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Reconstitution of Biosynthetic Machinery for Indole-Diterpene Paxilline in *Aspergillus oryzae*

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Supporting Information

ABSTRACT: Indole-diterpenes represented by paxilline share a common pentacyclic core skeleton derived from indole and geranylgeranyl diphosphate. To shed light on the detailed biosynthetic mechanism of the paspaline-type hexacyclic skeleton, we examined the reconstitution of paxilline biosynthetic machinery in *Aspergillus oryzae* NSAR1. Stepwise introduction of the six *pax* genes enabled us to isolate all biosynthetic intermediates and to synthesize paxilline. In vitro and in vivo studies on the key enzymes, prenyltransferase PaxC and cyclase PaxB, allowed us to elucidate actual substrates of these enzymes. Using the isolated and the synthesized epoxide substrates, the highly intriguing stepwide epoxidation/cyclization mechanism for the construction of core structure has been confirmed. In addition, we also demonstrated "tandem transformation" to simultaneously introduce two genes using a single vector (*paxG*/*paxB*, pAdeA; *paxP*/*paxQ*, pUNA). This may provide further option for the reconstitution strategy to synthesize more complex fungal metabolites.

Indole-terpenes are fungal and bacterial secondary metabolites with unique biological activities such as inhibitory activity of calcium-activated potassium channels,[1] tremorgenic activity,[2] potent and selective progesterone receptor agonistic activity,[3] and anti-MRSA activity.[4] The wide range of biological activities are dependent on their structural diversity. Indole-diterpenes share a common core structure consisting of a cyclic diterpene skeleton derived from indole and geranylgeranyl diphosphate (GGPP). Further modifications, such as prenylation, hydroxylation, and chlorination, increase members of this family to include compounds such as nodulisporic acid, lolitrem, thiersinine, and penitrem.

A series of feeding experiments with ^{13}C-labeled acetate revealed that the terpenoid core structure is derived from GGPP.[5,6] The structure of less modified indole diterpene paspaline (**2**) suggested that **2** is a cyclization product of a putative common intermediate 3-geranylgeranyl indole (GGI, **4**). This was experimentally supported by incorporation of deuterium labeled **4**.[7] In 2001, the first biosynthetic gene cluster of this family for paxilline (*pax*) was identified.[8] Then, a series of gene inactivation and gene transfer to paxilline negative deletion mutants confirmed that four *pax* genes, *paxG*, *paxC*, *paxM*, and *paxB*, are required for the biosynthesis of **2** (Figure 1A).[8,9] Further gene disruption studies of *paxP* and *paxQ*, a cytochrome P450 gene, indicated that PaxP and PaxQ catalyze multiple oxidation steps from paspaline (**2**) to paxilline (**1**) via 13-desoxypaxilline (**9**).[10] Recently, two more biosynthetic gene clusters for aflatrem (*atm*) and lolitrem (*ltm*) have been identified and added further information for the biosynthesis of indole diterpenes.[11,12] However, little is known about the detailed function of these four gene products.

Recently, we carried out heterologous expression of aphidicolin biosynthetic genes (four genes)[13] in *Aspergillus oryzae* NSAR1 strain, which accepts five expression vectors (pTAex3, pUSA, pAdeA, pPTR1, pUNA).[14−16] Transformation of plasmids harboring each gene into *A. oryzae* in stepwise manner enabled us to isolate all biosynthetic intermediates in addition to the natural product, aphidicolin, from corresponding transformants. Accumulated examples of heterologous production of fungal metabolites suggest that the *A. oryzae* expression system allows us to analyze the biosynthetic machinery of complex natural products. In this study, we applied this reliable expression system to analyze the functions of *paxG*, *paxC*, *paxM*, and *paxB* genes in the biosynthesis of **2** and achieved the total biosynthesis of **1**.

Among the four genes involved in paspaline biosynthesis, bioinformatics analysis indicated that the putative gene products PaxG, a geranylgeranyl diphosphate synthase, and PaxC, a prenyltransferase, participate in the construction of GGI **4**. To examine the functions of PaxG and PaxC, *A. oryzae* NSAR1 strain was transformed with pAdeA-*paxG* and pTAex3-*paxC*. Transformants, that efficiently produced novel compounds compared with the wild type strain, were screened by monitoring of their mycelial extracts by TLC or HPLC. HPLC analysis showed that the double transformant produced a new compound **4** eluting with the same retention time as authentic GGI (production of **4**: 7.7 mg/L) (Figure 1B). After purification, the structure was also confirmed by ^{1}H NMR analysis in comparison with authentic GGI. These experiments proved that *paxC* encodes a geranylgeranyl transferase using

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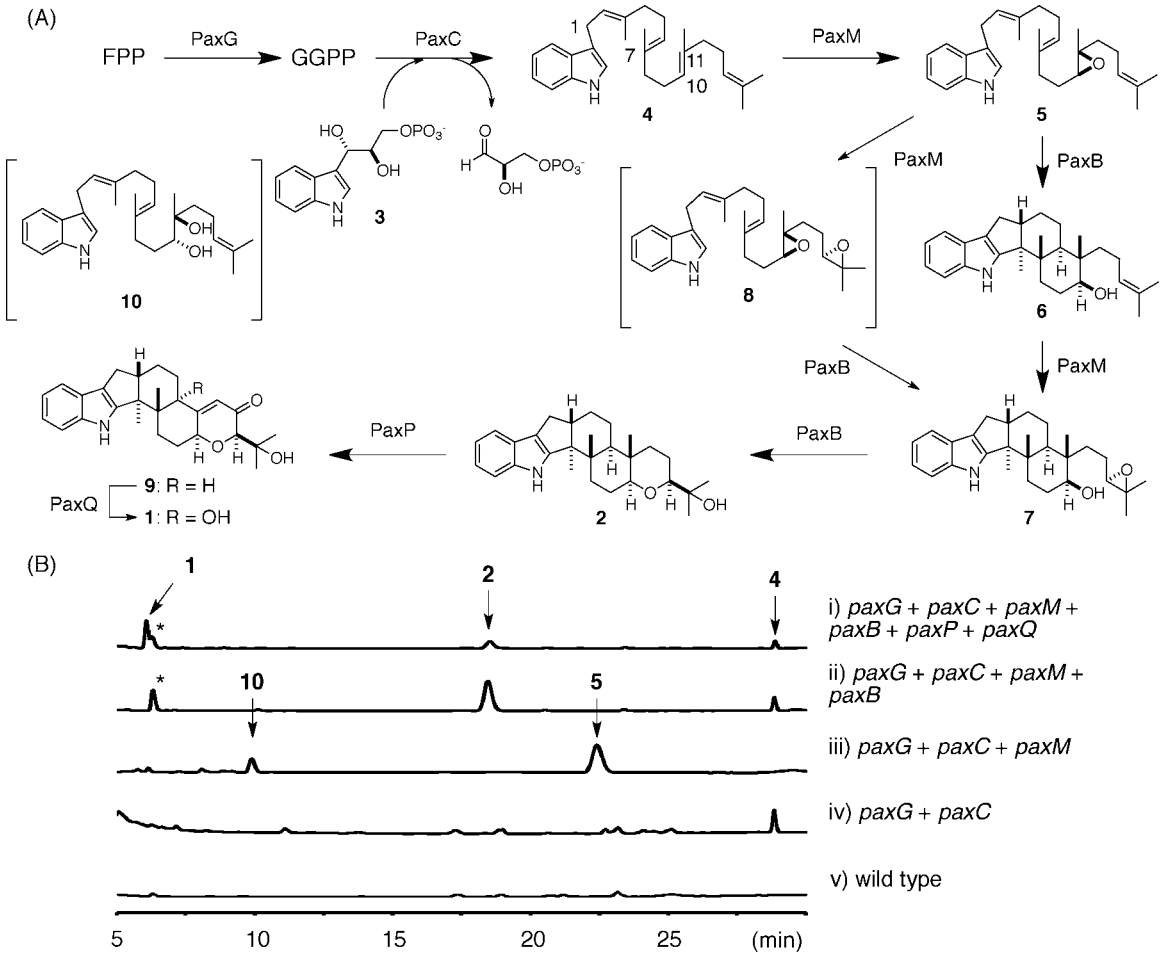

Figure 1. (A) Biosynthetic pathway of paxilline. (B) HPLC profiles of the extracts from *A. oryzae* transformant: (i) transformant harboring *paxG*, *paxC*, *paxM*, *paxB*, *paxP*, and *paxQ*; (ii) transformant harboring *paxG*, *paxC*, *paxM*, and *paxB*; (iii) transformant harboring *paxG*, *paxC*, and *paxM*; (iv) transformant harboring *paxG* and *paxC*; (v) wild type strain. *: impurity.

GGPP, and that, as suggested in our previous experiment,[7] GGI **4** is an intermediate in paxilline biosynthesis.

On the basis of the results of previous feeding experiments using tryptophan or anthranilic acid,[6,17] it was proposed that either tryptophan or its precursor, such as indole-3-glycerol phosphate (IGP: **3**), is a substrate in the GGI forming reaction. To determine the substrate of this prenylation step, we then carried out in vitro analysis using recombinant PaxC. *PaxC* cDNA was cloned into the pQE30 vector and expressed as an N-terminal His_6-tagged fusion protein. Recombinant PaxC was purified by Ni-NTA column chromatography (Figure S2). IGP **3** was subjected to PaxC reaction with GGPP and Mg^{2+}. Subsequent HPLC analysis of the reaction products showed the prominent peak of GGI **4** dependent on PaxC and Mg^{2+} (Figure S3). Importantly, **4** was also detected in the PaxC reaction with indole (Figure S4). The kinetic parameters revealed that IGP is a preferred substrate for PaxC (k_{cat}/K_M for **3**, 28.2 mM^{-1} s^{-1}; indole, 3.6 mM^{-1} s^{-1}) (Table S2). In contrast, trace amount of **4** was detected in the PaxC reaction with tryptophan (Figure S4). Further LC-MS analysis revealed that PaxC reaction accompanied the formation of glyceraldehyde 3-phosphate as a byproduct of PaxC reaction (Figure S5). These results indicated that PaxC catalyzed geranylgeranylation at C3 position of **3** followed by elimination of glyceraldehyde 3-phosphate (Figure S6). To our knowledge, the preceding example is an octaprenyltransferase MenA which catalyzes simultaneous prenylation and decarboxylation in the menaquinone biosynthesis of *Escherichia coli*.[18] PaxC shared a significant sequence similarity with typical polyprenyl synthetases having DDxxD motif but not with members of indole prenyltransferases belonging to the fungal tryptophan dimethylallyltransferase (DMATS) superfamily (Figure S7).[19]

We then turned our attention to the subsequent epoxidation and cyclization mechanism. As *paxM* showed a high similarity to flavin-dependent monooxygenase, which catalyzes various reactions including epoxidation,[20] the double transformant was then transformed with pUSA-*paxM*. Chromatograms of extracts from the triple transformant showed two new peaks (**5** and **10**) with typical absorption spectra of indole skeleton (production of **5**: 32 mg/L) that were not present in the double transformant (Figure 1B, Figure S8). ^1H NMR spectrum of **5** displayed characteristic signals corresponding to indole and terpene moieties. In particular, the triplet at 2.71 ppm indicated the presence of epoxide. HMBC and HSQC analysis revealed that the epoxide was installed at the C10−C11 position of GGI. Another metabolite **10** was confirmed as a diol analog of **5** based on a series of NMR analysis and LC-MS analysis (Figure S8, S9). Hydrolysis of the epoxide moiety in a heterologous host *A. oryzae* was reported previously.[21,22] To shed light on the final cyclization step, we then introduced the *paxB* gene (pAdeA-*paxB*), an integral membrane protein, in addition to the *paxG* gene (pAdeA-*paxG*) into double transformant (*paxC*

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+ *paxM*). HPLC analysis of the extracts from this quadruple transformant showed a new peak **2** (production of **2**: 57 mg/L) (Figure 1B). The ^{1}H NMR spectrum of **2** was identical to that of authentic paspaline.

Isolation of **5** and **2** from the triple and the quadruple transformants suggested that initial cyclization of **5** yields emindole SB (**6**)[23] and a second round of epoxidation/cyclization affords **2** (Figure 1A). To prove this stepwise epoxidation/cyclization mechanism, we carried out a biotransformation study using two epoxide substrates **5** and **8**. Bisepoxide **8** was synthesized by the sequential Sharpless asymmetric dihydroxylations in a highly stereocontrolled manner (9,10-epoxide, 90% d.e.; 13,14-epoxide, 98% d.e.) as shown in Supporting Information. Next, *A. oryzae* NSAR1 wild type strain was transformed with the expression plasmid pTAex3-*paxB*. Single *paxB* transformant was screened by semiquantitative RT-PCR (Figure S10),[8] monitoring the expression level of *paxB* at different growth times. This also enabled us to determine optimal fermentation period as 2.5 days for the biotransformation study. Incubation with **5** led to the formation of **6** as determined by HPLC analysis only in the extract from the transformant (Figure 2). Importantly this

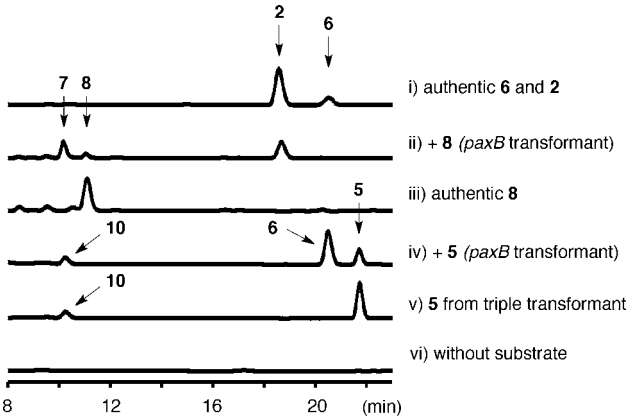

Figure 2. HPLC profiles of the biotransformation experiments: (i) authentic **2** and **6**; (ii) with **8**; (iii) authentic **8**; (iv) with **5**; (v) **5** from triple transformant; (vi) without substrate.

transformant also converted **8** into **2**. These results confirmed the stepwise epoxidation and cyclization mechanism and supported the involvement of the pentacyclic intermediate **7** (Figure 2). The core structure formation is different from that of ionophore polyether lasalocid that is biosynthesized from sequential epoxidation and subsequent cyclization.[20]

To date, biosynthetic gene clusters of three fungal indole diterpenes such as paxilline,[8] aflatrem,[11] and lolitrem[12] and one bacterial indole sesquiterpene, xiamycin,[24] were identified. Key genes for epoxidation and cyclization, *paxM* like flavin-dependent monooxygenase (aflatrem, *atmM*; lolitrem, *ltmM*; xiamycin, *xiaO*) and *paxB* like membrane protein (aflatrem, *atmB*; lolitrem, *ltmB*; xiamycin, *xiaH*) are highly conserved in all of these gene clusters. Therefore, the stepwise epoxidation/cyclization mechanism to construct the hexacyclic skeleton of paxilline can be extended to account for the biosynthetic machinery of these types of indole terpenes; scaffold A (C15, indosespene; C20, emindole SA) and scaffold B (C15, sespendole; C20, paspaline) (Figure 3). It should be noted that each core structure is formed by the regioselective epoxidation toward a third olefin from the indole ring followed

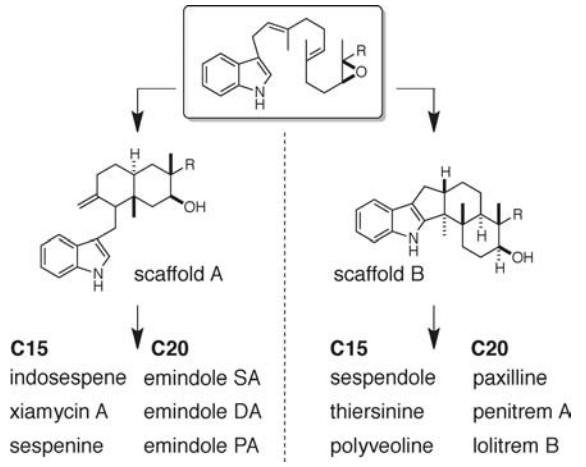

Figure 3. Biosynthetic model for various indole terpenes.

by two different types of cyclization (scaffold A and scaffold B). The characteristic THP ring system in indole diterpenes represented by paspaline is constructed by a second cycle of epoxidation/cyclization catalyzed by the same epoxidase and terpene cyclase. This adds further structural diversity to this family.

Finally, we focused on the heterologous production of paxilline (**1**) in *A. oryzae*. For this purpose, it would be necessary to introduce six genes, although currently only five vectors are available (pPTR1, pTAex3, pUNA, pUSA, pAdeA). Therefore, we examined "tandem transformation" using a single vector carrying two individual genes *paxP* and *paxQ* into the quadruple transformant which produces **2**. When selection was carried out on minimal medium lacking ammonium sulfate, the desired transformant harboring *paxP* and *paxQ* (four of seven positive clones) in addition to *paxP* and *paxQ* transformants (*paxP*, one clone; *paxQ*, two clones) were isolated. As expected, HPLC and ^{1}H NMR analysis showed that the *paxP* and the *paxP* + *paxQ* transformants produced **9** and **1**, respectively (production of **1**: 35 mg/L) (Figure 1B, Figure S11).

In this study, we successfully demonstrated stepwise reconstruction of biosynthetic machinery of the target natural product **1** by introducing six genes into *A. oryzae*. This methodology enabled us to characterize each gene and to obtain sufficient amount of intermediates for in vitro enzymatic transformation. The same strategy has been applied to synthesize natural products, such as polyketide-peptide hybrid tenellin (four genes),[25] diterpene aphidicolin (four genes),[13] and late biosynthetic intermediates, such as meroterpenoids pyripyropene (two + three genes)[21] and terretonin (five genes).[22] The synthesis of complex fungal metabolites involving more than ten biosynthetic genes requires practical methods for introduction of multiple genes. For this purpose, we performed "tandem transformation" to simultaneously indroduce two genes using a single vector (*paxG*/*paxB*, pAdeA; *paxP*/*paxQ*, pUNA). This "tandem transformation" in combination with the conventional method using single plasmids carrying two different genes enabled us to solve the vector problem for heterologous production of fungal metabolites.

In summary, we elucidated the detailed reaction mechanism of paspaline biosynthesis using stepwise introduction of biosynthetic genes. Bioconversion of the resultant transformants and in vitro study enabled determination of the

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actual intermediate of geranylgeranyl transferase PaxC and to establish two rounds of epoxidation/cyclization for construction of the characteristic paspaline scaffold. Our strategy can be applied to produce known or unknown natural products for which biosynthetic genes are available.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, HR-MS, NMR data, spectra and LC-MS data. 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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■ REFERENCES

(1) Knaus, H. −G.; McManus, O. B.; Lee, S. H.; Schmalhofer, W. A.; Garcia-Calvo, M.; Helms, L. M. H.; Sanchez, M.; Giangiacomo, K.; Ruben, J. P.; Smith, A. B., III.; Kaczorowski, G. J.; Garcia, M. L. *Biochemistry* **1994**, *33*, 5819.

(2) Miles, C. O.; Wilkins, A. L.; Gallagher, R. T.; Hawkes, A. D.; Munday, S. C.; Towers, N. R. *J. Agric. Food Chem.* **1992**, *40*, 234.

(3) Roll, D. M.; Barbieri, L. R.; Bigelis, R.; McDonald, L. A.; Arias, D. A.; Chang, L. -P.; Singh, M. P.; Luckman, S. W.; Berrodin, T. J.; Yudt, M. R. *J. Nat. Prod.* **2009**, *72*, 1944.

(4) Ogata, M.; Ueda, J.; Hoshi, M.; Hashimoto, J.; Nakashima, T.; Anzai, K.; Takagi, M.; Shin-ya, K. *J. Antibiot.* **2007**, *60*, 645.

(5) Acklin, W.; Weibel, F.; Arigoni, D. *Chimia* **1977**, *31*, 63.

(6) de Jesus, A. E.; Gorst-Allman, C. P.; Steyn, P. S.; van Heerden, F. R.; Vieggaar, R.; Wessels, P. L.; Hull, W. E. *J. Chem. Soc., Perkin Trans.* **1983**, 1863.

(7) Fueki, S.; Tokiwano, T.; Toshima, H.; Oikawa, H. *Org. Lett.* **2004**, *6*, 2697.

(8) Young, C.; McMillan, L.; Telfer, E.; Scott, B. *Mol. Microbiol.* **2001**, *39*, 754.

(9) Saikia, S.; Parker, E. J.; Koulman, A.; Scott, B. *FEBS Lett.* **2006**, *580*, 1625.

(10) (a) McMillan, L. K.; Carr, R. L.; Young, C. A.; Astin, J. W.; Lowe, R. G. T.; Parker, E. J.; Jameson, G. B.; Finch, S. C.; Miles, C. O.; McManus, O. B.; Schmalhofer, W. A.; Garcia, M. L.; Kaczorowski, G. J.; Goetz, M.; Tkacz, J. S.; Scott, B. *Mol. Gen. Genomics* **2003**, *270*, 9. (b) Saikia, S.; Parker, E. J.; Koulman, A.; Scott, B. *J. Biol. Chem.* **2007**, *282*, 16829.

(11) Nicholson, M. J.; Koulman, A.; Monahan, B. J.; Pritchard, B. L.; Payne, G. A.; Scott, B. *Appl. Environ. Microbiol.* **2009**, *75*, 7469.

(12) Young, C. A.; Bryant, M. K.; Christensen, M. J.; Tapper, B. A.; Bryan, G. T.; Scott, B. *Mol. Gen. Genomics* **2005**, *274*, 13.

(13) (a) Fujii, R.; Minami, A.; Tsukagoshi, T.; Sato, N.; Sahara, T.; Ohgiya, S.; Gomi, K.; Oikawa, H. *Biosci. Biotechnol. Biochem.* **2011**, *75*, 1813. (b) Efficient production of aphidicolin has observed in the fermentation of quadruple transformant using solid medium instead of normal liquid medium (>130 mg/kg rice).

(14) Jin, F. H.; Maruyama, J.; Juvvadi, P. R.; Akioka, M.; Kitamoto, K. *FEMS Microbiol. Lett.* **2004**, *239*, 79.

(15) Fujii, T.; Yamaoka, H.; Gomi, K.; Kitamoto, K.; Kumagai, C. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1869.

(16) Yamada, O.; Na Nan, S.; Akao, T.; Tominaga, M.; Watanabe, H.; Satoh, T.; Enei, H.; Akita, O. *J. Biosci. Bioeng.* **2003**, *95*, 82.

(17) Byrne, K. M.; Smith, S. K.; Ondeyka, J. G. *J. Am. Chem. Soc.* **2002**, *124*, 7055.

(18) Suvarna, K.; Stevenson, D.; Meganathan, R.; Hudspeth, M. E. S. *J. Bacteriol.* **1998**, *180*, 2782.

(19) (a) Li, S.-M. *Phytochemistry* **2009**, *70*, 1746. (b) Li, S.-M. *Nat. Prod. Rep.* **2010**, *27*, 57.

(20) Minami, A.; Shimaya, M.; Suzuki, G.; Migita, A.; Shinde, S. S.; Sato, K.; Watanabe, K.; Tamura, T.; Oguri, H.; Oikawa, H. *J. Am. Chem. Soc.* **2012**, *134*, 7246.

(21) Itoh, T.; Tokunaga, K.; Matsuda, Y.; Fujii, I.; Abe, I.; Ebizuka, Y.; Kushiro, T. *Nat. Chem.* **2010**, *2*, 858.

(22) Matsuda, Y.; Awakawa, T.; Itoh, T.; Wakimoto, T.; Kushiro, T.; Fujii, I.; Ebizuka, Y.; Abe, I. *ChemBioChem* **2012**, *13*, 1738.

(23) Nozawa, K.; Yuyama, M.; Nakajima, S.; Kawai, K.; Udagawa, S. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2155.

(24) Li, H.; Zhang, Q.; Li, S.; Shu, Y.; Zhang, G.; Zhang, H.; Tian, X.; Zhang, S.; Ju, J.; Zhang, C. *J. Am. Chem. Soc.* **2012**, *134*, 8996.

(25) Heneghan, M. N.; Yakasai, A. A.; Halo, L. M.; Song, Z.; Bailey, A. M.; Simpson, T. J.; Cox, R. J.; Lazarus, C. M. *ChemBioChem* **2010**, *11*, 1508.