Use of Chiral Malonates to determine the Absolute Configuration of the Hydrogen Atoms Eliminated during the Formation of 6-Methylsalicylic Acid by 6-Methylsalicylic Acid Synthase from *Penicillium patulum*

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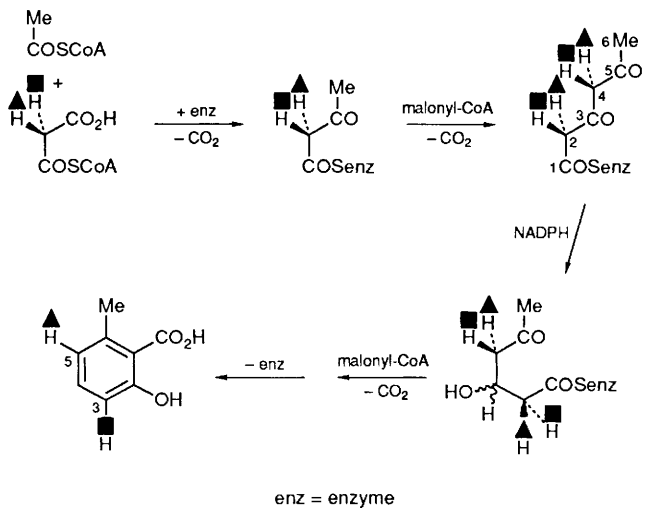
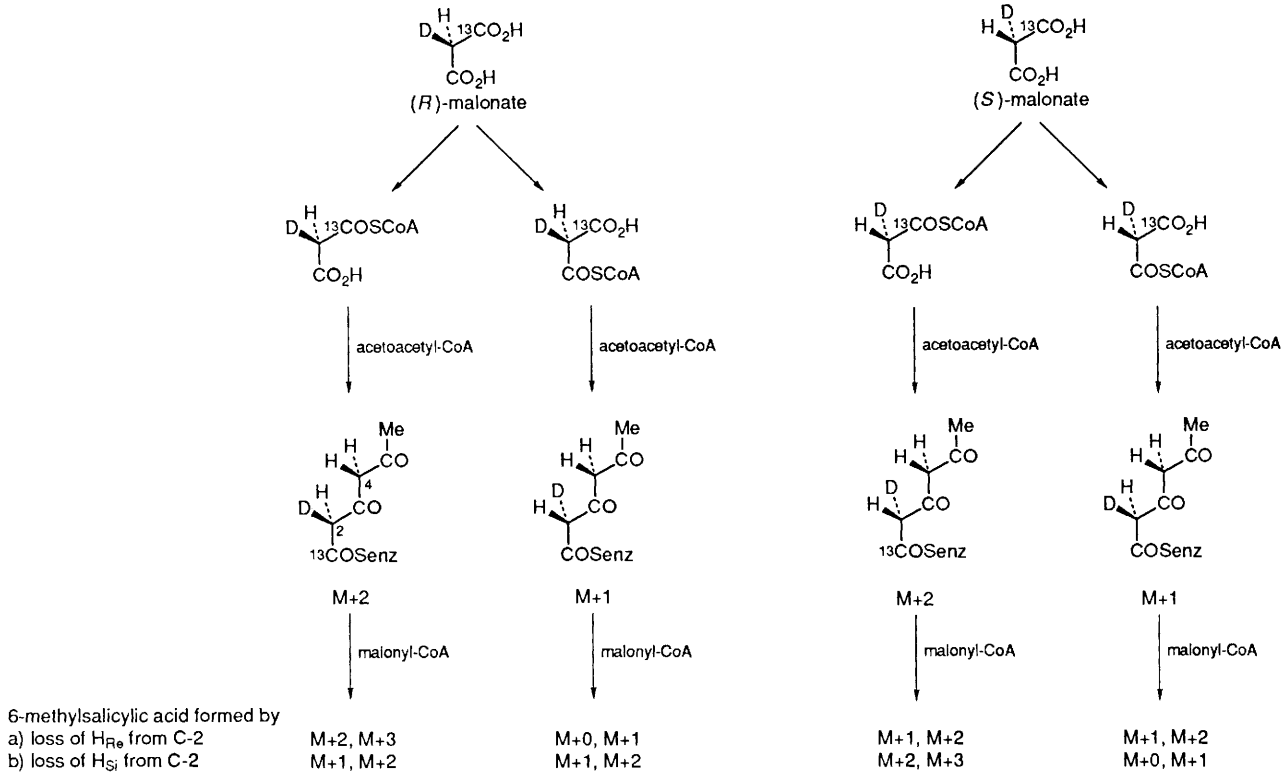
When acetoacetyl-CoA was used as the starter molecule together with chiral malonyl-CoA derivatives, prepared from (*R*)- and (*S*)-[1-^{13}C; 2-^{2}H]malonates, for the biosynthesis of 6-methylsalicylic acid using 6-methylsalicylic acid synthase from *Penicillium patulum*, mass spectrometric analysis of the products established that the hydrogen atom incorporated at the 3-position of 6-methylsalicylic acid originates from H_{Re} of malonyl-CoA allowing the deduction that the hydrogen atom at the 5-position must originate from H_{Si}.

6-Methylsalicylic acid synthase catalyses the reaction between acetyl-CoA and three malonyl-CoA molecules to yield 6-methylsalicylic acid.[1] Initially, acetyl-CoA and two malonyl-CoA molecules are utilised to form a putative enzyme-bound C-6 polyketide intermediate (Scheme 1). Further transformation of this intermediate by a complex series of stages including reduction, addition of a further C-2 unit from malonyl-CoA and cyclisation ultimately yields the aromatic ring.[2,3]

Central to our understanding of the mechanism for the formation of 6-methylsalicylic acid is a knowledge of the fate of the methylene hydrogen atoms, originally at C-2 of malonyl-CoA, during the transformation of the enzyme-bound C-6 polyketide intermediate into the product. The methylene hydrogen atoms of malonyl-CoA are indicated by ■ (H_{Re}) and ▲ (H_{Si}) in Scheme 1 and indicate that the Claisen condensation occurs by inversion. Using chiral malonic acids it was established that the hydrogen atoms at the 3- and 5-positions in 6-methylsalicylic acid, which arise from C-2 and C-4 of the putative C-6 polyketide intermediate respectively (Scheme 1), originate from opposite orientations of malonyl-



Scheme 1 Stages in the biosynthesis of 6-methylsalicylic acid



Scheme 2 Stereochemical details of 6-methylsalicylic acid formation from acetoacetyl-CoA and (*R*)- or (*S*)-[1-^{13}C; 2-^{2}H]malonates

CoA.[4] In these studies, however, it was not possible to assign the absolute configurations of the hydrogen atoms and two possible mechanisms were identified;[4] either H_{Re} and H_{Si} in the C-6 polyketide intermediate are removed from the 2- and 4-positions respectively or, conversely, H_{Si} and H_{Re} are eliminated. In order to distinguish between these two mechanisms it was necessary to develop an experimental approach which allowed the determination of the stereochemical events at one of these two positions in isolation.

The observation that the 6-methylsalicylic acid synthase will accept acetoacetyl-CoA as a starter molecule permits the regiospecific incorporation of the labelled methylene of chiral malonic acid into the C-2 position of the C-6 intermediate and, ultimately, into the 3-position of 6-methylsalicylic acid. Thus the stereochemical events which occur at this position may be studied independently. Although the third malonyl-CoA moiety will provide a ^{13}C label in 50% of the molecules biosynthesised, both hydrogen atoms of this C-2 unit are lost during cyclisation and aromatisation and thus do not contribute to the labelling of hydrogen atoms in the final product.

Our studies with chiral malonic acids[4,5] have shown that, as expected, each enantiomer is incorporated as a pair of malonyl-CoA derivatives. This results from the inability of the succinyl-CoA transferase to distinguish between the labelled and unlabelled substituents in the chiral malonic acids. The malonyl-CoA derivatives in each pair are, however, handled as chiral compounds resulting in a mass distribution for each C-2 unit incorporated into the product of M +1 (from ^{13}C or ^{2}H) and M +0 or M +2 (from neither or both labels).[6] Thus the (*R*)- and (*S*)-malonates may be distinguished by mass spectrometric analysis of the products. Using acetoacetyl-CoA as a starter molecule, and assuming that all the Claisen condensations occur with inversion,[7] the elimination of H_{Re} from position-2 of the C-6 intermediate (H_{Si} in malonyl-CoA) would give mass distributions of M +0, M +1, M +2 and M +3 for (*R*)-malonate and M +1 and M +2 for (*S*)-malonate as shown in Scheme 2a. Elimination of H_{Si} from the same position of the C-6 polyketide intermediate (H_{Re} in malonyl-CoA) would give the reverse mass distribution as shown in Scheme 2b with (*R*)-malonate yielding species of M +1 and M +2 and (*S*)-malonate species of M +0, M +1, M +2 and M +3.

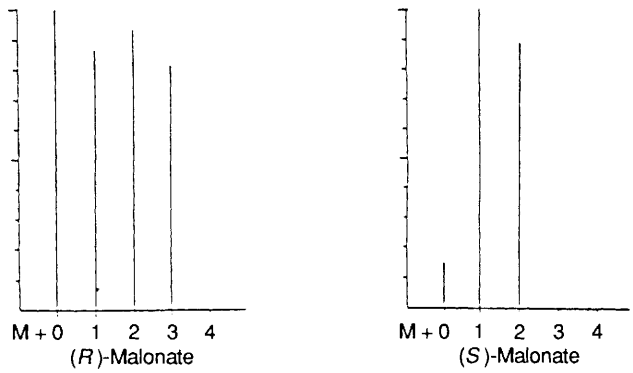


Fig. 1 Mass spectra of 6-methylsalicylic acid biosynthesised from acetoacetyl-CoA and (*R*)- or (*S*)-malonic acid using homogeneous 6-methylsalicylic acid synthase from *P. patulum*†

† Each chiral malonic acid was incubated with succinyl-CoA transferase, NADPH, acetoacetyl-CoA, Tris–H_2SO_4 buffer and homogeneous 6-methylsalicylic acid synthase in a final volume of 2 ml. The formation of 6-methylsalicylic acid was followed fluorimetrically. The 6-methylsalicylic acid was extracted into diethyl ether, converted into its trimethylsilyl derivative and purified by GLC. The derivatives were analysed using a VG Model TR10-1 mass spectrometer. The data shown have been corrected for natural abundance and represent M + 0 = 281 species arising from the loss of Me from one of the trimethylsilyl groups of the parent derivative M + 0 = 296.

Accordingly, (*R*)- and (*S*)-malonates were incubated in separate coupled experiments with succinyl-CoA transferase, acetoacetyl-CoA, NADPH and homogeneous 6-methylsalicylic acid synthase isolated from *P. patulum* and the resulting 6-methylsalicylic acid samples were analysed using GC–MS of the trimethylsilyl derivatives. The results in Fig. 1 show that the 6-methylsalicylic acid arising from (*R*)-malonate is made up largely from species of M +0, M +1, M +2 and M +3, whereas the 6-methylsalicylic acid arising from the (*S*)-malonate contains species of M +1 and M +2. This result establishes that the hydrogen atom (■) at the 3-position of 6-methylsalicylic acid originates from H_{Re} of malonyl-CoA, or H_{Si} in the C-6 polyketide intermediate, indicating that it is H_{Re} at the 2-position of the C-6 intermediate (▲ in Scheme 1) which is eliminated during 6-methylsalicylic acid biosynthesis. Since our previous experiments using acetyl-CoA as the starter molecule (which results in the labelling of hydrogen atoms at both the C-2 and C-4 positions of the C-6 intermediate) have shown that malonyl-CoA-derived hydrogen atoms with opposite orientations are removed from C-2 and C-4 of the C-6 intermediate,[4] it follows that the hydrogen atom eliminated at C-4 arises from H_{Si} of the polyketide intermediate (■ in Scheme 1) and that therefore the hydrogen atom incorporated at C-5 of 6-methylsalicylic acid (▲) originates from H_{Si} in malonyl-CoA.

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