ACS Catalysis

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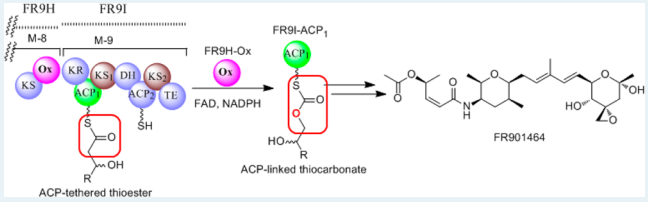
Baeyer–Villiger Oxidation of Acyl Carrier Protein-Tethered Thioester to Acyl Carrier Protein-Linked Thiocarbonate Catalyzed by a Monooxygenase Domain in FR901464 Biosynthesis

Man-Cheng Tang, Hai-Yan He, Feng Zhang, and Gong-Li Tang*

State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 345 Lingling Road, Shanghai, 200032, China

Supporting Information

ABSTRACT: Baeyer–Villiger monooxygenases (BVMOs), generally catalyzing the transformation of carbonylic compounds into the corresponding esters or lactones known as Baeyer–Villiger oxidation in organic chemistry, are widely distributed among microorganisms and have stimulated great interest as biocatalysts for organic synthesis. The physiological roles of this type of MOs are usually classified as degradation of organic compounds involved in primary metabolism. Recently, increasing numbers of BVMOs have been found to be involved in the biosynthesis of secondary metabolites, especially for postmodification; however, to date, none of them has been reported functionally as a tailoring domain within polyketide synthase (PKS) acting on carrier protein-tethered substrates. FR901464, an antitumor natural product that targets spliceosome and inhibits both splicing and nuclear retention of pre-mRNA, was elucidated to be biosynthesized by a hybrid acyltransferase-less PKS/nonribosomal peptide synthetase (NRPS) system. Within the hybrid system, an unprecedented domain that was proposed to mediate the chain release process was located in the termination module. In this paper, we report the in vitro biochemical characterization of this domain to be a BVMO tailoring domain that catalyzes the BV oxidation of an acyl carrier protein (ACP)-tethered thioester to an ACP-linked thiocarbonate, which represents the first example of BVMOs operating in cis within the PKS and NRPS biosynthetic paradigm.



KEYWORDS: *Baeyer–Villiger monooxygenase, tailoring domain, thioester, thiocarbonate, FR901464*

Baeyer–Villiger monooxygenases (BVMOs), typified by cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871, represent a specific class of monooxygenases that generally catalyze the transformation of ketones into esters or cyclic ketones into lactones, which is well-known as the Baeyer–Villiger (BV) oxidation reaction in organic chemistry.[1,2] Because they usually display high regio- and enantioselectivity, BVMOs have stimulated great interest as biocatalysts for organic synthesis, especially in the chiral BV oxidation reactions.[2,3] Since the first BVMO was purified to homogeneity and shown to be a flavoprotein with broad substrate tolerance in 1976,[1] a large number of BVMOs have been discovered, cloned, and characterized. Members of this type of enzymes are found to be widely distributed among different microorganisms, such as actinomycetes and filamentous fungi. Research in the past few decades has revealed that BVMOs are usually involved in the primary metabolism enabling microbes to grow on a variety of ketones,[4] and increasing numbers of BVMOs have also been discovered to be crucial for the biosynthesis of secondary metabolites, exemplified by the biochemical characterization of MtmOIV involved in the biosynthesis of mithramycin[5] and PtlE involved in the biosynthesis of neopentalenolactone.[6] In most cases, the substrates of BVMOs are small molecular compounds. Recently, a few numbers of BVMOs were proposed to catalyze the BV oxidation on acyl carrier protein (ACP)-tethered substrates in trans, exemplified by PedG in the pederin biosynthetic pathway[7] and AufJ in the aurafuron biosynthetic pathway;[8] however, to date, none of these BVMOs has been characterized to be a tailoring domain within polyketide synthase (PKS) acting in cis on ACP-tethered substrates.

FR901464 (Figure 1A), isolated from the fermentation broth of the bacterium *Pseudomonas* sp. no. 2663, is an antitumor natural product representing a new class of potent anticancer small molecules that target the spliceosome and inhibit both splicing and nuclear retention of pre-mRNA.[9] Recently, we identified the biosynthetic gene cluster of FR901464 and revealed that FR901464 is biosynthesized by a hybrid acyltransferase-less (AT-less) PKS/nonribosomal peptide synthetase (NRPS) system featuring both an isoprenoid-like β-branching process and incorporation of a glycolytic intermediate as the PKS start unit.[10] In addition, two different strategies were proposed to be employed to form the two tetrahydropyran moieties of FR901464. The first one was proposed to be formed by intramolecular Michael-type addition

Received: December 17, 2012
Revised: January 30, 2013
Published: February 8, 2013

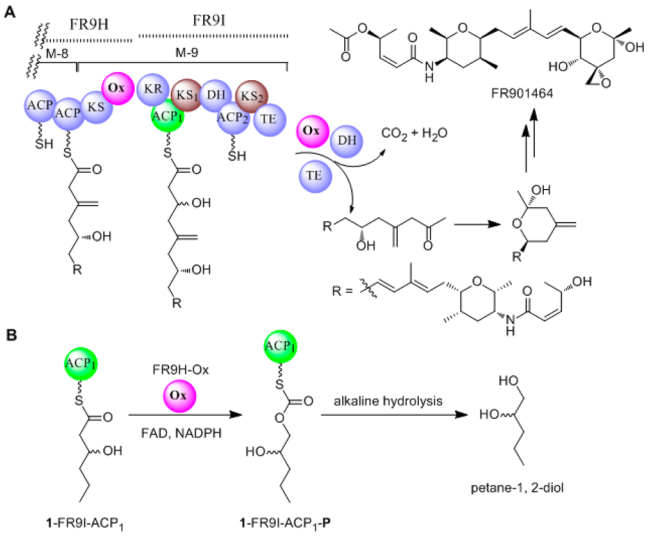


Figure 1. Chain termination module of the hybrid PKS/NRPS in FR901464 biosynthesis. (A) Annotation of the termination module (M-9) of the hybrid PKS/NRPS: ACP, acyl carrier protein; DH, dehydratase; KR, ketoreductase; KS, ketosynthase; Ox, putative monooxygenase; TE, thioesterase. (B) Proposed reaction catalyzed by FR9H-Ox with model substrate **1**-FR9I-ACP$_1$.

that was catalyzed by a putative cyclase, FR9Q. The second one was deduced to derive from a linear polyketide (PK)/nonribosomal peptide (NRP) hybrid precursor by intramolecular addition of a hydroxyl group to a ketone (Figure 1A).[10] Instead of a typical terminal carboxyl, aldehyde, or alcohol group found in PKs/NRPs,[11] this precursor contains a terminal methyl ketone moiety.

Moreover, sequence analysis of the hybrid PKS/NRPS assembly line revealed an unprecedented chain termination module (module 9, Figure 1A), which contains nine domains with unique organization, of which, the two β-ketoacyl synthase (KS) domains of FR9I were assigned inactive for decarboxylation-chain elongation because of a conserved catalytic triad of C−H−H replaced by C−Q−H or C−H−A.[10] In addition, a putative monooxygenase-like domain (FR9H-Ox) was flanked by a KS domain and a ketoreductase (KR) domain, which contains two Rossman fold motifs (GXGXXG) and a motif with the amino acid sequence FXGXXXHXXXY that are similar to the motifs described in flavin-containing monooxygenases (FMOs) and type I BVMOs.[10,12]

On the basis of the predicted full-length intermediate tethered to the first ACP domain of FR9I (FR9I-ACP$_1$, Figure 1A), a series of reactions, including thioester oxidation, dehydration, and decarboxylation, are required to form the terminal methyl ketone structure. According to the canonical function of the DH domain and the thioesterase (TE) domain, the FR9I-DH and FR9I-TE was proposed to catalyze the last steps (dehydration and decarboxylation) to release the intermediate from ACP. Therefore, the FR9H-Ox was the only candidate proposed to catalyze the thioester oxidation. Herein, we report the in vitro biochemical characterization of FR9H-Ox as a BVMO catalyzing the transformation of ACP-tethered thioester into ACP-linked thiocarbonate (Figure 1B), which represents the first characterized example of BVMOs operating in cis within the PKS and NRPS biosynthetic paradigm.

To validate the proposed function of FR9H-Ox, recombinant N-terminal His$_6$-tagged FR9H-Ox (single domain constructs) was cloned, expressed in *Escherichia coli*, and purified to homogeneity (Supporting Information Figure S1A). The purified protein solution was bright yellow (absorbance maxima at 376 and 444 nm, Supporting Information Figure S2A), suggesting that it may contain a flavin-like cofactor,[13] and the colored material was released from the denatured enzyme by heating. After removing the denatured enzyme by centrifugation, the supernatant was subjected to HPLC/MS analysis, and the colored agent expectedly turned out to be flavin adenine dinucleotide (FAD) (Supporting Information Figure S2B). These data confirmed that FR9H-Ox is a FAD-dependent enzyme.

Then, we sought to further characterize the deduced function of FR9H-Ox in vitro. FR9I-ACP$_1$ was cloned and overexpressed as C-terminal His$_8$-tagged single domain constructs (Supporting Information Figure S1B) in apo form to generate ACP-linked substrates. A simplified model substrate, 3-hydroxyhexanoyl-FR9I-ACP$_1$ (**1**-FR9I-ACP$_1$, Figure 1B), was designed to mimic the full-length chain intermediate tethered to FR9I-ACP$_1$ (Figure 1A), and the substrate **1**-FR9I-ACP$_1$ was generated by loading the acyl-CoA substrate (**1**-CoA) to the apo FR9I-ACP$_1$ by using Sfp, a promiscuous phosphopantetheinyl transferase from *Bacillus subtilis*.[14] We then incubated **1**-FR9I-ACP$_1$ with FR9H-Ox, FAD, and NADPH to directly test the oxidation reaction catalyzed by FR9H-Ox. By reversed-phase HPLC analysis, a peak eluting at 18.3 min appeared that depended on the presence of NADPH, and the production increased by more incubation time (Figure 2A). When subjected to quadrupole time-of-flight/MS (Q-TOF-MS) analysis, the unique product had a mass of 13 234.0 Da, 16

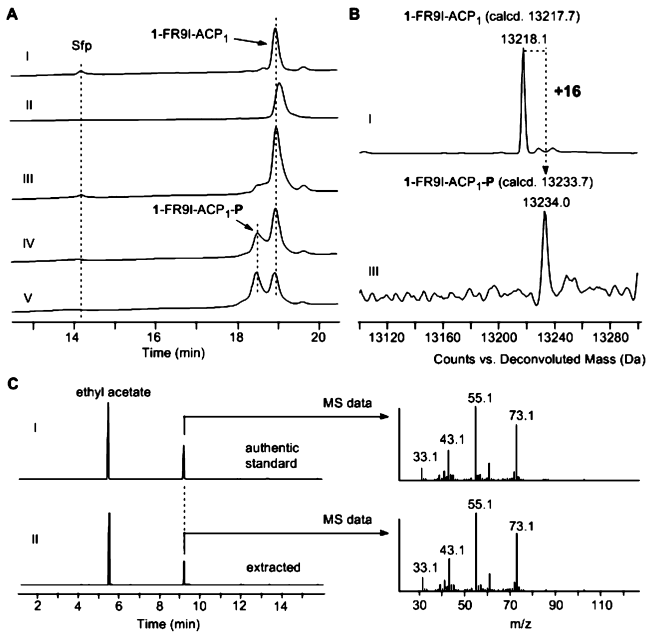


Figure 2. Analysis of FR9H-Ox reactions with **1**-FR9I-ACP$_1$. (A) HPLC analysis of **1**-FR9I-ACP$_1$ (I); FR9H-Ox (II); reaction mixtures of FR9H-Ox with **1**-FR9I-ACP$_1$ in the absence of NADPH (III, control) and in the presence of NADPH at different times (IV, 30 min; V, 60 min). (B) Q-TOF-MS analysis of **1**-FR9I-ACP$_1$ (I) and **1**-FR9I-ACP$_1$-**P** (II). (C) GC/MS data of small molecule compound extracted from the hydrolysis solution of **1**-FR9I-ACP$_1$-**P** (II) with authentic standard of pentane-1,2-diol (I).

dx.doi.org/10.1021/cs300819e | *ACS Catal.* 2013, 3, 444−447

Da larger than **1**-FR9I-ACP$_1$ (eluted at 18.9 min; mass detected: 13 218.1 Da (calcd. 13 217.7 Da)), which is in agreement with the mass increase for an oxygen insertion. To further investigate, the small molecular compound tethered to FR9I-ACP$_1$ was released by alkaline hydrolysis, extracted with ethyl acetate, and analyzed by GC/MS (see the Supporting Information). A peak eluting at 9.1 min was identified to be petane-1,2-diol by comparison with the authentic standard (Figure 2C). All these results indicated that a thiocarbonate linked to FR9I-ACP$_1$ (**1**-FR9I-ACP$_1$-**P**, Figure 1B) was formed after the treatment of **1**-FR9I-ACP$_1$ with FR9H-Ox in the presence of cofactors, which is similar to the transformation of BV oxidation catalyzed by BVMOs. Therefore, FR9H-Ox was definitely validated to be a FAD-dependent BVMO-tailoring domain that catalyzes the BV oxidation of thioester substrate tethered to ACP to form an ACP-linked thiocarbonate. To the best of our knowledge, this is the first time that a BVMO has been biochemically characterized to be a tailoring domain within PKSs catalyzing the BV oxidation of ACP-tethered thioester substrate.

To date, three types of BVMOs have been classified:[2] type I, containing a tightly bound FAD cofactor, NADPH-dependent, possessing a fingerprint motif sequence FXGXXXHXXXW (P/D), and belonging to class B FMOs; type II, a two-component system using FMN and NAD(P)H as cofactors, belonging to class C FMOs; and type "O" BVMOs, also known as "atypical" BVMOs, represented by MtmOIV, belonging to class A FMOs. Both sequence analysis (Supporting Information Figure S8) and the in vitro data indicated that FR9H-Ox belongs to type I BVMOs, which was further supported by phylogenetic analysis of FR9H-Ox with members from all three types of BVMOs (Figure 3). Moreover, the phylogenetic analysis results also indicated that, distinct from other members of type I BVMOs, which usually catalyze the BV oxidation of ketones or cyclic ketones, FR9H-Ox and its homologues, PedG (30% identity, from symbiont bacterium of *Paederus fuscipes*, AAS47561.1)[7] and BVMO bt (86% identity, from *Burkholderia thailandensis* MSMB43, ZP_02468377.1), fall into a clade of BVMOs that characterized or proposed to catalyze the BV oxidation of ACP-tethered thioester. These results also implied that FR9H-Ox-like BVMOs may be widespread in different microbes and involved in the biosynthesis of secondary metabolites.

As reported in the literature, type I BVMOs usually display a broad substrate acceptance profile,[2,3,15] and in the chain elongation process of PKS, the β-oxidation states of intermediates are various: β-keto; β-OH; α, β-enoyl; or β-CH$_2$. We thus prepared three other substrates (**2**-FR9I-ACP$_1$, **3**-FR9I-ACP$_1$, and **4**-FR9I-ACP$_1$; see Figure 4) with different β-oxidation states to test the substrate tolerance of FR9H-Ox. When tested on **2**-FR9I-ACP$_1$, a newly formed peak appeared in the HPLC trace (Supporting Information Figure S3A), and the corresponding MS data of this product was in good agreement with the expected BV oxidation product, **2**-FR9I-ACP$_1$-**P** (Supporting Information Figure S3B). The compound released from the product was identified as 1-hydroxypentan-2-one (Supporting Information Figure S3C), further supporting this BV oxidation catalyzed by FR9H-Ox. Similar results were also obtained when **3**-FR9I-ACP$_1$ was used as the substrate, although the conversion efficiency was low (Supporting Information Figure S4). However, when tested on **4**-FR9I-ACP$_1$, no BV oxidation product was detected under the same conditions (Supporting Information Figure S5). In addition, the relative reactivity of FR9H-Ox toward these substrates with

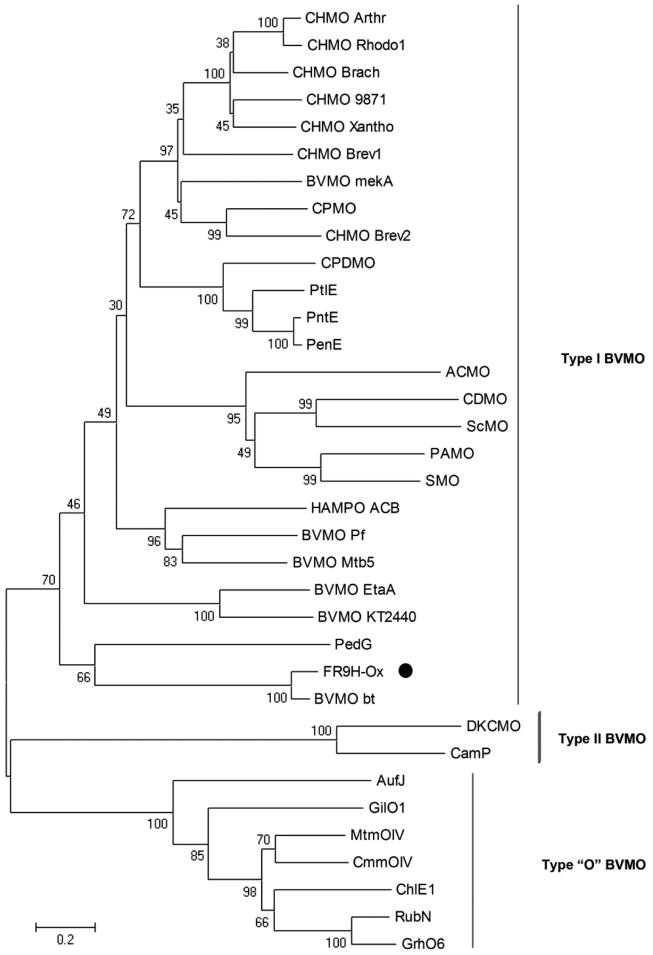
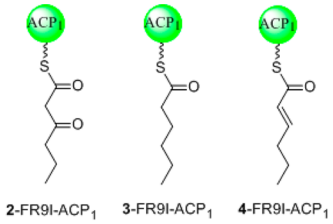


Figure 3. Phylogenetic analysis of FR9H-Ox with other members of BVMOs. ACMO, acetone monooxygenase; CDMO, cyclododecanone monooxygenase; CHMO, cyclohexanone monooxygenase; CPDMO, cyclopentadecanone monooxygenase; CPMO, cyclopentanone monooxygenase; DKCMO, diketocamphane monooxygenase; HAMPO, 4-hydroxyacetophenone monooxygenase; PAMO, phenylacetone monooxygenase; SMO, steroid monooxygenase. The neighbor-joining phylogenetic tree was constructed by using MEGA v5.0 with 500 bootstrap replicates. The scale 0.2 is the genetic distance. The detailed information about the selected BVMOs shown in this figure is presented in the Supporting Information.



Substrates	Relative activity
1-FR9I-ACP$_1$	++++
2-FR9I-ACP$_1$	+++
3-FR9I-ACP$_1$	+
4-FR9I-ACP$_1$	--

Figure 4. Other model substrates with different β-oxidation states prepared for testing the substrate tolerance of FR9H-Ox and their relative reactivity compared with **1**-FR9I-ACP$_1$.

different β-oxidation states (shown in Figure 4) was estimated from the time course experiments based on the HPLC analysis of the production of BV oxidation products. The results indicated that the model substrate with β-hydroxy (**1**-FR9I-ACP$_1$) was the preferred substrate, which was in good agreement with the proposed function of FR9H-Ox in vivo

dx.doi.org/10.1021/cs300819e | ACS Catal. 2013, 3, 444−447

that it catalyzes the BV oxidation of ACP-tethered substrate when the β-keto group was reduced to a hydroxy group. Moreover, these results not only indicated that, like other BVMOs, FR9H-Ox also displayed some substrate tolerance, but also suggested that the β-oxidation state of ACP-tethered substrates may play an important role in the BV oxidation process catalyzed by FR9H-Ox. It also implied that this novel BVMO tailoring domain might represent a new useful tool for combinatory biosynthesis.

To further investigate whether the interaction between FR9H-Ox and the ACP domain is essential for this BV oxidation biotransformation, acyl-*S*-*N*-acetylcysteamine (acyl-SNAC) derivatives (**1**-SNAC, **3**-SNAC, and **4**-SNAC) were chemically synthesized and tested in the in vitro assays; however, no expected BV oxidation products could be detected (Supporting Information Figure S6), suggesting the protein interaction of FR9H-Ox and the ACP domain is crucial for the catalysis of FR9H-Ox, which is further supported by the whole-cell biotransformation experiments of FR9H-Ox using the conventional BVMO substrates (cyclobutanone, cyclopentanone, and cyclohexanone) as the substrates that only trace amounts of BV oxidation products could be detected from the GC/MS analysis.

In conclusion, the putative monooxygenase-like domain, FR9H-Ox, was biochemically characterized to be a member of type I BVMOs that catalyzes the BV oxidation of ACP-tethered thioester to ACP-linked thiocarbonate. These findings provide the first example of BVMOs that act as a tailoring domain within the PKS/NRPS biosynthetic paradigm.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, SDS-PAGE, HPLC chromatograms, MS data of proteins, GC/MS data, and sequence alignments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: gltang@sioc.ac.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Prof. Zixin Deng's laboratory at Shanghai Jiao-Tong University for support in obtaining the MS data of the proteins. This work was supported in part by grants from the National Basic Research Program of China (973 Program) 2010CB833200 and 2012CB721100 and the National Natural Science Foundation of China (31200054 and 20921091).

■ REFERENCES

(1) Donoghue, N. A.; Norris, D. B.; Trudgill, P. W. *Eur. J. Biochem.* **1976**, *63*, 175.
(2) Leisch, H.; Morley, K.; Lau, P. C. *Chem. Rev.* **2011**, *111*, 4165.
(3) de Gonzalo, G.; Mihovilovic, M. D.; Fraaije, M. W. *ChemBioChem* **2010**, *11*, 2208.
(4) (a) Britton, L. N.; Markovetz, A. J. *J. Biol. Chem.* **1977**, *252*, 8561. (b) Ougham, H. J.; Taylor, D. G.; Trudgill, P. W. *J. Bacteriol.* **1983**, *153*, 140. (c) Darby, J. M.; Taylor, D. G.; Hooper, D. J. *J. Gen. Microbiol.* **1987**, *133*, 2137. (d) van der Werf, M. F.; Boot, A. M. *Microbiology* **2000**, *146*, 1129.
(5) (a) Gibson, M.; Nur-e-alam, M.; Lipata, F.; Oliveira, M. A.; Rohr, J. *J. Am. Chem. Soc.* **2005**, *127*, 17594. (b) Beam, M. P.; Bosserman, M. A.; Noinaj, N.; Wehenkel, M.; Rohr, J. *Biochemistry* **2009**, *48*, 4476.
(6) Jiang, J.; Tetzlaff, C. N.; Takamatsu, S.; Iwatsuki, M.; Komatsu, M.; Ikeda, H.; Cane, D. E. *Biochemistry* **2009**, *48*, 6431.
(7) Piel, J. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14002.
(8) Frank, B.; Wenzel, S. C.; Bode, H. B.; Scharfe, M.; Blöcker, H.; Müller, R. *J. Mol. Biol.* **2007**, *374*, 24−38.
(9) (a) Nakajima, H.; Sato, B.; Fujita, T.; Takase, S.; Terano, H.; Okuhara, M. *J. Antibiot.* **1996**, *49*, 1196. (b) Nakajima, H.; Sato, B.; Fujita, T.; Takase, S.; Terano, H.; Okuhara, M. *J. Antibiot.* **1996**, *49*, 1204. (c) Nakajima, H.; Takase, S.; Terano, H.; Tanaka, H. *J. Antibiot.* **1997**, *50*, 96. (d) Kaida, D.; Motoyo-shi, H.; Tashiro, E.; Nojima, T.; Hagiwara, M.; Ishigami, K.; Watanabe, H.; Kitahara, T.; Yoshida, T.; Nakajima, H.; Tani, T.; Horinouchi, S.; Yoshida, M. *Nat. Chem. Biol.* **2007**, *3*, 576. (e) Lo, C.-W.; Kaida, D.; Nishimura, S.; Matsuyama, A.; Yashiroda, Y.; Taoka, H.; Ishigami, K.; Watanabe, H.; Naka-jima, H.; Tani, T.; Horinouchi, S.; Yoshida, M. *Biochem. Biophys. Res. Commun.* **2007**, *364*, 573.
(10) Zhang, F.; He, H.-Y.; Tang, M.-C.; Tang, Y.-M.; Zhou, Q.; Tang, G.-L. *J. Am. Chem. Soc.* **2011**, *133*, 2452.
(11) Du, L.; Lou, L. *Nat. Prod. Rep.* **2010**, *27*, 255.
(12) (a) Fraaije, M. W.; Kamerbeek, N. M.; van Berkel, W. J. H.; Janssen, D. B. *FEBS Lett.* **2002**, *518*, 43. (b) van Berkel, W. J. H.; Kamerbeek, N. M.; Fraaije, M. W. *J. Biotechnol.* **2006**, *124*, 670.
(13) Karplus, P. A.; Fox, K. M.; Massey, V. *FASEB J.* **1995**, *9*, 1518.
(14) Quadri, L. E.; Weinreb, P. H.; Lei, M.; Nakano, M. M.; Zuber, P.; Walsh, C. T. *Biochemistry* **1998**, *37*, 1585.
(15) Mihovilovic, M. D.; Müller, B.; Stanetty, P. *Eur. J. Org. Chem.* **2002**, *22*, 3711.