

(-)-Glaciantarcin, a New Dipeptide and Some Secondary Metabolites from the Psychrophilic Yeast *Glaciozyma antarctica* PI12

((-)-Glasiantarsin, satu Dipeptida Terbaharu dan Beberapa Metabolit Sekunder
daripada Yis Psikrofilik *Glaciozyma antarctica* PI12)

ANDI R. ROSANDY, YEOH CHIANN YING, CHEAH YOKE KQUEEN, SENG JOE LIM, JALIFAH LATIP, A. MUNIR A. MURAD,
MUNTAZ A. BAKAR & ROZIDA M. KHALID*

ABSTRACT

A new dipeptide, (-)-glaciantarcin (**1**) and three known compounds, cyclo(-Pro-Gly) (**2**), 1-(2-deoxypentofuranosyl)-5-methyl-2,4(1H,3H)-pyrimidinedione (**3**) and vidarabine (Ara-A) (**4**), were isolated from *Glaciozyma antarctica* PI12, a cold-adapted yeast. The chemical structures were elucidated by FT-IR, NMR and mass spectrometry. The cytotoxicity and antioxidant activities of compounds **1-4** were evaluated by using the MTT bioassay on MCF-7 (human breast cancer cell line), PC-3 (human prostate cancer cell line) and HEK-293 (normal human embryonic kidney cell line) and DPPH free radical scavenging activity, respectively. At concentration of 400 μ M, all compounds showed the highest activity on MCF-7, with compound **1** at 65%, compound **2** (70%), compound **3** (66%) and compound **4** (58%) cell viability. All compounds exhibited weak antioxidant properties. To the best of our knowledge, this is the first report of compounds **1-4** from *Glaciozyma antarctica*.

Keywords: Biological activities; dipeptide; (-)-glaciantarcin; *Glaciozyma antarctica* PI12; psychrophilic yeast

ABSTRAK

Satu dipeptida terbaharu iaitu ((-)-glasiantarsin (**1**) dan tiga sebatian yang telah diketahui; siklo (-Pro-Gli) (**2**), 1-(2-Deoksipentofuranosil) -5-metil-2,4 (1H, 3H) -pirimidinedion (**3**) dan vidarabin (Ara-A) (**4**) telah diasingkan daripada *Glaciozyma antarctica* PI12, yis yang teradaptasi sejuk. Struktur kimia dijelaskan oleh spektroskopi inframerah (IM), ultra lembayung (UL), resonans magnetik nuklear (RMN) dan spektroskopi jisim. Aktiviti kesitotoksikan dan antioksidan daripada sebatian **1-4** dinilai menggunakan bioasai MTT ke atas MCF-7 (sel kanser payudara manusia), PC-3 (sel kanser prostat manusia) dan sel HEK-293 (sel normal) serta aktiviti perencatan radikal bebas DPPH. Pada kepekatan 400 μ M, semua sebatian menunjukkan aktiviti tertinggi ke atas MCF-7, iaitu masing-masing: sebatian **1** (62 ± 0.9), **2** (66 ± 1.6), **3** (62 ± 1.3) dan **4** (55 ± 1.7). Kesemua sebatian terencil menunjukkan sifat antioksidan yang lemah. Sepanjang pengetahuan kami, ini merupakan laporan pertama sebatian **1-4** daripada *Glaciozyma antarctica*.

Kata kunci: Aktiviti biologi; dipeptida; ((-)-glasiantarsin; *Glaciozyma antarctica* PI12; yis psikrofilik

INTRODUCTION

Since the 1960s, bioactive secondary metabolites were isolated and structurally characterised from microorganisms which have adopted strategies to grow in extreme terrestrial environments (Lesley-Ann & David 2015). *Glaciozyma antarctica* PI12 belongs to the family Kriegeriales (Di Menna 1960) and was isolated from a marine environment in Antarctica (Fell et al. 1969). Recently, this psychrophilic yeast was reclassified from *Leucosporidium antarcticum* to *Glaciozyma antarctica* PI12 (Turchetti et al. 2011) and was also isolated from various locations in Antarctica (Connell et al. 2008; Donachie 1995). Turkiewicz et al. (2005) reported that *G. antarctica* PI12 has optimum growth temperature of around 15°C and is able to tolerate higher temperatures up to 20°C (Morita 1975).

This paper reports the isolation and elucidation of secondary metabolites from *G. antarctica* PI12 fermented at 12°C with complete nutrient (optimised growth

conditions). A new dipeptide, (-)-glaciantarcin (**1**) and three known compounds, cyclo(-Pro,Gly) (**2**) (Ishizu et al. 1991), 1-(2-deoxypentofuranosyl)-5-methyl-2,4(1H,3H)-pyrimidinedione (Setyowati et al. 2008) and vidarabine (Ara-A) (**4**) (Lagoja 2005), were isolated from the ethyl acetate extract of *G. antarctica* PI12 and their structures were established by Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry techniques. The antioxidant properties and cytotoxicity activity of compounds **1-4** against two cancer cell lines, MCF-7 and PC-3 and the normal cell line HEK 293 are also included.

MATERIALS AND METHODS

GENERAL

Silica gel; Kieselgel 60 PF254 (art. no. 7749, Merck) and aluminum sheets 20 × 20 cm of the silica gel 60 F254

of 0.25 mm thickness (art. no. 5554, MERCK) were used for radial and thin layer chromatography, respectively. Structures of the pure compounds were determined based on the spectral data recorded on Shimadzu UV-260 spectrophotometer, optical rotation (Rudolph research analytical, Autopol VI), Jasco J-815 CD spectrometer, Frontier Perkin-Elmer FTIR/NIR spectrophotometer and Bruker NMR 700 MHz cryo-probe instrument that included 1-D and 2-D NMR. ESI-MS were recorded by using LCMS-Ultra performance liquid chromatography (UPLC), Brand WATER.

BIOLOGICAL MATERIAL AND CULTIVATION OF YEAST

The *G. antarctica* PI12 was originally isolated from Casey Research Station, in Antarctica. This yeast was identified based on its 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 (Hashim et al. 2014). Firstly, 10 µL of the *G. antarctica* PI12 glycerol stock was sub-cultured on the YPD agar with 50 µg/mL of ampicillin and 50 µg/mL kanamycin at 12°C until a single colony was obtained. Secondly, 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 kanamycin. Then it was 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 units of 250 mL conical flask were used) with two antibiotics and then cultured at 12°C at 180 rpm until stationary phase on day 14 (Hashim et al. 2013).

EXTRACTION AND ISOLATION

The fermented culture was centrifuged at 10,000 rpm, 4°C, for 5 min. The supernatant was filtered to remove cell debris and extracted thrice with ethyl acetate, to be concentrated under reduce pressure evaporator. The 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 mixtures of DCM and MeOH with increasing polarity (initially DCM/MeOH, 9.6 : 0.4). Eluents that showed the same profile on thin layer chromatography (TLC) chromatogram were combined to give three fractions (I-III). Purification of fraction I (1-4) (29.7 mg) was carried out by 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** (4 mg). Purification of fraction II (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) provided compound **2** (2 mg), compound **3** (1.5 mg) and compound **4** (7.5 mg).

CYTOTOXICITY ASSAY

Compounds **1-4** were subjected to anticancer screening by using breast adenocarcinoma cells (MCF-7) and human prostate cancer cells (PC-3). Toxicity assessment of the compounds was conducted by using human embryonic kidney normal cell line (HEK-293). All cell lines were grown in a suitable media and maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. For the assay, cells were detached, centrifuged and the viability of the cells was determined by Trypan blue dye exclusion technique. The cell suspension was diluted to give the optimal cell seeding density. Cytotoxic analysis of the cells was determined by using iCELLigence impedance-based, time-dependent cellular response profiles (TCRPs) system and further confirmed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The anticancer drug, doxorubicin was used as a positive control.

Cell viability was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which was based on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a purple formazan product. Firstly, cells were plated at 2.5 × 10⁴ cells/mL of density in 96-well plate. After an overnight growth, the cells were treated with Compounds **1-4**, with concentrations ranging from 12.5 to 400 µM for 48 h. Next, the cells were incubated with 10 µL of 4 mg/mL MTT dye at 37°C for 3 h. After the media was removed from the plate, 100 µL of DMSO was added, followed by absorbance measurement at 570 nm by using a microplate reader (Mosmann 1983).

ANTIOXIDANT (DPPH)

The stock concentration of each compound was 1 mg/mL in MeOH. For bioassay, 50 µL of each sample was added to 150 µL of 0.3 mM solution of DPPH. The mixture was vigorously shaken and allowed to stand in a dark place for 30 min; the absorbance at 517 nm was determined by using a UV spectrophotometer and the scavenging activity percentage was calculated. A blank solution of MeOH was prepared. BHT (butylated hydroxy toluene) and BHA (butylated hydroxy anisole) were used as positive controls (Brand-Williams et al. 1995).

(-)-GLACIANTARCIN (1)

White solid; -102.07 (c 0.10, MeOH); m.p 186-187°C; IR (ATR): 3044-2881, 1660, 1326, 1454, 1094, 886-758 cm⁻¹; ESI-MS: [M + H⁺] *m/z* 170.2350 (calcd. for C₇H₁₂N₃O₂: 170.1220); ¹H NMR (MeOD, 700 MHz) and ¹³C(APT) NMR (175 MHz): Table 1.

CYCLO(-PRO-GLY) (2)

Clear needle crystal; m.p 222-223°C; IR (ATR): 3191, 1640, 1453, 1332, 1294-1217, 1110, 1004, 922-764 cm⁻¹; ESI-MS: [M + H⁺] *m/z* 155.1750 (calcd. for C₇H₁₁N₂O₂: 155.0821); ¹H NMR (MeOD, 700 MHz): δ_H 4.13 (Ψ_d, 1H, H-3), 3.77

(*d*, 1H, H-3), 8.13 (*br-s*, 1H, 4-NH), 4.25 (Ψ t, $J = 7$ Hz, 1H, H-6), 2.34 (*t*, $J = 7$ Hz, 1H, H-7), 1.99 (*t*, $J = 7$ Hz, 1H, H-7), 2.04 (*m*, 1H, H-8), 1.95 (*m*, 1H, H-8), 3.57 (Ψ t, $J = 7$ Hz, 1H, H-9), 3.54 (Ψ t, $J = 7$ Hz, 1H, H-9); ^{13}C (APT) NMR (MeOD, 175 MHz): δ_{C} 165.1 (Cq-2), 45.6 (CH₂-3), 170.6 (Cq-2), 58.4 (CH-6), 27.9 (CH₂-7), 21.9 (CH₂-8), 44.9 (CH₂-9).

1-(2-DEOXPENTOFURANOSYL)-5-METHYL-2,4-(1*H*,3*H*)-PYRIMIDINEDIONE (3)

White solid; UV active; m.p 355-356°C; IR (ATR): 3500-3200, 2931-2839, 1678, 1421, 1272, 1029 cm⁻¹; ESI-MS: [M + H⁺] m/z 243.2240 (calcd. for C₁₀H₁₅N₂O₅: 243.0981); ^1H NMR (MeOD, 700 MHz): δ_{H} 7.83 (*s*, 1H, H-6), 1.90 (*s*, 3H, H-7), 6.29 (*t*, $J = 7$ Hz, 1H, H-2'), 2.24 (*m*, 2H, H-3'), 4.41 (*m*, 1H, H-4'), 3.92 (*q*, $J = 3.36$ Hz, 1H, H-5'), 3.81 (*dd*, $J_1 = 3.22$, $J_2 = 12.04$ Hz, 1H, H-6'), 3.78 (*dd*, $J_1 = 3.64$, $J_2 = 12.04$ Hz, 1H, H-6'); ^{13}C (APT) NMR (MeOD, 175 MHz): δ_{C} 151.1 (Cq-2), 165.0 (Cq-4), 110.3 (CH-5), 136.8 (Cq-6), 11.0 (CH₃-7), 84.8 (CH-2'), 39.8 (CH₂-3'), 70.8 (CH-4'), 87.4 (CH-5'), 61.4 (CH₂-6').

VIDARABINE (ARA-A) (4)

White solid; UV active; IR (ATR): 3400, 3300, 3134-2879, 1604-1420, 1577, 1100-1028 cm⁻¹; ESI-MS: [M + H⁺] m/z 268.3350 (calcd. for C₁₀H₁₄N₅O₄: 268.1047); ^1H NMR (MeOD, 700 MHz): δ_{H} 8.20 (*s*, 1H, H-2), 8.33 (*s*, 1H, H-8), 5.97 (*d*, $J = 7$ Hz, 1H, H-2'), 4.34 (*dd*, $J_1 = 7$, $J_2 = 14$ Hz, 1H, H-3'), 4.75 (*t*, $J = 7$ Hz, 1H, H-4'), 4.18 (*q*, $J = 7$ Hz, 1H, H-5'), 3.91 (*dd*, $J_1 = 7$, $J_2 = 14$ Hz, 1H, H-6'), 3.76 (*dd*, $J_1 = 7$, $J_2 = 14$ Hz, 1H, H-6'); ^{13}C (APT) NMR (MeOD, 175 MHz): δ_{C} 152.1 (Cq-2), 148.6 (Cq-4), 119.6 (Cq-5), 156.2 (Cq-6), 140.6 (Cq-8), 89.8 (CH-2'), 71.3 (CH-3'), 74.0 (CH-4'), 86.8 (CH-5'), 62.1 (CH₂-6').

RESULTS AND DISCUSSION

A new compound, (-)-glaciantarcin (**1**) was isolated as white amorphous solid (Figure 1). The molecular formula is C₇H₁₁N₃O₂, generated using MS [M + H⁺] at m/z 170.1034 (calc. for C₇H₁₂N₃O₂ 170.1220). Compound **1** was suggested to have a carbonyl group linked with the secondary and tertiary amide (1660 cm⁻¹) based on its IR. In addition, the IR spectra also showed a weak band presence for a primary amine (885-758 cm⁻¹) and secondary amine (1326 cm⁻¹), which supported by signals from ^1H -NMR at δ_{H} 4.60 (2H, *br-s*, 8-NH₂) and 8.02 (1H, *br-s*, 1-NH) ppm, respectively (Table 1). The ^1H -NMR showed signals of a methyl group at δ_{H} 1.45 (3H, *d*, $J = 7.0$ Hz, H-9) adjacent to methine group at δ_{H} 4.03 (1H, *q*, $J_1 = 7.0$, $J_2 = 14.0$ Hz, H-8). The olefinic protons were confirmed by signals at δ_{H} 5.63 (1H, *d*, $J = 7.0$ Hz, H-6) and 7.41 (1H, *d*, $J = 7.0$ Hz, H-7). The presence of two carbonyl groups was confirmed by ^{13}C -NMR at signals δ_{C} 170.2 (C-5) and 167.4 (C-2). Two signals of methines at δ_{C} 100.9 (C-6) and 142.0 (C-7) indicated the presence of an olefinic carbon, that is linked to a quaternary carbon C-5, while the other three shielded carbons (C-3, C-8, and C-9) showed the existence of an aliphatic amide in the molecule. The DBE value of C₇H₁₁N₃O₂ is four, indicating that the alkaloid consists of three double bonds (one olefinic and two carbonyls) and one ring of diazepine derivative.

The structure of Compound **1** was confirmed by using 2D NMR, including HMBC and H¹/H¹ COSY. The HMBC experiment showed that H-8 has correlations with carbonyl C-5 and methylene C-3, confirming the position of the 4-(1-aminoethyl) as a branch in this molecule (Figure 2).

Most probably Compound **1** was derived from the nonribosomal peptide synthetase (bimodular NRPS) pathway. The proposed mechanism was by using β -amino acid (3-amino-3-hydroxy propanoic acid) as the starter

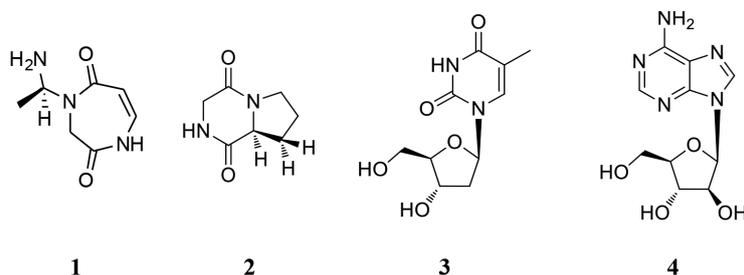


FIGURE 1. Compounds isolated from *G. antarctica* PI12

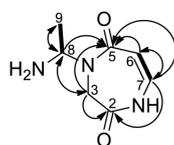


FIGURE 2. 2D Correlation NMR of (-)-glaciantarcin (**1**); HMBC (→), COSY (---)

TABLE 1. NMR data of (-)-glaciantarcin (**1**)

Position	1	
	δ_{H} (m, J in Hz)	δ_{C}
1-NH	4.60 (br-s)	-
2	-	167.4
3	3.94 (2H, d, $J = 7.0$ Hz)	44.0
5	-	170.2
6	5.63 (d, $J = 7.0$ Hz)	100.3
7	7.41 (d, $J = 7.0$ Hz)	142.1
8	4.03 (q, $J_1 = 7.0, J_2 = 14.0$ Hz)	50.3
8-NH ₂	8.02 (br-s)	-
9	1.45 (d, $J = 7.0$ Hz)	18.0

*HMBC correlations are from hydrogen(s) stated to the indicated carbon for 3-bond and 2-bond couplings

unit with glycine as extender (Figure 3). The utilisation of β -amino acid as a starter was well established in the microbial metabolites (Kudo et al. 2014). These substrates were tethered to the thiolation domains of the NRPS and underwent condensation reaction. Next, subsequent cyclisation and dehydration took place for the formation of the seven-membered diazepine ring **13**. Further modifications were needed for the production of Compound **1**.

Further purification with radial chromatography afforded another dipeptide (Compound **2**), and pyrimidine derivatives Compound **3** and Compound **4**. All these compounds were known, and were isolated from microbial sources. Compounds **2-4** were identified: Compound **2** was cyclo(Pro-Gly), Compound **3** was

1-(2-deoxypentofuranosyl)-5-methyl-2,4(1H,3H)-pyrimidinedione, and Compound **4** was vidarabine (Ara-A) by comparison of their NMR and MS data with the reported values in literature. This is the first report on Compounds **1-4** from *G. antarctica* PI12 (Figure 1).

Compound **2** was reported to be first isolated from fungi *Aspergillus fumigatus*, with antimicrobial activities (Furtado et al. 2005). The pyrimidine derivative Compound **3** was originally isolated from sponge, with *in vitro* cytotoxicity (HeLa, Raji and myolema cells) bioactivities (Setyowati et al. 2008). Compound **4** was originally developed as a synthetic analogue from spongouridine, a compound isolated from the Caribbean sponge *Tethya crypta* (Tethylidae) with good antiviral activity (Bergmann et al. 1951). However, Compound **4** was isolated from

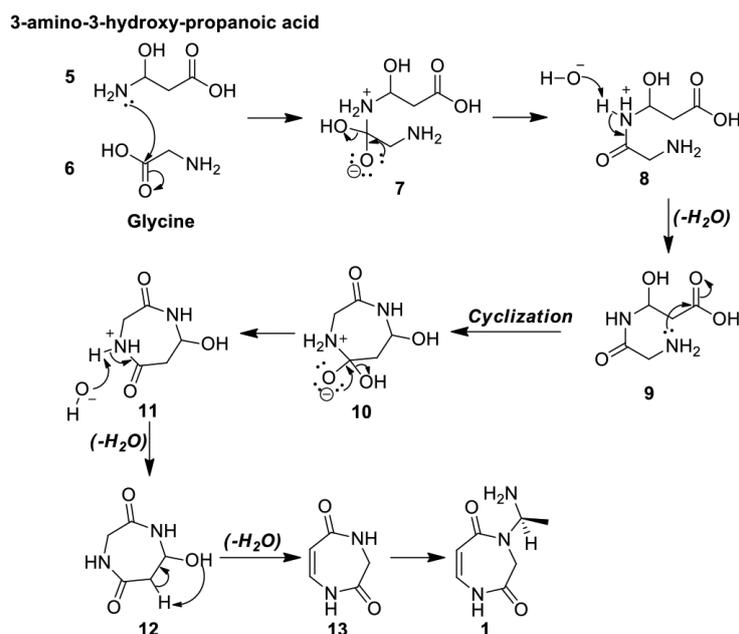


FIGURE 3. Proposed Biosynthesis of (-)-glaciantarcin (**1**)

sponge *Haliclona* sp., proving that this compound can also be synthesised by a biological system (Wang et al. 2009). All of the isolated Compounds (**1-4**) were assayed for cytotoxicity against human breast cancer (MCF-7) and human prostate cancer (PC-3) cell lines. Their toxicity towards normal cells was also tested by using a normal human embryonic kidney (HEK-293) cell line. At the highest concentration of 400 μ M, all compounds showed the best activity on MCF-7, with Compound **1** (65%), Compound **2** (70%), Compound **3** (66%) and Compound **4** (58%) cell viability. A lower concentration of all compounds gave more than 70% cell viability.

The DPPH radical scavenging activity of the compounds showed weak antioxidant activities at a fixed concentration of 100 μ M; with Compounds **1-4** at 7.49%, 24.83%, 11.56%, and 4.15%, respectively.

CONCLUSION

The investigation on the chemical constituents from EtOAc extract of psychrophilic yeast *Glaciozyma antarctica* PI12 resulted in the isolation of a new compound namely (-)-glaciantarcin (**1**) and three known compounds which were cyclo(-Pro-Gly) (**2**), 1-(2-deoxypentofuranosyl)-5-methyl-2,4(1H,3H)-pyrimidinedione (**3**) and vidarabine (Ara-A) (**4**). In cytotoxicity bioassay, at the concentration of 400 μ M, all compounds showed the highest activity on MCF-7, with Compounds **1-4** at 65%, 70%, 66% and 58% cell viability, respectively. All compounds showed weak DPPH radical scavenging activity. This study shows that *Glaciozyma antarctica* mainly produces nitrogen-based compounds when grown in optimised conditions.

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Andi R. Rosandy, Seng Joe Lim, Jalifah Latip, Muntaz A. Bakar
& Rozida M. Khalid*
School of Chemical Science and Food Technology
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
43600 UKM Bangi, Selangor Darul Ehsan
Malaysia

A. Munir A. Murad
School of Biosciences and Biotechnology
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
43600 UKM Bangi, Selangor Darul Ehsan
Malaysia

Yeoh Chiann Ying & Cheah Yoke Kqueen
Department of Biomedical Sciences
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
43400 UPM Serdang, Selangor Darul Ehsan
Malaysia

*Corresponding author; email: rozidakhalid@ukm.edu.my

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