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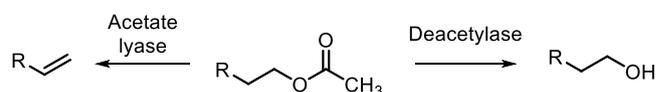
An Esterase-like Lyase Catalyzes Acetate Elimination in Spirotetronate/Spirotetramate Biosynthesis

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Abstract: Spirotetronate and spirotetramate natural products include a multitude of compounds with potent antimicrobial and antitumor activities. Their biosynthesis incorporates many unusual biocatalytic steps, including regio- and stereo-specific modifications, cyclizations promoted by Diels-Alderases, and acetylation-elimination reactions. Here we focus on the intriguing acetate elimination catalyzed by AbyA5, implicated in formation of the key Diels-Alder substrate to give the spirocyclic system of the antibiotic abyssomicin C. Using synthetic substrate analogues we show that AbyA5 catalyzes stereospecific acetate elimination, establishing the (*R*)-tetronate acetate as a biosynthetic intermediate. The X-ray crystal structure of AbyA5, the first of an acetate eliminating enzyme, reveals a deviant acetyl esterase fold. Molecular dynamics simulations and enzyme assays demonstrate use of a His-Ser dyad to catalyze either elimination or hydrolysis, via disparate mechanisms, under substrate control.

Acetylation is a ubiquitous chemical modification of major importance in biology. Acetylation state impacts protein stability, folding and localization, central metabolism, apoptosis, transcription, cytoskeletal organization, circadian regulation, bacterial cell wall architecture and integrity, natural product bioactivity, and antimicrobial resistance amongst others.^[1] The addition or removal of acetyl groups is facilitated by the coordinated action of acetylases and deacetylases, which function within stringently regulated cellular networks.^[2] Deacetylases have been the subject of considerable detailed investigation, and have been shown, without exception, to catalyze hydrolysis of the acetate *via* attack on the carbonyl group.^[3] Recently, studies of spirotetronate and spirotetramate biosynthetic pathways have revealed an alternative biocatalytic route to the processing of acetylated molecules. This involves the

action of free-standing acetate lyases; enzymes which employ a hitherto uncharacterized mechanism to eliminate acetate with formation of a double bond (Scheme 1).^[4] These enzymes are unique to spirotetronate/spirotetramate pathways, sharing <20% sequence identity to any protein of known structure. Given the role of acetate lyases in the biosynthesis of natural products of outstanding clinical potential, their enigmatic enzymology, and their potential utility as industrially relevant biocatalysts, these enzymes represent intriguing and important targets for detailed study.



Scheme 1. Enzyme catalyzed deacetylation and acetate elimination.

To elucidate the mechanistic details of enzyme catalyzed acetate elimination we focused on the putative acetate lyase AbyA5 from the abyssomicin C biosynthetic pathway. The antimicrobial natural product abyssomicin C (**1**) is a potent inhibitor of bacterial folate metabolism effective against a multitude of Gram-positive pathogens including *Mycobacterium tuberculosis* and multi-drug resistant strains of *Staphylococcus aureus*.^[5] The biosynthetic pathway to this compound, which comprises a multi-modular polyketide synthase (PKS) and associated tailoring and regulatory proteins, is encoded for within a single gene cluster (*aby*) spanning ~60 kb of the genome of the marine actinomycete *Verrucosispora maris* AB-18-032.^[6] Based on feeding studies, chemotyping of *V. maris* gene knock-out mutants, comparative bioinformatics analyses, and *in vitro* studies of homologous enzymes from other spirotetronate and spirotetramate pathways, a general mechanism for the biosynthesis of abyssomicin C has been proposed (Figure S1).^[7] One of the most intriguing features of this pathway is the formation and subsequent tailoring of the tetronate ring containing compound **2**, *via* the acetylated intermediate **3**, to yield **4**, which subsequently serves as a substrate for a Diels-Alderase catalyzed intramolecular [4 + 2] cycloaddition reaction (Figure 1).^[8] The conversion of **3** to **4** is postulated to proceed *via* elimination of acetate, yielding the 14,15-exocyclic double bond, but it is not known which enantiomer of **3** is involved. The introduction of this dienophile is implicitly required for the subsequent AbyU catalyzed cycloaddition reaction that forms the heterobicyclic ring nucleus of abyssomicin C.

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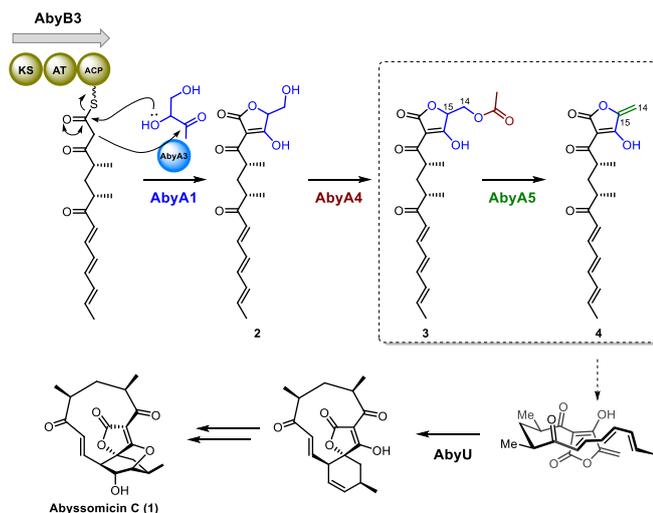


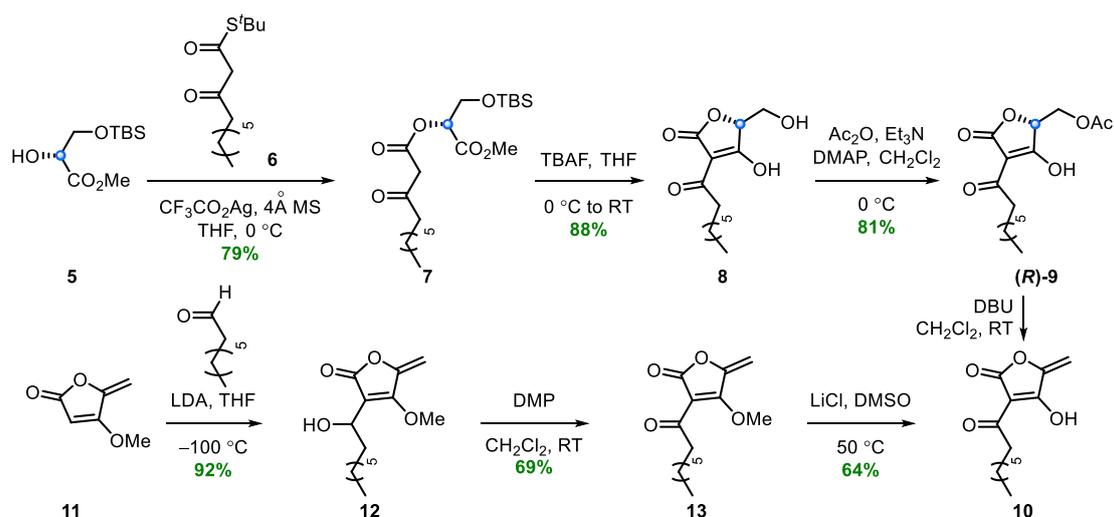
Figure 1. Tetronate ring formation and tailoring during abyssomicin C biosynthesis.

Amino acid sequence alignments of the known spirotetronate/spirotetramate acetate lyases Agg5 (agglomerin)^[4b] and QmnD4 (quartromicin),^[4a] and putative acetate eliminating enzymes from related biosynthetic pathways, with Open Reading Frames within the *aby* cluster, identify AbyA5 as the likely eliminating enzyme from the abyssomicin C pathway (Figure S2). To ascertain whether AbyA5 does indeed catalyze acetate elimination, this enzyme was recombinantly over-expressed in *E. coli* BL21(DE3) cells and purified to homogeneity (Figure S3). Recombinant AbyA5 was found to be a monomeric, well-folded, mono-disperse species in solution.

Both enantiomers of the unnatural substrate analogues of acetate **3** were synthesized. Hydroxy ester **5** was readily prepared

from D-mannitol diacetone in 4 steps according to the literature.^[9] Coupling with β -keto thioester **6** in the presence of $\text{CF}_3\text{CO}_2\text{Ag}$ gave keto ester **7** in 79% yield. The key TBAF mediated Dieckmann cyclization has wide precedent in the literature, however, difficulty has been reported using this method.^[10] In our hands this reaction proved challenging until the purified compound was washed with 1 M HCl according to Osada *et. al.*^[11] Following the cyclization the primary alcohol **8** was acetylated to give desired (*R*)-analogue (**R**)-**9**. To prepare a standard of the elimination product **10** for the enzyme assays, acetate (**R**)-**9** was reacted with DBU giving alkene **10**, other bases (eg. TBAF, imidazole and triethylamine) gave no reaction. An alternative approach to the synthesis of **10** is from **11** as shown in Scheme 2.

Incubation of (*R*)-acetate (**R**)-**9** with recombinant AbyA5 *in vitro* yielded a single product with a mass (m/z [M-H]⁻ = 237) and ¹H-NMR in keeping with alkene **10**, and consistent with the authentic synthetic standard (Figures 2 and S4), confirming that elimination had taken place. Control reactions lacking enzyme, or containing heat denatured AbyA5, showed no evidence of product formation. AbyA5 showed no activity against the (*S*)-enantiomer (**S**)-**9**, demonstrating that the enzyme is stereospecific for the (*R*)-form only. Steady-state kinetic characterization of AbyA5 with (**R**)-**9**, employing a spectrophotometric assay (supplementary information), yielded $k_{\text{cat}} = 1.8 \pm 0.13 \text{ min}^{-1}$, $K_m = 27 \pm 8.4 \mu\text{M}$, and $k_{\text{cat}}/K_m = 0.072 \pm 0.021 \text{ min}^{-1}\mu\text{M}$ (Figure 2). Together these data demonstrate the eliminating activity of AbyA5, unambiguously establish the role of this enzyme in abyssomicin C biosynthesis, and reveal that this reaction proceeds in a stereospecific manner.



Scheme 2. Synthesis of acetate elimination precursor (**R**)-**9** and product **10**. MS = molecular sieve, THF = tetrahydrofuran, TBAF = tetrabutylammonium fluoride, DMAP = 4-(dimethylamino)pyridine, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene, LDA = lithium diisopropylamide, DMP = Dess-Martin periodinane, DMSO = dimethylsulfoxide.

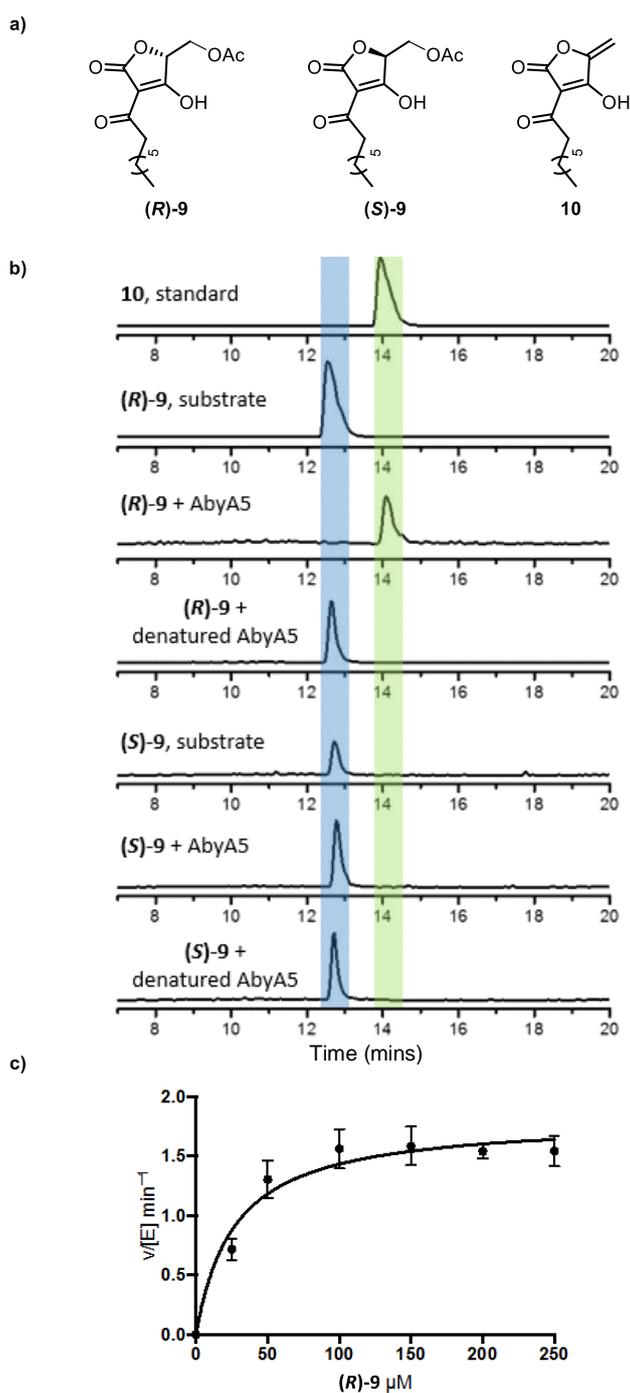


Figure 2. Acetate eliminating activity of AbyA5. a) Synthesized substrate analogues (**(R)**-9 and (**(S)**-9 and alkene **10**. b) HPLC-MS demonstrating the AbyA5 catalyzed conversion of (**(R)**-9 to **10** by AbyA5. c) Steady-state kinetic characterization of the conversion of (**(R)**-9 to **10**. Error bars are standard errors from the mean calculated from reactions run in triplicate.

To provide a structural framework of the AbyA5 catalyzed elimination reaction we determined the X-ray crystal structure of the enzyme. This was achieved using the single wavelength

anomalous dispersion (SAD) method, to 2.3 Å resolution, employing a selenomethionine labeled quadruple point mutant of AbyA5, within which the residues Leu66, Leu158, Leu195 and Leu295 were mutated to methionines (supplementary information). This structure was subsequently used as a molecular replacement search model to determine that of wild type AbyA5 to 2.5 Å resolution. The structure of AbyA5 is consistent with a monomeric α/β hydrolase (ABH), possessing a central, largely parallel 8-stranded β -sheet surrounded by α -helices (Figure 3). As with other ABHs, the second strand within the β -sheet runs antiparallel to the remaining 7 and the sheet possesses a left handed super-helical twist. The major structural features that distinguish AbyA5 from other ABHs are the large number of helices that decorate the central β -sheet core of the enzyme (15 in total) and the presence of a 4-helix subdomain that extends outwards from the top of AbyA5 in a fashion analogous to that reported in 2,6-dihydroxy-pseudo-oxynicotine hydrolase.^[12] Structurally, AbyA5 is most closely related to members of the acetyl esterase family of deacetylases, despite minimal amino acid sequence identity (<20%). Inspection of the AbyA5 crystal structure and superposition with structurally related acetyl esterases unambiguously identifies the location of the enzyme active site, which sits within an extended cleft on the surface of the enzyme of ~ 40 Å in length and ~ 10 Å in depth. The left side of the cleft houses a canonical ABH catalytic triad, comprised of the residues Ser198, Asp285 and His312 (Figure 3). Ser198 is located on a nucleophilic elbow formed by a loop linking $\beta 5$ and $\alpha 7$. His312 sits on the opposite side of Ser198, on a loop between $\beta 8$ and $\alpha 14$, directly above Asp285.

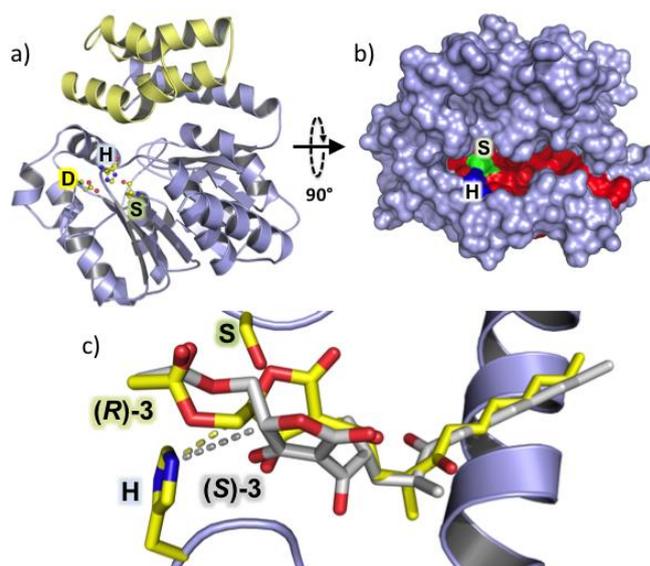


Figure 3. Crystal structure of AbyA5. a) Overall fold of the AbyA5 monomer showing the catalytic domain (blue) and 4 helix capping domain (yellow). S, Ser198; D, Asp 285; H, H312; b) Active site cleft on the enzyme surface (red). The capping domain has been removed for clarity. Asp285 is obscured by His312. c) Model of (**(R)**-3 and (**(S)**-3 docked into the active site of AbyA5.

To further investigate the acetyl elimination by AbyA5, a modelling study was performed. Molecular docking was conducted using the AbyA5 crystal structure with both **(R)-3** and **(S)-3**. This yielded a series of closely related models of the AbyA5-substrate complex, each of which positions the substrate directly within the AbyA5 active site cleft and locates the acetyl group in close proximity to Ser198 and His312 (Figure 3). The side chain of **3** occupies a largely hydrophobic portion on the right of the active site cleft. Given the minimal number of contacts between AbyA5 and substrate in this region it appears unlikely that chain length and composition is a major determinant of substrate selectivity. This is consistent with our *in vitro* assay data, which demonstrate that the substrate analogue **(R)-9**, which lacks the side chain functional group decoration of **3**, is readily acted upon by the enzyme. In contrast, appropriate positioning of the acetylated tetronate ring within the active site appears to play a significant role in substrate binding. This is achieved by a combination of shape and charge complementarity between the acetylated ring and the active site cavity, supported by a several hydrogen bonds. The point mutants AbyA5_H321A and Ser198A showed no activity with **(R)-9**, despite both proteins being well folded monodisperse species in solution (Figure S5), implying a critical role for these residues in substrate positioning and/or catalysis within the active site. Molecular dynamics simulations using docked poses of **(R)-3** reveal a distance of ~3.5 Å between His312 and C15 of the substrate, optimal for proton abstraction and consistent with the His dependent catalytic mechanism proposed previously for QmnD4 (Figure S6).^[4a] The distance between His312 and C15 for **(S)-3** docked poses is >4.5 Å, sufficient to preclude proton abstraction and thereby negate catalysis (Figure 3). Together, these data offer an explanation for the stereoselectivity of AbyA5 observed our *in vitro* enzyme assays.

The crystal structure of AbyA5 raises the intriguing possibility that this protein scaffold could support both acetate elimination and hydrolysis, though no **(R)-9** hydrolytic product **8** was detected in our functional assays. To test this hypothesis the ability of AbyA5 to deacetylate *p*-nitrophenolacetate (*p*-NPA) *in vitro* was monitored spectrophotometrically.^[13] AbyA5 was found to catalyze the hydrolytic deacetylation of *p*-NPA, with $k_{\text{cat}} = 44 \pm 2.4 \text{ min}^{-1}$, $K_{\text{m}} = 78 \pm 9.2 \text{ }\mu\text{M}$, and $k_{\text{cat}}/K_{\text{m}} = 0.62 \pm 0.31 \text{ min}^{-1}\mu\text{M}$ (Figure S7). Molecular dynamics simulations of AbyA5 in the absence of substrate reveal appropriate positioning of the His-Ser-Asp triad, as defined by the occupation of hydrogen bonds between these side chains, for ~10% of the simulation duration, consistent with the ability of the enzyme to catalyze ester bond cleavage *via* a canonical acetyl esterase mechanism (Figure S8).^[14] The infrequent adoption of this catalytically competent state accounts for the comparatively poor catalytic efficiency of AbyA5 for *p*-NPA as compared to naturally evolved acetyl esterases, with an up to 60 fold lower $k_{\text{cat}}/K_{\text{m}}$ (Table S2). Neither AbyA5_H321A nor Ser198A showed any activity with *p*-NPA. Docking studies confirmed that *p*-NPA can be readily accommodated within the AbyA5 active site.

In summary, we report the structural and functional characterization of the acetate lyase AbyA5, revealing the molecular details of the acetate elimination reaction catalyzed by this enzyme, and in doing so, establish explicitly the role of this biocatalyst in abyssomicin C biosynthesis. AbyA5 is shown to possess an acetyl esterase like fold, within which conserved catalytic machinery can be deployed to facilitate either acetate elimination or ester hydrolysis, in a manner dictated by substrate identity. Our studies establish the origins of substrate selectivity in AbyA5, revealing absolute stereoselectivity for the (*R*)-tetronate, but relaxed selectivity for the C3 chain. These findings hint at the potential general utility of AbyA5 as an acetate elimination biocatalyst. Although evolutionarily selected to catalyze elimination, AbyA5 exhibits kinetic parameters for ester hydrolysis comparable to naturally evolved acetyl esterases. Catalytic multifunctionality is an inherent feature of many biocatalysts, however; AbyA5 is unusual in its proficiency in performing a secondary non-cognate reaction.^[15] Our studies also further expand the breadth of transformations catalyzed by α/β -hydrolase fold enzymes, highlighting the utility of this protein scaffold in supporting a diverse array of biocatalytic reactions. Finally, given the high degree of sequence identify between AbyA5 and acetate eliminating enzymes from other spirotetronate/spirotetramate pathways, we conclude that many of the key findings reported herein will be directly applicable to acetate lyases from other biosynthetic pathways.

Acknowledgements

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Keywords: antibiotic • biocatalysis • enzymology • polyketides • enzyme structure

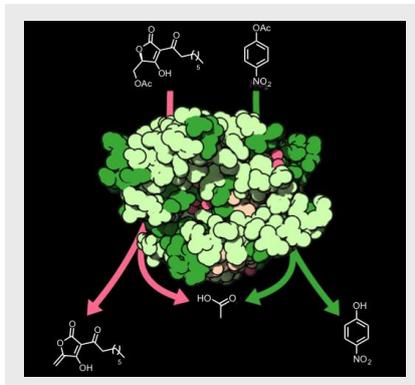
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Entry for the Table of Contents

COMMUNICATION

To eliminate, or to hydrolyze, that is the question: Structural, mechanistic and computational studies of the abyssomicin C pathway enzyme AbyA5 establish the molecular origins of enzyme catalyzed acetate elimination. The unexpected acetyl esterase-like scaffold of the protein is shown to support both acetate elimination and ester hydrolysis, in a manner dictated by substrate identity.



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An Esterase-like Lyase Catalyzes Acetate Elimination in Spirotetronate/Spirotetramate Biosynthesis