aso enhancing the dehydration from the C-4 hemiacetal intermediate. It was reported that the enzyme activity of reconstituted quinoprotein ethanol dehydrogenase with *N*(*1*)-alkyl-PQQs was very low (only about 1%).[29] This fact may indicate the importance of the pyrrole proton at N-1 for enzyme activity and is consistent with the present mechanism. After the C-4 acetal intermediate is formed by the attack of the substrate, the base-catalyzed

(29) Jongejan, J. A.; Groen, B. W.; Duine, J. A. In *PQQ and Quinoproteins (Proceedings of the First International Symposium on PQQ and Quinoproteins, Delft, The Netherlands, 1988)*; Jogenjan, J. A., Duine, J. A., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1998 ; p 205–216.

oxidative elimination of the substrate occurs to afford the reduced PQQ and formaldehyde, although the identity of the base catalyst for abstraction of the α-proton is unclear at this moment. Oxidation of the reduced species of PQQ regenerates the *p*-quinonoid intermediate, to which substrate methanol again attacks, resulting in a catalytic cycle.

According to the proposed mechanism (Scheme III), PQQ is covalently attached to the enzyme-active site through the ether linkage with a proximal serine or threonine hydroxyl function, which seems to be inconsistent with the reported fact that PQQ is noncovalently associated in the enzyme-active site. In the absence of methanol substrate (in the resting state of the enzyme), however, PQQ in the quinone form would be released easily from the catalytic cycle; *hydrolysis* of the *o*-quinonoid intermediate and the C-4 hemiacetal would predominate in the absence of methanol substrate. It was also demonstrated that PQQ was converted into the C-5 adduct of ring-opened cyclopropanol during the inhibition process of the enzyme by cyclopropanol.[21] This C-5 adduct formation is very reasonable, since it is an irreversible C–C bond formation reaction like the C-5 acetone adduct formation. A similar ionic mechanism through a C-4 amino ether-type intermediate was proposed by Forrest and co-workers, where they thought that the external primary amine, the activator of methanol dehydrogenase, was the nucleophilic catalyst activating the C-4 quinone carbonyl.[30]

There are still several problems to be solved in the mechanism of oxidation by quinoprotein methanol dehydrogenase.[31] A possibility of the hydride-transfer mechanism must also be examined.[32] But the present results give us possible implications for the mechanism of PQQ-containing alcohol dehydrogenases. Further investigations of the enzyme-active site and model studies of alcohol oxidation by PQQ will provide a more precise picture of the enzymatic oxidation mechanism.

Experimental Section

General Procedures. Trimethyl 4,5-dioxo-4,5-dihydro-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate (**3**), dimethyl 9,10-dioxo-9,10-dihydrobenzo[*f*]quinoline-2,4-dicarboxylate (**5**), trimethyl 4,5-dioxo-4,5-dihydro-1-methylpyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate (**6**), methyl 4,5-dioxo-4,5-dihydro-1*H*-pyrrolo[2,3-*f*]quinoline-2-carboxylate (**7**), methyl 4,5-dioxo-4,5-dihydrobenz[*g*]indole-2-carboxylate (**8**), and trimethyl 4-oxo-5-acetonyl-5-hydroxy-4,5-dihydro-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate (**17**) were obtained from previous studies.[17] The chemicals used in this study were purified by the standard methods,[33] if necessary. Melting points were determined with a Yamato MP-21 apparatus and were uncorrected. IR spectra were recorded with a Hitachi 270-30 spectrophotometer, and UV–vis spectra, with a Shimadzu UV-265 spectrophotometer equipped with a Shimadzu TCC-260 thermostated cell holder. Mass spectra were recorded with a JEOL JNX-DX303 HF mass spectrometer or a Shimadzu GCMS-QP2000 gas chromatograph mass spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL FT-NMR EX-270 spectrometer. Molecular orbital calculations were performed with the MOPAC program (version 6.1) using a CAChe work system (SONY Tektronix).[34]

Hemiacetal Formation. Equilibrium constants (K_{add}) for methanol addition to the quinones (4.0×10^{-4} M) were determined by spectrophotometric titration using a 1 mm path length UV cell in CH_3CN at 30 °C.

Trimethyl 4-oxo-5-hydroxy-5-methoxy-4,5-dihydro-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate (4) was crystallized from a methanol solution of **3** by allowing it to stand for a few days at room temperature: isolated yield 63%; IR (KBr) 3492 (OH), 1726 (ester C=O), 1710 (C=O), 1274, 1244, 1210 cm^{-1} (C—O); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.38 (3 H, s, $-OCH_3$), 3.96 (3 H, s, $-COOCH_3$), 4.04 (3 H, s, $-COOCH_3$), 4.13 (3 H, s, $-COOCH_3$), 5.60 (1 H, s, –OH), 7.44 (1 H, d, J = 2.2 Hz, H-3 (an NOE of 2% was detected when the methoxy group at C-5 (δ 3.38 ppm) was irradiated)), 8.75 (1 H, s, H-8), 12.65 (1 H, br s, NH).

Acetal Formation. Acetal derivatives **9**, **11–13**, and **15** were prepared by Corey's method.[25] Typically, the quinone (ca. 3–9 mM) was treated with 50 equiv of methyl orthoformate (ethyl orthoformate in the case of **15**) and a trace of *p*-toluenesulfonic acid in refluxing methanol for a few hours. The reaction mixture was extracted with CH_2Cl_2, the extract was washed with $NaHCO_3$ aqueous solution, and the organic layer was dried over $MgSO_4$. Crude products were purified by chromatography (SiO_2).

Trimethyl 4,4-dimethoxy-5-oxo-4,5-dihydro-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate (9): isolated yield 97% (pale yellow single crystals suitable for X-ray analysis were obtained by slow recrystallization from benzene); mp 227–229 °C; MS (EI) m/e 418 (M^+); IR (KBr) 3448, 3275 (NH), 1728 (ester C=O), 1690 (C=O), 1274, 1250, 1232, 1206 cm^{-1} (C—O); UV–vis (CH_3CN) λ_{max} 253 (ε = 17 1000), 364 nm (17 200 M^{-1} cm^{-1}); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.41 (6 H, s, $-OCH_3 \times 2$), 3.91 (3 H, s, $-COOCH_3$), 4.01 (3 H, s, $-COOCH_3$), 4.09 (3 H, s, $-COOCH_3$), 7.13 (1 H, d, J = 2.2 Hz, H-3 (an NOE of 12% was detected when the methoxyl group at C-4 (δ 3.41 ppm) was irradiated)), 8.71 (1 H, s, H-8), 12.22 (1 H, br s, NH); ^{13}C NMR (DMSO-d_6) 51.4 ($-OCH_3 \times 2$), 52.2 ($-COOCH_3$), 53.0 ($-COOCH_3$), 53.9 ($-COOCH_3$), 96.8 (C-4), 114.6, 122.3, 125.2, 125.3, 126.3, 128.8, 132.9, 145.7, 149.5 (nine aromatic carbons), 160.2 ($-COOCH_3$), 163.8 ($-COOCH_3$), 165.8 ($-COOCH_3$), 193.1 ppm (C-5).

When quinone **3** was treated in refluxing methanol in the presence of a catalytic amount of *p*-toluenesulfonic acid for a few hours, a mixture of the starting material **3** and products **9** and **10** was obtained in a 22:33:45 ratio after workup with CH_2Cl_2–0.1 M K_2CO_3. The ^{1}H NMR spectrum of the mixture suggested that product **10** is trimethyl 4,4-dimethoxy-5-hydroxy-5-methoxy-4,5-dihydro-1*H*-poyrrol[2,3-*f*]quinoline-2,7,9-tricarboxylate: ^{1}H NMR (270 MHz, $CDCl_3$) δ 2.96 (3 H, s, $-OCH_3$), 3.02 (3 H, s, $-OCH_3$), 3.70 (3 H, s, $-OCH_3$), 3.90 (3 H, s, $-COOCH_3$), 3.97 (3 H, s, $-COOCH_3$), 4.06 (3 H, s, $-COOCH_3$), 6.76 (1 H, d, J = 2.2 Hz, H-3), 8.62 (1 H, s, H-8), 12.0 (1 H, br s, NH).

Dimethyl 10,10-dimethoxy-9-oxo-9,10-dihydrobenzo[*f*]quinoline-2,4-dicarboxylate (11): isolated yield 66%; mp 57–59 °C; MS (FAB, positive) m/e 372 ($M^+ + 1$); IR (KBr) 1738 (ester C=O), 1700 (shoulder, C=O), 1258, 1205 cm^{-1} (C—O); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.40 (6 H, s, $-OCH_3 \times 2$), 3.94 (3 H, s, $-COOCH_3$), 4.03 (3 H, s, $-COOCH_3$), 7.29–7.34 (1 H, m, H-8 (an NOE of 4% was detected when the methoxyl group at C-10 (δ 3.40 ppm) was irradiated)), 7.40–7.48 (2 H, m, H-6 and H-7), 7.88–7.92 (1 H, m, H-5), 8.43 (1 H, s, H-3).

Trimethyl 5,5-dimethoxy-4-oxo-4,5-dihydro-1-methylpyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate (12): isolated yield 56%; mp 59–62 °C; MS (FAB, positive) m/e 433 ($M^+ + 1$); IR (KBr) 3460 (NH), 1734 (ester C=O), 1700 (shoulder, C=O), 1252 cm^{-1} (C—O); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.44 (6 H, s, $-OCH_3 \times 2$), 3.64 (3 H, s, $-N-CH_3$), 3.86 (3 H, s, $-COOCH_3$), 4.00 (3 H, s, $-COOCH_3$), 4.03 (3 H, s, $-COOCH_3$), 7.24 (1 H, s, H-3 (an NOE of 1% was detected when the methoxyl group at C-5 (δ 3.44 ppm) was irradiated)), 8.57 (1 H, s, H-8).

Methyl 4,4-dimethoxy-5-oxo-4,5-dihydrobenz[*g*]indole-2-carboxylate (13): isolated yield 84%; mp 75–77 °C; MS (FAB, positive) m/e 302 ($M^+ + 1$); IR (KBr) 3312 (NH), 1710 (shoulder, ester C=O), 1686 (C=O), 1300, 1280, 1224 cm^{-1} (C—O); ^{1}H NMR (270 MHz, $CDCl_3$) δ 3.41 (6 H, s, $-OCH_3 \times 2$), 3.93 (3 H, s, $-COOCH_3$), 7.11 (1 H, d, J = 2.2 Hz, H-3 (an NOE of 14% was detected when the methoxyl group at C-4 (δ 3.41 ppm) was irradiated)), 7.36 (1 H, dt, J = 1.5 and 7.3 Hz, H-7), 7.52 (1 H, dd, J = 1.5 and 7.3 Hz, H-9), 7.59 (1 H, dt, J = 1.5 and 7.3 Hz, H-8), 8.01 (1 H, dd, J = 1.5 and 7.3 Hz, H-6).

Trimethyl 4,4-diethoxy-5-oxo-4,5-dihydro-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate (15): isolated yield 94%; mp 188–190 °C; MS (FAB, positive) m/e 447 ($M^+ + 1$); IR (KBr) 3296 (NH), 1726 (C=O), 1274, 1250, 1228, 1206 cm^{-1} (C—O); UV–vis (CH_3CN) λ_{max} 253 (ε = 22 700), 365 nm (22 500 M^{-1} cm^{-1}); ^{1}H NMR (270 MHz, $CDCl_3$) δ 1.18 (6 H, t, J = 7.0 Hz, $-CH_3 \times 2$), 3.56, 3.59, 3.82, 3.85 (each 1 H, q, J = 7.0 Hz, methylene proton × 4), 3.93 (3 H, s, $-COOCH_3$), 4.02 (3 H, s, $-COOCH_3$), 4.10 (3 H, s, $-COOCH_3$), 7.16 (1 H, d, J = 2.4 Hz, H-3), 8.73 (1 H, s, H-8), 12.22 (1 H, br s, NH).

Trimethyl 4-Methoxy-5-hydroxy-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate (14). Acetal **9** (25.1 mg, 0.060 mmol) was treated with 100

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(31) Anthony, C. In *Principles and Applications of Quinoproteins*; Davidson, V. L., Ed.; Marcel Dekker, Inc.: New York, 1993; pp 17–45.

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(33) Perrin, D. D.; Armarego, W. L. F.; Perrin, D. R. *Purification of Laboratory Chemicals*; Pergamon Press: Elmsford, NY, 1966.

(34) (a) The MNDO method: Dewar, M. J. S.; Thiel, W. *J. Am. Chem. Soc.* **1977**, *99*, 4899. (b) The AM1 method: Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. P. *J. Am. Chem. Soc.* **1985**, *107*, 3902. (c) The PM3 method: Stewart, J. J. P. *J. Comput. Chem.* **1989**, *10*, 209.

equiv of triethylamine in refluxing CH_3CN (5 mL) for 5 h under anaerobic conditions. After removal of the excess amine and the solvent under reduced pressure, orange solids were obtained by flash chromatography (SiO_2/*n*-hexane–AcOEt) in 29% yield: mp 177–179 °C; IR (KBr) 3324 (NH), 1724 (ester C=O), 1264, 1210 cm^{-1} (C—O); UV–vis (CH_3CN) λ_{max} 324 nm (ϵ = 39 600 M^{-1} cm^{-1}); HRMS *m/e* 388.0888 (M^+), calcd for $C_{18}H_{16}O_8N_2$ 388.0907; ^1H NMR (270 MHz, $CDCl_3$) δ 4.01 (3 H, s, $-COOCH_3$), 4.09 (3 H, s, $-COOCH_3$), 4.17 (3 H, s, $-COOCH_3$), 4.32 (3 H, s, $-OCH_3$), 7.49 (1 H, d, J = 2.7 Hz, H-3 (an NOE of 6% was detected when the methoxyl group at C-4 (δ 4.32 ppm) was irradiated)), 8.14 (1 H, s, –OH), 8.85 (1 H, s, H-8), 12.3 (1 H, br s, NH).

Trimethyl 4-Ethoxy-5-hydroxy-1*H*-pyrrolo[2,3-*f*]quinoline-2,7,9-tricarboxylate (16). Acetal **15** (10.9 mg, 0.024 mmol) was treated with 100 equiv of triethylamine in refluxing CH_3CN (2.5 mL) for 5 h under anaerobic conditions. After removal of the excess amine and the solvent under reduced pressure, orange solids were obtained by flash chromatography (SiO_2/*n*-hexane–AcOEt) in 45% yield: mp 221–223 °C; IR (KBr) 3324 (NH), 1722 (ester C=O), 1262, 1210 cm^{-1} (C—O); UV–vis (CH_3CN) λ_{max} 324 nm (ϵ = 41 300 M^{-1} cm^{-1}); HRMS *m/e* 402.1061 (M^+), calcd for $C_{19}H_{18}O_8N_2$ 402.1063; ^1H NMR (270 MHz, $CDCl_3$) δ 1.49 (3 H, t, J = 7.3 Hz, $-CH_3$), 4.00 (3 H, s, $-COOCH_3$), 4.08 (3 H, s, $-COOCH_3$), 4.17 (3 H, s, $-COOCH_3$), 4.63 (2 H, q, J = 7.3 Hz, $-OCH_2-$), 7.48 (1 H, d, J = 2.7 Hz, H-3), 8.83 (1 H, s, H-8), 12.26 (1 H, br s, NH).

In order to detect the oxidation product, acetaldehyde, the same reaction was carried out again, and the reaction mixture was introduced into a solution of (2,4-dinitrophenyl)hydrazine (20 mg) in 6 N HCl (2.5 mL). The mixture was heated at 80 °C for 5 min, and then 5 mL of water was added. Orange precipitates ((2,4-dinitrophenyl)hydrazone of PQQTME) were removed by centrifugation, and the supernatant was extracted by CH_2Cl_2. After the solution was dried over $MgSO_4$, removal of the solvent gave a crude product in which (2,4-dinitrophenyl)hydrazone of acetaldehyde was detected by ^1H NMR (58% yield). An authentic sample of (2,4-dinitrophenyl)hydrazone of acetaldehyde was prepared by the reaction of paraldehyde with (2,4-dinitrophenyl)hydrazine in 6 N HCl.

X-ray Structure Determination of 4, 9, and 17. All the X-ray experiments were carried out on a Rigaku AFC-5R diffractometer with nickel-filtered Cu Kα radiation (λ = 1.541 78 Å). Since single crystals of **4** were not sufficiently stable against dryness, the intensity measurement was undertaken by sealing them in glass capillary tubes containing the mother liquid. The absorption effect was corrected empirically on the basis of azimuthal scans of three reflections. The crystal structures were solved by direct methods and refined by full-matrix least-squares techniques. The non-hydrogen atoms were refined anisotropically, while the hydrogen atoms located from the final stage of difference Fourier maps were included with isotropic thermal parameters. The weighting function used was $w = 4F_o^2/\sigma^2(F_o^2)$ for the function of $\sum w(|F_o| - |F_c|)^2$ to be minimized. All the calculations used the TEXSAN program package.[35] Summaries of the fundamental crystal data and experimental parameters for structure determinations are given in Table V. Atomic coordinates, thermal parameters, and intramolecular bond distances and angles have been deposited in the supplementary material.

(35) *TEXSAN, Single Crystal Structure Analysis Software*, Version 5.0; Molecular Structure Corp.: The Woodlands, TX 77381, 1989.

Table V. Summary of Crystallographic Data for **4**, **9**, and **17**

	4	**9**	**17**
empirical formula	$C_{18}H_{16}O_9N_2$	$C_{19}H_{18}O_9N_2$	$C_{20}H_{18}O_9N_2$
fw	404.3	418.4	430.4
crystal system	triclinic	monoclinic	monoclinic
space group	$P\bar{1}$ (No. 2)	$P2_1/n$ (No. 14)	$P2_1/c$ (No. 14)
a, Å	11.556(1)	10.728(1)	8.885(1)
b, Å	12.375(1)	10.658(1)	13.397(1)
c, Å	7.619(1)	16.963(1)	16.980(1)
α, deg	94.61(1)		
β, deg	94.11(1)	105.80(1)	103.83(1)
γ, deg	112.85(1)		
V, Å^3	994.5(1)	1866.2(2)	1962.6(2)
Z	2	4	4
F(000)	420	872	896
d_{calcd}, g/cm^3	1.350	1.489	1.456
T, °C	20	20	20
μ(Cu Kα), cm^{-1}	9.04	9.82	9.51
diffractometer	Rigaku AFC5R	Rigaku AFC5R	Rigaku AFC5R
radiation	Cu Kα	Cu Kα	Cu Kα
λ, Å	1.541 78	1.541 78	1.541 78
scan type	ω–2θ	ω–2θ	ω–2θ
2θ range, deg	4–120	4–120	4–120
scan width, deg	1.52 + 0.15 tan θ	1.57 + 0.15 tan θ	1.68 + 0.15 tan θ
scan rate in ω, deg/min	16.0	16.0	16.0
no. of reflns measd	2946	2932	3060
no. of reflns obsd [$I > 3\sigma(I)$]	2571	2307	2522
no. of variables	348	343	352
R	0.053	0.071	0.040
R_w	0.057	0.073	0.040

Acknowledgment. The present study was financially supported in part by a Grant-in-Aid for Scientific Research (No. 03403016) from the Ministry of Education, Science, and Culture of Japan. Thanks are due to the Instrumental Analysis Center, Faculty of Engineering, Osaka University, for assistance in obtaining mass spectra on a JEOL JMS-DX303 spectrometer.

Supplementary Material Available: Tables of fractional atomic coordinates, anisotropic temperature factors, intramolecular bond distances, and intramolecular bond angles for **4**, **9**, and **17** (15 pages). Ordering information is given on any current masthead page.