

Enzymatic Intermolecular Hetero-Diels–Alder Reaction in the Biosynthesis of Tropolonic Sesquiterpenes

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Supporting Information Placeholder

ABSTRACT: We report the isolation and characterization of the intermolecular hetero-Diels–Alderase, EupfF, involved in the biosynthesis of tropolonic sesquiterpenes. While many enzymatic intramolecular Diels–Alder reactions have been discovered, no natural intermolecular Diels–Alderase has been biochemically characterized previously. We demonstrate that a short-chain dehydrogenase EupfE catalyzed the reduction of stipitaldehyde (**4**) to form a hydroxymethyl containing tropolone (**5**) as a diene precursor; we characterized EupfF as the first intermolecular hetero-Diels–Alderase from fungi. EupfF catalyzes the dehydration of **5** to generate a reactive tropolone *o*-quinone methide (**6**) and controls the subsequent stereoselective intermolecular hetero-Diels–Alder reaction with 1*E*,4*E*,8*Z*-humulenol (**8**) to produce enantiomerically pure neosetophomone B (**1**).

Diels–Alder (DA) reactions are powerful synthetic methods for the synthesis of complex natural products.¹ Both intramolecular and intermolecular Diels–Alder cycloadditions are presumed to introduce structure complexity and biological activity in natural compounds. Although an increasing number of intramolecular Diels–Alderase or [4 + 2]-cyclases² such as LepI,³ SpnF,⁴ PyrE3,⁵ IccD,⁶ PvhB,⁷ and others⁸ have been discovered in Nature, no direct biochemical evidence for enzyme-catalyzed intermolecular DA reactions has been described.⁹ This is in spite of the fact that natural products probably biosynthesized *via* intermolecular Diels–Alder reaction are frequently reported from bacteria, fungi and plants (Figure S1).¹⁰ Natural products ainliatriolide A,¹¹ chrysanolide A,¹² ainsliatrimmer A,^{10c} et al., trimers or dimers likely formed *via* intermolecular DA reactions usually show significantly enhanced bioactivities over those of the monomers (Figure S2).

Several members of tropolone-sesquiterpenes family of fungal meroterpenoids (Figure S3) represented by neosetophomone B (**1**),¹³ epolone B (**2**),¹⁴ eupenifeldin (**3**)¹⁵, and pycnidione¹⁶ were proposed to be biosynthesized *via* one or two tandem hetero Diels–Alder reactions.¹⁷ All of them are tropolone-sesquiterpene or bistropolone-sesquiterpene adducts isolated as optically pure compounds from a variety of fungi. Structurally, these meroterpenoids possess an unique

central 11-membered macrocycle flanked by one or two dihydropyrans coupled to tropolones. Biologically, neosetophomone B (**1**) and eupenifeldin (**3**) were found as very potent antitumor agents with activity at the nanomolar level in *in vitro* tumor cell lines,^{13,15} while epolone B (**2**) and pycnidione, diastereomers of **1** and **3** respectively, are stromelysin inhibitor and potential antiarthritic drug (also induce erythropoietin gene expression).¹⁶

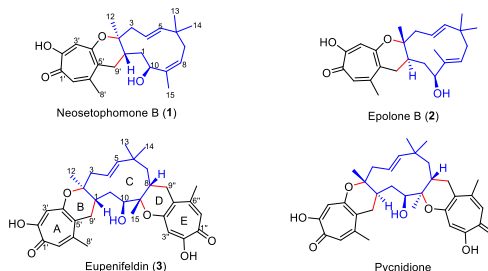


Figure 1. Representative tropolone-sesquiterpenes properly generated from intermolecular Diels–Alder reactions. The diene and dienophile moieties are shown in black and blue, respectively, and new bonds are shown in red.

The attempted biomimetic syntheses of tropolone-sesquiterpenes showed that the generation of monotropolone adducts *via* hetero Diels–Alder reaction under thermal conditions is successful only for unnatural model compounds.¹⁸ In fact, the total synthesis of these tropolone-sesquiterpene natural products is still a challenge. None of these natural products have been completely synthesized to date. We have explored the strategies and enzymes Nature uses to synthesize compounds of this family, especially the structure **1**.

Biosynthetically, hetero-Diels–Alder (hDA) reactions of a tropolone *o*-quinone methide with humulene were proposed for the biosynthesis of **1** and **2**, which further served as intermediate for the biosynthesis of eupenifeldin and pycnidione, respectively^{14, 19} (Figure 2a). While biosynthesis of the tropolone core in stipitonic acid has been identified (Figure 2a), including a non-reduced polyketide synthase (NR-PKS, TropA), a FAD dependent monooxygenase (FMO, TropB) and an α -

ketoglutarate-dependent dioxygenase (α -KGD, TropC),²⁰ the enzymatic basis for the DA reaction for **1** biosynthesis has remained unexplored. We report mapping of the cascade of enzymatic steps that furnish **1**, which include a cytochrome P450 monooxygenase (P450), a short-chain dehydrogenase (SDR) and an intermolecular hetero-Diels–Alderase (hDAase) as keys. We demonstrated that the hDAase is responsible for the formation of a highly reactive tropolone *o*-quinone methide, and further stereo-selectively controls the subsequent stereoselective intermolecular hDA cycloaddition with 1E,4E,8Z-humulenol (**8**) to produce **1**.

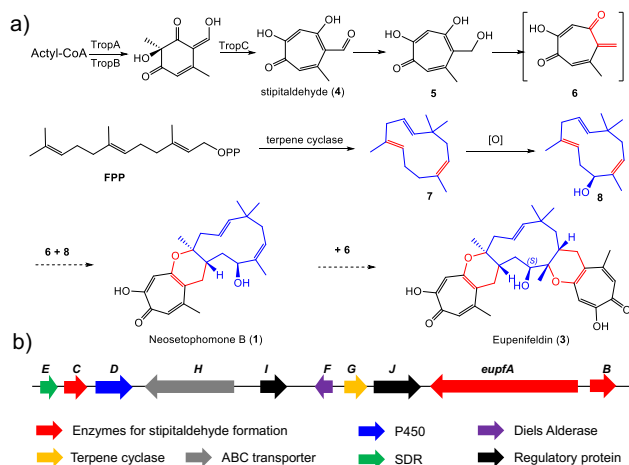


Figure 2. Proposed biosynthetic pathways of **1** and **3** (a) and the *eupf* gene cluster in *P. janthinellum* (b).

Recently, we co-isolated **1** and **3** from the wild type *Penicillium janthinellum* (FND-001), a producer of alanditrypinone,²¹ culturing in potato-dextrose broth (PDB) media. Analysis of the crude extract by liquid chromatography mass spectrometry (LC-MS) led to the observation of minor **1** and major **3** with characteristic UV absorption of tropolones, and molecular weight of 384 and 548, respectively (Figure S4). Purification and structural characterization by full NMR and Circular Dichroism (CD) spectra led to the identification of **1** and **3** as enantiomerically pure neosetophomone B and eupenifeldin, respectively. To investigate the biosynthetic pathway of **1**, the genome of the producing organism *P. janthinellum* was queried for the presence of *trop*-like gene cluster. Bioinformatics analysis led to the identification of only one *trop*-like NR-PKS gene cluster (named *eupf* cluster) in its genome (Figures 2b and S5). Besides three enzymes (EupfA–C) which showed homolog to TropA, TropB and TropC, respectively, the *eupf* cluster encodes additionally a cytochrome P450 monooxygenase (EupfD), a short-chain dehydrogenase (EupfE), two hypothetical enzymes (EupfF and EupfG), as well as ABC transporter and regulators (Figure 2b). EupfF and EupfG show high homolog to asR5 and asR6 (62% and 64%), respectively, which were recently suggested by Cox group as putative hetero-Diels–Alderase and humulene synthase, respectively, in the biosynthesis of xenovulene A.²² A biosynthetic gene cluster *eup* in *Phoma* sp. has been recently identified by gene deletion for the biosynthesis of eupenifeldin, and EupF, homolog to asR5, was also suggested as putative hDAase.²¹ However, the function of AsR5 and EupF has not been successfully characterized.

In the *eupf* gene cluster, it was found that EupfABC are responsible for the formation of the tropone, stipitaldehyde, which was a precursor a diene, while EupfG served as humulene synthase to form a sesquiterpene as dienophile. The only oxygenase EupfD catalyzes the hydroxylation of the terpene moiety at an uncertain stage. The putative hDAase EupfF, homologous to asR5 and EupF, was proposed to be

involved in the cycloaddition between diene and dienophile. However, the real function and order of these genes must be confirmed.

To verify the *eupf* gene cluster and elucidate the biosynthetic pathway of **1**, heterologous expression experiments in *Aspergillus nidulans* were conducted. As expected, expression of *eupfABC* in *A. nidulans* led to the major production of stipitaldehyde (**4**, Figure 3a). **4** was suggested as intermediate for biosynthesis of xenovulene A and other tropolone-sesquiterpenes. To form a diene precursor for DA reaction, the aldehyde moiety in **4** must first be reduced. As the only potential reductase in *eupf* cluster, *eupfE* was likely to be involved in this transformation. Although adding of *eupfE* to *A. nidulans* expressing *eupfABC* did not produce the expected diene or alcoholic product, the missing **4** was likely to be a substrate of EupfE (Figure S6). To verify the function of EupfE, intron-free *eupfE* was cloned from cDNA, heterologously expressed in *Escherichia coli* and purified to homogeneity (Figure S7). Control experiments showed that in the absence of EupfE or suitable co-factor no new product was generated (Figure 3b, trace i and Figure S8), incubation of EupfE with **4** in the presence of NADPH led to complete conversion to a new product **5** (Figure 3b, trace ii) with characteristic UV absorption of tropolone and MW of 182, 2Da more than that of **4**. The significant blue shift of UV absorption of **5** comparing to that of **4** (Figure S8) and the 2Da difference in molecular weight suggested that **5** was reductive product of **4**. Although **5** was not very stable enough, careful isolation and quick NMR analysis led to the full identification of **5** as expected (Figure 3d). Dehydration of **5** either spontaneously or enzymatically is proposed to generate the reactive tropolone *o*-quinone methide (**6**, Figure 4).

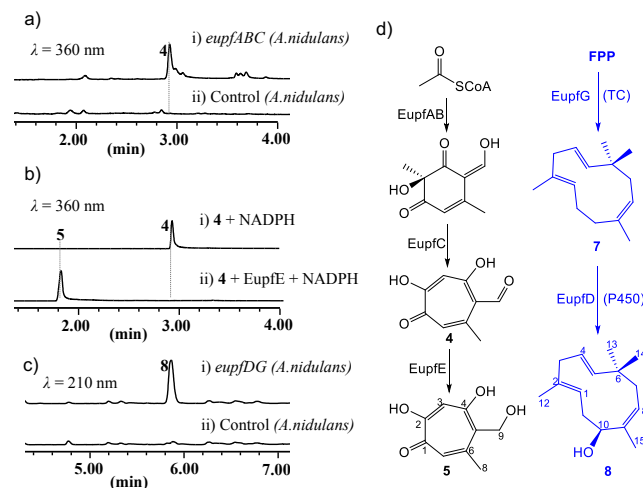


Figure 3. Generation of potential precursors in the biosynthesis of **1**. a–c) LCMS traces from heterologous expression of *eupfABC* and *eupfDG* in *A. nidulans* or from *in vitro* assay of EupfE. d) Biosynthetic pathway of **5** and **8**.

To identify the dienophile precursor, *eupfG*, the putative humulene synthase, was expressed in *A. nidulans*, which led to the detection of a compound **7** with same molecular weight (m/z 204) but different retention time to that of α -humulene (1E,4E,8E-humulene) standard indicated by GS-MS analysis (Figure S9), which suggested **7** was an isomer of α -humulene. Isolation of **7** was found to be difficult due to its high volatility. The structure of **7** was suggested by co-expression of *eupfG* and *eupfD* in *A. nidulans*, which led to the production of a sole compound **8** (Figure 3c, trace i) with MW of 220, 16Da more than that of α -humulene. **8** was fully characterized by MS and NMR spectra as (S,2Z,6E,9Z)-2,5,5,9-tetramethylcycloundeca-2,6,9-trien-1-ol, a hydroxylated product of **7**, which also supported that **7** was 1E,4E,8Z-humulene. Different from α -humulene in which all three olefins are E

form, compounds **7** and **8** possess a *Z* form of olefinic bond at $\Delta^{8,9}$, which was consistent with *Z* form of alkene in the structure of **1**. As a result, EupfG, highly homologous to the 1E,4E,8E-humulene synthase AsRS²², was identified as new sesquiterpene synthase producing 1E,4E,8Z-humulene.

We realized that **5** was *O*-methylated at C₉-OH gradually in methanol as evident in the time course analysis (Figure S10). Methylated **5** was isolated and fully characterized by NMR spectra as a tautomer mixture (2:1). The identification of methylated **5** (**6a**) as a tautomer mixture also suggested the spontaneous conversion from **5** to **6** occurred before it undergoes 1,4- or 1,6-addition by methanol (Figure S10). Due to the incomplete spontaneous conversion, we believed that enzyme was required to generate **6** from **5**, and EupfF, the putative hetero-Diels–Alderase, was presumed to involve in this process. However, repeated attempts to get soluble EupfF for biochemical assay failed. Fortunately, an enzyme, EupF, highly homologous to EupfF (81% similarity/67% identity) was found in the genome of *Phoma* sp., which was suggested but not characterized as putative hDAase for the biosynthesis of eupenifeldin²³. Attempts to obtain soluble EupF (42.0 kDa) with a N-terminal His-tag from *Escherichia coli* BL21(DE3) achieved success with a final yield of ~4 mg L⁻¹ (Figure S11). To explore if the EupF catalyzed the dehydration of **5**, we perform the *in vitro* EupF assay with **5** as the sole substrate. Since the expected product **6** was extremely unstable, we added 20% glycerol in the *in vitro* system to trap **6**. Consequently, 60% of **5** convert to two main products, **6b** and **6c**, with molecular weights of 256 and 346 respectively (Figure S12) in the presence of EupF after 3 hours, while only 5% of **5** could be transformed with boiled EupF. Based on the MS and UV, **6b** and **6c** were suggested to be 1,4-nucleophilic addition products of **6** with glycerol and **5**, respectively. The results from *in vitro* assay supported that dehydration of **5** could occur spontaneously, but EupfF remarkably accelerated this process. Hence, the diene precursor **6** could be identified as tropolone *o*-quinone methide, which was too reactive to be detected directly.

With **5** and **8** in hand, we were able to examine whether EupfF directly involved in the DA reaction during the biosynthesis of **1** and **3**. We performed an *in vitro* reaction containing 50 mM Bicine buffer (pH 7.0), 10 μ M EupF, 2.0 mM **5** and 1.0 mM **8**. Whereas the control reaction with boiled EupF did not lead to apparent new products (Figure 4a, traces ii), EupF catalyzed the conversion of **5** and **8** into **1** (Figure 4a, traces i). The feature of **1** as enantiopure compound indicated the hDA reaction is enzymatically controlled since racemic products would be expected from spontaneous reaction. Interestingly, when one of the natural substrate **8** is switched to the unnatural substrate humulenol (**10**), which was obtained from expression of EupfG in *A. nidulans* by feeding humulene (**9**), in the *in vitro* EupF assay, two products **2** and **11** (1:1 ratio) were detected with 8% of **10** consumed (Figure 4a, trace iii). To obtain enough of **2** and **11** for structural elucidation, **9** was fed to *A. nidulans* co-expressing EupfABCDEF. Finally, compounds **2** and **11** were fully characterized by NMR and CD as epolone B and its isomer named isoeopolone B (Figure 4c), respectively. Trace amount of **2** and **11** were also detected (Figure 4a, trace iv) in the absence of EupF with only 2% of **10** consumed. Hence, we suggested 1) the cycloaddition between the reactive tropolone *o*-quinone methide **6** and dienophile (**8** or **10**) occurs spontaneously; 2) EupfF directly controls the stereoselectivity of hDA reaction in the case of natural substrate **8**; 3) unnatural substrate humulenol **10** is not recognized by EupfF, but undergoes spontaneous cycloaddition with **6**.

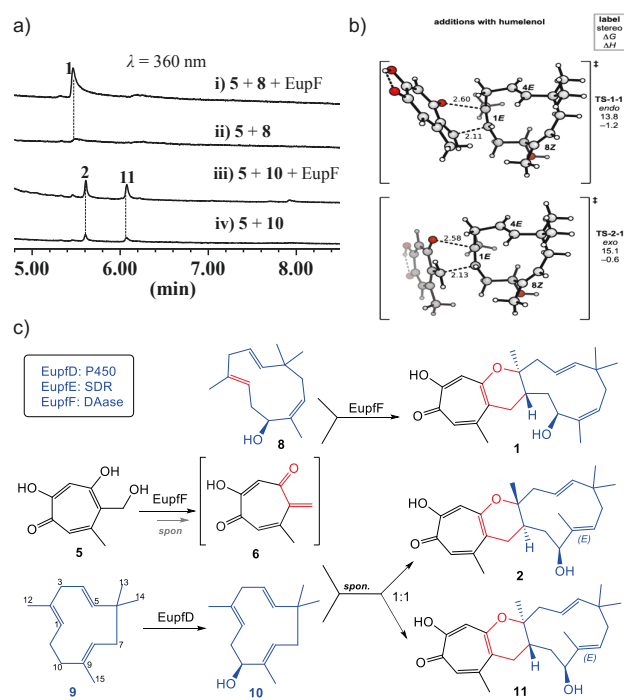


Figure 4. Biosynthesis of **1** and **2**. a) LCMS traces from *in vitro* assay of EupfF. b) Calculated transition state structures TS-1 and TS-2 and corresponding reaction barriers for hDA reaction between **6** and **8**. c) Biosynthetic pathway of **1**.

As a result, EupfF was fully characterized as the first hDAase in Nature, which differs from other intramolecular DAase in phylogenetic analysis (Figure S13) and has no recognizable cofactor-binding site. Model of EupfF (EupF) computed with SWISS-MODEL and template 2p4o suggest a predicted six-bladed propeller (Figure S14) which may provide suitable space for substrates.

To support the proposed pathway, we performed density functional theory (DFT) studies on the Diels–Alder reaction at the PBE0-D3(BJ)/def2-TZVP level of theory.^{24–29} These calculations indicate that this cycloaddition is very facile (Figure 4b, S15). The transition states TS-1 and TS-2 are highly- asynchronous with the two forming bonds differing by 0.49 and 0.45 Å, respectively. There is charge transfer in the transition state, in TS-1 the diene oxygen has a -0.4 partial charge and the 2-position of humulenol has a $+0.1$ partial charge. The charges are similar in TS-2. It is likely that the rate determining step is generation of the *o*-quinone methide **6**. After formation of **6**, the reaction barriers of TS-1 and TS-2 are purely entropic (TS-1 $\Delta H^\ddagger = -1.2$, $\Delta G^\ddagger = +13.8$ kcal/mol⁻¹, TS-2 $\Delta H^\ddagger = -0.6$, $\Delta G^\ddagger = +15.1$ kcal/mol⁻¹). These reactions are enthalpically barrier-less due to fact that the hetero-diene is highly electron-deficient and a highly stable aromatic product is formed. These results indicate that the role of the enzyme is to generate the highly reactive **6** and to orient it to react with the unactivated humulenol.

Unexpectedly, neither *in vitro* assay with **5** and **8** as substrates nor feeding experiments (Figure S15) led to production of **3** (only **1** was detected), which suggested that other or additional enzymes were required for the right side DA reaction for the biosynthesis of **3**. We are currently seeking to elucidate the biosynthetic pathways to bistropolone-sesquiterpenes, eupenifeldin and pycnidione.

In conclusion, we biochemically characterized EupfF (EupF) as the first intermolecular hetero-Diels–Alderase from fungi and demonstrated that EupfF catalyzed the generation of a reactive tropolone *o*-quinone methide and further stereo-selectively controlled the subse-

quent intermolecular hetero-Diels–Alder reaction with 1E,4E,8Z-humulenol to produce **1**. Our discovery expand the collection of increasing DAases from fungi and open a new strategy for mining and combinational biosynthesis of tropolone-sesquiterpenes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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Notes

The authors declare no competing financial interests.

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