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The catalytic mechanism of a natural Diels-Alderase revealed in molecular detail

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Supporting Information Placeholder

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.[1] This transformation is of major synthetic value for the preparation of substituted six-membered rings with the creation of up to four new stereocenters.[2–5] 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. Whilst a small number of natural enzymes have been shown to be capable of catalyzing [4+2] cycloaddition reactions[6–13] the molecular details of how these transformations are achieved, and whether they do indeed proceed *via* a formal Diels-Alder route, remain unknown.[14–16] Detailed mechanistic studies of protein-catalyzed Diels-Alder reactions have to date been restricted to *de novo* designed enzymes and catalytic antibodies.[17,18] These valuable test subjects, however, exhibit poor catalytic efficiencies, limiting their value as biocatalysts.[19,20] 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.[15-18,21]

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 *Verrucosispora maris* AB-18-032, is a potent inhibitor of bacterial folate metabolism effective against *Mycobacterium tuberculosis* and multi-drug resistant clinical isolates of *Staphylococcus aureus*.[22] The biosynthesis of this compound proceeds *via* the formation of a heterobicyclic ring system, comprising a tetronic acid ring (4-hydroxy-[5*H*]furan-2-one) spiro-linked to cyclohexene[23] (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**.[24,25] 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.[12,13] 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,[4,6–13] 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 over-expressed 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).

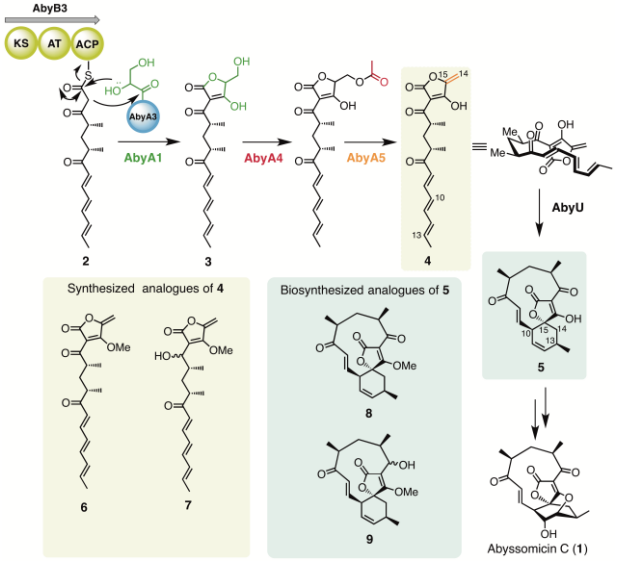

Figure 1. Proposed abyssomicin C biosynthetic pathway, synthetic substrate analogues (**6** and **7**) and biosynthesized spirotetronates (**8** and **9**). During the biosynthesis of abyssomicin C the heptaketide product **2** of the AbyB1-3 polyketide synthase is condensed with glycerate, presented on the free-standing acyl carrier protein AbyA3, to form a 5-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 spirotetronate abyssomicin C 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.

Substrate **6,** an analogue of the proposed AbyU substrate **4** (Figure 1) was synthesized and incubated with AbyU for 30 mins 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 ^{1}H-NMR of the crude material were in accord with the spirocyclic product **8** (Figure 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_{cat} = 564 ± 43 min^{-1}, K_m = 102 ± 17 μM and k_{cat}/K_m = 5.5 ± 0.2 min^{-1}μM^{-1}. This compares with a rate for the non-AbyU catalyzed reaction of k = 0.014 min^{-1}, consistent with a > 4 x 10^4 fold enhancement in rate in the presence of enzyme (Figure S3). Whilst 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 one week in chloroform[26] 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 hrs, nor heating of **7** in chloroform for 2 days gave the cycloadduct **9**. In contrast, incubation of **7** with AbyU for 30 mins 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 ^{1}H NMR data in accord with the synthetic spirotetronate **8**. No product was detected in control reactions with heat-denatured AbyU. Together, these data show that AbyU is capable of catalyzing [4+2] cycloaddition reactions, including one that cannot be readily achieved under standard conditions of prolonged heating.

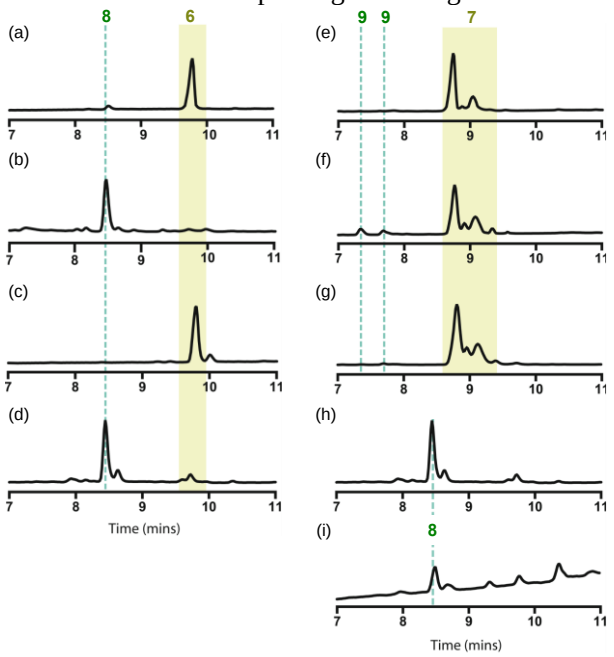

Figure 2. HPLC analysis demonstrating AbyU catalyzed [4+2] cycloaddition. Traces are shown for assay mixes comprising: (a), synthetic standard of diketone **6**; (b), **6** incubated with 280 μM AbyU; (c), **6** incubated with 280 μM heat denatured AbyU; (d), synthetic standard of Diels-Alder product **8**; (e), synthetic standard of diol **7** (as a mixture of epimeric alcohols); (f), **7** incubated with 280 μM AbyU; (g), **7** incubated with 280 μM heat denatured AbyU; (h), synthetic standard of Diels-Alder product **8**; (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 mins at 25 ºC.

To analyze the molecular basis of the AbyU-catalyzed cycloaddition, we determined the crystal structure of the enzyme (Figure 3).

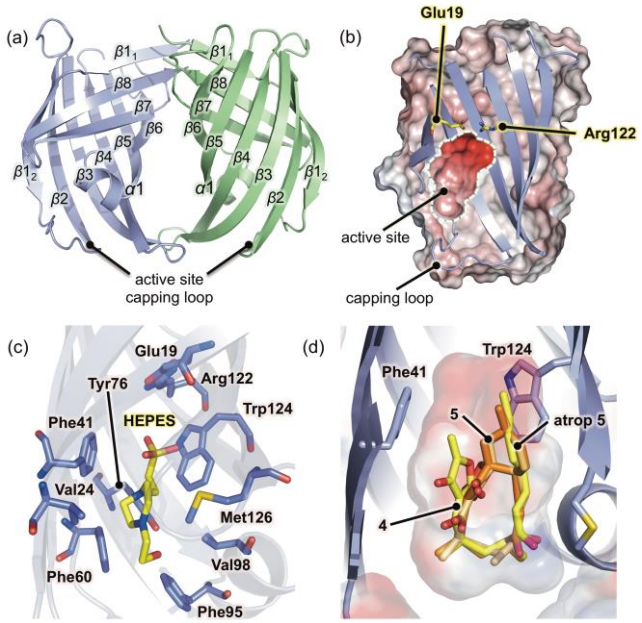

Figure 3. Crystal structure of AbyU and substrate binding mode. (a), Overall fold of the AbyU dimer. Individual monomers are colored blue and green respectively. (b), Cut-away view through crystal structure of AbyU, revealing the size and location of the enzyme active site. The protein is shown in space 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.

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 over-expressed 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 (SAD) 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 Cα 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 anti-parallel β-barrels with (+1)8 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 β1-β2 linker (residues Asp26-Gly36; Figure 3). Molecular dynamics simulations demonstrate the flexible nature of 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 ~720 Å^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 8 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 homolog is the allene oxide cyclase PpAOC2:[27] 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 Å, respectively. This indicates that the enzyme active site accommodates the substrate in a potentially reactive Michaelis conformation. Molecular dynamics (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 show 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) molecular dynamics simulations, which incorporate the effects of the enzyme on the reaction[28], 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 co-factor 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 Available. Experimental procedures, Figures S1-S11, Table S1, compound spectra and supplementary references.

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Notes

The authors declare no competing financial interest. During review of this manuscript the crystal structure of the spirotetramate cyclase PyrI4 was published.[29]

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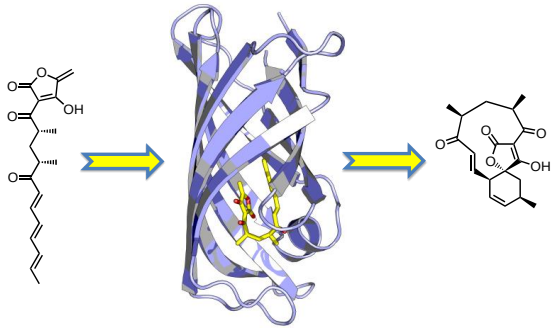

Diels-Alderase catalyzed [4+2] cycloaddition