

Streptomyces griseoruber Y1B, a Novel *Streptomyces* for 1-Hydroxyphenazine Production

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Received: April 11, 2014 Accepted: May 13, 2014 Published: May 20, 2014

Doi: 10.5296/jab.v2i2.5430 URL: <http://dx.doi.org/10.5296/jab.v2i2.5430>

Abstract

Strain Y1B was isolated from a soil sample collected from Wuxi in Jiangsu Province, China. The strain was identified as *Streptomyces griseoruber* based on phenotypic characteristics and 16S rRNA gene sequence analysis. *phzE* and *phzF* gene fragments were amplified by PCR from *S. griseoruber* Y1B, and showed 70-80% similarity to those of phenazine-producing *Pseudomonas* and *Streptomyces* species, indicating that this strain contained phenazine biosynthetic genes and had the potential to produce phenazine compounds. A crude extract, obtained from *S. griseoruber* Y1B fermentation broth by organic solvent extraction and evaporation under reduced pressure, showed significant antifungal activity against *Rhizoctonia solani*, *Pythiumultimum*, and *Fusarium oxysporum*, and had a broad spectrum of antifungal activity. A phenazine compound, 1-hydroxyphenazine, and a shikimic acid-derived metabolite, benzoic acid, were separated and purified from the crude extract by preparative high performance liquid chromatography. The effects of addition of intermediate metabolites on 1-hydroxyphenazine production implied that the phenazine biosynthesis pathway in *S. griseoruber* Y1B might branch off from the shikimate pathway, using phenazine-1-carboxylic acid as the phenazine precursor. Furthermore, this is the first demonstration that *S. griseoruber* can produce phenazine compounds. Therefore, as a novel *Streptomyces* strain, *S. griseoruber* Y1B might have potential applications for biocontrol in agricultural production.

Keywords: *Streptomyces griseoruber* Y1B, 1-Hydroxyphenazine, Antifungal activity, Phenazine biosynthetic gene, Isolation

1. Introduction

Natural phenazines are a kind of broad spectrum antibiotic. They are nitrogenous heterocyclic compounds with various substituent groups at different sites of the core phenazine ring, such as phenazine-1-carboxylic acid (PCA), 1-hydroxyphenazine (1-OH-PHZ), pyocyanin and endophenazines, which show a wide range of biological activities (Ligon et al., 2000; Krastel et al., 2002; Laursen & Nielsen, 2004; Mentel et al., 2009). Natural phenazine products are bioactive secondary metabolites that are mainly produced by *Pseudomonas* and *Streptomyces* species from soil and marine environments, which provide abundant resources to discover more phenazine-producing strains (Saleh et al., 2012).

The biosynthetic mechanism of natural phenazine compounds has been well studied, and the phenazine biosynthetic gene cluster has been characterized. For most phenazine-producing strains, phenazine biosynthesis begins with the shikimate pathway. The gene products of *phzC*, *phzE* and *phzD* catalyze the biosynthesis of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (DAHP), 2-amino-2-deoxyisochorismic acid (ADIC), and *trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHHA), respectively. *phzF* may be responsible for the isomerization of DHHA (McDonald et al., 2001; Mentel et al., 2009; Pierson III & Pierson, 2010). Consequently, these genes can be used as markers to detect phenazine-producing strains.

In this study, a novel phenazine-producing strain, Y1B, was isolated from soil and was identified as *S. griseoruber*. Here we describe the identification, taxonomy, phenazine biosynthetic genes, and antifungal activity of *S. griseoruber* Y1B, as well as the purification and chemical structures of its bioactive compounds. We also propose a phenazine biosynthesis pathway for this species. This is the first demonstration that *S. griseoruber* can produce phenazine compounds.

2. Materials and Methods

2.1 Collection of Soil Samples

Ten soil samples were collected from the rice fields of Wuxi in Jiangsu Province, China. The fresh soil was sampled from a depth of 10 cm from the soil surface. Each of the soil samples was placed in a sterilized plastic bag and stored at 4 °C for further study.

2.2 Isolation of Strain Y1B

The soil samples were carefully crushed and dried in a 60 °C incubator for 30 min. Five grams of the soil samples were added to 45 ml of sterilized water and were shaken for 30 min, followed by 10-fold serial dilutions of the soil suspension. Two hundred microliters of the 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} dilutions of each soil sample were spread in Gause's No. 1 synthetic medium (soluble starch 20 g l⁻¹, KNO₃ 1 g l⁻¹, K₂HPO₄ 0.5 g l⁻¹, MgSO₄·7H₂O 0.5 g l⁻¹, FeSO₄·7H₂O 0.01 g l⁻¹, NaCl 0.5 g l⁻¹, agar 20 g l⁻¹, pH 7.2-7.4) containing 0.1% (w/v) K₂Cr₂O₇ and incubated at 28 °C for 7-10 days. Single colonies picked from the primary medium were purified three times in SFM agar medium (soybean flour 20 g l⁻¹, mannitol 20 g l⁻¹, agar 20 g l⁻¹, pH 7.2-7.3).

phzE and *phzF* were selected as phenazine biosynthetic gene markers to screen novel phenazine-producing strains. Primers for each gene are listed in Table 1. PCR reaction was performed in the 50 µl reaction system containing 100 ng of DNA template, 2 × GC Buffer II, 2.5 mM dNTP Mixture, 20 µM of each primer, 5 U/µl LA Taq polymerase with the PCR Kit (TAKARA Biotech). PCR amplification conditions for *phzE* and *phzF* fragments were as follows: initial denaturation at 94 °C for 2 min, followed by 36 cycles of 94 °C for 1 min, 54.7 °C (*phzE*) and 57 °C (*phzF*) for 1 min, and 72 °C for 2 min, and a final extension at 72 °C for 7 min. PCR products were purified and sequenced by Sangon Biotech. Strain Y1B was isolated with positive PCR amplification results.

Table 1. Primers used in this study

Primer	Sequence	Function	Reference
<i>phzE</i> -F	5'-GAAGGCGCCAACCTTCGTYATCAA-3'	PCR and sequencing of <i>phzE</i>	(Schneemann et al., 2011)
<i>phzE</i> -R	5'-GCCYTCGATGAAGTACTCGGTGTG-3'		
<i>phzF</i> -F	5'-ATCTTCACCCCGGTCAACG-3'	PCR and sequencing of <i>phzF</i>	(Schneemann et al., 2011)
<i>phzF</i> -R	5'-CCRTAGGCCCGGTGAGAAC-3'		
7f	5'-CAGAGTTTGATCCTGGCT-3'	PCR and sequencing of the 16S rRNA gene	(Brosius et al., 1978)
1450r	5'-AGGAGGTGATCCAGCCGCA-3'		

2.3 Taxonomy of Strain Y1B

Strain Y1B was cultured on SFM agar medium at 28 °C for 7 days, and colonies were collected for fatty acid analysis. The whole-cell fatty acids were saponified, methylated and extracted, and the composition of fatty acids was analyzed by comparing their retention times with Gas Chromatography (GC), according to the standard Sherlock Microbial Identification System (MIDI) (Eerola & Lehtonen, 1988; Kroppenstedt et al., 1990).

Genomic DNA was extracted from strain Y1B using the EasyPure Genomic DNA Kit (TransGen Biotech). The 16S rRNA gene was amplified by PCR using the primers listed in Table 1. Based on the PCR reaction system of *Par. 2.2*, PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 35 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 8 min. 16S rRNA PCR products were purified and sequenced by Sangon Biotech.

2.4 In Vitro Antifungal Activity Assay

Four plant pathogens *viz.*, *Rhizoctonia solani*, *Pythiummultimum*, *Fusarium oxysporum*, and *Stevia rebaudiana Bertoni* spot blight, were used as target pathogens to determine the antifungal activity of strain Y1B. Each of the plant pathogens was cultured on potato dextrose agar (PDA) medium (potato 200 g l⁻¹, glucose 20 g l⁻¹, agar 15 g l⁻¹) at 28 °C for 7 days (Shirzad et al., 2012).

The crude extract of strain Y1B fermentation broth was used to test antifungal activity against the four plant pathogens. Strain Y1B was cultured in shake flasks in GYM medium (glucose 4 g l⁻¹, yeast extract 4 g l⁻¹, malt extract 10 g l⁻¹, pH 7.2) at 28 °C for 6 days on a rotary shaker (180 rpm). Following fermentation, the supernatant was separated from the fermentation broth by centrifugation at 8225 × g for 10 min (Centrifuge 5804R, Eppendorf). The culture supernatant was extracted three times with an equal volume of ethyl acetate. The organic phase was separated and evaporated under reduced pressure to obtain the crude extract.

Antifungal activity was tested by the mycelial growth rate method (Gorgolous & Dekker, 1982). Briefly, the crude extract was dissolved in dimethylsulfoxide (DMSO), and the extract-DMSO solution was mixed with PDA medium to make test plates containing a crude extract concentration of 50 mg l⁻¹. An equal volume of sterile water and DMSO was mixed with PDA medium to make a blank control plate and a solvent control plate, respectively. An 8-mm mycelial plug from a fresh plant pathogen culture was placed in the middle of a test plate, as well as on a blank control plate and a solvent control plate. Following incubation at 28 °C for 5 days, the diameter of the mycelial plug was measured to calculate relative inhibition rate using the following formula:

$$\text{Relative inhibition rate (\%)} = \frac{\text{Diameter of solvent control} - \text{Diameter of sample test}}{\text{Diameter of blank control} - \text{Initial diameter}} \times 100\%$$

where the initial diameter of the mycelial plug is 8 mm.

2.5 Purification of the Active Compound

Strain Y1B was cultivated in 25 L batches using GYM medium in shaker incubator. After cultivation for 6 days, the supernatant was separated by centrifugation and was extracted with ethyl acetate as *Par. 2.4*.

The crude extract of strain Y1B fermentation broth was purified by preparative High Performance Liquid Chromatography (HPLC, Agilent 1260) on a C18 reversed-phase column (Agilent Eclipse XDB-C18, 5 μm, 4.6×250 mm), eluting with CH₃CN-H₂O (50:50, v/v) as the mobile phase at a flow rate of 0.6 ml min⁻¹. The purification yielded compound 1 and fraction I at retention times of 9.8 and 12.4 min, respectively.

Fraction I was further purified by preparative HPLC (Agilent 1260) on the same C18 column, eluting with CH₃OH-0.1% CH₃COOH aqueous solution (55:45, v/v) as the mobile phase at a flow rate of 0.6 ml min⁻¹. The purification yielded compound 2 at the retention time of 5.5 min.

2.6 Structure Determination of the Active Compound

The electrospray ionization MS (ESI-MS) and UV absorption spectra of compound 1 were measured on an Agilent HPLC 1290-MS 6230 apparatus. The ESI-MS and ultra-performance liquid chromatography tandem MS (UPLC-MS-MS) spectra of compound 2 were measured on an ACQUITY UPLC & Q-TOF MS Premier machine. ¹H NMR spectra were recorded on a Bruker Advance III 400 MHz spectrometer.

2.7 Production of Phenazine with the Addition of Intermediate Metabolites

Based on the phenazine biosynthesis and shikimate pathways, PCA, L-phenylalanine and compound 2 were selected as intermediate metabolites, and were added on day 4 of fermentation in GYM at final concentrations of 0.02 mM, 0.04 mM, 0.06 mM, 0.08 mM and 0.1 mM. After 6 days of fermentation, the crude extract was dissolved in methanol for further analysis. Compound 1 was detected at 254 nm by preparative HPLC (Agilent 1260) on the same C18 column, eluting with CH₃CN-H₂O (50:50, v/v) at a flow rate of 0.6 ml min⁻¹.

2.8 Data Analysis

The phylogenetic tree of strain Y1B and its relatives based on 16S rRNA gene sequence was constructed using MEGA software version 4.0 with the neighbor-joining method (Saitou & Nei, 1987; Tamura et al., 2007).

3. Results and Discussions

3.1 Identification of Strain Y1B

The 16S rRNA gene sequence (1400 bp, GenBank accession no. KF991392) of strain Y1B was aligned with sequences from the GenBank database by BLAST analysis (Tatusova & Madden, 1999). The results indicated that strain Y1B belonged to the genus *Streptomyces*, and that the 16S rRNA gene sequence of strain Y1B showed 100% similarity to that of *S. griseoruber*. A phylogenetic tree was constructed using the neighbor-joining method with different type strains of the genus *Streptomyces* (Figure 1). The tree also showed that strain Y1B was closely related to *S. griseoruber*, and shared a branch with *S. griseoruber* JCM 4642. Consequently, we designated the strain *S. griseoruber* Y1B.

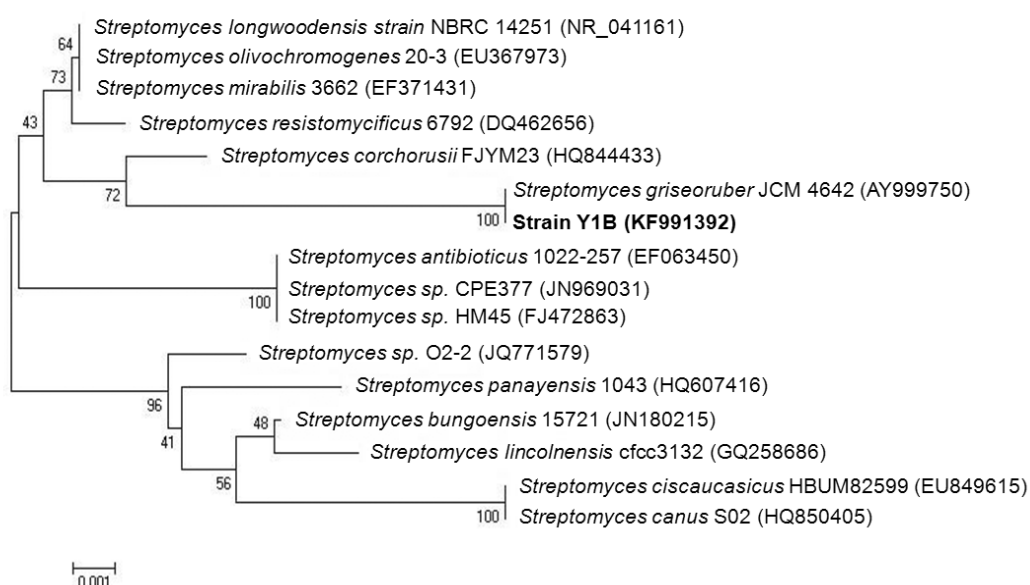


Figure 1. Phylogenetic tree of strain Y1B and its relatives based on 16S rRNA gene sequence analysis using the neighbor-joining method. The tree was constructed by MEGA software version 4.0. Bootstrap values are based on 1000 replicates. The scale bar represents 1 substitution per 1000 bases

3.2 Taxonomy of Strain Y1B

S. griseoruber Y1B grew on SFM agar medium and formed grey colonies that were circular, smooth, and dry. As shown in Table 2, the cellular fatty acids of *S. griseoruber* Y1B mainly included anteiso-C15:0 (23.03%), iso-C16:0 (16.04%), anteiso-C17:0 (12.24%), C16:0 (10.96%), and iso-C15:0 (10.33%). This is the first description of fatty acid analysis of *Streptomyces griseoruber*.

Table 2. Fatty acid profile of *S. griseoruber* Y1B

Fatty acids	Content (%)
anteiso-C13:0	0.28
iso-C14:0	2.45
C14:0	0.73
iso-C15:0	10.33
anteiso-C15:0	23.03
C15:1 B	0.30
C15:0	1.10
iso-C16:1 H	3.59
iso-C16:0	16.04
C16:1 cis 9	5.67
C16:0	10.96
C16:0 9 methyl	2.21
anteiso-C17:1 C	3.45
iso-C17:0	3.07
anteiso-C17:0	12.24
cyclo-C17:0	3.90
C17:0	0.25
C18:3 cis 6,12,14	0.38

3.3 Identification of Phenazine Biosynthetic Gene Fragments from Strain Y1B

phzE and *phzF* fragments were amplified by PCR, with fragment sizes of 390 bp and 399 bp, respectively. The nucleotide sequences of the *phzE* and *phzF* fragments from *S. griseoruber* Y1B were submitted to GenBank under accession numbers KF991393 and KF991394, respectively. The alignment of the *S. griseoruber* Y1B *phzE* and *phzF* fragments with those of phenazine-producing *Pseudomonas* and *Streptomyces* species showed 70–80% similarity in both regions (Table 3). Among these phenazine-producing strains, *S. anulatus* 9663 and *S.*

cinnamomensis DSM1042 can produce endophenazines (Seeger et al., 2011; Saleh et al., 2012), and *P. chlororaphis* GP72, *P. aeruginosa* M18, *P. aeruginosa* PAO1, and *P. aeruginosa* PA7 mainly produce simple carboxyl- and hydroxyl-substituted phenazine derivatives (Mavrodi et al., 2001; Huang et al., 2011; Li et al., 2011). These results indicated that *S. griseoruber* Y1B contained phenazine biosynthetic genes and may be able to produce phenazine compounds.

Table 3. Comparison of nucleotide similarity in the phenazine biosynthetic gene cluster between *S. griseoruber* Y1B and other phenazine-producing strains

Phenazine-producing strains	Accession no.	Gene similarity	
		<i>phzE</i>	<i>phzF</i>
<i>Streptomyces anulatus</i> 9663	FN178498	79%	72%
<i>Streptomyces cinnamomensis</i> DSM1042	AM384985	80%	72%
<i>Pseudomonas chlororaphis</i> GP72	HM594285	76%	70%
<i>Pseudomonas aeruginosa</i> M18	CP002496	80%	71%
<i>Pseudomonas aeruginosa</i> PAO1	AE004091	80%	71%
<i>Pseudomonas aeruginosa</i> PA7	CP000744	80%	72%

3.4 Antifungal Activity Test In vitro

The antifungal activity of *S. griseoruber* Y1B was characterized by its ability to inhibit the growth of four plant pathogens. As shown in Figure 2, the crude extract of *S. griseoruber* Y1B, at 50 mg l⁻¹, showed inhibitory effect against *Rhizoctonia solani*, *Pythiummultimum*, *Fusarium oxysporum* and *Stevia rebaudiana* Bertoni spot blight, with relative inhibition rates of 47.7%, 20.6%, 31.0% and 6.4%, respectively. The results indicated that *S. griseoruber* Y1B exhibited different inhibition effect against different plant pathogens, and had a broad spectrum of antifungal activity. In addition, *S. griseoluteus* P510, which had been reported as another phenazine-producing *Streptomyces* species, with the concentration of crude extract at 70 mg l⁻¹, showed relative inhibition rates of 22.2% and 11.1% against *Rhizoctonia solani* and *Pythiummultimum*, respectively (Wang et al., 2011). Therefore, as a novel *Streptomyces* strain, *S. griseoruber* Y1B might have potential applications for biocontrol in agricultural production.

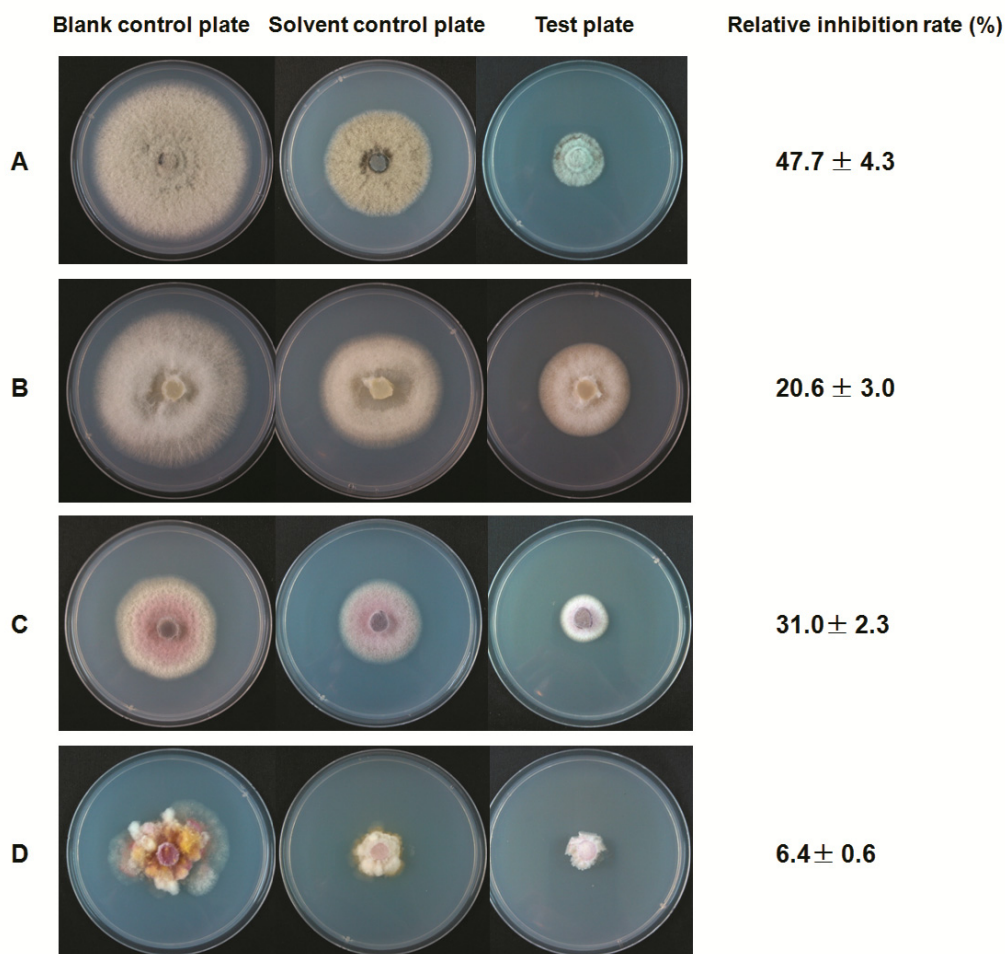


Figure 2. Inhibitory effect of the crude extract of *S. griseoruber* Y1B fermentation broth against different plant pathogens. (a) *Rhizoctonia solani*. (b) *Pythiummultimum*. (c) *Fusarium oxysporum*. (d) *Stevia rebaudiana* Bertoni spot blight. The data shown are the averages of triplicate experiments with standard deviations

3.5 Structure Elucidation of the Active Compound

The isolation scheme of compounds 1 and 2 is shown in Figure 3. The yield of dry crude extract from 25 L of fermentation broth was 2.6 g, and compounds 1 (3.8 mg) and 2 (5.2 mg) were collected from by preparative HPLC (Figures S1 and S2).

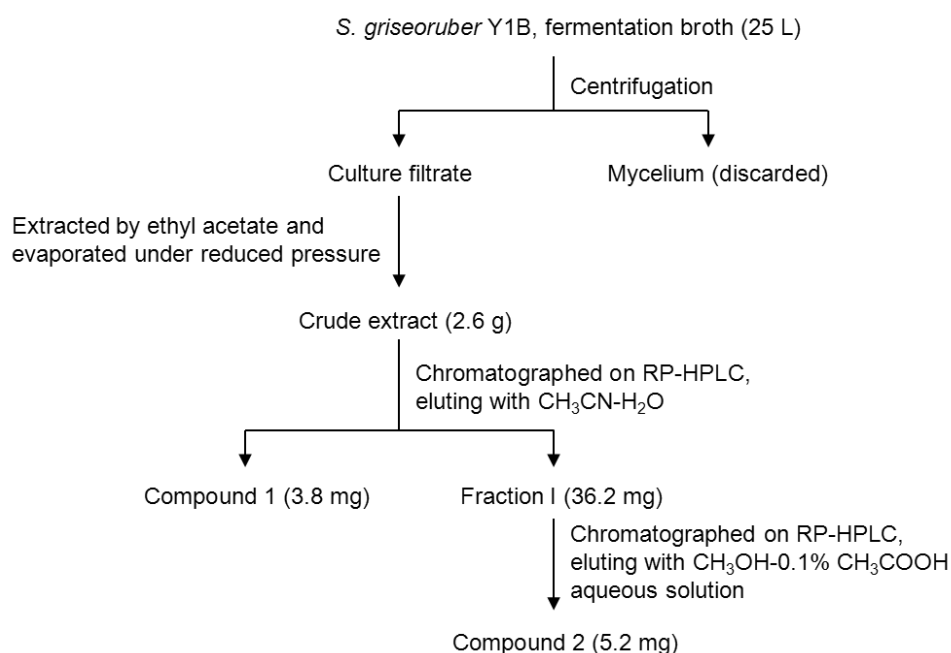


Figure 3. Separation and purification scheme of active compounds from *S. griseoruber* Y1B

Compound 1 was obtained as an orange, amorphous powder. High-resolution ESI-MS displayed a positive peak at m/z 197.0809 $[M + H]^+$, and three distinct peaks at 203 nm, 259 nm, and 367 nm were found in the UV absorption spectrum (Figure S3), indicating that compound 1 may have an aromatic ring structure. The 1H NMR spectrum of compound 1 showed chemical shifts of H protons at δ 7.19, 7.69, 7.79, 7.91, 8.18, and 8.31 (Figure S4). Based on the comparison of the molecular mass, UV absorption spectrum, and 1H NMR spectrum with published data (Mavrodi et al., 2001; Dharni, 2012), the structure of compound 1 was identified as 1-hydroxyphenazine (1-OH-PHZ, $C_{12}H_8N_2O$, MW = 196.20).

Compound 2 was obtained as a white, needle-like crystal. High-resolution ESI-MS exhibited a negative peak at m/z 121.0281 $[M - H]^-$, as shown in Figure S5, and the UPLC-MS-MS spectrum displayed a base peak at m/z 77.0396, which indicated the presence of a benzene ring (C_6H_6). The 1H NMR spectrum of compound 2 showed chemical shifts of H protons at δ 7.45, 7.58, and 8.01 (Figure S6). According to the molecular mass, UPLC-MS-MS, and 1H NMR spectrum, the structure of compound 2 was identified as benzoic acid (BA, $C_7H_6O_2$, MW = 122.12).

S. griseoruber has previously been reported as a kind of antibiotic-producing strain in the medical field. For example, *S. griseoruber* MTCC8121_CKM5, *S. griseoruber* 4620, and *S. griseoruber* 1618-306 can produce actinomycin D, rhodomycin, and anthracyclinone, respectively, which have showed antitumor activity in the clinical application (Podojil et al., 1980; Příkrylová et al., 1980; Bakina et al., 1989; Praveen & Tripathi, 2009). In this study, *S. griseoruber* Y1B was shown to produce 1-OH-PHZ, which is the first description of the production of a phenazine compound by *S. griseoruber* in the agricultural field.

3.6 Effects of Intermediate Metabolites on Phenazine Compound Production

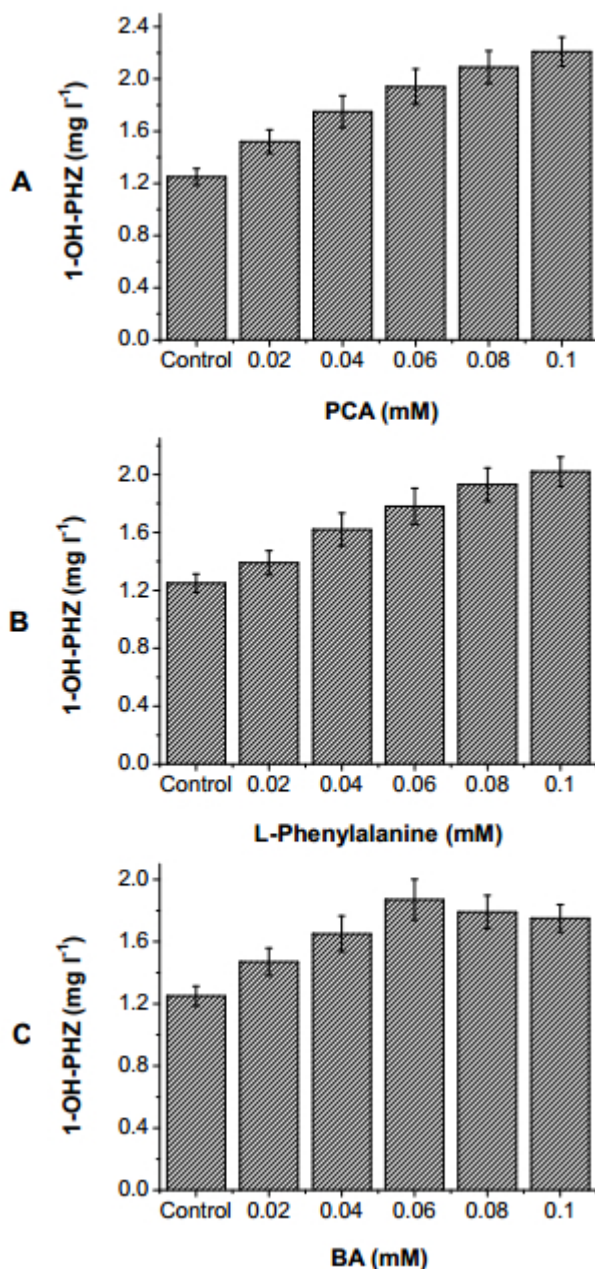


Figure 4. Production of 1-OH-PHZ with the addition of PCA (a), L-phenylalanine (b), and BA (c) in 250 ml shake flasks containing 60 ml of GYM medium. Results are averages of triplicate experiments and error bars indicate standard deviations of the means

The effects of intermediate metabolites on 1-OH-PHZ production are shown in Figure 4. The production of 1-OH-PHZ increased from 1.25 mg l⁻¹ to 2.21 mg l⁻¹ with the addition of 0.1 mM PCA. In the phenazine biosynthesis pathway, the biosynthesis of natural phenazine derivatives is the consequence of different modifications of a limited number of phenazine precursors, a biosynthesis mechanism that is conserved in all bacterial phenazine producers. These phenazine precursors might be PCA and phenazine-1,6-dicarboxylic acid (PDC), and

the gene product of *phzS* is involved in the conversion of PCA into 1-OH-PHZ (Mentel et al., 2009; Pierson III & Pierson, 2010). Therefore, the addition of PCA contributes to the production of 1-OH-PHZ.

In general, addition of L-phenylalanine and BA increased the production of 1-OH-PHZ to some extent. L-phenylalanine is an important intermediate metabolite in the shikimate pathway, and L-phenylalanine can be degraded into BA (Moerkercke et al., 2009). The addition of L-phenylalanine and BA might inhibit L-phenylalanine metabolism, and promote an increase of branched metabolic flux in the shikimate pathway, including the phenazine biosynthesis pathway. At the same time, BA itself could have a toxic effect on the microbial cells and inhibit the growth of *S. griseoruber* Y1B, which might lead to the decline in production of 1-OH-PHZ with the addition of BA at 0.08 mM and 0.1 mM.

Consequently, it can be inferred that the phenazine biosynthesis pathway in *S. griseoruber* Y1B might branch off from the shikimate pathway, and that PCA and L-phenylalanine might be biosynthesized as the precursors of 1-OH-PHZ and BA, respectively. The proposed phenazine biosynthesis pathway in *S. griseoruber* Y1B is shown in Figure 5.

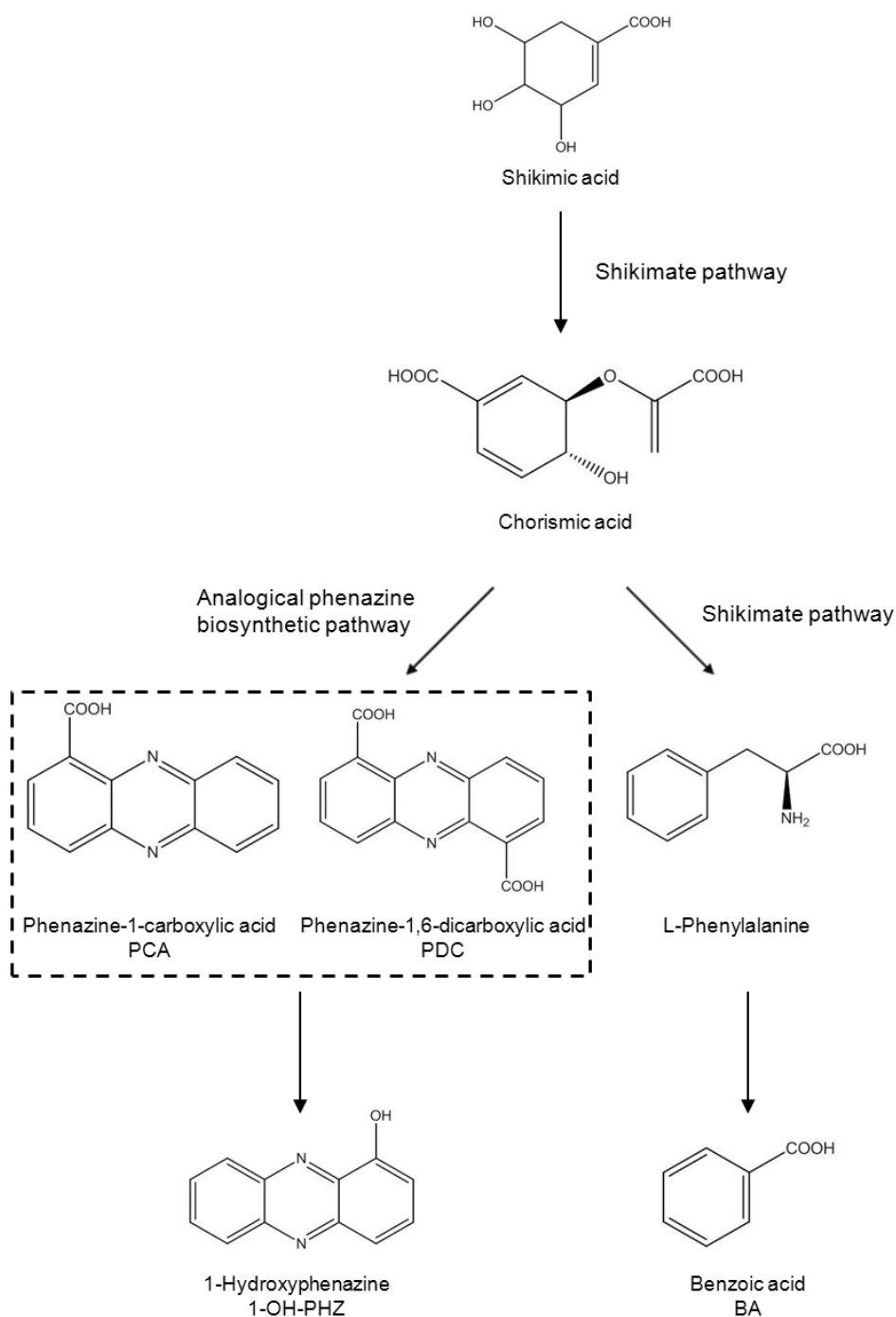


Figure 5. Schematic illustration of the proposed phenazine biosynthesis pathway in *S. griseoruber* Y1B

4. Conclusion

A novel phenazine-producing strain named Y1B was isolated from soil in this study. Based on phenotypic characteristics and 16S rRNA gene sequence analysis, strain Y1B was identified as *S. griseoruber*. In addition, we analyzed the fatty acid composition of *S.*

griseoruber Y1B to provide the first fatty acid profiles of a *S. griseoruber* strain. *phzE* and *phzF* were selected as phenazine biosynthetic gene markers to detect the phenazine-producing potential of *S. griseoruber* Y1B. *phzE* and *phzF* gene fragments were amplified by PCR, and showed 70-80% similarity to those of phenazine-producing *Pseudomonas* and *Streptomyces* species. The crude extract obtained from *S. griseoruber* Y1B fermentation broth showed significant activity against *Rhizoctonia solani*, *Pythiumultimum* and *Fusarium oxysporum*, with relative inhibition rates of 47.7%, 20.6% and 31.0%, respectively. These results indicated that *S. griseoruber* Y1B contained phenazine biosynthetic genes and has a broad spectrum of antifungal activity.

S. griseoruber Y1B could produce 1-OH-PHZ and BA, and might use PCA as the phenazine precursor to biosynthesize phenazine compounds, which is the first description of the production of a phenazine compound by *S. griseoruber*. The discovery of *S. griseoruber* Y1B offers a new source for the isolation of phenazine-producing strains in the agricultural field. As a new *Streptomyces* strain, *S. griseoruber* Y1B may play an important role in biocontrol in the future.

Acknowledgments

Financial supports from the 863 Programs of China (Grant No. 2012AA022107) and the National Natural Science Foundation of China (NSFC project No. 20706037) are gratefully acknowledged.

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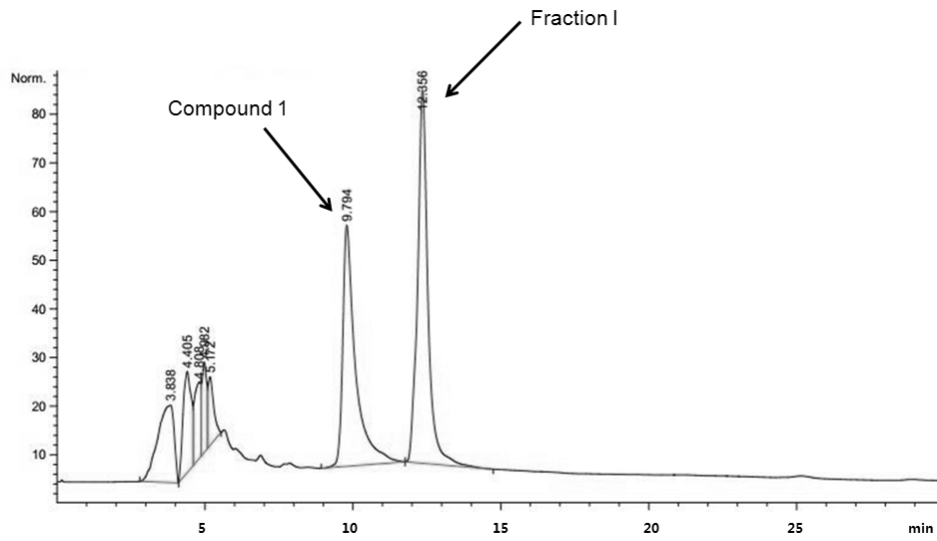


Figure S1. HPLC analysis of dry crude extract from *S. griseoruber* Y1B fermentation broth. Detection wavelength: 254 nm

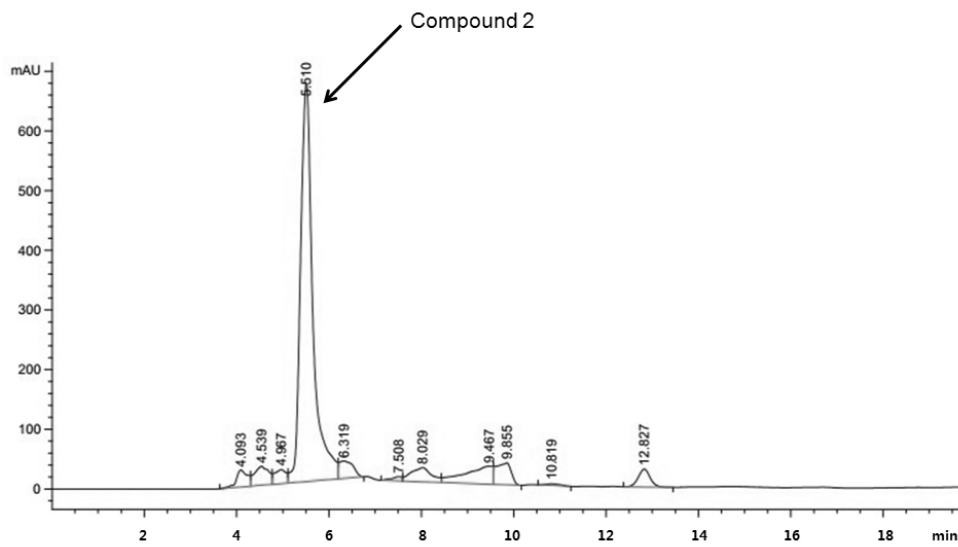


Figure S2. HPLC analysis of fraction I. Detection wavelength: 254 nm

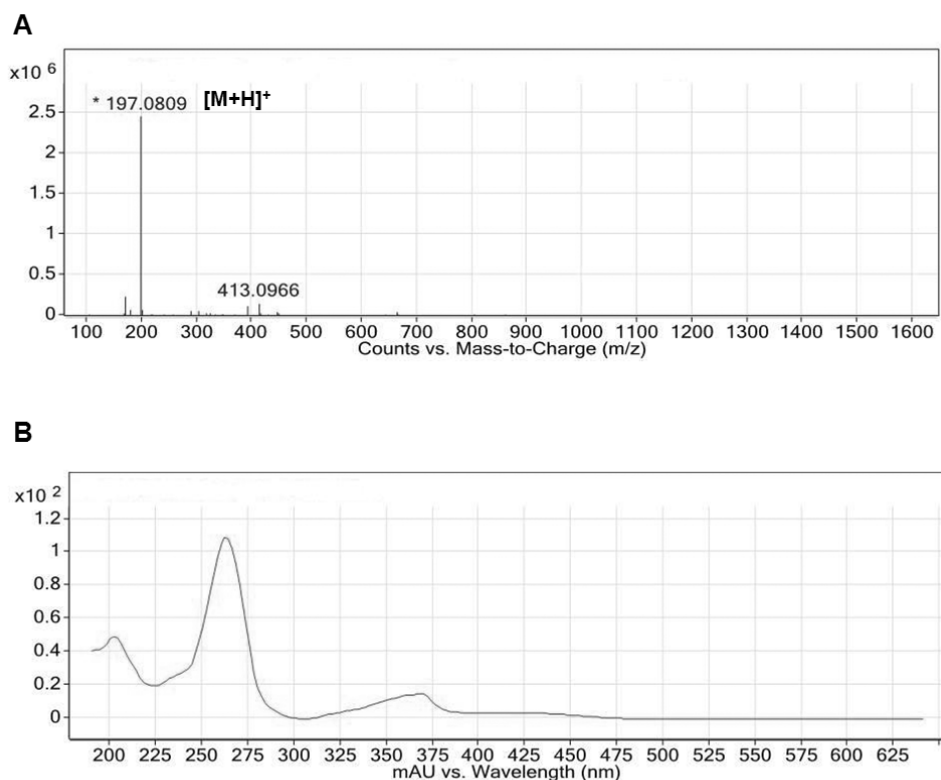


Figure S3. ESI-MS spectrum (a) and UV absorption spectrum (b) of compound 1

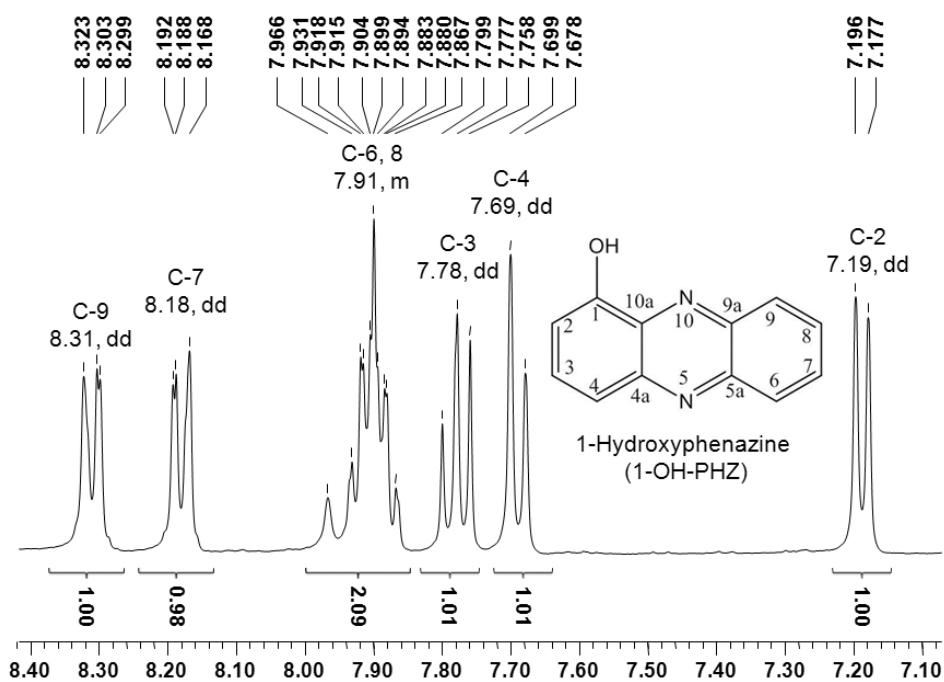


Figure S4. ¹H NMR data of compound 1 in CD₃OD

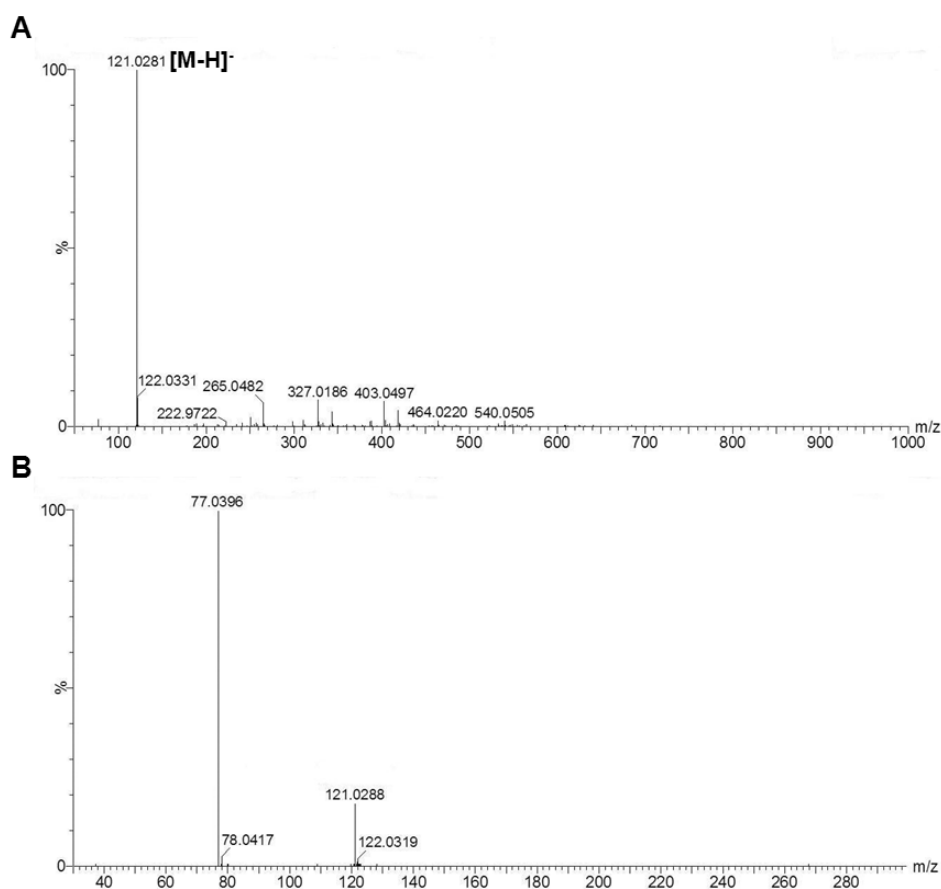


Figure S5. ESI-MS spectrum (a) and UPLC-MS-MS spectrum (b) of compound 2

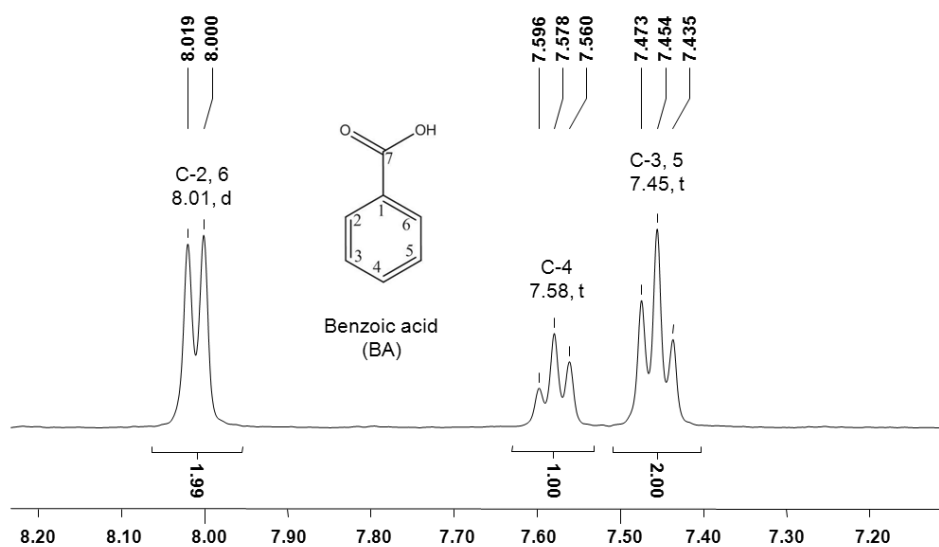


Figure S6. ¹H NMR data of compound 2 in CD₃OD