Investigation of the Mechanism and Steric Course of the Reaction Catalyzed by 6-Methylsalicylic Acid Synthase from *Penicillium patulum* Using (*R*)-[1-^{13}C;2-^{2}H]- and (*S*)-[1-^{13}C;2-^{2}H]Malonates†

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ABSTRACT: Chiral malonyl-CoA derivatives, enzymically synthesized from (*R*)- and (*S*)-[1-^{13}C;2-^{2}H]-malonates using succinyl-CoA transferase, were incorporated into 6-methylsalicylic acid with homogeneous 6-methylsalicylic acid synthase isolated from *Penicillium patulum*. Analysis of the 6-methylsalicylic acid formed established that the hydrogen atoms at the 3- and 5-positions are derived from opposite absolute configurations in malonyl-CoA. When acetoacetyl-CoA was used as the starter molecule, a single hydrogen atom is incorporated from the chiral malonates into the 3-position of the 6-methylsalicylic acid. Mass spectrometric analysis of the 6-methylsalicylic acid indicates that this hydrogen atom originates from H_{Re} of malonyl-CoA or H_{Si} in the polyketide intermediate. It is thus concluded that the hydrogen atom at the 5-position of 6-methylsalicylic acid originates from H_{Si} of malonyl-CoA or H_{Re} in the polyketide intermediate. During the reaction the enzyme also catalyzes the stereospecific exchange of hydrogen atoms in the polyketide intermediates. The impliciations of the stereochemical information from these experiments are discussed in relation to the mechanism of the 6-methylsalicylic acid synthase reaction.

The biosynthesis of the aromatic ring occurs by two main routes—the shikimate pathway and the polyketide pathway. The intermediates of the shikimate pathway are well documented, and many of the enzymes involved in their biosynthesis have been isolated and studied at both the mechanistic and structural levels (Dewick, 1989). In contrast, our understanding of polyketide-derived aromatic ring biosynthesis is not nearly as well understood, largely because the intermediates are labile and are enzyme-bound up to, and including, the point of aromatic ring release from the enzyme system. One of the simplest aromatic compounds, 6-methylsalicylic acid, is assembled from one molecule of acetyl-CoA and three molecules of malonyl-CoA by the multifunctional enzyme 6-methylsalicylic acid synthase (Dimroth et al., 1976). 6-Methylsalicylic acid is the first stable intermediate to be released into solution during the biosynthesis of patulin, a major secondary metabolite produced by *Penicillium patulum*. The 6-methylsalicylic acid synthase reaction follows many of the mechanistic principles involved in fatty acid biosynthesis, although, unlike fatty acid formation, there is only a single reduction step with the result that the remaining keto functions permit cyclization to the aromatic ring (Scheme I).

Polyketide synthases may be considered as belonging to two overlapping categories—first, those that catalyze a nonrepetitive synthesis in which a separate catalytic activity is involved at each biosynthetic stage and, second, those that catalyze a repetitive synthesis in which each catalytic function is involved several times. The enzyme deoxyerythronilide B synthase falls into the former category (Bevitt et al., 1992; Donadio & Katz, 1992), whereas 6-methylsalicylic acid synthase is representative of the latter. In this respect 6-methylsalicylic acid synthase has important similarities to fatty acid synthase, in which the catalytic sites recognize repetitively the same classes of intermediates attached to the acyl carrier protein during each of the seven reaction cycles (Lynen, 1980). 6-Methylsalicylic acid synthase, however, differs from fatty acid synthase in that after the addition of each malonyl-CoA, a different reaction cycle occurs.

The precise detail of what dictates the order of events in the 6-methylsalicylic acid synthase reaction relies on a comprehensive understanding of the stereochemical events at the methylene positions of the enzyme-bound intermediates since their conformation at the catalytic site is likely to have a direct bearing on the mechanistic course of the reaction at each stage of the assembly process. A study by Abell and Staunton (1981) has addressed this aspect by following the incorporation of [1-^{13}C;2-^{2}H$_3$]acetate into 6-methylsalicylic acid in cultures of *P. patulum*. NMR analysis of the 6-methylsalicylic acid showed that 80% retention of deuterium at position C-3 and 70% in position C-5 had occurred (see Scheme I). Further experiments in which [1-^{13}C;2-^{2}H]acetate was incorporated into 6-methylsalicylic acid, presumably via the two epimeric monodeuterated malonyl-CoA species, led to a 50% incorporation of deuterium label into the product (Abell & Staunton, 1984). Although this study provided no information about the absolute stereochemical course of the reaction, Abell and Staunton concluded that the methylene hydrogen atoms in the polyketide intermediates are handled stereospecifically by the 6-methylsalicylic acid synthase enzyme.

In 6-methylsalicylic acid synthesis a single reduction stage generates a hydroxy group at the 3-position of the C-6 intermediate. The equivalent step in the fatty acid synthase reaction has always been shown to result in the formation of a (3*R*)-hydroxy intermediate (Sedgwick et al., 1978; Reese et al., 1988). However, in polyketide biosynthesis examples with *R* and *S* configurations have been described. For instance,

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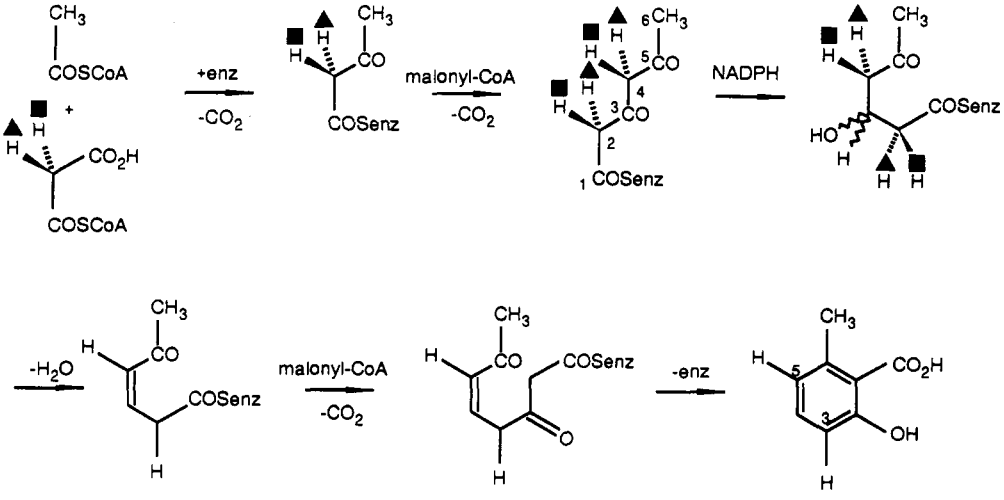
Scheme I: Biosynthesis of 6-Methylsalicylic Acid [after Bu'Lock (1979)]



Vederas and co-workers (Reese et al., 1988) have investigated the absolute configuration at positions C-14 and C-3 of cladosporin and found both to be of the *S* configuration on the growing chain. More recently, in vivo studies with the polyketide synthase aspyrone synthase, of *Aspergillus niger*, have shown that the enzyme accepts only the (3*R*)-hydroxybutyryl-*N*-acetylcysteamine derivative, suggesting that the enzyme-bound acetoacetyl moiety is reduced to the (3*R*)-3-hydroxybutyrate intermediate (Jacobs et al., 1991).

Despite the elegance of these studies, the steric events at the methylene groups during the earlier stages in polyketide biosynthesis can only be determined by the incorporation of stereospecifically labeled malonyl-CoA of known chirality. The major drawback to this approach is the fact that the methylene hydrogen atoms of malonyl-CoA are extremely labile, leading to the rapid racemization of the chiral center. This problem may be overcome by the use of (*R*)-[1-^{13}C;2-^{2}H]- and (*S*)-[1-^{13}C;2-^{2}H]malonates which are an order of magnitude more stable than the CoA thiol esters (Huang et al., 1986; Jordan et al., 1986). The conversion of each chiral malonate into its pair of malonyl-CoA derivatives is easily accomplished by enzymic means, allowing the controlled formation of the malonyl-CoA derivatives for incorporation experiments (Jordan et al., 1986; Spencer & Jordan, 1993). Despite this, experiments with chiral malonates need to be carried out rapidly to prevent undue exchange and racemization of the deuterium label (Jordan et al., 1986; Spencer & Jordan, 1993) and require the use of highly purified enzymes to permit the rapid formation of sufficient product for analysis. Studies using chiral malonic acids have been carried out to investigate the steric course of the polyketide synthase involved in the biosynthesis of orsellinic acid (Woo et al., 1989; Spencer & Jordan, 1992b). This paper describes the synthesis of 6-methylsalicylic acid from (*R*)-[1-^{13}C;2-^{2}H]- and (*S*)-[1-^{13}C;2-^{2}H]malonates to elucidate the stereochemical course of hydrogen elimination during the reaction. The mechanistic implications of the results are discussed. Preliminary accounts of this work have been published (Jordan & Spencer, 1990; Spencer & Jordan, 1990).

RESULTS AND DISCUSSION

Synthesis of (R)- and (S)-[1-^{13}C;2-^{2}H]Malonic Acids. The *R* and *S* chiral samples of malonic acids were synthesized from (2*S*,3*R*)-[1,4-$^{13}C_2$;3-^{2}H]malic acid and (2*S*,3*S*)-[1,4-$^{13}C_2$;2,3-$^{2}H_2$]malic acid, respectively, by oxidation with potassium permanganate as described previously (Jordan et al., 1986; Jordan & Spencer, 1991; Spencer & Jordan, 1993). Relevant details are also given under Experimental Procedures.

Determination of Conditions That Minimize Exchange of the Methylene Hydrogens of Malonate during Its Incorporation into 6-Methylsalicylic Acid. The incorporation of malonate into 6-methylsalicylic acid was achieved by a coupled reaction in which malonate was first converted into malonyl-CoA (Jordan et al., 1986; Spencer & Jordan 1993) in a reaction catalyzed by succinyl-CoA transferase (eq 1). The resulting malonyl-CoA was then incorporated into 6-methylsalicylic acid by purified 6-methylsalicylic acid synthase.

succinyl-CoA + malonic acid → malonyl-CoA + succinic acid (1)

A prerequisite for any stereochemical investigations on the biosynthesis of 6-methylsalicylic acid from chiral malonate is the establishment of conditions under which succinyl-CoA transferase and 6-methylsalicylic acid synthase can be coupled to minimize the exchange of the methylene hydrogens of malonate. Preliminary experiments were carried out, therefore, to determine the level of deuterium that was incorporated into 6-methylsalicylic acid from [2-$^{2}H_2$]malonate. The deuterium atom located at position 5 of 6-methylsalicylic acid is derived from the first malonyl-CoA molecule to be condensed by 6-methylsalicylic acid synthase, while that at position 3 is from the second malonyl-CoA molecule (Scheme I). If exchange does take place under the experimental conditions, singly labeled and unlabeled species of 6-methylsalicylic acid would be expected. The level of exchange of the methylene deuteriums of malonate can therefore be calculated from the relative abundance of M + 0, M + 1, and M + 2 ions of 6-methylsalicylic acid produced. The third malonyl-CoA molecule loses both of its methylene hydrogen atoms during the formation of the aromatic ring and thus does not contribute deuterium label to the product.

The mass spectrum of 6-methylsalicylic acid, derived from [2-$^{2}H_2$]malonate under conditions determined to be most favorable for preventing exchange of the methylene hydrogens, is shown in Figure 1a. This spectrum indicates that the major species of product has a mass of M + 2, although the presence of M + 0 and M + 1 species indicates that some loss of label has occurred. A theoretical spectrum generated by mathematical modeling to simulate 15% exchange (Figure 1b) is close to the experimental observation.

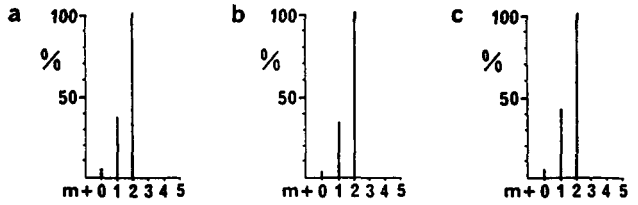


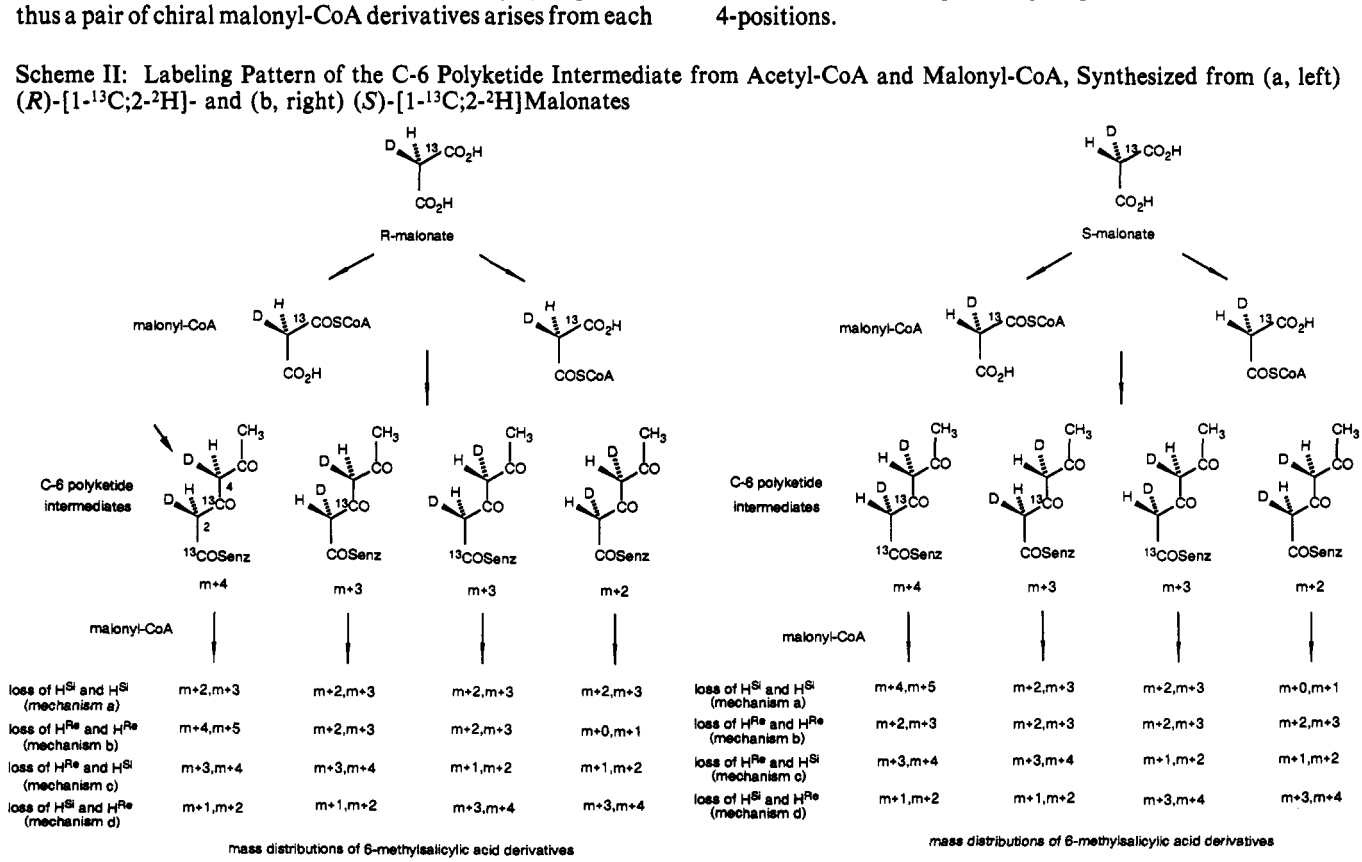
FIGURE 1: Mass spectra of 6-methylsalicylic acid enzymically synthesized with acetyl-CoA as a starter from (a) [2-$^2H_2$]malonate, (b) theoretical spectrum predicted for (a), and (c) malonic acid generated from (2-*RS*)-[3-$^2H_2$]malate. 6-Methylsalicylic acid was analyzed as the bis(trimethylsilyl) derivative ($m + 0 = 281$). Details of the incubation conditions and analytical methods are provided under Experimental Procedures.

To ascertain whether the conditions of the permanganate oxidation result in the exchange of the methylene hydrogen atoms of malonic acid, the synthesis of 6-methylsalicylic acid was repeated under the same conditions as above except that [2-$^2H_2$]malonate was generated by permangante oxidation of (2*RS*)-[3-$^2H_2$]malate. The mass spectrum of 6-methylsalicylic acid so derived (Figure 1c) is broadly similar to that from the enzymic experiment shown in Figure 1a. This indicates that the conditions employed for both the permanganate oxidation and the enzymic incorporation do not cause significant exchange of the methylene hydrogens of malonate and that the byproducts of the oxidation do not interfere with the enzymic reactions.

Investigation of the Enzymic Synthesis of 6-Methylsalicylic Acid from Chiral Malonates. 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 into each molecule of product. The succinyl-CoA transferase enzyme used for the formation of the malonyl-CoA samples cannot, despite the chiral nature of the substrate, distinguish between the two malonate carboxyl groups, and thus a pair of chiral malonyl-CoA derivatives arises from each chiral malonate. The condensation of malonyl-CoA, in which the two hydrogen atoms are indicated by ■ (H_{Re}) and ▲ (H_{Si}), would be expected to proceed by inversion of configuration. Such a steric course has been demonstrated in fatty acid synthase (Sedgwick et al., 1978), the monocerin polyketide synthase from *Dreschlera ravenelli* (T. Simpson, personal communication) and in all other Claisen condensations studied to date (Hanson & Rose, 1975). In the case of 6-methylsalicylic acid synthase, inversion at each condensation would therefore result in the incorporation of both hydrogen atoms into the putative enzyme-bound C-6 polyketide intermediate as indicated in Scheme I.

On purely statistical grounds, eight labeled enzyme-bound intermediates will arise (Scheme II) 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 each will contain a ^{2}H label at the 2- and 4-positions of the C-6 polyketide intermediate. Once incorporated into the malonyl-CoA derivatives, the subsequent manipulations of the ^{13}C and ^{2}H labels will be sterically interrelated in every molecule. Thus, whatever the stereochemical course of the subsequent events, four C-6 intermediates with unique complements of ^{2}H and ^{13}C label will be formed from each chiral malonate for any given mechanism. On reaction with the third molecule of malonyl-CoA, each C-6 intermediate will have a 50% chance of incorporating a further ^{13}C label and will thus give rise to a pair of 6-methylsalicylic acid molecules, differing by 1 mass unit from the other, leading to eight possible labeling patterns in 6-methylsalicylic acid for each chiral malonate. Since two of the four malonyl-CoA-derived methylene hydrogen atoms in the C-6 intermediate are removed in the overall transformation into 6-methylsalicylic acid, four broad mechanistic routes are possible, designated mechanisms a–d, each of which involves the loss of a different pair of hydrogen atoms from the 2- and 4-positions.

Scheme II: Labeling Pattern of the C-6 Polyketide Intermediate from Acetyl-CoA and Malonyl-CoA, Synthesized from (a, left) (*R*)-[1-^{13}C;2-^{2}H]- and (b, right) (*S*)-[1-^{13}C;2-^{2}H]Malonates



The fate of the hydrogen atoms arising from chiral (*R*)-[1-^{13}C;2-^{2}H]malonate by these mechanisms is first considered in the C-6 intermediate arrowed in Scheme IIa. In mechanism a, H_{Si} (H_{Re} in malonyl-CoA) is eliminated from the 2- and 4-positions of the C-6 intermediate; thus *either* ^{13}C *or* ^{2}H, *but not both labels*, from each malonate-derived C-2 unit is 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 mechanism a 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 labeled 6-methylsalicylic acid samples with masses of M + 2 and M + 3, although the positions of the labels will be different in each case. 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 exhibit mass distributions extending from M + 0 to M + 5 when compared to those arising from the (*R*)-[1-^{13}C;2-^{2}H]malonate (Scheme IIa).

In mechanism b, however, in which H_{Re} (H_{Si} in malonyl-CoA) is removed from the 2- and 4-positions of the arrowed C-6 intermediate in Scheme IIa, the converse labeling patterns to those for mechanism a would occur. Two other mechanisms, mechanism c in which H_{Re} is lost from the 2-position and H_{Si} from the 4-position and mechanism d where H_{Si} is lost from the 2-position and H_{Re} from the 4-position, complete the four possibilities and are shown in Scheme IIa. In the same way the labeling derived from chiral (*S*)-[1-^{13}C;2-^{2}H]malonate for the four mechanistic possibilities is shown in Scheme IIb.

The above considerations would thus give, for mechanism a, relative intensities in the product for the M + 0, M + 1, M + 2, M + 3, M + 4, and M + 5 ions of 0, 0, 4, 4, 0, and 0, using (*R*)-malonate and 1, 1, 2, 2, 1, and 1 for (*S*)-malonate, respectively. Mechanism b would give similar, but reversed, intensities. In mechanisms c and d both (*R*)- and (*S*)-malonates will give similar mass distributions of 0, 2, 2, 2, 2, and 0. The relative abundance of the various labeled species of 6-methylsalicylic acid produced from (*R*)- and (*S*)-malonates by the four mechanisms in Scheme II is summarized in Figure 2. Mechanisms a and b may be thus readily distinguished from one another and also from mechanisms c and d by mass spectrometry.

The results obtained from experiments in which (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate were incubated in separate experiments with succinyl-CoA transferase and 6-methylsalicylic acid synthase are shown in parts a and b, respectively, of Figure 3. 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 (Figure 3d,e) expected for a mechanism in which hydrogen atoms with opposite absolute orientations are removed stereospecifically (mechanism c or d). The presence of 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, than species with M + 0 and M + 5 would have been far more evident. The cumulative considerations thus point to a mechanism involving the loss of hydrogen atoms with opposite orientations from the C-6 intermediate during the formation of 6-methylsalicylic acid through either mechanism c or d.

Although a small amount of the M + 0 species is produced, this can be accounted for by exchange (about 15%), which tends to increase the species of lower mass and diminish those with higher mass. Exchange of this magnitude was also noted

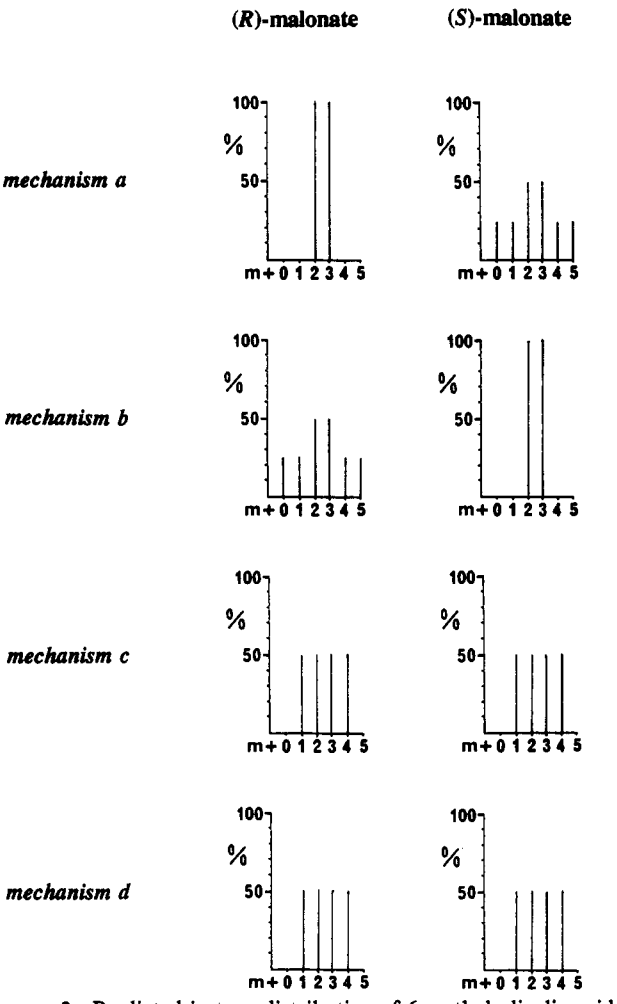


FIGURE 2: Predicted isotope distribution of 6-methylsalicylic acid derived from acetyl-CoA and either (*R*)-[1-^{13}C;2-^{2}H]- or (*S*)-[1-^{13}C;2-^{2}H]malonate by mechanisms a–d.

in the preliminary experiments with [2-$^{2}H_2$]malonate described above. The very small amount of M + 5 can be accounted for by a limited amount of racemization. It is interesting that the results show that the exchange of the methylene hydrogen atoms is greater than their racemization, suggesting that the enzyme is catalyzing a stereospecific deprotonation reaction.

Biosynthesis of 6-Methylsalicylic Acid from (R,S)-[1-^{13}C;2-^{2}H]Malonate. To eliminate the possibility that the mass spectra of 6-methylsalicylic acid derived from (*R*)- and (*S*)-malonate are the same because of extensive racemization of these samples, a crucial experiment was performed in which (*R,S*)-[1-^{13}C;2-^{2}H]malonate, prepared by permanganate oxidation of a mixture composed of equal quantities of (2*S*,3*R*)-[1,4-$^{13}C_2$;3-^{2}H]malic acid and (2*S*,3*S*)-[1,4-$^{13}C_2$;2,3-$^{2}H_2$]-malic acid, was transformed into 6-methylsalicylic acid. Such an experiment should give all 32 possible permutations of labeled 6-methylsalicylic acid (Scheme II). Thus, the predicted mass spectra of 6-methylsalicylic acids derived from (*R,S*)-[1-^{13}C;2-^{2}H]malonate would have relative intensities for M + 0, M + 1, M + 2, M + 3, M + 4, and M + 5 peaks of 1, 5, 10, 10, 5, and 1 respectively. The experimental results from this experiment, shown in Figure 3c, are close to the predicted mass distributions if an allowance is made for 15% exchange (Figure 3f). These results prove that extensive racemization of the samples does not occur and that the reaction has proceeded with a very high degree of steric control. The uncorrected raw data relating to the spectra in Figure 3a–c are shown in Figure 3g–i for completeness.

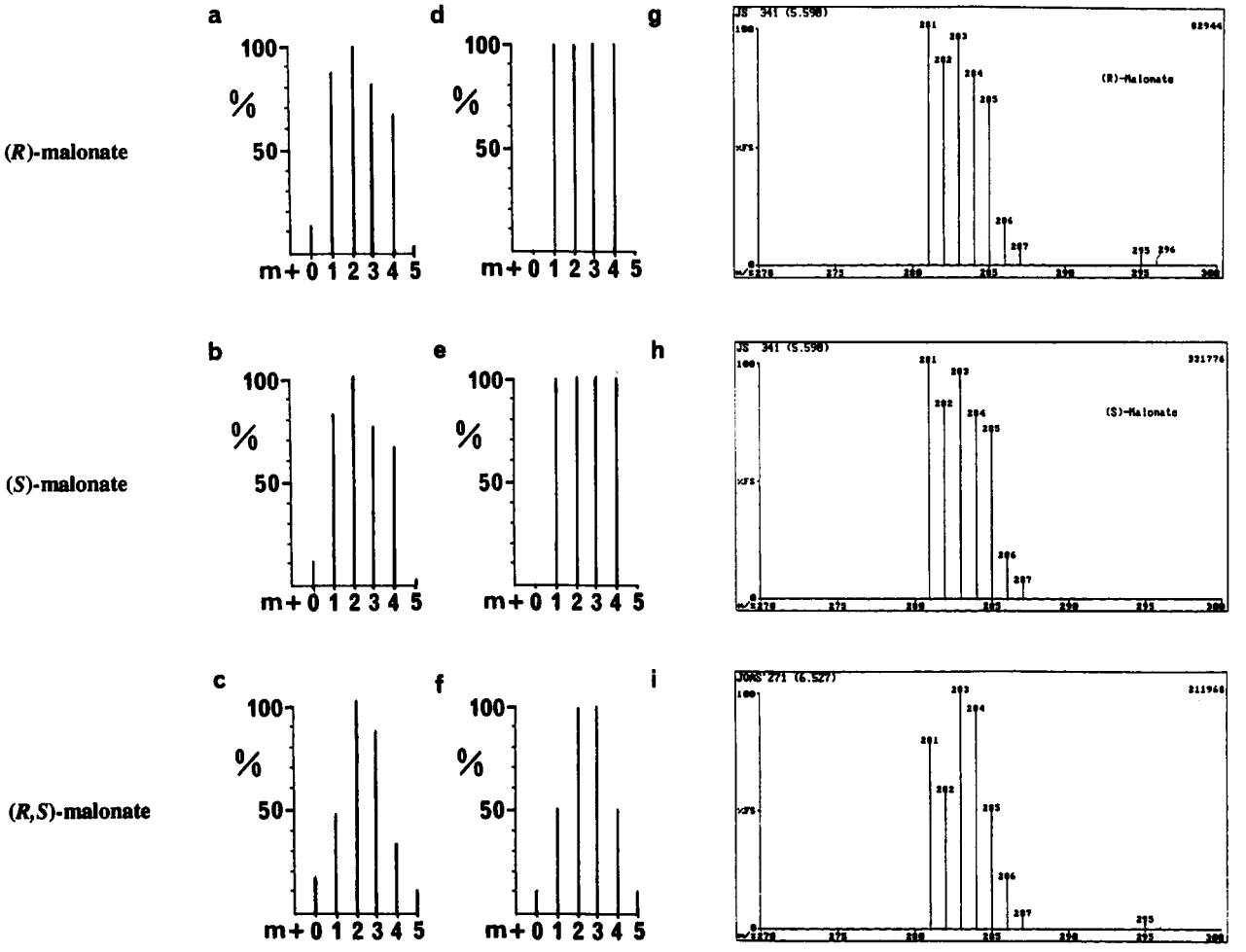


FIGURE 3: Mass spectra of 6-methylsalicylic acid enzymically synthesized from malonic acid with acetyl-CoA as a starter molecule from (a) (*R*)-malonate, (b) (*S*)-malonate, and (c) a mixture of (*R*)-malonate and (*S*)-malonate. Spectra are corrected for natural abundance. Theoretical spectra predicted for (a), (b), and (c) are represented by (d), (e), and (f), respectively, assuming mechanism c or d. Raw uncorrected data for (a), (b), and (c) are shown in (g), (h), and (i), respectively. 6-Methylsalicylic acid was analyzed as the bis(trimethylsilyl) derivative ($m + 0 = 281$). Details of the incubation conditions and analytical methods are provided under Experimental Procedures.

Incorporation of Acetoacetyl-CoA as a Starter Molecule for 6-Methylsalicylic Acid Synthase. The two sets of isotopically labeled 6-methylsalicylic acid molecules derived from (*R*)- and (*S*)-malonate cannot be resolved from one another by mass spectrometry. To distinguish between mechanisms c and d, acetoacetyl-CoA, which is incorporated into 6-methylsalicylic acid at about 5% of the rate of acetyl-CoA, was used to bypass the first condensation between acetyl-CoA and malonyl-CoA. The use of acetoacetyl-CoA permits the regiospecific incorporation of the methylene carbon atom of chiral malonic acid solely into the C-2-position of the C-6 intermediate and thus incorporates ^2H label only into the 3-position of 6-methylsalicylic acid. Although the third malonyl-CoA moiety will provide a ^{13}C label in 50% of the molecules synthesized, both hydrogens of this C-2 unit are lost during cyclization and aromatization and thus do not contribute to the labeling of hydrogens in the final product.

Preliminary experiments in which acetoacetyl-CoA was incorporated with [2-^{13}C]malonic acid resulted in substantial amounts of 6-methylsalicylic acid with mass of M + 3, showing that the acetoacetyl-CoA was being cleaved by thiolase present as a contaminant of the succinyl-CoA transferase (Figure 4a). To overcome this problem, thiolase-free succinyl-CoA transferase was prepared using high-resolution ion-exchange chromatography. When such steps were taken, 6-methylsalicylic acid containing only two ^{13}C atoms was generated from [2-^{13}C]malonic acid, yielding a spectrum with only the expected M + 2 species, indicating that the acetoacetyl-CoA had been incorporated intact (Figure 4b).

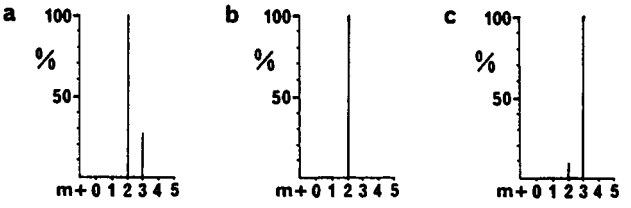
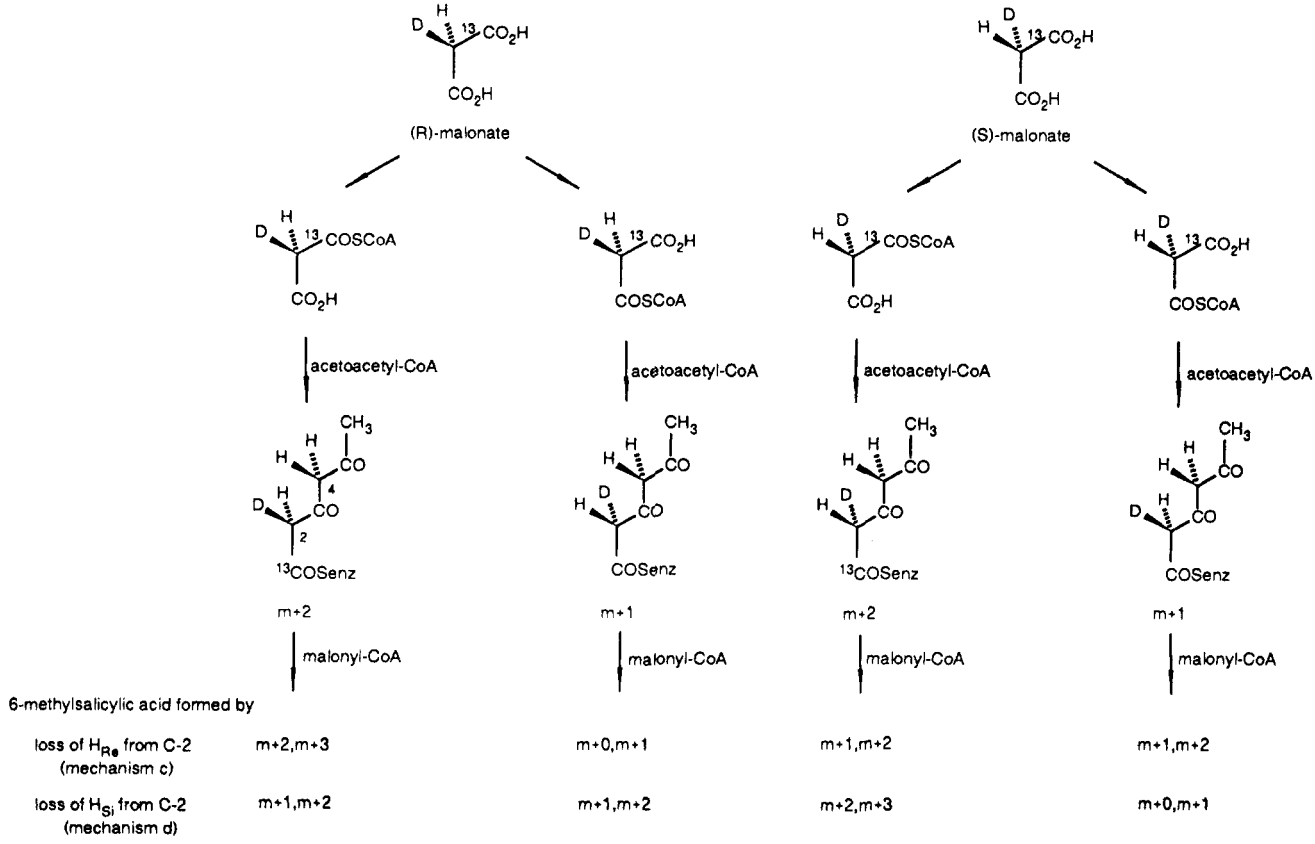


FIGURE 4: Mass spectra of 6-methylsalicylic acid enzymically synthesized with acetoacetyl-CoA as the starter molecule from (a) [2-^{13}C]malonic acid in the presence of commericially produced succinyl-CoA transferase containing contaminating thiolase, (b) [2-^{13}C]malonic acid, as in (a) but in the presence of highly purified thiolase-free succinyl-CoA transferase, and (c) [2-^{13}C;2-^{2}H]malonic acid. Spectra are corrected for natural abundance. 6-Methylsalicylic acid was analyzed as the bis(trimethylsilyl) derivative ($m + 0 = 281$). Details of the incubation conditions and analytical methods are provided under Experimental Procedures.

Further experiments were carried out to determine conditions under which exchange of the methylene hydrogens originally in malonate could be minimized. This was achieved by measuring the incorporation of deuterium into 6-methylsalicylic acid from [2-^{13}C;2-$^2H_2$]malonate. The ^{13}C label was included in the deuterated malonate to shift the 6-methylsalicylic acid spectrum to a position 2 mass units higher. The most favorable incubation conditions resulted in a 9:100

Scheme III: Labeling Patterns of 6-Methylsalicylic Acid from Acetoacetyl-CoA and Malonyl-CoA, Synthesized from (*R*)-[1-^{13}C;2-^{2}H]- and (*S*)-[1-^{13}C;2-^{2}H]Malonates



ratio of undeuterated 6-methylsalicylic acid:deuterated 6-methylsalicylic acid with masses of 283:284 as shown in Figure 4c. Therefore, only 8% exchange of the deuterium atoms of [2-^{13}C;2-$^{2}H_2$]malonate occurs, significantly less than that when acetyl-CoA was used as a starter unit. This high incorporation of label ensures that the mass spectra of 6-methylsalicylic acid derived from acetoacetyl-CoA and chiral (*R*)- or (*S*)-malonates will be meaningful.

It is interesting that a greater (15% per position average) exchange was observed when acetyl-CoA and [2-$^{2}H_2$]malonate were the substrates compared to the experiments when acetoacetyl-CoA was used. This suggests that there is more rapid exchange at C-4 (22%) than at C-2 (8%) in the C-6 intermediate. Although the results from the two experiments should be compared with caution, it is significant that a similar observation was made previously in NMR studies on 6-methylsalicylic acid synthase (Abell & Staunton, 1984).

Determination of the Absolute Configuration of the Hydrogens Eliminated during the Biosynthesis of 6-Methylsalicylic Acid from Chiral Malonate Using Acetoacetyl-CoA as a Starter Molecule. Using acetoacetyl-CoA as a starter molecule with (*R*)- or (*S*)-[1-^{13}C;2-^{2}H]malonic acid mechanism c, involving the elimination of H_{Re} from position 2 of the C-6 intermediate, 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 III and Figure 5a,c. Elimination of H_{Si} from the 2-position of the C-6 intermediate, mechanism d, would give the opposite mass distributions for the (*R*)- or (*S*)-[1-^{13}C;2-^{2}H]malonic acid, respectively, as shown in Scheme III and Figure 5b,d. Accordingly, (*R*)- and (*S*)-malonates were incubated in separate coupled experiments with succinyl-CoA transferase, acetoacetyl-CoA, NADPH, and homogeneous 6-methylsalicylic acid synthase, and the resulting 6-methylsalicylic acid samples were analyzed as their trimethylsilyl derivatives using GLC/MS. The results 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 (Figure 6a), whereas the 6-methylsalicylic acid arising from the (*S*)-malonate contains species of M + 1 and M + 2 (Figure 6b). The mass spectra are extremely close to those theoretically predicted for mechanism c (Figure 6c,d). The uncorrected data are shown in Figure 6e,f. This result establishes that the hydrogen atom (■) at the 3-position of 6-methylsalicylic acid originates from H_{Re} of malonyl-CoA (H_{Si} in the designated C-6 polyketide intermediate in Schemes II and III), indicating that the H_{Re} (▲) is eliminated from the 2-position of the C-6 intermediate

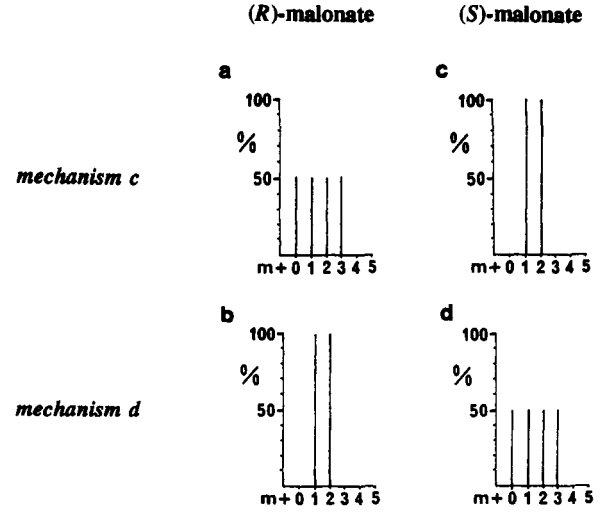


FIGURE 5: Predicted isotope distribution of 6-methylsalicylic acid derived from acetoacetyl-CoA and either (*R*)-[1-^{13}C;2-^{2}H]- or (*S*)-[1-^{13}C;2-^{2}H]malonate by mechanisms c and d.

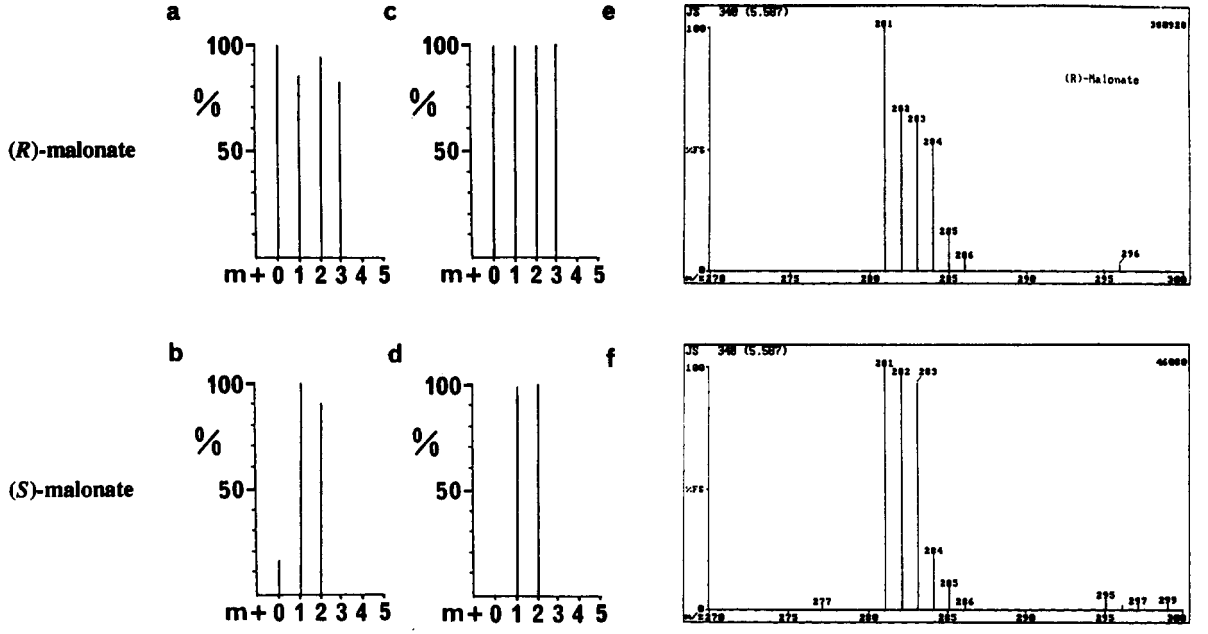
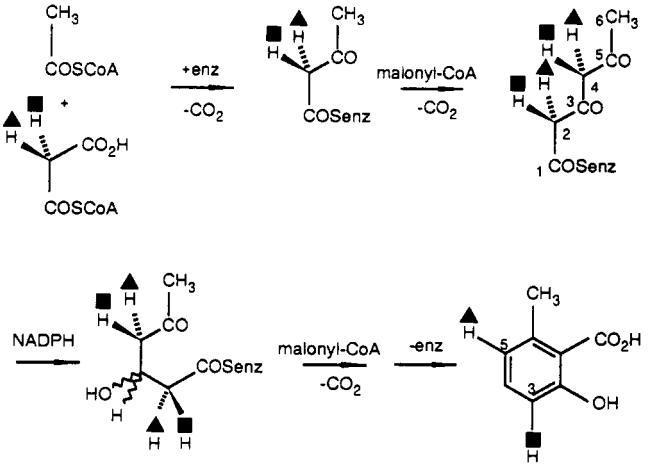


FIGURE 6: Mass spectra of 6-methylsalicylic acid enzymically synthesized from malonic acid with acetoacetyl-CoA as the starter molecule from (a) (*R*)-malonate and (b) (*S*)-malonate. Spectra are corrected for natural abundance. Theoretical spectra predicted for (a) and (b) are represented by (c) and (d), respectively, assuming mechanism c. Raw uncorrected data from (a) and (b) are shown in (e) and (f), respectively. 6-Methylsalicylic acid was analyzed as the bis(trimethylsilyl) derivative ($m + 0 = 281$). Details of the incubation conditions and analytical methods are provided under Experimental Procedures.

Scheme IV: Labeling of the 3- and 5-Positions in 6-Methylsalicylic Acid Deduced from Incorporation Experiments with Malonyl-CoA, Synthesized from (*R*)-[1-^{13}C;2-^{2}H]- and (*S*)-[1-^{13}C;2-^{2}H]Malonates (See also Table I)



(Scheme IV). Since the previous experiments, using acetyl-CoA as the starter molecule, which results in the labeling 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, it follows that the hydrogen atom eliminated at C-4 arises from H_{Si} of the polyketide intermediate. Therefore, the hydrogen atom incorporated at C-5 of 6-methylsalicylic acid (▲) must originate from H_{Si} in malonyl-CoA (Scheme IV). This experiment eliminates mechanism d. The stereochemical conclusions of the above experiments are summarized in Table I and in Scheme IV.

Mechanism of 6-Methylsalicylic Acid Formation. It is generally considered that 6-methylsalicylic acid synthase from *P. patulum* catalyzes the condensation reactions with acetyl-CoA and malonyl-CoA substrates by reactions closely analogous to the fatty acid synthase reactions. This is borne out by the strong sequence similarities between the primary protein

Table I: Summary of the Retention and Loss of Labeled Hydrogen Atoms from Malonyl-CoA and the Putative C-6 Polyketide Intermediate during the Biosynthesis of 6-Methylsalicylic Acid Catalyzed by 6-Methylsalicylic Acid Synthase from *P. patulum* Determined from Experiments with (*R*)-[1-^{13}C;2-^{2}H]- and (*S*)-[1-^{13}C;2-^{2}H]Malonates (See also Scheme IV)

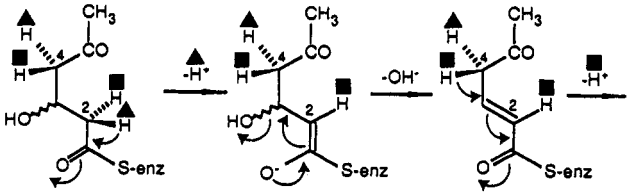
final position of label in 6-methyl-salicylic acid	absolute configuration of hydrogen atoms at C-2 of malonyl-CoA		position of label in C-6 polyketide intermediate	absolute configuration of hydrogen atoms in the C-6 polyketide intermediate	
	retained	lost		retained	lost
C-3	H_{Re} ■	H_{Si} ▲	C-2	H_{Si} ■	H_{Re} ▲
C-5	H_{Si} ▲	H_{Re} ■	C-4	H_{Re} ▲	H_{Si} ■

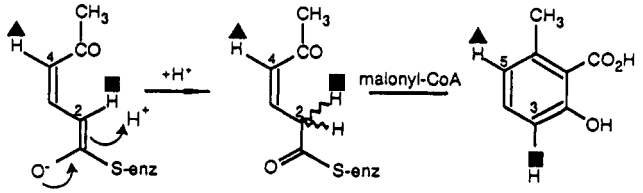
structures derived from the nucleotide sequences of rat fatty acid synthase (Witkowski et al., 1991) and 6-methylsalicylic acid synthase (Beck et al., 1990). The protein sequences specifying acyl transferase, β-ketoacyl synthase, and acyl carrier protein domains, all of which are required for the condensation reactions, are present in both enzyme systems. Support for a closely related three-dimensional arrangement between the sulfhydryl groups of the β-ketoacyl synthase and acyl carrier protein domains in rat fatty acid synthase and 6-methylsalicylic acid synthase may also be inferred from their similar reactions with the cross-linking reagent 1,3-dibromopropan-2-one (Stoops & Wakil, 1981; Spencer & Jordan, 1992a), suggesting that the catalytic unit of both enzyme systems is a functional dimer. It is assumed, for the purposes of the discussion, that all the Claisen condensations have proceeded with inversion of configuration (Scheme I) as is the generally accepted rule (Hanson & Rose, 1975).

The stereochemical findings from the above experiments on 6-methylsalicylic acid synthase may now be considered in the context of three broad mechanisms.

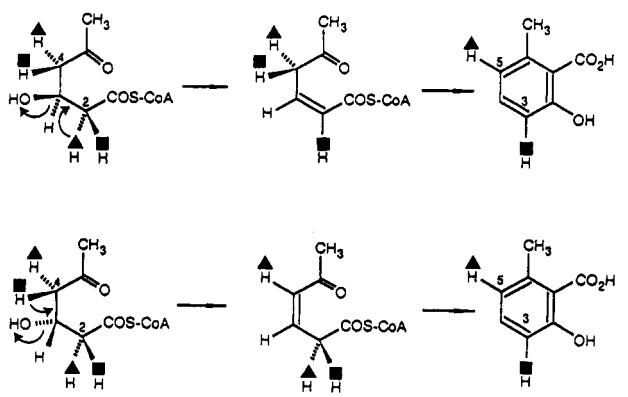
Mechanism A is analogous to the reaction catalyzed by β-hydroxyacyl-decanoyl-CoA dehydratase (Scheme V) and results in the formation of a cis double bond (Scott et al., 1974). This reaction initially involves the elimination of water,

Scheme V: Mechanism A—Formation of a Cis Double Bond by an Elimination–Isomerization Mechanism [after Scott et al. (1974)]





Scheme VI: Mechanism B—One-Step Elimination of Water To Form a Cis Double Bond: (a) Removal of H_{Re} from Position C-2 of the C-6 Polyketide Intermediate; (b) Removal of H_{Si} from Position C-4 of the C-6 Polyketide Intermediate[a]

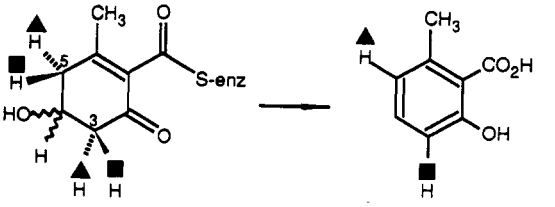


[a] An anti-elimination in (a) would require the (*S*)-alcohol. An anti-elimination in (b) would require the (*R*)-alcohol.

in a similar manner to that catalyzed by fatty acid synthase, to form a trans double bond. However, this intermediate is then transformed into Δ^3-*cis*-decanoyl-CoA by the enzyme. The steric course of the allylic rearrangement catalyzed by 3-hydroxydecanoyl-CoA dehydratase has been shown to be a suprafacial process, consistent with a "one enzymic base" mechanism (Schwab & Klassen, 1984). The results of the experiments described in our studies with chiral malonate indicated that H_{Re} (▲) is eliminated from the 2-position and H_{Si} (■) from the 4-position of the C-6 intermediate. Although these hydrogen atoms are of opposite absolute configurations, if the C-6 intermediate assumes a conformation as portrayed in Scheme V, the two hydrogen atoms eliminated are placed on the same face of the molecule and, in principle, could be removed by a single strategically placed basic group of the enzyme. If such a process occurs, of the two hydrogen atoms situated at position 2 in the "new" C-6 intermediate it is the one originating from H_{Re} of malonyl-CoA (■) that must be retained during the subsequent reactions.

Mechanism B (Scheme VI) involves the direct formation of either a Δ^2- or Δ^3-cis-double bond from the C-6 polyketide intermediate. The formation of a cis double bond in an acyclic system is more likely to proceed by anti-elimination (although a cis-elimination cannot be excluded). Our results show that H_{Re} (▲) is lost from position 2 of the polyketide intermediate; therefore, anti-elimination of water to form the Δ^2-cis double bond would require the 3-position to be of *S* configuration (Scheme VIa). If, on the other hand, the anti-elimination occurs to form the Δ^3-cis double bond, the loss of H_{Si} (■) from position 4 observed in our experiments would mandate the 3-position to be of *R* configuration (Scheme VIb).

Scheme VII: Mechanism C—Formation of the Cis Double Bond after Cyclization[a]



[a] The species portrayed is one of many possibilities, and no particular preference is given.

Mechanism C (Scheme VII) involves the retention of the hydroxyl group until after the reaction with the third malonyl-CoA molecule. Once cyclization has occurred, the driving force toward aromatization may be utilized for the elimination of the hydroxyl group at C-5 in the tetraketide. This mechanism also has the advantage that a conjugated ketone, produced in mechanisms A and B by the formation of a Δ^3-cis double bond, is not involved, thus making the ring closure more facile. If the cyclizing intermediate was in the keto form, as shown in Scheme VII, the observed isotopic labeling of 6-methylsalicylic acid would require the loss of the hydrogen atoms from opposite faces of the ring system.

The experiments discussed above represent the first successful study of a polyketide synthase using chiral (*R*)-[1-^{13}C;2-^{2}H]- and (*S*)-[1-^{13}C;2-^{2}H]malonates. The results highlight the fact that, despite their reactivity, a remarkable degree of steric control exists during the manipulation of the enzyme-bound polyketide intermediates, or their equivalents, at the catalytic site of 6-methylsalicylic acid synthase. The findings from the stereochemical analysis have an important bearing on the mechanism and steric course of the reduction, dehydration, and aromatization stages in the formation of 6-methylsalicylic acid. The overall stereochemical course is likely to have significant implications for the understanding of conformational regulation and its influence on the molecular programming of this and other polyketide synthase reactions.

EXPERIMENTAL PROCEDURES

Materials. Acetoacetyl-CoA, acetyl-CoA, coenzyme A, malonyl-CoA, NADPH, and succinyl-CoA transferase were purchased from Sigma Chemical Co. [2-^{13}C]Malonic acid and bis(trimethylsilyl)trifluoroacetamide were from Aldrich Chemical Co., and [1-^{14}C]acetyl-CoA was from Amersham International, U.K. (*R*)-[1-^{13}C;2-^{2}H]Malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate were synthesized by published methods (Jordan et al., 1986; Jordan & Spencer, 1991). 6-Methylsalicylic acid was a gift from Dr. A. Berry (Cambridge University). All other chemicals were of analytical grade and were purchased from BDH, Poole, Dorset, U.K.

Enzymes. 6-Methylsalicylic acid synthase was purified from synchronous cultures grown from a spore inoculum as described by Spencer and Jordan (1992b). The enzyme was stored in aliquots at −70 °C in 100 mM potassium phosphate buffer, pH 7.5, containing 15% glycerol. Succinyl-CoA transferase was further purified from commercially available enzyme (Sigma) as follows. The ammonium sulfate was removed by dialysis, and the enzyme was applied to a MonoQ

(Pharmacia) anion-exchange column equilibrated previously in 30 mM tris(sulfate) buffer pH 7.2. The enzyme was eluted with a linear gradient of NaCl (0–0.5 M; total volume 20 mL) in the same buffer. The enzyme was precipitated by the addition of ammonium sulfate to 60% of saturation, and the enzyme was stored at –4 °C until required.

Incubation of Labeled Malonate with Succinyl-CoA Transferase and 6-Methylsalicylic Acid Synthase Using Acetyl-CoA as a Starter Unit. A quartz cuvette (1 cm) containing succinyl-CoA transferase (2.4 units), 6-methylsalicylic acid synthase (1.5 units), bovine serum albumin (0.25 mg), acetyl-CoA (0.2 mM), succinyl-CoA (0.4 mM), NADPH (0.4 mM), 30 000 dpm of 6-methylsalicylic acid (specific activity 0.8 mCi/mmol), and 90 mM tris(sulfate) buffer, pH 8.4, to a final volume of 2.0 mL, was incubated at 25 °C with stirring for 2 min. The labeled malonate samples were mixed with the above incubation, and 0.5 mL was immediately removed from the cuvette, adjusted to pH 1.5 with 5 M HCl to prevent any biosynthesis of 6-methylsalicylic acid taking place, and used in the determination of the endogenous concentration of 6-methylsalicylic acid. The malonate samples used were as follows: (a) potassium [2-$^2H_2$]malonate (20 μmol), dissolved in 0.5 mL of 90 mM tris(sulfate) buffer, pH 8.4, immediately prior to addition to the incubation mixture; (b) either potassium (*R*)- or (*S*)-[1-^{13}C;2-^{2}H]malonate (≈20 μmol, in 0.5 mL of K_2CO_3, pH 9.3) that had been stored frozen in liquid nitrogen and brought to 25 °C immediately prior to addition to the incubation mixture; (c) potassium (*R,S*)-[1-^{13}C;2-^{2}H]malonate (≈20 μmol, volume 0.5 mL) that had been prepared by oxidization of a mixture composed of equal quantities of (2*S*,3*R*)-[1,4-$^{13}C_2$;3-^{2}H]malic acid and (2*S*,3*S*)-[1,4-$^{13}C_2$;2,3-$^2H_2$]malic acid and stored frozen.

The incubations were carried out in the fluorescence cell in a Perkin-Elmer CS-3B fluorescence spectrometer, and the appearance of 6-methylsalicylic acid was monitored by setting the excitation wavelength to 310 nm and the emission wavelength to 390 nm. The reaction was stopped after 4 min by adjusting the incubation mixture to pH 1.5 with 5 M HCl. The 6-methylsalicylic acid was then extracted as described below.

Incubation of Labeled Malonate with Succinyl-CoA Transferase and 6-Methylsalicylic Acid Synthase Using Acetoacetyl-CoA as a Starter Unit. The cuvette contained succinyl-CoA transferase (0.6 unit), 6-methylsalicylic acid synthase (3 units), bovine serum albumin (0.25 mg), NADPH (0.4 mM), [7-^{14}C]-6-methylsalicylic acid (30 000 dpm of specific activity 0.8 mCi/mmol), and 90 mM tris(sulfate) buffer, pH 8.4, to a final volume of 2.0 mL. Incubation was at 25 °C with stirring for 2 min. The labeled malonate sample was prepared and added to the incubation mixture with 2 μmol of acetoacetyl-CoA as follows: (a) potassium [2-^{13}C]-malonate or potassium [2-^{13}C;2-$^2H_2$]malonate (20 μmol) dissolved in 0.5 mL of 90 mM tris(sulfate) buffer immediately prior to addition to the incubation mixture; (b) (*R*)- or (*S*)-[1-^{13}C;2-^{2}H]malonate (volume 0.5 mL ≈ 20 μmol) that had been stored frozen in liquid nitrogen and brought to 25 °C immediately prior to addition to the above incubation mixture.

Incubation and sampling was carried out as described in the previous section.

Extraction and Derivatization of 6-Methylsalicylic Acid. After adjustment of the incubation mixture to pH 1.5, the precipitated protein was removed by centrifugation. Each sample was then diluted to 5 mL by the addition of distilled water, and the 6-methylsalicylic acid was extracted with 4 × 10 mL of diethyl ether. The extract was dried over sodium sulfate and evaporated to dryness, and the residue was dried under vacuum for several hours. Each sample of 6-methylsalicylic acid was converted into the bis(trimethylsilyl) derivative by the addition 25 μL of a solution containing ethyl acetate–bis(trimethylsilyl)trifluoroacetamide–pyridine (2:2:1 v/v) followed by reaction at 50 °C in a heating block for 30 min. This solution was then used directly for injection into the GLC/MS.

Synthesis of [7-^{14}C]-6-Methylsalicylic Acid. [7-^{14}C]-6-Methylsalicylic acid was prepared from [2-^{14}C]acetyl-CoA using homogeneous 6-methylsalicylic acid synthase as follows. To a quartz cuvette (1 cm) were added [2-^{14}C]acetyl-CoA (specific activity 50 mCi/mmol; 0.7 μmol), malonyl-CoA (0.8 μmol), NADPH (0.4 μmol), bovine serum albumin (0.25 mg), 6-methylsalicylic acid synthase (1 unit), and 90 mM tris-(sulfate) buffer, pH 7.6, to a final volume of 2 mL. The reaction was terminated after 20 min by the addition of 0.5 mL of 5 M KOH. Carrier 6-methylsalicylic acid (1 mg) was added, and the remaining [2-^{14}C]acetyl-CoA was hydrolyzed by heating the solution at 80 °C for 30 min. Precipitated protein was removed by centrifugation, and the supernatant was adjusted to pH 1.5 with 5 M HCl and lyophilized to remove the [2-^{14}C]acetic acid. The residue was dissolved in 5 mL of distilled water and the pH readjusted to 1.5 with 5 M HCl. 6-Methylsalicylic acid was extracted with 4 × 10 mL of diethyl ether, which was then dried over sodium sulfate. The extract was evaporated to a small volume and spotted onto a silica TLC plate (8 × 15 cm). The plate was developed in benzene–dioxane–glacial acetic acid (90:25:4 v/v). 6-Methylsalicylic acid, visualized as a blue fluorescent spot under UV light, was eluted from the silica with 3 × 5 mL of methanol; the solution was evaporated to a small volume and adjusted to 5 mL with distilled water. The concentration of 6-methylsalicylic acid was determined fluorometrically. The specific activity of the [7-^{14}C]-6-methylsalicylic acid was calculated to be 0.8 mCi/mmol. No further purification was necessary. The location of the 7-position is shown in Scheme I.

Mass Spectrometric Analysis of 6-Methylsalicylic Acid. The most intense ion containing all atoms of interest of the bis(trimethylsilyl) derivative of 6-methylsalicylic acid (6-methyl-2-*O*-(trimethylsilyl)benzoic acid trimethylsilyl ester) has a mass of 281. This ion arises from the loss of Me^- from one of the trimethylsilyl groups of the parent bis(trimethylsilyl) derivative M + 0 = 296. This ion was used to determine the isotopic distribution of 6-methylsalicylic acid derived from labeled malonate.

The bis(trimethylsilyl) derivative of 6-methylsalicylic acid was analyzed using either a VG Model Ts250, 70SEQ, or TRIO 1 mass spectrometer. The ionization of the molecules was achieved by electron impact (70 eV). The capillary column was a DB-1 (J&W Scientific Capillary Gas Chromatography Products) and was 30 m in length. Both full scans and single ion recording of the samples were taken. Comparison between the two different methods of analysis produced similar intensities for each ion. This confirmed that the GLC retention times for unlabeled and deuterated bis(trimethylsilyl) derivatives were the same and hence that the full scan was a reliable method for determining the isotopic distribution of 6-methylsalicylic acid.

The determination of the isotope enrichment of each ion requires correction for the endogenous and natural abundance contribution from the ions of lower mass. Purified 6-methylsalicylic acid synthase is associated with significant quantities of its product. Therefore, the M + 0 ion at 281 is a mixture of unlabeled 6-methylsalicylic acid derived from that made

during the experiment together with that introduced with 6-methylsalicylic acid synthase. Although M + 0 is not an important ion with regard to distinguishing which mechanism is operating, an attempt was made to remove the contribution from endogenous 6-methylsalicylic acid. The inclusion of [7-^{14}C]-6-methylsalicylic acid in the incubation allows the accurate determination of the yield of 6-methylsalicylic acid extracted in the blank compared to that of the sample. Comparing the area of the single ion chromatogram of the bis(trimethylsilyl) derivative of 6-methylsalicylic acid from the blank and that of the sample allows the calculation of the percentage of the intensity of the 281 ion that is due to endogenous 6-methylsalicylic acid. The accuracy of this method cannot be relied on to be any better than ±15%. If an accurate value for M + 0 is required, the biosynthesized 6-methylsalicylic acid must be shifted away from the endogenous 6-methylsalicylic acid by the inclusion of an additional ^{13}C label in one of the substrates. This method was used in the experiment where the level of deuterium incorporation was established using acetoacetyl-CoA and [2-^{13}C,2-$^{2}H_2$]-malonate (see Figure 4c).

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