

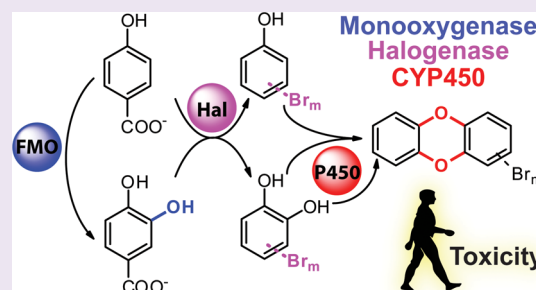
Enzymatic Synthesis of Polybrominated Dioxins from the Marine Environment

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S Supporting Information

ABSTRACT: Polyhalogenated dibenzo-*p*-dioxins are arguably among the most toxic molecules known to man. In addition to anthropogenic sources, marine invertebrates also harbor polybrominated dibenzo-*p*-dioxins of as yet unknown biogenic origin. Here, we report that the *bmp* gene locus in marine bacteria, a recently characterized source of polybrominated diphenyl ethers, can also synthesize dibenzo-*p*-dioxins by employing different phenolic initiator molecules. Our findings also diversify the structural classes of diphenyl ethers accessed by the *bmp* biosynthetic pathway. This report lays the biochemical foundation of a likely biogenetic origin of dibenzo-*p*-dioxins present in the marine metabolome and greatly expands the toxicity potential of marine derived polyhalogenated natural products.



The marine metabolome is rich in small molecule natural products that have attracted attention as environmental pollutants and toxins. Prominent examples include the ozone damaging halomethanes sourced to marine algae,¹ and a large repertoire of highly potent mammalian toxic polyether compounds derived from marine cyanobacteria, dinoflagellates, and algae.² Additionally, marine invertebrates are prolific sources of potentially toxic halogenated natural products that include polybrominated molecules unique to the marine metabolome.³ Polybrominated marine natural products, such as the polybrominated diphenyl ethers (PBDEs), have been extensively isolated from marine invertebrates.^{4–6} Structurally characterized marine PBDEs reveal a preponderance of hydroxylated derivatives (OH-BDEs) that distinguish these naturally produced organobromines⁷ from anthropogenic PBDEs that were once commonly employed as flame retardant chemicals.⁸ OH-BDEs are ubiquitous across all tropic levels in the marine food web and can be detected in even humans where they can exert a myriad of toxicities.⁹

We recently reported the molecular bases for the biosynthesis of OH-BDEs and structurally related polybrominated biphenyls by the highly conserved *bmp* gene locus present in numerous and geographically disperse marine γ -proteobacteria that are commonly associated with marine Eukarya (Figure 1a).¹⁰ The biosynthesis of OH-BDEs proceeds from the primary metabolite 4-hydroxybenzoic acid (1), which is derived from chorismic acid by the chorismate lyase enzyme Bmp6. The decarboxylative-brominase flavoenzyme Bmp5 converts 1 to its principal bromophenol product, 2,4-dibromophenol (2). Then, 2 is coupled to other bromophenols by the CYP450 Bmp7 via ether and C–C linkages to generate OH-BDEs and biphenyls, respectively.

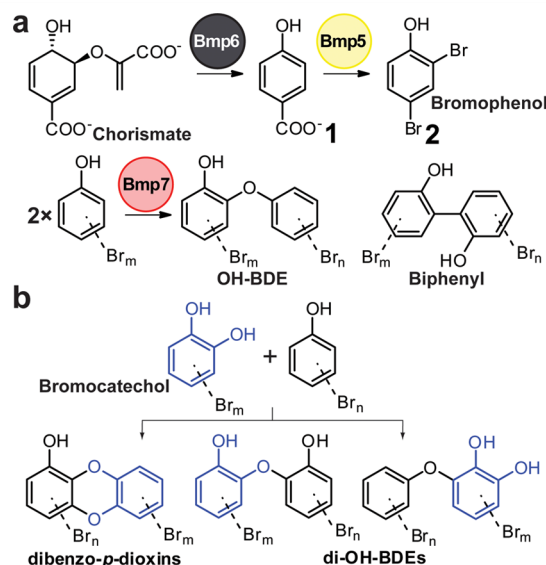


Figure 1. Biosynthetic schemes for polybrominated aryl marine natural products. (a) The biosynthesis of OH-BDEs and biphenyls via the *bmp* pathway involves three basic enzymological reactions catalyzed by Bmp5–7. (b) Basic structural skeletons of marine dibenzo-*p*-dioxins and di-OH-BDEs that are hypothesized to be derived from the heterocoupling of bromophenols and bromocatechols.

While OH-BDEs are potent inhibitors of mammalian nuclear hormone mediated signaling pathways,^{11,12} alternate bioinhibi-

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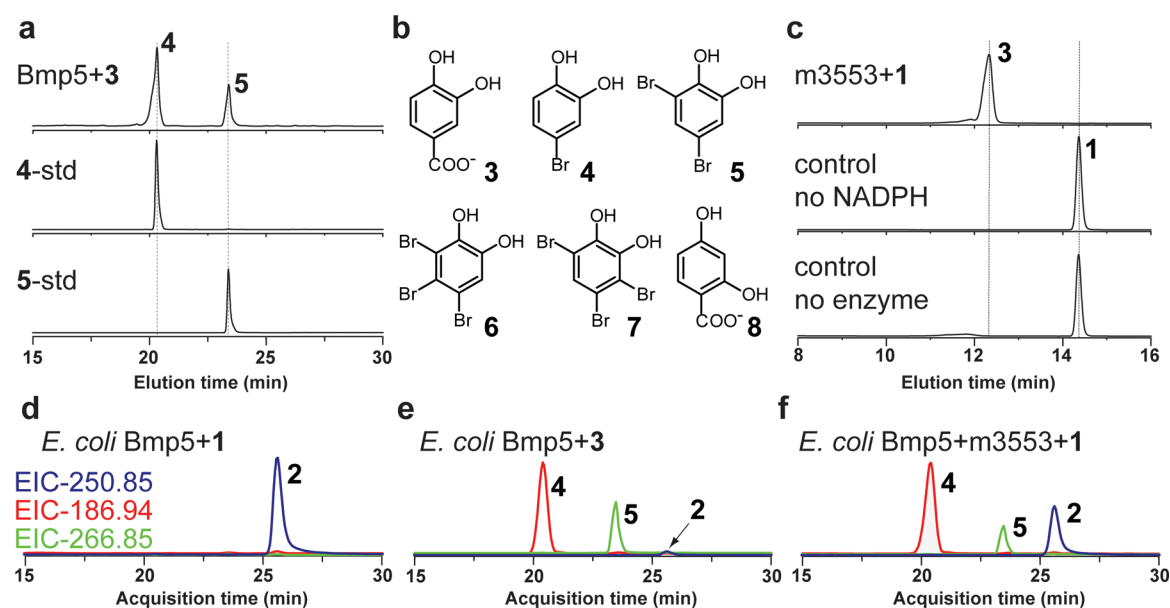


Figure 2. Engineered biogenetic route for production of bromocatechols. (a) HPLC analysis of the extract of Bmp5 reaction with 3 identifies 4–5 as the major products by comparison to authentic standards. Absorbance was monitored at 214 nm. (b) Chemical structures for 3–8. (c) HPLC analysis for NADPH-dependent hydroxylation of 1 to 3 catalyzed by m3553. Absorbance was monitored at 280 nm. (d–f) Extracted ion chromatograms (EICs) for the most abundant $[M-H]^-$ ions for 2 (in blue, $m/z = 250.85$), 4 (in red, $m/z = 186.94$), and 5 (in green, $m/z = 266.85$) for culture extracts of *E. coli* expressing (d) Bmp5 in the presence of 0.5 mM 1 in the culture medium, (e) Bmp5 in the presence of 0.5 mM 3, and (f) Bmp5 and m3553 in the presence of 0.5 mM 1. All EICs were generated within 10 ppm tolerance. Note that relative peak heights in EICs are not indicative of relative amounts of metabolites present in an analyte.

tory mechanisms are employed by marine dihydroxylated-BDEs (di-OH-BDEs) (Figure 1b).^{13,14} The toxicity potential for dibenzo-*p*-dioxins, also isolated from marine sponges,^{15,16} and detected in other marine invertebrates^{17,18} is unquestioned as they are structurally analogous to some of the most toxic anthropogenic molecules ever produced.¹⁹ Noticeably, di-OH-BDEs and dibenzo-*p*-dioxins as products were absent from the initial characterization of the *bmp* pathway in which polybrominated phenolic products were strictly derived from 1. As the *bmp* gene locus does not harbor a physiological oxygenase, the origin of the extra oxygen atom in these seemingly dimeric phenolic molecules was not immediately apparent. Our current study presents an experimental basis for the diversity of polybrominated phenols, diphenyl ethers, and dibenzo-*p*-dioxins found in the marine environment by rationally expanding the repertoire of simple phenolic substrates for the Bmp5 and Bmp7 enzymes.

RESULTS AND DISCUSSION

A hypothetical scheme for the biosyntheses of dibenzo-*p*-dioxins and di-OH-BDEs from 1, and consequently from bromophenols such as 2, would require hydroxylation of OH-BDEs to generate di-OH-BDEs, followed by an intramolecular cyclization to generate dibenzo-*p*-dioxins. An alternative mechanism could involve heteromeric coupling of bromocatechol and bromophenol monomers to generate the requisite phenolic skeletons (Figure 1b). Two lines of evidence lead to our enthusiasm for the latter scenario: first, rates of nonspecific hydroxylation for OH-BDEs have been reported to be too low to support bioaccumulation and subsequent isolation of di-OH-BDEs and dibenzo-*p*-dioxins.⁹ Second, bromocatechols and bromoresorcinols have themselves been isolated from marine sources,⁵ thus supporting the postulate that marine bacteria can

sample phenolic molecules other than 1 to initiate biosynthesis of diverse polybrominated compounds.

From an enzymatic synthesis point-of-view, we rationalized that bromocatechols could be derived from the decarboxylative-bromination of 3,4-dihydroxybenzoic acid (3), analogous to the biosynthesis of 2 from 1 by Bmp5. To test this hypothesis, we incubated Bmp5 with 3 in the presence of bromide and a continuous NADPH regeneration system. LC-MS analysis of the reaction extract demonstrated the production of two major bromocatechol products 4–5. Identity of the products was determined by NMR spectroscopy (Supporting Information Figures 1–7) and comparison to synthetic standards (Figure 2a–b). Two minor tribrominated bromocatechol products were also identified as 6–7 (Supporting Information Figures 8–10). Of the four bromocatechols generated by Bmp5, 5 has been previously reported as a sponge-derived marine natural product.⁵

A time course analysis for the Bmp5 reaction revealed that the rate of disappearance of 3 as a substrate was much slower than that for 1. However, 2,4-dihydroxybenzoic acid (8) was consumed by Bmp5 at a rate greater than that for 1 (Supporting Information Figure 11). We hypothesize that the decrease in catalytic efficiency of Bmp5 for 3 can be attributed to antagonistic effects of the catechol ortho-hydroxyls, while the resorcinol meta-hydroxyls of 8 direct electrophilic additions synergistically. This hypothesis is further supported by the near identical rates of substrate disappearance for 3-methyl-4-hydroxybenzoic acid and 2-methyl-4-hydroxybenzoic acid to 1 (Supporting Information Figure 11).

Having established an *in vitro* biosynthetic scheme for bromocatechols 4–7, we queried the applicability for invoking 3 as a physiological substrate for Bmp5. Compound 3, commonly referred to as protocatechuic acid, is a ubiquitous microbial metabolite derived from the shikimic acid pathway

for incorporation into secondary metabolites such as siderophores.²⁰ It is also an intermediate in the oxidative biodegradation of phenols. One of the numerous enzymological routes to **3** is the ortho-hydroxylation of **1**, catalyzed by the flavin-dependent 4-hydroxybenzoate-3-hydroxylase. A homologue of this enzyme, henceforth referred to as m3553, is present in the genome of *Marinomonas mediterranea* MMB-1, a melanogenic marine bacterium previously reported to be a producer of OH-BDEs.¹⁰ We could indeed confirm the NADPH-dependent *in vitro* conversion of **1** to **3** by m3553 (Figure 2c), while no change in the product profile could be observed upon addition of catalase or superoxide dismutase to the assay (Supporting Information Figure 12). We next explored whether m3553 could be coupled to Bmp5 *in situ* to produce bromocatechols. As a control, *Escherichia coli* expressing Bmp5, when grown in the presence of bromide and **1**, produced **2**¹⁰ but no bromocatechols at detectable levels (Figure 2d). In the presence of exogenous **3**, **4** and **5** are produced, in addition to **2**, as *E. coli* harbors the *ubiC* gene for the production of **1** from chorismate (Figure 2e).²¹ However, *E. coli* expressing both Bmp5 and m3553 produced **2**, as well as **4** and **5** when **1** is added to the culture medium (Figure 2f), thus confirming an *in vivo* bromocatechol biosynthetic system.

While **2** and **5** have individually been detected in marine invertebrates, there is no report describing the concomitant presence of the two compounds in a marine eukaryotic sample. To address this knowledge gap, we generated and analyzed a methanol extract of the Fijian marine sponge *Dysidea* spp., a genus with an extensive precedence for harboring PBDEs synthesized by symbiotic marine bacteria.^{4–6} Using LC-MS, we could clearly detect the presence of both **2** and **5** in the sponge extract (Supporting Information Figure 13). This finding thus supports our premise that both bromophenol and bromocatechol modalities are simultaneously accessible to PBDE biosynthesizing bacteria in the marine metabolome.

We next explored the bromocatechol-bromocatechol coupling activity of CYP450 Bmp7. Upon incubation of Bmp7 with **5** in the presence of electron transfer partners Bmp9–10 and NADH, we observed the production of six major products (Figure 3a–b) that correspond to two distinct sets of isomers (denoted by ▲ and ◆ in Figure 3b). **9**–**12** and **13**–**14** bear the molecular formulas $C_{12}H_7Br_3O_4$ and $C_{12}H_5Br_3O_4$, respectively. The presence of **9**–**12** and **13**–**14** in a single EIC is due to the characteristic isotopic distribution for polybrominated molecules (Figure 3c–d). Supported by MS/MS fragmentation (Supporting Information Figure 14), we established the structures of **9**–**12** by NMR spectroscopy (Figure 3e and Supporting Information Figures 15–30). These structures correspond to para-OH-BDEs (**9**, **11**) and ortho-OH-BDEs (**10**, **12**), two classes of OH-BDEs that were described previously.¹⁰

NMR spectra (Supporting Information Figures 31–40), together with the trimethylsilyl derivitization and GC-MS analysis for **13**–**14** (Supporting Information Figure 41) established the existence of an additional ether bond, leading to the loss of one aryl and one hydroxyl proton as compared to **9**–**12**. Thus, **13**–**14** were proposed to be dibenzo-*p*-dioxins. Guided by literature,^{6,15,16} we firmly established the structures for **13**–**14** by NMR spectroscopy (Figure 3e). To the best of our knowledge, this is the first report for an enzymatic synthesis of polybrominated dibenzo-*p*-dioxins. In contrast to the Bmp7 catalyzed coupling of bromophenols,¹⁰ no biphenyls were detected as products during the coupling of bromocatechol **5**.

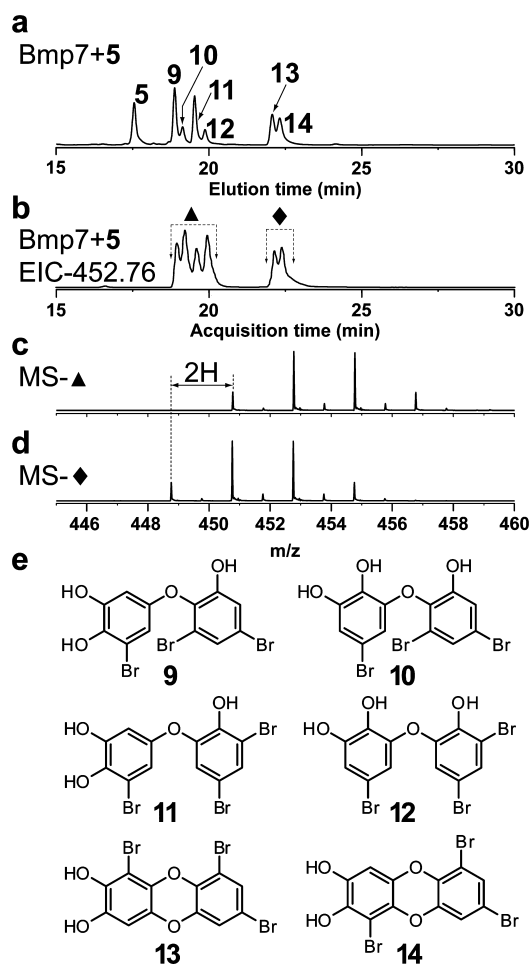


Figure 3. CYP450 Bmp7 mediated homocoupling of bromocatechol **5**. (a) HPLC elution curve demonstrating the production of **9**–**14** upon coupling of **5** by Bmp7. Absorbance was monitored at 214 nm. (b) EIC [M-H]⁻ $m/z = 452.76$ also identifies six products. (c) MS1 spectra corresponding to **9**–**12** and (d) **13**–**14**. Note that the isotopic distribution leads to a major molecular ion corresponding to 452.76 for both sets of isomers. (e) Chemical structures of **9**–**14**.

As established by the coexpression of Bmp5 and m3553 in *E. coli* (Figure 2f), a plausible physiological scenario entails both bromophenols and bromocatechols to be available simultaneously to Bmp7 for coupling. Thus, we explored the Bmp7 catalyzed coupling of **2** and **5** *in vitro*. As both **2** and **5** individually generate at least six homocoupling products each (ref 10 and Figure 3a), we relied on mass spectrometry based dereplication to distinguish the bromophenol–bromocatechol heterocoupling products from the bromophenol–bromophenol and bromocatechol–bromocatechol homocoupling products. We observed two distinct peaks in the heterocoupling reaction in an EIC [M-H]⁻ $m/z = 436.78$ that were not present in the homocoupling reactions (Figure 4a). This molecular ion is diagnostic for both molecular formulas, $C_{12}H_7O_3Br_3$ and $C_{12}H_5O_3Br_3$. Based on MS/MS fragmentation and relative HPLC elution times, one of the heterocoupled products (labeled by ■, Figure 4b) can be identified as a di-OH-BDE (Supporting Information Figure 42) while the other represents a tribrominated dibenzo-*p*-dioxin species (labeled by ●, Figure 4c).

We next queried whether the *in situ* bromophenol–bromocatechol production system, as described in Figure 2f,

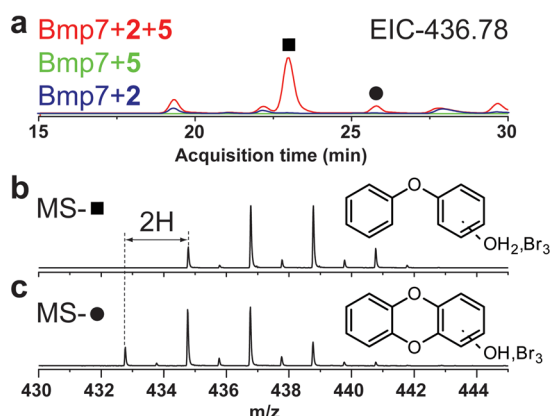
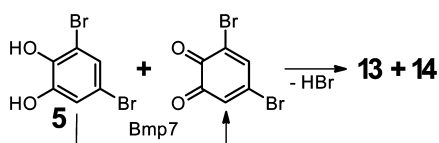


Figure 4. CYP450 Bmp7 mediated heterocoupling of bromophenol **2** and bromocatechol **5**. (a) Comparison of EIC $[M-H]^-$ $m/z = 436.78$ for Bmp7 catalyzed *in vitro* reactions for both **2** and **5** present simultaneously (in red), and for **5** (in green) and **2** (in blue) individually. MS1 spectra corresponding to (b) dihydroxylated-tribrominated diphenyl ether and (c) tribrominated dibenzo-*p*-dioxin corresponding to species denoted by ■ and ● in panel a. Assignment of ■ as a di-OH-BDE is supported by MS/MS fragmentation.

could drive the *in vivo* production of dibenzo-*p*-dioxins and di-OH-BDEs. Upon coexpression of Bmp5, Bmp7, and m3553, and supplementation of the culture medium with exogenous **1**, we indeed observed the production of bromophenols, bromocatechols, mono- and dihydroxylated dibenzo-*p*-dioxins, mono- and di-OH-BDEs, as well as biphenyls (Supporting Information Figure 43). Thus, for the first time, all structural classes of naturally detected polybrominated marine natural products, as depicted in Figure 1, were made accessible via a rationally engineered *bmp* pathway. This *in vivo* experiment closely mirrored the extract from the *Dysidea* sponge, which is also profuse in different structural classes of polybrominated phenolic molecules (Supporting Information Figure 44), including, albeit in low abundance, never before reported dihydroxylated dibenzo-*p*-dioxin corresponding to species denoted by ◆ in Figure 3b.

It is intriguing that dioxins were observed as products only in the presence of bromocatechol substrates. Both bromophenols and bromocatechols are expected to undergo an identical radical initiation step by the homolytic scission of the hydroxyl oxygen–hydrogen bond. However, specific for catechols, the semiquinone radical can rearrange to a 1,2-benzoquinone intermediate. Illustratively, starting from **5**, a 3,5-dibromo-1,2-benzoquinone intermediate would be generated following radical initiation at either of the two catechol hydroxyls. The electrophilic quinone may then undergo coupling with a bromophenol or a bromocatechol partner to generate dibenzo-*p*-dioxins in a manner such that both 1,4-dioxin oxygen atoms would be derived from the benzoquinone (Scheme 1). This scheme is reminiscent of synthetic hetero-Diels–Alder coupling

Scheme 1. Proposed Enzymatic Synthesis of Dibenzo-*p*-dioxins from Bromocatechol Monomers Catalyzed by CYP450 Bmp7



of orthoquinones to enamines for installation of 1,4-benzodioxin scaffolds²² and is also supported by comparison of previously reported structures of marine dibenzo-*p*-dioxins^{15,16} and **13–14**, in which the 3,5-dibromo-1,2-ethylenedioxybenzene moiety is found to be strictly conserved. Driven by the relative lack of substrate specificity of the CYP450 Bmp7, it is likely that the structural diversity of naturally produced dibenzo-*p*-dioxins is constrained primarily by the bromophenol and bromocatechol products generated by Bmp5.

In conclusion, we have further expanded the already vast chemical space explored by the products derived from the *bmp* gene locus. Discovery of a likely biogenetic route for the biosynthesis of dibenzo-*p*-dioxins puts into perspective the immense toxicological potential of these polybrominated marine natural products, particularly in light of recent proposals for the trophic transfer of these polybrominated marine natural products to human population.^{23–25}

METHODS

Purification and enzymatic assays for *M. mediterranea* MMB-1 Bmp5 and Bmp7 are described in detail in the Supporting Information. The open reading frame corresponding to m3553, which is encoded by gene *marme_3553* within the genome of *M. mediterranea* MMB-1 (GenBank: CP002583.1), was amplified by PCR using genomic DNA from *M. mediterranea* MMB-1 as template. The PCR product was purified, digested with NdeI and HindIII restriction enzymes, and ligated to an NdeI–HindIII digested pET28a(+) vector. The ligation reaction was transformed into *Escherichia coli* DH5α cells, plated on LB-Agar plates supplemented with 50 μg/mL kanamycin, and incubated at 37 °C for 12 h. Individual colonies were grown in 5 mL LB media supplemented with kanamycin for 12 h, verified by sequencing of isolated plasmid DNA, and plasmid DNA from a positive clone was transformed into *E. coli* BL21Gold(DE3) cells for protein expression. A 1 L Terrific Broth culture, supplemented with 50 μg mL^{−1} kanamycin was grown at 30 °C. When the optical density reached 0.6, the temperature was reduced to 18 °C and protein expression was induced by the addition of 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After overnight growth, the bacterial cells were harvested by centrifugation and resuspended in 20 mM Tris-HCl (pH 8.0) 500 mM NaCl 10% glycerol buffer. Cells were lysed by sonication and the lysate clarified by centrifugation. The supernatant was applied to a 5 mL Ni-NTA affinity column using a AKTA purification system. The column was extensively washed using 20 mM Tris-HCl (pH 8.0) 1 M NaCl 30 mM imidazole buffer and bound protein was eluted using a linear gradient to 250 mM imidazole concentration across 10 column volumes. The purity of the protein was verified by SDS-PAGE. Pure fractions were pooled and dialyzed in 20 mM Tris-HCl (pH 8.0) 10% (v/v) glycerol buffer with the addition of 1 unit/mg thrombin protease at 4 °C. After overnight dialysis, the protein was concentrated using a 10 kDa centrifugal filter and desalted to 20 mM Tris-HCl (pH 8.0) 10% (v/v) glycerol buffer using a PD-10 column. Protein concentration was measured using a standardized Bradford assay.

Purified m3553 (1 μM) was incubated with 0.5 mM **1** and 2 mM NADPH in 20 mM Tris-HCl (pH 8.0) buffer at 30 °C. Assays were also conducted in the presence of 7.5 units of commercially available catalase (Sigma-Aldrich C-40) or 10 units of superoxide dismutase (Sigma-Aldrich S7446) in addition to m3553. Then, 50 μL of reaction was quenched by the addition of 20 μL MeCN + 0.35% TFA and heated at 50 °C. The quenched reaction was centrifuged and 25 μL was injected on a Phenomenex Luna C18 5 μ (4.6 × 100 mm) analytical column operating on an Agilent 1260 analytical HPLC setup at RT. Water + 0.1% (v/v) TFA was used as buffer A, and MeCN + 0.1% (v/v) TFA was used as buffer B. The elution profile was as follows. Elution gradient I: 5% buffer B for 5 min, linear gradient to 95% buffer B across 25 min, step increase to 100% buffer B, 100%

buffer B for 3 min, linear decrease to 5% buffer B across 2 min, 5% buffer B for 5 min. Absorbance was monitored at 280 nm wavelength. Under these reaction conditions, stoichiometric conversion of **1** to **3** was achieved within 1 h. Identity of the product was confirmed by comparison of retention time and UV absorbance profile against an authentic synthetic standard of **3** analyzed under identical chromatographic conditions. Negative control reactions were set up by replacing NADPH and enzyme with buffer in the assay.

■ ASSOCIATED CONTENT

■ Supporting Information

Additional methods and figures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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