Small-molecule inhibitors of the tuberculosis target, phenylalanyl-tRNA synthetase from Penicillium griseofulvum CPCC-400528

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Small-molecule inhibitors of the tuberculosis target, phenylalanyl-tRNA synthetase from *Penicillium griseofulvum* CPCC-400528

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**Abstract:** Phenylalanyl-tRNA synthetase (PheRS), a member of aminoacyl-tRNA synthetase family, was the new target of anti-tubercular drug discovery. In an attempt to fully exploit the new potential anti-tuberculosis drugs presented in micro-organisms, we developed a high-throughput screening assay against *Mycobacterium tuberculosis* (*Mtb*) PheRS and then screened a library consisting of 32,000 strains and 1500 natural product-derived compounds. One potent hit extract of *Penicillium griseofulvum* CPCC-400528 was identified. In this study, isopatulin (1), (+)-epiepoformin (2) and gentisyl alcohol (3), three patulin-producing intermediates, together with three indole-tetramic acids, α-cyclopiazonic acid (4), β-cyclopiazonic acid (5) and iso-α-cyclopiazonic acid (6), were isolated and identified as bioactive constituents from *P. griseofulvum* CPCC-400528. Their structures were elucidated on the basis of spectroscopic data. Compounds 1, 3, 4, and 5 exhibited *Mtb* PheRS-inhibiting activities, as well as moderate to weak anti-tuberculosis activities against *Mtb* H37Rv.

**Subjects:** Infectious Diseases; Medicinal & Pharmaceutical Chemistry; Pulmonary Medicine

**Keywords:** phenylalanyl-tRNA synthetase; isopatulin; cyclopiazonic acid; *Mycobacterium tuberculosis*; *Penicillium griseofulvum*

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**PUBLIC INTEREST STATEMENT**

Tuberculosis is an infectious bacterial disease caused by *Mycobacterium tuberculosis* (*Mtb*), which most commonly affects the lungs. Tuberculosis occurs in every part of the world. According to a WHO Global Tuberculosis Report (2015), about 19% of reported Tuberculosis cases occurred in China in 2014. The emergence of multidrug resistant and extensively drug-resistant strains of *Mtb*, have amplified the incidence of tuberculosis. There is an urgent need to develop new, effective, and inexpensive anti-tubercular agents. We thus selected Phenylalanyl-tRNA synthetase (PheRS) as a potential target for the development of the *Mtb*-specific inhibitor. Subsequently, six small molecules were isolated and identified as bioactive constituents from the active strain CPCC-400528. At last, Compounds 1, 3, 4, and 5 exhibited the enzymatic inhibiting activity of PheRS in vitro, as well as moderate to weak anti-tuberculosis activities against *Mtb* H37Rv.
1. Introduction

*Mycobacterium tuberculosis* (*Mtb*), the etiologic agent of tuberculosis (TB), continues to be the leading cause of death due to bacterial infection worldwide (Gandhi et al., 2010; Lienhardt et al., 2012; World Health Organization, 2013). The emergence of multidrug-resistant and extensively drug-resistant strains of *Mtb* have amplified the incidence of TB. There is an urgent need to develop new, effective, and inexpensive anti-tubercular agents (Falzon et al., 2013; Lemos & Matos, 2013; Lynch, 2013; Migliori et al., 2013; Skrahina et al., 2013; Zhao et al., 2012).

Aminoacyl-tRNA synthetases (AaRSs) are being pursued as targets for new drugs (Delarue, 1995). Blocking AaRS function inhibits the proper charging of tRNAs leading to disruption of translation. This leads to protein synthesis inhibition, which in turn causes cell growth arrest (Tao & Schimmel, 2000). With the aim of developing a new anti-tuberculosis drug, we selected Phenylalanyl-tRNA synthetase (PheRS) as a potential target. PheRS is a member of subclass Iic of the AaRSs which have been considered as promising targets due to their central role in cell metabolism, the significant sequence differences between the prokaryotic and eukaryotic enzymes, the availability of enzyme material and access to structural information (Delarue, 1995; Gallant, Finn, Keith, & Wendler, 2000; Mosyak, Reshetnikova, Goldgur, Delarue, & Safro, 1995; Schimmel, Tao, & Hill, 1998). Fortunately, Mupirocin, one of AaRS-specific inhibitor, has been used as a currently topical antibiotic (Sutherland et al., 1985).

Recently, we have reported the establishment and application of a high throughput screening (HTS) assay for inhibitors of *Mtb* PheRS (Zhang et al., 2012). During our campaign to discover PheRS inhibitors, the culture broth of *Penicillium griseofulvum* CPCC-400528 showed PheRS inhibitory activity and thus was selected for further study of chemical components. Bioassay guided purification of the extract of *P. griseofulvum* CPCC-400528 afforded isopatulin (1), (+)-epiepoformin (2), and gentisyl alcohol (3), three patulin-producing metabolites, together with three indole-tetramic acids, α-cyclopiazonic acid (4), β-cyclopiazonic acid (5), and iso-α-cyclopiazonic acid (6) (Figure 1). Four of them exhibited activity in a range of 8–256 μg/ml against *Mtb*. Although these patulins and cyclopi-azonic acids were recognized as mycotoxins and limited by some governments, investigations of their bioactivity and structural characteristics continue owing to their significant biological activities. To our knowledge, a comprehensive evaluation of the anti-tuberculosis effects of these compounds has not been systematically studied. Herewith, we reported the isolation, structure elucidation, PheRS inhibitory activity of these small-molecule inhibitors 1–6.

Figure 1. Chemical structures of compounds 1–6.
2. Results and discussion

Though HTS assay for inhibitors of Mtb PheRS (Zhang et al., 2012), we have identified six active strains, which can inhibit enzymatic activity of PheRS in vitro and growth of Mycobacterium smegmatis with low cytotoxicity. Among them, fungal strain CPCC-400528 was isolated from soil collected in Kanas lake, Sinkiang, China. Based on the morphological and molecular analysis, the producing strain CPCC-400528 was identified as *P. griseofulvum* Dierckx. The ITS rRNA sequence of the strain CPCC-400528 obtained in this study was deposited in GenBank under the accession number AB733349.

Compounds 1–6 were all obtained from fermentation of the fungus *P. griseofulvum* CPCC-400528 by processes involving silica gel column chromatography followed by reversed-phase HPLC. The lactone isopatulin (1) was isolated as very unstable colorless oil, which was deduced to have the molecular formula C7H8O4 from the HREIMS of it and NMR data (Table 1). The UV spectrum of 1 showed the presence of an α, β, γ, δ unsaturated carboxyl in the two system (274 nm). The 1H and 13C NMR spectra of 1 showed signals attributable to one lactone (δ 171.6, C-1), two trisubstituted double bonds ([δ 6.06, H-2], [δ 110.5, C-2], [δ 154.3, C-3], [δ 6.04, H-6], [δ 111.8, C-6], [δ 148.7, C-7]), one oxygenated methylene ([δ 4.67, 4.00, H-4), [δ 61.1, C-4]), and one hemiacetal group ([δ 5.98, H-5], [δ 90.7, C-5]). Isopatulin (1) is an isomer of the well-known fungal toxin, patulin (Mikami et al., 1996; Moss & Long, 2002). Finally, Interpretation of 2D NMR (COSY, HMOC, and HMBC) confirmed the presence of functional groups noted above and led to structure 1.

ESIMS and NMR data established the molecular formula of (+)-epiepoformin (2), [α]D = +310.0 (c 0.27, ethanol), as C19H16O7 was isolated. Analysis of 1H and 13C NMR data for 2 (Table 1) revealed the characteristic resonances for one lactone (δ 194.0, C-1), one 2,3-disubstituted oxirane ([δ 3.78, H-2], [δ 53.3, C-2], [δ 3.52, H-3], [δ 57.6, C-3]), one oxygenated methylene ([δ 4.67, H-4], [δ 63.4, C-4]) one trisubstituted double bonds ([δ 6.46, H-5], [δ 134.7, C-5], [δ 138.6, C-6]), and one methyl group ([δ 1.86, H-7]). Interpretation of 2D NMR (COSY, HMOC, and HMBC) confirmed the presence of functional groups noted above and led to structure 2. Compound 2 has the positive optical rotation consistent with (+)-epiepoformin, whereas (-)-epiepoformin has the negative one in ethanol (Gioer & Truckenbrod, 1988). Therefore, 2 was determined to be (+)-type.

The structures of three indole-tetramic acids, α-cyclopiazonic acid (4), β-cyclopiazonic acid (5), and iso-α-cyclopiazonic acid (6) were also unequivocally identified by ESIMS, 1D NMR and 2D NMR spectroscopic analysis (Table 2), and comparisons with data reported in the literatures (Beyer, 2011; Losito, Monaci, Aresta, & Zambonin, 2002; Nolte, Steyn, & Wessels, 1980). The 3-acetylpyrroline-2,4-diones (tetramic acids) existed in solution as pairs of internal tautomers and as a pair of acylated external tautomers ([α and β], [5α and 5β]). The disproportionate integration area was easily observed in the 1D NMR spectra of 4 and 5 (Table 2). Since Iso-α-cyclopiazonic acid (6) was isolated in only quite small amounts, sharp resonances in the 1H-NMR spectrum were not obtained.

| No. | 1       | 2       | 3       |
|-----|---------|---------|---------|
| δH (J/Hz) | δC     | δH (J/Hz) | δC     | δH (J/Hz) | δC     |
| 1   | 171.6   | 184.2   | 129.4   |
| 2   | 6.06 (s) | 111.8 | 3.50 (dd, 3.6, 1.2) | 53.4 | 6.71 (d, 3.0) | 115.8 |
| 3   | 148.7   | 3.78-3.80 (m) | 57.6 | 151.0 |
| 4   | 5.98 (s) | 90.7 | 4.67 (t, 6.0) | 63.4 | 6.49 (dd, 8.4, 3.0) | 115.5 |
| 5   | 4.67 (dd, 17.3, 2.5) | 61.1 | 6.46-6.47 (m) | 138.8 | 6.56 (d, 8.4) | 116.7 |
| 6   | 4.40 (dd, 17.3, 3.6) | 6.04 (m) | 110.5 | 134.6 | 148.9 |
| 7   | 154.3 | 1.86 (s) | 15.9 | 4.54 (s) | 60.0 |

Notes: Recorded in CDCl3, and chemical shifts are expressed as δ ppm. s, singlet; d, doublet; t, triplet; m, multiplet.
The structures of the bacterial PheRSs are well conserved but differ significantly from their eukaryotic counterparts (Ling et al., 2012). Compounds 1, 3–5 were evaluated for inhibition of the amidation activity of PheRS derived from both Mtb and human mitochondria (Hm). They were further tested for antibacterial activity against replicating Mtb H37Rv strain using a microplate Alamar blue assay (MABA) method, following our previously validated protocol (Zhang et al., 2012). Table 3 shows the enzyme and whole cell activities. The patulin-producing metabolite 1 yielded with $I_{50}$ (0.161 mM) for Mtb PheRS with high selectivity over Hm PheRS ($I_{50} =$ 5.503 mM). While the other patulin-producing compound 3 showed lower enzymatic activity for Mtb PheRS ($I_{50} =$ 0.262 mM) than 1, whereas higher activity was observed for Hm PheRS ($I_{50} =$ 0.648 mM). The two indole-tetramic acids 4 and 5 showed no significant differences with both enzymes (Table 3). In vitro antibacterial activity against Mtb H37Rv assays were done to assess those compounds potencies (Table 3). Compounds 1 and 3 were effective in antibacterial activity against Mtb H37Rv with minimum inhibitory concentration (MIC) of 8 and 16 μg/mL, respectively, as compared with compounds 4 and 5 (MIC = 128 and 64 μg/mL). As mentioned above, it appeared that the patulin-producing metabolites were more potent Mtb PheRS inhibitors than the indole-tetramic acids.

### Table 2. $^1$H (600 MHz) and $^{13}$C (150 MHz) NMR spectroscopic data for compounds 4–6

| No. | 4α | 4β | 5α | 5β |
|-----|----|----|----|----|
| δH (J/Hz) | δC | δH (J/Hz) | δC | δH (J/Hz) | δC | δH (J/Hz) | δC |
| 1 | 8.09 (brs) | 175.2 | 8.09 (brs) | 175.2 | 8.20 (brs) | 105.6 | 8.23 (brs) | 104.6 |
| 2 | 195.2 | 195.2 | 194.5 | 200.2 |
| 3 | 4.08 (d, 10.8) | 71.8 | 4.25 (d, 6.6) | 71.8 | 4.08 (dd, 10.8, 2.4) | 63.5 | 4.25 (d, 9.0) | 60.5 |
| 4 | 184.4 | 184.4 | 185.4 | 189.3 |
| 5 | 19.7 | 2.54 (3H, s) | 19.7 | 2.49 (3H, s) | 19.6 | 2.51 (3H, s) | 20.5 |
| 6 | 2.46 (3H, s) | 53.0 | 3.68 (brs) | 53.5 | 3.65 (dd, 15.0, 1.8) | 32.2 | 3.65 (dd, 15.0, 1.8) | 32.3 |
| 7 | 123.0 | 123.0 | 122.6 | 122.6 |
| 8 | 6.93 (s) | 116.5 | 6.88 (s) | 116.5 | 6.91 (dd, 7.2) | 120.0 |
| 9 | 110.0 | 110.0 | 111.4 | 110.9 |
| 10 | 120.8 | 120.8 | 7.00 (brs) | 123.0 | 7.00 (brs) | 123.3 | 7.07 (brs) |
| 11 | 133.4 | 133.4 | 137.2 | 137.2 |
| 12 | 108.7 | 108.7 | 109.5 | 109.5 |
| 13 | 7.21 (d, 7.8) | 109.6 | 6.88 (d, 6.6) |
| 14 | 7.12 (dd, 7.8, 7.2) | 122.6 | 6.75 (dd, 8.4, 3.0) |
| 15 | 6.91 (d, 7.2) | 120.4 | 6.84 (d, 6.4) |
| 16 | 128.6 | 128.6 | 134.6 | 134.4 |
| 17 | 125.9 | 125.9 | 124.6 | 124.5 |
| 18 | 3.00 (2H, brs) | 26.5 | 3.10 (2H, brs) | 26.5 | 3.75 (dd, 22.8, 15.6, 6.6) | 30.0 | 3.75 (dd, 22.8, 15.6, 6.6) | 30.1 |
| 19 | 2.66 (s) | 36.1 | 2.66 (s) | 36.4 | 5.31 (t, 6.6) | 123.2 | 5.31 (t, 6.6) | 123.3 |
| 20 | 63.4 | 63.4 | 132.8 | 132.8 |
| 21 | 1.70 (3H, s) | 26.3 | 1.76 (3H, s) | 26.1 | 1.76 (3H, s) | 25.7 | 1.76 (3H, s) | 25.7 |
| 22 | 1.53 (3H, s) | 24.4 | 1.67 (3H, s) | 24.8 | 1.74 (3H, s) | 18.1 | 1.74 (3H, s) | 18.1 |

Notes: Recorded in CDCl₃ and chemical shifts are expressed as δ ppm. s, singlet; d, doublet; t, triplet; m, multiplet, o, overlap.
To understand the interactions between the most active compound 1 and Mtb PheRS, we performed molecular docking calculations using the molecular operating environment (MOE) program. Structural models of Mtb PheRS A and B were built from the Escherichia coli PheRS (PDB code 3PCO) crystal structure. Compound 1 was found to favorably interact with Mtb PheRS A (S-value −10.0437), occupying the phenylalanyl-adenylate binding site, as shown in Figure 2. 1 formed three hydrogen bonding interactions with the amino acid residues Arg 312 and His 209, electronic interactions with Glu 279, Thr 208, Arg 201, Gly 307, Gln 215, and Gly 309, and hydrophobic interactions with Phe 213 and Met 323. Compound 1 has no interact with Mtb PheRS B.

Docking test of Mtb PheRS showed that the binding mode was well conserved in prokaryotes. Mtb PheRS inhibitors were thus suspected to have the broad antibacterial activities. The patulin-producing compounds 1 and 3 with better activities against Mtb PheRS were evaluated in vitro antibacterial activities against 37 other important human pathogens, followed our previously validated protocol (Ling et al., 2012). The obtained results were represented in Table 4, where it could be noticed that the tested compounds showed mild activity in comparison to the reference drug levofloxacin.

### 3. Experimental

#### 3.1. General experiment

NMR spectra were measured on a Bruker Avance DRX-600 spectrometer operating at 600 ($^1$H) and 150 ($^{13}$C) MHz with TMS as internal standard. HREIMS spectra were obtained on a Waters GCT system mass spectrometer. HRESIMS were carried out on a LTQ-Orbitrap XL. HPLC was performed on an Agilent 1200 liquid chromatography with a ZORBAXSB-C18 Column (9.4, 250, and 5 mm). All solvents used were of analytical grade. Silica gel (200−300 mesh, Qingdao Haiyang Chemical Co., Ltd., Qingdao, People’s Republic of China and Sephadex LH-20 (25−100 mm; Pharmacia Biotek, Denmark) were used for column chromatography. TLC was carried out with high-performance TLC plates pre-coated with silica gel GF$_{254}$ (Qingdao Haiyang Chemical Co., Ltd.). Spots of TLC were visualized within UV light or by spraying with H$_2$SO$_4$-EtOH (1:9) followed by heating.

| Compound | $IC_{50}$ (mM) ($^a$Mtb PheRS) | $IC_{50}$ (mM) ($^a$Hm PheRS) | MIC (μg/ml) (Mtb H$_3$7Rv) |
|----------|--------------------------------|--------------------------------|----------------------------|
| 1        | 0.161                          | 5.503                          | 8                          |
| 3        | 0.262                          | 0.648                          | 16                         |
| 4        | 1.345                          | 0.967                          | 128                        |
| 5        | 1.338                          | 0.193                          | 64                         |
| Rifampin | -                              | -                              | 0.125                      |

**Table 3. Inhibition of Mtb PheRS, Hm PheRS and minimum inhibitory concentration (MIC) of the isolates (1, 3−5)**
3.2. Fungi

Fungal strain CPCC-400528 was isolated from soil collected in Kanas lake, Sinkiang, China. Based on the morphological and molecular analysis, the producing strain CPCC-400528 was identified as *P. griseofulvum Dierckx*. The ITS rRNA sequence of the strain CPCC-400528 obtained in this study was deposited in GenBank under the accession number AB733349.
3.3. Cultures of *P. corylophilum* stressed with *P. griseofulvum* Dierckx

Made the medium with the ingredients (per liter), 4 g of malt extract, 10 g of malt extract, 4 g of glucose, 18 g of agar, and turned the pH to 6.0. Then dispensed the medium into test tubes, sterilized at the condition of 121°C, 30 min, made Slant medium while it was still hot.

3.4. Extraction and isolation

Fermentation of the fungus *P. griseofulvum* CPCC-400528 was carried out at 26°C for 96 h under agitation speed 150 rpm. The whole fractionation was guided by a bioassay for PheRS inhibitory and antibacterial activities. The cultured broth was separated into supernatant and mycelium by centrifugation. About 3 liters of supernatant was chromatographed on an RP-18 silica gel column (Merck, 70–150μm, MeOH/H₂O, 0:1 to 1:0) to fractions 1–50. Two active fractions, 2 and 48 were collected and concentrated in vacuo to yield ca. 2.0 g and 0.2 g of brownish oils, respectively. Fraction 2 (1.8 g) was further purified over HPLC (Agilent 1100 isopump, Agilent 1200 VWD detector (270 nm), and Phenomenex Luna 5 μm C18 column (250 × 4.60 mm), MeOH/H₂O 2.5:97.5, 0.8 mL/min) to yield 1 (1.5 g), 2 (2.3 mg), and 3 (23.0 mg). Fraction 48 (180 mg) was further purified over HPLC (Agilent 1100 isopump, Agilent 1200 VWD detector (230 nm), and Phenomenex Luna 5 μm C18 column (250 × 4.60 mm), MeOH/H₂O 76.5:23.5, 0.8 mL/min) to yield 4 (23.0 mg), 5 (12 mg) and 6 (0.3 mg). The structures of compounds 1–6 were identified as isopatulin (1), (+)-epiepoformin (2), gentisyl alcohol (3), α-cyclopiazonic acid (4), β-cyclopiazonic acid (5) and iso-α-cyclopiazonic acid (6), respectively, by comparing the spectral (MS, NMR) data with the values reported in the literature (Beyer, 2011; Gloer & Truckenbrod, 1988; Losito et al., 2002; Nolte et al., 1980).

3.5. Strains and antibiotic

*Mtb* H37Rv is clinical isolates (purified and identified according to standard procedures) from clinical. All other antibiotics used in this study were bought from sigma.

3.6. Molecular docking

MOE was used for ligand and protein preparation, docking calculations and molecular structure viewing. The crystal structure was obtained from the PDB with the accession code 3PCO.

3.7. PheRS and *M. tuberculosis* inhibition studies

The PheRS inhibition determined using a luciferase chemiluminescence-based assay. In this assay, Phenylalanyl, bulk *E. coli* tRNA, and ATP are incubated with PheRS enzyme in the presence of test compounds. MICs against replicating *Mtb* were determined by the MABA. The fluorescence was read at an excitation of 530 nm and an emission of 590 nm.

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