Mutasynthesis of Fluorinated Pactamycin Analogues and Their Antimalarial Activity
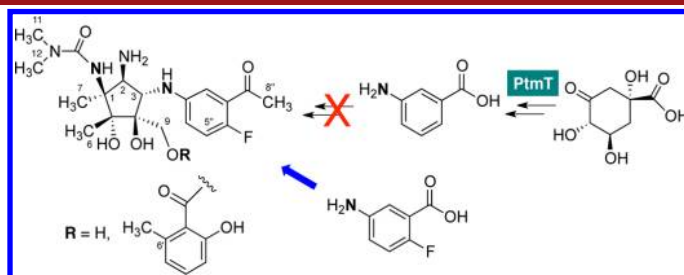
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ABSTRACT

A mutasynthetic strategy has been used to generate fluorinated TM-025 and TM-026, two biosynthetically engineered pactamycin analogues produced by *Streptomyces pactum* ATCC 27456. The fluorinated compounds maintain excellent activity and selectivity toward chloroquine-sensitive and multidrug-resistant strains of malarial parasites as the parent compounds. The results also provide insights into the biosynthesis of 3-aminobenzoic acid in *S. pactum*.

Pactamycin, a bacterial-derived natural product discovered by the Upjohn Company in the early 1960s, has been shown to have broad-spectrum growth inhibitory activity against bacteria,[1] mammalian cells,[2] viruses,[3] and protozoa.[4] This broad-spectrum activity is primarily due to its strong binding to a conserved region within the ribosome of most organisms, leading to nonselective inhibition of protein synthesis.[5,6] Consequently, its wide-ranging cytotoxicity, coupled with stability issues and difficulties to generate analogues of pactamycin through organic synthesis, have hampered its further development. As one of the most complex aminocyclitol natural products, pactamycin had presented great synthetic challenges; although, through

seminal work of Hanessian and co-workers, more recently, this densely functionalized aminocyclitol antibiotic has finally surrendered to total synthesis.[7,8] In addition, a number of synthetic methodologies to access the aminocyclopentitol moiety of pactamycin have also been reported.[9,10] However, long synthetic routes and low overall yields remain major limitations of the synthetic approach to generate pactamycin analogues for clinical uses.

Recently, using biosynthetic engineering technology, we were able to generate a number of mutant strains of *Streptomyces pactum* ATCC 27456 that produce novel analogues of pactamycin with improved chemical and biological properties.[11,12] One of the mutants, in which

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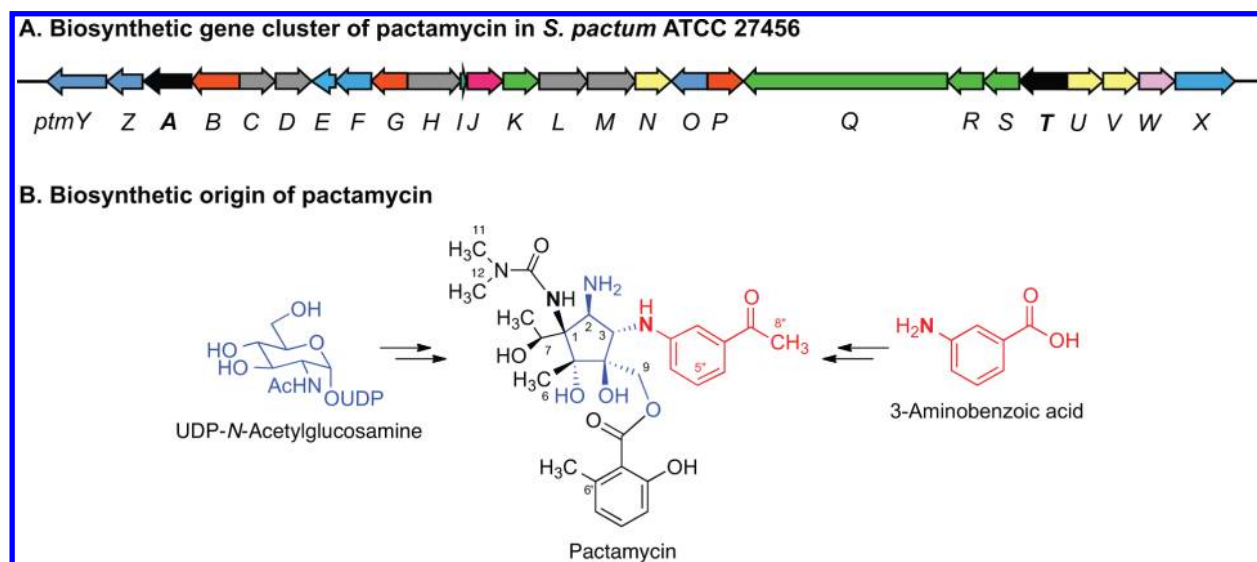
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Figure 1. Identification of genes responsible for the production of pactamycin precursor.

ptmH (a gene that encodes a radical SAM-dependent protein) (Figure 1A) was inactivated, produces two novel analogues of pactamycin, TM-025 (de-6MSA-7-demethyl-7-deoxypactamycin) and TM-026 (7-demethyl-7-deoxy-pactamycin). These new analogues are chemically more stable and are produced in 10- to 20-fold higher yield than pactamycin. More significantly, they showed potent anti-malarial activity at low nanomolar concentrations, but in contrast to pactamycin have no significant antibacterial activity and reduced cytotoxicity against human colorectal HCT-116 cells.[12]

To further improve the pharmacological properties of these analogues, we explored the application of the muta-synthetic approach to generate fluorinated TM-025 and TM-026 by blocking the formation of pactamycin biosyn-thetic precursor in *S. pactum* and performing precursor-directed structure modifications. Fluorinated compounds are rare in nature; however, the fluorine atom has been widely used in medicinal chemistry to improve drug prop-erties. An estimated 15−20% of new pharmaceutical leads contains the fluorine atom in their structures.[13]

Previously, Rinehart and co-workers demonstrated that feeding of *S. pactum* var. *pactum* with 3-amino-5-fluoro-benzoic acid resulted in the production of a fluorinated pactamycin analogue.[14] The product was reported to have a comparable biological activity as pactamycin, but the production yield was inferior, as the fluorinated precursor has to compete with the intrinsic precursor 3-aminoben-zoic acid (Figure 1B) in the cells. Therefore, we set out to engineer a mutant strain of *S. pactum* that lacks the ability to produce 3-aminobenzoic acid.

On the basis of isotope incorporation studies using ^{13}C-labeled glucose, it has been proposed that 3-aminoben-zoic acid is derived from an intermediate of the shikimate

pathway (either dehydroquinate or dehydroshikimate) in-volving a transamination reaction.[15] However, no genetic and biochemical data are available to support that notion. Our recent investigations of the pactamycin biosynthetic gene cluster revealed the presence of two genes, *ptmA* and *ptmT*, that encode PLP-dependent aminotransferases in the cluster.[11] However, it is unclear if any of these genes is involved in 3-aminobenzoic acid formation.

To investigate the role of the aminotransferase genes in pactamycin biosynthesis, we carried out in-frame deletion of *ptmA* and *ptmT* individually from the *S. pactum* genome and analyzed the metabolic profiles of the resulting mu-tants. The mutants were constructed by cloning two 1-kb PCR fragments upstream and downstream of the *ptmA* and *ptmT* genes, respectively, and separately cloned into the plasmid pTMN002.[12] The products pTMW034 (for *ptmA*) and pTMW037 (for *ptmT*) were individually intro-duced into *S. pactum* by conjugation. Apramycin resistant strains representing single crossover mutants were ob-tained and subsequently grown on nonselective manni-tol-soy flour agar containing MgCl$_2$ to allow the formation of double crossover recombinants. Apramycin sensitive colonies were counter-selected by replica plating onto MS-Mg agar with and without apramycin, and the result-ing double-crossover candidate strains were confirmed by PCR amplification (Figure S1, Supporting Information).

HPLC (Figure 2) and MS analyses of the extracts of those mutants showed that both mutants lack the ability to produce pactamycin or its intermediates. While the results suggest that both *ptmA* and *ptmT* are critical for pactamy-cin biosynthesis, it was not clear if *ptmA* or *ptmT* are involved in the formation of 3-aminobenzoic acid.

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To investigate if any of the genes are involved in the formation of 3-aminobenzoic acid, we carried out chemical complementation experiments with 3-aminobenzoic acid. The compound was pulse-fed to the cultures of ΔptmA and ΔptmT mutants, and the products were harvested and analyzed by HPLC and MS. The results showed that feeding ΔptmT mutant with 3-aminobenzoic acid did rescue the production of pactamycin (detected as pactamycate),[11] whereas that of ΔptmA mutant did not (Figure 2).
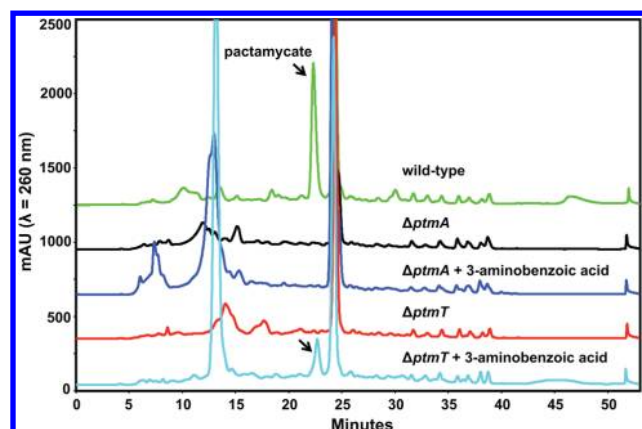

Figure 2. HPLC analysis of EtOAc extracts of the wild-type and the mutant strains of *S. pactum*.

The results suggest that *ptmT*, not *ptmA*, is the one involved in the formation of 3-aminobenzoic acid. To confirm this incorporation result, we subsequently fed the mutant with [7-^{13}C]-3-aminobenzoic acid, and MS analysis of the EtOAc extract of the culture broths revealed the incorporation of ^{13}C in the final products (Figure 3C), confirming the direct involvement of 3-aminobenzoic acid in pactamycin biosynthesis and PtmT is a dedicated enzyme for 3-aminobenzoic acid biosynthesis.

To produce fluorinated TM-025 and TM-026 as well as other possible analogues by a mutasynthesis approach, we generated a ΔptmT/H double mutant strain. This mutant strain was obtained by introducing the *ptmT* knockout plasmid pTMW037 into ΔptmH mutant (which produces TM-025 and TM-026)[12] by conjugation. The product (ΔptmT/H mutant) was cultured in the production medium and confirmed for its lack of TM-025 and TM-026 production (Figure 3D). Further chemical complementation experiments with 3-aminobenzoic acid resulted in the recovery of TM-025 and TM-026 production (Figure 3E). The result confirmed full functionality of the biosynthetic machinery in this mutant except for the genes that have been inactivated.

To explore the possibility of using ΔptmT/H mutant to produce pactamycin analogues with different amino positions of aminoacetophenone, we fed the mutant with 2-, 3-, and 4-aminobenzoic acids individually. The mutant was pulse-fed with the compounds (50 μmol) at 16, 28, 40, 52, and 64 h, and the products were isolated using *n*-BuOH

and analyzed by MS. The results showed that only feeding experiments with 3-aminobenzoic acid gave the expected products TM-025 and TM-026 (Figure S2B, Supporting Information), whereas feeding with 2- and 4-aminobenzoic acids did not give any corresponding products (Figures S2A and S2C, Supporting Information), suggesting substrate rigidity of some of the enzymes in the pathway.
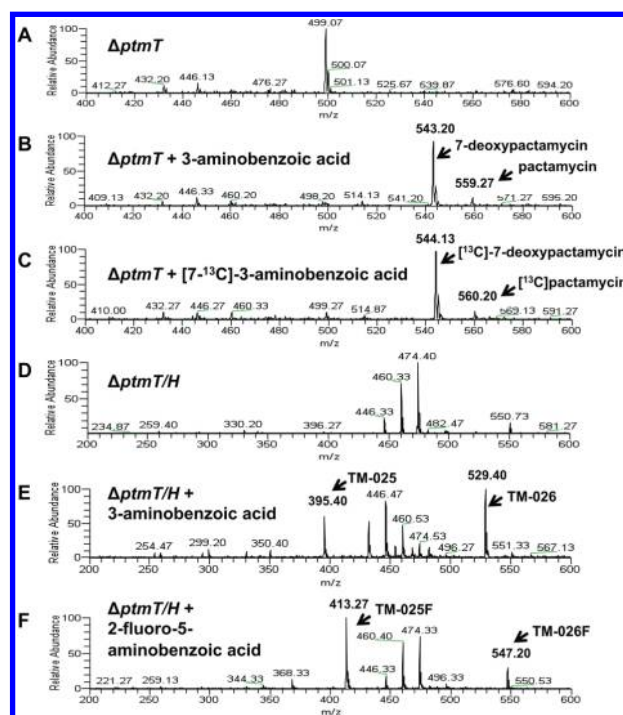

Figure 3. Mass spectral data for EtOAc (A−C) and *n*-BuOH (D−F) extracts of *S. pactum* mutants with and without chemical complementations.

To determine if fluorinated TM-025 and TM-026 analogs can be produced by mutasynthesis, we fed ΔptmT/H mutant with 2-fluoro-5-aminobenzoic acid and 5-fluoro-4-methyl-3-aminobenzoic acid. Both compounds were chemically prepared from commercially available 2-fluoro-5-nitrobenzoic acid and 5-fluoro-4-methyl-3-nitrobenzoic acid, respectively, by catalytic hydrogenation (Figure S3, Supporting Information). The ΔptmT/H mutant was pulse-fed with the compounds, and the products were analyzed. The results showed that feeding experiments with 2-fluoro-5-aminobenzoic acid gave rise to fluorinated TM-025 and TM-026, designated as TM-025F and TM-026F (Figures 3F and 4). These two compounds gave quasimolecular ions *m/z* 413 and 547, respectively, 18 atom mass unit (amu) higher than those of TM-025 and TM-026 (Figure S4, Supporting Information). Further confirmation of the products was carried out by comparing their MS/MS fragmentation patterns with those of TM-025 and TM-026 (Figure S4, Supporting Information) and by NMR analyses (Figures S9−S12, Supporting Information). Whereas 2-fluoro-5-aminobenzoic acid was incorporated in relatively high yields, giving rise to 4−5 mg/L of each TM-025F and TM-026F,
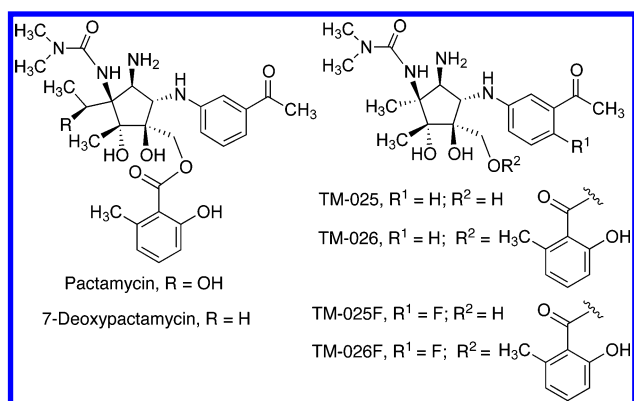
Figure 4. Chemical structures of pactamycin analogues.

Table 1. Antimalarial Activity of Pactamycin Analogues[a]

compd	IC$_{50}$ (nM)		
	D6	Dd2	7G8
pactamycin	4.9	3.9	4.2
TM-025	7.7	10.5	7.1
TM-026	11.5	14.0	9.1
TM-025F	12.3	16.8	12.4
TM-026F	26.5	39.1	24.9
chloroquine	10.6	114	89.5

[a] Key: D6, chloroquine-sensitive *P. falciparum*; Dd2 and 7G8, multidrug-resistant *P. falciparum*.

feeding experiments with 5-fluoro-4-methyl-3-aminobenzoic acid did not give any new products (Figure S2F, Supporting Information).

To test the antimalarial activity of TM-025F and TM-026F, the compounds were subjected to an in vitro antimalarial activity assay. The compounds, along with TM-025, TM-026, and pactamycin were tested against chloroquine sensitive (D6) and multidrug-resistant (Dd2 and 7G8) strains of *Plasmodium falciparum*. The results showed that TM-025F and TM-026F maintain strong activity against malarial parasites with IC$_{50}$ in low nM concentrations (Table 1). The results also revealed that fluorine atom substitution in the aminoacetophenone moiety of TM-025 and TM-026 does not significantly affect their antimalarial activity in vitro.

To examine if the fluorinated analogues retain the target selectivity of TM-025 and TM-026, antibacterial activity tests were performed against both Gram-positive (*Mycobacterium smegmatis*, *Staphylococcus aureus*, *Bacillus subtilis*) and Gram-negative (*Pseudomonas aeruginosa* and *E. coli*) bacteria in an agar diffusion assay. Whereas pactamycin is active against all bacteria tested, neither TM-025F nor TM-026F showed any significant activity at the concentrations used (Figure S13, Supporting Information), suggesting their high selectivity for plasmodial cells, but not for bacteria.

In conclusion, we have used an efficient mutasynthetic strategy to generate fluorinated TM-025 and TM-026, two promising pactamycin analogues, in *S. pactum*. Similar to the parent compounds, the fluorinated analogues maintain excellent activity and selectivity toward chloroquine-sensitive and multidrug-resistant strains of *P. falciparum*. The results also provide insight into the biosynthesis of 3-aminobenzoic acid, which is relatively obscure, as well as substrate preferences of some of the enzymes in the pathway.

Supporting Information Available. Experimental procedures, Table S1, Figures S1−S13. This material is available free of charge via the Internet at http://pubs.acs.org.