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First *in vitro* directed biosynthesis of new compounds by a minimal type II polyketide synthase: evidence for the mechanism of chain length determination†

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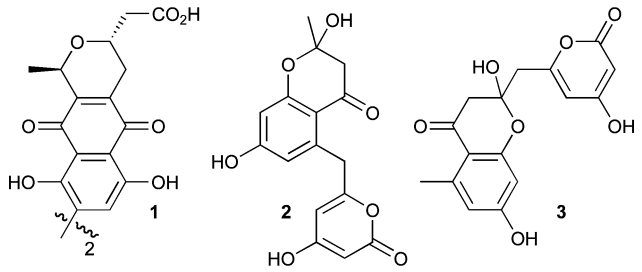
The minimal actinorhodin polyketide synthase bearing two point mutations (KS_β Q161A, ACP C17S) was chemically modified to carry novel C_4 to C_8 starter units on the ACP: on incubation with an excess of malonyl CoA new 16-carbon polyketides are made, supporting a measuring mechanism.

Actinorhodin **1** is the archetypical dimeric 16-carbon polyketide produced by a type II polyketide synthase (PKS). In *Streptomyces coelicolor*, a series of genes encode several discrete proteins which associate to form the functional actinorhodin PKS.[1]



Experiments reported by ourselves,[2] and others,[3] have identified a minimal *in vitro* PKS, consisting of an acyl carrier protein (ACP) together with two β-ketoacylsynthase (KS) components (KS_α and KS_β). KS_α is responsible for carbon–carbon bond formation, while KS_β has been shown to decarboxylate malonyl ACP to provide the acetyl ACP starter unit which initiates chain assembly.[4] Thus incubation of KS_α, KS_β and ACP with malonyl ACP yields three C_{16} polyketides SEK4 **2**, SEK4b **3** and dehydro-SEK4b.

Early *in vivo* experiments involving expression of sets of type II PKS genes from different organisms suggested that KS_β controlled the length of the polyketide carbon chain.[5] This component was thus annotated chain length factor (CLF). However, more recent experiments suggest that other PKS components can influence the final carbon-chain length.[6] It therefore seems likely that chain-length determination is a function of the entire PKS complex.

Two different strategies could be envisaged for chain length determination. One mechanism would be to count the number of condensation reactions (seven in the case of the octaketides **1**, **2** and **3**). An alternative would be to measure the length of the chain. Measuring strategies are known to operate in related synthase systems such as fatty acid biosynthesis, where defined protein clefts limit the size of the growing fatty acid,[7] and in chalcone synthases where the size of the active site pocket controls the overall size of the product.[8] Counting strategies are less common, but a rare possible example comes from experiments with 6-methylsalicylic acid synthase (6-MSAS) which will accept a range of starter units and extend them by two condensations in each case to make pyrones in the absence of NADPH.[9]

† Electronic supplementary information (ESI) available: LCMS spectra and detailed experimental procedures for all reported experiments. See http://www.rsc.org/suppdata/cc/b3/b300847a/

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In order to investigate how the actinorhodin minimal PKS determines chain length we have examined its activity with different starter units. We have recently developed synthetic methods for the preparation of a wide range of acylated C17S actinorhodin ACPs required for the experiments we wished to perform.[10,11]

Incubation of malonyl ACP with KS_α/KS_β results in rapid production of C_{16} polyketides **2**, **3** and dehydro-**3** (Fig. 1B).[2] Because malonyl ACP is required as a chain extender unit, and KS_β rapidly decarboxylates malonyl ACP to make the usual chain-priming acetyl ACP, it is not possible to supplement this system with alternative starter units as they are out-competed by the native acetyl ACP production for the synthesis of **2** + **3**. We thus turned to a mutant KS_α/KS_β system in which starter unit production was impaired by the introduction of the KS_β mutation Q161A.[4] In the presence of malonyl ACP alone, polyketide production by this sytem is very slow because of the low decarboxylase activity, and hence rate of starter unit production.[4]

In initial experiments we synthesised hexanoyl ACP **4** and β-keto-octanoyl ACP **5**.[11] These ACPs were then included in separate assay mixtures with *holo*-ACP, malonyl CoA and KS_α/KS_β(Q161A). In both cases, after 2 h at 30 °C, the produced polyketides (*ca* 1 nM) were extracted, concentrated and examined by LCMS. Compounds **2**, **3** and dehydro-**3** were produced under these conditions, mirroring our previous results. However new compounds were also synthesised (Fig. 1C). Three of the major compounds were present in sufficient quantity (*ca* 100 pM each) for analysis by ESMS.

ESMS analysis (Fig. 1E–G) showed that the new compound eluted at 14.5 min possessed a *m*/*z* of 309 Da for the protonated molecular ion (MH^+). Facile dehydration was indicated by $[M - H_2O]H^+$ at *m*/*z* 291 Da. High resolution mass analysis under chemical ionisation (CI) conditions showed that the $(M - H_2O)H^+$ ion had *m*/*z* 291.123734, corresponding to a molecular formula of $C_{16}H_{18}O_5$ (calc. 291.123249, −1.7 ppm). The hydrated compound therefore has a molecular formula of $C_{16}H_{20}O_6$. MS analysis also indicated that the 15.0 min compound was related to the 14.5 min compound by loss of water. Similarly the 21.9 min peak yielded to analysis of $C_{16}H_{18}O_5$.†

In a second round of experiments we made butyryl ACP[11] and β-keto-hexanoyl ACP.[10] When used as surrogate starter units, both of these acyl species were incorporated into a new range of polyketides differing from those observed when hexanoate/β-keto-octanoate were used as starters. MS analysis showed the empirical formula of these compounds to be $C_{16}H_{16}O_6$.

The results of these experiments clearly show, for the first time, that a type II minimal *in vitro* PKS is capable of synthesising new compounds when supplemented with synthetic starter units and when the native ability to manufacture acetyl ACP from malonyl ACP is slowed.

All of the new compounds possess 16 carbon atoms. Although the exact structures have yet to be fully determined, the molecular formulas are consistent with polyketides analogous to **2** and **3**. Use of the starter units hexanoate **4** and β-keto-octanoate **5** resulted in the same 16-carbon products, showing

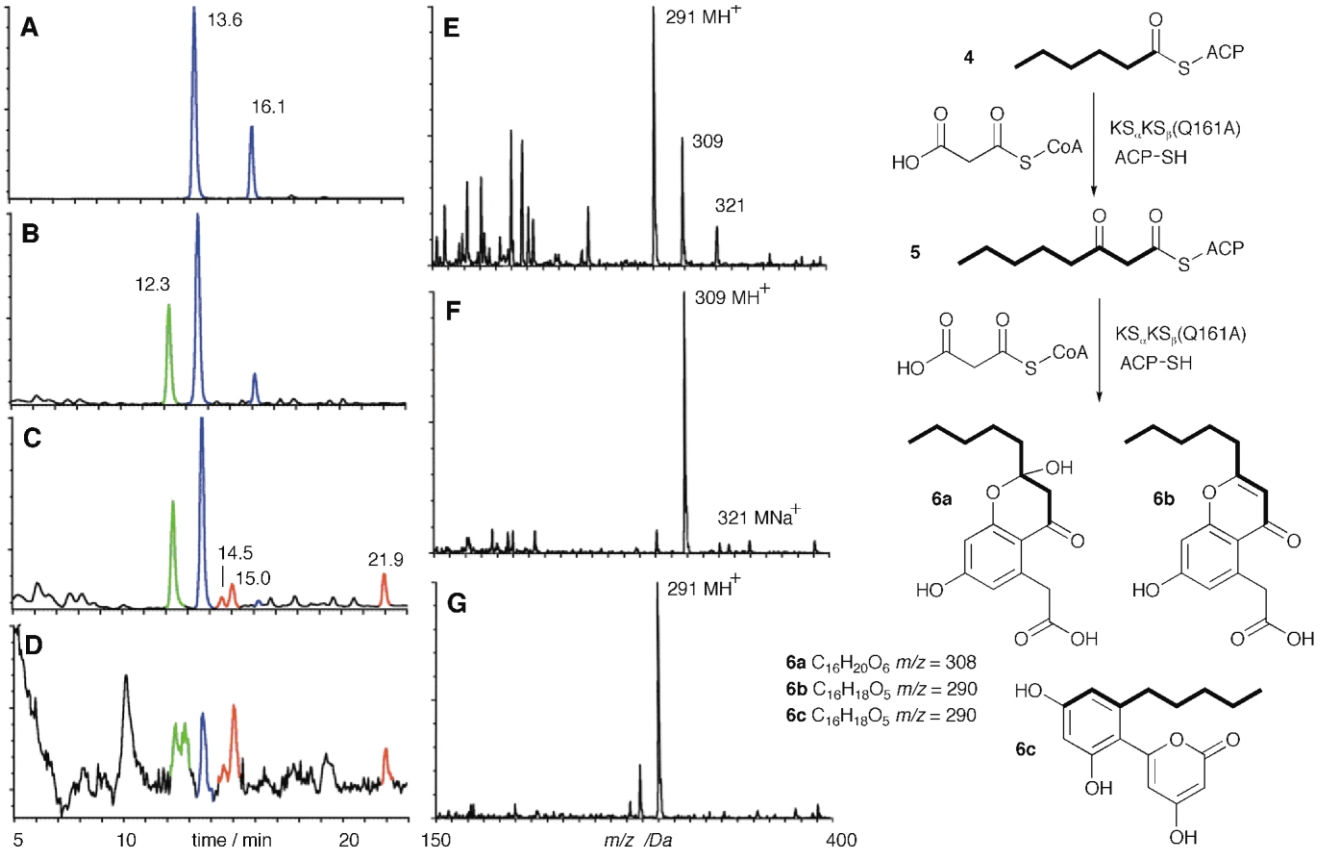
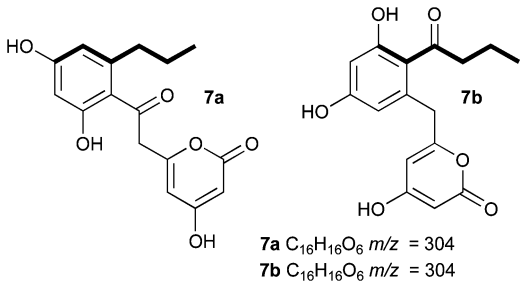


Fig. 1 LCMS Data for compounds produced by KS_{α} and KS_{β}(Q161A) *in vitro*. **A**, UV detection (218 nm) for standard **3** (13.6 min) and dehydro-**3** (16.1 min) shown in blue; **B**, UV detection of incubation with ACP and malonyl CoA alone with the WT actinorhodin PKS showing production of **2** (green) and **3**; **C**, UV detection for incubation with ACP, malonyl CoA and β-keto-octanoyl ACP **5**, new compounds are indicated in red; **D**, total ion current detected in ES^+ mode for experiment C; **E**, ESMS of 14.5 min compound; **F**, ESMS of 15.0 min compound; **G**, ESMS of 21.9 min compound.



that hexanoate is extended first to β-keto-octanoate, and then on to the final products by *four* more condensations (possible structures **6a–c** are indicated in Fig. 1). Likewise, butyrate is extended to β-keto-hexanoate before being extended by *five* more condensations to give compounds such as **7a,b**. Chain length in each case is limited to 16 carbons, despite the differing numbers of condensation reactions catalysed. The results of these experiments suggest that the actinorhodin minimal PKS governs chain length by measurement. The range of compounds produced likely reflects the range of possible aldol/Claisen type cyclisation patterns available to putative poly-β-keto intermediates, and the facile dehydration to form pyrones and lactones, mirroring the observation that at least three C_{16} polyketides are produced when acetate is the starter unit.

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