



Phenazine-1-carboxylic acid (PCA) from cold-adapted yeast *Glaciozyma antarctica* PI12

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ABSTRACT

A phenazine alkaloid, phenazine-1-carboxylic acid (PCA) was isolated from the ethyl acetate extract of cold-adapted yeast *Glaciozyma antarctica* PI12 (Kriegeriales). The compounds were isolated by several chromatography techniques i.e. radial chromatography (RC) and thin-layer chromatography (TLC). The chemical structure was elucidated by ultraviolet (UV), infrared (IR), nuclear magnetic resonance (1D and 2D NMR) spectroscopy and mass spectrometry.

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1. Introduction

The search for secondary metabolites is still going on, particularly from the extremophilic microorganisms (Rosandy *et al.*, 2019). The psychrophilic can be a good tool to explore the new bioactive metabolites of pharmaceutical importance (Archer *et al.*, 2008) due to uniqueness of their habitat and changes in the metabolic systems, amenable for their adaptation to extreme cold environmental conditions (Morita 1975; De Maayer *et al.*, 2014). Early studies regarding the chemical constituents of cold-adapted yeast *Glaciozyma antarctica* PI12 (Di Menna 1960; Fell *et al.*, 1969; Donachie 1995; Turkiewicz *et al.*, 2005; Connell *et al.*, 2008; Turchetti *et al.*, 2011) revealed the presence of a new diketopiperazine i.e. (-)-Glaciantarcin, together with five known diketopiperazine and two pyrimidine (Rosandy *et al.*, 2017 & 2018; Alwi *et al.*, 2017s).

This paper reported for the first time the isolation and detailed characterization of a PCA from yeast *G. antarctica* PI12

2. Materials and Methods

2.1. General

Thin-layer chromatography, aluminium sheets 20 × 20 cm of the silica gel 60 F254 of 0.25 mm thickness (art. no. 5554, MERCK) and Silica gel; Kieselgel 60 PF254 (art. no. 7749, Merck) (radial chromatography, Chromatotron-7924T-01 USA). The structure of the isolated compound was determined based on the spectral data

recorded on UV-Vis (Perkin-Elmer Lambda 35, Waltham, MA, USA), FTIR (Perkin-Elmer USA) spectrophotometer and NMR 700 MHz cryo-probe (Bruker Germany). ESI-MS was recorded by using Ultra performance liquid chromatography (UPLC-LCMS, WATER USA).

2.2. Biological materials, cultivation of yeast and isolation of compound

The procedures of cultivation of yeast *G. antarctica* PI12 and isolation of compound were described in Rosandy *et al.* (2017, 2018).

2.3. Preliminary phytochemical screening

The standard procedures of chemicals screening was described by Trease and Evans (Trease & Evans 1989).

2.3.1. Saponin

One milligram of the extract was diluted with 20 ml of distilled water and shaken in a graduated cylinder for 5 to 10 min. The formation of a foam layer indicates the presence of saponins.

2.3.2. Phenols

One milligram of the extract was diluted with 1 ml of distilled water in a test tube and 2 drops of Iron (III) chloride (FeCl₃) was added. Blue, green, red or purple colour is a positive test.

2.3.3. Glycosides

One milligram of the extract was taken in 1 ml of distilled water in a test tube and a drop of aqueous NaOH were added. A yellow colouration indicates the presence of glycosides.

2.3.4. Flavonoids

Two to five drops of concentrated HCl were added to 1 mg of the extract. A red colour indicates the presence of flavonoids.

2.3.5. Alkaloids

One to two millilitres of extract were taken in a test tube together with 0.2 mL dilute HCl and 1 ml of Meyer's reagent. A yellowish colouration indicates alkaloid's presence.

2.3.6. Tannins

Two to five millilitres of the extract were placed in a test tube together with 2 ml of 5% of FeCl₃ solution. A greenish-black precipitate indicates the presence of tannins.

2.3.7. Terpenoids

One millilitre of extract was added in a test tube containing 2 ml of CHCl₃. This is then followed by the addition of 1 to 3 ml H₂SO₄ which forms a layer. The reddish-brown colouration of the interface indicates terpenoids.

3. Results and discussion

The chemicals screening of the extract of *G. antarctica* PI12 showed the presence of saponin, phenol, glycoside alkaloid and terpenoid. Flavonoid and tannin were not found to be present in this yeast extract (Table 1).

Table 1. Results of chemical screening of *G. antarctica* PI12

Test	Result
Saponin	+
Phenol	+
Glycoside	+
Flavonoid	-
Alkaloid	+
Tannin	-
Terpenoid	+

Two milligrams of the pure compound were isolated as yellow crystals with melting point 240 to 242°C. The molecular formula C₁₃H₈N₂O₂Na was identified using ESI-MS [M+Na]⁺ ion at m/z 247.0359 (Fig. 1).

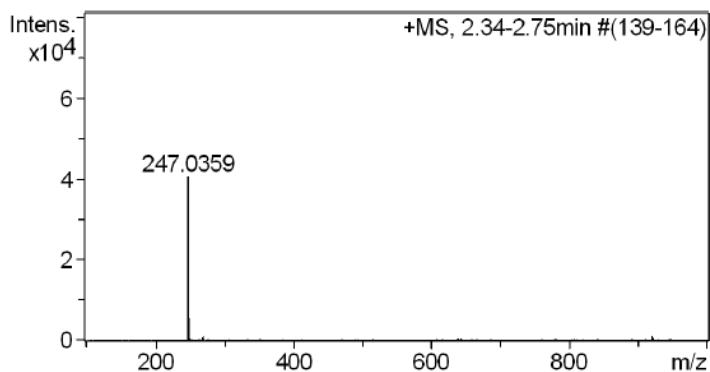


Fig. 1. ESI-MS spectrum of isolated compound.

The UV spectrum displays absorbance peaks at 256 and 368 nm indicating of the double bonds conjugated system in the molecule. The FT-IR spectral data (Fig. 2) exhibited strong absorption peaks at 1727 cm⁻¹ (carboxylic acid vibration), 1467 cm⁻¹ (aromatic C=C stretch), while 861 cm⁻¹ and 747cm⁻¹ (C-H bending vibrations).

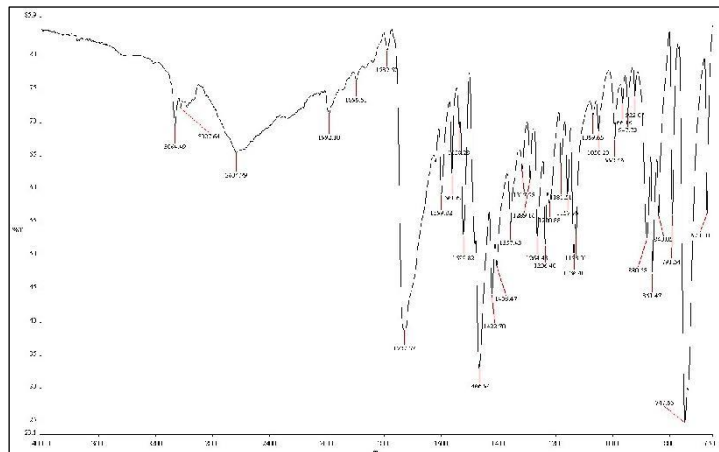


Fig. 2. FT-IR spectrum of isolated compound.

The presence of functional groups is supported by NMR data. The APT (Attached Proton Test) spectrum (Fig. 3) reveals that the compound contains 13 carbons consisting of seven methines and six quaternary carbons. The most highly deshielded signal at δ C 165.9 (C11) integrating for one-carbon is attributed to a carboxylic acid. The highly shielded carbons at δ C 135.1 (C2), 130.3 (C3), 137.5(C4), 130.1 (C6), 131.8 (C7), 133.2 (C8) and 128.0 (C9) represent the existence of two aromatic rings in the molecule. The other five carbons at δ C 125.0 (C1), 144.1 (C4a), 140.1 (C5a), 139.9 (C9a) and 143.4 (C10a) are fused the aromatic ring. The DBE (Double Bond Equivalent) value of C₁₃H₈N₂O₂ is 11, indicating that the compound consists of eight double bonds (seven olefinic and one carbonyl) and three rings.

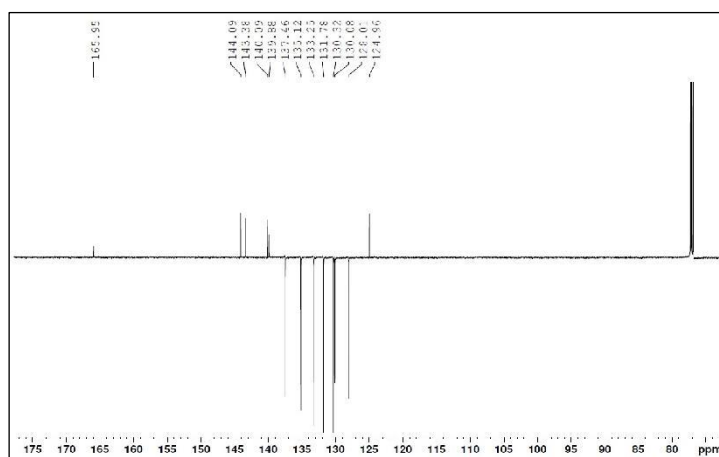


Fig. 3. APT-NMR spectrum of isolated compound.

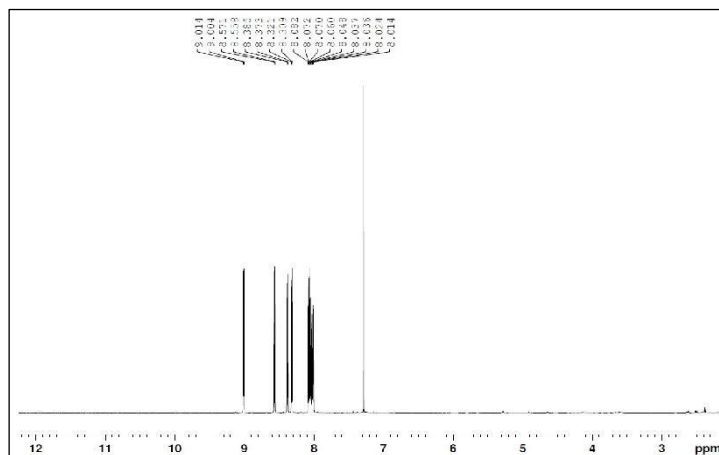


Fig. 4. ¹H-NMR of isolated compound.

Meanwhile, seven proton signals consisting of seven protons aromatic appeared in the $^1\text{H-NMR}$ spectrum (Fig. 4) as shown in Table 2. Three of the signals at δ_{H} 8.57 (d, 7) H2, 8.08 (t, 7) H3 and 9.02 (d, 7) H4 came from aromatic ring A, while the other four signals at δ_{H} 8.38 (d, 7) H6, 8.02 (d, 7) H7, 8.04 (d, 7) H8 and 8.32 (d, 7) H9 are fused in the aromatic ring C.

The structure is confirmed using 2D NMR. The HMBC experiment shows $^2J_{\text{C-H}}$ couplings between proton methine olefinic H-2 at δ_{H} 8.57 (d, 7) with C-1 and C-3, while H-2 has $^3J_{\text{C-H}}$ couplings with C-11, thus confirming the position of carboxylic acid (C-11).

The other two methines olefinic, H-4 has correlations with two quaternary carbons at C-4a and C-10a, while H-6 generates $^2J_{\text{C-H}}$ couplings with C-5a and $^3J_{\text{C-H}}$ couplings with C-9a. Correlation between H-7 and C-8 also appears in the HMBC spectrum. All correlations showed that the isolated compound is phenazine-1-carboxylic acid (PCA) (Abraham et al., 2015). This compound previously isolated from *Streptomyces antibioticus* strain Tü 2706 (Geiger et al., 1988), *Pseudomonas aeruginosa* (Jayatilake et al., 1996) and *Pseudomonas aeruginosa* UPMP3 (Lee et al., 2018) and *Streptomyces kebangsaanensis* (Remali et al., 2019).

Table 2. NMR data of phenazine-1-carboxylic acid (PCA) (Abraham et al., 2015)

	δ_{C} (ppm)		δ_{H} (mult. J in Hz) ΣH		HMBC ($^1\text{H} \leftrightarrow ^{13}\text{C}$)
	PCA	*	PCA	*	PCA
1	125.0	124.9	-	-	-
2	135.1	135.1	8.57 (d, 7) 1H	8.54	C-1, C-4, C-11
3	130.3	130.2	8.08 (t, $J=7$) 1H	8.06	C-1, C-4, C-10a
4	137.5	137.4	9.02 (d, 7) 1H	8.99	C-2, C-3, C-4a
4a	144.1	144.1	-	-	-
5-N	-	-	-	-	-
5a	140.1	140.1	-	-	-
6	130.1	130.1	8.38 (d, 7) 1H	8.36	C-8, C-9a,
7	131.8	131.7	8.02 (d, 7) 1H	8.00	C-5, C-6
8	133.2	133.2	8.04 (d, 7) 1H	8.02	C-7, C-9, C-9a
9	128.0	127.9	8.32 (d, 7) 1H	8.29	C-7
9a	139.9	139.8	-	-	-
10-N	-	-	-	-	-
10a	143.4	143.4	-	-	-
11	165.9	165.9	-	-	-

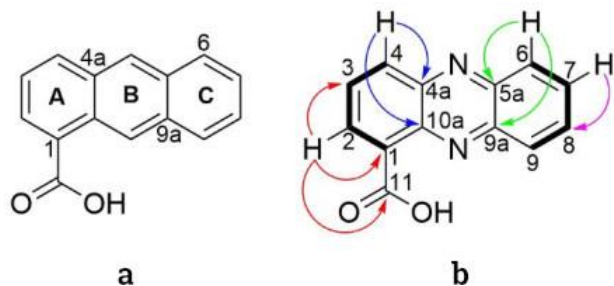


Fig. 5. (a) The structure of PCA and (b) correlation NMR; HMBC (→), COSY (—)

4. Conclusion

A phenazine alkaloid, PCA was isolated and reported for the first time from cold-adapted yeast *G. antarctica* PI12. This isolated compound was not subjected to further bioactivity studies due to insufficient amounts.

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