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Diketopiperazine and alloxazine alkaloids from the antarctic bacteria, *Pseudorhodobacter psychrotolerans* sp. nov

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ABSTRACT

Chemical investigation of the ethyl acetate extract of the Antarctic bacteria, *Pseudorhodobacter psychrotolerans* sp. nov. led to the isolation of five diketopiperazines (**1–5**), an alloxazine (**6**), *p*-hydroxybenzoic acid (**7**), and 2-methylbutanedioic acid (**8**). The structures of these compounds were confirmed by 1D and 2D-NMR and MS experiments, as well as by comparison with published values. This is the first phytochemical study of *Pseudorhodobacter psychrotolerans* sp. nov. Furthermore, all the compounds have been isolated for the first time from the genus *Pseudorhodobacter* and the family Rhodobacteraceae. The chemotaxonomic significance of the isolated compounds is discussed.

1. Subject and source

The genus *Pseudorhodobacter* (Rhodobacteraceae) has been isolated from intertidal sandy sediments, seawater, and wood falls (Uchino et al., 2002; Jung et al., 2012; Chen et al., 2013; Lee et al., 2013). The genus *Pseudorhodobacter* contains four species with validly published names: *P. ferrugineus* (Rüger and Höfle, 1992), *P. aquimaris* (Jung et al., 2012), *P. antarcticus* (Chen et al., 2013) and *P. wandonensis* (Lee et al., 2013). In a recent study, *P. psychrotolerans* sp. nov. has been newly found from the Antarctic terrestrial soil (Lee et al., 2016). (see Fig. 1)

A bacterial strain, PAMC 27389, isolated from the Antarctic terrestrial soil was collected from King George Island, Antarctica (628 12.379 S 588 47.409 W), on 12 February 2011, and the bacterial isolate was identified as *P. psychrotolerans* sp. nov. (PAMC 27389) by phylogenetic analysis of the 16s rDNA sequence (Lee et al., 2016). Pure cultures of the bacterial isolates used in this study are preserved at −80 °C in KOPRI institute.

2. Previous work

In previous studies of the family Rhodobacteraceae, oligosaccharides (Kwon and Jung, 2011) and bacteriochlorophyll (Kim et al., 2015) from *Rhodobacter sphaeroides*, N-acylhomoserine lactones and N-acyl alanine methyl ester from *Roseovarius tolerans* (Bruns et al., 2013, 2018), and tropodithietic acid from *Phaeobacter inhibens* (Brock et al., 2014) have been reported. However, most of the genera in the Rhodobacteraceae family have been poorly investigated. In particular, there are no chemical or biological studies on the genus *Pseudorhodobacter* as well as *P. psychrotolerans* sp. nov. Herein, we report first time secondary metabolites isolated from the Antarctic bacteria, *P. psychrotolerans* sp. nov.

3. Present study

The fungal strain, *P. psychrotolerans* sp. nov. was cultivated at 25 °C in 2 L modified yeast-malt-glucose medium consisted of 4 g/L malt extract, 10 g/L glucose and 4 g/L yeast extract for 30 days by using five 4-L Penicillin flasks (Duran, Germany). Cells were removed from the cultures by centrifugation and filtration through a 0.45-μm filter, and the filtrate (10 L) was passed through an Amberlite XAD-20 resin (40 × 8 cm), using a gradient solvent system of H_2O-MeOH (100:0 to 0:100) to give crude extracts (4.0 g). The extracts were diluted with H_2O (1 L) and partitioned using ethyl acetate (EtOAc) (0.5 L, 3 times) to yield EtOAc extracts (1.5 g). The EtOAc extracts (1.3 g) were separated by column chromatography (CC) over a C_{18} gel column and eluted with MeOH:H_2O (10:90–100% MeOH) to obtain 12 subfractions (ER1 to ER12).

Combined subfractions ER3 to ER5 (100 mg) based on TLC patterns were subjected to an open Sephadex LH 20 (50 g) gel column using solvent mixtures (MeOH:H_2O, 10:90–100% MeOH), and purified by

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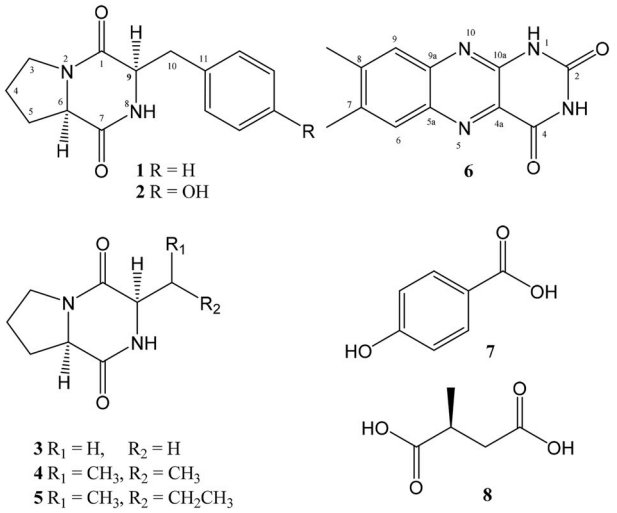

Fig. 1. Chemical structures of **1–8** isolated from *P. psychrotolerans* sp. nov.

HPLC on a semi-preparative C18 gel column, using MeOH:H_2O mixtures, from 20:80 to 90:10, as the solvent system, to yield **3** (1.5 mg, t_R 85 min), **4** (1.0 mg, t_R 80 min), and **5** (2 mg, t_R 78 min). Subfractions ER7 and ER8 (60 mg) were purified over a Sephadex LH 20 (50 g) gel column using solvent mixtures (MeOH:H_2O, 10:90–100% MeOH), to yield five subfractions (ER7L1 to ER7L5). Subfraction ER7L4 (15 mg) was subjected to separation on a semi-preparative C18 column by HPLC, using MeOH:H_2O mixtures (from 30:70–100% MeOH) as the solvent system, to yield compounds **6** (5 mg, t_R 90 min) and **1** (1.5 mg, t_R 95 min). Compound **2** (2.0 mg) was purified from subfraction ER10 (15 mg) by a semi-preparative C18 column and HPLC method, using MeOH:H_2O, from 20:80 to 90:10, as the solvent system. Fraction ER12 (50 mg) was subjected to repeated chromatography on a Sephadex LH-20 and RP-18 gel columns and eluted with a MeOH:H_2O (from 0:100 to 50:50) solvent system, to give five subfractions (ER12R1 to ER12R5). Subfractions ER12R2 and R3 (20 mg) were purified by semi-preparative HPLC on a C18 column, using MeOH:H_2O solvent mixtures (from 20:80–100% MeOH), to yield **7** (3.0 mg, t_R 67 min) and **8** (1.0 mg, t_R 70 min).

The compounds were identified as *cyclo*-(L-Pro-L-Phe) (**1**) (Jayatilake et al., 1996), *cyclo*-(L-Pro-L-Tyr) (**2**) (Jayatilake et al., 1996), *cyclo*-(L-Ala-L-Pro) (**3**) (Chen et al., 2015), *cyclo*-(L-Pro-L-Val) (**4**) (Adamczeski et al., 1995), *cyclo*-(L-Pro-L-Ile) (**5**) (Jayatilake et al., 1996), 7,8-dimethylalloxazine (**6**) (Kwon et al., 2004), 4-hydroxybenzoic acid (**7**) (Lo et al., 2002), and (2*S*)-2-methylbutanedioic acid (**8**) (Henrikson et al., 2009), by comparison of their physical and spectral properties with published values.

4. Chemotaxonomic significance

The family Rhodobacteraceae contains more than 180 genera, including the genus *Pseudorhodobacter*. The phytochemical study on the family Rhodobacteraceae has only reported N-acylhomoserine lactones and N-acyl alanine methyl ester from *Roseovarius tolerans* (Bruns et al., 2013, 2018), tropodithietic acid from *Phaeobacter inhibens* (Brock et al., 2014), and oligosaccharides and bacteriochlorophyll from *Rhodobacter sphaeroides* (Kwon and Jung, 2011; Kim et al., 2015). However, there are few chemical studies on the other genera, including the genus *Pseudorhodobacter*. The current study reports on the isolation and chemotaxonomic significance of five diketopiperazines (**1–5**), an alloxazine (**6**), *p*-hydroxybenzoic acid (**7**), and 2-methylbutanedioic acid (**8**) from the bacterium, *P. psychrotolerans* sp. collected in the Antarctic terrestrial soil.

Cyclic peptides, known as diketopiperazines comprise an important class of natural product, many of which are known to exhibit a wide range of biological activity (Yoshida et al., 1988). They occur widely throughout nature and are generally biosynthesized from proteinogenic L-α-amino acids, where cyclisation of a parent L,L-dipeptide affords the core skeleton of the diketopiperazine (Birch and Russell, 1972). About 90% of Gram-negative bacteria produce diketopiperazines and they have also been isolated from fungi and higher organisms (Fenical, 1993).

Diketopiperazines (**1–5**) were previously isolated from an Antarctic sponge associated bacterial strain of *Pseudomonas aeruginosa* (Pseudomonadaceae) (Jayatilake et al., 1996). Compounds **3–5** have even been found in *Micrococcus* sp. (Micrococcaceae) with sponge *Tedania ignis* (Stierle et al., 1988). In particular, *cyclo*-(L-Pro-L-Phe) (**1**) with other diketopiperazine derivatives have been isolated from a *Psychrobacter* species (Moraxellaceae), and reported to have protective effect against *Vibrio vulnificus* (Le et al., 2008). In addition, *cyclo*-(L-Phe-L-Pro) (**1**), *cyclo*-(L-Pro-L-Val) (**3**), and *cyclo*-(L-Leu-L-Pro) (**4**), together with their derivatives, *cyclo*-(Gly-L-Pro), *cyclo*-(L-Pro-L-Pro), *cyclo*-(L-Leu-L-*trans*-4-OH-Pro), and *cyclo*-(L-Phe-L-*trans*-4-OH-Pro), were isolated from the fermentation broth of an *Aspergillus fumigatus* (Trichocomaceae) (Furtadoa et al., 2005). However, the diketopiperazines, **1–5** have not been reported from the family Rhodobacteraceae and the genus *Pseudorhodobacter*.

Flavins are redox-active chromophores that are widely found in animal and plant systems, where they play key roles in enzymes and photoreceptors (Walsh, 1980; Conrad et al., 2014; Losi, 2007). Due to their extensive and complex biochemical roles, flavins have been the focus of many photophysical and photochemical studies, especially in aqueous solution (Penzkofer, 2016; Tyagi and Penzkofer, 2010; Kondo et al., 2006). Riboflavin (RF) is essential vitamin for human nutrition and animal feeding as precursor of two coenzymes: riboflavin-50-phosphate (flavin mononucleotide (FMN)) and riboflavin-50-adenosine diphosphate (flavin adenine dinucleotide (FAD)). The flavin coenzymes are involved in a wide range of biochemical processes, particularly in mitochondrial electron transport, photosynthesis, fatty acid oxidation, metabolism of vitamins B_6, B_9 (folates) and B_{12}. FMN and FAD play a pivotal role in the dehydrogenation of metabolites in one-and two-electron transfer reactions, in the activation of oxygen in oxidase, oxygenase and hydroxylase reactions. Therefore, flavins are widely used in medicine and are also important as biochemical reagents (Fischer and Bacher, 2005; Joosten and vanBerkel, 2007; Powers, 2003).

In previous studies, 7,8-dimethylalloxazine (**6**) has been reported from a fungal strain, *Paecilomyces* sp. J300 (Trichocomaceae) (Kwon et al., 2004) and the entomopathogenic fungus, *Beauveria bassiana* (Cordycipitaceae) (Andrioli et al., 2017). Meanwhile, various alloxazine derivatives are mostly synthesized by the reaction of diamines and alloxans (Richtar et al., 2018).

Primary metabolites, *p*-hydroxybenzoic acid (**7**) and 2-methylbutanedioic acid (**8**) have also been isolated for the first time in this genus as well as *P. psychrotolerans* sp.

In conclusion, diketopiperazines (**1–5**) and an alloxazine (**6**), including primary metabolites, *p*-hydroxybenzoic acid (**7**) and 2-methylbutanedioic acid (**8**) have not been found in this genus or in the family Rhodobacteraceae. Therefore, these secondary metabolites (**1–6**) could be important taxonomic markers for the identification of *P. psychrotolerans* sp., and might suggest chemotaxonomic relationship of *Pseudorhodobacter* with other genera within this family in the further studies related.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bse.2019.04.010.

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