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Biosynthesis of Polycyclic Natural Products from Conjugated Polyenes via Tandem Isomerization and Pericyclic Reactions

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Abstract

We report biosynthetic pathways that can synthesize and transform conjugated octaene and nonaene into complex natural products. The biosynthesis of (−)-PF1018 involves an enzyme PfB which can control the regio-, stereo-, and periselectivity of multiple reactions starting from a conjugated octaene. Using PfB as a lead, we discovered a homologous enzyme, BruB, that facilitates diene isomerization, tandem 8π-6π-electrocyclization, and a 1,2-divinylcyclobutane Cope rearrangement to generate a new-to-nature compound.

Graphical Abstract

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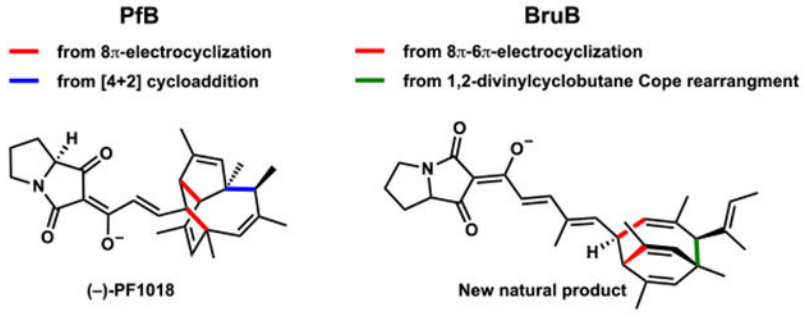
All authors have given approval to the final version of the manuscript.

Supporting Information

Additional experimental details, materials, and methods, including photographs of experimental setup. 1D, 2D NMR spectra for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.



Pericyclic reactions are powerful transformations to construct complex organic molecules.[1] Recently discovered enzymes from biosynthetic pathways that catalyze pericyclic reactions have been collectively name “pericyclases”.[2–18] While a number of well-known pericyclic reactions have corresponding pericyclases in Nature, enzymes facilitating electrocyclization have not been identified definitively (Figure 1A). Electrocyclization can convert conjugated polyenes to unsaturated rings or skipped polyenes, which can undergo additional pericyclizations to afford polycyclic scaffolds.[19–21] Nonenzymatic thermal 8π-6π-electrocyclization cascades are reported in the biosynthesis of molecules like SNF4435s[22] and shimalactones,[23] and the products are isolated as diastereomer pairs (Figure S2). Putative enzymatic 6π-electrocyclization have been reported in the formation of benzene rings,[24–26] although the roles of enzymes in regio- and stereocontrol of the final aromatic products are not clear. Therefore, discovering an enzyme that can control the stereochemical outcome of the electrocyclization reaction remains unresolved.

One biosynthetic puzzle that requires a stereoselective electrocyclization is (−)-PF1018 (**1**) isolated from *Humicola* sp. PF1018 (Figure 1B),[27] which has a tricyclo[6.3.1.$0^{5,9}$]-dodeca-2,6,10-triene core that features six stereocenters. Biomimetic synthesis of **1** by the Trauner group, together with computation by the Houk group, showed that a possible biosynthetic precursor could be a polyene such as the pre-PF1018 (**2**).[20] The linear **2** can undergo three regioselective *E* to *Z* isomerizations to trigger an 8π-electrocyclization, followed by an intramolecular Diels-Alder cyclization (Figure 1B).[20] The isolation, synthetic and computational studies collectively point to the involvement of one or more enzymes controlling the pericyclic reactions. First, **1** is isolated as a single diastereomer, unlike all other known natural products derived from 8π-electrocyclization.[23,28] This suggests the 8π step is stereoselective; second, transition state calculations of a model substrate showed 6π-electrocyclization would occur exclusively following the 8π-electrocyclization, when the conjugated olefin to the dienophile is unsubstituted. Indeed, only careful tuning of the protecting group in the synthesis achieved periselectivity towards the [4+2] reaction required for the formation of **1**. Hence, enzyme control of periselectivity at this step is also necessary. We show here that a single enzyme can transform **2** to **1**.

The polyene **2** is likely synthesized by a polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS). The PKS module should contain a methyltransferase (MT) domain, and should not contain an enoylreductase (ER) domain since no α,β–enoylreduction is required. The NRPS module would incorporate L-proline, while a

releasing domain would perform a Dieckmann cyclization to give the pyrrolo-tetramate. We sequenced the genome of *Humicola* sp. PF1018 and identified three biosynthetic gene clusters (BGCs) that encode PKS-NRPSs (Figure S3). One PKS-NRPS (PfA) that contains a MT domain but no ER domain, was selected for functional reconstitution (Figure 2A). Expression of PfA in the heterologous host *Aspergillus nidulans* ΔEMΔST[29] led to the biosynthesis of a new product with λ_{max}=426 nm and molecular weight (MW) that is consistent with that of **2** (Figure 2B, i). This compound was unstable and was purified under dark conditions (4.3 mg/L) (Supporting methods). NMR analysis confirmed **2** to be the proposed biosynthetic polyene precursor with an all *E* configuration and methyl groups at the five terminal olefins (Table S4 and Figures S34–S39).

We evaluated the reactivity of the polyene under thermal conditions by incubating **2** in a MeOH solution at 40 °C for three days, followed by product analysis (Figures 2C, iv and S10). A number of compounds were formed, with one minor product matched with a standard of **1** purified from the producing host (Table S3 and Figures S29, S30, and S33). Scaled-up reaction led to the isolation of the most abundant products **3-7** (Figures S11–S14). Structural characterizations with NMR and DFT calculation of ^{13}C NMR chemical shifts were performed. The major product **3** (Table S5 and Figures S40–S45) is a bicyclo[4.2.0]octadiene formed from isomerization of C6–C7 and C8–C9 olefins to **11**, followed by tandem 8π-6π-electrocyclization (Figure 2E). Isomerization of only the C8–C9 olefin to *Z* configuration in **12**, followed by 6π-electrocyclization is proposed to form the cyclohexadiene product **4** (Table S6 and Figures S46–S51). **5** contains a tricyclo[3.2.1.$0^{2,7}$]oct-3-ene core (Table S7 and Figures S52–S57). The stereochemistry of the substituents of the C6–C11 bond suggests that **5** is formed from a disrotatory 6π-electrocyclization of the 6*Z*,8*Z*-**11** to the cyclohexadiene **13**, followed by an intramolecular Diels-Alder cycloaddition.[30]

6 and **7** are diastereomers that feature the bicyclo[3.3.1]nona-2,6-diene core, also found in fungal natural product rugulosone (Tables S8, S9, S13, and S14 and Figures S58–S69).[31,32] We propose that **6** and **7** are each formed via a cationic cyclization mechanism from the diastereomeric cyclooctatriene **9** and **14**, respectively, which are products of nonenzymatic 8π-electrocyclization. This electrocyclization requires isomerization of **2** to give the 10*Z*,12*Z*,14*Z*-polyene **8**. Note that **8** and **9** are proposed intermediates to **1** (Figure 1B). Other minor products from the nonenzymatic reaction of **2** can be detected, but are present at quantities too minute for structural characterization. These studies show clearly that while **2** can be nonenzymatically converted to **1** under thermal conditions, a number of competing isomerization/electrocyclization/cyclization routes can take place without enzymatic control. Dedicated enzyme(s) that control the regioselective isomerization of **2** to **8**, followed by stereoselective electrocyclization to **9** and periselective [4+2] cycloaddition, must be functional during the biosynthesis of **1**.

The *pfB* gene immediately juxtaposed to *pfA* encodes a hypothetical protein PfB without any conserved domain or cofactor binding site. However, HHpred analysis of PfB showed high structural homology (>90 % probability) to NTF2-like enzymes such as NsrQ[33] (aa identity: 17 %) and AusH (15 %).[34] NTF2-like enzymes have been noted to catalyze a variety of reactions.[35,36] We coexpressed PfA and PfB together in *A. nidulans*. Which

led to production of **1** (0.6 mg / L) (Table S3, Figures 2B, ii, and Figures S31–S32). No diastereomers of **1** or other polyene derived-products can be detected in the extract, suggesting that PfB specifically controls the formation of **1** from **2**. To confirm the role of PfB, the C-His-tagged protein was purified from *Escherichia coli* BL21(DE3). When 1 mM **2** was incubated with 100 μM PfB in PBS (pH 8.0) at room temperature, **2** was completely converted to **1** in less than 1 hour, with no formation of shunt products **3-7** (Figures 2C, i–iii, and Figures S5–S6). Temperature, pH, and buffer conditions did not significantly affect the activity of PfB (Figures S7–S9).

The *in vivo* and *in vitro* data demonstrate that PfB is involved in three reactions converting the reactive polyene portion of **2** to the tricyclo[6.3.1.$0^{5,9}$]dodeca-2,6,10-triene seen in **1**: 1) PfB facilitates regioselective isomerization of **2** to the 10*Z*,12*Z*,14*Z*-isomer **8**, which eliminates formation of shunt products **3-5**. As in previous examples of polyene isomerization in biosynthesis,[37] the proximities of the C10, C12, and C14 methyl groups results in a nonplanar polyene that may lead to more facile *E*/*Z* isomerization. In addition, these methyl groups may also promote protonation of the olefins to yield tertiary carbocations for isomerization; 2) Whereas nonenzymatic 8π-electrocyclization gives an equal mixture of conrotatory stereoisomers, such as in the case of shimalactone,[23] PfB controls stereoselective 8π-electrocyclization through a helical conrotatory transition state[38] to yield the single cyclooctatriene **9** and prevents formation of **14** (and **7**); 3) PfB can facilitate the isomerization of the C6–C7 olefin in **9** to **10**, followed by the [4+2] cycloaddition to afford the final product **1**. This suppresses the cationic rearrangement that forms **6**, as well as the computationally predicted, energetically favorable 6π-electrocyclization.[20] Therefore, PfB can be considered as a multifunctional isomerase/pericyclase, and is the first example of an enzyme that facilitates stereoselective electrocyclization.

The pairing of PfA and PfB in biosynthesis of **1** demonstrates an efficient chemical logic in generation of polycyclic scaffold. We searched for similar gene pairings that may produce new polyene-derived compounds. Eight hits, mainly distributed in Sordariaceae (Figure S4), were identified from the NCBI fungal genome database. One pair (*bruA* and *bruB*) (Figure 3A), from *Sphaerosporella brunnea* was chosen for heterologous expression due to strain availability. Expression of the PKS-NRPS BruA (55% sequence identity to PfA) resulted in the formation of a new pyrrolo-tetramate polyene **15** with λ_{max}=451 nm (3.8 mg/L) (Figure 3B, i). Detail NMR and HRESIMS analyses of **15** isolated from *A. nidulans* confirmed **15** has one additional conjugated olefin compared to **2** (Table S10 and Figures S70–S75). Similar to **2**, **15** can undergo nonenzymatic transformations under thermal conditions, but the metabolites were not characterized (Figure S15). Coexpression of the hypothetical protein BruB (41 % sequence identity to PfB) led to formation of **18** and **19**, both with λ_{max} = 370 nm (Figure 3B, ii). However, the titers of these two compounds from the heterologous host were insufficient for isolation and characterization.

We next examined *in vitro* reaction with **15** and recombinant BruB expressed from *E. coli*. Surprisingly, incubation of **15** and BruB at 20 °C led to formation of only **18** (Figure S16), while increasing temperature to 37 °C led first to the formation of **18**,

followed by the formation of **19** (Figures 3C and S17). This suggests that **18** may be an intermediate to **19**. Using **15** isolated from *A. nidulans*, we performed *in vitro* reaction in the presence of BruB, followed by HPLC isolation of **18** and **19** for NMR characterization (Tables S11–S12, Figures S76–S87). In **18**, the polyene portion of **15** is cyclized into the bicyclo[4.2.0]octadiene that is substituted with 3-methyl-2,4-hexadiene at C13 (Figure 3E). Therefore, **18** is formed by olefin isomerization to the 8*Z*,10*Z*-intermediate **16** under the control of BruB, followed by stereoselective 8π-6π-electrocyclization. Using purified **18**, we performed a second enzymatic reaction with BruB (Figures 3D and S18). At higher temperatures, we observed BruB-dependent conversion of **18** to **19**. Extensive NMR analysis revealed **19** contains a bicyclo[4.2.2]-deca-2,5,8-triene core in which a new C10–C15 bond is formed, while the C12–C13 bond in **18** is broken.

We propose BruB, in addition to facilitating the isomerization and controlling the electrocyclization reactions to convert **15** to **18** (Figure 3E), can subsequently catalyze a [3,3]-Cope rearrangement of the 1,5-diene system (between C10 and C15) in **18** to give **19** at elevated temperature. The transformation from **18** to **19** is the classic 1,2-divinylcyclobutane Cope rearrangement through a boat-like transition state.[39,40] Computation analysis confirmed the rearrangement is energetically concerted, with **18** and **19** having nearly the same energy (Figure S19). However, the $\Delta G^{\ddagger}$ of the Cope reaction transition state is ~33 kcal/mol, making it a highly challenging transformation in the absence of BruB. Indeed, heating **18** in water at 100 °C did not lead to any detectable amount of **19** (Figure S20). The rearrangement catalyzed by BruB is slow with k_{cat} = 0.060 ± 0.004 min^{-1} and K_M = 86.6 ± 19.7 μM (Figure S21), and the activity of BruB-catalyzed reactions is not affected by pH or buffer conditions (Figures S22–S25). In addition, the reverse Cope rearrangement from **19** to **18** can also be observed only in the presence of BruB (Figure S26). Therefore, the BruB-catalyzed conversion of **18** into **19** represents the first example of an enzyme-catalyzed, reversible [3,3]-Cope rearrangement. Lastly, mixing and matching of **2** made by PfA with BruB, or **15** made by BruA with PfB, did not yield any cyclized products (Figures S27–S28), suggesting these polyene-modifying enzymes are substrate specific.

In summary, although the mechanisms of rate acceleration and stereoselectivity by PfB and BruB are not resolved in the absence of cocrystal structure complexes, our results demonstrate how a single enzyme can dramatically morph an extended and reactive polyene into specific polycyclic products. These findings add electrocyclization to the growing list of pericyclic reactions that can be controlled by enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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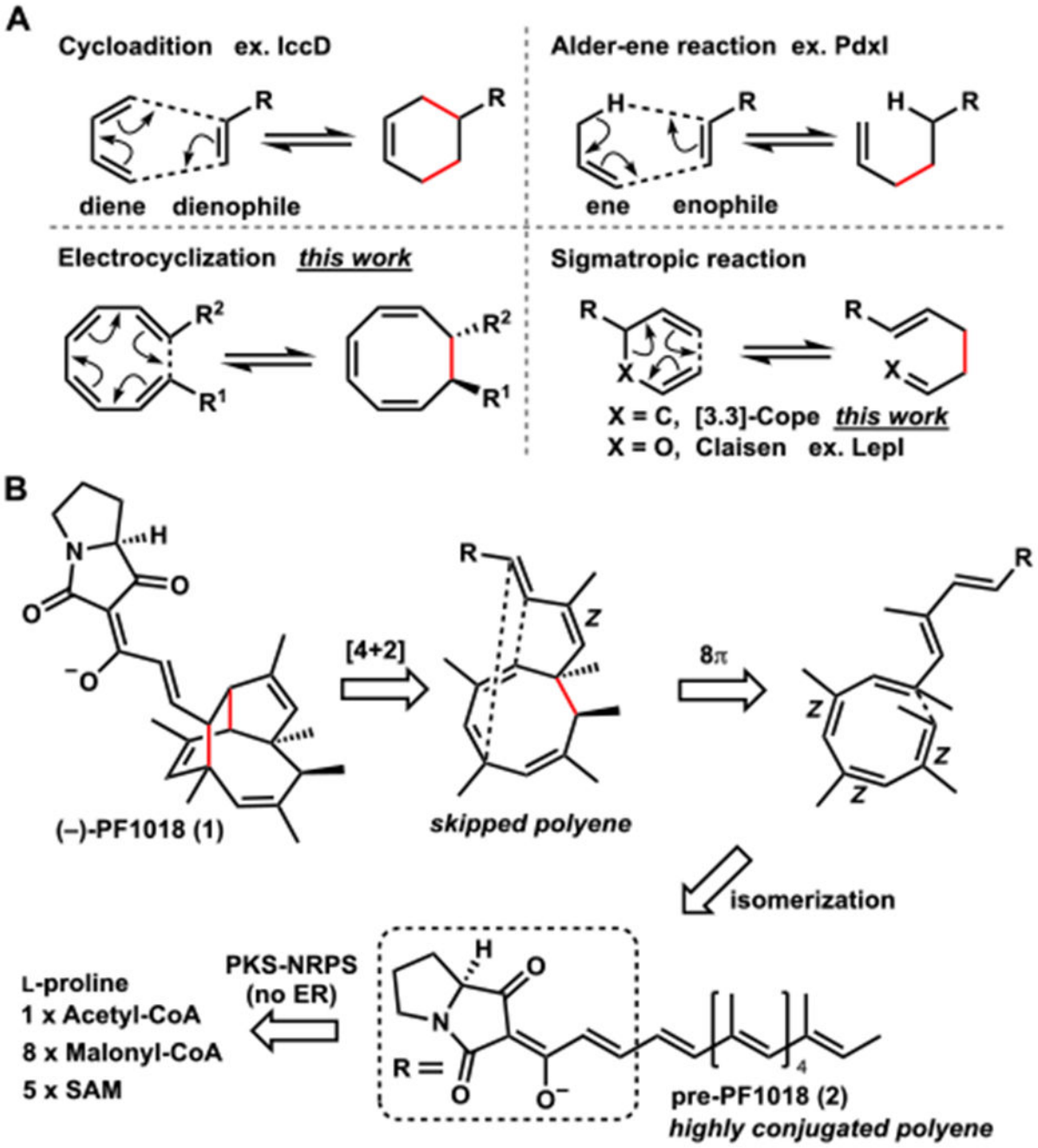

Figure 1.

(**A**) Representative enzymatic pericyclic reactions; (**B**) Retro-biosynthetic analysis of **1**.

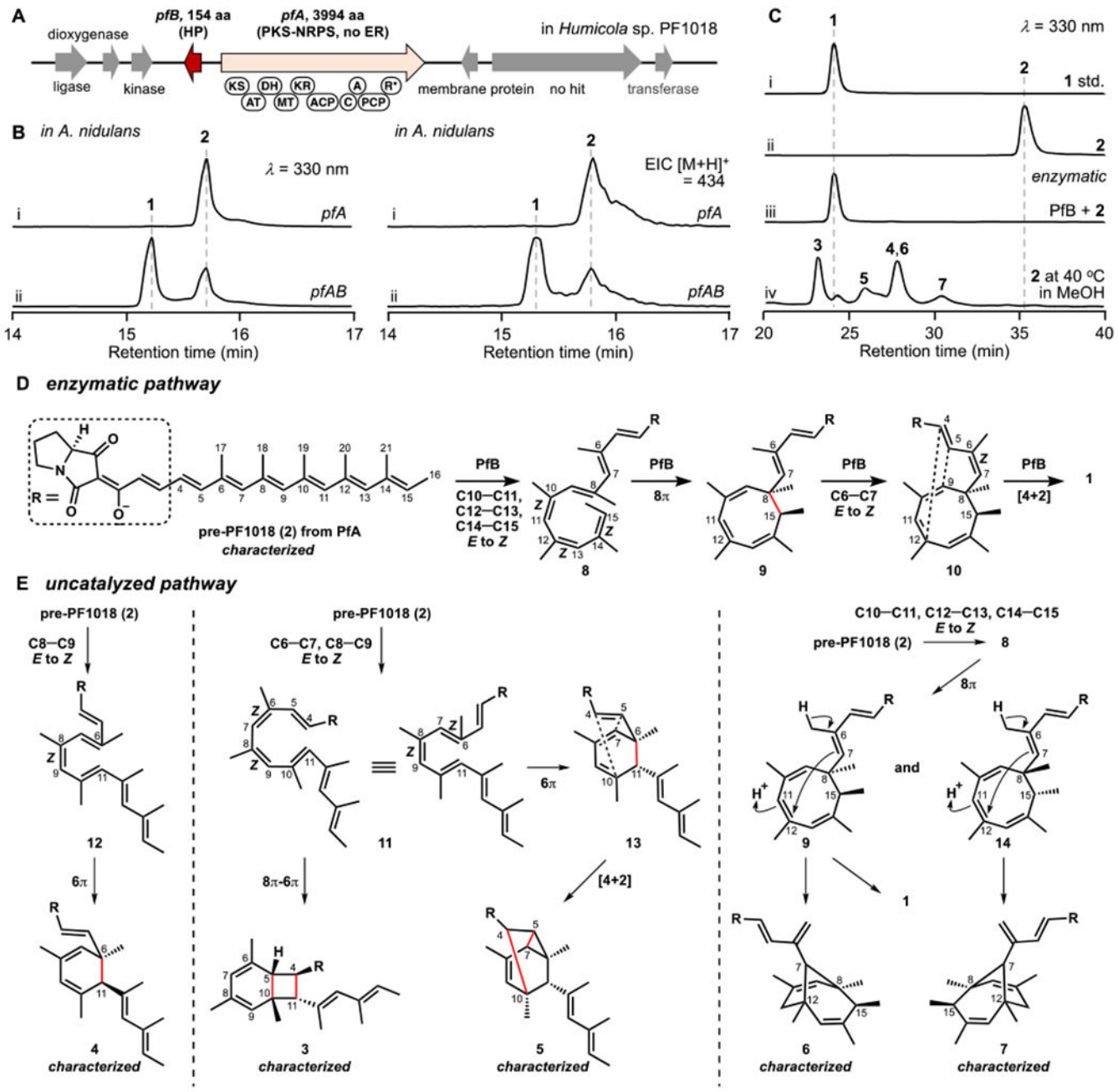

Figure 2.

Characterization of (−)-PF1018 (**1**) biosynthesis. (**A**) (−)-PF1018 BGC. The *pf* cluster encodes a PKS-NRPS and a hypothetical protein (HP). (**B**) Analysis of metabolites from *A. nidulans* upon overexpression of (i) *pfA*. (ii) *pfA* and *pfB*. (**C**) *in vitro* reaction of 1 mM **2** with 100 μM PfB for 1 h at room temperature. (i) standard of **1**. (ii) **2** in buffer at room temperature. (iii) **2** with PfB. (iv) HPLC profile for heating **2** in MeOH/H_2O (1:1) at 40 °C for 3 days. (**D**) PfB-controlled transformation of **2** to **1**. (**E**) Uncatalyzed transformations of **2**.

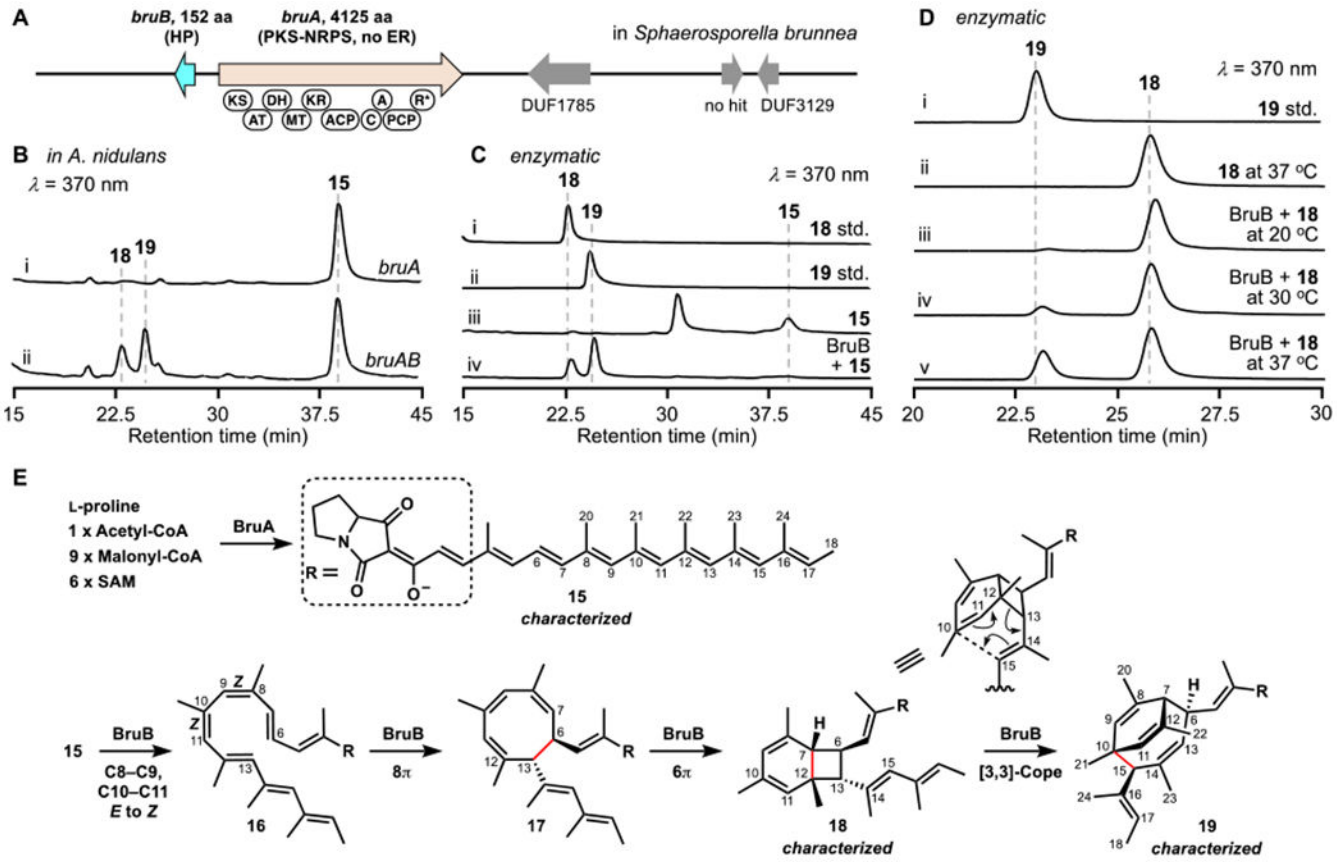

Figure 3.

Genome mining of new polyene-derived compounds. (**A**) The *bru* BGC. (**B**) Analysis of metabolites from *A. nidulans* upon overexpression of (i) *bruA*. (ii) *bruA* and *bruB*. (**C**) *in vitro* reaction of 1 mM **15** with 100 μM BruB for 3 h at 37 °C. (i) standard of **18**. (ii) standard of **19**. (iii) **15** in buffer at 37 °C. (iv) **15** with BruB. (**D**) *in vitro* reaction of 1 mM **18** with 100 μM BruB for 3 h at different temperature. (i) standard of **19**. (ii) **18** in buffer at 37 °C. (iii) **18** with BruB at 20 °C. (iv) **18** with BruB at 30 °C. (v) **18** with BruB at 37 °C. (**E**) Proposed reactions catalyzed by BruA and BruB.