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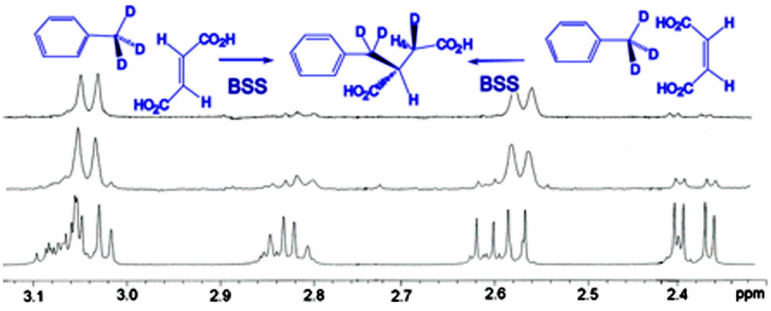

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Mechanism of Benzylsuccinate Synthase: Stereochemistry of Toluene Addition to Fumarate and Maleate

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Benzylsuccinate synthase (BSS) catalyzes a remarkable chemical reaction: the addition of toluene across the double bond of fumarate to produce (*R*)-benzylsuccinate (Scheme 1).

This reaction is the first step in the anaerobic metabolism of toluene by various denitrifying and sulfate-reducing bacteria such as *Thauera aromatica* and *Desulfobacula toluolica*, which are able to live on toluene as their sole source of carbon and energy.[1,2] The degradation of aromatic compounds, both natural and man-made, is of great importance in the environment. It was initially thought that oxidative pathways that require molecular oxygen to functionalize otherwise inert aromatic hydrocarbons were the only routes to degrade such compounds. However, the recent discovery of anaerobic pathways for the degradation of aromatic compounds has aroused much interest, both for the potential they offer for bioremediation in situations where oxygen is scarce and because of the novel chemistry involved.[3,4]

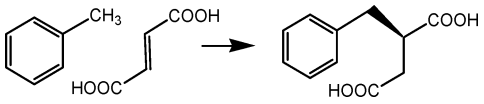
Sequence similarities with pyruvate formate-lyase (PFL) and anaerobic ribonucleotide reductase initially identified BSS as a member of the growing class of glycyl radical-containing enzymes.[5−9] Furthermore, EPR studies show that the resting enzyme harbors an organic radical similar to that observed in PFL and ribonucleotide reductase.[10] Like all glycyl radical enzymes, BSS is extremely oxygen sensitive, and exposure to air results in oxidative cleavage of the α subunit at the site of the presumed glycyl radical.[5]

Although there is strong evidence that the BSS-catalyzed reaction involves radical intermediates, the rapid loss of activity encountered during attempts to purify the enzyme[5] means that virtually nothing is known of the mechanism. It has been shown that BSS produces (*R*)-benzylsuccinate stereospecifically and that deuterium in the methyl group of toluene is retained in the product.[11] However, the position of the label was not established. We therefore decided to determine the stereochemistry of hydrogen transfer to benzylsuccinate, as this might provide valuable mechanistic information.

Here we describe experiments in which BSS was reacted with [d_3-methyl]toluene and either fumarate or its cis stereoisomer, maleate, which also serves as a substrate for the enzyme.[12] We demonstrate that when fumarate is the cosubstrate, deuterium is transferred from toluene to the C-3 pro-(*S*) position of benzylsuccinate, implying that the addition of toluene to the double bond of fumarate is syn. Most interestingly, however, when maleate is the cosubstrate, the addition of toluene occurs in an anti fashion. This is consistent with the formation of the C-3 radical of benzylsuccinate as an intermediate, in which rotation about the C-2−C-3 bond can occur to relieve the sterically unfavorable cis conformation of the carboxylate groups when maleate is the cosubstrate.

Enzymatic Synthesis of Deuterium-Labeled Benzylsuccinate. Cell-free extracts of toluene-grown *T. aromatica*[6] were prepared under rigorously anaerobic conditions in 10 mM triethanolamine/Cl buffer, pH 8.0, containing either 5 mM fumaric acid or 5 mM maleic acid, and 10% glycerol. Typically, 500 μL of cell extract

Scheme 1. Reaction Catalyzed by Benzylsuccinate Synthase



was incubated with ∼40 mM deuterium-labeled toluene (1 μL) at 4 °C for 24 h. The reaction mixture was acidified with H_2SO_4 and the benzylsuccinic acid extracted with CH_2Cl_2. The crude material was further purified by reverse phase HPLC. To analyze the stereochemistry of deuterium transfer, the benzylsuccinic acid was converted to benzylsuccinic anhydride by reaction with acetyl chloride[13] and the NMR spectrum of the deuterated material compared with that of unlabeled standard material prepared by cyclization of commercially available racemic benzylsuccinic acid.

Assignment of the NMR Spectrum of Benzylsuccinic Anhydride. The proton spectrum of unlabeled benzylsuccinic anhydride exhibits five resonances in the aliphatic region (Figure 1). The multiplet at 3.46 ppm may be assigned to H_c. The resonances at 3.25 and 3.03 ppm were assigned to the benzylic protons H_a and H_b; these protons exhibit a geminal coupling constant of 19 Hz between them, and vicinal coupling constants of 4.5 and 8 Hz respectively to H_c. The resonances at 2.96 and 2.73 ppm were assigned to the protons at C-3 of the five-membered ring. These protons exhibit a geminal coupling constant of 19.5 Hz between them, whereas H_d exhibits a coupling constant of 9.5 Hz to H_c, and H_e exhibits a coupling constant of 6.0 Hz to H_c.

The magnitudes of syn and anti coupling constants are well studied for protons in five-membered rings. In particular, studies of syn and anti disubstituted succinic anhydrides have established that syn coupling constants lie in the range of 9−10 Hz, whereas

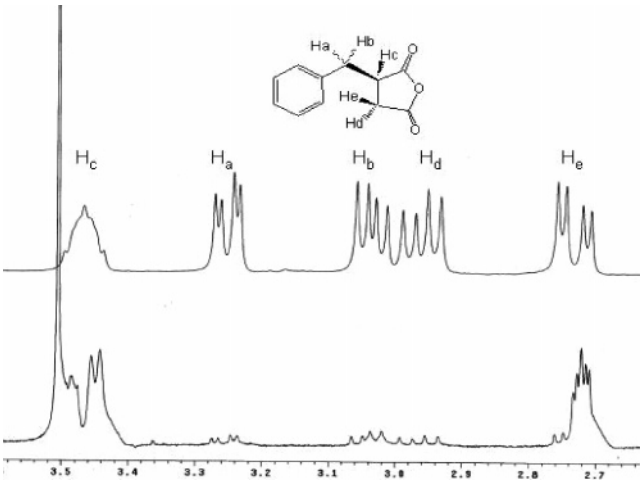


Figure 1. (Top) Assignment of the NMR spectrum of authentic benzylsuccinic anhydride. (Bottom) Spectrum of benzylsuccinic anhydride derived from the enzymatic reaction of fumarate with deuterated toluene. The strong peak at 3.5 ppm is a solvent contaminant.

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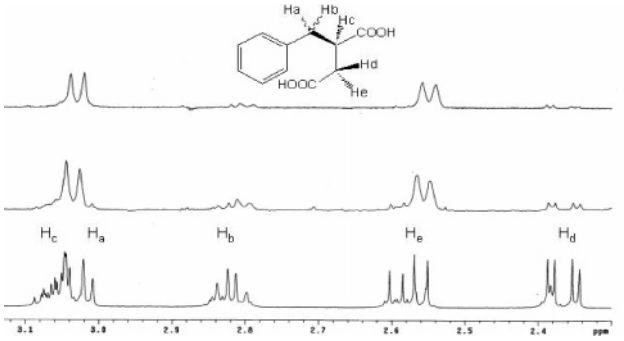

Figure 2. (Top) NMR spectrum of benzylsuccinic acid produced by the enzymatic reaction of fumarate with d_3-toluene. (Middle) Spectrum of benzylsuccinic acid produced by enzymatic reaction of maleate and d_3-toluene. (Bottom) Assignment of NMR spectrum for authentic benzylsuccinic acid.

anti coupling constants lie between 5 and 6 Hz.[14] We may therefore be confident in assigning H_d as being syn to H_c and H_e as being anti.

Stereochemistry of Hydrogen Transfer. The NMR spectrum of deuterated benzylsuccinic anhydride derived from BSS-catalyzed addition of deuterated toluene to fumarate shows only two proton resonances in the aliphatic region (Figure 1). (Small peaks are also evident from unlabeled benzylsuccinic anhydride that are most likely due to residual unlabeled material in the cell extracts.) As expected, the signals due to H_a and H_b are absent, as is the signal at 2.96 ppm assigned to H_d. Of the remaining two protons, H_e is multiplet, consistent with its coupling both to H_c and to the geminal deuterium atom to give an overlapping doublet of triplets. H_c is simplified to a doublet with a coupling constant of 9 Hz, confirming its syn relationship to H_e; the doublet is broadened by weak J-3 coupling to deuterium. These results demonstrate the stereospecific nature of hydrogen transfer and imply that the hydrogen atom and the benzyl group are transferred to the same face of the double bond of fumarate.

The results of this deuterium-labeling experiment also allowed the spectrum of benzylsuccinic acid to be assigned (Figure 2). In the deuterated material the multiplet at 3.05 ppm simplifies to a broad doublet and is assigned to H_c, whereas the double doublets at 3.03 and 2.82 ppm are missing and therefore belong to H_a and H_b. The double doublet at 2.58 ppm simplifies to a broad doublet and is assigned to the 3-pro-(*S*) hydrogen (H_e); the double doublet at 2.36 ppm is absent in the deuterated material and is assigned to the 3-pro-(*R*) hydrogen (H_d) of benzylsuccinate.

Reaction with Maleate. Maleate appeared to be a poorer substrate for BSS, as significantly less benzylsuccinic acid was obtained with this substrate. The deuterated benzylsuccinic acid synthesized in this reaction had an NMR spectrum that exactly matched that of the deuterated material synthesized from fumarate and toluene (Figure 2). Thus, again, it is H_d that becomes deuterated, implying that the overall addition of toluene to maleate must be anti. This result is most readily explained by a two-step mechanism in which the benzylic radical formed by deuterium abstraction from toluene adds to the double bond of maleate, transiently generating a radical at C-3 of benzylsuccinic acid. This allows rotation about the C-2−C-3 single bond to relieve the unfavorable cis conformation of the carboxylate groups. Subsequent transfer of hydrogen from the enzyme to the product results in overall anti addition of toluene.

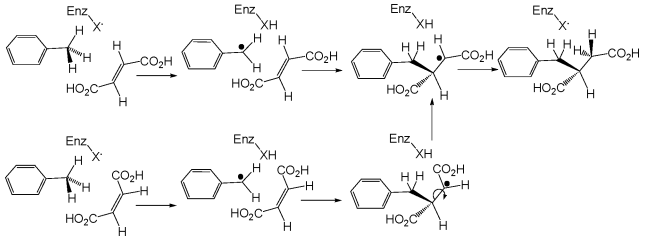

Figure 3. Mechanism for the BSS-catalyzed addition of toluene to fumarate (top scheme) and maleate (bottom scheme)

Conclusions. These results provide the first mechanistic insights into this extremely unusual enzymatic reaction. They support a mechanism, shown in Figure 3, in which an enzyme-based radical, most likely a cysteinyl radical, initially abstracts hydrogen from toluene to generate a benzylic radical that subsequently undergoes addition to fumarate. The overall *syn* addition of toluene to fumarate suggests an active-site geometry in which the cysteinyl radical and the methyl group of toluene are disposed on the same face of fumarate. Furthermore, the inversion of the stereochemical course of hydrogen transfer when maleate is the substrate provides strong evidence for the formation of the C-3 radical of benzylsuccinate as an intermediate, in which rotation about the central C−C bond of succinate is possible.

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Supporting Information Available: Details of the preparation of cell-free extracts, purification of benzylsuccinic acid, and full NMR spectra of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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