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In vitro reconstitution of the radical SAM enzyme MqnC involved in the biosynthesis of futalosine-derived menaquinone

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Abstract

The radical *S*-adenosylmethionine (SAM) enzyme MqnC catalyzes conversion of dehypoxanthine futalosine (DHFL) to the unique spiro-compound cyclic DHFL in the futalosine pathway for menaquinone biosynthesis. This study describes the *in vitro* reconstitution of [4Fe-4S]-cluster-dependent MqnC activity and identifies the site of hydrogen atom abstraction from DHFL by the adenosyl radical.

Keywords

Menaquinone; futalosine; radical SAM enzyme

Menaquinone (MK, vitamin K_2) is a lipid-soluble molecule that shuttles electrons between membrane-bound protein complexes in the respiratory chain. In the classic *Escherichia coli* pathway, it is biosynthesized from chorismate by eight enzymes (MenA to MenH).[1–3] Recently, an alternative route for menaquinone production was discovered, termed the futalosine pathway (Scheme 1).[4] Chorismate (**1**)-derived futalosine (**2**) is converted to dehypoxanthine futalosine (DHFL, **3**) by futalosine hydrolase MqnB, and **3** is transformed into cyclic dehypoxanthine futalosine (CDHFL, **4**) by the radical SAM enzyme MqnC. MqnD then converts **4** to 1,4-dihydroxy-6-naphthoic acid (**5**). In the later steps of the pathway, based on the annotation and clustering of the open reading frames in *Streptomyces coelicolor* A3(2), it is possible that SCO4491 (prenylation) and SCO4556 (methylation) could be involved in completing the biosynthetic pathway for production of menaquinone (**6**). The early and late steps in the pathway are currently unknown and under investigation. Humans and commensal intestinal bacteria lack this pathway therefore it represents an attractive target for the development of chemotherapeutic compounds.

Enzymes belonging to the radical SAM superfamily are typically characterized by the presence of a cysteine-rich CX_3CX_2C motif that ligates a [4Fe-4S] cluster.[5,6] The iron-sulfur cluster is responsible for the reductive cleavage of SAM, to generate a 5′-deoxyadenosyl (5′-dA) radical and methionine. Amino acid sequence analysis revealed that

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ASSOCIATED CONTENT

Supporting Information

Supporting Information Available: Detailed procedures for cloning the *mqnC* expression construct, MqnC expression and purification, DHFL synthesis, CDHFL isolation, and HPLC and LC-MS methods are available in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Contributions

Lisa E. Cooper was responsible for the biochemical studies. Dmytro Fedoseyenko, Sameh Abdelwahed, and Soong-Hyun Kim synthesized DHFL. Lisa E. Cooper and Tadhg P. Begley designed the experiments and prepared the manuscript.

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MqnC contains this characteristic CX_3CX_2C iron-sulfur cluster motif. Therefore, a recombinant plasmid encoding *mqnC* from *Bacillus halodurans* C-125 was introduced into *E. coli* BL21(DE3)-T1R containing the *E. coli suf* operon for co-expression of His_6-MqnC with iron-sulfur cluster biogenesis proteins.[7] Anaerobic purification by nickel affinity chromatography yielded pure brown protein with 3 irons and 2 sulfides per monomer, suggesting incomplete reconstitution of the cluster. The UV-Vis spectrum of MqnC had an absorbance maximum at approximately 410 nm that disappeared upon exposure of the protein to oxygen (Figure 1A).[8] Electron paramagnetic resonance (EPR) spectra and g-values for MqnC treated with excess sodium dithionite were consistent with a protein-bound $[4Fe\text{-}4S]^{1+}$ cluster (Figure 1B).

Based on gene disruption studies in *S. coelicolor*, MqnC is predicted to catalyze the conversion of **3** to **4**.[4] To test the activity of MqnC, DHFL was synthesized as illustrated in Scheme 2 (experimental methods are described in detail in the supporting information). When MqnC was incubated under anaerobic conditions with sodium dithionite, DHFL, and SAM (**11**) in phosphate buffer, pH 7.5, production of CDHFL and 5′-deoxyadenosine (5′-dAd) was observed by HPLC as confirmed by co-elution with authentic standards (Figure 2A). CDHFL was not observed in control reactions in which sodium dithionite, DHFL, SAM, or MqnC were omitted. Assay and control samples were also analyzed by LC-MS using an Agilent 1200 LC (ChemStation) with a Bruker Daltonics micrOTOF-Q II ESI-Qq-TOF mass spectrometer (HyStar). The observed m/z for cyclic DHFL in negative ion mode was 293.1 as compared to the theoretical exact mass of 293.1 m/z ($[M\text{-}H]^-$, Figure 2C). The authentic standard for CDHFL was purified from the previously described *S. coelicolor* SCO4326-disruptant strain (observed 293.1 m/z).[4] These results confirm the predicted role of MqnC as the DHFL cyclase.

Deuterium labeling of DHFL was used to elucidate the site of hydrogen atom abstraction by the 5′-dA (**13**) radical during MqnC catalysis. DHFL that was site-specifically deuterated at the C4′ position was synthesized and used as a substrate for MqnC. Deuterium transfer from [4-^{2}H]-DHFL to the 5′-dA radical (**13**) was monitored by LC-MS (Figure 3). The data revealed an increase of a single mass unit for 5′-dAd, thus demonstrating that the hydrogen from the C4′ position of DHFL is abstracted by the 5′-dA radical. The low efficiency of label transfer is most likely due to the substantial levels of uncoupled 5′-dAd formation (10:1, Figure S5) as well as a primary isotope effect on the initial hydrogen atom abstraction, which would favor consumption of [4-^{1}H]-DHFL over [4-^{2}H]-DHFL.

A mechanistic proposal for the MqnC catalyzed reaction is outlined in Scheme 3. Reductive cleavage of SAM generates the adenosyl radical **13**. This then abstracts a hydrogen atom from DHFL **3** to give radical **15**. Cyclization gives **16**, which after deprotonation transfers an electron back to the $[4Fe\text{-}4S]^{2+}$ cluster to give CDHFL **4**.

In summary, the activity of the radical SAM enzyme MqnC was successfully reconstituted. EPR studies confirmed that MqnC contains a [4Fe-4S] cluster and isotope labeling studies demonstrated that the C4′ hydrogen atom of DHFL **3** is abstracted by the 5′-deoxyadenosyl radical. A mechanistic proposal for the MqnC-catalyzed reaction is described. Experiments are in progress to test this proposal.

Supplementary Material

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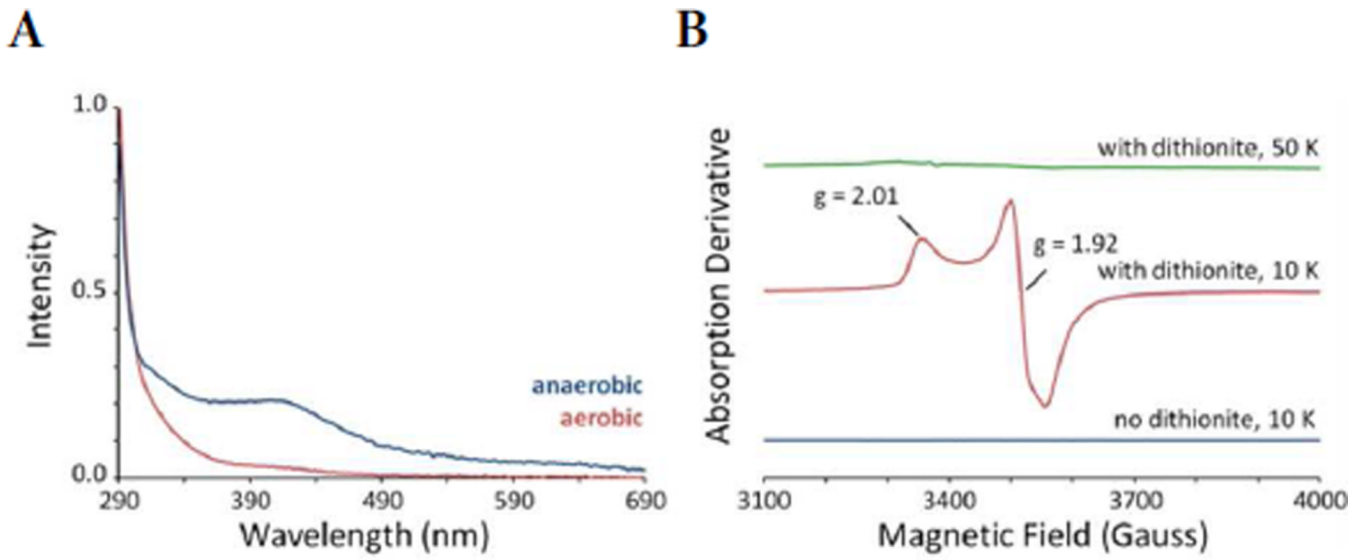

Figure 1.

Spectroscopic properties of MqnC. (A) UV-Vis absorbance of purified Fe/S-reconstituted MqnC (160 μM) in 100 mM phosphate buffer, pH 7.5 before (blue) and after (red) exposure to oxygen. (B) EPR spectra of purified Fe/S-reconstituted MqnC (280 μM) treated with (red and green) or without (blue) dithionite.

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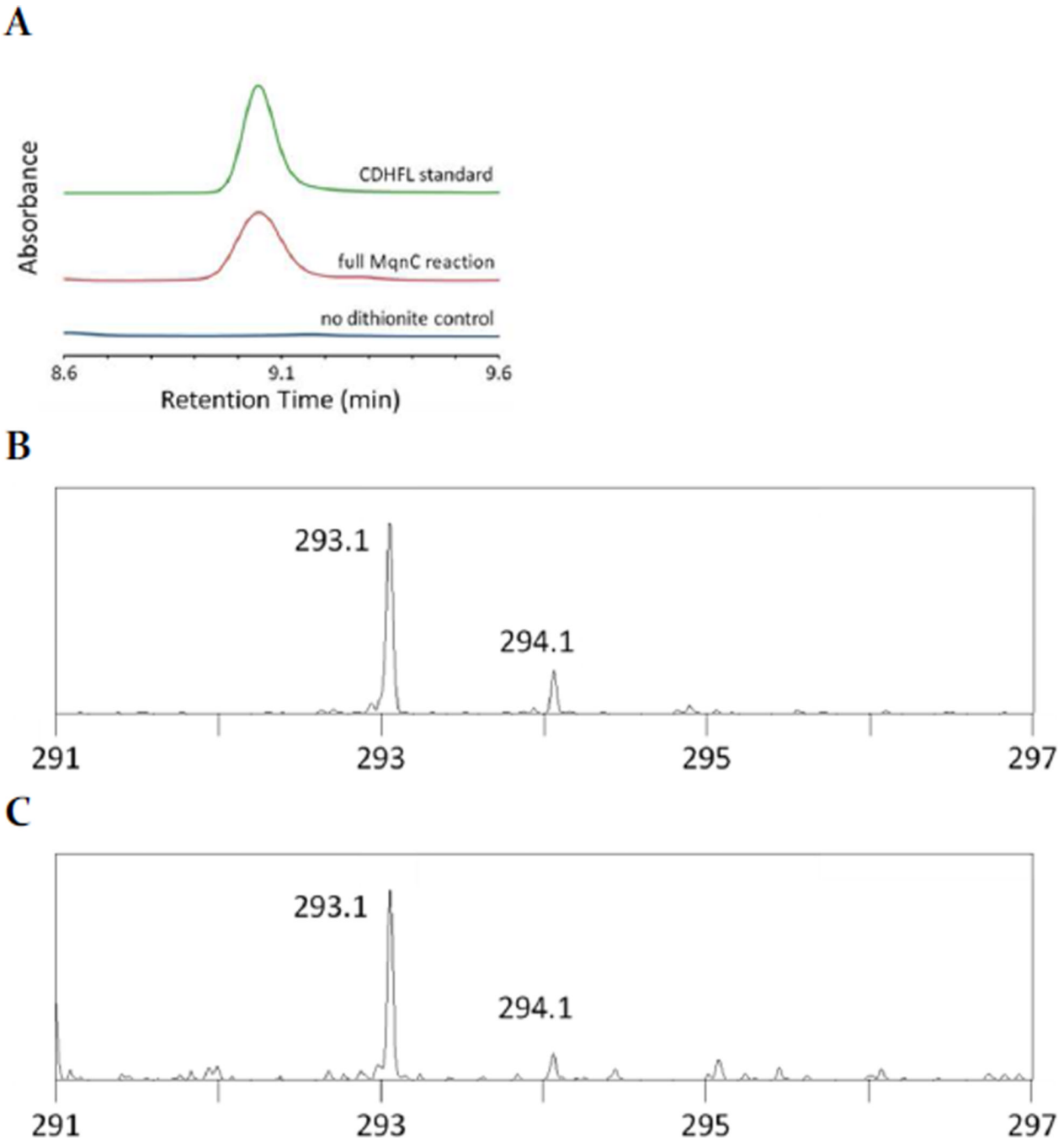

Figure 2.

MqnC activity *in vitro*. (A) HPLC chromatograms monitoring absorbance at 250 nm. (B) Mass spectrum data for CDHLF purified from *S. coelicolor mqnD*-disruptant strain. (C) Mass spectrum for CDHFL from full MqnC reaction. Calculated exact mass of CDHFL: 293.1 m/z ($[M-H]^-$).

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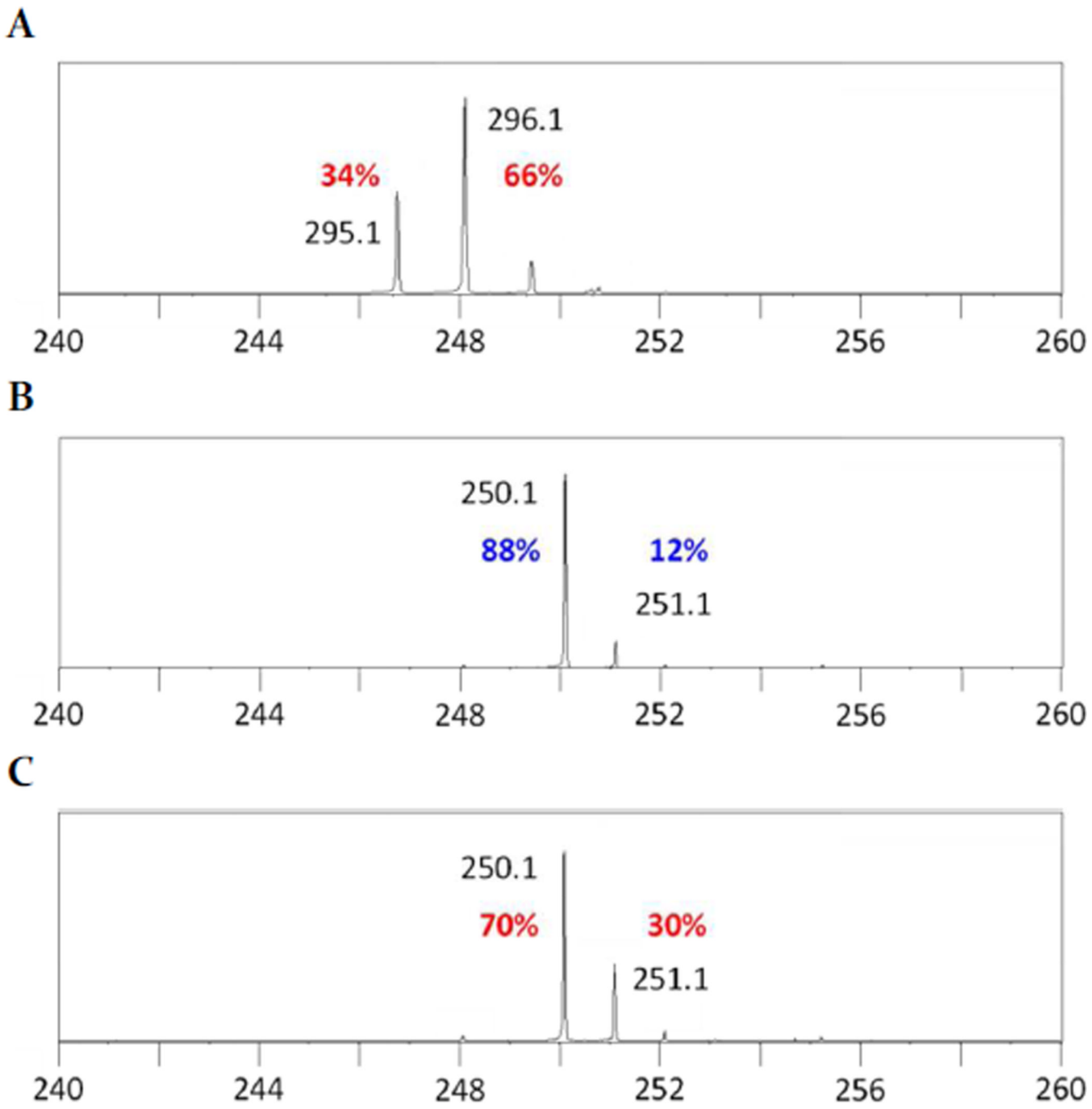
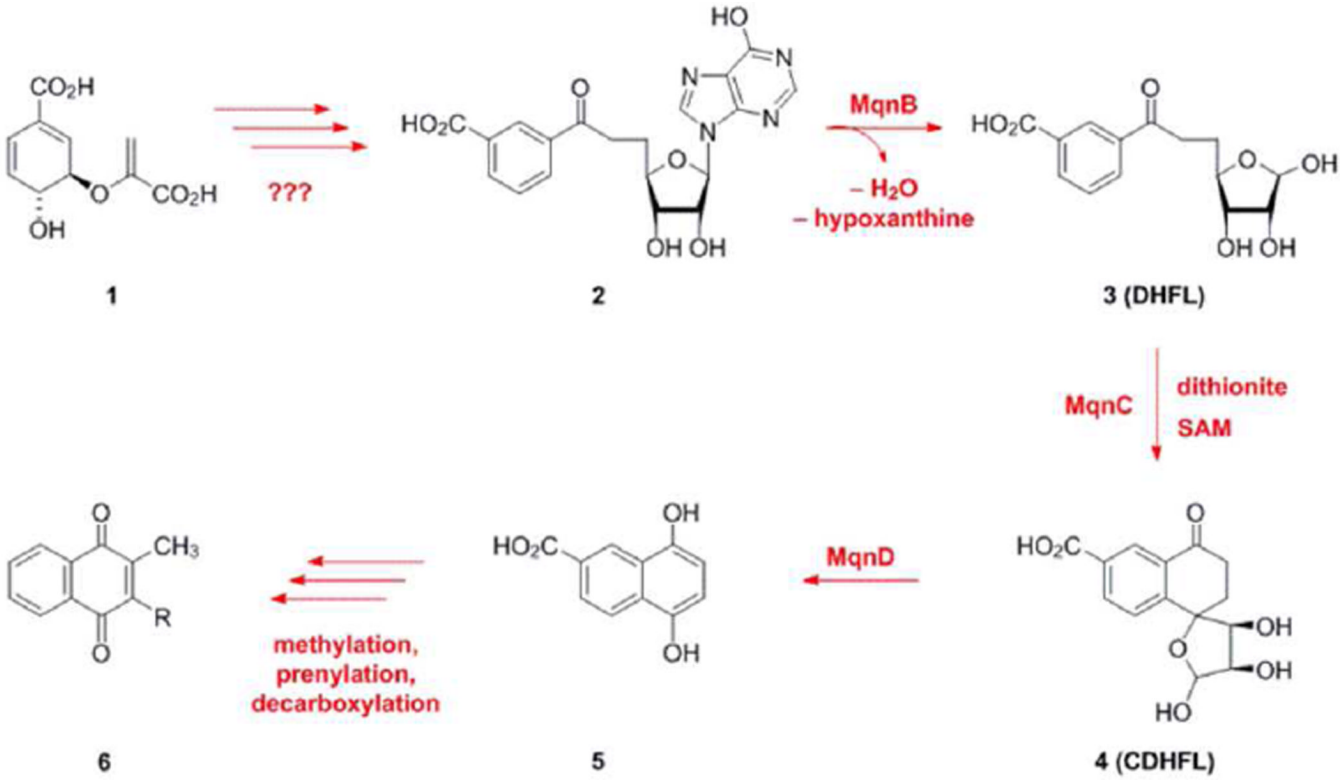


Figure 3.

MqnC-mediated hydrogen atom abstraction by the 5′-dA radical occurs at the C4′-position of DHFL. (A) Mass spectrum demonstrating 66% deuterated [4-^{2}H]-DHFL. (B) 5′-dAd derived from MqnC reaction using DHFL as the substrate. (C) 5′-dAd derived from MqnC reaction using [4-^{2}H]-DHFL as the substrate. Calculated exact mass of DHFL: 295.1; [4-^{2}H]-DHFL: 296.1; and 5′-dAd: 250.1 m/z ([M-H]$^{-}$).



Scheme 1.
Menaquinone biosynthesis via the futalosine pathway.

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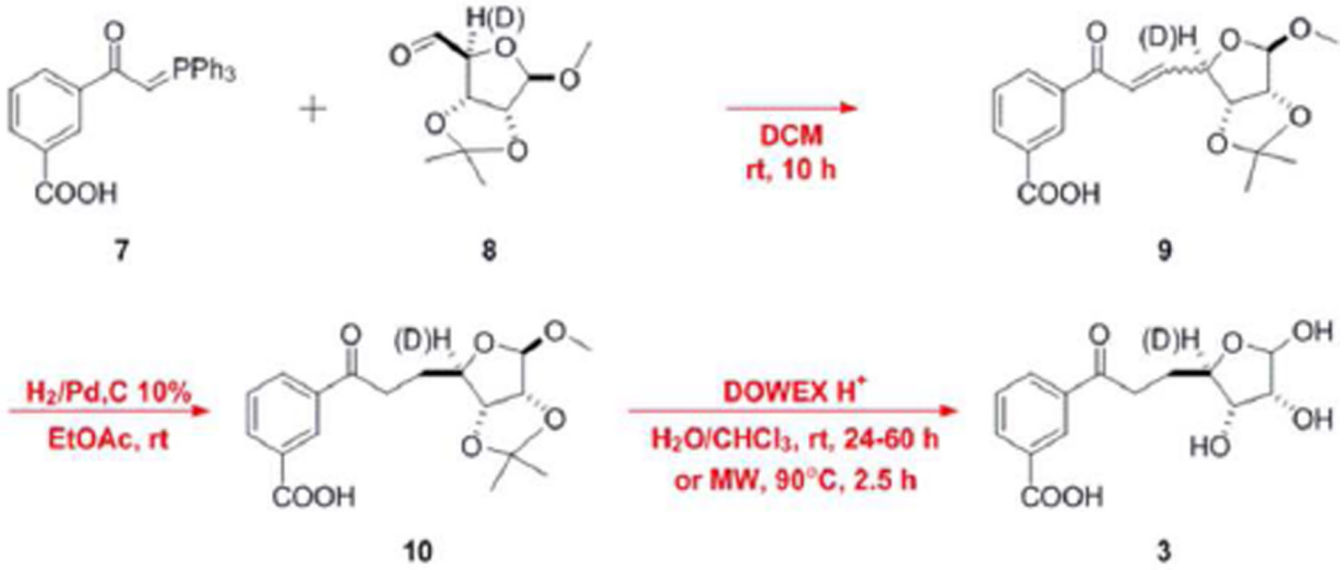

Scheme 2.
Synthesis of DHFL and [4-^{2}H]-DHFL.

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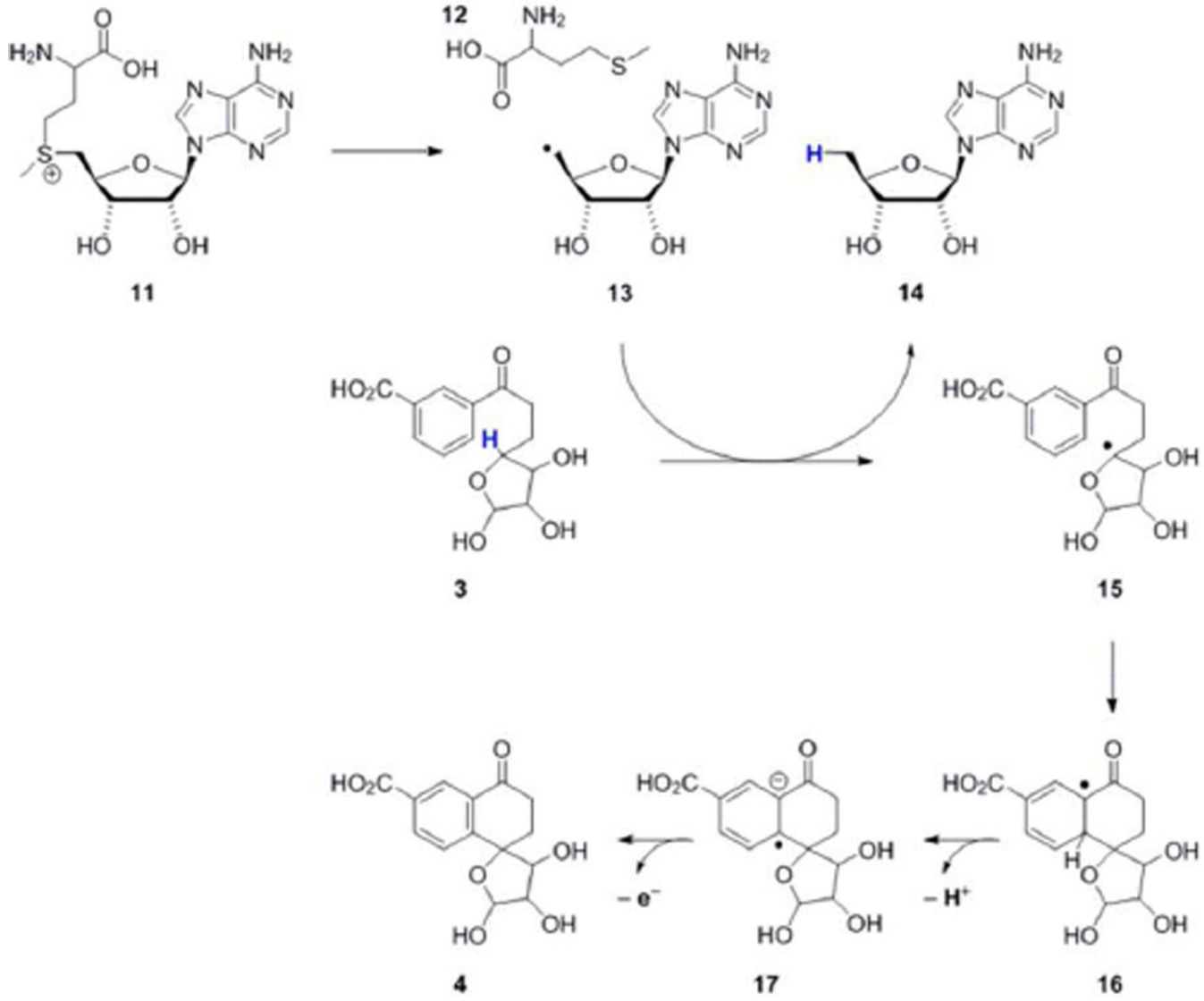

Scheme 3.
Proposed MqnC reaction mechanism.