Supporting information for:

A Natural Diels-Alder Biocatalyst Enables Efficient [4 + 2] Cycloaddition Under Harsh Reaction Conditions

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Materials and Methods

1. Recombinant over-expression of AbyU

The gene encoding AbyU was amplified from *Verrucosispora maris* AB-18-032 genomic DNA and inserted into the plasmid pOPINF[1] as described previously.[2] This construct (*abyU*::pOPINF) encodes an N-terminally hexa-histidine tagged variant of AbyU. Cultures of *E. coli* BL21(DE3) cells harbouring *abyU*::pOPINF were grown in 1 L Luria-Bertani (LB) medium, supplemented with 100 µg/mL carbenicillin, at 37 °C with shaking, until the optical density of the cultures at 600 nm had reached 0.6. Protein expression was induced by the addition of IPTG to a final concentration of 1 mM. Cultures were grown for an additional 16 hours with shaking at 20 °C, following which cells were harvested by centrifugation at 4,600 g, supernatants removed, and remaining cell pellets flash frozen in liquid nitrogen and stored at -80 °C.

2. Purification of AbyU

Frozen cell pellets were thawed on ice and re-suspended in 35 mL of His-load buffer (50 mM Tris-HCl, 150 mM NaCl, 20 mM imidazole, pH 7.5). Cells were lysed using a Microfluidizer (Constant Systems, UK) and the resulting lysate clarified by centrifugation at 38,000 g. Following centrifugation the supernatant was decanted and loaded onto a 5 mL His-Trap chelating column preloaded with nickel (GE Healthcare Life Sciences). The column was washed with 10 column volumes of load buffer to remove non-specifically bound proteins. Hexa-histidine tagged AbyU was eluted using a linear gradient of 0 - 50% His-load to His-elute buffer (50 mM Tris-HCl, 150 mM NaCl, 1 M imidazole, pH 7.5) with fractionation throughout (1 mL). The absorbance of the column eluent was monitored continuously at 280 nm and was used to guide the selection of candidate fractions containing recombinant AbyU. 10 µL samples were removed from candidate fractions and subjected to SDS-PAGE analysis to assess protein purity and quantity. Fractions found to contain AbyU were pooled and concentrated by ultrafiltration to a final volume of 5 mL. Concentrated protein samples were loaded onto a Superdex S75 gel filtration column (GE Healthcare) pre-equilibrated with size

exclusion chromatography (SEC) buffer comprising either, 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, for samples used for fluorescence analyses and *in vitro* enzyme assays; or 50 mM NaCl, 20 mM $NaPO_4$, pH 7.5, for samples used for circular dichroism analysis. Eluted fractions found to contain recombinant AbyU based on continuous monitoring of the absorbance of the column eluent at 280 nm followed by SDS-PAGE analysis, were pooled, concentrated to 10 mg/mL, flash frozen in liquid nitrogen and stored at -80 °C. SDS-PAGE and size exclusion chromatography analysis of AbyU was consistent with the protein being a dimeric, homogeneous species in solution. Protein samples used for all biophysical analyses were of at least 95% purity, as estimated from SDS-PAGE analysis (Figure S1).

3. Circular dichroism spectroscopy

Thermal and chemical denaturant induced circular dichroism (CD) unfolding studies of AbyU were conducted using a Jasco J-1500 spectrophotometer fitted with a Peltier temperature control unit. CD spectra were collected from 200 µL samples of purified AbyU (0.15 mg/mL) in assay buffer (50 mM NaCl, 20 mM $NaPO_4$, pH 7.5), using a 1 mm path length spectroscopic grade cuvette (Hellma Analytics) pre-purged with nitrogen gas to cleanse any contaminants prior to sample analysis. For all experiments a High-Tension (HT) voltage of < 700 V was taken as the quality threshold for the CD signal.[3] Thermal denaturation was achieved by heating samples from 25 °C to 95 °C in 5 °C increments, with continual monitoring of sample ellipticity from 190 - 260 nm throughout. Once the sample had reached 95 °C it was progressively cooled to 25 °C in 5 °C increments with sample ellipticity monitored as outlined above. For chemically induced unfolding CD experiments AbyU at 0.1 mg/mL was incubated for 30 minutes with denaturant (GuHCl; 0 - 6 M, 0.5 M increments) followed by assessment of sample ellipticity (190 - 260 nm). For both thermal and chemical unfolding experiments fraction folded values were calculated from mean sample ellipticity readings at 200 nm derived from three repeats of the same experiment. Errors bars reported are standard errors from the mean. Thermal unfolding profiles were fitted to a Boltzman sigmoid equation assuming a two-state model using GraphPad Prism. Chemical denaturation profiles were fitted to the Fersht equation[4] without sloping baselines using GraphPad Prism, where:

$$[D] = \frac{([D] + [N])e^{(\{m_{D-N}.[Denaturant] - \Delta G_{D-N}\}.RT)}}{1 + e^{(\{m_{D-N}.[Denaturant] - \Delta G_{D-N}\}/RT)}}$$

4. Fluorescence unfolding studies

For fluorescence unfolding studies 20 µL of AbyU (10 mg/mL) was mixed with 480 µL of SEC buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) supplemented with GuHCl (0 - 6 M, 0.5 M increments). Samples were incubated for 30 minutes at 25 °C prior to analysis. Fluorescence

was recorded using an Agilent Cary Eclipse Spectrophotometer with excitation at 295 nm and emission recorded between 305 – 405 nm. For refolding experiments AbyU (10 mg/ml) was incubated in SEC buffer supplemented with 6 M GuHCl for 15 minutes at 25 °C. Following incubation 20 μL aliquots of this protein sample was diluted into 480 μL of SEC buffer containing the desired concentration of GuHCl (0 - 6 M, 0.5 M increments). Samples were incubated for 15 minutes at 25 °C followed by analysis by fluorescence. All readings were taken using a 10 mm pathlength fluorescence quality quartz cuvette (Agilent technologies). For GuHCl unfolding experiments fraction folded values were calculated from fluorescence emission readings at 343 nm derived from three repeats of the same experiment. Errors bars reported are standard errors from the mean. For solvent induced unfolding studies 20 μL of AbyU (10 mg/mL) was diluted into 480 μL of SEC buffer supplemented with 0 - 100% v/v acetonitrile (MeCN), dimethyl sulphoxide (DMSO) or methanol (MeOH), in 10% increments. Samples were incubated for 30 minutes at 25 °C prior to analysis. Fluorescence was recorded as outlined above with excitation at 295 nm and emission recorded between 305 – 405 nm. All readings were normalized by subtraction of the solvent contributed fluorescence signal at each concentration.

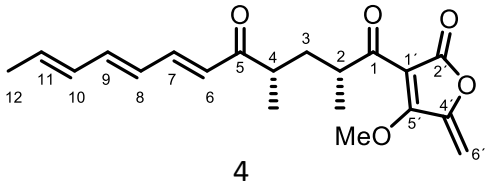
5. Kinetic unfolding studies

Stopped-flow kinetic unfolding experiments were performed using an Applied Photophysics SX-17MV stopped-flow fluorimeter fitted with a 150 W Xenon-Mercury lamp, using a monochromatic slit width of 2 mm. Denaturant solutions comprised SEC buffer supplemented with GuHCl (4 - 8 M; 0.5 M increments). Purified AbyU (10 mg/mL) was rapidly mixed with buffered GuHCl solutions in the stopped-flow apparatus in a ratio of 1:25, giving a final protein concentration of 0.4 mg/mL. All samples were injected into a thermally regulated chamber maintained at 25 °C throughout. Excitation photons of wavelength 295 nm were used, and a monochromatic filter cut-off of 320 nm applied. All data was collected at 345 nm using a photomultiplier set to 300 V sensitivity. Instrument sample chambers were equilibrated with buffered denaturant or protein samples as appropriate prior to analysis. Data are presented as a mean average of four replicates, with the first data point excluded from curve fitting to remove a persistent mixing artefact. Data were fit to the sum of exponential functions using GraphPad Prism and the quality of each fit judged by residual plots (Figure S5).

6. Substrate synthesis

The AbyU substrate analogue **4** and Diels-Alder adduct **5** were synthesized as follows, using the methods described previously.[2]

(2*R*,4*S*,6*E*,8*E*,10*E*)-2,4-Dimethyl-(5'-methoxy-4'-methylene-2'-oxo-2',4'-dihydrofuran-1'-yl)-dodeca-6,8,10-triene-1,5-dione 4.

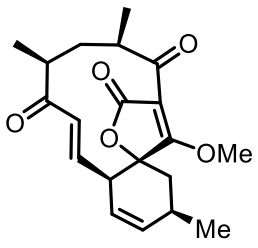


4

Dess-Martin periodinane (673 mg, 0.159 mmol) was added to a solution of tetronate **4** (50 mg, 0.144 mmol) in CH_2Cl_2 (6 mL) and stirred at room temperature until TLC confirmed complete consumption of starting material to a less polar product (approx. 1 hour). The reaction was quenched with 5:1 mixture of sat. $Na_2S_2O_{3(aq)}$ and sat. $NaHCO_{3(aq)}$ and left to stir for 30 minutes. The resulting mixture was separated and the aqueous layer extracted with CH_2Cl_2 (2 × 6). The combined organic extracts were dried over $MgSO_4$ and concentrated *in vacuo*. The crude material was purified by flash column chromatography (10-20% EtOAc/Hex) to give **4** as a pale-yellow oil (28.5 mg, 57 %) as a 5:1 mixture of diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$): (major) δ_H 7.30 – 7.21 (1H, overlapping m, 7-H), 6.58 (1H, dd, *J* 15.0, 10.5, 9-H), 6.30 – 6.11 (3H, m, 6-H, 8-H and 10-H), 6.01 – 5.92 (1H, m, 11-H), 5.26 (1H, d, *J* 3.0, 6'H*H*), 5.21 (1H, d, *J* 3.0, 6'-*H*H), 4.11 (3H, s, OCH_3), 3.68 – 3.60 (1H, m, 2-H), 2.86 – 2.77 (1H, m, 4-H), 2.25 – 2.18 (1H, m, 3-H*H*), 1.83 (3H, d, *J* 7.0, 12-CH_3), 1.39 – 1.17 (1H, m, 3-*H*H), 1.15 (3H, d, *J* 6.5, 2-CH_3), 1.13 (3H, d, *J* 6.5, 4-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 203.4 (C-5), 200.7 (C-1), 168.7 (C-5'), 166.4 (C-2 '), 148.9 (C-4'), 143.4 (C-7), 142.3 (C-9), 135.5 (C-11), 131.5 (C-10), 128.3 (C-8), 127.4 (C-6), 104.8 (C-1'), 95.9 (C-6'), 62.8 (O*C*H_3), 42.4 (C-4), 42.2 (C-4), 35.8 (C-3), 18.7 (C-12), 18.0 (2-CH_3), 17.1 (4-CH_3).

All data is in accordance with the literature.[5]

Diels Alder Adduct



5

Tetronate **4** (10 mg, 0.0290 mmol) and hydroquinone (0.1 mg) was dissolved in $CHCl_3$ (2 mL) and heated in a sealed tube at 75 °C for 2 days. The reaction mixture was cooled to room temperature and concentrated *in vacuo*. The crude material was purified by flash column chromatography (10-20% EtOAc/Hex) to give Diels-Alder adduct **5** as a yellow oil (6.4 mg, 64%). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 6.47 (1H, dd, *J* 16.5, 6.0, 9-H), 6.25 (1H, d, *J* 16.5, 8-H), 5.86 (1H, app. dt, *J* 10.0, 3.0, 12-H), 5.68 (1H, app. dt, *J* 10.0, 3.0, 11-H), 3.91 (3H, s, OMe),

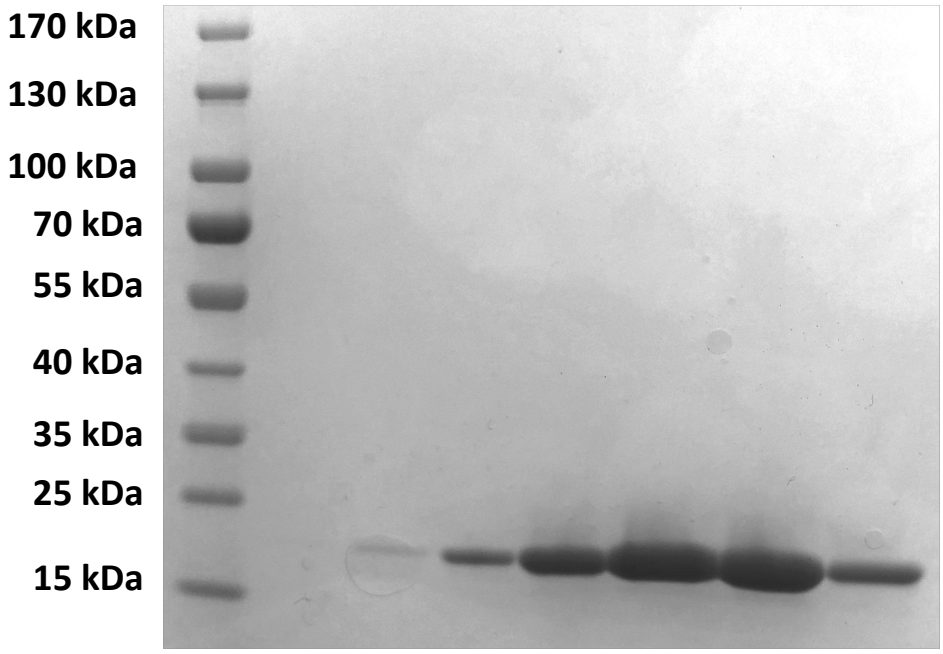
3.47 – 3.43 (1H, m, 10-H), 3.16 – 3.09 (1H, m, 4-H), 2.98 – 2.91 (1H, m, 6-H), 2.68 – 2.60 (1H, m, 13-H), 2.40 (1H, dd, *J* 14.5, 8.0, 14-H*H*), 1.87 (1H, ddd, *J* 15.5, 6.0, 4.0, 5-H*H*), 1.82 (1H, dd, *J* 14.5, 4.5, 14-*H*H), 1.21 (3H, d, *J* 7.0, 6-CH_3), 1.19 (3H, d, *J* 7.0, 4-CH_3), 1.17 – 1.13 (1H, overlapping m, 5-*H*H), 1.15 (3H, d, *J* 7.5, 13-CH_3). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 204.3 (C-7), 200.6 (C-3), 178.2 (C-16), 169.9 (C-1), 141.5 (C-9), 136.7 (C-12), 131.6 (C-8), 121.8 (C-11), 107.0 (C-2), 86.1 (C-15), 61.7 (O*C*H_3), 46.65 (C-4), 46.60 (C-6), 44.6 (C-10), 39.0 (C-5), 36.6 (C-14), 29.3 (C-13) , 21.1 (13-*C*H_3), 17.0 (6-*C*H_3), 16.6 (4-*C*H_3).
All data is in accordance with the literature.[5]

7. LC-MS assays

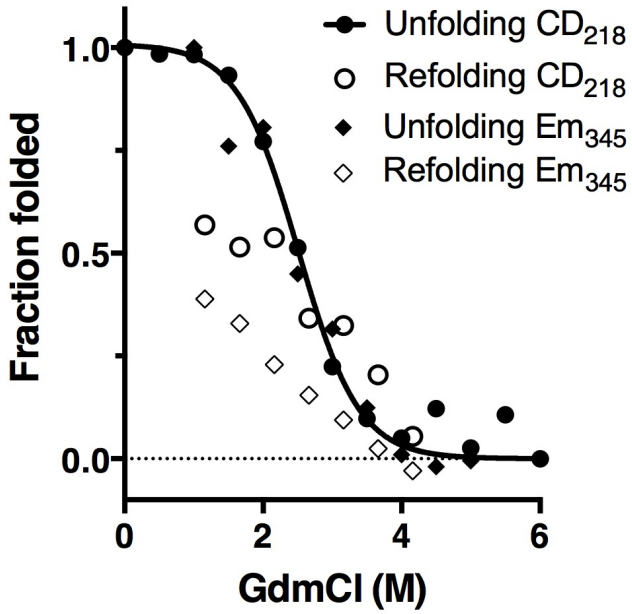
The ability of AbyU to accept compound **4** as substrate for [4 + 2] cycloaddition was assessed in 20 µL assay mixes comprising 280 µM AbyU, 10 mM **4**, 20 mM Tris-HCl, 150 mM NaCl, 10% methanol, pH 7.5, as described previously.[2] Assays were initiated by the addition of cold enzyme and incubated for 30 minutes at 25 °C, following which they were quenched by the addition of 30 µL of ice-cold acetonitrile and centrifuged at 11300 g to remove precipitated protein. Control experiments were performed as above but excluding enzyme, Assay components were extracted in 2 x 1 mL of ethyl acetate and dried under a stream of nitrogen gas. Resulting residues were re-suspended in 100 µL of acetonitrile, of which 50 µL was subjected to LC-MS analysis. Chromatographic separations were performed using a Phenomenex LUNA column (5 µ, C18, 100 Å, 4.6 x 250 mm), using a linear gradient of 10-90% acetonitrile/0.5% formic acid. Mass spectrometry was performed using a Waters ZQ Micromass spectrometer.

8. Enzyme activity kinetic assays

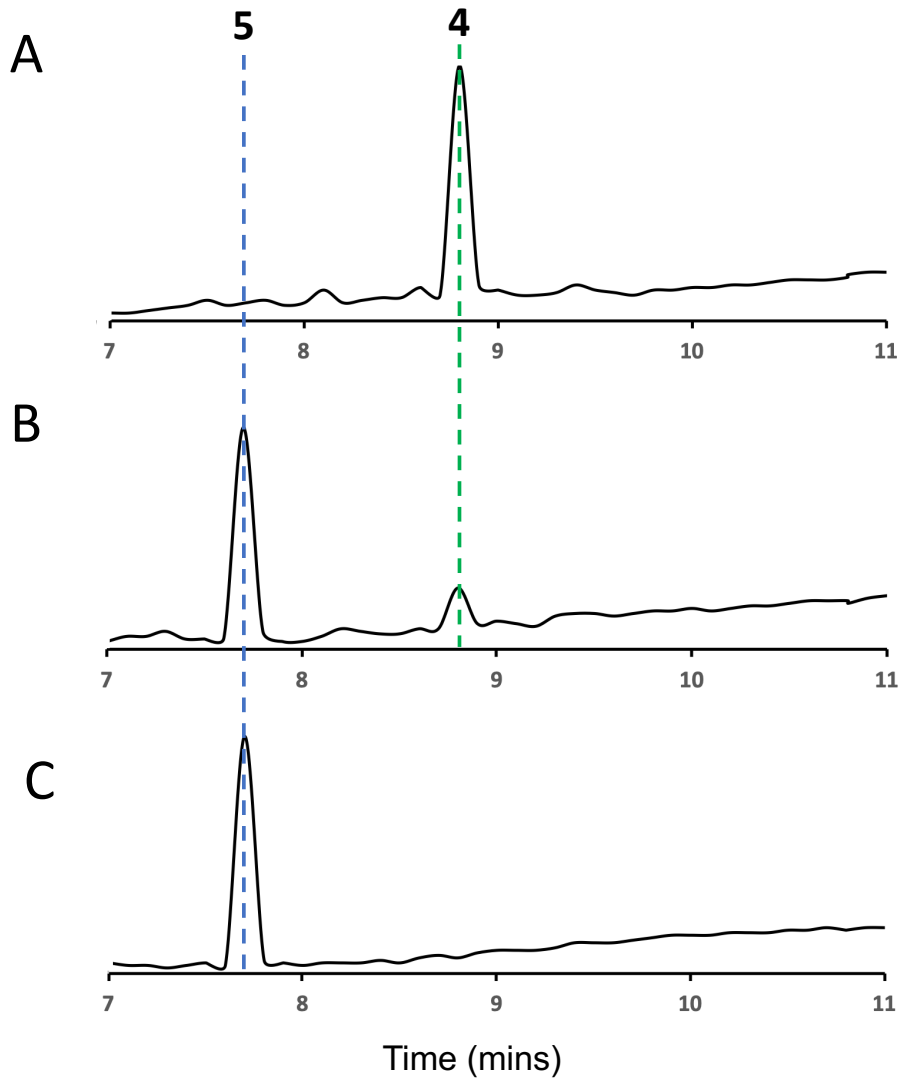
Reaction rates for the AbyU catalyzed conversion of **4** to **5** in the presence of increasing denaturant concentration were measured spectrophotometrically by monitoring the disappearance of **4** at 300 nm ($\varepsilon_{300\,nm}$ = 18400 $M^{-1}cm^{-1}$). All assays were performed in reaction buffer comprising 20 mM Tris-HCl, 150 mM NaCl, pH 7.5, at 25 °C, using a 1 mm pathlength quartz cuvette (Hellma Analytics), with a 5 mM stock solution of **4** dissolved in acetonitrile. Assay mixes comprised AbyU (0.78 µM final concentration), diketone **4** (1 mM; ~x10 the K_m for the AbyU catalyzed reaction[2]) and increasing concentrations of MeOH, MeCN, DMSO or GuHCl, dissolved in reaction buffer. Assays were initiated by the addition of diketone **4** following a 10 min pre-incubation of enzyme with denaturant. Initial rates were calculated by monitoring the change in absorbance for at least 1 minute, following which rates for the non-enzymatically catalysed conversion of **4** to **5** at each substrate concentration were subtracted.



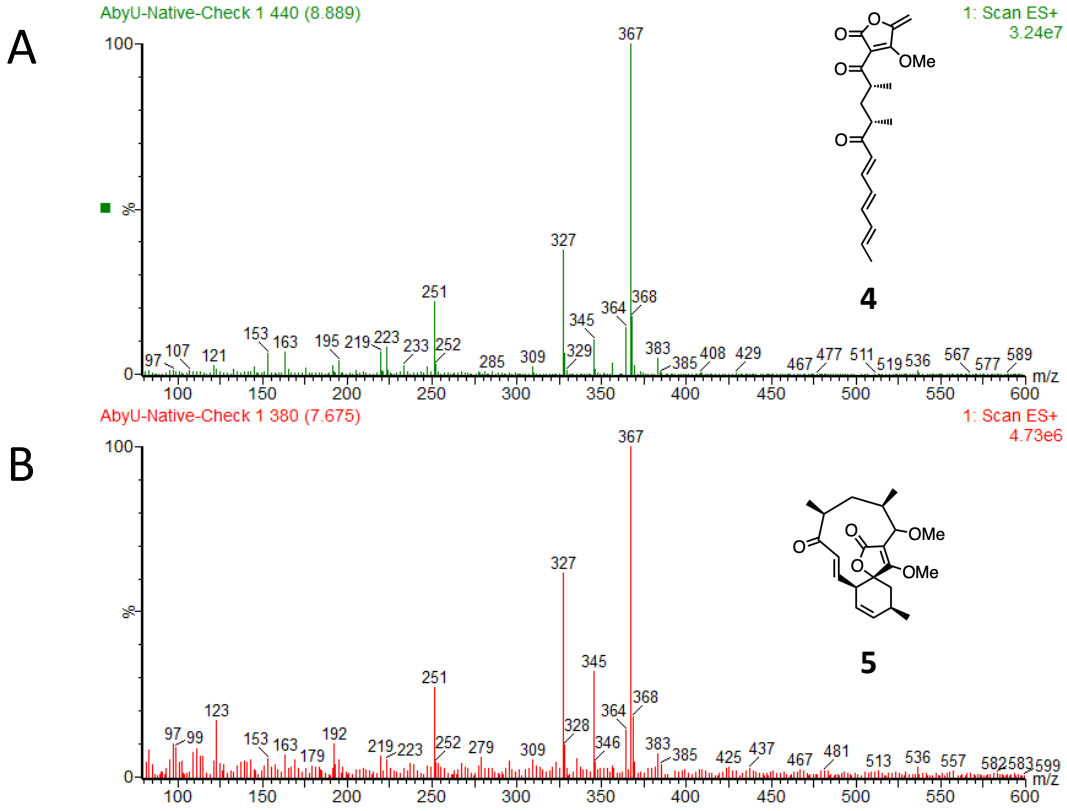
Supplementary Figure 1. SDS-PAGE analysis of recombinant AbyU following purification by SEC. Each lane shows a 10 μL sample from a 1 mL fraction collected in sequence during purification (L-R). The theoretically calculated molecular mass of an AbyU monomer based on amino acid composition is 17.7 kDa.



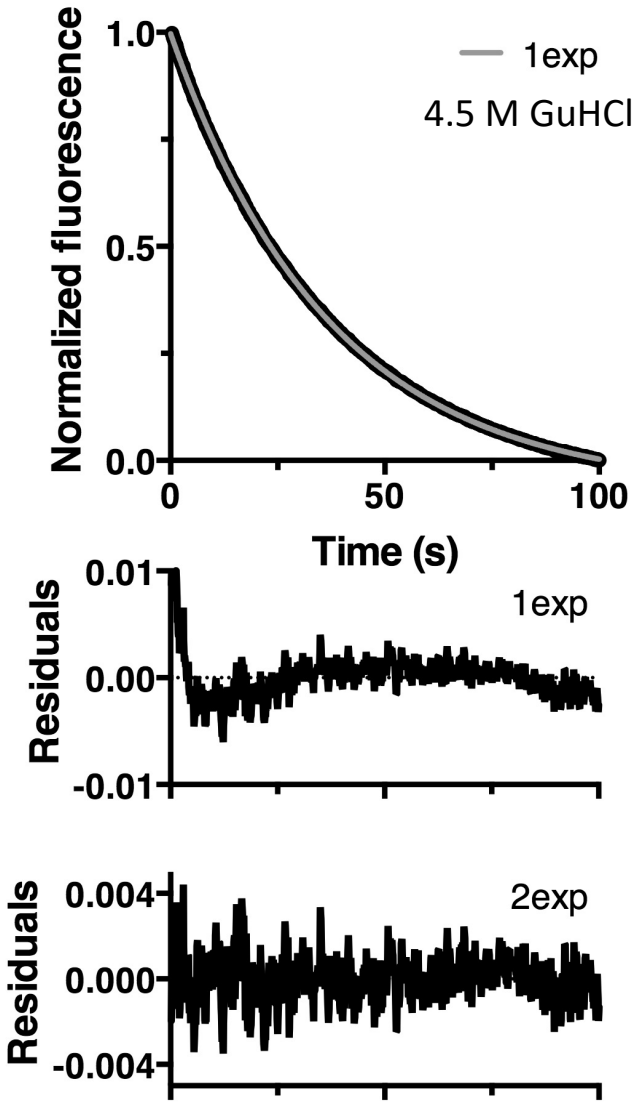
Supplementary Figure 2. Equilibrium unfolding and refolding of AbyU. Unfolding (filled symbols) follows a cooperative transition that is non-reversible in the absence of denaturant (open symbols).



Supplementary Figure 3. HPLC analysis demonstrating the AbyU catalyzed conversion of diketone **4** to spirocyclic **5**. (A) Synthetic standard of diketone **4**. (B) **4** incubated with 280 μM AbyU. (C) Synthetic standard of Diels-Alder product **5**.



Supplementary Figure 4. (A) Mass spectrum of synthetic standard of diketone **4**. (B) Mass spectrum of the AbyU cycloaddition product **5**.

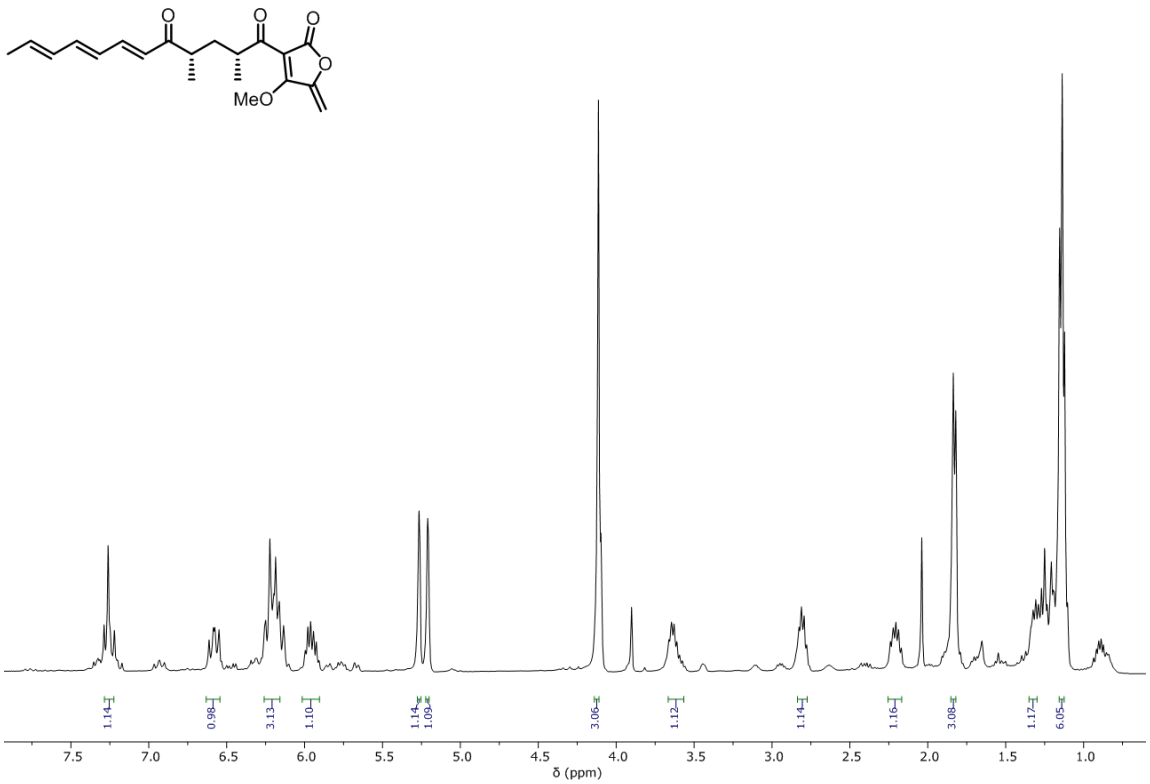


Supplementary Figure 5. Kinetics of AbyU unfolding. Representative unfolding time-course at 4.5 M GuHCl. Residual traces show that the data are best fit to two exponents. The amplitude of the faster phase contributes only 2% of the total signal change.

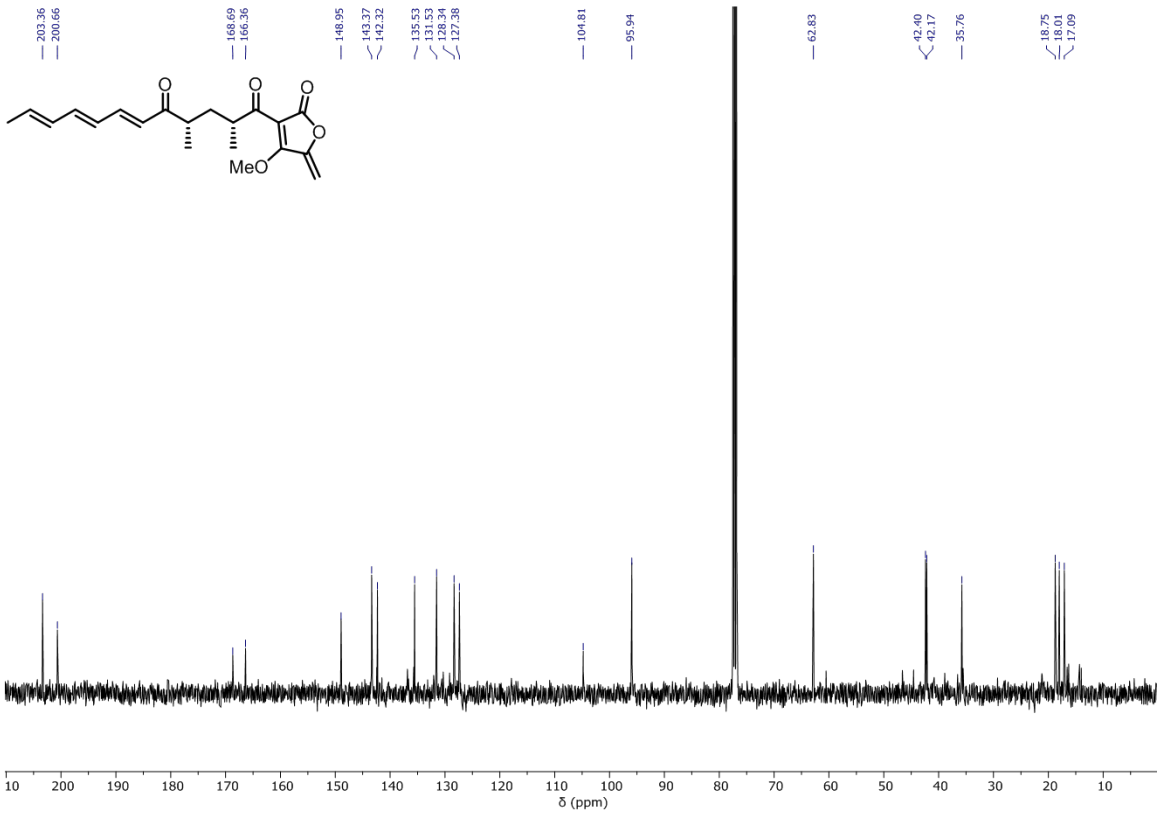
NMR Spectra

(2*R*,4*S*,6*E*,8*E*,10*E*)-2,4-Dimethyl-(5′-methoxy-4′-methylene-2′-oxo-2′,4′-dihydrofuran-1′-yl)-dodeca-6,8,10-triene-1,5-dione 4.

^{1}H NMR (500 MHz, $CDCl_3$)

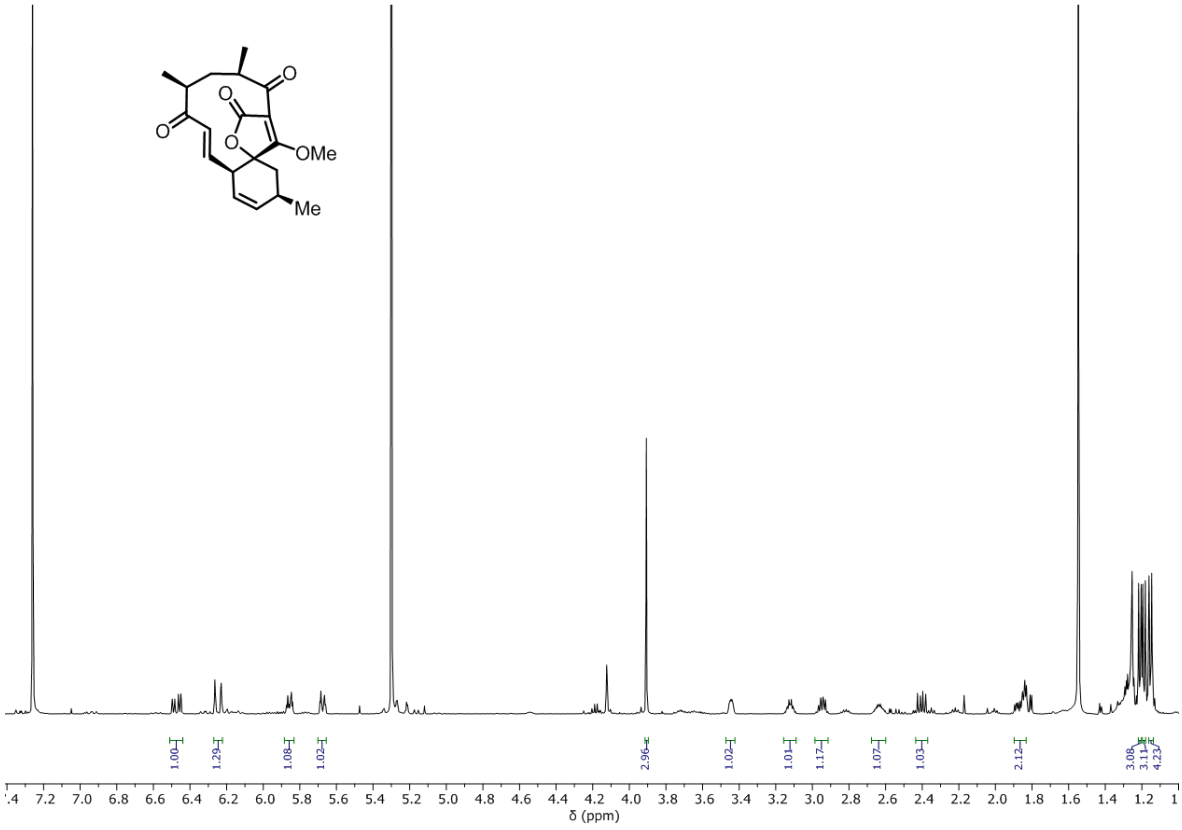


^{13}C NMR (125 MHz, $CDCl_3$)

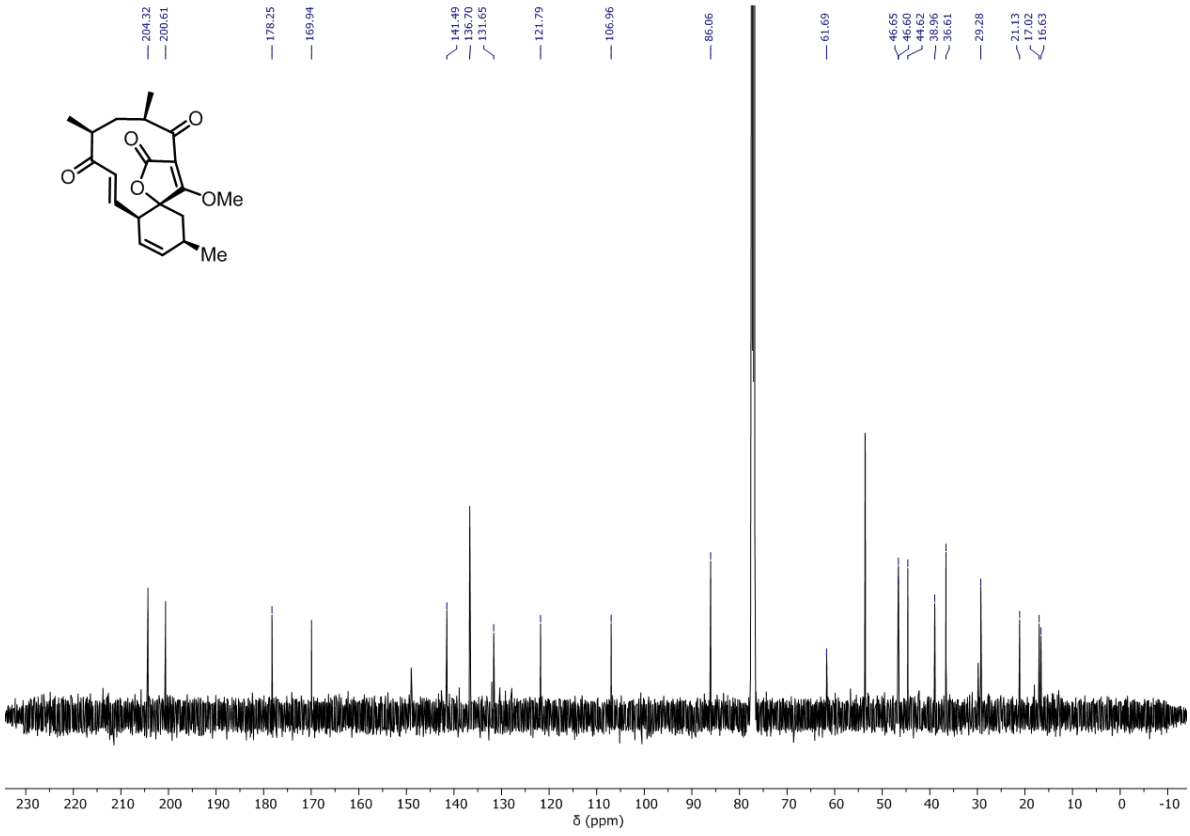


Diels Alder Adduct

^{1}H NMR (500 MHz, $CDCl_3$)



^{13}C NMR (125 MHz, $CDCl_3$)



Supplementary References

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