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Investigation of the early stages in soraphen A biosynthesis

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The unusual benzoate starter unit in soraphen A derives from phenylalanine *via* cinnamate in a β-oxidative (plant-like) pathway; 3-phenyl-3-hydroxypropanoate incorporates directly into soraphen by loading onto module 2 of the PKS and indirectly from the β-oxidative pathway to generate benzoyl CoA.

Soraphen A **1** is a novel 18-membered macrolide characterised by an unsubstituted phenyl group and by a cyclic hemiketal moiety. It is assembled by a type I polyketide synthase (PKS), the genes for which have been cloned and sequenced.[1] The first module in the sorPKS contains both the loading domain and the first extension module. This 'starter module' has the function of loading the benzoyl CoA starter unit and a malonyl CoA extender unit, condensing the two units together and reducing the β-keto moiety to produce the diketide intermediate, 3-hydroxy-3-phenylpropanoate **2**, bound to the PKS as a thioester. Benzoate is a rare starter unit in polyketide biosynthesis and has been found to date in only one other bacterial polyketide biosynthetic pathway, the type II enterocin PKS from the marine bacterium *Streptomyces maritimus*.[2] The enterocin benzoate starter unit is derived from phenylalanine *via* cinnamate. Five activities are required for this transformation which generates benzoyl CoA using a β-oxidative pathway; the putative soraphen diketide **2**, but not benzoic acid, is an intermediate in this pathway (Fig. 1). In anaerobic bacteria, phenylalanine is converted to benzoate *via* phenylpyruvate; cinnamate is not an intermediate.[3] We had previously observed labelling of the phenyl ring from [1,3-$^{13}C_2$]-glycerol consistent with the shikimate pathway[4] and Höfle and Reichenbach reported that phenylalanine, but not cinnamate or benzoate, labelled the phenyl ring of **1**.[5] However, a hybrid soraphen/erythromycin bimodular PKS was able to produce a phenyl substituted lactone product only when benzoyl CoA was provided within the cell as a specific precursor.[6] To investigate the origin of the starter unit and diketide intermediate in soraphen A biosynthesis, ^{13}C- and ^{2}H-labelled precursors were administered to *Sorangium cellulosum*. The results of these labelling experiments are reported herein.

Initial experiments focused on the β-oxidative pathway to produce benzoate and 3-[^{13}C]-phenylalanine, [2,3-$^{13}C_2$]-cinnamate‡ and 1-^{13}C-benzoic acid were administered to *S. cellulosum*.§ For all of these compounds we observed incorporation of ^{13}C label into position 17 of soraphen (Table 1). We observed that the incorporation of benzoate, while significant, was much lower than for cinnamate. The most likely explanation for this is that oxidative cleavage of cinnamate gives benzoyl CoA directly and that the low levels of benzoate incorporation into soraphen A arises from low levels of ligase activity and/or from toxicity (benzoate is a known antibacterial agent). A benzoyl CoA ligase has been reported in enterocin biosynthesis, in addition to the β-oxidative pathway from phenylalanine, which provides two possible routes to the starter unit.[2b] No specific benzoyl CoA ligase has been reported in *S. cellulosum* to date[1] and consequently transformation of benzoate to benzoyl CoA would require sequestering a ligase from primary metabolism. To overcome this problem, benzoyl *N*-acetyl cysteamine (NAC) thioester was prepared¶ (these thioesters have been shown to facilitate incorporation of precursors onto polyketide synthases).[7] Unfortunately, we found that NAC thioesters were incompatible with the adsorber resin used in the production media and growing the organism in the absence of resin resulted in a reduction of the soraphen titre from *ca.* 30 to 3 mg litre^{-1}. Consequently, we observed only 5 fold enrichment of [1-^{13}C]-benzoyl NAC thioester into **1**. In conclusion, we have shown that the benzoate starter unit in soraphen A biosynthesis is derived from the deamination of phenylalanine to give cinnamate followed by β-oxidative cleavage to furnish benzoyl CoA in a plant like pathway (Fig. 1). This is the same biosynthetic route observed for the type II polyketide metabolite enterocin.[2]

The benzoyl CoA starter unit is then extended and processed by the starter module of the soraphen PKS to give the diketide, 3-hydroxy-3-phenylpropanoate **2** bound to the PKS as a thioester; note that **2** is also an intermediate in the conversion of cinnamate to benzoyl CoA (Fig. 1). We decided to label the carbinol site with both ^{13}C and ^{2}H so that we could delineate between intact incorporation of **2** (resulting from the substrate being accepted by the PKS) and incorporation *via* degradation of **2** to benzoyl CoA; while it is possible for the ^{13}C label to be incorporated into soraphen by either pathway, the deuterium label will be incorporated into **1** *only* if the diketide precursor loads onto the PKS directly. Despite the problems encountered

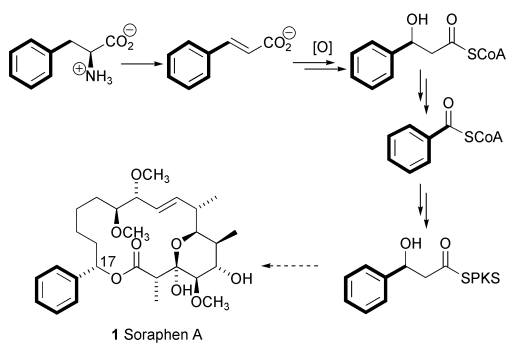

Fig. 1 Early stages in soraphen **1** biosynthesis.

Table 1 Incorporation of precursors into C-17 of soraphen A **1**

Compound	^{13}C Enrichment at C-17 of **1**[a]	^{1}H : ^{2}H[b] (ppm)	$\Delta\delta_C$[c]
[3-^{13}C]-Phenylalanine	23		
[2,3-$^{13}C_2$]-Cinnamic Acid	65		
[1-^{13}C]-Benzoic Acid	10		
[1-^{13}C]-Bz NAC thioester	5		
rac-[3-^{13}C,^{2}H]-Diketide **2a**	2.5	2.3 : 0.2	−0.34
rac-[3-^{13}C,^{2}H]-Diketide **2b**	3.3		
rac-[3-^{13}C,^{2}H]-Diketide **2c**	3		

[a] Expressed as number of fold increase in the height of the carbon-13 resonance relative to unlabelled **1**, with correction by normalising each signal to C-3′,5′ in both labelled and unlabelled samples. [b] Ratio of carbon peak intensities for α-isotope shifted signal. [c] α-Isotope shift due to ^{2}H.

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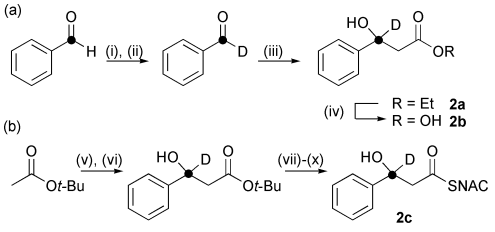
with the use of NAC thioesters described above, it was thought that their ability to facilitate incorporation onto the PKS coupled with lower substrate degradation would outweigh the lower soraphen titre. 3-(*R*,*S*)-[3-^{13}C,^{2}H]-3-hydroxy-3-phenylpropanoate NAC thioester **2c** was prepared (Scheme 1b) and administered to the producing organism. As expected, the soraphen titre was low and 0.5 mg of purified **1** was isolated from a 500 mL culture. Analysis of this sample with ^{13}C NMR resulted in a spectrum with only two signals: We were able to detect ^{13}C enrichment at C-17 of soraphen A (*ca.* 3 fold) in addition to the chloroform peak. No upfield triplet corresponding to intact incorporation of the ^{13}C–^{2}H bond was detected. In the ^{13}C proton and deuterium decoupled NMR spectrum, no additional peak was detected upfield from the C-17 signal. The deuterium NMR spectrum was also inconclusive from this sample. Hence, we decided to repeat the experiment but this time presenting the diketide to the producing organism as the corresponding free acid **2b** and ethyl ester **2a**. This would enable us to use the adsorber resin during the growth of the micro-organism increasing the titre back to 20–30 mg litre^{-1}. However, gain in soraphen production would be counteracted by degradation of the substrate. We chose a literature procedure[2a] to synthesise the target molecule, 3-(*R*,*S*)-[3-^{13}C,^{2}H]-3-hydroxy-3-phenylpropanoate ethyl ester **2a**, from 1-[^{13}C,^{2}H]-benzaldehyde‖ and ethyl bromoacetate; saponification of the ethyl ester gave the corresponding acid **2b** (Scheme 1a). As expected, these feeding experiments resulted in the isolation of between 10 and 15 mg of soraphen from a 500 mL culture. Intact incorporation of the ^{13}C–^{2}H bond was observed in the ^{13}C proton decoupled NMR spectrum of the soraphen sample isolated from the 3-(*R*,*S*)-[3-^{13}C,^{2}H]-diketide ethyl ester **2a** feeding experiment (Fig. 2): a triplet (J = 22 Hz) α-shifted 0.3 ppm upfield was observed which collapsed to a singlet when the deuterium atom was decoupled. We also observed significant degradation of the substrate as a 2.3 fold enrichment of the C-17 signal was observed. For the soraphen sample isolated from the corresponding acid **2b** feed, only ^{13}C enrichment at C-17 was observed (Table 1). We could see no triplet upfield from this signal, and upon simultaneous deuterium decoupling, no new signal upfield from C-17 was detected.

The intact incorporation of the ^{13}C–^{2}H bond of the racemic diketide ethyl ester **2a** into soraphen A demonstrates that it is possible to by-pass the starter module and load an advanced precursor directly onto the second module of the soraphen PKS. Incorporation of the ^{13}C label only from all three diketide precursors demonstrates that the major pathway of incorporation is *via* the β-oxidative pathway to generate the benzoyl CoA starter unit as 3-hydroxy-3-phenylpropanoyl CoA is an intermediate between cinnamate and benzoyl CoA; presumably, the anabolic and catabolic pathways are differentiated by the stereochemistry of the carbinol site.

In summary, feeding experiments with labelled intermediates have shown that the starter unit in soraphen A biosynthesis is benzoyl CoA which is biosynthesised by the plant-like pathway from phenylalanine. Intact incorporation of the racemic advanced intermediate, 3-hydroxy-3-phenylpropanoate, has demonstrated that it is possible to by-pass the 'starter module' of the soraphen PKS.



Scheme 1 Synthesis of 3-(*R*,*S*)-[3-^{13}C,^{2}H]-diketides **2a–c**. (i) $LiAlD_4$; (ii) MnO_4; (iii) $BrCH_2CO_2Et$, $CrCl_2$, LiI; (iv) 1M NaOH; (v) LDA; (vi) Ph^{13}CDO; (vii) *t*-$BuPh_2SiCl$, imidazole; (viii) TFA; (ix) *N*-acetyl cysteamine (NAC), DCC, DMAP; (x) HF–py/THF/py.

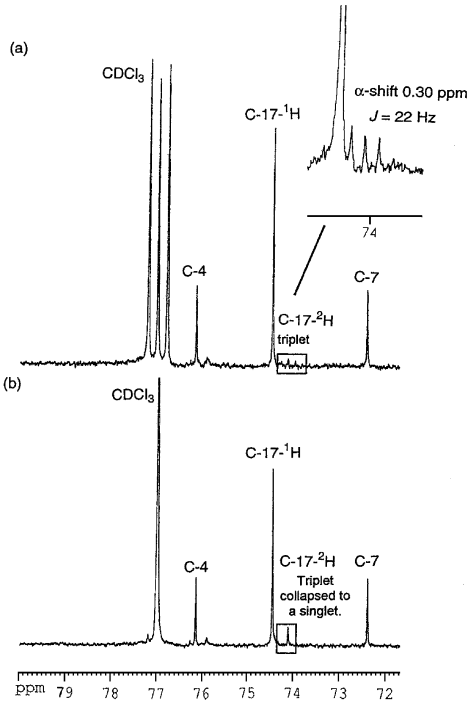


Fig. 2 Intact incorporation of 3-(*R*,*S*)-[3-^{13}C,^{2}H]-diketide ethyl ester **2a** into soraphen A **1**.

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Notes and references

‡ [2,3-$^{13}C_2$]-Cinnamic acid was prepared from a Horner–Emmons reaction between ^{13}C-benzaldehyde and [1-^{13}C]-triethylphosphonoacetate followed by saponification using sodium hydroxide.

§ Cultures of a kanamycin and streptomycin resistant strain of *S. cellulosum* (mutant over-producing strain SJ3) were used for the diketide **2a** and **2b** experiments and the kanamycin resistant strain of *S. cellulosum* (wild-type strain M15) was used for the starter unit experiments following the established protocol.[4] [3-^{13}C]-Phenylalanine (250 mg), [2,3-$^{13}C_2$]-cinnamic acid (100 mg), and 1-[^{13}C]-benzoic acid (1 g) were added as sterile aqueous solutions with a few drops of NaOH to aid dissolution. The diketides **2a** (100 mg) and **2b** (60 mg) were dissolved in water with a minimum amount of ethanol to aid dissolution. The [1-^{13}C]-benzoyl NAC thioester (500 mg) and diketide **2c** (208 mg) were added as 50% aqueous ethanolic solutions.

¶ [1-^{13}C]-benzoyl NAC thioester was prepared from *carboxy*-^{13}C-benzoic acid and *N*-acetylcysteamine with DCC and DMAP.

‖ [1-^{13}C,^{2}H]-benzaldehyde (99% D) was prepared by reduction of [1-^{13}C]-benzaldehyde with $LiAlD_4$ followed by oxidation with MnO_2.

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