Isolation and Identification of Metabolites from the Gram-negative Proteobacteria of
Burkholderia cenocepacia and Serratia marcescens
(Pengasingan dan Pengenalpastian Metabolit daripada Proteobakteria Gram-negatif
Burkholderia cenocepacia dan Serratia marcescens)

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ABSTRACT

Burkholderia cenocepacia and Serratia marcescens are Gram-negative proteobacteria commonly found in the natural environment and are also opportunistic pathogens that caused a number of human diseases. The fermentation culture of Burkholderia cenocepacia yielded three compounds, 4-(2-hydroxyethoxy)-phenol (1), Maculosin (2) and methyl myristate (3). Compound 2 was also isolated together with cyclo(L-Leu-L-Pro) (4) from Serratia marcescens. Compound 1 was isolated from a natural source for the first time and the first isolation of compounds 2-4 was also reported from both Burkholderia cenocepacia and Serratia marcescens.

Keywords: Burkholderia cenocepacia; Malaysia microorganism natural products; proteobacteria metabolites; Serratia marcescens

INTRODUCTION

The Burkholderia cenocepacia and Serratia marcescens are classified as Gram-negative proteobacteria. B. cenocepacia is commonly found as an endophytic bacterial species in the natural environment and is also a well-known opportunistic pathogen that causes cystic fibrosis in humans. Burkholderia sp. are known to be natural antagonists of fungal infections in agriculture due to their ability to produce antifungal compounds such as pyrrolnitrine and phenylacetic acid (El-Banna & Winkelmann 1998; Mao et al. 2006). S. marcescens is also an opportunistic pathogen known to cause nosocomial infection (de Boer et al. 2008; Hejazi & Falkiner 1997; Su et al. 2003) and to produce the characteristic red pigment prodigiosin and other bioactive metabolites such as althiomycin and marcescin (Fuller & Horton 1950). This bacterial species also produced biosurfactants such as serratomolide and serrawettin which were desirable in the petroleum industry, oil recovery and bioremediation (Matsuyama et al. 2011). S. marcescens was capable of metabolizing a wide range of substrates such as vanillin (Perestelo et al. 1989) glucose (Bouvet et al. 1989) the explosive 2,4,6-trinitrotoluene (Montpas et al. 1997; Araujo et al. 2010) and pentachlorophenol, an ingredient used in pesticides, fungicides and herbicides (Singh et al. 2007). We have previously reported the metabolites isolated from the proteobacteria Enterobacter cloacae (Yap et al. 2015a, 2015b) and in continuation of our interest, we report here the metabolites isolated from two more Malaysian proteobacteria, B. cenocepacia and S. marcescens.

MATERIALS AND METHODS

GENERAL EXPERIMENTAL PROCEDURES

NMR spectra data were obtained from 600 MHz Bruker AVANCE III (Bruker, Fällanden, Switzerland) NMR spectrometers with chemical shifts expressed in ppm and TMS as an internal standard in CDCl₃. HRESIMS data were obtained from the Agilent 6530 Q-TOF (Agilent Technologies, Santa Clara, CA, USA) mass-spectrometer equipped with the Agilent 1200 series Rapid Resolution LC system. The UV data were recorded using Varian Cary Eclipse Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) using quarts cell. IR was carried out on the Perkin-Elmer RXI FT-IR (Perkin Elmer, Waltham, MA,
USA) using NaCl cell. Optical rotation was measured on the Jasco P-1020 digital polarimeter (Jasco, Tokyo, Japan).

BACTERIAL SOURCE

The B. cenocepacia strain pp9q (GenBank accession number FJ870663.1) and S. marcescens strain MH6 (GenBank accession number: FJ853424.1) were isolated from the soil of Rimba Ilmu, University of Malaya, Kuala Lumpur, Malaysia.

FERMENTATION AND EXTRACTION

The bacteria were cultured on Luria Bertani (LB) agar plate. A seed culture of 100 mL LB broth buffered with 10 mM of 3-morpholinopropane-1-sulfonic acid (MOPS) was prepared by inoculation of a single bacterial colony from the LB plate. The seed culture was incubated at 28°C in a shaking incubator for a day. Then, a large scale fermentation was carried out by inoculating 10 mL of seed culture (OD

whereas the precipitated cells were lysed and extracted from the fermentation of S. marcescens yielded 189.4 mg and were purified by CC with a gradient solvent system of chloroform to chloroform-methanol (4:1) which resulted in three fractions (A, B and C). Further purification on fraction B by using CC with gradient solvent system of hexane-chloroform (1:1) to chloroform yielded fraction B-1 that contained compound 4 and fraction B-2 which was then purified with 5% of methanol in chloroform by CFC to give compound 2. The yields of the compounds were as follow: 1 (2.0 mg), 2 (11.7 mg from B. cenocepacia and 8.0 mg from S. marcescens) 3 (1.9 mg) and 4 (2.5 mg) (Figure 1).

4-(2-HYDROXYETHOXY)-PHENOL (1)

Yellowish oil; RF (CHCl3/MEOH 95:5) 0.62; UV (EtOH) λ

NMR (CDCl3, 150 MHz) δ 144.3 (C, C-1 and C-4), 120.9 (CH, C-3 and C-5), 115.4 (CH, C-2 and C-6), 72.5 (CH2, C-8), 62.0 (CH2, C-7); HMBC: 1J C-1 to H-2; C-1 to H-6; C-2 to H-3; C-3 to H-2; C-4 to H-3; C-4 to H-5; C-5 to H-6; C-6 to H-5; 1J C-1 to H-3; C-1 to H-5; C-4 to H-2; C-4 to H-6; HRESIMS with m/z 109.0295 [M - C6H12O2]+ (calcd for C6H10O3 - C2H4O2, m/z 109.0295).

MACULOSIN (2)

Light yellowish oil; RF (CHCl3/MEOH 98:2) 0.35; [α]D

-43.1 (c 0.14, EtOH); UV (EtOH) λ

IR (NaCl) ν

3248, 2927, 2853, 1747, 1658, 1449, 1252, 1174, 1114, 1017, 858 cm-1; 1H NMR (CDCl3, 600 MHz) δ 7.05 (2H, d, J = 8.3 Hz, H-2' and H-6'), 6.77 (2H, d, J = 8.3 Hz, H-3' and H-5'), 5.69 (1H, s, H-1), 4.19 (1H, dd, J = 3.5 and 10.5 Hz, H-9), 4.06 (1H, t, J = 8.3 Hz, H-6), 3.62 (1H, dt, J = 7.9 and 12.0 Hz, H-3a), 3.54 (1H, ddd, J = 3.0, 9.0 and 12.0 Hz, H-3b), 3.48 (1H, dd, J = 3.5 and 14.5 Hz, H-10a), 2.73 (1H, dd, J = 10.5 and 14.5 Hz, H-10b) 2.32 (1H, m, H-5a), 2.00 (1H, m, H-4a),
1.99 (1H, m, H-5b), 1.88 (1H, m, H-4b). $^1$H NMR (CDCl$_3$, 150 MHz) $\delta$ 169.8 (C, C-7), 165.4 (C, C-1), 155.5 (C, C-4'), 130.6 (CH, C-2' and C-6'), 127.7 (C, C-1'), 116.4 (CH, C-3' and C-5'), 59.4 (CH, C-6), 56.4 (CH, C-9), 45.7 (CH$_2$, C-3), 36.0 (CH$_2$, C-10), 28.6 (CH$_3$, C-5), and 22.7 (CH$_3$, C-4). 

METHYL MYRISTATE (3)

Light yellowish oil; Rf (Hexane) 0.61; UV (EtOH) $\lambda$$_{max}$ (log e) 222 (2.42) nm; IR (NaCl) $\nu$$_{max}$ 2925, 2854, 1716, 1541, 1270, 1158, 1109, and 1028 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 600MHz) $\delta$ 3.64 (3H, s, -OCH$_3$); 1.97 (10H, s, CH$_3$-13), 14.4 (CH, C-14).

CYCLO (L-LEU-L-PRO) (4)

Light yellowish oil; Rf (CHCI$_3$/Hexane 1:1) 0.59; [α]$_D$ +28.1 ($\epsilon$ 0.032, EtOH); UV (EtOH) $\lambda$$_{max}$ nm (log e) 212 (2.90) nm; IR (NaCl) $\nu$$_{max}$ 3222, 2958, 2930, 2872, 1686, 1676, 1426, 1302, 1275, 1235, 1157, 1102, 1032, 996 and 919 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 600MHz) $\delta$ 5.85 (1H, s, NH-8), 4.10 (1H, t, $J$ = 8.3 Hz, H-6), 4.00 (1H, dd, $J$ = 4.0, 9.5 Hz, H-9), 3.57 (1H, m, H-3b), 3.53 (1H, m, H-3a), 2.33 (1H, m, H-5b), 2.11 (1H, m, H-5a), 2.05 (1H, m, H-10b), 2.00 (1H, m, H-4b), 1.88 (1H, m, H-4a), 1.71 (1H, m, H-11), 1.50 (1H, dd, $J$ = 4.0, 9.5 and 14.7 Hz, H-2a), 0.98 (3H, d, $J$ = 6.8 Hz, H-12), 0.94 (3H, d, $J$ = 6.8 Hz, H-13); $^1$C NMR (CDCl$_3$, 150 MHz) $\delta$ 170.0 (C, C-1), 166.2 (C, C-7), 59.0 (CH, C-6), 53.4 (CH, C-9), 45.5 (CH$_2$, C-3), 38.6 (CH$_2$, C-10), 28.1 (CH$_3$, C-5), 24.7 (CH, C-11), 23.3 (CH$_3$, C-12), 22.8 (CH$_2$, C-4), 21.2 (CH$_2$, C-13); HRESIMS m/z 211.1433 [M + H]$^+$ (calcd for C$_{16}$H$_{14}$O$_3$ + H, 211.1441).

RESULTS AND DISCUSSION

Both B. cenocepa and S. marcescens were extracted from soil of the Rimba Ilmu, University of Malaya, Kuala Lumpur, Malaysia and were subjected to chemical constituent analysis. 4-(2-Hydroxyethoxy)-phenol (1) was isolated from B. cenocepa in addition to two other known compounds, maculosin (2) and methyl myristate (3). Maculosin (2) was also isolated together with cyclo(L-Leu-L-Pro) (4) from S. marcescens through extensive chromatographic purifications. The structures of compounds 1 - 4 were characterized using NMR, HRESIMS, IR and UV spectroscopy.

4-(2-Hydroxyethoxy)-phenol (1) is a phenolic compound isolated for the first time from a natural sources. It was obtained as yellowish oil and the HRESIMS showed m/z 109.0295 [M - C$_5$H$_9$OH] consistent with the molecular formula C$_8$H$_7$O$_3$ and 4 degrees of unsaturation.

The IR spectrum showed bands attributed to the hydroxyl (3364 cm$^{-1}$) and ether (2868 cm$^{-1}$) functional groups. The $^1$H NMR spectrum of 1 indicated the presence of four aromatic methines and two methylenes protons, while the $^1$C NMR spectrum exhibited three overlapped aromatic carbon signals (two methines and a quaternary carbon) and two methylenes carbon in agreement with the molecular formula. The observation of the three overlapped aromatic carbons in the $^1$C NMR spectrum suggested the presence of a symmetric 1,4-disubstituted aromatic ring. The COSY spectrum of compound 1 exhibited three partial structures, i.e. two CH=CH moieties and CH$_2$-CH$_2$, corresponding to C(2)=C(3), C(5)=C(6) and O-C(7)-C(8)-O, respectively. The $^1$C NMR signals carbon-7 and carbon-8 ($\delta_c$ 62.0 and 72.5, respectively) suggested that carbon-8 is attached to a terminal hydroxyl group, i.e. O-C(7)-C(8)-OH. The HMBC spectrum (Figure 2) showed $J$ correlations from C(1) to H(4) and H(8) and C(3) to H(5) and H(7). The other HMBC results were also consistent with the structure and identity of compound 1 as 4-(2-Hydroxyethoxy)-phenol. This is the first report of isolation of 4-(2-hydroxyethoxy)-phenol from a natural sources to the best of our knowledge.
the methine protons is syn configuration, indicating the S configuration for C(6). The experimental NMR data of Maculosin (2) was in good agreement with those reported in literature (Stierle et al. 1989). Maculosin (2) which has the cyclo(L-Pro-L-Tyr) structure is a known signalling molecule for communication between bacterial species was isolated for the first time from both B. cenocepacia and S. marcescens (Holden et al. 1999; Stierle et al. 1989). In addition to quorum sensing, maculosin (2) also possesses host-specific phytotoxicity activity against spotted knapweed, antifungal and antibacterial properties (Cimmino et al. 2014; Stierle et al. 1989).

Compound 3 is a methyl myristate with the molecular formula C_{15}H_{32}O_2. Compound 3 showed UV absorption maximum at 206 nm and the IR spectrum showed absorptions for aliphatic (2925 and 2854 cm\(^{-1}\)) and carbonyl (1716 cm\(^{-1}\)). Methyl myristate (3) is a common aliphatic fatty acid frequently encountered in the natural resources. The experimental NMR data of compound 3 is in good agreement with those reported in literature (Read & Miller 1932).

![HMBC and NOESY correlations of compound 2](image)

Compound 4 was isolated as white powder and the HRESIMS showed a m/z 211.1433 [M+H]\(^{+}\) consistent with the molecular formula C\(_{12}\)H\(_{18}\)O\(_{2}\)N, and 4 degrees of unsaturation. The IR spectrum showed bands attributed to the amine (3222 cm\(^{-1}\)), aliphatic carbons (2958, 2930, 2872 cm\(^{-1}\)) and amide (1686, 1676 cm\(^{-1}\)) functional groups. The \(^1\)H NMR spectrum of compound 4 indicated the presence of three methines, four methylenes and two methyls and an NH signal (\(\delta_\text{H} 5.85\)), while the \(^13\)C NMR spectrum exhibited the presence of 11 carbon signals corresponding to two methyls, four methylenes, three methines and two quaternary carbons. The observation of the quaternary carbons at \(\delta_\text{C} 166.2\) and 170.0 suggested that these carbon signals were due to the carbonyl carbon of the amide groups. The COSY spectrum of compound 4 exhibited two partial structures, i.e. CH-CH\(_2\)-CH\(_2\)-CH\(_3\) and CH-CH\(_2\)-CH(CH\(_3\))CH\(_2\). The two partial structures were also consistent with the HMBC spectrum which showed \(^2\)J correlation from H(4) and H(5) to \(^2\)J correlation from H(5) to C(6); and \(^2\)J correlation from H(10) to C(1), hence suggesting the presence of partial structures N-C(7)-O-C(6)-C(5)-C(4)-C(3)-N and N-C(1)-O-C(9)-C(10)-C(11)-C(12)-C(13), respectively. Other key HMBC correlations were shown in Figure 4. The experimental NMR data of compound 4 was in good agreement with those reported in literature (Yan et al. 2004). Cyclo(L-Leu-L-Pro) (4) inhibits the productions of the toxic aflatoxin (Yan et al. 2004) and is reported for first time from S. marcescens.

![HMBC correlations of compound 4](image)

**CONCLUSION**

In the present report, 4-(2-Hydroxyethoxy)-phenol (1) was isolated and reported for the first time from a natural product source. Compound 1 was also being isolated with 2 other compounds, i.e. maculosin (2) and methyl myristate (4) from the fermentation culture of B. cenocepacia. In addition, maculosin (2) and cyclo(L-Leu-L-Pro) (4) was isolated and characterized for the first instance from the Gram-negative proteobacteria S. marcescens. All the compounds reported in the present study were not subjected to further bioassays testing due to the low amounts isolated insufficient for bioassays.

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