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The Biosynthesis of Ubiquinone: Synthesis and Enzymatic Modification of Biosynthetic Precursors

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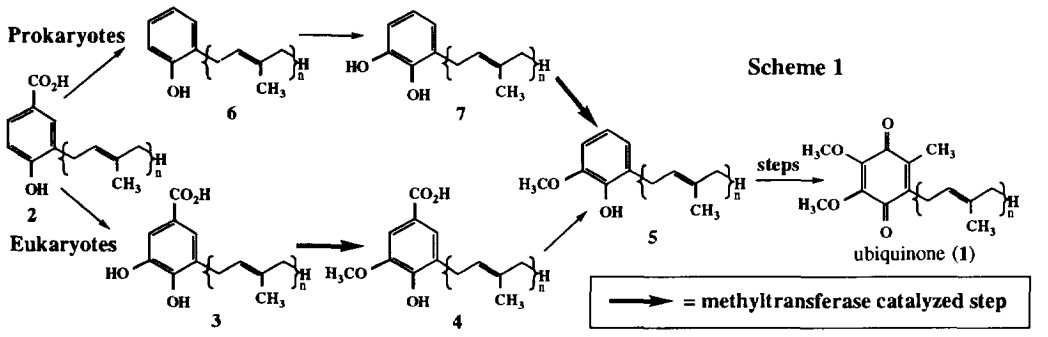
Abstract: The synthesis of key intermediates in the eukaryotic biosynthetic pathway of ubiquinone (Q) and the biotransformation of these materials with yeast (*S. cerevisiae*) mitochondria are described. The synthesis of Q aromatic precursors **2**, **3**, and **4** (n = 3 in all cases) relies on the palladium (0) catalyzed coupling of farnesyl tributylstannane with suitably functionalized aryl halides. Preliminary experiments show that incubation of synthetic substrate **3** with mitochondria from yeast containing the 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase gene (*COQ3*) and S-[*methyl*-^{3}H]adenosyl-L-methionine yields radiolabeled **4**.

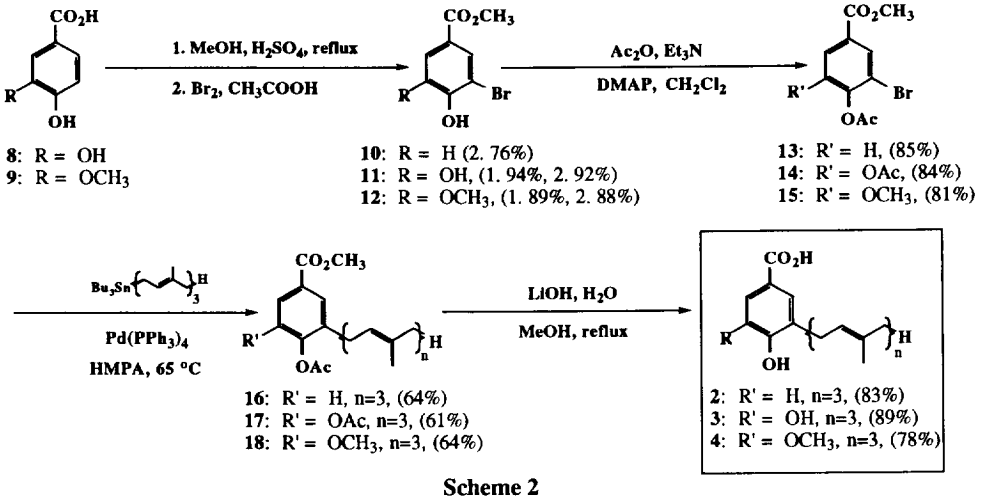
Ubiquinone (**1**) (Q) is an essential component in the mitochondrial respiratory chain of eukaryotes and in the plasma membrane of many prokaryotes.[1] In addition, Q is present in other intracellular membranes and in human low density lipoprotein.[2] More recently, the hydroquinone form of Q (QH_2) has been implicated as a defense mechanism against oxidative stress.[3] Despite these important roles played by Q/QH_2, the enzymes that catalyze their biosynthesis are not fully characterized.[4] Shown in Scheme 1 are the proposed early intermediates in the biosynthesis of Q. In both prokaryotes and eukaryotes, the elements of the biosynthesis are similar, differing only in the order of steps. In eukaryotes, 4-hydroxy-3-polyprenylbenzoic acid (**2**) is oxidized and methylated prior to decarboxylation. In contrast, the prokaryotic pathway proceeds via initial decarboxylation of **2** followed by oxidation and methylation. Each pathway to Q contains three transformations promoted by methyltransferases, two resulting in formation of C-O bonds and one leading to a C-C bond. In this letter we describe the synthesis of key intermediates in the eukaryotic biosynthetic pathway of Q and show that these materials serve as methyl-acceptor substrates in *in vitro* assays with mitochondria isolated from the yeast, *S. cerevisiae*.



To investigate the first methyltransferase-catalyzed step in the eukaryotic biosynthetic pathway, compounds **3** and **4** (Scheme 1), were our first synthetic objectives. Substrates for the methyltransferase-catalyzed steps in the biosynthesis of Q were prepared via a Pd(0) promoted coupling of the prenyl side chain to suitably functionalized aromatic head groups. The required 3-bromobenzoic acids **11** and **12** were synthesized via standard procedures from commercially available materials (Scheme 2).[5] The bromoesters were obtained as crystalline solids after aqueous work-up and concentration.[6] Acylation of the phenols then set the stage for the palladium promoted coupling of the prenyl side chain.

Between species, and even within species, there is a wide range in the length of the prenyl tail. This evidence suggests that the length of the prenyl tail is not a major factor in the recognition of substrate during the methyl transfer reaction.[7] Since farnesyl bromide is commercially available, we chose to convert this material to its tributylstannyl analog (Bu_3SnLi, THF, -78°C) to access precursors of Q(3) (**1**, n = 3).

Under conditions described by Stille and co-workers, treatment of farnesyl tributylstannane and the aryl bromides with $Pd(Ph_3P)_4$ in HMPA at 65°C furnished the desired farnesylated benzoates **17** and **18** in good yields (see Scheme 2).[8] Exhaustive hydrolysis of the methyl ester and acetates furnished the putative precursors to Q_3 **3** (n=3)[9] and **4** (n=3). The preparation of **2** (n=3) was also achieved using the same synthetic stategy. These air and light sensitive materials could be purified via flash chromatography (silica gel, 1:1 hexanes:ethyl acetate), but were routinely used in their crude forms.



Scheme 2

We chose to study the eukaryotic biosynthesis of Q using yeast (*S. cerevisiae* JM45) as the model system.[10] Yeast *coq3* mutants are unable to synthesize Q and instead accumulate 3,4-dihydroxy-5-hexaprenylbenzoate **3** (n = 6) (DHHB).[11] Transformation of yeast *coq3* mutants with plasmids containing the yeast *COQ3* gene restores both respiration and the synthesis of Q.[10] The amino acid sequence predicted by the yeast *COQ3* gene contains a consensus sequence for binding of the methyl donor S-adenosylmethionine.[10] These findings suggest that the *COQ3* gene encodes the dihydroxyhexaprenylbenzoate (DHHB) methyltransferase, since the mutant is unable to carry out this methyl transfer. To test this hypothesis and determine the viability of **3** as a substrate for the DHHB methyltransferase, a series of *in vitro* experiments with yeast mitochondrial extracts was conducted.[12]

Extracts from four yeast strains were used for these assays: 1.) a respiratory competent strain (JM45), 2.) the *coq3* deletion mutant harboring the plasmid vector as a control (JM45Δ*coq3*:pQM), 3.) a

rescued mutant with a single copy plasmid encoding *COQ3* driven by the *CYC1* promoter (JM45Δ*coq*3:pAH-3), and 4.) a rescued mutant with a multicopy plasmid encoding *COQ3* driven by the *CYC1* promoter (JM45Δ*coq*3:pCH-3). In all cases, the methyl donor was S-[*methyl*-^{3}H]adenosyl-L-methionine (SAM). Mitochondria were incubated with SAM and Q precursor **3** for 1 h at 30°C.[13] After quenching with acetic acid, the mixtures were extracted with chloroform. The organic extracts were then concentrated, resuspended in methanol, and analyzed by reverse-phase HPLC (C_{18}, 9:1 MeOH:Water). Under these conditions SAM, **3**, and **4** elute at 4.0, 7.0, and 8.5 min, respectively. Incorporation of the radiolabeled methyl group in **4** was quantitated by scintillation counting of the HPLC fractions. Extracts from mitochondria isolated from respiration-competent wild-type yeast, showed a high level of radioactivity that co-eluted with **4**, confirming the presence of **4** in the assay sample (Panel A, Figure 1). When mitochondria prepared from the *coq3* deletion mutant yeast were incubated with **3** and SAM, only residual SAM was observed in the HPLC analysis (Panel B, Figure 1). Transformation of the *coq*3 deletion mutant yeast with either single copy (Panel C) or multi-copy plasmids (Panel D), harboring the *COQ3* gene, restored the ability to generate the methylated product. Assays in which either mitochondria or substrate (compound **3**) was omitted showed no methyltransferase activity.

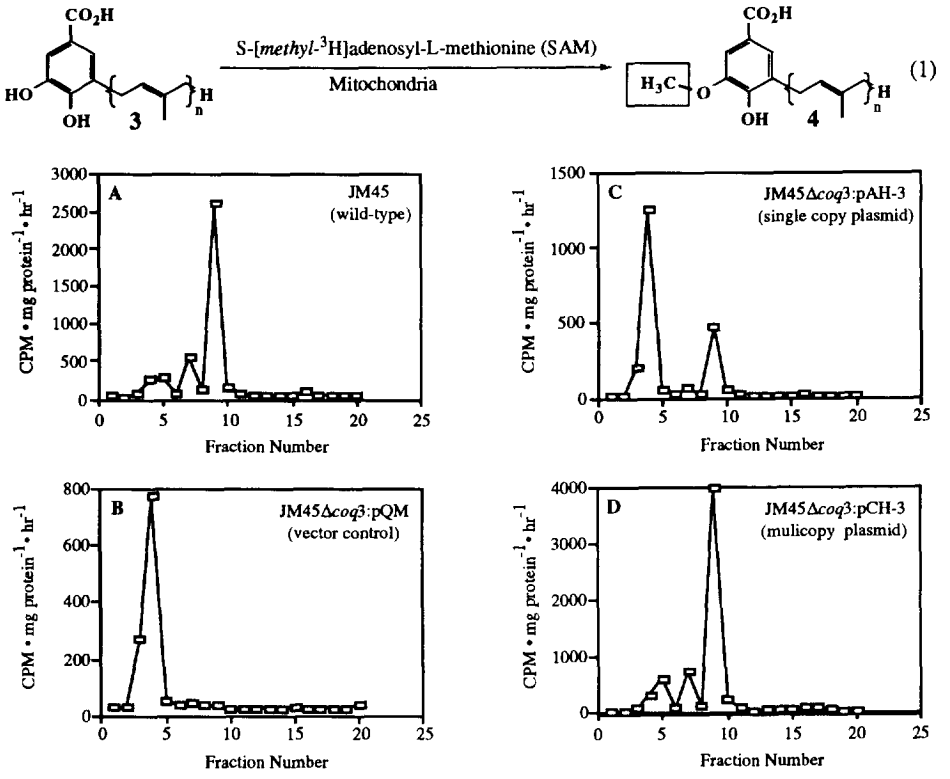


Figure 1. HPLC Analysis of Mitochondrial Extracts. Isocratic reversed-phase HPLC analysis was carried out over C18 stationary phase with 9:1 methanol:water as eluent. Fractions were collected at 1 min intervals at a flow rate of 1 mL•min^{-1}. Panel A: JM45 (wild-type yeast). Panel B: JM45Δ*coq*3:pQM (vector control). Panel C: JM45Δ*coq*3:pAH-3 (single copy *coq*3 plasmid). Panel D: JM45Δ*coq*3:pCH-3 (multicopy *coq*3 plasmid).

These findings show that the yeast *COQ3* gene encodes a methyltransferase which catalyzes the methyl transfer from S-[*methyl*-^{3}H]adenosyl-L-methionine to the substrate 3,4-dihydroxy-5-farnesylbenzoic acid (**3**), to form the methylated product, 3-methoxy-4-hydroxyl-5-benzoic acid (**4**). The native substrate for this enzyme possesses six prenyl units in its side chain. Thus, these results indicate that the length of the prenyl tail does not play a significant role in recognition of substrate. The extent to which the prenyl chain can be modified and the substitution on the aromatic moiety can be manipulated are currently under investigation.[14]

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9 Data for **3**: ^{1}H NMR ($CDCl_3$) δ: 1.59 (s, 6H); 1.66 (s, 3H); 1.77 (s, 3H); 2.10 (m, 8H); 3.39 (d, 2H, *J*= 6.9 Hz); 5.08 (m, 2H); 5.33 (m, 1H); 7.49 (br s, 2H); acid and phenolic protons not observed. ^{13}C NMR ($CDCl_3$, 90 MHz) δ: 16.03, 16.24, 17.67, 25.68, 26.38, 26.88, 29.15, 39.68 (2 carbons), 114.91, 121.02, 121.07, 123.68, 124.31, 124.36, 127.28, 131.36, 135.58, 138.58, 143.36, 147.60, 171.22. HRMS (EI) $C_{22}H_{30}O_4$ calculated 358.2144, found 358.2147.

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12 Details of the *in vitro* assays will be disclosed in a following paper: Poon, W.; Shepherd, J. A.; Clarke, C. F.; Myles, D. C. manuscript in preparation.

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14 Shepherd, J. A.; Poon, W.; Myles, D. C.; Clarke, C. F. unpublished results.

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