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Mechanistic and Stereochemical Investigation of Fatty Acid and Polyketide Biosynthesis using Chiral Malonates

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Abstract Chiral malonates, (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate, were synthesised and characterised as malonyl-CoA derivatives with yeast fatty acid synthase using mass spectrometric analysis of the palmitic acid produced. The chiral malonates were used to investigate the steric course of fatty acid synthase from rat liver and the fatty acid synthase and 6-methylsalicylic acid synthase from *Penicillium patulum*.

Malonic acid, activated in the form malonyl-CoA, is a key building block for the biosynthesis of many naturally occurring compounds including fatty acids, flavanones and polyketides[1]. The methylene carbon atom of the malonyl-CoA, with its two hydrogen atoms, is of particular interest since it is at this position that many of the crucial mechanistic events occur during the assembly of these compounds. Detailed stereochemical information about the fate of the hydrogen atoms at this position during the incorporation of malonyl-CoA into these natural products is thus an essential requirement for the understanding of the mechanism and steric course of the enzymic steps involved.

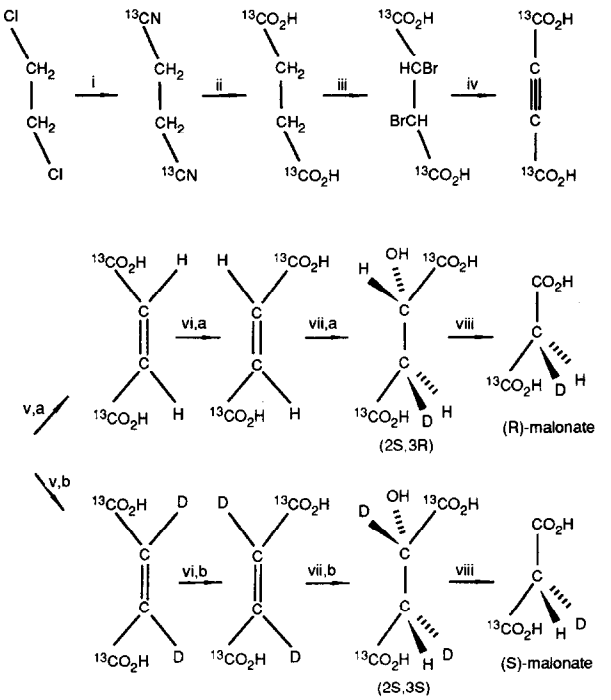
This problem was originally addressed by elegant studies in which the tritiated enantiomers of malonyl-CoA were synthesised and used to elucidate the mechanism of yeast fatty acid synthase[2]. Difficulties encountered in this work with the extreme lability of the methylene protons suggested that the use of malonyl-CoA derivatives had severe limitations. This prompted us to devise an alternative strategy based on the synthesis of the more stable chiral malonates although this approach, as will be clear, introduces cryptic stereochemistry which complicates the analytical methodology. This stems from the fact that malonic acid exhibits *pro-pro*-chiral stereochemistry, having the structure Ca_2b_2. Thus even if the malonic acid is made chiral, by labeling the paired constituents isotopically with ^{13}C and ^{2}H in the same molecule, the labeled groups will remain essentially indistinguishable to an enzyme. Therefore each chiral malonate will yield a unique pair of malonyl-CoA derivatives, even when activated enzymically. Once formed, each of the malonyl-CoA derivatives will be recognised as a *pro*-chiral molecule by an enzyme and the labeled substituents will be manipulated stereospecifically. Such aspects have been discussed thoughtfully elsewhere[3]. This paper discusses the synthesis of (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate and their use to study the mechanism and stereochemistry of fatty acid synthases and the polyketide synthase 6-methylsalicylic acid synthase.

RESULTS AND DISCUSSION

The synthesis of (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate

The two enantiomers of malonate, (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate, were synthesised *via* [1,4-$^{13}C_2$]acetylenedicarboxylic acid by the route shown in Scheme 1, the ^{13}C label having been introduced initially by the reaction of 1,2-dichloroethane with K^{13}CN in a high yielding reaction. Hydrolysis of the succinylnitrile to succinate, bromination to give 2,3-dibromosuccinate, and subsequent

elimination yielded the acetylenedicarboxylic acid labeled in both carboxyl groups with ^{13}C. Lindlar catalyst promoted reduction of the labeled acetylenedicarboxylic acid introduced either ^{1}H or ^{2}H (route a or b, Scheme 1) and furnished the two maleic acids which, after isomerisation to the fumarates, were hydrated stereospecifically using fumarase in the presence of the desired species of water using published methods[4] to give the two chiral malates. The doubly labeled malic acids (98% atom excess ^{2}H and 99% atom excess ^{13}C) were obtained in approximately 35% overall yield from dichloroethane. The malic acids provided stable intermediates from which the chiral malonic acids could be generated readily by permanganate oxidation[5]. A summary of the reagents and conditions employed at each stage are described in the Experimental Section.



Scheme 1. The synthesis of (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate

Analysis of (*R*)- and (*S*)-[1-^{13}C;2-^{2}H]malonates with yeast fatty acid synthase and mass spectrometry

One of the most convenient means for the determination of the chirality of the (*R*)- and (*S*)-[1-^{13}C;2-^{2}H]malonates is by using yeast fatty acid synthase the mechanism of which is known to involve the loss of H_{Re} of the precursor malonyl-CoA. Initially, the malonates were activated by conversion into their respective malonyl-CoA derivatives, a reaction catalysed by succinyl-CoA transferase which, providentially, also accepts malonate as a substrate in a related reaction as shown in Scheme 2.

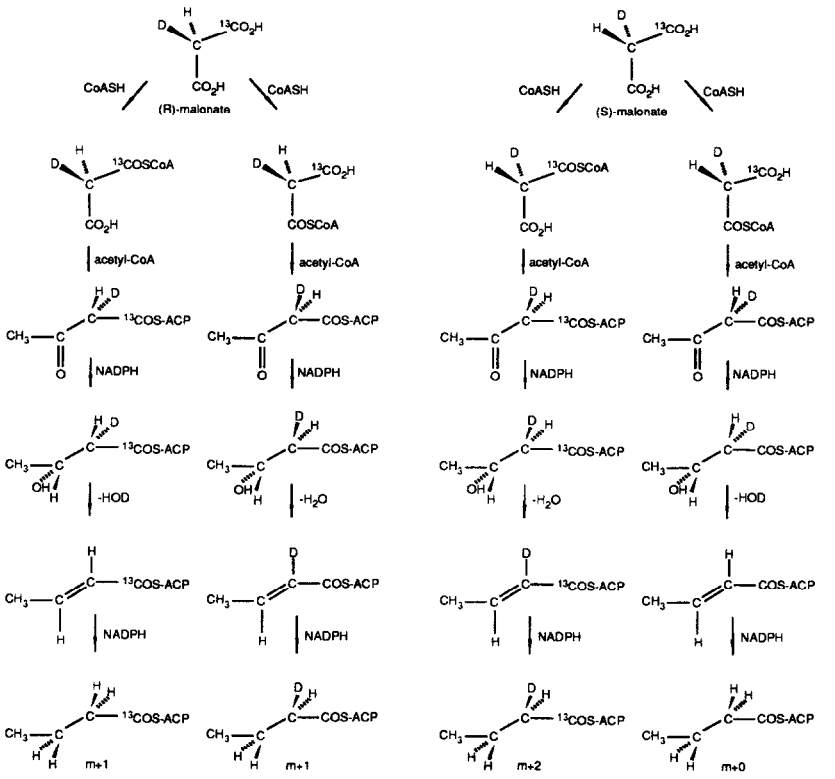
In order to minimise the exchange of the sensitive methylene hydrogen atoms of malonyl-CoA, the succinyl-CoA transferase reaction was coupled directly to yeast fatty acid synthase so that malonyl-CoA was rate-limiting in the reaction. The resulting palmitic acid samples were extracted, methylated, purified by g.l.c., and subjected to mass spectrometric analysis as described in the Experimental Section.

succinyl-CoA + acetoacetic acid = acetoacetyl-CoA + succinate

succinyl-CoA + malonic acid = malonyl-CoA + succinate

Scheme 2. Reactions catalysed by succinyl-CoA transferase

Because the two carboxyl groups of chiral malonate cannot be distinguished from one another by the transferase enzyme, **two** chiral malonyl-CoA species will arise from **each** chiral malonate (Scheme 3). Since the yeast fatty acid synthase is known to follow a mechanistic course in which H_{Re}, originally in malonyl-CoA, is stereospecifically removed at each dehydration stage[2], the chiral malonates may be distinguished from one another by analysis of the palmitic acids produced. It is clear that during the synthesis of palmitic acid from (*R*)-[1-^{13}C;2-^{2}H]malonate, there would be an equal chance of incorporating **either** ^{13}C **or** ^{2}H, but **not** both labels, each time a C-2 unit is introduced during chain elongation (Scheme 3). Conversely, the (*S*)-[1-^{13}C;2-^{2}H]malonate-derived palmitic acid would be made up of C-2 units, **half** of which contain ^{13}C **and** ^{2}H labels and **half** of which contain **no** label (Scheme 3). All Claisen condensations are expected to proceed by inversion of configuration since this appears to be an obligatory mechanistic feature of such reactions[6]. For simplicity, the fatty acid synthase reaction is shown as far as the formation of the enzyme-bound butyryl-thioester, although the outcome of incorporating seven malonyl-derived C-2 units into palmitic acid can be deduced by similar reasoning.



Scheme 3. Incorporation of (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate into fatty acids.

From the above considerations the palmitic acids derived from each chiral malonate may be readily differentiated from one another by their mass distributions. Theoretically, in the case of the (*R*)-malonate enantiomer the derived palmitic acid would be expected to show a single peak at m+7 if there was no isotopic dilution (Figure 1a). In contrast, the palmitic acid derived from the (*S*)-malonate enantiomer would be expected to show a broad envelope of peaks two mass units apart extending from m+0 to m+14 (Figure 1b). In practice, the presence of a small amount of singly labeled species present in the original substrate or arising from the exchange of ^{2}H in the malonyl-CoA intermediates would have the effect of shifting the mass spectral profile of the palmitic acids to lower mass in the case of the (*R*)-malonate sample (Figure 1c) and would generate odd number mass peaks in the spectrum of the palmitic acid derived from the (*S*)-isomer (Figure 1d). The results from experiments in which (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate were incubated with the coupled succinyl-CoA transferase/fatty acid synthetase enzyme systems are shown in Figures 1 e) and f) respectively. The mass distributions in the palmitic acid samples, as their methylpalmitates, obtained from both isomers show a close correlation to those predicted on the basis of the above theoretical considerations.

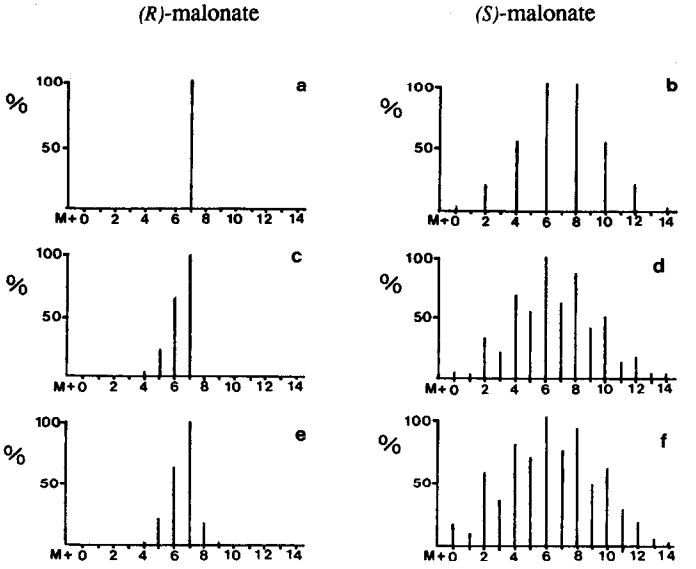


Figure 1. Mass spectra of methylpalmitates derived from (*R*)-[1-^{13}C;2-^{2}H]-malonate, a), c) and e) and from (*S*)-[1-^{13}C;2-^{2}H]malonate, b), d) and f). Theoretical spectra, a) and b); simulated results on the basis of 15% exchange, c) and d); experimental data corrected for natural abundance and unlabelled species of methylpalmitate (m+0 = 270) derived from (*R*)-[1-^{13}C;2-^{2}H]-malonate, e); methylpalmitate derived from (*S*)-[1-^{13}C;2-^{2}H]malonate, f).

From the results obtained, which confirm that H_{Re} of malonyl-CoA is lost during the biosynthesis, it may be concluded that the synthetic routes for the preparation of the chiral malonates afford samples of high stereochemical purity. In addition, the close similarity of the results to those expected indicates that during the activation of malonic acid to malonyl-CoA, and the subsequent conversion into palmitic acid, the exchange of the CH_2 protons is no more than 15%. The results also show that there is virtually no racemisation during the enzymic incubations. This is largely as a result of using highly purified enzymes and the adjustment of the levels of the two enzymes to ensure that malonyl-CoA generation is rate limiting in the overall reaction. In an independent study

by Floss and his colleagues[7], chiral malonates were prepared by a related methodology involving a combination of chemical and enzymic steps although in contrast to our methodology their resolution was performed by chemical derivatisation and n.m.r. analysis.

Investigation of the steric course of fatty acid synthases from rat liver and *Penicillium patulum* using (*R*)- and (*S*)-[1-^{13}C;2-^{2}H]malonates

A similar coupled enzyme approach to that outlined above was used to investigate the unknown steric course of the fatty acid synthases isolated from rat liver and *Penicillium patulum*. The results of incubations in which these fatty acid synthases were substituted for the yeast enzyme are shown in Figure 2.

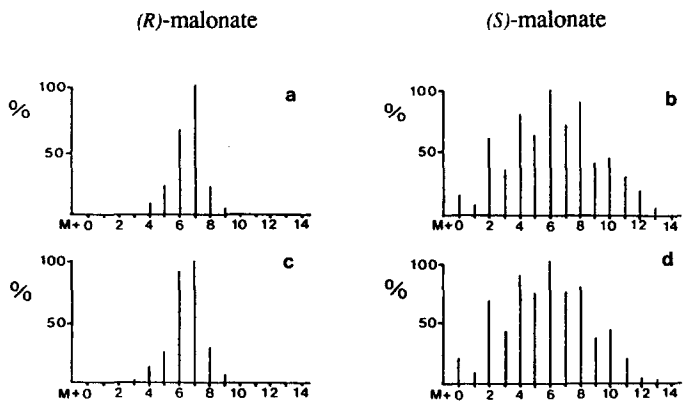


Figure 2. Mass spectra of methylpalmitates derived from (*R*)-[1-^{13}C;2-^{2}H]-malonate, a) and c) and from (*S*)-[1-^{13}C;2-^{2}H]malonate, b) and d) using fatty acid synthases isolated from rat liver, a) and b) and *Penicillium patulum*, c) and d).

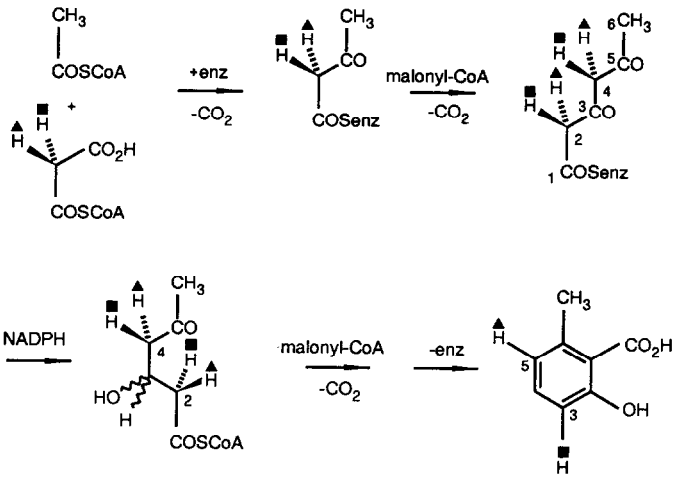
The results are similar to those obtained with the yeast fatty acid synthase the mechanism of which has been shown to involve the loss of H_{Re} originating from malonyl-CoA during the dehydration of the β-hydroxyacyl intermediate (Scheme 3). One may thus conclude from these results that the same stereochemical course operates in the fatty acid synthases from yeast, rat liver[5] and *Penicillium patulum*. It is interesting to note that the derived primary protein sequences of eukaryotic fatty acid synthases are closely related suggesting that the individual components of fatty acid synthases have a similar tertiary structure and may arise from a common evolutionary origin[8]. It is most likely that structurally related enzymes would follow similar mechanistic courses.

Studies on the stereochemical course of 6-methylsalicylic acid synthesis

Chiral malonates offer their greatest potential for the investigation of the mechanistic and stereochemical events during polyketide biosynthesis since a knowledge of the fate of the methylene hydrogen atoms originally at C-2 of malonyl-CoA can provide crucial information about the manipulation of enzyme-bound polyketide intermediates as they are transformed into products. The remainder of this paper will thus deal with a prototype study on the enzyme 6-methylsalicylic acid synthase using the same experimental approach as that described above for fatty acid synthases.

6-Methylsalicylic acid is one of the simplest of the polyketide derived natural products being made up of one acetyl-CoA and 3 malonyl-CoA units (Scheme 4). The 6-methylsalicylic acid synthase from *Penicillium patulum* has been chosen because the enzyme has been characterised[9], mechanistic studies have provided evidence for enzyme-bound intermediates[10] and the product 6-methylsalicylic acid is a stable product

amenable to mass spectrometric analysis. Initially, acetyl-CoA and two malonyl-CoA molecules are utilised to form a putative enzyme-bound C-6 polyketide intermediate. Further transformation of this intermediate by a complex series of stages including reduction, addition of a further C-2 unit from malonyl-CoA and cyclization ultimately yields the aromatic ring as shown in Scheme 4. The methylene hydrogen atoms of malonyl-CoA are indicated by ■ (H_{Re}) and ▲ (H_{Si}).



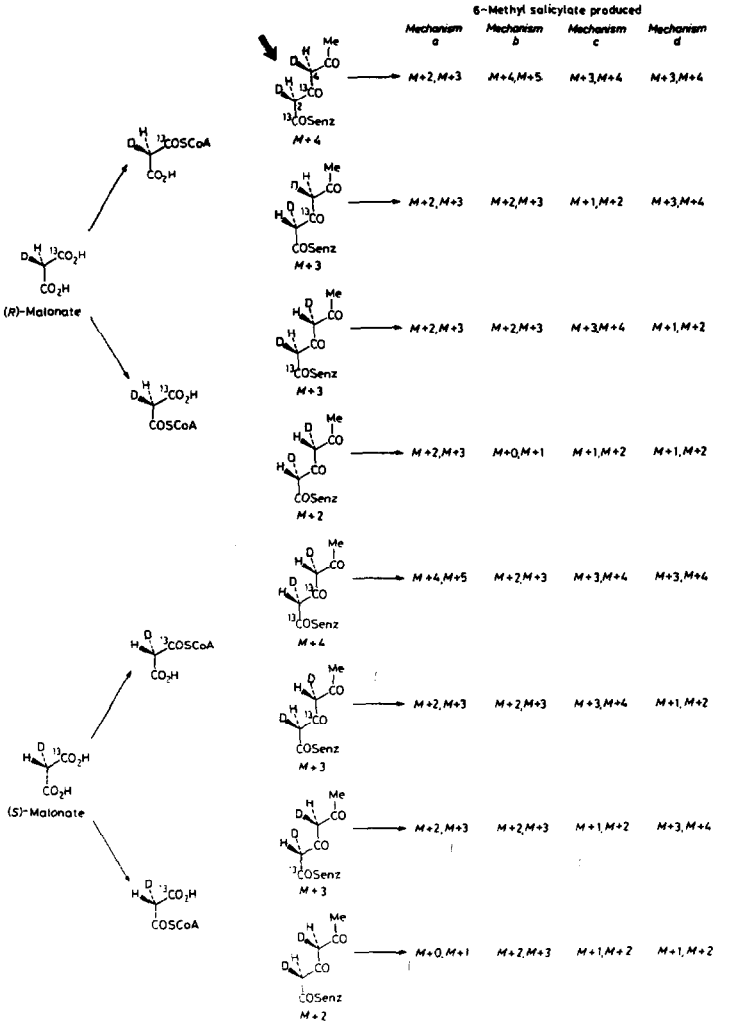
Scheme 4. Stages in the biosynthesis of 6-methylsalicylic acid from acetyl-CoA and malonyl-CoA

Earlier mechanistic studies, using deuterated acetate[11], have provided evidence that during the biosynthesis of 6-methylsalicylic acid the polyketide intermediates are handled by stereospecific processes. However, little information is known about the absolute stereochemical mechanism of any polyketide synthase, except for a report on the steric course of the *enoyl*-reductase step in the biosynthesis of the polyketide cladosporin[12,13] and the apparant lack of steric control during the biosynthesis of orsellinic acid[14].

Since 6-methylsalicylic acid arises from 3 molecules of malonyl-CoA, the use of malonyl-CoA derived from (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate will result in the incorporation of up to three ^{13}C atoms and up to two ^{2}H atoms into each molecule of product. As discussed above each Claisen condensation would be expected to occur with inversion of configuration[6] and to result in the incorporation of the intact methylene hydrogen atoms. These hydrogen atoms in the initial C-6 intermediate are indicated by ▲ (H_{Re}) or ■ (H_{Si}) in Scheme 4. On purely statistical grounds, eight labelled enzyme-bound C-6 intermediates will arise (Scheme 5) from one molecule of acetyl-CoA and two molecules of chiral malonyl-CoA, derived in turn from either (*R*)-[1-^{13}C;2-^{2}H]malonate or (*S*)-[1-^{13}C;2-^{2}H]malonate each containing a ^{2}H label at the 2- and 4-positions (indicated by arrows on the **left-hand** structure in Scheme 5). The subsequent transformation of these intermediates can occur by different mechanisms each of which will lead to product with a unique complement of ^{2}H label. On reaction with the third and last molecule of malonyl-CoA, each C-6 intermediate will have a 50% chance of incorporating an additional ^{13}C label, and each will thus give rise to a pair of 6-methylsalicylic acid molecules, differing by one mass unit (Scheme 5) giving a total of 16 possible labeled products from each chiral malonate.

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 possible, 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 5). In *mechanism a*, H_{Si} is removed from the 2- and 4-positions of this intermediate and therefore **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 labeled 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 5, *mechanism a*).



Scheme 5. Incorporation of (*R*)-[1-^{13}C;2-^{2}H]malonate and (*S*)-[1-^{13}C;2-^{2}H]malonate into polyketide intermediates and their transformation into 6-methylsalicylic acid with homogeneous 6-methylsalicylic acid isolated from *Penicillium patulum*.

In contrast, for *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 when compared to those arising from the (*R*)-[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 C-6 intermediate (arrowed in Scheme 5) the overall labeling in the products would be such that the mass spectrometric analysis would yield converse patterns (Scheme 5, *mechanism b*). Two other mechanisms, *mechanism c* in which H_{Re} is lost from the 2-position and H_{Si} from the 4-position would give the pattern shown in Scheme 5, *mechanism c*. *Mechanism d*, where H_{Si} is lost from the 2-position and H_{Re} from the 4-position (Scheme 5, *mechanism d*) completes 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 (Figure 3a) and 1,1,2,2,1,1 for (*S*)-malonate (Figure 3b). *Mechanism b* will give converse patterns (Figure 3c) and d)). In *mechanisms c* and *d* both (*R*)- and (*S*)-malonates will give similar mass distributions of 0,2,2,2,2,0 (Figure 3e), f), g) and h)). *Mechanisms a* and *b* may be thus be readily distinguished from one another and from *mechanisms c* and *d* by mass spectrometric analysis.

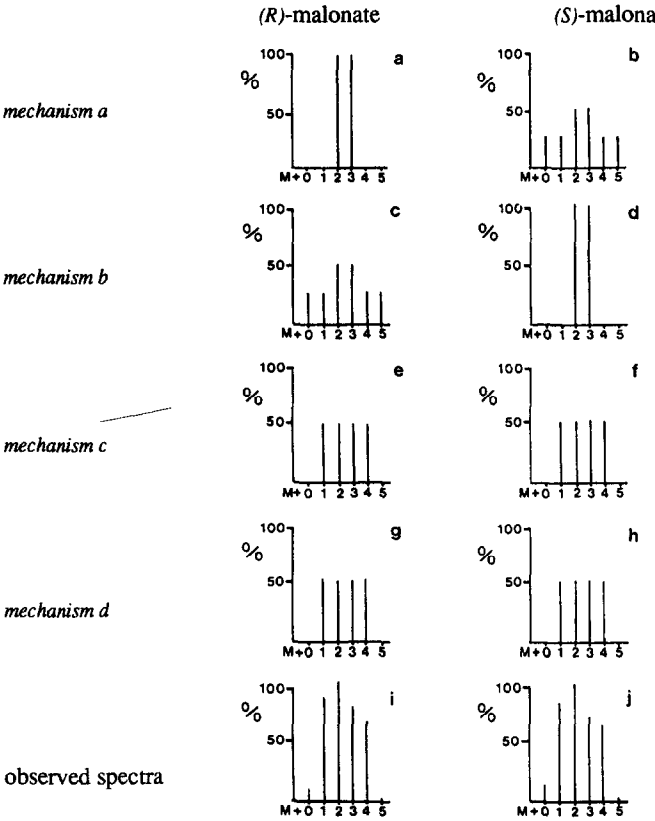


Figure 3. Mass spectra of 6-methylsalicylic acid synthesised from (*R*)-[1-^{13}C; 2-^{2}H] malonate and (*S*)-[1-^{13}C; 2-^{2}H]malonate predicted respectively for *mechanism a*, a) and b); *mechanism b*, c) and d); *mechanism c*, e) and f); *mechanism d*, g) and h). Observed mass-spectra for 6-methylsalicylic acid biosynthesised from (*R*)-[1-^{13}C;2-^{2}H]malonate i) and (*S*)-[1-^{13}C;2-^{2}H]malonate j). All observed data are corrected for natural abundance and represent the m+0 = 281 species arising from the loss of CH_3- from one of the trimethylsilyl groups of the parent trimethylsilyl derivative, m+0 = 296.

It is important when considering absolute configurations that H_{Re} and H_{Si} refer to the C-6 intermediate on the left side of Scheme 5. The introduction of ^{13}C and ^{2}H into other positions of this intermediate changes the priority and gives rise to complications in the designation of absolute configuration. It is thus essential to consider the symbols ■ and ▲ for labeling the paired hydrogen atoms, as shown in Scheme 4, rather than to use absolute configuration.

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 the coupled succinyl-CoA transferase and 6-methylsalicylic acid synthase enzymes are shown in Figure 3i) and j). The spectra with 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 5, *mechanism c* or *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 overall findings from our investigations thus eliminate *mechanisms a* and *b* where the hydrogen atoms with the same absolute configurations in malonyl-CoA are removed. Therefore *mechanism c* or *mechanism d* must operate in which **either** H_{Re} and H_{Si} **or** H_{Si} and H_{Re} are removed from the 2- and 4-positions respectively in the C-6 polyketide intermediate[15].

Determination of the absolute stereochemistry of hydrogen atoms eliminated during the biosynthesis of 6-methylsalicylic acid

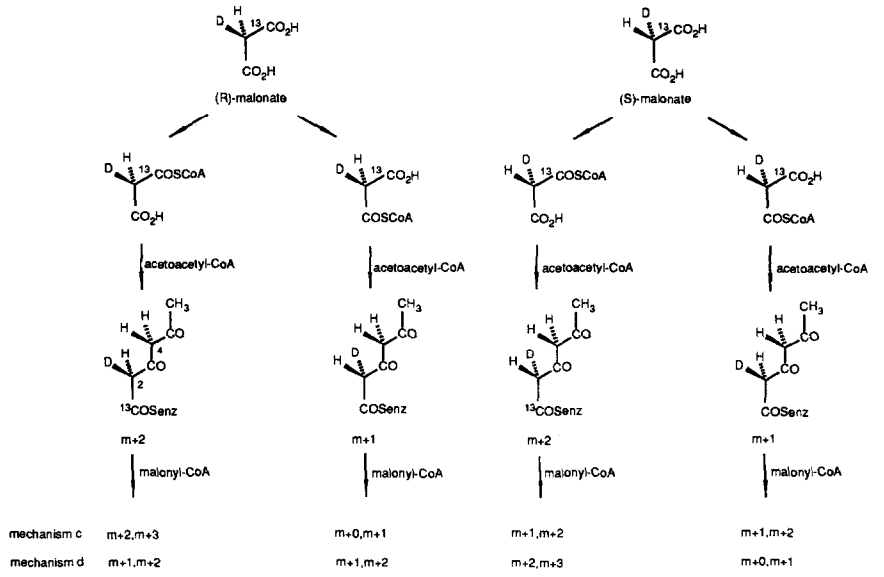
Since the experimental results described above do not permit a distinction between *mechanisms c* and *d*, an experimental approach which allows the determination of the stereochemical events which occur at the 2- or 4-positions, in isolation, is required. This type of analysis could, in principle, be carried out by an n.m.r. study in which the coupling between labeled atoms could be used to distinguish between the two mechanisms. In reality, it is not possible to obtain sufficient biosynthetic material to employ the n.m.r. approach and thus an alternative method was developed.

The observation that the 6-methylsalicylic acid synthase will accept acetoacetyl-CoA as a starter molecule permits the regiospecific incorporation of the labeled 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 manipulations of the hydrogen atoms at a **single** position may be studied independently. As before, the final malonyl-CoA moiety to be incorporated will provide a ^{13}C label in 50% of the molecules biosynthesised, however, since both hydrogen atoms of this C-2 unit are lost during cyclisation and aromatisation they do not contribute to the labeling of hydrogen atoms in the final product (Scheme 6).

Using similar reasoning to that described above, both species of each pair of malonyl-CoA derivatives will be 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). Using acetoacetyl-CoA as a starter molecule 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 6 (see also Scheme 5, *mechanism c*). Elimination of H_{Si} from the same position of the C-6 polyketide intermediate (H_{Re} in malonyl-CoA) would give the converse mass distribution 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 as shown in Scheme 6 (see also Scheme 5, *mechanism d*).

When *(R)*- and *(S)*-malonates were incubated in separate coupled experiments with succinyl-CoA transferase, acetoacetyl-CoA, NADPH and 6-methylsalicylic acid synthase

isolated to homogeneity from *Penicillium patulum* and the resulting 6-methylsalicylic acid samples were analysed by g.l.c/m.s. the results shown in Figure 4 were obtained.



Scheme 6. 6-Methylsalicylic acid formation from acetoacetyl-CoA and *(R)*- or *(S)*-malonates

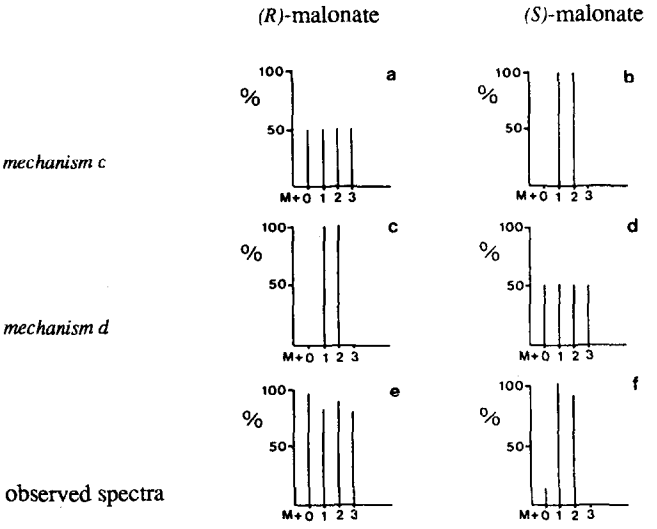


Figure 4 Mass spectra of 6-methylsalicylic acid biosynthesised from acetoacetyl-CoA and *(R)*- or *(S)*-malonic acids respectively. Theoretical spectra predicted for *mechanism c*, a) and b); for *mechanism d*, c) and d). Experimental data, e) and f), obtained from *(R)*- and *(S)*-malonic acids respectively using homogeneous 6-methylsalicylic acid synthase from *Penicillium patulum*. The data shown have been corrected for natural abundance and represent m+0 = 281 species arising from the parent derivative, m+0 = 296.

The results in Figure 4 show that the 6-methylsalicylic acid arising from *(R)*-malonate is made up almost exclusively from species of m+0, m+1, m+2 and m+3, whereas the 6-methylsalicylic acid arising from the *(S)*-malonate contains largely the species m+1 and m+2. This result establishes that *mechanism c* is operative in which the hydrogen atom (■) at the 3-position of 6-methylsalicylic acid originates from H_{Re} of malonyl-CoA (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 4) which is eliminated during 6-methylsalicylic acid biosynthesis[16]. Since our previous experiments[15] using acetyl-CoA as the starter molecule have shown that malonyl-CoA-derived hydrogen atoms with opposite orientations are removed from C-2 and C-4 of the C-6 intermediate, it follows that the hydrogen atom eliminated at C-4 arises from H_{Si} of the polyketide intermediate (■ in scheme 4) and that therefore the hydrogen atom incorporated into the C-5 position of 6-methylsalicylic acid (▲) originates from H_{Si} in malonyl-CoA[16] (*mechanism c*, Scheme 5).

Summary

The results described above establish that the mechanism of fatty acid synthases from rat liver and *Penicillium patulum* follow similar stereochemical courses to that of the yeast enzyme in which the H_{Re} from malonyl-CoA is lost during the dehydration of the enzyme-bound β-hydroxyacyl intermediate. In 6-methylsalicylic acid synthesis, it has been established that H_{Si} and H_{Re} of malonyl-CoA are lost from the C-2 and C-4 positions respectively in the putative C-6 polyketide intermediate. Fatty acid synthase and 6-methylsalicylic acid synthase both accept acetyl-CoA and malonyl-CoA as substrates and the initial transacylation, condensation and reductase stages[17] are likely to be related. Since it is not known whether the dehydration of the β-hydroxyacyl intermediate occurs before the final malonyl-CoA is added to the enzyme or if it is the aromatisation reaction which provides the driving force for dehydration it is not fruitful to speculate further on the detailed mechanism. It should be noted, however, that in the putative C-6 hydroxyacyl-intermediate, as drawn in Scheme 4, the two hydrogen atoms which are lost are positioned on the **same** face of the intermediate and therefore, in principle, could be manipulated by a single strategically placed enzyme group.

The foregoing account suggests that the application of chiral malonates to the study of the mechanistic and stereochemical course of other polyketide synthases is likely to be possible. However, despite the fact that the chiral malonates are at least an order of magnitude more stable than their corresponding CoA-derivatives it is unlikely that such clear-cut results will be obtained from *in vivo* feeding experiments and that such investigations will most probably need to be confined to polyketide synthases which can be isolated to high purity.

EXPERIMENTAL

Synthesis of *(R)*-[1-^{13}C;2-^{2}H] malonic acid and *(S)*-[1-^{13}C;2-^{2}H] malonic acid

(R)-[1-^{13}C;2-^{2}H] Malonic acid and *(S)*-[1-^{13}C;2-^{2}H] malonic acid were synthesised from dichloroethane by a combination of chemical and enzymic stages[5] as shown in Scheme 1.

[1,4-$^{13}C_2$] Succinic acid.

Dichloroethane (1.52g), dry acetonitride (18ml), 18-crown-6-ether (0.4g) and 2g of K^{13}CN (99% enriched) were refluxed together at 80°C for four hours. The solution was filtered, reduced to one third of the volume and 30ml of chloroform and 30ml of NaCl solution (saturated) were added. The solution was extracted several times with chloroform (4 x 20ml), the extract was dried over anhydrous Na_2SO_4 and evaporated to leave 1.2g of succinonitride. The succinonitride was hydrolysed with 1.4g of potassium hydroxide in 36ml of water:methanol (2:1 v/v). The mixture was stirred at room temperature for 1 hr then refluxed overnight at 60°C. The solution was evaporated to remove the methanol and the remaining mixture was lyophilised. The resulting solid was dissolved in a minimum of water and acidified to produce white crystals of succinic acid which were purified by sublimation at 180°C and 0.05mm Hg. The overall yield

from potassium cyanide was 1.44g (78-81%), m.p 186-188°C.

[1,4-$^{13}C_2$] 2,3-Dibromosuccinic acid.

To 0.8ml of water were added 0.2ml of hydrogen bromide (constant boiling point), 2.7g of bromine and 1g of finely powdered [1,4-$^{13}C_2$] succinic acid. This mixture was placed in a sealed glass tube under vacuum and heated for two days at 100°C[18]. The resulting white crystals of [1,4-$^{13}C_2$] 2,3-dibromosuccinic acid were washed over suction with cold distilled water (3 x 5ml) and dried in air, yield 92-95%, m.p. 275°C (sub).

[1,4-$^{13}C_2$] Acetylenedicarboxylic acid.

[1,4-$^{13}C_2$] 2,3-Dibromosuccinic acid (2g) was added to KOH (2.44g in 14ml of 95% methanol) and reflux was carried out for 1hr 15mins. The reaction mixture was cooled and filtered. The mixed salts were washed with 10ml of cooled (0°C) methanol and dried in air. The product (2.4g) was dissolved in 5.4ml of water and the acid salt was precipitated by the addition of 0.16ml of concentrated sulphuric acid. After standing the mixture overnight it was filtered under suction. The acid salt was then dissolved in 2.4ml of water, to which 1.2ml of concentrated sulphuric acid had been added and the solution was extracted with ether. The extract was evaporated to dryness and placed over phosphorus pentoxide, yield 83-86%, m.p 175-176°C (dec).

[1,4-$^{13}C_2$] Maleic acid and [1,4-$^{13}C_2$;2,3-$^{2}H_2$] maleic acid.

[1,4-$^{13}C_2$] Acetylenedicarboxylic acid (0.5g) and 0.25g of Lindlar catalyst in 10ml of ethanol (deuterated ethanol for the deuterium reaction) were hydrogenated until 105% of the theoretical amount of H_2 (or $^{2}H_2$) gas had been consumed. The resulting labeled maleic acid solution was filtered through Celite and evaporated to dryness, yield 84-86%, m.p. 130-131°C.

[1,4-$^{13}C_2$] Fumaric acid and [1,4-$^{13}C_2$;2,3-$^{2}H_2$] fumaric acid.

To 1.4 ml of 8M HCl (8M ^{2}HCl in the case of the isomerisation of [1,4-$^{13}C_2$; 2,3-$^{2}H_2$] maleic acid) 400mg of maleic acid were added and the mixture was heated at 85°C for 1hr 15mins. Fumaric acid crystallised from the solution during the reactions and upon cooling. The fumaric acid was filtered, washed with cold water and purified further by sublimation at 140°C (0.05mm Hg) for 30mins. Overall yield 92-94%, m.p. 290 (dec).

(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.

A mixture of 200mg of [1,4-$^{13}C_2$] fumaric acid and 235mg of potassium dihydrogen phosphate was dissolved in water, adjusted to pH 7.4 and lyophilised. The dried residue was redissolved in 10ml of 99.8% $^{2}H_2O$ and lyophilised again. This procedure was repeated twice to achieve maximum exchange of deuterium for protium. The dried material was dissolved in $^{2}H_2O$ and adjusted to a final volume of 8ml to yield a solution of p^{2}H 7.0 as measured with a glass electrode. Fumarase (25 units) was equilibrated in $^{2}H_2O$ by passing it down a Pharmacia PD 10 gel filtration column, previously washed with 0.1M potassium phosphate, p^{2}H 7.0, in 15ml of $^{2}H_2O$ and incubated with the labeled fumaric acid at 30°C for ten minutes when the reaction had reached equilibrium (81% malate and 19% fumarate). The enzyme was inactivated at 90°C for two minutes and the precipitated protein was removed by centrifugation. The reaction mixture was adjusted to pH 10 with NaOH and each malic acid was separated from the fumaric acid on a Dowex-1X8-formate (200-400 mesh) column. Fumaric acid thus recovered was reincubated with fumarase to give a final overall yield of malic acid of 86-89%, m.p. 98-100°C.

(*R*)-[1-^{13}C;2-^{2}H] Malonic acid and (*S*)-[1-^{13}C;2-^{2}H] malonic acid.

Malic acid (67mg, 0.5mmol) was dissolved in a solution of 1ml of distilled water containing 27.6mg of potassium carbonate and the pH was adjusted to 9.5 with potassium hydroxide. This solution was mixed with potassium permanganate (63mg, 0.4mmol) and reacted at 25°C until the permanganate had been consumed. Cold distilled water (1ml) was added and the solution was filtered immediately through Celite to remove the manganese dioxide. A further 1ml of cold distilled water was washed

through the Celite and the filtrate was quickly separated into five aliquots, each of which contained approximately 2mg of malonic acid. The aliquots were frozen in liquid nitrogen until required.

The above procedure is shown in Scheme 1. A summary of the reagents used at each stage are: i, $K^{13}CN$, MeOH, 18-crown-6; ii, KOH, MeOH; iii, Br_2, HBr in a sealed tube heated for 2 days; iv, KOH, H_20, MeOH; v, Lindlar catalyst, a) H_2, EtOH, b) D_2, EtOD; vi, heat, a) 8MHCl, b) 8MDCl; vii, fumarase, a) D_2O, b) H_2O; viii, $KMnO_4$, H_2O, pH 9.5, 0°C, 10 min.

Isolation of enzymes

Fatty acid synthases were isolated and assayed by published procedures[19,20]. 6-Methylsalicylic acid synthase was isolated essentially as previouly described[9].

Enzymic synthesis of palmitic acid from chiral malonates.

Each chiral malonate sample (20 μmol) was incubated with succinyl-CoA transferase (4.8 units), succinyl CoA (0.8 μmol), fatty acid synthase (3-5 units), NADPH (0.8 μmol), acetyl CoA (0.4 μmol), tris/H_2SO_4 buffer, pH 8.3 (180μmoles) in a final volume of 2ml in a spectrophotometer cell. The enzyme reaction was followed by the disappearance of NADPH at 340 nm and the reaction was terminated after 4 min. Fatty acids were extracted into ether and methylated prior to purification by g.l.c. The methyl palmitate samples were analysed using a MS 30 mass spectrometer. The data shown are corrected for natural abundance and unlabeled species at m+0 = 270.

Enzymic synthesis of 6-methylsalicylic acid from chiral malonates and acetyl-CoA.

Each chiral malonate sample was incubated with succinyl-CoA (0.8μmol), succinyl-CoA transferase (2.4 units), NADPH (0.8μmol), acetyl-CoA (0.4μmol), tris/H_2SO_4 buffer, pH 8.4 and homogeneous 6-methylsalicylic acid synthase (0.6 unit) in a volume of 2ml. 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 g.l.c. 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 CH_3- from one of the trimethylsilyl groups of the parent trimethylsilyl derivative m+0 = 296.

Enzymic synthesis of 6-methylsalicylic acid from chiral malonates and acetoacetyl-CoA.

Each chiral malonate sample was incubated with succinyl-CoA transferase (0.4 unit), NADPH (0.4μmol), acetoacetyl-CoA (2μmol), tris/H_2SO_4 buffer, pH 8.4 and homogeneous 6-methylsalicylic acid synthase (1.2units) in a final volume of 2ml. 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 g.l.c. The derivatives were analysed using a VG Model TRIO-1 mass spectrometer.

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