Biosynthesis of 6-Methylsalicylic Acid; the Combined Use of Mono- and Tri-deuteriated Acetate Precursors to investigate the Degree of Stereocontrol in the Aromatisation Sequence

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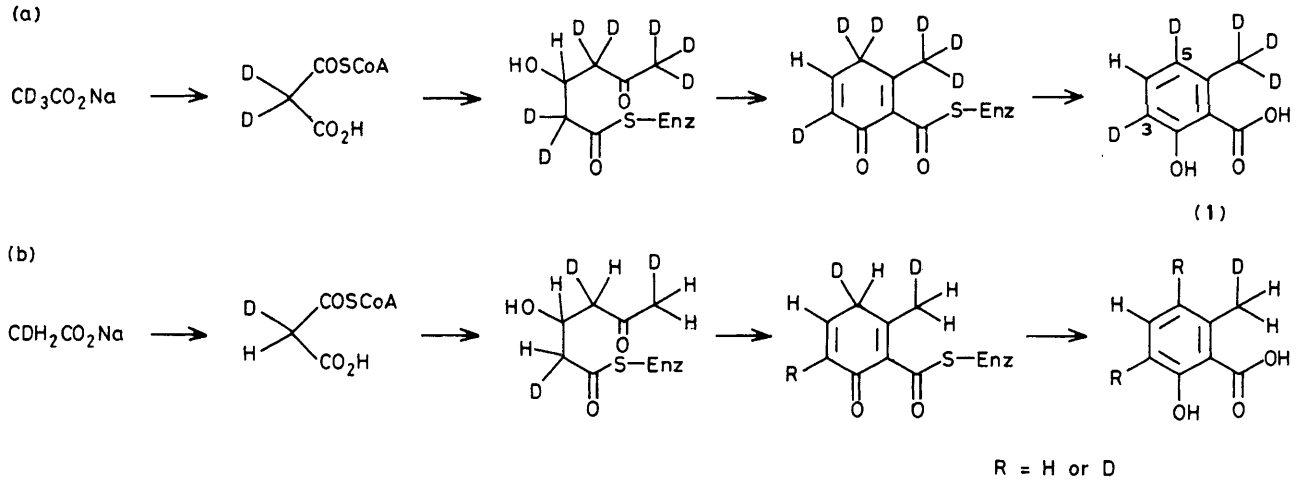
The results from a novel combination of incorporation experiments using mono- and tri-deuteriated acetate precursors indicate that the aromatisation sequence in 6-methylsalicylic acid biosynthesis occurs stereospecifically and therefore is under enzymic control.

Many details of the biosynthesis of the tetraketide 6-methylsalicylic acid (6-MSA) (**1**) are still unknown. In a preliminary study aimed at probing the later stages of the biosynthesis, we described the results of incorporation experiments with [2-$^{2}H_3$]- and [1-^{13}C,2-$^{2}H_3$]-acetate.[1] We now report an extension of this study using monodeuteriated acetate as a precursor. This unprecedented approach has revealed that the aromatisation sequence involves stereospecific hydrogen removal and therefore probably occurs under enzymic control. This conclusion, which is independent of the exact sequence of reactions, supports the assumed role of polyketide-synthesising enzymes in aromatisation reactions.[2]

A possible sequence of late enzyme-bound intermediates on the pathway to 6-MSA is outlined in Scheme 1. At some stage one of the two hydrogens on each of the methylene positions destined to become C-3 and C-5 of (**1**) is removed. It is possible that one of these hydrogen removals is the last step in the aromatisation sequence, for instance enolisation to generate the phenol. Such a reaction could occur spontaneously in a non-stereospecific process without the intervention of a catalytic site on the enzyme (as in the N.I.H. shift[3]). Alternatively there could be one or more specially adapted active sites to catalyse every stage of the aromatisation, with the consequence that there would be stereospecific hydrogen removal at both sites.

The monodeuteriated acetates used in the present study were prepared by reduction of the appropriate bromoacetic acid using zinc in deuterium oxide,[4] and administered to *Penicillium griseofulvum*. Analysis of the ^{2}H n.m.r. spectrum of 6-MSA (as the methyl ester), obtained after incorporation



Scheme 1

Table 1. Relative deuterium distribution at C-3 and C-5 of 6-methylsalicylic acid derived from deuteriated acetate precursors.[a]

Experiment	Precursor	C–3	C–5
1	[2–$^2H_1$]acetate	1.2	1
2[b]	[2–$^2H_3$]acetate	1.2	1

[a] Deuterium retention measured by comparing signal intensities in ^2H n.m.r. spectra of the methyl ester of 6-MSA. [b] From reference 1.

Table 2. Percentage deuterium retention measured relative to ^{13}C at C-3 and C-5 of 6-methylsalicylic acid.[a]

Experiment	Precursor	C–3	C–5
3	[1–^{13}C, 2–$^2H_1$]acetate	21±8 (26)[b]	25±8 (23)[b]
4[c]	[1–^{13}C, 2–$^2H_3$]acetate	80±5	70±5

[a] ^{13}C N.m.r. spectra recorded on methyl ester of 6-MSA. Deuterium retention measured by comparing intensities of β-shifted and unshifted resonances, allowing for the contribution to the latter from ^{13}C at natural abundance. Pulse width 30°, relaxation delay 30 s. [b] Calculated deuterium retention in absence of isotope effects and assuming the same degree of exchange as in experiment 4. [c] From reference 1.

of [2-$^2H_1$]acetate, revealed that the relative retention of deuterium at C-3 and C-5 is 1.2:1 (Table 1). Furthermore quantitative analysis of the ^{13}C n.m.r. spectrum of 6-MSA labelled by [1-^{13}C,2-$^2H_1$]acetate showed a deuterium retention relative to ^{13}C of approximately 25% at each site (Table 2). The results from the previously reported incorporation study with trideuterioacetates[1] are also included in the tables. It should be noted that although the deuterium retention relative to ^{13}C is higher following incorporation of trideuterioacetate than monodeuterioacetate, the relative deuterium retention at C-3 and C-5 is exactly the same in both experiments.

To interpret the above results it is necessary to consider the fate of deuterium from tri- and mono-deuteriated acetates as the molecules are incorporated into the polyketide. Carboxylation of trideuterioacetate generates [2-$^2H_2$]malonyl CoA which acts as the chain extending unit and doubly labels methylene positions on the polyketone chain. These deuterium atoms can be lost by exchange but a significant number are retained.[5] Upon enolisation or dehydration one of the two deuterons at the appropriate methylene position is lost whilst the other is carried forward into the aromatic product (Scheme 1a).

Monodeuterioacetate of course undergoes the same reactions as the trideuteriated species but the presence of the single label imparts a subtle difference in its behaviour (Scheme 1b). Carboxylation of acetyl CoA proceeds with a very small isotope effect[6] to generate three malonyl species, the two epimeric monodeuteriated compounds, and undeuteriated material, all of which are used to assemble the polyketone chain. However, any methylene position on the chain can now only be labelled by one deuterium atom, stereorandomly. The degree of retention or loss of isotope from such a site depends on the stereospecificity of the subsequent hydrogen removal step in the aromatisation sequence. Stereospecific removal from a prochiral methylene removes a certain hydrogen atom regardless of whether it is a protium or deuterium, so that half the label originally present is lost. A non-stereospecific process will more frequently remove protons than deuterons if there is a normal kinetic isotope effect, and so more than half the deuterium atoms will be retained. A very large isotope effect might be expected for the depicted enolisation by analogy with the nonstereospecific enolisation in the conversion of phenylalanine into tyrosine ($k_H/k_D = 10$).[3] Expression of even a small isotope effect in the hydrogen removal at either C-3 or C-5 would result in enrichment of label at that site, and give a different value for the ratio of deuterium retention at C-3 and C-5 from that measured in the trideuterioacetate experiment. This was not observed (Table 1).

It is also possible, but less probable, that hydrogen removal at both C-3 and C-5 proceeds non-stereospecifically with similar isotope effects at both loci, and so maintains the same relative deuterium distribution at the two positions. This possibility was discounted by measuring the deuterium enrichment relative to ^{13}C. The β-shift experiment shows that the deuterium retention corresponds closely to the value calculated if hydrogen removal occurs with no isotope effect (Table 2). This value is arrived at by assuming the same degree of exchange at each site as was measured in the trideuterioacetate experiment.

The ^{13}C and ^2H n.m.r. results are consitent with stereospecific hydrogen removal at C-3 and C-5 in the aromatisation sequence. This in turn suggests that the aromatisation occurs in the chiral environment provided by the active site of an enzyme, presumably part of the same enzyme system responsible for assembling the polyketone chain. Although such a role has long been assumed for polyketide synthases, this study provides the first direct evidence to support that assumption.

Instead of using chiral precursors to investigate the stereospecificity of hydrogen removal the above experiments used three kinds of complementary data. Two precursors were used, two sites of labelling were compared, and two analytical techniques were used. The method is simple and convenient enough to be used on a routine basis for the screening of many different polyketide synthases.[7] While it does not determine the absolute chirality of the hydrogen removal, the technique does provide the essential information which is needed for mechanistic conclusions to be drawn concerning the degree of enzyme involvement.

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