Acta Cryst B
STRUCTURAL SCIENCE
CRYSTAL ENGINEERING
MATERIALS
ISSN 2052-5206

Synthesis and crystal structure of pyrroloquinoline quinol ($PQQH_2$) and pyrroloquinoline quinone (PQQ)

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Received 20 December 2016
Accepted 10 February 2017

Edited by A. Katrusiak, Adam Mickiewicz University, Poland

Keywords: pyrroloquinoline quinone; pyrroloquinoline quinol; cofactor; growth factor.

CCDC references: 1487730; 1487731

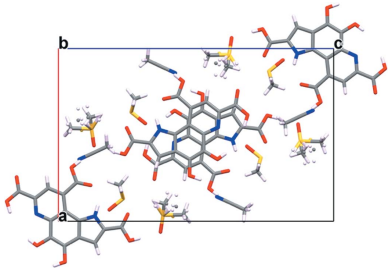
Supporting information: this article has supporting information at journals.iucr.org/b

Pyrroloquinoline quinone (PQQ) is a water-soluble quinone compound first identified as a cofactor of alcohol- and glucose-dehydrogenases (ADH and GDH) in bacteria. For example, in the process of ADH reaction, alcohol is oxidized to the corresponding aldehyde, and inversely PQQ is reduced to pyrroloquinoline quinol ($PQQH_2$). PQQ and $PQQH_2$ molecules play an important role as a cofactor in ADH and GDH reactions. However, crystal structure analysis has not been performed for PQQ and $PQQH_2$. In the present study, the synthesis of $PQQH_2$ powder crystals was performed under air, by utilizing vitamin C as a reducing agent. By reacting a trihydrate of disodium salt of PQQ ($PQQNa_2{\cdot}3H_2O$) with excess vitamin C in H_2O at 293 and 343 K, yellowish brown and black powder crystals of $PQQH_2$ having different properties were obtained in high yield, respectively. The former was $PQQH_2$ trihydrate ($PQQH_2{\cdot}3H_2O$) and the latter was $PQQH_2$ anhydrate ($PQQH_2$). Furthermore, sodium-free red PQQ powder crystal (a monohydrate of PQQ, $PQQ{\cdot}H_2O$) was prepared by the reaction of $PQQNa_2{\cdot}3H_2O$ with HCl in H_2O. Single crystals of $PQQH_2$ and PQQ were prepared from Me_2SO/CH_3CN mixed solvent, and we have succeeded in the crystal structure analyses of $PQQH_2$ and PQQ for the first time.

1. Introduction

Pyrroloquinoline quinone (PQQ; Fig. 1) has received much attention in recent years, owing to its several interesting physiological functions (Stites *et al.*, 2000; Rucker *et al.*, 2009; Akagawa *et al.*, 2016, and references therein). PQQ acts as a growth factor and contributes to mammalian cell growth (Killgore *et al.*, 1989; Steinberg *et al.*, 2003; Kimura *et al.*, 2012). It has also been reported to show neuroprotective effects (Jensen *et al.*, 1994; Zhang *et al.*, 2006; Hara *et al.*, 2007). A small amount of PQQ has been found not only in microorganisms, but also in human and rat organs or tissues, the highest especially in human milk (Kumazawa *et al.*, 1992; Mitchell *et al.*, 1999). A further small amount of PQQ is also found in daily foods and beverages (Kumazawa *et al.*, 1995; Noji *et al.*, 2007).

PQQ is known as a cofactor of alcohol- and glucose-dehydrogenases (ADH and GDH) in bacteria (Duine *et al.*, 1979; Salisbury *et al.*, 1979; de Beer *et al.*, 1983; McIntire, 1998; Yamada *et al.*, 2003; Toyama *et al.*, 2004; Mustafa *et al.*, 2008). In the process of ADH reaction, alcohol is oxidized to the corresponding aldehyde, and inversely PQQ is reduced to pyrroloquinoline quinol ($PQQH_2$), indicating that both the PQQ and $PQQH_2$ molecules play an important role as a cofactor in enzymatic reactions (Oubrie *et al.*, 1999, 2002;



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Anthony & Williams, 2003; Anthony, 2004; Williams *et al.*, 2005; Sakuraba *et al.*, 2010; Rozeboom *et al.*, 2015). The mechanism of ADH and GDH was discussed based on the result of X-ray crystal structure analysis of quinoprotein. X-ray crystal structure analysis of PQQ itself was performed for the pentahydrate of PQQ disodium salt ($PQQNa_2{\cdot}5H_2O$) and trihydrate of $PQQNa_2$ ($PQQNa_2{\cdot}3H_2O$; Ishida *et al.*, 1989; Ikemoto *et al.*, 2012). However, the crystal structure of sodium-free PQQ and $PQQH_2$ has not been reported. $PQQH_2$ is generally unstable in solution under air and thus the preparation of single crystals is difficult.

Previous studies demonstrated that $PQQH_2$ exhibits antioxidative capacity in *in vitro* examinations (Miyauchi *et al.*, 1999; He *et al.*, 2003; Ouchi *et al.*, 2009, 2013; Mukai *et al.*, 2011). It has been reported that $PQQNa_2$ is easily reduced to $PQQH_2$, by reacting $PQQNa_2$ with glutathione and cysteine in buffer solution (pH 7.4) under a nitrogen atmosphere (Ouchi *et al.*, 2009). Furthermore, recently we found that PQQ is reduced to $PQQH_2$ by vitamin C (Vit. C), and $PQQH_2$ produced is recycled to PQQ by air oxidation in buffer solution at pH 7.4 (Mukai *et al.*, 2016). However, when a high concentration of Vit. C coexists in buffer solution, $PQQH_2$ is not easily oxidized and remains in solution as the reduced form under air. The result suggests that PQQ is reduced by Vit. C, glutathione and cysteine and functions as an antioxidant in biological systems, because it has been reported that $PQQH_2$ shows high free radical scavenging and singlet oxygen ($^1O_2$)-quenching activities in buffer solutions at pH 7.4 (Ouchi *et al.*, 2009; Mukai *et al.*, 2011). On the other hand, free radical scavenging activity of $PQQNa_2$ is low and almost negligible, because the catechol moiety in $PQQH_2$ is lost in PQQ. The results obtained suggest that PQQ exists as a reduced form throughout the cell and plays a role as an antioxidant. In experiments using cultured cells, it is reported that PQQ prevents oxidative stress-induced neuronal death (Hara *et al.*, 2007; Nunome *et al.*, 2008). Moreover, marked decreases in the ischemia damage are found in *in vivo* models such as cardiovascular or cerebral ischemia models (Zhu *et al.*, 2006; Zhang *et al.*, 2006). Furthermore, it was reported that PQQ prevents cognitive deficit caused by oxidative stress in rats (Ohwada *et al.*, 2008; Takatsu *et al.*, 2009).

In the present study, the synthesis of $PQQH_2$ powder crystals was performed by the reduction of $PQQNa_2$ with Vit. C in H_2O. Yellowish brown and black powder crystals of $PQQH_2$, having different properties, were obtained in high yield depending on the reaction temperatures (293 and 343 K), respectively. The former was $PQQH_2$ trihydrate ($PQQH_2{\cdot}3H_2O$) and the latter was $PQQH_2$ anhydrate ($PQQH_2$). In addition, sodium-free red PQQ crystal (a monohydrate of PQQ, $PQQ{\cdot}H_2O$) was prepared from $PQQNa_2$ salt by the reaction with HCl in H_2O. Furthermore, $PQQH_2$ and PQQ single crystals were prepared using DMSO/CH_3CN mixed solvent, and we have succeeded in X-ray crystal structure analyses of these compounds. From the results obtained, detailed comparison between the molecular structures of $PQQH_2$ and PQQ was performed.

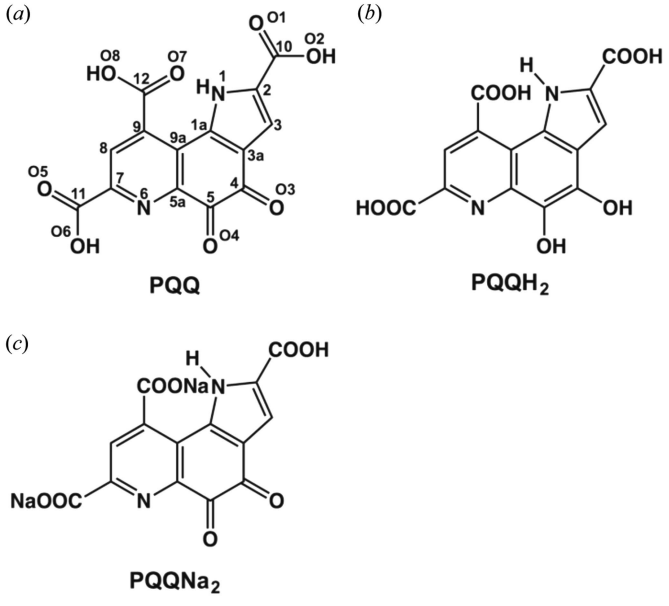

Figure 1
Molecular structures of $PQQH_2$, PQQ and $PQQNa_2$ with atom-numbering scheme.

2. Experimental

2.1. Materials

Unless otherwise stated, all chemicals were purchased as the highest purity commercially available and were used without further purification. All aqueous solutions were prepared using distilled water treated with a Millipore Q system. The trihydrate of the disodium salt of PQQ ($PQQNa_2{\cdot}3H_2O$) is commercially available as BioPQQ™ from Mitsubishi Gas Chemical Co., Inc. (Tokyo, Japan; Akagawa *et al.*, 2016, and references therein).

2.2. Synthesis of yellowish-brown colored $PQQH_2$ crystal ($PQQH_2{\cdot}3H_2O$ crystal)

A solution including Vit. C (30.0 g, 1.70×10^{-1} mol), H_2O (120 ml) and 2*N* HCl (2.50 g) was prepared, and temperature was adjusted to 285 K. A H_2O (1200 ml) solution of $PQQNa_2{\cdot}3H_2O$ (3.00 g, 7.01×10^{-3} mol) was added dropwise slowly during 2 h under stirring, where the pH of the solution was 3.0. After the addition of $PQQNa_2{\cdot}3H_2O$ solution, the reaction was continued for 18 h with stirring at 293 K. Orange crystals of $PQQH_2$ started to precipitate in the solution after $\sim$ 1 h stirring. To the solution, 2*N* HCl (2.50 g) was added, and stirring was continued for a further 1 h. The orange crystals precipitated were filtered using a Büchner funnel, and washed with 2*N* HCl (5.00 g) and then ethanol/H_2O (1:1, *w*/*w*; 8.00 ml) solution. The precipitate was dried for 20 h at room temperature under vacuum, and the yellowish-brown powder crystals (2.25 g) with metallic luster were obtained.

The result of the elemental analysis indicates the formation of $PQQH_2{\cdot}3H_2O$ crystal: m.p. > 573 K; 2.25 g, 83% yield; UV λ_{max} (DMSO) nm (ε_{max} $M^{-1}cm^{-1}$): 325 (32 500), 458 (853); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.42 (d, J = 4 Hz), 8.62, 9.2

broad, 12.43 (d, J = 2 Hz) p.p.m. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 105.3, 110.7, 119.0, 122.6, 123.2, 127.7, 130.9, 133.8, 137.5, 140.5, 142.1, 161.8, 165.0, 169.7 p.p.m. Anal.: calc. for $C_{14}H_{14}N_2O_{11}$ (that is, $PQQH_2{\cdot}3H_2O$): C 43.53, H 3.66, N 7.25; found: C 43.43, H 3.89, N 7.64%.

The water content (13.99%) calculated for a trihydrate of $PQQH_2$ ($PQQH_2{\cdot}3H_2O$) based on the result of the elemental analysis shows good agreement with that (13.71%) determined by the titration of H_2O due to Karl Fisher's reagent.

2.3. Synthesis of black colored $PQQH_2$ crystal ($PQQH_2$ anhydrate)

Similarly, the synthesis of the black colored $PQQH_2$ crystal was performed by reacting $PQQNa_2$ with Vit. C. However, the reaction was performed at a higher temperature (343 K) as follows: A solution including Vit. C (30.0 g, 1.70×10^{-1} mol), H_2O (120 ml) and 2N HCl (2.50 g) was prepared. A H_2O (1200 ml) solution of $PQQNa_2{\cdot}3H_2O$ (3.00 g, 7.01×10^{-3} mol) was added dropwise during 2 h under stirring, where the pH of the solution was 3.0. The reaction was continued for 96 h at 343 K. Black crystals of $PQQH_2$ started to precipitate in the solution after $\sim$ 1 h stirring. To the solution, 2N HCl (2.50 g) was added, and stirring was continued for a further 1 h. The black crystals precipitated were filtered using a Büchner funnel, and washed with 2N HCl (5.00 g) and then ethanol/H_2O (1:1, w/w; 8.00 ml) solution. The precipitate was dried for 16 h at 343 K under vacuum, and black powder crystals (2.32 g) were obtained.

The result of the elemental analysis indicates the formation of $PQQH_2$ anhydrate crystals: m.p. > 573 K; 2.32 g, 99.6% yield; UV λ_{max} (DMSO) nm (ε_{max} M^{-1} cm^{-1}): 324 (37 900), 460 (984), $\sim$ 380sh. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.40 (d, J = 3 Hz), 8.60, 12.39 (d, J = 2 Hz) p.p.m. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 105.3, 110.6, 118.9, 122.5, 123.2, 127.7, 130.8, 133.8, 137.5, 140.4, 142.0, 161.8, 164.9, 169.7 p.p.m. Anal.: calc. for $C_{14}H_6N_2O_8$ (that is, $PQQH_2$ anhydrate): C 50.61, H 2.43, N 8.43; found: C 50.19, H 2.69, N 8.25%.

The ^{1}H and ^{13}C NMR δ values of black $PQQH_2$ crystals in DMSO-d_6 were in good agreement with those obtained for yellowish-brown crystals of $PQQH_2$, except that a weak broad absorption at 9.2 p.p.m. of ^{1}H proton due to two OH groups in the yellowish-brown $PQQH_2$ crystal was not observed in the case of black $PQQH_2$ crystals. The water content (0%) calculated for $PQQH_2$ anhydrate based on the result of the elemental analysis shows a good agreement with the water content (1.28%) determined by the titration of H_2O due to Karl Fisher's reagent.

2.4. Synthesis of red colored sodium-free PQQ crystal ($PQQ{\cdot}H_2O$)

$PQQNa_2{\cdot}3H_2O$ (15.0 g, 3.50×10^{-2} mol) was added to H_2O (1.50 L) at room temperature, and resolved by heating the solution at 343 K. Concentrated HCl (30.0 g) was added to the solution, and reaction with $PQQNa_2$ was continued under stirring at 343 K. Red powder crystals of PQQ started to precipitate in the solution after $\sim$ 2 h stirring. The reaction was continued for 1 d at 343 K. The red powder crystals that precipitated were filtered using a Büchner funnel at room temperature. The powder crystals of PQQ (13.0 g) were added to H_2O (200 ml) and left at 343 K for 18 h in order to eliminate any NaCl salt and HCl remaining in the powder crystals. The precipitate was filtered and dried for 18 h at room temperature under vacuum, and red powder crystals of PQQ (12.0 g) were obtained.

Measurement of Na^+ content was performed using a compact ion meter (LAQUA Twin Compact Ion Meter, Horiba Ltd, Japan); for example, 10.0 mg of PQQ powder sample was dissolved in 1.00 ml of 2.5% (46 wt% in H_2O) choline hydroxide aqueous solution (Sigma Aldrich). The result indicated that Na^+ is not included in the PQQ powder sample.

The result of the elemental analysis indicates the formation of $PQQ{\cdot}H_2O$ crystal: m.p. > 573 K; 12.0 g, 99% yield; UV λ_{max} (DMSO) nm (ε_{max} M^{-1} cm^{-1}): 273 (22 300), 335 (14 500), 430 (1710). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.21 (d, J = 2 Hz), 8.61 p.p.m. ^{13}C NMR (75 MHz, DMSO-d_6) δ: 113.4, 124.4, 126.3, 127.5, 129.0, 134.1, 136.0, 146.7, 148.7, 160.8, 164.9, 168.7, 173.3, 177.7 p.p.m. Anal.: calc. for $C_{14}H_8N_2O_9$ (that is, $PQQ{\cdot}H_2O$, monohydrate of PQQ): C 48.29, H 2.32, N 8.05; found: C 47.93, H 2.55, N 7.90%.

The water content (5.17%) calculated for a monohydrate of PQQ ($PQQ{\cdot}H_2O$) based on the result of the elemental analysis shows good agreement with that (4.70%) determined by the titration of H_2O due to Karl Fisher's reagent.

2.5. Preparation of single crystals of $PQQH_2$ and PQQ

$PQQH_2$ is unstable in water under air. On the other hand, $PQQH_2$ is comparatively stable in organic aprotic solvent (such as DMSO and CH_3CN), even under an air atmosphere (Miyauchi *et al.*, 1999). Preparation of single crystals of $PQQH_2$ and PQQ is as follows: $PQQH_2$ and PQQ are soluble in DMSO. On the other hand, $PQQH_2$ and PQQ show low solubility in CH_3CN. Consequently, a single crystal of $PQQH_2$ was prepared by utilizing the difference in solubilities of $PQQH_2$ in DMSO and CH_3CN. A yellow crystal of $PQQH_2$ ($PQQH_2{\cdot}3H_2O$) (50 mg) was dissolved in DMSO (1.0 ml) under air. The DMSO solution of $PQQH_2{\cdot}3H_2O$ (0.10 ml) was added to CH_3CN (0.80 ml) at room temperature. Orange needle crystals of $PQQH_2$ were obtained keeping the solution at 277 K in a refrigerator for 4 d.

Similarly, a single crystal of PQQ was prepared. A red PQQ ($PQQ{\cdot}H_2O$; 50 mg) crystal was dissolved in DMSO (1.0 ml) at 343 K under air. The DMSO solution of $PQQ{\cdot}H_2O$ (0.10 ml) was added to CH_3CN (1.2 ml) at 343 K. Orange block crystals of PQQ were obtained keeping the solution at room temperature for 1 d.

2.6. Measurements

^{1}H and ^{13}C NMR spectra were recorded on 400 MHz and 75 MHz spectrometers, respectively. Chemical shifts (δ) are given in p.p.m. relative to TMS, and coupling constants (J) are given in Hertz. The spectra were recorded in DMSO-d_6

Table 1
Experimental details.

	(I)	(II)
Crystal data		
Chemical formula	$2C_7H_4NO_4{\cdot}2C_2H_6OS{\cdot}C_2H_3N$	$C_{14}H_6N_2O_8{\cdot}3C_2H_6OS$
M_r	529.53	564.59
Crystal system, space group	Orthorhombic, *Pnma*	Orthorhombic, $P2_12_12_1$
Temperature (K)	100	100
a, b, c (Å)	14.563 (5), 6.748 (2), 23.299 (9)	6.901 (5), 12.371 (8), 28.674 (18)
V (Å^3)	2289.6 (14)	2448 (3)
Z	4	4
Radiation type	Mo $K\alpha$	Mo $K\alpha$
μ (mm^{-1})	0.30	0.37
Crystal size (mm)	0.24 × 0.03 × 0.02	0.05 × 0.05 × 0.02
Data collection		
Diffractometer	Rigaku Saturn724	Rigaku Saturn724
Absorption correction	Multi-scan REQAB (Rigaku, 1998)	Multi-scan REQAB (Rigaku, 1998)
T_{min}, T_{max}	0.912, 0.994	0.910, 0.993
No. of measured, independent and observed [$I > 2\sigma(I)$] reflections	38156, 2990, 2846	41911, 5885, 4968
R_{int}	0.051	0.077
(sin $\theta/\lambda)_{max}$ (Å^{-1})	0.661	0.660
Refinement		
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.060, 0.134, 1.07	0.070, 0.185, 1.04
No. of reflections	2990	5885
No. of parameters	267	337
No. of restraints	236	0
H-atom treatment	H atoms treated by a mixture of independent and constrained refinement	H atoms treated by a mixture of independent and constrained refinement
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.48, −0.36	1.43, −0.78
Absolute structure	–	Flack x determined using 1648 quotients $[(I+) - (I-)]/[(I+) + (I-)]$ (Parsons *et al.* (2013)
Absolute structure parameter	–	0.12 (3)

Computer programs: *CrystalClear* (Rigaku Inc., 2008), *SIR2011* (Burla, *et al.*, 2012), *SHELXL2016*/6 (Sheldrick, 2016), *CrystalStructure* 4.2.4 (Rigaku, 2016).

solvent at room temperature. TMS served as an internal standard (δ = 0 p.p.m.). Measurements of the UV–vis absorption spectrum of PQQ, $PQQH_2$ and $PQQNa_2$ were performed in DMSO using a Shimadzu UV-2100S spectrophotometer. Elemental analysis was performed using a C, H, N analyzer, and the results were found to be within ±0.3% of the calculated values.

The X-ray measurements of the $PQQH_2$ and PQQ crystals were carried out on a Rigaku Saturn724 diffractometer using multi-layer mirror monochromated Mo $K\alpha$ (λ = 0.71069 Å) radiation. The structure was solved by direct methods. The crystallographic data and the parameters of structure refinement are given in Table 1 and Table S1 of the supporting information.

3. Results and discussion

3.1. Synthesis of yellowish-brown and black colored $PQQH_2$ crystals

The preparation of $PQQH_2$ has been performed by the reduction of PQQ using many reducing agents in previous studies (Duine *et al.*, 1981; Itoh *et al.*, 1986, 1987; Toyama *et al.*, 2007). Earlier $PQQH_2$ has been prepared by the reduction of PQQ with phenylhydrazine (pH 3.1) and catalytic hydrogenation of PQQ (H_2 (1 atom)/PtO_2, pH 6.7) under a nitrogen atmosphere by Duine *et al.* (1981). Itoh *et al.* (1986, 1987) prepared $PQQH_2$ by the reaction of PQQ with 1-benzyl-1,4-dihydronicotinamide, sodium dithionite, sodium borohydride and thiols (such as thiophenol, mercaptoethanol, cysteine and 1,4-butanedithiol) under a nitrogen atmosphere. The spectral data of the product were in good agreement with those reported by Duine *et al.* (1981). Reduction of PQQ to $PQQH_2$ has also been performed using dithiothreitol with two SH groups in the same molecule (Toyama *et al.*, 2007).

In the present study, the synthesis of $PQQH_2$ crystals was performed by the reaction of $PQQNa_2$ with a high concentration of Vit. C. By reacting $PQQNa_2$ with excess Vit. C under the coexistence of 2*N* HCl in H_2O at two different temperatures (293 and 343 K), yellowish brown and black crystals of $PQQH_2$ were obtained. Hereafter, 'yellowish brown crystal' is abbreviated to 'brown crystal' for simplicity. The result of elemental analysis of brown and black powder crystals indicated that the former is $PQQH_2$ trihydrate ($PQQH_2{\cdot}3H_2O$) and the latter is $PQQH_2$ anhydrate ($PQQH_2$), respectively. Further, the result of the titration of H_2O due to Karl Fisher's reagent also supported the above result. Impurities such as Vit. C and Na^+ were not included in brown and black powder crystals. Brown and black $PQQH_2$ crystals are stable at room temperature under air. However, the color of the brown crystal easily changes to black by dry-heating or in water under air. The color of the brown $PQQH_2$ crystal changed to black by heating the crystal at 453 K under vacuum.

The chemical shift values for the brown $PQQH_2$ crystal obtained by ^1H and ^{13}C NMR measurements in DMSO-d_6 solvent showed good agreement with those reported by Duine *et al.* (1981). The brown and black $PQQH_2$ crystals showed almost the same ^1H and ^{13}C NMR spectra in DMSO-d_6, except a weak broad absorption at 9.2 p.p.m. in the ^1H NMR spectrum due to two OH protons of $PQQH_2$ molecules observed for the brown crystal disappearing in the black crystal.

On the other hand, the solubilities (0.13 and 8.5 mg ml^{-1}) of the brown crystal in H_2O and C_2H_5OH are 6.8 and 6.1 times

Table 2
Values of UV–visible absorption maxima (λ^{i}_{max}) and molar extinction coefficients (ε_i) of $PQQH_2{\cdot}3H_2O$, $PQQH_2$, $PQQ{\cdot}3H_2O$ and $PQQNa_2{\cdot}3H_2O$ in DMSO solution and $PQQH_2$ and $PQQNa_2$ in buffer solution (pH 7.4).

Compound	Solvent	λ^{1}_{max}, nm (ε^{1}/M^{-1} cm^{-1})	λ^{2}_{max}, nm (ε^{2}/M^{-1}cm^{-1})	λ^{3}_{max}, nm (ε^{3}/M^{-1} cm^{-1})	λ^{4}_{max}, nm (ε^{4}/M^{-1} cm^{-1})
$PQQH_2{\cdot}3H_2O$ (yellowish brown crystal)	DMSO	325 (32 500)	458 (853)	–	–
$PQQH_2$ (black crystal)	DMSO	324 (37 900)	460 (984)	–	–
$PQQ{\cdot}H_2O$ (red crystal)	DMSO	273 (22 300)	335 (14 500)	430 (1710)	–
$PQQNa_2{\cdot}3H_2O$ (orange crystal)	DMSO	273 (21 900)	326 (13 800)	458 (1060)	–
$PQQH_2$	Buffer (pH 7.4)†	304 (40 000)	340 sh (11 500)	405 sh (2410)	499 (1170)
$PQQNa_2$	Buffer (pH 7.4)†	249 (26 600)	267 sh (20 500)	331 (12 700)	477 (692)

† The values reported by Ouchi *et al.* (2009).

higher than the corresponding values (0.019 and 1.4 mg ml^{-1}) of the black crystal. Further, the brown crystal showed a high dispersibility in ethanol, and gelation phenomena occurred at the concentration higher than the solubility of $PQQH_2$.

Recently, in the light of its beneficial physiological effects, $PQQNa_2{\cdot}3H_2O$ (BioPQQ^{TM}) has been identified as a potential candidate for its use in the dietary supplements (Nakano *et al.*, 2013, 2014; Health Canada, 2012). Vit. C is well known as a representative natural antioxidant. As described above, we have succeeded in synthesizing significant quantities of $PQQH_2$ crystals in high yield, using safe and cheap Vit. C as a reducing agent. As Vit. C is water soluble, it was not necessary to use a generally harmful organic solvent for the synthesis of $PQQH_2$ crystal. PQQ does not show antioxidant activity. On the other hand, $PQQH_2$ shows very high free radical scavenging and singlet oxygen-quenching activity in solution (Ouchi *et al.*, 2009, 2013; Mukai *et al.*, 2011). Consequently, we may use $PQQH_2$ in cosmetics to protect the degradation of skin induced by the reactive oxygen species.

3.2. Synthesis of red colored sodium-free PQQ crystal

X-ray crystal structure analysis was performed for the pentahydrate of $PQQNa_2$ ($PQQNa_2{\cdot}5H_2O$) and trihydrate of $PQQNa_2$ ($PQQNa_2{\cdot}3H_2O$; Ishida *et al.*, 1989; Ikemoto *et al.*, 2012.) However, the crystal structure of sodium-free PQQ has not been reported. Therefore, in the present study, first, sodium-free PQQ crystal was synthesized, and then crystal structure analysis was performed for the PQQ crystal, in order to compare the crystal structure of sodium-free PQQ with that of $PQQH_2$.

The synthesis of sodium-free PQQ crystal was performed by reacting $PQQNa_2{\cdot}3H_2O$ with HCl in water at 343 K for 1 d. The red powder crystals of PQQ obtained were added to water, and left at 343 K for 18 h, in order to eliminate NaCl salt and HCl remaining in the powder crystals. The precipitate was filtered and dried for 18 h at room temperature under vacuum. It was ascertained that Na^+ is not included in PQQ powder sample, using a compact ion meter. The results of the titration of H_2O due to Karl Fischer's reagent and elemental analysis indicated that the red colored PQQ crystal obtained is a monohydrate of PQQ ($PQQ{\cdot}H_2O$).

The ^{1}H and ^{13}C NMR spectra of the red PQQ crystal are almost the same as those of PQQ reported by Duine *et al.* (1981), showing the same chemical shifts in DMSO-d_6, except for the disappearance of a broad absorption (at 13.3 p.p.m.) of ^{1}H NMR due to the three COOH protons of PQQ molecule in the red crystal.

3.3. UV–vis absorption spectra of yellowish brown and black $PQQH_2$, red PQQ and orange $PQQNa_2$ crystals in DMSO solution

As reported in a previous study, measurements of UV–vis absorption spectra for $PQQNa_2$ and $PQQH_2$ were performed in 0.01 *M* phosphate buffer solution (pH 7.4) under a nitrogen

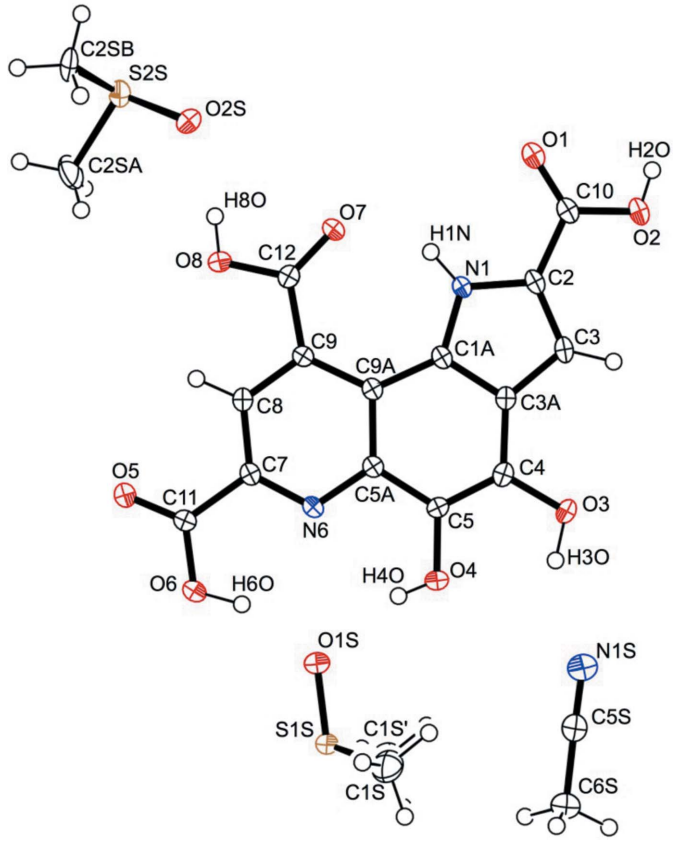

Figure 2
Molecular structure of $PQQH_2{\cdot}2DMSO{\cdot}CH_3CN$ with atom-numbering scheme. Displacement ellipsoids of C, N, O and S are set at 50% probability.

Table 3
Selected structural data for $PQQH_2{\cdot}2DMSO{\cdot}CH_3CN$, $PQQ{\cdot}3DMSO$ and $PQQNa_2{\cdot}3H_2O$.

	$PQQH_2{\cdot}2DMSO{\cdot}CH_3CN$	$PQQ{\cdot}3DMSO$	$PQQNa_2{\cdot}3H_2O$†
Bond	Catechol moiety (Å)	Orthoquinone moiety (Å)	Orthoquinone moiety (Å)
O3—C4	1.364 (3) s‡ (C—OH)	1.217 (6) d§ (C═O)	1.221 (4) (Å) d§ (C═O)
O4—C5	1.356 (3) s (C—OH)	1.207 (7) d (C═O)	1.215 (3) d (C═O)
C9*A*—C1*A*	1.428 (4) conj. double bond¶	1.455 (8) s‡ (C—C)	1.412 (4) s‡ (C—C)
C1*A*—C3*A*	1.413 (4) conj. double bond	1.409 (7) d (C═C)	1.397 (4) d (C═C)
C4—C3*A*	1.421 (4) conj. double bond	1.450 (7) s (C—C)	1.440 (4) s (C—C)
C4—C5	1.367 (4) conj. double bond	1.563 (7) s (C—C)	1.554 (3) s (C—C)
C5—C5*A*	1.428 (4) conj. double bond	1.515 (8) s (C—C)	1.501 (4) s (C—C)
C5*A*—C9*A*	1.444 (4) conj. double bond	1.415 (8) conj. double bond¶	1.411 (4) conj. double bond¶
	Pyrrole moiety	Pyrrole moiety	Pyrrole moiety
N1—C1*A*	1.364 (3)	1.355 (7)	1.351 (4)
N1—C2	1.365 (4)	1.386 (7)	1.378 (3)
C3—C3*A*	1.416 (4)	1.425 (7)	1.420 (4)
C2—C3	1.376 (4)	1.375 (7)	1.373 (5)
	Pyridine moiety	Pyridine moiety	Pyridine moiety
C7—N6	1.322 (3)	1.343 (7)	1.338 (4)
C5*A*—N6	1.351 (3)	1.342 (7)	1.335 (3)
C7—C8	1.391 (4)	1.401 (8)	1.379 (4)
C8—C9	1.387 (4)	1.391 (8)	1.392 (3)
C9—C9*A*	1.431 (4)	1.424 (8)	1.412 (4)
	3 COOH groups	3 COOH groups	2 COONa and 1 COOH groups
O5—C11	1.213 (3): d (C═O)	1.224 (7): d (C═O)	1.285 (5): d (C═O)
O6—C11	1.327 (3): s (C—OH)	1.308 (7): s (C—OH)	1.236 (4) s (C—ONa)
O7—C12	1.211 (3): d (C═O)	1.239 (7): d (C═O)	1.269 (3): d (C═O)
O8—C12	1.319 (3): s (C—OH)	1.275 (7): s (C—OH)	1.241 (4): s (C—ONa)
O1—C10	1.209 (4): d (C═O)	1.215 (8): d (C═O)	1.240 (4): d (C═O)
O2—C10	1.329 (4): s (C—OH)	1.327 (7): s (C—OH)	1.290 (3): s (C—OH)

† The value reported by Ikemoto *et al.* (2012). ‡ 's' represents 'single bond'. § 'd' represents 'double bond'. ¶ '(conj. double bond)' represents 'conjugated double bond.

atmosphere, and the values of maximum wavelength (λ_{max}) and molar extinction (ε_{max}) of $PQQNa_2$ and $PQQH_2$ were determined (see Table 2; Ouchi *et al.*, 2009). In the present work, measurements of λ_{max} and ε_{max} for brown $PQQH_2$, black $PQQH_2$, red PQQ and orange $PQQNa_2$ crystals were performed in DMSO solvent, because $PQQH_2$ is comparatively stable in aprotic DMSO solvent under air (Miyauchi *et al.*, 1999).

As listed in Table 2, the brown $PQQH_2$ crystal shows almost the same λ^1_{max} and λ^2_{max} values as the corresponding values of the black crystal. Apparent ε^1_{max} (32 500 M^{-1} cm^{-1}) and ε^2_{max} (853 M^{-1} cm^{-1}) values of brown $PQQH_2$ ($PQQH_2{\cdot}3H_2O$) are 0.86 and 0.87 times smaller than the corresponding values (37 900 and 984 M^{-1} cm^{-1}) of black $PQQH_2$ ($PQQH_2$ anhydrate). However, the intrinsic ε^1_{max} (37 800 M^{-1} cm^{-1}) and ε^2_{max} (992 M^{-1} cm^{-1}) values due to the $PQQH_2$ molecule in the brown $PQQH_2$ crystal calculated by taking the ratio of the molecular weight (M_r) of $PQQH_2{\cdot}3H_2O$ (M_r 386.27) and $PQQH_2$ anhydrate (M_r 332.22) into account, showing good agreement with the values observed for black $PQQH_2$. Although the apparent color of brown and black crystals is different from each other, the crystals consist of the same $PQQH_2$ molecule.

Measurement of the UV–vis absorption spectrum of PQQ was performed in DMSO solvent. The values of λ^1_{max} (273 nm), λ^2_{max} (335 nm) and λ^3_{max} (430 nm) of $PQQ{\cdot}H_2O$ in DMSO solvent are different from the corresponding values [λ^1_{max} (273 nm), λ^2_{max} (326 nm) and λ^3_{max} (458 nm)] of $PQQNa_2{\cdot}3H_2O$ (Table 2), because the two protons of the two COOH groups in $PQQNa_2{\cdot}3H_2O$ are ionized. Similarly, the λ_{max} and ε_{max} values of $PQQNa_2$ in DMSO are different from those of $PQQNa_2$ in buffer solution (pH 7.4), because all three COOH groups of $PQQNa_2$ will take the ionized structure (COO^-) in buffer solution. The λ_{max} and ε_{max} values of brown and black $PQQH_2$ crystals in DMSO are quite different from those of $PQQH_2$ in buffer solution (pH 7.4), because one of two OH groups in $PQQH_2$ will take the ionized structure (O^-H^+) in buffer solution (Mitani *et al.*, 2008).

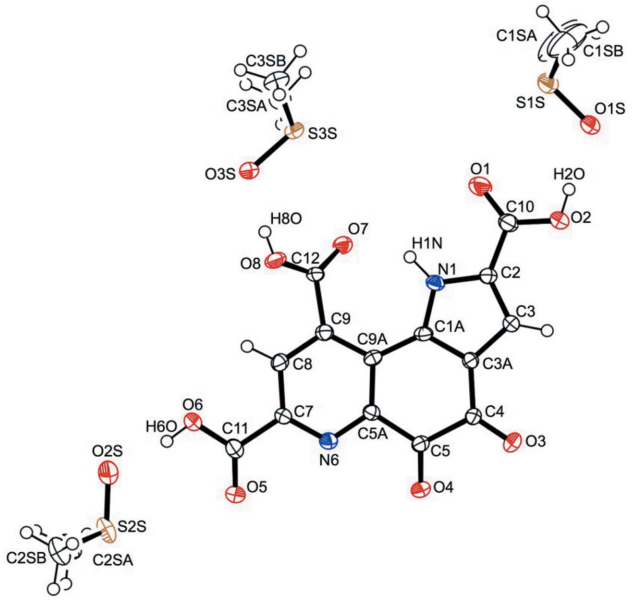

Figure 3
Molecular structure of PQQ·3DMSO with atom-numbering scheme. Displacement ellipsoids of C, N, O and S are set at 50% probability.

3.4. Crystal structures of $PQQH_2$ and PQQ

Single crystals of $PQQH_2$ and PQQ were prepared as described in §2. The crystal structure could be determined for the $PQQH_2$ and PQQ crystals, as listed in Table S1. $PQQH_2$ includes DMSO and CH_3CN molecules in the crystal with a ratio of $PQQH_2$:DMSO:CH_3CN = 1:2:1. On the other hand, PQQ includes only the DMSO molecule in the crystal with a ratio of PQQ:DMSO = 1:3. The unit cell of $PQQH_2$ and PQQ

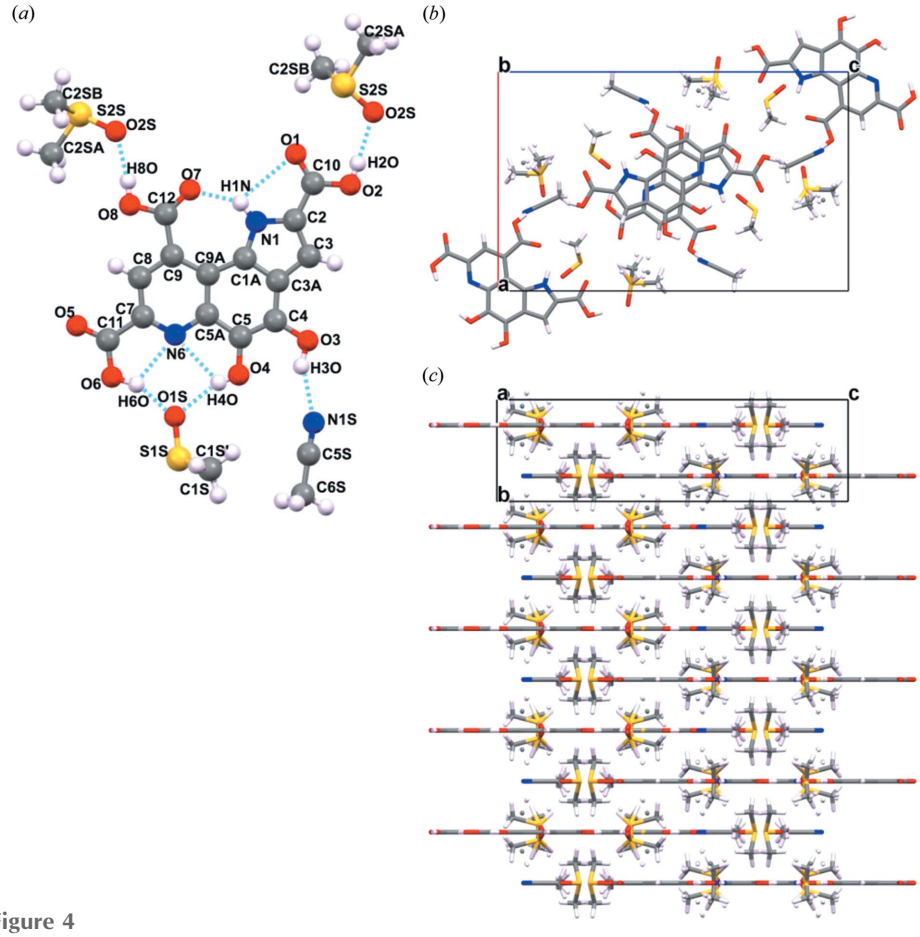

Figure 4
(*a*) Molecular structure of $PQQH_2{\cdot}2DMSO{\cdot}CH_3CN$ with atom-numbering scheme. The intra- and intermolecular hydrogen bonds are marked as dashed lines (see Table S3). (*b*) and (*c*) Molecular packing in $PQQH_2{\cdot}2DMSO{\cdot}CH_3CN$, showing the formation of strong π–π interactions between neighboring $PQQH_2$ molecules. (*b*) View from the top; (*c*) side view.

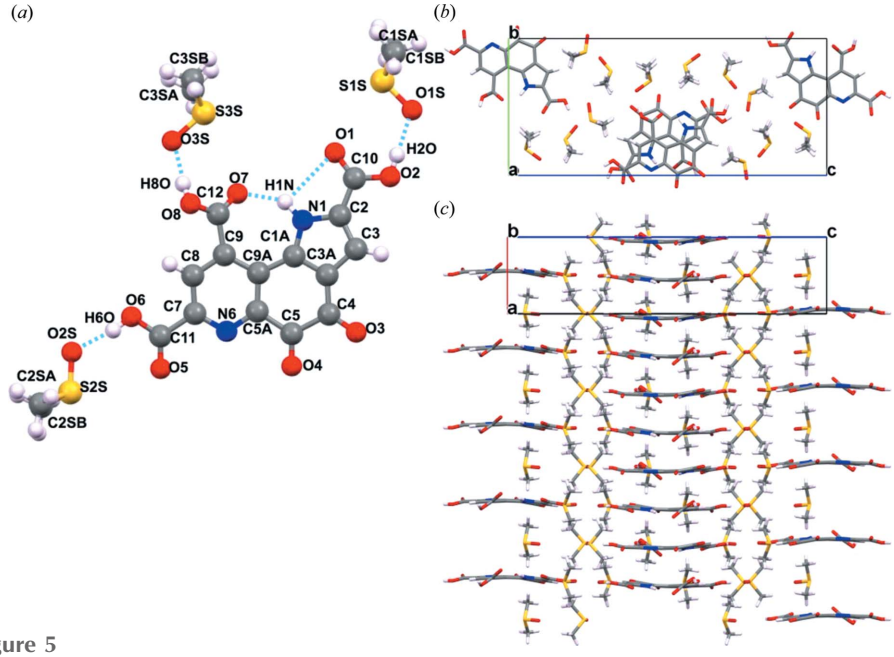

Figure 5
(*a*) Molecular structure of PQQ·3DMSO with atom-numbering scheme. The intra- and intermolecular hydrogen bonds are marked as dashed lines (see Table S5). (*b*) and (*c*) Molecular packing in $PQQH_2{\cdot}3DMSO$, showing the formation of strong π–π interactions between neighboring PQQ molecules. (*b*) View from the top; (*c*) side view.

crystals contains four $PQQH_2$ and PQQ molecules, respectively (Table S1). In Figs. 2 and 3, we show the solid-state structures of $PQQH_2{\cdot}2DMSO{\cdot}CH_3CN$ and PQQ·3DMSO crystals, respectively.

The selected bond lengths for $PQQH_2{\cdot}2DMSO{\cdot}CH_3CN$ and PQQ·3DMSO are listed in Table 3, together with those reported for $PQQNa_2{\cdot}3H_2O$ (Ikemoto *et al.*, 2012). As expected from the molecular structure of PQQ and $PQQNa_2$, the bond lengths of PQQ and $PQQNa_2$ are similar to each other, except for the difference in those of the COOH group in PQQ and COO^-Na^+ group in $PQQNa_2$. On the other hand, as the molecular structures of $PQQH_2$ and PQQ show, the bond lengths of the catechol moiety in $PQQH_2$ are very different from the corresponding values of the *ortho* quinone moiety in PQQ. Especially the bond lengths of O3—C4 [1.364 (3) Å] and O4—C5 [1.356 (3) Å] bonds having single bond character (that is the C—OH bond) of $PQQH_2$ are ∼ 0.15 Å larger than those of O3—C4 [1.217 (6) Å] and O4—C5 [1.207 (7) Å] bonds having double-bond character (*i.e.* the C═O bond) of PQQ.

It is interesting that all the torsion angles in the $PQQH_2$ molecule of $PQQH_2{\cdot}2DMSO{\cdot}CH_3CN$ are 0.0 or 180.0° (Table S2), although heterocyclic pyridine and pyrrole ring moieties and three COOH groups are included in the molecule. The result shows high planarity of the $PQQH_2$ molecule (see Fig. 4*c*). One of two DMSO molecules included in $PQQH_2{\cdot}2DMSO{\cdot}CH_3CN$ showed a disorder in the crystal, although only one orientation of the DMSO molecule was shown in Fig. 2 for the sake of simplicity. Optimization of the orientation of the DMSO molecule was performed, and, at least, three kinds of different conformations were observed for one DMSO molecule (see Fig. S1 and Table S3). On the other hand, such a disorder was not observed for three

DMSO molecules in the PQQ·3DMSO crystal, as shown in Fig. 3.

The possible hydrogen bonds in $PQQH_2$·2DMSO·CH_3CN are listed in Table S3. The result indicates that three kinds of intramolecular hydrogen bonds [(i) O6—H6O···N6, (ii) N1—H1N···O7 and (iii) N1—H1N···O1], including three COOH groups, may contribute to the high planarity of the $PQQH_2$ molecule, as shown in Figs. 2 and 4(*a*), where possible hydrogen bonds are shown by dotted lines. Four kinds of intermolecular hydrogen bonds exist between one $PQQH_2$ molecule and three DMSO molecules (see Table S3) which connect neighboring $PQQH_2$ molecules (see Fig. 4*a* and Fig. S2). Such a hydrogen bond may also contribute to the high planarity. Furthermore, an intermolecular hydrogen bond exists between $PQQH_2$ and CH_3CN molecules.

The molecular packing of $PQQH_2$·2DMSO·CH_3CN is shown in Figs. 4(*b*) and (*c*). Each $PQQH_2$ molecule in $PQQH_2$ crystal is surrounded by four DMSO and one CH_3CN molecules, forming hydrogen bonds, respectively (see Fig. S2). The $PQQH_2$ molecules in $PQQH_2$·2DMSO·CH_3CN form a linear chain with an interplane distance of 3.370 Å along the *b* axis (see Figs. 4*b* and *c*). The intermolecular contact of 3.370 Å is very similar to that (3.35 Å) of graphite. The result indicates the existence of strong π–π interactions between neighboring $PQQH_2$ molecules having a large π-electron system.

Molecular packing of PQQ·3DMSO is shown in Figs. 5(*b*) and (*c*). The PQQ molecule also takes the planar structure as a whole. Each PQQ molecule in the PQQ crystal is surrounded by three DMSO molecules, forming hydrogen bonds with these DMSO molecules (Table S5); possible hydrogen bonds are shown by dotted lines in Fig. 5(*a*). The PQQ molecules in PQQ·3DMSO also form a linear chain with an interplane distance of 3.389 Å along the *a* axis (see Figs. 5*b* and *c*), where the calculation of mean distance was performed for the plane consisting of 15 atoms in a PQQ molecule except for three COOH groups. The mean distance 3.389 Å is a little longer than those (3.37 and 3.35 Å) for $PQQH_2$·2DMSO·CH_3CN and graphite, respectively, because the planarity of the PQQ molecule is lower than those of $PQQH_2$ and graphite, as described above. However, the result also indicates the existence of strong π–π interactions between neighboring PQQ molecules.

4. Conclusion

As described in §1, X-ray crystal structure analysis of PQQ was performed for sodium salts of PQQ (Ishida *et al.*, 1989; Ikemoto *et al.*, 2012). However, crystal structure analysis has not been performed for $PQQH_2$, because $PQQH_2$ is generally unstable in solution under air. In the present study we succeeded in the synthesis of a significant quantity of $PQQH_2$ powder crystals in high yield under air, by utilizing safe and cheap Vit. C as a reducing agent. Single crystals of $PQQH_2$ and PQQ were prepared from DMSO/CH_3CN mixed solvent, and we succeeded in the crystal structure analysis of $PQQH_2$ and PQQ for the first time. From the results obtained, detailed comparison between the molecular structures of $PQQH_2$ and PQQ was performed.

Acknowledgements

We are grateful to Professor Shinichi Nagaoka of Ehime University for his kind support of this work. We are also grateful to Dr Aya Ouchi of Ehime University for her kind help in the measurement of UV–vis absorption spectra.

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