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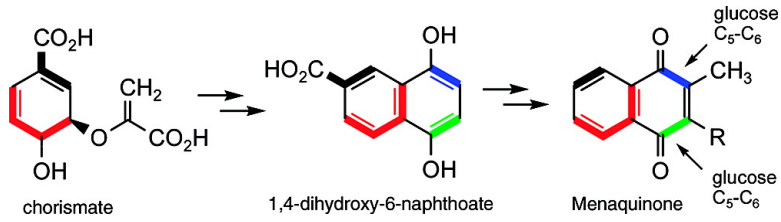
J. Am. Chem. Soc., **2008**, 130 (17), 5614-5615 • DOI: 10.1021/ja710207s • Publication Date (Web): 08 April 2008



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Published on Web 04/08/2008

Studies on A New Biosynthetic Pathway for Menaquinone

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Ubiquinone and menaquinone (MK) (Figure 1) are lipid-soluble molecules that shuttle electrons between the membrane-bound protein complexes in the electron transport chain.[1] In prokaryotes, the electron transport chain utilizes MK,[2] and thus, its biosynthesis is essential for survival of *Bacillus subtilis*.

The biosynthesis of MK was mainly studied in *Escherichia coli*.[2] Chorismate, derived from phosphoenolpyruvate (PEP) and D-erythrose-4-phosphate via the shikimate pathway, is initially converted into isochorismate and finally to MK through *o*-succinylbenzoate and 1,4-dihydroxy-2-naphthoate (Figure 1, path A). These reactions are catalyzed by proteins MenA to MenG.

In 2005, however, Borodina et al.[3] reported the absence of the known menaquinone biosynthetic pathway genes (*men*B to *men*F) in *Streptomyces coelicolor* A3(2)[4] and *Streptomyces avermitilis*[5] by analyzing the entire genomes of these organisms, suggesting the operation of a novel pathway for the biosynthesis of MK in the genus Streptomycetes. Interestingly, some pathogenic microorganisms such as *Helicobacter pylori* and *Campylobacter jejuni* that are known to cause gastric carcinoma[6] and diarrhea,[7] respectively, also lacked these *men* gene homologues, though they were reported to synthesize MK.[8,9]

In order to reveal this new biosynthetic pathway for MK, we started ^{13}C-labeling experiments by using *Streptomyces* sp. CL190 since we had utilized this organism[10] to reveal the origin of IPP (isopentenyl diphosphate) that is the precursor of the prenyl side chain of MK [through the mevalonate pathway or the MEP pathway (nonmevalonate pathway)] and had observed an unusual incorporation pattern of [U−^{13}C$_6$]glucose into the naphthoquinone moiety of MK that could not be explained by the known MK biosynthetic pathway.

At the onset of the studies, we established unequivocal assignment of the naphthoquinone moiety of MK with a highly symmetric structure, as shown in Figure 2, by HSQC and CT-HMBC[11] experiments (Figures S1 and S2), the latter enabled us to analyze very closely spaced signals by giving sharp cross peaks.

Since distinction between C-4a and C-8a (δ 132.20 and 132.16) and C-6 and C-7 (δ 133.33 and 133.27) could not be made by 2D NMR techniques due to insufficient separation of these signals, they were assigned based on ^{13}C−^{13}C couplings observed with MK labeled with [U−^{13}C$_6$]glucose (Figure S18) and incorporation pattern of [5-^{13}C]glucose and [6-^{13}C]glucose.

With the established assignment in hand, we analyzed ^{13}C-labeled MKs that were prepared and purified as described

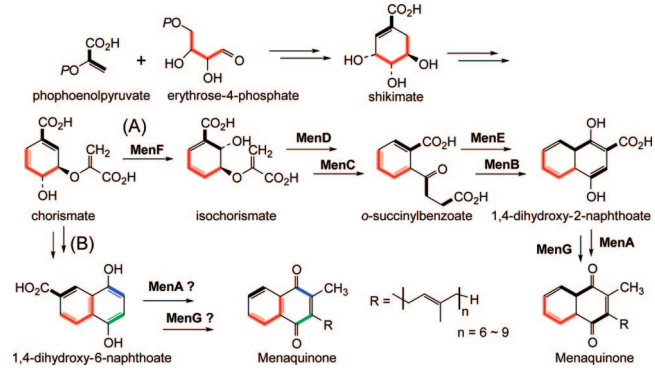

Figure 1. Menaquinone biosynthetic pathways. (A) Known pathway, (B) new pathway. Green and blue bold lines indicate two carbon units derived from C-5 and C-6 of glucose via different metabolic pathways.

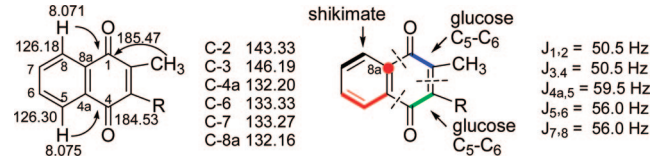

Figure 2. NMR assignment of menaquinone and its incorporation pattern from [U−^{13}C$_6$]glucose.

before.[10] The ^{13}C NMR spectrum of MK labeled with [U−^{13}C$_6$]glucose (incorporation yield 3−4%, Figure S15) showed the ^{13}C−^{13}C couplings as shown in Figure 2. This labeling pattern can be explained by incorporation of [U−^{13}C$_6$]glucose into MK through the shikimate pathway by condensation of phosphoenolpyruvate (PEP) (into C-7 and C-8) and of erythrose-4-phosphate (into C-8a, -4a, -5, and -6) (Figure 1).

The unusual singlet pattern of C-8a (Figure S18) can be explained by assuming a metabolism of glucose through glycolysis and the pentose phosphate cycle to erythrose-4-phosphate ([U−^{13}C$_6$]glucose → [U−^{13}C$_3$]dihydroxyacetone phosphate → [1,2,3-^{13}C$_3$]glucose → [1-^{13}C]erythrose). Incorporation of this singly labeled erythrose would cause labeling of C-8a of MK. Similar metabolism of glucose was reported in the biosyntheses of axenomycin,[12] orsellides,[13] quinolactacin A,[14] and polyketomycin.[15]

In order to obtain more information on the precursors, we carried out labeling experiments using glucoses labeled with ^{13}C at different positions. The incorporation ratio of the precursors into MK is summarized in Table 1. Specific incorporation of [3-^{13}C], [4-^{13}C], [5-^{13}C], and [6-^{13}C]glucoses into C-8a, -4a, -5, and -6, respectively (indicated by bold letters

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10.1021/ja710207s CCC: $40.75

Table 1. Incorporation of ^{13}C-Labeled Glucose (GL)[a] into MK by *Streptomyces* sp. CL190

	carbon									
precursor	1	2	3	4	4a	5	6	7	8	8a
[3-^{13}C]GL	1.3	2.2	2.9	2.1	3.2	2.4	3.1	3.0	1.0	**8.0**
[4-^{13}C]GL	1.0	2.1	2.7	1.1	**6.6**	3.3	2.7	2.6	1.5	3.1
[5-^{13}C]GL	*b*			**5.9**		**4.9**		**4.5**	1.0	
[6-^{13}C]GL	1.0	**4.9**	**15.4**	1.0	1.4	2.0	**7.7**	2.8	**6.0**	1.7

[a] Signal intensities were normalized to that of the methyl signal at C-2, and the intensity of the weakest signal was set to 1.0. [b] Due to poor production yield, peak intensities of the remaining signals could not be determined.

in Table 1, Figure S13), is compatible with incorporation of glucose into MK, via the shikimate pathway. Enrichments of C-7 and C-8 by [5-^{13}C]glucose and [6-^{13}C]glucose, respectively, supported the incorporation of these precursors via PEP through the shikimate pathway. These results established the formation mechanism of the aromatic ring of MK from the shikimate pathway.

The remaining problem was to define the precursors of the quinone ring. No specific incorporation was observed with [1,2-$^{13}C_2$]acetate, [2-^{13}C]alanine, and [2-^{13}C]serine into the chromophore moiety of MK (data not shown). An important clue for the origin of the two carbon units (C-1/C-2 and C-3/C-4) was obtained by incorporation of C-5 and C-6 of glucose with retention of the C-5/C-6 bond; high enrichment of C-3 and C-4 may be ascribed to the incorporation of C-3 and C-2 of PEP into these positions. Higher incorporations of [5-^{13}C]glucose and [6-^{13}C]glucose into the unit C-3/C-4 than into the unit C-1/C-2 indicated the different metabolic fate of glucose before incorporation. This result was fortified by the following experiments. When [U$-^{13}C_6$]glucose was added at 0 h, incorporation of the label was considerably suppressed with C-3 but not with C-2 (peak ratio, C-2/C-3 = 1:1 at 16 h, 1:0.3 at 0 h). Addition of 5 equiv of nonlabeled serine into the fermentation medium caused stimulation and suppression of the incorporation [U$-^{13}C_6$]glucose into C-3/C-4 and C-1/C-2, respectively, of MK (Figure S21, enrichment ratio; without serine, C-2 4.2%, C-3 4.0%; with serine C-2 2.2%, C-3 5.0%), while no specific incorporation of [2-^{13}C]serine was observed (data not shown). This result may be explained by inhibition of the metabolic flux from glucose to serine via 3-phosphoglycerate by external serine, thereby driving the metabolism of 3-phosphoglycerate to PEP, causing efficient enrichment of C-3 and C-4 of MK. Similar results were obtained with aspartic acid, glutamic acid, and glyceric acid (Figures S22−S27).

Therefore, the precursors of the quinone ring moiety may be PEP (into C-3/C-4) and a still unknown metabolite originated from C-5 and C-6 of glucose (into C-1/C-2).

Very high ^{13}C incorporation of the C-2 methyl group (12.7% enrichment) confirmed this methyl group's origin from methionine following [$^{13}CH_3$]methionine feeding experiments (Figure S14).

In order to confirm the presence of the novel pathway in *S. coelicolor*, we carried out the labeling experiment using [U$-^{13}C_6$]glucose. The incorporation pattern of the precursor (Figure S19) was completely identical with that obtained with *Streptomyces* sp. CL190 and thus suggested the presence of the novel pathway in *S. coelicolor* (Figure 1, pathway B).

Uneven incorporation of ^{13}C precursors into the upper half and lower half of the naphthoquinone nucleus clearly excluded the possibility of 1,4-naphthoquinone as a biosynthetic intermediate. In view of the known MK biosynthetic pathway, where 1,4-naphthoquinone was converted to MK by prenylation (catalyzed by MenA) followed by methylation (catalyzed by MenG),[16] it was most reasonable to assume that the same reaction sequence takes place in the new biosynthetic pathway since the same prenylation genes (SCO4491 and SAV4810) and methylation genes (SCO4556 and SAV4831) are present in *S. coelicolor* A3(2)[4] and *S. avermitilis*.[5] Thus, we postulated that the intermediate naphthoquinone to be prenylated and methylated in the new pathway was most likely 1,4-naphthoquinone-6-carboxylic acid (Figure 1). This compound was prepared by $Ce(SO_4)_2$ oxidation of naphthalene-2-carboxylic acid (Supporting Information). This synthesized acid enabled the production of MK by an MK auxotroph of *S. coelicolor* and recovered its growth (Supporting Information), suggesting that 1,4-naphthoquinone-6-carboxylic acid is the biosynthetic intermediate of the new MK pathway. On the other hand, 1,4-naphthoquinone-2-carboxylic acid (an intermediate of the known MK biosynthetic pathway) was toxic and did not show any effect.

The evidence described above clearly showed the presence of a new pathway for the biosynthesis of MK (pathway B shown in Figure 1) in *Streptomyces* and presumably in several pathogenic bacteria mentioned above. Attempts to identify intermediates of the new biosynthetic pathway for MK by analyzing several kinds of blocked mutants are underway in our laboratories.

Acknowledgment. This work was supported in part by a Grant-in-Aid for Scientific Research (B) to H.S., and by a Grant-in-Aid for Scientific Research on Priority Areas "Applied Genomics" from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Urakami Foundation, the Skylark Food Science Institute to T.D.

Supporting Information Available: Complete ref 4, NMR analysis of MK, preparation of 1,4-naphthoquinone-6-carboxylic acid, preparation and properties of blocked mutants of *S. coelicolor*, HSQC and CT-HMBC spectra of MK, ^{13}C NMR spectra of MKs labeled with singly labeled ^{13}C glucoses, ^{13}C NMR spectra of MKs labeled with [U$-^{13}C_6$]glucose in the presence of several amino acids. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA710207S