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gem-Diprenylation of Acylphloroglucinols by a Fungal Prenyltransferase of the Dimethylallyltryptophan Synthase Superfamily

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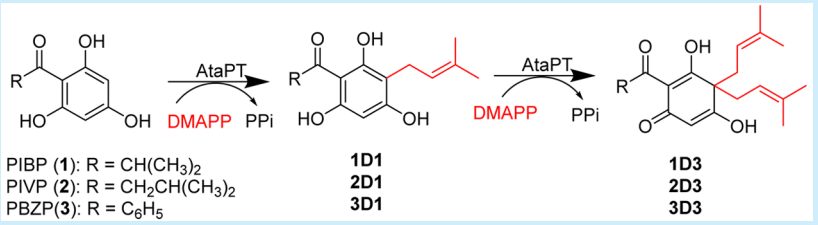
Supporting Information



ABSTRACT: *Aspergillus terreus* aromatic prenyltransferase (AtaPT) catalyzes predominantly C-monoprenylation of acylphloroglucinols in the presence of different prenyl diphosphates. With dimethylallyl diphosphate (DMAPP) as prenyl donor, *gem*-diprenylated products 1D3, 2D3, and 3D3 were also detected. High conversion of 1D1 to 1D3, 2D1 to 2D3, and 3D1 to 3D3 was demonstrated by incubation with AtaPT and DMAPP. The first example of *gem*-diprenylation by a member of the dimethylallyltryptophan synthase superfamily is provided.

Prenylated acylphloroglucinols (APs) are characteristic constituents of several plant families. Compounds of this class have fascinating chemical structures and intriguing biological and pharmacological activities.[1−5] Main structural features of prenylated APs are highly oxygenated and densely decorated with prenyl such as dimethylallyl and geranyl moieties. Polyprenylated APs like α- and β-bitter acids (Figure 1) from *Humulus lupulus* (Cannabinaceae), commonly known

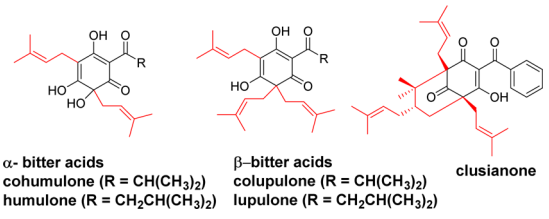
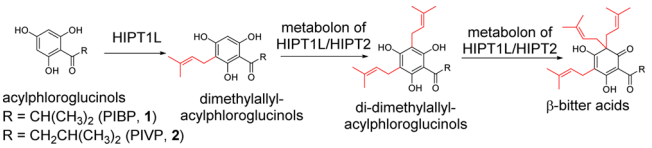


Figure 1. Examples of polyprenylated APs from plants.

as hops, are considered as multipotent bioactive compounds including their sedative effects.[4] Clusianone (Figure 1) and its 7-epimer from different plants such as *Garcinia brasiliensis* and *Clusia torresii*, both from the family Clusiaceae, exhibit anti-HIV and antitumor activities.[6−9] An exceptional structure feature of polyprenylated APs is the *gem*-diprenylation at C-atoms.

Biogenetically, cohumulone and colupulone are prenylated phlorisobutyrophenone (PIBP, **1**, Scheme 1), while humulone and lupulone prenylated phlorisovalerophenone (PIVP, **2**,

Scheme 1. Prenylation Steps in the Biosynthesis of β-Bitter Acids in *Humulus lupulus*



Scheme 1). Clusianone carries the skeleton of phlorbenzophenone (PBZP, **3**, Scheme 2). The AP cores of these compounds are tetraketides and formed by condensation of three malonyl-CoA molecules with different start units, i.e., isobutyryl-CoA in the case of **1**, isovaleryl-CoA in the case of **2**, and benzoyl-CoA in the case of **3**. The responsible polyketide synthases have been characterized.[10−12] In comparison, little is known about the enzymes for the multiple prenylation steps. Only the prenyltransferases involved in the biosynthesis of bitter acids in *H. lupulus* have been reported.[12,13] Tsurumaru et al.[13] reported the overproduction of the membrane-bound prenyltransferase HIPT-1 from *H. lupulus* and its biochemical characterization. It was found that this enzyme catalyzed the prenylation of **2** in the presence of DMAPP and also accepted **1** as prenylation acceptor. Recently, Li et al.[12] identified two membrane-bound prenyltransferases HIPT1L and HIPT2 from *H. lupulus*.

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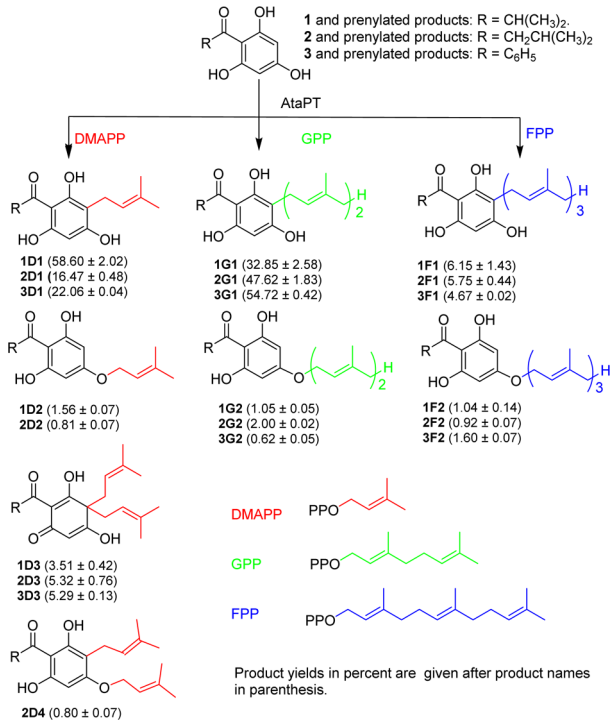
Scheme 2. Prenylations of 1−3 by AtaPT in the Presence of DMAPP, GPP, and FPP



Coexpression of different genes in *Saccharomyces cerevisiae* revealed that HIPT1L and HIPT2 catalyzed three sequential prenylation steps in the ß-bitter acid pathway. HIPT1L was confirmed to be an orthologue of HIPT1 identified by Tsurumaru et al.[13] Interestingly, HIPT2 was only active when it was co-expressed with HIPT1L. This led to the hypothesis that HIPT1L and HIPT2 form a metabolon as the catalytic unit.[12]

In recent years, significant progress has been achieved for the members of the dimethylallyltryptophan synthase (DMATS) superfamily, mainly from ascomycetous fungi.[14] These soluble enzymes use predominantly tryptophan and other indole derivatives as prenyl acceptors but also accept a broad spectrum of aromatic compounds as substrates.[14] Diprenylation of indole derivatives by one DMATS enzyme was observed in several cases.[15−17] However, a *gem*-diprenylation has not been reported for such enzymes or for other soluble prenyltransferases. The sole example of *gem*-diprenylation of an aromatic substrate was described for the metabolon of HIPT1L and HIPT2 mentioned above.[12]

In a previous study,[18] we demonstrated the prenylation of APs **1**−**3** by the soluble fungal prenyltransferase AnaPT from *Neosartorya fischeri*, which catalyzed the prenylation of (*R*)-benzodiazepinedinone, a cyclic dipeptide of tryptophan, and anthranilic acid.[19] The observed activities of AnaPT toward these substrates are much higher than that of a microsomal fraction containing the overproduced prenyltransferase HIPT1.[13] However, only monoprenylated derivatives were obtained in the presence of DMAPP, and the conversion yields of **1**−**3** with GPP as prenyl donor were very low.[18]

Very recently, a soluble prenyltransferase AtaPT from *Aspergillus terreus* strain A8-4 was demonstrated to carry an unprecedented promiscuity toward diverse aromatic acceptors and prenyl donors including DMAPP, geranyl diphosphate (GPP), and farnesyl diphosphate (FPP). AtaPT shares high sequence identity with the hypothetical protein EAU34068 encoded by ATEG_04999 from *A. terreus* NIH2624 and differs at only three residues. Among the tested aromatic substrates, AtaPT also consumed **3** in the presence of DMAPP, GPP, and FPP, although no noteworthy sequence homology exits between AtaPT and HIPT1L or HIPT2. These reactions were not studied in detail.[20]

In this study, we investigate the behavior of AtaPT toward the three APs **1**−**3**, which serve as precursors of most polyprenylated APs,[1−3,5] in the presence of DMAPP, GPP, and FPP. We hope to increase the structure diversity of prenylated APs by high conversion yields with GPP and FPP as well as by multiple prenylations, especially *gem*-diprenylations.

The coding sequence of AtaPT orthologue from *A. terreus* DSM 1958 was amplified by PCR and cloned into the expression vector pQE-70, resulting in the expression construct pCaW7 (see the Supporting Information for details). Sequence analysis revealed that differences at only four residues were found between AtaPT and its orthologue from DSM 1958. S230, T290, A292, and N373 in AtaPT were replaced by A230, K290, E292, and S373 in that of strain DSM 1958, respectively. Because these residues are not located in the active sites,[20] it can be expected that the orthologue from DSM 1958 will fulfill the function of AtaPT, and we therefore use hereafter the name AtaPT also for this enzyme. Gene expression in *E. coli* and purification of the soluble protein resulted in a predominant band on SDS-PAGE with a migration above the 45 kDa size marker, corresponding well to the calculated mass of 48.7 kDa for AtaPT-His$_6$. The protein yield was calculated to be 29 mg of purified protein per liter of culture (see the SI for details).

To compare the activities of AnaPT mentioned above and AtaPT toward **1**−**3**, incubations on a 100 μL scale, containing 20 μg of protein, DMAPP, GPP, or FPP, were carried out at 37 °C for 2 h. HPLC analysis revealed that **1**−**3** were much better converted by AtaPT than by AnaPT in all enzyme assays. Total conversion yields of 63.67 ± 2.43, 23.4 ± 1.26, and 27.35 ± 0.25% were calculated for **1**, **2**, and **3** with AtaPT and DMAPP, respectively. These values are significantly higher than those of AnaPT, with conversion yields of 3.64 ± 0.77, 4.33 ± 0.52, and 7.66 ± 0.49%, respectively (Scheme 2; see the SI for details). The poor acceptance of GPP by AnaPT was confirmed in this study, and product formation was only detected in the reaction mixtures of **1** and **3** with GPP. In contrast, GPP served as a very good prenyl donor for the AtaPT reactions, with product yields of 32.85 ± 2.58, 47.62 ± 1.83, and 54.72 ± 0.42% for **1**, **2**, and **3**, respectively (Scheme 2; see the SI for details). FPP was accepted by AtaPT with conversion yields of 7.19 ± 1.57, 6.66 ± 0.51, and 6.27 ± 0.09% for **1**, **2**, and **3**, respectively (Scheme 2; see the SI for details).

Only one product each was detected in the AnaPT reactions of **1**−**3** with DMAPP as well as those of **1** and **2** with GPP. These products were identified as predominant ones in the AtaPT reactions. Isolation and structure elucidation by NMR and MS analyses (see the SI for details) proved the main products of the AtaPT reactions to be monoprenylated derivatives; that is **1D1**−**3D1** with DMAPP, being consistent with the AnaPT products,[18] **1G1**−**3G1** with GPP, and **1F1**−**3F1** with FPP (Scheme 2, see the SI for details).

Additional product peaks with longer retention times were also detected in the reaction mixtures of AtaPT with **1**−**3** in the presence of all three prenyl donors (see the SI for details). The intensities of these peaks are clearly increased in the incubation mixtures with 50 μg of protein at 37 °C for 2 h, which were

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detected by LC−MS analysis (Figure 2). In the incubation mixtures of **1**−**3** with GPP and FPP, two monoprenylated

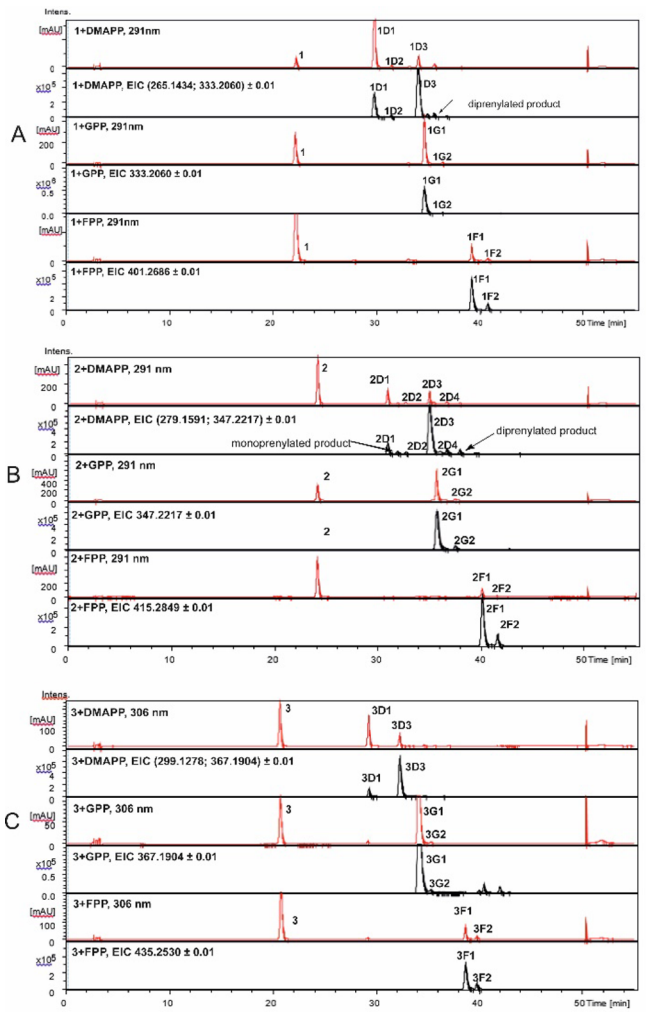


Figure 2. LC−MS analysis of the reaction mixtures of DMAPP, GPP, or FPP with **1** (A), **2** (B), and **3** (C) after incubation with 50 μg of AtaPT per 100 μL at 37 °C for 2 h. Red lines are UV absorptions, and black lines are extracted positive-ion chromatograms (EIC).

derivatives each were detected. In addition to the *C*-prenylated **1G1**−**3G1** and **1F1**−**3F1**, one *O*-prenylated derivative each, **1G2**, **2G2**, **3G2**, **1F2**, **2F2**, or **3F2** ,was identified by NMR and MS analyses as a minor product after isolation and structure elucidation (Scheme 2 and Figure 2; see the SI for details).

In the incubation mixtures of **1** with DMAPP, two mono- and two diprenylated derivatives were detected. In comparison, three mono- and three diprenylated derivatives were found in the reaction mixture of **2** with DMAPP and one mono- and one diprenylated products of **3** with DMAPP. Isolation and structure elucidation proved the presence of *O*-prenylated **1D2** and **2D2** in the reaction mixtures of **1** and **2**, respectively. Interestingly, *gem*-diprenylated derivatives **1D3**, **2D3**, and **3D3** were identified in the reaction mixtures of **1**, **2**, and **3**, respectively (Figure 2 and Scheme 2; see the SI for details).

These results proved the ability of AtaPT for a *gem*-dipenylation of acylphloroglucinols and encouraged us to investigate the conversion of the monoprenylated **1D1**, **2D1**, and **3D1** by AtaPT. **1D1**, **2D1**, and **3D1** were then incubated in the presence of DMAPP with 50 μg of AtaPT at 37 °C for 2 h. LC−MS analysis revealed clear conversion by detection of the *gem*-diprenylated derivatives **1D3**, **2D3**, and **3D3** as predominant products, with product yields of 4.88 ± 0.15, 57.18 ± 1.07, and 33.31 ± 0.23%, respectively (Figure 3). Better

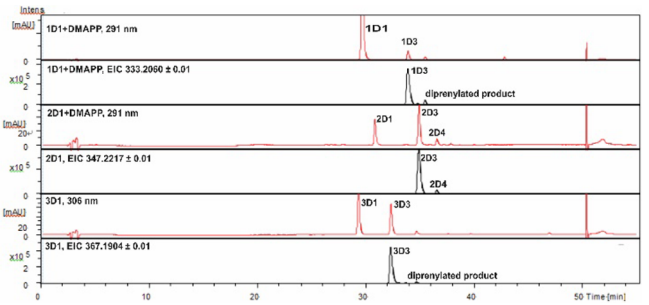
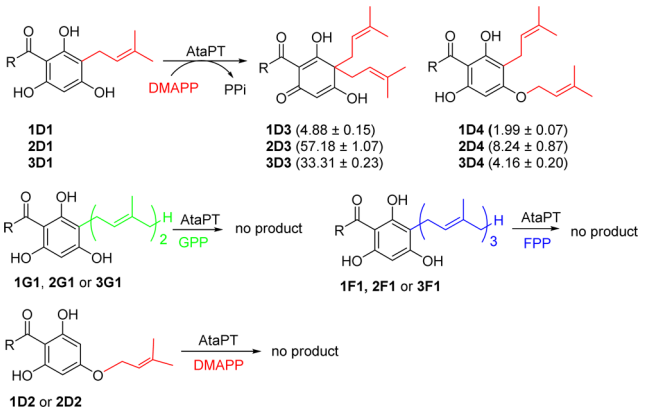


Figure 3. LC−MS analysis of AtaPT reactions (50 μg per 100 μL, 37 °C for 2 h) with DMAPP and **1D1**, **2D1**, or **3D1**. Red lines are UV absorptions, and black lines are extracted positive-ion chromatograms (EIC).

conversion of **2D1** and **3D1** than **1D1** confirmed the higher product yields of **2D3** and **3D3** in the incubation mixtures of **2** and **3** than **1D3** in that of **1** (Figure 2). A minor diprenylated product each with larger retention times and product yields of 1.99 ± 0.07, 8.24 ± 0.87, and 4.16 ± 0.20%, respectively, was also detected. The second product **2D4** from the incubation mixture of **2D1** was proven to be a *C*- and *O*-diprenylated derivative, which was also isolated and identified from the incubation mixture of **2** with DMAPP (Figures 2 and 3 and Schemes 2 and 3; see the SI for details). The second

Scheme 3. Reactions of Monoprenylated Derivatives with AtaPT



diprenylated derivative of the reaction mixtures with **1D1** and **3D1** could not be isolated and identified. However, on the basis of their retention times and UV spectra together with LC−MS data, it can be speculated that these compounds are also *C*- and *O*-diprenylated derivatives.

These results provide evidence for successive diprenylations of **1**, **2**, and **3** by AtaPT, which was also confirmed by time dependence of the product formation in the incubation mixtures of **2** and **3** with DMAPP. The formation of **2D3** and **3D3** increased continuously, while **2D1** and **3D1** reached their maxima in short time and decreased after that (see the SI for details). No product formation was detected in the incubation mixtures of **1D1**, **2D1**, and **3D1** with AnaPT and DMAPP under the same conditions (data not shown).

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As mentioned above, no diprenylated product was detected in the incubation mixtures of AtaPT with **1**−**3** and GPP or FPP (Figure 2 and Scheme 2). This was confirmed by incubation of their *C*-monoprenylated derivatives. Product formation was not observed for the reaction mixtures of **1G1**, **2G1**, and **3G1** with AtaPT in the presence of GPP or those of **1F1**, **2F1**, and **3F1** with FPP (Scheme 3). Incubation of **1D1**, **2D1**, and **3D1** with AtaPT and GPP or FPP did not result in product formation. A previous study showed that AtaPT also catalyzed *C*-diprenylations of several aromatic acceptors in the presence of GPP and FPP, although no *gem*-prenylated products were detected.[20] No formation of diprenylated **1**−**3** with these donors resulted from their orientations in the reaction cavity. No product formation was detected by LC−MS analysis after incubation of **1D2** and **2D2** with 50 μg of AtaPT and DMAPP at 37 °C for 2 h (Scheme 3), excluding the possible formation of *C*- and *O*-diprenylated derivatives from *O*-monoprenylated products.

To obtain more insights into the catalytic efficiency of the tetrameric AtaPT, kinetic parameters including Michaelis−Menten constants (K_M) and turnover numbers (k_{cat}), were determined at pH 7.5 for **1**, **2**, and **3** in the presence of DMAPP, GPP, and FPP as well as DMAPP with **1** and GPP with **3** (see the SI for details). The catalytic efficiency of AtaPT toward **1** is 32-fold of that of AnaPT in the presence of DMAPP. compound **1** was better consumed by AtaPT in the presence of DMAPP and FPP than **2** and **3**. Compound **3** was most efficiently consumed in the presence of GPP. The determined k_{cat}/K_M values for **1**−**3** are in the range of 170−300 s^{-1} M^{-1} in the presence of GPP and between 17 and 37 s^{-1} M^{-1} in the presence of FPP. With **1** as acceptor, a 95-fold k_{cat}/K_M value of that of AnaPT was determined for DMAPP with AtaPT (see Table S8 for details).

In conclusion, we have provided in this study the first example of *gem*-diprenylation of APs by a member of the DMATS superfamily, proving their unprecedented application potential. AtaPT could be an interesting candidate for production of polyprenylated APs like β-bitter acids by synthetic biology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03594.

Experimental procedures, detailed NMR data, HR-ESI-MS data, determination of kinetic parameters, as well as NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Ciochina, R.; Grossman, R. B. *Chem. Rev.* **2006**, *106*, 3963.
(2) Richard, J. A.; Pouwer, R. H.; Chen, D. Y. *Angew. Chem., Int. Ed.* **2012**, *51*, 4536.
(3) Wu, S.-B.; Long, C.; Kennelly, E. J. *Nat. Prod. Rep.* **2014**, *31*, 1158.
(4) Van Cleemput, M.; Cattoor, K.; De Bosscher, K.; Haegeman, G.; De Keukeleire, D.; Heyerick, A. *J. Nat. Prod.* **2009**, *72*, 1220.
(5) Kobayashi, J.; Tanaka, N. *Heterocycles* **2015**, *90*, 23.
(6) Garnsey, M. R.; Matous, J. A.; Kwiek, J. J.; Coltart, D. M. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2406.
(7) Sales, L.; Pezuk, J. A.; Borges, K. S.; Brassesco, M. S.; Scrideli, C. A.; Tone, L. G.; dos Santos, M. H.; Ionta, M.; de Oliveira, J. C. *BMC Complementary Altern. Med.* **2015**, *15*, 393.
(8) Piccinelli, A. L.; Cuesta-Rubio, O.; Chica, M. B.; Mahmood, N.; Pagano, B.; Pavone, M.; Barone, V.; Rastrelli, L. *Tetrahedron* **2005**, *61*, 8206.
(9) Nagalingam, S. V.; Wai-Ling, K.; Teng-Jin, K. *Planta Med. Lett.* **2016**, *3*, e10−e13.
(10) Beerhues, L.; Liu, B. *Phytochemistry* **2009**, *70*, 1719.
(11) Okada, Y.; Ito, K. *Biosci., Biotechnol., Biochem.* **2001**, *65*, 150.
(12) Li, H.; Ban, Z.; Qin, H.; Ma, L.; King, A. J.; Wang, G. *Plant Physiol.* **2015**, *167*, 650.
(13) Tsurumaru, Y.; Sasaki, K.; Miyawaki, T.; Uto, Y.; Momma, T.; Umemoto, N.; Momose, M.; Yazaki, K. *Biochem. Biophys. Res. Commun.* **2012**, *417*, 393.
(14) Winkelblech, J.; Fan, A.; Li, S.-M. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 7379.
(15) Fan, A.; Li, S.-M. *Adv. Synth. Catal.* **2013**, *355*, 2659.
(16) Liu, C.; Noike, M.; Minami, A.; Oikawa, H.; Dairi, T. *Biosci., Biotechnol., Biochem.* **2014**, *78*, 448.
(17) Winkelblech, J.; Li, S.-M. *ChemBioChem* **2014**, *15*, 1030.
(18) Zhou, K.; Ludwig, L.; Li, S.-M. *J. Nat. Prod.* **2015**, *78*, 929.
(19) Yin, W.-B.; Grundmann, A.; Cheng, J.; Li, S.-M. *J. Biol. Chem.* **2009**, *284*, 100.
(20) Chen, R.; Gao, B.; Liu, X.; Ruan, F.; Zhang, Y.; Lou, J.; Feng, K.; Wunsch, C.; Li, S.-M.; Dai, J.; Sun, F. *Nat. Chem. Biol.* **2016**, DOI: 10.1038/nchembio.2263.

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