

# The Catalytic Mechanism of a Natural Diels–Alderase Revealed in Molecular Detail

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## Supporting Information

**ABSTRACT:** The Diels–Alder reaction, a [4 + 2] cycloaddition of a conjugated diene to a dienophile, is one of the most powerful reactions in synthetic chemistry. Biocatalysts capable of unlocking new and efficient Diels–Alder reactions would have major impact. Here we present a molecular-level description of the reaction mechanism of the spirotetronate cyclase AbyU, an enzyme shown here to be a bona fide natural Diels–Alderase. Using enzyme assays, X-ray crystal structures, and simulations of the reaction in the enzyme, we reveal how linear substrate chains are contorted within the AbyU active site to facilitate a transannular pericyclic reaction. This study provides compelling evidence for the existence of a natural enzyme evolved to catalyze a Diels–Alder reaction and shows how catalysis is achieved.

The Diels–Alder reaction is a [4 + 2] cycloaddition that involves the reorganization of a six-electron system to form a cyclohexene.<sup>1</sup> This transformation is of major synthetic value for the preparation of substituted six-membered rings with the creation of up to four new stereocenters.<sup>2–5</sup> Development of protein catalysts for these reactions is a major goal in biocatalysis and would potentially enable new, efficient, and “green” synthetic routes to a wide variety of valuable bioactive compounds. While a small number of natural enzymes have been shown to be capable of catalyzing [4 + 2] cycloaddition reactions,<sup>6–13</sup> the molecular details of how these transformations are achieved, and whether they do indeed proceed via a formal Diels–Alder route, remain unknown.<sup>14–16</sup> Detailed mechanistic studies of protein-catalyzed Diels–Alder reactions have to date been restricted to de novo designed enzymes and catalytic antibodies.<sup>17,18</sup> These valuable test subjects, however, exhibit poor catalytic efficiencies, limiting their value as biocatalysts.<sup>19,20</sup> Establishing whether natural enzymes have evolved Diels–Alderase activity, and in particular, how such catalysis is achieved at the molecular level, is crucial for the development of efficient protein Diels–Alder catalysts.<sup>15–18,21</sup>

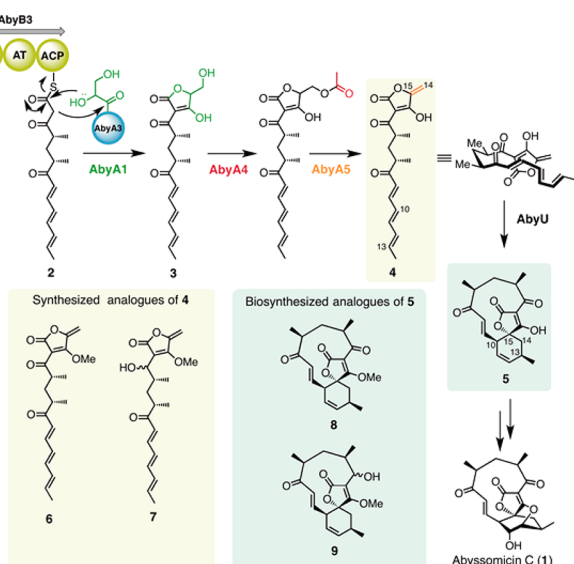
We focus on the putative natural Diels–Alderase AbyU, from the abyssomicin C biosynthetic pathway. The spirotetronate antibiotic abyssomicin C (1), first isolated from the marine actinomycete *Verrucosipora maris* AB-18-032, is a potent inhibitor of bacterial folate metabolism effective against *Mycobacterium tuberculosis* and multidrug resistant clinical isolates of *Staphylococcus aureus*.<sup>22</sup> The biosynthesis of this compound proceeds via the formation of a heterobicyclic ring system, comprising a tetronic acid ring (4-hydroxy-[5H]furan-2-one) spiro-linked to cyclohexene<sup>23</sup> (Figure 1). Formation of this carbocycle is postulated to occur via an enzyme-catalyzed intramolecular [4 + 2] cycloaddition between the exocyclic methylene group and conjugated diene of 4.<sup>24,25</sup> This transformation could conceivably progress via a formal Diels–Alder reaction. Studies of enzymes from other spirotetronate biosynthetic pathways have demonstrated the presence of a stand-alone cyclase that facilitates [4 + 2] cycloaddition.<sup>12,13</sup> However, the molecular basis of how this reaction is performed and whether it does indeed progress via a concerted mechanism are unknown. Given that spirotetronate cyclases share no amino acid sequence identity to other putative natural Diels–Alderases,<sup>4,6–13</sup> it is likely that these enzymes represent a distinct protein scaffold in which a Diels–Alder reaction may take place.

An N-terminally hexa-histidine tagged variant of AbyU was recombinantly overexpressed in *E. coli* B834(DE3) cells and purified to homogeneity. Purified recombinant AbyU was found to be a homogeneous, dimeric species in solution, of >95% purity (Figure S1).

Substrate 6, an analogue of the proposed AbyU substrate 4 (Figure 1), was synthesized and incubated with AbyU for 30 min at 25 °C. Analysis of the resulting reaction mixture by HPLC indicated that a single product had been formed with the same retention time as a standard of 8, prepared by heating diketone 6 for 2 days in chloroform (Figure 2). The mass of this product ( $m/z = 367.53$ ) and <sup>1</sup>H NMR of the crude material were in accord with the spirocyclic product 8 (Figure

Received: January 18, 2016

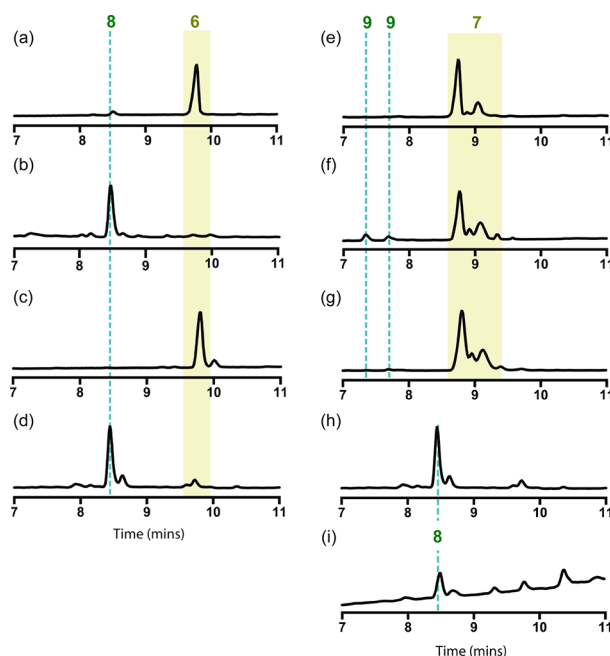
Published: May 3, 2016



**Figure 1.** Proposed 1 biosynthetic pathway, synthetic substrate analogues (6 and 7), and biosynthesized spiroketones (8 and 9). During the biosynthesis of 1 the heptaketide product 2 of the AbyB1–3 polyketide synthase is condensed with glycerate, presented on the free-standing acyl carrier protein AbyA3, to form a five-membered ring in 3. Acetylation of 3 followed by elimination, catalyzed by the AbyA4/AbyA5 enzyme couple, introduces the exocyclic methylene of 4. Intramolecular [4 + 2] cycloaddition of 4 is catalyzed by AbyU and yields the spiroketone 1 precursor 5. Structural and functional studies of AbyU reported herein were conducted using substrate analogues 6 and 7, which were enzymatically converted to 8 and 9, respectively.

S2 and SI). There was no evidence of cyclization occurring in control reactions lacking enzyme or containing heat-denatured AbyU (Figure 2). Steady-state kinetic characterization of the AbyU-catalyzed conversion of 6 to 8 gave  $k_{\text{cat}} = 564 \pm 43 \text{ min}^{-1}$ ,  $K_{\text{m}} = 102 \pm 17 \mu\text{M}$ , and  $k_{\text{cat}}/K_{\text{m}} = 5.5 \pm 0.2 \text{ min}^{-1} \mu\text{M}^{-1}$ . This compares with a rate for the non-AbyU catalyzed reaction of  $k = 0.014 \text{ min}^{-1}$ , consistent with  $a > 4 \times 10^4$ -fold enhancement in rate in the presence of enzyme (Figure S3). While these data give compelling support for an AbyU mediated [4 + 2] cycloaddition, diketone 6 is known to slowly undergo a Diels–Alder reaction at room temperature (40% conversion after 1 week in chloroform<sup>26</sup> and Figure S3). To further explore the potential value of AbyU, a substrate less prone to undergo cyclization was investigated.

Since in general, electron-deficient dienophiles are favored in Diels–Alder reactions, a mixture of epimeric alcohols 7, the synthetic precursor of diketone 6, was selected. Neither incubation of 7 in aqueous buffer for 24 h nor heating of 7 in chloroform for 2 days gave the cycloadduct 9. In contrast, incubation of 7 with AbyU for 30 min at 25 °C yielded two products (Figure 2) with the correct mass ( $m/z = 369.63$ ) for the epimeric cycloaddition products 9 (Figure S2), indicating that an intramolecular [4 + 2] cycloaddition had taken place. No synthetic standard of 9 was available for comparison. To circumvent this, the mixture of 9 from the AbyU-catalyzed reaction was purified by preparative TLC and oxidized using Dess–Martin periodinane to yield a less polar product (Figure 2 and SI), which exhibited a retention profile, mass spectrum ( $m/z = 367.53$ ), and <sup>1</sup>H NMR data in accord with the synthetic spiroketone 8. No product was detected in control reactions with heat-denatured AbyU. Together, these data show that

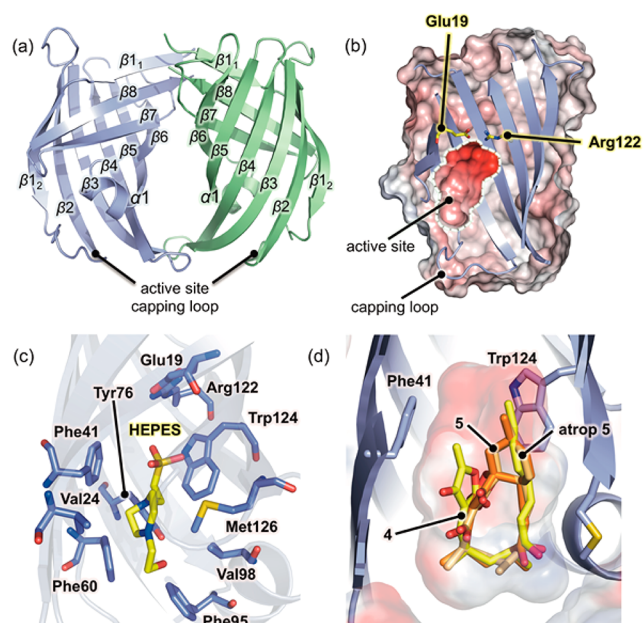


**Figure 2.** HPLC analysis demonstrating AbyU catalyzed [4 + 2] cycloaddition. Traces are shown for assay mixes comprising: (a) synthetic standard of diketone 6; (b) 6 incubated with 280  $\mu\text{M}$  AbyU; (c) 6 incubated with 280  $\mu\text{M}$  heat denatured AbyU; (d) synthetic standard of Diels–Alder product 8; (e) synthetic standard of diol 7 (as a mixture of epimeric alcohols); (f) 7 incubated with 280  $\mu\text{M}$  AbyU; (g) 7 incubated with 280  $\mu\text{M}$  heat denatured AbyU; (h) synthetic standard of Diels–Alder product 8; and (i) product from the purification of 9 isolated from (f) followed by oxidation using Dess–Martin periodinane to give 8. Standard incubation conditions comprised 10 mM substrate with or without AbyU for 30 min at 25 °C.

AbyU is capable of catalyzing [4 + 2] cycloaddition reactions, including one that cannot be readily achieved under standard conditions of prolonged heating. To analyze the molecular basis of the AbyU-catalyzed cycloaddition, we determined the crystal structure of the enzyme (Figure 3).

For the purposes of phase determination, a mutant of AbyU (AbyU\_DM) was generated in which the residues Leu73 and Leu139 were replaced with selenomethionines, using methionine encoding codons (Figure S4). AbyU\_DM was recombinantly overexpressed in *E. coli* and purified to homogeneity. As with AbyU, AbyU\_DM was found to be dimeric in solution (Figure S1). The crystal structure of AbyU\_DM was determined to 1.7 Å resolution using the single wavelength anomalous dispersion method as applied to selenomethionine (SeMet)-labeled crystals of AbyU\_DM. This structure was subsequently used as a molecular replacement search model to elucidate the crystal structure of AbyU. The *C $\alpha$*  RMSD between AbyU\_DM and AbyU is 0.4 Å (Figure S5). Both unlabeled and SeMet labeled AbyU\_DM catalyzed the conversion of 6 to 8 (Figure S6), confirming their catalytic competency.

AbyU is a homodimer comprised of two eight-stranded antiparallel  $\beta$ -barrels with (+)18 topology (Figure 3). The central channel of each barrel is sealed at one end by a salt bridge formed by the side-chains of Glu19 and Arg122 and is capped at the other by a largely hydrophobic loop formed by the  $\beta$ 1– $\beta$ 2 linker (residues Asp26–Gly36; Figure 3). Molecular dynamics (MD) simulations demonstrate the flexible nature of



**Figure 3.** Crystal structure of AbyU and substrate binding mode. (a) Overall fold of the AbyU dimer. Individual monomers are colored blue and green, respectively. (b) Cut-away view through crystal structure of AbyU, revealing the size and location of the enzyme active site. The protein is shown in space filling representation and is colored by electrostatic surface potential. The residues Glu19 and Arg122 are shown in stick format and colored by atom. (c) Detailed view of the AbyU active site highlighting key residues and bound molecule of HEPES. Coloring is by atom. (d) Superposition of the computationally predicted binding modes of 4, 5, and the atropisomer of 5 within the active site of AbyU.

the capping loop and its ability to gate active site access (Figure S7). The central channel of the barrel forms an extended largely hydrophobic cavity of  $\sim 720 \text{ \AA}^3$  that constitutes the active site of the enzyme, formed by the side chains of the residues Val21, Phe41, Phe60, Tyr76, Phe95, Tyr106, Trp124, and Met126 (Figure 3). Electron density corresponding to a single bound HEPES molecule is observed in the active sites of each of the eight copies of AbyU and three of the four copies of AbyU\_DM that comprise their respective asymmetric units (Figures 3 and S5).

AbyU's closest structural homologue is the allene oxide cyclase PpAOC2:<sup>27</sup> a more elaborated eight-stranded barrel with a significantly different active site architecture (Figure S8). Comparison of the sequence of AbyU with those of other known spirotetronate cyclases reveals minimal sequence conservation (Figure S9), raising the intriguing possibility that mutation of the internal cavity of barrel-like structures may offer a route to generating enzymes capable of performing [4 + 2] cycloaddition reactions using a range of substrates.

To establish the catalytic mechanism of AbyU, we first investigated the binding mode of the substrate associated with the reaction. Docking of 4, 5, and the atropisomer of 5 in the active site supports a binding mode where the substrate dienophile is positioned next to Phe41 and the diene close to Trp124 (Figures 3 and S10). In this binding mode, a hydrogen bond is formed between Tyr76 and the lactone carbonyl, which may contribute to substrate specificity. Binding is largely hydrophobic, with good steric complementarity between the enzyme and substrate. In the docked conformation of 4, the C13–C14 and C10–C15 distances (bonds formed in the

product) are 3.6 and 3.9  $\text{\AA}$ , respectively. This indicates that the enzyme active site accommodates the substrate in a potentially reactive Michaelis conformation. MD simulations of the enzyme with product 5 bound show that the binding mode obtained from docking is stable. Little structural change of the enzyme is required to accommodate the substrate; comparison of MD simulations of the *apo* and substrate-bound enzymes primarily shows changes in the loop covering the active site cavity (residues 26–36), which becomes more ordered on substrate binding (Figure S7).

Quantum mechanics/molecular mechanics (QM/MM) MD simulations, which incorporate the effects of the enzyme on the reaction,<sup>28</sup> reveal that the reaction in the enzyme proceeds via a concerted, asynchronous Diels–Alder mechanism (Figures S10 and S11). They show that the substrate can react from the binding mode described above. The structural and electronic properties of the transition state (Figures S10 and S11) indicate that AbyU is indeed a true Diels–Alderase. Free-energy profiles of the reaction were calculated at the SCC-DFTB/ff14SB QM/MM level, with the reaction simulated in the reverse direction, 5 to 4. The active site is well organized to catalyze the reaction, and the interactions noted above are maintained throughout. In the transition state, C13–C14 bond formation is more advanced than C10–C15 bond formation, which occurs predominantly on the downhill path to the product (Figure S10). Higher-level density functional theory calculations (M06-2X/6-31G(d,p)) confirm the identification of the transition-state structure and the order of bond formation (Figure S11). Both levels of theory thus show that the [4 + 2] cycloaddition of 4 to 5 occurs via an asynchronous concerted Diels–Alder mechanism. The transition-state structures calculated for the reaction of the isolated substrate are similar to those in the enzyme, which indicates that the primary catalytic function of the enzyme is to provide a preorganized active site that binds a reactive conformation of the substrate, from which the reaction can occur with a relatively low free-energy barrier.

In summary, we report the structural and functional characterization of the spirotetronate cyclase AbyU, establishing the molecular basis of the [4 + 2] cycloaddition reaction catalyzed by this enzyme. AbyU is a cofactor-independent, stand-alone Diels–Alderase with a low molecular weight  $\beta$ -barrel scaffold. The simplicity of this enzyme makes it a compelling and practical target for engineering. It has further been shown capable of accepting and acting upon non-natural substrates, including those that do not readily cyclize upon heating. This study presents unequivocal evidence of the existence of a natural enzyme capable of catalyzing a formal Diels–Alder reaction and paves the way for the exploitation of AbyU as a biocatalyst for industrial applications and the designed synthesis of novel bioactive compounds.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b00232.

Experimental details and data (PDF)

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### Notes

The authors declare no competing financial interest.



## ■ ACKNOWLEDGMENTS

This work was supported by BBSRC and EPSRC through the BrisSynBio Synthetic Biology Research Centre (BB/L01386X/1), Ph.D. studentships awarded to M.J.B. (BBSRC, BB/D526037/1) and N.R.L. (EPSRC, EP/G0367641/1 and GSK), and a BBSRC David Phillips Fellowship to M.W.vdK. (BB/M026280/1). The authors thank Professors Tom Simpson FRS and Varinder Aggarwal FRS for fruitful discussions. During review of this manuscript the crystal structure of the spirotetramate cyclase PyrI4 was published.<sup>29</sup>

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