DOI: 10.1002/ange.201301680

Tetronate Antibiotics

Unusual Acetylation–Elimination in the Formation of Tetronate Antibiotics**

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Tetronate antibiotics comprise an important and growing family of polyketide natural products possessing a characteristic tetronate (4-hydroxy-[5*H*]furan-2-one) ring system. They have been isolated from both terrestrial and marine bacteria, and show a diverse range of biological activities.[1] They include the tetronate polyethers tetronomycin[2] and tetronasin,[3] the fatty acyltetronate antibiotic agglomerin,[4] and the structurally closely related protein phosphatase inhibitor RK-682[5] (Figure 1). Of particular interest are the structurally intriguing spirotetronates (Figure 1 a), including the antibacterial compounds chlorothricin[6] and abyssomicin,[7] the antiviral compound quartromicin,[8] and the antitumour compounds tetrocarcin[9] and kijanimicin.[10] These compounds appear to arise through an enzyme-catalyzed Diels–Alder reaction[11] after specific dehydration of an initially formed tetronate precursor, as shown for *atrop*-abyssomicin C[7] in Figure 1 b.

Analysis of the biosynthetic gene clusters for several of these natural products[3,5–10] has highlighted the presence of a set of highly conserved genes unique to tetronate biosynthesis, whose predicted products include candidate enzymes that might catalyze formation of the tetronate ring C–C and C–O bonds. Reconstitution of RK-682 biosynthesis in vitro has been used to show that RkE is a glyceryl-*S*-acyl carrier protein (ACP) synthase,[12] and that the ketoacyl-*S*-ACP synthase FabH-like RkD is necessary and sufficient to catalyze formation of the tetronate ring in vitro starting from a 3-ketoacyl thioester and glyceryl-*S*-ACP.[5] Similar results were recently obtained for the FabH-like QmnD5 in quartromicin biosynthesis[8] and it seems highly likely that most (if not all)[13] tetronates follow an analogous biosynthetic pathway.

Until now, the course of the dehydration step in spirotetronate biosynthesis, which provides the dienophile for the ensuing Diels–Alder-like reaction, has remained obscure. We show here, by cloning and analysis of the gene cluster for biosynthesis of agglomerins A–D in *Pantoea agglomerans* (formerly *Enterobacter agglomerans*) PB-6042,[4] its heterologous expression in *Escherichia coli*, and the total reconstitution of agglomerin biosynthesis in vitro, that the mechanism of dehydration actually involves two steps after formation of the tetronate ring: *O*-acetylation catalyzed by Agg4, followed by elimination of acetic acid to form the exocyclic double bond catalyzed by Agg5. We propose that the biosynthesis of spirotetronates involves the same two-step reaction sequence, catalyzed by enzymes homologous to Agg4 and Agg5.

The agglomerin biosynthetic pathway of *P. agglomerans* provided an attractive system in which to study these key steps, given the relative simplicity of the structures of agglomerin A and its congeners agglomerins B–D, which differ from each other only in the nature of the fatty acyl sidechain (Figure 1).[4] Cosmid and whole-genome sequencing of *P. agglomerans* PB-6042 was used to reveal a circular chromosome of approximately 4.3 Mbp (bp = base pairs), within which the agglomerin cluster was readily identified through its similarity to that of RK-682 (Supporting Information, Figure S1).

A 12 kbp DNA sequence encodes seven ORFs that could be plausibly assigned to the cluster. As well as the expected high homology between several genes in the *agg* and *rk* clusters, key differences in enzymology could also be inferred from the comparison of these clusters: whereas RK-682 obtains its linear precursor from palmitic acid, which is activated and then elongated on a modular polyketide synthase (PKS) to give 3-oxo-stearoyl-*S*-ACP,[12] no counterpart of the *rkC* PKS could be found anywhere on the *P. agglomerans* chromosome. The precursors for agglomerins A–D appear to be taken directly from primary metabolism, probably as the corresponding 3-oxoacyl-CoA thioesters. In support of this, when the seven genes of the putative

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[**] This research was supported by grants from the Biotechnology and Biological Sciences Research Council (BBSRC BB/D018943/1), and the 973 and 863 programs from the Ministry of Science and Technology, the National Natural Science Foundation of China, the Ministry of Education. C.K. acknowledges a stipend from the Higher Education Commission of Thailand, W.T. acknowledges funding from the China Postdoctoral Science Foundation (CPSF 2012M521461), and F.H. funding as an EU Marie Curie Postdoctoral Fellow. F.H. is an Emmy Noether Fellow of the Deutsche Forschungsgemeinschaft, and P.F.L. is a Research Awardee of the Alexander von Humboldt Foundation. We thank Prof. Dr. R. D. Süssmuth and Dr. F. Huang for helpful discussions.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201301680.

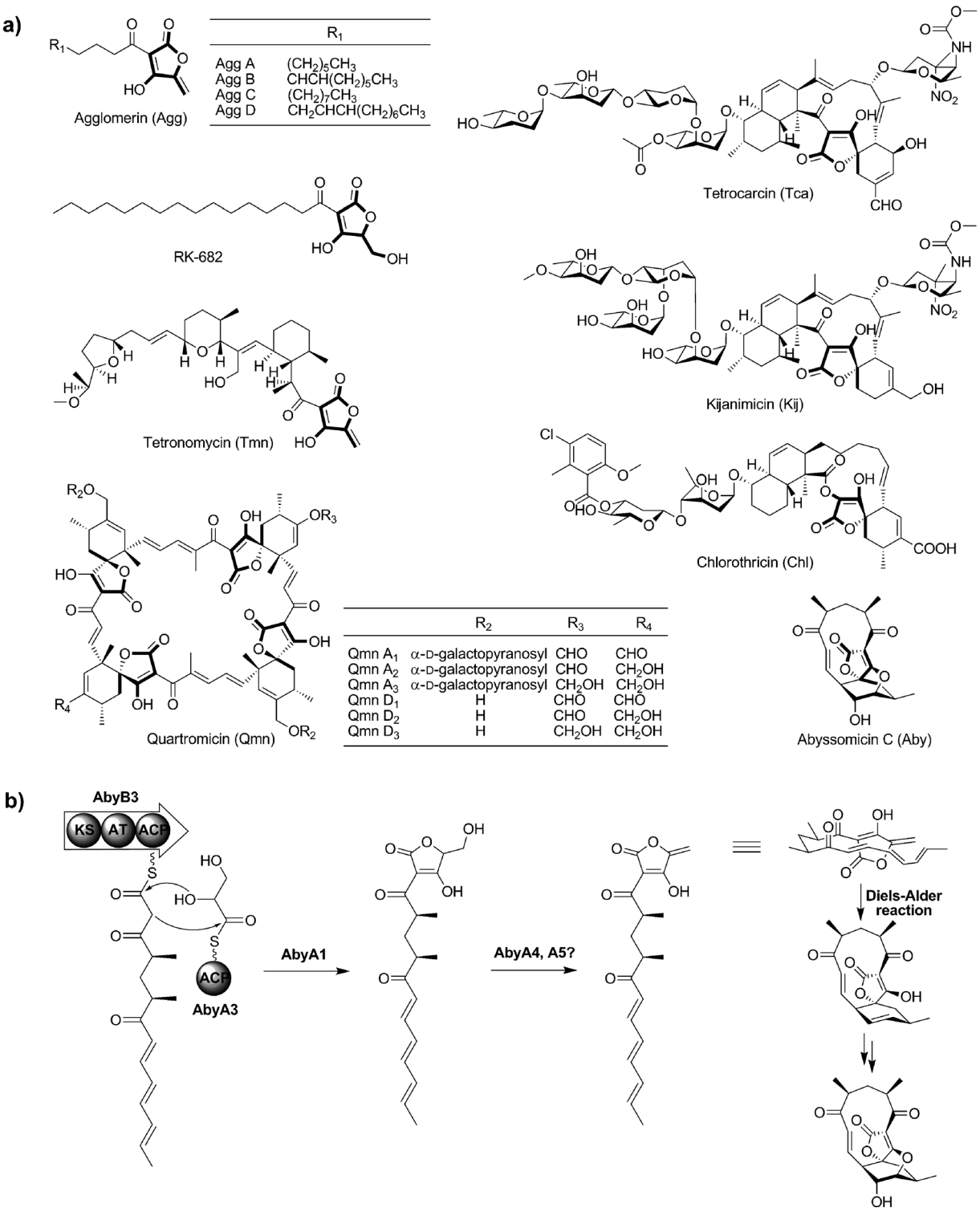


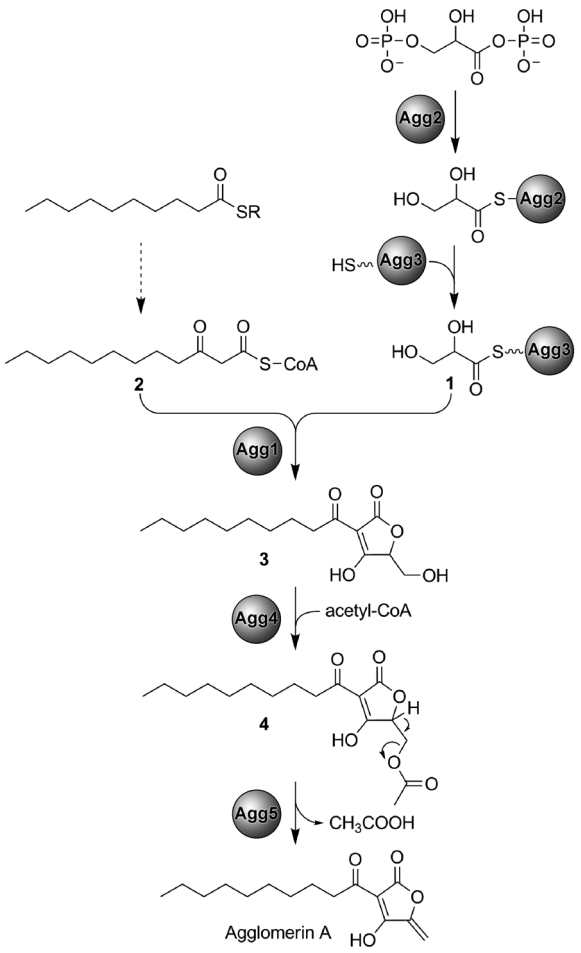
Figure 1. a) The structures of agglomerins, RK-682, tetronomycin and of representative spirotetronate antibiotics whose gene cluster has been identified. b) Proposed common mechanism of spirotetronate biosynthesis exemplified for abyssomicin (adapted from Ref. [7]).

agg cluster were cloned into *E. coli*, the resulting recombinant strain produced agglomerins A–D (Supporting Information). Strikingly, the *agg* cluster does contain two adjacent genes (*agg4* and *agg5*) not found in the *rk* cluster, but which have counterparts in all characterized spirotetronate gene clusters (Supporting Information). We therefore particularly wished to define the contribution of these genes to agglomerin biosynthesis.

First, we individually expressed in *E. coli* each of Agg1 (FabH-like ketosynthase), Agg2 (glyceryl-*S*-ACP synthase), Agg3 (ACP), Agg4, and Agg5. All five were obtained as purified recombinant proteins, although the yields of Agg4

and Agg5 were significantly lower than those of the other proteins (Supporting Information). We have previously reported[2] that the Agg4 homologue in tetronomycin biosynthesis, Tmn7, has significant sequence similarity to the C-terminal catalytic domain of the E2 (acyltransferase) component of 2-oxoacid dehydrogenase multienzymes,[14] and that all the conserved acyltransferase active-site residues are present in Tmn7. The E2 domain has a known tertiary and quaternary structure that closely resembles that of acetyl-CoA:chloramphenicol acetyltransferase,[14] making it an attractive idea that acetyl-CoA might be a co-substrate for the enzymatic activity of Agg4 as well.

To test this idea, glyceryl-*S*-Agg3 (**1**; Scheme 1) was produced in vitro as previously described[5,12] by generating the glycolytic intermediate 1,3-bisphosphoglycerate in situ in the presence of Agg2 and the acylcarrier protein Agg3 (Supporting Information). The other partner needed for tetronate formation to form "hydroxy-agglomerin A" (**3**) was predicted to be 3-keto-dodecanoyl-CoA (**2**). This was synthesized in six steps starting from decanoic acid (Supporting Information). When glyceryl-*S*-Agg3 was incubated with **2** in the presence of purified Agg1, it led to the formation of a species whose molecular mass, as judged by HPLC-MS (Figure 2 and Supporting Information), exactly matched that predicted for hydroxy-agglomerin A (3-decanoyl-5-hydroxymethyl-tetronate; **3**; Scheme 1). This result supports the view that **2** is the natural substrate for the ring-forming reaction.



Scheme 1. Proposed pathway for tetronate formation and dehydration in agglomerin biosynthesis. Intermediate **2** is likely to be derived from fatty acid biosynthesis.

When purified **3** was incubated with both Agg4 and acetyl-CoA, a species was produced with a molecular mass (Figure 2 and Supporting Information) exactly matching that predicted for acetyl-agglomerin A (3-decanoyl-5-acetoxymethyl-tetronate; **4**). No reaction occurred if either Agg4 or acetyl-CoA were omitted from the incubation. Likewise, no reaction occurred when Agg5 alone was incubated with **3**. In contrast, when acetyl-CoA, Agg4 and Agg5 were all present, the product of the reaction was a species with a molecular mass that exactly matched that of authentic agglomerin A (Figure 2). When [$^2H_3$]-acetyl-CoA was used instead of acetyl-CoA in the incubation with Agg4, the observed molecular mass of the putative acetyl-agglomerin A (**4**) was shifted higher by three mass units (Supporting Information), as expected. The high resolution masses of **3** (calculated: 283.1551; found: 283.1549) and **4** (calculated: 325.1657; found 325.1657), as well as their MS^2 fragmentation patterns (Supporting Information), were in full agreement with the structures assigned to them. These results taken together support a two-step mechanism (Scheme 1) for formation of the exocyclic double bond in agglomerins: first, Agg4 catalyzes the acetylation of the primary hydroxy group at C-6 of pre-agglomerin; and Agg5 then catalyzes proton abstraction from C-4 and concomitant departure of acetate as a good leaving group. This mirrors the well-established chemical transformation of RK-682 and its analogs into their agglomerin counterparts through mesylation and subsequent base-catalyzed elimination.[15] To our knowledge, this mechanism for elimination has no direct enzymatic precedent, but it is strongly reminiscent of the phosphorylation–elimination sequence that is invoked for the formation of dehydro-Ser and dehydro-Thr residues during lantibiotic biosynthesis.[16,17]

We next wished to establish whether the proposed mechanism for formation of the exocyclic double bond is likely to operate in vivo. To address this, we developed a system for carrying out specific gene deletion in *P. agglomerans*; and used it to create mutant strains specifically lacking *agg4* and/or *agg5* (see Supporting Information). Fermentation of the mutants was carried out under conditions where agglomerins A–D are produced by wild-type *P. agglomerans*, and ethyl acetate extracts of the fermentation media were analyzed by HPLC-MS. The analysis showed that under these conditions the Δagg4 and Δagg4Δagg5::cat strains produced only the respective hydroxy-agglomerins A–D, while the Δagg5::cat strain produced as major metabolites the respective acetyl-agglomerins A–D (Supporting Information), consistent with the intermediacy of these species in agglomerin biosynthesis and with the roles ascribed to Agg4 and Agg5.

When a mixture of hydroxy-agglomerins A–D, purified from the Δagg4 strain, was incubated in vitro with purified Agg4 and acetyl-CoA, all were converted to the respective acetyl-agglomerins. When Agg5 was also included, the product mixture was identical in HPLC-MS and $HPLC\text{-}MS^2$

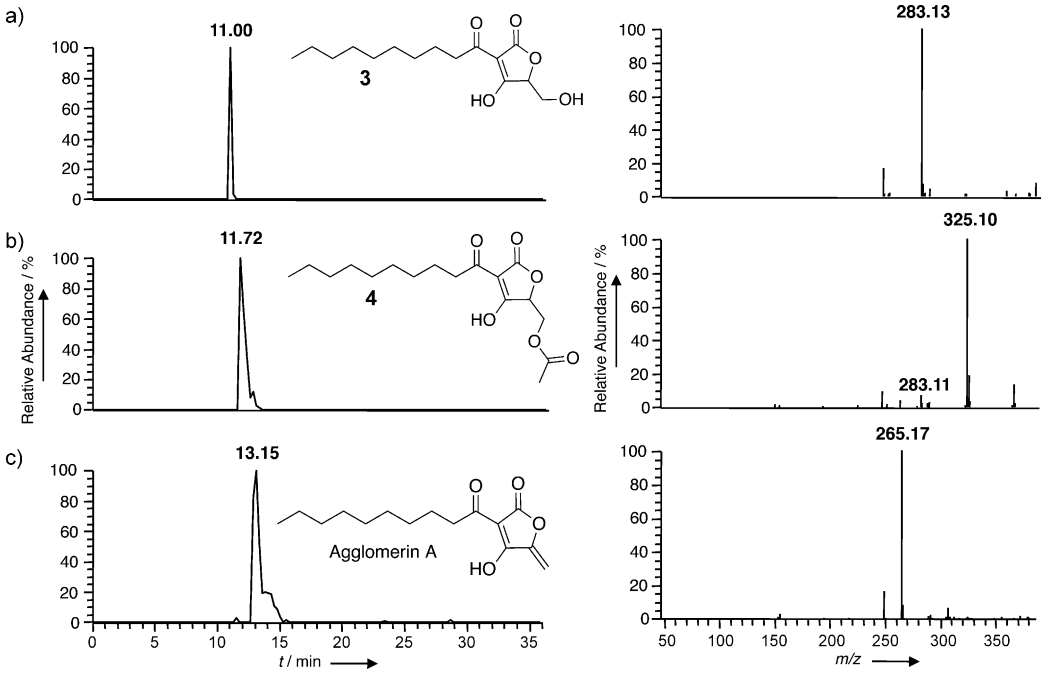


Figure 2. HPLC-MS analysis of tetronate intermediates in agglomerin biosynthesis in vitro: a) **3** formed in the presence of Agg1, glyceryl-*S*-Agg3 and 3-keto-dodecanoyl-CoA in 50 mM Tris-HCl, pH 7.2; b) **4** formed from **3** in the presence of Agg4 and acetyl-CoA; c) agglomerin A formed from **3** in the presence of Agg4, Agg5, and acetyl-CoA.

behavior with the respective agglomerins (Supporting Information).

We reasoned that Agg4 and Agg5 might be sufficiently versatile to accept RK-682 (3-palmitoyl-5-hydroxymethyltetronate) as an alternative substrate to give a novel agglomerin. When RK-682 was incubated with purified Agg4 and acetyl-CoA, a species was formed with the expected mass of acetyl-RK-682 (calculated 409.2596; found 409.2598); and inclusion of Agg5 in the incubation led to the appearance of a new species with the expected mass of dehydro-RK-682 (3-palmitoyl-5-methylidene-tetronate), the predicted novel agglomerin with the fatty acyl chain characteristic of RK-682 (calculated 349.2384; found 349.2382). The products of incubation of Agg4 and acetyl-CoA with RK-682 were also analyzed by ^{1}H NMR spectroscopy (Supporting Information). The deduced structure was in full accord with our proposed mechanism. In particular, the acetyl group in the acetyl-RK-682 is found to be attached via the C-6 hydroxy, as expected.

Our present results establish both the identity and the chemical competence of the intermediates in agglomerin biosynthesis shown in Scheme 1, and identify the respective roles of the acetyltransferase Agg4 and the eliminating enzyme Agg5. Although the predicted protein fold of Agg5[17] places it within the α/β hydrolase superfamily, it has no significant sequence similarity to proteins of known function apart from homologues in known or suspected tetronate biosynthetic pathways. We propose that enzymes homologous to Agg4 and Agg5 carry out the dehydration steps in all spirotetronate biosynthetic pathways. If this proves correct, it may assist knowledge-based engineering of these pathways to create novel analogues. Our identification of the role of these novel enzymes should also now allow facile chemoenzymatic synthesis of authentic substrates that can be used to probe directly the mechanism of the subsequent Diels–Alder rearrangement in spirotetronate biosynthesis, the catalyst for which remains unknown.

Received: February 26, 2013
Published online: April 18, 2013

Keywords: antibiotics · biosynthesis · dehydration · polyketide · tetronate

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