



DIKETOPIPERAZINE PRODUCED BY PSYCHROPHILIC YEAST *Glaciozyma antarctica* PI12

(Diketopiperazin Dihasilkan oleh Yis Psikropilik *Glaciozyma antarctica* PI12)

Andi Rifki Rosandy¹, Muntaz Abu Bakar¹, Nurul Nadiah Abdul Rahman¹, Abdul Munir Abdul Murad²,
Azira Muhamad³, Rozida Mohd. Khalid^{1*}

¹School of Chemical Science and Food Technology, Faculty of Science and Technology

²School of Biosciences and Biotechnology, Faculty of Science and Technology
Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

³Malaysia Genome Institute (MGI),
National Institute of Biotechnology Malaysia, Jalan Bangi, 43000 Kajang, Selangor, Malaysia

*Corresponding author: rozidakhalid@ukm.edu.my

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Abstract

Four diketopiperazine derivatives; cyclo(-Pro-Val) (1), (-)-cyclo(-Pro-Tyr) (2), (-)-cyclo(-Pro-Phe) (3) and (+)-cyclo(-Pro-Leu) (4) were isolated from the ethyl acetate extract of *Glaciozyma antarctica* PI12, a cold-adapted yeast that belongs to family Kriegeriales. The compounds were isolated by radial chromatography and thin layer chromatography techniques and the chemical structures were elucidated by infrared (IR), ultraviolet (UV), nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry.

Keywords: *Glaciozyma antarctica* PI12, diketopiperazine, Kriegeriales

Abstrak

Empat terbitan diketopiperazin; siklo(-Pro-Val) (1), (-)-siklo(-Pro-Tir) (2), (-)-siklo(-Pro-Phe) (3) and (+)-siklo(-Pro-Leu) (4) yang dipencilkan daripada ekstrak etil asetat *Glaciozyma antarctica* PI12, merupakan yis adaptasi sejuk yang terdiri dari famili Kriegeriales. Sebatian – sebatian terencil telah diasingkan menggunakan teknik kromatografi pepejal dan kromatografi lapisan nipis dan struktur kimia dijelaskan oleh spektroskopi infra merah (IM), ultra lembayung (UL), resonans magnetik nuklear (RMN) dan spektrometri jisim.

Kata kunci: *Glaciozyma antarctica* PI12, diketopiperazin, Kriegeriales

Introduction

Antarctica is a continent with extreme low temperature and nutrients, with high UV radiation, low water availabilities and frequent freeze-thaw cycle [1]. Therefore, the biology of Antarctica more than other continents, is dominated by microorganisms [2], with a high level of adaptation and able to withstand extreme condition [3]. However, temperature is one of major factors affecting the survival of microorganisms in Antarctica [4]. Cold adapted fungi that grow optimally at temperatures less than 15 °C and cannot grow above 20 °C are called “psychrophilic fungi or cold-adapted fungi” [5]. Since the 1960s, bioactive secondary metabolites have been isolated and structurally characterised from psychrophiles [6] such as bioactive non-ribosomal peptides (NRPs),

compounds derived from the NRP-synthetase that were developed into antibiotics i.e. bacitracin, polymyxin B and E [7].

Glaciozyma antarctica PI12 belongs to the family Kriegeriales [8] and was isolated from a marine environment in Antarctica [9]. Recently, this psychrophilic yeast was reclassified from *Leucosporidium antarcticum* to *Glaciozyma antarctica* PI12 [10] and has also been isolated from various locations in Antarctica [11, 12]. *Glaciozyma antarctica* PI12 was widely investigated in recent years in most antifreeze protein production and cold-active enzyme, including an extracellular serine proteinase [13]. The main objective of this paper is to describe the isolation and structure characterisation from psychrophilic yeast *G. antarctica* PI12.

Materials and Methods

Biological materials

The *G. antarctica* PI12 sample used was a gift from Prof. Dr. Nazalan Najimudin from Universiti Sains Malaysia in Penang, Malaysia. It was identified based on the biochemical characteristics and the sequence of the internal transcribed spacer (ITS) as well as the LSU rRNA (Accession numbers JX89896955 and JX896956) by the National Collection of Yeast Cultures, Norwich, UK. The strain was cultured on Tryptic Soy Agar for 10 days at 4 °C until a single colony was obtained [14]. The culturing of *G. antarctica* PI12 was conducted in collaboration with the Malaysia Genome Institute.

Cultivation of yeast

10 µL of the *G. antarctica* PI12's glycerol stock was sub-cultured on the Yeast Extract-Peptone-Dextrose (YPD) agar with 50 µg/mL of ampicillin and 50 µg/mL of kanamycin at 12 °C until a single colony was obtained. Next, a starter culture of *G. antarctica* PI12 was prepared by inoculating a single colony of *G. antarctica* PI12 from the agar plate into 10 mL of YPD medium (50 mL falcon tube) with 50 µg/mL of ampicillin and 50 µg/mL of kanamycin. It was then cultured at 12 °C at 180 rpm until mid-log phase (OD₆₀₀: 1.0 to 1.8) (5 days). Thirdly, a fixed amount (10⁶ yeast cells/mL) of starter culture was inoculated into 50 mL of YPD medium (three 250 mL of conical flask were used) with two antibiotics, and then cultured at 12 °C at 180 rpm until stationary phase at day 14 [15].

Extraction and isolation

The production culture was centrifuged (10,000 rpm, 4 °C, 5 min). The supernatant was filtered under vacuum to remove any cells that were not pelleted out and its pH was adjusted to 4.0. The supernatant was extracted thrice with ethyl acetate, and concentrated using a rotary evaporator. The ethyl acetate (EtOAc) extract (200.7 mg) of *G. antarctica* PI12 was subjected to radial chromatography (RC) with 1 mm thickness silica gel on a round glass plate and eluted with the mixtures of dichloromethane (DCM) and methanol (MeOH) with increasing polarity (started with DCM/MeOH, 9.6: 0.4). The eluates showing the same profile on thin layer chromatography (TLC) were combined to give three fractions (I-III). Purification of Fraction I (1-4) (29.7 mg) was carried out using RC with a silica gel plate of 0.5 mm thickness eluted with DCM and MeOH (9.2: 0.8) in 5% polarity increment to yield Compound **1** (1 mg). Purification of Fraction III (5-7) (48.8 mg) was conducted by utilising another RC with silica gel plate of 0.5 mm thickness. Elution with DCM and MeOH (8.4: 1.6) produced Compound **2** (1 mg), Compound **3** (1.5 mg), and Compound **4** (2 mg).

Results and Discussion

Characterization study

Cyclo(-Pro-Val) (**1**) is a white amorphous solid. ESI-MS $[M + H]^+$ at m/z : 197. IR $\bar{\nu}_{\max}$ (ATR) cm^{-1} : 3191, 2956, 1640, 1453, 1110, 922. ¹H NMR (MeOD, 700 MHz) δ_{H} (ppm) and ¹³C NMR (MeOD, 175 MHz) δ_{C} (ppm) data are tabulated in Table 1.

(-)-Cyclo(-Pro-Tyr) (**2**) is a white amorphous solid. $[\alpha]_{\text{D}}^{20}$ -43.1 (c 0.14, ethanol). ESI-MS $[M + H]^+$ ion at m/z : 261. IR $\bar{\nu}_{\max}$ (ATR) cm^{-1} : 3248, 2927, 2853, 1747, 1658, 1449, 1252, 1174, 1114, 1017, 858. ¹H NMR (MeOD, 700 MHz) δ_{H} (ppm) and ¹³C NMR (MeOD, 175 MHz) δ_{C} (ppm) data are tabulated in Table 1.

(-)-Cyclo(-Pro-Phe) (**3**) is a white amorphous solid. $[\alpha]_D^{20}$ -72 (c 0.7, methanol). ESI-MS $[M + H]^+$ ion at m/z : 169. IR $\bar{\nu}_{\max}$ (ATR) cm^{-1} : 3357, 2956, 2108, 1647, 1449, 1318 and 1087. ^1H NMR (MeOD, 700 MHz) δ_{H} (ppm) and ^{13}C NMR (MeOD, 175 MHz) δ_{C} (ppm) data are tabulated in Table 1.

(+)-Cyclo(-Pro-Leu) (**4**) is a white powder. $[\alpha]_D^{20}$ +28.1 (c 0.032, ethanol). ESI-MS $[M + H]^+$ ion at m/z : 211. IR $\bar{\nu}_{\max}$ (ATR) cm^{-1} : 3222, 2958, 2930, 2872, 1686, 1676, 1426, 1302, 1275, 1235, 1157, 1102, 1032, 996-919. ^1H NMR (MeOD, 700 MHz) δ_{H} (ppm) and ^{13}C NMR (MeOD, 175 MHz) δ_{C} (ppm) data are tabulated in Table 1.

Table 1. ^1H and ^{13}C NMR data (700 and 175 MHz, MeOD) for compounds **1-4**

Position	APT (ppm)				^1H (mult., ΣH)			
	1	2	3	4	1	2	3	4
1-N	-	-	-	-	-	-	-	-
2	165.8	165.7	165.5	167.1	-	-	-	-
3	59.9	56.6		53.1	3.92 (<i>d</i> , 1H)	4.37 (<i>m</i> , 1H)	4.25 (<i>t</i> , 1H)	4.00 (<i>t</i> , 1H)
4-NH	-	-	-	-	7.93 (<i>br-s</i> , 1H)	7.65 (<i>s</i> , 1H)	7.62 (<i>s</i> , 1H)	7.99 (<i>br-s</i> , 1H)
5	170.9	169.6	169.3	171.1	-	-	-	-
6	58.7	58.7	58.6	58.9	4.11 (<i>t</i> , 1H)	4.05 (<i>m</i> , 1H)	4.14 (<i>t</i> , 1H)	4.18 (<i>t</i> , 1H)
7	28.3	28.0	28.0	27.9	1.82 (<i>m</i> , 1H)	2.33 (<i>q</i> , 1H)	2.29 (<i>m</i> , 1H)	2.12 (<i>q</i> , 1H)
					2.14 (<i>m</i> , 1H)	1.99 (<i>q</i> , 1H)	1.92 (<i>q</i> , 1H)	1.89 (<i>q</i> , 1H)
8	22.4	21.4	21.3	22.9	1.80 (<i>m</i> , 1H)	2.01 (<i>m</i> , 1H)	2.01 (<i>m</i> , 1H)	1.91 (<i>m</i> , 1H)
					1.86 (<i>m</i> , 1H)	1.93 (<i>m</i> , 1H)	1.93 (<i>m</i> , 1H)	1.80 (<i>m</i> , 1H)
9	45.1	44.6	44.5	45.3	3.33 (<i>t</i> , 1H)	3.63 (<i>m</i> , 1H)	3.68 (<i>m</i> , 1H)	3.36 (<i>t</i> , 1H)
					3.38 (<i>t</i> , 1H)	3.57 (<i>m</i> , 1H)	3.58 (<i>m</i> , 1H)	3.37 (<i>t</i> , 1H)
10	28.2	36.3	36.3	38.1	2.32 (<i>m</i> , 1H)	3.46 (<i>dd</i> , 1H)	3.47 (<i>dd</i> , 1H)	1.75 (<i>t</i> , 1H)
						2.79 (<i>dd</i> , 1H)	2.79 (<i>dd</i> , 1H)	1.36 (<i>t</i> , 1H)
11	18.7	-	-	24.5	0.99 (<i>d</i> , 3H)	-	-	1.85 (<i>m</i> , 1H)
12	16.7	-	-	23.2	0.83 (<i>d</i> , 3H)	-	-	0.86 (<i>d</i> , 3H)
13	-	-	-	22.3	-	-	-	0.85 (<i>d</i> , 3H)
1'	-	126.3	126.2	-	-	-	-	-
2'/6'	-	130.7	130.7	-	-	7.05 (<i>dd</i> , 2H)	7.02 (<i>dd</i> , 2H)	-
3'/5'	-	115.0	130.3	-	-	6.79 (<i>dd</i> , 2H)	6.73 (<i>dd</i> , 2H)	-
4'	-	156.1	128.2	-	-	5.35 (<i>br-s</i> , 1H), -OH	7.24 (<i>d</i> , 1H)	-

Compound **1** was isolated as white amorphous solid. Its molecular formula was determined as $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_2$, obtained by ESI-MS $[M + H]^+$ m/z 197. The IR spectrum showed the presence of carbonyl groups from amide (1640 cm^{-1}), and also saturated methylene groups at the range of $2956\text{--}2889\text{ cm}^{-1}$. The ^{13}C -APT NMR spectrum showed the location of ten signals representing two quaternary, three methines, three methylenes and two methyls carbons. The double bond equivalent (DBE) value of four indicated that this compound contained two double bonds and two cyclic aliphatic. The most deshielded carbons at δ_{C} 170.9 and 165.8 ppm were indicated as an asymmetrical carbonyl group. Three saturated non-equivalent methylenes signals at δ_{C} 28.3 (C-7), 22.4 (C-8) and 45.1 (C-9) were attributed to a pyrrolidine ring. A methine carbon at δ_{C} 58.7 ppm (C-6) was the specific of methine signal of diketopiperazine alkaloid and another one methine at δ_{C} 59.9 ppm (C-3) indicated the presence of a methine existed

between carbonyl and secondary amide while the two non-equivalent gem-dimethyls at δ_C 18.7 ppm (C-11) and δ_C 18.7 ppm (C-12) bound a saturated methine at δ_C 28.2 ppm (C-10).

Meanwhile, 12 proton signals representing 16 protons appeared in the ^1H -NMR spectrum as shown in Table 1. Six of the signals came from the three non-equivalent methylenes proton at H-7, H-8 and H-9, forming a basic pyrrolidine ring. An aminated methine proton, H-6 (δ_H 4.11 ppm) resonated as a triplet, indicating that C-6 was adjacent to a methylene group (C-7). Another methine proton at H-10 (δ_H 2.32 ppm) was resonated as a multiplet, because it was bound to two non-equivalent gem-dimethyls at C-11 and C-12. Comparison of the ^1H and ^{13}C -APT NMR spectra of the compound suggested that it was Cyclo(-Pro-Val) (Fig. 1) [16]. Thus, the structure of Cyclo(-Pro-Val) (**1**) was previously isolated from *Aspergillus fumigatus* [17], *Pseudomonas aeruginosa* [18], and *Stenotrophomonas* sp. [19].

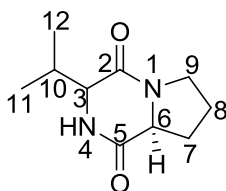


Figure 1. Chemical structure of compound **1**

Compounds **2** and **3** (Figure 2) were found in Fraction III as a UV absorbing spot, which stained to violet with anisaldehyde/sulphuric acid. The ^1H NMR spectrum of Compound **2** showed two *ortho*-coupled signals at δ_H 7.05 (H-2', 6') and 6.79 (H-3', 5'), which pointed to an AA'-BB' system of a 1,4-disubstituted aromatic ring, as well as two signals at δ 4.37 and 4.05 for two methines attached to electron withdrawing substituents. The spectrum proton of Compound **2** showed also two doublet of doublets for an ABX system of a methylene group at δ_H 3.46 and 2.79 (CH₂-10), as well as three methylene multiplets (CH₂-7) (δ_H 2.33, 1.99) attached to a heteroatom and CH₂-8, 9 (δ_H 2.01, 1.93, 3.63, 3.57). The ESI mass spectra determined the molecular weight of Compound **2** as 261 Dalton by (+)-ESI mode. The structure was further confirmed as (-)-cyclo(-Pro-Tyr) (**2**) by comparison with an authentic sample and spectra from our collection [20]. (-)-cyclo(-Pro-Tyr) (**2**) was previously isolated from *Pseudomonas fluorescens* GcM5-1A [21], *Streptomyces* sp. H7372 and ML 1532 [22, 23], *Pseudomonas aeruginosa* [18] and *Lysobacter capsici* AZ78 [24]. The difference between Compound **2** and Compound **3** lay on C-4', where Compound **2** had a hydroxy at C-4' (Figure 2).

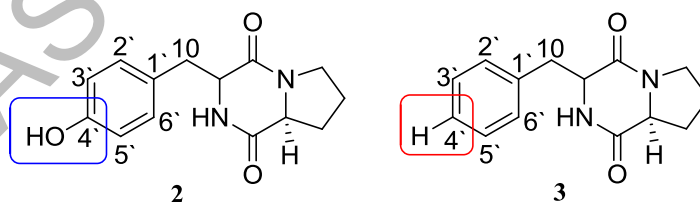


Figure 2. Structure of compounds **2** and **3**

From the spectroscopy data and comparison with the literature, compound **3** was confirmed as (-)-cyclo(-Pro-Phe) (**3**) [17] previously isolated from microbial sources, including *Streptomyces rochei* 87051-3 [16], *Streptomyces sudanensis*. A4.4 [25], *Tyridiomyces formicarum* [26], and *Alternaria alternata* [27].

(+)-Cyclo(-Pro-Leu) (**4**), [α]_D²⁰ +28.1 (*c* 0.032, ethanol), was isolated as a white amorphous material with molecular formula C₁₁H₁₈N₂O₂, obtained by ESI-MS [M + H]⁺ *m/z* 211. The IR spectrum showed bands attributed to the aliphatic carbons (2958, 2930, 2872 cm⁻¹) and amide (1686, 1676 cm⁻¹) functional groups. The ^1H NMR spectrum

of compound **4** indicated the presence of three methines at H-3, H-6 and H-11; four methylenes at H-7, H-8, H-9 and H-10; and two non-equivalent dimethyls at H-12 and H-13 (Table 1). ^{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} 167.1 (C-2) and 171.1 (C-5) suggested that these carbon signals were due to the carbonyl carbon of the amide groups. The DBE value of four indicated that this compound was containing two double bonds and two cyclic aliphatic. (+)-cyclo(Pro-Leu) (**4**) previously isolated from the Gram-negative Proteobacteria of *Burkholderia cenocepacia* and *Serratia marcescens* [28], *Alternaria alternata* [29], the bacteria *Pseudoalteromonas* sp. [30] and *Vibrio alginolyticus* [31].

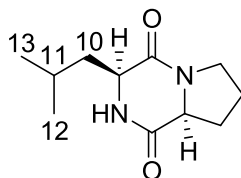


Figure 3. Structure of compounds **4**

Conclusion

Four diketopiperazine derivatives, i.e. cyclo(-Pro-Val) (Compound**1**), (-)-cyclo(-Pro-Tyr) (Compound **2**), (-)-cyclo(-Pro-Phe) (Compound**3**) and (+)-cyclo(-Pro-Leu) (Compound**4**) were isolated and reported for the first time from psychrophilic yeast *G. antarctica* PI12. All the compounds reported in the present study were not subjected to further bioactivity studies due to insufficient amounts.

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