Novel Oxidized Derivatives of Antifungal Pyrrolnitrin from the Bacterium *Burkholderia cepacia* K87

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Abstract The screening of antifungal active compounds from the fermentation extracts of soil-borne bacterium *Burkholderia cepacia* K87 afforded pyrrolnitrin (**1**) and two new pyrrolnitrin analogs, 3-chloro-4-(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one (**2**) and 4-chloro-3-(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one (**3**). Pyrrolnitrin showed strong antifungal activity against *Rhizoctonia solani* but the analogs (**2** and **3**) were found to be marginally active. The isolates, **2** and **3**, are believed to be biodegraded derivatives of pyrrolnitrin.

Keywords *Burkholderia cepacia* K87, metabolite, pyrrolnitrin, 3-chloro-4-(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one, 4-chloro-3-(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one

Introduction

Certain genera of microorganisms like *Pseudomonas*, *Penicillium*, *Bacillus*, and *Streptomyces* have been known to produce many famous bioactive compounds with antibacterial, antifungal, and antitumor activities. The antimicrobial agents are used to treat the infectious diseases of human beings as well as to control infectious plant pathogens [1].

Introduction of active microbial populations into the soil around seeds and roots of agricultural plants is becoming of great concern with the aim of enhancing plant productivity

because those microorganisms stimulate plant growth, control or inhibit the activity of plant pathogens, and improve soil structure [2]. Also, biological control of soil-borne plant diseases using rhizospheric microorganisms has been attracting public interest over the use of long-lasting chemicals in the environment in general. For this purpose, pseudomonads, especially fluorescent *Pseudomonas* species, are the best known microorganisms with plant-growth-promoting-rhizobacteria (PGPR) activity as well as antimicrobial activity [3, 4]. A variety of different classes of compounds like *N*-containing heterocycles, unusual amino acids, peptides, and glycolipids have been isolated from pseudomonads [5–7]. *Pseudomonas* species belonging to rRNA group II of the genus *Pseudomonas* have been changed to the new genus *Burkholderia* [8].

In continuation of our research to identify plant growth promoting and antifungal compounds from the rhizospheric soil bacteria in the roots of plants, we isolated pyrrolnitrin and its oxidized derivatives from *Burkholderia cepacia* K87. The strain K87 induced systemic resistance in cucumber anthracnose caused by *Colletotrichum orbiculare* and showed antifungal activity against major fungal pathogens like *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, and *Sclerotinia sclerotiorum*.

The methanolic extract of the solid culture of *B. cepacia* K87 was purified to isolate antifungal pyrrolnitrin [3-chloro-4-(2-nitro-3-chlorophenyl)pyrrole] and two apparently bio-oxidized structures of pyrrolnitrin. In this paper, we describe the isolation and structure-determination

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Fig. 1 Chemical structures of the isolated compounds **1**–**3**.

of two pyrrolnitrin-like compounds (**2** and **3**) along with pyrrolnitrin (**1**) (Fig. 1).

Materials and Methods

General

High resolution TOF mass spectra were measured in positive ESI mode on a Waters LCT Premier mass spectrometer (Waters Corporation; Manchester, UK) coupled with a Waters Alliance HPLC system, with data acquisition achieved using MassLynx software, version 4.1. Optical rotations were measured on a Perkin-Elmer 341-LC polarimeter (Perkin-Elmer; Wellesley, MA, USA). UV and IR spectra were measured on a Shimadzu UV-2401 PCR spectrometer (Shimadzu, Kyoto, Japan) and a Perkin-Elmer BX FT-IR spectrometer (Perkin-Elmer Limited, England, UK), respectively. ^1H - and ^{13}C -NMR spectra were recorded on a Varian Mercury 400 spectrometer (Varian Inc.; Palo Alto, CA, USA), with standard pulse sequences operating at 400 MHz for ^1H -NMR and 100 MHz for ^{13}C spectra. The chemical shifts were given in ppm (δ) and were referenced relative to methanol- d_4 (δ 3.30 and 49.15 ppm for ^1H - and ^{13}C -NMR, respectively). 2D NMR spectra (COSY, HSQC, HMBC, and NOESY) were recorded using the manufacturer's software VNMR 6.1C. Flash column chromatography was carried out on a column packed with reversed phase C-18 silica gel (40–63 μm , 90 $\times$ 100 mm, Merck, Darmstadt, Germany). Medium-pressure liquid chromatography (MPLC) was carried out on a Yamazen instrument (model GR-200, pump 540, Yamazen, Osaka, Japan) using a column packed with reversed phase C-18 silica gel (Cosmosil 40 C-18 Prep, 40 $\times$ 320 mm, Nacalai Tesque Inc, Kyoto, Japan) at a flow rate of 5 ml/minute. TLC was performed on precoated silica gel plates (Kieselgel 60, F₂₅₄, 20 $\times$ 20 cm, 0.25 mm thick, Merck, Darmstadt, Germany). Spots were visualized under UV light at 254 and 365 nm or by dipping in a methanolic solution of *p*-anisaldehyde-sulfuric acid (3 : 1) followed by heating. Preparative HPLC was performed on a Waters 600

model system (Waters, MA, USA) with a 996 photodiode array UV detector and a reversed phase C-18 column (Chromosep, Microsorb 100-5 C-18, 21.4 $\times$ 250 mm, Varian, CA, USA) at a flow rate of 7 ml/minute at 210 nm. The fungal strain *R. solani* AG-4 (NIAS B-1 strain) was received from the National Institute of Agricultural Science and Technology (Suwon, Korea). Potato dextrose agar (PDA), tryptic soy broth (TSB), and King's B agar (KBA) were purchased from Becton Dickinson and Company (Sparks, MD, USA).

Identification and Culture Conditions of the Bacterial Strain, *B. cepacia* K87

The strain K87 was isolated from the rhizosphere of Chinese cabbage and identified as *B. cepacia* by sequence analysis of 16S rRNA gene using the NCBI BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>). *B. cepacia* K87 was maintained at -80°C in the TSB media amended with 20% glycerol. The strains streaked from -80°C were pre-cultured at 28°C and inoculated onto Petri dishes containing solidified media (KBA). The dishes were incubated at 28°C for 7 days.

Extraction and Isolation

The solid culture media (10 liters) were harvested and crushed into smaller pieces by using a mechanical stirrer. The media were soaked in MeOH (20 liters) for 2 days at room temperature. The methanol layer was decanted and the remaining solids were washed with an additional amount of MeOH (10 liters). The combined methanolic extracts were concentrated to yield a brownish residue (180 g). The residue was suspended in water (100 ml) and subjected to reversed phase C-18 flash column chromatography with a stepwise gradient elution of a mixture of water and MeOH to afford eleven fractions (1 liter/each fraction). On the basis of TLC, the eluates from 60–80% aq MeOH (fraction 7, 8, and 9) were combined and evaporated under reduced pressure to yield a brown oily residue (578.7 mg). The residue was further chromatographed on a reversed phase C-18 column (MPLC) with a gradient elution of 50% aq MeOH to 100% MeOH for 180 minutes to afford four subfractions. The active subfractions 3 and 4 were combined (279.4 mg) and purified by reversed phase HPLC (30–60% aq MeCN for 70 minutes) to afford compounds, **2** (6.0 mg, R_t =35.1 minutes), **3** (3.2 mg, R_t =37.7 minutes), and **1** (14.4 mg, R_t =68.9 minutes).

3-Chloro-4-(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one (**2**)

Light brown oil; $[\alpha]_D^{20}$ -2.4 (c 0.25, MeOH); UV (MeCN)

$\lambda_{\max}$ (log ϵ) nm 214 (4.15), 303 (3.21); IR (KBr) $\nu_{\max}$ cm^{-1} 3448, 2926, 1718, 1654, 1541, 1508, 1458, 1061; ^1H -NMR (400 MHz, CD_3OD) δ 7.80 (1H, d, $J=8.0$ Hz, H-4'), 7.69 (1H, t, $J=8.0$ Hz, H-5'), 7.57 (1H, d, $J=8.0$ Hz, H-6'), 5.49 (1H, s, H-5), 3.31 (3H, s, OCH_3); ^{13}C -NMR (100 MHz, CD_3OD) δ 167.0 (C, C-2), 149.4 (C, C-2'), 145.6 (C, C-4), 133.3 (CH, C-4'), 133.2 (CH, C-5'), 132.2 (C, C-3), 130.7 (CH, C-6'), 127.0^a (C, C-1'), 126.9^a (C, C-3'), 87.2 (CH, C-5), 55.6 (CH_3 , OCH_3) (^a values are interchangeable); HRTOFMS (+ESI mode) $[\text{M}+\text{H}]^+$ m/z 302.9931 (calcd. for $\text{C}_{11}\text{H}_9\text{Cl}_2\text{N}_2\text{O}_4+\text{H}$, 302.9939); Rf (3 : 7 hexane - EtOAc) 0.23.

4-Chloro-3-(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one (**3**)

Light brown oil; $[\alpha]_{\text{D}}^{20} +11.90$ (c 0.063, MeOH); UV (MeCN) $\lambda_{\max}$ (log ϵ) nm 214 (3.98), 306 (3.06); IR (Nujol) cm^{-1} 3423, 2932, 1719, 1541, 1363, 1204, 1132; ^1H -NMR (400 MHz, CD_3OD) δ 7.78 (1H, dd, $J=8.0$, 1.2 Hz, H-4'), 7.70 (1H, t, $J=8.0$ Hz, H-5'), 7.52 (1H, dd, $J=8.0$, 1.2 Hz, H-6'), 5.58 (1H, s, H-5), 3.31 (3H, s, OCH_3); ^{13}C -NMR (100 MHz, CD_3OD) δ 169.4 (C, C-2), 149.2 (C, C-2'), 148.1 (C, C-4), 133.5^a (C, C-3), 133.5^a (CH, C-4'), 133.5^a (CH, C-5'), 131.6 (CH, C-6'), 127.7^b (C, C-1'), 126.3^b (C, C-3'), 87.3 (CH, C-5), 51.8 (CH_3 , OCH_3) (^a values are overlapped, ^b values are interchangeable); HRTOFMS (+ESI mode) $[\text{M}+\text{H}]^+$ m/z 302.9929 (calcd. for $\text{C}_{11}\text{H}_9\text{Cl}_2\text{N}_2\text{O}_4+\text{H}$, 302.9939). Rf (3 : 7 hexane - EtOAc) 0.26.

Antifungal Assay

The antifungal activity against *R. solani* was determined by paper disc diffusion assay. In brief, a small mycelial plug cut from the actively growing front of a week-old colony of *R. solani* was placed on the center of the Petri dish with sterilized autoclaved agar media (PDA 3.9 g in 100 ml water). After incubation of the dishes at 28°C for a day the filter paper discs (8 mm in diameter) impregnated with specified concentrations of samples were placed near the mycelium. The zone of fungal growth inhibition was measured in diameter after 3-day incubation at 28°C.

Results and Discussion

The bacterial strain *B. cepacia* K87 was fermented in KBA media at 28°C for 7 days. The solid culture media (10 liters) were soaked with MeOH. The resulting crude extract was concentrated and subjected to a series of chromatographic purification procedures like reversed phase C-18 medium pressure and C-18 HPLC.

Compounds, **1**, **2**, and **3** were obtained in yield of 14.4, 6.0, and 3.2 mg, respectively, from the crude extract (180 g).

Compound **1** was isolated as a light brown oil and established as pyrrolnitrin by comparison of the spectroscopic data with those reported in the literature [9~11]. Compound **2** was obtained as a light brown oil with a specific rotation of -2.4 (c 0.25, MeOH). The mass spectrum (HRTOFMS) displayed a protonated molecular ion $[\text{M}+\text{H}]^+$ at m/z 302.9931 (calcd. for $[\text{M}+\text{H}]$, 302.9939), indicating of a molecular formula of $\text{C}_{11}\text{H}_9\text{Cl}_2\text{N}_2\text{O}_4$. The UV maxima were observed at 214 and 303 nm. The IR spectrum showed the characteristic peaks at 3448, 1718, and 1654 cm^{-1} , suggesting of the presence of OH (or NH), C=O, and C=C bonds. The ^1H -NMR spectrum of compound **2** showed proton signals [δ 7.69 (t, $J=8.0$ Hz), 7.80 (d, $J=8.0$ Hz), and 7.57 (d, $J=8.0$ Hz)] for a characteristic ABC spin pattern of a trisubstituted benzene ring, a singlet proton signal (δ 3.31) for a methoxy group, and a singlet proton signal (δ 5.49) for a methine group. In the ^1H - ^1H COSY spectrum, the aromatic proton at δ 7.69 (H-5') was coupled with the protons at δ 7.80 (H-4') and 7.57 (H-6'). Their corresponding carbon signals were assigned from the HSQC spectrum as δ 133.2 (C-5'), 133.3 (C-4'), 130.7 (C-6'), respectively. In the HBMBC spectrum, the protons (H-4' and H-6') showed correlations with the quaternary carbon signal at δ 149.4 (C-2'); the proton (H-5') with two quaternary aromatic signals at δ 126.9 (C-3') and 127.0 (C-1'). From these NMR data analysis the trisubstituted benzene moiety could be deducible to be 3-chloro-2-nitrophenyl group as the same moiety as that in compound **1**, as the benzene proton signals in compound **2** are relatively well resolved unlike those in compound **1** in which they appeared overlapped. In addition, the HMBC spectrum revealed that the singlet proton at δ 5.49 (H-5) attached to the carbon C-5 (δ 87.2) showed long range correlations with the remaining three quaternary carbons at δ 167.0 (C-2), 145.6 (C-4), and 132.2 (C-3), and the methoxy carbon at δ 55.6. This moiety could be assignable to a $\text{HC}(\text{OMe})-\text{C}=\text{C}-\text{C}=\text{O}$ group with a long range connection of the methine carbon to the C=O group, thus, from the molecular formula, a 4-substituted 5-methoxy-3-chloro-pyrrolin-2-onyl group. The quaternary carbon at 145.6 (C-4) in this pyrrolinone moiety showed an important correlation with the proton (H-6') in the 3-chloro-2-nitrophenyl group. This information establishes the direct connectivity between the two moieties (Fig. 2). In addition, a weak NOE was observed between the aromatic proton (H-6') and the methoxy protons (H-6), confirming that those groups are in vicinity in their spatial arrangement. From the above spectral analyses, the structure of compound **2** was determined to be 3-chloro-4-

Fig. 2 ¹H- (underline) and ¹³C-NMR assignments (values with asterisks interchangeable with each other within the structure), and HMBC (solid arrows; H to C) and NOE (dashed curve) correlations in compounds **2** and **3**.

(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one.

Compound **3** was obtained as a light brown oil ($[\alpha]_D^{20} +11.9$, c 0.063, MeOH). The UV spectrum was similar with that of compound **2**. In the IR spectrum, the OH (or NH), C=O, and C=C bonds were also indicated. The mass spectrum (HRTOFMS) of compound **3** indicated its molecular formula as the same as that of compound **2**. The ¹H- and ¹³C-NMR data of the aromatic region of compound **3** was quite similar with those of compound **2**, indicating of the presence of 3-chloro-2-nitrophenyl group. In the HMBC, the proton (H-5) at δ 5.58 showed long range correlations with three quaternary carbons at δ 169.4 (C-2), 148.1 (C-4), and 133.5 (C-3), and methoxy carbon (C-6) at δ 51.8, which indicates again the presence of pyrrolinone group (Fig. 2). Unlike compound **2**, no NOE was observed between the proton (H-6') and methoxy protons (H-6) at δ 3.31. From these data we suggest the structure of compound **3** could be determined to be 4-chloro-3-(3-chloro-2-nitrophenyl)-5-methoxy-3-pyrrolin-2-one, a regioisomer of compound **2**. It should be noted that the long range correlation of the proton (H-6') at δ 7.52 with the quaternary carbon (C-3) in the HMBC was not unambiguous because the C-3 carbon appeared overlapped with the other two carbons (C-4' and C-5'). It is reasonable to accept that the α,β -unsaturated C=O groups in the pyrrole moiety of compounds **2** and **3** seem influenced the chemical shifts of the β -carbons at δ 145.6 (C-3) in compound **2** and δ 148.1 (C-4) in compound **3** to shift down-field. However, in compound **2**, there was no significant red shift in the UV nor down field chemical shifts of the aromatic carbons (C-2', C-4', and C-6') due to the carbonyl group, which suggests that the benzene and pyrrolinone rings are not fairly in the same plane, as indicated in the case of pyrrolnitrin **1** [12]. The absolute

configuration at C-5 in compounds **2** and **3** was not determined.

The isolated pyrrolnitrin (**1**) exhibited strong antifungal activity against *R. solani* at a concentration of 50 μ g/disc (25 mm of inhibition zone in diameter) by paper disc diffusion assay, but compounds, **2** and **3**, showed a relatively low activity even at 250 μ g/disc (10 mm of inhibition zone). For the antifungal assay, ketoconazole was used as a positive control, which showed 11 mm of inhibition zone at 30 μ g/disc. It has been reported that pyrrolnitrin (**1**) was isolated from many pseudomonads like *Pseudomonas*, *Burkholderia*, and *Serratia* species showing antifungal activity [10, 11, 13~15].

Although compound **1** had been shown to be strongly active against fungi, yeasts, and Gram-positive bacteria, it was not sufficiently stable upon exposure to sunlight resulting in a loss of its antibiotic activity for agricultural purpose [9]. It was also reported that the pyrrole ring of compound **1** was readily metabolized *in vivo* in rat and *in vitro* by enzymes like mixed-function oxidases to succinimide, pyrrolinone, and maleimide derivatives, resulting in no pyrrolnitrin-like antifungal activity [16]. Much of the work in structural modification of this compound has been focused on an increase of its photostability. Pyrrolnitrin derivatives such as 3-cyano-4-aryl pyrroles have been developed to improve photostability for agricultural purpose [17]. Recently, the UV irradiation to compound **1** was reported to produce 7,4'-dichloro-spiro[1,3-dihydrobenzo(c)isoxazole-3,3'-pyrrolin-2'-one] in an aprotic solvent *via* intramolecular oxidation of the pyrrole ring by the triplet-excited nitro group and 3,7-dichloro-8-hydroxy-8,8a-dihydropyrrolo[2,3-*b*]indol-2-one in an aqueous solvent *via* intramolecular cyclization by the singlet-excited nitro group followed by hydroxylation.

Scheme 1 Postulated pathways to compounds **2** and **3** from compound **1** (electron movements in compound **1a** to compound **2** for heterolytic cleavage are shown).

These photoproducts were reported to show no remarkable antifungal activities [18]. The two new isolates, **2** and **3**, could be biosynthetically generated from compound **1** by oxidation during fermentation *via* a [4+2] cycloaddition (dioxine **1a**) of the diene group of the pyrrole ring with oxygen followed by rearrangement (pyrrolinones **1b** and **1c**) and ketalization with MeOH (or methylation of the hemiketal OH group), as shown in Scheme 1. The suggested pyrrolinone intermediate **1c** has been isolated previously as a metabolite from compound **1** *in vitro* using rat liver microsomes supplemented with NADPH [16]. As an analogy in the mechanism shown in the Scheme 1, it was reported that 1,2-dioxines were readily prepared chemically from dienes with oxygen by photooxidation using a photosensitizer under UV light. The dioxines are readily ring-opened homolytically *via* use of transition metal catalysts like Co(II) and Ru(II) or heterolytically under basic condition to yield γ -hydroxy enones [19–21].

In conclusion, pyrrolnitrin-like compounds, **2** and **3**, were isolated from the fermentation broth of *B. cepacia* K87. They would be biosynthetic intermediates for degradation of compound **1** *via* oxidation during fermentation causing of a loss of antifungal activity. The proposed degradation pathway would suggest the inactivation route of antibiotic **1**.

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Supporting Information Available IR, UV, ^1H , ^{13}C , COSY, HSQC, and HMBC NMR spectra, HPLC profile, and MS spectra for compounds **1–3** (only MS and ^1H -NMR for compound **1**).

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