The Pentaketide Hydroxyacyl Intermediate in Aspyrone Biosynthesis in *Aspergillus melleus* is shown to be the (*S*)-Enantiomer using Deuterium-labelled Precursors and ^{2}H NMR

Alison M. Hill and James Staunton*

Department of Chemistry, Lensfield Road, Cambridge, UK CB2 1EW

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

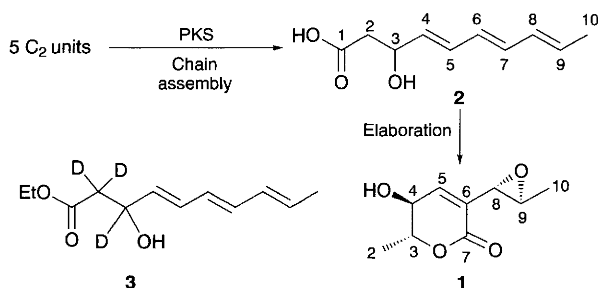
In the assembly of a polyketide chain by a polyketide synthase (PKS) enzyme system successive building blocks are added to a growing acyl chain until an intermediate of the desired length has been assembled. All the intermediates up to that point are thought to remain bound to the PKS *via* thioester links. Finally, the completed chain is released so that it can be further processed by other enzymes to produce the natural product. In terms of the enzymology of the pathway, therefore, the final product of the chain assembly process on the PKS is a pivotal intermediate which links the chain assembly phase of the biosynthetic pathway with the elaboration phase.

The biosynthetic pathway leading to the polyketide aspyrone **1**† is outlined in Scheme 1. Five acetate units are linked head-to-tail on a PKS to form the linear pentaketide intermediate **2**.[1] The corresponding ethyl ester derivative **3**, in the labelled form shown, gave a positive incorporation into aspyrone with the retention of all three deuteriums at the predicted sites. Therefore, the hydroxy acid produced in the fourth chain extension cycle is almost certainly the final PKS product. In this paper we describe further experiments which confirm that the hydroxyacyl pentaketide **2** is formed on the PKS and also demonstrate which enantiomer is active on the pathway. The stereochemistry is of interest because it is already known that the first three hydroxyacyl intermediates of chain assembly have the same absolute configuration at the carbinol centre.[2,3]

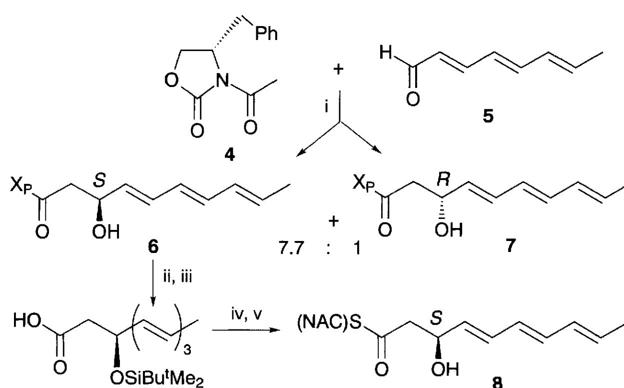
Incorporation experiments were carried out with chiral derivatives of the hydroxyacyl chain derivatised as a thioester of *N*-acetylcysteamine (NAC). The synthesis of the (*S*)-enantiomer **8** is shown in Scheme 2. The acetyl derivative **4** of an Evans chiral auxiliary[4] underwent aldol condensation with 2*E*,4*E*,6*E*-octatrienal **5** to give one enantiomer **6** of the required acyl chain in a useful yield (46%). The good stereoselectivity (up to 8 : 1) made it necessary to prepare the opposite enantiomer **7** by an independent parallel synthesis using the optical antipode of the chiral auxiliary. In both series the major diastereoisomer produced in the aldol reaction was separated by chromatography and converted by standard methods to the *N*-acetylcysteamine thioester. The absolute configurations of the products in the two enantiomeric series were established by conversion of **6** to the known (3*R*)-3-hydroxydecanoic acid ($[\alpha]_D^{20}$ −22.1; lit.[5] $[\alpha]_D^{20}$ −21). The various deuterium-labelled analogues of the potential precursors described below were prepared by simple variations on the synthetic scheme using appropriately labelled forms of the synthetic precursors **4** and **5**.

Initial studies were carried out with the labelled analogue **9** and its enantiomer. The deuterium label at C-3 served as a test of the integrity of the carbinol centre and that at C-2 as an internal standard. Both enantiomers were administered to the growing organism following the standard protocol at the time of aspyrone production.[3] Duplicate experiments were carried out to check for reproducibility. The aspyrone samples derived from both enantiomers proved to be labelled with deuterium according to the ^{2}H NMR spectra. For the samples derived from the (*R*)-enantiomer of the pentaketide only the D-2/10 signal was significantly enriched and the overall levels of enrichment were lower than those observed for the (*S*)-enantiomer. The enrichment at D-2/10 can be explained by degradation of the administered test compound to acetate labelled in the methyl group. The absence of an enriched signal for D-3 rules out intact incorporation of the precursor.

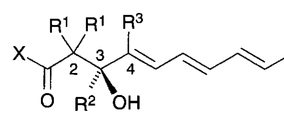
In the aspyrone samples derived from the (*S*)-enantiomer **9** there were signals from other labelled sites in addition to the strong signal attributed to D-2/10. The intensities of the various peaks in the ^{2}H NMR spectra relative to the key signal arising from D-3 are given in Table 1. The strong signal from D-3 is consistent with intact incorporation of molecules of **9** without oxidation of the chiral centre. The significant levels of activity at other sites in the aspyrone derived from **9** show that competing degradative pathways leading ultimately to incorporation of smaller labelled fragments are also in operation. The enrichments of D-4 and D-8 can be attributed, as in past investigations,[6] to breakdown of the precursor to deuterium-labelled acetate which would then label these methyl-derived sites [these sites incorporate deuterium from acetate much less



Scheme 1



Scheme 2 *Reagents and conditions*: i, LDA; ii, Bu^tMe_2SiCl, imidazole; iii, LiOH, H_2O_2; iv, NAC, DCC, DMAP; v, HF, pyridine, THF



9 X = (NAC)S, R^1 = R^2 = D, R^3 = H
10 X = (NAC)S, R^1 = H, R^2 = R^3 = D

Table 1 ^{2}H NMR spectra of aspyrone **1** derived from pentaketide analogues **9** and **10**

Precursor	Incorporation (%)	Intensity of ^{2}H NMR signals relative to D-3				
		D-2/10	D-4	D-5	D-8	D-9
9	0.23[a]	3.3	0.6	0.7	—	0.4
9	0.25[a]	5.2	0.6	0.4	0.4	0.4
10	0.08[a]	2.3	1.4	—	—	—
10	0.06[a]	2.9	1.0	—	—	0.6

[a] Based on the total amount of deuterium at C-3 in the isolated **1** compared with that at the corresponding position, C-3, of the precursor.

efficiently than D-2/10, so labelling was not detectable at them in the less strongly labelled samples derived from the (*R*)-enantiomer]. The excess activity at the control position D-2/10 (average 4.3 instead of the value, 2.0, expected for exclusive intact incorporation of the labelled precursor) can be similarly explained. More surprising is the unprecedented enrichment of D-5 and D-9. These signals arise from carboxyl-derived sites which would be expected to derive the attached hydrogen by transfer of hydride from a nicotinamide coenzyme. We therefore suggest that some of the labelled precursor was oxidised at C-3 by a nicotinamide coenzyme and that the deuterium label was subsequently transferred to carboxyl-derived carbons of other polyketide chains undergoing synthesis at the time of the experiment. It is significant that the levels of enrichment at these sites, which would not be expected to be labelled directly by the precursor, were consistently less than that at C-3.

In view of this unusual transfer of deuterium to carboxyl-derived sites we carried out complementary experiments with an alternative labelled form **10** of the bioactive enantiomer in which the control label was placed at C-4 of the pentaketide rather than at C-2. This would allow better discrimination between direct and indirect incorporation, because the extent of background labelling in the metabolite caused by degradation of the precursor to labelled acetate is much lower at D-4 than at D-2/10 under given conditions. The deuterium NMR of the resulting aspyrone samples from duplicate experiments showed as expected a strongly enhanced signal arising from D-4 as well as D-3 with the two signals being equal in intensity within experimental error (Table 1), as would be expected for an intact incorporation of the added precursor. A strong signal from D-2/10 showed that competing breakdown of the precursor had given rise to labelled acetate (from C-4 of the precursor). Other weak signals were observed for D-5, D-8 and D-9 but they were too close to the noise for reliable integration.

Taken together, the results of these feedings confirm that the acyl residue of the (*S*)-enantiomer **8** of the proposed pentaketide is capable of direct incorporation into the biosynthetic pathway. The relatively strong labelling with deuterium at C-3 in the aspyrone confirms that this enantiomer can be incorporated without oxidation to the keto analogue. Even though a substantial proportion of the test compound is degraded prior to incorporation, however, the evidence is strong that the (*S*)-enantiomer is an obligatory intermediate on the pathway.

Hence, it has been demonstrated that the (*S*)-enantiomer of the pentaketide is an acceptable substrate for aspyrone biosynthesis and the (*R*)-isomer is not. The (*S*)-enantiomer of the pentaketide has the same absolute configuration as the equivalent hydroxyacyl intermediates in the first three chain extension cycles. The ketoacylreductase activity of the PKS therefore has the same stereospecificity throughout the chain extension process. This is consistent with a PKS in which there is a single set of enzymes for all the chain extension cycles, as is the case with the 6-methylsalicylic acid synthase,[7] and there is no cause to invoke a separate set of enzymes for each cycle, as is found for the erythromycin PKS.[8] The absolute configuration of the hydroxyacyl intermediates produced by the aspyrone PKS have the same conformation as the equivalent intermediates in fatty acid biosynthesis.[9] The aspyrone PKS may therefore have evolved from a saturated fatty acid synthase simply by disruption or deletion of the enoyl reductase component.

We thank Trinity College, Cambridge for financial support.

Received, 23rd January 1995; Com. 5/00383K

Footnote

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

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