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Characterization of Yatakemycin Gene Cluster Revealing a Radical *S*-Adenosylmethionine Dependent Methyltransferase and Highlighting Spirocyclopropane Biosynthesis

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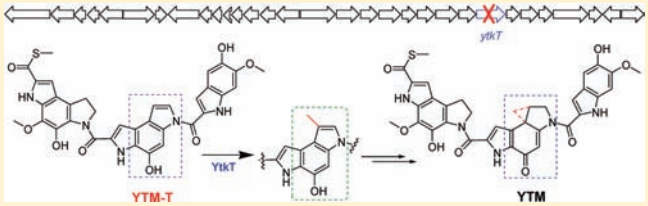
Supporting Information

ABSTRACT: Yatakemycin (YTM), an antitumor natural product, represents the most potent member of a class of potent anticancer natural products including CC-1065 and duocarmycins. Herein we describe the biosynthetic gene cluster of YTM, which was identified by genome scanning of *Streptomyces* sp. TP-A0356. This cluster consists of 31 open reading frames (ORFs) and was localized to a 36 kb DNA segment. Moreover, its involvement in YTM biosynthesis was confirmed by cluster deletion, gene replacement, and complementation. Inactivation of *ytkT*, which encodes a radical *S*-adenosylmethionine (SAM) protein, created a mutant strain that failed to produce YTM but accumulated a new metabolite, which was structurally elucidated as a precursor that was related to the formation of the cyclopropane ring. More importantly, biochemical characterization of the radical SAM-dependent enzyme YtkT revealed that it is a novel C-methyltransferase and contributes to an advanced intermediate during formation of the cyclopropane ring through a radical mechanism in the YTM biosynthetic pathway. On the basis of *in silico* analysis, genetic experiments, structure elucidation of the novel intermediate, and biochemical characterization, a biosynthetic pathway for yatakemycin was proposed, which sets the stage to further investigate the novel enzymatic mechanisms and engineer the biosynthetic machinery for the production of novel analogues.



INTRODUCTION

DNA-targeting antitumor natural products play an important role in the treatment of cancer.[1] Yatakemycin (YTM, **1**, Figure 1), isolated from the culture broth of *Streptomyces* sp. TP-A0356, is a novel antitumor antibiotic belonging to the family of CC-1065 (**2**, Figure 1) and duocarmycins (**3**, **4**, Figure 1), which are known to be naturally derived DNA alkylating agents.[2] Among the spirocyclopropane family of antibiotics, YTM exhibits the most potent cytotoxicity against cancer cell lines, with an IC_{50} of 3 pM in the L1210 cell line. It also has been shown to inhibit the growth of pathogenic fungi, such as *Aspergillus fumigatus* and *Candida albicans*, with MIC values of 0.01−0.03 μg/mL.[2] Therefore, YTM is more potent than amphotericin B (MIC of 0.1−0.5 μg/mL) or itraconazole (MIC of 0.03−0.2 μg/mL). DNA alkylation studies of this family have shown that both the shape-selective recognition and the shape-dependent catalysis that contribute to the alkylation selectivity are dependent on the DNA minor groove shape and size characteristics of an AT-rich sequence.[3] Recently, it was reported that not only free DNA but also nucleosomal DNA could be effectively accessed, recognized, and selectively alkylated by this family of potential anticancer agents, even at sites that seem completely occluded by histones.[4,5]

The structure of YTM was first elucidated by NMR and CID-MS/MS experiments[2] and further revised on the basis of total synthesis.[6] Despite the presence of a dienone cyclopropane ring, which is responsible for DNA alkylation and is the typical structural property of this family, YTM is a remarkable hybrid of the preceding natural products and incorporates a central active subunit that is identical to that of **3**, a left subunit that is identical to the DNA-binding subunit of **2**, and a right subunit that is similar to that found in **4**. It represents the first naturally occurring member possessing a DNA binding subunit that flanks each side of the alkylation subunit in a characteristic "sandwiched" arrangement.[7] The unique structure and potent biological activity of YTM have drawn considerable interest of this compound for chemical synthesis and medical development.[8,9] Total synthesis of YTM and its analogues have been extensively investigated,[6,7,10−13] highlighting the remarkable and fundamental relationships between structure, reactivity, and biological potency embodied in this class of natural products.[14]

Combinatorial biosynthesis offers a promising alternative to prepare complex "un-natural" natural products biosynthetically and complements the creation of structural diversity within

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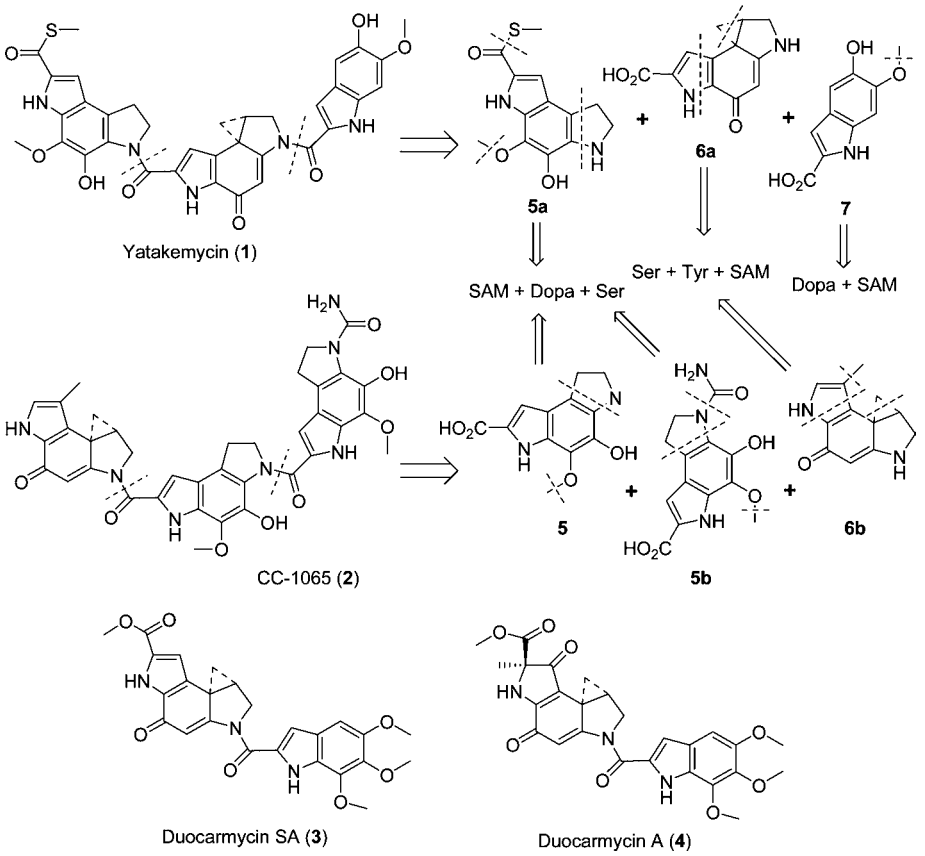

Figure 1. Structures of yatakemycin (YTM, **1**) as well as related natural products, and retro-biosynthetic analysis of YTM and CC-1065.

natural products chemically.[15] The success of this strategy depends on the cloning and characterization of the biosynthetic pathway of the target natural product genetically and biochemically. However, little is known regarding the biosynthesis of these natural products to date, except that the biological origin of **2** has been roughly investigated by radioactive isotope precursor feeding in combination with chemical degradation.[16] Due to the unusual structural features, we have been interested in the biosynthesis of YTM family natural products for several years. First, the subunits **7** and **5a** (or **6a**) require the cyclization of tyrosine (Tyr) analogues to form C−N bonds, which should require a different enzymatic process for amino acid derivatization (Figure 1). Second, the "sandwiched" arrangement of three subunits is connected by amide bonds, while the formation of these linkages is most likely catalyzed by another enzymatic system, except for nonribosomal peptide synthetase (NRPS). Next, the unusual *S*-methylthiol ester of YTM is unique in this family, which provides another opportunity to understand the biosynthetic logic. Finally, the central dienone cyclopropane ring is the active center, and therefore, it provides a challenging but fascinating topic for exploration of how this moiety is synthesized in nature. Herein, we report the cloning and identification of the gene cluster of YTM through the genome-scanning strategy, genetic characterization of the gene cluster, further structure elucidation of a novel intermediate, and biochemical assessment of a radical S-adenosylmethionine (SAM)-dependent methyltransferase. Together, these results support the proposal of a biosynthetic pathway of YTM, which represents the first member of this family of natural products.

MATERIALS AND METHODS

General. A YTM producer, *Streptomyces* sp. TP-A0356, was cultured as previously described.[2] *Escherichia coli* DH5α competent cells were used for routine subcloning and plasmid preparations. The pJTU2554 vector[17] was used for cosmid library preparations. *E. coli* cells were grown in LB medium with appropriate antibiotics when necessary. PCR amplification was carried out using either *Taq* DNA polymerase or PfuUltra DNA polymerase with genomic DNA or fosmid used as a template and the specific primers listed in Supporting Information (SI) Table S1. Primer synthesis was performed at the Invitrogen Shanghai Center. DNA sequencing was performed at the Shanghai GeneCore Biotechnology Inc. The general genetic manipulations of *Streptomyces* were preformed following previously described methods.[18] UV spectra analysis was performed on a Varian Cary 100 UV−visible spectrophotometer. IR spectra were recorded on a Nicolet 380 FT/IR spectrometer. NMR spectra was measured on a Bruker AV-500 with the residual C_5H_5N (δ_H 8.74, 7.58, 7.22 ppm; δ_C 150.35, 135.91, 123.87 ppm) as an internal standard. ESIMS was recorded on a ThermoScientific LCQ Fleet ion trap spectrometer with an Agilent LC system. High-resolution mass spectral analysis was acquired on an IonSpec HiRes MALDIFT-mass spectrometer. A commercial silica gel (Yan Tai Jiang You Silica Gel Development Co., Ltd., 200−300 mesh) was used for column chromatography.

Construction of a Genomic Library and PCR-Based Screening for Selected Sequences. A genomic library of *Streptomyces* sp. TP-A0356 was constructed in the cosmid vector pJTU2554 (derived from pOJ446[19] by replacing the SCP2* replicon with $int^{\phi C31}$)[17] according to the manufacturer's protocol. *Streptomyces* sp. TP-A0356 genomic DNA was prepared according to the protocol. Half of the clones from the genomic library (4.0×10^3 clones) were isolated and stored in 96-well plates, and the other half of the clones were combined. The genomic library was screened by PCR for the desired gene sequence using YTM-G-For and YTM-G-Rev as primers, and the resulting positive clones were further confirmed by PCR amplification

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using primers YTM-L-For/YTM-L-Rev and YTM-N-For/YTM-N-Rev. All PCR primers are summarized in SI Table S1.

Sequence Analysis. Whole genome sequencing of *Streptomyces* sp. TP-A0356 and annotation were provided by BGI-Shenzhen, China, and completed sequences and annotations have been deposited into Genbank with accession no. JF429418. The initial functional annotation combined the search results of COGs, SwissProt/TrEMBL, and KEGG databases using a set of specific rules. The open reading frames (ORFs) were deduced from the sequence by performing the FramePlot 4.0beta program (http://nocardia.nih.go.jp/fp4) and BLAST methods. Amino acid sequence alignments were performed by the CLUSTALW method from BIOLOGYWORKBENCH 3.2 software (http://workbench.sdsc.edu).

Construction of Gene Replacement Mutants. To delete the entire YTM biosynthetic gene cluster, a 3.0 kb *Hin*dIII/*Xba*I fragment (YTM-GD1-For and YTM-GD1-Rev as PCR primers; SI Table S1) and a 3.1 kb *Xba*I/*Eco*RI fragment (YTM-GD2-For and YTM-GD2-Rev as PCR primers; SI Table S1) were successively ligated and cloned into *Hin*dIII/*Eco*RI sites of pKC1139[19] to yield pTG1303. The resulting plasmid pTG1303 was introduced into *Streptomyces* sp. TP-A0356, and apramycin-resistant (50 μg/mL) colonies were identified as single-crossover mutants. The single-crossover mutants were further incubated without antibiotics and then screened for apramycin-sensitive clones. Among these clones, the double-crossover mutant strain TG1301 was selected and verified by PCR analysis (primers YTM-L-For and YTM-L-Rev, YTM-N-For and YTM-N-Rev, and YTM-GKT-For and YTM-GKT-Rev; SI Figure S1).

The *ytkE*, *ytkT*, *ytkJ*, *orf(+1)*, and *orf(+2)* genes were inactivated by REDIRECT technology.[20] First, the selected cosmid pTG1301 or pTG1302 was modified by inactivation of $int^{\phi C31}$ to meet the requirements of this technology. A spectinomycin resistance gene *aadA/oriT* cassette was used to replace an internal region of each target gene (*ytm-ed*-For and *ytm-ed*-Rev, *ytm-jd*-For and *ytm-jd*-Rev, *orf(+1)d*-For and *orf(+1)d*-Rev, *orf(+2)d*-For and *orf(+2)d*-Rev, and *ytm-td*-For and *ytm-td*-Rev as PCR-targeting primers; SI Table S1). The cosimds pTG1304 (Δ*ytkE*), pTG1305 (Δ*ytkJ*), pTG1306 (Δ*orf+1*), pTG1307 (Δ*orf+2*), and pTG1308 (Δ*ytkT*) were constructed and introduced into *Streptomyces* sp. TP-A0356 for gene inactivation by conjugation from *E. coli* S17−1. The double-crossover mutants that are spectinomycin-resistant (100 μg/mL) and apramycin-sensitive (50 μg/mL) were selected and named *Streptomyces* sp. TG1302 (Δ*ytkE*), TG1303 (Δ*ytkJ*), TG1304 (Δ*orf+1*), TG1305 (Δ*orf+2*), and *Streptomyces* sp. TG1306 (Δ*ytkT*), respectively. The genotypes of these mutants were verified by PCR analysis (SI Figure S2, *ytm-E*-For and *ytm-E*-Rev, *ytm-J*-For and *ytm-J*-Rev, *orf(+1)*-For and *orf(+1)*-Rev, *orf(+2)*-For and *orf(+2)*-Rev, and *ytm-T*-For and *ytm-T*-Rev as PCR primers; SI Table S1).

Complementation of the Δ*ytkT* Mutant TG1306 in trans. To express the *ytkT* gene in mutant *Streptomyces* sp. TG1306, a 1.5 kb *Eco*RI/*Nde*I fragment (amplified with primers YTM-tC-For and YTM-tC-Rev) containing the intact *ytkT* gene was cloned into the pSET152[19] vector to yield plasmid pTG1309. It was introduced into *Streptomyces* sp. TG1306 by conjugation to produce complemented strain TG1307, and the fermentation broth of *Streptomyces* sp. TG1307 was analyzed by HPLC for YTM production as described below. The wild-type strain was used as a control.

Fermentation and Analysis of the Production of YTM. *Streptomyces* sp. TP-A0356 wild-type and mutant strains fermentation was performed as a two-step process. First, 0.05 mL of preserved seeding culture was inoculated in 50 mL of seed medium (tryptic soy broth) in a 250 mL flask and incubated at 28 °C and 220 rpm for 48 h. Second, 5 mL of this seed culture was transferred into a 250 mL flask containing 50 mL of fermentation medium (International *Streptomyces* Project medium 2) and incubated at 28 °C for 72 h. For YTM isolation, each 50 mL of the culture broth was filtered and extracted twice with 50 mL of ethyl acetate. The extracts were then combined and concentrated to 100 μL in a vacuum.

High performance liquid chromatography (HPLC) analysis was carried out on a Microsorb-MV C18 column (5 μ, 4.6 × 250 mm, GraceSmart RP18). The column was equilibrated with 50% solvent A (H_2O) and 50% solvent B (CH_3CN) and developed with the following program: 0−3 min, constant 85% A/15% B; 3−6 min, a linear gradient from 85% A/15% B to 60% A/40% B; 6−12 min, constant 60% A/40% B; 12−19 min, a linear gradient from 60% A/40% B to 45% A/55% B; 19−22 min, a linear gradient from 45% A/55% B to 15% A/85% B; 22−27 min constant 15% A/85% B; 27−28 min, a linear gradient from 15% A/85% B to 85% A/15% B; 28−30 min, constant 85% A/15% B. This was conducted at a flow rate of 1 mL/min with UV detection at 385 nm using an Agilent 1100 HPLC system. The identity of the compound was confirmed by liquid chromatography−mass spectrometry (LC-MS) analysis performed on an LCMS-2010 A (liquid chromatography mass spectrometry, SHIMADZU, JP) under the same conditions. YTM showed $(M + H)^+$ ion at m/z = 680.17, consistent with the molecular formula $C_{35}H_{29}N_5O_8S$.

Production, Isolation, and Elucidation of YTM-T (8). Compound **8** was produced by *Streptomyces* sp. TG1306 using the same culture procedures as described for wild-type *Streptomyces* sp. TP-A0356. For preparation of compound **8**, all culture plates (2 L) were extracted with acetone (1.5 L × 3) exhaustively at room temperature and the aqueous acetone solution was evaporated to generate a residue. The residue was partitioned between ethyl acetate and H_2O. The ethyl acetate solution was concentrated under reduced pressure to create a brown residue (312 mg), which was subjected to gradient silica gel column chromatography and eluted with a step gradient (0−50% MeOH in $CHCl_3$) to yield the pure compound **8** (9.5 mg). Yellow powder; ν_{max} $(CDCl_3)/cm^{-1}$ 3344, 2920, 2851, 1743, 1665, 1575, and 1260; UV (MeOH) λ_{max} (ε) 359 nm (2565); ^1H NMR and ^{13}C NMR, see SI Table S2; HR (+) MALDI-MS m/z 666.1653 $[(M + H)^+]$, calcd for $[C_{34}H_{28}N_5O_8S]^+$ 666.1659.

Cloning, Expression, and Purification of YtkT. The *ytkT* gene was PCR-amplified from plasmid pTG1302 as a template with the primers YtkT-For and YtkT-Rev listed in SI Table S1. The purified PCR product was cloned into the *Bam*HI/*Eco*RI site of the expression plasmid pRSET B to generate pTG1310. The insertion fragment was verified by sequencing. *N*-terminal His_6-tagged YtkT was overexpressed in *E.coli* BL21 (DE3) plysE. A 3 mL starter culture was grown overnight from a single colony in LB media with 50 μg/mL ampicillin and 25 μg/mL chloramphenicol at 37 °C with shaking and then used to inoculate 1 L of LB medium at 37 °C with 50 μM of $Fe(NH_4)_2(SO_4)_2$, 400 μM of cysteine, 100 μg/mL of ampicillin, and 25 μg/mL of chloramphenicol for 2 h. The cultures were then cooled to 16 °C when the OD_{600} reached 0.5, induced with 0.2 mM IPTG, and incubated at 16 °C for an additional 24 h.

All protein purification steps took place in a glovebox in an environment where the oxygen was under 2 ppm. The protein purification buffers contained 300 mM NaCl, 50 mM HEPES adjusted to pH 7.5, 10% glycerol, and increasing concentrations of imidazole. Buffers A (lysis), B (wash), and C (elution) contained 0, 50, and 500 mM imidazole, respectively. The cells were harvested by centrifugation (5000 rpm for 10 min at 4 °C) and resuspended in buffer A. The cells were then incubated with 1 mg/mL lysozyme on ice and lysed by sonication (10 × 60 s pulsed cycles), and the debris was removed by centrifugation (12000 rpm for 60 min at 4 °C). Soluble protein was collected and purified on a Ni-NTA column by washing with sufficient buffer B and eluting with 10 mL of buffer C. The eluant was concentrated and desalted into buffer A using a PD-10 column (GE Healthcare, USA). The purified proteins were concentrated using an Amicon Ultra-4 (10K, GE Healthcare) and stored as 10% glycerol stocks at −80 °C for further use. Protein purity was assessed by 12% acrylamide SDS-PAGE. Protein concentration was determined by the Bradford method[21] using a BSA calibration curve.

In Vitro Enzymatic Assays of YtkT. Before the enzyme assay, the protein was thawed and reconstituted by incubating with 10 mM DTT for 15 min followed by 1 mM $Fe(NH_4)_2(SO_4)_2$ for 5 min and finally 1 mM Na_2S for 30 min on ice. The reconstituted protein was desalted by passing it through the PD-10 column again. Standard assay mixtures (50 μL) at 25 °C were composed of 50 mM HEPES buffer, pH 7.5, 1 mM SAM, 10 mM $Na_2S_2O_4$, 1 μM YTM-T, 10 μL of DMSO, and 20 μM YtkT. The reactions were carried out in the glovebox overnight

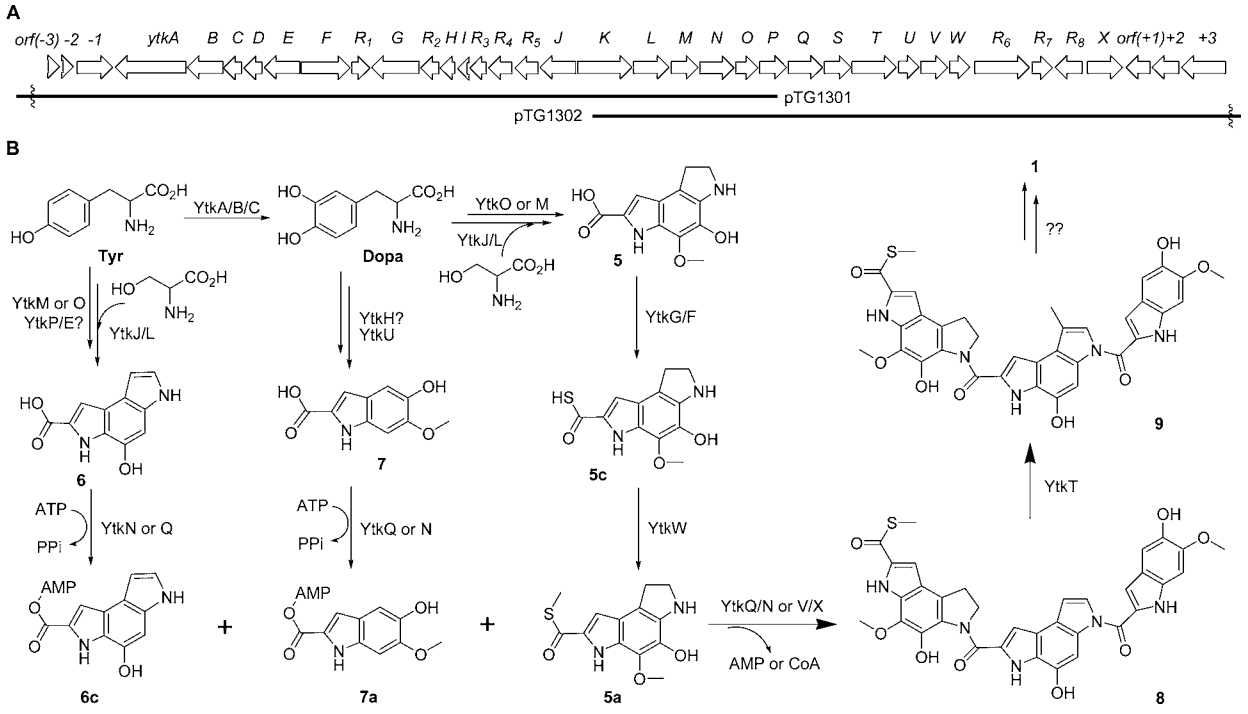

Figure 2. Biosynthetic pathway of YTM. (A) Organization of gene cluster. (B) Proposed model for biosynthesis of YTM.

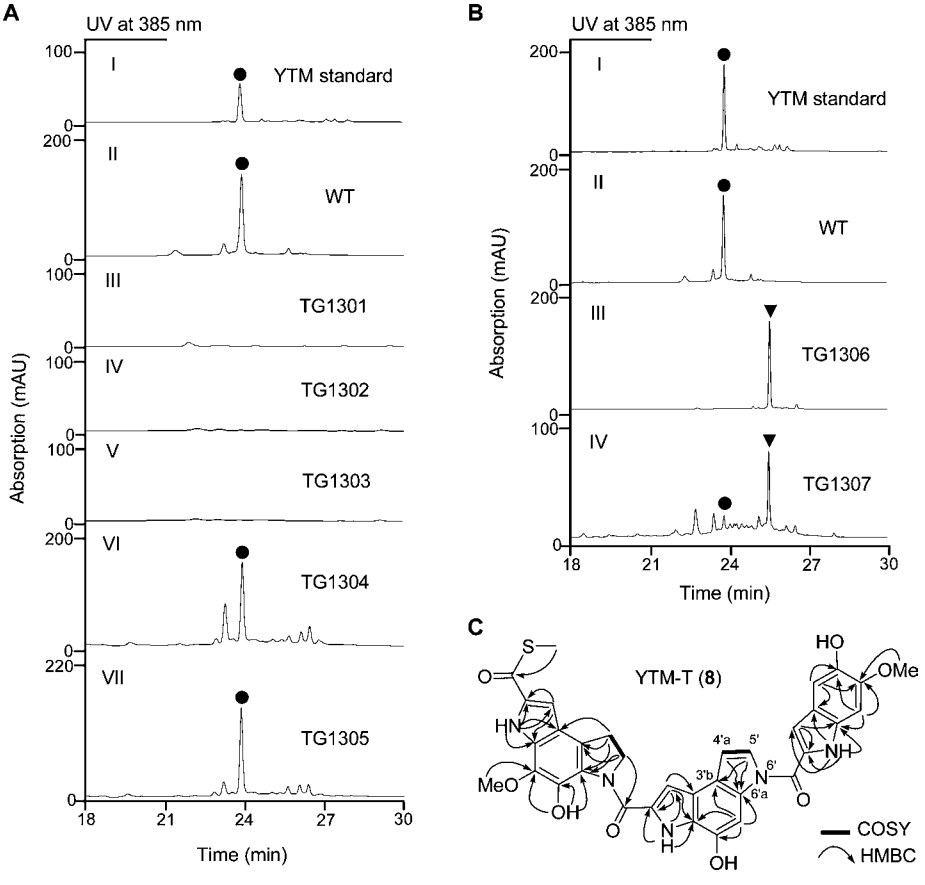

Figure 3. Genetic characterization of YTM gene cluster in vivo. (A) HPLC analysis of YTM production from wild-type and selected recombinant strains: (I) YTM standard; (II) wild-type *Streptomyces* sp. TP-A0356; (III) mutant *Streptomyces* sp. TG1301 (Δ*cluster*); (IV) mutant *Streptomyces* sp. TG1302 (Δ*ytkE*); (V) mutant *Streptomyces* sp. TG1303 (Δ*ytkJ*); (VI) mutant *Streptomyces* sp. TG1304 (Δ*orf+1*); (VII) mutant *Streptomyces* sp. TG1305 (Δ*orf+2*). (B) HPLC analysis of YTM production from wild-type and selected recombinant strains: (I) YTM standard; (II) wild-type *Streptomyces* sp. TP-A0356; (III) mutant *Streptomyces* sp. TG1306 (Δ*ytkT*); (IV) mutant *Streptomyces* sp. TG1307 (TG1306 harboring the *ytkT* expression plasmid pTG1309). (●) YTM (**1**); (▼) YTM-T (**8**). (C) Selected $^{1}H-^{1}H$ COSY and HMBC correlations of YTM-T (**8**).

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Table 1. Deduced Functions of ORFs in YTM Biosynthetic Gene Cluster

gene	Aa[a]	protein homologue (accession no.); origin	similarity/identity (%)	proposed function
orf(−6)[b]	269	hydrolase (BAC74288); *S. avermitilis* MA-4680	80/66	hydrolase
orf(−5)[b]	351	kinase (BAC68745); *S. avermitilis* MA-4680	80/69	kinase
orf(−4)[b]	54	50S ribosomal protein L33 (ZP_05543341); *S. griseoflavus* Tu4000	90/85	ribosomal protein
orf(−3)[b]	86	ribosomal protein L31 (ADI04616); *S. bingchenggensis* BCW-1	87/81	ribosomal protein
orf(−2)[b]	40	ribosomal protein L36 (ZP_04683835); *S. ghanaensis* ATCC 14672	97/87	ribosomal protein
orf(−1)[b]	354	SnoP (CAB59005); *S. nogalater*	89/83	protein ligase
ytkA	735	oxidase/dehydrogenase (AAZ63857); *Ralstonia eutropha* JMP134	61/48	hydroxylase
ytkB	329	dehydrogenase (ACC83370); *Nostoc punctiforme* PCC 73102	68/54	hydroxylase
ytkC	172	oxidoreductase (BAB49178); *Mesorhizobium loti* MAFF303099	86/74	hydroxylase
ytkD	193	GNAT family (EEP12476); *Streptosporangium roseum* DSM 43021	79/71	acetyltransferase
ytkE	316	hypothetical protein (ABW31363); *Acaryochloris marina* MBIC11017	48/33	unknown
ytkF	619	PdtI (ACZ86411); *Pseudomonas putida*	53/44	oxidoreductase
ytkR1	212	AraC type regulator (ABB40495); *Desulfovibrio desulfuricans* sp. G20	53/41	regulator
ytkG	543	PdtJ (AAQ01713); *Pseudomonas putida*	60/45	AMP-binding ligase
ytkR2	251	COG4912 (ZP_00380470); *Brevibacterium linens* BL2	57/43	DNA repair enzyme
ytkH	198	azoreductase (BAB85975); *Geobacillus stearothermophilus*	60/41	reductase
ytkI	75	ferredoxin (ACB76771); opitutus terrae PB90-1	68/50	ferredoxin
ytkR3	213	xanthine phosphoribosyl-transferase (ACA70711); *Pseudomonas putida* W619	40/25	hydrolase
ytkR4	298	DNase (ABQ86610); *Methanobrevibacter smithii* ATCC 35061	48/28	deoxyribonuclease
ytkR5	278	AP endonuclease (ACA56002); *Clostridium botulinum* A3	65/42	endonuclease
ytkJ	460	tryptophan synthase-*β*, (ABQ26707); *Geobacter uraniireducens* Rf4	66/51	PLP-dependent synthase
ytkK	698	molybdopterin oxidoreductase (ACA86297); *Shewanella woodyi* ATCC 51908	63/45	oxidoreductase
ytkL	428	PLP-dependent decarboxylase (ACA86291); *Shewanella woodyi* ATCC 51908	53/34	decarboxylase
ytkM	374	zinc-binding dehydrogenase (CAL83684); *Clostridium botulinum* A str. ATCC 3502	54/34	dehydrogenase
ytkN	407	coenzyme F390 synthetase (ACA86294); *Shewanella woodyi* ATCC 51908	41/28	ATP-dependent ligase
ytkO	317	NADPH-dependent F420 reductase (BAC73647); *S. avermitilis* MA-4680	52/40	dehydrogenase
ytkP	368	3-dehydroquinate synthase (EDT85045); *Clostridium botulinum* Bf	55/35	dehydroquinate synthase
ytkQ	459	coenzyme F390 synthetase (ACA86300); *Shewanella woodyi* ATCC 51908	55/36	ATP-dependent ligase
ytkS	391	oxidoreductase (ACA86297); *Shewanella woodyi* ATCC 51908	45/28	oxidoreductase
ytkT	506	coproporphyrinogen III oxidase (ACA86286); *Shewanella woodyi* ATCC 51908	52/36	O_2-independent oxidase
ytkU	248	methyltransferase (ACA86285); *Shewanella woodyi* ATCC 51908	54/33	methyltransferase
ytkV	317	carbon−nitrogen hydrolase (ACA86293); *Shewanella woodyi* ATCC 51908	62/41	hydrolase
ytkW	209	NodS-like methyltransferase (ACC40642); *Mycobacterium marinum* M	49/33	*S*-methyltransferase
ytkR6	783	ChaT1 protein (CAH10178); *S. chartreusis*	57/37	transporter
ytkR7	201	unknown protein (EEP40663); *Sphaerobacter thermophilus* DSM 20745	66/50	regulator
ytkR8	305	transcriptional regulator (ABB40495); *Desulfovibrio desulfuricans* G20	46/26	regulator
ytkX	365	triacylglycerol lipase (EFF88568); *S.* sp. e14	78/64	lipase/esterase
orf(+1)[b]	197	FMN reductase (ZP_05535732); *S. viridochromogenes* DSM 40736	87/82	reductase
orf(+2)[b]	281	oxidoreductase (BAC74944); *S. avermitilis* MA-4680	88/81	oxidoreductase
orf(+3)[b]	424	membrane protein (BAC68752); *S. avermitilis* MA-4680	84/74	transporter

[a]Amino acid. [b]*orfs* beyond gene cluster.

and terminated by adding 50 μL of methanol. Identical assays with buffer and denatured protein YtkT were carried out as negative controls. Following centrifugation to remove protein, the reactions were analyzed by HPLC as mentioned above.

RESULTS AND DISCUSSION

Identification and Validation of Biosynthetic Gene Cluster. After several unsuccessful attempts to probe genes involved in the biosynthesis of YTM using a degenerate primer-based PCR approach, genome scanning[22] was finally used to identify the gene cluster. The genomic DNA of producer *Streptomyces* sp. TP-A0356 was subjected to solexa sequencing and yielded 8.17 Mb of sequence on 77 scaffolds. Previously reported radioactive isotope feeding experiments had established that Tyr, dihydroxyphenylalanine (Dopa), serine (Ser), and SAM are precursors of **2**;[16] we therefore hypothesized that the same precursors should be used for YTM biosynthesis (Figure 1) and at least two methyltransferases should be involved in the biosynthetic process. Following this idea, bioinformatic analysis permitted the identification of a gene cluster encoding protein homologues including several methyltransferases, tryptophan synthase, amino acid decarboxylase, and coenzyme A ligases, which all seem to be necessary for the biosynthesis of YTM. We then constructed a genomic cosmid library (approximately 4,000 clones) of the *Streptomyces* sp. TP-A0356. A PCR screen of the library isolated two overlapping cosmids, pTG1301 and pTG1302 (Figure 2A), which span a 49.7 kb DNA region covering the entire gene cluster.

To confirm the involvement of this gene cluster in YTM production, a gene replacement plasmid pTG1303 was constructed, in which the gene cluster was inactivated by deleting a 24.2 kb DNA region from *ytkF* to *ytkR7*. The plasmid pTG1303 was introduced into *Streptomyces* sp. TP-A0356 by

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conjugation to select for the resulting double crossover mutant *Streptomyces* sp. TG1301, and the genotype of this mutant strain was confirmed by PCR analysis (SI Figure S1). The gene cluster deleted mutant strain, TG1301, completely lost its ability to produce YTM (Figure 3A, III), which was confirmed by HPLC and LC-MS analysis. This result unambiguously proved that the cloned cluster was necessary for YTM biosynthesis. Further systematic analysis of the region revealed 40 ORFs, 31 of which were proposed to constitute the YTM gene cluster from *ytkA* to *ytkX* according to functional assignment of their deduced products and series of gene knockout experiments (Figure 3A, IV, VI, and VII). The analysis results are summarized in Table 1, and the sequence of the gene cluster was deposited into GenBank under accession no. JF429418. The YTM gene cluster includes 22 biosynthetic genes encoding enzymes for precursor formation, subunit ligation, and subsequent tailoring steps, three regulatory genes, five resistance genes, and one gene with unknown function (Figure 2A).

Functional Analysis of Individual Genes in the Biosynthesis Cluster. Given the fact that Tyr, Dopa, Ser, and SAM are biosynthetic origins of **2**,[16] thus, a reasonable hypothesis is that the left subunit of YTM **5a** and central subunit **6a** are derived from Tyr and Ser, and the right subunit **7** originated from Tyr (Figure 1). In addition, the biosynthesis of subunits **5a** and **7** should involve the conversion of the Dopa intermediate from Tyr. The *ytkA*, *ytkB*, and *ytkC* genes encode enzymes showing high sequence homology to aldehyde oxidase or xanthine dehydrogenase, including a molybdenum subunit (YtkA), a FAD-binding subunit (YtkB), and an iron−sulfur center (YtkC).[23] This three-component hydroxylase was proposed to catalyze the hydroxylation of Tyr into Dopa (Figure 2B), and the similar hydroxylation of the aromatic ring was also observed in *Pseudomonas putida* KT2440 for the aerobic nicotinic acid degradation pathway.[24] The *ytkJ* gene encodes a pyridoxal-5′-phosphate (PLP)-dependent tryptophan synthase beta superfamily, and the *ytkL* gene encodes a PLP-dependent decarboxylase,[25] both of which were predicted to work together for condensation and decarboxylation of hydroxylated indole and Ser (Figure 2B). To further prove *ytkJ* is involved in YTM biosynthesis, a gene replacement plasmid pTG1305 was constructed and introduced into *Streptomyces* sp. TP-A0356 to select double crossover mutants *Streptomyces* sp. TG1303 (SI Figure S2). As expected, the mutant strain failed to produce YTM (Figure 3A, V). Seven genes, including *ytkP*, *ytkH*, *ytkI*, *ytkK*, *ytkM*, *ytkO*, and *ytkS*, encode dehydroquinate synthase, oxidoreductase, dehydrogenase, or relative enzymes, which may be involved in the cyclization and modification to form subunit **5** and **6** or other tailoring steps. However, the exact function of these enzymes and the order of these tailoring steps remain to be determined experimentally.

Additional analyses indicated that YtkG, YtkF, and YtkW may be involved in the *S*-methylthiol ester **5a** biosynthesis (Figure 2B). YtkG belongs to the AMP-dependent synthetase or ligase family, and YtkF exhibits two conserved CoA-transferase motifs. This pair of enzymes shows high sequence homology (44% identity and 53−60% similarity) to PdtJ and PdtI, which catalyze the synthesis of the thiocarboxylic acid group through the following scheme: −COOH → −CO∼AMP → −COSOH → −COSH.[26,27] Otherwise, the sulfur incorporation may originate from persulfides according to the following scheme: −COOH → −CO∼AMP → −COS-SR → −COSH, which occurs in the formation of a thiocarboxylate at the C terminus of small sulfur carrier proteins during thiamin biosynthesis.[28] Two genes, *ytkW* and *ytkU*, were identified in this cluster that encode SAM-dependent methyltransferases, both of which have a conserved SAM-binding motif. We assigned YtkU as an *O*-methyltransferase in the biosynthesis of **5** and **7** and YtkW as an *S*-methyltransferase in the biosynthesis of **5a** (Figure 2B), because YtkW shows strong sequence homology with thiopurine *S*-methyltransferase, which is a cytosolic enzyme that catalyzes *S*-methylation of aromatic and heterocyclic sulfhydryl compounds, including anticancer and immunosuppressive thiopurines.

The NRPSs exist widely in nature to catalyze amide bond formation, and they use the ATP-dependent adenylation of the carboxyl group and require the carrier protein-tethered substrates.[29] Very recently, two alternative strategies including a NRPS and an N-acetyltransferase were proposed to form the two amide bonds in the amicetin biosynthetic pathway.[30] Additionally, a transglutaminase homologue, AdmF, which catalyzes the formation of the first amide bond in andrimid biosynthesis,[31] a carboxyl methyltransferase CapS/lactamase CapW pair, which uses an ATP-independent manner to form an amide bond in the A-503083s pathway,[32] and a tRNA-dependent peptide formation involved in the biosynthesis of pacidamycin[33] represent three novel strategies of amide bond formation. The YTM cluster contains two genes, *ytkN* and *ytkQ*, which encode two proteins with sequence homology to coenzyme F390 synthetase or phenylacetate-CoA ligase. This type of enzyme usually activates the carboxylic acid phenylacetate through ATP-dependent adenylation for the subsequent attack of the terminal thiol of CoA. There are two possible mechanistic logics for assembly of **5a**, **6c**, and **7a** by amide bond formation (Figure 2B). The carboxylic acids of **6** and **7** were activated through an ATP-dependent manner catalyzed by YtkN and YtkQ, perhaps followed by nucleophilic attack of the amino group for direct amide bond formation without the CoA-linked thioester intermediates. This family of ATP-dependent amide synthases have been characterized in the biosynthesis of secondary metabolites exemplified by novobiocin,[34] coumermycin A1,[35] some siderophores,[36−38] and dapdiamide.[39] Otherwise, the CoA-linked thioester species will first be produced by YtkN and YtkQ; then, a hydrolase (YtkV) with sequence homology to nitrilase[40] and an esterase/lipase (YtkX) may be responsible for coupling between the acyl CoAs and the amino group to generate the amide bond linkage. Similar CoA-dependent strategies have been proposed in the biosynthesis of pyrrole-amide antibiotic for amide bond formation[41] and in the neocarzinostatin pathway for ester bond formation.[42]

Genetical Characterization of *ytkT* and Elucidation of an Important Intermediate YTM-T. The biosynthesis of cyclopropane rings has attracted significant attention for many years and may provide new ideas for the study of enzymatic mechanisms.[43] Given that the methylene group needed to form the cyclopane ring of **2** was derived from SAM by the feeding experiments,[16] other SAM-related enzymes, except YtkW and YtkU in YTM biosynthesis, were expected to participate in the formation of spirocyclopropane. This hypothesis led to identification of the *ytkT* gene, which encodes a protein with a conserved CxxxCxxC motif that coordinates the conserved iron−sulfur cluster and generates radicals by combining a 4Fe-4S cluster and SAM in close proximity to each other.[44] To investigate the role of YtkT in YTM production, the gene

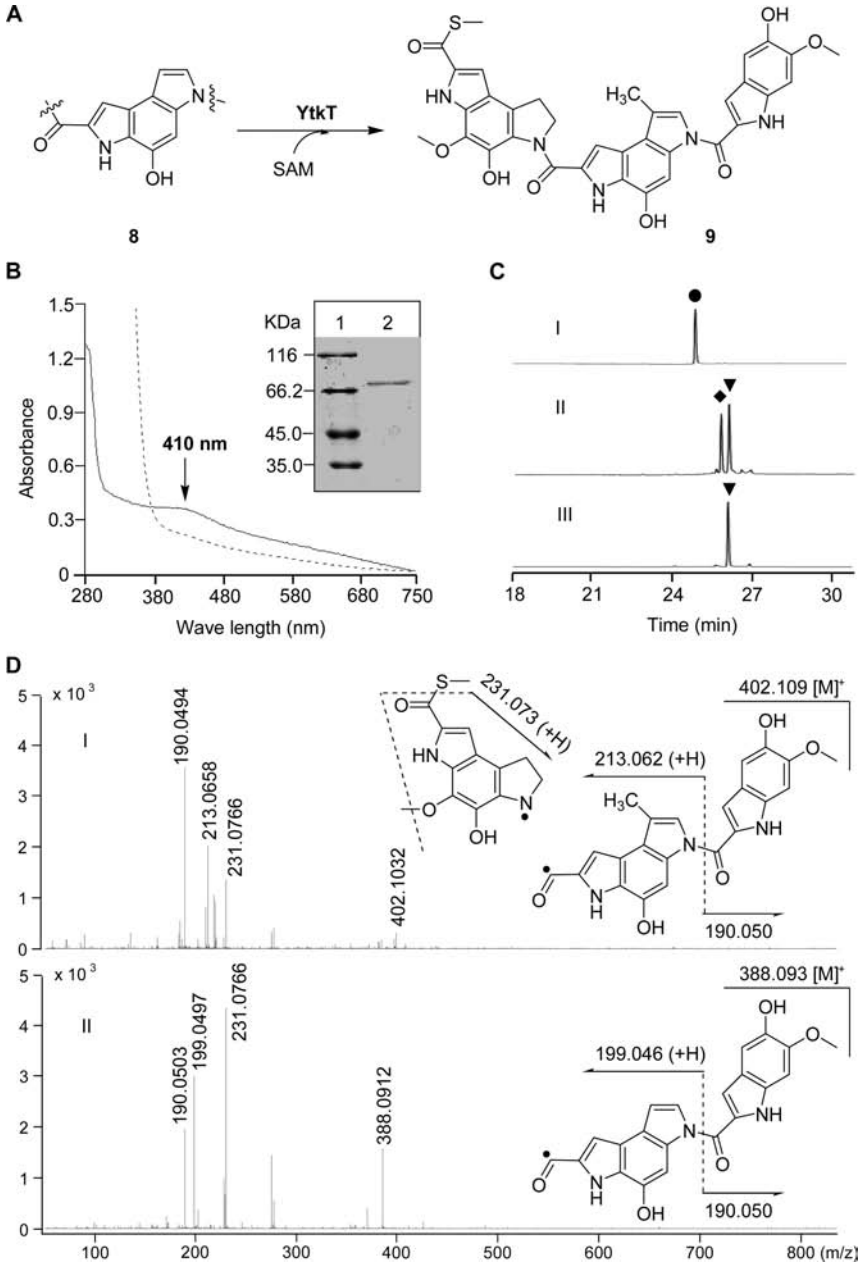

Figure 4. Biochemical characterization of recombinant YtkT as a radical SAM-dependent C-methyltransferase in vitro. (A) Reaction catalyzed by YtkT. (B) UV−vis spectra of the reconstituted YtkT (solid line), the reconstituted YtkT reduced with sodium dithionite (dashed line), and the SDS-PAGE of the purified YtkT. (C) HPLC analysis of the methylation of YTM-T catalyzed by YtkT: (I) YTM standard (●); (II) reaction with YtkT and SAM; (III) substrate YTM-T (▼) with denatured YtkT or (◆) methylated product **9**. (D) Comparative tandem MS analysis of **9** (I) and **8** (II).

replacement plasmid pTG1308 was constructed, in which *ytkT* was inactivated by gene replacement with the spectinomycin resistance gene (*aadA*). Plasmid pTG1308 was introduced into *Streptomyces* sp. TP-A0356 by conjugation to select for the resulting double crossover mutants *Streptomyces* sp. TG1306, which were spectinomycin-resistant and apramycin-sensitive. The genotype of this mutant strain was confirmed by PCR analysis (SI Figure S2). The *ytkT* gene replacement mutant strain, TG1306, completely abolished YTM production (Figure 3B, III). However, HPLC analysis of the TG1306 metabolite profile revealed one major peak at 25.5 min, which suggested a new compound was produced by this mutant that was different from YTM (Figure 3B, III). To genetically complement the mutant strain, the pTG1309 construct expressing a functional copy of *ytkT* in trans was created and introduced into TG1306 to afford strain TG1307. The analysis of the metabolite accumulated by this mutant was performed by HPLC (Figure 3B, IV) and LC-MS, which revealed that the production of YTM was partially restored. In addition, when compared to YTM, the MS/MS spectrum of the new compound showed the presence of the same ion peaks at *m*/*z* 277, but different ion peaks at *m*/*z* 475 and 664 to 489 and 678 of YTM (SI Figure S3), which were consistent with the chemical structure of YTM-T (**8**). The new compound was substantially extracted with ethyl acetate and purified by silica gel column chromatography, and the resulting yellow powder showed somewhat similar physicochemical properties to YTM. The molecular formula of YTM-T was determined as $C_{34}H_{28}N_5O_8S$

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on the basis of the HR-MALDI-MS, which gave a $[M + H]^+$ ion at m/z 666.1653 (calculated for 666.1659). The structure was completely elucidated by NMR spectroscopy (Figure 3C and SI Figure S4−S9 and Table S2). The genetic characterization of *ytkT* and chemical elucidation of intermediate YTM-T not only demonstrated that *ytkT* was necessary for YTM biosynthesis but also suggested that YtkT was a C-methyltransferase likely through a radical SAM-dependent process.

As expected, the new compound **8** was not cytotoxic against the HeLa cell line and did not inhibit the growth of *Saccharomyces cerevisiae* Y190 (SI Figure S10). These results were also consistent with the finding that the spirocyclopropane ring is the key moiety of DNA alkylation.[9,15]

Biochemical Characterization of Radical SAM-Dependent C-Methyltransferase YtkT and Insights into Cyclopropane Ring Formation. To investigate the biochemical process catalyzed by this radical SAM-dependent enzyme, the recombinant YtkT was expressed in *E. coli* BL21 (DE3) plysE. The N-terminal His_6-tagged enzyme was then purified under anaerobic conditions by Ni-NTA affinity chromatography and found to migrate as a single band at the predicted size on the basis of SDS-PAGE analysis (Figure 4B). When anaerobically purified YtkT was treated with Fe-$(NH_4)_2(SO_4)_2$ and Na_2S in the presence of dithiothreitol, the reconstituted enzyme showed absorption maxima at approximately 410 nm (Figure 4B), which is characteristic for the $[4Fe\text{-}4S]^{2+}$ cluster containing radical SAM proteins.[45] After further treatment with sodium dithionite, the absorption around 410 nm was bleached (Figure 4B), as described for other radical SAM enzymes.[46] When the reconstituted YtkT was incubated under anaerobic conditions with substrate **8** in the presence of SAM as a methyl donor and sodium dithionite as a source of the electron required for the reduction of the $[4Fe\text{-}4S]^{2+}$ cluster to the +1 oxidation state, a new compound **9** with different elution time from **8** or YTM was detected by HPLC analysis (Figure 4C). HR-MALDI-MS analysis of the enzymatic production yielded a $[M + H]^+$ ion at m/z 680.1812 (SI Figure S11), which suggested the molecular formula of this compound as $C_{35}H_{29}N_5O_8S$ (calculated for 680.1770). Further comparative tandem MS spectrum analysis demonstrated the presence of the same ion peaks at m/z 190 and 231 but different ion peaks at m/z 213 and 412 of **9** to 199 and 388 of **8** (Figure 4D), which were consistent with the chemical structure of the enzymatic product as methylated YTM-T (**9**, Figure 4A).

Recently, the biochemical characterization of CmaC/CmaB and KtzA/KtzD pairs used two distinct enzymatic strategies that were both chlorination-dependent for the generation of the thioester enolate needed to initiate cyclopropane formation.[47−49] Genetical analysis of the quinomycin gene cluster revealed a PLP-dependent aminotransferase (Swb6) and a radical SAM dehydrogenase (Swb7) pair for cyclopropanation using a radical process from L-Val.[50] In addition, a 10-enzyme assembly, including a halogenase domain, a 3-hydroxy-3-methylglutaryl β-branching enzyme cassette, and an enoyl reductase domain, was biochemically elucidated to catalyze formation of a cyclopropane moiety in curacin A biosynthesis.[51] The observation of cyclopane ring formation with participation from SAM in **2** looks similar to the biosynthesis of cyclopropane fatty acid (CFA) or cyclopropane-containing sterols,[43] which involve an enzymatic methyl-transfer reaction from SAM to a double bond. Biochemical and theoretical studies of CFA synthase support the following two-step mechanism: an S_N2 transfer of a methyl group from SAM to the substrate double bond, followed by the transfer of a proton from the methyl group to bicarbonate coupled with ring closing.[52−54] However, YtkT has no sequence homologue to CFA synthase but shares significant sequence identity with coproporphyrinogen III oxidase (36% identity and 52% similarity), which is an enzyme that catalyzes the oxygen-independent conversion of coproporphyrinogen-III to protoporphyrinogen-IX, one of the last steps in haem biosynthesis.[55] This family of enzymes was recently grouped into class C radical SAM-dependent methyltransferases,[56] including NosN,[57] NocN,[58] and TpdU,[59] in the biosynthesis of some thiopeptide members, and Blm-Orf8,[60] Tlm-Orf11,[61] and Zbm-Orf26,[62] in the biosynthetic pathway of the bleomycin family of antibiotics. However, this class of radical-mediated enzymatic methylation has never been biochemically characterized in vitro.[56] The biochemical studies of YtkT suggested that this C-methyltransferase contributes to an advanced intermediate during the formation of the cyclopropane ring through a radical mechanism in the YTM biosynthetic pathway (Figure 2B). Currently, more and more studies showed that this family of radical SAM-dependent enzymes could catalyze highly complex and chemically unusual transformations.[63,64] Very recently, two SAM-dependent enzymes, RlmN and Cfr, revealed a novel radical-associated mechanism for methylation of the electrophilic sp^2-hybridized carbons in ribosome methylation.[65−67] Although YtkT catalyzes the radical SAM-dependent methylation of a sp^2-carbon of the aromatic rings of YTM-T (**8**), it remains to be determined whether YtkT catalyzes this process using a different mechanism.

■ CONCLUSION

In summary, we have identified the biosynthetic gene cluster and genetically and biochemically characterized several steps in the biosynthesis of YTM, which is an antitumor natural product representing a new class of potent anticancer small molecules that target not only free DNA but also nucleosomal DNA, even at sites that seem completely occluded by histones. The isolation and elucidation of YTM-T, an intermediate of YTM biosynthesis from the *ytkT* gene replacement mutant, and the biochemical characterization of YtkT as a radical SAM-dependent C-methyltransferase provide new insight into the mechanism of spirocyclopropane formation. The availability of the YTM gene cluster and proposed biosynthetic pathway provides an excellent opportunity to investigate the enzymatic mechanism and biosynthetic machinery, including Tyr derivation, the formation of "sandwiched" linkages, unusual *S*-methylthiol esters, and the dienone cyclopropane ring, which will enrich our current knowledge of natural products biosynthesis. The work described here also sets the stage for engineering the biosynthetic pathway for novel analogues to develop useful anticancer drugs or biological probes to explore nucleosomal DNA.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

List of primers and summary of NMR data of YTM-T; construction and genotype analysis of gene replacement mutants; MS/MS and NMR data of YTM-T; bioassay of YTM and YTM-T; and HR-MALDI-MS data of the enzymatic production catalyzed by YtkT. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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