Stereospecific Manipulation of Hydrogen Atoms with Opposite Absolute Orientations during the Biosynthesis of the Polyketide 6-Methylsalicylic Acid from Chiral Malonates in *Penicillium patulum*

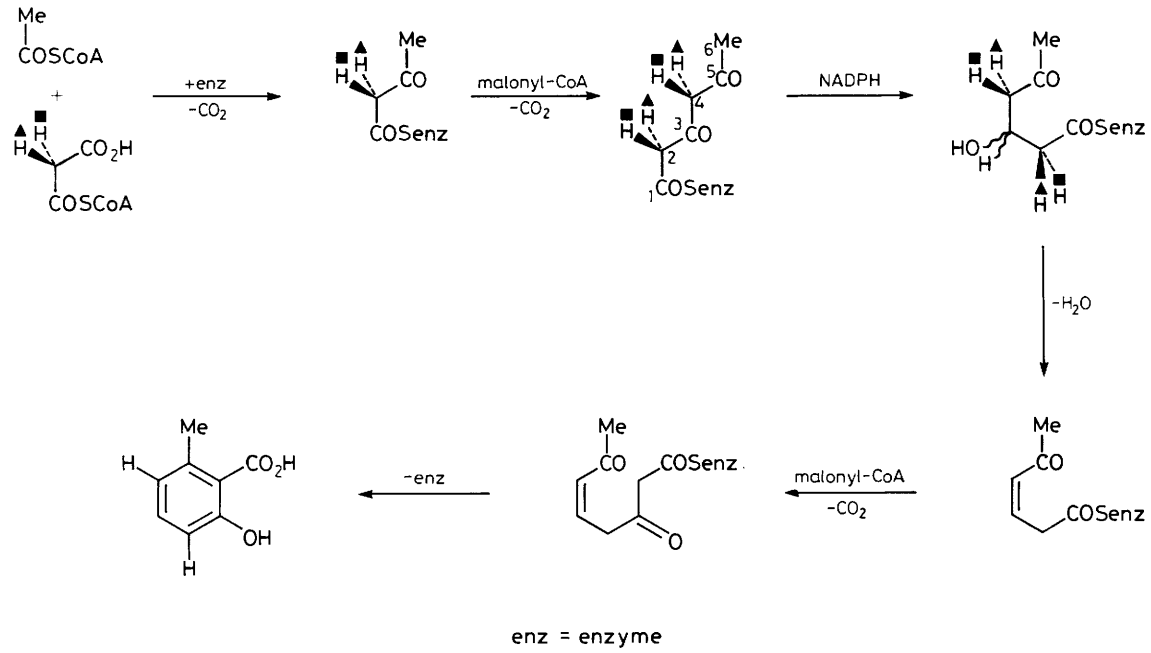
Peter M. Jordan*† and Jonathan B. Spencer

Department of Biochemistry, University of Southampton, Southampton SO9 3TU, U.K.

(*R*)- and (*S*)-[1-^{13}C; 2-^{2}H]malonate have been synthesized chemically and transformed, using succinyl-CoA transferase, into their respective paired malonyl-CoA derivatives; incorporation into 6-methylsalicylic acid has been achieved using homogeneous 6-methylsalicylic acid synthase isolated from *Penicillium patulum*; mass spectra of the resulting 6-methylsalicylic acids revealed that the hydrogen atoms removed from the two methylene groups at the 2- and 4-positions in the putative polyketide intermediate, have opposite absolute stereochemistry.

Malonic acid, as its malonyl-CoA derivative, is an important building unit for a large number of natural products which are thought to arise *via* polyketide intermediates.[1] Malonic acid shows *pro-pro*-chiral stereochemistry (Ca_2b_2) and, as such, its paired hydrogen and carboxy groups are indistinguishable by enzyme systems. The *pro-pro*-chiral centre in malonic acid has been synthesized in chiral form by preparing malonic acids, doubly labelled with ^{13}C and ^{2}H in the same molecule.[2,3] The stereospecific incorporation of both (*R*)- and (*S*)-[1-^{13}C; 2-^{2}H]malonate, as their respective malonyl-CoA derivatives, into palmitic acid using fatty acid synthase has been demonstrated in our laboratory.[2] The analysis of the products arising from such transformations is complex and requires either mass spectrometry[2] or NMR, or a combination of both techniques.

† *Present address*: Biochemistry Laboratory, School of Biological Sciences, Queen Mary College, University of London, Mile End Road, London E1 4NS, U.K.



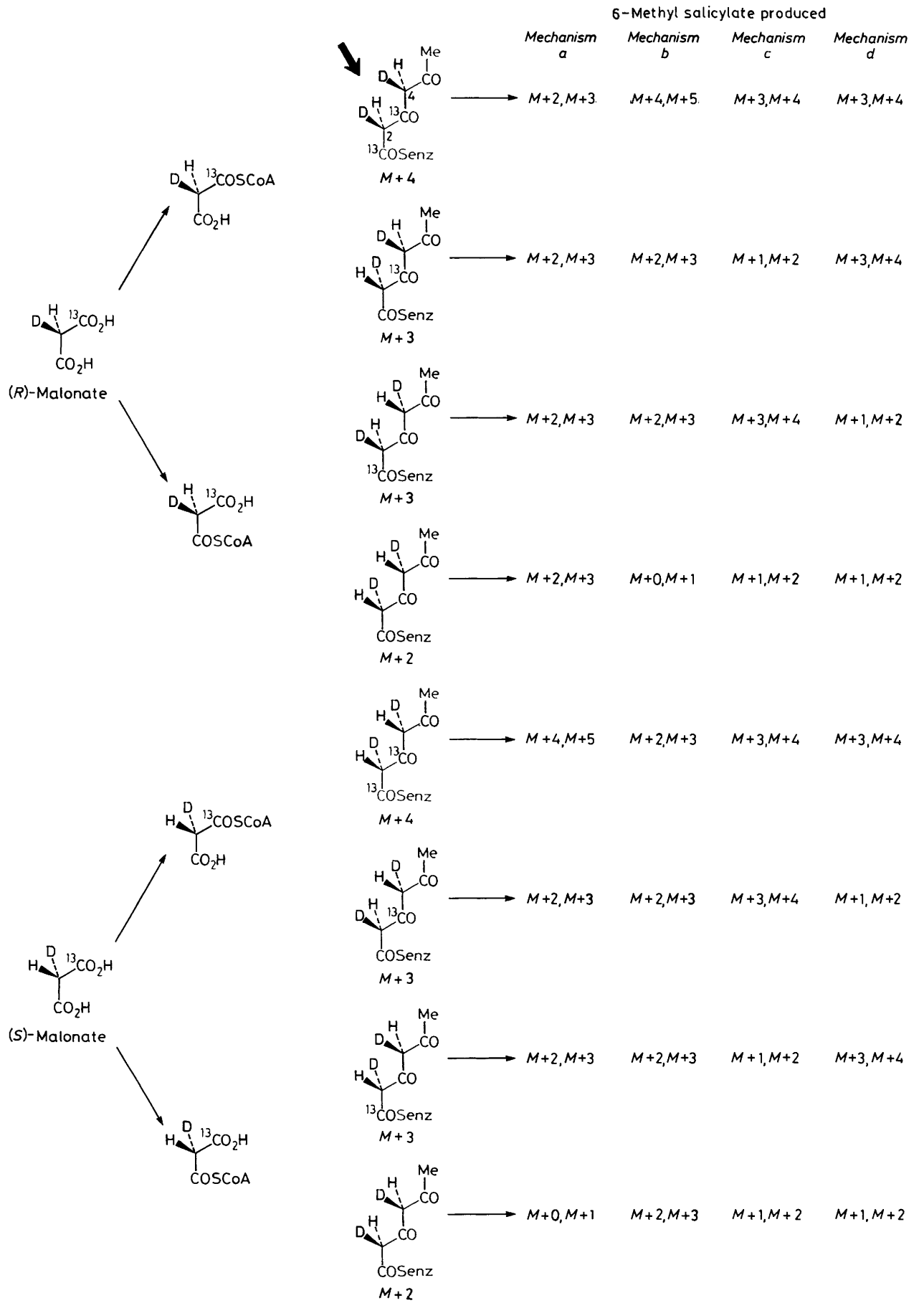
Scheme 1. Stages in the biosynthesis of 6-methylsalicylic acid.

6-Methylsalicylic acid is one of the simplest polyketide-derived natural products, being made up of one acetyl-CoA unit and 3 malonyl-CoA units, and represents a good model system for the study of mechanistic and stereochemical aspects of polyketide biosynthesis. The 6-methylsalicylic acid synthase from *Penicillium patulum* has been partially characterized[4] and the nature of enzyme-bound intermediates[5] has been investigated. Scheme 1 outlines the stages in the biosynthesis of 6-methylsalicylic acid. Mechanistic studies, using deuteriated acetate,[6,7] have provided evidence that the polyketide intermediates are processed stereospecifically, however, virtually no information is known about the absolute stereochemical course of any polyketide synthase, except for a report on the *enoyl*-reductase step in the biosynthesis of the polyketide cladosporin.[8,9]

The availability of (*R*)- and (*S*)-[1-^{13}C; 2-^{2}H]malonate, the methodology for their conversion into malonyl-CoA derivatives[2] using the enzyme succinyl-CoA transferase, which accepts malonic acid as a substrate, together with a strategy for the analysis of labelled products by mass spectrometry allowed us to investigate the mechanism and steric course of 6-methylsalicylic acid biosynthesis using the enzyme isolated from *Penicillium patulum*. Since 6-methylsalicylic acid arises from three molecules of malonyl-CoA, the use of malonyl-CoA derived from (*R*)- and (*S*)-[1-^{13}C; 2-^{2}H]malonate will result in the incorporation of up to three ^{13}C atoms and two ^{2}H atoms in each molecule of product. A study of the incorporation pattern of the ^{13}C and ^{2}H atoms provided information about the mechanism and steric course of the 6-methylsalicylic acid reaction.

As discussed previously[2,10] each Claisen condensation would be expected to occur with inversion of configuration and to result in the incorporation of the intact methylene hydrogen atoms (Scheme 1). The hydrogen atoms in the initial C-6 intermediate are indicated by ▲ (H_{Re}) or ■ (H_{Si}). On purely statistical grounds, eight labelled enzyme-bound intermediates will arise (Scheme 2) from one molecule of acetyl-CoA and two molecules of chiral malonyl-CoA, derived in turn from either (*R*)- or (*S*)-[1-^{13}C; 2-^{2}H]malonate, and will contain a ^{2}H label at the 2- and 4-positions (indicated by an arrow on the structure in Scheme 2, *top line*). The subsequent transformation of these intermediates can occur by different mechanisms each of which will lead to a product with a unique complement of ^{2}H label. On reaction with the third molecule of malonyl-CoA, each C-6 intermediate will have a 50% chance of incorporating a ^{13}C label, and will thus give rise to a pair of 6-methylsalicylic acid molecules, each differing by one mass unit.

Since two of the four hydrogen atoms in the C-6 intermediate are removed in the overall transformation into 6-methylsalicylic acid, four broad mechanistic routes (*mechanisms a, b, c,* and *d*) are required, each of which involves the loss of a different pair of hydrogen atoms from the 2- and 4-positions. Let us first consider the fate of the hydrogen atoms in the C-6 intermediate arising from chiral (*R*)-[1-^{13}C; 2-^{2}H]malonate (arrowed in Scheme 2). In *mechanism a*, in which H_{Si} is removed from the 2- and 4-positions of this intermediate, either ^{13}C *or* ^{2}H, but not both labels from each malonate-derived C-2 unit, are carried through to 6-methylsalicylic acid. Since the third malonyl-CoA unit contributes no ^{2}H and has a 50% chance of incorporating one ^{13}C atom, the 6-methylsalicylic acid molecules generated by this route will exhibit a mass of $M + 2$ and $M + 3$. The four possible C-6 intermediates arising from (*R*)-[1-^{13}C; 2-^{2}H]malonate will thus give eight labelled 6-methylsalicylic acid samples with masses of $M + 2$ and $M + 3$, although the position of the label will be different in each case (Scheme 2, *mechanism a*). Conversely, in *mechanism a*, (*S*)-[1-^{13}C; 2-^{2}H]malonate will contribute C-2 units, half of which contain *both* ^{13}C and ^{2}H labels and half of which contain *no* label. By similar considerations, the resulting 6-methylsalicylic acid samples will have very different mass distributions (Scheme 2, *mechanism a*) when compared to those arising from the (*R*)-[1-^{13}C; 2-^{2}H]malonate.



Scheme 2. Incorporation of (*R*)- and (*S*)-[1-^{13}C; 2-^{2}H]malonate into polyketide intermediates and their transformation into 6-methylsalicylic acid.

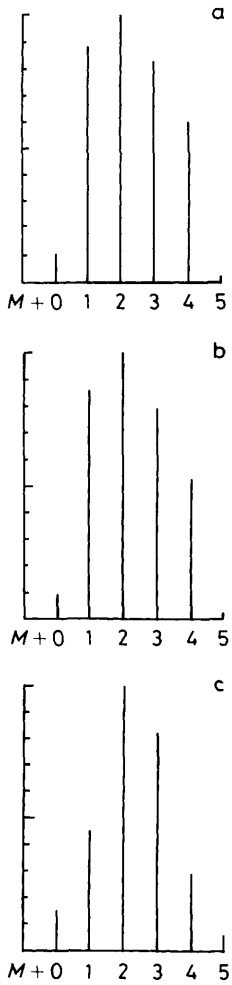


Figure 1. Observed mass spectra for 6-methylsalicylic acid biosynthesised from (a) (*R*)-[1-^{13}C; 2-^{2}H]malonate, (b) (*S*)-[1-^{13}C; 2-^{2}H]malonate, and (c) a mixture of (*R*)- and (*S*)-[1-^{13}C; 2-^{2}H]malonate.

In *mechanism b*, however, in which H_{Re} is removed from the 2- and 4-positions of the intermediate (arrowed in Scheme 2), the opposite result would occur, although the overall labelling in the products would be such that the mass spectrometric analysis would yield similar patterns (Scheme 2). Two other mechanisms, *mechanism c* in which H_{Re} is lost from postion 2 and H_{Si} from position 4 would give the pattern shown in Scheme 2, and *mechanism d*, where H_{Si} is lost from position 2 and H_{Re} from position 4, complete the four possibilities.‡ The above considerations would thus give, for *mechanism a*, relative intensities for the $M + 0$, $M + 1$, $M + 2$, $M + 3$, $M + 4$, and $M + 5$ peaks of 0,0,4,4,0,0, using (*R*)-malonate and 1,1,2,2,1,1 for (*S*)-malonate respectively. *Mechanism b* will give similar, but reversed, patterns. In both *mechanisms c* and *d* both (*R*)- and (*S*)-malonates will give similar mass distributions of 0,2,2,2,2,0. *Mechanisms a* and *b* may be thus readily distinguished from *mechanisms c* and *d* by mass spectrometry.

The results obtained from experiments in which (*R*)- and (*S*)-[1-^{13}C; 2-^{2}H]malonate were incubated in separate experiments with the coupled succinyl-CoA transferase and 6-methylsalicylic acid synthase enzymes are shown in Figure 1(a) and (b) respectively.§ The mass distributions indicate $M + 1$, $M + 2$, $M + 3$, and $M + 4$ as the major species from *both* isomers and are close to those expected for a mechanism in which the hydrogen atoms with opposite absolute orientations are stereospecifically removed (Scheme 2, *mechanisms c* and *d*). The presence of the $M + 1$ and $M + 4$ species in both sets of data is particularly diagnostic for *mechanisms c* and *d*. Furthermore, had *mechanism a* or *b* been operative then species with $M + 0$ and $M + 5$ would have been far more evident. Although a small amount of the $M + 0$ species is produced, this can be accounted for by exchange (15—20%) which tends to increase the species with lower mass and diminish those with higher mass. The very small amount of $M + 5$ can be accounted for by a limited amount of racemization. The cumulative considerations thus point to a mechanism involving the loss of hydrogen atoms with opposite orientations.

In order to secure further evidence for a mechanism in which the two hydrogen atoms arising from the opposite absolute orientations in malonate are removed in stereospecific processes, the (*R*)- and (*S*)-malonates were incubated together in a mixed experiment with 6-methylsalicylic acid synthase as before. Such an experiment would give all 32 possible permutations shown in Scheme 2. The result of mass spectrometric analysis of the resulting 6-methylsalicyclic acid derivatives are shown in Figure 1(c). The mass distributions interface perfectly with the data in Figure 1(a) and (b), and are close to the predicted mass distribution from the species in Scheme 2, indicating unambiguously that the reaction has proceeded with a high degree of steric control. These findings are, interestingly, in complete contrast to those found for the biosynthesis of the related aromatic polyketide-derived product, orsellenic acid, in which the results of incubating chiral malonic acids with extracts containing orsellinic acid synthase have been interpreted to indicate a mechanism involving the non-stereospecific manipulation of the methylene protons in the putative polyketide intermediate.[11]

The overall findings from our investigations thus eliminate *mechanisms a* and *b* where the hydrogen atoms with the same absolute configuration in malonic acid are removed. The experimental results presented do not, however, permit a distinction between *mechanisms c* and *d*, in which hydrogen atoms with different absolute configurations are lost,¶ and work is currently under way to resolve this matter.

‡ The configurations H_{Re} and H_{Si} refer to the C-6 intermediate on the top line of Scheme 2. The introduction of ^{13}C and ^{2}H into other positions of the intermediate change the priority and give rise to complications in the designation of absolute configuration. It is thus preferential to consider the symbols ▲ and ■ for labelling the paired hydrogen atoms rather than absolute configuration as shown in Scheme 1.

§ Each chiral malonate sample was incubated with succinyl-CoA, succinyl-CoA transferase, NADPH, acetyl-CoA, Tris/H_2SO_4 buffer, and homogeneous 6-methylsalicylic acid synthase, in 2 ml. The formation of 6-methylsalicylic acid was followed fluorimetrically. The 6-methylsalicylic acid was extracted into ether, converted into its trimethylsilyl derivative and purified by GLC. The derivatives were analysed using a V.G. Model 70SEQ mass spectrometer. All data shown are corrected for natural abundance and represent the $M + 0$ = 281 species arising from the loss of Me- from one of the trimethylsilyl groups of the parent trimethylsilyl derivative, $M + 0$ = 296.

¶ Although the results point to the loss of hydrogen atoms with the opposite orientations from the original malonates, it should be noted that in the putative C-6 hydroxy-intermediate shown in Scheme 1, the hydrogen atoms arising from opposite orientations are positioned on the same face of the intermediate and in principle could be manipulated by a single strategically placed enzyme group.

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