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Elucidating the Stereochemistry of Enzymatic Benzylsuccinate Synthesis with Chirally Labeled Toluene

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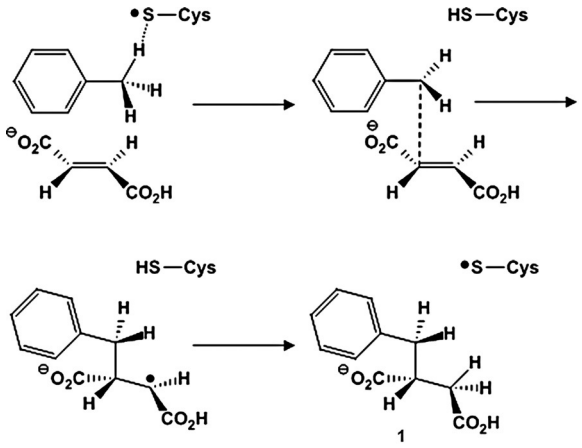
Abstract: *Benzylsuccinate synthase is a glycyl radical enzyme that initiates anaerobic toluene metabolism by adding fumarate to the methyl group of toluene to yield (R)-benzylsuccinate. To investigate whether the reaction occurs with retention or inversion of configuration at the methyl group of toluene, we synthesized both enantiomers of chiral toluene with all three H isotopes in their methyl groups. The chiral toluenes were converted into benzylsuccinates preferentially containing ^{2}H and ^{3}H at their benzylic C atoms, owing to a kinetic isotope effect favoring hydrogen abstraction from the methyl groups. The configuration of the products was analyzed by enzymatic CoA-thioester synthesis and stereospecific oxidation using enzymes involved in benzylsuccinate degradation. Assessment of the configurations of the benzylsuccinate isomers based on loss or retention of tritium showed that inversion of configuration at the methyl group occurs when the chiral toluenes react with fumarate.*

The glycyl radical enzyme benzylsuccinate synthase (BSS) replaces dioxygen-dependent mono- or di-oxygenases in initiating bacterial toluene degradation under anaerobic conditions. It represents one of many enzymes characterized in recent years that use radical mechanisms to catalyze chemically demanding reactions in biochemistry, and presents a model case for how radical-based reactions can still be performed stereospecifically.[1] BSS is activated to the radical state by a special activating enzyme that belongs to the "radical SAM" family and requires *S*-adenosylmethionine for converting a conserved glycine within the catalytic subunit of BSS to a glycyl radical. Activated BSS then catalyzes radical addition of a fumarate co-substrate to the methyl group of toluene, leading to the stereospecific synthesis of (*R*)-benzylsuccinate (**1**, see Scheme 1).[1] The reaction pathway starts with a hydrogen atom transfer from a conserved active-site cysteine to the glycyl radical, thereby generating a reactive thiyl radical. With BSS, the thiyl radical is proposed to abstract a hydrogen atom from the methyl group of toluene to form an enzyme-bound benzyl radical as an intermediate,



Scheme 1. Proposed mechanism of BSS.

which is poised to undergo addition to the *Re-Re* face of the double bond of a bound fumarate to yield an intermediate (*R*)-benzylsuccinyl radical. This radical is quenched by hydrogen transfer from the active-site cysteine, and after re-establishing the stable glycyl radical state of BSS, the product is released and new substrates are bound[2] (Scheme 1). In accordance with the proposed mechanism, the initially abstracted hydrogen atom is returned to the intermediate radical in a *syn*-addition mode.[3]

In recent years, BSS has become a paradigm of many paralogous fumarate-adding enzymes for aromatic or aliphatic hydrocarbons.[4] In this study, we explore the "cryptic stereochemistry"[5] of BSS towards the methyl group of toluene, that is, does the reaction lead to retention or inversion of configuration at this alkyl group? This kind of study was pioneered in 1969 with chiral acetates containing all three H isotopes in their methyl groups.[6] In this work, we synthesized both enantiomers of chiral toluene and converted them into benzylsuccinate with ^{14}C-labeled fumarate. The respective stereochemical configurations of the benzylsuccinate products were analyzed by converting them with the next two enzymes of toluene degradation, benzylsuccinate CoA-transferase (BS-CT) and benzylsuccinyl-CoA dehydrogenase

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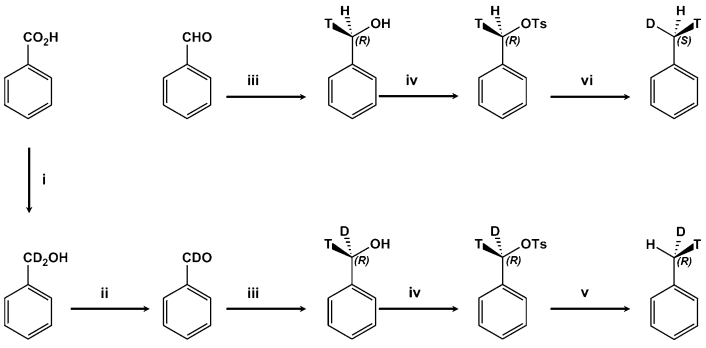
(BS-DH), which results in either retention or loss of the ^{3}H-label.

Our experiments depended on a strong primary kinetic isotope effect (KIE) for the action of BSS on the chiral toluenes, with preferential removal of the (^{1}H) substituent and enrichment of stereochemically defined [1-^{3}H],(1-^{2}H)benzylsuccinates (note that full isotope occupancy is indicated by round brackets, partial occupancy by square brackets). Since KIE values were previously only reported from a different BSS isoenzyme from *Thauera aromatica* strain T1,[7] we determined the KIE of BSS from *T. aromatica* strain K172, which was used in this study. Specific BSS activities of 4.0 and 16 nmol min^{-1} (mg protein)$^{-1}$ were measured with ($^2H_8$)toluene and unlabeled toluene, respectively, thus indicating a KIE value of 4.0. Control assays with unlabeled toluene and (2,3-$^2H_2$)fumarate yielded a specific activity of 18 nmol min^{-1} (mg protein)$^{-1}$. Therefore, we used a KIE value of 4.0 between ^{1}H and ^{2}H for our further calculations, rather than the somewhat lower values of 2.9 to 3.1 reported for BSS from strain T1.[7] According to the Swain–Schaad equation, this corresponds to a KIE value of 7.4 between ^{1}H and ^{3}H.[8] This rather high intermolecular KIE should provide a reliable basis to obtain significant differences between the two toluene enantiomers, although the experiments actually depend on the intramolecular KIE values of the chiral toluenes, which are not available and may deviate slightly from the intermolecular KIE values[9] (see the Supporting Information for QM-based KIE estimates).

(*R*)- and (*S*)-[1-$^3H_1$],(1-$^2H_1$,1-$^1H_1$)toluene (hereafter called (*R*)- and (*S*)-toluene) were synthesized through a sequence of chemical and enzymatic reactions (Scheme 2). (1-$^2H_1$)benzaldehyde, the starting material for (*R*)-toluene, was synthesized by reducing benzoic acid to dideuterated benzyl alcohol, followed by MnO_2-mediated oxidation to give (1-$^2H_1$)benzaldehyde containing a high ^{2}H content (99.3%) with a yield of 70.5% relative to benzoic acid (see the Supporting Information). To obtain both enantiomers of chiral toluenes, we set up two parallel routines starting with either unlabeled benzaldehyde or (1-$^2H_1$)benzaldehyde, which were converted into (*S*)- or (*R*)-toluene, respectively, in a three-step reaction sequence. The first reaction was stereoselective reduction with [^{3}H]NADH by yeast alcohol dehydrogenase,[10] resulting in *Re*-addition of ^{3}H to the benzaldehyde isotopomers. [^{3}H]Formate and formate dehydrogenase from *Candida boidinii* were used to generate [^{3}H]NADH in situ (see Supporting Information). This procedure yielded the (*R*)-enantiomers of [1-^{3}H],(1-^{1}H) and [1-^{3}H],(1-^{2}H)benzyl alcohol, respectively, with yields of 72%. After this step, the isotopically labeled benzyl alcohols were diluted with unlabeled benzyl alcohol, which does not interfere with the planned experiments because the ^{3}H-labeled chiral centres necessary to evaluate the results are already established. The labeled benzyl alcohols were activated to the corresponding tosylates, which were reduced with inversion of configuration[11] to the desired chiral toluenes by using either $LiAlH_4$ (for (*R*)-toluene) or $LiAl(^2H)_4$ (for (*S*)-toluene) as the reductant (Scheme 2). The final yields were 0.51 g (5.4 mmol) of (*S*)-toluene containing 32.2 kBq of radioactivity (specific activity 6.0 kBq mmol^{-1}), and 0.45 g (4.8 mmol) of (*R*)-toluene containing 38.9 kBq of radioactivity (specific activity 8.1 kBq mmol^{-1}), which represents about 24% relative to the starting amounts of the benzaldehyde isotopomers. The amounts and specific activities of the obtained chiral toluenes were sufficient to distinguish between retention and inversion.

The chiral toluene enantiomers (100 Bq) were converted into benzylsuccinate by incubating them anaerobically for 16 h with added ^{14}C-labeled fumarate (33 Bq) and extracts of toluene-grown *T. aromatica* cells at room temperature as described previously.[12] The assays were then adjusted to pH 1.5 by adding aqueous HCl and the benzylsuccinate formed was removed by solid-phase extraction using a silica-based C_{18} column. The specific activities of BSS in the various replicate assays were determined to be 10 to 20 nmol min^{-1} (mg protein)$^{-1}$, and the yields of benzylsuccinate in the conversion assays were between 0.32 to 0.54 μmol. These products (**2a**/**2b**) are expected to contain mainly the ^{2}H and ^{3}H atoms from the former methyl group of toluene (Figure 1), and their contents of ^{3}H and ^{14}C were determined by scintillation counting. The relative retention or loss of ^{3}H during the further conversion reactions (Figure 1) was based on their ^{3}H/^{14}C ratios.



Scheme 2. Synthesis of chiral toluenes. Reagents and solvents: i. $LiAlD_4$/diethyl ether; ii. MnO_2/CH_2Cl_2; iii. yeast alcohol dehydrogenase and formate dehydrogenase/[^{3}H]formate/NAD^+/aq. phosphate buffer (pH 7.0) under argon; iv. *p*-toluenesulfonyl chloride/K_2CO_3; v. $LiAlH_4$/tetraglyme; vi. $LiAlD_4$/tetraglyme (see the Supporting Information for details). NAD^+ = oxidized nicotinamide adenine dinucleotide.

To evaluate their configurations, the extracted benzylsuccinates (**2a**/**2b**) were converted along the pathway of benzylsuccinate β-oxidation by reacting them with purified BS-CT[13] and BS-DH[14a] (Figure 1 A), giving the benzylidenesuccinyl-CoA thioesters **4a**/**4b** via CoA-thioesters **3a**/**3b**. To achieve this, we used BS-CT from the toluene-degrading species *Geobacter metallireducens* mixed with BS-DH from *T. aromatica*. The extracted doubly labeled benzylsuccinates (**2a**/**2b**) from conversion of the chirally labeled toluenes were measured for their initial contents of ^{3}H and ^{14}C by scintillation counting and incubated with the two auxiliary enzymes under conditions favoring their complete conversion into benzylidenesuccinyl-CoA (**4a**/**4b**; see the Supporting Information). The reaction was complete after 3 h, as confirmed by HPLC analysis (data not shown). The solutions were acidified with HCl

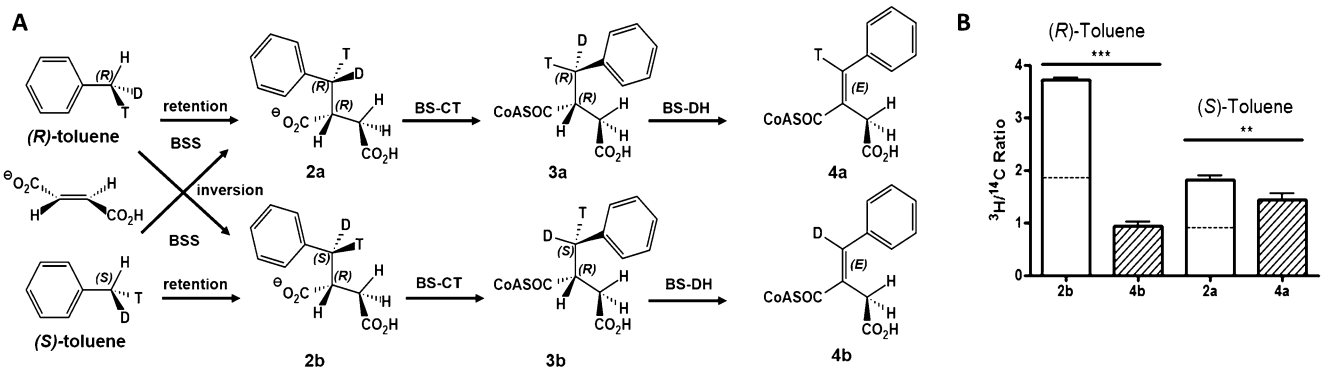


Figure 1. Configurational analysis of benzylsuccinates generated from chirally labeled toluenes. A) Possible results of benzylsuccinate synthesis with chiral toluenes and the further enzymatic conversion reactions. B) $^3H/^{14}C$ ratios of benzylsuccinate and benzylidenesuccinyl-CoA derived from chiral toluenes. Mean values and standard errors are from four independent experiments performed with three parallel measurements each. Mean retention values of tritium in benzylidenesuccinyl-CoA are 25% for (*R*)- and 79% for (*S*)- toluene. The dotted lines show the expected values in the respective products in a non-stereoselective reaction or with racemic **2a**/**2b**. Total radioactivity in the analyzed samples varied from 12–18 Bq ^3H and 2–5 Bq ^{14}C with (*R*)-toluene and from 13–21 Bq ^3H and 2–12 Bq ^{14}C with (*S*)-toluene.

to pH 1.5 and the organic acid products obtained by solid-phase extraction were again analyzed for their ^3H and ^{14}C content. The results revealed that 75% of the ^3H content of **2b** derived from (*R*)-toluene was lost in the corresponding intermediate **4b**, whereas **4a** derived from (*S*)-toluene retained 79% of the ^3H content (Figure 1B). Since BS-DH catalyzes an *anti* elimination of the hydrogen atoms at C2 and C3,[14] this demonstrates that the configuration of the methyl group of toluene is inverted during benzylsuccinate synthesis. Using KIE values of 4.0 and 7.4 for $^1H/^2H$ and $^1H/^3H$, respectively, the initial attack of BSS should occur at 72% of the ^1H, 18% of the ^2H, and 10% of the ^3H substituents.

Any initially abstracted ^3H will be re-donated to benzylsuccinate at a position not affected by BS-DH, thereby resulting in expected ^3H-retention values in benzylidenesuccinyl-CoA of 82% (**4a**) and 28% (**4b**) for pure (*S*)- and (*R*)-toluene, respectively. The experimentally observed values of 79% and 25% (Figure 1B) are fully consistent with the expected values for enantiomer purities of the chiral toluenes of more than 90% (see the Supporting Information). The mean $^3H/^{14}C$ ratios for both pairs of the respective benzylsuccinate and benzylidenesuccinyl-CoA intermediates (**2a** and **4a** vs. **2b** and **4b**) were found to be significantly different ($p < 0.05$) by paired two-tailed t-tests (Figure 1B).

The same stereospecificity of fumarate addition to toluene was predicted by the recently solved X-ray structure of the enzyme containing both substrates and by a quantum mechanics (QM) modeling approach to the BSS reaction mechanism[15] (see Figures S1, S2 in the Supporting Information). The observed result is also consistent with a recent report on methylpentylsuccinate synthase, a paralogue of BSS that is involved in anaerobic alkane degradation and adds fumarate to the C2 methylene group of *n*-hexane.[4c] By using chirally labelled hexane containing ^1H and ^2H at the C-2 and C-5 positions, the resulting methylpentylsuccinate adduct was shown to originate through inversion of the configuration at C-2/C-5.[16]

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