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Bacillus methylotrophicus ASWU-C2, a strain inhabiting hot desert soil, a new source for antibacterial bacillopyrone, pyrophen, and cyclopeptides

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Abstract: A strain of *Bacillus methylotrophicus* was isolated from a soil sample collected in Aswan eastern desert, which is known for its extremely arid climate. After fermentation of the strain in liquid culture and subsequent extraction, a bioassay-guided isolation procedure yielded five compounds: 2-benzyl-4*H*-pyran-4-one, named bacillopyrone (**1**), pyrophen (**2**), macrolactin A (**3**) and the cyclopeptides malformin A1 (**4**), and bacillopeptin A (**5**). The structures were determined by interpretation of nuclear magnetic resonance (NMR) spectroscopy and high resolution mass spectrometry (HR-MS) data. This is the first report on the isolation of compounds **1** and **2** from *Bacillus* species; compound 1 was reported previously as synthetic product. Bacillopyrone (**1**) exhibited moderate activity against the Gram-negative *Chromobacterium violaceum* with minimum inhibitory concentration 266.6 μg/mL, while macrolactin A (**3**) and malformin A1 (**4**) inhibited *Staphylococcus aureus* (minimum inhibitory concentrations 13.3 and 133.3 μg/mL, respectively).

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1 Introduction

Soil microorganisms inhabiting arid regions are exposed to prolonged tough environmental extremes, in which high temperatures with low moisture in arid deserts lead to enrichment of microbial communities, which can survive extreme variations in temperature and moisture [1]. Species of the genus *Bacillus* are Gram-positive bacteria that are especially predominant in soil. To date more than 850 natural products were obtained from *Bacillus*. They are well known as antibiotic producers with antagonistic activity against fungal and bacterial pathogens. *Bacillus methylotrophicus*, well-studied Gram-positive bacteria, has previously been identified as plant growth promoting [2]. Nevertheless, few reports describe the isolation and characterization of its secondary metabolites; they are mainly lipopeptides [3]. In the course of our studies to detect novel bioactive substances from the hot soil microorganisms [4], herein we report the isolation, structural elucidation, and antimicrobial activity of five natural products from *B. methylotrophicus* ASWU-C2.

Several chromatographic techniques were used to purify the methanol extract of the culture of *B. methylotrophicus*, which lead to isolation of compounds (**1–5**) (Figure 1).

Compound 1 was obtained as a white powder; its molecular formula was assigned as $C_{12}H_{10}O_2$ on the basis of high resolution mass spectrometry (HR-MS). Nuclear magnetic resonance (NMR) spectroscopy data revealed the presence of 9 protonated carbons (including one methylene) and three sp^2 carbons. A mono-substituted aromatic ring was determined by interpretation of its correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) data (see Supplementary Material, Fig. S1). Five of the remaining carbons were assigned to a C-6 substituted γ-pyrone ring on the basis of the characteristic chemical shifts and 2D NMR assignment. Finally, HMBCs from H-7 to C-5/C-9/C-13 showed that the two structure moieties were connected by a methylene bridge. Thus, the structure of

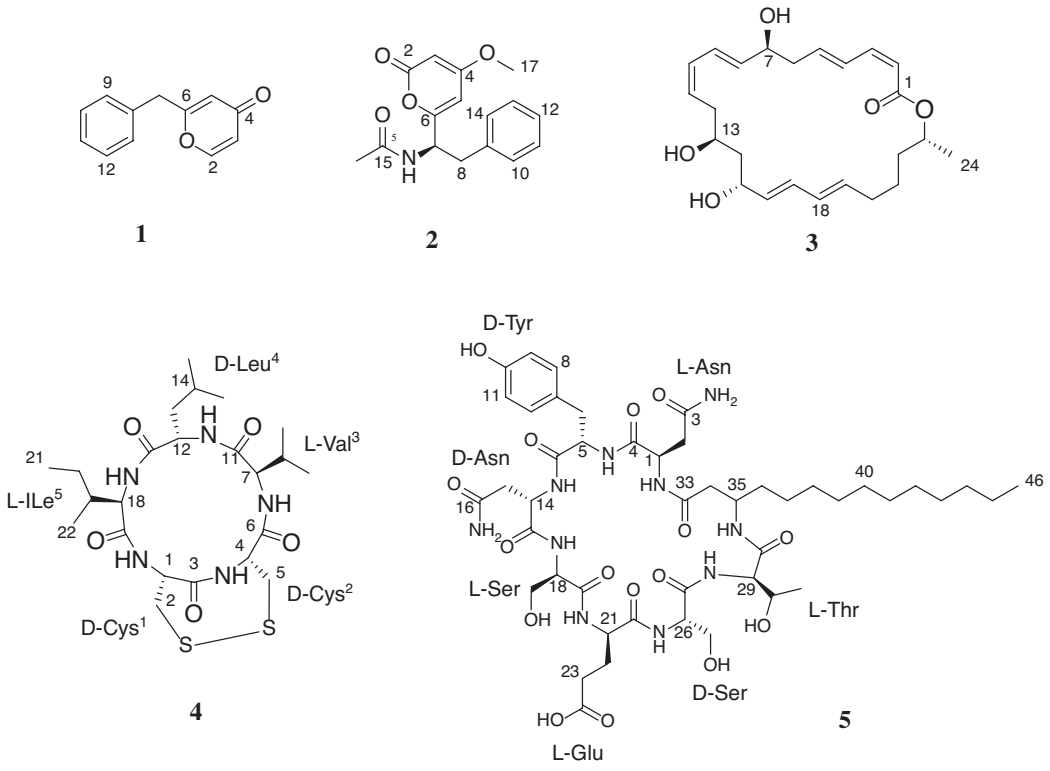

Figure 1: Structures of bacillopyrone (**1**), pyrophen (**2**), macrolactin A (**3**), malformin A1 (**4**), and bacillopeptin A (**5**).

1 was determined as 2-benzyl-4*H*-pyran-4-one and named bacillopyrone. 2-Benzyl-4*H*-pyran-4-one was previously reported as synthetic product [5] and was recently mentioned in a report written in Chinese as natural product from marine mangrove endophytic fungus [6]. It was not possible to get the Chinese report for comparison. Nevertheless, the NMR data of bacillopyrone (**1**) were in good agreement with those reported for similar compounds [7]. This is the first report on the isolation of bacillopyrone from *Bacillus*.

Compound **2** has a molecular formula of $C_{16}H_{17}NO_4$. A full structure proposed to the compound revealed that **2** is 4-methoxy-6-(1′-acetamido-2′-phenylethyl)-2*H*-pyran-2-one. The NMR data for **2** were in accordance with those reported for pyrophen, a 4-methoxy-2-pyrone derivative of L-phenylalanine, isolated previously from cultures of *Aspergillus niger* [8]. The optical rotation value for **2** $[\alpha]_D^{20}$ –12.6° ($c = 0.1$, in $CHCl_3$) indicated the same configuration for **2** as that reported for pyrophen ($[\alpha]_D^{20}$ –13.8°, $c = 0.1$, in $CHCl_3$) (Table S1). This is the first report on the isolation of pyrophen from *Bacillus*.

The molecular formula of compound **3** was determined as $C_{24}H_{34}O_5$. A series of COSY correlations from H-2 to H-24 supported by the HMBC correlations revealed the 24-carbon chain. Key HMBC correlation from H-24 to C-1 allowed the construction of a 24-membered lactone ring (see Supplementary Material, Fig. S1). The NMR data and the specific rotation value of **3** matched those reported in literature for macrolactin A, a C_{24} macrolide antibiotic [9].

In addition, two cyclopeptides, malformin A1 (**4**) and the lipopeptide bacillopeptin A (**5**), were isolated from the same extract. Their structures were assigned by comparison of their NMR spectral data, which were identical with those reported in the literature [10, 11].

α-Pyrone metabolites are highly abundant in bacteria, fungi, plants, and animals. They exhibit a wide range of biological activities [12, 13]. To date, the majority of secondary metabolites bearing 2-benzyl-4*H*-pyran-4-one skeletons have been reported from black aspergilli [7]. γ-Pyrone derivatives are distributed widely in a variety of natural and synthetic biologically active compounds [14]. Neither antimicrobial activities nor cytotoxicity was reported for pyrophen [15]. Furthermore, malformin A1 was found to be strongly cytotoxic against several human cancer cell lines [16]. Macrolactin A was reported to have antibacterial activity [17]. Bacillopeptin A was isolated from *Bacillus subtilis* as antifungal agent [11].

The minimum inhibitory concentrations (MICs) toward bacteria, yeasts, and filamentous fungi of the pure compounds **1–4** were recorded in a serial dilution assay. Bacillopyrone (**1**) exhibited a moderate inhibitory effect against the Gram-negative *Chromobacterium violaceum*

with MIC 266.6 μg/mL, while no activity against *Candida albicans*, *Micrococcus luteus*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa* was found. Macrolactin A (**3**) and malformin A1 (**4**) inhibited *S. aureus* (MICs 13.3 and 133.3 μg/mL, respectively) (Tables S2 and S3). Pyrophen (**2**) was not active against all the tested pathogens as previously reported, and bacillopeptin A (**5**) was not tested due to lack of material and because its activities have been previously evaluated.

1.1 General experimental procedure

Optical rotations were determined with a PerkinElmer 241 MC polarimeter (PerkinElmer, Rodgau, Germany). High performance liquid chromatography (HPLC)-diode array detection (DAD)/MS analysis was performed using an amaZon speed ETD ion trap mass spectrometer (Brucker Daltonics, Bremen, Germany), HR-electrospray ionization (ESI)-MS spectra were recorded on a maXis ESI TOF mass spectrometer (Brucker Daltonics, Bremen, Germany), and preparative HPLC purification was performed at room temperature on an Agilent 1100 series preparative HPLC system (Agilent Technologies, Waldbronn, Germany) as described in our previous report [18]. NMR spectra were recorded on Bruker 500 MHz Avance III spectrometer with a BBFO (plus) SmartProbe (^{1}H 500 MHz, ^{13}C 125 MHz) and a Bruker 700 MHz Avance III spectrometer (Brucker Daltonics, Bremen, Germany) with a 5-mm TCI cryoprobe (^{1}H 700 MHz, ^{13}C 175 MHz), locked to the deuterium signal of the solvent.

1.2 Strain collection from soil sample

The strain was isolated by using a standard suspension method [19]. About 1 g of a dried soil sample, collected from Aswan Eastern desert, was suspended in 100 mL sterile water. The suspension was agitated vigorously at room temperature, then 1 mL volume of the sample was applied onto plates and 20 mL of melted starch casein agar (SCA) medium was added to it. The SCA medium consists of soluble starch, 10.0 g; casein hydrolysate, 0.3 g; KNO_3, 2.0 g; NaCl, 2.0 g; K_2HPO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; agar, 18.0 g; and 1 L water (pH 7). The medium was sterilized at 121 °C for 15 min by autoclaving. The plates were incubated at 35 °C and monitored for 1–2 weeks. Thereafter, each different colony was transferred onto a new SCA medium plate and then was identified using molecular phylogenetic methods.

1.3 Taxonomy of the producing strain

The bacterial DNA was extracted by Takara DNA extraction kit (Takara Shuzo Co. Ltd, Japan). 16S rRNA genes were amplified with the universal primers, 27F (5′-CCTATCCC CTGTGTGCCTTGGCAGTCTCAG-3′) and 1525R (5′-AAGGAG-GTGWTCCARCC-3′) with some modification [20]. Each 50 μL polymerase chain reaction (PCR) contained 1 μL DNA extract, 1.5 μM $MgCl_2$, 0.2 μM of each dNTPs (Ecogen), 0.4 μM of each primer, and 1.5 U Taq DNA polymerase (Ecogen) with 1X PCR buffer. Amplification was performed in a thermal cycler TP600 (Takara Bio, Shiga, Japan) using the program: initial denaturation at 98 °C for 3 min followed by 30 cycles at 94 °C for 1 min, annealing at 52 °C for 1 min, and primer extension at 72 °C for 2 min followed by a final extension at 72 °C for 10 min. Sequencing reactions were performed employing the BigDye Terminator v. 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were determined using an ABI Prism 3100 genetic analyzer (Applied Biosystems). The sequence data were analyzed using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) at NCBI (National Center for Biotechnology Information, Bethesda, MD, USA). The strain was identified as *B. methylotrophicus* and coded (ASWU_C2). A slant culture was deposited at the Department of Botany-Section Microbiology, Aswan University, Aswan, Egypt.

1.4 Fermentation and isolation

A seed culture of *B. methylotrophicus* (ASWU-C2) was grown in 250 mL of SCA medium incubated at 35 °C for 4 days and used as inoculation for the scale-up fermentation of 4 L, for which the following medium was used: 10 g glucose, 20 g starch, 5 g yeast extract, 5 g casein peptone, 1 g $CaCO_3$, and 1 L water (pH 7.6). The strain was incubated at 35 °C for 10 days while vigorously shaken. The biomass was removed by filtration, and the culture filtrate was applied on an Amberlite XAD-16 column. The organic material was eluted from the XAD-16 adsorber resin using 2 L of methanol, and the eluate was concentrated in *vacuo* to give 5.35 g of the crude extract. The extract was then loaded on a Flash chromatography column (3.5×50 cm) using silica gel 60 as stationary phase. The separation was performed by a linear gradient using methylene chloride/methanol. All obtained fractions were assayed for their antibacterial activity against five bacterial strains (*Escherichia coli*, *Neisseria lactamica*, *B. subtilis*, and *S. aureus*). The active subfractions were further purified by silica gel column (2.5×60 cm). Methylene chloride:methanol

(9.5:0.5) was used as eluent. Fractions were collected every 20 min. Fraction ZA5 (28 mg), which contained compounds **1**, **2**, and **4**, was purified using preparative HPLC with a Kromasil RP C_{18} column (MZ-Analysentechnik, Mainz, Germany), water (A) and acetonitrile (B) as mobile phase and a gradient of 15%–70% B in 30 min, 70%–100% B for 5 min, 100% B isocratic for 5 min. Compound **1** (3 mg) was obtained at a retention time (t_R = 16.5 min), compound **2** (2 mg) was eluted at t_R = 15.5 min, and compound **4** (3 mg) at t_R = 23.3 min. Fraction ZA6 (62 mg) was further purified by the aforementioned HPLC gradient to yield compound **3** (3 mg) eluted at t_R = 7.8 min. Finally, the fraction that contained compound **5** (ZB21, 50 mg) was purified using the preparative HPLC with gradient of 40%–50% solvent B in 30 min, 50%–100% B for 5 min, 100% B isocratic for 5 min, and beside several impure fractions obtained, compound **5** (1.5 mg) was eluted at t_R = 10.6 min.

Bacillopyrone (**1**):
White powder; LCMS: *m/z* 187 $[M+H]^+$ (100); HR-ESI-MS: *m/z* 187.0747 $[M+H]^+$ (calcd for $C_{12}H_{11}O_2^+$, 187.0754); ^{1}H NMR (500 MHz, DMSO-d_6) 8.08 (d, *J* = 5.8 Hz, H-2), 6.22 (dd, *J* = 5.8, 2.6 Hz, H-3), 6.18 (d, *J* = 2.6 Hz, H-5), 3.89 (s, H_2-7), 7.30 (m, H-9 and H-13), 7.35 (m, H-10 and H-12), 7.29 (m, H-11); ^{13}C NMR (125 MHz, DMSO-d_6) 158.5 (CH, C-2), 115.9 (CH, C-3), 177.9 (C, C-4), 114.8 (CH, C-5), 168.1 (C, C-6), 38.7 (CH_2, C-7), 135.7 (C, C-8), 129.0 (2CH, C-9/C-13), 128.7 (2CH, C-10/C-12), 127.1 (CH, C-11).

1.5 Biological activities of the pure compounds

The biological activities of the pure compounds were conducted in accordance with literature descriptions; the MICs toward bacteria, yeast, and filamentous fungi were determined by a serial dilution technique using 96-well microtiter plates [21].

2 Conclusions

Five compounds including 2-benzyl-4*H*-pyran-4-one, named bacillopyrone, were isolated from hot soil inhabiting *B. methylotrophicus* ASWU-C2. This is the first report on the isolation of compounds (**1**) and (**2**) from *Bacillus*. Bacillopyrone (**1**) exhibited activity against the Gram-negative *C. violaceum*. Macrolactin A (**3**) and malformin A1 (**4**) inhibited *S. aureus*.

Supplementary Material: Physicochemical properties, HR-ESI-MS, NMR data for the compounds, and the antimicrobial results are available as supporting information.

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