Biosynthesis of Pyrroloquinoline Quinone. 1. Identification of Biosynthetic Precursors Using ^{13}C Labeling and NMR Spectroscopy

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Pyrroloquinoline quinone (PQQ, 2,7,9-tricarboxy-1*H*-pyrrolo-[2,3-*f*]quinoline-4,5-dione) was first recognized as a cofactor for the pyridine nucleotide-independent methanol dehydrogenase in methylotrophic bacteria.[1] Quinoproteins represent a novel class of dehydrogenases distinct from the well-known pyridine nucleotide and flavoprotein dehydrogenases.[2] The quinoproteins and their coenzyme PQQ are now recognized to be widely distributed in nature, including in mammals.[3] However, nothing is known about the biosynthetic origin of PQQ. We have used ^{13}C labeling and NMR spectroscopy to probe the biosynthesis of PQQ in the methylotrophic bacterium *Methylobacterium* AM1.

Methylobacterium AM1 (*Pseudomonas* AM1, ATCC 14718) was cultured in a standard mineral medium[4] with methanol (0.5%) or ethanol (0.5%) as the carbon source. PQQ was isolated from the clarified culture broth in three chromatographic steps[5] and analyzed for ^{13}C enrichment by NMR spectroscopy (Bruker AM-200 WB or AF-250). The yield of PQQ was typically 1 mg/L of culture broth. Amino acids were obtained from protein hydrolysates[6] and separated by ion exchange chromatography.[7] The one-to-one assignments (Table I) for the 14 ^{13}C NMR signals from natural abundance PQQ (Figure 1A) were determined from the ^1H–^{13}C coupling patterns ($^1J_{CH}$ and $^3J_{CH}$) and carbon–carbon correlations. These data agree with the partial assignments made by Duine and co-workers.[8] Carbon–carbon couplings were observed with a sample of [U-^{13}C]PQQ (90+ %^{13}C) isolated from cultures grown on [^{13}C]methanol (99.7%). The complete assignment was achieved by selecting for one-bond ^{13}C coupling interactions ($^1J_{C\text{-}C}$ = 55 Hz) in ^{13}C COSY experiments.[9]

During growth on methanol, *Methylobacterium* AM1 derives essentially all of its carbon from the methanol;[10] therefore, it is difficult to extract information pertinent to the biosynthesis of PQQ from experiments with use of [^{13}C]methanol as a precursor. A more useful labeled precursor is ethanol because, as described below, one can determine which carbons in PQQ are derived from C-1 and/or C-2 of ethanol. The ^{13}C enrichments (Table II) of alanine, aspartate, and glutamate derived from [1-^{13}C]ethanol are consistent with published radiolabeling data[11,12] and are characteristic of organisms that have an incomplete TCA cycle.[13]

Table I. Chemical Shift Assignments and ^{13}C Enrichments of PQQ

carbon	δ,[a] ppm	$^1J_{CC}$, Hz ($^1J_{CH}$, Hz)	^{13}C enrichments[b] (atom % ^{13}C) from: [1-^{13}C]-ethanol	[2-^{13}C]-ethanol
1a	136.7	61, 62	16	54
2	127.6	65, 87	23	68
2′	161.3	87	82	24
3	113.8	61, 63 (178)	16	65
3a	123.4	60, 63	16	59
4	173.4	58, 61	13	59
5	179.2	60	27	46
5a	148.1	59	54	27
7	146.5	80, 59	16	64
7′	165.4	80	59	24
8	130.3	57, 58 (170)	17	61
9	142.2	58, 59, 65	n.o.	76
9′	167.2	65	101	n.o.
9a	126.1	56	35	37

[a] Chemical shifts of a natural abundance sample (17.2 mg/mL in DMSO-d_6 at 25 °C) of PQQ obtained from Fluka Chemical Co. ^{13}C NMR spectra were obtained at 25 °C with a 45° pulse and with the ^1H decoupler gated off for 10 s to minimize NOE effects. Biosynthetic samples of PQQ contained 10 mg/3.0 mL of DMSO. [b] ^{13}C Enrichments were determined from the relative integrals of ^{13}C NMR resonances which were obtained by Lorentzian line shape analysis and normalized to the enrichment at C-8 which was determined by ^1H NMR analysis to be 12% from [1-^{13}C]ethanol and 61% from [2-^{13}C]-ethanol. Data were not corrected for partial T_1 saturation effects; the enrichment of C-9′ is overestimated for this reason. [1-^{13}C]- and [2-^{13}C]ethanol were 92 and 98 atom% ^{13}C, respectively; n.o. denotes resonance not observed above noise.

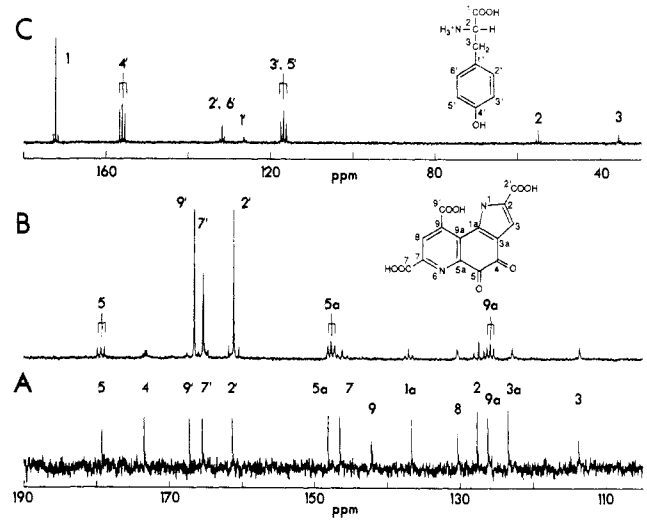

Figure 1. Proton-decoupled ^{13}C NMR spectra of (A) a natural abundance sample of PQQ, (B) PQQ derived from [1-^{13}C]ethanol, and (C) tyrosine (10 mg in 3 mL of deuterium oxide, pH 1.0) derived from [1-^{13}C] ethanol. Spectra were obtained with parameters described in the legend of Table I.

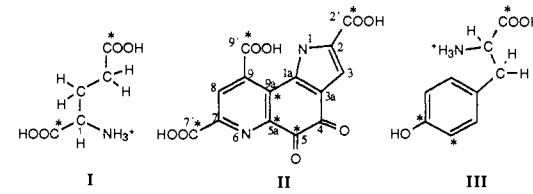

Figure 2. Biosynthetic precursors of PQQ. The primary sites in glutamate (I), PQQ (II), and tyrosine (III) labeled by [1-^{13}C]ethanol are marked (*).

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Table II. Labeling of Amino Acids in *Methylobacterium* AM1 by [1-^{13}C]Ethanol

amino acid	^{13}C enrichments from [1-^{13}C]ethanol[a] (atom % ^{13}C)				
	C-1	C-2	C-3	C-4	C-5
alanine	58	14	15		
aspartate	68	17	17	68	
glutamate	52	13	13	2	72

amino acid	^{13}C enrichments from [1-^{13}C]ethanol[a] (atom % ^{13}C)						
	C-1	C-2	C-3	C-1′	C-2′ (C-6′)	C-3′ (C-5′)	C-4′
tyrosine	73	17	17	17	18 (18)	61 (17)	61

[a] [2-^{13}C]Ethanol labeled the alternate positions with the exception that C-3′ (C-5′) of tyrosine was labeled by both C-1 and C-2 of ethanol. ^{13}C NMR spectra were obtained with the ^{1}H decoupler gated off (60–300 s, 45° pulse). ^{13}C Enrichments were determined as in Table I and normalized to the enrichment at the α-carbons as determined by ^{1}H NMR.

[1-^{13}C]Ethanol labels the phenol ring of tyrosine at C-3′ and C-4′ yielding an NMR spectrum that exhibits $^{1}J_{C-C}$ coupling (Figure 1C); this labeling pattern is identical with that observed in tyrosine isolated from *E. coli* cultured on [1-^{13}C]lactate.[14] The adjacent labeling of C-3′ and C-4′ of tyrosine is diagnostic of compounds that arise from the shikimate pathway.

The ^{13}C NMR spectrum of PQQ isolated from *Methylobacterium* AM1 cultures containing [1-^{13}C]ethanol is shown in Figure 1A; the relative peak intensities are a clear indication that incubation with [1-^{13}C]ethanol selectively labels PQQ. The ^{13}C enrichments in PQQ based on analysis of these NMR intensities are summarized in Table I. C-1 of ethanol labels predominantly the three carboxylates (C-2′, -7′, and -9′) and carbons 5, 5a, and 9a. Obviously, the biosynthesis of PQQ does not involve the "head-to-tail" joining of acetate units characteristic of fatty acids or polyketides.[15] The predominantly singlet character of the carboxylates indicates that they are incorporated into positions in which their neighbors arise from C-2 of ethanol. Carbons 5, 5a, and 9a each yield three resonances which are the combination of a singlet from singly labeled species and doublet ($^{1}J_{C-C}$ = 60 Hz) from species labeled at C-5 and C-5a or C-9a and C-5a. The [1-^{13}C]ethanol labeling experiment coupled with the obvious structural homologies provide a working hypothesis for the biosynthetic origins of PQQ (Figure 2). We propose that glutamate provides N-6 and carbons 7′, 7, 8, 9, and 9′, while the remaining nine carbons and N-1 are donated by an amino acid from the shikimate pathway.

The precursors were identified by comparing the selective ^{13}C-labeling patterns in PQQ with those observed in amino acids. In PQQ, C-1 of ethanol significantly labels C-7′ (59%) and C-9′ (>99%) but not C-9 (<2%); similarly, C-2 of ethanol labels PQQ at C-7 (64%), C-8 (61%), and C-9 (76%) but not C-9′. These labeling patterns are essentially identical with those observed in glutamate (Table II). The incorporation of C-1 of ethanol into C-2, 5, 5a, and 9 of PQQ is equivalent to its incorporation into C-1, 3′, and 4′ of tyrosine. The adjacent labeling evident from the high degree of ^{13}C coupling at C-4′ and C-3′ in tyrosine is also observed in the orthoquinone-containing ring in PQQ. Tyrosine C-3′ and C-5′ are biosynthetically inequivalent because the aromatic ring is a product of asymmetric synthesis via the shikimate pathway;[16] C-3′ arises from ethanol C-1, whereas C-5′ arises from ethanol C-2. PQQ derived from [1-^{13}C]ethanol has adjacent ^{13}C labeling (doublets) at C-5a and C-5 or C-5a and C-9a. This labeling implies that the orthoquinone-containing ring arises from a symmetric compound (C_2 axis through C-1′ and C-4′) and predicts that C-5 and C-9a will be labeled equivalently and to an intermediate extent by both C-1 and C-2 of ethanol. Indeed, [2-^{13}C]ethanol labels C-5 and C-9a but not C-5a. This symmetric labeling pattern rules out indole as a precursor for that portion of PQQ containing the orthoquinone and pyrrole rings.

As demonstrated by Gould and co-workers,[17] the quinoline system of streptonigrin is biosynthesized by condensing three carbons of D-erythrose with 4-aminoanthranilate, a novel product of the shikimate pathway. Our data indicate that the quinoline portion of PQQ is formed by a novel condensation of N-1, C-2, -3, and -4 of glutamate with a symmetrical six-carbon ring derived from the shikimate pathway. It is most likely that tyrosine is the shikimate-derived precursor, since the pyrrole could be formed by the internal cyclization of the amino acid backbone. This is analogous to the cyclization of dopaquinone to form dopachrome.[18] Dopaquinone is a product of the oxidation of tyrosine (via dopa) in reactions catalyzed by monophenol monooxygenase (EC 1.14.18.1).

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Synthesis of a Remarkably Stable Bicyclo[7.3.1]diynene Esperamicin A_1/Calicheamicin γ System. Structural Requirements for Facile Formation of a 1,4-Diyl

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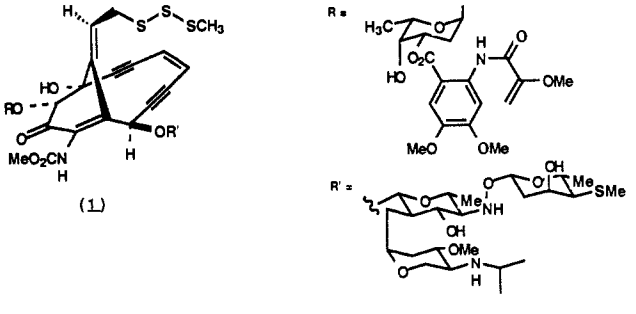
In our previous paper which described a model for the proposed mechanism of action of the potent antitumor agents esperamicin A_2/calicheamicin γ_1 **1**[1] we showed that oxidative decomplexation



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