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Avermectin Biosynthesis. Intact Incorporation Of A Diketide Chain-Assembly Intermediate Into The Polyketide Macrocyclic Ring

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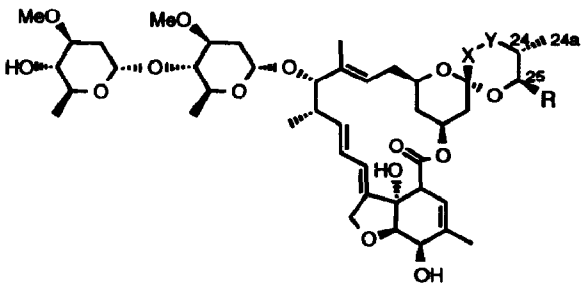
Abstract: *Incorporation experiments in* Streptomyces avermitilis *with synthetic analogues of the proposed diketide intermediate support the processive mechanism of chain assembly in the biosynthesis of the polyketide core of the avermectin structure. Specific incorporation of a ^{13}C labelled precursor was established by ^{13}C NMR; intact incorporation of two deuterium-labelled analogues was established by electrospray mass spectrometry (ESMS).*

The naturally occurring avermectins are a family of 8 macrolide polyketides isolated from *Streptomyces avermitilis*.[1] Two representative structures, **1a** and **1b**, named avermectins B1b and B2b, respectively, are shown below. The structures were determined by NMR spectroscopy, mass spectrometry, and X-ray crystallography.[2]

The polyketide aglycone core is biosynthesised by head to tail condensation of seven acetate and five propionate units onto a starter acid derived from *L*-valine or *L*-isoleucine.[3] The later stages of the pathway have been investigated using blocked mutants, and research on the genes encoding avermectin polyketide synthase (PKS)[4] has supported the overall plan of a proposed pathway.[5]

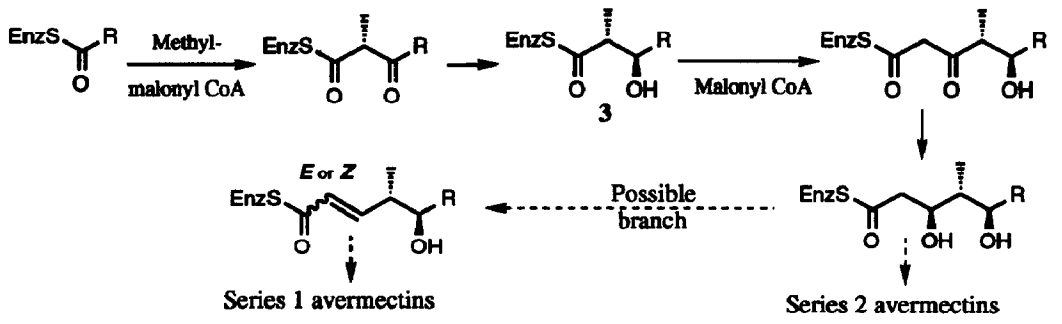


Avermectin		R	X-Y
1a	B1b		CH=CH
1b	B2b		CH_2-CHOH
2a	CHB1		CH=CH
2b	CHB2		CH_2-CHOH

Recently, the range of avermectin structures has been greatly extended by use of a mutant strain of *S. avermitilis* which lacks a branched chain ketoacid decarboxylase responsible for production of the starter acids used by the PKS.[6] In these mutants, avermectin biosynthesis is suppressed but is restored when natural starter acids (such as isobutyric acid for **1a** and **1b**) are introduced into the fermentation medium. A wide range of unnatural starter acids also proved effective, leading to the production of a corresponding range of novel avermectins differing from the natural products in the group attached to C25. One candidate, Doramectin (**2a**), produced by adding cyclohexane carboxylic acid, is an antiparasitic agent under development at Pfizer, and is marketed as Dectomax®.

A possible pathway for the early steps of polyketide chain assembly on the PKS is proposed in Scheme 1. To test this hypothesis, labelled analogues of the diketide **3** were synthesised for biosynthetic incorporation experiments. The synthetic polyketide acyl intermediates were attached to *N*-acetyl cysteamine (NAC) residues

for the feeding experiments to aid transfer of the acyl group onto the PKS, following precedents set in investigations of other polyketide pathways.[7]



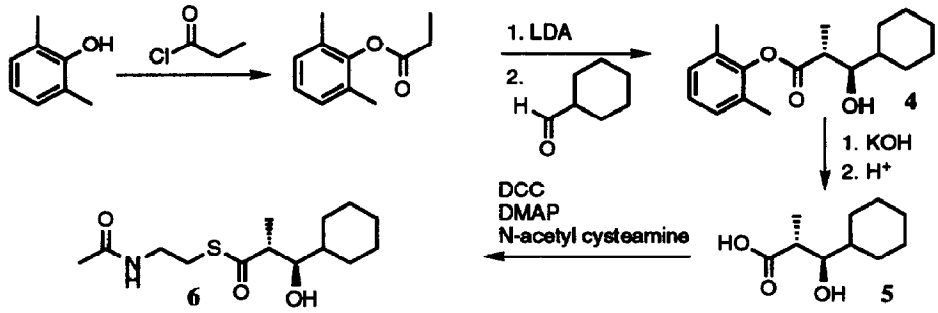
Scheme 1. Postulated early intermediates in chain assembly on the PKS

The deficient mutant of *S. avermitilis* offers an important advantage for the planned biosynthetic study in that natural avermectin production is virtually suppressed. Consequently dilution of the isotopic label in any isolated avermectins by naturally produced material at natural abundance is greatly reduced. The sites of enrichment can therefore be established with greater sensitivity by NMR spectroscopy or mass spectrometry. In this preliminary report the results relating to the cyclohexyl derivatives, **2a** and **2b** are presented.

Results and Discussion

Synthesis of Precursors

The synthetic route to the NAC analogue **6** of the proposed diketide intermediate is shown in Scheme 2. Introduction of the required *threo* stereochemistry was carried out using Heathcock's anti-aldol chemistry to give the ester **4**.[8] Hydrolysis followed by recrystallisation of the dibenzylammonium salt gave a single diastereoisomer of **5** which was converted to **6**. Three labelled versions of **6** (shown in Figures 1, 2 and 3) were prepared by logical variations of the route starting from appropriately labelled starting materials.



Scheme 2. Synthesis of the Diketide Precursor **6** of Doramectin **2a**

Incorporation Experiments

Our initial incorporation experiments, in which the trideuterated precursor **7** was fed on the first day of the 14 day production cycle gave **2a** and **2b** in reasonable amounts, but the metabolites appeared to be unlabelled by deuterium NMR. Mass spectrometry is a more sensitive technique and is potentially useful for isotopic analysis as long as enrichment levels are sufficiently high for any enhanced peaks to show above coincident peaks in the natural abundance spectrum. Because of their structural complexity, avermectins are almost completely fragmented when ionised by electron impact or fast atom bombardment. We have found that

electrospray mass spectrometry (ESMS), a much softer ionisation process, leads to the formation of strong molecular ions (by addition of a sodium or potassium ion) with very little competing fragmentation. All the mass spectroscopic analysis described in this paper was carried out on the sodium adducts, the composition of the ions being confirmed by accurate mass analysis. In the ESMS spectrum of **2b**, for example, there was a strong parent ion (found $[M+Na]^+$, 939.508 ±0.010; $C_{50}H_{76}O_{15}Na$ requires 939.508). The expected ions for the corresponding ^{13}C isotopomers were observed one, two, three, and four mass units higher (calculated relative intensities with respect to $[M+Na]^+ = 100$: 55, 30, 16, 9). ESMS analysis of the avermectin samples isolated from the biosynthetic experiment described above showed no significant variation from this pattern, hence confirming the evidence from deuterium NMR that isotopic labels from the deuteriated precursors had not been incorporated to any significant extent. To explain production of the unlabelled metabolite we reasoned that the precursor had been catabolised by enzymes of fatty acid degradation to produce cyclohexane carboxylic acid. This would initiate the observed biosynthesis in the normal way without incorporation of deuterium.[6]

Thinking that the capacity of the micro-organism to degrade added precursors might be reduced later in the period of production when the bacteria become senescent, as we had already demonstrated in parallel studies of tetronasin biosynthesis,[9] we fed **7** on day 10 of the production period. Avermectins **2a** and **2b** which were isolated after further incubation. Both metabolites now gave prominent $[M+3+Na]^+$ peaks by ESMS, and accurate mass analysis showed these to be due to incorporation of three deuterium atoms (found for $[M+3+Na]^+$, 942.535; $C_{50}H_{73}D_3O_{15}Na$ requires 942.528). The cluster of parent ions for **2b** is shown in Figure 1. In addition to the peak at 941.5 Da, the peak at 941.5 Da is stronger than would be expected for the $^{13}C_2$ isotopomer of **2b**. This is attributed to the presence of a minor impurity which was not identified and could not be removed. It appeared in variable amounts from incubation to incubation, but never in sufficient quantity to vitiate the mass spectrometric analysis.

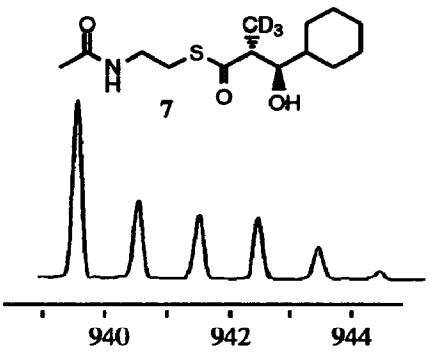


Figure 1. Peaks in the ESMS Spectrum of **2b** Following Incorporation of **7**

No signal could be detected by deuterium NMR spectroscopy of the labelled sample, however, and no suitable fragmentation was obtainable by electron impact (e.i.) mass spectrometry as there was insufficient material. There was therefore no direct evidence from this experiment for specific incorporation of a CD_3 group at a single site in the avermectin structure. As deuterium NMR was not sufficiently sensitive for detection of the site of the deuterium label in the small quantities of avermectins obtained, we turned to the use of ^{13}C as tracer isotope. When incorporation experiments are carried out using wild type or overproducing organisms, ^{13}C labelling is a less useful technique than deuterium labelling because natural production of the metabolite can dilute the ^{13}C label to undetectable levels in the metabolite. With the mutant organism, however, very little dilution should occur.

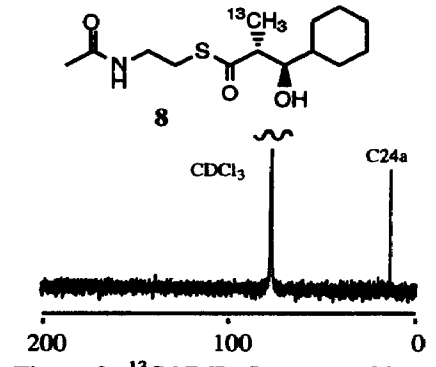


Figure 2. ^{13}C NMR Spectrum of **2b** Following Incorporation of **8**

The ^{13}C-labelled precursor **8** was introduced to the fermentation on day 11, and subsequently small quantities of cyclohexyl avermectins were isolated. An enhanced $[M+1+Na]^+$ peak was present in the ESMS spectrum of **2a**, but the amount of metabolite was insufficient for detecting the site of label by ^{13}C NMR spectroscopy. The second product **2b** also gave an enhanced $[M+1+Na]^+$ peak by ESMS, and the composition of the ion was confirmed by accurate mass measurement (found for $[M+1+Na]^+$, 940.520; $C_{49}{}^{13}CH_{76}O_{15}Na$ requires 940.512). The ^{13}C NMR spectrum (Figure 2) of this metabolite showed a single detectable peak at 13.8 ppm. This confirms the specific incorporation of the label at the predicted position, C24a.[10]

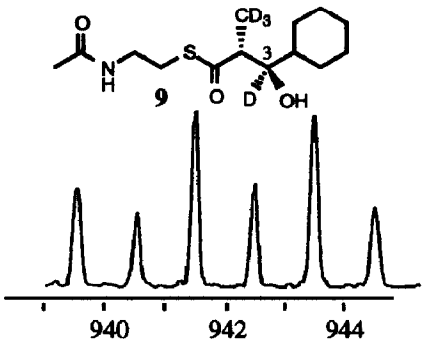


Figure 3. Peaks in the ESMS Spectrum of **2b** Following Incorporation of **9**

Next, the tetradeuterated precursor **9** was prepared. A deuterium label was placed at C3 to investigate the level of oxidation at that site in the course of its incorporation into avermectin. Any obligatory oxidation to a ketone would result in loss of this label; the CD_3 group serves as an internal standard. The precursor was introduced to the fermentation on day 11 and small quantities of **2a** and **2b** were isolated. No molecular ion could be detected from the sample of **2a** but **2b** gave a satisfactory spectrum (Figure 3). As expected, there was an enhanced $(M+4+Na)^+$ peak shown to be due to 4 deuterium atoms by high resolution ESMS (found for $[M+4+Na]^+$, 943.545; $C_{50}H_{72}D_4O_{15}Na$ requires 943.533). The $[M+5+Na]^+$ at 944.5 Da was also enhanced to the extent that would be predicted for the corresponding ^{13}C isotopomer. These two strong ions are attributed to intact incorporation of molecules of the multiply labelled precursor **9** without loss of deuterium. Oxidation of the carbinol site is not therefore essential to incorporation of the diketide precursor.

Any competing reversible oxidation at the C3 carbinol centre would lead to the production of avermectin molecules labelled only with a CD_3 group and hence to the enhancement of the peak at 942.5 Da for $[M+3+Na]^+$. This was indeed enhanced compared with the natural abundance spectrum but the analysis is complicated by the existence of the impurity mentioned above which gives a strong ion at 941.5 Da. The observed intensity in the peak at 942.5 Da can be satisfactorily accounted for by the presence of the ^{13}C isotopomer of this impurity and the $^{13}C_2$ isotopomer of undeuterated molecules of avermectin in our sample. Any contribution to this peak from molecules of avermectin labelled only with a CD_3 group would be at most very small, so reversible oxidation at the carbinol centre does not occur to a significant extent. The impurity does not contribute significantly to the peaks at 943.5 and 944.5 Da, so the evidence for intact incorporation of **9** is compelling. The mechanism of chain assembly in the first chain extension cycle on the avermectin PKS therefore follows the processive mode, as does that of the ionophore tetronasin.

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9 Preceeding papers in this series.

10 Pfizer Ltd., unpublished characterisation data of avermectin cyclohexyl B2.

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