Cloning and Structure-Function Analyses of Quinolone- and Acridone-producing Novel Type III Polyketide Synthases from *Citrus microcarpa*

Received for publication, June 17, 2013, and in revised form, August 8, 2013. Published, JBC Papers in Press, August 20, 2013, DOI 10.1074/jbc.M113.493155

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Background: Type III polyketide synthases (PKSs) synthesize various polyketide and alkaloid scaffolds.

Results: QNS synthesizes quinolone as the single product, whereas ACS produces acridone as the major product.

Conclusion: QNS and ACS are novel quinolone- and acridone-producing type III PKSs, respectively.

Significance: Structure-function analyses of QNS and ACS provide insights into molecular bases for alkaloid biosyntheses.

Two novel type III polyketide synthases, quinolone synthase (QNS) and acridone synthase (ACS), were cloned from *Citrus microcarpa* (Rutaceae). The deduced amino acid sequence of *C. microcarpa* QNS is unique, and it shared only 56–60% identity with *C. microcarpa* ACS, *Medicago sativa* chalcone synthase (CHS), and the previously reported *Aegle marmelos* QNS. In contrast to the quinolone- and acridone-producing *A. marmelos* QNS, *C. microcarpa* QNS produces 4-hydroxy-N-methylquinolone as the “single product” by the one-step condensation of N-methylanthraniloyl-CoA and malonyl-CoA. However, *C. microcarpa* ACS shows broad substrate specificities and produce not only acridone and quinolone but also chalcone, benzophenone, and phloroglucinol from 4-coumaroyl-CoA, benzoyl-CoA, and hexanoyl-CoA, respectively. Furthermore, the x-ray crystal structures of *C. microcarpa* QNS and ACS, solved at 2.47- and 2.35-Å resolutions, respectively, revealed wide active site entrances in both enzymes. The wide active site entrances thus provide sufficient space to facilitate the binding of the bulky *N*-methylanthraniloyl-CoA within the catalytic centers. However, the active site cavity volume of *C. microcarpa* ACS (760 Å³) is almost as large as that of *M. sativa* CHS (750 Å³), and ACS produces acridone by employing an active site cavity and catalytic machinery similar to those of CHS. In contrast, the cavity of *C. microcarpa* QNS (290 Å³) is significantly smaller, which makes this enzyme produce the diketide quinolone. These results as well as mutagenesis analyses provided the first structural bases for the anthranilate-derived production of the quinolone and acridone alkaloid by type III polyketide synthases.

The members of the chalcone synthase (CHS) superfamily of type III polyketide synthases (PKSs) are distributed in diverse organisms, including plants, fungi, and bacteria, and are responsible for the synthesis of various biologically and pharmacologically important natural products (1, 2). They are structurally simple 40–45-kDa homodimeric enzymes that utilize a conserved Cys-His-Asn catalytic triad in an active site to catalyze the iterative condensations of C₂ units derived from malonyl-CoA to a CoA-linked starter molecule. The functional diversity of the type III PKSs is basically derived from small modifications within the active site architectures of the enzymes, which influence the starter substrate selection, the number of chain extensions, and the cyclization reaction mechanisms (1, 2). As one of the characteristic features of the type III PKSs, the enzymes show broad substrate promiscuity and catalytic versatility in *in vitro* reactions and convert structurally and chemically distinct CoA starters into various molecular scaffolds (2–6).

The quinolone and acridone alkaloids have been investigated as *N*-methyl-d-aspartate (NMDA) and serotonin 5-hy-
The greatest abundance of these alkaloids is found in the Rutaceae plants, and \( \text{N} \)-methylanthranilic acid is thought to be a key intermediate in their biosynthesis (10–12). Among the growing number of reported type III PKSs, two kinds of type III PKSs specifically involved in the biosynthesis of the anthranilate-derived alkaloids have been obtained from Rutaceae plants. One is acridone synthase (ACS) from \( \textit{Ruta graveolens} \), which catalyzes the iterative condensations of \( \text{N} \)-methylanthraniloyl-CoA with three molecules of malonyl-CoA to produce the tetraketide 1,3-dihydroxy-\( \text{N} \)-methylacridone (Fig. 1A) (10, 11). Although the chalcone-forming CHS does not accept the bulky \( \text{N} \)-methylanthraniloyl-CoA starter (4, 11, 13–15), \( \textit{R. graveolens} \) ACS can accept 4-coumaroyl-CoA as a starter substrate to produce naringenin chalcone after three condensations with malonyl-CoA (Fig. 1B) (13, 15).

The other type III PKS is the recently reported quinolone synthase (QNS) from \( \textit{Aegle marmelos} \), which produces the diketide 4-hydroxy-\( \text{N} \)-methylquinolone by the one-step condensation of \( \text{N} \)-methylanthraniloyl-CoA with one molecule of malonyl-CoA (Fig. 1A) (12). The two type III PKS enzymes thus catalyze the \( \text{C}–\text{N} \) bond-forming reactions, in addition to the \( \text{C}–\text{C} \) bond formation, to generate the anthranilate-derived alkaloid scaffolds. Notably, \( \textit{A. marmelos} \) QNS does not produce the diketide.
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quinoine specifically but also generates the tetraketide acridone from N-methylantraniloyl-CoA in an in vitro enzyme reaction (Fig. 1A). Thus, A. marmelos QNS could be regarded as another acridine-producing ACS, which simply yields the diketide quinolone as a by-product. This is analogous to the tetraketide chalcone-forming ACS, which also produces triketide and tetraketide lactone by-products in vitro (Fig. 1B).

However, we previously demonstrated that benzalacetone synthase (BAS) from Rheiunum palmatum, which normally produces the diketide benzalacetone by the one-step condensation of 4-coumaryl-CoA with malonyl-CoA, also accepts N-methylantraniloyl-CoA as a starter substrate to produce the quinolone by condensation with malonyl-CoA (Fig. 1B) (16).

We now report the first QNS that produces 4-hydroxy-N-methoxyquinoline from N-methylantraniloyl-CoA, as well as another acridine-producing ACS, from the leaves of the natural anti-inflammatory Rutaceae plant Citrus microcarpa. Remarkably, in contrast to the previously reported quinolone-and-acridone-forming A. marmelos QNS, C. microcarpa QNS produces the diketide quinolone as the "single product" by the one-step condensation of N-methylantraniloyl-CoA with malonyl-CoA (Fig. 1A). However, the newly obtained C. microcarpa ACS shows quite promiscuous substrate specificities and produces not only the anthranilate-derived acridone and quinolone but also accepts various starter substrates to generate distinct molecular scaffolds, including chalcone, benzophenone, and phloroglucinol (Fig. 1 (1, 2, 17). To clarify the mechanistic details of the enzyme reactions, we also solved the x-ray crystal structures of C. microcarpa QNS and ACS, at 2.47- and 2.35-Å resolutions, respectively. A comparison of the crystal structures revealed the unique active site architectures of these two enzymes and provided the first structural basis for the production of the anthranilate-derived quinolone and acridone alkaloids by type III PKSs.

EXPERIMENTAL PROCEDURES

Materials—The [2-14C]malonyl-CoA (55 mCi/mmol) and [1-14C]acetyl-CoA (52.5 mCi/mmol) were purchased from Moravek Biochemicals (Brea, CA). The 4-coumaryl-CoA and N-methylantraniloyl-CoA were chemically synthesized as described previously (4, 18). Malonyl-CoA, hexanoyl-CoA, and benzoyl-CoA were purchased from Sigma. Authentic samples of the enzyme reaction products were obtained in our previous work (4, 18).

Gene Cloning of QNS and ACS—C. microcarpa Bunge leaves were harvested from the Matakihi farm in Okinawa, Japan, and immediately frozen in liquid nitrogen. Total RNA was extracted with an RNaseasy plant mini kit (Qiagen) and was reverse-transcribed using Superscript II RT (Invitrogen) and the oligo(dT) primer (RACE 32mers, 5'-CCCAACCAAGATG-3') according to the manufacturer’s protocol. The single strand cDNA thus obtained was used as the template for the PCRs with inosine-containing degenerate oligonucleotide primers, designed on the basis of the conserved sequences of known CHSs, as described previously (4, 19–21): 112S = 5’-(A/G)A(A/G)GCI-ITI(A/C)A(A/G)GA(A/G)GGGGGICA-3’; 174S = 5’-GCIAAA- (A/G)GA(T/C)ITICIGCA(A/G)AA(T/C)AA-3’; 368A = 5’-CCC(C/A)(A/T)ITCIA(A/G)ICICITCICITG3’; and 380A = 5’-TCIA(T/C)IGTIA(A/G)ICICICG ICC(A/G)AA-3’ (the primer’s number indicates the amino acid number within M. sativa CHS). Following the conditions described previously (4, 19–21), nested PCR was performed with the primer sets 112S and 380A and then with 174S and 368A to amplify the respective 550-bp core fragments of C. microcarpa QNS and ACS. The 3’-RACE, using two gene-specific primers (209S, 5’-ACA- CTGTGTTGGGGAAC(M)CTGTGTG-3’; and 252S, 5’-ACCAAACAGTTTATCAGGCGACAGTG-3’ for QNS, and 297S, 5’-GATTCTCTAGTGGTGGAGCTTT-3’, and 245S, 5’-GCTCTCTGAGTAATCGTGAGTCAAA-3’ for ACS), and the oligo(dT) primer RACE32, was used to amplify the QNS’s 472-bp cDNA and the ACS’s 642-bp fragment. The 5’-RACE was performed using the 5’-RACE system (Invitrogen) and two gene-specific primers (209A, 5’-TTTCAATGCTGTGA-CAAACATTTGTTGTT-3’, and 298A, 5’-GAAAACATGCTTTGAGTCAAA-3’ for QNS, and 253A, 5’-ATGCACTGACGAAAGCGC-3’; and 215A, 5’-AAAGAGCCT-GACCCTAGAGATC-3’ for ACS) to amplify QNS’s 643-bp and ACS’s 681-bp DNA fragments. The full-length cDNA encoding M. microcarpa QNS was obtained using N- and C-terminal primers as follows: 5’-GCGAGATCATATGGAATC-3’ (sense, the XhoI site is underlined). The amplified full-length QNS’s 472-bp cDNA and the ACS’s 681-bp fragment. The full-length cDNA encoding C. microcarpa ACS was finally obtained using N- and C-terminal-specific primers as follows: 5’-GCGAGATCATATGGAATC-3’ (sense, the BglII site is underlined) and 5’-CCACTCAAGTCCAGGCAAAGA-TCAATGAGGACC-3’ (antisense, the Xhol site is underlined). The full-length cDNA encoding C. microcarpa ACS was finally obtained using N- and C-terminal-specific primers as follows: 5’-GCGAGATCATATGGAATC-3’ (sense, the BglII site is underlined) and 5’-GCGCTCATTGCTTCTTAATAGGGAGATGGAATC-3’ (antisense, the Xhol site is underlined). The amplified full-length C. microcarpa QNS and ACS cDNA fragments were digested with BglII/Xhol and cloned into the BamHI/Xhol sites of pQE80L (Novagen), respectively. Both recombinant enzymes thus contain an additional N-terminal hexahistidine tag. The nucleotide sequence was determined using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Phylogenetic Tree—A total of 52 type III PKS amino acid sequences were aligned, and a phylogenetic tree was developed with the ClustalW (1.8) program (DNA Data Bank of Japan), as reported previously (4).

Expression and Purification of C. microcarpa QNS and ACS—The plasmids containing the full-length cDNAs encoding C. microcarpa QNS and ACS were individually transformed into Escherichia coli M15. The cells harboring the plasmids were cultured to an A600 of 0.6 in LB medium containing 100 μg ml−1 ampicillin at 23 °C, and 1.0 mM isopropyl-β-D-galactopyranoside was then added to induce protein expression. The culture was incubated further at 23 °C for 16 h. All of the following procedures were performed at 4 °C. The E. coli cells were harvested by centrifugation at 5,000 × g and resuspended in 50 mM Tris-HCl buffer (pH 7.5), containing 0.2 M NaCl, 5% (v/v) glycerol, and 5 mM imidazole (buffer A). The cells were disrupted by sonication, and the lysate was centrifuged at 12,000 × g for 30 min. The supernatant was loaded onto a nickel-Sepharose 6 Fast Flow column (GE Healthcare) equilibrated with buffer A. After washing the resin with 50 mM Tris-
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HCl buffer (pH 7.5), containing 0.2 mM NaCl, 5% (v/v) glycerol, and 10 mM imidazole (buffer B), the recombinant protein was subsequently eluted with buffer B containing 300 mM imidazole. The protein solution was then diluted 5-fold with 50 mM Tris-HCl buffer (pH 7.5), containing 5% (v/v) glycerol and 2 mM DTT (buffer C), and applied to a Resource-Q column (GE Healthcare). The column was washed with buffer C containing 50 mM NaCl, and the protein was subsequently eluted using a linear gradient of 50–300 mM NaCl. The protein solution was concentrated to 5 mL, purified to homogeneity by gel filtration chromatography on a Superdex 200 HR column (16/60 GL; GE Healthcare), and concentrated to 10 mg/mL in 20 mM Tris-HCl buffer (pH 7.5), containing 100 mM NaCl and 2 mM DTT.

Enzymatic Reactions of C. microcarpa QNS and ACS—The standard reaction mixture contained 54 μmol of starter CoA, 108 μmol of malonyl-CoA, and 20 μg of the purified recombinant enzyme, in a final volume of 500 μL of 100 mM potassium phosphate buffer (pH 7.0). The reactions were incubated at 30 °C for 60 min and stopped by adding 50 μL of 20% HCl. The products were then extracted three times with 500 μL of ethyl acetate, concentrated by evaporation, and analyzed by RP-HPLC on a COSMOSIL 30% MeOH; 5–17 min, 30–60% MeOH; 17–25 min, 60% MeOH; 25–27 min, 60–70% MeOH; 27–35 min, 70% MeOH; and 35–40 min, 70–100% MeOH. On-line LC-ESI-MS spectra were measured with an Agilent Technologies (Santa Clara, CA) HPLC 1100 series coupled to a Bruker Daltonics (Bremen, Germany) Esquire 4000 ion trap mass spectrometer fitted with an ESI source, as described previously (4–6). The enzyme reaction products were identified by direct comparisons to authentic compounds.

Enzyme Kinetics—Steady-state kinetic parameters were determined using [2-14C]malonyl-CoA (1.8 mCi/mmol) as the substrate. The experiments were performed in triplicate, using five concentrations (51.2, 25.6, 12.8, 6.4, and 3.2 μmol) of starter-CoA and 4 μg of purified enzyme, in a final volume of 100 μL of 100 mM Tris-HCl (pH 8.0). The reactions were incubated at 30 °C for 30 min. The reaction products were extracted twice with 100 μL of ethyl acetate and separated by TLC (Merck 1.11798 silica gel 60 F254; ethyl acetate/hexane/ACOH = 63:27:5, v/v/v). Radioactivities were quantified by autoradiography, using a BAS-2000II bioimaging analyzer (Fujifilm, Tokyo, Japan). Lineweaver-Burk plots of data were employed to derive the apparent K_m and k_cat values (average of triplicates ± S.D.) using the EnzFitter software (BIOSOFT, Cambridge, UK).

Crystallization and Structure Refinement of QNS and ACS—Initial crystallization attempts and optimization of the crystallization conditions for both enzymes were performed at 20 °C, using the sitting-drop vapor diffusion method. First, 0.5 μL of either the purified recombinant QNS or ACS protein and an equal volume of reservoir solution were mixed and equilibrated against 50 μL of reservoir solution, using a 96-condition crystallization screen originally designed by Mitsubishi Chemical Corp. Crystals of QNS appeared the next day in a crystallization solution consisting of 100 mM Tris-HCl (pH 7.0) and 18% PEG 3350. Crystals of ACS were observed a few days later in a crystallization solution consisting of 100 mM HEPES-NaOH (pH 7.5), 1,400 mM ammonium sulfate, and 2 mM CoASH. Further attempts to crystallize C. microcarpa QNS and ACS were performed, using Additive Screen (Hampton Research), at various pH values, 18% PEG 3350, 100 mM HEPES-NaOH (pH 7.5), and 1,400 mM ammonium sulfate as a precipitant, respectively. Diffraction quality crystals of C. microcarpa QNS were finally obtained in 50 mM Tris-HCl (pH 7.0), 18% PEG 3350, and 4% (v/v) 1-propanol, and those of C. microcarpa ACS were finally obtained in 100 mM HEPES-NaOH (pH 7.5), 1,400 mM ammonium sulfate, 2 mM NiCl2, and 2 mM CoASH using the sitting-drop vapor diffusion method at 20 °C.

The crystals of C. microcarpa QNS were transferred into a cryoprotectant solution, consisting of 50 mM Tris-HCl (pH 7.0), 18% PEG 3350, 4% (v/v) 1-propanol, and 18% glycerol. After a few seconds, the crystals were picked up in a nylon loop and then flash-cooled at −173 °C in a nitrogen gas stream. X-ray diffraction data sets were collected on beamline BL17A at the Photon Factory (wavelength 0.9800 Å) using an ADSC Quantum 315 CCD detector, with a distance of 250 mm between the crystal and the detector. A total of 180 frames was recorded, with a 1° oscillation angle and 1-s exposure time.

The crystals of C. microcarpa ACS were transferred into a cryoprotectant solution, consisting of 100 mM HEPES-NaOH (pH 7.5), 1,400 mM ammonium sulfate, 2 mM NiCl2, and 20% glycerol. After a few seconds, the crystals were picked up in a nylon loop and then flash-cooled at −173 °C in a nitrogen gas stream. X-ray diffraction data sets were collected on beamline NW-12 of the Photon Factory-AR (wavelength 1.00000 Å) using an ADSC Quantum 210 CCD detector, with a distance of 150 mm between the crystal and the detector. A total of 180 frames were recorded, with a 1° oscillation angle and 1-s exposure time. The data were indexed, integrated, and scaled with the HKL-2000 program package (22).

Structure Refinement—The initial phases of the C. microcarpa QNS and ACS structures were determined by molecular replacement, using the M. sativa CHS structure (Protein Data Bank code 1CGK) as the search model. Molecular replacement was performed with MOLREP in the CCP4 suite (23, 24). The structure was modified manually with Coot (25) and refined with PHENIX (26). The final model of C. microcarpa QNS consisted of residues 1–389 of monomers A–D, two molecules of glycerol, and 198 molecules of water. The final model of C. microcarpa ACS consisted of residues 1–389 with 11 artificial residues, including an artificial Met as the initiation codon and the N-terminal His6 tags of monomers A and B, two molecules of CoA-SH, nine molecules of nickel, three molecules of SO4, and 331 molecules of water. The qualities of the final models were assessed with MolProbity (27). A total of 97.1% of the residues in the C. microcarpa QNS structure are in the favored regions of the Ramachandran plot, and 2.9% are in the allowed regions, although a total of 97.5% of the residues in the C. microcarpa ACS structure are in the most favored regions of the Ramachandran plot, and 2.5% are in the allowed regions. The coordinates and structure factors have been deposited in the Protein Data Bank (Protein Data Bank code 3WPD8 for the
C. microcarpa QNS apo structure and code 3WD7 for the C. microcarpa ACS CoA-SH complexed structure).

A structural similarity search was performed, using the Dali program (28). The cavity volume and the active site entrance area were calculated with the program CASTP. All crystallographic figures were prepared with PyMOL (DeLano Scientific).

Site-directed Mutagenesis—The plasmids expressing the mutants of C. microcarpa QNS (Y197A) and C. microcarpa ACS (S132M, T194M, and T197Y) were constructed with a QuikChange site-directed mutagenesis kit (Stratagene), according to the manufacturer’s protocol, using the following pairs of primers (mutated codons are underlined): Y197A (5’-H11032 GATATCATGAACATGGCTTTTCATGAGCCG-3’/H11032 and 5’/H11032 CGGCTCATGAAAAGCCATGTTCATGATATC-3’/H11032), for C. microcarpa QNS, and S132M (5’/H11032 CATCTCATTTTCTGCACAATGGCAGGCGTCGACATGCC-3’/H11032 and 5’/H11032 GGCATGTCGACGCCTGCCATTGTGCAGAAAATGAGATG-3’/H11032), T194M (5’/H11032 GTTGTCCTCGAGAATACTGATCCCACTTTTCGGT-3’ and 5’/H11032 -CATCTCATTTTCTGCACAATGGCAGGCGTCGACATGCC-3’ and 5’/H11032 GGCATGTCGACGCCTGCCATTGTGCAGAAAATGAGATG-3’/H11032), and T197Y (5’/H11032 GAGAACACAATCCCCTATTCCGTGGGCCG-3’/H11032 and 5’/H11032 CGGCCCACGGAAATAGGGATTGTTGTTTCTC-3’/H11032) for C. microcarpa ACS. The mutant enzymes were expressed and purified with the same procedures as described for the wild-type enzymes and used for the enzyme reaction.

RESULTS

Sequence Analyses of QNS and ACS—Two full-length cDNAs encoding novel type III PKSs, QNS and ACS, were cloned and sequenced from the leaves of C. microcarpa by the RT-PCR method (the details of the method are described under “Experimental Procedures”). The full-length QNS and ACS cDNAs contained 1,191- and 1,176-bp open reading frames encoding M<sub>r</sub> 43,331 and 42,830 proteins with 396 and 391 amino acids, respectively (GenBank™ accession numbers AB823730 and AB823699). Notably, the deduced amino acid sequence of C. microcarpa QNS is quite unique and shared 60% identity to that of the acridone-producing C. microcarpa ACS, 56% identity to R. graveolens ACS (10), 59% identity to the quinolone-producing A. marmelos QNS (12), 60% identity to the chalcone-producing M. sativa CHS (29), and 58% identity to the benzalacetone-producing R. palmatum BAS (Fig. 2) (19). No additional cDNAs encoding other type III PKS isomers were obtained in this study.

The sequence analysis revealed that the C. microcarpa QNS retains the CHS’s active site residues, such as Gly-211, Pro-375, and the “gatekeeper” Phe-215, as well as the conserved catalytic residues, Cys-164, His-303, and Asn-336. However, half of the conserved active site residues are uniquely altered in C. microcarpa QNS. Thus, Thr-132, Ser-133, and the gatekeeper Phe-265 in M. sativa CHS are simultaneously substituted with Met, respectively.
Ala, and Leu, respectively (Fig. 2). In addition, Met-137, Thr-194, Thr-197, Gly-256, and Ser-338 of *M. sativa* CHS are characteristically altered to Ile, Met, Tyr, Ala, and Gly, respectively. The G256A/S338G substitutions are also found in *A. marmelos* and *S. aucuparia* biphenyl synthase (30) and *Hypericum androsaemum* benzophenone synthase (17). The conserved active site residues Thr-197/Gly-256/Ser-338 are altered in a number of functionally different type III PKSs and are thought to be crucial for governing the substrate and product specificities of the enzyme reactions (1, 2). However, in *C. microcarpa* ACS, Thr-132, Ser-133, and Phe-265 are substituted with Ser, Ala, and Val, respectively, as in the case of *R. graveolens* ACS and *A. marmelos* QNS (Fig. 2). These three residues are reportedly crucial for the substrate and product specificities of the enzyme reactions in *A. marmelos* QNS and *R. androsaemum* ACS (12, 15). A phylogenetic tree analysis grouped *C. microcarpa* QNS and ACS with the non-chalcone-producing enzymes and the closely related biphenyl- and alkaloid-producing type III PKSs, respectively (Fig. 3).

**In Vitro Analysis of *C. microcarpa* QNS Activity**—The sequence analyses suggested that both of the newly obtained *C. microcarpa* type III PKSs are functionally distinct from the regular CHS and could possess interesting catalytic activities. The recombinant *C. microcarpa* QNS was functionally expressed in *E. coli* as an N-terminally His_{6}-tagged protein and was purified and subjected to enzyme reactions using *N*-methylanthraniloyl-CoA, 4-coumaroyl-CoA, benzoyl-CoA, hexanoyl-CoA, and malonyl-CoA as substrates. Although the phylogenetic tree analyses predicted a close relationship between *C. microcarpa* QNS and the biphenyl-producing biphenyl synthase (Fig. 3), the LC-ESI-MS analyses of the enzyme reaction products demonstrated that it does not produce biphenyl from benzoyl-CoA (Fig. 4C). Instead, *C. microcarpa* QNS efficiently accepts *N*-methylanthraniloyl-CoA as a substrate to produce the diketide 4-hydroxy-N-methylquinolone as the single product by the condensation of one molecule of malonyl-CoA (Fig. 4A). Notably, *C. microcarpa* QNS did not accept 4-coumaroyl-CoA as a substrate (Fig. 4B) and produced only triketide lactones from benzoyl-CoA and hexanoyl-CoA as starters (Fig. 4, C and D). No additional products were detected in all of the enzyme reactions tested, even with changes in the reaction pH, temperature, and time. The steady-state kinetics values for quinolone formation by *C. microcarpa* QNS were $K_{m} = 37.8 \, \mu M$, $k_{cat} = 15.7 \, \text{min}^{-1}$, and $k_{cat}/K_{m} = 416 \, \text{min}^{-1} \cdot \mu M^{-1}$ for *N*-methylanthraniloyl-CoA, with a pH optimum at 8.0 within a range of 6.5–8.5. The steady-state kinetics values for quinolone formation were better than those of *R. palmatum* BAS ($K_{m} = 23.7 \, \mu M$, $k_{cat} = 1.5 \, \text{min}^{-1}$, $k_{cat}/K_{m} = 62.4 \, \text{min}^{-1} \cdot \mu M^{-1}$) (16) but not as good as those of *A. marmelos* QNS ($K_{m} = 2.9 \, \mu M$, $k_{cat} = 3.8 \, \text{min}^{-1}$, $k_{cat}/K_{m} = 1290 \, \text{min}^{-1} \cdot \mu M^{-1}$) (Table 1) (12).

**In Vitro Analysis of *C. microcarpa* ACS Activity**—The phylogenetic tree analyses suggested that *C. microcarpa* ACS is closely related to the acridone-producing *R. graveolens* ACS and *A. marmelos* QNS (Fig. 3). Indeed, *C. microcarpa* ACS effi-
sufficiently accepted N-methylanthraniloyl-CoA as the starter substrate to produce the tetraketide 1,3-dihydroxy-N-methylacridone after sequential condensations with three molecules of malonyl-CoA, along with 4-hydroxy-N-methylquinolone and N-methylanthraniloyltriacetic acid lactone (Fig. 4A). The steady-state kinetics values for acridone formation by C. microcarpa ACS were $K_{m} = 4.3 \mu M$, $k_{cat} = 1.4 \text{ min}^{-1}$, and $k_{cat}/K_{m} = 324 \text{ min}^{-1}\text{mM}^{-1}$ for N-methylanthraniloyl-CoA, with a pH optimum at 8.0 within a range of 6.5–8.5. However, with respect to the quinolone forming activity, the kinetics values were $K_{m} = 34.4 \mu M$, $k_{cat} = 4.0 \text{ min}^{-1}$, and $k_{cat}/K_{m} = 117 \text{ min}^{-1}\text{mM}^{-1}$ for N-methylanthraniloyl-CoA, with a pH optimum at 8.0 within a range of 6.5–8.5 (Table 1). Thus, the catalytic efficiency for the formation of the acridone is 2.8-fold higher than that of the quinolone. In addition, C. microcarpa ACS also accepted 4-coumaroyl-CoA as a starter substrate to yield naringenin chalcone, along with triketide and tetraketide lactone derailment by-products (Fig. 4B). When benzoyl-CoA

### TABLE 1

| Product | $K_{m}$ (μM) | $k_{cat}$ (min⁻¹) | $k_{cat}/K_{m}$ (min⁻¹ μM⁻¹) | Ref. |
|---------|-------------|------------------|-----------------------------|-----|
| N-methylanthraniloyl-CoA | | | | |
| C. microcarpa QNS Quinolone | 37.8 ± 3.2 | 15.7 ± 2.0 | 416 | This paper |
| A. marmelos QNS Quinolone | 2.9 | 3.8 | 1290 | 12 |
| R. palmatum BAS Quinolone | 23.7 | 1.5 | 624 | 16 |
| C. microcarpa ACS Quinolone | 37.4 ± 2.4 | 4.0 ± 0.6 | 117 | This paper |
| C. microcarpa ACS Acridone | 4.3 ± 1.7 | 1.4 ± 0.0 | 324 | This paper |
| 4-Coumaroyl-CoA | | | | |
| C. microcarpa ACS Naringenin | 13.6 ± 3.1 | 6.8 ± 2.2 | 500 | This paper |
| M. sativa CHS Naringenin | 5.1 | 6.1 | 843 | 13 |
| C. microcarpa QNS Y197A Benzalacetone | 28.3 ± 5.7 | 1.3 ± 0.2 | 45.5 | This paper |
| R. palmatum BAS Benzalacetone | 10.0 | 1.8 | 179 | 19 |

FIGURE 4. HPLC elution profiles of the enzyme reaction products of C. microcarpa QNS and ACS. A–D, enzyme reaction products of C. microcarpa (Cm) ACS and QNS from N-methylanthraniloyl-CoA (A), 4-coumaroyl-CoA (B), benzoyl-CoA (C), and hexanoyl-CoA, and malonyl-CoA (D). Note that by acid treatment naringenin chalcone is converted to racemic naringenin (5,7,4′-trihydroxyflavanone) through a nonstereospecific ring-C closure.
or hexanoyl-CoA was tested as the starter substrate, C. microcarpa ACS afforded the aromatic tetraketides 2,4,6-trihydroxy-3,5,7-trimethylchroman and 2-(1-keto-hexyl)phloroglucinol, but much less efficiently (Fig. 4, C and D). With respect to the chalcone forming activity, C. microcarpa ACS showed $k_{\text{cat}} = 13.6 \mu \text{m} \text{m}^{-1} \text{s}^{-1}$, and $k_{\text{cat}}/K_m = 500 \text{m} \text{m}^{-1} \text{m}^{-1} \text{m}^{-1}$ for 4-coumaroyl-CoA, and the latter value is 1.7-fold lower than that of M. sativa CHS ($K_m = 5.1 \mu \text{m}$, $k_{\text{cat}} = 6.9 \text{m} \text{m}^{-1} \text{s}^{-1}$, and $k_{\text{cat}}/K_m = 843 \text{m} \text{m}^{-1} \text{m}^{-1} \text{m}^{-1}$) (Table 1).

### Overall Structures of C. microcarpa QNS and ACS

The overall structures of the recombinant C. microcarpa QNS and the crystal structure of the recombinant C. microcarpa ACS complexed with CoA-SH were solved at 2.47 and 2.35 Å resolution, respectively. The crystallographic data and refinement statistics are summarized in Table 2. The asymmetric units of C. microcarpa QNS and ACS contained four and two nearly identical monomers, respectively, and significant backbone changes were not observed between the monomers in these structures. The structures of C. microcarpa QNS and ACS, sharing 56% amino acid sequence identity, are nearly identical, with root-mean-square deviations (r.m.s.d.) of 0.9 Å. The overall structures of C. microcarpa QNS and ACS revealed the conservation of the $\alpha_6\beta_6\alpha_6$-fold, observed in all structurally characterized type III PKSs (Fig. 5). The catalytic triad consisting of Cys-164, His-303, and Asn-336 is buried deep within each monomer, at the intersection of a characteristic 16-Å-long CoA binding tunnel and a large cavity, in a location and orientation very similar to those of the other plant type III PKSs (6, 31–35). Ile-137 in C. microcarpa QNS and Met-137 in C. microcarpa ACS, corresponding to Met-137 of M. sativa CHS, protrude into the other monomer and form part of the active site wall, as in the case of M. sativa CHS. The overall structures of C. microcarpa QNS and ACS are highly homologous to those of the structurally characterized plant type III PKSs (r.m.s.d. 0.7–1.3 Å and 0.5–1.2 Å for C. microcarpa QNS and ACS, respectively). A structure-based similarity search using the Dali program revealed that the overall structures of M. sativa CHS, Huperzia serrata PKS1, and R. palmatum BAS, which are functionally related to C. microcarpa QNS and ACS, exhibited r.m.s.d. of 0.9, 0.9, and 0.7 Å to that of C. microcarpa QNS, and 0.5, 0.7, and 0.6 Å to that of C. microcarpa ACS, respectively.

**Active Site Structure of C. microcarpa QNS**—Similar to C. microcarpa ACS, the CHS's conserved gatekeeper Phe-265 is substituted with Leu in C. microcarpa QNS. The aromatic side chain of the highly conserved Phe-265 in the plant type III PKSs usually blocks the access of the residues behind it to the active site cavity. In addition, Thr-132, Ser-133, Met-137, Thr-194, Thr-197, Gly-256, and Ser-338 in M. sativa CHS are characteristically replaced by Met, Ala, Ile, Met, Tyr, Ala, and Gly, respectively. No such simultaneous substitutions were observed in the primary structures of the other known type III PKSs, suggesting that these changes control the unique substrate and product specificities of C. microcarpa QNS. These residues are spatial analogs among the structures of C. microcarpa QNS and M. sativa CHS. The F265L substitution in C. microcarpa QNS not only grossly changes the shape of the active site cavity, but also permits Leu-265, behind Leu-263, to form part of the active site entrance (Fig. 6A). Furthermore, because of the absence of the aromatic moiety, the side chain of Phe-215 protrudes more toward Leu-265, as compared with that of Phe-215 in M. sativa CHS, thus widening part of the active site entrance.

The structural comparison also revealed the significant conformational differences of residues 159–164 (r.m.s.d. of 1.1 Å for the Ca atoms) and the slight displacement of an $\alpha$-helix consisting of residues 164–179 in C. microcarpa QNS. These conformational changes are presumably caused by various neighboring amino acid replacements in C. microcarpa QNS, such as the large-to-small M159I and small-to-large A166I substitutions in these regions. As a result, the Ca atom of the catalytic residue Cys-164 in C. microcarpa QNS is displaced by 1.7 Å toward the outside of the CHS's active site cavity. This alters the shape and size of the active site cavity and also widens the active site entrance. The estimated total area of the active site entrance of QNS is 47 Å², which is 2.8 and 1.5 times larger than those of M. sativa CHS (17 Å²) and C. microcarpa ACS (33 Å²) (Fig. 6, A–C), respectively.

However, the side chain of Met-132 of C. microcarpa QNS, corresponding to Thr-132 in M. sativa CHS, occupies the so-called coumaroyl-binding pocket that accommodates the aromatic moiety of the coumaroyl starter in M. sativa CHS (Fig. 7, A and C). The side chain of Met-194 in C. microcarpa QNS protrudes toward Gly-338, as compared with CHS's Thr-194. The coumaroyl-binding pocket in M. sativa CHS is thus absent from the active site cavity of C. microcarpa QNS, along with slight differences in the various surrounding amino acids.

Interestingly, the side chain of Tyr-197 in C. microcarpa QNS, corresponding to Thr-197 in M. sativa CHS, occupies the bottom of the active site cavity of M. sativa CHS, is inserted in front of the side chain of Met-263, corresponding to Leu-263 in M. sativa CHS, and its terminal hydroxyl group forms a hydrogen bond with the sulfur atom of Met-132 (Fig. 7, A and C). The substitution of Thr-197 with Tyr in C. microcarpa QNS thereby drastically narrows the

### TABLE 2

| Data collection and refinement statistics | C. microcarpa QNS | C. microcarpa ACS |
|------------------------------------------|------------------|------------------|
| **Data collection**                      |                  |                  |
| Space group                             | P2₁              | P2₁              |
| $a$, $b$, $c$                           | 51.7, 135.9, 107.6 Å | 106.0, 106.0, 346.5 Å |
| Resolution range                        | 50 to 2.47 Å (2.51 to 2.47 Å) | 50 to 2.35 Å (2.39 to 2.35 Å) |
| Completeness                            | 99.4% (98.7%)    | 99.9% (100%)     |
| **Refinement**                          |                  |                  |
| Resolution                              | 20.0 to 2.47 Å   | 34.5 to 2.35 Å   |
| Overall $R_{\text{work}}$               | 96.2%            | 18.3%            |
| Overall $R_{\text{free}}$               | 24.1%            | 21.8%            |
| Total atoms                             | 12238            | 6624             |
| No. of protein atoms                    | 12028            | 6173             |
| No. of water                            | 198              | 331              |
| No. of ligand                           | 12               | 120              |
| Average B-factors                       |                  |                  |
| Protein atoms                           | 39.1 Å²          | 33.2 Å²          |
| Waters                                   | 33.3 Å²          | 33.3 Å²          |
| Ligands                                  | 49.6 Å²          | 56.2 Å²          |
| r.m.s.d. from ideal standard            |                  |                  |
| Bond length                              | 0.01 Å           | 0.009 Å          |
| Bond angles                              | 1.301°           | 1.208°           |

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active site cavity of *C. microcarpa* QNS, especially at the bottom. Additionally, the side chain of Ala-256, which is the spatial analog of Gly-256 of *M. sativa* CHS in *C. microcarpa* QNS, protrudes toward Tyr-197 and constricts the bottom part of the active site cavity of *C. microcarpa* QNS, along with the side chain of Tyr-197. In contrast, the large-to-small S338G and M137I substitutions in *C. microcarpa* QNS slightly expand its active site wall near the active site entrance, as compared with *M. sativa* CHS. The total cavity volume (290 Å³) of the active site of *C. microcarpa* QNS is about 2.6 times smaller than those of *M. sativa* CHS (750 Å³) and *C. microcarpa* ACS (760 Å³) (Fig. 7, A–C).

**Active Site Structure of *C. microcarpa* ACS**—One of the characteristic features of the *C. microcarpa* ACS sequence is the characteristic substitution of the CHS’s conserved active site residues Thr-132, Ser-133, and Phe-265 with Ser, Ala, and Val, respectively. As in the case of *C. microcarpa* QNS, the location of Val-265 in the active site cavity of *C. microcarpa* ACS is similar to that of Phe-265 in *M. sativa* CHS. The loss of the aromatic moiety at this position obviously changes the shape of the active site cavity of *C. microcarpa* ACS. Furthermore, the side chain C81 of Leu-267, corresponding to Leu-267 behind the aromatic side chain of Phe-265 in *M. sativa* CHS, participates in the formation of the active site entrance, with a slight displacement of its Cα atom and a significant shift of its torsion angle toward Val-265 (Fig. 6B). In addition, presumably because of the loss of the aromatic side chain at this position and of several slight conformational differences between both enzymes, such as the G216A substitution in *C. microcarpa* ACS, the backbone torsion angle of Phe-215 (−53, 136), as compared with that of Phe-215 (−79, 138) in *M. sativa* CHS, is shifted by a φ angle of −26° and a ψ angle of +2°. This is accompanied by a slight displacement of its Cα atom, and the side chain of Phe-215 protrudes toward Val-265 (Fig. 6B). These conformational differences expand the entrance to the active site cavity of *C. microcarpa* ACS, as compared with that of *M. sativa* CHS. As a result, the estimated total area of the *C. microcarpa* ACS’s entrance is 33 Å², which is twice as large as that of *M. sativa* CHS (17 Å²) (Fig. 6, B and C).

In addition to these significant conformational changes, the side chains of Val-265 and Ser-132, as compared with those of Phe-265 and Thr-132 in *M. sativa* CHS, slightly protrude toward the inside and the outside of the active site cavity of *C. microcarpa* ACS, respectively (Fig. 7, B and C). With the slight displacements of their Cα atoms and the backbone torsion angles, these conformational changes contribute toward alter-
ing the shape of the active site cavity of *C. microcarpa* ACS. However, Ala-133 occupies almost the same position as the active site residue Ser-133 of *M. sativa* CHS, with an orientation very similar to that of Ser-133. The only difference is the steric bulk between the side chains of Ala and Ser at this position. No other significant conformational differences were observed between both structures. The estimated total cavity volume of *C. microcarpa* ACS is 760 Å³, which is almost the same as that of *M. sativa* CHS (750 Å³) (Fig. 7, B and C).

**Structure-based Mutagenesis of *C. microcarpa* QNS and ACS—** To further clarify the structure-function relationship of *C. microcarpa* QNS and ACS, we performed site-directed mutagenesis and investigated the mechanistic consequences of the point mutations. First, to test the hypothesis that the bulky Tyr-197 plays a crucial role in the active site architecture of *C. microcarpa* QNS, we constructed the Y197A mutant of *C. microcarpa* QNS, in which the bulky Tyr-197 was substituted with a small Ala. As a result, the Y197A mutant produced the diketide quinolone, from N-methylanthraniloyl-CoA, almost as efficiently as the wild-type enzyme; however, interestingly, the QNS mutant now accepted 4-coumaroyl-CoA as the starter substrate to produce the diketide benzalacetone and triketide lactone (Fig. 8A). The kinetics values for benzalacetone formation by the Y197A mutant were $K_m = 28.3 \mu M$, $k_{cat} = 1.3 \text{min}^{-1}$,
and $k_{\text{cat}}/K_m = 45.5 \text{ min}^{-1}\text{mM}^{-1}$. Thus, the catalytic efficiency for the formation of the benzalacetone is 3.9-fold lower than that of the native *R. palmatum* BAS ($K_m = 10.0 \mu\text{M}, k_{\text{cat}} = 1.8 \text{ min}^{-1}, k_{\text{cat}}/K_m = 179 \text{ min}^{-1}\text{mM}^{-1}$) (Table 1).

However, the crucial active site residues, Ser-132, Thr-194, and Thr-197 in *C. microcarpa* ACS, were replaced with Met, Met, and Tyr, respectively, as in the case of *C. microcarpa* QNS. Notably, consistent with our hypothesis, all the point mutants efficiently produced quinolone as the single product and lost the tetraketide acridone and lactone producing activities from the *N*-methylanthraniloyl-CoA starter (Fig. 8B). Furthermore, as in the case of *C. microcarpa* QNS, all the ACS mutants no longer accepted 4-coumaroyl-CoA as a substrate and produced only triketide lactones from benzoyl-CoA and hexanoyl-CoA as starter substrates (Fig. 1, B–D). These results suggested that *C. microcarpa* QNS can be regarded as a dedicated quinolone-producing enzyme. In contrast, the previously reported *A. marmelos* QNS did not produce the quinolone specifically, because it also generated the tetraketide 1,3-dihydroxy-*N*-methylacridone from *N*-methylanthraniloyl-CoA (12). Thus, *A. marmelos* QNS could be regarded as another acridone-producing ACS, which simply yields the diketide quinolone as a by-product. This is analogous to the tetraketide chalcone-forming CHS, which also produced triketide and tetraketide lactone by-products in the *in vitro* enzyme reactions. Furthermore, another major difference is that the quinolone- and acridone-forming *A. marmelos* QNS also accepted 4-coumaroyl-CoA to produce the diketide benzalacetone (12). However, we previously reported that the diketide-forming *R. palmatum* BAS also accepted both 4-coumaroyl-CoA and *N*-methylanthraniloyl-CoA as starter substrates to produce the benzalacetone and quinolone scaffolds, respectively (16, 19). These observations indicated that the sub-

**FIGURE 8. HPLC elution profiles of the enzyme reaction products of *C. microcarpa* QNS and ACS mutants. A, enzyme reaction products of *C. microcarpa* QNS, wild-type, and Y197A mutant, from 4-coumaroyl-CoA. B and C, enzyme reaction products of *C. microcarpa* ACS, wild-type, and mutants (S132M, T194M, and T197Y), from *N*-methylanthraniloyl-CoA (B), and 4-coumaroyl-CoA (C). Note that by acid treatment naringenin chalcone is converted to racemic naringenin (**5,7,4'-trihydroxyflavanone**) through a nonstereospecific ring-C closure.
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substrate preferences of C. microcarpa QNS, A. marmelos QNS, and R. palmatum BAS are significantly different, due to the modifications of their active site architectures, as discussed below.

Interestingly, the crystal structure revealed that C. microcarpa QNS has an unusually wide active site entrance (Fig. 6A). Similar expansions of the active site entrances have also been reported for the structures of the R. palmatum BAS (34) and acridone-producing M. sativa F215S CHS mutant (13), which both accept the bulky substrate N-methylanthraniloyl-CoA.

The active site entrance of C. microcarpa QNS (47 Å²) is larger than that of R. palmatum BAS (34 Å²) and is almost as large as that of the M. sativa F215S CHS mutant (41 Å²). In the structure of C. microcarpa QNS, the broadening of the active site entrance is caused by the F265L substitution and the deviation of the catalytic residue Cys-164 toward the outside of the active site cavity, as compared with the other type III PKSs. However, in R. palmatum BAS, the F208L substitution and its conformational changes expand the active site entrance. The wide active site entrance thus provides enough space to facilitate the access of the bulky N-methylanthraniloyl-CoA starter to the catalytic center of the enzymes.

In addition, the active site cavity volume of C. microcarpa QNS is quite small (290 Å³) and is even smaller than that of the diketide-forming R. palmatum BAS (350 Å³) (Fig. 7, A and D) (34). Furthermore, the crystal structure revealed the absence of the CHS’s coumaroyl-binding pocket in the active site of C. microcarpa QNS, due to the unique substitutions of CHS’s conserved Thr-132, Thr-194, and Thr-197 with Met, Met, and Tyr, respectively. This is the reason why C. microcarpa QNS does not accept 4-coumaroyl-CoA as a substrate. In contrast, although R. palmatum BAS also lacks the coumaroyl-binding pocket, this enzyme utilizes novel alternative pockets to bind the coumaroyl starter and produce the diketide benzalacetone (Fig. 7D) (34). Thus, the shape and the cavity volume of C. microcarpa QNS restrict the binding of the coumaroyl starter and the malonyl-CoA extender. Indeed, the mutagenesis analyses revealed that the bulky Tyr-197 drastically narrows down the active site cavity of C. microcarpa QNS and thereby controls the product chain length and specificity of the enzyme reaction. It is remarkable that the large-to-small Y197A mutant of C. microcarpa QNS gained the function of benzalacetone and triketide lactone-producing activities by the single amino acid substitution. Notably, similar steric contraction of the active site cavity was also observed for Gerbera hybrida 2-methylpyrone synthase, which utilizes the small acetyl-CoA as a starter substrate to produce triacetic acid lactone (31). The active site cavity of G. hybrida 2-pyrone synthase is even smaller (250 Å³) and also lacks the coumaroyl-binding pocket (36, 37). These observations support the unique catalytic activity of C. microcarpa QNS, which accepts the N-methylanthraniloyl-CoA starter through its wide active site entrance and catalyzes the condensation with malonyl-CoA. The chain elongation reaction is terminated at the diketide stage due to the steric contraction of the active site cavity, and this is followed by the N/C1 intramolecular lactamization of the Cys-bound linear diketide intermediate and concomitant thioester bond cleavage to produce the quinolone scaffold.

The crystal structure of C. microcarpa ACS also revealed a wide active site entrance (37 Å²), as in the case of C. microcarpa QNS (47 Å²) (Fig. 6B). However, unlike C. microcarpa QNS, the broadening of the active site entrance of C. microcarpa ACS is caused by the F265V substitution and the conformational changes of Phe-215 and Leu-267 (Fig. 6B). Furthermore, the cavity volume of C. microcarpa ACS (760 Å³) is 2.6 times larger than that of C. microcarpa QNS (290 Å³) and almost as large as that of M. sativa CHS (750 Å³). The wide active site entrance and the large active site cavity clearly affect the starter substrate preference and the product specificity of C. microcarpa ACS. As a result, C. microcarpa ACS accepts the bulky N-methylanthraniloyl-CoA as the starter substrate and catalyzes the iterative condensations with three molecules of malonyl-CoA to produce the acridone scaffold, by employing an active site cavity and catalytic machinery similar to those of CHS.

Furthermore, as in the cases of the other type III PKSs, C. microcarpa ACS also exhibits promiscuous substrate specificity and accepts various starter substrates to produce distinct molecular scaffolds, including acridone, quinolone, chalcone, benzophenone, and phloroglucinol (Fig. 1, A–D). The site-directed mutagenesis studies suggested that three active site residues Ser-132, Thr-194, and Thr-197 play a crucial role for the enzyme activity of C. microcarpa ACS. The S132M, T194M, and T197Y point mutations resulted in the loss of the tetraketide acridone and chalcone forming activities and production of the diketide quinolone as the single product. Thus, C. microcarpa ACS was functionally converted into QNS. These results clearly supported our hypothesis that the volume and shape of the active site cavity control the product specificity of these enzyme reactions.

In the generation of the acridone tricyclic ring system, one of the intriguing points is the timing of the C–N bond formation, and the C-ring-forming reaction involving Claisen-type C–C bond formation and concomitant thioester bond cleavage of the linear tetraketide intermediate from the active site Cys. Interestingly, previously reported experiments for the formation of rutacridone with [1-C¹³], [2-C¹³], and [1,2-C¹³]acetate revealed two different labeling patterns within the C-ring (38). This result indicated that the C-ring is indeed derived from three acetate units and suggested that the different labeling patterns were caused by the rotation of a bicyclic N-methylanthraniloylbenzophenone intermediate (or a nonaromatized anthraniloyl-trione intermediate) prior to C–N bond formation. In fact, many bicyclic N-methylanthraniloylbenzophenones have been isolated from various Rutaceae plants (39–42). Our docking simulations based on the crystal structure of C. microcarpa ACS predicted that the active site cavity is large enough to accommodate the linear or cyclized tetraketide intermediate; however, there is not enough space for the rotation. The docking simulation with the linear tetraketide intermediate also predicted that the C1 carbon resides near the C6 carbon in the active site cavity. Therefore, we propose that the formation of the acridone tricyclic ring system proceeds by the initial cyclization of the linear tetraketide intermediate by Claisen-type condensation to produce the bicyclic N-methylanthraniloylbenzophenone (or a nonaromatized anthraniloyltrione), which is fol-
lowed by the C–N bond formation to generate the acridone scaffold.

Finally, despite the high sequence identity with C. microcarpa ACS and R. graveolens ACS, both accepting 4-coumaroyl-CoA as the starter substrate to produce chalcone, the previously reported acridone/quinolone-producing A. marmelos QNS does not produce chalcone from 4-coumaroyl-CoA, although these three enzymes share the simultaneous substitutions of the CHS’s conserved active site residues Thr-132, Ser-133, and Phe-265 with Ser, Ala, and Val, respectively. Site-directed mutagenesis studies of R. graveolens ACS and A. marmelos QNS indicated that the three residues play important roles in the substrate and product specificities of the two enzymes (12, 15). For example, the S132T/A133S double mutant of A. marmelos QNS produced chalcone from 4-coumaroyl-CoA, whereas the S132T/A133S/V265F triple mutant lost the enzyme activity, and no longer accepted the coumaroyl-CoA, whereas the S132T/A133S/V265F triple mutant of R. graveolens ACS caused full conversion into a functionally distinct chalcone-forming enzyme (15). These observations suggested that subtle structural differences exist in the active site architectures of these three acridone-producing enzymes.

In conclusion, C. microcarpa QNS is a novel type III PKS that produces the diketide quinolone by the one-step condensation of N-methylantraniloyl-CoA and malonyl-CoA, whereas C. microcarpa ACS is a multifunctional PKS that produces not only acridone, but also chalcone, benzophenone, and phloroglucinol. The x-ray crystal structures of C. microcarpa QNS and ACS revealed the wide active site entrances of both enzymes, which facilitate the binding of the bulky N-methylantraniloyl-CoA starter. The active site cavity of C. microcarpa QNS is significantly smaller than that of ACS, which leads to the specific production of the quinolone scaffold, whereas C. microcarpa ACS utilizes a large cavity to yield the tetraakide acridone, by employing an active site cavity and catalytic machinery similar to those of CHS. These results have provided the first structural bases for the production of the anthranilate-derived quinolone and acridone alkaloids by the type III PKSs. These findings will enable further engineering of the enzymes to create novel, structurally distinct, and biologically active molecular scaffolds for drug discovery.

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