J|A|C|S
ARTICLES
Published on Web 04/17/2007

Structure-Based Engineering of a Plant Type III Polyketide Synthase: Formation of an Unnatural Nonaketide Naphthopyrone

Ikuro Abe,*[,†,¶] Hiroyuki Morita,[§] Satoshi Oguro,[†] Hisashi Noma,[†] Kiyofumi Wanibuchi,[†] Nobuo Kawahara,[‡] Yukihiro Goda,[‡] Hiroshi Noguchi,[†] and Toshiyuki Kohno*[,§]

Contribution from the School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422-8526, Japan, PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan, National Institute of Health Sciences (NIHS), Setagaya, Tokyo 158-8501, Japan, and Mitsubishi Kagaku Institute of Life Sciences (MITILS), Machida, Tokyo 194-8511, Japan

Received January 18, 2007; E-mail: abei@ys7.u-shizuoka-ken.ac.jp; tkohno@mitils.jp

Abstract: Pentaketide chromone synthase (PCS) from *Aloe arborescens* is a novel plant-specific type III polyketide synthase (PKS) that produces 5,7-dihydroxy-2-methylchromone from five molecules of malonyl-CoA. On the basis of the crystal structures of wild-type and M207G mutant PCS, the F80A/Y82A/M207G triple mutant was constructed and shown to produce an unnatural novel nonaketide naphthopyrone by sequential condensations of nine molecules of malonyl-CoA. This is the first demonstration of the formation of a nonaketide by the structurally simple type III PKS. A homology model predicted that the active-site cavity volume of the triple mutant is increased to 4 times that of the wild-type PCS.

Introduction

Pentaketide chromone synthase (PCS) from *Aloe arborescens* (Liliaceae) is a novel plant-specific chalcone synthase (CHS) (EC 2.3.1.74) superfamily type III polyketide synthase (PKS) that catalyzes sequential decarboxylative condensation of five molecules of malonyl-CoA (**1**) to produce 5,7-dihydroxy-2-methylchromone (**2**), which is a biosynthetic precursor of the anti-asthmatic furochromones including khellin and visnagin (Figure 1A).[1,2] *A. arborescens* PCS shares 50−60% amino acid sequence identity with those of other type III PKSs of plant origin. In PCS, CHS's conserved active-site Thr197, Gly256, and Ser338 (numbering in *M. sativa* CHS), sterically altered in a number of divergent type III PKSs, are uniquely replaced with Met, Leu, and Val, respectively (Figure 2). Remarkably, substitution of the Met207 of PCS (corresponding to the CHS's Thr197) with Gly yielded a mutant that efficiently catalyzes successive condensation of eight molecules of malonyl-CoA to produce octaketides, SEK4 (**3**) and SEK4b (**4**), the products of the minimal type II (subunit type) PKS for the benzoisochromanequinone actinorhodin (*act* from *Streptomyces coelicolor*) (Figure 1B).[2,3] The pentaketide-forming PCS was thus functionally transformed into an octaketide synthase by the simple steric modulation of the chemically inert single residue lining the active-site cavity. Indeed, recently solved crystal structures of wild-type and M207G mutant PCS revealed that the large-to-small substitution dramatically increase the volume of the polyketide elongation tunnel by opening a gate to newly found hidden pockets behind the active site of the enzyme (Figure 3A,B).[4] To further manipulate the PCS enzyme reaction, here we extended the polyketide elongation tunnel of the M207G mutant by simultaneously replacing two aromatic residues, Phe80 and Tyr82, forming the bottom of the novel pocket, with Ala. A homology model based on the crystal structure of the PCS M207G mutant predicted that the active-site cavity volume of the F80A/Y82A/M207G triple mutant (1031 Å^3) is increased to 4 times that of the wild-type PCS (247 Å^3) (Figure 3).

Experimental Section

Chemicals. [2-^{14}C]Malonyl-CoA (48 mCi/mmol) was purchased from Moravek Biochemicals (California). Authentic samples of SEK4/SEK4b and aloesone were obtained in our previous works.[2,3]

Site-Directed Mutagenesis. *Aloe arborescens* PCS F80A/Y82A/M207G mutant was constructed with the QuickChange Site-Directed

† University of Shizuoka.
¶ PRESTO, Japan Science and Technology Agency.
‡ NIHS.
§ MITILS.

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

(2) Abe, I.; Utsumi, Y.; Oguro, S.; Morita, H.; Sano, Y.; Noguchi, H. *J. Am. Chem. Soc*. **2005**, *127*, 1362−1363.

(3) (a) Abe, I.; Oguro, S.; Utsumi, Y.; Sano, Y.; Noguchi, H. *J. Am. Chem. Soc*. **2005**, *127*, 12709−12716. (b) Abe, I.; Watanabe, T.; Lou, W.; Noguchi, H. *FEBS J*. **2006**, *273*, 208−218. (c) Abe, I.; Watanabe, T.; Morita, H.; Kohno, T.; Noguchi, H. *Org. Lett*. **2006**, *8*, 499−502. (d) Abe, I. In ACS Symposium Series 955; American Chemical Society: Washington, DC, 2007; pp 109−127.

(4) (a) Morita, H.; Kondo, S.; Abe, T.; Noguchi, H.; Sugio, S.; Abe, I.; Kohno, T. *Acta Crystallogr*. **2006**, *F62*, 899−901. (b) Morita, H.; Kondo, S.; Oguro, S.; Noguchi, H.; Sugio, S.; Abe, I.; Kohno, T. *Chem. Biol*. **2007**, in press.

10.1021/ja070375l CCC: $37.00

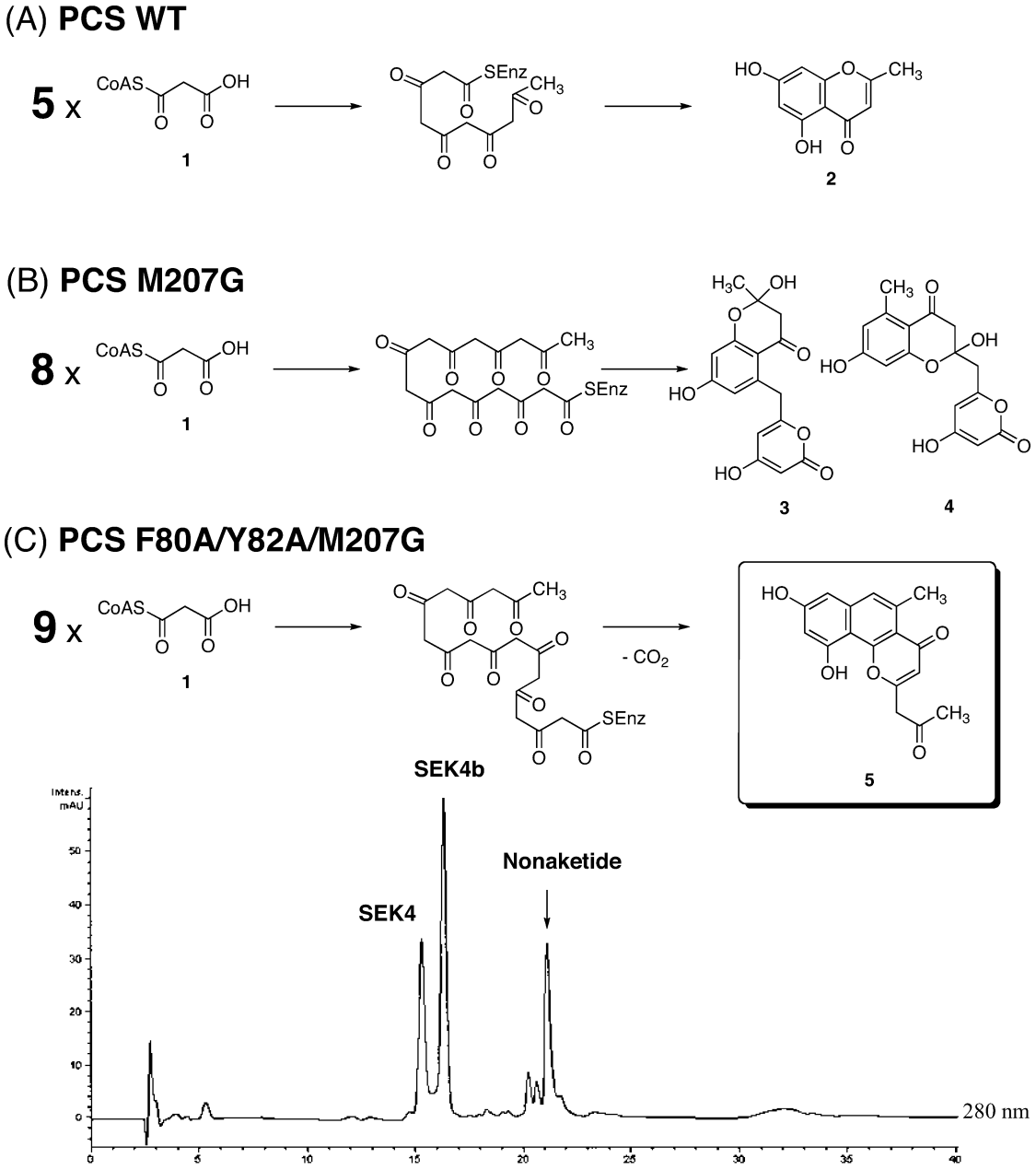


Figure 1. Enzyme reaction of (A) wild-type PCS, (B) PCS M207G mutant, and (C) PCS F80A/Y82A/M207G mutant. HPLC elution profile of the reaction products of the triple mutant.

Mutagenesis Kit (Stratagene), according to manufacturer's protocol, using a pair of primers (mutated codons are underlined): sense 5′-GGGAAGCGTTAGCCAACGCTGACGAGGAGTTC-3′ and antisense as a sense primer, 5′-GAACTCCTCGTCAGCGTTGGCGTAACGCTTCCC-3′, and the PCS M207G expression plasmid as the template.

Enzyme Expression and Purification. After confirmation of the sequence, protein expression, extraction, and initial purification by glutathione Sepharose 4B affinity chromatography (Amersham) were carried out as previously described by PreScission protease digestion to remove the GST tag.[4] The resultant PCS mutant protein thus contains three additional residues (GPG) at the N-terminal flanking region derived from the PreScission protease recognition sequence. After affinity purification and the GST tag removal, the protein solution containing the mutant PCS was diluted 5-fold with 50 mM HEPES/NaOH buffer (pH 7.0) containing 5% glycerol and 2 mM DTT and then was applied to a Resource-Q column (Amersham). The column was washed with the HEPES/NaOH buffer containing 50 mM NaCl, and the protein was subsequently eluted using 50−200 mM NaCl linear gradient. Finally, the mutant PCS protein solution was further purified to homogeneity by Superdex 200HR (10/100GL) (Amersham) and concentrated to 10 mg/mL in 20 mM HEPES/NaOH (pH 7.0) containing 100 mM NaCl and 2 mM DTT.

Enzyme Reaction. The standard reaction mixture contained 216 μM of malonyl-CoA and 10 μg of enzyme in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 6.0. Incubations were carried out at 30 °C for 1 h. The products were extracted with 1000 μL of ethyl acetate and analyzed by reverse-phase HPLC on a TSK-gel ODS-80TsQA column (2.0 × 150 mm) (TOSOH, Tokyo, Japan) with a flow rate of 0.8 mL/min. Gradient elution was performed with H_2O and MeOH, both containing 0.1% TFA: 0−5 min, 30% MeOH; 5−17 min, 30−60% MeOH; 17−25 min, 60% MeOH; 25−27 min, 60−70% MeOH; 27−35 min, 70% MeOH; 35−40 min, 70−100% MeOH. Retention time: SEK4 (15.3 min), SEK4b (16.3 min), and the novel nonaketide (21.1 min). On-line LC-ESIMS spectra were measured with a Agilent Technologies HPLC 1100 series coupled to a Bruker Daltonics

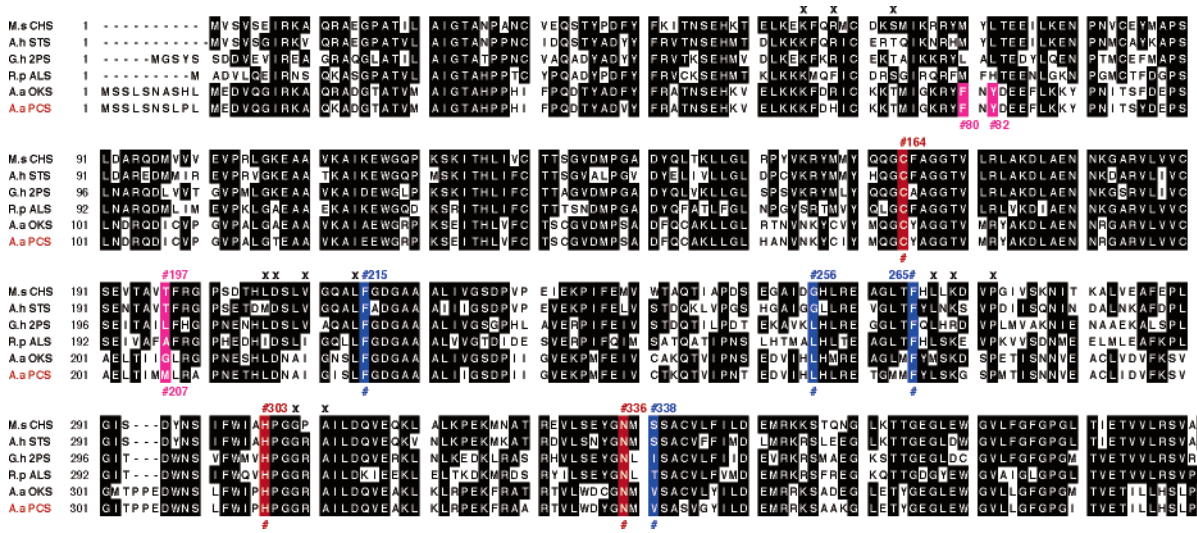


Figure 2. Comparison of the amino acid sequences of *Aloe arborescens* PCS and other CHS superfamily type III PKSs. M.s CHS, *Medicago sativa* CHS; A.h STS, *Arachis hypogaea* stilbene synthase; G.h 2PS, *Gerbera hybrida* 2PS; R.p ALS, *Rheum palmatum* ALS; A.a OKS, *A. arborescens* octaketide synthase; A.a PCS, *A. arborescens* PCS. The critical active-site residue 197 (in pink), the catalytic triad (Cys164, His303, and Asn336) (in red), and the residues lining the active site (Phe215, Gly256, F265, and Ser338) (in blue) are marked with # (numbering in *M. sativa* CHS), and residues for the CoA binding with +. Phe80 and Tyr82 of *A. arborescens* PCS are also marked (in pink).

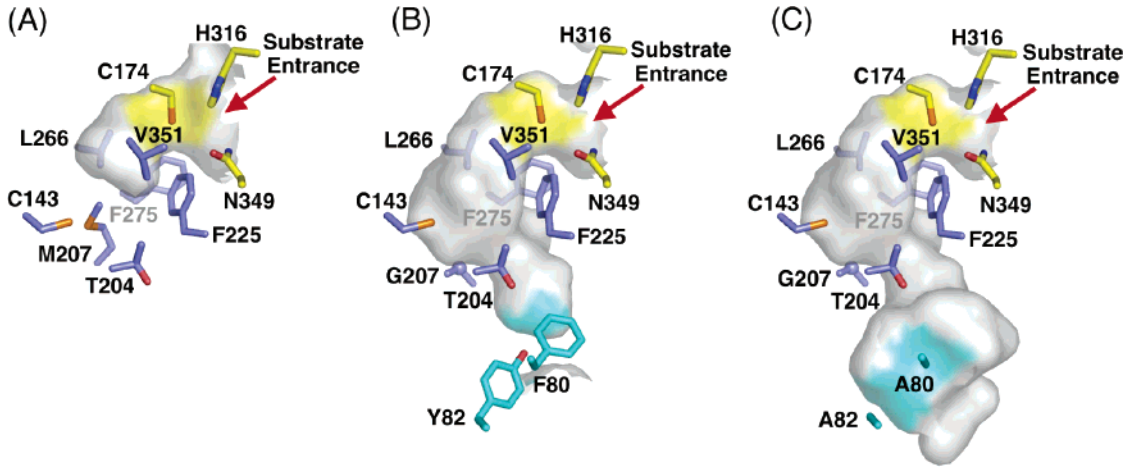


Figure 3. Comparison of the active-site cavities of (A) wild-type PCS (PDB code 2D3M), (B) PCS M207G mutant (PDB code 2D52), and (C) PCS F80A/Y82A/M207G mutant (homology model). The residues lining the cavity (blue and orange) are shown with the catalytic triad (yellow). The bottoms of the pockets of the mutants are highlighted in light blue. The cavity volumes are calculated to be 247, 649, and 1031 Å, respectively.

esquire4000 ion trap mass spectrometer fitted with an ESI source. HPLC separations were carried out under the same conditions as described above. The ESI capillary temperature and capillary voltage were 320 °C and 4.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in both negative and positive mode, over a mass range of *m*/*z* 50−600, at a range of one scan every 0.2 s. The collision gas was helium, and the relative collision energy scale was set at 30.0% (1.5 eV).

For a large-scale reaction, 24 mg of malonyl-CoA was incubated with 10 mg of the enzyme in 40 mL of 100 mM phosphate buffer, pH 6.0. Incubations were carried out at 30 °C for 12 h. The products were extracted with 100 mL of ethyl acetate and separated by reverse-phase HPLC as described. The experiment was repeated 10 times to get ca. 1 mg of pure compound for the NMR measurement. 2-Acetonyl-8,10-dihydroxy-5-methyl-4*H*-naphtho[1,2-*b*]pyran-4-one (**5**): ^{1}H NMR (800 MHz, CD_3OH) δ 7.20 (s, 1H), 6.60 (s, 1H), 6.52 (s, 1H), 6.31 (s, 1H), 3.94 (s, 2H), 2.77 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (200 MHz, CD_3OH) δ 204.6, 181.6, 161.8, 161.4 158.9, 140.7, 135.8, 126.7, 117.5, 114.7, 108.3, 104.2, 102.8, 48.1, 30.2, 23.3; UV λ_{max} 240, 272, 360 nm; LRMS (FAB) *m*/*z* 299 $[M + H]^+$, 277, 185, 93, 75; HRMS (FAB) found for $[C_{17}H_{15}O_5]^+$ 299.0893, calcd 299.0920.

Homology Modeling. The model of *A. arborescens* PCS F80A/Y82A/M207G mutant was generated with the CPHmodels 2.0 package[5] (http://www.cbs.dtu.dk/services/CPHmodels/) based on the crystal structure of the PCS M207G mutant[4] (PDB code: 2D52). The model quality was checked using PROCHECK.[6] The cavity volume was calculated on the basis of the residues composing the active-site cavity wall, by using CASTP (http://cast.engr.uic.edu/cast/).

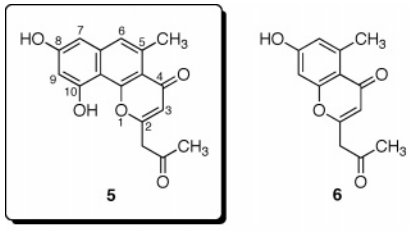
Results and Discussion

A. arborescens PCS F80A/Y82A/M207G triple mutant was functionally expressed in *Escherichia coli* at levels comparable with the wild-type enzyme and purified to homogeneity as in the case of the wild-type PCS.[4] When incubated with malonyl-CoA as a substrate, the triple mutant afforded a novel product (0.2% yield, which was calculated by incubation with [2-^{14}C]-malonyl-CoA as a substrate under the standard assay condition;

(5) Lund, O.; Nielsen, M.; Lundegaard, C.; Worning, P. Abstract at the CASP5 conferenceA102, 2002.
(6) Laskowski, R. A.; MacArthur, M. W.; Moss, D. S.; Thornton, J. M. *J. Appl. Crystallogr.* **1993**, *26*, 283−291.

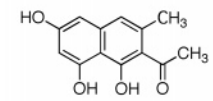
10 times less efficient than that of the pentaketide production by the wild-type PCS) in addition to SEK4 (**3**) and SEK4b (**4**) (Figure 1C). The product gave a UV spectrum (λ_{max} 240, 272, and 360 nm) and a parent ion peak $[M + H]^+$ at *m*/*z* 299 on LC-ESIMS, indicating formation of a nonaketide. The nonaketide-forming activity was maximum at pH 6.0, but decreased to ca. 30% under alkaline pH. In contrast, the SEK4/SEK4b-forming activity of the PCS mutants showed a broad pH optimum within a range of 6.0−8.0. Thus, the pH change did not significantly affect the yield and the ratio of SEK4/SEK4b (1:4). Only the nonaketide-forming activity suffered sharp decline under the alkaline pH.

The ^{1}H NMR spectrum of the nonaketide product obtained from a large-scale incubation revealed the presence of three aromatic protons (δ 7.20, 6.60, and 6.52, each 1H, s), one α,β-unsaturated olefinic proton (δ 6.31, 1H, s), one methylene (δ 3.94, 2H, s), and two methyl protons (δ 2.77 and 2.34, each 3H, s). A structure with a naphthopyrone skeleton was uniquely consistent with both biogenetic reasoning and NMR spectroscopic data, including heteronuclear correlation spectroscopy (HMQC and HMBC). The structure of the novel nonaketide was thus determined to be 2-acetonyl-8,10-dihydroxy-5-methyl-4*H*-naphtho[1,2-*b*]pyran-4-one (**5**) (Figure 1C), which has been isolated until now neither from the aloe plant, a rich source of aromatic polyketides such as pharmaceutically important aloenin (hexaketide), aloesin (heptaketide), and barbaloin (octaketide),[2] nor from other natural sources.

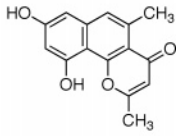


5

6



7



8

Interestingly, the structure of the novel nonaketide **5** showed close similarity with heptaketides aloesone (2-acetonyl-7-hydroxy-5-methylchromone) (**6**)[7] produced by the aloe plant and 6-hydroxymusizin (2-acetyl-1,6,8-trihydroxy-3-methylnaphthalene) (**7**) from rhubarb (Polygonaceae),[8] and an octaketide eleutherinol (8,10-dihydroxy-2,5-dimethyl-4*H*-naphtho[1,2-*b*]-pyran-4-one) (**8**), a constituent of another medicinal plant *Eleutherine bulbosa* (Iridaceae),[9] suggesting that these aromatic polyketides are produced by closely related type III PKSs. Indeed, we have previously reported that the G207A mutant of *A. arborescens* octaketide synthase produced aloesone from seven molecules of malonyl-CoA.[3a] In contrast, the structure of the nonaketide naphthopyrone **5** is apparently different from that of the pentaketide 5,7-dihydroxy-2-methylchromone (**2**), the normal product of PCS.

It is remarkable that the PCS F80A/Y82A/M207G mutant not only catalyzed condensation of nine molecules of malonyl-CoA but also altered the mechanism of the cyclization to produce the unnatural novel "nonaketide" naphthopyrone **5**, which is the longest polyketide generated by the structurally simple type III PKS. Significant part of the reactions was, however, terminated at the octaketide stage to afford SEK4/SEK4b as shunt products. The low yield of the enzyme reaction products could be attributed to the possible conformational changes caused by the triple mutation. In addition, it should be noted that the entrance to the newly formed polyketide tunnel is still narrow in the triple mutant (Figure 3). Further optimization of the active-site structure would lead to improvement of the yield of the nonaketide product. On the other hand, despite the structural similarity with eleutherinol (**8**), formation of the "octaketide" naphthopyrone was not detected either with the M207G point mutant or with the triple mutant, which was confirmed by the LC-ESIMS analysis. The naphthalene ring-forming activity was thus only attained by the F80A/Y82A/M207G triple mutation.

As mentioned above, our homology model predicted that the replacement of the three residues (Phe80, Tyr82, and Met197) resulted in dramatic increase in the active-site cavity volume. The functional conversion appeared to be caused by the simple steric modulation of the active site accompanied by conservation of the Cys-His-Asn catalytic triad. Interestingly, a similar active-site architecture with the downward expanding polyketide tunnel has been reported for a bacterial "pentaketide" naphthalene-producing type III PKS, 1,3,6,8-tetrahydroxynaphthalene synthase (THNS) from *S. coelicolor*[10] that shares only ca. 20% amino acid sequence identity with the plant enzyme. On the basis of the crystal structure of *S. coelicolor* THNS, the possible involvement of an additional catalytic Cys residue has been proposed for the unusual naphthalene ring formation reaction.[10] However, such an additional Cys residue is not present at the catalytic center of *A. arborescens* PCS; only the conventional Cys174 (corresponding to CHS's Cys164) serves as the covalent attachment site for the growing polyketide intermediates, suggesting a different mechanism of the enzyme reaction in the PCS F80A/Y82A/M207G mutant.

The proposed mechanism of the formation of the nonaketide naphthopyrone **5** with a fused tricyclic ring system involves a consecutive intramolecular aldol condensation (Figure 4A). Presumably, the enzyme catalyzes the first aromatic ring formation reaction after the sequential decarboxylative condensations of nine molecules of malonyl-CoA. One of the most important points here is the timing of the cyclization reaction and the thioester bond cleavage of the nonaketide intermediate bound to the active-site Cys. Further, it is not certain whether all three ring formations are enzymatic or not. At least, the final pyranone ring formation, as in the case of that of SEK4/SEK4b (Figure 1B), is likely to be a nonenzymatic process. The partially cyclized aromatic intermediates would be released from the active site and undergo subsequent spontaneous cyclizations, thereby completing the formation of the fused ring system.

(7) Abe, I.; Utsumi, Y.; Oguro, S.; Noguchi, H. *FEBS Lett.* **2004**, *562*, 171−176.
(8) Tsuboi, M.; Minami, M.; Nonaka, G.; Nishioka, I. *Chem. Pharm. Bull.* **1977**, *25*, 2708−2712.
(9) (a) Birch, A. J.; Donovan, F. W. *Aust. J. Chem.* **1953**, *6*, 373−378. (b) Ebnother, A.; Meijer, Th. M.; Schmid, H. *Helv. Chim. Acta* **1952**, *35*, 910−928.
(10) Austin, M. B.; Izumikawa, M.; Bowman, M. E.; Udwary, D. W.; Ferrer, J.-L.; Moore, B. S.; Noel, J. P. *J. Biol. Chem.* **2004**, *279*, 45162−45174.

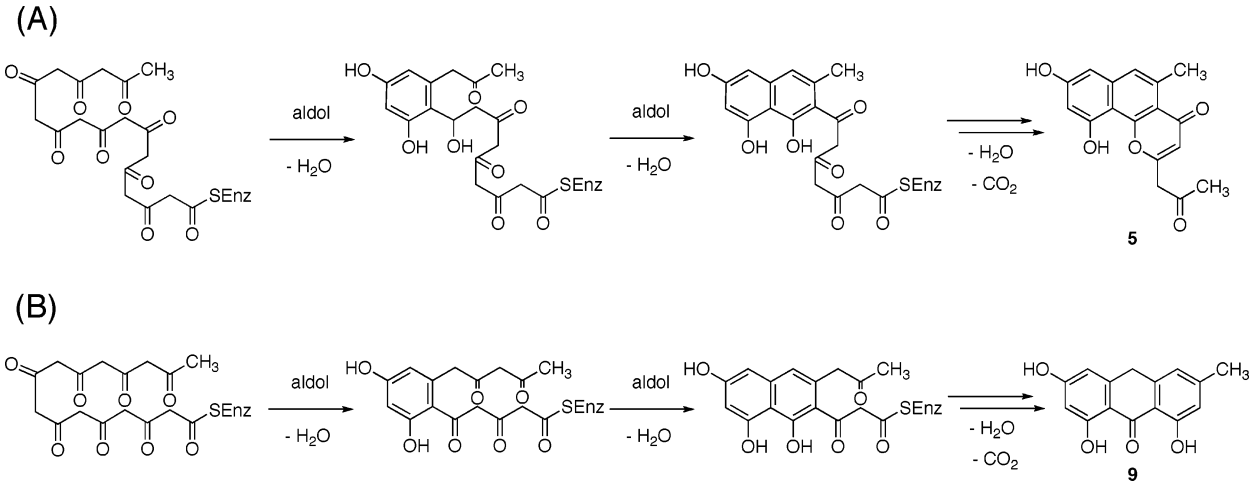


Figure 4. Proposed mechanism for the formation of (A) naphthopyrone **5** from a nonaketide intermediate, and (B) emodin anthrone (**9**) from an octaketide intermediate.

Interestingly, Harris and co-workers have developed a methodology for chemical syntheses of polycyclic polyketides; β-polyketones having the two terminal carbonyl groups protected as ketals are efficiently and directly cyclized into aromatic polyketides including 6-hydroxymusizin (**7**) and eleutherinol (**8**).[11] Finally, it should be noted that the formation of the nonaketide naphthopyrone with the fused ring system by the *A. arborescens* PCS mutant strongly suggests further involvement of the CHS superfamily type III PKS in the biosynthesis of anthrones and anthraquinones including emodin anthrone (**9**) and barbaloin in the aloe plant (Figure 4B).[3a]

In summary, this is the first demonstration of the formation of a nonaketide by the structurally simple type III PKS. Structure-based engineering of the CHS superfamily type III PKS enzymes would thus lead to further production of chemically and structurally disparate unnatural novel polyketides.

Acknowledgment. This work was supported by the PRESTO program from Japan Science and Technology Agency, Grant-in-Aid for Scientific Research (Nos. 18510190 and 17310130), and the COE21 program from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Supporting Information Available: A complete set of NMR spectra of nonaketide naphthopyrone (**5**) (^{1}H and ^{13}C NMR, DEPT135, HMQC, and HMBC). This material is available free of charge via the Internet at http://pubs.acs.org.

JA070375L

(11) (a) Harris, T. M; Wittek, P. J. *J. Am. Chem. Soc.* **1975**, *97*, 3270−3271. (b) Harris, T. M.; Harris, C. M. *Pure Appl. Chem.* **1986**, *58*, 283−294.