SUPPORTING INFORMATION

Mutasynthesis of Fluorinated Pactamycin Analogs and Their Antimalarial Activity

Khaled H. Almabruk,^{1,§} Wanli Lu,^{1,§} Yuexin Li,² Mostafa Abugreen,¹ Jane X. Kelly,² and Taifo Mahmud^{1,*}

¹Department of Pharmaceutical Sciences, Oregon State University, Corvallis, OR 97331-3507; ²Veterans Affairs Medical Center, Portland, OR 97239

Taifo.Mahmud@oregonstate.edu

§These authors contributed equally

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Experimental Section

Bacterial strains and plasmids. Pactamycin producing *S. pactum* ATCC 27456 was purchased from American Type Culture Collection (ATCC). *Escherichia coli* DH10B was used as a host strain for the construction of recombinant plasmids. *E. coli* ET12567 was used as a donor strain in conjugation experiments. pJET1.2 (Fermentas) and pGEM-T (Promega) were used as cloning vectors. pTMN002 is a pJTU1278+ derivative containing an OriT transfer element required for conjugation.^{1, 2} All bacterial strains and plasmids used in this study are listed in Table 1.

General DNA manipulations. Genomic DNA of *S. pactum* ATCC 27456 was prepared by standard protocol³ or using the DNeasy Tissue Kit (QIAGEN). DNA fragments were recovered from an agarose gel by using the QIAquick Gel Extraction Kit (QIAGEN). Restriction endonucleases were purchased from Invitrogen or Promega. Preparation of plasmid DNA was done by using a QIAprep Spin Miniprep Kit (QIAGEN). All other DNA manipulations were performed according to standard protocols.^{3, 4} PCR was performed in 30 cycles using a Mastercycler gradient thermocycler (Eppendorf) and Platinum *Taq* DNA polymerase (Invitrogen) or Platinum *Pfx* DNA polymerase (Invitrogen). Oligodeoxyribonucleotides for PCR primers were synthesized by Sigma-Genosys, and are shown in Table 2. The nucleotide sequences of the gene fragments were determined at the Center for Genome Research and Biocomputing (CGRB) Core Laboratories, Oregon State University.

Construction of *ptmA* and *ptmT* knock-out plasmids. For the construction of *ptmA* knock-out plasmid, two 1 kb PCR fragments upstream and downstream of the *ptmA* gene were generated with primers *ptmA*-F1/*ptmA*-R1 and *ptmA*-F2/*ptmA*-R2 (see Table 2), respectively, and separately cloned into pJET1.2 cloning vector for DNA sequencing. HindIII and EcoRI digested upstream DNA fragment, EcoRI and XbaI digested downstream DNA fragment, and HindIII and XbaI digested pTMN002 were ligated to create pTMW034, which was introduced into *S. pactum* by conjugation with the *E. coli* donor strain ET12567 (pUZ8002). Apramycin resistant strains representing single crossover mutants were obtained and subsequently grown on nonselective mannitol-soy flour agar containing MgCl₂ (10 mM, MS-Mg) 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 (50 µg mL⁻¹). The resulting double-crossover candidate strains were confirmed by PCR amplification with external primers (*ptmA*-P1 and *ptmA*-P2, Table 2) flanking the targeted *ptmA* gene. The resulting PCR fragment from putative double crossover mutants was subcloned into pGEM-T Easy vector and sequenced to confirm that part of *ptmA* has been removed from the genomic DNA. A similar approach was used for the construction of *ptmT* knock-out plasmid pTMW037, except that the PCR fragments upstream and downstream of the *ptmT* gene were generated with primers *ptmT*-F1/*ptmT*-R1 and *ptmT*-F2/*ptmT*-R2, respectively. pTMW037 was introduced into *S. pactum* wild type strain or $\Delta ptmH$ strain, separately, to generate *S. pactum* $\Delta ptmT$ or *S. pactum* $\Delta ptmT/H$ mutant strains.

Feeding experiments with 3-aminobenzoic acid and [7-¹³C]-3-aminobenzoic acid.

The $\Delta ptmA$ and $\Delta ptmT$ mutants were streaked on BTT agar [glucose (1%), yeast extract (0.1%), beef extract (0.1%), casein hydrolysate (0.2%), agar (1.5%), pH 7.4] and incubated at 30 °C for 3 days. Spores of the $\Delta ptmA$ and $\Delta ptmT$ mutants were individually grown in two Erlenmeyer flasks (125 mL) containing seed medium [glucose (1%), yeast extract (0.1%), beef extract (0.1%), casein hydrolysate (0.2%), pH 7.4] (50 mL) for 3 days at 28°C and 200 rpm. Each of these seed cultures (10 mL each) was used to inoculate six Erlenmeyer flasks (500 mL) containing modified Bennett medium (100 mL). After incubation for 16 h under the same conditions, the cultures were grouped into two groups; the first group was fed with 3-aminobenzoic acid (5 mM, 200 μ L) and the second group was fed with [7-¹³C]-3-aminobenzoic acid (5 mM, 200 μ L). 3-aminobenzoic acid was purchased from Aldrich and [7-¹³C]-3-aminobenzoic acid was a gift from Prof. Heinz G. Floss. The feeding was repeated every 12 h for 2 days. After five days of incubation, the cultures were centrifuged and the pH was adjusted to 7.5 using NaOH solution (1 M). The metabolites of each group were extracted with *n*-BuOH (3 x 300 mL). The organic solvent was evaporated using rotary evaporator and the products were analyzed by MS.

Feeding experiments with 2-, 3-, and 4-aminobenzoic acids. The $ptmT/H$ mutant was streaked on BTT agar [glucose (1%), yeast extract (0.1%), beef extract (0.1%), casein hydrolysate (0.2%), agar (1.5%), pH 7.4] and incubated at 30 °C for 3 days. Spores of the $\Delta ptmT/H$ mutant were initially grown in three Erlenmeyer flasks (125 mL) containing seed medium [glucose (1%), yeast extract (0.1%), beef extract (0.1%), casein hydrolysate (0.2%), pH 7.4] (50 mL) for 3 days at 28°C and 200 rpm. This seed culture (10 mL each) was used to inoculate nine Erlenmeyer flasks (500 mL) containing modified Bennett medium (100 mL). After incubation for 16 h under the same conditions, the cultures were grouped into three groups; the first group was fed with 2-aminobenzoic acid (5 mM, 200 μ L), the second group was fed with 3-aminobenzoic acid (5 mM, 200 μ L) and the third group was fed with 4-aminobenzoic acid. 2- and 3-aminobenzoic acids were purchased from Aldrich and 4-aminobenzoic acid was chemically synthesized from methyl 4-amino-benzoate by refluxing the compound with 3 N NaOH for 1 h. The feeding was repeated every 12 h for 2 days. After five days of incubation, the cultures were centrifuged and the supernatants of each group were pooled and the pH was adjusted to 7.5 using NaOH solution (1 M). The metabolites of each group were extracted with *n*-BuOH (3 x 300 mL). The organic solvent was evaporated using rotary evaporator and the products were analyzed by MS.

Synthesis of 2-fluoro-5-aminobenzoic acid. 2-Fluoro-5-nitrobenzoic acid (100 mg, 0.5405 mmol) was dissolved in EtOH-6N HCl (1:1, 1 mL) and then Pd/C (10%, 15 mg) was added and the mixture was stirred under hydrogen atmosphere for 3 h. The reaction was monitored by TLC (visualized by ninhydrin) until the starting material has converted completely to the product. The mixture was passed through celite and the eluted solution was evaporated to dryness and subjected to Sephadex LH-20. Fractions containing the product were pooled and dried to afford the title compound (80 mg). ¹H NMR (300 MHz, CD₃OD): δ_H 8.00 (dd, *J* = 6, 3 Hz, 6-H, 1H), 7.67 (dt, *J* = 9, 3 Hz, 4-H, 1H), 7.41 (dd, *J* = 10, 9 Hz, 3-H, 1H). ¹³C NMR (75 MHz, CD₃OD): δ_C 165.6 (d, *J*_{C-F} =

3.4 Hz, 1C), 162.9 (d, J_{C-F} = 265.5 Hz, 1C), 130.4, (d, J_{C-F} = 9.6 Hz, 1C), 128.2 (d, J_{C-F} = 4.3, Hz, 1C), 128.1 (d, J_{C-F} = 1.9 Hz, 1C), 121.2 (d, J_{C-F} = 11 Hz, 1C), 119.1 (d, J_{C-F} = 19 Hz, 1C). LRMS (EI+) m/z 155 [M^+]. HRMS (EI+) m/z 155.03811 (calcd for $C_7H_6FNO_2$ [M^+]: 155.03825).

Synthesis of 3-amino-4-methyl-5-fluorobenzoic acid. 3-Nitro-4-methyl-5-fluorobenzoic acid (30 mg, 0.150 mmol) was dissolved in EtOH-6N HCl (1:0.1, 1 mL) and then Pd/C (10%, 10 mg) was added and the mixture was stirred under hydrogen atmosphere for 3 h. The reaction was monitored by TLC until the starting material has converted completely to the product. The mixture was passed through celite and the eluted solution was evaporated to dryness and subjected to Sephadex LH-20. Fractions containing the product were pooled and dried to afford the title compound (22 mg). 1H NMR (300 MHz, CD_3OD): δ : 7.41 (d, J = 10 Hz, 6-H, 1H), 6.60 (d, J = 6 Hz, 2-H, 1H), 2.27 (d, J = 1.4 Hz, 4-CH₃, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ_C : 167.6, 161.2 (d, J_{C-F} = 245 Hz, 1C), 133.9 (d, J_{C-F} = 19 Hz, 1C), 130.4, 128.4 (d, J_{C-F} = 5 Hz, 1C), 123.8, 119.3 (d, J_{C-F} = 25 Hz, 1C), 14.7 (d, J_{C-F} = 3 Hz, 1C). LRMS (TOF MS ES-) m/z : 186.0 [$M-H$]⁻. HRMS (TOF MS ES-) m/z : 168.0455 (calcd for $C_8H_7FNO_2$ [$M-H$]⁻: 168.0461).

Feeding experiments with 2-fluoro-5-aminobenzoic acid and 3-amino-4-methyl-5-fluorobenzoic acid. The procedure for feeding experiments with 2-fluoro-5-aminobenzoic acid and 3-amino-4-methyl-5-fluorobenzoic acid is similar to that for 2-, 3-, and 4-aminobenzoic acids.

Isolation of TM-025F and TM-026F. Five days culture broths (500 mL) of the $\Delta ptmT/H$ mutant fed with 2-fluoro-5-aminobenzoic acid (5 mM, 5 mL) were centrifuged and the supernatant was adjusted to pH 7.5. The metabolites were extracted three times with EtOAc (500 mL each) and 2 times with *n*-BuOH (500 mL each). The organic solutions were dried over anhydrous Na₂SO₄ and the solvents were dried *in vacuo* to afford EtOAc extract (90.2 mg) and *n*-BuOH extract (280 mg). The EtOAc extract was subjected to a silica gel column and eluted with 100% CHCl₃ followed by CHCl₃-MeOH (9:1) and CHCl₃-MeOH (8:2). Fractions containing TM-026F were pooled and the organic solvent was evaporated to give pure TM-026F (4 mg/L). Yellowish powder, 1H NMR (700 MHz, CryoProbe, CD_3OD) δ : 7.15 (t, J = 8 Hz, 1H, H-4'), 7.07 (dd, J = 6.3, 3.5 Hz, 1H, H-2''), 6.86 (dt, J = 9, 3.5 Hz, 1H, H-4''), 6.70 (dd, J = 11 Hz, J = 9 Hz, 1H, H-5''), 6.62 (d, J = 8 Hz, 1H, H-5'), 6.58 (d, J = 7.5 Hz, 1H, H-3'), 4.85 (d, 1H, H-8a), 4.32 (d, J = 12 Hz, 1H-H-8b), 4.06 (d, J = 9 Hz, 1H, H-3), 3.65 (d, J = 9 Hz, 1H, H-2), 2.97 (s, 6H, N(CH₃)₂), 2.41 (d, J = 5 Hz, 3H, COCH₃), 2.25 (s, 3H, 7'-CH₃), 1.64 (s, 3H, 7-CH₃), 1.40 (s, 3H, 6-CH₃). ^{13}C NMR (175 MHz, CryoProbe, CD_3OD) δ_C : 197.8 (C-7''), 170.7 (C-8'), 160.6 (C-9), 159.7, 156.3 (d, J_{C-F} = 243 Hz, C-6''), 145.6, 141.2, 133.5, 125.7 (d, J_{C-F} = 12 Hz, 1C), 123.3, 120.7 (d, J_{C-F} = 7 Hz, 1C), 117.9 (d, J_{C-F} = 24 Hz, 1C), 117.1, 115.3, 112.7, 84.0, 82.9, 67.0, 66.6, 65.9, 65.7, 36.7 (C-10, C-11), 31.4 (d, J_{C-F} = 8.7 Hz, C-8''), 22.3 (C-6''), 16.2 (C-6), 14.9 (C-7). LRMS (TOF MS ES+) m/z 547.3 [$M+H$]⁺. HRMS (TOF MS ES+) m/z 547.2560 (calcd for $C_{27}H_{35}FN_4O_7$ [$M+H$]⁺: 547.2568).

The *n*-BuOH extract was first dissolved in MeOH and then CHCl₃ (three volumes) was added. The mixture was loaded onto a silica gel column and let stand for 10 min.

Subsequently, the metabolites were eluted with CHCl₃-MeOH (10:1) and the ratio was change incrementally to CHCl₃-MeOH (6:4). Fractions containing the product were pooled and dried to give TM-025F (4.5 mg/L).

Yellowish powder, ¹H NMR (300 MHz, CD₃OD) δ: 7.16 (bd, *J* = 5.1 Hz, 1H, H-2''), 7.00 (m, 2H, H-4'', H-5''), 4.05 (d, *J* = 11.4 Hz, 1H, 8-Ha), 3.98 (bd, *J* = 7.5 Hz, 1H, 3-H), 3.6 (m, 1 H, 2-H), 3.45 (d, *J* = 11.4 Hz, 1H, H-8b), 2.96 (s, 6H, N(CH₃)₂), 2.56 (d, *J* = 4.5 Hz, COCH₃), 1.61 (s, 3H, 7-CH₃), 1.41 (s, 3H, 6-CH₃). ¹³C NMR (75 MHz, CD₃OD): δ_C 198.9 (C-7''), 160.7 (C-9), 156.3 (d, *J*_{C-F} = 242 Hz, C-6''), 146.2, 126.7 (d, *J*_{C-F} = 14 Hz, 1C), 120.8 (d, *J*_{C-F} = 8 Hz, 1C), 118.2 (d, *J*_{C-F} = 25 Hz, 1C), 113.5, 83.6, 82.8, 66.9, 66.5, 65.2, 63.4, 36.8 (C-10, C-11), 31.4 (d, *J*_{C-F} = 7.8 Hz, C-8''), 16.9 (C-6), 14.9 (C-7). LRMS (TOF MS ES+) *m/z* 413.2 [M+H]⁺. HRMS (TOF MS ES+) *m/z* 413.2195 (calcd for C₁₉H₂₉FN₄O₅ [M+H]⁺: 413.2200).

***In vitro* Antimalarial Activity Assay.** *P. falciparum* strains D6, Dd2, and 7G8 were cultured in human erythrocytes at 2% hematocrit in RPMI 1640 containing 0.5% Albumax, 45 µg/L hypoxanthine, and 50 µg/L gentamicin, as previously described.⁵ *In vitro* antimalarial activity was determined by the malaria SYBR Green I-based fluorescence (MSF) assay described previously⁶ with slight modification.⁵ Stock solutions of each test drug were prepared in sterile distilled water at a concentration of 10 mM. The drug solutions were serially diluted with culture medium and distributed to asynchronous parasite cultures on 96-well plates in quadruplicate in a total volume of 100 µL to achieve 0.2% parasitemia with a 2% hematocrit. Automated pipetting and dilution were carried out with a programmable Precision 2000 robotic station (Bio-Tek, Winooski, VT). The plates were then incubated for 72 h at 37 °C. After incubation, 100 µL of lysis buffer with 0.2 µl/ml SYBR Green I was added to each well. The plates were incubated at room temperature for an hour in the dark and then placed in a 96-well fluorescence plate reader (Spectramax Gemini-EM; Molecular Diagnostics) with excitation and emission wavelengths at 497 nm and 520 nm, respectively, for measurement of fluorescence. The 50% inhibitory concentration (IC₅₀) was determined by nonlinear regression analysis of logistic dose-response curves (GraphPad Prism software).

Antibacterial Activity Assay. Antibacterial activity of pactamycin and its analogs was determined by an agar diffusion assay. *M. smegmatis*, *S. aureus*, *B. subtilis*, *P. aeruginosa*, and *E. coli* were streaked on nutrient agar (Difco) and grown overnight at 37 °C. Colonies were transferred to nutrient broth and incubated at 37 °C for 24 h. Turbidity of the inoculum was measured to a proper density at 600 nm (BioRad, SmartSpec 3000). For plate preparation, inoculum (500 µL) was mixed thoroughly with warm nutrient agar (50 mL) and poured to petri disks. The agar plates were allowed to solidify and dry for 30 min before assay. Sterile blank paper disks (Becton-Dickinson) were impregnated with pactamycin and its analogs (20 µL of 1 mM solution) and dried at room temperature. The disks were placed onto inoculated agar plates and incubated at 37 °C for 24 h. In order to produce a contrast background of the inhibition zone, 0.25% MTT developing dye (1 mL) was added over the plates.

Supplemental Data

Table 1. Strains and plasmids used in this study

Strains	Relevant genotype/comments	Source/Ref
<i>Escherichia coli</i> DH10B	<i>F mcrA Δ(mrr-hsdRMS-mcrBC)φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻ rspL nupG</i>	GibcoBRL
<i>E. coli</i> ET12567(pUZ8002)	<i>dam dcm hsdS</i> , pUZ8002	⁷
<i>Streptomyces pactum</i> ATCC 27456	Wild-type pactamycin producing strain	ATCC
<i>S. pactum</i> Δ <i>ptmH</i>	<i>ptmH</i> disruption mutant	⁸
<i>S. pactum</i> Δ <i>ptmA</i>	<i>ptmA</i> disruption mutant	This study
<i>S. pactum</i> Δ <i>ptmT</i>	<i>ptmT</i> disruption mutant	This study
<i>S. pactum</i> Δ <i>ptmH-ptmT</i>	<i>ptmH</i> and <i>ptmT</i> double mutant	This study
Plasmids		
pJET1.2	pMBI-based PCR product cloning vector; T7 promoter; <i>bla</i> .	Fermentas
pGEM-T	High copy number PCR cloning vector containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the alpha-peptide coding region of the enzyme beta-galactosidase; <i>bla</i> .	Promega
pJTU1278+	pHZ1358 derivative containing <i>lacZ</i> and MCS	¹
pTMN002	pJTU1278+ derivative containing a 1 kb <i>aac(3)/V</i> apramycin resistance cassette	²
pTMW032	1 kb DNA fragment containing downstream PCR product of <i>ptmA</i> gene in pJET1.2	This study
pTMW033	1 kb DNA fragment containing upstream PCR product of <i>ptmA</i> gene in pJET1.2	This study
pTMW034	Two 1 kb PCR fragments upstream and downstream of the <i>ptmA</i> gene in pTMN002	This study
pTMW035	1 kb DNA fragment containing downstream PCR product of <i>ptmT</i> gene in pJET1.2	This study
pTMW036	1 kb DNA fragment containing upstream PCR product of <i>ptmT</i> gene in pJET1.2	This study
pTMW037	Two 1 kb PCR fragments upstream and downstream of the <i>ptmT</i> gene in pTMN002	This study
TIP3	Fosmid clone containing part of the <i>ptm</i> cluster	²
5A7	Fosmid clone containing part of the <i>ptm</i> cluster	²
24H11	Fosmid clone containing part of the <i>ptm</i> cluster	²

Table 2. Primers used in this study

Primer	Sequence ^(a)
<i>ptmA</i> -F1	5'- CCC <u>AAGCTT</u> GCTGCTCGCCTTCGTGGA -3'
<i>ptmA</i> -R1	5'- CCG <u>GAATTC</u> CCAGGGCTCGTACCGCATC -3'
<i>ptmA</i> -F2	5'- CCG <u>GAATTC</u> ATCGTCGCCACCCTCCGCTCG -3'
<i>ptmA</i> -R2	5'- TGCT <u>TCTAGAC</u> GGCGTGACGGGCGTCGTT -3'
<i>ptmA</i> -P1	5'- CGACCACTCGCTGGGCTTCA -3'
<i>ptmA</i> -P2	5'- GACGATGTCCGCCGACCATT -3'
<i>ptmT</i> -F1	5'- CCC <u>AAGCTT</u> CTCTGGCCCGTGATGAATCCG -3'
<i>ptmT</i> -R1	5'- CCG <u>GAATTC</u> GTTGGCACCCACTGAGAACCTC -3'
<i>ptmT</i> -F2	5'- CCG <u>GAATTC</u> CAGCGGTCCCTGGTCGGA -3'
<i>ptmT</i> -R2	5'- TGCT <u>TCTAGAT</u> CGCATCGGCCCTCGTCG -3'
<i>ptmT</i> -P1	5'- CAGCCGCACGCAGGTCAGTT -3'
<i>ptmT</i> -P2	5'- GACGTTGATGCAGGTGGACAGC -3'

^(a) nucleotides underlined refer to restriction sites

Figure S1. Gel electrophoreses of PCR products that confirm Δ *ptmA* and Δ *ptmT* mutant strains of *S. pactum*.

Figure S2. MS data for *n*-BuOH extracts from $\Delta ptmT/H$ cultures complemented with external precursors.

Figure S3. Preparation of fluorinated precursors and chemical complementation experiments with $\Delta ptmT/H$ mutants.

Figure S4. MS/MS analysis of TM-025, TM-026, and their fluorinated analogs

Figure S5. ^1H NMR spectrum of 2-fluoro-5-aminobenzoic acid.

Figure S6. ^{13}C NMR spectrum of 2-fluoro-5-aminobenzoic acid.

Figure S7. ^1H NMR spectrum of 3-amino-4-methyl-5-fluorobenzoic acid.

F-M-ABA-SUB

Figure S8. ^{13}C NMR spectrum of 3-amino-4-methyl-5-fluorobenzoic acid.

Figure S9. ^1H NMR spectrum of TM-026F.

Figure S10. ^{13}C NMR spectrum of TM-026F.

Figure S11. ^1H NMR spectrum of TM-025F.

Figure S12. ¹³C NMR spectrum of TM-025F.

Figure S13. Agar diffusion assay of pactamycin analogs. **1**, TM-025; **2**, TM-026; **3**, TM-025F; **4**, TM-026F; **P**, pactamycin; **Am**, ampicillin.

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