Investigation of the Stereochemistry of the Dehydration of the Diketide, (3*R*)-3-Hydroxybutyrate to Crotonate, in the First Chain Extension Cycle on the Aspyrone Polyketide Synthase in Intact Cells of *Aspergillus melleus*

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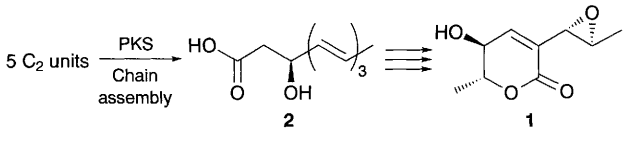
When samples of the diketide, (3*R*)-3-hydroxybutyrate, labelled stereospecifically with deuterium at C-2 in the *Re* and *Si* positions, are administered to *Aspergillus melleus*, and the resulting aspyrone examined by ^2H NMR, the (2*R*)-[2-$^2H_1$]-enantiomer gives a much higher level of incorporation at the expected position which is consistent with a *syn* elimination mechanism.

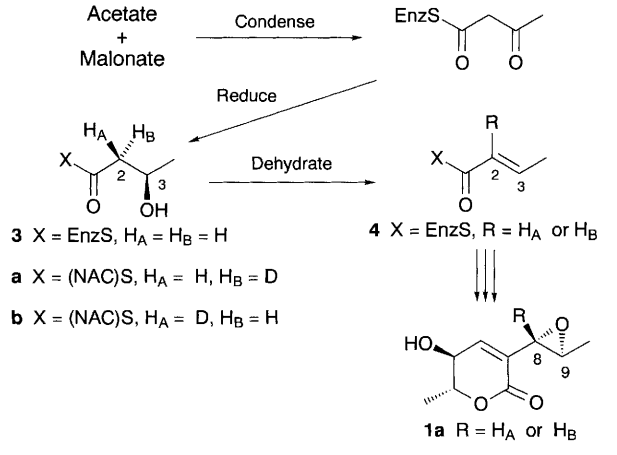
In the early stages of the biosynthetic pathway to the polyketide antibiotic aspyrone **1**, outlined in Scheme 1, a polyketide synthase (PKS) assembles the building blocks to produce a linear pentaketide intermediate **2** by head-to-tail linkage of the five C_2 units. Subsequently, the unbranched carbon skeleton of **2** is elaborated to the branched skeleton of **1** by a series of oxidation and rearrangement reactions.

All the chain extension cycles carried out by the PKS follow the processive mode. At the start of each chain extension cycle the existing acyl group is extended by addition of a C_2 unit to produce a β-ketoacyl intermediate, which is then modified to the corresponding alkene functionality present in **2** prior to further chain extension.[1,2] The established sequence of intermediates for the first cycle is shown in Scheme 2.[3] All the acyl intermediates up to **2** remain bound to the enzyme system *via* thioester links, as indicated. This paper describes an investigation of the stereochemistry of the elimination reaction in which a dehydratase converts **3** to **4**.

The problem can be approached by labelling the two diastereotopic hydrogens, H_A and H_B, of the C-2 methylene group of **3** separately with deuterium. The stereochemistry of elimination will then be established by determining whether H_A or H_B is retained at C-2 of **4**, and ultimately at C-8 of aspyrone **1a**. Two diastereotopically labelled analogues **3a** and **3b** were prepared as their *N*-acetylcysteamine (NAC) thioesters to aid transfer onto the active thiol of the protein to which the acyl chain is bound at the time of the dehydration reaction. Each of the diastereotopically labelled analogues was mixed with a sample of the racemic NAC analogue of **3** labelled with deuterium at C-3. This deuterium, which is incorporated into **4** at C-3, and eventually at C-9 of aspyrone, serves as an internal standard for assessing the degree of retention of H_A or H_B. The relative amounts of deuterium at C-8 and C-9 of **1** can be conveniently measured by running the ^2H NMR spectrum of the labelled aspyrone. The two signals for these positions are well resolved at δ 3.4 and 2.7, respectively, and can be separately integrated. As an additional basis of comparison, an experiment was carried out with the (2*RS*)-[2-$^2H_1$] *N*-acetylcysteamine analogue of **3**.



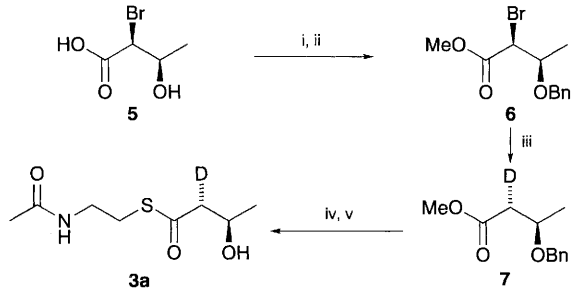
Scheme 1



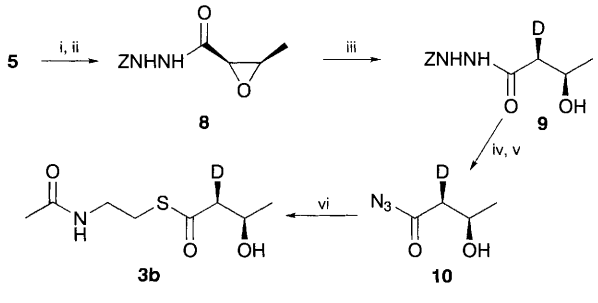
Scheme 2

The synthetic route to **3a**, the (2*R*,3*R*)-labelled analogue of **3**, is presented in Scheme 3. The 2-bromo-3-hydroxybutyrate, derived from L-threonine,[4] was protected as a methyl ester and as an *O*-benzyl ether at the C-3 hydroxy group. With the two residues which might interfere with the stereochemistry of a nucleophilic displacement at C-2 rendered inactive in **6**, the way was open to displace the bromine at C-2 with deuterium by treatment with sodium borodeuteride. The reaction was expected to proceed with inversion of configuration to give **7**. The remaining steps involved removal of the two protecting groups and addition of the NAC residue to give **3a**. The product was shown to be stereochemically pure by the ^1H NMR spectrum, which showed only one of the two signals attributed to the diastereotopic hydrogens at C-2.

The route to the (2*S*,3*R*)-diastereoisomer **3b** shown in Scheme 4 also commenced with the bromo acid **5**. This was converted by treatment with base to the epoxide **8**, which was then derivatised as the benzyloxycarbonyl-protected hydrazide



Scheme 3 *Reagents and conditions*: i, MeOH, HCl; ii, benzyl trichloracetamidate, triflic acid; iii, $NaBD_4$, Me_2SO; iv, Me_3SiI; v, NAC, DCC, DMAP



Scheme 4 *Reagents and conditions*: i, NaH; ii, DCC, $ZNHNH_2$; iii, lithium triethylborodeuteride; iv, TFA; v, HNO_2; vi, NAC; vii, heat

9. The key step involved stereospecific attack by deuteride at C-2, this time with lithium triethylborodeuteride. Because of the double displacement in this scheme, the anticipated configuration of the product is as indicated in **9**. This was then converted to the azide **10** which gave the NAC derivative **3b**. The ^{1}H NMR spectrum confirmed that the configuration at C-2 was opposite to that in **3a**, and that the product was enantiomerically pure.

The biosynthetic precursors were administered to the growing organism following the standard protocol at the time of aspyrone production.[5] The resulting aspyrone was then isolated and purified before being submitted for analysis by ^{2}H NMR to measure the ratio of enrichment of D-8 relative to D-9. The results of the experiments are presented in Table 1. The first two columns show the expected results for an *anti* elimination mechanism and a *syn* elimination, respectively. The third column shows the experimental outcome.

The *trend* of the incorporation levels from a high level of deuterium retention at C-8 for the 2-pro-*R* labelled isomer **3a** to a low retention from the pro-*S*-labelled isomer **3b** parallels that predicted for *syn* elimination rather than *anti* elimination. As expected, the observed retention value for the (2*RS*,3*R*) precursor falls approximately midway between the two extremes. However, all three values differ markedly from the predicted value for a stereospecific reaction. The fact that the aspyrone derived from the (2*RS*,3*R*)-isomer retains only 60% of the predicted amount of deuterium points to a significant washout of deuterium by exchange at C-2 with the medium. This effect would also account for the equivalent lowering of the retention value for the (2*R*,3*R*)-isomer. In contrast, the retention of deuterium at C-8 from the (2*S*,3*R*)-isomer is increased from the predicted value. This effect can also be accommodated if it is assumed that the exchange process at C-2 is accompanied by some racemisation. A proportion of the administered (2*S*,3*R*) isomer would then be converted to the (2*R*,3*R*) isomer by removal and readdition of a proton at C-2, and so lead ultimately to unexpected retention of deuterium by the *syn* elimination mechanism. A plausible reaction sequence which could achieve these effects is reversible oxidation of the hydroxy group to a keto group by transfer of the hydrogen species at C-3 to a nicotinamide coenzyme, followed by non-stereospecific chemically induced exchange at C-2 on the resulting β-ketoester. The oxidation process would have to be reversed by readditon of the hydrogen species from the coenzyme to a keto group at C-3, but not necessarily in the same molecule.

Table 1 ^{2}H NMR spectra of aspyrone **1** following incorporation of NAC analogues of **3** labelled with deuterium at C-2 and C-3

	Ratio of intensities of signals for D-8 and D-9		
Precursor	Calculated for *anti* elimination[a]	Calculated for *syn* elimination[a]	Observed
3a	0	2.0	1.1
Equal mixture of **3a** plus **3b**	1.0	1.0	0.61
3b	2.0	0	0.24

[a] In each experiment the [2-^{2}H$_1$]-labelled material was mixed with an equal amount of [3-^{2}H$_1$]-labelled material as a control. The C-2 labelled material was homochiral, whereas the C-3 labelled material was racemic. The ratio of enrichments at the corresponding sites of aspyrone should therefore be 2.0 : 1.0 because only half of the C-3 labelled material has the correct configuration at C-3 for incorporation.

An alternative explanation which involves competing elimination reactions, the dominant one proceeding with *syn* elimination, the minor one with *anti*, does not fit the results because the balance between the competing reactions would be subject to isotope effects. It can be predicted that the amount of retention for the (2*S*,3*R*) isomer resulting from the minor *anti* elimination reaction would be greater than the amount of loss for the (2*R*,3*R*) isomer resulting from the same process, because the former involves loss of a proton and the latter the loss of a deuterium. In practice the observed lowering of the retention level for the 2-pro-*R* deuterium (45% of the predicted value) is much greater than the raising of the retention level of the 2-pro-*S* label (12% of the predicted value for complete retention).

Although the competing exchange process reduces the discrimination between the two diastereoisomers **3a** and **3b**, the trend of the retention values is unambiguous, and it provides strong evidence that the preferred stereochemistry of the elimination reaction is *syn*. It is likely that the reaction is completely stereospecific, as would be expected for an enzyme catalysed reaction, but this is not proved by our results. The observed stereochemistry for the elimination process matches that established for the equivalent reaction in fatty acid biosynthesis.[6] This further strengthens our suggestion that the aspyrone PKS may have evolved from a saturated fatty acid synthase by loss of the enoyl reductase activity.

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