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Panosialins, Inhibitors of Enoyl-ACP Reductase from *Streptomyces* sp. AN1761

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In the continued search for inhibitors of enoyl-acyl carrier protein (ACP) reductase, we found that four acyl-benzenediol sulfate metabolites from *Streptomyces* sp. AN1761 potently inhibited bacterial enoyl-ACP reductases of *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis*. Their structures were identified as panosialins A, B, wA, and wB by MS and NMR data. They showed stronger inhibition against *S. aureus* FabI and *S. pneumoniae* FabK with IC_{50} of 3–5 μM than *M. tuberculosis* InhA with IC_{50} of 9–12 μM. They also exhibited a stronger antibacterial spectrum on *S. aureus* and *S. pneumoniae* than *M. tuberculosis*. In addition, the higher inhibitory activity of panosialin wB than panosialin B on fatty acid biosynthesis was consistent with that on bacterial growth, suggesting that they could exert their antibacterial activity by inhibiting fatty acid synthesis.

Key words: Panosialin, enoyl-ACP reductase, inhibitor, *Staphylococcus aureus*, antibacterial

Bacterial fatty acid synthesis (FAS II) is an attractive antibacterial target since FAS is organized differently in bacteria and mammals [2, 4]. Bacterial enoyl-ACP reductase catalyzes the final and rate-limiting step in bacterial fatty acid synthesis [8, 11]. There are three isoforms, FabI, FabK, and FabL, in enoyl-ACP reductase. FabI is distributed broadly throughout the majority of bacteria including *S. aureus*, whereas *Streptococcus pneumoniae* contains only FabK, *Enterococcus faecalis* and *Pseudomonas aeruginosa* contain both FabI and FabK, and *Bacillus subtilis* contains both FabI and FabL. InhA is a FabI homolog in *M. tuberculosis*. Bacterial enoyl-ACP reductase has been validated as an excellent target for antibacterial drug development since FabI and InhA have been identified as the antibacterial target of triclosan and isoniazid, respectively [7, 9]. Triclosan is a broad-spectrum biocide used in a wide range of consumer goods, and isoniazid has been utilized for 50 years in the treatment of tuberculosis. Therefore, inhibitors of enoyl-ACP reductase could be interesting lead compounds for treatment of multidrug-resistant bacteria.

In our continued screening of inhibitors of enoyl-ACP reductase from microbial resources [6, 12], four acyl-benzenediol sulfate metabolites were isolated from *Streptomyces* sp. AN1761. The isolated compounds were identified as panosialins A (**1**), B (**2**), wA (**3**), and wB (**4**), rare microbial metabolites, by spectral analysis (Fig. 1) [5]. Panosialins A and B have been reported as the inhibitors of viral sialidase [1] and panosialins A, B, wA, and wB as inhibitors of glycosidases together with brief antibacterial activity [10]. Their antibacterial activity, however, has yet to be thoroughly examined. In this study, we report the isolation, inhibitory activity against bacterial enoyl-ACP reductases and intracellular fatty acid biosynthesis, and antibacterial activity of **1**–**4**.

The strain AN1761 was isolated from a soil sample collected from a mountain located near Gongju-city, Chungcheongnam-do, Korea. This strain was identified as a *Streptomyces* species on the basis of its 16S rDNA sequence. Fermentation was conducted in a liquid culture medium containing soluble starch 1%, glucose 2%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.025%, and $CaCO_3$ 0.2% (adjusted to pH 7.2 before sterilization). A piece of the AN1761 strain from a mature plate culture was inoculated into a 500 ml Erlenmeyer flask containing 80 ml of the above sterile seed liquid medium and cultured for 3 days on a rotary shaker (150 rpm) at 28°C. For the production of active compounds, 5 ml of the seed culture was transferred into 500 ml Erlenmeyer flasks (65 flasks) containing 100 ml of the same medium, and then cultivated for 7 days at

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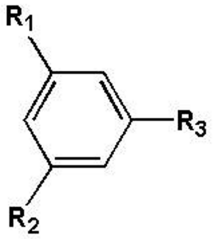

	R_1	R_2	R_3
Panosialin A (1)	OSO_3Na	OSO_3Na	$(CH_2)_{12}$-$CH(CH_3)_2$
Panosialin B (2)	OSO_3Na	OSO_3Na	$(CH_2)_{14}$-CH_3
Panosialin wA (3)	OSO_3Na	OH	$(CH_2)_{12}$-$CH(CH_3)_2$
Panosialin wA (4)	OSO_3Na	OH	$(CH_2)_{14}$-CH_3
Urushil (5)	OH	OH	$(CH_2)_{14}$-CH_3

Fig. 1. Structures of panosialins A (**1**), B (**2**), wA (**3**), and wB (**4**), and related compound.

28°C. The fermented whole medium (2 L) was extracted with 50% acetone and the extract was concentrated *in vacuo* to an aqueous solution, which was then extracted three times with an equal volume of EtOAc. The EtOAc extract was concentrated to dryness *in vacuo*. The crude extract was then subjected to SiO_2 column chromatography followed by stepwise elution with $CHCl_3$-MeOH (10:1, 5:1, 3:1, 1:1) to afford two active fractions. The first active fractions eluted with $CHCl_3$-MeOH (5:1) were pooled and concentrated *in vacuo*. The residue dissolved in MeOH was purified *via* preparative SiO_2 thin-layer chromatography developed with $CHCl_3$-MeOH (3:1) containing 0.1% TFA to yield an active band (12.7 mg) at an R_f of 0.35. The active band was further purified by reversed-phase HPLC. The column was eluted with ACN–25 mM sodium phosphate (pH 7.0) (65:35) at a flow rate of 7 ml/min to afford **3** and **4**, with retention times of 14.8 and 16.2 min, respectively. They were desalted by a MCI gel column to yield 2.6 mg of **3** and 4.7 mg of **4**. The second active fractions eluted with $CHCl_3$-MeOH (3:1) were pooled and concentrated *in vacuo*. The residue dissolved in MeOH was again applied to a Sephadex LH-20 column and then eluted with MeOH. The active fractions residue dissolved in MeOH was purified *via* preparative SiO_2 thin-layer chromatography developed with $CHCl_3$-MeOH (2:1) containing 0.1% TFA to yield an active band (16.5 mg) at an R_f of 0.2. The active band was further purified by reversed-phase HPLC chromatography eluted with ACN-50 mM sodium phosphate (pH 7.0) (78:22) at a flow rate of 3.5 ml/min to afford **1** and **2**, with retention times of 34.8 and 38.5 min, respectively. They were also desalted by a MCI gel column to yield 2.5 mg of **1** and 5.1 mg of **2**. The structures of **1–4** were determined as panosialins A, B, wA, and wB, respectively, by NMR and MS spectral data [5, 10]. The ^{1}H- and ^{13}C-NMR data of panosialins A and B were reported for the first time in this study. NMR spectra were recorded on a Bruker Biospin Avance 500 spectrometer (Korea Basic Science Institute). ESI-MS data were obtained with a Finnigan Navigator spectrometer.

Compound **1**: $C_{21}H_{34}Na_2O_8S_2$, a white powder. ^{1}H-NMR (500 MHz, CD_3OD) : δ 7.05 (1H, dd, J = 2.0, 2.0, H-2), 6.99 (2H, d, J = 2.0, H-4 and 6), 2.58 (2H, t, J = 7.5, H_2-1'), 1.61 (2H, m, H_2-2'), 1.51 (1H, q, J = 7.0, H_2-13'), 1.28 (18H, m, H_2-3' to 11'), 1.17 (2H, m, H_2-12'), 0.87 (6H, d, J = 7.0, H_3-14' and 15'); ^{13}C-NMR (200 MHz, CD_3OD) : δ 154.3 (C-1 and 3), 145.9 (C-5), 118.9 (C-4 and 6), 113.4 (C-2), 40.4 (C-12'), 37.0 (C-1'), 32.5 (C-2'), 30.6 – 31.2 (C-3' to 10'), 29.3 (C-13'), 28.7 (C-11'), 23.2 (C-14' and 15'); ESI-MS: 547.3 $[M+Na]^+$, 501.3 $[M-Na]^-$.

Compound **2**: $C_{21}H_{34}Na_2O_8S_2$, a white powder. ^{1}H-NMR (500 MHz, CD_3OD) δ 7.05 (1H, dd, J = 2.0, 2.0, H-2), 6.99 (2H, d, J = 2.0, H-4 and 6), 2.58 (2H, t, J = 7.5, H_2-1'), 1.61 (2H, m, H_2-2'), 1.28 (24H, m, H_2-3' to 14'), 0.89 (3H, d, J = 7.5, H_3-15'); ^{13}C-NMR (200 MHz, CD_3OD) δ: 154.3 (C-1 and 3), 145.9 (C-5), 118.9 (C-4 and 6), 113.4 (C-2), 37.0 (C-1'), 33.2 (C-13'), 32.5 (C-2'), 30.6 – 30.9 (C-3' to 12'), 23.9 (C-14'), 14.6 (C-15'); ESI-MS: 547.3 $[M+Na]^+$, 501.3 $[M-Na]^-$.

Compound **3**: $C_{21}H_{35}NaO_5S$, a white powder. ^{1}H-NMR (500 MHz, CD_3OD) : δ 6.62 (1H, dd, J = 2.1, 2.0, H-4), 6.60 (1H, dd, J = 2.1, 2.0, H-2), 6.42 (1H, dd, J = 2.1, 2.0, H-6), 2.50 (2H, t, J = 7.5, H_2-1'), 1.58 (2H, m, H_2-2'), 1.52 (1H, q, J = 7.0, H_2-13'), 1.28 (18H, m, H_2-3' to 11'), 1.16 (2H, m, H_2-12'), 0.87 (6H, d, J = 7.0, H_3-14' and 15'); ^{13}C-NMR (200 MHz, CD_3OD) : δ 159.1 (C-1), 154.8 (C-3), 146.2 (C-5), 113.8 (C-4), 113.1 (C-6), 107.2 (C-2), 40.4 (C-12'), 37.1 (C-1'), 32.5 (C-2'), 30.5 – 31.2 (C-3' to 10'), 29.3 (C-13'), 28.7 (C-11'), 23.2 (C-14' and 15'); ESI-MS : 445.4 $[M+Na]^+$, 399.4 $[M-Na]^-$.

Compound **4**: $C_{21}H_{35}NaO_5S$, a white powder. ^{1}H-NMR (500 MHz, CD_3OD) : δ 6.61 (1H, dd, J = 2.1, 2.0, H-4), 6.59 (1H, dd, J = 2.1, 2.0, H-2), 6.42 (1H, dd, J = 2.1, 2.0, H-6), 2.50 (2H, t, J = 7.5, H_2-1'), 1.58 (2H, m, H_2-2'), 1.28 (24H, m, H_2-3' to 14'), 0.88 (3H, d, J = 7.5, H_3-15'); ^{13}C-NMR (200 MHz, CD_3OD) : δ 159.0 (C-1), 154.8 (C-3), 146.1 (C-5), 113.8 (C-4), 113.1 (C-6), 107.1 (C-2), 37.0 (C-1'), 33.2 (C-13'), 32.5 (C-2'), 30.5 – 30.9 (C-3' to 12'), 23.9 (C-14'), 14.6 (C-15'); ESI-MS: 445.4 $[M+Na]^+$, 399.4 $[M-Na]^-$.

Urushil was purchased from Alfa Aesar (Ward Hill, MA, USA). Triclosan was purchased from Sigma. [1-^{14}C] Acetate (57 μCi/mol) and L-[U-^{14}C] leucine (306 μCi/mol) were purchased from Amersham.

S. aureus FabI, *S. pneumoniae* FabK, and *M. tuberculosis* InhA enzymes were cloned, overexpressed, and purified as

previously described [6, 13]. Assays were conducted in half-area, 96-well microtiter plates. The compounds were evaluated in 100 μl assay mixtures containing components specific to each enzyme (see below). The reduction of the *trans*-2-octenoyl *N*-acetylcysteamine (t-o-NAC thioester) substrate analog was spectrophotometrically measured by following the utilization of NADH or NADPH at 340 nm at 30°C over the linear period of the assay. *S. aureus* FabI assays contained 50 mM sodium acetate, pH 6.5, 400 μM t-o-NAC thioester, 200 μM NADPH, and 150 nM *S. aureus* FabI. The rate of decrease in the amount of NADPH in each reaction well was measured using a microtiter ELISA reader using SOFTmax PRO software (Molecular Devices, CA, USA). The inhibitory activity was calculated *via* the following formula: % of inhibition = 100 × [1−(rate in the presence of compound/ rate in the untreated control)]. The IC_{50} values were calculated by fitting the data to a sigmoid equation. An equal volume of dimethyl sulfoxide solvent was used for the untreated controls. FabK assays contained 100 mM sodium acetate, pH 6.5, 2% glycerol, 200 mM NH_4Cl, 50 μM t-o-NAC thioester, 200 μM NADH, and 150 nM *S. pneumoniae* FabK. InhA assays contained 30 mM PIPES buffer, pH 8.0, 100 μM t-o-NAC thioester, 100 μM NADPH, and 500 nM *M. tuberculosis* InhA.

Whole-cell antimicrobial activity on *S. aureus,* methicillin-resistant *S. aureus* (MRSA), *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* was determined *via* broth microdilution, as previously described [13]. The test strains were grown to mid-log phase in Mueller–Hinton broth and diluted 1,000-fold in the same medium. Cells (10^5/mL) were inoculated into Mueller–Hinton broth, except that *S. pneumoniae* was inoculated into tryptic soy broth containing 5% sheep blood, and then dispensed at 0.2 ml/well in 96-well microtiter plates. MICs were determined in triplicate *via* the serial dilution of test compounds. The MIC was defined as the concentration of a test compound required to completely inhibit cell growth at 30°C. Bacterial growth was determined by measuring the absorption at 650 nm with a microtiter ELISA reader. The minimal inhibitory concentration against *Mycobacterium tuberculosis* (Mtb) H37Rv was determined by the green fluorescent protein (GFP) reporter microplate assay [3]. Mtb H37Rv-GFP was grown in 10 ml of Middlebrook 7H9 broth (Difco, Sparks, MD, USA) supplemented with 50 mg/l kanamycin, 0.2% (v/v) glycerol (Sigma Chemical Co., Saint Louis, MO, USA), 1.0 g of Casitone (Difco) per liter, 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase; Difco), and 0.05% (v/v) Tween 80 (Sigma) until its optical density at 600 nm reached 0.4. Test compound solution was added to 7H9 media, and 2-fold serial dilutions were made in 7H9 broth in the microplates. The culture was diluted 1:50 in 7H9, and was inoculated to yield 2 × 10^5 CFU/ml in plate wells. The plates containing compounds dilutions and Mtb were incubated at 37°C for 7 days, and then fluorescence was measured in a Fluostar Optima microplate fluorometer (BMG Labtech, Ortenberg, Germany) in the bottom-reading mode with excitation at 485 nm and emission at 520 nm. The MRC was defined as the lowest concentration of extracts that inhibited fluorescence by 50% comparing with the fluorescence of bacteria-only wells.

The effect of FabI inhibitors on the incorporation of [1-^{14}C]acetate and L-[U-^{14}C]leucine in *S. aureus* was measured as described previously [13]. *S. aureus* was grown to the mid-log phase in LB medium. Each 1 ml culture was treated with drugs for 10 min. An equal volume of DMSO solvent was added to the untreated control. For [1-^{14}C]acetate incorporation, 2 μCi of [1-^{14}C] acetate was then added to the cultures and incubated at 37°C for 1 h in a shaker. After being harvested by centrifugation, the cell pellets were washed twice with PBS. The total cellular lipids were then extracted with chloroform-methanol-water. The incorporated radioactivity in the chloroform phase was measured by scintillation counting. For L-[U-^{14}C]leucine incorporation, 0.6 μCi of L-[U-^{14}C]leucine was added to the cultures and incubated at 37°C for 1 h in a shaker. The incorporation was terminated by the addition of 10% (w/v) TCA and cooling on ice for 20 min. The precipitated material was collected

Table 1. Comparison of the inhibitory activity of compounds **1–5** against enoyl-ACP reductases, bacterial viability, and intracelluar fatty acid biosynthesis in *S. aureus*.

Compounds	IC_{50} (μM)			MIC (μg/ml)					IC_{50} (μM)
	saFabI[a]	spFabK[b]	mtInhA[c]	S.a.[d]	MRSA[e]	S.p.[f]	P.a.[g]	M.t.[h]	[^{14}C]-acetate
1	4.3	3.9	11.8	128	128	256	256	256	n.t.[i]
2	5.4	5.2	8.5	128	128	256	256	256	55.3
3	3.0	5.2	9.6	16	16	64	64	128	n.t.
4	4.6	5.5	9.1	16	16	64	64	128	26.3
5	>100	>100	>100	>256	>256	>256	>256	>256	>500
Triclosan	0.66	>100	>100	0.01	0.01	>32	>64	16	0.04

[a]*S. aureus* FabI; [b]*S. pneumoniae* FabK; [c]*M. tuberculosis* InhA; [d]*S. aureus* RN4220; [e]MRSA CCARM 3167; [f]*S. pneumoniae* KCTC 5412; [g, h]MRC, the lowest concentration that prevented more than 50% of the bacterial growth, against *P. aeruginosa* KCTC 2004 and *M. tuberculosis* H37Rv, respectively; [i]not tested.

on Whatman GF/C glass microfiber filters, washed with TCA and ethanol, dried, and counted in a scintillation counter. Total counts incorporated at 1 h of incubation without inhibitors ranged from >6,000 for [^{14}C]leucine to >10,000 for [^{14}C]acetate.

Compounds **1**–**4** strongly inhibited *S. aureus* FabI in a dose-dependent fashion with IC_{50} of 3–5 μM (Table 1). They also showed the inhibitory activity on *S. pneumoniae* FabK with a similar potency, but exhibited two-times weaker inhibition on *M. tuberculosis* InhA. In order to determine whether **1**–**4** inhibit the bacterial cell growth, their antibacterial activity against *S. aureus,* methicillin-resistant *S. aureus* (MRSA), *S. pneumoniae*, *P. aeruginosa,* and *M. tuberculosis* were evaluated. Interestingly, **3** and **4** exhibited higher antibacterial activity against *S. aureus* RN4220 and MRSA CCARM 3167 with a MIC of 16 μg/ml than **1** and **2** with a MIC of 128 μg/ml (Table 1). Similarly, **3** and **4** exhibited higher antibacterial activity against *S. pneumoniae* and *M. tuberculosis* than **1** and **2**.

In order to determine whether the antibacterial effects of panosialins are attributable to the inhibition of intracellular fatty acid biosynthesis, we attempted to ascertain whether the compounds blocked the incorporation of acetate into membrane fatty acids *in vivo*. We measured the effect of the compounds on the incorporation of [1-^{14}C] acetate into the membrane fatty acids in *S. aureus*. Indeed, **4** inhibited fatty acid synthesis *in vivo* as compared with the untreated cells (Fig. 2). In addition, **4** showed stronger activity with an IC_{50} of 26.3 μM than **2** with an IC_{50} of 55.3 μM (Table 1). The higher inhibitory activity of **4** against the intracellular fatty acid biosynthesis than **2** is consistent with its higher antibacterial activity, suggesting that the antibacterial activity of panosialins could be due to the inhibition of fatty acid synthesis. Triclosan, used as a positive control, inhibited acetate incorporation. In contrast, the incorporation of leucine into proteins was not influenced by **4** at 30 μM. Even though **4** started to inhibit protein biosynthesis at 100 μM, the compound clearly evidenced selective inhibition for fatty acid synthesis. On the other hand, urushil (**5**), the desulfated derivative of **4**, did not show any inhibition against enoyl-ACP reductases, fatty acid synthesis, and bacterial growth. It suggested that the sulfate moiety in the panosialins could be involved in the activity. In the Lineweaver–Burk plot analysis (Fig. 3), **4** evidenced a

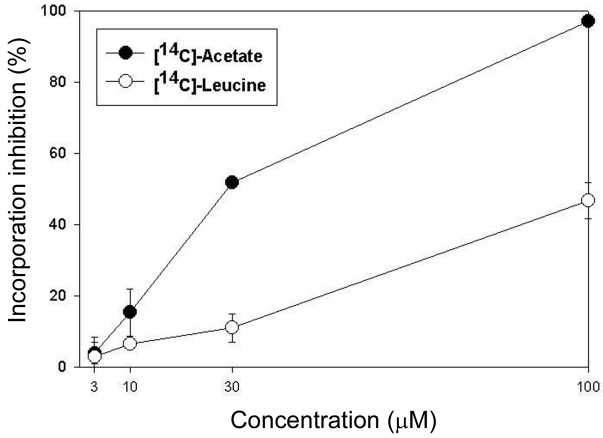

Fig. 2. Effects of panosialin wB (**4**) on fatty acid biosynthesis and protein biosynthesis in *S. aureus.*
The values were represented as the mean ± SD in duplicates obtained from two independent experiments.

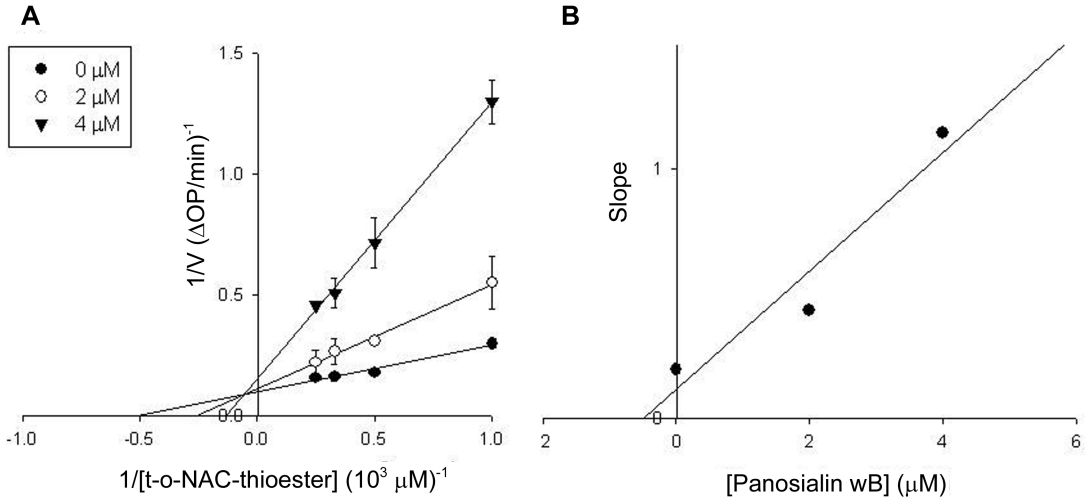

Fig. 3. The mechanism of inhibition of *S. aureus* FabI by panosialin wB (**4**) respective to t-o-NAC thioester (**A**) and K_i determination of panosialin wB (**B**).
(**A**) The reciprocals of the initial reaction and substrate concentrations are plotted. (**B**) The slope values of the lines from graph A are plotted *versus* the inhibitor concentrations affording a line obtained by linear regression. The intercept point of this line with the x-axis gives an approximate K_i value of 0.49 μM for panosialin wB. The values were represented as the mean ± SD in triplicates obtained from two independent experiments.

mixed-type inhibition against *S. aureus* FabI with respect to the substrate, t-o-NAC-thioester, with a K_i value of 0.49 μM. Interestingly, **3** and **4** showed the similar inhibitory activity on the enoyl-ACP reductases, but had higher inhibition on fatty acid synthesis as well as bacterial growth than **1** and **2**, which might be attributed to the higher membrane permeability of **3** and **4**, monosulfated, than **1** and **2**, disulfated.

Panosialins A–C have been reported as the inhibitors of viral sialidase, acid phosphatase, and polygalacturonase [1]. Yamada *et al.* [10] have re-isolated panosialins A–D and wA–wD as inhibitors of glycosidases including α-mannosidase, α-glucosidase, and β-glucosidase, from *Streptomyces* sp. OH-5186. Panosialins wA–wD were reported to show mitogenic activity in spleen cells. Inhibitory activity of panosialins on FabI and fatty acid synthesis was reported for the first time in this study. Since an inhibitor targeting multiple enoyl-ACP reductases is expected to exhibit a broader spectrum of activity, panosialins that inhibit both FabI and FabK, and especially InhA, may have potential for the development of a new broad-spectrum antibacterial.

Acknowledgments

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