ORGANIC LETTERS

2009
Vol. 11, No. 3
551−554

Enzymatic Formation of Unnatural Novel Chalcone, Stilbene, and Benzophenone Scaffolds by Plant Type III Polyketide Synthase

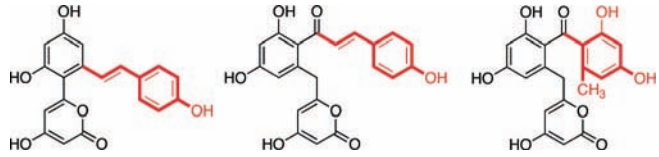
She-Po Shi,[†] Kiyofumi Wanibuchi,[†] Hiroyuki Morita,[†] Kohei Endo,[†] Hiroshi Noguchi,[†] and Ikuro Abe*[,†,‡]

School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan, and PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan

abei@u-shizuoka-ken.ac.jp

Received November 11, 2008

ABSTRACT



A C_{19} hexaketide stilbene and a C_{21} heptaketide chalcone were synthesized by *Aloe arborescens* octaketide synthase (OKS), a plant-specific type III polyketide synthase (PKS). Remarkably, the C_{21} chalcone-forming activity was dramatically increased in a structure-guided OKS N222G mutant that produces a C_{20} decaketide SEK15 from 10 molecules of malonyl-CoA. The findings suggested further strategies for production of unnatural polyketides by combination of the precursor-directed biosynthesis and the structure-guided engineering of type III PKS.

Chalcone synthase (CHS) and stilbene synthase (STS) are plant-specific type III polyketide synthases (PKSs) that catalyze sequential condensations of 4-coumaroyl-CoA with three molecules of malonyl-CoA to produce naringenin chalcone and resveratrol, respectively (Scheme 1).[1] The enzyme reactions are initiated by binding of 4-coumaroyl-CoA, which is followed by three rounds of iterative decarboxylative condensation with malonyl-CoA. Then, Claisen-type cyclization of the enzyme-bound intermediate produces the C_{15} tetraketide chalcone, while aldol-type cyclization and decarboxylation leads to formation of the C_{14} tetraketide stilbene.[1,2] Recent crystallographic studies have demonstrated that electronic effects of the "aldol-switch" hydrogen bond network balance the competing cyclization specificities from the common tetraketide intermediate.[3] Remarkably, type III PKSs exhibit unusually broad, promiscuous substrate specificities; the structurally simple homodimeric proteins accept a variety of nonphysiological substrates, including aromatic and aliphatic CoA thioesters, to produce an array of chemically and structurally divergent unnatural polyketides.[4,5]

Octaketide synthase (OKS) from *Aloe arborescens* is a plant-specific type III PKS catalyzing sequential condensations of eight molecules of malonyl-CoA to yield SEK4 and SEK4b (Scheme 1C).[6] The C_{16} aromatic octaketides are known to be the shunt products of the minimal type II PKS

[†] University of Shizuoka.
[‡] PRESTO.

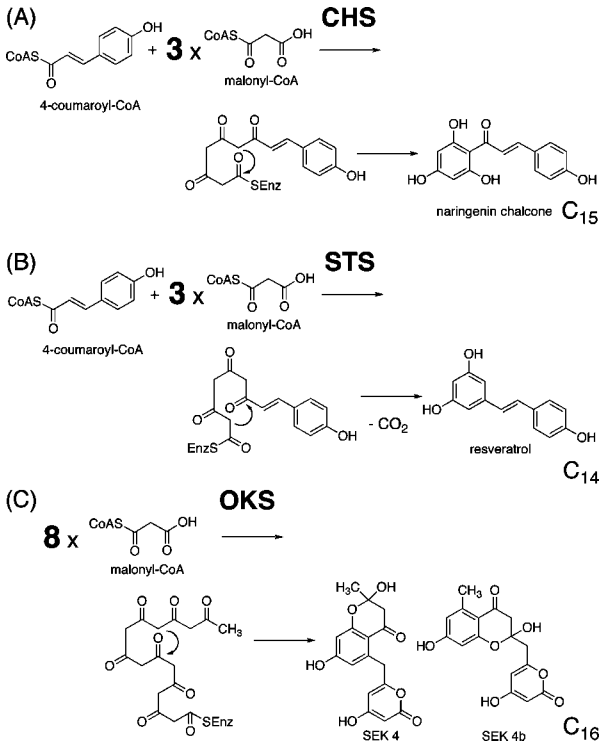
(1) For recent reviews, see: (a) Austin, M. B.; Noel, J. P. *Nat. Prod. Rep.* **2003**, *20*, 79–110. (b) Schröder, J. In *Comprehensive Natural Products Chemistry*; Elsevier: Oxford, 1999; Vol. 2, pp 749−771,

(2) (a) Ferrer, J. L.; Jez, J. M.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Nat. Struct. Biol.* **1999**, *6*, 775–784. (b) Jez, J. M.; Ferrer, J. L.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Biochemistry* **2000**, *39*, 890–902. (c) Jez, J. M.; Noel, J. P. *J. Biol. Chem.* **2000**, *275*, 39640–39646. (d) Jez, J. M.; Bowman, M. E.; Noel, J. P. *Biochemistry* **2001**, *40*, 14829–14838. (e) Tropf, S.; Kärcher, B.; Schröder, G.; Schröder, J. *J. Biol. Chem.* **1995**, *270*, 7922–7928. (f) Suh, D. Y.; Fukuma, K.; Kagami, J.; Yamazaki, Y.; Shibuya, M.; Ebizuka, Y.; Sankawa, U. *Biochem. J.* **2000**, *350*, 229–235.

(3) Austin, M. B.; Bowman, M. E.; Ferrer, J.-L.; Schröder, J.; Noel, J. P. *Chem. Biol.* **2004**, *11*, 1179–1194.

10.1021/ol802606w CCC: $40.75
Published on Web 01/05/2009

Scheme 1. Formation of (A) Naringenin Chalcone by CHS, (B) Resveratrol by STS, and (C) SEK4/SEK4b by *A. arborescens* OKS



for actinorhodin (Act from *Streptomyces coelicolor*).[7] Here we report that the octaketide-producing OKS also catalyzes condensations of 4-coumaroyl-CoA with malonyl-CoA to produce an unnatural C_{21} heptaketide chalcone and a C_{19}

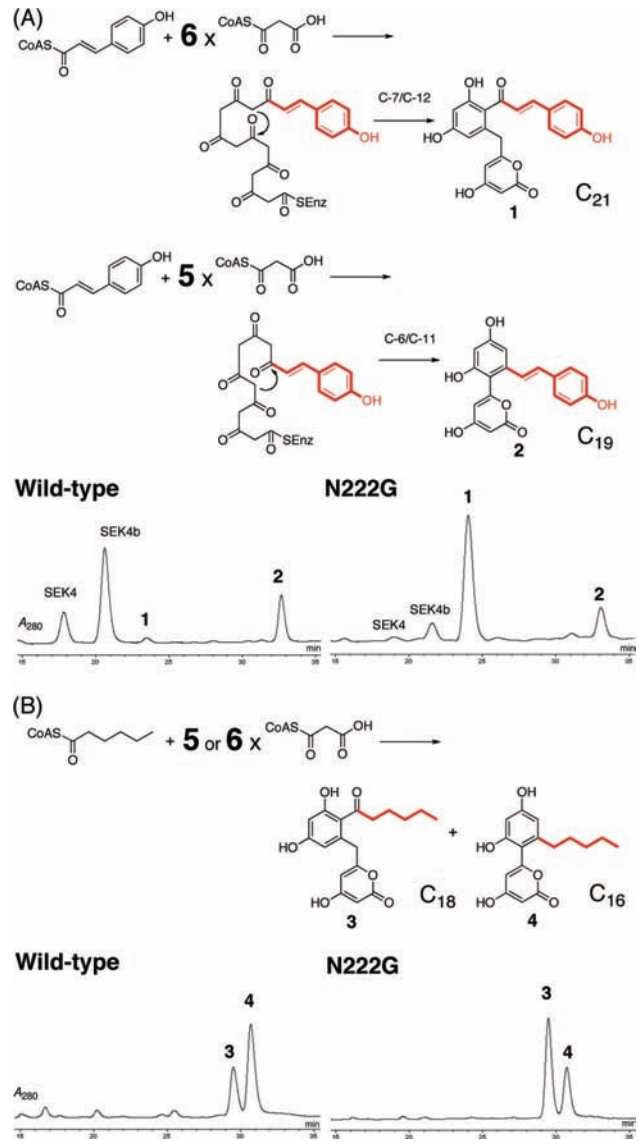


Figure 1. Enzymatic formation of unnatural polyketides (and HPLC elution profiles) from (A) 4-coumaroyl-CoA and (B) hexanoyl-CoA by *A. arborescens* wild-type OKS (left) and N222G mutant (right).

hexaketide stilbene, novel polyketide scaffolds generated by the structurally simple type III PKS. It is remarkable that the C_{21} chalcone-forming activity was dramatically increased in a structure-guided OKS N222G mutant that produces a C_{20} decaketide SEK15 from 10 molecules of malonyl-CoA. In addition, we also report enzymatic formation of an unnatural C_{18} phloroglucinol and a C_{16} resorcinol from hexanoyl-CoA/malonyl-CoA by *A. arborescens* OKS.

When recombinant wild-type OKS was incubated with 4-coumaroyl-CoA and malonyl-CoA as substrates, most of the enzyme reactions were initiated by malonyl-CoA and the octaketides SEK4 and SEK4b were obtained as major products (Figure 1A). However, careful examination of the reaction products led to isolation of two less polar novel products. The parent ion peaks $[M + H]^+$ at *m*/*z* 381 and 339 on LC-ESIMS indicated formation of a coumaroyl-

(4) (a) Abe, I.; Morita, H.; Nomura, A.; Noguchi, H. *J. Am. Chem. Soc.* **2000**, *122*, 11242–11243. (b) Morita, H.; Takahashi, Y.; Noguchi, H.; Abe, I. *Biochem. Biophys. Res. Commun.* **2000**, *279*, 190–195. (c) Morita, H.; Noguchi, H.; Schröder, J.; Abe, I. *Eur. J. Biochem.* **2001**, *268*, 3759–3766. (d) Abe, I.; Takahashi, Y.; Noguchi, H. *Org. Lett.* **2002**, *4*, 3623–3626. (e) Abe, I.; Takahashi, Y.; Lou, W.; Noguchi, H. *Org. Lett.* **2003**, *5*, 1277–1280. (f) Abe, I.; Sano, Y.; Takahashi, Y.; Noguchi, H. *J. Biol. Chem.* **2003**, *278*, 25218–25226. (g) Abe, I.; Watanabe, T.; Noguchi, H. *Phytochemistry* **2004**, *65*, 2447–2453. (h) Oguro, S.; Akashi, T.; Ayabe, S.; Noguchi, H.; Abe, I. *Biochem. Biophys. Res. Commun.* **2004**, *325*, 561–567. (i) Abe, T.; Noma, H.; Noguchi, H.; Abe, I. *Tetrahedron Lett.* **2006**, *47*, 8727–8730. (j) Abe, I.; Abe, T.; Wanibuchi, K.; Noguchi, H. *Org. Lett.* **2006**, *8*, 6063–6065. (k) Abe, I.; Watanabe, T.; Lou, W.; Noguchi, H. *FEBS J.* **2006**, *273*, 208–218. (l) Wanibuchi, K.; Zhang, P.; Abe, T.; Morita, H.; Kohno, T.; Chen, G.; Noguchi, H. *FEBS J.* **2007**, *274*, 1073–1082.

(5) (a) Schüz, R.; Heller, W.; Hahlbrock, K. *J. Biol. Chem.* **1983**, *258*, 6730–6734. (b) Zuurbier, K. W. M.; Leser, J.; Berger, T.; Hofte, A. J. P.; Schröder, G.; Verpoorte, R.; Schröder, J. *Phytochemistry* **1998**, *49*, 1945–1951. (c) Jez, J. M.; Bowman, M. E.; Noel, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5319–5324. (d) Samappito, S.; Page, J.; Schmidt, J.; De-Eknamkul, W.; Kutchan, T. M. *Planta* **2002**, *216*, 64–71. (e) Samappito, S.; Page, J. E.; Schmidt, J.; De-Eknamkul, W.; Kutchan, T. M. *Phytochemistry* **2003**, *62*, 313–323. (f) Springob, K.; Samappito, S.; Jindaprasert, A.; Schmidt, J.; Page, J. E.; De-Eknamkul, W.; Kutchan, T. M. *FEBS J.* **2007**, *274*, 406–417. (g) Karppinen, K.; Hokkanen, J.; Mattila, S.; Neubauer, P.; Hohtola, A *FEBS J.* **2008**, *275*, 4329–4342.

(6) (a) Abe, I.; Oguro, S.; Utsumi, Y.; Sano, Y.; Noguchi, H. *J. Am. Chem. Soc.* **2005**, *127*, 12709–12716. (b) Abe, I.; Watanabe, T.; Morita, H.; Kohno, T.; Noguchi, H. *Org. Lett.* **2006**, *8*, 499–502. (c) Abe, I. *ACS Symp. Ser.* **2007**, *955*, 109–127. (d) Abe, I. *Chem. Pharm. Bull.* **2008**, *56*, 1505–1514.

(7) (a) Fu, H.; Ebert-Khosla, S.; Hopwood, D. A.; Khosla, C. *J. Am. Chem. Soc.* **1994**, *116*, 4166–4170. (b) Fu, H.; Hopwood, D. A.; Khosla, C. *Chem. Biol.* **1994**, *1*, 205–210.

derived heptaketide and a hexaketide, respectively. Spectroscopic data (^{1}H NMR, ^{13}C NMR, MS, and UV) showed good agreement with those of naringenin chalcone and resveratrol, respectively, except the signals due to the terminal α-pyrone ring. The structures of the minor products were determined to be a novel C_{21} heptaketide chalcone (**1**) (0.6 mg, 0.96% yield) and a novel C_{19} hexaketide stilbene (**2**) (1.6 mg, 2.9% yield), which was uniquely consistent with both biogenetic reasoning and NMR data including HMQC and HMBC. The C_{21} chalcone was thus produced by a C-7/C-12 aldol-type cyclization of a heptaketide intermediate, while a C-6/C-11 aldol-type cyclization of the intermediate folded in a different conformation yielded the C_{19} stilbene (Figure 1A).

In contrast, hexanoyl-CoA was found to be a better substrate for the enzyme reaction;[8] *A. arborescens* OKS efficiently accepted hexanoyl-CoA as a starter to produce a 3:4 mixture of a novel C_{18} heptaketide phloroglucinol (**3**) (1.5 mg, 13.0% yield) and a novel C_{16} hexaketide resorcinol (**4**) (2.0 mg, 21.2% yield) as major products after condensations with five and six molecules of malonyl-CoA, respectively (Figure 1B).[8] The two products gave the parent ion peaks $[M + H]^+$ at *m*/*z* 333 and 291 on LC-ESIMS, respectively, and their structures were unambiguously elucidated by NMR spectroscopic analysis. Thus, as in the case of the coumaroyl-derived products, the C_{18} phloroglucinol was produced by a C-7/C-12 aldol-type cyclization of a heptaketide intermediate, while a C-6/C-11 aldol-type cyclization of a hexaketide intermediate yielded the C_{16} resorcinol (Figure 1B). The C_{16} resorcinol has been previously proposed as one of the possible reaction products of the minimal type II PKS from hexanoyl-ACP/malonyl-CoA.[9] Here it should be noted that the reaction products **1**−**4** have not been isolated from either the aloe plant or other natural sources. On the other hand, as previously reported, *A. arborescens* OKS also accepts long chain (C_{10}−C_{20}) fatty acyl-CoAs as a starter and carries out condensations with malonyl-CoA only to produce triketide and tetraketide α-pyrones without formation of an aromatic ring system.[6a]

Recently, a resolved X-ray crystal structure of *A. arborescens* OKS at 2.6 Å resolution revealed that OKS shares active-site architecture similar to that of the previously reported M207G mutant of *A. arborescens* PCS, which also produces SEK4/SEK4b from eight molecules of malonyl-CoA.[10,11] In the CHS/STS enzyme reaction, it has been proposed that 4-coumaroyl-CoA first binds to the so-called "coumaroyl binding pocket";[2] however, in OKS/PCS, the conserved S338 of CHS/STS (numbering in *Medicago sativa*

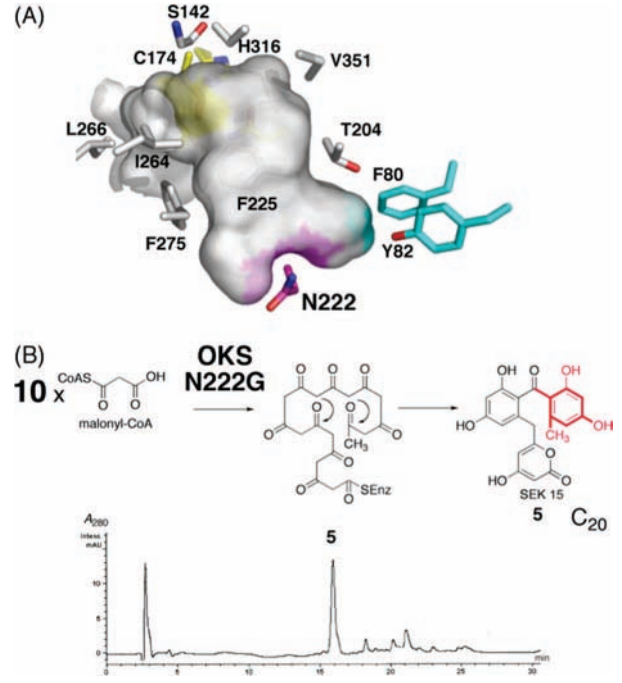


Figure 2. (A) Active-site cavity of *A. arborescens* OKS (wild-type). The residues lining the cavity are shown with the catalytic triad (yellow). The bottom of the pocket and N222 are highlighted in purple. (B) Formation of SEK 15 by N222G mutant and a HPLC profile of the enzyme reaction products.

CHS) is uniquely substituted with hydrophobic Val351, which causes loss of the binding pocket. As a result, OKS/PCS no longer produce the tetraketide chalcone/stilbene from the coumaroyl starter, but instead, both the OKS and the PCS M207G mutant utilize novel buried pockets that extend into the traditional active-site cavity for the production of SEK4/SEK4b (Figure 2).[11b] Therefore, it is likely that the formation of the coumaroyl- and hexanoyl-derived heptaketide/hexaketide product is also dependent upon the presence of the novel buried pockets.

Indeed, it was clearly demonstrated that a structure-based OKS N222G mutant, in which the buried pocket was expanded by a large-to-small substitution of N222 at the bottom of the polyketide chain elongation tunnel, efficiently produced the C_{21} heptaketide chalcone **1** as a major product from coumaroyl-CoA/malonyl-CoA (Figure 1A).[12] Interestingly, when incubated with malonyl-CoA as a sole substrate, the OKS N222G mutant produced a C_{20} decaketide benzophenone, SEK15 (**5**),[7a] by condensations of 10 molecules of malonyl-CoA (Figure 2), which was confirmed by direct comparison with an authentic compound. The decaketide benzophenone has been previously reported as a product of genetically engineered type II PKSs[7a] and is the longest polyketide produced by the structurally simple type III

(8) Steady state kinetic analysis revealed that $K_M = 42.5$ μM and $k_{cat} = 0.211$ min^{-1} for hexanoyl-CoA for the C_{16} resorcinol-forming activity, while $K_M = 94.0$ μM and $k_{cat} = 0.094$ min^{-1} for malonyl-CoA for the SEK4b-forming activity of OKS.[6a]

(9) Nicholson, T. P.; Winfield, C.; Westcott, J.; Crosby, J.; Simpson, T. J.; Cox, R. J. *Chem. Commun.* **2003**, 686–687.

(10) Morita, H.; Kondo, S.; Kato, R.; Wanibuchi, K.; Noguchi, H.; Sugio, S.; Abe, I.; Kohno, T. *Acta Crystallogr.* **2007**, *F63*, 947–949.

(11) (a) Abe, I.; Utsumi, Y.; Oguro, S.; Morita, H.; Sano, Y.; Noguchi, H. *J. Am. Chem. Soc.* **2005**, *127*, 1362–1363. (b) Morita, H.; Kondo, S.; Oguro, S.; Noguchi, H.; Sugio, S.; Abe, I.; Kohno, T. *Chem. Biol.* **2007**, *14*, 359–369. (c) Abe, I.; Morita, H.; Oguro, S.; Noma, H.; Wanibuchi, K.; Kawahara, N.; Goda, Y.; Noguchi, H.; Kohno, T. *J. Am. Chem. Soc.* **2007**, *129*, 5976–5980.

(12) Steady state kinetic analysis revealed that $K_M = 32.1$ μM and $k_{cat} = 0.045$ min^{-1} for 4-coumaroyl-CoA for the C_{21} chalcone-forming activity, and $K_M = 54.4$ μM and $k_{cat} = 0.027$ min^{-1} for malonyl-CoA for the SEK15-forming activity.

PKS.[11c] Presumably, after the chain elongation, *A. arborescens* OKS catalyzes the first aromatic ring formation reaction at the middle of the polyketide intermediate. The partially cyclized aromatic intermediates would then be released from the active site and undergo subsequent spontaneous cyclizations, thereby completing the formation of the terminal α-pyrone ring. Alternatively, the α-pyrone ring could also be formed in the active site of the enzyme; the pyrone ring formation could be an important process for the release of the polyketide products from the thioester-linked active-site Cys residue.

In summary, the present work describes the enzymatic formation of unnatural coumaroyl- and hexanoyl-derived novel polyketides, as well as a decaketide benzophenone, by the structurally simple plant type III PKS. Manipulation of the enzyme reaction by combination of the precursor-directed biosynthesis and structure-guided engineering of the enzyme would thus lead to further production of unnatural novel polyketide scaffolds.

Acknowledgment. The authors thank Professor Chaitan Khosla (Stanford University) for the gift of SEK15. This work was supported by the PRESTO program from Japan Science and Technology Agency, and Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. S.-P.S. and K.W. are recipients of the JSPS Postdoctoral Fellowship for Foreign Researchers (no. P07048) and the JSPS Fellowship for Young Scientists (no. 207112), respectively.

Supporting Information Available: Experimental details and a complete set of NMR data and charts. This material is available free of charge via the Internet at http://pubs.acs.org.

OL802606W