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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

ABSTRAK

Burkholderia cenocepacia *dan* Serratia marcescens *adalah proteobakteria Gram-negatif yang biasa dijumpai di alam semula jadi dan juga patogen yang boleh membawa penyakit kepada manusia. Tiga metabolit telah diasingkan daripada kultur fermentasi* Burkholderia cenocepacia*, iaitu 4-(2-hidroksietoksi)-fenol (**1**), Maculosin (**2**) dan metil miristat (**3**). Sebatian **2** juga diasingkan bersama siklo(L-Leu-L-Pro) (**4**) daripada* Serratia marcescens. *Sebatian **1** diasingkan untuk pertama kali daripada sumber hasil semula jadi dan pengasingan untuk sebatian **2**-**4** juga dilaporkan buat pertama kali daripada* Burkholderia cenocepacia *dan* Serratia marcescens.

Kata kunci: Burkholderia cenocepacia*; metabolit proteobakteria; sebatian semula jadi mikroorganisme Malaysia;* 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, Fallanden, Switzerland) NMR spectrometers with chemical shifts expressed in ppm and TMS as an internal standard in $CDCl_3$. 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 RX1 FT-IR (Perkin Elmer, Waltham, MA,

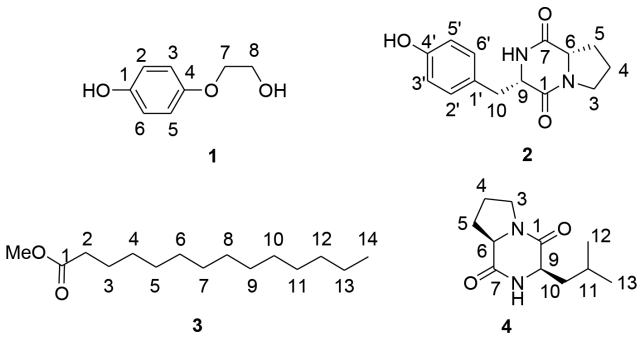

FIGURE 1. Compound **1 – 4**

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_{600} = 1) into 1 L of freshly prepared LB broth with 10 mM MOPS and subsequently incubated at 28°C in a shaking incubator for 2 days. The 1 L fermentation broth was then centrifuged (Sigma 3-16PK, SIGMA Laborzentrifugen GmbH, Osterode, Germany) at 8000 g to separate the bacterial cells from the broth. The supernatant was extracted with ethyl acetate whereas the precipitated cells were lysed and extracted with methanol.

ISOLATION

Column chromatography (CC) (Silica gel 60 0.040-0.063 mm, Merck, Darmstadt, Germany), thin layer chromatography (TLC) (Silica gel 60G PF_{254}, Merck, Darmstadt, Germany) and centrifugal chromatography (CFC) (Silica gel 60G PF_{254} containing gypsum, Merck, Darmstadt, Germany) were extensively used for the isolation and purification of compounds. A total of 64.8 mg from the ethyl acetate and methanol extracts were yielded from the fermentation of *B. cenocepacia*. The extract was purified by using CC with a solvent system chloroform-methanol (19:1) to give three fractions (D, E and F). Fraction D was purified by using CFC with the solvent chloroform to give compound **3**. Fraction E was purified by using CFC with a gradient solvent system chloroform to chloroform-methanol (1:4) to give compound **1**. Fraction F contained the pure compound **2**. All the purification by CC and CFC were guided by TLC. The combined ethyl acetate and methanol extracts 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 ($CHCl_3$/MeOH 95:5) 0.62; UV (EtOH) λ_{max} nm (log ε) 206 (2.42) and 279 (0.55) nm; IR (NaCl) ν_{max} 3364, 2931, 2868, 1684, 1520, 1395, 1372, 1274, 1254, 1056, 920, 856, and 749 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.83 (2H, dd, *J* = 3.4 and 6.0 Hz, H-2 and H-6), 6.74 (2H, dd, *J* = 3.4 and 6.0 Hz, H-3 and H-5), 3.74 (2H, t, *J* = 4.5 Hz, H-7), 3.59 (2H, t, *J* = 4.5 Hz, H-8); ^{13}C NMR ($CDCl_3$, 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 (CH_2, C-8), 62.0 (CH_2, C-7); HMBC: ^2J 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; ^3J 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 - C_2H_4OH]$^-$ (calcd for $C_8H_{10}O_3 - C_2H_4OH$, *m/z* 109.0295).

MACULOSIN (2)

Light yellowish oil; Rf ($CHCl_3$/MeOH 98:2) 0.35; $[\alpha]_D$ -43.1 (*c* 0.14, EtOH); UV (EtOH) λ_{max} (log ε) 229 (3.1) and 278 (0.7) nm; IR (NaCl) ν_{max} 3248, 2927, 2853, 1747, 1658, 1449, 1252, 1174, 1114, 1017, 858 cm^{-1}; ^{1}H NMR ($CDCl_3$, 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, NH-8), 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). ^{13}C NMR ($CDCl_3$, 150 MHz) δ 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_2, C-5), and 22.7 (CH_2, C-4); HRESIMS *m/z* 259.1098 $[M - H]^-$ (calcd for $C_{14}H_{16}N_2O_3$–H, 259.1088), and 519.2238 $[2M - H]^-$ (calcd for $2(C_{14}H_{16}N_2O_3)$ - H, 519.2249).

METHYL MYRISTATE (3)

Light yellowish oil; Rf (Hexane) 0.61; UV (EtOH) λ_{max} (log ε) 206 (2.42) nm; IR (NaCl) ν_{max} 2925, 2854, 1716, 1541, 1270, 1158, 1109, and 1028 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600MHz) δ 3.64 (3H, s, $-OCH_3$), 2.28 (2H, t, *J* = 7.3 Hz, H-2), 1.59 (2H, m, H-3), 1.29 (20H, m, H-(4-13)), 0.86 (3H, t, *J* = 7.3 Hz, H-14); ^{13}C NMR ($CDCl_3$, 150MHz) δ 174.4 (C, C-1), 51.7 (CH_3, C-1'), 34.3 (CH_2, C-2), 32.2 (CH_2, C-12), 28.9-30.4 (CH_2, C-(4-11)), 25.2 (CH_2, C-3), 22.9 (CH_2, C-13), 14.4 (CH_3, C-14).

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

Light yellowish oil; Rf ($CHCl_3$/Hexane 1:1) 0.59; $[\alpha]_D$ +28.1 (*c* 0.032, EtOH); UV (EtOH) λ_{max} nm (log ε) 212 (2.90) nm; IR (NaCl) ν_{max} 3222, 2958, 2930, 2872, 1686, 1676, 1426, 1302, 1275, 1235, 1157, 1102, 1032, 996 and 919 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 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, ddd, *J* = 4.0, 9.5 and 14.7 Hz, H-10a), 0.98 (3H, d, *J* = 6.8 Hz, H-12), 0.94 (3H, d, *J* = 6.8 Hz, H-13); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 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_2, C-5), 24.7 (CH, C-11), 23.3 (CH_3, C-12), 22.8 (CH_2, C-4), 21.2 (CH_3, C-13); HRESIMS *m/z* 211.1433 $[M + H]^+$ (calcd for $C_{11}H_{18}O_2N_2$ + H, 211.1441).

Results and Discussion

Both *B. cenocepacia* 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. cenocepacia* 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 $[M - C_2H_4OH]^-$ consistent with the molecular formula $C_8H_{10}O_3$ 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 ^{13}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 ^{13}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 ^{13}C NMR signals carbon-7 and carbon-8 (δ_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 ^3J 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.

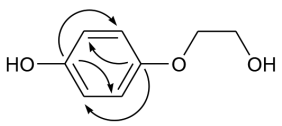

FIGURE 2. HMBC correlations of compound **1**

Compound **2** was obtained as white amorphous solid with a molecular formula $C_{14}H_{16}N_2O_3$ determined by HRESIMS with *m/z* 259.1098 $[M - H]^-$ and 519.2238 $[2M - H]^-$ with $[\alpha]_D$ -43.1 (*c* 0.14, EtOH). The UV spectrum showed absorption maximum at 229 and 278 nm. The IR spectrum showed absorptions band attributed to amine, hydroxyl and carbonyl functionalities. The ^{1}H NMR spectrum of compound **2** indicated the presence of six methines, four methylenes and an NH signals (δ_H 5.69), while the ^{13}C NMR spectrum exhibited the presence of 14 carbon signals corresponding to four methylenes, six methines and four quaternary carbons. The HMBC spectrum showed correlation between the proline and tyrosine moieties through ^3J correlation from the carbonyl C(1) of the tyrosine moiety and C(6) of the proline moiety, to the amine proton NH-8. In addition, the H(10) showed ^2J correlation to the aromatic C(1') and ^3J correlation C(2') and C(6'). Other key HMBC correlations are showed in Figure 3. The NOESY spectrum (Figure 3) showed the spatial proximity between the chiral centre H(9) with the aromatic H(2'), H(6') and the NH-8 protons. However, the absence of the NOESY correlation between H(6) with the H(10) indicated that the stereochemistry at the stereogenic centre C(9) is *S* configuration. The observation of NOESY correlation between H(6) and H(9) indicated that both

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 phytotoxin 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 $C_{15}H_{30}O_2$. 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).

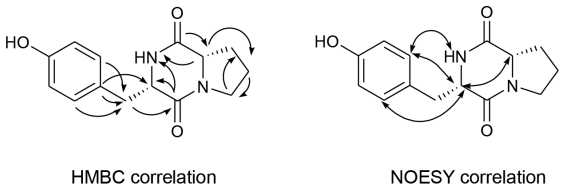

FIGURE 3. HMBC and NOESY correlations of compound **2**

Compound **4** was isolated as white powder and the HRESIMS showed a *m/z* 211.1433 $[M+H]^+$ consistent with the molecular formula $C_{11}H_{18}O_2N_2$ 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 signals (δ_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 δ_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_2 and CH-CH_2-CH(CH_3)-CH_3. The two partial structures were also consistent with the HMBC spectrum which showed ^3J correlation from H(4) and H(5) to ^2J correlation from H(5) to C(6); and ^3J 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 showed 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*.

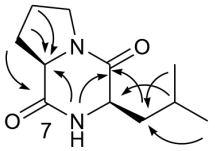

FIGURE 4. HMBC correlations of compound **4**

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 cylco(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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