Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

J Am Chem Soc. Author manuscript; available in PMC 2018 April 19.

Published in final edited form as:

J Am Chem Soc. 2017 April 19; 139(15): 5317–5320. doi:10.1021/jacs.7b02432.

Collaborative Biosynthesis of Maleimide- and Succinimide-Containing Natural Products by Fungal Polyketide Megasynthases

Michio Sato[1,3], **Jacob E. Dander**[2], **Chizuru Sato**[1], **Yiu-Sun Hung**[1], **Shu-Shan Gao**[1], **Man-Cheng Tang**[1], **Leibniz Hang**[2], **Jaclyn M. Winter**[1,4], **Neil K. Garg**[2,*], **Kenji Watanabe**[3,*], and **Yi Tang**[1,2,*]

[1]Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, California 90095, United States

[2]Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90095, United States

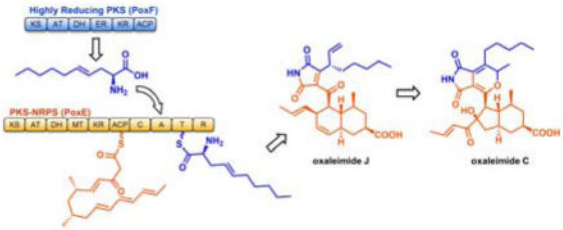
[3]Department of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan

[4]Medicinal Chemistry Department, University of Utah, Salt Lake City, Utah, 84112

Abstract

Fungal polyketide synthases (PKSs) can function collaboratively to synthesize natural products of significant structural diversity. Here we present a new mode of collaboration between a highly reducing PKS (HRPKS) and a PKS-nonribosomal peptide synthetase (PKS-NRPS) in the synthesis of oxaleimides from the *Penicillium* species. The HRPKS is recruited in the synthesis of an olefin-containing free amino acid, which is activated and incorporated by the adenylation domain of the PKS-NRPS. The precisely positioned olefin from the unnatural amino acid is proposed to facilitate a scaffold rearrangement of the PKS-NRPS product to forge the maleimide and succinimide cores of oxaleimides.

Graphical Abstract



Corresponding Author: yitang@ucla.edu, kenji55@u-shizuoka-ken.ac.jp, neilgarg@chem.ucla.edu.

Notes

The authors declare no competing financial interests.

Supporting Information

Experimental details, spectroscopic and computational data. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Fungal polyketides, synthesized by iterative polyketide synthases (IPKSs), comprise a structurally diverse family of natural products, displaying a wide range of biological activities.[1] Standalone IPKSs are capable of assembling complete polyketide scaffolds, such as aromatic compounds by nonreducing PKSs (NRPKS),[1c] or more reduced products by highly reducing PKSs (HRPKSs).[1b] Nature can increase structural diversity of polyketides by collaboratively pairing IPKSs in the same biosynthetic pathway.[1a] For example, pairing of a HRPKS and a NRPKS in series can lead to the synthesis of compounds with both aromatic and reduced structural features, such as radicicol[2] and chaetoviridin A (Figure 1A).[3] Collaborative pairing of two HRPKSs also enables the parallel construction of two separate parts of the final natural product, as illustrated in the biosynthesis of lovastatin.[4] Collaborative IPKSs are frequently colocalized in the same biosynthetic gene cluster, a genetic feature that can be utilized in the genome mining of new natural products.[5]

One strategy to install nitrogen-containing functional groups in fungal polyketides is used by hybrid megasynthases known as IPKS-nonribosomal peptide synthetases (PKS-NRPSs).[6] The *cis*-acting NRPS module can amidate the completed polyketide acyl chain with a specific amino acid selected by the adenylation domain. The aminoacyl polyketide can then be cyclized into compounds that contain a nitrogen heterocycle, such as tetramate. Hence, one would expect pairing a PKS-NRPS with other IPKSs can lead to additional structural diversity. Here we demonstrate a new mode of collaboration between a HRPKS and a PKS-NRPS, in which the HRPKS is responsible for biosynthesis of a free, unnatural amino acid that is subsequently incorporated by the PKS-NRPS to generate new succinimide and maleimide natural products.

During our genome mining efforts, we found two homologous biosynthetic gene clusters in *Penicillium oxalicum* (*pox*) and *Penicillium aethiopicum* (*pae*) that encode a conserved pair of HRPKS (*poxF*) and PKS-NRPSs (*poxE*) (Figure 2A and Table S2). In addition, several other notable tailoring enzymes are also conserved (>80% identity), including a dissociated enoylreductase (ER) as partner of the PKS-NRPS (*poxP*), a decalin-forming Diels-Alderase (*poxQ*),[7] an α/β-hydrolase (*poxO*), three P450 monooxygenases (*poxC, poxD* and *poxM*), and an aminotransferase (*poxL*). RT-PCR analysis showed that both clusters were transcriptionally silent when cultured under laboratory conditions (See SI and Figure S6). To activate transcription of the *pox* cluster, we overexpressed a Gal4-like transcription factor, PoxB, that is encoded in the gene cluster.[8] The *poxB* gene was placed under the P*gpdA* promoter and was ectopically integrated into the genome of *P. oxalicum* to give the strain POX3. RT-PCR analysis of POX3 grown on PDA showed that the *pox* genes were transcribed (Figure S6). Subsequently, metabolites from POX3 were extracted and analyzed by LC/MS. Compared to the wild type strain, which produced secalonic acid D (*)[9] and linolenic acid (**) as major products (Figure 2B, ii), POX3 produced an array of compounds (**1–10**) upon PoxB overexpression (Figure 2B, i). We then selectively inactivated either the HRPKS *poxF* or the PKS-NRPS *poxE* in the POX3 strain (Supporting Information). Both strains lost the ability to produce all of the compounds (Figure 2B, iii and iv), establishing the necessity of both megasynthases in the biosynthesis of **1–10**.

Each of the ten compounds were characterized to be new natural products. The most abundant compounds oxaleimides A (**1**), B (**2**) and C (**3**) all contain an unusual disubstituted

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

succinimide moiety.[10] At the 3 position of the succinimide ring in **1** and **2** (or C18 in Figure 1), both compounds contain a branched aliphatic chain that has a terminal olefin (oct-1-en-3-yl). **1** contains a substituted *trans*-decalin ring that is carboxylated at C7. To determine the absolute stereochemistry at C7 in **1,** the carboxylate group was converted into (*R*)- and (*S*)-PGME amides (**11a** and **11b**),[11] which allowed assignment of the absolute configuration at C7 to be *R* (Figure S7). This stereochemistry was further confirmed by X-ray crystal structure of **11a** (Figure 2C). The absolute stereochemistry of the remaining compounds was assigned based on that of **1.** In contrast to **1**, the southern portion of **2** is a 5–6 bicyclic ring system. Compound **3** was isolated as a diastereomeric pair (**3** and **3′**) and contains a pyran ring fused to the succinimide.

Oxaleimide I (**9**) is nearly identical to **1**, except that the succinimide core is oxidized to a maleimide. A related compound TAN-1813 isolated from *Phoma* sp. FL-41510 (Figure 1B) is a Ras-farnesyltransferase inhibitor.[12] Compared to TAN-1813, **9** differs in substituents around the *trans*-decalin ring. Oxaleimide J (**10**) results from migration of the maleimide double bond in **9**. Lastly, compounds **4–8** are likely derived from oxidation of the decalin ring in **1** (Figure 3). We tested the cytotoxicity of the compounds toward HeLa cells using a cell viability assay (Supporting Information). Whereas **1** and **2** exhibited no activity, the maleimide **9** displayed cytotoxicity with an IC_{50} of 0.32 μM. Compounds **3** and **10** showed weaker toxicity with IC_{50} values ~ 4.0 μM (Figure S8).

The *trans*-decalin compounds isolated in this study, such as **1**, **9**, and **10** are biosynthetically related to other compounds derived from the combined actions of PKS-NRPSs and Diels-Alderases, including equisetin[13] and myceliothermophin.[7b] The key difference, is the replacement of the tetramate or 3-pyrrolin-2-one, derived from Dieckmann or Knoevenagel condensation of the aminoacyl precursor, respectively, with succinimide or maleimide. Additionally, the substituted allyl group at C18 is an unusual feature not seen among fungal polyketides. To probe the origin of the aliphatic group, we performed a feeding experiment using [1,2-$^{13}C_2$]-acetate and determined the ^{13}C enrichment of **1** (Figure 1B, S17 and Table S4). The labeling studies showed that the substituent at C18 is of polyketide origin. Notably, the terminal olefin is derived from one intact unit of acetate, suggesting that a carbon skeletal rearrangement is required in the biosynthesis of **1**.

To test this hypothesis, we chemically synthesized **14** using a two-step metathesis, deprotection reaction sequence from *N*-Fmoc-(L)-allylglycine, and supplemented it to either the Δ*poxE* or Δ*poxF* mutant. Whereas no oxaleimide production was restored in the former, addition of **14** led to near complete restoration of oxaleimide production in Δ*poxF* (Figure 2F, iii). These results suggested that the HRPKS PoxF is directly involved in the biosynthesis of **14**. A notable example of a HRPKS involved in the biosynthesis of an unnatural amino acid is that of 4*R*-4-[(*E*)-2-butenyl]-4-methyl-threonine (Bmt) synthase found in the cyclosporine pathway (Figure S9).[15] A P450 and an aminotransferase, encoded in tandem in the cyclosporine cluster,[16] were proposed to catalyze α-oxidation and transamination of the polyketide to yield Bmt, respectively. In the *pox* pathway, the same tandem arrangement of genes encoding a P450 (*poxM*) and an aminotransferase (*poxL*) is also observed (Figure 2A), providing further support for a dedicated pathway in the synthesis of **14.**

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

The functions of PoxF, M and L were assayed using *A. nidulans* as a heterologous host (Figure 2E). Expressing the HRPKS alone did not yield any detectable product (Figure 2E, ii), while expressing PoxF and PoxM led to the formation of two new products: 2-hydroxydec-4-enoic acid (**15**) and 2-oxodec-4-enoic acid (**16**) (Figure 2E, iii). Further addition of the aminotransferase PoxL led to production of **14** (Figure 2E, iv and v). The role of the PoxL was verified in an enzyme assay during which **16** was reductively aminated to **14** using aspartic acid as the optimal amine donor (Figure S10). The intermediacy of **15** was confirmed when addition of **15** to *P. oxalicum* Δ*poxF* restored the biosynthesis of **1–10** (Figure 2F, ii). We propose a biosynthetic pathway to **14** as shown in Figure 2G. The HRPKS PoxF is recruited by the pathway to synthesize an ACP-bound dec-4-enoate, which was not detected as a free acid in the extract of *A. nidulans* expressing PoxF only. PoxM-catalyzed oxidation at the α-position creates the enzyme-bound 2-hydroxydec-4-enoyl-ACP thioester, which may be prone to spontaneous hydrolysis to yield **15** due to increased electrophilicity of the carbonyl. **15** can then be further oxidized by PoxM to yield the α-ketoacid **16**, which is reductively aminated by PoxL to yield **14.**

The HRPKS PoxF hence serves in a new mode of collaborative biosynthesis with the PKS-NRPS PoxE, by providing the olefin containing amino acid **14**. The internal olefin is essential, as feeding of the saturated 2-aminodecanoic acid to *P. oxalicum* Δ*poxE* did not restore biosynthesis of **1–10**. Therefore, the pathway strategically uses the highly programmed nature of PoxF to introduce a single double bond at a specific position. The importance of the double bond in the subsequent steps is shown in the proposed biosynthetic pathway (Figure 3). Following release of aldehyde **13**, the Knoevenagel condensation affords **18** which is then oxidized at the 5-position of the pyrrolidone ring to give **19**. This modification is observed in several related natural products.[7b, 17] We propose the presence of the olefin from the amino acid building block allows for migration of the substituted allyl group to occur. This allylic transposition reaction takes place in a conjugate addition, semipinacol-like fashion to yield the succinimide **21**. This rearrangement is consistent with the labeling pattern established through [1,2-$^{13}C_2$]-acetate feeding experiments. Iterative two-electron oxidations of the C7 methyl in **21** to the carboxylic acid can be catalyzed by one of two remaining P450s (PoxC or PoxD) to yield **1**. Subsequent oxidation yields the maleimide scaffold **9.** Both **1** and **9** can undergo oxidative modifications in the decalin ring to yield the series of products **2–8**. As shown for **9,** we propose ring contraction of the *trans*-decalin ring can be initiated by oxidation at C2 and C3 to give **22**, which can undergo a semipinacol rearrangement to afford **23**. Isomerization of the terminal olefin in **23** to **24**, followed by 6π-electrocyclization to give the pyran **3** (and **3′**). **2** is likely the terminal product of the same oxidation/rearrangement cascade starting from **1**, although a number of shunt products **4–8** were isolated.

In summary, we have discovered a set of new natural products, named oxaleimides, of which the maleimide members are cytotoxic. Maleimide containing natural products are rare and only a few examples are known.[12, 18] Formation of the maleimide requires a new mode of collaboration between two fungal megasynthases. Such collaboration expands the biosynthetic capabilities of fungal PKSs and the chemical space of polyketide natural products.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the NIH (1DP1GM106413 and 1R35GM118056 to YT, R01GM117016 to NKG and T32GM067555 to JED) and JSPS Program for Advancing Strategic International Networks to Accelerate the Circulation of Talented Researchers (No. G2604 to K.W.). M.S. is supported by Fellowship of Astellas Foundation for Research on Metabolic Disorders. We thank S.I. Khan for assistance in solving the X-ray structures. Chemical characterization studies were supported by shared instrumentation grants from the NSF (CHE-1048804) and the NIH NCRR (S10RR025631).

References

1. (a) Chooi YH, Tang Y. J Org Chem. 2012; 77:9933. [PubMed: 22938194] (b) Cox RJ. Org Biomol Chem. 2007; 5:2010. [PubMed: 17581644] (c) Crawford JM, Townsend CA. Nat Rev Microbiol. 2010; 8:879. [PubMed: 21079635]
2. (a) Zhou H, Qiao K, Gao Z, Vederas JC, Tang Y. J Biol Chem. 2010; 285:41412. [PubMed: 20961859] (b) Xu Y, Zhou T, Zhou Z, Su S, Roberts SA, Montfort WR, Zeng J, Chen M, Zhang W, Lin M, Zhan J, Molnar I. Proc Natl Acad Sci U S A. 2013; 110:5398. [PubMed: 23509261]
3. (a) Winter JM, Sato M, Sugimoto S, Chiou G, Garg NK, Tang Y, Watanabe K. J Am Chem Soc. 2012; 134:17900. [PubMed: 23072467] (b) Chiang YM, Szewczyk E, Davidson AD, Keller N, Oakley BR, Wang CC. J Am Chem Soc. 2009; 131:2965. [PubMed: 19199437]
4. Campbell CD, Vederas JC. Biopolymers. 2010; 93:755. [PubMed: 20577995]
5. Ziemert N, Alanjary M, Weber T. Nat Prod Rep. 2016; 33:988. [PubMed: 27272205]
6. Boettger D, Hertweck C. Chembiochem. 2013; 14:28. [PubMed: 23225733]
7. (a) Sato M, Yagishita F, Mino T, Uchiyama N, Patel A, Chooi YH, Goda Y, Xu W, Noguchi H, Yamamoto T, Hotta K, Houk KN, Tang Y, Watanabe K. Chembiochem. 2015; 16:2294. [PubMed: 26360642] (b) Li L, Yu P, Tang MC, Zou Y, Gao SS, Hung YS, Zhao M, Watanabe K, Houk KN, Tang Y. J Am Chem Soc. 2016; 138:15837. [PubMed: 27960349]
8. Bergmann S, Schumann J, Scherlach K, Lange C, Brakhage AA, Hertweck C. Nat Chem Biol. 2007; 3:213. [PubMed: 17369821]
9. Steyn PS. Tetrahedron. 1970; 26:51. [PubMed: 5415401]
10. Li XW, Ear A, Nay B. Nat Prod Rep. 2013; 30:765. [PubMed: 23640165]
11. Yabuuchi T, Kusumi T. J Org Chem. 2000; 65:397. [PubMed: 10813947]
12. Ishii T, Hayashi K, Hida T, Yamamoto Y, Nozaki Y. J Antibiot. 2000; 53:765. [PubMed: 11079798]
13. Kato N, Nogawa T, Hirota H, Jang JH, Takahashi S, Ahn JS, Osada H. Biochem Biophys Res Commun. 2015; 460:210. [PubMed: 25770422]
14. (a) Fujii R, Minami A, Gomi K, Oikawa H. Tetrahedron Lett. 2013; 54:2999.(b) Song Z, Bakeer W, Marshall JW, Yakasai AA, Khalid RM, Collemare J, Skellam E, Tharreau D, Lebrun MH, Lazarus CM, Bailey AM, Simpson TJ, Cox RJ. Chem Sci. 2015; 6:4837.
15. Offenzeller M, Santer G, Totschnig K, Su Z, Moser H, Traber R, Schneider-Scherzer E. Biochemistry. 1996; 35:8401. [PubMed: 8679598]
16. Bushley KE, Raja R, Jaiswal P, Cumbie JS, Nonogaki M, Boyd AE, Owensby CA, Knaus BJ, Elser J, Miller D, Di Y, McPhail KL, Spatafora JW. PLoS Genet. 2013; 9:e1003496. [PubMed: 23818858]
17. Singh SB, Goetz MA, Jones ET, Bills GF, Giacobbe RA, Herranz L, Stevensmiles S, Williams DL. J Org Chem. 1995; 60:7040.
18. (a) Bush JA, Long BH, Catino JJ, Bradner WT. J Antibiot. 1987; 40:668. [PubMed: 3112080] (b) Elsebai MF, Nazir M, Kehraus S, Egereva E, Ioset KN, Marcourt L, Jeannerat D, Gütschow M, Wolfender J, König GM. Eur J Org Chem. 2012:6197.(c) Nakagawa Y, Kano H, Tsukuda Y, Koyama H. Tetrahedron Lett. 1967; 42:4105. [PubMed: 6062169] (d) Steglich W, Steffan B, Kopanski L, Eckhardt G. Angew Chem Int Ed. 1980; 19:459.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

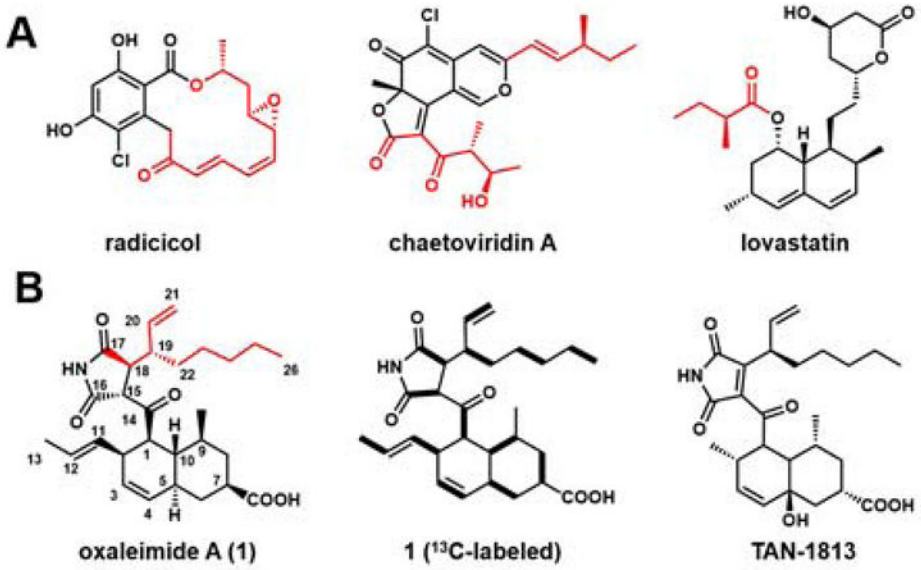


Figure 1.

Structures of (**A**) fungal polyketides derived from collaborative actions of two IPKSs. (**B**) oxaleimide **1** and TAN-1813. Bold lines show enrichment from [1,2-^{13}C]-acetate.

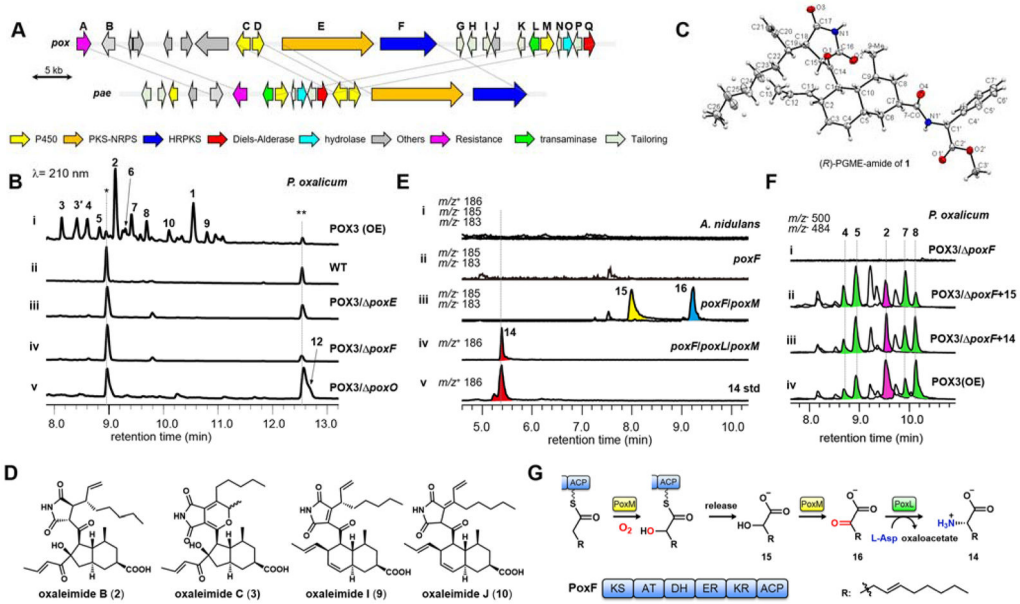


Figure 2.

Characterization of the natural products from the *pox* cluster in *P. oxalicum*. (**A**) The gene clusters of interest. (**B**) HPLC traces of the metabolic extracts from different *P. oxalicum* cultures: (i) POX3 (transcriptionally activated through overexpression of PoxB) (ii) wild-type, (iii) Δ*poxE* of POX3, (iv) Δ*poxF* of POX3 and (v) Δ*poxO* of POX3. *: secalonic acid D; **: linolenic acid that overlaps with **12**. (**C**) X-ray crystal structure of **11a**, (*R*)-PGME-amide of **1.** (**D**) Structures of oxaleimide **2**, **3**, **9** and **10**. (**E**) LC/MS analysis of the metabolic extracts from the culture of (i) *A. nidulans* A1145 (*An*), (ii) *An* expressing *poxF*, (iii) *An* expressing *poxF* and *poxM*, (iv) *An* expressing *poxF*, *poxM* and *poxL*, and (v) standard of **14**, containing trace of olefin isomer. Extracted LC traces corresponding to the m/z^+ for **14** (m/z^+ = 186), m/z^- for **15** (m/z^- = 185) and m/z^- for **16** (m/z^- = 183). (**F**) Chemical complementation of POX3/*ΔpoxF* using **14** or **15**. Extracted LC traces corresponding to the m/z^- for **2** (m/z^- = 500) and **4–8** (m/z^- = 484) of (i) POX3/*ΔpoxF*, (ii) POX3/*ΔpoxF* supplemented with **15**, (iii) POX3/*ΔpoxF* supplemented with **14** and (iv) POX3. (**G**) Proposed biosynthesis of **14**.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

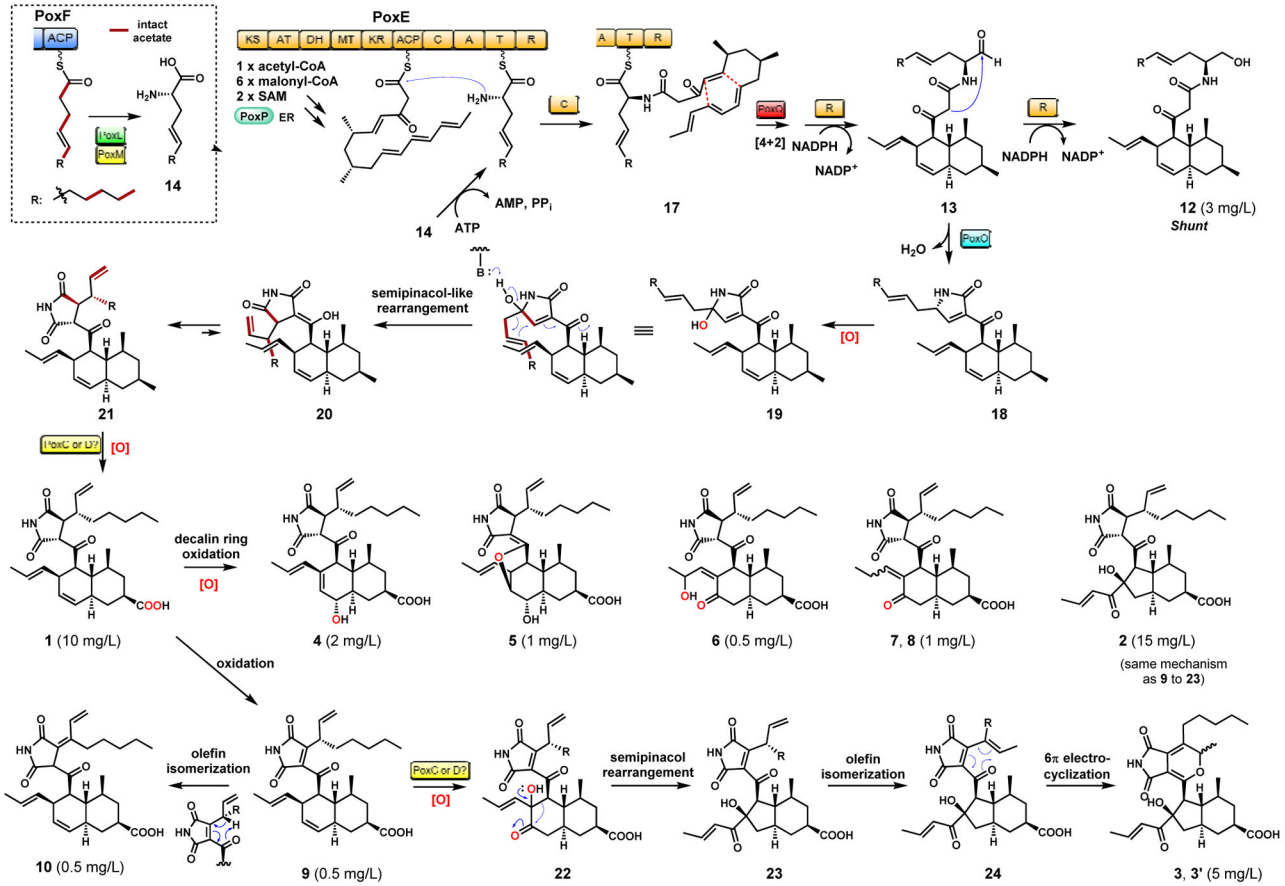


Figure 3.

Proposed biosynthetic pathway of compound **1** to **10**. PKS-NRPS domain abbreviations: KS: ketosynthase; MAT: malonyl-CoA:ACP transacylase; DH: dehydratase; MT: methyltransferase; KR: ketoreductase; ACP: acyl carrier protein; C: condensation; A: adenylation; T: thiolation; R: reductase; ER: enoylreductase. To determine the biosynthetic origin of the C18 substituent, we looked for a blocked mutant that may accumulate a precursor to **1** and **9** prior to rearrangement. Among the mutant constructs, we isolated a new compound **12** upon knockout of the α/β-hydrolase *poxO* (Figure 2b, v). **12** is the *trans*-decalin containing alcohol depicted in Figure 3. The alcohol can be formed from the over-reduction of the aldehyde **13**. This could occur when the Knoevenagel condensation of **13** to form **18** does not take place. Formation of similar over-reduced products from PKS-NRPKS has been observed.[14] Hence, this suggests that PoxO is responsible for the Knoevenagel condensation. More importantly, the structure of **12,** and that inferred for **13,** reveal that the aliphatic chain may be derived from incorporation of a nonproteinogenic amino acid, (*S, E*)-2-aminodec-4-enoic acid (**14**), by the NRPS domain of PoxE (Figure 3). Condensation between the octaketide product of the PKS and the amino group of **14** yields an aminoacyl product **17**, which can be cyclized by the Diels-Alderase PoxQ and reductively released by the reductive (R) domain of PoxE to yield **13.**

Author Manuscript Author Manuscript Author Manuscript Author Manuscript