Structure determination of reaction products of pyrroloquinoline-quinone (PQQ) with L-tryptophan *in vitro* and their effects for microbacterial growth

Eiji Kawamoto,[a] Takashi Amano,[a] Jyutoku Kanayama,[a] Yasuko In,[a] Mitsunobu Doi,[a] Toshimasa Ishida,*[,a] Takashi Iwashita[b] and Kyosuke Nomoto*[,b]

[a] *Department of Physical Chemistry, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki, Osaka 569-11, Japan*
[b] *Suntory Institute for Bioorganic Research, 1-1-1 Shimamoto-cho, Mishima-gun, Osaka 618, Japan*

The *in vitro* reaction of PQQ with L-tryptophan was investigated under an aerobic condition. Three different reaction products were isolated from the reaction solution in phosphate buffer (pH 6.5), and their chemical structures were investigated by UV, mass and NMR spectroscopies. By virtue of the structure of imidazolopyrroloquinoline (IPQ) of 4 isolated as the main product, which was determined by the NMR spectroscopic and X-ray single crystal analyses, the other two products of 5 and 6 were shown to be the indolyl and indolylmethyl derivatives of IPQ, respectively. Based on this information, a possible production pathway of 4–6 from PQQ and L-tryptophan was proposed. These products were shown to exhibit the potential growth-stimulating activity for the micro-organism, *Acetobacter aceti*, which was more active than PQQ when compared on a molar basis, indicating their biological significance in living cells.

Pyrroloquinolinequinone (PQQ, **1**) is one of several *o*-quinones that serve as prosthetic groups in quinoproteins.[1] In addition to the growth-stimulating, pharmaceutical and nutritional activities,[2] PQQ itself also behaves as a catalyst for the non-enzymatic reaction of biomolecules *in vitro*.[3] Since PQQ is very reactive towards nucleophiles, its reaction with nucleophillic biomolecules is interesting from a physiological point of view.

For the non-enzymatic reaction of PQQ with amino acids, it is known that PQQ catalyses, *via* Schiff base formation, the oxidative decarboxylation or oxidative dealdolation of the amino acid.[4] However, it is also recognized that PQQ itself is gradually converted into an inactive compound as the catalytic cycle progresses, due to the formation of reaction products of PQQ and the amino acid. The oxazolopyrroloquinoline (OPQ, **2**), or its derivative (**3**), has been suggested to be the major product.[3–5]

In the process of elucidating the structures and biological activities of the *in vitro* reaction products of PQQ and L-tryptophan (Trp) the molecular interaction[6] and reaction mechanism between PQQ and Trp in phosphate buffer (pH 6.7, 37 °C) under aerobic conditions have been investigated with the isolation of three kinds of products (**4–6**). Furthermore, recently, we succeeded in analysing the single crystal structure of **4**, which is the main reaction product; preliminary X-ray results have already been published.[7] The UV, mass and ^{1}H and ^{13}C NMR spectral analyses of the respective products, together with the X-ray crystal analysis of **4**, made it possible to deduce their structures and to propose an *in vitro* production pathway. In this paper, we report these results, together with the growth-stimulating activity of these products for *Acetobacter aceti*. Although the *in vitro* reaction of PQQ with amino acids, and the structure and biological activities of the reaction products have been investigated by several groups,[3–5] little is known about the reaction with Trp, and thus the results described here provide useful information on the chemical properties of PQQ and its reaction with biomolecules.

Experimental

In vitro reaction of PQQ and Trp and isolation of products

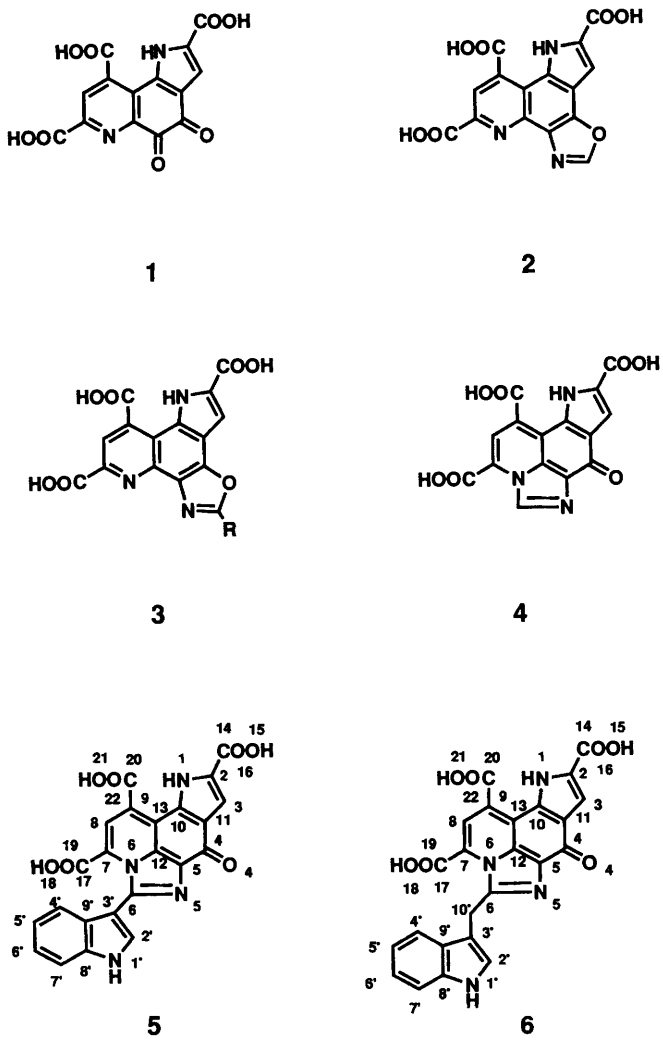
A reaction solution (50 ml) consisting of PQQ (30 μmol) and L-tryptophan (300 μmol) in 50 mmol dm^{-3} phosphate buffer (pH 6.5) was incubated for 24 h at 30 °C under aerobic conditions in a dark room. The isolation of products from the reaction solution was done by the elution through a DEAE Cellulofine A200 column [0–2 M linear gradient of triethylammonium acetate (TEAA)], thus separating the reaction products from the unreacted Trp and PQQ. The product fraction was further separated into the three different products by elution through an ODS column (5–8% linear gradient of acetonitrile in 10 mmol dm^{-3} TEAA, pH 7.0): **4** (the major product with orange colour), **5** (red–brown) and **6** (orange). The purities of these compounds were checked by HPLC using a reversed-phase column (Cosmosil ODS 5C18).

Spectroscopy

Ultraviolet and visible (UV–VIS) absorption spectra were recorded on a JASCO spectrophotometer at 20 °C, the sample solutions were prepared with 0.5 M phosphate buffer (pH 7.0). Mass spectra were recorded on a JEOL HX110A spectrometer with a MALDI (matrix assisted laser desorption ionization) system. ^{1}H NMR spectra were recorded at 27 °C on a Bruker DMX-500 spectrometer (500 MHz for ^{1}H) and ^{13}C NMR spectra were obtained on a JEOL GSX-400 spectrometer (100 MHz for ^{13}C). A sample of about 5–6 mg was dissolved in 0.5 ml [$^2H_6$]dimethyl sulfoxide ([$^2H_6$]DMSO), and the chemical shifts (in ppm) were measured by reference to the standard value of ^{1}H (2.49 ppm) or ^{13}C (39.5 ppm) of DMSO. Two-dimensional NMR spectra were measured in the phase-sensitive mode using the standard pulse schemes and software programs.

X-Ray crystal analysis

Single crystals of **4** (dark orange-coloured plates) were obtained from DMSO solution at room temperature. Since the crystals



The numbering system of **4** is the same as that for **5** and **6**

become opaque in the air, they were sealed in glass capillaries containing some mother liquid.

Crystal data. $C_{15}H_7N_3O_7 \cdot 3(CH_3)_2SO$, $M = 875.624$, triclinic, space group $P\bar{1}$, $a = 13.451(1)$ Å, $b = 14.697(2)$ Å, $c = 7.308(1)$ Å, $\alpha = 91.26(1)°$, $\beta = 103.37(1)°$, $\gamma = 66.79(1)°$, $V = 1287.9(3)$ Å^3, $Z = 2$, $D_x = 1.484$ g cm^{-3}, λ(Cu-Kα) = 1.5418 Å, μ(Cu-Kα) = 3.16 mm^{-1}, $F(000) = 600$. Determining the structure by the direct method with refinement by the least-squares method using anisotropic temperature factors for non-H atoms led to a discrepancy index $R_1 = 0.058$ using 1575 independent reflections of $F_0 \geqslant 2\sigma(F_0^2)$. The details of the crystal structure will be published elsewhere.[8]

Growth-stimulating assay

A basal medium for assaying growth-stimulating activities of compounds **4–6** was prepared according to the proposal of Adachi *et al.*[9] Each product (100 μg l^{-1}) was added to 500 ml of the medium in a 500 mL flask and a cell suspension (0.4 ml) of *Acetobacter aceti* IFO 3284 (Institute for Fermentation, Osaka), having an optical density of 0.1 to 0.2 at 660 nm, was added. As a control experiment, a culture without the product was similarly prepared. Incubation was carried out at 25 °C for the period indicated and the bacterial growth was estimated from the turbidity at 660 nm.

Results

Isolation of reaction products of PQQ and Trp

Chromatograms at the isolation and purification steps of the reaction products from PQQ and Trp are shown in Fig. 1.

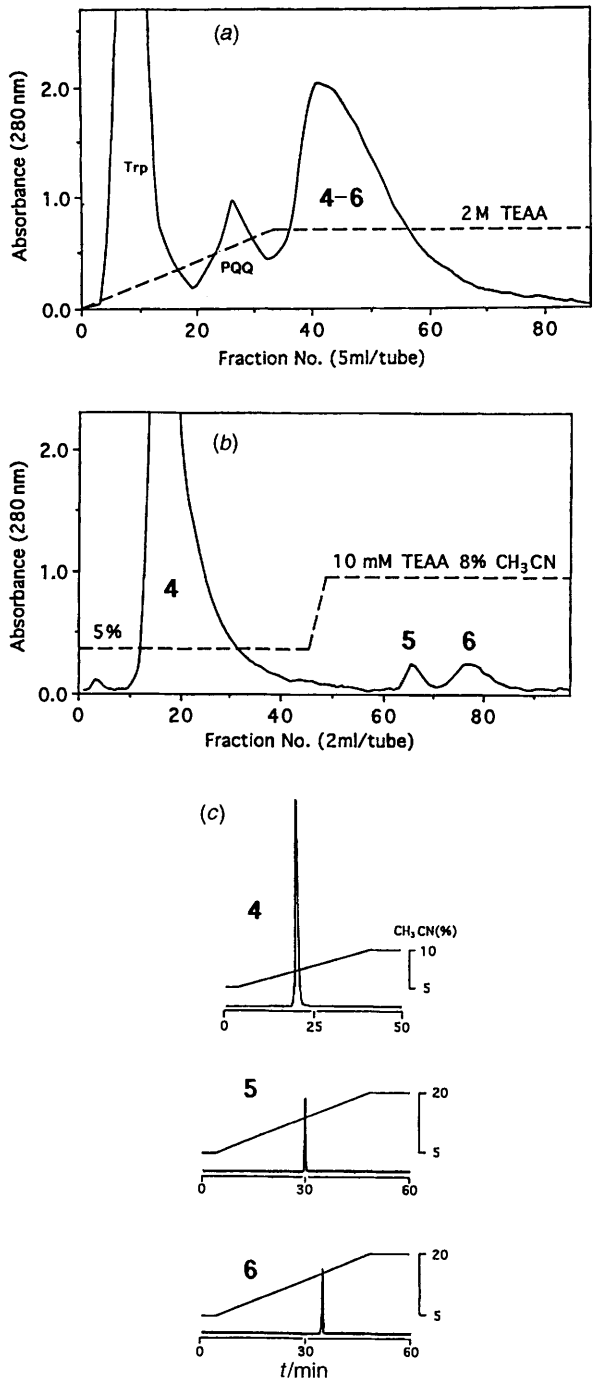


Fig. 1 (*a*) Elution profile of the reaction solution of PQQ with Trp by reversed-phase chromatography (0–2 M linear gradient of TEAA), (*b*) separation profile of the three reaction products (**4–6**) by reversed-phase chromatography (5–8% linear gradient of CH_3CN in 10 mmol dm^{-3} TEAA) and (*c*) purification of **4–6** by HPLC (column, Cosmosil ODS 5C18; flow rate, 0.5 ml min^{-1}; solvent, 10 mmol dm^{-3} TEAA–CH_3CN (5–10%); detection, 280 nm)

Respective fractions of the products and unreacted Trp and PQQ were separated by elution of the reaction solution through a DEAE Cellulofine A200 column [Fig. 1(*a*)]. The product fraction was further separated into three compounds (**4–6**) by elution through an ODS column; the purification of these products by HPLC is shown in Fig. 1(*c*), the product ratio being *ca.* 120:1:2 for **4**:**5**:**6**, respectively. As judged from the respective retention times, the hydrophobicity is in the order of **4** < **5** < **6**, and this also reflects the order of the molecular weight if their hydrophobicities are nearly the same.

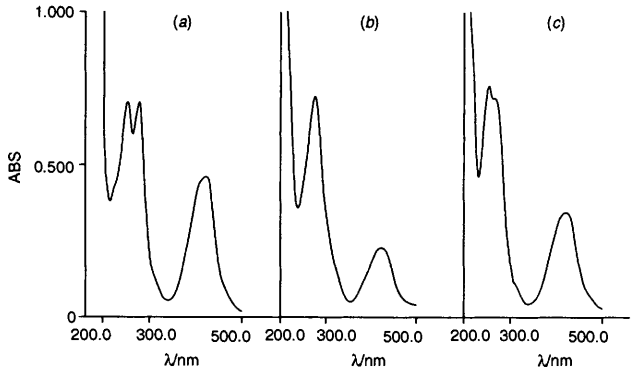


Fig. 2 UV–VIS spectra of **4** (*a*), **5** (*b*) and **6** (*c*). The spectra were measured in 0.5 M phosphate buffer (pH 7.0).

Table 1 ^{1}H NMR data of reaction products **4–6**

Proton	4	5	6
H-1	12.90 (d, 2.4)[a]	14.22 (d, 1.8)	14.01 (d, 2.4)
H-3	7.21 (d, 2.4)	7.32 (d, 1.8)	7.29 (d, 2.4)
H-6	9.19 (s)		
H-8	8.24 (s)	8.04 (s)	7.95 (s)
H-1′		11.62 (s)	10.95 (s)
H-2′		7.72 (s)	6.96 (s)
H-4′		7.73 (d, 7.9)	7.17 (d, 7.9)
H-5′		7.11 (t, 7.9)	6.84 (t, 7.9)
H-6′		7.20 (t, 7.9)	7.00 (t, 7.9)
H-7′		7.49 (d, 7.9)	7.29 (d, 7.9)
H-10′			4.78 (s)

[a] Chemical shift (δ) (mult, J in Hz).

Table 2 ^{13}C NMR data of reaction products **4–6**

Carbon	4	5[a]	6[a]
C-2	127.66	126.95	126.76
C-3	111.70	111.80	111.73
C-4	173.10	173.55	173.49
C-5	132.68	131.14	129.85
C-6	133.96	141.14	145.49
C-7	121.30	—	—
C-8	121.12	120.69	120.41
C-9	124.15	128.25	127.78
C-10	128.77	130.31	130.13
C-11	127.35	126.63	126.72
C-12	130.88	133.76	134.46
C-13	120.79	118.59	119.06
C-14	160.70	160.38	161.32
C-17	161.85	162.09	162.58
C-20	166.98	166.90	166.84
C-2′		125.92	124.18
C-3′		106.36	108.11
C-4′		119.85	118.14
C-5′		119.85	118.48
C-6′		121.97	121.08
C-7′		111.80	111.35
C-8′		135.97	136.22
C-9′		125.97	126.94
C-10′			27.03

[a] The peak of C-7 was not assigned because it overlapped with those of the C-2, C-11 and C-2′ atoms.

Absorption, mass and NMR spectra of 4–6

The UV–VIS spectra of **4–6** are shown in Fig. 2. The spectral data of **4–6** in 0.5 M phosphate buffer (pH 7.0) are as follows: 251 nm (log ε = 4.36), 276 nm (log ε = 4.35) and 422 nm (log ε = 4.17) for **4**; 277 nm (log ε = 4.36) and 420 nm (log ε = 3.88) for **5** and 258 nm (log ε = 4.37), 272 nm (log ε = 4.35), 422 nm (log ε = 4.06) for **6**. The mass spectra of **4–6** showed molecular ion peaks of m/z = 342, 457 and 471, respectively. Characteristically, the molecular fragments of **5** and **6** showed similar distribution patterns, while that of **4** differed notably (data are not shown).

Peak assignments for the respective NMR spectra of **4–6** are given in Tables 1 and 2 and are based on (i) a comparison with the ^{1}H and ^{13}C NMR spectra of PQQ and Trp, and (ii) a combination of two-dimensional DQF-COSY, ROESY and ^{1}H–^{13}C shift correlation HSQC and HMBC measurements; the ^{1}H and ^{13}C NMR spectra of **5** are exemplified in Fig. 3.

X-Ray crystal structure of reactant 4

The molecular structure of **4** (main product) from the crystal structure determination is shown in Fig. 4, and it is shown to be an imidazolopyrroloquinoline (IPQ). The values of the bond lengths and angles suggested that the three carboxy groups of the structure are all in a neutral state with C(4)–O(4) taking the keto form. The molecular structure of **4** is essentially planar with the three carboxy groups and the keto oxygen atom almost coplanar with the aromatic ring resulting in the resonance state being expanded over the whole structure.

Growth-stimulating activity

The growth-stimulating activities of PQQ and **4–6** on the growth of *A. aceti* are shown in Fig. 5. Since the maximum levels of microbial growth in the presence of these compounds are nearly the same as that in the control, except for the appearance of a marked reduction of the lag phase, it appears that these compounds are not necessarily the essential growth factors against the bacteria, but rather function as the acceleration factors in the initial stage of cell growth.

Discussion

Structure determinations of 4–6

The structure of **4** was undoubtedly shown to be IPQ by its X-ray crystal analysis (Fig. 4). However, it is very important to note that the UV–VIS and mass spectral data, and the HPLC retention time of **4** agreed completely with those of OPQ (**2**) which was synthesized according to the method of Itoh *et al.*[4] Thus, the present X-ray result indicates that the main product should be IPQ or its C-6 derivative, although OPQ (**2**), or its derivative (**3**), has been considered to be the main reaction product of PQQ and the amino acid.[3,4] The coexistence of IPQ and OPQ structures is probable in solutions of **4**, because these structures are isomeric. However, the NMR spectra suggest the presence of a single structure for **4** from the respective single peaks for the protons and carbons (Tables 1 and 2). Furthermore, the NMR peak assignment suggested the IPQ structure for **4**, rather than the OPQ structure. For example, as was already stated in ref. 7, the chemical shift at δ 9.19, which is assigned to the oxazole ring proton in the case of OPQ, occurs at a lowfield, compared with the usually observed value (δ ~8.10),[10] and the peak at δ 8.24, assigned to the H-8 proton of OPQ, is to some extent shifted to a higher field, compared with that of PQQ (δ ~ 8.60). As far as the ^{13}C NMR spectrum of **4** is concerned, the peak which should be assigned to the carbonyl carbon of OPQ is revealed at a lower field (173.10 ppm)[10] than the three peaks corresponding to carboxy carbons (δ 160.70, 161.85 and 166.98).

The peak assignment for **4** given in Tables 1 and 2 was carried out according to the following logic. One labile proton was observed at δ 12.90, which was thought to be the amine proton (H-1) by observation of a long-range coupling with the proton at δ 7.21 (H-3). Based on these protons, the carbons at δ 111.70, 127.35, 127.66 and 128.77 were assigned to the pyrrole ring part of the molecule using the HSQC and HMBC data. The proton at δ 8.24 was assigned to that (H-8) of the pyridine ring part, because the proton possessed a long-range coupling to the two carboxy carbons at δ 161.85 and 166.98. The carbon at δ 166.98 should be C-20 because it is deshielded by the hydrogen

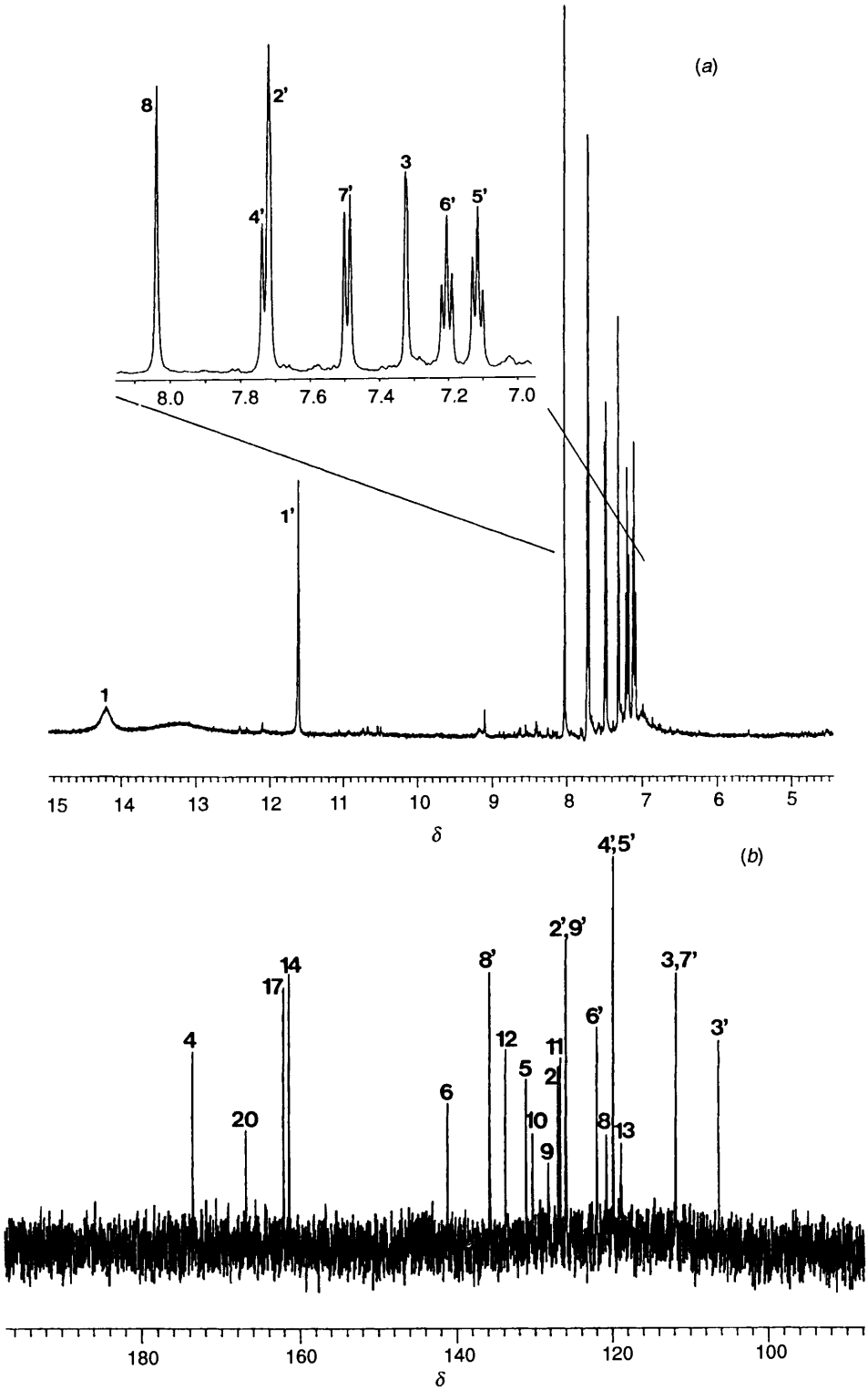


Fig. 3 (*a*) ^{1}H and (*b*) ^{13}C NMR spectra of **5** in [$^{2}H_6$]DMSO solution at 20 °C. Numbers correspond to those shown in the structure of **5**.

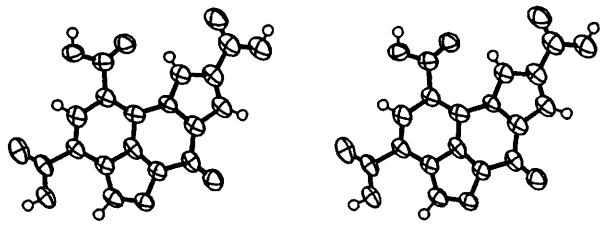


Fig. 4 Stereoscopic view of the molecular conformation of **4**

bonding with the amine proton. The remaining carboxy carbon was by elimination thought to be on the pyrrole ring, which was at δ 160.70. From the proton at δ 9.19, the carbons at δ 130.88, 132.68 and 133.96 were thought to be in the imidazole ring from the HSQC and HMBC data, but the assignments of the δ 130.88 and 132.68 cabons are ambiguous and are tentatively assigned. The remaining two carbons at δ 121.30 and 124.15 were also tentatively assigned to the pyridine ring; the positions of these carbons were not determined from the HMBC data.

The structure determinations of **5** and **6** were done by reference to the IPQ structure of **4**. It is obvious from Fig. 2 that both compounds **5** and **6** consist of an IPQ derivative containing an indole ring, because the band ranging from 280 to 300 nm reflects an absorption due to an indole ring, and

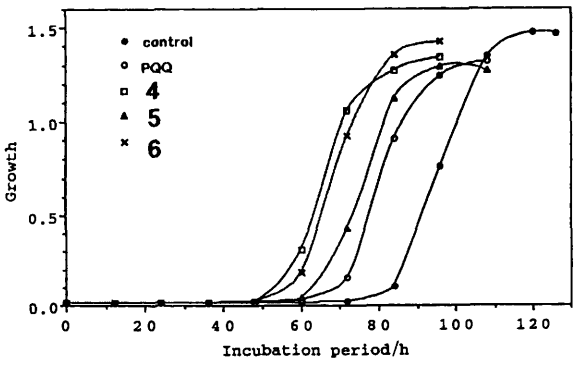
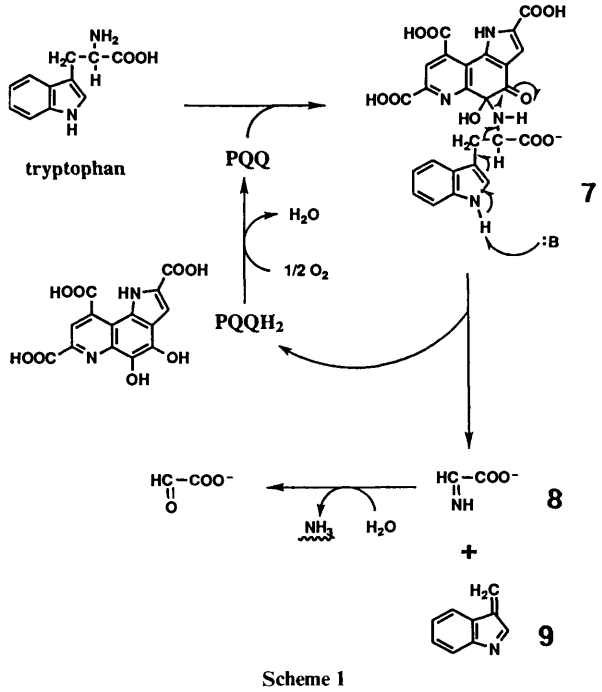


Fig. 5 Effect of **4**–**6** on growth of *Acetobacter aceti* IFO 3284, together with that of PQQ

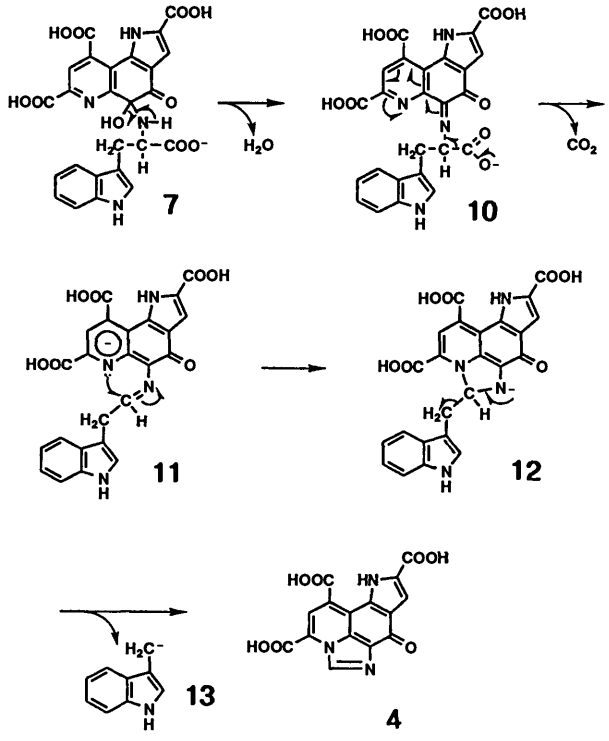
the absorption at around 422 nm is characteristic of an IPQ aromatic ring;[4,5] the existence of the indole ring was also ascertained by the Ehrlich test. Since the mass spectra of **5** and **6** showed similar fragmentation patterns, their structures could be thought to be very similar to each other, except for that **6** has an additional CH_2 group, which corresponds to the difference between the molecular ion peaks of **5** (m/z = 457) and **6** (m/z = 471). In addition to these data, the peak assignment (Tables 1 and 2) of the ^1H and ^{13}C NMR spectra suggested the most probable structures of **5** and **6** to be as shown. Similar logic to that used for **4** was applied for the NMR peak assignments of **5** and **6**. Two labile protons were detected in the down-field region for both products, which were assigned to the amino protons. The labile proton at *ca.* δ 14 was assigned to the pyrrole part of the molecule, because the proton (H-1) has a long-range coupling with the pyrrole proton (H-3). Thus, the proton at *ca.* δ 11 was assigned to the indole amine of the molecule. Based on the indole amine proton, the protons and carbons of the indole part of the molecule were assigned by DQF-COSY, ROESY, HSQC and HMBC measurements. All protons and carbons, except one carbon (C-7), were finally assigned.

Possible production pathway of 4–6 from PQQ and Trp *in vitro*

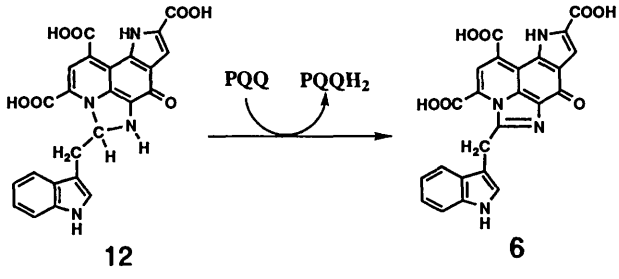
Possible production pathways of **4**–**6** from PQQ and Trp are shown in Schemes 1–4. Under aerobic conditions, two reaction pathways would be possible for PQQ: the catalytic pathway (Scheme 1) of PQQ and its inactivation pathway (Schemes 2–4).



Scheme 1



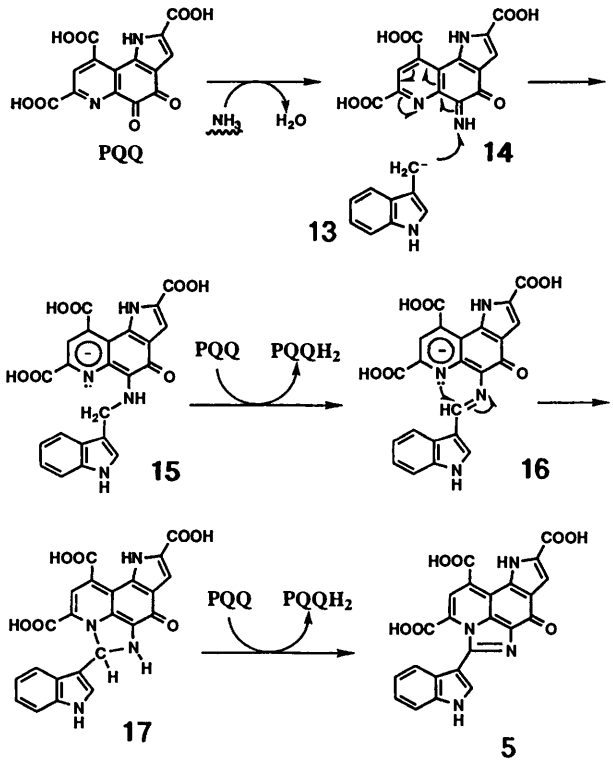
Scheme 2



Scheme 3

The first step in these *in vitro* reactions is the aminomethanol-type formation of **7**; the amine oxidation by PQQ has already been demonstrated to proceed *via* an ionic mechanism involving an aminomethanol-type intermediate.[4,11] From the results reported for the oxidative dealdolation of amino acids, in particular the catalytic reaction of PQQ with tryptophan, tyrosine and β-hydroxyamino acid[4,12] the catalytic cycle for PQQ catalysis shown in Scheme 1 is proposed, where Cα–Cβ bond cleavage (oxidative dealdolation) of Trp takes place to produce **8** and **9**. As the catalytic cycles progress, however, PQQ is gradually converted into inactive compounds; in fact, the major reaction product from PQQ and Trp was shown to be IPQ (**4**). The conversion into **4** would be very significant in anaerobic conditions. Schemes 2 and 3 show the proposed reaction pathways for the production of inactive **4** and **6**, respectively, from PPQ. Based on the insights obtained so far,[4,5b,7] it can be considered that after the formation of iminoquinone-type **10** by the direct dehydration of **7**, followed by its decarboxylation, the ring closure of the Schiff base (**11**) takes place *via* the pyridine nitrogen atom to produce **12**. As judged from the chemical structure of **4** or **6**, the release of the indolylmethyl anion (**13**) from **12** or the reduction of **12** by PQQ could be proposed as a possible formation pathway of **4** or **6**, respectively.

For the formation of **5**, on the other hand, the reaction pathway shown in Scheme 4 is proposed. Ammonia, produced by the deamination of Trp by PQQ (Scheme 1), reacts with PQQ to form the iminoquinone **14**; the formation of **14** has already been reported as the reaction product of PQQ with ammonia.[13] The reaction of **14** with **13** would form intermediate **15**. After



Scheme 4

the reduction of **15** by PQQ, the ring closure of **16** *via* the pyridine nitrogen atom would afford **17**. The reduction of **17** by PQQ would then lead to the production of **5**. This reaction scheme is supported by the fact that the production of **5** was significantly promoted by the addition of an ammonium salt to the reaction solution of PQQ and Trp.

Growth-stimulating activity and biological implications

Fig. 5 shows that **4** and **6** exhibit a much more potent growth-stimulating effect than PQQ and **5**, although the latter compounds still exert a marked effect, compared with the control data. Based on these results, it would be interesting to consider the possible relation between the chemical features and the growth-stimulating activities of PQQ and **4**–**6**. Provided that the activity is directly dependent on the structure itself, it would be considered from (i) no notable difference between the activity of **4** and **6** and (ii) the very similar structures of **5** and **6**, that the IPQ structure itself is important for the activity, rather than the PQQ structure, and that the greater conformational flexibility of the IPQ structure in **6** than that in **5** is necessary for the activity; the indole ring itself would not effect the activity directly.

PQQ has been shown to be a cofactor of various quinoproteins. The present results indicated that PQQ itself also reacts with amino acids under physiological conditions and easily produces products, as have already been reported in other groups.[4,5b] Since these products are reported to be less active as the prosthetic group for the quinoprotein than intact PQQ,[5a] they may function as regulators controlling the catalytic reaction of quinoprotein. However, reaction products such as **4** and **6** have been shown to have more potent growth-stimulating activity against the microorganism than PQQ itself. This may imply that the reaction products of the reaction of PQQ with biomolecules play important biological functions.

Acknowledgements

Authors thank Drs H. Noshi and Y. Itagaki, Suntory Institute for Bioorganic Research, for the measurement of mass spectra.

References

1 J. A. Duine, *Eur. J. Biochem.*, 1991, **200**, 271.
2 (*a*) M. Ameyama, K. Matsushita, E. Shinagawa and O. Adachi, *Vitam. Horm. (NY)*, 1991, **46**, 229; (*b*) J. Killgore, C. Smidt, L. Duich, N. Romero-Chapman, D. Tinker, K. Reiser, M. Melko, D. Hyde and R. B. Rucker, *Science*, 1989, **245**, 850; (*c*) W. Watanabe, N. Hobara and T. Tsuji, *Curr. Ther. Res.*, 1988, **44**, 896; (*d*) Y. Hamagishi, S. Murata, H. Kamei, T. Oki, O. Adachi and M. Ameyama, *J. Pharmacol. Exp. Thera.*, 1990, **255**, 980; (*e*) H. Nishigori, M. Yasunaga, M. Mizumura, J. W. Lee and M. Iwatsuru, *Life Sci.*, 1989, **45**, 593.
3 (*a*) J. A. Duine, Jzn. J. Frank and J. A. Jongejan, *Adv. Enzymol. Relat. Areas. Mol. Biol.*, 1986, **59**, 169; (*b*) Y. Ohshiro and S. Itoh, *Yuki Gosei Kagaku Kyokaishi*, 1989, **47**, 855; S. Itoh and Y. Ohshiro, *Nat. Prod. Rep.*, 1995, **12**, 45.
4 S. Itoh, M. Mure, A. Suzuki, H. Murao and Y. Ohshiro, *J. Chem. Soc., Perkin Trans. 2*, 1992, 1245.
5 (*a*) O. Adachi, K. Okamoto, E. Shinagawa, K. Matsushita and M. Ameyama, *BioFactors*, 1988, **1**, 251; (*b*) M. A. G. van Kleef, J. A. Jongejan and J. A. Duine, *Eur. J. Biochem.*, 1989, **183**, 41.
6 T. Ishida, M. Doi, K. Tomita, H. Hayashi, M. Inoue and T. Urakami, *J. Am. Chem. Soc.*, 1989, **111**, 6822.
7 T. Ishida, E. Kawamoto, Y. In, T. Amano, J. Kanayama and M. Doi, *J. Am. Chem. Soc.*, 1995, **117**, 3278.
8 T. Ishida, Y. In, E. Kawamoto, T. Amano, J. Kanayama, M. Doi, T. Iwashita and K. Nomoto, *Chem. Pharm. Bull.*, in the press.
9 O. Adachi, K. Okamoto, K. Matsushita, E. Shinagawa and M. Ameyama, *Agric. Biol. Chem.*, 1990, **54**, 2751.
10 E. Pretsch, T. Clerc, J. Seibl and W. Simon, *Tabellen zur Strukturaufklarung Organischer Verbindungen mit Spektroskopischen Methoden*, Springer–Verlag, Berlin, 1981.
11 (*a*) E. J. Rodriguez and T. C. Bruice, *J. Am. Chem. Soc.*, 1989, **111**, 7947; (*b*) M. Mure, S. Itoh and Y. Ohshiro, *Tetrahedron Lett.*, 1989, **30**, 6875; (*c*) S. Itoh, M. Mure, M. Ogino and Y. Ohshiro, *J. Org. Chem.*, 1991, **56**, 6857.
12 M. Mure, A. Suzuki, S. Itoh and Y. Ohshiro, *J. Chem. Soc., Chem. Commun.*, 1990, 1608.
13 (*a*) R. H. Dekker, J. A. Duine, Jzn. J. Frank, P. E. J. Verwiel and J. Westerling, *Eur. J. Biochem.*, 1982, **125**, 69; (*b*) P. R. Sleath, J. B. Noar. G. A. Eberlein and T. C. Bruice, *J. Am. Chem. Soc.*, 1985, **107**, 3328.

Paper 6/00594B
Received 25*th January* 1996
Accepted 28*th February* 1996