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PYRROLOQUINOLINEQUINONE (P.Q.Q.)—A NOVEL COENZYME

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Pyrroloquinolinequinone (P.Q.Q.) or methoxatin—a new coenzyme, which has a unique tricyclic-*o*-quinone structure, has been discovered recently. It occurs in many dehydrogenases, which are not NAD (P) or flavin-dependent and thus represent a third class of dehydrogenases. Methanol dehydrogenase—a P.Q.Q.-dependent enzyme utilises it for the oxidation of methanol. This enzyme has biotechnological significance in the commercial production of single cell protein. The high redox potential of P.Q.Q. and its discovery in a mammalian enzyme points to a wider role. Three different chemical synthesis of methoxatin have been described. Biomimetic micellar oxidation of amines has been carried out successfully using synthetic methoxatin. P.Q.Q. directed inhibitors could be important in chemotherapy. Studies on the distribution, biosynthesis and biochemistry of the new coenzyme are expected to reveal its special role.

Key Words : Methanol Dehydrogenase; Methoxation; Tricyclic-*o*-quinone; "Umpolung" Japp-Klingemann Reaction and Kozikowski Modification; Linear or Convergent Synthesis; Biomimetic Micellar Oxidations

INTRODUCTION

IN a recent survey of the progress in the study of enzyme mechanisms, Walsh[1] highlighted the discovery of a new coenzyme, methoxatin in methylotrophs (methane oxidising bacteria).[2] This new cofactor in C_1 biochemistry (i.e. biochemical reactions involving molecules containing one carbon atom) has a novel tricyclic pyrroloquinoline quinone (P.Q.Q.) structure and has been shown to be fairly widespread in its occurrence. P.Q.Q. occurs in many dehydrogenases e.g., methanol dehydrogenase which utilises it for oxidising methanol. The name quinoproteins has been suggested[3] for these enzymes in analogy with flavoproteins, hemoproteins, etc., since these enzymes utilise an *ortho*-quinone and thus represent a third class of dehydrogenases apart from the well-known NAD (P) or flavin-dependent ones. The isolation and structure elucidation of P.Q.Q. has been quickly followed by three elegant syntheses and the relatively small, yet complex molecule has provided enough challenges to the synthetic organic chemist. Great excitement has been provided by the very recent report of the discovery of P.Q.Q. in a mammalian enzyme.[3] The ready availability of P.Q.Q. by chemical synthesis now makes it possible for the biochemical role to be elucidated in detail. The significance of these P.Q.Q.-dependent enzymes e.g. methanol dehydrogenase in the commercial

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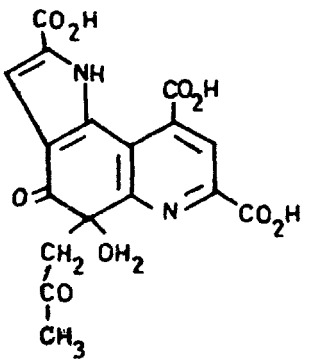
production of single cell proteins or metabolites makes the study of such enzyme systems extremely relevant for biotechnology, as well.[4–7] In fact, a commercial process, has been described for synthesis of single cell protein from methanol by such a microorganism.[8]

The Discovery

As early as in 1964, it was pointed out that the enzyme glucose dehydrogenase from *Acinetobacter calcoaceticus* contains an unusual coenzyme which is different from the well-known NAD(P) or flavin. This report was, however, largely overlooked. Anthony and Zatman[10] who isolated a methanol dehydrogenase from *Pseudomonas*. M 27 obtained a fluorescent low molecular weight compound upon denaturation of the enzyme. Based on its fluorescent properties they suggested that this coenzyme had a pteridine structure. Later, it was reported to be a neopterin cyclic phosphate but again based on the spectral properties of its photodegradation product, it was considered to be a lumazine derivative.[11] Many even considered methanol dehydrogenase to be a pteridoprotein.[11,12] A methnol dehydrogenase was purified from *Hyphomicrobium* X and its co-factor isolated with methanol.[2] They conclusively proved that the co-factor was not a pteridine or lumiazine but was an *ortho*-quinone by analysing the ESR spectrum of the free radical derived from it. However, they could not establish its structure at that stage. A year later Salisbury *et al.*,[13] isolated an acetone adduct of the coenzyme from the facultative methylotroph *Pseudomonas* TPI using the intact bacteria. By X-ray diffraction analysis of the orange crystals, they established the structure of the acetone adduct as (1) and based on it suggested the structure (2) for the coenzyme itself (which was not isolated in pure form by them). They also introduced the name methoxatin for it and even put down its systematic name as 4,5-dihydro-4, 5-dioxo-1H-pyrrolo[2,3-f]-quinoline-2, 7,9-tricarboxylic acid.



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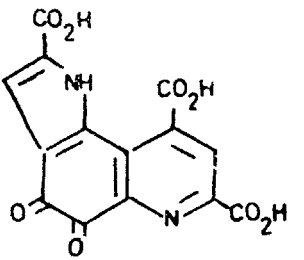


2

Finally, the coenzyme itself was isolated by Duine and coworkers[14] who recorded its NMR, Mass and ESR spectra and confirmed its *o*-quinone structure. They also showed that the quinone moiety was essential for its activity.

Occurrence

P.Q.Q. has been shown to occur in dehydrogenases e.g., glucose dehydrogenase and methanol dehydrogenase. The former is important industrially in oxidation of glucose to gluconic acid, while the latter oxidises methanol. They have been isolated from *Gluconobacter oxydans* and methylotrophic bacteria, respectively. Primary amines also serve as substrates for P.Q.Q. dependent enzymes of methyl amine dehydrogenase, which converts methylamine into formaldehyde and ammonia. It has been shown to contain a covalently bound P.Q.Q. derivative. A reconstitutive apoenzyme from glucose dehydrogenase has provided a very sensitive test for P.Q.Q. The surprisingly widespread nature of P.Q.Q. has been shown by using this test [*see* Table taken from Duine (*loc cit.*)].[15]

Distribution of P.Q.Q. in bacterial dehydrogenases

Enzyme	*Organism*
Methanol dehydrogenase	Methylotroplic bacteria
Alcohol dehydrogenase	*Acinetobacter calcoaceticus*
Alcohol dehydrogenase	Acetic acid bacteria
Glucose dehydrogenase	*Acinetobacter calcoaceticus*
Glucose dehydrogenase	*Pseudomonas aeruginosa*
Glucose dehydrogenase	*Gluconobacter oxydans*
Methylamine dehydrogenase	*Pseudomonas AM1*
Methylamine dehydrogenase	*Thiobacillus A2*
Lactate dehydrogenase	*Propionibacterium pentosaeum*
	Eubacterium limosum

Duine and co-workers[15] have determined even very low amounts of P.Q.Q. in culture supernatants by reaction with butyraldehyde, reduction ($NaBH_4$) and $NaIO_4$ oxidation and fluorescence studies. P.Q.Q. has been very recently detected in a mammalian enzyme by Lobenstein *et al.*[16] who studied Bovine serum oxidase and isolated a derivative of P.Q.Q. by reaction with 2,4-DNP (2,4-dinitrophenylhydrazine). This derivative compared very well with a model compound prepared from the coenzyme itself. P.Q.Q. also reacts with amino-acids to give several derivatives[17] Serum oxidase converts spermine and spermidine into aldehydes, which are believed to function in regulation of cell division. P.Q.Q.-directed inhibitors could be designed which are important for chemotherapy.[18] Dijkstra *et al.*[19] have shown that cyclopropanol and cyclopropanone hydrate inhibit alcohol dehydrogenase containing the oxidised form of P.Q.Q.

Biochemical Role

Pyroloquinolinequinone occurs in the enzyme in its reduced form P.Q.Q. H_2 and artificial oxidants like phenazine methosulphate inactivate it. Dehydrogenases containing P.Q.Q. can oxidise methanol (CH_3OH) since it removes hydrogen and gets converted into $PQQH_2$. However, the $PQQ/PQQH_2$ couple has a very high redox potential (+0.09 volt). As shown in the diagram below (Fig. 1), NADP on the other hand is not well suited for oxidation of CH_3OH as it has a higher negative

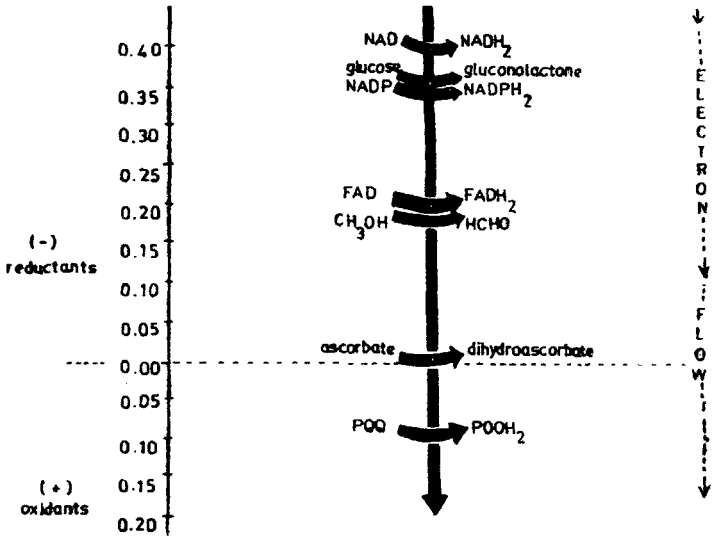


FIG 1

(*Note*: $POOH_2$ at below right may be read as $PQQH_2$. Below $PQQH_2$, the symbol e^- may be added.)

redox potential (—0.35 volt), compared to the redox potential of the CH_3OH/HCHO couple (— 0.18 volt). Electrons can thus readily flow from CH_3OH to PQQ but not to NADP. Since oxidation is essentially electron removal CH_2OH can be readily oxidised with PQQ, rather than with NADP. PQQ is thus a mild oxidant. It is quite possible that these oxidoreductases have been conserved during evolution and have a specialised role, as yet to be explained.

SPECTRAL AND OTHER CHARACTERISTICS

P.Q.Q., an *ortho*-quinone is a red solid and can be analysed by thin-layer chromatography (TLC) [Cellulose, 2 percent aq. NH_4OAC-propanol (1:1)]. Reverse phase-High pressure liquid chromatography (RP-HPLC) on a Water associates $^{c}18$-μ-Bondapak column using 95:5 water-methanol containing 0.1 per cent acetic acid (*p*H ca 4.5) was eluted at 3.55 retention volumes. Various colour tests for *ortho*-quinones have been described.[14,20] The test using 3,4-dimethoxy aniline is however, specific for inner ring quinones.[21]

U.V. spectrum of the synthetic coenzyme in aqueous solution at *p*H 5.5 shows λ_{max} 247, 330 with a shoulder at 270nm. At *p*H 2.5 λ_{max} 250, 340nm were observed. Excitation of the synthetic compound at 265nm in water resulted in fluorescence at 483nm.

The absorption spectrum of the prosthetic group and its reduced form at *p*H 7.0 are shown in (Fig. 2).

^{1}H-NMR of the prosthetic group in D_2O (*p*H 8) showed only 2 aromatic signals at 7.14ppm (broadened singlet, 3-H) and 8.16ppm (broad 8-H).

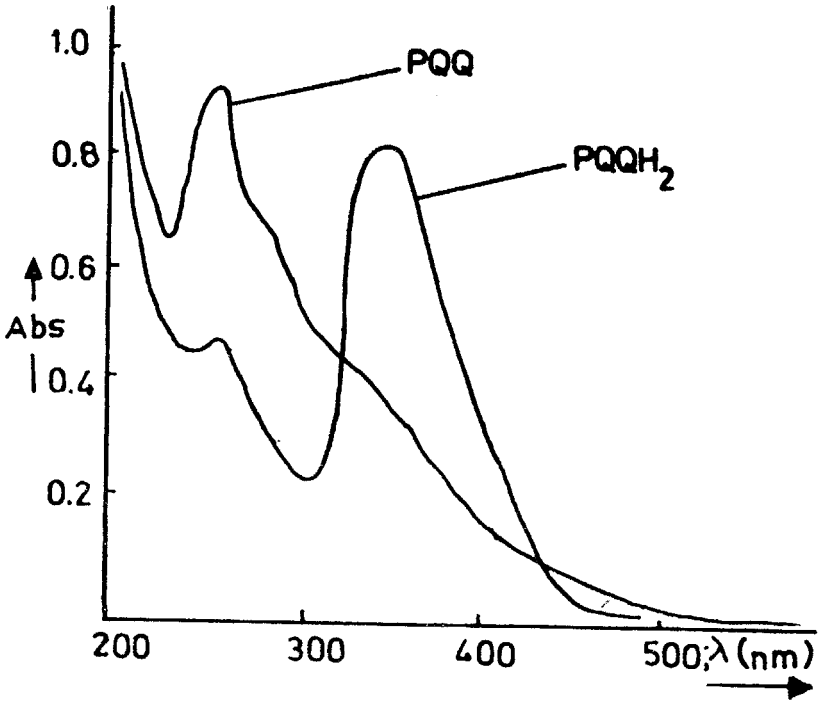


FIG 2

^{13}C-NMR-of PQQ (synthetic) in DMSO-d_6 showed peaks at δ(ppm) 113.86, 122.76, 125.97, 127.71, 130.68, 137.60, 144.63, 146.41, 147.62, 161.25, 165.48 166.45, 173.30and 180.00.

The mass spectrum of the trimethyl ester shows a M^+ ion at m/e 374 (69 per cent relative intensity) and fragments at *m/e* 372 (15), 344(32), 342(94), 318(26), 314 (41), 286(47), 282 (72), 254(100), 239(11), and 228(23). However, the field desorption mass spectrum showed a M^+. ion at $m/e = 372$. This difference in the two observations could be due to the generation of $(M+2)^+$ peak by reduction of PQQ by residual water in the inlet system, a behaviour observed for many quinones, esp. *o*-quinones.[22]

The X-band ESR spectrum of methanol dehydrogenase (Fig. 3) did not show any hyperfine structure. For this, the radical obtained from the coenzyme, had to be disconnected from the enzyme and allowed to rotate freely in solution. This was achieved by dissolving the lyophilised enzyme in a concentrated alkaline salicylate buffer under anaerobic conditions. ESR of this free radical showed hyperfine structure (Fig. 4).

ESR spectrum of the isolated prosthetic group was also recorded and found to be identical. But since it was in oxidised form it was reduced with alkaline salicylate buffer before the ESR spectrum could be recorded. The *g*-value (2.0046) is in

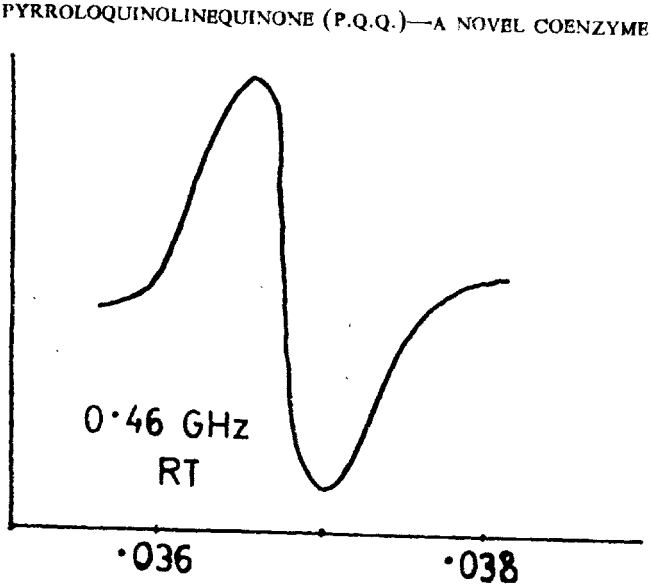


FIG 3

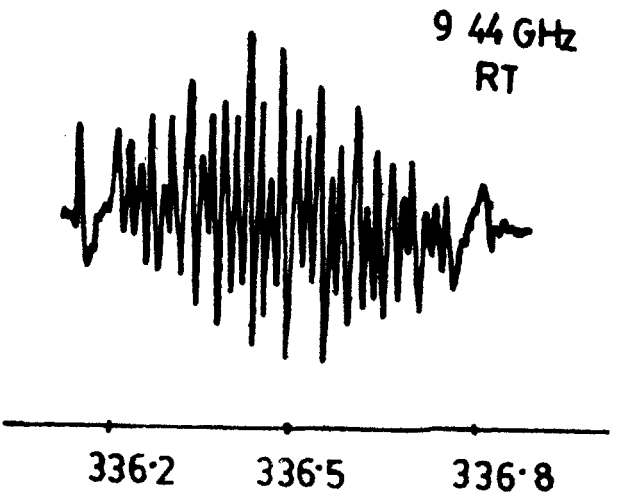


FIG 4

agreement with a quinone radical but not with a pteridine or lumazine structure. The total width between the outerlines of the ESR spectrum is 0.69mT. This is a value within the usual range of 0.6 to 2mT for many quinones. However, it is incompatible with values for pteridines and lumazines. Duine *et al.* in fact interpreted the hyperfine structure and though in 1978 they did not know the structure of the coenzyme, correctly concluded from the hyperfine structure that it contained 2 nitrogen atoms ($I = 1$) and 3 hydrogen atoms ($I = \frac{1}{2}$) and calculated the 5 different coupling constants which caused the splitting of the ESR signal.

General Strategies for Synthesis of Methoxatin

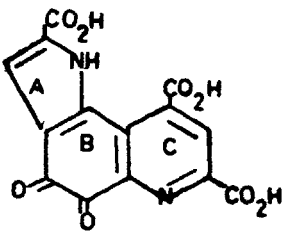
(*i*) *The Need for a Chemical Synthesis*

The small, yet complex molecule of pyrroloquinoline quinone has provided enough challenges to the synthetic organic chemist. Moreover, it is a very sensitive compound. In fact, the meagre amounts from biochemical sources hindered the study of its mechanism and this prompted attempts at organic chemical synthesis in the laboratory.

(*ii*) *Linear* vs *Convergent Synthesis*

Different strategies could be envisaged for the synthesis of this molecule. Three different syntheses to this new coenzyme have been described since 1981.

a. A linear synthesis could be carried out starting with a compound which would be the eventual middle ring *B* of the target molecule and then ring *A* and *C* could be built up one by one. Two different approaches are possible here viz. either one could build up the quinoline ring first and then construct the pyrrole ring system. This was the approach exploited by Gainor and Weinreb.[23] Corey & Tramontano[24] however used the other alternative approach and made the substituted indole first and then built up the pyridine ring.

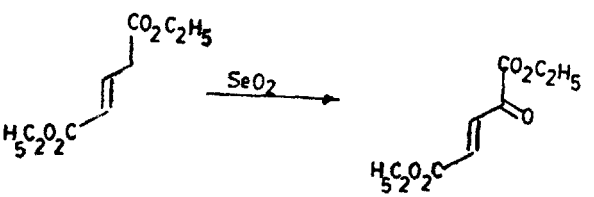


b. One could also carryout a convergent synthesis where the outer rings (*A* and *C*) could be made first followed by the middle ring *B*. One year later, Hendrickson[25] carried out such a synthesis.

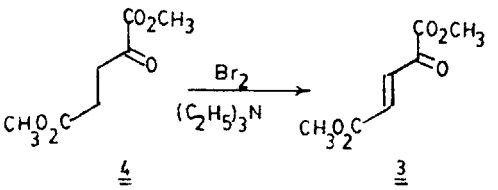
(*iii*) *Important Intermediates/Reactions involved*

Synthetic approahes towards PQQ has necessitated the use of many interesting starting materials and has involved the use of several important reactions. Highlighted below are a few of these :-

a. Synthesis of dimethyl 2-oxo-glutaconate—Conforth and Conforth reported the synthesis of diethyl-2-oxo-glutaconate in 2 per cent yield by SeO_2 oxidation



However, the synthesis of dimethyl-2-oxo-glutaconate (3) in 97 per cent yield by the bromination of dimethyl-2-oxo-glutarate (4), removal of hydrogen bromide and dehydrobromination using triethyl amine.[24]



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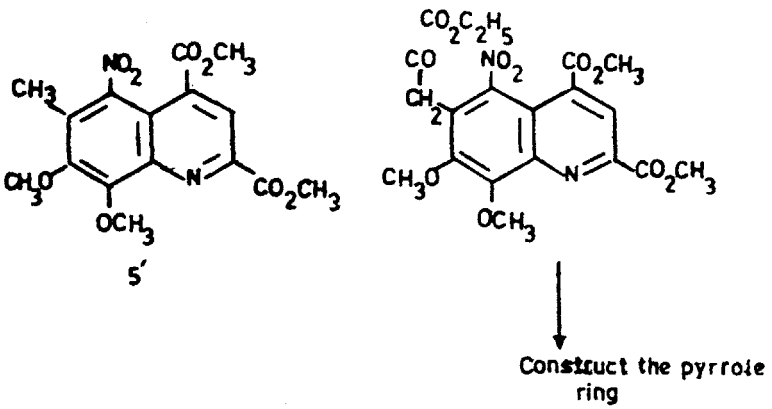
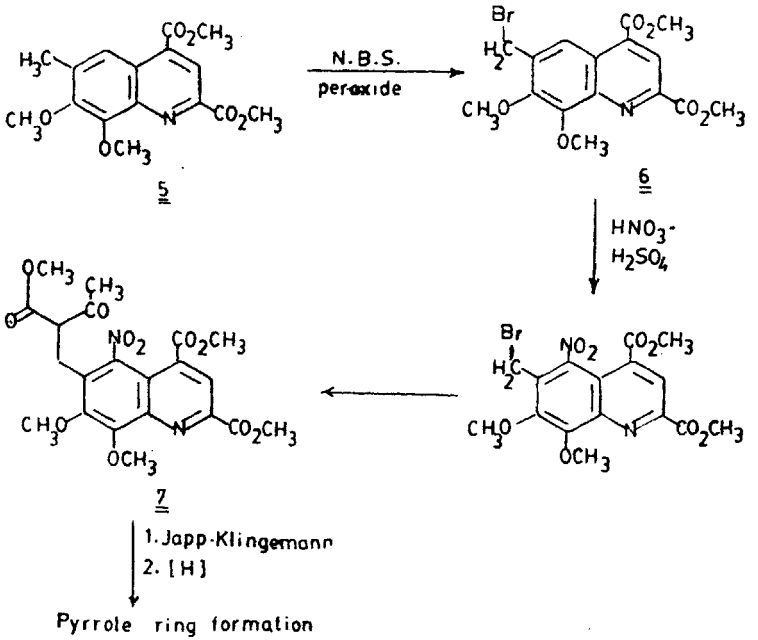
b. "Umpolung" Variation of Reissert Synthesis—In one of the synthesis by Gainor[23] a Reissert Synthesis using the Synthon (5) was planned.

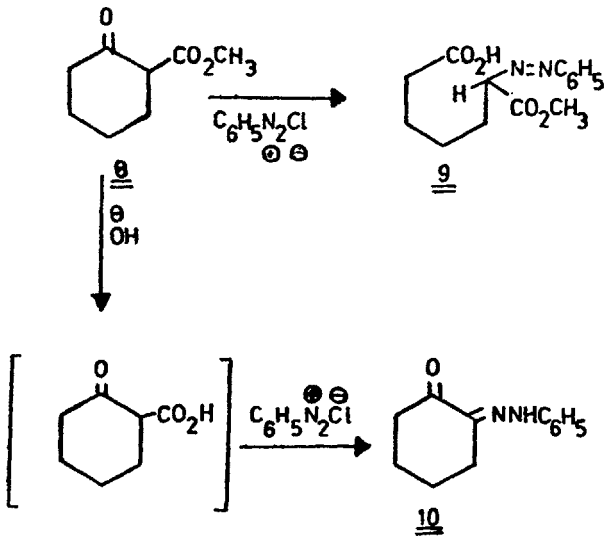


However, the condensation with dimethyl oxalate did not occur as the nitro group is twisted out of plane of the aromatic ring due to "overcrowding". This thus prevented the resonance stabilisation of the benzylic carbonion and consequently the $-CH_3$ group was not acidic enough for the condensation. An "Umpolung" variation of this synthesis had therefore to be carried out. This was achieved by treatment of synthon (5) with N-bromo-succinimide (N.B.S.) in the presence of peroxide. The substituted benzyl bromide (6) thus obtained was nitrated and then easily condensed with methyl acetoacetate to give (7). The pyrrole ring could then be constructed using a Japp-Klingeman reaction followed by reduction.

c. Japp-Klingemann Reaction and the Kozikowski's Modification—Two of the three syntheses of P.Q.Q. carried out in the recent past have involved a Japp-Klingemann reaction at some stage. The reaction deals with the interaction between a β-Ketoester and a diazonium salt.[25–27] Two different possible pathways are available depending upon whether one reacts the β-keto ester itself or the β-keto acid obtained after an initial hydrolysis. The former pathway leads to the splitting of the acyl group and the latter to the loss of the ester group.

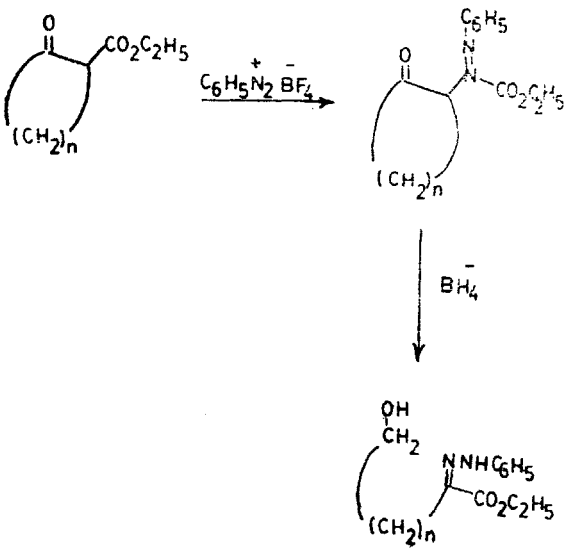


The two possible pathways for cyclic β-keto esters like methyl 2-cyclohexanecarboxylate (8) are shown below:



It can lead to the open chain compound (9) by fission of the acyl bond or alternatively lead to the monophenylhydrazone of cyclohexane 1,2-dione (10) after an initial step of saponification followed by decarboxylation.

Japp-Klingeman reaction could thus be used both for the synthesis of amino acids and indoles. The phenyl hydrazones obtained through the Japp-Klingeman reaction are converted into the Indoles by Fischer Indole synthesis. A modification of the Japp-Klingeman reaction involves the use of diazonium fluoroborates[28] (rather than the chlorides) and the generation of the azo compounds under milder conditions (lower temperature and use of a mixture of pyridine and water) followed by reduction with $NaBH_4$. Phenylhydrazones with a ω-hydroxylic group are the final products (11).



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The following table cites a few illustrative examples of products obtained from the different β-keto esters shown on the next page :

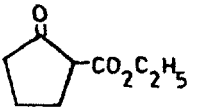
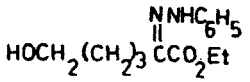
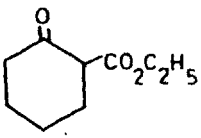
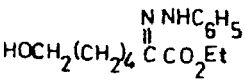
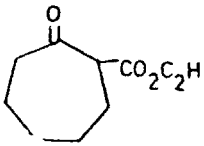
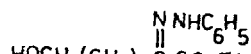
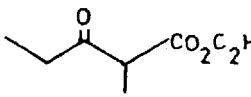
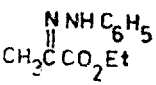
d. The generation of the O-Quinone System—Yet another general question to be answered is as to how and when should the rather sensitive *o*-quinone system be generated. Each of the three synthesis reported has preferred has a different strategy.

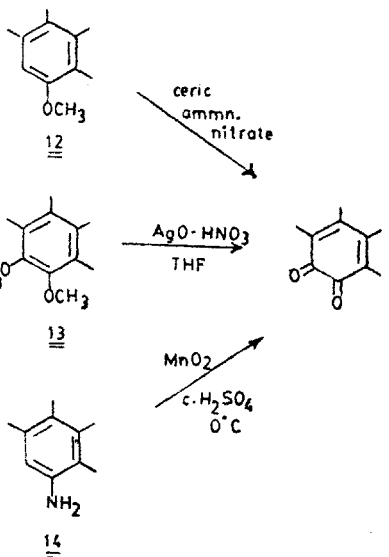
Corey[24] preferred the reagent, ceric ammonium nitrate and generated the o-benzo quinone from a phenolic monomethyl ether (12).

Gainor[23] generated the *o*-quinone system from a catechol dimethyl ether (13) using and AgO-HNO_3-THF at the room temperature for 10 min.[29]

Hendrickson[25] generated the *o*-quinone using MnO_2-conc. H_2SO_4 at 0 °C from an amine (14).

Kozikowski

Keto ester	Product (11)	Yield%
		62%
		97%
		88%
		96%

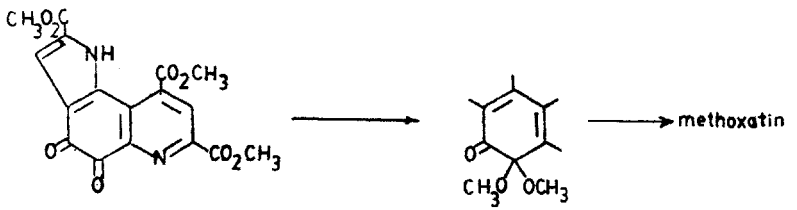


However, in all the three syntheses the sensitive *o*-quinone system was generated only after the tricyclic framework had been already built up. It is thus advisable to delay the generation of this sensitive part of the molecule as far as possible. The concept of "latent functionality" has been beautifully illustrated here and a phenolic

methyl ether, catechol dimethyl ether and an aromatic amino group have all been used to generate on *o*-quinone system in a subsequent step.

e. Hydrolysis of the Ester Groups in the Sensitive Trimethyl ester of P.Q.Q.— The hydrolysis of the trimethyl ester of P.Q.Q. obtained in all the three synthesis posed a major hurdle.

Corey[24] overcame this hurdle by converting the *o*-quinone into a mono-acetal (15) and then hydrolysing the ester group under mild conditions with 0.5M aq. K_2CO_3, followed by acidification. Weinreb[23] employed LiOH-H_2O, THF at room temperature for this purpose.



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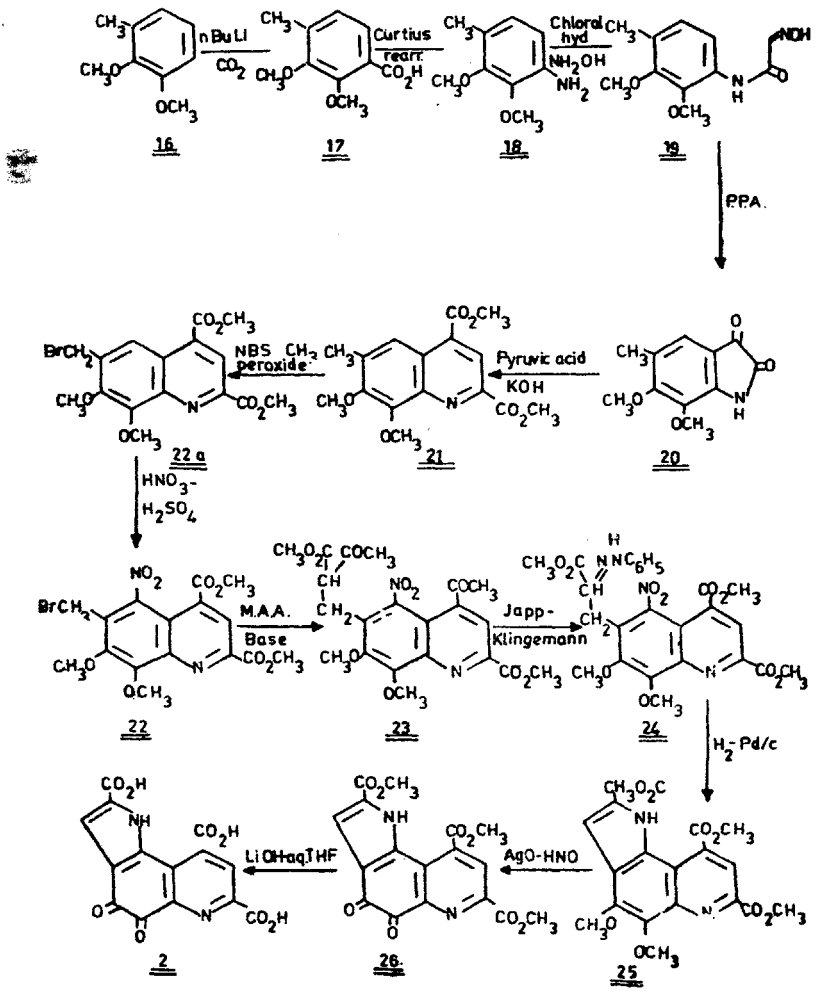
VII. Synthesis of Methoxatin

(*i*) *Gainor and Weinreb's Synthesis*[23]

2,3-dimethoxytoluene (16) was carboxylated using *n*-butyllithium and carbon dioxide to give the benzoic acid derivative (17). A curtius rearrangement then converted the carboxylic acid into the amine (18), which has not been described in the literature earlier. The quinoline ring was built up first by these workers using the Sandmeyer isatin synthesis followed by a Pfitzinger quinoline synthesis. Thus the amine (18) was first converted into the oxime (19) using chloral hydrate, and hydroxylamine. The oxime (19) was cyclised using polyphosphoric acid and isatin (20) obtained was reacted with pyruvic acid and alkali (Pfitzinger synthesis). The quinoline dicarboxylic acid formed was not isolated but converted into the dimethyl ester (21) with methanol and conc. sulphuric acid.

To construct the third ring of methoxatin, a sequence using the Reissert reaction was planned. As this did not succeed, the "Umpolung" variation was developed. Compound (21) was treated with N-bromosuccinimide (N.B.S.) and dibenzoyl peroxide. The benzyl bromide thus obtained was nitrated to give compound (22). This readily condensed with methyl acetoacetate in the presence of base and compound (23) resulted. Kozikowski's modification of Japp-Klingemann reaction led to the phenylhydrazone (24). Attempted catalytic reduction of the nitro group fortunately led to the formation of the pyrrolering. The tricyclic compound (25) was thus obtained. Oxidation of the catechol dimethyl ether (25) with AgO in the presence of nitric acid and THF furnished the corresponding *o*-quinone (26). This



Note: Between structures (26) & (25), the compound may be read as $\xleftarrow[THF]{Ago\text{-}HNO_3}$.

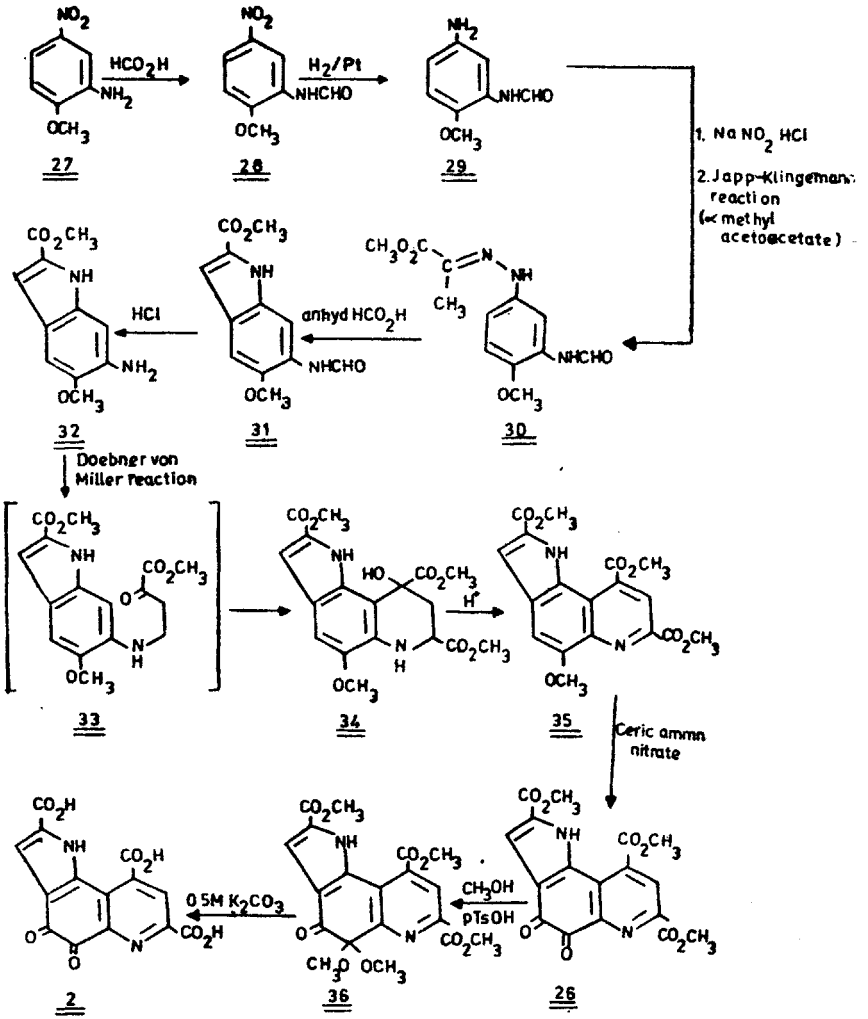
on saponification with lithium hydroxide in aqueous THF afforded the final product methoxatin (2).

(*ii*) *Corey and Tramontano's Synthesis*[24]

2-methoxy-5-nitroaniline (27) was formylated and the formyl derivative (28) catalytically reduced to the amino formanilide (29). This was then diazotised and the diazonium salt used in a Japp-Klingemann reaction with α-methylacetoacetate, when the phenylhydrazone (30) was obtained. It may be noted the acetyl group of the β-keto ester was lost in this step involving ann acyl fission. Hydrazone (30) when heated with anhydrous formic acid underwent Fischer Indole Synthesis and Indole (31) formed contained the required pyrrole ring.

Indole (31) was then deformylated with hydrochloric acid to give the amino-indole (32) which was reacted with dimethyl 2-oxo-glutaconate (Doebner-von Miller

annulation). The intermediate (33) cyclised to the piperidinol (34) which readily lost a molecule of water and aromatised to (35) when treated with acid. Ceric ammonium nitrate oxidation of (35) gave the *o*-quinone (26).



Saponification of methoxatin trimethyl ester (26) was carried out by converting it into the monoacetal (36) with methyl orthoformate-CH_3OH and *p*-toluenesulphonic acid. The acetal was then treated with 0.5M potassium carbonate. On acidification with dilute hydrochloric acid, methoxatin (2) was obtained as a deep red solid.

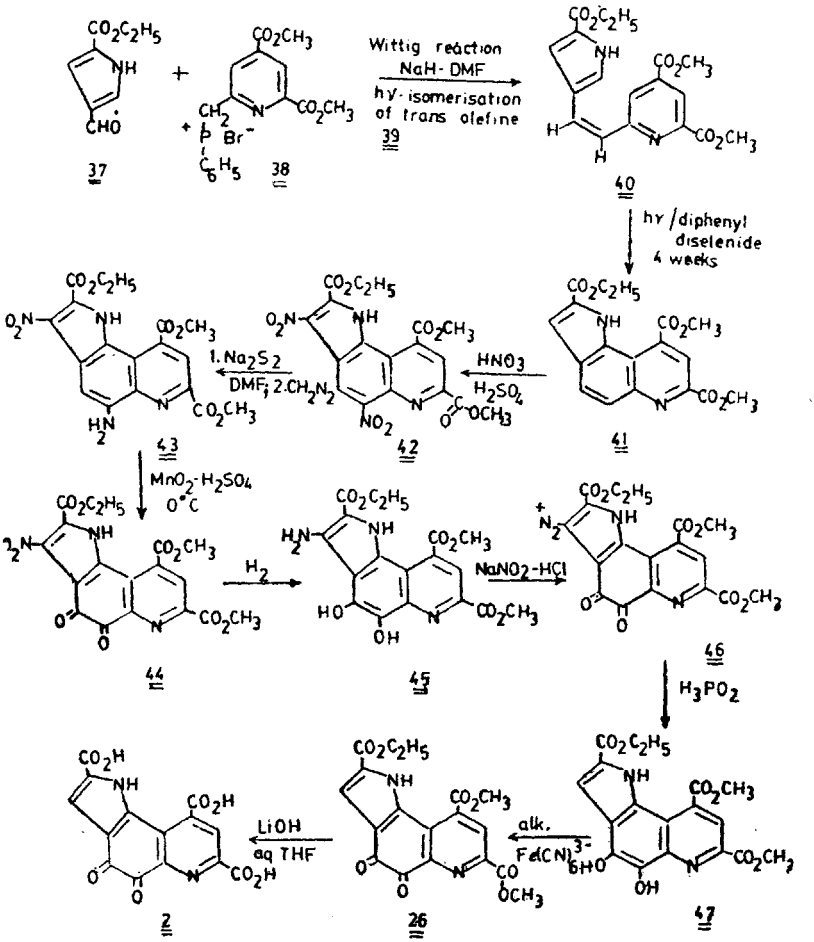
(*iii*) *Hendrickson and de Vries Synthesis*[25]

Wittig reaction of the phosphonium bromide (38) obtained from dimethyl uvitonate with 4-pyrrole aldehyde-2-carboxylic ester (37) in the presence of NaH-

DMF led to the formation of the *trans* olefin (39), *Cis/trans* isomerisation was carried out photochemically to give the *cis* olefin (40).

The carbon-carbon bond formation leading to the tricyclic compound (41) was more difficult to achieve. This photochemical reaction took place in the presence of diphenyl diselenide in 4 weeks time in 40 per cent yield.[30] Photocyclisation of stilbenes are well known but this photocyclisation involving a pyrrole ring in a molecule is unique. The central double bond in (41) (unlike in phenanthrene) did not readily get oxidised to the *o*-quinone.

This was achieved finally by a sequence involving nitration, reduction and oxidation. Thus the dinitro compound (42) was first prepared. Zinin reduction of the dinitro compound[31] with Na_2S_2-DMF gave a monoamine (43) (the nitro group on the pyrrole ring remained unaffected). In this step, the ester group at C-9 was also hydrolysed. This was re-esterified with diazomethane. MnO_2-H_2SO_4 oxidation of compound (43) yielded the *o*-quinone (44).



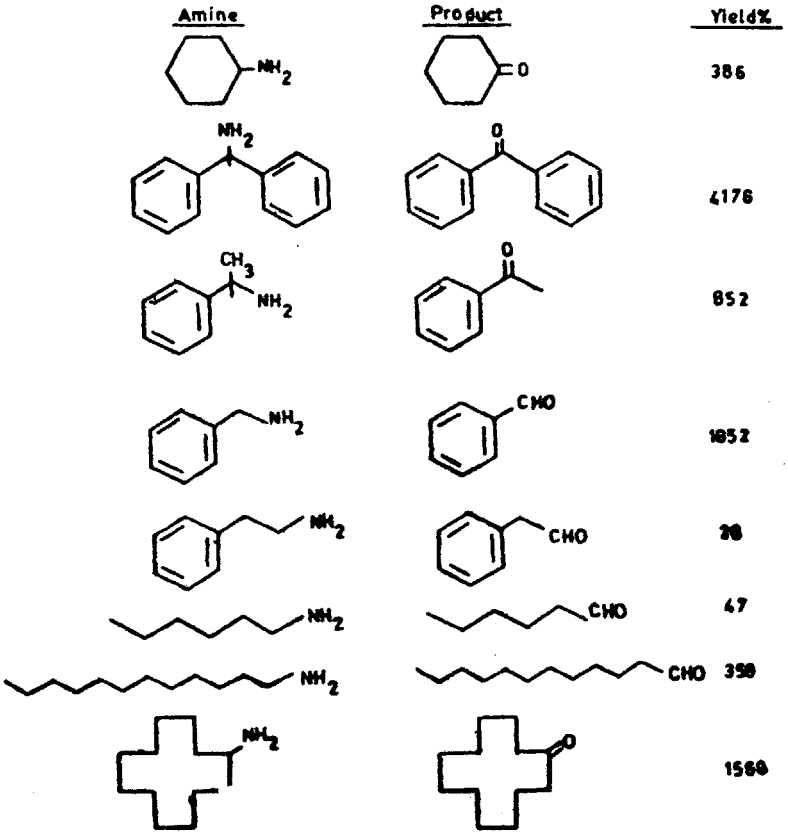
The nitro group was reduced to the amine (the quinone group was also reduced) and the quinol (45) resulted. Diazotisation ($NaNO_2$-HCl) gave the diazoquinone (46). The diazonium group was replaced by hydrogen using phosphorous acid. The quinone group was once again reduced in the process and quinol (47) was obtained. The quinone group was regenerated by washing with alkaline ferricyanide solution and methoxatin trimethyl ester (26) was obtained.

Methoxatin (2) was then obtained by hydrolysis of methoxatin trimethyl ester (26) by Gainor and Weinreb's procedure using lithium hydroxide and aqueous THF.

Biomimetic Reactions Using P.Q.Q.

Recently, using 0.05 N Na_2CO_3 (12hr, room temperature.)[32] P.Q.Q. was generated '*in situ*' from its trimethyl ester, synthesised by Corey's method (*loc. cit.*). They further showed that micelles (only cationic) like hexadecyl trimethyl ammonium bromide (CTAB) drastically enhanced the oxidation of amines e.g. cyclohexylamine was oxidised by P.Q.Q. in the presence of air to cyclohexanone in 22 per cent yield only but the yield increased to 386 per cent when the reaction was carried out in the presence of CTAB (2×10^{-2} M). The reaction rate was very highly *p*H-depen-

Oxidation of Amines with P.Q.Q. & CTAB



dent. At *p*H 7–8 the yield was the highest (778 per cent) and at *p*H 12 it dropped to 152 per cent but at *p*H 1.6 it was completely depressed. The reactions of many other amines were also similarly facilitated by P.Q.Q. and CTAB. These are shown below.

Conclusion

The tricyclic, pyrroloquinolinequinone (P.Q.Q.) or methoxatin occurs in a number of bacterial dehydrogenases. The *o*-quinone nature of this novel coenzyme was first conclusively shown by its ESR spectrum and chemical structure indicated by X-ray diffraction study of an acetone adduct. These studies were quickly followed by three elegant chemical syntheses of methoxatin which helped not only confirm its structure but also provided larger amounts for biochemical studies. P.Q.Q. has a very high redox potential and P.Q.Q. containing enzymes readily oxidise molecules like methanol and thus represent a third class of dehydrogenases, apart from the well-known NAD (P) and flavin-dependent ones. Recently, it has been shown to be present in a mammalian enzyme. Many interesting questions are thus raised. Can it be made by mammals? Is it an unsuspected vitamin? .In any case research on the chemistry, biosynthesis of P.Q.Q. and the distribution and biochemistry of P.Q.Q. containing enzymes appears to be an interesting and fertile field for the immediate future.

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