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海 外 特 別 研 究 員 最 終 報 告 書

独立行政法人日本学術振興会 理事長　殿

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海外特別研究員としての派遣期間を終了しましたので、下記のとおり報告いたします。
なお、下記及び別紙記載の内容については相違ありません。

記

1-1. 用務地（派遣先国名）用務地：　University of California, Los Angeles（国名：　米国　　）

1-2. 研究課題名（和文）※研究課題名は申請時のものと違わないように記載すること。

モレキュラーグルー創出を目指した、多機能型 electrocyclase の解明

1-3. 派遣期間:令和　5 年　4 月　1 日 ～ 令和 6 年　7 月　5 日（ 462 日間）

1-4.　受入研究機関名及び部局名

受入研究機関名：　University of California, Los Angeles

部局名：　chemistry and bio engineering

1-5. 海外特別研究員の研究課題の遂行状況…書式任意　**書式任意（A4 判相当 3 ページ以上、英語で記入も可）**

【記載事項】

・　研究・調査実施状況及びその成果の発表・関係学会への参加状況等

Pericyclic reactions are powerful transformations to construct contiguous stereogenic carbon centers.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). Electrocycliza-tion can convert conjugated polyenes to unsaturated rings or skipped polyenes, which can undergo addi-tional pericyclizations to afford polycyclic scaffolds.19–21 Nonenzymatic 8π-6π-electrocyclization cascades are reported in the biosynthesis of molecules like SNF4435s22 and shimalactones,23 and the products are isolated as diastereomer pairs (Figure S2). Putative en-zymatic 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 en-zyme that can control the stereochemical outcome of the electrocyclization reaction remains unresolved.

One biosynthetic puzzle that requires a stereoselec-tive electrocyclization is (−)-PF1018 (1) isolated from Humicola sp. PF1018 (Figure 1B),27 which has a tricy-clo[6.3.1.05,9]-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), which can undergo three regioselective E to Z isomerizations to trigger an 8π-electrocyclization, followed by an intramo-lecular Diels-Alder cyclization (Figure 1B).20 Collective-ly, isolation, synthetic and computational studies strong-ly point to the involvement of one or more enzymes con-trolling 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 tun-ing of the protecting group in the synthesis achieved periselectivity towards the [4+2] reaction required for the formation of 1. Hence, enzyme control of periselec-tivity 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 methyltrans-ferase (MT) domain, while should not contain an enoylreductase (ER) domain since no □□□–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 do-main, was selected for functional reconstitution (Figure 2A). Expression of pfA in the heterologous host Asper-gillus nidulans □EM□ST29 led to the biosynthesis of a new product with □max=426 nm and molecular weight (MW) that is consistent with that of 2 (Figure 2B, i). This com-pound was unstable and was purified under dark con-ditions (4.3 mg/L) (Supporting methods). NMR analy-sis 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 Fig-ures S34-S39).

We evaluated the reactivity of the polyene under thermal conditions by incubating 2 in a MeOH solution at 40 oC 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 stand-ard 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 13C 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, fol-lowed by tandem 8π-6π-electrocyclization (Figure 2E). Isomerization of only the C8–C9 olefin to Z configura-tion in 12, followed by 6π-electrocyclization is proposed to form the cyclohexadiene product 4 (Table S6 and Figures S46-S51). 5 contains a tricyclo[3.2.1.02,7]oct-3-ene core (Table S7 and Figures S52-S57). The stereo-chemistry of the substituents of the C6–C11 bond sug-gests that 5 is formed from a disrotatory 6π-electrocyclization of the 6Z,8Z-11 to the cyclohexadiene 13, followed by an intramolecular Diels-Alder cycload-dition.30

6 and 7 are diastereomers that feature the bicy-clo[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 8□-electrocyclization. This electrocyclization requires isomerization of 2 to give the 10Z,12Z,14Z-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 num-ber of competing isomerization/electrocyclization/cyclization routes can take place without enzymatic control. Dedicated enzyme(s) that control the regioselective isomerization of 2 to 8, fol-lowed by stereoselective electrocyclization to 9 and periselective [4+2] cycloaddition, must be functional during the biosynthesis of 1.

The 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 NsrQ33 (aa identity: 17 %) and AusH (15 %).34 NTF2-like enzymes have been noted to catalyze a variety of reactions.35,36 We co-expressed 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 □M PfB in PBS (pH 8.0) at room temperature, 2 was completely con-verted 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 didn't affect the activi-ty of PfB-catalyzed conversion of 2 to1 (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[6.3.1.05,9]dodeca-2,6,10-triene seen in 1: 1) PfB facilitates regioselective isomerization of 2 to the 10Z,12Z,14Z-isomer 8, which eliminates formation of shunt products 3-5. As in previ-ous examples of polyene isomerization in biosynthe-sis,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) Where-as 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 transi-tion state38 to yield the single cyclooctatriene 9 and pre-vents 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, energetical-ly favorable 6π-electrocyclization.20 Therefore, PfB can be considered as a multifunctional isomer-ase/pericyclase, and is the first example of an enzyme that facilitates stereoselective electrocyclization.

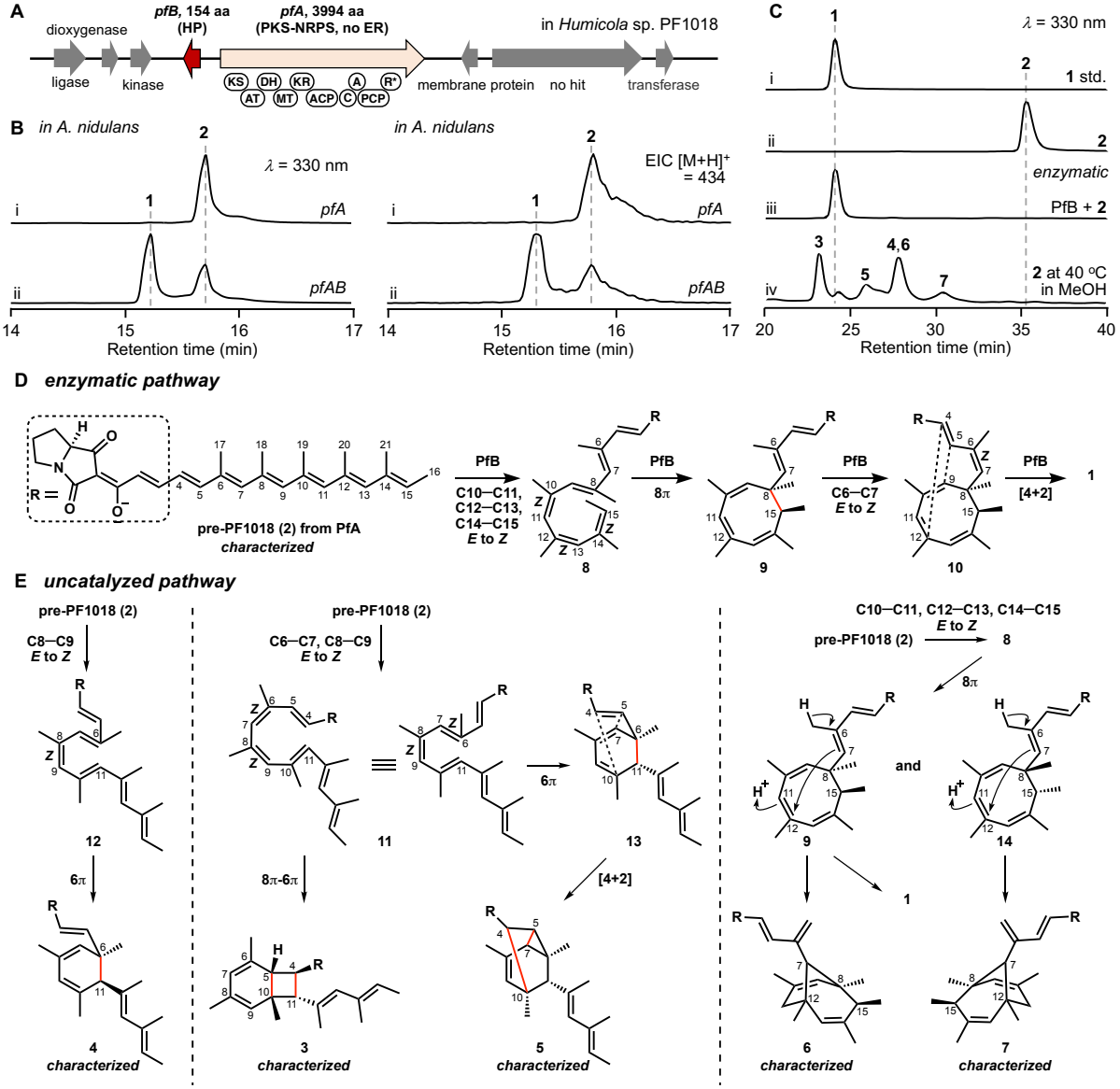

Figure 2. Characterization of (−)-PF1018 (**1**) biosynthesis. (**A**) (−)-PF1018 BGC. The *pf* cluster encodes a PKS-NRPS and a hypothetical protein (HP). (**B**) Analysis of metabolites from *A. nidulans* upon overexpression of (i) *pfA*. (ii) *pfA* and *pfB*. (**C**) *in vitro* reaction of 1 mM **2** 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**.

The pairing of PfA and PfB in the biosynthesis of 1 demonstrates an efficient chemical logic in generation of complex polycyclic scaffold. We next searched for similar gene pairings that may produce new polyene-derived compounds. Eight hits, mainly distributed in Sordariaceae, were identified from the NCBI fungal genome database. One pair (bruA and bruB), from Sphaerosporella brunnea was chosen for heterologous expression

due to availability of the strain. Expression of the PKS-NRPS BruA (55 % sequence identity to PfA) resulted in the formation of a new pyrrolo-tetramate polyene 15 with lmax=451 nm (3.8 mg/L). Detail NMR and HRESIMS analyses of 15 isolated from A. nidulans confirmed 15 has one additional conjugated olefin compared to 2.

We next examined in vitro reaction with 15 and recombinant BruB expressed from E. coli. Surprisingly, incubation of 15 and BruB at 20 oC led to only formation of 18, while increasing temperature to 37 oC led first to the formation of 18, followed by the formation of 19. This suggests 18 is an intermediate to 19. Using 15 isolated from A. nidulans, we performed larger scale in vitro reaction in the presence of BruB, followed by HPLC isolation of 18 and 19 for NMR characterization. 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, similar to that in 3. Therefore, 18 is clearly the product of olefin isomerization to the 8Z,10Z-intermediate 16, followed by stereoselective 8p-6p-electrocyclization under the control of BruB. With purified 18 in hand, we performed a second enzymatic reaction with BruB at different temperatures. At higher temperatures (30 oC and 37 oC), we observed BruB-dependent conversion of 18 to 19. Extensive NMR analysis of 19 purified from the scaled-up reaction revealed 19 contains a new 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 catalyzing the isomerization and electrocyclization reactions to convert 15 to 18, 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.

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 multifunctional isomerase/pericyclase can dramatically morph an extended polyene into polycyclic structures. These findings add electrocyclization to the growing list of pericyclic reactions that can be catalyzed and controlled by enzymes.

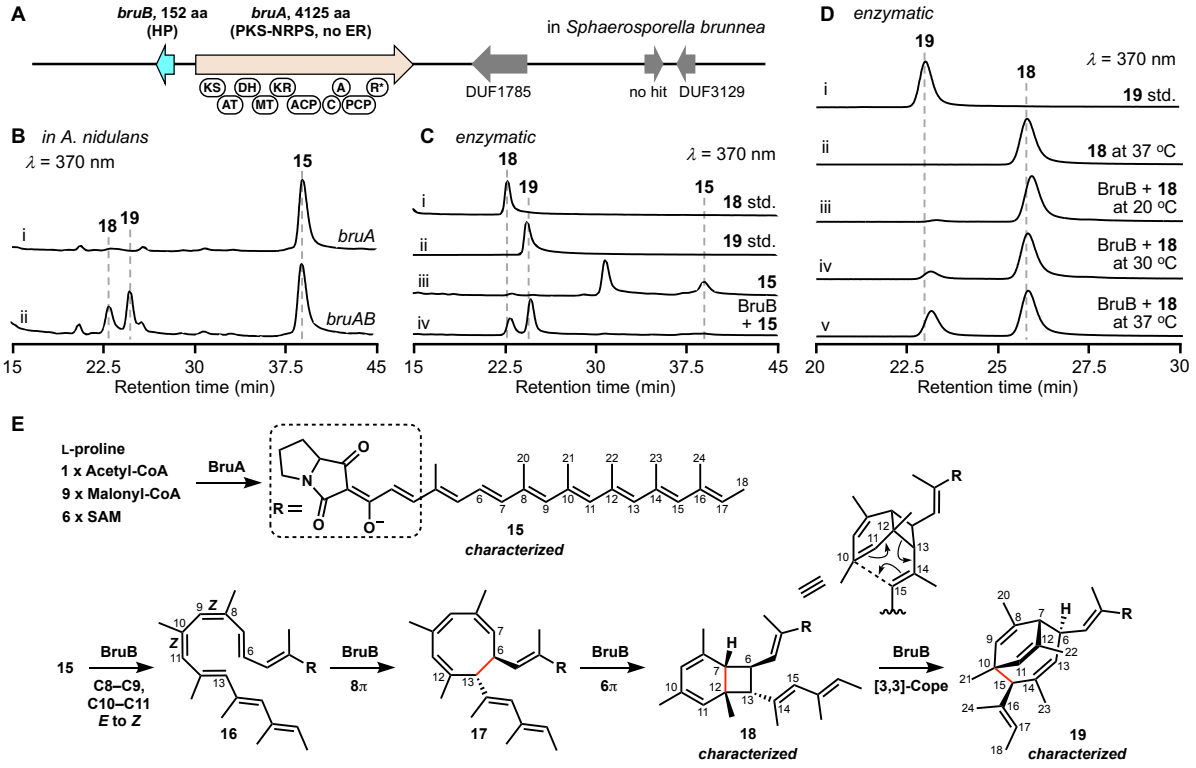

Figure 3. Genome mining of new polyene-derived compounds. (**A**) The *bru* BGC. (**B**) Analysis of metabolites from *A. nidulans* upon overexpression of (i) *bruA*. (ii) *bruA* and *bruB*. (**C**) *in vitro* reaction of 1 mM **15** with 100 μM BruB for 3 h at 37 °C. (i) standard of **18**. (ii) standard 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.

(reference)

Niwa, K., Ohashi, M.*, Xie, K., Chiang, C. Y., Jamieson, C. S., Sato, M., Watanabe, K., Liu, F., Houk, K. N.*, Tang, Y.* "Biosynthesis of Polycyclic Natural Products from Conjugated Polyenes via Tandem Isomerization and Pericyclic Reactions." **2023**, *J. Am. Chem. Soc.* 145, 13520–13525.