Biocatalysis Hot Paper

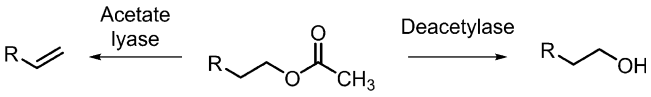
International Edition: DOI: 10.1002/anie.201812105
German Edition: DOI: 10.1002/ange.201812105

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

Nicholas R. Lees[+], Li-Chen Han[+], Matthew J. Byrne[+], Jonathan A. Davies, Alice E. Parnell, Pollyanna E. J. Moreland, James E. M. Stach, Marc W. van der Kamp, Christine L. Willis, and Paul R. Race**

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 acetate elimination catalyzed by AbyA5, implicated in the formation of the key Diels–Alder substrate to give the spirocyclic system of the antibiotic abyssomicin C. Using synthetic substrate analogues, it is shown 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 show the 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 the 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 the formation of a double bond (Scheme 1).[4] These enzymes are



Scheme 1. Enzyme-catalyzed deacetylation and acetate elimination.

unique to the spirotetronate/spirotetramate pathways, sharing less than 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.

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 and is 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 approximately 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 (Supporting Information, 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

[*] N. R. Lees,[+] Dr. L.-C. Han,[+] J. A. Davies, Prof. C. L. Willis
School of Chemistry, University of Bristol
Bristol, BS8 1TS (UK)
E-mail: Chris.Willis@bristol.ac.uk
Dr. M. J. Byrne,[+] Dr. A. E. Parnell, Dr. M. W. van der Kamp, Dr. P. R. Race
School of Biochemistry, University of Bristol
Bristol, BS8 1TD (UK)
E-mail: Paul.Race@bristol.ac.uk
N. R. Lees,[+] Dr. L.-C. Han,[+] Dr. M. J. Byrne,[+] J. A. Davies, Dr. A. E. Parnell, Dr. M. W. van der Kamp, Prof. C. L. Willis, Dr. P. R. Race
BrisSynBio Synthetic Biology Research Centre, University of Bristol
Bristol, BS8 1TQ (UK)
P. E. J. Moreland, Dr. J. E. M. Stach
School of Biology, Newcastle University
Newcastle-upon-Tyne, NE1 7RU (UK),
and
Centre for Synthetic Biology and the Bioeconomy
Newcastle-upon-Tyne, NE2 4AX (UK)
[+] These authors contributed equally to this work.
Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201812105.

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 the 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.

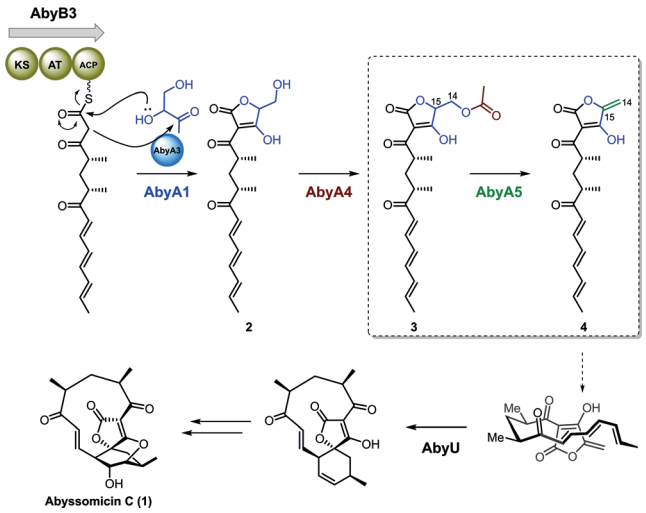
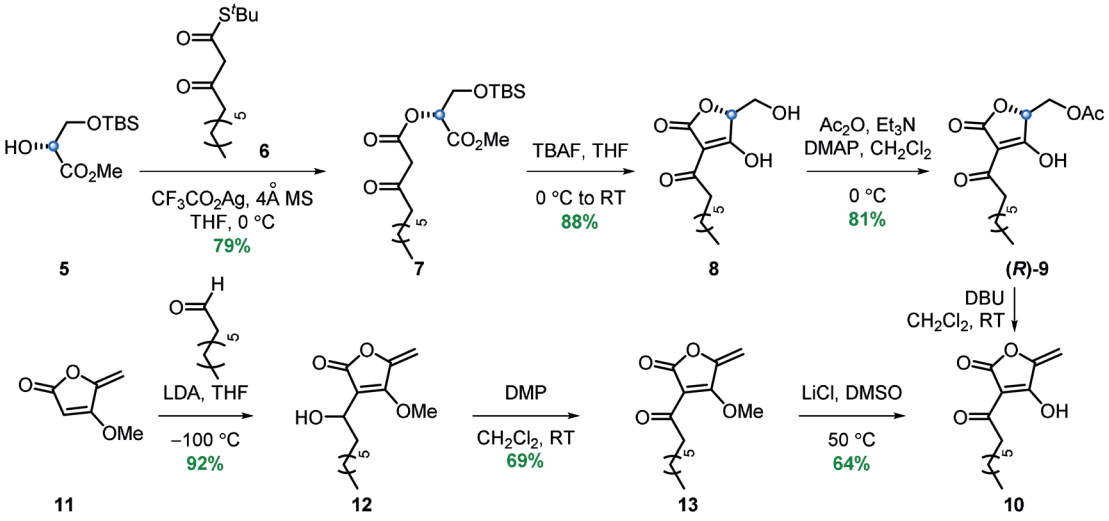


Figure 1. Tetronate ring formation and tailoring during abyssomicin C biosynthesis.

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 (Supporting Information, 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 (Supporting Information, Figure S3). Recombinant AbyA5 was found to be a monomeric, well-folded, monodisperse species in solution.

Both enantiomers of the unnatural substrate analogues of acetate **3** were synthesized. Hydroxy ester **5** was readily prepared from D-mannitol diacetonide in 4 steps according to the literature.[9] Coupling with β-ketothioester **6** in the presence of CF_3CO_2Ag gave keto ester **7** in 79% yield. The key TBAF-mediated Dieckmann cyclization has wide precedent in the literature, however, difficulty has been reported when using this method.[10] In our hands this reaction proved challenging until the purified compound was washed with 1M HCl according to Osada et al.[11] Following the cyclization, the primary alcohol **8** was acetylated to give the 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 (for example, TBAF, imidazole, and triethylamine) gave no reaction. An alternative approach to the synthesis of **10** is from **11** as shown in Scheme 2.

The incubation of (*R*)-acetate ***(R)*-9** with recombinant AbyA5 in vitro yielded a single product with a mass (*m*/*z* $[M-H]^- = 237$) and ^{1}H-NMR in keeping with alkene **10**, and consistent with the authentic synthetic standard (Figure 2 and Supporting Information, Figure 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 (See the Supporting Information for details), yielded $k_{cat} = 1.8 \pm 0.13\ min^{-1}$, $K_m = 27 \pm 8.4\ \mu M$, and $k_{cat}/K_m = 0.072 \pm 0.021\ min^{-1}\,\mu M$ (Figure 2). Together these data demonstrate



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.

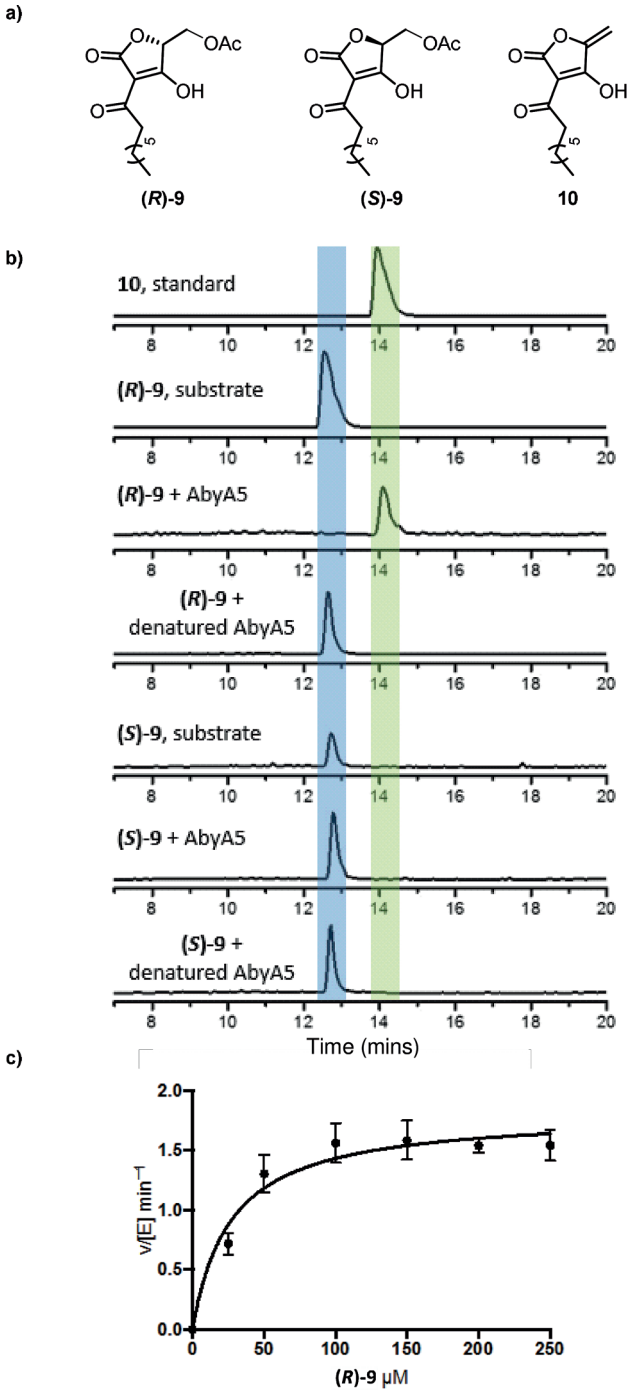


Figure 2. Acetate-eliminating activity of AbyA5. a) Synthesized substrate analogues **(*R*)-9**, **(*S*)-9**, and alkene **10**. b) HPLC-MS demonstrating the AbyA5-catalyzed conversion of **(*R*)-9** to **10**. c) Steady-state kinetic characterization of the conversion of **(*R*)-9** to **10**. Error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

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.

To provide a structural framework of the AbyA5-catalyzed elimination reaction, the X-ray crystal structure of the enzyme was determined. This was achieved using the single-wavelength anomalous dispersion (SAD) method, to 2.3 Å resolution, employing a selenomethinone-labeled quadruple point mutant of AbyA5, within which the residues Leu66, Leu158, Leu195, and Leu295 were mutated to methionines (See the Supporting Information for details). 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 eight-stranded β-sheet surrounded by α-helices (Figure 3). As with other ABHs, the second strand

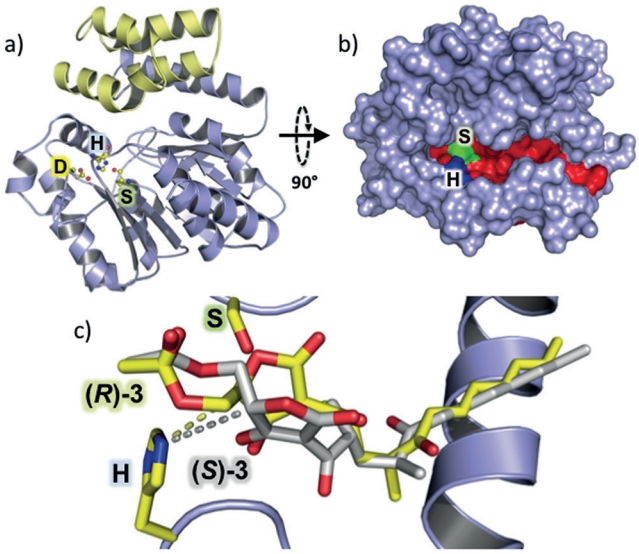


Figure 3. X-ray crystal structure of AbyA5. a) Overall fold of the AbyA5 monomer showing the catalytic domain (blue) and four-helix capping domain (yellow). S, Ser198; D, Asp 285; H, H312; b) Active site cleft on the enzyme surface (red). The capping domain has been removed for clarity. Asp285 is obscured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

within the β-sheet runs antiparallel to the remaining seven 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 four-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 (less than 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 approximately 40 Å in length and approximately 10 Å in depth. The left side of the cleft houses a canonical ABH catalytic triad, comprising of the residues Ser198, Asp285, and His312 (Figure 3). Ser198 is located on a nucleophilic elbow formed by a loop linking β5 and α7. His312 sits on the opposite side of Ser198, on a loop between β8 and α14, directly above Asp285.

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 the 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 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 (Supporting Information, 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 approximately 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 (Supporting Information, Figure S6).[4a] The distance between His312 and C15 for **(*S*)-3** docked poses is greater than 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 in 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 $k_{cat} = 44 \pm 2.4\ min^{-1}$, $K_m = 78 \pm 9.2\ \mu M$, and $k_{cat}/K_m = 0.62 \pm 0.31\ min^{-1}\ \mu M$ (Supporting Information, Figure S7). Molecular dynamics simulations of AbyA5, in the absence of substrate, reveal the appropriate positioning of the His-Ser-Asp triad, as defined by the occupation of hydrogen bonds between these side chains, for approximately 10 % of the simulation duration, consistent with the ability of the enzyme to catalyze ester bond cleavage via a canonical acetyl esterase mechanism (Supporting Information, 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_{cat}/K_m (Supporting Information, 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 enzyme 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] These 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 identity 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

This study was supported by BBSRC and EPSRC through the BrisSynBio Synthetic Biology Research Centre (BB/L01386X/1), PhD studentships awarded to N.R.L. (EPSRC, EP/G036764/1 and GSK), M.J.B. (BBSRC, BB/D526037/1), J.A.D. (EPSRC, EP/L015366/1), P.E.J.M. (BBSRC, BB/J014516/1), and a BBSRC David Phillips Fellowship to M.W.v.d.K (BB/M026280/1).

Conflict of interest

The authors declare no conflict of interest.

Keywords: antibiotics · biocatalysis · enzyme structure · enzymology · polyketides

[1] a) A. Hirano, Y. H. Fu, L. J. Ptacek, *Nat. Struct. Mol. Biol.* **2016**, *23*, 1053 – 1060; b) R. Janke, A. E. Dodson, J. Rine, *Annu. Rev. Cell Dev. Biol.* **2015**, *31*, 473 – 496; c) H. Aksnes, A. Drazic, M. Marie, T. Arnesen, *Trends Biochem. Sci.* **2016**, *41*, 746 – 760; d) A. V. Zamaraev, G. S. Kopeina, E. A. Prokhorova, B. Zhivotovsky, I. N. Lavrik, *Trends Cell Biol.* **2017**, *27*, 322 – 339; e) T. Kouzarides, *EMBO J.* **2000**, *19*, 1176 – 1179; f) P. J. Moynihan, D. Sychantha, A. J. Clarke, *Bioorg. Chem.* **2014**, *54*, 44 – 50; g) I. A.

Murray, W. V. Shaw, *Antimicrob. Agents Chemother.* **1997**, *41*, 1 – 6; h) J. Davies, G. D. Wright, *Trends Microbiol.* **1997**, *5*, 234 – 240.
[2] a) A. Drazic, L. M. Myklebust, R. Ree, T. Arnesen, *Biochim. Biophys. Acta Mol. Cell Res.* **2016**, *1864*, 1372 – 1401; b) E. Seto, M. Yoshida, *Cold Spring Harbor Perspect. Biol.* **2014**, *6*, a018713.
[3] a) P. M. Lombardi, K. E. Cole, D. P. Dowling, D. W. Christianson, *Curr. Opin. Struct. Biol.* **2011**, *21*, 735 – 743; b) C. M. Grozinger, S. L. Schreiber, *Chem. Biol.* **2002**, *9*, 3 – 16; c) U. T. Bornscheuer, *FEMS Microbiol. Rev.* **2002**, *26*, 73 – 81.
[4] a) L. F. Wu, H. Y. He, H. X. Pan, L. Han, R. Wang, G. L. Tang, *Org. Lett.* **2014**, *16*, 1578 – 1581; b) C. Kanchanabanca, W. Tao, H. Hong, Y. Liu, F. Hahn, M. Samborskyy, Z. Deng, Y. Sun, P. F. Leadlay, *Angew. Chem. Int. Ed.* **2013**, *52*, 5785 – 5788; *Angew. Chem.* **2013**, *125*, 5897 – 5900; c) Q. Zheng, Z. Wu, P. Sun, D. Chen, Z. Tian, W. Liu, *Org. Biomol. Chem.* **2017**, *15*, 88 – 91.
[5] J. Riedlinger, A. Reicke, H. Zahner, B. Krismer, A. T. Bull, L. A. Maldonado, A. C. Ward, M. Goodfellow, B. Bister, D. Bischoff, R. D. Sussmuth, H. P. Fiedler, *J. Antibiot.* **2004**, *57*, 271 – 279.
[6] E. M. Gottardi, J. M. Krawczyk, H. von Suchodoletz, S. Schadt, A. Muhlenweg, G. C. Uguru, S. Pelzer, H. P. Fiedler, M. J. Bibb, J. E. Stach, R. D. Sussmuth, *ChemBioChem* **2011**, *12*, 1401 – 1410.
[7] L. Vieweg, S. Reichau, R. Schobert, P. F. Leadlay, R. D. Sussmuth, *Nat. Prod. Rep.* **2014**, *31*, 1554 – 1584.
[8] M. J. Byrne, N. R. Lees, L. C. Han, M. W. van der Kamp, A. J. Mulholland, J. E. Stach, C. L. Willis, P. R. Race, *J. Am. Chem. Soc.* **2016**, *138*, 6095 – 6098.
[9] a) S. Ladame, M. Bardet, J. Perie, M. Willson, *Bioorg. Med. Chem.* **2001**, *9*, 773 – 783;
[10] L. J. Montgomery, G. L. Challis, *Synlett* **2008**, *14*, 2164 – 2168.
[11] M. Sodeoka, R. Sampe, S. Kojima, Y. Baba, T. Usui, K. Ueda, H. Osada, *J. Med. Chem.* **2001**, *44*, 3216 – 3222.
[12] C. Schleberger, P. Sachelaru, R. Brandsch, G. E. Schulz, *J. Mol. Biol.* **2007**, *367*, 409 – 418.
[13] A. J. Alder, G. B. Kistiakowsky, *J. Am. Chem. Soc.* **1962**, *84*, 695 – 703.
[14] a) G. Dodson, A. Wlodawer, *Trends Biochem. Sci.* **1998**, *23*, 347 – 352; b) H. Jochens, M. Hesseler, K. Stiba, S. K. Padhi, R. J. Kazlauskas, U. T. Bornscheuer, *ChemBioChem* **2011**, *12*, 1508 – 1517.
[15] a) M. López-Iglesias, V. Gotor-Fernández, *Chem. Rec.* **2015**, *15*, 743 – 759; b) Y. Miao, M. Rahimi, E. M. Geertsema, G. J. Poelarends, *Curr. Opin. Chem. Biol.* **2015**, *25*, 115 – 123.

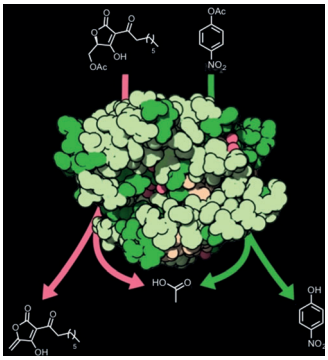
Manuscript received: October 24, 2018
Version of record online: ■■ ■■, ■■■■

Communications

Biocatalysis

N. R. Lees, L.-C. Han, M. J. Byrne, J. A. Davies, A. E. Parnell, P. E. J. Moreland, J. E. M. Stach, M. W. van der Kamp, C. L. Willis,* P. R. Race* _____ ■■■–■■■

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



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.

