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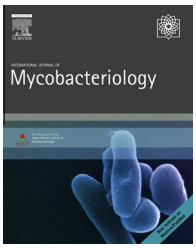

Anti-mycobacterial activity of polyketides from *Penicillium* sp. endophyte isolated from *Garcinia nobilis* against *Mycobacterium smegmatis*

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ABSTRACT

Objective/background: According to estimates by the World Health Organization, there were 9.6 million new tuberculosis (TB) cases in 2014: 5.4 million among men, 3.2 million among women, and 1.0 million among children. There were also 1.5 million TB deaths. Although there are potent anti-TB molecules, the misuse of these drugs in addition to inconsistent or partial treatment have led to the development of multidrug-resistant TB and extensively drug-resistant TB. It is established that plants harbor microorganisms, collectively known as endophytes, which also produce metabolites. Exploring the as-yet untapped natural products from the endophytes increases the chances of finding novel and active compounds. The present study was aimed to investigate the antimycobacterial activity of the crude extract and compounds isolated from *Penicillium* sp. endophyte associated with *Garcinia nobilis* against *Mycobacterium smegmatis*.

Methods: Liquid culture obtained from the fermentation of *Penicillium* sp. was extracted using ethylacetate and the liquid chromatography–mass spectrometry monitored fractionation of crude extracts yielded six compounds. Their structures were elucidated with spectroscopic analyses including two-dimensional nuclear magnetic resonance, high resolution mass spectrometry by dereplication using Antibase, and by comparison to literature data. All compounds and the crude extract from the liquid medium were evaluated for their antimycobacterial activity against *M. smegmatis*.

Results: In this study, the activity of penialidins A–C (1–3), citromycetin (4), *p*-hydroxy phenyl glyoxalaldoxime (5), and Brefeldin A (6) were tested against nonpathogenic *M. smegmatis*. Penialidin C was the most active compound with a minimum inhibitory concentration of 15.6 μg/mL.

Conclusion: Isolated compounds from *Penicillium* sp. harbored in *G. nobilis* exhibited promising antimycobacterial activity against *M. smegmatis* thus supporting the immensity of the

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potential of antimycobacterial drug discovery from endophytes from medicinal plants. Penialidin C could further be investigated for antimycobacterial drug development.

Introduction

Tuberculosis (TB) is currently a major public health problem due to the advent of multidrug-resistant (MDR) forms of bacilli as well as human immunodeficiency virus epidemics. Global efforts are underway to eradicate TB using new drugs with new modes of action, higher activity, and fewer side effects. For this reason, unexplored new sources and previously explored sources were examined and around 353 antimycobacterial compounds [1] have been previously reported. To develop drugs from these new sources, additional work is required for preclinical and clinical evaluation. In 2014, there were an estimated 9.6 million new TB cases: 5.4 million among men, 3.2 million among women, and 1.0 million among children. Also, there were 1.5 million TB deaths [2,3]. A potent antitubercular drug rifampicin (RMP), which was introduced 50 years ago, is still currently being used for treatment in combination with isoniazid (INH), ethambutol (EMB), and pyrazinamide (PZA) as a multidrug regimen for a period of at least 6 months. The misuse of these drugs in addition to inconsistent or partial treatment has led to the development of MDR-TB and extensively drug-resistant TB. The resistant strains coupled with drug hepatotoxicity and lengthy therapy paved the way for a TB therapeutic crisis [4–7]. This situation presently acts as a serious challenge to the health care system. Thus, it becomes necessary to prioritize the search for new antimycobacterial agents worldwide [8,9]. As part of our research activities in discovering potent anti-TB compounds [10] and based on our previous investigation on the antibacterial activity of some compounds [11], we further our investigation with the determination of the activities of metabolites produced by endophytes on mycobacteria, subject of this report.

Materials and methods

General experimental procedures

The high resolution mass spectra were obtained with an LTQ-Orbitrap Spectrometer (Thermo Fisher, Waltham, MA, USA) equipped with a HESI-II source. The spectrometer was operated in positive mode (1 spectrum/s; mass range: 100–1000) with nominal mass resolving power of 60,000 at *m*/z 400 with a scan rate of 1 Hz) with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal standard, bis (2-ethylhexyl) phthalate: *m*/z = 391.28428. The spectrometer was attached with an Agilent (Santa Clara, CA, USA) 1200 High Performance Liquid Chromatography (HPLC) system consisting of a LC-pump, photodiode array detector (λ = 260 nm), auto sampler (injection volume 5 μL), and column oven (30 °C). The following parameters were used for experiments: spray voltage 5 kV, capillary temperature 260 °C, and tube lens 70 V. Nitrogen was used as a sheath gas (50 arbitrary units) and auxiliary gas (5 arbitrary units). Helium served as the collision gas. The high resolution mass spectra of compounds was performed on a preparative HPLC using a Nucleodur C18 gravity column (50 mm × 2 mm, 1.8-μm particle size) with a H_2O (+0.1% HCOOH; A)/acetonitrile (+0.1% HCOOH; B) gradient (flow rate 350 μL/min). Samples were analyzed using a gradient program as follows: 95% A isocratic for 10 min, linear gradient to 100% B over 14 min, after 100% B isocratic for 4 min, the system returned to its initial condition (95% A) within 0.5 min, and was equilibrated for 4.5 min (flow rate 350 μL/min). The purification was carried out using a preparative HPLC run for 20 min on a Gilson apparatus with UV detection at 220 nm using a Nucleodur C18 Isis column (Macherey–Nagel, Düren, Germany), 5 μm (250 mm × 16 mm) with a H_2O (A)/CH_3OH (B) gradient (flow rate 4 mL/min). Samples were separated using a gradient program as follows: 60% A isocratic for 2 min, linear gradient to 100% B over 18 min, after 100% B isocratic for 5 min, the system returned to its initial condition (60% A) within 0.5 min, and was equilibrated for 4.5 min. Silica gel (Merck, Kenilworth, NJ, USA [0.063–0.200 mm]) was used for column chromatography. The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX-500 MHz, Bruker BioSpin, 34 RUE DE L INDUSTRIE. 67160, Wissembourg, France, spectrometer.

Sampling of Garcinia nobilis

Leaves of *G. nobilis* were collected in Mount Etinde in the Southwest Region of Cameroon. The plant material was identified by Mr. Victor Nana, botanist at the Cameroon National Herbarium (Yaoundé, Cameroon) under a voucher specimen (50779/HNC/Cam/Mt Zamangoué).

Isolation and identification of the endophytic fungus

The healthy leaf was first cleaned by washing it several times under running tap water and then cut into small slices, followed by successive surface sterilization in 70% ethanol and NaOCl (6–14% active chlorine) for 2 min and finally with sterile distilled water for two to three times. Plant material was then dried in between the folds of sterile filter papers and deposited on a petri dish containing potato dextrose agar medium (20 g of potato, 20 g of dextrose, and 15 g of agar in 1 L of H_2O [supplemented with 100 mg/L of chloramphenicol to suppress bacterial growth]) and incubated at 25 °C until the growth of endophytic fungi was discerned. Hyphal tips originating from plant segments were transferred to potato dextrose agar. Each fungal isolate was checked for purity and transferred to the new medium using the hyphal tip method. One of the isolates, CAM64, was selected for further

studies based on its morphotype and the liquid chromatography–mass spectrometry profile of its crude extract from small scale fermentation. The fungus was identified as *Penicillium* sp., as previously described [11].

Fermentation, extraction, and isolation of secondary metabolites

Fermentation and extraction from potato dextrose broth medium were described during our previous investigation of this fungus. Compounds tested herein, penialidins (A–C) and citromycetin (1–4; Fig. 1) were obtained as we previously reported for their isolation and characterization from potato dextrose broth medium of the investigated fungus [11].

Antimycobacterial assay

The activities were screened using the Alamar Blue method as previously described. A modified twofold serial dilution of extract/compound in 96-well microtiter plates was used to detect antimycobacterial activity [12,13]. Isolated compounds were serially diluted (100 μL) with albumin dextrose saline supplemented with Middlebrook 7H9 broth in the wells of a microtiter plate before mycobacterial culture (100 μL) was added to each well. The combined anti-TB drugs RMP 150 mg, INH 75 mg, EMB 275 mg, and pyrazinamide 400 mg was used as a positive control, and media controls were included. Various dilutions were tested in triplicate and the entire experiment was repeated.

The microplates were sealed with parafilm and placed in an environmental shaker; this was incubated at 37 °C for 48 h. Minimal inhibitory concentration (MIC) values were detected using a resazurin indicator at 0.2 mg/mL. The color reaction after the addition of resazurin generally occurred after a 2-h incubation period. MIC values were read as those concentrations where a pronounced change of color formation was noticed (from blue to orange).

Results

From the fermentation of this fungus, the crude extract (3.2 g) was first partitioned with cyclohexane to remove fatty material. The resulted polar fraction (1.8 g) was then subjected to column chromatography using Sephadex LH-20 to yield four main fractions (Fr1–Fr4) after thin layer chromatography monitoring. Fractions Fr2 (96 mg) and Fr3 (105 mg) were purified by preparative HPLC eluting with a gradient of MeOH–H_2-O+ 0.1%TFA to yield *p*-hydroxy-phenyl glyoxalaldoxime (5) $C_8H_8O_3N$ (4.6 mg, t_R = 6.73 min, HRESIMS at 166.05022 $[M+H]^+$: (calculated 166.05042), and Brefeldin A (6) $C_{16}H_{24}O_4$ (3.5 mg, t_R = 12.06 min, HRESIMS at 281.17957 $[M+H]^+$: (calculated 281.17528). Their structures (Fig. 1) were determined from their high resolution mass spectrometry combined to ^1H and ^{13}C NMR, by introducing these data into Antibase [14] and finally confirmed by two-dimensional NMR spectra. The chemical shifts were in agreement with those reported in the literature [15].

^1H NMR and ^{13}C NMR data of compounds 5 and 6

p-Hydroxy-phenyl glyoxalaldoxime (5): ^1H NMR (500 MHz, CD_3OD, δ [ppm], *J* [Hz]): δ 8.05 (1H, d, 8.8, H-2/2′), 6.87 (2H, m, H-3/3′), 8.04 (1H, s, H-6), and ^{13}C NMR (500 MHz, CD_3OD,δ [ppm]): δ 138.9 (C-1), 147.6 (C-2/2′), 115.1 (C-3/3′), 163.2 (C-4), 187.7 (C-5), 105.0 (C-6).

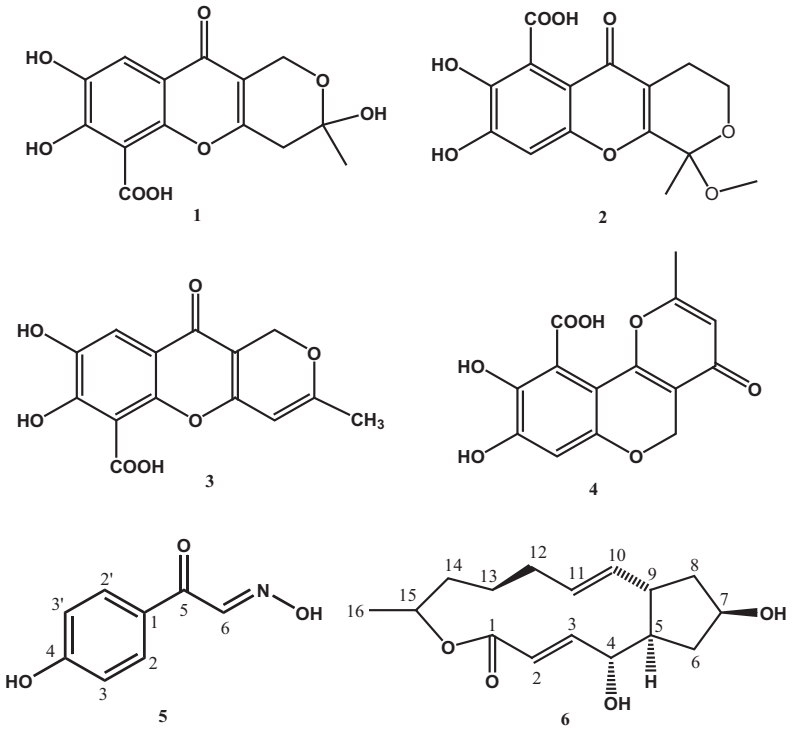

Fig. 1 – Structures of penialidin A–C (1–3), citromycetin (4), *p*-hydroxy-phenyl-glyoxalaldoxime (5), and Brefeldin A (6).

Table 1 – Antimycobacterial activity of compounds 1–6 compared with a standard reference.

Test samples	MIC (μg/mL)
Crude extract (liquid medium)	62.5
1. Penialidin A	62.5
2. Penialidin B	62.5
3. Penialidin C	15.6
4. Citromycetin	31.2
5. *p*-Hydroxy-phenyl-glyoxalaldoxime	62.5
6. Brefeldin A	250
RMP/INH/EMB/PZA	0.62

Note. All values were derived from experiments done in triplicates. EMB = ethambutol; INH = isoniazid; PZA = pyrazinamide; RMP = rifampicin.

Brefeldin A (6): ^{1}H NMR (500 MHz, CD_3OD, δ [ppm], *J* [Hz]): δ 7.47 (1H, dd, 15.6, 3.1, H-2), 5.85 (1H, dd, 15.6, 1.9, H-3), 4.06 (1H, dt, 4.6, 2.5, H-4), 1.86 (1H, m, H-5), 2.04 (1H, m, H-6a), 1.83 (1H, m, H-6b), 4.24 (1H, m, H-7), 2.16 (1H, m, H-8a), 1.47 (1H, m, H-8b), 2.40 (1H, m, H-9), 5.29 (1H, m, H-10), 5.76 (1H, m, H-11), 2.04 (1H, m, H-12a), 1.88 (1H, m, H-12b), 0.95 (2H, m, H-13), 1.79 (1H, m, H-14a), 1.61 (1H, m, H-14b), 4.82 (1H, d, 1.1, H-15), 1.27 (3H, d, 6.3, H-16), and ^{13}C NMR (500 MHz, CD_3OD,δ [ppm]): δ 167.3 (C-1), 154.1 (C-2), 116.8 (C-3), 75.2 (C-4), 52.1 (C-5), 40.5 (C-6), 71.9 (C-7), 43.1 (C-8), 44.0 (C-9), 137.0 (C-10), 130.9 (C-11), 31.9 (C-12), 10.5 (C-13), 34.0 (C-14), 72.1 (C-15), and 20.0 (C-16).

All compounds and the crude extract from the liquid medium were tested for their antimycobacterial activities and compared with the positive control. Results are provided in Table 1. Penialidin C was the most active compound with a MIC of 15.6 μg/mL, followed by citromycetin with a MIC of 31.2 μg/mL and RMP/INH/EMB/PZA used as a reference drug in the assay having a MIC value of 0.62 μg/mL.

Discussion

Our study showed that penialidin C, isolated from the endophyte *Penicillium* sp. harbored by *G. nobilis*, has antimycobacterial activity.

G. nobilis is found in the Southwest Region of Cameroon and is known to be a rich source of bioactive xanthones [16,17]. Similar to the small peptides, polyketides constitute a large proportion of industrial antibiotics. More than 300 novel antitubercular agents were identified and characterized from biological sources between 2003 and 2005, while there were a further 450 novel entities identified from 2006 to 2009. Furthermore, there have been 28 novel compounds isolated from microbial sources between 2008 and 2012. Of these, 11 were polyketides or polyketide derivatives [18].

WHO declared TB as global emergency because of the increase of TB cases with the advent of human immunodeficiency virus/AIDS infection and the emergence of MDR and extensively drug-resistant TB strains [19]. No new antimycobacterial has been introduced in the past 30 years; hence, there is an urgent need to develop novel, safe, effective, and affordable antitubercular agents. There has been a renewed interest in microbes as a source of novel bioactive compounds in the past decade. Thus, endophytes have been investigated for various pharmacological effects including antimycobacterial activity [20,21]. Medicinal plants are recognized as repository for fungal endophytes with metabolites containing novel molecular structures and biologically active compounds against various human pathogenic diseases for potential use in modern medicine [22]. A number of endophytic fungi from some *Garcinia* species have been found to produce new antitubercular secondary metabolites [23,24].

The observed activities of these compounds support the immensity of the potential of antimycobacterial drug discovery from endophytes from medicinal plants. Penialidin C was identified in our study as having antimycobacterial activity and could be further investigated for its development as an anti-TB drug.

Conflicts of interest

We declare that we have no conflicts of interest.

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