Investigation of the Stereochemistry of the Tri- and Tetra-ketide Hydroxyacyl Intermediates in the Biosynthesis of the Polyketide Aspyrone in *Aspergillus melleus* using Deuterium Labelling and Deuterium NMR Spectroscopy

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Following administration of both enantiomers of the hydroxyacyl tri- and tetra-ketide intermediates in the biosynthesis of aspyrone labelled with deuterium to *Aspergillus melleus*, it is shown by ^{2}H NMR that in each case only the (*S*)-isomer is incorporated intact into the natural product.

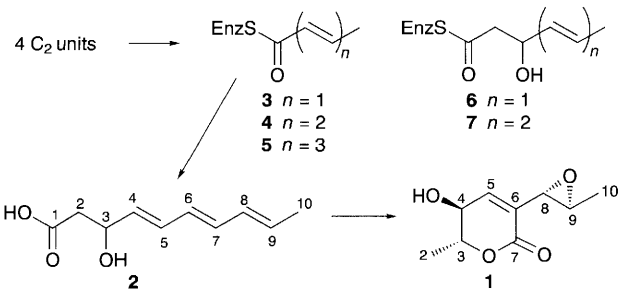
Aspyrone **1**,† a weak broad spectrum antibiotic produced by the mould *Aspergillus melleus*, is a polyketide derived from five C_2 units. The carbon skeleton is derived from five C_2 units which are initially joined head-to-tail to form the linear pentaketide intermediate **2** (Scheme 1).[1] Based on analogy with fatty acid biosynthesis, it is thought that the intermediates leading to **2** are bound by thioester links to the polyketide synthase (PKS) responsible for chain assembly.

Intermediates can be infiltrated onto PKS systems *in vivo* as long as they are administered as *N*-acetylcysteamine thioesters.[2,3] Using this strategy, a succession of unsaturated esters **3**, **4** and **5** have been identified as intermediates leading to **2**.[4] In this paper we prove that the immediate biosynthetic precursors of **4** and **5** are **6** and **7**, respectively, and that the β-hydroxy residues in these tri- and tetra-ketides have the same absolute configuration as the equivalent diketide intermediate, (3*R*)-3-hydroxybutanoic acid.[5]

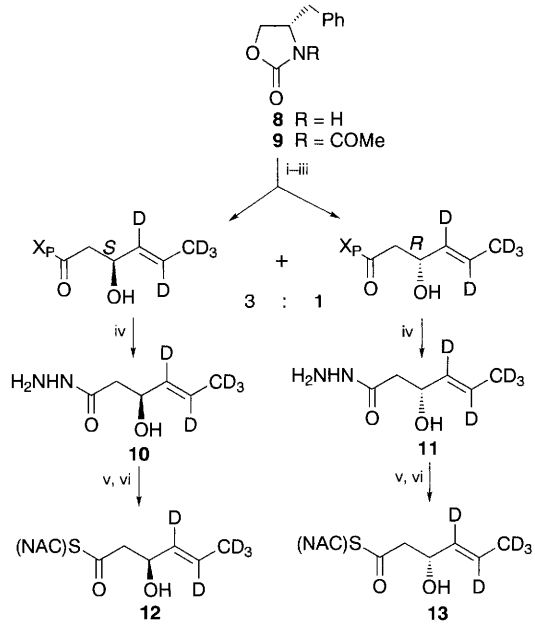
The synthetic route to the putative triketide enantiomers is shown in Scheme 2. Aldol condensation of the acetate derivative **9** of the Evans chiral auxiliary[6] **8** with crotonaldehyde gave a mixture of diastereoisomers which were separated and converted to the respective hydrazides **10** and **11**. An auxiliary was selected that gave a low diastereoselectivity in the aldol reaction so as to provide useful quantities of both enantiomers of the hydrazide from a single preparation. Each of these was converted to its *N*-acetylcysteamine thioester by diazotization followed by nucleophilic attack with the free thiol. The absolute stereochemistry of the major aldol product was determined by converting **10** to the corresponding (3*R*)-hydroxyhexanoyl hydrazide and comparing its optical rotation ($[\alpha]_D^{20}$ −15.0) with the literature value[7] ($[\alpha]_D^{20}$ −15.6). For the biosynthetic tracer experiment, deuterium labels were incorporated into the precursors **12** and **13** by using the multiply-deuteriated crotonaldehyde **14**.[8] With this labelling pattern, an intact incorporation can be inferred if the aspyrone is labelled only at equivalent sites and without change from the isotopic ratio at the various sites in the synthetic precursor.

The synthesis of the putative tetraketides from the diene aldehyde **15** is shown in Scheme 3. The chiral induction in the aldol reaction was again sufficiently low to give useful amounts of both diastereoisomers. A different strategy was adopted in forming the *N*-acetylcysteamine thioester derivatives to avoid α,β-elimination. The absolute stereochemistry of the major aldol product was determined by converting it to methyl (3*R*)-hydroxyoctanoate and comparing its optical rotation ($[\alpha]_D^{20}$ −22.7) with the literature value[7] ($[\alpha]_D^{20}$ −18.4). Two sets of labelled enantiomers were produced, the triply-labelled analogues **16** and **17**, starting from **9a**, and also a pair of singly-labelled analogues, **18** and **19**, starting from **9b**.

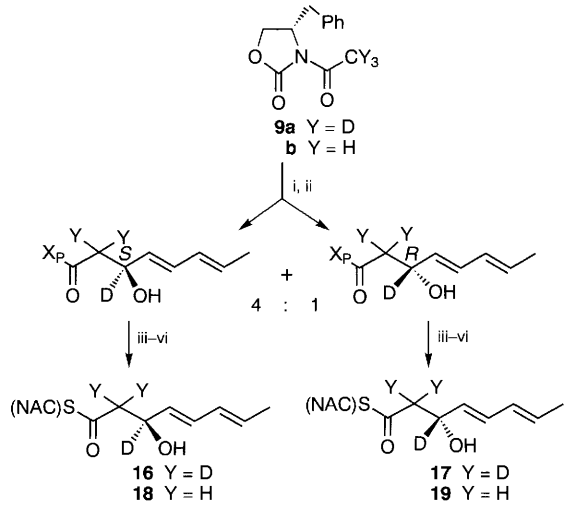
Following an established protocol,[4] each of the two enantiomers of each putative precursor was administered in duplicate



Scheme 1



Scheme 2 *Reagents and conditions*: i, BuLi, MeCOCl; ii, LDA; iii, $CD_3CD{=}CDCHO$ **14**: iv, $N_2H_4{\cdot}H_2O$; v, H_2SO_4, $NaNO_2$; vi, NEt_3, *N*-acetylcysteamine (NAC)



Scheme 3 *Reagents and conditions*: i, LDA; ii, MeCH=CHCH=CHCDO **15**; iii, Bu^tMe_2SiCl, imidazole; iv, H_2O_2, LiOH, 1.5 mol dm^{-3} Na_2SO_3; v, *N*-acetylcysteamine, DCC, DMAP; vi, HF–pyridine

parallel experiments to separate growing cultures. The resulting labelled aspyrone samples were analysed by ^2H NMR.

All four samples of aspyrone **1**, isolated following administration of triketide analogues **12** and **13**, showed intense deuterium NMR spectra. The intensities of the various key signals (relative to D-9) and total incorporation levels are shown in Table 1. Three sites in the metabolite, D-8, D-9 and D-10, which correspond to the labelled sites in the precursor **12**, were the only significant sites of enrichment following incorporation of the (*S*)-enantiomer **12**. Integration of the NMR signals showed that the distribution of deuterium over the three sites is approximately 1 : 1 : 4, which is close to the distribution observed in the precursor (1 : 1 : 3). The extra intensity of the signal at D-10 can be attributed to competing breakdown of the precursor **12** to CD_3-labelled acetate, which would enrich this site and also D-2 whose signal overlaps with that for D-10. A small signal from D-4 can also be attributed to this indirect pathway for incorporation.

In contrast, the aspyrone samples derived from the (*R*)-enantiomer **13** showed much lower levels of incorporation. The ^2H NMR spectra showed that there was substantial isotopic enrichment at sites not expected to be labelled following specific incorporation of the precursor, including D-4, demonstrating that significant breakdown of the precursor to labelled acetate had occurred followed by incorporation of deuterium at several methyl-derived sites. This confirms that the (*S*)-enantiomer is the more efficient precursor. The incorporation of deuterium at D-9 from the 'wrong' enantiomer demonstrates that there is also a minor pathway for intact incorporation of its labelled fragment. The central result remains clear, however: the (*S*)-enantiomer **12** is a more efficient precursor than **13** and it is incorporated intact.

Incorporation studies with the potential tetraketides followed the same protocol beginning with the singly labelled precursors, **18** and **19**, carrying deuterium at the carbinol centre. The aspyrone **1** derived from the (*R*)-enantiomer **19** showed no enriched signals in its spectrum whereas the sample derived from the (*S*)-enantiomer **18** showed a strong signal at δ 6.7 which arises from deuterium at D-5, the expected site of enrichment following intact incorporation. This is strong evidence that the natural tetraketide hydroxyacyl intermediate has the (*S*)-configuration.

Subsequently, the multiply labelled precursors **16** and **17** were tested. The extra labelled site measured the extent to which the precursor was degraded in competition with direct incorporation; any oxidative degradation would now give rise to labelled acetate and so lead to strong deuterium incorporation at D-2 and D-10. The aspyrone **1** derived from the 'wrong' precursor **17** produced a strong ^2H NMR signal for D-2/10 but none for D-5, showing that the precursor had entered the cell and had been degraded but that none of the administered acyl chain had been incorporated intact. The metabolite derived from the 'correct' precursor **16** showed strong signals arising from D-5 and D-4 with the ratio of intensities (1.5 : 1) unchanged from that for corresponding sites in the precursor, within experimental error (±0.2). This is consistent with intact incorporation. In addition there was also a strong signal at D-2/10 showing that, in this case also, there was significant competing breakdown of the precursor to acetate followed by random incorporation of label. Once again, the degree of degradation was not sufficient to throw doubt on the evidence for direct incorporation and it is clear that the natural tetraketide hydroxyacyl intermediate has the (*S*)-configuration.

In summary, the hydroxyacyl intermediates in the second and third chain extension cycles have the (*S*)-configuration which is equivalent to the (*R*)-configuration already established for the corresponding diketide intermediate, 3-hydroxybutyrate, in the first chain extension cycle.

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Table 1 ^2H NMR spectra of aspyrone **1** derived from triketide analogues **12** and **13**

Precursor	Incorporation (%)[a]	Intensity of ^2H NMR signals relative to D-9		
		D-4	D-8	D-2/10
12	0.3	0.03	1.0	4.8
12	0.2	0.08	0.9	3.9
13	0.04	0.3	1.1	6.1
13	0.03	0.6	1.3	5.9

[a] Based on the total amount of deuterium at C-9 in the isolated **1** compared with that at the corresponding position, C-5, of the precursor.

Footnote

† The numbering system for **1** has been chosen so that carbons have the same number as biosynthetically equivalent carbons in structure **2**.

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