Supplementary Information

*Exo*-selective intermolecular Diels-Alder reaction by PyrI4 and AbnU on non-natural substrates

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Supplementary Methods and Results

**Materials**: All chemicals required for experiments were purchased from Merck (Sigma-Aldrich, USA). All molecular biology enzymes were purchased from New England Biolabs, USA. Details about the equipment, clones, reagents, columns etc. used in this study are referred in experimental part as and when required.

1. **Sequence and structural analyses the β-barrel proteins**

   Structures of some β-barrel proteins known for their Diels-Alderase or cyclase activity have been retrieved from the PDB\(^1\) and aligned in Pymol\(^2\). They share though very low sequence similarity but share high structural homology (Supplementary Figure 1). Sequence of PyrI4, AbyU and AbnU were aligned to understand the sequence identity between three homologues (Supplementary Figure 2).

**Supplementary Figure 1**: Structures of representative β-barrel proteins that carryout Diels-Alder reactions. PDB ID’s are given in the brackets.

![Structures of β-barrel proteins](image)
**Supplementary Figure 2:** Sequence alignment of AbnU with its closest homologue AbyU (PDB: 5DYV) and PyrI4 (PDB: 5BU3) depicting the conserved residues generated by Jalview 2.10.5 software. The associated NCBI accession codes and their respective sequence identity to AbnU are also shown for both the homologs.

| Accession | Identity | NCBI Accession Code |
|-----------|----------|---------------------|
| AbnU/1–131| 100%     | OHW405703.1          |
| AbnU/1–141| 51.3%    | WP_013733860.1       |
| PyrI4/1–184| 26%     | AFY713381.1          |

2. Gene Cloning

Genes encoding PyrI4, PyrI4-Δ10 (both from *Streptomyces rugosporus*) and AbnU (NCBI sequence id: WP_157905384.1, *Frankia sp. Cc1.17*, 393 bp) were codon optimized and synthesized by Genscript USA in pET28a (+) vector with N-terminal polyhistidine-tag. PyrI4 and PyrI4-Δ10 were inserted between restriction sites NdeI and HindIII. AbnU was inserted between restriction sites NdeI and XhoI. Single-point mutations were introduced by using site-directed mutagenesis strategy utilizing PCR-based methods followed by DpnI (New England Biolabs Inc.) digestion, transformation into Dh5α *E. coli* strain, and screening of positive clones by nucleotide sequencing. All primers used for site-directed mutagenesis are summarized in the table below (Supplementary Table 1).

| Primer     | Sequence                      |
|------------|-------------------------------|
| PyrI4-E65A-for | GAGCTGACCAGCGTTCGCGAGAAACTGTTGAGTCAC |
| PyrI4-E65A-rev  | CTCCGCCAACGCCGAGTGTCATCCAGCACAATGCG |
3. Protein expression

All clones (AbnU, PyrI4, PyrI4-Δ10 and all mutants) were transformed into *Escherichia coli* BL21 (DE3). Cultures of transformed *E. coli* BL21 (DE3) cells were grown in 1 L Luria-Bertani (LB) medium, supplemented with 50 µg/mL kanamycin, at 37 °C with shaking, until the optical density of the cultures at 600 nm had reached 0.8. Protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM. Cultures were grown for an additional 12 h with shaking at 37 °C, following which the cells were harvested by centrifugation and the cell pellet stored at −80 °C for further purification using Ni-NTA resin with a standard affinity purification protocol (as mentioned below).

4. Protein purification and characterization

All recombinant proteins were purified using the same general strategy. For purification, harvested cells were thawed and suspended in the lysis buffer A (50 mM Tris, 200 mM NaCl [pH 8.0] supplemented with 1 mM PMSF). Bacterial cells were lysed by sonication (pulse of 1 sec ON, 3 sec OFF) and then centrifuged (SS-34 rotor) at 38,724 g for 40 minutes at 4 °C. The supernatant was loaded on to a 5 mL His Ni-NTA column (Sigma). The column was equilibrated with binding buffer A prior to loading the supernatant. Washed the column with buffer A to remove impurities and eluted with a step gradient of 2%, 5%, 10%, 20%, 50%, and 100% of buffer B (50 mM Tris, 200 mM NaCl [pH 8.0], 500 mM imidazole, pH 8.0). Fractions found to contain the target protein of interest, as determined by monitoring the absorbance of the column eluent at 280 nm followed by SDS-PAGE analysis, were dialyzed against the buffer C (20 mM Tris [pH 8.0], 100 mM NaCl) to remove imidazole. Dialyzed proteins were concentrated to at least 10 mg/ml. The concentration was estimated by Bradford assay and flash-frozen in liquid nitrogen for storage at -80 °C. PyrI4 protein was stored with 1mM DTT and 1mM EDTA (for crystallization purpose only). Pure concentrated proteins were analyzed on SDS-PAGE for purity (>95% pure) (Supplementary Figure 3a-b).

Size exclusion chromatography: To characterize the oligomer state of AbnU in solution, we performed size exclusion chromatography (SEC) using the Bio-Rad NGC chromatography system. S200 column (XK-16/70 (GE Life Sciences) packed manually) was pre-calibrated with blue dextran.
for the void volume and a calibration standard (Bio-Rad gel filtration standard, cat: 1511901) were
run for standard plot as shown in Supplementary Figure 3c. Concentrated AbnU protein (20 mg/ml)
was spinned and loaded onto a pre-equilibrated S200 column with buffer E (20 mM Tris, [pH 8.0],
100 mM NaCl). SEC fractions were eluted, monitored at 280 nm (multi wavelength detector; an
inbuilt system feature) and analyzed on SDS gel (Supplementary Figure 3c) for purity and
monomer mass. Mass of AbnU, PyrI4 and PyrI4-Δ10 is determined by MALDI-reTOF/TOF
UltrafleXtreme (Bruker Daltonics, Bremen, Germany) (Supplementary Figure 3d).

**Thermal shift assay:** 20 µM of protein (AbnU, PyrI4, PyrI4-Δ10 and mutants) in buffer D (20 mM
Tris [pH 8.0] and 50 mM NaCl) and 6.25x SYPRO Orange dye (5000x stock concentration in
DMSO, Sigma) reaction was setup to a volume of 20 µl in a 96-well PCR plate. PCR plate was
centrifuged at 200 x g for 5 minutes before monitoring the change in SYPRO Orange fluorescence
by real-time PCR instrument (Biorad CFX96 Real-Time System). Fluorescence was monitored with
a temperature scan from 4 °C to 95 °C with an increment of 1 °C /min. The differential of the
fluorescence intensity (dF/dT) versus temperature plot gave an estimate of melting temperature (T_m)
of all the individual proteins and hence their thermal stability (Supplementary Figure 3e).
Supplementary Figure 3: SDS-PAGE gel analysis of purified recombinant proteins PyrI4 and its mutant: a) PyrI4 and its mutant protein with a protein standard marker (M) on 12% SDS-PAGE gel b) Comparative gel (15% SDS-PAGE gel) of purified PyrI4 and PyrI4-Δ10 mutant showing a difference of about 1.0 kDa corresponding to the lack of N-terminal 10 residues in PyrI4-Δ10.

c) Size exclusion chromatogram and SDS-PAGE gel analysis of the purified recombinant protein AbnU with the standard gel filtration standard plot.
d) Positive mode MALDI-reTOF/TOF (linear mode) mass spectra of A) AbnU, B) PyrI4, and C) PyrI4-Δ10 using 2-Cyano-3-(2-thienyl)acrylic Acid (CTA) as matrix

![Mass Spectra for AbnU, PyrI4, and PyrI4-Δ10](image)

e) Thermal shift assay for AbnU, PyrI4 and its mutants depicted by plot of differential of the fluorescence intensity (dF/dT) versus temperature plot. Corresponding T<sub>m</sub> are included in °C.
5. Protein Crystallization

The initial attempts failed to crystallize the PyrI4 full length protein using published conditions. We screened all recombinant proteins, namely AbnU, PyrI4 and PyrI4Δ10 using 12 different crystallization conditions (Hampton Research and Molecular Dimensions), which correspond to about 1200 reagents. 10 mg/ml of protein was used to set up the crystallization screen by mixing 0.2 µl of protein and 0.2 µl of reservoir solution via the sitting drop vapor diffusion method. Crystal trays were kept at 25 °C in a temperature-controlled and vibration free cabinet. Crystallization conditions for all proteins were identified. Diffraction quality crystals were obtained by further optimization of the hit conditions with the hanging drop vapor diffusion method at 25°C. Diffraction quality co-crystals for PyrI4 were grown in reservoir solution comprising of reservoir solutions 0.02 M calcium acetate hydrate, 0.1 M sodium cacodylate pH 6.5, 42% v/v PEG 300 in the presence of 1 mM 3a. Reservoir for PyrI4Δ10 comprised of 6% Polypropylene glycol P400 and 2% glycerol and 1 mM 3a. Reservoir buffer for AbnU protein contained 1M Imidazole, pH 7.0 and 20% ethanol. Diffraction quality crystal appeared in 24-48 hrs. Both co-crystallization and soaking techniques were employed to determine the ligand bound crystals.

6. Optimization of the chromatographic method and Mass spectrometry

a) Quantitative study:
Molecular separation was carried out using a UHPLC system (Ultimate 3000, Thermo Scientific, Bremen, Germany) equipped with a quaternary pump, an in-line degasser, and an auto-injector and column compartment. The reversed-phase chromatographic separations were performed on a Hypersil Gold C18 (Dimensions 100 x 2.1 (mm), 1.9 (µ) particle size, Thermo scientific) using 0.1 % formic acid in water (A) as buffer and acetonitrile (B) as organic modifier for gradient elution. The flow rate was 0.5 mL/min., the column compartment temperature was maintained at 25 °C, the autosampler was kept at 7 °C and the injection volume was 10 µl. The elution gradient was optimized at: (time in min/ % of solvent B): 0-0.3/5, 0.3-4/95, 4-4.6/95, 4.6-4.7/5, and 4.7-5.5/5. The column was equilibrated for 2 min with an initial gradient before the next injection and three independent measurements were taken. The eluent from the UHPLC column was directed into the mass spectrometer (TSQ Altis, Thermo Scientific, Bremen, Germany) through heated electrospray ionization (HESI) interface operated in the positive ion mode. The interface conditions were as follows: vaporizer temperature 350 °C, ion transfer tube temperature 325 °C, sheath gas flow 40 (arbitrary units), auxiliary gas flow gas flow 10 (arbitrary units), sweep gas 0 (arbitrary units), positive ion voltage 3500 V and negative ion voltage 2500 V. Nitrogen was used as the sheath gas and auxiliary gas. The LC-MS/MS data were
acquired in the selected reaction-monitoring (SRM) mode. The resolutions of Q1 and Q3 were set as 0.7 FWHM, the CID gas (Argon gas) pressure was 1.5 mTorr. Every analyte has resulted in two SRM transitions, one was selected as the quantifier, and the other as the qualifier. All the transitions are listed in Supplementary Table 2 below. The acquired data were processed using Thermo Xcalibur software (version 4.1.31.9).

Supplementary Table 2: SRM transition parameters for Diels-Alder Product (3a)

| Analytes              | Precursor ion (m/z) | Product ion (m/z) | Collision energy (V) | Dwell time (ms) | RF Lens (V) | Retention Time (min) |
|-----------------------|---------------------|-------------------|----------------------|-----------------|-------------|----------------------|
| Diels-Alder Product   | 361.15              | 152.15            | 14                   | 198.717         | 51          | 4.55                 |
|                       |                     | 149.05            | 24                   | 198.717         |             |                      |
| Tri-ethyl phosphate   | 183.15              | 126.84            | 11.9                 | 198.717         | 35          | 3.99                 |
| (IS)                  |                     | 98.98             | 19.4                 | 198.717         |             |                      |

b) Stereoselectivity study:

Chromatographic separations of Diels-Alder reaction product were achieved on a Surveyor UHPLC system (Thermo Scientific, Germany) consisting of a quaternary gradient pump, an auto-injector and an in-line degasser. The column compartment was maintained at a temperature of 25 °C. The chromatographic separation method was developed in normal phase mode with chiral column (0.46 × 25 cm; Chiralpak AD-H, Daicel Chemical Ind., Ltd.). Varying the selectivity determination factors such as pH of the mobile phase, ratios of polarity change and flow rate optimized the method. Finally, the acceptable separation was achieved using 0.1% TFA in isopropanol (A) and 0.1% TFA in hexane (B) in the ratio of 30:70, v/v with a flow rate of 0.6 mL min⁻¹ in isocratic elution mode. The column was equilibrated with 20 column volumes of mobile phase at the composition prior to sample injection and the injection volume was 1µl.

Mass spectral analysis was carried out on Thermo Orbitrap Exactive mass spectrometer (Thermo Scientific, Bremen, Germany). The eluent from the UHPLC system was directed into the mass spectrometer via heated electrospray ionization (HESI) interface and operated in the positive ion mode. The mass spectrometer was calibrated before analysis using the manufacturer's calibration solution (ProteoMass LTQ/FN-Hybrid ESI Pos. Mode Cal Mix, SUPELCO) to obtain the mass range in external calibration mode. Parameters of the ion source were as follows: ion spray voltage 4.20 kV, heater temperature 275 °C, capillary temperature 320 °C, capillary voltage 67.50 V, tube lens voltage 140 V, skimmer voltage 20 V, sheath gas flow 45 (arbitrary units), auxiliary gas flow 10 (arbitrary units) and sweep gas 0 (arbitrary units). Nitrogen was used as the sheath and auxiliary
gas in the ion source. The instrument was operated in full scan FTMS over the range m/z 350-400 at a resolving power of 1,00,000 (full width at half maximum). Mass spectral data were processed using Thermo Xcalibur software (version 2.2).

**Supplementary Figure 4:** APCI-MS (negative mode) spectrum of 1a ([M-H]⁻; m/z 260.42) and 1b ([M-H]⁻; m/z 246.37). ESI-HRMS spectra of 2 ([M+H]⁺; m/z 100.076, [M+Na]⁺; m/z 411.1889), 3a ([M+H]⁺; m/z 361.1769.0756, [M+Na]⁺; m/z 383.1580), 3b ([M+H]⁺; m/z 347.1593, [M+Na]⁺; m/z 369.1410), 4 ([M+H]⁺; m/z 128.1064, [M+Na]⁺; m/z 150.0883), 5 ([M+H]⁺; m/z 142.1221, [M+Na]⁺; m/z 164.0676), 6 ([M+H]⁺; m/z 389.2070, [M+Na]⁺; m/z 411.1889), 7 ([M+H]⁺; m/z 403.2216, [M+Na]⁺; m/z 425.1670).
7. Diels-Alder reaction by DAse

The Diels-Alder reaction was performed in PBS buffer with 1 mM of Diene (1a), 10 mM of dienophile (2), 4% DMSO (vol/vol) and 300 µM of an enzyme i.e. AbnU or PyrI4 with 10% glycerol (volume 100 µl). In the same way, control reaction (background) was carried out in the absence of the enzyme. Reactions were incubated at 37 °C for 2.5 h and quenched with 250 µl ice-cold acetonitrile and then kept at -80 °C for 10 minutes. Acetonitrile layer was separated (200 µl) and internal standard (triethyl phosphate, 275 nM) was added prior to Liquid Chromatography-Selected Reaction Monitoring (LC-SRM) analysis. Standard DA product was also spiked in the buffer without an enzyme and followed the above procedure. Calculated recovery percentage was 97 %. For the absolute quantification, the standard plot was drawn (R$^2 = 0.99$) with the authentic product concentrations ranging from 5.6 nM to 2.22 µM.

8. Kinetic Analysis

Diels alder reactions were performed in PBS buffer at 37°C with 4% DMSO (vol/vol) and 40µM of PyrI4/20 µM of AbnU with 10% glycerol. Diene concentrations were fixed (50, 100, 200, 450 and 600 µM for AbnU and 150, 200, 300, 400 and 600 µM for PyrI4) with varying concentration of dienophile (0, 6, 12, 20, 60, and 100 mM for AbnU and 5, 10, 20, 40, 100 and 150 mM for PyrI4) in the presence or absence of enzyme. Reaction was then incubated for 2.5 h before quenching the reaction with ice-cold acetonitrile and LC-SRM measurement as described above. The experimental data was analysed as described in the previous report.

9. Stereoselectivity of Diels-Alderases

Diels-Alder reactions were performed in PBS buffer at 37 °C with 4% DMSO (vol/vol) and 300 µM of enzymes (AbnU, PyrI4, PyrI4-Δ10, and mutant proteins) with 10% glycerol. A reaction with 1 mM of diene and 10 mM dienophile was run for 165 h with and without enzyme. Both the reactions were quenched with an equal volume of a solution of acetonitrile:water (80:20) with 0.1% formic acid. The above mixture was incubated for 5 minutes and half the volume of ethyl acetate was added for the extraction of product (organic layer formed by centrifuge). Extracted product in
ethyl acetate was dried under a stream of nitrogen gas and then analyzed by chiral LC-HRMS as described above.

**Supplementary Figure 5:** Chiral HPLC spectra of synthetic products. Stereoselectivity pattern for all the ortho isomers (exo-3S,4S, exo-3R,4R, endo-3S,4R and endo-3R,4S) of 3a and 3b separated by Chiral-HPLC as reported in the literature. Synthesis of 3a and 3b used in this experiment is described later in this document. Retention time and the pattern were used as a standard for all the stereoselectivity experiments. We have used the previous literature in assigning the individual peaks to a specific isomer.⁷

**Supplementary Table 3:** Integrated peak areas for the stereoselectivity data a) AbnU with 3a, b) AbnU with 3b c) PyrI4 with 3a d) PyrI4-Δ10 with 3a e) PyrI4 with 3b.

|                | Integrated Area of Peaks | Relative Peak Area |
|----------------|--------------------------|--------------------|
|                | Exo (3S,4S)               | Exo (3R,4R)        | Endo (3S,4R) | Endo (3R,4S) | Exo (3S,4S)    | Exo (3R,4R)    | Endo (3S,4R) | Endo (3R,4S) |
| 3a (synthetic product) | 5901803                  | 35234229           | 37623516     | 7500314      | 6.84%          | 40.84%         | 43.62%      | 8.70%        |
| Background reaction      | 538514                   | 736272             | 625306       | 731288       | 20.47%         | 27.98%         | 23.76%      | 27.79%       |
| AbnU + 1a + 2          | 3012412                  | 186047             | 308059       | 365835       | 77.79%         | 4.80%          | 7.96%       | 9.45%        |
| AbnU Enhancement     | 2473898                  | -550225            | -317247      | -365453      | 199.35%        | -44.34%        | -25.56%     | -29.45%      |

⁷
### Integrated Area of Peaks

|          | Exo (3S,4S) | Exo (3R,4R) | Endo (3S,4R) | Endo (3R,4S) |
|----------|-------------|-------------|--------------|--------------|
| **3b**   | 11988195    | 12913001    | 3763906      | 3497066      |
| Background reaction | 1613043 | 494691 | 1521197 | 1588433 |
| AbnU + 1b + 2 | 8824441 | 1545087 | 4286487 | 4102267 |
| AbnU Enhancement | 7211398 | 1050396 | 2765290 | 2513834 |

### Relative Peak Area

|          | Exo (3S,4S) | Exo (3R,4R) | Endo (3S,4R) | Endo (3R,4S) |
|----------|-------------|-------------|--------------|--------------|
| **3b**   | 37.27%      | 40.15%      | 11.70%       | 10.87%       |
| Background reaction | 30.92% | 9.48% | 29.16% | 30.45% |
| AbnU + 1b + 2 | 47.04% | 8.25% | 22.85% | 21.87% |
| AbnU Enhancement | 53.26% | 7.76% | 20.42% | 18.56% |

### Integrated Area of Peaks

|          | Exo (3S,4S) | Exo (3R,4R) | Endo (3S,4R) | Endo (3R,4S) |
|----------|-------------|-------------|--------------|--------------|
| **3a**   | 5901803     | 35234229    | 37623516     | 7500314      |
| Background reaction | 65727261 | 22433920 | 34291872 | 96049331 |
| PyrI4 + 1a + 2 | 164308250 | 37443568 | 52740078 | 67583575 |
| PyrI4 Enhancement | 98580989 | 15009648 | 18448206 | -28465756 |

### Relative Peak Area

|          | Exo (3S,4S) | Exo (3R,4R) | Endo (3S,4R) | Endo (3R,4S) |
|----------|-------------|-------------|--------------|--------------|
| **3a**   | 6.84%       | 40.84%      | 43.62%       | 8.70%        |
| Background reaction | 30.08% | 10.27% | 15.69% | 43.96% |
| PyrI4 + 1a + 2 | 51.01% | 17.30% | 17.30% | 21.49% |
| PyrI4 Enhancement | 95.18% | 14.49% | 17.81% | -27.48% |

### Integrated Area of Peaks

|          | Exo (3S,4S) | Exo (3R,4R) | Endo (3S,4R) | Endo (3R,4S) |
|----------|-------------|-------------|--------------|--------------|
| **3a**   | 5901803     | 35234229    | 37623516     | 7500314      |
| Background reaction | 65727261 | 22433920 | 34291872 | 96049331 |
| PyrI4-Δ10 + 1a + 2 | 126446477 | 33146744 | 45113286 | 56019695 |
| PyrI4-Δ10 Enhancement | 60719216 | 10712824 | 10821414 | -40029636 |

### Relative Peak Area

|          | Exo (3S,4S) | Exo (3R,4R) | Endo (3S,4R) | Endo (3R,4S) |
|----------|-------------|-------------|--------------|--------------|
| **3a**   | 6.84%       | 40.84%      | 43.62%       | 8.70%        |
| Background reaction | 30.08% | 10.27% | 15.69% | 43.96% |
| PyrI4-Δ10 + 1a + 2 | 48.50% | 16.38% | 17.30% | 21.49% |
| PyrI4-Δ10 Enhancement | 95.18% | 14.49% | 17.81% | -27.48% |

### Integrated Area of Peaks

|          | Exo (3S,4S) | Exo (3R,4R) | Endo (3S,4R) | Endo (3R,4S) |
|----------|-------------|-------------|--------------|--------------|
| **3b**   | 11988195    | 12913001    | 3763906      | 3497066      |
| Background reaction | 1613043 | 494691 | 1521197 | 1588433 |
| PyrI4 + 1b + 2 | 4115294 | 963833 | 2769367 | 3229729 |
| PyrI4 Enhancement | 2502251 | 469142 | 1248170 | 1641296 |
|                  | Exo (3S,4S) | Exo (3R,4R) | Endo (3S,4R) | Endo (3R,4S) |
|------------------|-------------|-------------|--------------|--------------|
| **3b** (synthetic product) | 37.27% | 40.15% | 11.70% | 10.87% |
| Background reaction | 30.92% | 9.48% | 29.16% | 30.45% |
| PyrI4 + 1b + 2    | 37.15% | 8.70% | 25.01% | 29.15% |
| PyrI4 Enhancement | 42.69% | 8.00% | 21.30% | 28.00% |
**Supplementary Figure 6**: Stereoselectivity data for background (non-enzymatic), AbnU, PyrI4 and PyrI4-Δ10 (only for 3a) represented by overlay of the extracted ion chromatogram (EIC) of a) 3a \([m/z \, 361 \, [M+H]^+ \text{ and } m/z \, 383 \, [M+Na]^+]\) and b) 3b \([m/z \, 347 \, [M+H]^+ \text{ and } m/z \, 369 \, [M+Na]^+]\) respectively.

a)

![Stereoselectivity Data Graph](image)

b)
10. Diffraction data collection and structure determination

Apo PyrI4 crystals for diffraction data collection were mounted in an appropriately sized litholoops (Molecular Dimensions Ltd) and directly flash-cooled in liquid nitrogen prior to analysis. PyrI4-Δ10 protein crystals were submerged in mother liquor supplemented with 10% glycerol followed by flash freezing in liquid nitrogen prior to analysis. The diffraction data for the PyrI4 and PyrI4-Δ10-3a complex were collected at the home source on a MARS CCD detector using a home source X-ray diffractometer (Rigaku Micromax-007) with R-axis IV++ detector. AbnU protein crystal was submerged in mother liquor supplemented with 15% ethylene glycol followed by flash freezing in liquid nitrogen prior to analysis. X-ray data for AbnU were collected on beamline XRD2 at Elettra synchrotron, Trieste, Italy.

Diffraction data were processed using XDS. Data were further scaled and merged using AIMLESS as implemented within the CCP4 suite of programs. About 5% of the data were set aside for the calculation of R_free. Molecular replacement was performed by MOLREP using PDB ID: 5BTU for PyrI4 as a model while PDB ID: 5DYV for AbnU as a model in the CCP4 suite of programs. Further several iterative rounds of manual model building and refinement were carried out using COOT and REFMAC5. Crystallographic data for AbnU and PyrI4-Δ10 are summarized in the table below (Supplementary Table 4). Unfortunately, PyrI4 crystals did not diffract better than 3.0 Å and had very high mosaicity which did not permit us to solve the structure.
## Supplementary Table 4: Crystallographic table of AbnU and PyrI4-Δ10

|                  | AbnU                  | PyrI4-Δ10-3a             |
|------------------|-----------------------|-------------------------|
| **Wavelength**   | 0.974                 | 1.54                    |
| **Resolution range** | 41.34 - 2.003 (2.074 - 2.003) | 46.04 - 2.601 (2.694 - 2.601) |
| **Space group**  | P 6(4)22              | P 4(3)2(1)2             |
| **Unit cell**    | 104.44 104.44 67.66 90 90 120 | 102.96 102.96 168.80 90 90 |
| **Total reflections** | 552015 (51514)        | 290143 (28180)          |
| **Unique reflections** | 15136 (1464)         | 28575 (2764)            |
| **Multiplicity** | 36.5 (35.2)           | 10.2 (10.2)             |
| **Completeness (%)** | 99.89 (99.19)       | 99.68 (98.36)           |
| **Mean I/σ(I)**  | 42.85 (7.94)          | 21.18 (5.76)            |
| **Wilson B-factor** | 34.32                 | 40.67                   |
| **R-merge**      | 0.06271 (0.5703)      | 0.0861 (0.3639)         |
| **R-meas**       | 0.06361 (0.5787)      | 0.09072 (0.3831)        |
| **R-pim**        | 0.01055 (0.09681)     | 0.0281 (0.1181)         |
| **CC1/2**        | 1 (0.982)             | 0.999 (0.949)           |
| **CC***          | 1 (0.995)             | 1 (0.987)               |
| **Reflections used in refinement** | 15134 (1464)        | 28560 (2764)            |
| **Reflections used for R-free** | 724 (76)             | 1402 (120)              |
| **R-work**       | 0.19                  | 0.21                    |
| **R-free**       | 0.24                  | 0.27                    |
| **Number of non-hydrogen atoms** | 1265                 | 5053                    |
| **macromolecules** | 1099                  | 4869                    |
| **ligands**      | 96                    | 134                     |
| **solvent**      | 70                    | 50                      |
| **Protein residues** | 134                   | 652                     |
| **RMS(bonds)**   | 0.04                  | 0.29                    |
| **RMS(angles)**  | 2.03                  | 5.08                    |
| **Ramachandran favored (%)** | 98.48             | 96.12                   |
| **Ramachandran allowed (%)** | 1.52               | 3.88                    |
| **Ramachandran outliers (%)** | 0.00               | 0.00                    |
| **Rotamer outliers (%)** | 5.22                | 10.44                   |
| **Clashscore**   | 5.02                  | 9.29                    |
| **Average B-factor** | 39.30                | 44.08                   |
| **macromolecules** | 37.48                 | 43.33                   |
| **ligands**      | 55.20                 | 76.45                   |
| **solvent**      | 45.99                 | 30.60                   |
| **PDB ID**       | 7DVI                  | 7DVK                    |
**Supplementary Figure 7**: Energy minimized three-dimensional structure and molecular surface representation (dot) of 3a a) linear *Exo*-3S,4S and b) U-shaped *Endo*-3R,4S generated by ChemDraw version 19.
11. Confirmation of product bound PyrI4-Δ10 via mass spec

Crystals of PyrI4-Δ10 co-crystallized with 3a were examined for the presence of the product. Crystals were thoroughly washed in the well solution to remove any ligand bound on the surface. Then the crystals were crushed in ethyl acetate and mixed thoroughly to separate the aqueous layer with protein and buffer, and organic layer. The organic layer was subjected to mass spectral analysis (Supplementary Figure 8a and 8b).

Supplementary Figure 8: Mass spectral analysis of product 3a extracted from PyrI4-Δ10-3a co-crystal a) ESI-HRMS spectrum data depicting the presence of product 3a in PyrI4-Δ10-3a co-crystal b) Chiral Liquid Chromatography-Selected Reaction Monitoring (LC-SRM) analysis of product 3a in PyrI4-Δ10-3a co-crystal showing the presence of both the exo adduct with the retention time matching with the synthetic standard 3a.
Supplementary Figure 9: Crystal structure of AbnU determined at 2.0 Å resolution depicted by ribbon and the surface representation of dimer of AbnU.
12. **Tryptophan fluorescence spectroscopy:**

All tryptophan fluorescence spectroscopy experiments were performed using Varioskan FLASH multimode reader (Thermo Scientific) and 96-well black flat bottom microtiter plate (Corning Inc., USA) at ambient temperature. The excitation wavelength was fixed to 285 nm and emission spectra were collected between 300 and 500 nm with a slit width of 5 nm.

To study the AbnU-ligand interactions, recombinant AbnU protein at 20 µM was equilibrated in PBS buffer prior to experiment. Ligands included the Dienes 1a and 1b, Dienophile 2 and the Diels-Alder adduct 3a. AbnU-ligand interaction was tested with increasing concentrations of 1a and 1b from 0 µM to 3000 µM, 2 from 0 mM to 1000 mM and Diels-Alder product 3a from 0 µM to 3000 µM. Control buffer titration experiments were always performed in parallel to ensure background subtraction, if any.

The cumulative effect of the binding of both the substrates was analyzed by titrating fixed concentration of 1a at 500 µM and 2 at 5 mM in the presence or absence of 20 µM AbnU. Control fluorescence of the individual substrate and the buffer titration was performed in triplicate.

Triplicate experimental data were analyzed for the background subtraction and was further analyzed by using nonlinear regression with ‘One Site - Specific Binding’ model with the following equation:

\[ Y = B_{\text{max}} \times \frac{X}{K_D + X} \]

where X is the ligand concentration, Y is the fluorescence intensity, \( B_{\text{max}} \) is the maximum specific binding and \( K_D \) is the equilibrium binding constant. \( K_D \) values were determined by fitting the data to the above equation in GraphPad Prism 8.1.0.

**Supplementary Figure 10:** Intrinsic tryptophan fluorescence (\( \lambda_{\text{ex}}=285 \text{ nm}, \lambda_{\text{em}}=330 \text{ nm} \)) profile for a) AbnU in the presence and absence of DMSO showing no effect on its fluorescence b) Comparison of tryptophan fluorescence between AbnU and PyrI4
13. Substrate Scope

Diels alder reactions were performed in PBS buffer at 37°C with 4% DMSO (vol/vol), fixed concentration of 1a (0.2 mM) and different dienophiles namely 2, 4 and 5 (each 10 mM) were incubated with and without 60 uM of AbnU and PyrI4 in 10% glycerol. The reaction was incubated for 2.5 hr and quenched with ice-cold acetonitrile. Relative quantification of the analytes was carried out by using UHPLC coupled with ESI-SRM with the flow rate as 0.4 ml/min. The elution gradient was modified and optimized at: (time in min/proportion of solvent B): 0-0.3/5, 0.3-4/95, 4-5.9/95, 5.9-6.0/5, and 6.0-8.0/5. Same SRM parameters (Supplementary Table 4) were used for the analyte (3a) without the internal standard. Data were analyzed by Thermo Xcalibur software.

Supplementary Figure 11: Substrate selectivity of AbnU and PyrI4 for different dienophiles namely 2, 4 and 5. Data shown here represent the comparison between background (non-enzymatic) and enzyme catalyzed activity. All error bars reflect the standard deviation obtained from independent triplicate experiments.
14. Chemical synthesis dienes, dienophiles and products

General Procedure:

Methyl (E)-4-(buta-1, 3-dien-1-ylcarbamoyl)oxy)methyl)benzoate (1a): All the synthetic protocols were based on the previous report.\(^2\) 2,4-pentadienoic acid (A) (1 eq) dissolved in 30 mL of toluene and N,N-diisopropylethylamine was added drop wise at room temperature under nitrogen. The resulting clear solution was stirred and cooled to 0 °C in ice-bath. A solution of ethyl chloroformate (2 eq) in 90 mL of toluene was added over 30 minutes while the temperature was maintained at 0 °C. Stirring was continued for 30 minutes at 0 °C, after sodium azide (2 eq) was added. The solution was stirred for additional 30 minutes at 0 °C and poured into separatory funnel containing 150 mL of ice-water. The acylazide was isolated in mother liquor and dried over Na\(_2\)SO\(_4\). Above isolated acylazide (toluene) treated with methyl 4-(hydroxymethyl)benzoate (B) (1.2 eq) stirred at 110 °C. The reaction was monitored by TLC and after the indicated reaction time; first the cooled mixture and quenched with saturated ammonium chloride solution and partitioned between ice-cold water (30 mL) and ethyl acetate (50 mL). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (2 × 30 mL). The combined organic layer was washed with brine and dried over Na\(_2\)SO\(_4\). The solvent was removed in \textit{vacuo}. The residue was purified by column chromatography on silica gel to afford the desired product (1a) (Supplementary Scheme 1).

![Supplementary Scheme 1. Synthesis of 1a](image-url)
**Diels Alder reaction general procedure.**

Methyl (E)-4-(buta-1, 3-dien-1-ylcarbamoyl)oxy)methyl)benzoate (1a) (1eq), dissolved in 10 mL toluene and dienophile (2) (1.2 eq) were added at room temperature and the reaction was stirred under reflux conditions for 4 days. The reaction was monitored by TLC and after the completion of the reaction indicated; first the mixture was cooled and quenched with saturated ammonium chloride solution and partitioned between ice-cold water (30 mL) and ethyl acetate (50 mL). The organic layer was separated, and the aqueous layer was extracted with ethyl acetate (2 × 30 mL). The combined organic layer was washed with brine and dried over Na₂SO₄. The solvent was removed in vacuo. The residue was purified by column chromatography on silica gel to afford the desired product (3a) (Supplementary Scheme 2).

**Supplementary Scheme 2. Synthesis of 3a**

**Ester hydrolysis**

A solution of LiOH.H₂O (3 eq) in 5 mL of water was added to a solution of 1a or 3a in 15 mL of THF. The biphasic system was stirred at room temperature for 24 h until it became homogeneous. The solution was acidified by the addition of 1M HCl, and extracted with ethyl acetate (2 × 30 mL). The organic layer dried over Na₂SO₄ and evaporated in vacuo. The yellow crude product was purified by flash chromatography on silica gel to give as a white solid (1b or 3b) (Supplementary Scheme 3).
Supplementary Scheme 3. Synthesis of 3b

Methyl (E)-4-((Buta-1, 3-dien-1-ylcarbamoyl)oxy)methyl)benzoate (1a) White solid, IR (KBr): 3281, 3016, 2958, 2930, 1726 (C=O), 1697 (C=O) cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.03 (d, \(J = 8.2\) Hz, 2H), 7.41 (d, \(J = 8.0\) Hz, 2H), 6.79 – 6.70 (m, 1H), 6.54 (d, \(J = 12.3\) Hz, 1H), 6.28 (dt, \(J = 17.8, 10.3\) Hz, 1H), 5.79 – 5.66 (m, 1H), 5.21 (s, 2H), 5.05 (d, \(J = 19.2\) Hz, 1H), 4.93 (d, \(J = 11.0\) Hz, 1H), 3.92 (s, 3H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 166.74, 153.12, 140.85, 134.31, 129.91, 127.70, 126.77, 113.81, 112.55, 66.56, 52.23; LC-MS: (M+H)\(^+\) = 262.10 (Supplementary Figure 12a and 12b).

(E)-4-(((Buta-1, 3-dien-1-ylcarbamoyl)oxy) methyl) benzoic acid (1b) White solid, IR (KBr): 3330, 2954, 1702 (C=O), 1659 (C=O) cm\(^{-1}\); \(^1\)H NMR (300 MHz, CDCl\(_3\)+DMSO): \(\delta\) 9.47 (d, \(J = 10.0\) Hz, 1H), 8.07 (d, \(J = 8.0\) Hz, 2H), 7.49 (d, \(J = 8.0\) Hz, 2H), 6.82 – 6.65 (m, 1H), 6.30 (dt, \(J = 16.9, 10.4\) Hz, 1H), 5.99 – 5.75 (m, 1H), 5.25 (s, 2H), 5.04 (d, \(J = 16.9\) Hz, 1H), 4.89 (d, \(J = 10.3\) Hz, 1H), 2.65 (s, 1H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)+DMSO) \(\delta\) 168.53, 153.91, 140.84, 134.31, 131.74, 129.98, 128.35, 127.59, 112.59, 112.15, 66.04. LC-MS: (M-H)\(^-\) = 246.10 (Supplementary Figure 13a and 13b).
Methyl 4-(((6-(Dimethylamino) cyclohex-2-en-1-yl) carbamoyl) oxy) methyl) benzoate (3a)

White solid, IR (KBr): 3287, 3016, 2958, 2930, 1726 (C=O), 1697 (C=O) cm⁻¹; ¹H NMR (400 MHz, CDCl₃): δ 8.01 (d, J = 8.0 Hz, 2H), 7.39 (d, J = 6.1 Hz, 2H), 5.81 (d, J = 9.4 Hz, 1H), 5.66 (d, J = 8.8 Hz, 1H), 5.11 (dd, J = 26.6, 12.7 Hz, 2H), 4.37 (s, 1H), 3.92 (s, 3H), 3.12 (dd, J = 25.4, 2.7 Hz, 1H), 2.98 (s, 3H), 2.93 (s, 3H), 2.13 (s, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 173.77, 166.82, 155.13, 141.87, 129.77, 128.49, 128.34, 127.50, 65.63, 52.18, 50.43, 41.31, 37.22, 35.76, 25.08, 24.24; LC-MS: (M+H)⁺ = 361.15 (Supplementary Figure 14a and 14b).

4-(((6-(dimethylamino)cyclohex-2-en-1-yl)carbamoyl) oxy) methyl) benzoic acid (3b) White solid, ¹H NMR (300 MHz, CDCl₃): δ 8.07 (dd, J = 16.5, 8.2 Hz, 4H), 7.84 (d, J = 7.7 Hz, 1H), 7.52 – 7.37 (m, 4H), 6.11 (t, J = 8.8 Hz, 1H), 5.98 – 5.87 (m, 1H), 5.86 – 5.64 (m, 2H), 5.50 – 5.35 (m, 1H), 5.20 – 5.07 (m, 2H), 5.03 (s, 1H), 4.80 (s, 1H), 4.59 – 4.52 (m, 1H), 4.38 – 4.37 (m, 1H), 3.18 (s, 3H), 3.01 (s, 3H), 2.94 (s, 6H), 2.15 (s, 4H), 2.07 – 1.98 (m, 2H), 1.83 (s, 4H), 0.98 – 0.74 (m, 2H); LC-MS: (M+H)⁺ = 347.10 (Supplementary Figure 15).

N,N-diethylacrylamide (4) ¹H NMR (400 MHz, CDCl₃): δ 6.55 (dd, J = 16.7, 10.4 Hz, 2H), 6.34 (dd, J = 16.7, 2.1 Hz, 2H), 5.66 (dd, J = 10.4, 2.1 Hz, 2H), 3.42 (dq, J = 24.5, 7.1 Hz, 9H), 1.18 (dt, J = 15.9, 7.1 Hz, 14H). ¹³C NMR (101 MHz, CDCl₃): δ 165.63, 127.93, 127.53, 42.21, 40.82, 14.85, 13.12. LC-MS: (M+H)⁺ = 128.15 (Supplementary Figure 16a and 16b).
**1-morpholinoprop-2-en-1-one (5)**

{\( ^1H \text{ NMR (400 MHz, CDCl}_3 \): \( \delta \) 6.62 – 6.50 (m, 1H), 6.31 (d, J = 16.8, 3.8, 1.9 Hz, 1H), 5.72 (d, J = 10.5, 3.5, 1.7 Hz, 1H), 3.64 (d, J = 44.8 Hz, 8H).}

{\( ^{13}C \text{ NMR (101 MHz, CDCl}_3 \): \( \delta \) 165.48, 128.37, 127.02, 77.09, 66.80, 46.22, 42.28.}

{LC-MS: (M+H)+ = 142.21 (Supplementary Figure 17a and 17b).}

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**Methyl 4-(((6- (Diethylcarbamoyl) cyclohex-2-en-1-yl) carbamoyl) oxy) methyl) benzoate (6)**

{\( ^1H \text{ NMR (400 MHz, CDCl}_3 \): \( \delta \) 8.00 (d, J = 8.1 Hz, 3H), 7.38 (d, J = 7.5 Hz, 3H), 5.81 (d, J = 8.0 Hz, 1H), 5.66 (d, J = 9.1 Hz, 1H), 5.16 (d, J = 12.8 Hz, 3H), 5.04 (d, J = 12.9 Hz, 1H), 4.35 (s, 1H), 3.92 (s, 5H), 3.41 (dt, J = 14.1, 7.0 Hz, 1H), 3.27 (qd, J = 14.0, 7.1 Hz, 4H), 3.10 (s, 1H), 2.13 (t, J = 16.7 Hz, 3H), 1.81 (dd, J = 8.4, 4.4 Hz, 3H), 1.07 (t, J = 7.0 Hz, 8H).}

{\( ^{13}C \text{ NMR (101 MHz, CDCl}_3 \): \( \delta \) 173.26, 166.83, 155.12, 141.89, 129.85, 129.75, 128.82, 128.13, 127.58, 65.54, 52.18, 50.84, 41.87, 41.37, 40.48, 26.05, 24.34, 14.89, 13.13.}

{LC-MS: (M+H)+ = 389.2071 (Supplementary Figure 18a and 18b).}

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**Methyl 4-(((6- (morpholine-4-carbonyl) cyclohex-2-en-1-yl) carbamoyl) oxy) methyl) benzoate (7)**

{\( ^1H \text{ NMR (400 MHz, CDCl}_3 \): \( \delta \) 8.01 (d, J = 8.2 Hz, 2H), 7.39 (d, J = 8.1 Hz, 2H), 5.89 (d, J = 9.7 Hz, 1H), 5.72 – 5.65 (m, 1H), 5.27 (d, J = 9.7 Hz, 1H), 5.13 (dd, J = 27.8, 13.3 Hz, 2H), 4.46 (d, J = 4.2 Hz, 1H), 3.91 (s, 3H), 3.80 (dd, J = 13.2, 7.1 Hz, 1H), 3.70 – 3.38 (m, 6H), 2.97 (dd, J = 12.5, 6.2 Hz, 1H), 2.16 (dd, J = 18.1, 4.3 Hz, 1H), 2.08 – 1.99 (m, 1H), 1.87 – 1.78 (m, 2H).}

{\( ^{13}C \text{ NMR (101 MHz, CDCl}_3 \): \( \delta \) 170.94, 166.86, 155.62, 141.92, 130.38, 129.76, 129.60, 127.19, 126.42, 66.62, 66.36, 65.81, 52.18, 46.05, 45.98, 41.99, 40.71, 23.98, 21.45.}

{LC-MS: (M+H)+ = 403.1864 (Supplementary Figure 19a and 19b).}
Supplementary Figure 12: a) $^1$H NMR (400 MHz, CDCl$_3$) Spectrum of compound 1a

b) $^{13}$C NMR (100 MHz, CDCl$_3$) Spectrum of compound 1a
Supplementary Figure 13: a) $^1$H NMR (300 MHz, CDCl$_3$+DMSO) Spectrum of compound 1b

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{\text{\text{\text{\text{H}}}N} & \quad \text{\text{\text{\text{\text{H}}}N} \\
\end{align*}
\]

b) $^{13}$C NMR (100 MHz, CDCl$_3$+DMSO) Spectrum of compound 1b
Supplementary Figure 14: a) $^1$H NMR (400 MHz, CDCl$_3$) Spectrum of compound 3a

b) $^{13}$C NMR (100 MHz, CDCl$_3$) Spectrum of compound 3a
Supplementary Figure 15: $^1$H NMR (300 MHz, CDCl$_3$) Spectrum of compound 3b
**Supplementary Figure 16:**

(a) $^1$H NMR (400 MHz, CDCl$_3$) Spectrum of compound N, N-diethylacrylamide (4)

(b) $^{13}$C NMR (100 MHz, CDCl$_3$) Spectrum of compound N,N-diethylacrylamide (4)
**Supplementary Figure 17:** a) $^1$H NMR (400 MHz, CDCl$_3$) Spectrum of compound 1-morpholinoprop-2-en-1-one (5)

![H NMR spectrum](image)

b) $^{13}$C NMR (100 MHz, CDCl$_3$) Spectrum of compound 1-morpholinoprop-2-en-1-one (5)

![C NMR spectrum](image)
Supplementary Figure 18: a) $^1$H NMR (400 MHz, CDCl$_3$) Spectrum of compound 6

b) $^{13}$C NMR (101 MHz, CDCl$_3$) Spectrum of compound 6
Supplementary Figure 19: a) $^1$H NMR (400 MHz, CDCl3) Spectrum of compound 7

b) $^{13}$C NMR (100 MHz, CDCl3) Spectrum of compound 7
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