The Polyketide Synthase (PKS) of Aspyrone Biosynthesis: Evidence for the Enzyme Bound Intermediates from Incorporation Studies with *N*-Acetylcysteamine Thioesters in Intact Cells of *Aspergillus melleus*

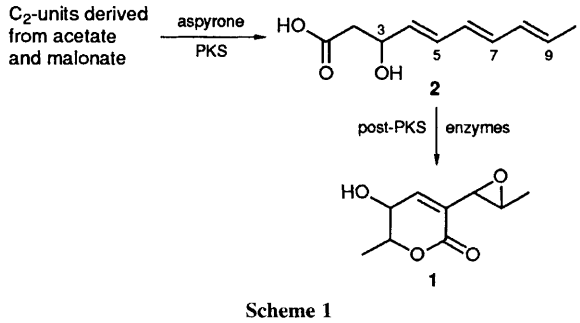
James Staunton* and Andrew C. Sutkowski

University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, UK

The intact incorporation of deuterium labelled *N*-acetylcysteamine thioesters of crotonic, (2*E*, 4*E*)-hexadienoic and (2*E*, 4*E*, 6*E*)-octatrienoic acids into aspyrone support the hypothesis that the aspyrone polyketide synthase resembles a fatty acid synthase in its mode of operation, and that it follows a processive mode of operation.

The distinctive feature of a polyketide biosynthetic pathway is found in the initial steps in which small biosynthetic building blocks (usually C_2 or C_3 units) are successively linked to form an extended carbon chain.[1] The enzymes which operate in this stage of the pathway are collectively known as the polyketide synthase (PKS). The product of the PKS, which has a characteristic linear carbon skeleton and array of functional groups, is occasionally the end product of the pathway. Usually, however, it is elaborated by further biosynthetic reactions to produce a different final metabolite, sometimes with an extensively reorganised carbon skeleton. The proposed pathway leading to aspyrone **1**, shown in Scheme 1, conforms to this plan. The hydroxy acid **2**, which has been demonstrated[2] to be an advanced intermediate, has a linear carbon skeleton consistent with a polyketide biosynthesis from five C_2 units. In subsequent steps there is a major reorganisation of the molecule by cleavage, rearrangement and oxidative processes to produce the branched and truncated carbon skeleton of aspyrone.

The focus of interest in this paper is the mode of operation of the aspyrone PKS. Despite great efforts very little is known about the detailed mechanism of any such enzyme system. Current speculations are guided by the widely held view that PKS systems probably resemble fatty acid synthases. In fatty acid biosynthesis the chain extension is carried out by means of repeated operation of the standard set of reactions shown in Scheme 2.[3] Initially, the starter acid [usually acetyl coenzyme A (CoA)] is bound as a thioester to a thiol at the active site of a ketone synthase which catalyses step 1. The second reactant, malonate, is also bound to the enzyme system, *via*, an acyl carrier protein (ACP). In step 1, a condensation reaction takes place, with concomitant decarboxylation of the malonate residue, to yield acetoacetate. In subsequent steps the β-keto



Scheme 1

group is converted to a methylene. The extended chain is then transferred to the ketone synthase thiol and the next chain extension cycle can commence. All the intermediates remain covalently bound to the system of enzymes until the final completed chain is released. It is this peculiarity of these processes which has made their study exceptionally challenging.

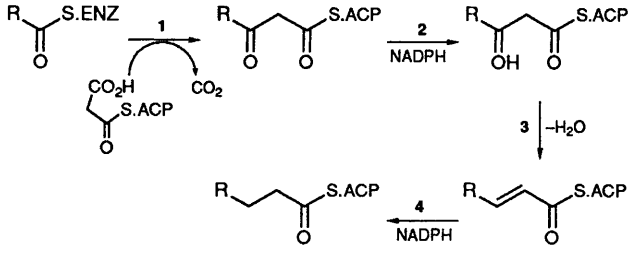
The reactions of a standard fatty acid synthase, with appropriate variations, can account for the formation of **2**, if it is assumed that the aspyrone PKS is 'programmed' in some way to terminate the first three chain extension cycles after step 3 of Scheme 2, and the fourth cycle after step 2. This is the so-called 'processive' mode of operation, in which the new keto group generated in each cycle is appropriately modified prior to the commencement of the next chain extension cycle.[4] Alternatively, a 'non-processive' strategy may operate, in which a 3,5,7,9-tetraketo analogue of **2** is formed by repeated operation of identical chain extension cycles using only step 1 of Scheme 2. The array of functional groups in **2** would then be produced by subsequent modification of the carbonyl groups. It is also possible that the mode of operation lies between these two extremes.

Having identified the advanced precursor **2** of the aspyrone pathway, we are now in a position to test these suggestions.[2] If a processive strategy operates, the sequence of intermediates shown in Scheme 3 would be formed at the end of successive chain extension cycles on the PKS. Analogues of the proposed intermediates **3**, **4** and **5** were therefore synthesised in suitably labelled forms and administered to intact growing cultures of *A. melleus* at the time of aspyrone biosynthesis.

Both free acids and *N*-acylcysteamine thioesters were investigated. The utility of these thioester analogues for the study of PKS systems was demonstrated in the pioneering work of Scott[5] and Lynen[6] on the synthase responsible for 6-methylsalicylic acid biosynthesis. An *N*-acylcysteamine residue is capable of mimicking the thiol terminus of the pantotheine unit which is present in CoA and the ACP components of the PKS systems. In suitable cases, an acyl group attached to the thiol group of the *N*-acylcysteamine can therefore be infiltrated onto the ACP thiol group of the PKS under investigation, and is then ready to be carried through subsequent chain extension steps. Recently, there have been a number of reports that these thioester derivatives are capable of passing intact across cell walls in a number of microorganisms, thus opening the way to incorporation experiments with intact organisms.[4,7]

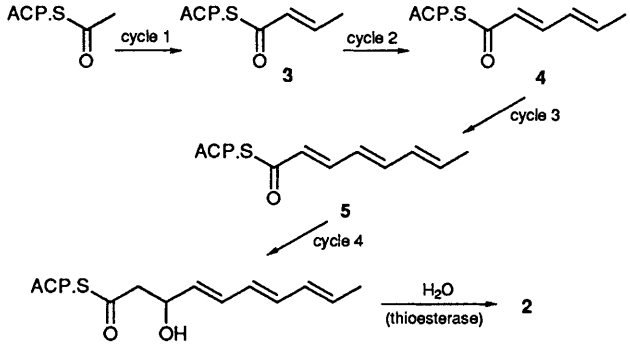
The strategy adopted in this investigation is indicated in Scheme 4. Potential intermediates were synthesised with an array of strategically placed deuterium labels. The rationale behind this approach was twofold: firstly, intact incorporation of the carbon skeleton precursor can be inferred if the expected sites in the metabolite become specifically enriched without change in isotopic ratio; secondly, use of a deuterium label at an appropriate site, rather than ^{13}C, can reveal information concerning possible changes taking place in the functionality of the carbon chain. The latter point is crucial in discriminating between a processive and non-processive synthetic strategy on the PKS.

^{2}H NMR spectroscopy was used to determine the distribution of deuterium in both precursors and the metabolite. Where appropriate, the aspyrone was degraded by ozonolysis to the glycerol **6** (purified and analysed as its tribenzoate derivative) and the epoxy acid **7** (purified as its *p*-bromophenacyl ester).[8] These fragments are derived from different halves of aspyrone and will carry different deuterium labels as indicated, thus allowing the unambiguous determination of the degree of enrichment at the various individual sites of **1** following incorporation of labelled precursors. For the ester of



HS.ENZ = a thiol attached to a condensing enzyme (ketone synthase)
HS.ACP = a thiol attached to an acyl carrier protein (ACP)
NADPH = reduced nicotinanide adenine dinucleotide phosphate

Scheme 2

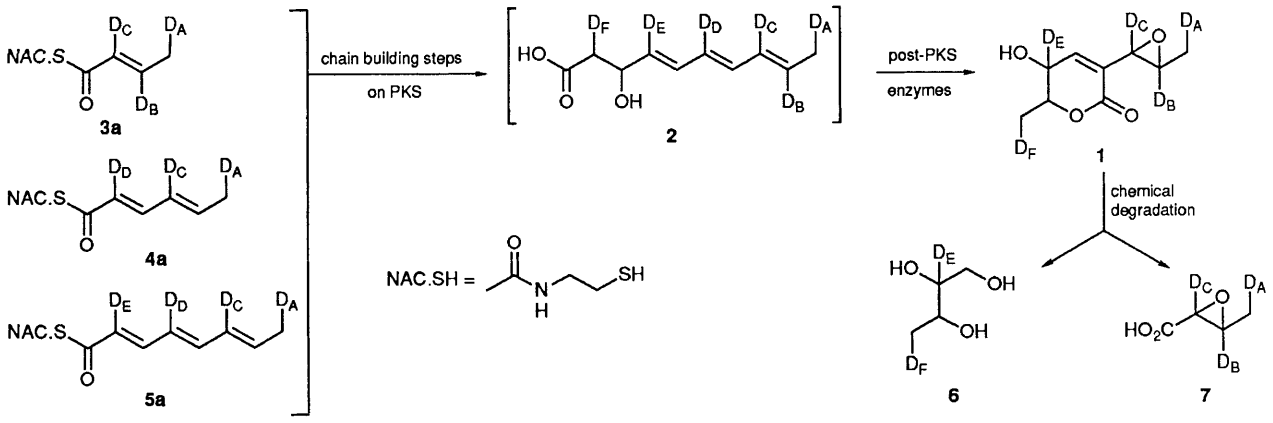


Scheme 3

Table 1 Relative intensities of peaks in the ^{2}H NMR spectra of aspyrone following incorporation of ^{2}H labelled precursors

		Relative enrichments at sites of deuterium labelling[a]									
		Precursor					Aspyrone				
Expt	Precursor	D_A	D_B	D_C	D_D	D_E	D_A	D_B	D_C	D_E	D_F
1	CD_3CO_2Na	100	—	—	—	—	100[b]	0	5	14	100[b]
2	CD_3COS·NAC	100	—	—	—	—	100[b]	0	5	14	100[b]
3	**3a**	100	0	15	—	—	100[b]	0	13	0	100[b]
4	**3a**	100	12	25	—	—	100[c]	14[c]	25[c]	0[c]	0[c]
5	**3**[d]	100	0	33	—	—	100[b]	0	21	12	100[b]
6	**3**[e]	100	0	15	—	—	100[b]	0	5	16	100[b]
7	**4a**	100	0	0[f]	133[f]	—	100[b]	0	0	0	100[b]
8	**4a**	100	0	30[f]	29[f]	—	100[c]	0[c]	27[c]	0[c]	0[c]
9	**4**[e]	100	0	74[b]	74[b]	—	100[b]	0	5	13	100[b]
10	**5a**	100	0	12[f]	27[f]	59	100[c]	0[c]	10[c]	52[c]	0[c]

[a] Measured relative to resonance for D_A = 100. [b] Pairs of peaks not resolved (values quoted are the total intensity of the composite peak). [c] Peaks measured individually on the appropriate degradation product, **6** or **7**. [d] Administered as the *N*-caproylcysteamine analogue of **3**. [e] Administered as the free acid analogue. [f] Signals resolved by use of a shift reagent [Eu(fod)3].



Scheme 4

7 a shift reagent [Eu(fod)$_3$] (fod = 1,1,1,2,2,3,3,-heptafluoro-7,7-dimethyloctane-4,6-dione) was necessary to resolve signals from D_B and D_C.

Initially, as a control experiment, CD_3CO_2Na was tested as a precursor. As in previous investigations a non-uniform but highly reproducible distribution of deuterium was observed (experiment 1, Table 1).[9] The resonances for D_A and D_F of aspyrone overlap and so a composite result is given. This uncertainty could have been overcome by chemical degradation but it was not considered necessary in this case because the more significant result was the relatively high intensity of the resonance for D_E. In experiments with analogues of potential intermediates, **3**, **4** and **5**, this position acts as a control for detecting significant degradation of administered analogues to deuterium labelled acetate prior to incorporation. This is a likely competing fate of these test compounds in an intact organism if the enzymes of fatty acid degradation are accessible and active. As a further control, the *N*-acetylcysteamine (NAC) thioester of deuterioacetate was fed (experiment 2) with similar results.

Experiments 3–6 show the results of various experiments to test the intermediacy of crotonate. The first two, using NAC thioesters, show that this acyl residue is indeed incorporated intact with no significant competing breakdown to acetate. In contrast, the *N*-caproylcysteamine analogue shows evidence of a significant degree of competing breakdown (experiment 5), with the key resonance D_E showing significant incorporation. Finally, in experiment 6, using the free acid as the precursor, the distribution of deuterium in the metabolite is the same as that observed in experiments 1 and 2, showing that prior oxidative breakdown of the precursor to deuterium-labelled acetate is the dominant route of incorporation.

Experiment 4 deserves further comment on the grounds that the precursor is labelled at D_B, a carboxy derived site. The distribution of deuterium in the resulting aspyrone was rigorously established for all the relevant sites by degradation to **6** and **7**. The retention of deuterium D_B without significant loss rules out the possibility of conversion of the precursor to acetoacetate prior to incorporation and therefore strongly supports the proposal that a processive strategy operates in the first chain extension cycle.

Three experiments, 7–9, were carried out to test the proposed intermediacy of the diene **4**. In the first two, NAC thioester analogues with different distributions of deuterium in the diene residue were used. In each case the ^2H NMR of the resulting metabolite was consistent with faithful reproduction of the labelling pattern at the expected sites (the label D_D was necessarily lost). This conclusion was rigorously confirmed for experiment 8 by degradation of the labelled metabolite. Hence the acyl chain of **4** is incorporated intact when administered as its NAC thioester, but experiment 9 shows that the corresponding free acid is completely degraded to acetate prior to incorporation of deuterium labels. Finally, the NAC thioester analogue of **5** was administered (experiment 10), and the results confirm an intact incorporation of this acyl residue also.

In conclusion, the evidence presented in this paper supports the proposal that **3**, **4** and **5** are intermediates generated at the end of successive cycles on the PKS and therefore that a processive mode of chain building is followed. The possibility that the compounds are first oxidised to β-ketones before incorporation into a sequence of polyketone intermediates on a non-processive pathway (a possibility not specifically excluded in most earlier experiments of this type) is most unlikely, because deuterium exchange with the medium would be expected to occur at any methylene flanked by two carbonyl groups. Additional rigorous proof for the processive strategy has been provided for the first cycle by retention of a strategically placed deuterium label in the precursor at a site which would be required to be oxidised to a ketone in the non-processive mode of operation. These experiments therefore provide the strongest evidence yet that a processive mode of operation applies, and not just in early cycles but throughout the operations of the PKS. In the following paper we investigate the nature of the intermediates generated within the first chain extension cycle on this PKS system, and provide further strong support for the processive mode of operation.

We thank Dr A. Bassindale and Dr D. Wilson who acted as internal supervisors to A. C. S. while he was an external research student at the Open University, Milton Keynes, UK.

Received, 15th March 1991; Com. 1/01254A

References

1 J. D. Bu'Lock, *Comprehensive Organic Chemistry*, eds. D. H. R. Barton and W. D. Ollis, Pergamon Press, Oxford, 1979, vol. 5, p. 927.
2 J. Staunton and A. C. Sutowski, *J. Chem. Soc., Chem. Commun.*, 1991, preceding communication.
3 *Fatty Acid Metabolism and Its Regulation*, ed. S. Numa, Elsevier, Amsterdam, 1984, p. 29.
4 S. Yue, J. S. Duncan, Y. Yamamoto and C. E. Hutchinson, *J. Am. Chem. Soc.*, 1987, **109**, 1253.
5 A. I. Scott, L. C. Beadling, N. H. Georgopapadakou and C. R. Subbarayan, *Bioorg. Chem.*, 1974, **3**, 238.
6 P. D. Dimroth, H. Walker and F. Lynen, *Eur. J. Biochem.*, 1970, **13**, 98.
7 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.
8 R. J. Copeland, R. A. Hill, D. J. Hinchliffe and J. Staunton, *J. Chem. Soc., Perkin Trans. 1*, 1984, 1013.
9 M. J. Garson and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1981, 2584.