Aspyrone Biosynthesis in *Aspergillus melleus:* Identification of the Intermediates Formed on the Polyketide Synthase (PKS) in the First Chain Extension Cycle Leading to Crotonate

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Incorporation studies, using intact cells of *Aspergillus melleus* and deuterium labelled precursors in association with ^2H NMR spectroscopy as method of analysis, have shown that the enzyme-bound intermediates generated in the first two steps of the chain extension cycle catalysed by the aspyrone polyketide synthase (PKS) are thioesters of acetoacetic acid and (*R*)-3-hydroxybutyric acid.

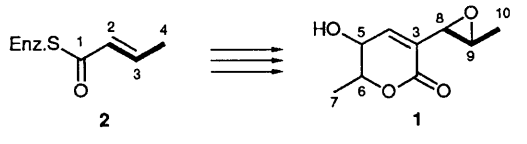
In the previous paper[1] of this series, evidence was presented to support the hypothesis that a thioester of crotonate **2** is the product of the first chain extension cycle carried out by the polyketide synthase (PKS) associated with the chain assembly steps of the biosynthetic pathway leading to aspyrone 1 in *Aspergillus melleus*. The C_3 residue of crotonate, C-2, C-3 and C-4, is incorporated without rearrangement into aspyrone to provide carbons, C-8, C-9 and C-10, respectively, as indicated in Scheme 1. In this paper we are concerned with the biosynthetic steps leading from acetate, the chain starter acid, to crotonate.

It is widely accepted that in the early stages of a biosynthetic pathway leading to a polyacetate derived metabolite such as aspyrone, a linear carbon chain is built up by repeated addition of C_2 units. The individual enzymic steps responsible for these synthetic operations are thought to be based on the chain extension cycle which functions in the biosynthesis of saturated fatty acids.[2] This is shown in Scheme 2. First, a β-ketoacyl residue **4** is generated by condensation of a starter acyl group **3** with a malonate unit, with concomitant decarboxylation. Reduction of the keto group gives the corresponding (*R*)-3-hydroxyacyl analogue **5**, which is then dehydrated to the enoyl derivative **6**. Finally, a further reduction step leads to the saturated acyl compound **7** with a carbon skeleton two carbons longer than that of the starting acyl residue **3**. This basic sequence of reactions is repeated with **7** in place of **3** as the starter acyl group, and so on, until a chain of the appropriate length is reached.

At least four catalytic sites are required for the steps of the chain extension cycle, respectively, a ketone synthase (KS), a ketone reductase (KR), a dehydratase (DH), and an enoyl reductase (ER). These enzymes may be separable, or they may be covalently linked to form a large multifunctional protein, depending on the organism.[2] Collectively they are termed the fatty acid synthase (FAS). Every intermediate generated by the FAS system remains bound to the enzyme system by a thioester link, until the completed chain is finally released. At least two thiol groups are required in the first step, one to bind the starter acyl residue, the other to bind the chain extension unit, malonate. A specialised acyl carrier protein (ACP) serves the latter role and also to carry the subsequent intermediates through the remaining steps of the chain extension cycle. At the end of the cycle, the acyl group is transferred from the ACP to the starter unit binding (Enz·SH) site so that the next cycle can commence.

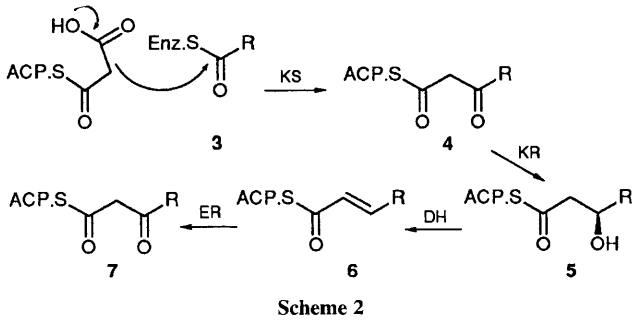
The close parallel between fatty acid biosynthesis and the chain building steps of polyketide biosynthesis has long been recognised, but efforts to provide experimental proof by isolating and purifying active PKS extracts have, with one exception, 6-methylsalicylic acid synthase,[3] been thwarted by the size, complexity, and instability of the constituent proteins. In addition, classical methods of biosynthetic study using intact organisms and labelled precursors have been frustrated by the fact that intermediates need to become bound to the enzyme system by covalent bonds rather than by relatively simple non-covalent molecular interactions. A number of groups, working on various metabolites, have shown that this barrier to incorporation in intact cells can sometimes by overcome by administering the proposed acyl intermediates of the PKS as thioesters of *N*-acetylcysteamine **8**.[4] How this incorporation is achieved is still unclear, but it is likely that this small residue can effectively mimic the thiol terminus of the pantotheine group of the ACP of the synthase, allowing the added intermediate to be elaborated. It may be significant that all experiments so far reported have involved intermediates generated at the end of the relevant chain extension cycle. In these cases the added precursor may be transferred from the *N*-acetylcysteamine residue to the binding site for the starter acid using the normal mechanism for transferring the growing chain from the ACP. No such direct route for attachment to the synthase is available for an *N*-acetylcysteamine analogue of an intermediate generated in one of the reactions within a chain extension cycle, because acyl transfer is not an obligatory process at these stages. In the event this has not proved to be a limitation of the approach, as will become clear from the results presented in this communication.

In this paper we describe the first direct investigation of the intermediates produced within a chain extension cycle on a PKS. In the case of the aspyrone PKS, the final product of the first chain extension cycle is crotonate **6** (R = Me), and

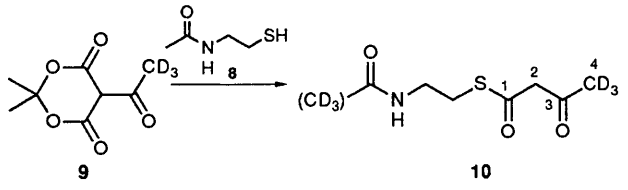


HS. Enz = a thiol attached to a condensing enzyme (ketone synthase)

Scheme 1



Scheme 2

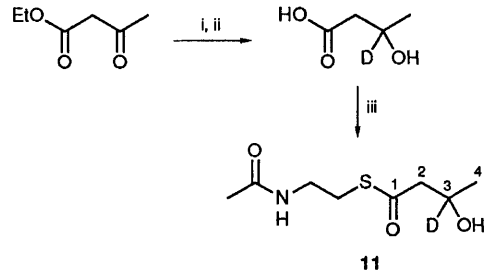


Scheme 3

Table 1 Relative intensities of signals in ^2H NMR spectra of aspyrone following incorporation of ^2H-labelled precursors

		Relative enrichments of ^2H at labelled sites[a]						
		Acyl group of precursor			Aspyrone **1**			
Expt	Precursor	C-2	C-3	C-4	C-5[b]	C-8	C-9	C-10[c]
1	CD_3CO_2H	—	—	—	14	5	0	100
2	**10**	0	0	100	4	<1	0	100
3	**10**	0	0	100	6	<1	0	100
4	(*S*)-**11**	0	100	0	0	0	0	0
5	(*R*)-**11**	0	100	0	0	0	100	0

[a] Measured by ^2H NMR; 0 signifies that there was no detectable signal. [b] Peak overlaps with any signal from deuterium at C-6. [c] Peak overlaps with any signal from deuterium at C-7.



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Scheme 4 *Reagents:* i, $NaBD_4$; ii, NaOH; iii, **8**, dicyclohexylcarbodiimide

therefore the relevant intermediates are expected to be acetoacetate **4** (R = Me), and (3*R*)-hydroxybutyrate **5**, (R = Me) or its (*S*)-isomer. *N*-Acetylcysteamine thioester analogues of these compounds were prepared with strategically placed deuterium labels. The method adopted for the acetoacetate analogue **10** is shown in Scheme 3. The starting material **9** was prepared by acetylating Meldrum's acid with trideuterioacetyl chloride. Reaction with *N*-acetylcysteamine **8** gave the desired product directly. There was some scrambling of acetyl residues between the *C*-acetate and *N*-acetate groups as indicated, but this did not affect the specificity of the labelling in the acetoacetate residue itself. The synthetic approach to the labelled hydroxy acid analogues **11** is shown in Scheme 4. This allowed deuterium labels to be introduced at C-3 by use of $NaBD_4$ in the reduction step. Optically active samples of **11** were prepared by resolution of the hydroxy acid intermediate using quinine as resolving agent. Stereochemical purity was shown to be greater than 98% by use of chiral shift reagents and ^1H NMR spectroscopy.

The results of the incorporation experiments are presented in Table 1. The first experiment, with deuterium-labelled acetate as the precursor, serves as a control for monitoring potential breakdown of larger precursors by enzymes which breakdown fatty acids to acetate; in each case deuterium-labelled acetate would result, and its incorporation would lead to the characteristic and reproducible distribution of deuterium enrichments reported for experiment 1. Note that the reported enrichment for C-10 also includes intensity from the C-7 deuterium signal, and similarly the value reported for C-5 will be inflated if any deuterium is located at C-6. These overlaps do not prejudice the analyses given below, however, because our assessment relies on the difference between the result of the acetate incorporation and that obtained with other precursors. Experiments 2 and 3 with the acetoacetate thioester show a reproducible distribution of deuterium which is significantly different from that observed in experiment 1. The small but significant incorporations at C-8 and C-5 of aspyrone show that some (as much as 50%) of the deuterium incorporation may have resulted from labelled acetate produced by degradation of the precursor. However, the marked selective enrichment of C-10, which would be expected for an intact incorporation of the C-4 precursor, shows that at least half of the labelled molecules of aspyrone have been formed in this way.

The precursors used in experiments 4 and 5 would not be degraded to labelled acetate as a consequence of fatty acid degradation pathway because the isotopic label is placed at a site which would ultimately be converted to the carboxy group of acetate rather than the methyl. The significant result here is the striking contrast between experiment 4 in which no significant deuterium enrichment was observed, and the other in which a high and specific enrichment at the expected site was the outcome. We can therefore conclude that on this PKS system the (*R*)-isomer of 3-hydroxybutyrate is accepted as an intermediate in the first chain extension cycle leading to crotonate but that the (*S*)-isomer is not.

These results support the view that the chain extension reactions of the aspyrone PKS are equivalent to those used in fatty acid biosynthesis. It is interesting that the PKS uses the same isomer (3-*R*) of 3-hydroxybutyrate as the FAS systems investigated to date.[2] It may be that the aspyrone PKS is an incomplete FAS which lacks the active site, enoyl reductase, employed in the final step of the normal FAS chain extension cycle. It will be interesting, therefore, to discover whether the hydroxyacyl intermediates formed in subsequent cycles on the aspyrone PKS have the same absolute configuration at the hydroxy bearing carbon.

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References

1 J. Staunton and A. C. Sutkowski, *J. Chem. Soc., Chem. Commun.*, 1991, preceding communication.
2 *Fatty Acid Metabolism and Its Regulation*, ed. S. Numa, Elsevier, Amsterdam, 1984, p. 29.
3 P. D. Dimroth, H. Walker and F. Lynen, *Eur. J. Biochem.*, 1970, **13**, 98; A. I. Scott, L. C. Beadling, N. H. Georgopapadakou and C. R. Subbarayan, *Bioorganic Chemistry*, 1974, **3**, 2387.
4 S. Yue, J. S. Duncan, Y. Yamamoto and C. R. Hutchinson, *J. Am. Chem. Soc.*, 1987, **109**, 1253; D. E. Cane and C. C. Yang, *J. Am. Chem. Soc.*, 1987, **109**, 1255; Z. M. Spavold and J. A. Robinson, *J. Chem. Soc., Chem. Commun.*, 1988, 4; Y. Yoshizawa, Z. Li, P. B. Reese and J. C. Vederas, *J. Am. Chem. Soc.*, 1990, **112**, 3212.