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In vitro reconstruction of tetronate RK-682 biosynthesis

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Abstract

The protein phosphatase inhibitor RK-682 is one of a number of potentially valuable tetronate polyketide natural products. Understanding how the tetronate ring is formed has been frustrated by the inaccessibility of the putative substrates. We report the heterologous expression of *rk* genes in *Saccharopolyspora erythraea* and reconstitution of the RK-682 pathway using recombinant enzymes, and show that RkD is the enzyme required for RK-682 formation from acyl carrier protein-bound substrates.

Natural products remain an outstandingly important source of leads and inspiration for drug discovery and their importance has been boosted by rapid advances in our ability to clone and manipulate the gene clusters that govern their production1. A growing family of polyketide natural products, characterized by the presence of the unusual five-membered tetronate ring, is known to interact with novel, diverse targets to mediate either apoptotic or antibacterial effects2-8. Intensive genetic investigations of several tetronate biosynthetic clusters2-6 have highlighted candidate enzymes that might catalyze formation of the tetronate C-C and C-O bonds, but the evidence remains inconclusive in the absence of biochemical evidence9-12. However, there have been no examples of modular polyketide synthase multienzymes being successfully used *in vitro* to generate advanced polyketide intermediates as substrates for unusual biosynthetic enzymes. The search for a tractable system in which to study tetronate formation led us to examine the biosynthesis of the 3-hexadecanoyl-5-hydroxymethyltetronic acid RK-682 (**1**)8. This compound is a potent inhibitor of protein phosphatases13 and of HIV-1 proteinase14. We initially predicted that RK-682 would be pieced together from a C18 3-oxoacyl thioester (from fatty acid biosynthesis) and a glyceryl-*S*-acyl carrier protein (ACP) thioester derived from glycolytic intermediates15. Glyceryl-*S*-ACP has been previously synthesized *in vitro*16 using recombinant enzymes encoded by biosynthetic genes for the polyether tetronate antibiotic tetronomycin (**9**)4 and homolog of these enzymes are also found in the chlorothricin (**10**)2, kijanimicin (**11**)5, and tetrocarcin A (**12**)6 biosynthetic gene clusters. We now propose the detailed biosynthetic scheme for **1** shown in Fig. 1, in which the unusual enzyme RkD plays a central role.

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AUTHOR CONTRIBUTIONS

Y.S., H.O. and P.F.L. formulated the project; Y.S., Y.D. and J.C. carried out cloning and analysis of the gene cluster; Y.S. carried out gene knock-outs, heterologous expression and *in vitro* reconstitution; F.H. carried out chemical synthesis and part of the *in vitro* reconstitution; M.T. carried out high resolution mass analysis; Y.S., H.O. and P.F.L. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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To clone the **1** biosynthetic genes, we screened a cosmid library of *Streptomyces* sp. 88–682 with the FkbH-like glyceryl-*S*-ACP synthase gene *tmn16* from the **9** biosynthetic gene cluster of *Streptomyces longisporoflavus* A1984. We subjected a positively-hybridizing cosmid to shotgun sequencing. Bioinformatic analysis identified, within an 11.8 kbp region, nine predicted open reading frames (*orfs*) designated *rkA-rkI,* whose putative functions suggested a role in **1** biosynthesis (Fig. 2a and Supplementary Table 1). The sequence data have been deposited in the EMBL/GenBank databases under the accession number GQ332353. To confirm the identity of the cluster, we undertook in-frame deletion of *rkD* (Supplementary Methods). The resulting ΔrkD mutant produced no **1**. Complementation with *rkD* under either a constitutive promoter P*ermE** or a pathway-specific activator promoter P*actII-ORF4* restored synthesis of **1** (Supplementary Methods and Supplementary Fig. 1).

We then determined the minimal set of genes that is both necessary and sufficient for **1** biosynthesis in a heterologous actinomycete host strain. We constructed a series of plasmids harboring portions of the **1** cluster and expressed them in *Saccharopolyspora erythraea* JCB2 (Fig. 2b and Supplementary Fig. 2). This strain lacks the ability to biosynthesize erythromycin A (**13**)17 and provides a clean background for the detection of **1**. Each gene cassette was placed under the P*actII-ORF4* promoter using the integrative vector pCJR12418 (Supplementary Fig. 2). Strain JCB2:pYH281, which carries the six genes *rkA-rkF*, produced **1** although the production level was only about 1% that of the wild-type producer strain under comparable conditions (Supplementary Fig. 3 and Supplementary Methods). In contrast, neither JCB2::pYH266, which only contained *rkA* to *rkE*, nor JCB2::pYH264, which only contained *rkB* to *rkE* (Fig. 2b) produced **1**. The six genes *rkA-rkF* therefore comprised a minimal set essential for **1** biosynthesis. In this minimal gene cassette, the homologous ACP gene *tmn7a* from the **9** gene cluster4,16 can replace the ACP gene *rkF* (Supplementary Fig. 2, Supplementary Fig. 4 and Supplementary Methods).

We then probed the roles of the adjacent *orfs, rkH* (encoding a putative antibiotic efflux protein) and *rkI* (homologous to the TetR-family transcriptional regulator gene). As shown in Supplementary Fig. 5 and Supplementary Fig. 6, an in-frame deletion mutant ΔrkH produced no **1**. In addition, co-expression of *rkA-rkB-rkC-rkD-rkE-rkF* and *rkH-rkI* on pYH284 in JCB2 gave a dramatically increased level of production of **1** to about nine-fold compared with pYH281 without *rkH-rkI* (Fig. 2b). These results suggest that *rkH*, and possibly also *rkI*, increase **1** biosynthesis and/or export of the antibiotic. Since the ΔrkH mutant also lost the ability to produce a brown pigment (Supplementary Fig. 6c), *rkH* may regulate additional pathways. The gene *rkG*, encoding a discrete typeII thioesterase likely acts to remove misacylated ACP domains and stalled polyketide intermediates19,20. An in-frame deletion mutant ΔrkG (Fig. 2b and Supplementary Fig. 7) produced **1** at only about 26% of wild-type levels. Consistent with this, the heterologous expression of pYH282 containing *rkA* to *rkG* resulted in an increase of **1** production of about four-fold compared with pYH281 (Fig. 2b); and addition of *rkG* to the cassette containing the eight genes *rkA-rkB-rkC-rkD-rkE-rkF* and *rkH-rkI* (to give pYH285) boosted **1** production to levels comparable to the wild-type 88–682 strain (Supplementary Fig. 8). These results strongly suggest that this cassette of nine contiguous genes comprises the **1** biosynthetic gene cluster.

Having defined the genes that contribute to **1** production *in vivo*, we expressed and purified candidate Rk proteins from recombinant *Escherichia coli*, and attempted to reconstitute portions of the biosynthetic pathway *in vitro*. First, we tested the ability of RkE and RkF to form glyceryl-*S*-RkF from D-1,3-bisphosphoglycerate (**14**, D-1,3-BPG), a reaction we have shown in the **9** biosynthetic pathway16. Accordingly, we incubated RkE and holo-RkF (RkF containing the 4′-phosphopantethinyl prosthetic group) with ATP, $MgCl_2$, D-3-phosphoglycerate (**15**, D-3-PG) and D-3-phosphoglyceric acid phosphokinase (D-PGK), and

analysed the mixture by LC-MS (Supplementary Methods). As shown in Supplementary Fig. 9, a distinct new peak appeared with a mass corresponding to the molecular ion for glyceryl-*S*-RkF (11,241 Da), consistent with substantial transfer of the glyceryl group from D-1,3-BPG to holo-RkF catalyzed by RkE.

Another discrete ACP gene (*rkB*) is located between *rkA* and *rkC* (Fig. 2a). To investigate directly the possibility that this ACP participates in glyceryl-*S*-ACP formation, RkB was incubated with each of the FkbH-like glyceryl-*S*-ACP synthases RkE and Tmn1616. However, no glyceryl-*S*-RkB was formed (Supplementary Table 2), and we therefore sought an alternative role for RkB.

We were initially surprised to uncover a single-module polyketide synthase (PKS) multienzyme (RkC) in the **1** gene cluster, because the fatty acid biosynthetic pathway could have directly furnished from a 3-oxo-stearoyl-*S*-ACP thioester. Instead, it appeared that *rkA*, whose gene product shows a high degree of homology to authentic acyl-CoA synthetases21 might activate palmitic acid (**2**) and load it onto the ACP RkB before elongation on RkC. To test this, we added holo-RkB to a mixture containing **2**, CoA, ATP, $MgCl_2$ and RkA. In support of the proposed function of this protein, a substantial conversion (about 80%) of **2** into palmitoyl-*S*-RkB took place, while no acylation occurred in a control assay without RkA (Supplementary Fig. 10). Consistent with these *in vitro* results, an in-frame deletion of *rkA* gave an approximately 2,000-fold decrease in **1** production (Supplementary Fig. 11 and Fig. 2b). The ΔrkA mutant still produced a trace of **1** which can be explained if another enzyme can (albeit feebly) substitute for RkA in activation of **2**. Expression of the intact PKS RkC (subunit molecular weight 108,446 Da) in soluble form in *E. coli* was initially unsuccessful even after co-expression with the flanking *rkB, rkD* and *rkE* genes. Finally, by employing heat-shock and co-expression with *mtaA* (Supplementary Methods), a gene encoding a 4′-phosphopantetheinyl transferase Sfp from *Stigmatella aurantiaca*22, we obtained small amounts of purified soluble RkC. We reasoned that the product of the RkC-catalysed elongation might remain tethered to the multienzyme, which would make it difficult to confirm by LC-MS directly that elongation had occurred. To circumvent this, we supplemented RkC with additional recombinant holo-ACP(RkC), expressed and purified as a single domain from *E. coli*, which should compete23 with the integral ACP domain of RkC for malonyl extender units and subsequently also as a substrate for chain elongation. This would give rise to 3-oxo-stearoyl-*S*-ACP(RkC) (Supplementary Fig. 12), whose formation we could monitor precisely by LC-MS. After addition of the holo-ACP(RkC) domain to a reaction mixture containing intact holo-RkC, purified palmitoyl-*S*-RkB and malonyl CoA (**16**), a new and significant peak was seen at 39.01 min, with a mass corresponding to the expected 3-oxo-stearoyl-*S*-ACP(RkC) (Supplementary Fig. 13a). This species did not form in the absence of **16** (Supplementary Fig. 13b). This result clearly demonstrated that RkC is a fully functional PKS that can carry out one round of polyketide chain elongation.

For catalysis of the assembly of **1** from 3-oxo-stearoyl-*S*-RkC and glyceryl-*S*-RkF, the only plausible candidate remaining was the gene product of *rkD*, which bears sequence similarity to 3-oxoacyl-(acyl carrier protein) synthase III (FabH), an essential enzyme in fatty acid biosynthesis which initiates fatty acid elongation by decarboxylative condensation of a malonyl-ACP and an acyl-CoA. RkD and its homologs in other tetronate biosynthetic pathways all contain the active site cysteine residue typical of FabH, but they lack the conserved histidine residue implicated in decarboxylation by FabH2, 4-6 (Supplementary Fig. 14). However, in all these other clusters, there are additional unassigned genes which might participate in tetronate ring formation, and indeed disruption of *chlM* has been reported not to affect **10** production2. For our reconstitution experiments *in vitro*, we incubated RkD over-expressed in *E. coli* as an N-terminally His_6-tagged protein with

glyceryl-*S*-RkF and 3-oxo-stearoyl-*S*-ACP(RkC) created as described in Supplementary Methods. Analysis of the reaction products using LC-MS showed the presence of a new compound, with a retention time and an MS^n fragmentation pattern which were identical to that of authentic **1** (HRMS, calc.: 369.2641, found: 369.2629) (Fig. 3a,b). The compound was not found in the absence of RkD. These observations provided the first direct experimental evidence that RkD is necessary and sufficient to accomplish tetronate ring formation in **1**, as summarized in the mechanistic proposal of Fig. 1.

The recognition that 3-oxo-stearoyl-*S*-ACP(RkC) is a competent substrate for RkD prompted us to undertake the chemical synthesis of 3-oxo-stearoyl-*S*-CoA (**8**, Supplementary Methods, Supplementary Scheme 1 and Supplementary Fig. 15-20), to provide this intermediate in quantity. Incubation of the synthetic **8** with apo-ACP(RkC) (RkC lacking the prosthetic group) and 4′-phosphopantetheinyltransferase Sfp (to form 3-oxo-stearoyl-*S*-ACP(RkC)), together with glyceryl-*S*-RkF and RkD, again efficiently gave **1** (HRMS, calc.: 369.2641, found: 369.2629) (Fig. 3c). This product did not form in the absence of RkD. In conclusion, we have identified RkD as the key enzyme involved in tetronate ring formation. We propose that RkD first catalyzes C-C bond formation, followed by either catalyzed or spontaneous C-O bond formation24,25. It remains possible that, in other tetronate biosynthetic pathways, additional enzymes are involved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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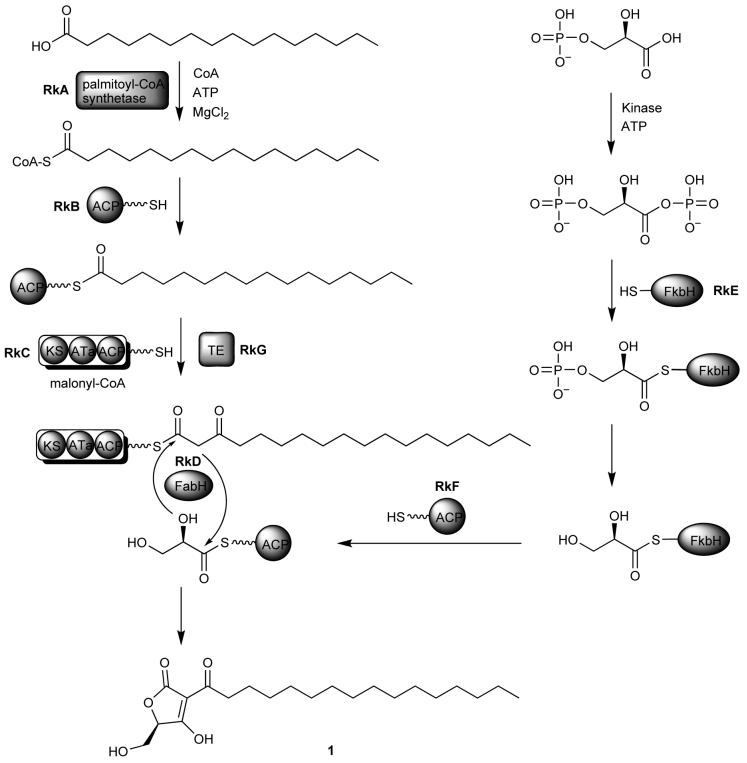

Figure 1.

Biosynthesis of tetronate RK-682. Proposed biosynthetic pathway to RK-682, based on the results presented here. The Rk enzymes shown as involved at each stage are discussed in the text and in the legend to Fig. 2.

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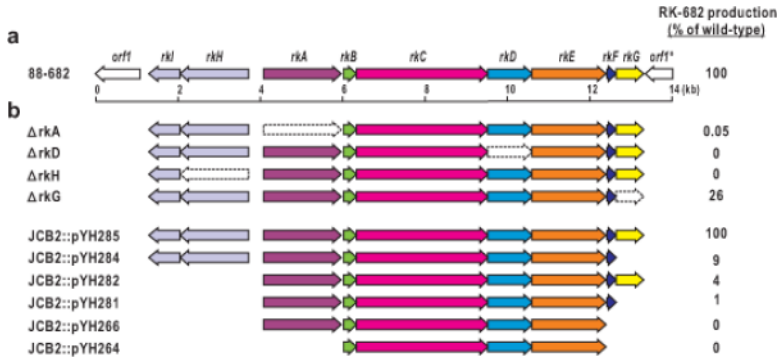

Figure 2.

Genetic analysis of the RK-682 biosynthetic gene cluster. (**a**) Organization of the gene cluster in *Streptomyces* sp. 88–682, showing the size and direction of transcription of each gene. The genes and their predicted products are as follows: *rkI*, TetR-family transcriptional regulator; *rkH*, antibiotic efflux protein; *rkA*, palmitoyl-CoA synthetase; *rkB* and *rkF*, discrete acyl carrier protein; *rkC*, modular PKS containing domains for ketosynthase (KS), malonyltransferase (AT) and acyl carrier protein (ACP); *rkD*, FabH-like 3-oxoacyl-ACP synthase III; *rkE*, FkbH-like glyceryl-*S*-ACP synthase; *rkG*, discrete type II thioesterase (TEII); *orf1* (upstream) and *orf1** (downstream) are likely not involved in RK-682 biosynthesis. (**b**) RK-682 production from modified *rk* gene sets expressed either in strain 88–682 (genes deleted in-frame are shown in dotted lines) or in the heterologous host strain *Saccharopolyspora erythraea* JCB2.

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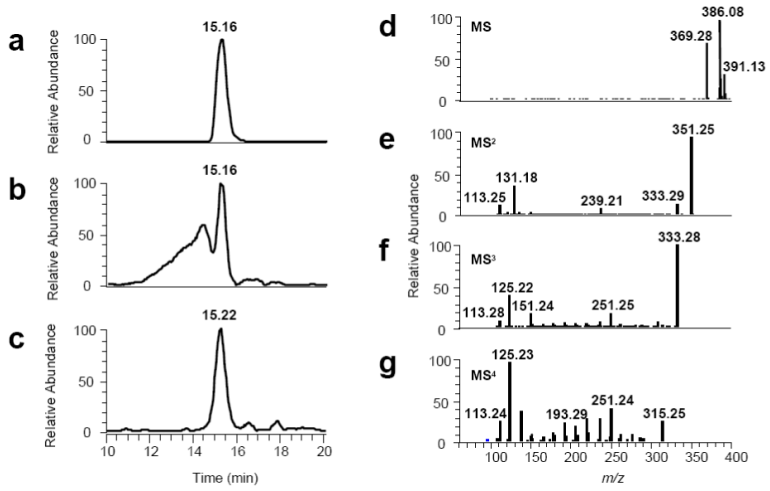

Figure 3.

In vitro reconstitution of RK-682 biosynthesis. LC-MS analysis of (**a**) authentic RK-682; (**b**) the reaction products of RkD, glyceryl-*S*-RkF, MgCl2, TCEP and 3-oxo-stearoyl-*S*-RkC created as described in Supplementary Methods, and (**c**) the reaction products of RkD, glyceryl-*S*-RkF, MgCl2, TCEP, 3-oxo-stearoyl-*S*-CoA, apo-ACP(RkC) and 4′-phosphopantetheinyl transferase Sfp (Supplementary Methods). The MSn fragmentation patterns (**d**, **e**, **f** and **g**) of the peak in (**a**), (**b**) and (**c**) were indistinguishable from one another. (**d**) RK-682 pseudo-molecular ion ([M+H]+) at *m/z* 369.3; (**e**) MS2 of ion at *m/z* 369.3; (**f**) MS3 of ion at *m/z* 351.3 produced from MS2 of the pseudo-molecular ion (*m/z* 369.3); (**g**) MS4 at *m/z* 333.3 produced from MS3 of the pseudo-molecular ion (*m/z* 351.3).

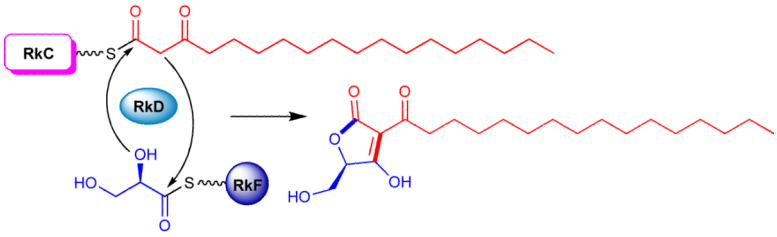

Figure 4.

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