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Structurally Diverse Chaetophenol Productions Induced by Chemically Mediated Epigenetic Manipulation of Fungal Gene Expression

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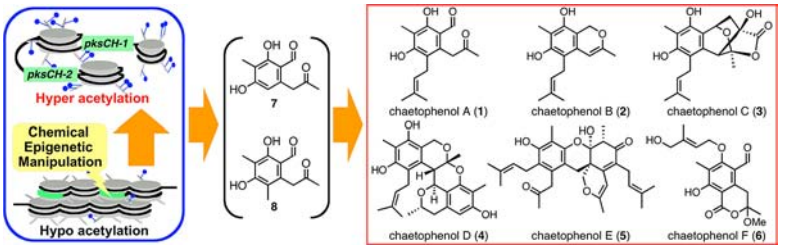
ABSTRACT



Epigenetic manipulation of gene expression in *Chaetomium indicum* using a HDAC inhibitor led to the isolation of structurally diverse chaetophenols, and 3, 4 and 5 bear unprecedented polycyclic skeletons. The expression of two silent genes (*pksCH-1* and *pksCH-2*) for nonreducing PKSs involved in chaetophenol biosynthesis was associated with an increase of histone acetylation level. The heterologous gene expression study in *Aspergillus oryzae* revealed *pksCH-2* to be the NR-PKS gene for 8.

As fungal genome sequencing has progressed, it has become apparent that fungi possess far more polyketide-encoding biosynthetic gene clusters than was evident from previous chemical studies.[1] This observation suggests that many of the gene clusters that produce uncharacterized natural products are transcriptionally suppressed under normal laboratory culture conditions.[2] One promising approach to activate the attenuated or silenced genes relies on the fact that chromatin-based epigenetic regulation of gene expression is deeply involved in fungal secondary metabolism.[3] Histone deacetylases (HDACs) have been found to inactivate biosynthetic gene expression in the genera *Aspergillus*.[4] Small molecule HDAC inhibitors have been shown to induce transcriptional up-regulation of many PKS genes in a model fungus, *A. niger*.[5] Epigenetic manipulation of fungal gene expression using HDAC inhibitors was thus expected to be an effective method for accessing natural products obtained from cryptic biosynthetic

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(1) (a) Sanchez, J. F.; Somoza, A. D.; Keller, N. P.; Wang, C. C. C. *Nat. Prod. Rep.* **2012**, *29*, 351–371. (b) Khaldi, N.; Seifuddin, F. T.; Turner, G.; Haft, D.; Nierman, W. C.; Wolfe, K. H.; Fedorova, N. D. *Fungal Genet. Biol.* **2010**, *47*, 736–741.

(2) Scherlach, K.; Hertweck, C. *Org. Biomol. Chem.* **2009**, *7*, 1753–1760.

(3) (a) Strauss, J.; Reyes-Dominguez, Y. *Fungal Genet. Biol.* **2011**, *48*, 62–69. (b) Gacek, A.; Strauss, J. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 1389–1404. (c) Brakhage, A. A. *Nat. Rev.* **2013**, *11*, 21–32.

(4) (a) Shwab, E. K.; Bok, J. W.; Tribus, M.; Galehr, J.; Graessle, S.; Keller, N. P. *Eukaryot. Cell* **2007**, *6*, 1656–1664. (b) Lee, I.; Oh, J. H.; Shwab, E. K.; Dagenais, R. T.; Andes, D.; Keller, N. P. *Fungal Genet. Biol.* **2009**, *46*, 782–790.

(5) Fisch, K. M.; Gillaspy, A. F.; Gipson, M.; Henrikson, J. C.; Hoover, A. R.; Jackson, L.; Najar, F. Z.; Wägele, H.; Cichewicz, R. H. *J. Ind. Micrbiol. Biotechnol.* **2009**, *36*, 1199–1213.

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pathways. Indeed, the studies using the method were carried out to give a handful of new polyketides from several *Aspergillus* and *Penicillium* fungi.[6] To further develop the method and search for novel skeletal polyketides to enlarge the chemical space composed of natural products, we applied HDAC inhibitors in our search of novel skeletal fungal polyketides and showed that the method is quite versatile.[7] Experiments are needed to provide direct evidence that induction of fungal secondary metabolites production by HDAC inhibitors is directly associated with chromatin-based epigenetic regulation through histone acetylation.

In our continuing search, we found that the cultivation of *Chaetomium indicum* in the presence of suberoyl bishydroxamic acid (SBHA), an HDAC inhibitor, yielded significantly enhanced polyketide productions and led to the isolation of six novel prenylated aromatic polyketides, chaetophenols A−F (**1**−**6**) (Figure 1). Among these, compounds **3**, **4**, and **5** featured unprecedented polycyclic skeletons. Here, we report the structures of **1**−**6** and their plausible biosynthetic relationships. In addition, we showed that HDAC inhibitor awoke silent nonreducing PKS (NR-PKS) genes that may be involved in the biosynthesis for common precursors (**7** and **8**) of novel compounds with increment of the extent of histone acetylation at both the gene loci by using an ChIP analysis (Figure 4).

The IR spectrum of **1** ($C_{16}H_{20}O_4$), a polyketide structure that forms the basis of the chaetophenols, revealed a saturated ketone (1716 cm^{-1}), an intramolecular hydrogen-bonded α,β-unsaturated aldehyde (1621 cm^{-1}), and hydroxyls (3420 cm^{-1}). The ^1H and ^{13}C NMR spectra revealed the presence of a vinylic methyl [δ_C 7.3 (C-11); δ_H 2.11 (s, H_3-11)], an aldehyde [δ_C 193.0 (C-10); δ_H 9.91 (s, H-10), a 2-oxopropyl [δ_C 29.6 (C-1), 204.6 (C-2), 42.3 (C-3); δ_H 2.24 (s, H_3-1), 4.05 (s, H_2-3)], and a 3,3-dimethylallyl group [δ_C 135.0 (C-14), 121.4 (C-13), 25.7 (C-16), 25.4 (C-12), 18.0 (C-15); δ_H 5.02 (t, J = 6.6 Hz, H-13), 3.29 (d, J = 6.6 Hz, H_2-12), 1.81 (s, H_3-15), 1.73 (s, H_3-16)] (Table S1, Supporting Information). The hexasubstituted benzene ring was also shown in the molecule from the remaining six signals due to aromatic carbons without protons [δ_C 162.6 (C-8), 160.4 (C-6), 134.5 (C-4), 119.0 (C-5), 113.0 (C-9), 110.4 (C-7)], and its substitution pattern was clearly deduced from the 3-bond H−C long-range couplings between the hydrogens in the substituents and aromatic carbons in the HMBC spectrum (H_2-3/C-5, C-9; H_2-12/C-4, C-6; 6-OH/C-5, C-7; H_3-11/C-6, C-8; 8-OH/C-7, C-9; H-10/C-4, C-8) (Figure 2).

As with compound **1**, the ^1H and ^{13}C NMR spectra of **2**, **3**, and **4** indicated the presence of a hexasubstituted benzene ring (C-4−C-9) with a 3,3-dimethylallyl group (C-12−C-16), two phenolic hydroxy groups, and a methyl group at C-5, C-6 and C-8, and C-7, respectively (Tables S1 and S2, Supporting Information). The presence of an oxymethylene [δ_C 63.8 (C-10); δ_H 5.10 (s, H_2-10), a trisubstituted olefin [δ_C 155.1 (C-2), 98.2 (C-3); δ_H 5.70 (s, H-3)], and a vinylic methyl group [δ_C 19.9 (C-1); δ_H 1.93 (s, H_3-1)] in compound **2** ($C_{16}H_{20}O_3$) implied that **2** could be formed by the cyclization reaction of **1** through the carbonyls at C-2 and C-10, followed by reduction. The HMBC correlations between H-3/C-5, C-9; H_2-10/C-2, C-4, C-8; H_3-1/C-3 allowed us to substantiate the structure of **2** (Figure 2).

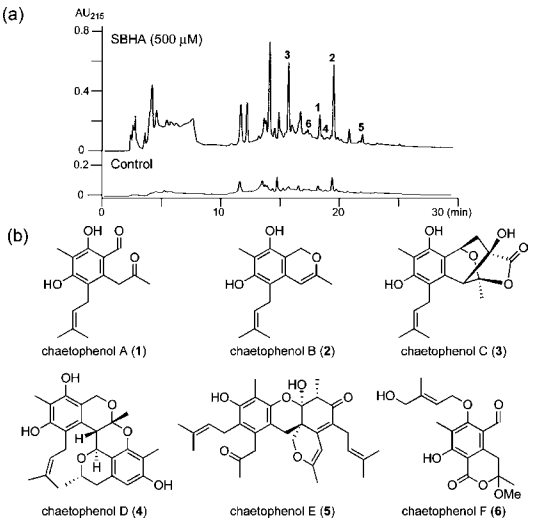


Figure 1. (a) HPLC profiles of the EtOAc extracts of *C. indicum* cultivated in the presence of SBHA 500 μM (upper) and control (bottom), as detected by UV absorption at 215 nm. (b) Structures of **1**−**6**.

The third novel polyketide **3** was thought to be produced *via* the condensation of **2** with pyruvic acid, based on its molecular formula, $C_{19}H_{22}O_6$. The ^{13}C NMR and DEPT spectra displayed signals corresponding to substituents at C-4 and C-9 of the common hexasubstituted benzene ring which were composed of an acetal, an ester carbonyl, an oxygen-bearing quaternary, an oxymethine, a methine, a methylene, and a methyl carbons (Table S1, Supporting Information). The nine degrees of unsaturation indicated that **3** possessed a substituent involving a three-ring system. The HMBC correlations of H_3-1/C-2, C-3; H-3/C-5, C-9; H-10/C-2, C-4, C-8 suggested that **3** included a 3-methyl-1*H*-isochroman core (C-1−C-10). The presence of a γ-lactone moiety was suggested by the IR band at 1770 cm^{-1} and the ^{13}C chemical shift for C-1′ ester carbonyl. The connectivities of C-3′−C-10, C-1′−C-2′−C-3′, and C-3−C-2′ were indicated by the $^1H-^1H$ COSY and HMBC spectra (Figure 2). The relative stereochemistry of **3** was deduced to be 2*S**,2′*R**,3*R**,10*R** based on the structural requirements for the construction of a restricted tricyclic ring system including a γ-lactone ring and a NOE for H_3-1/H-3 (Figure 2). The experimental IR and VCD of **3** measured

(6) (a) Williams, R. B.; Henrikson, J. C.; Hoover, A. R.; Lee, A. E.; Cichewicz, R. H. *Org. Biomol. Chem.* **2008**, *7*, 1895–1897. (b) Henrikson, J. C.; Hoover, A. R.; Joyner, P. M.; Cichewicz, R. H. *Org. Biomol. Chem.* **2009**, *7*, 435–438. (c) Wang, X.; Sena Filho, J. G.; Hoover, A. R.; King, J. B.; Ellis, T. K.; Powell, D. R.; Cichewicz, R. H. *J. Nat. Prod.* **2010**, *73*, 942–948.

(7) (a) Asai, T.; Chung, Y. M.; Sakurai, H.; Ozeki, T.; Chang, F. R.; Yamashita, K.; Oshima, Y. *Org. Lett.* **2012**, *14*, 513–515. (b) Asai, T.; Yamamoto, T.; Oshima, Y. *Org. Lett.* **2012**, *14*, 2006–2009. (c) Asai, T.; Morita, S.; Shirata, N.; Taniguchi, T.; Monde, K.; Sa-kurai, H.; Ozeki, T.; Oshima, Y. *Org. Lett.* **2012**, *14*, 5456–5459.

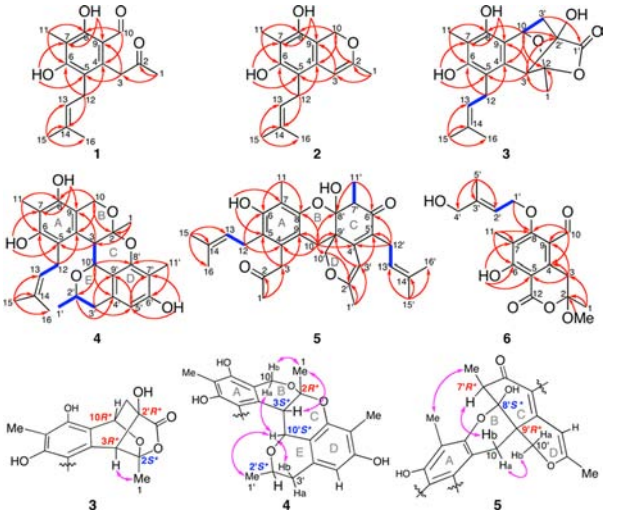


Figure 2. Key HMBC (arrow) and $^1H-^1H$ COSY (blue bold line) correlations of **1**–**6** and relative stereochemistries of **3**–**5** on the basis of 1D NOE experiments (purple arrow).

in $CDCl_3$ agreed well with the calculated spectra for (2*S*,2′*R*,3*R*,10*R*)-**3**, as determined using DFT calculations (Figure 3), thereby supporting the absolute stereochemistry of **3**.

The molecular formula of **4** ($C_{27}H_{32}O_6$) required 12 degrees of unsaturation in the molecule. The ^{13}C NMR and DEPT spectra showed 12 quaternary sp^2, 2 tertiary sp^2, an acetal, 3 methine, 3 methylene, and 6 methyl carbons (Table S2, Supporting Information). The 12 degrees of unsaturation in **4**, coupled with the ^{13}C NMR data revealing 14 sp^2 carbons, indicated that **4** must possess 4 rings in addition to the common hexasubstituted benzene ring (C-4–C-9) bearing the same substitutions as were present in **1**, **2**, and **3** (Tables S1 and S2, Supporting Information). The HMBC correlations revealed 2 1*H*-isochroman cores composed of A,B- (C-2–C-10) and D,E-rings (C-2′–C-10′) (Figure 2). The C-3–C-10′ linkage was clear from the spin coupling of H-3/H-10′ (J = 10.0 Hz). The functionality described above accounted for 11 of the 12 degrees of unsaturation, suggesting the presence of a C-ring formed by the linkage between the acetal C-2 and C-8′ through an ether bond. The relative configuration of **4** was determined as shown in Figure 2 based on the 1D-NOE experiments. The NOE of H_3-1/H-3 revealed a *cis*-fused BC ring. The correlations of Hb-3′/H-10′; H_3-1′/H-10′ indicated that the E-ring adopted a *pseudo*-boat conformation in which C-1′, Hb-3′, and H-10′ were situated on the same side. The NOEs of Ha-10/H-10′ and H_3-1/Hb-10 were also detected. These NOE data indicated the *trans* relationship of H-3 and H-10′. The absolute stereochemistry was determined to be 2*R*,2′*S*,3*S*,10′*S* by VCD spectral analysis, as in the case of **3** (Figure 3).

The polyketide **5** was characterized by the molecular formula $C_{32}H_{40}O_6$, corresponding to a dimeric compound

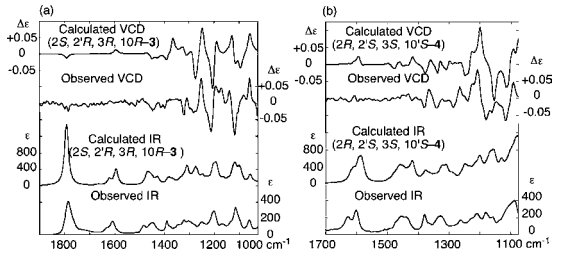


Figure 3. Comparison of IR (lower flame) and VCD (upper flame) spectra: (a) observed for **3** (*c* 0.07 M in $CDCl_3$) with those calculated for 2*S*,2′*R*,3*R*,10*R*–**3**; (b) observed for **4** (*c* 0.05 M in $CDCl_3$) with those calculated for 2*R*,2′*S*,3*S*,10′*S*–**4**.

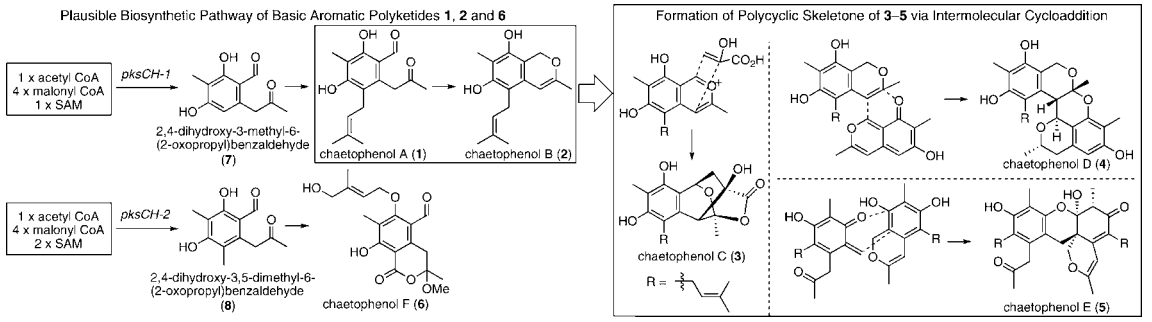
of **2**. The ^1H and ^{13}C NMR spectral data unambiguously allowed us to deduce that the A-ring bore C-4, -5, -6, and -7 substituents (C-1–C-16) and an additional 3,3-dimethylallyl moiety (C-12′–C-16′) (Table S2, Supporting Information). The HMBC correlations from H-3′, H-10′, H-11′, and H-12′ to the carbons on a cyclohexenone ring provided good evidence that the C-ring would be derived from **2** (Figure 2). In addition, the HMBC correlations of H_2-10/C-4′, C-9′; H_2-10′/C-2′; H_3-1′/C-2′, C-3′; H-3′/C-9′ indicated that the D- ring was composed of a dihydropyran ring (Figure 2), and the H–C couplings between H-10 and the carbons on the A- and B-rings revealed the presence of a C-ring bearing a C-8–C-8′ ether bond. The relative configuration was shown by the NOEs of Hb-10/H-7′, H_3-11/H_3-11′, and Ha-10/Hb-10′ (Figure 2). **5** was thought to be racemic due to the observation of a minimal optical rotation ($[\alpha]_D^{25}$ = ± 0.00 (*c* = 0.29, $CHCl_3$)), and no remarkable Cotton effects were observed in its CD spectrum.

The $^1H-^1H$ COSY (H_2-1′/H-2′) and the HMBC correlations (H_3-5′/C-2′, C-3′, C-4′) of **6** indicated the presence of a 4-hydroxy-3-methylbut-2-enyloxy moiety (C-1′–C-5′). The hexasubstituted benzene ring (C-4–C-9) was determined based on the HMBC correlations from Ha-3, 6-OH, H_3-11, and H-10 (Figure 2). The long-range H–C correlation of H_2-1′/C-8; H_3-1/C-2, C-3; OMe/C-2 revealed the *O*-prenylated C-8 and C-1–C-2(OMe)–C-3 connections. The sequence of the C-5–C-12–O–C-2 linkage was clear based on the molecular formula. The *E*-geometry of the C-2′/C-3′ double bond was elucidated based on the NOE of H_2-4′/H-2′. The absolute stereochemistry at C-2 was expected to be racemic due to the observation of a minimal optical rotation ($[\alpha]_D^{25}$ = 0.00 (*c* = 0.99, EtOH)) and the absence of remarkable Cotton effects in the CD spectrum.

A plausible biosynthetic pathway for **1**–**6** is proposed in Scheme 1. The unprecedented polycyclic architectures of **3**, **4**, and **5** could potentially be generated via an intermolecular cycloaddition. Inspection of the structures suggested that **1**–**5** and **6** arose from the benzaldehyde intermediates

(8) Ahuja, M.; Y. Chiang, M.; Chang, S. L.; Praseuth, M. B.; Entwistle, R.; Sanchez, J. F.; Lo, H. C.; Yeh, H. H.; Oakley, B. R.; Wang, C. C. *J. Am. Chem. Soc.* **2012**, *134*, 8212–8221.

Scheme 1. Plausible Biosynthetic Pathway for **1**–**6**



7 and **8**, respectively, requiring two NR-PKSs in the pathways. Recently, PkeA was characterized as NR-PKS for **7** in *A. nidulans*.[8] Using PkeA amino acid sequence, we scanned the draft genome of *C. indicum* and found only two genes for NR-PKS possessing the same domain structure [SAT −KS−AT−PT−ACP−CMeT−R] as that of PkeA (Table S4, Supporting Information). This finding pointed to the two genes, named as *pksCH-1* and *pksCH-2*, as the corresponding biosynthetic NR-PKS genes for **7** and **8** in *C. indicum*. RT-PCR analysis of *pksCH-1* and *pksCH-2*, which were transcriptionally suppressed under normal culture conditions, revealed that the expression of both NR-PKS genes was clearly activated by the HDAC inhibitor, SBHA (Figure 4a). To verify the enrichment of histone acetylation at the NR-PKS gene loci by HDAC inhibitor, we applied chromatin immunoprecipitation (ChIP) using acetylated histone 4 (H4Ac) antibody followed by real-time quantitative PCR.[9] ChIP analysis showed that SBHA, clearly increased histone acetylation at both the loci in the fungus (Figure 4b). It means that HDAC inhibitor altered histone modifications from transcriptionally inactive hypo-acetylated to active hyper-acetylated form at both the silencing NR-PKS gene loci and awoke their gene expressions through chromatin-based epigenetic regulation, resulting the enhancement of diverse polyketide productions in the fungus.

To characterize the functions of *pksCH-1* and *pksCH-2*, we applied heterologous expression in *A. oryzae* using pTAex3 overexpression plasmid vector.[10] Considering the induction level of gene expressions by SBHA (Figure 4a), heterologous expression of *pksCH-2* was initially carried out. After preparing expression plasmids for *pksCH-2* using the pTAex3 vector, the resultant plasmids were transformed into *A. oryzae* M-2−3 strain.[11] The transfomant was cultivated in CDS (Czapek-Dox containing starch) and the EtOAc extract of the culture medium showed a new peak in the HPLC analysis that was not found in a control culture of the wild-type strain (Figure 4c). After isolation of the compound, it was identified to be **8** (Figure S1, Supporting Information).

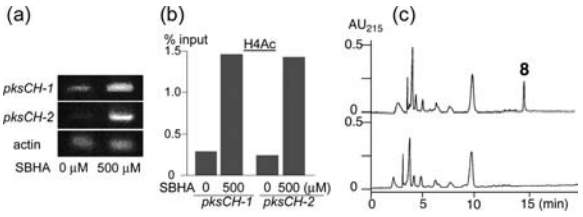


Figure 4. (a) Transcriptional analysis by RT-PCR of *pksCH-1* and *pksCH-2* in *C. indicum* cultivated under each condition. The actin gene was used as an internal control. (b) Examination of histone H4 acetylation in *C. indicum*, cultivated under each condition, by chromatin immunoprecipitation (ChIP) analyses of *pksCH-1* and *pksCH-2*. (c) HPLC analysis of the EtOAc extracts of the culture media from *A. oryzae* M-2-3 *pksCH-2* transformant (upper) and wild type (bottom).

This result confirmed *pksCH-2* to be the NR-PKS for **8**. Even though a number of trials to express *pksCH-1* in *A. oryazae* have not yet succeeded in its functional expression, the gene might code **7** from the similarity of its amino acid sequences and domain structures to those of *pkeA*, and the results shown in Figure 4.

In conclusion, we successfully isolated structurally diverse polyketides from *C. indicum* by chemically epigenetic method. Among them, chaetophenols C−E (**3**−**5**) possessed unprecedented polycyclic skeletons. Our study also provided the evidence that the induction of fungal polyketide productions by an HDAC inhibitor was directly associated with chromatin-based epigenetic regulation through histone acetylation.

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Supporting Information Available. Figures, Experimental methods, full spectroscopic data and NMR spectra of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

(9) Furuhashi, H.; Takasaki, T.; Rechtsteiner, A.; Li, T.; Kimura, H.; Checchi, P. M.; Strome, S.; Kelly, W. G. *Epigenet. Chromatin* **2010**, *3*, 15.
(10) Gomi, K.; Iimura, Y.; Hara, S. *Agric. Biol. Chem.* **1987**, *51*, 2549–2555.
(11) Seshime, Y.; Juvvadi, P. R.; Kitamoto, K.; Ebizuka, Y.; Nonaka, T.; Fujii, I. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 4785–4788.