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Novel soraphens from precursor directed biosynthesis†

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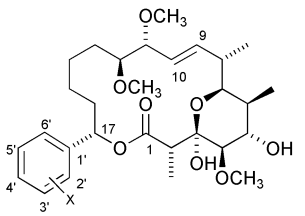
Six novel halogenated soraphen analogues have been isolated from the wild-type producing organism using precursor directed biosyntheses; the best 'delivery vehicle' for the novel starter acids was cinnamate but *ortho* substituents were not tolerated by the soraphen PKS.

The polyketides are a rich source of bioactive compounds and novel non-natural polyketide structures have been produced by combining DNA from different gene clusters and expressing in heterologous and/or wild type hosts.[1] An alternative approach to combinatorial biosynthesis is to supplement the fermentation broth with novel starter acids to produce analogues of the parent compound. Precursor-directed biosynthesis has been used to generate the avermectin analogue Doramectin from a mutant strain of *Streptomyces avermitilis*[2] and to produce novel fluorinated enterocin analogues from the wild type *S. hygroscopicus A-5294*.[3]

The polyketide metabolite soraphen A **1** is a highly efficient inhibitor of fungal acetyl CoA carboxylase and is active against a number of plant pathogens.[4] To date soraphen A has not been commercialised because of its teratogenic effects in animals. A number of soraphen analogues, prepared by derivatisation of the parent compound, have been reported but none of the analogues possessed a better bioprofile than **1**.[5] The unusual benzoyl CoA starter unit in soraphen A[6] is loaded and processed by the starter module in the soraphen PKS.[7] We were interested in whether non-natural starter units could be loaded and processed by the soraphen PKS to produce novel analogues that may lack the adverse teratogenic properties of soraphen, but retain its potent antifungal properties.

The benzoyl CoA starter unit in soraphen A is derived from phenylalanine *via* cinnamate and in isotopic labelling studies we had observed that benzoate incorporated poorly compared to cinnamate.[6] Hence, initial experiments focussed on determining the best delivery vehicle for the incorporation of non-natural starter units into the soraphen skeleton. We chose to use ^{19}F-labelled precursors as fluorine can be detected by ^{19}F NMR which would enable us to quickly determine whether novel fluorine containing compounds had been produced.



X = H, soraphen A **1**
X = 3'-F, 3'-fluoro soraphen A **2**
X = 4'-F, 4'-fluoro soraphen A **3**
X = 3',4'-F_2, 3',4'-difluoro soraphen A **4**
X = 3',5'-F_2, 3',5'-difluoro soraphen A **5**
X = 4'-Cl, 4'-chloro soraphen A **6**
X = 4'-Br, 4'-bromo soraphen A **7**

Fluorinated phenylalanine, cinnamate and benzoate were administered to the wild type organism§ and the cell counts and soraphen titre determined. Unlabelled substrates were used as a positive control to ascertain their effect on metabolism. Soraphen A was produced in all of the cultures, and higher soraphen titres were observed for cultures supplemented with unlabelled phenylalanine or cinnamate. Analysis of the crude extracts from the cultures fed fluorinated benzoate by ^{19}F NMR and LC-MS showed that no fluorine-containing soraphens were present. The cell counts and soraphen titres were unaffected by the addition of fluorinated benzoate which suggests that substrate toxicity was not a problem. The lack of incorporation of the fluorinated benzoates may be due to the necessity to convert the acid to its CoA thioester; the ligase activity required for this transformation is either absent or unable to process the fluorinated substrate.

We observed significantly lower cell counts and soraphen titres for the cultures fed fluorinated phenylalanines suggesting that these compounds were toxic to the organism, however, analysis of the crude broth extract by ^{19}F NMR and LC-MS showed that novel fluorine-containing compounds were present for the cultures fed 3- and 4-fluorophenylalanine. No new compounds were detected for the cultures administered with 2-fluorophenylalanine suggesting that the *ortho* substituent could not be processed by the enzymes which convert cinnamate to benzoyl CoA and/or the soraphen PKS. The cell count and soraphen titre were not affected by the addition of fluorinated cinnamates and analysis of the crude extracts showed that fluorinated compounds were present only for 3- and 4-fluorocinnamates. Once again, the *ortho* fluorine substituent was not accepted and no new fluorinated soraphens were isolated from the cultures fed 2-fluorocinnamate or pentafluorocinnamate. The novel fluorinated soraphens were extracted from the cultures with soraphen A and purified from soraphen A using preparative HPLC. Approximately 1 mg of **2** and **3** were isolated and fully characterised by ^{1}H, ^{13}C (Fig. 1), ^{19}F NMR, mass spectrometry¶ and their biological profile evaluated by Novartis (now Syngenta) against the fungus *Botrytis cinerea*. Unfortunately, **2** and **3** were found to be slightly less active than soraphen A and so further biological testing was not carried out.

These initial experiments demonstrated that it was possible to generate novel soraphen analogues by feeding substituted *meta*- and *para*-fluoro-phenylalanine and cinnamate to the producing organism. Higher cell counts and soraphen titres were obtained from the experiments using cinnamates and consequently, subsequent experiments used substituted cinnamates as the 'delivery vehicle' for the novel starter units. The rejection of pentafluorocinnamate was consistent with the inability of the organism to process *ortho*-substituted cinnamates but did not establish whether more than one substituent could be accomodated on the phenyl ring. To determine whether two fluorine atoms could be incorporated into the phenyl ring of soraphen, 3,4- and 3,5-difluorocinnamates were fed to *S. cellulosum*. The crude soraphen extracts were analysed in each case by ^{19}F NMR and LC-MS and were shown to contain a novel difluoro soraphen. 3′,4′-Difluoro soraphen A **4** was purified by preparative HPLC and fully characterised,¶ however there was insufficient 3′,5′-difluoro soraphen **5** produced for it to be isolated and so it was identified by ^{19}F NMR and LC-MS only.‖

† Electronic supplementary information (ESI) available: full spectroscopic data for novel fluorinated soraphens **2–4**, Table S1: LC-MS and ^{19}F NMR data for soraphen analogues. See http://www.rsc.org/suppdata/cc/b3/b303543n/

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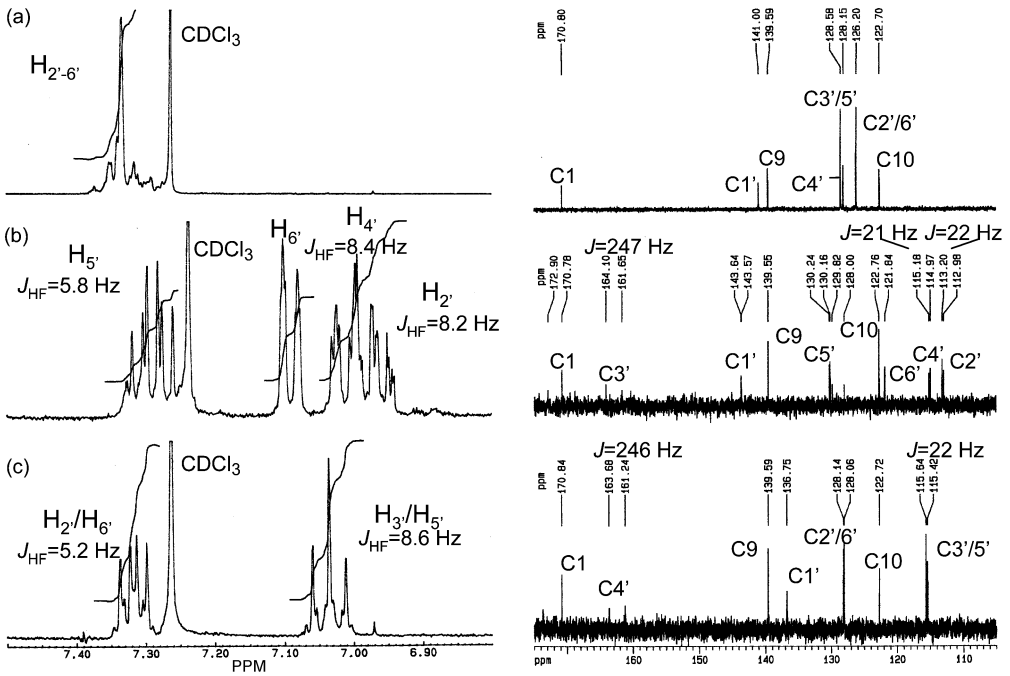

Fig. 1 ^{1}H NMR (aromatic region) and ^{13}C NMR (105–175 ppm) spectra for (a) soraphen A **1** (b) 3′-fluoro soraphen A **2** and (c) 4′-fluoro soraphen A **3**.

Finally, we were interested in whether other halogens could be accepted and processed by the soraphen PKS so 4-chloro and 4-bromocinnamate were administered to the producing organism. The crude extracts were analysed by LC-MS and novel mono-chlorinated and mono-brominated soraphen analogues were detected, however isolation of these compounds was not possible due to the minute quantities obtained. We observed molecular ions with the characteristic isotopic abundance and distribution within each sample in the mass spectra of **6** and **7**.‖

In summary, we have shown that the soraphen loading domain can accept and process some novel halogenated starter units. Only *meta* and *para* substituents were tolerated on the phenyl ring. We found that the best way to present the non-natural starter units was as the appropriately substituted cinnamate and consequently, the substituents are tolerated by all the enzymes which convert cinnamate to benzoyl CoA as well as the soraphen PKS and post-PKS activities. These results suggest that combinatorial biosynthetic experiments with the soraphen loading module should enable halogenated aromatic compounds to be generated in addition to phenyl substituted ones.

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Notes and references

§ Halogenated substrates (250 mg) were administered to cultures of a kanamycin resistant strain of *S. cellulosum* (SJ3) (500 mL) following the established protocol.[8] Substrates were added as sterile aqueous solutions with a few drops of NaOH to aid dissolution. The XAD-1180 resin was extracted with Pr^{i}OH and the crude mixture analysed by LC-MS and ^{19}F NMR. The crude soraphen mixture was purified by flash chromatography (SiO_2, 1 : 1 hexane/ethyl acetate) and fractions containing soraphen (R_f 0.3) and adjacent fractions were combined. Preparative reverse phase HPLC (C-18) was used to separate soraphen A from the novel halogenated soraphens.

¶ Full spectroscopic data for novel fluorinated soraphens **2–4** is given in the supplementary information.†

‖ Refer to Table S1† for LC-MS data for **1–7** and ^{19}F NMR data for **2–4**.

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