Articles

Engineered Biosynthesis of Novel Polyketides: Regiospecific Methylation of an Unnatural Substrate by the tcmO *O*-Methyltransferase†

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ABSTRACT: TcmO is an *O*-methyltransferase that methylates the C-8 hydroxyl of Tcm B3, a four-ring aromatic intermediate in the tetracenomycin biosynthetic pathway of *Streptomyces glaucescens*. The gene encoding this enzyme was expressed in *Streptomyces coelicolor* CH999 together with the actinorhodin polyketide synthase (PKS) gene cluster, which is responsible for the biosynthesis of 3,8-dihydroxy-methylanthraquinone carboxylic acid (DMAC) and its decarboxylated analog, aloesaponarin. The resulting recombinant strain produced approximately equal quantities of aloesaponarin and a new product but no DMAC. Spectroscopic analysis revealed that the novel polyketide was the 3-O-methylated analog of DMAC. An *in vitro* radioisotopic assay was developed for tcmO. The enzyme requires *S*-adenosylmethionine as a co-substrate. It has a K_m of 3 μM and a k_{cat} of 2.7 min^{-1} for DMAC. A series of monocyclic, bicyclic, and tricyclic aromatic compounds were also tested as candidate substrates *in vitro*. Remarkably, none was modified by tcmO within detectable limits of the assay. Together, these results highlight the interesting molecular recognition features of this enzyme. On one hand, there appears to be some flexibility in the number and structures of unreactive rings, since both Tcm B3 and DMAC are good substrates. However, 6-methylsalicylic acid, a monocyclic analog of the reactive ring, is not recognized by the enzyme. Likewise, neither aloesaponarin (which only differs from DMAC in the reactive ring) nor carminic acid (which only differs in the distal nonreactive ring) is modified. Thus, the binding energy for the tcmO-catalyzed methyl transfer appears to involve significant contributions from both the aromaticity and the functionality of polycyclic substrates.

Polyketides are a large family of structurally diverse natural products possessing a broad range of biological activities including antibiotic and pharmacological properties. Biosynthetic and molecular genetic studies have demonstrated that the early steps in polyketide biosynthetic pathways are catalyzed by polyketide synthases (PKSs). These multifunctional enzymes, which are structurally and mechanistically related to each other and to fatty acid synthases (FASs), catalyze repeated decarboxylative condensations between acylthioesters (Hutchinson & Fujii, 1995; Katz & Donadio, 1993; O'Hagan, 1991). In addition to varying the chain length, PKSs also vary the extent of a reductive cycle comprising a ketoreduction, dehydration, and enoylreduction on each β-keto group of the growing polyketide chain. Following release from the PKS complex, the intermediate is typically acted upon by additional enzymes in the pathway (oxidoreductases, group transferases, etc.), leading to the final natural product. Thus, molecular diversity arises from controlled enzymatic variation at each stage in the biosynthetic cascade. Mimicking this remarkable biological process to generate novel "unnatural" natural products has been an important goal over the past decade (Tsoi & Khosla, 1995).

The genes responsible for the biosynthesis of tetracenomycin C (Tcm C), an antitumor antibiotic, have been cloned and sequenced (Bibb et al., 1989; Summers et al., 1992, 1993). Sequence analysis revealed that the tetracenomycin (*tcm*) gene cluster consists of 12 open reading frames (Figure 1). The biosynthetic pathway of Tcm C is illustrated in Figure 2 (Motamedi & Hutchinson, 1987; Summers et al., 1993). The minimal *tcm* PKS, which is composed of tcmK, a β-ketoacyl synthase (KS), tcmL, a chain length factor (CLF), and tcmM, an acyl carrier protein (ACP), utilizes acetyl CoA as the primer unit and nine molecules of malonyl CoA as extender units to form a decaketide backbone. In the presence of tcmN (and possibly tcmJ) this backbone cyclizes to yield Tcm F2. Conversion of Tcm F2 into Tcm F1 is catalyzed by tcmI. Subsequent steps in the pathway are catalyzed by tcmH (a monooxygenase), the C-terminal domain of tcmN (C-3 *O*-methyltransferase), tcmO (C-8 *O*-methyltransferase), tcmP (methyltransferase), and tcmG (hydroxylase).

Within the past few years, several reports have demonstrated the feasibility of engineered biosynthesis of novel aromatic polyketides through combinatorial manipulation of PKS subunits (Bartel et al., 1990; Fu et al., 1994a−d; McDaniel et al., 1993a,b, 1994a,b, 1995a,b; Shen et al., 1995). As a step toward exploring the potential for recruiting

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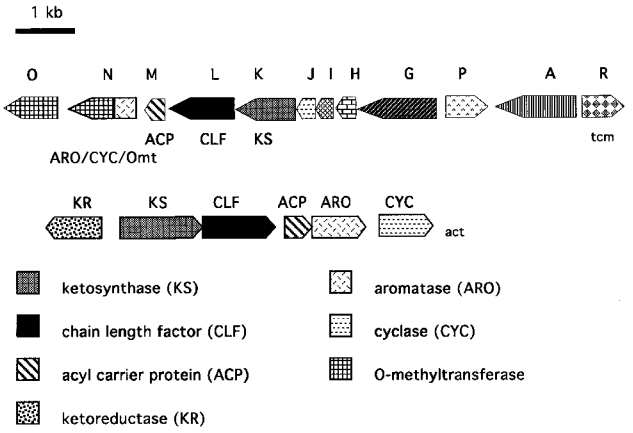

FIGURE 1: The *act* and *tcm* PKS gene clusters. Each "minimal" PKS includes a ketosynthase/putative acyltransferase (KS), a chain length determining factor (CLF), and an acyl carrier protein (ACP). The *act* cluster also contains a ketoreductase (KR), which reduces a specific carbonyl (C-9) of the actinorhodin polyketide backbone. Both gene clusters also encode aromatases (ARO) and cyclases (CYC) involved in cyclization and aromatization of the nascent polyketide backbone. The *tcm* cyclase/aromatase (TcmN) is unusual, since it is similar to the *act* (and other) aromatases only in the N-terminal half of the polypeptide, while its C-terminal half-resembles *O*-methyltransferases (Summers et al., 1992). Other genes in tcm gene cluster include *tcm*O (an *O*-methyltransferase), *tcm*H (a monooxygenase), *tcm*P (a methyltransferase), and *tcm*G (a hydroxylase).

post-PKS enzymes to expand upon this source of molecular diversity, a series of recombinant strains expressing the *tcmO O*-methyltransferase gene, together with different PKS gene clusters, were constructed and analyzed. While tcmO was unable to recognize most of the products synthesized *in vivo*, it could regiospecifically modify an octaketide-derived anthraquinone. We also report on the development of a cell-free enzyme preparation that allowed us to determine the kinetic parameters for this reaction *in vitro* as well as to study the substrate specificity of tcmO.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The following three plasmids carrying recombinant gene clusters were constructed and introduced via transformation into *Streptomyces coelicolor* CH999: pZP4 (containing *tcm*K, *tcm*L, *act* ACP, *act* KR, *act* ARO, *act* CYC, and *tcm*O), pZP5 (containing *act* KS, *act* CLF, *act* ACP, *act* KR, *act* ARO, *act* CYC, and *tcm*O), and pZP6 (containing *fren* KS, *fren* CLF, *act* ACP, *act* KR, *act* ARO, *act* CYC, and *tcm*O). For nomenclature of genes, see Figure 1. The general procedures for strain design and construction have been described elsewhere (McDaniel et al., 1993a). The *tcm*O gene was derived from the 3 kb *Bam*HI−*Bgl*II fragment of the tetracenomycin gene cluster cloned into the *Bam*HI site of pGEM3f (Promega), which encodes the *tcm*O and *tcm*N genes (Summers et al., 1992). First, the *tcm*N gene was eliminated by digesting this plasmid with *Xho*I and *Sph*I followed by religation after treatment with the Klenow fragment of DNA polymerase. The *Sph*I−*Eco*RI fragment from the resulting plasmid was inserted into the corresponding sites of plasmids pRM20, pRM5, and pRM18 that lie immediately downstream of the *act* CYC gene. The resulting plasmids were designated pZP4, pZP5, and pZP6, respectively.

Isolation and Purification of Polyketide Products from S. coelicolor CH999/pZP4, CH999/pZP5, and CH999/pZP6. CH999/pZP4, CH999/pZP5, and CH999/pZP6 were grown on R2YE agar plates (20 plates each, ∼30 mL/plate) at 30 °C for 7 days. The agar was chopped and extracted with ethyl acetate in the presence of 5% acetic acid (250 mL × 3). Following evaporation of the solvent under vacuum, the products were purified on a Beckman HPLC using a preparative C-18 reverse-phase column and acetonitrile/water (1:4 to 3:2 over a period of 50 min) as the mobile phase. Product appearance was monitored at 280 and 410 nm.

Generation of Cell-Free Preparations of tcmO. Spores of CH999/pZP4 harvested from five agar plates were inoculated into 400 mL of SMM medium (Hopwood et al., 1985) in a 2 L flask and grown at 275 rpm at 30 °C for 42 h. The mycelium was harvested by centrifugation at 3210*g*, washed with 0.5 M NaCl and water, collected by filtration, frozen at −70 °C, and lyophilized to yield 0.75 g of dry mycelium. To the dry mycelium was added 9 mL of disruption buffer (0.17 M $NaHPO_4$, 0.2 M NaCl, 3 mM DTT, 1 mM benzamidine in 33% glycerol, pH 7.1). The mixture was sonicated using a Branson Sonifier 450 for 5 × 30 s at constant cycle. The crude cell-free preparation was centrifuged at 47 900*g* for 60 min. The supernatant was collected, 0.45 mL of polyethylenimine (3.9% solution) was slowly added, and the resulting solution was stirred at 4 °C for 15 min. The precipitate was removed after centrifugation at 47 900*g* for 25 min, and the supernatant was kept. The protein in this supernatant was recovered as a pellet by ammonium sulfate precipitation (75% saturation), dissolved in minimal amount of buffer (100 mM $NaHPO_4$, 1 mM DTT in 15% glycerol, pH 7.1), and desalted on a Pharmacia PD-10 column using 100 mM $NaHPO_4$, 5 mM DTT, and 5 mM EDTA as elution buffer.

Cell-Free Assays of tcmO. 200 μL of the above enzyme preparation was added to 300 μL of buffer containing 100 mM $NaHPO_4$ (pH = 7.1), 5 mM DTT, and 5 mM EDTA. The relevant substrate and *S*-adenosyl-1-methionine (SAM) were then added to a final concentration of 150 μM each. The mixture was incubated at room temperature overnight and extracted two times with 0.5 mL of ethyl acetate (1% acetic acid). The solvent was evaporated under vacuum and the extract was separated using thin layer chromatography (TLC). Resolved products synthesized in adequately high yields were detected using fluorescence detection. Alternatively, when ^{14}C-labeled SAM was added to the reaction mixture, ^{14}C-labeled products were detected and quantified using a PhosphorImager SI (Molecular Dynamics) and Image Quant software. In cases where 3,8-dihydroxymethylanthraquinone carboxylic acid (DMAC, **1**) was found to be converted to 8-methyl-DMAC (Met-DMAC, **2**), authentic Met-DMAC, isolated from CH999/pZP5, was used as the reference. Total protein concentrations were measured using the Bradford method. Compounds **11**, **12**, and **14**−**17** were from Aldrich Chemical Co. Compounds **1**, **6**, and **13** were isolated as reference biosynthetic products from engineered strains, as reported previously (Bedford et al., 1995; McDaniel et al., 1993b).

Mass and NMR Spectroscopy. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrometer under fast atom bombardment (FAB) conditions. NMR spectra were recorded on a Varian XL-400 using DMSO-d_6 (0.03% TMS) as the solvent. Spectra were referenced internally, to TMS in ^1H NMR and to the solvent in ^{13}C NMR. Met-DMAC. ^1H NMR (DMSO-d_6, 400 MHz)

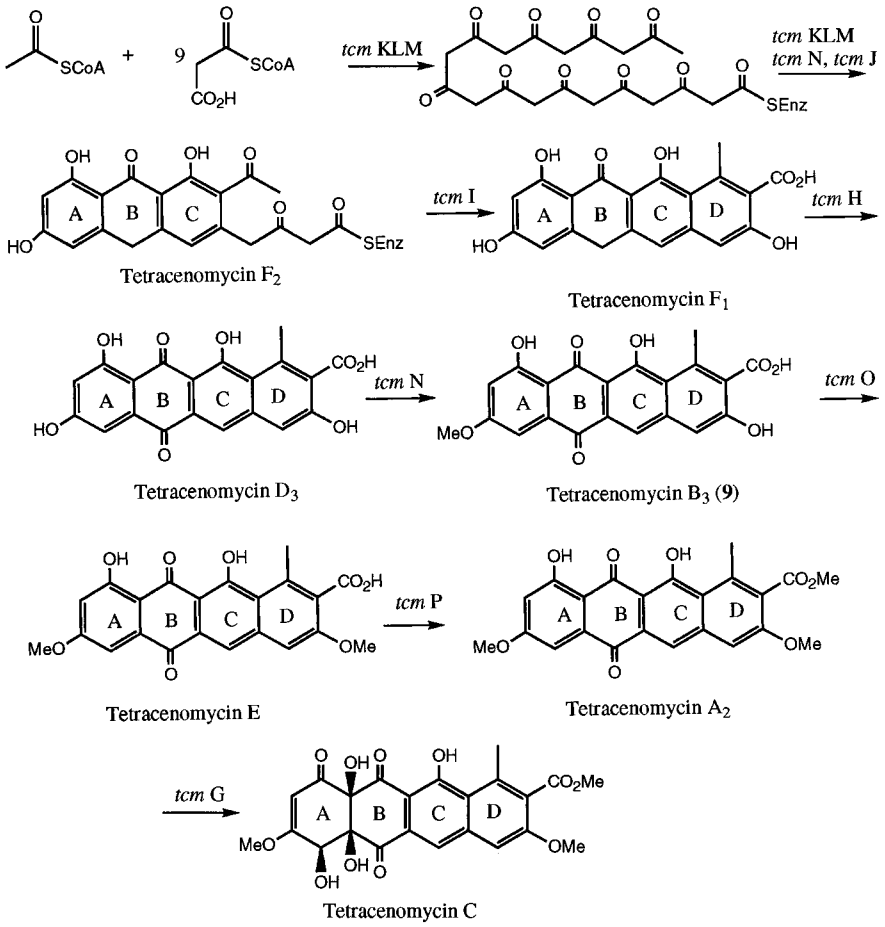

FIGURE 2: Biosynthetic pathway of Tcm C.

δ (ppm): 2.73 (s, 3H), 4.09 (s, 3H), 7.40 (d, J = 7.36 Hz, 1H), 7.71 (d, J = 6.72 Hz, 1H), 7.71 (s, 1OH), 7.80 (dd, J = 6.72, 7.36 Hz, 1H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 19.6, 56.5, 107.4, 116.7, 118.5, 124.2, 124.5, 132.3, 132.5, 136.3, 136.5, 139.2, 159.0, 161.4, 167.7, 181.6, 189.4.

RESULTS

Analysis of Recombinant Strains Expressing tcmO. CH999/pZP4 was found to produce no new metabolites, as judged by TLC and HPLC; its two most abundant products were identified as RM20b (**3**) (~45 mg) and RM20c (**4**) (~7 mg) (Figure 3) (Fu et al., 1994d). [The yield of RM20 (**5**), which was also detected, was not quantified.] In contrast, both CH999/pZP5 and CH999/pZP6 were found to produce substantial amounts of the same new metabolite (~15 mg) in addition to aloesaponarin (**6**) (~15 mg) (Figure 3). [CH999/pZP6 also produced substantial amounts of RM18 (**7**) and RM18b (**8**), both products of the *fren* min PKS and *actKR* (McDaniel et al., 1993b).] Surprisingly, no DMAC could be detected in these strains, even though DMAC is the major product of control strains containing the same sets of PKS genes but lacking *tcmO* (McDaniel et al., 1993a,b). These results suggested that the new metabolite, which had a higher R_f value than DMAC on silica gel TLC, was a derivative of DMAC.

Structure Determination of the New Metabolite Produced by CH999/pZP5 and CH999/pZP6. Comparison of ^{1}H and ^{13}C NMR spectra of the new product with those of DMAC indicated that this compound was derived from DMAC with one of the hydroxyl groups methylated. High-resolution mass spectroscopy of Met-DMAC under fast atom bombardment (FAB) conditions gave a molecular weight of 313.0720 (M + H$^+$), consistent with $C_{17}H_{12}O_6$ (calculated M + H$^+$, 313.0712). Nuclear Overhauser effect (NOE) experiments were carried out to confirm that the hydroxyl group ortho to the carboxyl was methylated (Figure 4).

Determination of $K_{m,DMAC}$ and k_{cat} for tcmO. In order to study the substrate preferences of tcmO, a cell-free assay was developed for this enzyme using extracts derived from CH999/pZP4, which expresses the *tcmO* gene but does not produce an appropriate substrate for this enzyme (see above). The preparation and assay of an active cell-free extract are described in Materials and Methods. In the presence of DMAC (purified from a producer strain) and unlabeled SAM as substrates, sufficient amounts of Met-DMAC were synthesized so as to be detected as an orange fluorescent product with the correct R_f value via TLC. (For comparison, authentic Met-DMAC, derived from CH999/pZP5, was used.) Furthermore, after an overnight incubation, no DMAC could be detected via TLC, suggesting that most of the substrate was converted into Met-DMAC. To determine the kinetic parameters for tcmO, the concentration of SAM was fixed at 107.1 μM [80 μM unlabeled SAM and 27.1 μM (CH_3-^{14}C)SAM (Moravek Biochemicals); final specific activity = 14.9 Ci/mol] while that of DMAC was varied between 0.5 and 100 μM. Each reaction was carried out in a total volume of 500 μL and contained 300 μL of the cell-free enzyme preparation. The reaction was stopped by quenching 100 μL aliquots with 10 μL of glacial acetic acid, and the amount of Met-DMAC was quantified using TLC/phosphorimaging. The K_m and V_{max} were determined to be 3 μM

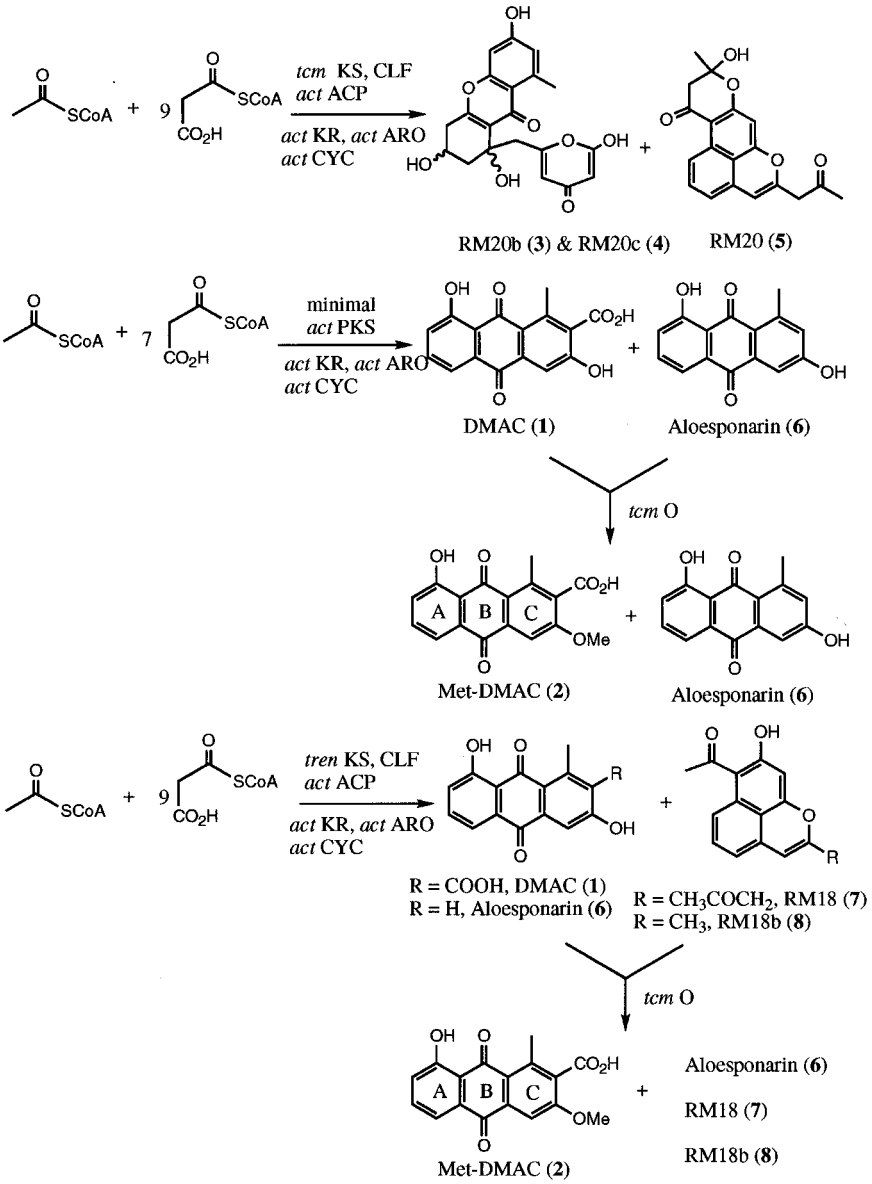

FIGURE 3: Products generated from our recombinant strains, CH999/pZ4, CH999/pZP5, and CH999/pZP6. pZP4 contains *tcm* KS, *tcm* CLF, *act* ACP, *act* KR, *act* ARO, *act* CYC, and *tcm*O while pZP5 contains *act* KS, *act* CLF, *act* ACP, *act* KR, *act* ARO, *act* CYC, and *tcm*O and pZP6 contains *fren* KS, *fren* CLF, *act* ACP, *act* KR, *act* ARO, *act* CYC, and *tcm*O.

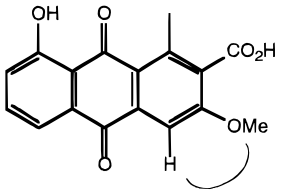

FIGURE 4: Summary of NOE data from met-DMAC.

and 6.4×10^{-2} μM min^{-1}, respectively. The concentration of total protein in the cell-free preparation was determined to be 15 mg/mL. The amount of tcmO, determined via SDS−PAGE densitometry, was found to be 1% of the total protein. Thus, the k_{cat} of the enzyme for DMAC is estimated at 2.7 min^{-1}.

Substrate Specificity of tcmO. DMAC and the natural substrate of tcmO, Tcm B3 (**9**), share common structural features. The D ring of Tcm B3 is identical to the C ring of DMAC. Furthermore, both molecules possess a highly conjugated aromatic planar structure. In order to understand the precise determinants for molecular recognition, a series of compounds (**6** and **10**−**17**; Figure 5) were tested as potential substrates for tcmO. Included in this set of compounds was aloesaponarin purified from a biosynthetic source. Each compound (150 μM) was individually incubated with [^{14}C]SAM (107.1 μM; final specific activity, 14.9 Ci/mol) and tcmO under the assay conditions described in Materials and Methods. The appearance of new radiolabeled products was examined after overnight incubations via TLC/autoradiography. Within the limits of detectability of this radioisotopic assay, none of the above molecules, including aloesaponarin, was recognized as a substrate by the enzyme. Together these compounds provide insights into the molecular recognition features of tcmO (see below).

DISCUSSION

The remarkable molecular diversity observed in the polyketide family of natural products arises from controlled enzymatic variation in multistep biosynthetic pathways. In particular, *O*-methyltransferase activities are known to exist in many polyketide pathways. The genes encoding several such *O*-methyltransferases have been recently cloned (Connors & Strohl, 1990; Paulus et al., 1990; Shafiee et al., 1994;

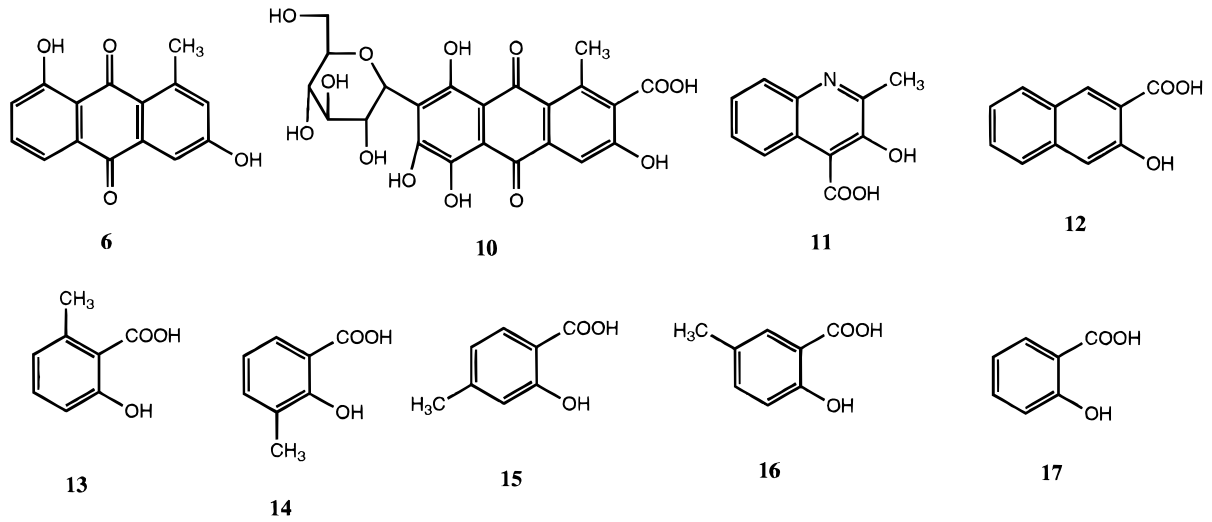

FIGURE 5: Molecules tested as potential substrates of *tcmO*.

Summers et al., 1992). Sequence comparisons have revealed the presence of SAM-binding domains, suggesting that many such enzymes are SAM-dependent. Several of these *O*-methyltransferases have been highly purified and studied *in vitro*. For example, 31-*O*-desmethyl-FK-506 *O*-methyltransferase from *Streptomyces* sp. MA6858 was found to be able to use several analogs of FK506 as substrates (Shafiee et al., 1994). Likewise, carminomycin 4-*O*-methyltransferase from *Streptomyces* sp. strain C5 recognized 13-dihydrocarminomycin as an alternative substrate (Connors & Strohl, 1990, 1993). Thus, although these enzymes normally recognize and selectively modify a unique hydroxyl group on their natural substrates, available evidence suggests that they may have relatively broad specificity toward structurally distinct but related polyketides. The result that tcmO, which normally methylates Tcm B3 (**9**), can also efficiently methylate DMAC (**1**) *in vivo* is consistent with such a notion, and augurs well for the potential of harnessing post-PKS enzymes in combinatorial biosynthesis. In this context it is also noteworthy that tolerance of SAM-dependent methyltransferases toward non-natural metabolites is not unique to polyketide substrates. For example, methyltransferases have been used *in vitro* to prepare a whole series of "unnatural" C-methylated natural products including Factor S_3 (Ozaki et al., 1993) and the di- and trimethylated porphyrinoids derived from the unnatural type I, II, and IV templates, where "false" substrates containing a special array of carboxylates were recognized regio- and stereospecifically by a methyltransferase (Warren et al., 1990).

In order to evaluate the potential for utilizing polyketide pathway-derived *O*-methyltransferases for combinatorial biosynthesis, a better understanding of their molecular recognition features is essential. The development of a cell-free assay for tcmO and its use to explore the range of substrates that can be modified by this enzyme represent a step in this direction. The complete inability of tcmO to methylate aloesaponarin (*in vivo* and *in vitro*) demonstrates an absolute requirement for a carboxyl group ortho to the reactive hydroxyl. Likewise, the absence of activity against **12** suggests that the methyl group para to this hydroxyl may also be important. However, these substituents alone are insufficient in a candidate substrate, as illustrated by the failure of tcmO to recognize 6-methylsalicylic acid (**13**). Thus, the presence of a fused aromatic ring system appears to be important, although some flexibility must exist, since Tcm B3 (**9**) and DMAC (**1**) differ in both the number and structures of unreactive rings. Lastly, the failure of tcmO to modify carminic acid (**10**) argues in favor of an active site unable to recognize aromatic molecules that possess bulky nonplanar substituents, even on the unreactive rings. Further studies with a more systematic series of DMAC and Tcm B3 derivatives, as well as structural studies on the enzyme itself, should shed light on the recognition features of this remarkable and potentially useful enzyme.

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