Stereospecific Elimination of Hydrogen Atoms with Opposite Absolute Orientations during the Biosynthesis of Orsellinic Acid from Chiral Malonates in *Penicillium cyclopium*

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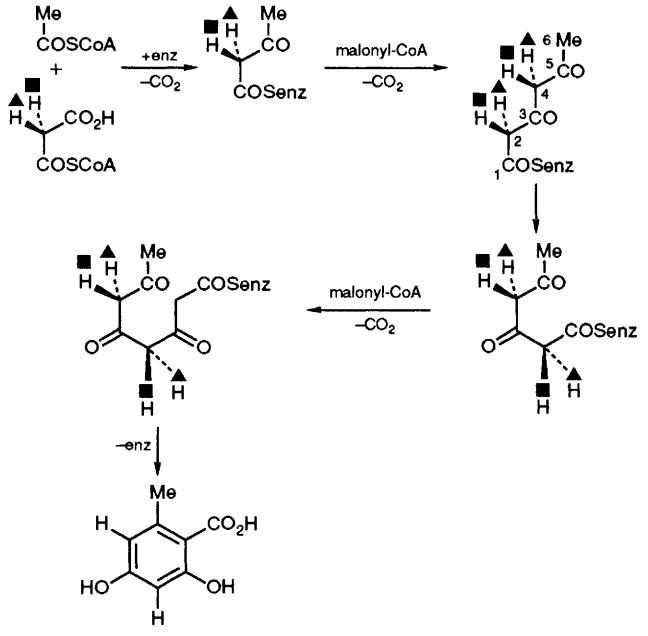
(*R*)-[1-^{13}C; 2-^{2}H]Malonate and (*S*)-[1-^{13}C; 2-^{2}H]malonate are transformed, using succinyl-CoA transferase, into their paired chiral malonyl-CoA derivatives; incorporation of the malonyl-CoA derivatives into orsellinic acid is acomplished using homogeneous orsellinic acid synthase from *Penicillium cyclopium*; mass spectrometric analysis of the orsellinic acid reveals that the hydrogen atoms eliminated from the methylene groups at the 2- and 4-positions of the putative polyketide intermediate are from opposite absolute orientations in malonyl-CoA.

Malonyl-CoA is an important building unit for a large family of natural products which are assembled *via* polyketide intermediates.[1] The parent molecule, malonic acid, shows *pro-pro*-chiral stereochemistry (CX_2Y_2) and thus its paired hydrogen and carboxy groups are indistinguishable from one another by enzyme systems.[2] Similarly, (*R*)-[1-^{13}C; 2-^{2}H]malonate and (*S*)-[1-^{13}C; 2-^{2}H]malonate, in which ^{13}C and ^{2}H isotopes are located within the same molecule,[3,4] although chiral, also are not discriminated from one another enzymically. However, once the (*R*) and (*S*) chiral malonates each are converted into their pairs of malonyl-CoA derivatives, the isotopic labels are manipulated stereospecifically. Thus (*R*)-[1-^{13}C; 2-^{2}H]malonate and (*S*)-[1-^{13}C; 2-^{2}H]malonate, as malonyl-CoA derivatives, have been incorporated successfully into palmitate using fatty acid synthases from yeast, rat and *Penicillium patulum*[3,5] permitting the steric course of all three of these enzymes to be investigated. The chiral malonates have also been incorporated into 6-methylsalicylic acid with highly purified 6-methylsalicylic acid synthase from *P. patulum* and the absolute stereochemistry of hydrogen-atom elimination from the methylene groups originating from malonyl-CoA has been determined.[5–7] These investigations have established that the incorporation of chiral malonyl-CoA into natural products can provide valuable information about the mechanism and stereochemical course of the reactions.

Orsellinic acid, like 6-methylsalicylic acid, is one of the simplest polyketide-derived natural products, being made up of one acetyl-CoA unit and three malonyl-CoA units (Scheme 1). The enzyme responsible for its biosynthesis, orsellinic acid synthase, has been partially characterised in *P. madriti*[8] and also demonstrated in *P. cyclopium*.[9] The biosynthesis of orsellinic acid differs from that of 6-methylsalicylic acid in that no NADPH-dependent reduction step is present with the result that hydroxy functions occur at both the 2- and 4-positions. Consequently, the hydrogen atoms arising from malonic acid at the 3- and 5-positions of the aromatic ring are highly susceptible to base-catalysed keto–enol exchange. Thus, before attempting incorporation experiments with chiral malonates, comprehensive NMR studies were necessary in order to determine the stability of these hydrogen atoms under the conditions required for the enzymic synthesis and subsequent analysis. These experiments established the necessity for highly purified orsellinic acid synthase to maximise the transformation of malonyl-CoA into orsellinic acid and the importance of derivatising the orsellinic acid immediately after its formation before significant exchange of label could obscure sterochemical information.

Orsellinic acid arises from three molecules of malonyl-CoA, thus the incorporation of malonyl-CoA derived from (*R*-[1-^{13}C; 2-^{2}H]malonate or (*S*)-[1-^{13}C; 2-^{2}H]malonate will result in



Scheme 1 Stages in the biosynthesis of orsellinic acid

a maximum of three ^{13}C atoms and two ^{2}H atoms in each molecule of product. The reaction is expected to proceed through a C-6 polyketide intermediate with each Claisen condensation resulting in the inversion of configuration at the methylene carbon atoms (Scheme 1). All Claisen condensations, including that catalysed by fatty acid synthase,[9] have been shown without exception to proceed with inversion of absolute configuration.[10] In view of the very close similarity between the condensing enzymes of polyketide synthases and fatty acid synthase,[1] it may thus be argued, by analogy, that the condensation reaction catalysed by orsellinic acid synthase also is accompanied by inversion. The isotopically labelled hydrogen atoms in the C-6 polyketide intermediate are indicated by ▲ (H_{Re}) or ■ (H_{Si}).† As discussed previously[6] the incorporation of ^{2}H and ^{13}C label from the same molecule of malonyl-CoA into the product are interdependent and will thus reflect the steric course of the subsequent reaction.

Two of the four hydrogen atoms in the C-6 intermediate are removed in the overall transformation into orsellinic acid, thus four broad mechanistic routes may be formulated. In *mechanism a*, H_{Si} ■ (H_{Re} in malonyl-CoA) is eliminated from both the 2- and 4-positions of the polyketide intermediate generating species of $m + 0$, $m + 1$, $m + 2$, $m + 3$, $m + 4$, and $m + 5$ with peaks of relative intensity 0,0,4,4,0,0 using (*R*)-malonate and 1,1,2,2,1,1 for (*S*)-malonate respectively. In *mechanism b* the elimination of H_{Re} ▲ (H_{Si} in malonyl-CoA) from both positions of the polyketide intermediate will give similar but reversed patterns. In *mechanisms c* and *d*, in which either H_{Re} and H_{Si} or H_{Si} and H_{Re} are eliminated from the 2- and 4-positions of the C-6 polyketide intermediate, respectively, both (*R*)- and (*S*)-malonates will give similar mass distributions of 0,2,2,2,2,0 in orsellinic acid. *Mechanisms a* and *b* may thus be distinguished readily from *mechanisms c* and *d* by mass spectrometric analysis.

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 orsellinic acid synthase enzymes are shown in Fig. 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, close to the pattern predicted for a mechanism in which the hydrogen atoms with opposite absolute orientations are stereospecifically removed. The presence of the $m + 1$ and $m + 4$ species in both sets of data is particularly diagnostic for *mechanisms c* and *d* since these species are not formed in *mechanisms a* or *b*. Although some $m + 0$ species is produced, this can be accounted for by unavoidable exchange of orsellinic acid (approximately 30%), which tends to increase the level of species with lower mass and diminish those with higher mass. The cumulative considerations thus point to either *mechanism c* or *d* involving the stereospecific elimina-

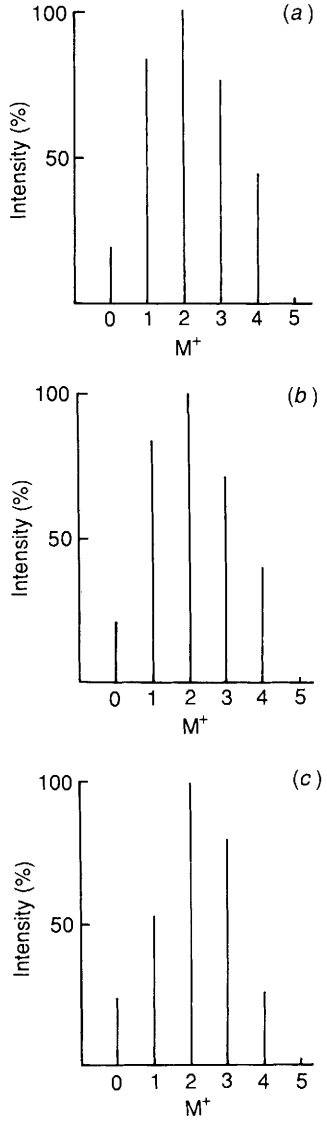


Fig. 1 Observed mass spectra for orsellinic acid biosynthesised from (*a*) (*R*)-[1-^{13}C; 2-^{2}H]malonate, (*b*) (*S*)-[1-^{13}C; 2-^{2}H]malonate and (*c*) a 1 : 1 mixture of (*R*)-[1-^{13}C; 2-^{2}H]malonate and (*S*)-[1-^{13}C; 2-^{2}H]malonate.

Each chiral malonate sample was incubated with succinyl-CoA (0.4 mmol dm^{-3}), succinyl-CoA transferase (2 units), acetyl-CoA (0.2 mmol dm^{-3}), Tris/H_2SO_4 buffer (0.1 mol dm^{-3}), pH 7.8, and homogeneous orsellinic acid synthase isolated from *P. cyclopium* (0.4 units) in a volume of 2 ml. The formation of orsellinic acid was followed fluorimetrically. The orsellinic acid was rapidly converted into the trisilyl 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 + 369 species (loss of Me from one of the trimethylsilyl groups of trimethylsilylorsellinic acid).

† The configurations H_{Re} and H_{Si} refer to the malonyl-CoA and the C-6 intermediate in Scheme 1. The generation of multiple labelled intermediates containing ^{13}C and ^{2}H leads to changes in priority and gives rise to complications in the designation of absolute configuration. It is thus preferential to relate all discussions to unlabelled malonyl-CoA.

tion of malonyl-CoA derived hydrogen atoms with opposite orientations.‡

To reinforce further the above conclusion and to eliminate the remote possibility that the mass spectra of orsellinic acid derived from (*R*) and (*S*) malonates were similar because of extensive racemisation rather than the operation of *mechanism c* or *d*, a 1 : 1 mixture of (*R*)-[1-^{13}C; 2-^{2}H]malonate and (*S*)-[1-^{13}C; 2-^{2}H]malonate was incubated with orsellinic acid synthase under similar conditions. This experiment should yield orsellinic acid with relative intensities for the $m + 0$, $m + 1$, $m + 2$, $m + 3$, $m + 4$, and $m + 5$ peaks of 1,5,10,10,5,1, respectively, since all 32 possible permutations of labelled orsellinic acid are possible. The mass spectrometric analysis of the orsellinic acid shown in Fig. 1(*c*) is close to the predicted mass distribution if an allowance is made, as before, for 30% exchange of deuterium, which results in the enrichment of species with lower mass. This experiment proves conclusively that extensive racemisation of the samples has not taken place and that the enzymic synthesis of orsellinic acid proceeds with a high degree of steric control. These findings contrast those found for the biosynthesis of orsellenic acid by Floss and coworkers,[11] 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.

Although our investigations eliminate *mechanism a* and *b*, in which the hydrogen atoms with the same absolute configuration in malonyl-CoA are lost, the results presented do not permit a distinction between *mechanisms c* and *d*, in which malonyl-CoA hydrogen atoms with opposite absolute configurations are lost and further studies are in progress to address this question.

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‡ In the event of the Claisen condensations catalysed by orsellinic acid synthase occurring with retention of configuration, the results given by *mechanism a* and *b* would be reversed. Since *mechanisms c* and *d* give identical results no effect would be seen in the mass spectrometric measurements.

References

1 D. A. Hopwood and D. H. Sherman, *Ann. Rev. Genet.*, 1990, **24**, 37.
2 H. G. Floss, M.-D. Tsai and R. W. Woodward, *Top. Stereochem.*, 1984, **15**, 253.
3 P. M. Jordan, J. B. Spencer and D. L. Corina, *J. Chem. Soc., Chem. Commun.*, 1986, 911.
4 S. H. Huang, J. M. Beale, P. J. Keller and H. G. Floss, *J. Am. Chem. Soc.*, 1986, **108**, 1100.
5 P. M. Jordan and J. B. Spencer, *Tetrahedron*, 1991, **47**, 6015.
6 P. M. Jordan and J. B. Spencer, *J. Chem. Soc., Chem. Commun.*, 1990, 238.
7 J. B. Spencer and P. M. Jordan, *J. Chem. Soc., Chem. Commun.*, 1990, 1704.
8 G. M. Gaucher and M. G. Shepherd, *Biochem. Biophys., Res. Commun.*, 1968, **32**, 664.
9 B. Sedgwick, D. Morris, and S. J. French, *J. Chem. Soc., Chem. Commun.*, 1978, 193.
10 K. R. Hanson and I. A. Rose, *Acc. Chem. Res.*, 1975, **8**, 1.
11 E.-R. Woo, I. Fujii, Y. Ebizuka, U. Sankawa, A. Kawaguchi, S. Huang, J. M. Beale, M. Shibuya, U. Mocek and H. G. Floss, *J. Am. Chem. Soc.*, 1989, **111**, 5498.