ORGANIC LETTERS

2007
Vol. 9, No. 23
4765−4767

Determination of the Stereochemistry of 2-Succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate, a Key Intermediate in Menaquinone Biosynthesis

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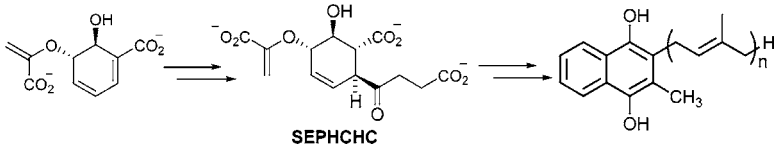
Received August 29, 2007

ABSTRACT



SEPHCHC

The turnover product of the committed step of menaquinone biosynthesis was isolated and determined to be (1*R*,2*S*,5*S*,6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate. Structural determination of this key intermediate represents a critical step to complete elucidation of the biosynthetic pathway.

Menaquinone is a lipid-soluble molecule that serves important biological functions.[1] In human and animals, it is a vitamin (K_2) that has to be acquired from the diet or bacteria in the gut;[2] it is crucial to blood clotting through its involvement in the γ-carboxylation of glutamate residues.[3] In bacteria, menaquinone is an electron-transporting molecule that is synthesized from chorismate of the shikimate pathway.[4] It serves as the only electron carrier in the respiratory chain of aerobic Gram-positive bacteria and most anaerobic bacteria.[1] It is also needed in the respiration of facultative bacteria, such as *Escherichia coli*, during their anaerobic growth.[1] The bisoynthesis of menaquinone is an attractive target for development of antibiotics to treat microbial pathogens due to its absence in humans and animals. For example, the biosynthetic pathway has been suggested as a valuable target for antituberculosis drug discovery[5] because the Gram-positive pathogen, *Mycobacterium tuberculosis*, is an obligate aerobe and exclusively uses the naphthoquinone in the respiratory chain.[6]

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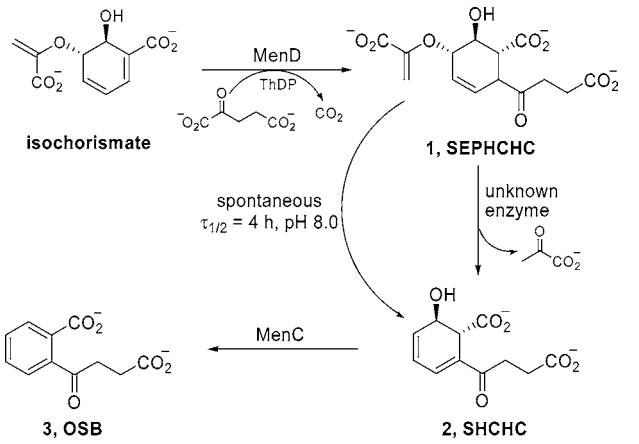
10.1021/ol702126m CCC: $37.00
Published on Web 10/23/2007

The biosynthesis of menaquinone is highly conserved among bacterial species.[7] It involves conversion of the common branch-point intermediate—chorismate—of the shikimate pathway to *o*-succinylbenzoate (OSB, **3**), which is further transformed to the reduced form of vitamin K_2 by five enzymes.[1,7] The first step of the biosynthesis is catalyzed by a dedicated isochorismate synthase (MenF).[8] The transformation from isochorismate to OSB, however, is not completely understood. For a long time, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC) has been mistaken as the product of the second biosynthetic enzyme, MenD.[1,7,9,10] Recently, we found that the correct MenD product is 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate (SEPHCHC, **1**) and an additional enzyme is required to convert this new intermediate to SHCHC (**2**) for OSB synthesis by MenC (Scheme 1).[11]

Scheme 1. Conversion of Isochorismate to *o*-Succinylbenzoate (OSB) in the Biosynthesis of Menaquinone



In the revised biosynthetic pathway, MenD is responsible for the first irreversible step, catalyzing the decarboxylation of 2-ketoglutarate to form a succinic semialdehyde-thiamine diphosphate (ThDP) anion for a Michael-type addition on the isochorismate substrate.[10] This central role in menaquinone biosynthesis makes MenD an attractive target for discovery of drugs to treat menaquinone-dependent microbial pathogens. However, the stereochemistry at C-2 of the MenD product (**1**) is not known. The configurations of the other three chiral centers in the intermediate can be inferred to be (1*R*,5*S*,6*S*) from its formation from isochorismate or its conversion to SHCHC. Since these chiral centers are not involved in the transformations, the C-5 and C-6 configurations are identical to that of the corresponding stereogenic centers in isochorismate, while C-1 configuration is known from the stereochemistry of SHCHC.[9f] As a step toward complete understanding of the biosynthetic pathway and to facilitate mechanistic studies on MenD, we purified the intermediate and determined its stereochemistry by nuclear magnetic resonance techniques.

We overexpressed EntC, an isochorismate synthase in enterobactin biosynthesis,[12] and MenD as hexahistidine-tagged proteins and purified them to >90% purity for preparation of high-purity SEPHCHC (**1**) from chorismate. To avoid spontaneous decomposition of SEPHCHC (**1**),[11] the enzymatic preparation was performed in 1.1 mL of 50 mM sodium phosphate buffer (pH 6.5) containing 75 mM chorismate, 500 mM 2-ketoglutarate, 5 mM $MgCl_2$, 60 μM ThDP, 0.16 mg/mL EntC, and 0.52 mg/mL MenD. The reaction mixture was incubated at room temperature (24 °C) for 5 h to ensure complete consumption of chorismate, quenched by addition of trifluoroacetic acid (final pH $\approx$ 2.0), and cooled down to 0 °C in an ice bath. After removal of the enzymes by ultrafiltration, the reaction product was fractionated by HPLC under acidic conditions. Fractions of SEPHCHC (**1**) were identified by adjusting a small portion of each fraction (200 μL out of 5.0 mL) to pH 12−13 for detection of SHCHC formation at 290 nm. A colorless oily compound was obtained after lyophilizing the collected fractions, which was verified to be SEPHCHC by FAB mass spectrometry (found: $m/z = 328.9$ $[M + H]^+$; calcd: 328.1).

The obtained SEPHCHC was dissolved in CD_3OD and subjected to analysis by nuclear magnetic resonance spectroscopy. One-dimensional ^1H NMR spectrum indicated that the sample contained much less impurity in comparison to a previous preparation,[11] exhibiting small impurity peaks at ~8.0, 3.2, 1.5, and 1.3 ppm. Consistent with our preliminary NMR characterization of the intermediate,[11] signals for 11 protons could be clearly identified, whereas the signal for another proton was missing in the spectrum. Two-dimensional $^1H-^1H$ TOCSY spectrum revealed that the missing proton signal was hidden in the water signal at $\delta = 4.85$. The 12 nondissociable protons of the compound form three spin systems: one consisting of two protons belonging to

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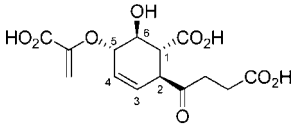
the methylene group in the 5-enolpyruvyl group, another one consisting of the four protons in the 2-succinyl group, and the last one consisting of the six protons on the cyclohexene ring. Combining these results and structural information provided by ^{13}C spectra (DEPT-135 and DEPT-90), $^{1}H-^{1}H-$COSY spectrum, and $^{1}H-^{13}C$ HMQC and HMBC spectra, all ^{1}H and ^{13}C signals were unambiguously assigned, and all $^{1}H-^{1}H$ coupling constants, except those between the four protons of the 2-succinyl group, were determined (Supporting Information).

The coupling constants between the six protons on the cyclohexene ring are listed in Table 1. Interestingly, any two vincinal protons of the four chiral centers have a coupling constant greater than 7.0 Hz, indicating that they are in *trans* and that all the protons occupy an axial position.[13] This relative stereochemistry allows the assignment of *S*-configuration to C-2 in SEPHCHC (**1**) on the basis of the known absolute configurations at C-1, C-5, and C-6.

Table 1. *J*-Coupling Constants between Protons on the Cyclohexene Ring of SEPHCHC (**1**)



1, SEPHCHC

entry	value (Hz)	entry	value (Hz)
J_{56}	7.92	J_{24}	2.91
J_{16}	11.29	J_{34}	10.17
J_{12}	10.03	J_{35}	1.83
J_{23}	2.13	J_{45}	1.60

NOESY spectra of SEPHCHC (**1**) showed a medium-intensity cross-peak between H-5 and H-1 (Figure 1A), consistent with the known *cis*-relationship between them. A cross-peak with a comparable intensity was also found between H-2 and H-6, suggesting that the distance between them is similar to that between the axial H-5 and H-1 and thus providing support for the *trans* arrangement for H-1 and H-2. The disparity in the intensity of the cross-peaks may indicate that the axial protons are distorted because of the ring double bond and other substituents. Indeed, H-2 is slightly twisted toward H-1 in a MM2-minimized conformation of SEPHCHC (**1**) (Figure 1B). This shift of H-2 results in a slightly larger distance between *cis* H-2 and H-6 in comparison to that between *cis* H-5 and H-1, leading to a weaker NOE interaction between the former two protons. These results provide further support for the *S*-configuration at C-2.

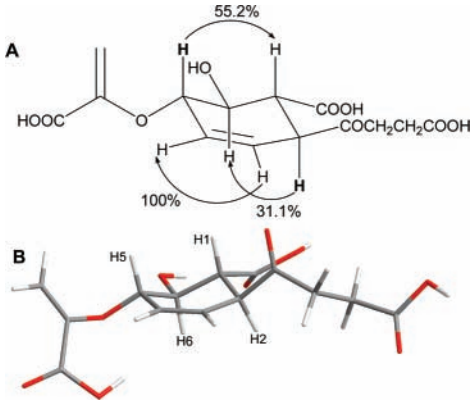


Figure 1. Relative intensities of NOE interaction of H-2 and H-5 with selected adjacent protons (A) and MM2-minimized structure of protonated SEPHCHC (B). Carbon, hydrogen, and oxygen atoms are colored in gray, white, and red, respectively.

In summary, we have collected spectroscopic evidence to show that the MenD product, SEPHCHC (**1**), is (1*R*,2*S*,5*S*,6*S*)-2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic acid. Determination of the stereochemistry of this intermediate will facilitate mechanistic studies on MenD to assist discovery of drugs against menaquinone-dependent microbial pathogens. In addition, it also represents a crucial step to complete elucidation of the pathway of menaquinone biosynthesis.

Acknowledgment. This work was supported by HKUST and RGC of the government of Hong Kong SAR.

Supporting Information Available: Experimental procedures and full spectral data for SEPHCHC (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

OL702126M

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