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A Flavin-Dependent Decarboxylase–Dehydrogenase–Monooxygenase Assembles the Warhead of α,β-Epoxyketone Proteasome Inhibitors

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Supporting Information

ABSTRACT: The α,β-epoxyketone proteasome inhibitor TMC-86A was discovered as a previously unreported metabolite of *Streptomyces chromofuscus* ATCC49982, and the gene cluster responsible for its biosynthesis was identified via genome sequencing. Incorporation experiments with [^{13}C-methyl]L-methionine implicated an α-dimethyl-β-keto acid intermediate in the biosynthesis of TMC-86A. Incubation of the chemically synthesized α-dimethyl-β-keto acid with a purified recombinant flavin-dependent enzyme that is conserved in all known pathways for epoxyketone biosynthesis resulted in formation of the corresponding α-methyl-α,β-epoxyketone. This transformation appears to proceed via an unprecedented decarboxylation–dehydrogenation–monooxygenation cascade. The biosynthesis of the TMC-86A warhead is completed by cytochrome P450-mediated hydroxylation of the α-methyl-α,β-epoxyketone.

TMC-86A **1** belongs to a family of peptide natural products that irreversibly inhibit the proteasome (Figure 1).[1] Other members of this family include eponemycin **2**, epoxomicin **3**, macyranone A **4**, and landepoxins A/B **5**/**6** (Figure 1).[2−5] The α,β-epoxyketone pharmacophore of these metabolites covalently modifies the catalytically essential N-terminal Thr residue of the proteasome β-subunit.[6] This inspired the development of carfilzomib **7**, a synthetic proteasome inhibitor that has recently been approved for the treatment of multiple myeloma (Figure 1).[7] Although several biosynthetic gene clusters for metabolites belonging to this group have recently been identified and genetically investigated,[4,5,8] the mechanism for assembly of the epoxyketone pharmacophore remains unclear.

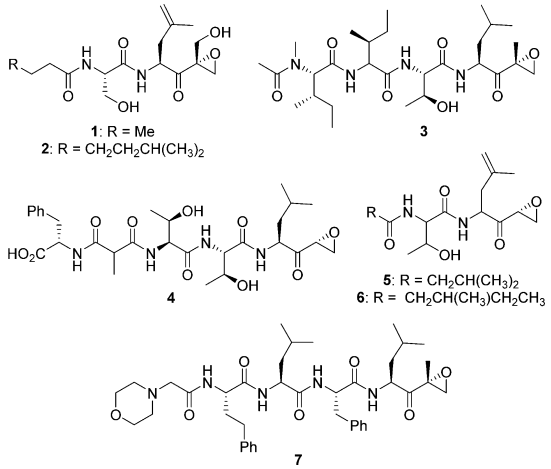

Figure 1. Structures of natural and synthetic proteasome inhibitors containing the α,β-epoxyketone pharmacophore.

During the course of a genomics-driven natural product discovery program, we generated a near-complete genome sequence of the herboxidiene-producer *Streptomyces chromofuscus* ATCC49982 and showed that TMC-86A **1** is a previously unknown metabolite of this organism (see Supporting Information).[9] Analysis of the *S. chromofuscus* genome sequence identified the putative TMC-86A biosynthetic gene cluster (BGC), which shows a high degree of similarity to the *epn* gene cluster reported to direct the biosynthesis of eponemycin in *Streptomyces hygroscopiscus* ATCC53709 (Figure 2).[8] The *tmcH* gene in *S. chromofuscus* was replaced with an apramycin resistance gene. TMC-86A production was abrogated in the resulting mutant (Figure 3), confirming the involvement of the *tmc* cluster in the biosynthesis of this metabolite.

By analogy with the pathway suggested by Kaysser and co-workers for eponemycin biosynthesis,[8] we propose that the nonribosomal peptide synthetase (NRPS) TmcG catalyzes the condensation of butanoyl-TmcE **8** with L-serine and L-leucine to give *N*-acyl-dipeptidyl thioester **9** (Figure 2). Elongation of *N*-acyl-dipeptidyl thioester **9** with a malonyl-CoA-derived extender unit by the modular polyketide synthase (PKS) TmcH yields the corresponding β-ketothioester. It was unclear whether the C-methyl transferase (CMT) domain of TmcH catalyzes mono- or dimethylation of this β-ketothioester. To investigate this, we fed [^{13}C-methyl]-L-methionine to *S. chromofuscus* and examined its incorporation into TMC-86A. ^{13}C NMR spectroscopic analysis showed that both methylene carbons of the α-hydroxymethyl-α,β-epoxyketone moiety of TMC-86A are labeled (Figure 4 and Supporting Information). This indicates that β-ketothioester is

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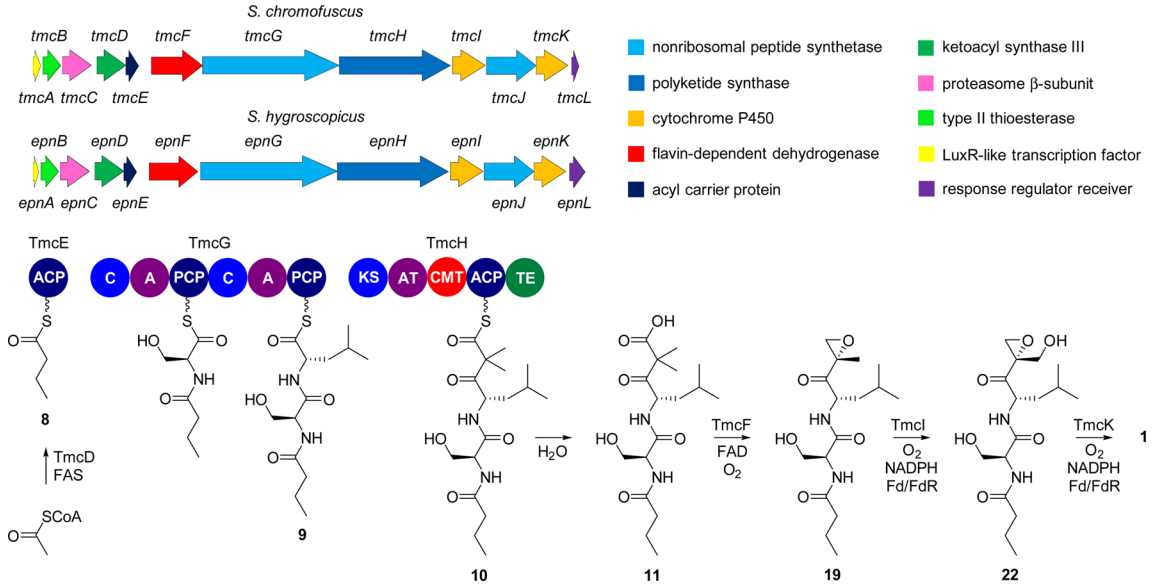

Figure 2. Organization of the *S. chromofuscus* TMC-86A and *S. hygrosopicus* eponomycin BGCs, and proposed pathway for TMC-86A biosynthesis. Desaturation of the leucine side chain occurs after loading of L-leucine onto TmcG, but the timing of this reaction is unclear.

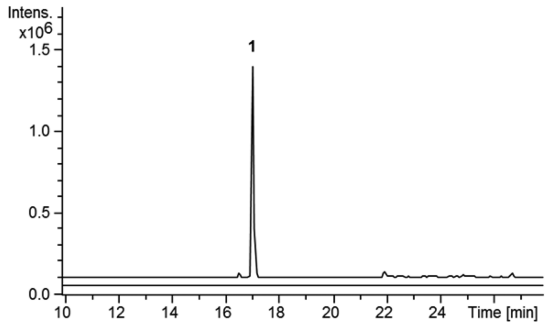

Figure 3. Extracted ion chromatograms at m/z = 343.18, corresponding to $[M + H]^+$ for TMC-86A, from UHPLC-ESI-TOF-MS analyses of wild type *S. chromofuscus* (top trace) and the *tmcH* mutant (bottom trace).

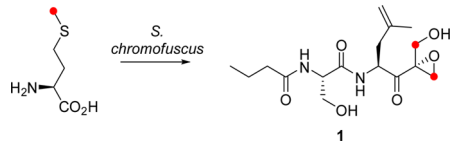

Figure 4. Sites of incorporation of [^{13}C-methyl]-L-methionine into TMC-86A determined by ^{13}C NMR spectroscopic analysis.

dimethylated by the CMT domain, yielding α-dimethyl-β-ketothioester **10**, which is hydrolytically released from TmcH by the thioesterase (TE) domain to give α-dimethyl-β-keto acid **11** (Figure 2). A similar conclusion has recently been reached for eponemycin biosynthesis on the basis of analogous incorporation experiments.[10,11]

Comparison of the genes encoding tailoring enzymes in the *tmc* cluster with those in other epoxyketone BGCs,[4,5,8] identified *tmcF* as the only tailoring gene that is conserved in all clusters. We therefore hypothesized that TmcF and its homologues in the other clusters are responsible for assembly of the epoxyketone pharmacophore. TmcF shows sequence similarity to flavin-dependent dehydrogenases, which typically catalyze α,β-desaturation of acyl-CoA or acyl-ACP thioesters.[12] To examine the role played by TmcF in TMC-86A biosynthesis, we attempted to overproduce it in *Escherichia coli*. However, it resided exclusively within insoluble inclusion bodies. We thus cloned and overexpressed *epnF*, the homologue from the *S. hygroscopicus* eponemycin BGC,[8] in *E. coli*. The resulting N-terminal hexahistidine fusion protein was produced in soluble form and was purified by Ni-affinity chromatography. The identity of the purified protein was confirmed by ESI-MS and peptide mass fingerprinting, and gel filtration chromatography showed that it is a homodimer. Solutions of the purified protein were bright yellow, and UV−vis spectroscopic analysis revealed an absorbance maximum at ∼450 nm, consistent with the presence of a flavin cofactor (see Supporting Information).

Given the propensity of β-keto acids to undergo spontaneous decarboxylation and the possibility that the TE domain of TmcH could catalyze this process,[13,14] we initially surmised that isopropyl ketone **12**, resulting from decarboxylation of α-dimethyl-β-keto acid **11**, was most likely to be the substrate of TmcF/EpnF. We thus synthesized 4-amino-2,6-dimethyl-heptan-3-one **14** by reacting the Weinreb amide of *N*-BOC-L-leucine **13** with ^{i}PrMgCl and subsequent deprotection with HCl in 1,4-dioxane.[15] BOP-mediated condensation of **14** with *N*-butanoyl-L-serine **16**, derived from DCC coupling of L-serine methyl ester **15** with butanoic acid and subsequent saponification with H_2O_2/LiOH, gave isopropyl ketone **12** (Scheme 1).[16] NMR spectroscopic analysis indicated that this material is a mixture of diastereomers resulting from epimerization of the ketone α-stereocenter.

No detectable products were formed when **12** was incubated with EpnF. We thus turned our attention to the synthesis of α-dimethyl-β-keto acid **11** (Scheme 2), the presumed product of TmcH. EDCI/DMAP-mediated coupling of N-BOC-L-leucine with Meldrum's acid, followed by thermolysis in the presence of BnOH, gave β-keto ester **17**.[17] Methylation of **17** with excess K_2CO_3 and MeI gave the corresponding α-dimethyl-β-ketoester,[18] which was deprotected with TFA and coupled with *N*-butanoyl-L-serine **16** to give the benzyl ester **18** of α-dimethyl-β-keto acid **11**. Hydrogenolysis of **18** over Pd/C gave a mixture of **11** and its decarboxylation product **12**. The ratio of **11** to **12** obtained from this reaction varied, but was ∼9:1 in the best

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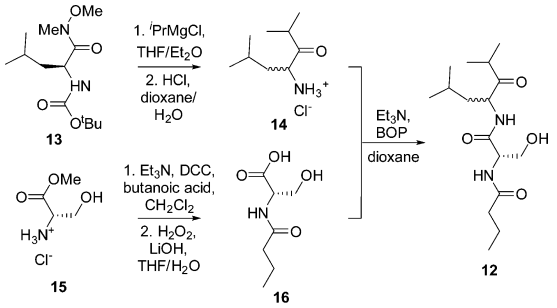
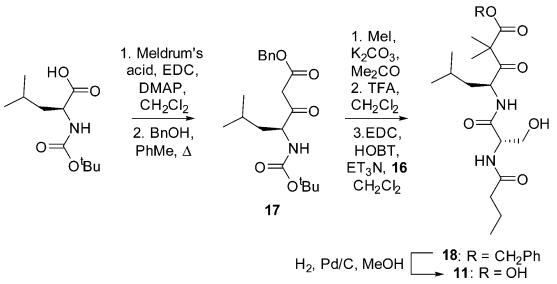
Scheme 1. Synthesis of Isopropyl Ketone 12



Scheme 2. Synthesis of α-Dimethyl-β-keto Acid 11



cases. Consistent with the anticipated propensity of **11** to undergo spontaneous decarboxylation, a significant proportion was converted to **12** during spectroscopic analysis.

Incubation of a mixture of **11** and **12** with EpnF resulted in formation of a new compound with a molecular formula corresponding to epoxyketone **19** (Figure 5). This compound

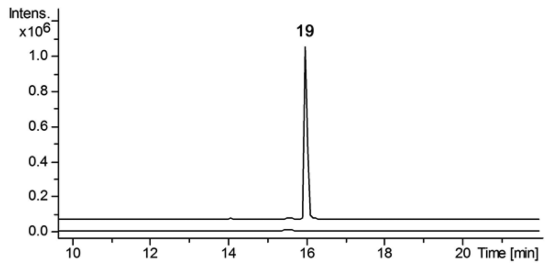


Figure 5. Extracted ion chromatograms at $m/z = 329.19$, corresponding to $[M + H]^+$ for **19**, from UHPLC-ESI-TOF-MS analyses of reactions containing a mixture of **11** and **12**, and native EpnF (top trace) or the heat-denatured enzyme (bottom trace).

was absent in control experiments from which the enzyme had been omitted, or added after heat denaturation. High resolution MS/MS analyses confirmed that **19** is the product of the EpnF-catalyzed reaction (see Supporting Information). These findings are consistent with the recent report that heterologous expression of *epnF*, *epnG*, and *epnH* in *E. coli* results in the production of the *N*-octanoyl analogue of **19**.[10]

The catalytic mechanism of acyl-CoA dehydrogenases typically involves deprotonation of the thioester substrate by an active site base to generate an enolate intermediate that transfers hydride from the β-carbon atom to the bound flavin cofactor.[12] The observation that EpnF can utilize **11**, but not **12**, as a substrate indicates that it employs a unique mechanism involving decarboxylation of **11** to generate the enolate intermediate **20**. Elimination of hydride from one of the β-carbon atoms of **20** would reduce the flavin cofactor, yielding α,β-unsaturated ketone **21**. Reaction of the reduced flavin with molecular oxygen forms the flavin hydroperoxide, which could convert **21** to the epoxyketone **19** via a conjugate addition−1,3-elimination mechanism (Figure 6).

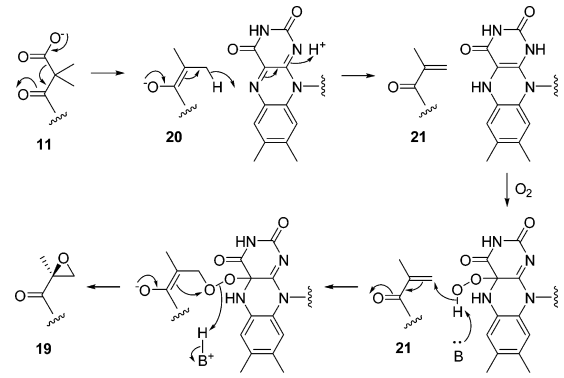


Figure 6. Proposed mechanism of the EpnF-catalyzed reaction.

To investigate whether α,β-unsaturated ketone **21** can dissociate from EpnF during the conversion of **11** to **19**, we included *N*-acetylcysteamine (NAC) in the reaction mixture. Only the product resulting from opening of the epoxide in **19** with NAC could be detected in LC-MS analyses (see Supporting Information). None of the product resulting from conjugate addition of NAC to α,β-unsaturated ketone **21** was observed. Thus, **21** is likely unable to dissociate from EpnF before undergoing epoxidation.

Hydroxylation of the methyl group in the α-methyl-α, β-epoxyketone moiety of **19** could be catalyzed by either of the cytochrome P450s encoded by *tmcI* and *tmcK*. Sequence comparisons show that *tmcK* orthologs are only present in BGCs for epoxyketones that contain a dehydroleucine residue. Thus, TmcK is proposed to be involved in desaturation of the side chain of the leucine residue incorporated into TMC-86A, but the timing of this reaction remains unclear. One scenario involves TmcJ, which contains MbtH, adenylation (A), and peptidyl carrier protein (PCP) domains. Loading of L-Leu by the A domain of TmcJ onto the adjacent PCP domain would afford the corresponding aminoacyl thioester. TcmK-catalyzed desaturation of this thioester, followed by hydrolytic release, would generate 4,5-dehydro-L-Leu, which could be loaded onto the second PCP domain of TmcG by the adjacent A domain. Other nonproteinogenic amino acid NRPS substrates are known to be biosynthesized via a similar mechanism.[19] Alternatively, the second A domain of TmcG could load L-Leu directly onto the adjacent PCP domain and TmcK could catalyze side chain desaturation at either this point or a later stage in the assembly of TMC-86A. To discriminate between these possibilities, we overproduced the second A-PCP didomain of TmcG in *E. coli* as a soluble C-terminal hexahistidine fusion. The substrate specificity of the A domain within the purified protein was assessed using a pyrophosphate release assay. While the TmcG A domain could adenylate 4,5-dehydro-L-leucine, it was found to greatly prefer L-leucine as a substrate (see Supporting Information). Thus, it appears that TmcK acts at some point after loading of L-leucine onto the second PCP domain of TmcG. This conclusion is supported by the observation that both eponemycin and its leucine-containing derivative are produced when the *epn* cluster is expressed in *Streptomyces albus*.[11]

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By a process of elimination, we hypothesized that TmcI catalyzes hydroxylation of the methyl group in the α-methyl-α,β-epoxyketone moiety of **19**. To test this hypothesis, we overproduced TmcI in *E. coli* as a soluble N-terminal hexahistidine fusion. The identity of the purified protein was confirmed by ESI-MS, and it was shown to exist as a monomer containing a heme prosthetic group (see Supporting Information). Incubation of a mixture of **11** and **12** with EpnF, TmcI, spinach ferredoxin (Fd) and ferredoxin reductase (FdR), and NADPH resulted in production of a compound with a molecular formula corresponding to α-hydroxymethyl-α,β-epoxyketone **22**. This product was absent from control reactions containing heat-denatured EpnF and TmcI (Figure 7). Comparison of high resolution MS/MS spectra for **1** and the reaction product confirmed the structure of the latter as **22** (see Supporting Information).

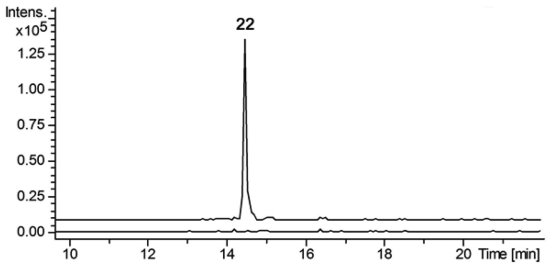

Figure 7. Extracted ion chromatograms at m/z = 367.17, corresponding to $[M + Na]^+$ for **22**, from UHPLC-ESI-TOF-MS analyses of reactions containing a mixture of **11** and **12**, spinach ferredoxin and ferredoxin reductase, NADPH and native (top trace) or heat-denatured (bottom trace) EpnF and TmcI.

In conclusion, using a genomics-led approach, we have identified a TMC-86A biosynthetic gene cluster in *Streptomyces chromofuscus* ATCC49982. Incorporation of [^{13}C-methyl]L-methionine into both of the methylene groups of the α-hydroxymethyl-α,β-epoxyketone warhead of TMC-86A implicated an α-dimethyl-β-keto acid intermediate in its biosynthesis. This α-dimethyl-β-keto acid was synthesized in eight steps and shown to be converted to the corresponding α-methyl-α,β-epoxyketone by a flavin-dependent enzyme that is conserved in all known pathways for epoxyketone biosynthesis. The isopropyl ketone resulting from decarboxylation of the α-dimethyl-β-keto acid was also synthesized and shown not to be a substrate of the enzyme. Thus, we conclude that the pharmacophore of α,β-epoxyketone proteasome inhibitors is biosynthesized by a remarkable decarboxylase−dehydrogenase−monooxygenase that employs a catalytic flavin cofactor. To the best of our knowledge, no other flavin-dependent enzyme is known to catalyze a similar sequence of reactions. The α-methyl-α,β-epoxyketone pharmacophore initially formed in TMC-86A biosynthesis undergoes additional elaboration via hydroxylation of the α-methyl group. We have shown that this is catalyzed by the cytochrome P450 TmcI. Homologues of TmcI are encoded by genes within the eponemyin **2** and epoxomicin **3** BGCs,[8] although in the latter the TmcI homologue appears to be nonfunctional.[11]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b01619.

Details of experimental procedures, additional LC-MS, MS/MS, NMR and protein characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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