Malaysian Journal of Analytical Sciences, Vol 23 No 4 (2019): 748 - 762
DOI: https://doi.org/10.17576/mjas-2019-2304-21

MALAYSIAN JOURNAL OF ANALYTICAL SCIENCES
Published by The Malaysian Analytical Sciences Society
ISSN 1394 - 2506

PSYCHROTOLERANT ACTINOBACTERIA *Barrientosiimonas humi* 39^T AS A SOURCE OF DIKETOPIPERAZINE

(Aktinobakteria Psikrotoleran *Barrientosiimonas humi* 39^T Sebagai Sumber Sebatian Diketopiperazin)

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Received: 31 March 2018; Accepted: 17 April 2019

Abstract

Barrientosiimonas humi 39^T (*B. humi*) is a psychrotolerant actinobacteria from the family Dermacoccaceae, was first isolated from Antarctica. Five diketopiperazine derivatives: cyclo(-Pro-Val) (**1**), (-)-cyclo(-Pro-Tyr) (**2**), cyclo(-Pro-Phe) (**3**), (+)-cyclo(-Pro-Leu) (**4**), and L-tyrosine (**5**) were isolated from the crude extract of *B. humi*, and this is the first reported compounds isolated from *B. humi*. The crude extracts were obtained *via* liquid-liquid fractionation using ethyl acetate as the organic solvent. The compounds were isolated and purified using chromatographic methods such as vacuum liquid chromatography and radial chromatography. The chemical structures were elucidated by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry.

Keywords: *Barrientosiimonas humi* 39^T, diketopiperazine, *Dermacoccaceae*

Abstrak

Barrientosiimonas humi 39^T (*B. humi*) merupakan aktinobakteria psikrotoleran termasuk dalam famili Dermacoccaceae, asalnya dipencilkan dari Antartika. Lima terbitan diketopiperazin; siklo(-Pro-Val) (**1**), (-)-siklo(-Pro-Tir) (**2**), (-)-siklo(-Pro-Phe) (**3**), (+)-siklo(-Pro-Leu) (**4**) dan L-tirosina (**5**) diasingkan daripada ekstrak mentah *B. humi*, dan ini merupakan laporan pertama sebatian dipencilkan dari *B. humi.* Ekstrak mentah diperolehi melalui fraksi cecair-cecair menggunakan etil asetat sebagai pelarut. Sebatian-sebatian dipencilkan dan ditulenkan menggunakan kaedah kromatografi seperti kromatografi cecair vakum dan kromatografi jejari. Struktur kimia ditentukan menggunakan spektroskopi resonans magnetik nuklear (RMN) dan spektrometri jisim.

Kata kunci: *Barrientosiimonas humi* 39^T, diketopiperazine, *Dermacoccaceae*

Introduction

Psychrotolerant microorganisms are mostly present in the extremely cold environment [1, 2] but exhibit slower growth rates, as they automatically encounter number of growths limiting conditions such as reduced efficiency of

nutrient uptake, membrane disorders and decrease in the enzyme activity [3]. The psychrotolerants survive at low temperature due to their better nutritional adaptability [4] and have unique cold shock and cold acclimation proteins and enzymes [5]. In this century, actinobacteria derived from polar region have yielded an array of interesting new metabolites, genes, proteins, and other products with potential for commercial uses [6]. Polar actinobacteria often provide plentiful and diverse two bioactive alkaloids which are medicinally important such as nitrosporeusines A and nitrosporeusines B produced by *Streptomyces nitrosporeus* CQT14-24 [7].

The search for new and bioactive secondary metabolites is still going on, particularly from the extremophilic microorganisms. The psychrotolerant actinobacteria can be a good tool to explore the new bioactive metabolites of pharmaceutical importance due to uniqueness of their habitat and changes in the metabolic systems, amenable for their adaptation to extreme cold environmental conditions. The genus *Barrientosiimonas* (Bar.ri.en.to.si.i.mo′nas. N.L. n. Barrientosia Barrientos Island, an island in the Antarctic; L. fem. n. *monas* a unit, a monad; N.L. fem. n. *Barrientosiimonas* a bacterium isolated from Barrientos Island) was validly published early in the year 2013 with *Barrientosiimonas humi* 39^T (actinobacteria/gram-positive) as the type and only species [8]. *Barrientosiimonas humi* 39^T belongs to the family *Dermacoccaceae* [9] and was isolated from soil in Barrientos Island, Antarctica [8]. It is growth optimally at temperatures less than 15 °C and able to grow above 20 °C was called psychrotolerant [10]. So far, the chemical structures from *Barrientosiimonas humi* 39^T have not reported. The main objective of this paper is to describe the isolation and structure characterization from the ethyl acetate extract of *Barrientosiimonas humi* 39^T.

Materials and Methods

Biological materials

The *B. humi* 39^T was isolated from soil sampled at Barrientos Island (Antarctica) (62° 24′ 26.0″ S 59° 44′ 49.1″ W, 62° 24′ 24.4″ S 59° 44′ 44.2″ W and 62° 24′ 28.5″ S 59° 44′ 52.3″ W) during the XI Ecuadorian Antarctic Expedition in 2007. The cultural characteristics of strain 39^T were determined following growth on tap-water agar [11], solidified rich R medium [12] and ISP 2–7 [13] for 7 days at 28 °C. The culturing of *B. humi* 39^T was conducted in collaboration with the Department of Biomedical Sciences, UPM, Serdang Malaysia.

Cultivation of yeast

A single bacterial colony of *B. humi* 39^T will be inoculated into an Erlenmeyer flask (500 mL) containing 100 mL of actinomyces broth and incubated in an orbital shaker at 28 °C, 200 rpm for 72 hours. The seed culture (5%) will be then inoculated into Erlenmeyer flasks (1 L × 4) containing same media (300 mL × 4) and incubated at 28 °C with shaking at 200 rpm for 7 days [13, 14].

Extraction and isolation

The production culture was centrifuged (10,000 rpm, 4 °C, 5 minutes). The supernatant was extracted three times with ethyl acetate, and concentrated using a rotary evaporator. The EtOAc extract (300 mg) of *B. humi* 39^T was subjected to radial chromatography (RC) with 1 mm thickness silica gel on a round glass plate and eluted with the mixtures of $CHCl_3$ and MeOH with increasing polarity (started with $CHCl_3$/MeOH, 9 :1). 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 $CHCl_3$ and MeOH (9.2:0.8) in 5% polarity increment to yield compound **1** (1 mg).and **2** (1.5 mg). Purification of fraction III (5-7) (48.8 mg) was conducted utilizing another RC with silica gel plate of 0.5 mm thickness. Elution with $CHCl_3$ and MeOH (8.5:1.5) produced compounds **3** (1.5 mg), **4** (2 mg), and **5** (2 mg), respectively.

Results and Discussion

Characterization study

Cyclo(-Pro-Val) (**1**) is a needle crystal. m.p.: 218-219 °C. ESI-MS $[M + H]^+$ at *m/z* : 197. IR $\bar{\upsilon}_{max}$ (ATR) cm^{-1}: 3191, 2956, 1640, 1453, 1110, 922. ^1H NMR (DMSO, 700 MHz): δ_H 3.92 (*d, J* = 7 Hz, 1H, H-3), 7.93 (*br-s*, 1H, 4-NH), 4.11 (*t, J* = 7 Hz, 1H, H-6), 1.82 (*m*, 1H, H-7), 2.14 (*m*, 1H, H-7), 1.86 (*m*, 1H, H-8), 1.80 (*m*, 1H, H-8), 3.33 (*t, J* = 7 Hz, 1H, H-9), 3.38 (*t, J* = 7 Hz, 1H, H-9), 2.32 (*m*, 1H, H-10), 0.99 (*d, J* = 7 Hz, 3H, H-11), 0.83 (*d, J* = 7 Hz, 3H, H-12) ppm. NMR APT (DMSO,175 MHz): δ_C 165.8 (Cq-2), 59.9 (CH-3), 170.9 (Cq-5), 58.7 (CH-6), 28.3 (CH_2-7), 22.4 (CH_2-8), 45.1 (CH_2-9), 28.2 (CH-10), 18.7 (CH_3-11), 16.8 (CH_3-12) ppm.

(-)-Cyclo(-Pro-Tyr) (**2**) is a colorless amorphous solid. $[\alpha]_D^{20}$ -43.1 (c 0.14, ethanol). ESI-MS $[M + H]^+$ ion at *m/z* : 261. IR $\bar{\upsilon}_{max}$ (ATR) cm^{-1}: 3248, 2927, 2853, 1747, 1658, 1449, 1252, 1174, 1114, 1017, 858. ^1H NMR (CD_3OD, 700 MHz): δ_H 4.37 (*t, J* = 7 Hz, 1H, H-3), 7.65 (*br-s*, 1H, 4-NH), 4.05 (*t, J* = 7 Hz, 1H, H-6), 1.25 (*q, J*$_1$ = 7 Hz, 1H, H-7), 2.12 (*m*, 1H, H-7), 1.82 (m, 2H, H-8), 3.57 (*t, J* = 7 Hz, H-9), 3.36 (*t, J* = 7 Hz, H-9), 3.07 (*d, J* = 7 Hz, 2H, H-10), 7.06 (*d, J* = 7 Hz, 2H, H-2`/6`), 6.74 (*d, J* = 7 Hz, 2H, H-3`/5`), 9.15 (*br-s*, 1H, 4`-OH) ppm. NMR APT ($CD_3OD$, 175 MHz): $\delta_C$ 165.7 (Cq-2), 56.6 (CH-3), 169.6 (Cq-5), 58.7 (CH-6), 28.0 ($CH_2$-7), 21.4 ($CH_2$-8), 44.6 ($CH_2$-9), 36.3 ($CH_2$-10), 126.3 (Cq-1`), 130.7 (CH-2`/6`), 115.0 (CH-3`/5`), 156.1 (Cq-4`) ppm.

(-)-Cyclo(-Pro-Phe) (**3**) is a colorless solid. m.p.: 144-145 °C. $[\alpha]_D^{20}$-70.00 (*c* 0.02, MeOH). Turned to yellow with anisaldehyde/sulphuric acid. ESI-MS $[M + H]^+$ ion at *m/z* : 245. ^1H NMR (CD_3OD, 700 MHz): δ_H 4.38 (*t*, 7) 1H (H-3), 7.78 (*br-s*) 1H (4-NH), 4.06 (*t*, 7) 1H (H-6), 1.23 (*m*) 1H (H-7a), 2.11 (*m*) 1H (H-7b), 1.82 (*m*) 2H (H-8), 3.57 (*m*) 1H (H-9a), 3.37 (*m*) 1H (H-9b), 3.30 (*dd*, 7, 14) 1H (H-10a), 2.98 (*dd*, 7, 14) 1H (H-10b), 7.22 (*d*, 7) 2H (H-2`/6`), 7.27 (*t*, 7) 2H (H-3`/5`), 7.32 (*t*, 7) 1H (H-4`) ppm. NMR APT ($CD_3OD$, 175 MHz): $\delta_C$ 165.6 (Cq-2), 56.5 (CH-3), 169.4 (Cq-5), 58.7 (CH-6), 28.0 ($CH_2$-7), 21.3 ($CH_2$-8), 44.5 ($CH_2$-9), 38.9 ($CH_2$-10), 126.2 (Cq-1`), 130.4 (CH-2`/6`), 126.9 (CH-3`/5`), 128.1 (CH-4`) ppm.

(+)-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 $\bar{\upsilon}_{max}$ (ATR) cm^{-1}: 3222, 2958, 2930, 2872, 1686, 1676, 1426, 1302, 1275, 1235, 1157, 1102, 1032, 996-919. ^1H NMR (DMSO, 700 MHz): δ_H . 4.00 (*t, J* = 7 Hz, 1H, H-3), 7.99 (*br-s*, 1H, 4-NH), 4.18 (*t, J* = 7 Hz, 1H, H-6), 2.12 (*m*, 1H, H-7), 1.89 (*m*, 1H, H-7), 1.80 (*m*, 2H, H-8), 3.37 (*t, J* = 7 Hz, 1H, H-9), 3.36 (*t, J* = 7 Hz, 1H, H-9), 1.75 (*m*, 1H, H-10), 1.36 (*m*, 1H, H-10), 1.85 (*m*, 1H, H-11), 0.86 (*d, J* = 7 Hz, 3H, H-12), 0.85 (*d, J* = 7 Hz, 3H, H-13) ppm. NMR APT (DMSO, 175 MHz): δ_C 167.1 (Cq-2), 53.1 (CH-3), 171.1 (Cq-5), 58.9 (CH-6), 27.9 (CH_2-7), 22.9 (CH_2-8), 45.3 (CH_2-9), 38.1 (CH_2-10), 24.5 (CH-11), 23.2 (CH_3-12), 22.3 (CH_3-13) ppm.

L-Tyrosine (**5**) is a colorless amorphous solid. m.p.: 343-344 °C. ESI-MS $[M + H]^+$ at *m/z* 182. ^1H NMR (CD_3OD, 700 MHz): δ_H 6.84 (*d, J* = 7 Hz, 2H, H-2/6), 6.68 (*d, J* = 7 Hz, 2H, H-3/5), 9.23 (*br-s*, 1H, 4-OH), 2.51 (*d, J* = 7 Hz, H-7), 2.11 (*dd, J*$_1$ = 7 Hz, *J*$_2$ = 14 Hz, 1H, H-7), 3.85 (*t, J* = 7 Hz, 1H, H-8), 7.77 (*br-s*, 2H, 8-NH_2), 10.88 (*br-s*, 1H, 9-OH) ppm. NMR APT (CD_3OD, 175 MHz): δ_C 127.0 (Cq-1), 131.2 (CH-2/6), 115.5 (CH-3/5), 156.5 (CH-4), 39.3 (CH_2-7), 56.2 (CH_2-8), 166.7 (Cq-9) ppm.

Fraction I was applied to PF254 Gypsum (Merck 7749, size 0.040-0.063 mm) followed by TLC to afford compound **1** that turned to blue with chlorine/anisidine and violet when sprayed with anisaldehyde/sulphuric acid. The ^1H NMR (Figure 1) spectrum of **1** contained three methine proton signals; two methines δ_H 4.11 (H-6), 3.92 (H-3) were possibly connected to heteroatoms. A further methine signal was found at δ_H 2.32 (H-10) bind a geminal dimethyl proton at δ_C 18.7 (CH_3-11) and 16.8 (CH_3-12) (Figure 2). Additionally, three saturated non-equivalent methylenes signals at δ_H 1.82-2.14 (H-7), (H-7), 1.80-1.86 (H-8), and 3.33-3.38 (H-9) were attributed to a pyrrolidine ring. The ESI mass spectrum (Figure 3) showed molecular ion peaks at *m/z* ESI-MS $[M + H]^+$ which fixed the molecular weight as *m/z* 197. Compound **1** was found to have a molecular formula of $C_{10}H_{16}N_2O_2$ by LRESIMS. A search in the Anti-Base using all of the spectroscopic data above led to cyclo(-Pro-Val) (**1**) as a result. Cyclo(-Pro-Val) (**1**) was previously isolated from *Alternaria altenata* [12], *Aspergillus fumigatus* Brazilian strain [13] and *Pseudomonas aeruginosa* [14].

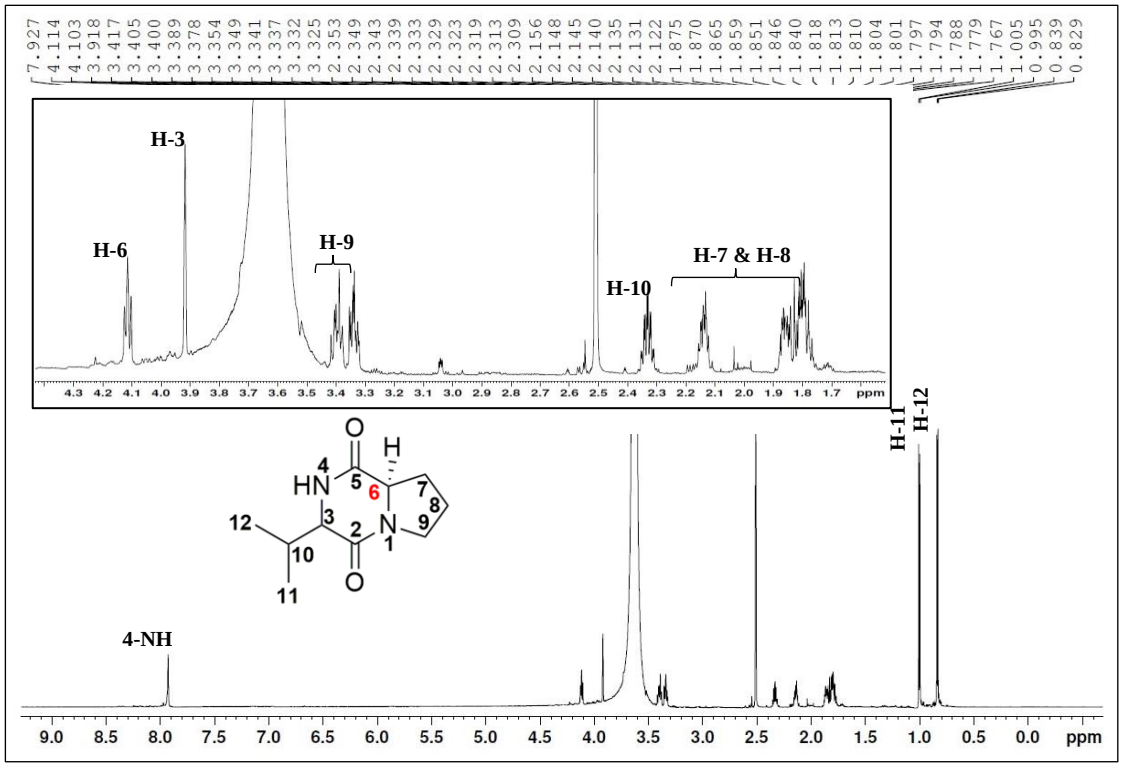

Figure 1. The ^{1}H-NMR (DMSO, 700 MHz) Spectrum of Cyclo(-Pro-Val) (**1**)

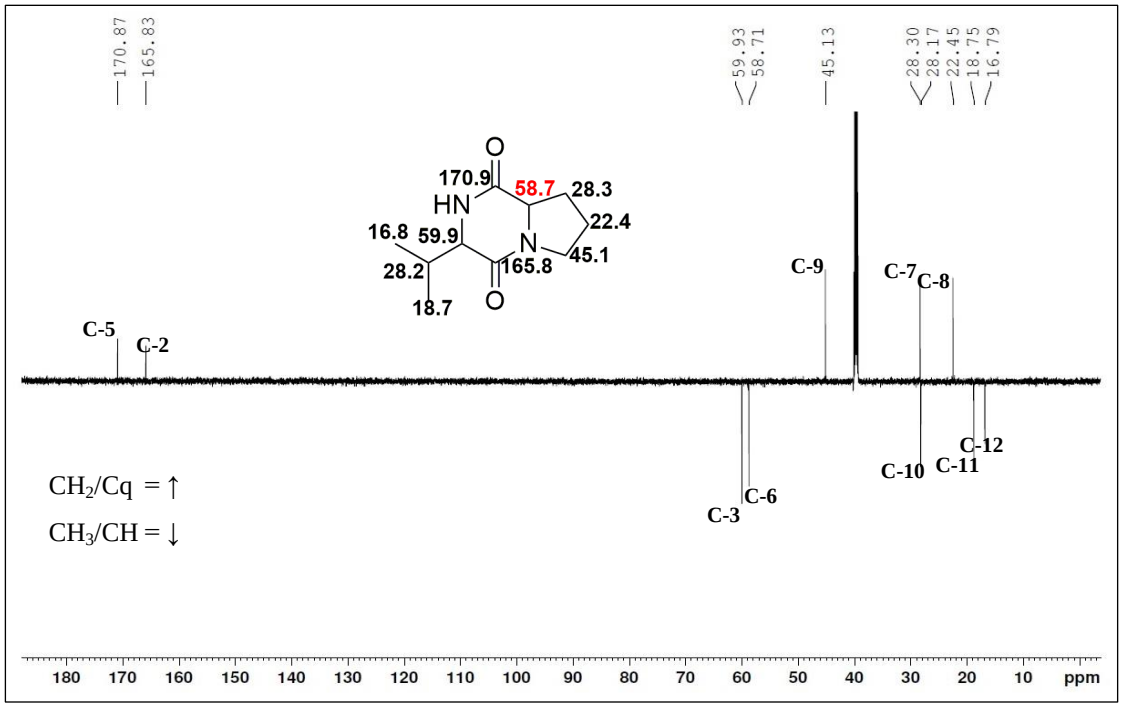

Figure 2. The ^{13}C NMR-APT (DMSO, 175 MHz) Spectrum of Cyclo(-Pro-Val) (**1**)

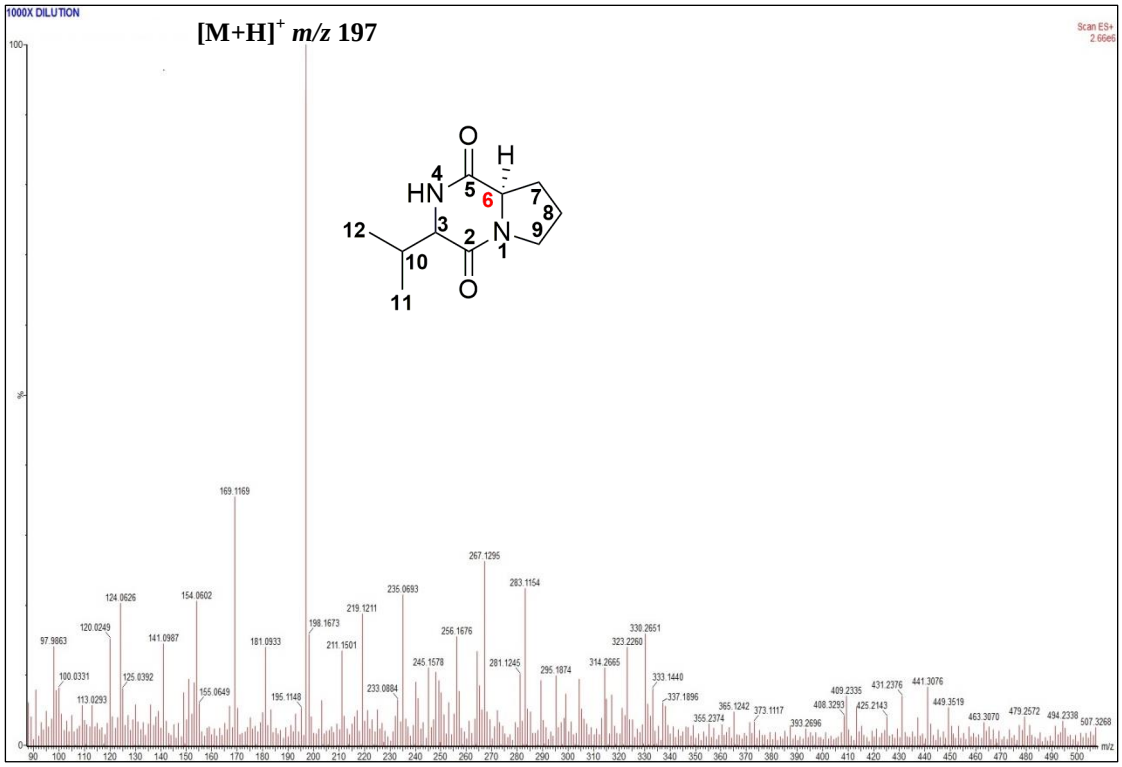

Figure 3. The LC-MS Spectrum of Cyclo(-Pro-Val) (**1**)

Compounds **2** was found in Fraction I as a UV absorbing spot, which stained to violet with sulphuric acid. The ^{1}H NMR and 13C NMR spectrum (Figure 4 and Figure 5) of Compound **2** showed two *ortho*-coupled signals at δ_H 7.05 (H-2', 6') and 6.79 (H-3', 5'), which illustrated as an AA'-BB' system of a 1,4-disubstituted aromatic ring, as well as two signals at δ_H 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 (CH_2-7) (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 (Figure 6) determined the molecular weight of Compound **2** as *m/z* 261 by (+)-ESI mode. The structure was further confirmed as cyclo(-Pro-Tyr) (**2**) was previously isolated from *Pseudomonas fluorescens* GcM5-1A [15], *Streptomyces* sp. H7372 and ML 1532 [16, 17], *Pseudomonas aeruginosa* [14] and *Lysobacter capsici* AZ78 [18].

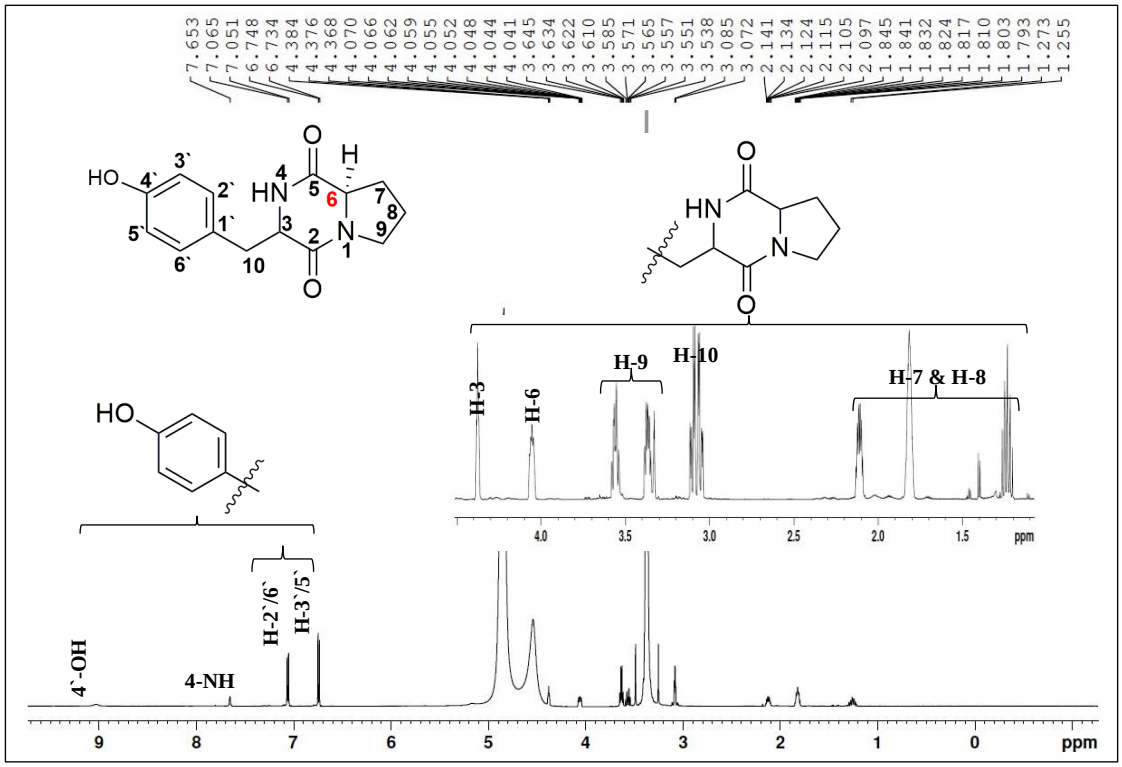

Figure 4. The ^{1}H-NMR (CD_3OD, 700 MHz) Spectrum of (-)Cyclo(-Pro-Tyr) (**2**)

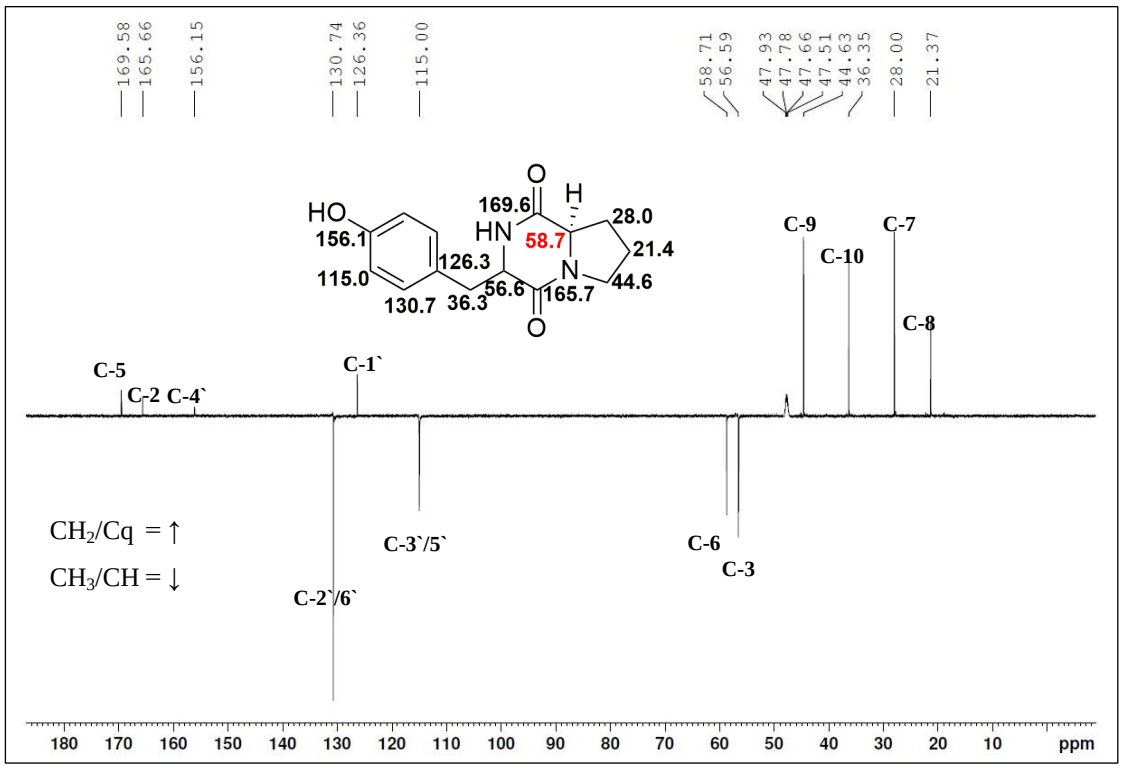

Figure 5. The NMR-APT(CD_3OD, 175 MHz) Spectrum of (-)Cyclo(-Pro-Tyr) (**2**)

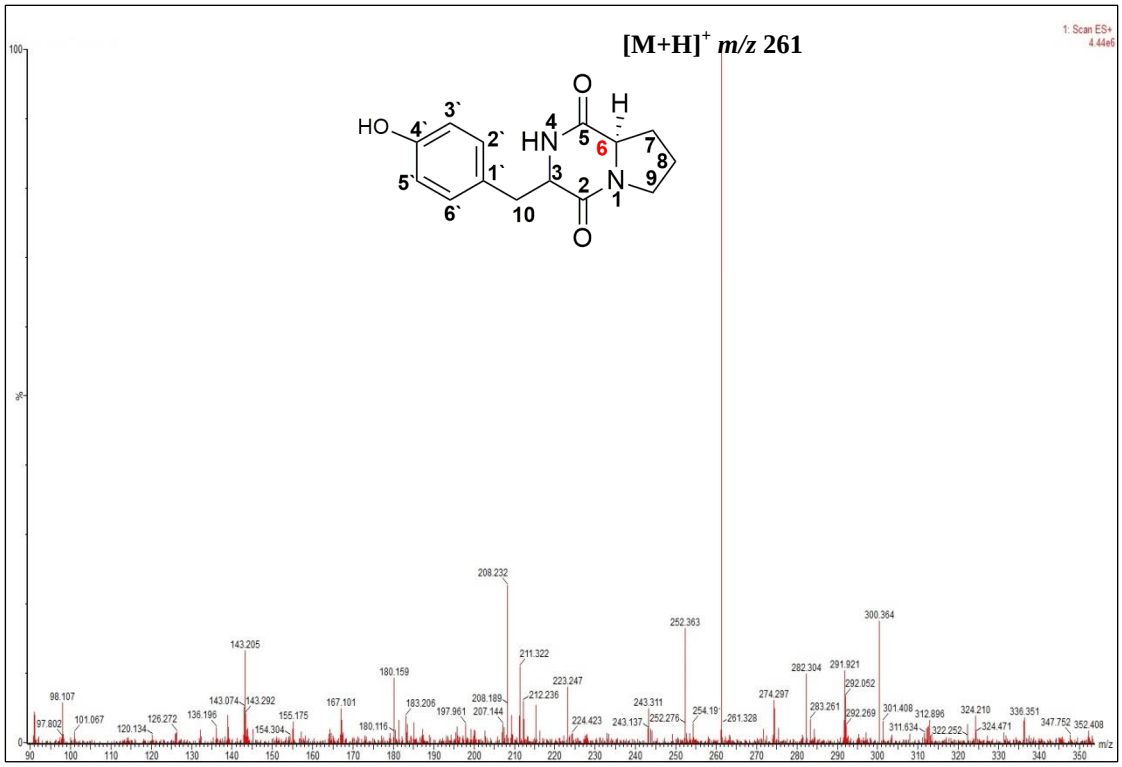

Figure 6. The LC-MS Spectrum of (-)Cyclo(-Pro-Tyr) (**2**)

Compounds **3** were found in Fraction III as a UV absorbing spot, which stained to violet with anisaldehyde/sulphuric acid. The ^{1}H NMR and ^{13}C NMR spectrum (Figure 7 and Figure 8) of compound **2** showed two ortho-coupled signals at δ_H 7.22 (H-2', 6') and 7.27 (H-3', 5'), which pointed to an AA'-BB' system of a 1,4-disubstituted aromatic ring, as well as two signals at δ_H 4.38 and 4.06 for two methines attached to electron withdrawing substituents. The spectrum proton of Compound **2** also depicted two doublet of doublets for an ABX system of a methylene group at δ_H 3.30 and 2.98 (CH_2-10), as well as three methylene multiplets (CH_2-7) (δ_H 1.23, 2.11) attached to a heteroatom and CH_2-8, 9 (δ_H 1.82, 3.57, 3.37). The ESI mass spectra (Figure 9) determined the molecular weight of Compound **3** as *m/z* 245 by (+)-ESI mode. The structure was further confirmed as (-)-cyclo(-Pro-Phe) (**3**) previously isolated from microbial *Streptomyces sudanensis*. A4.4 [19].

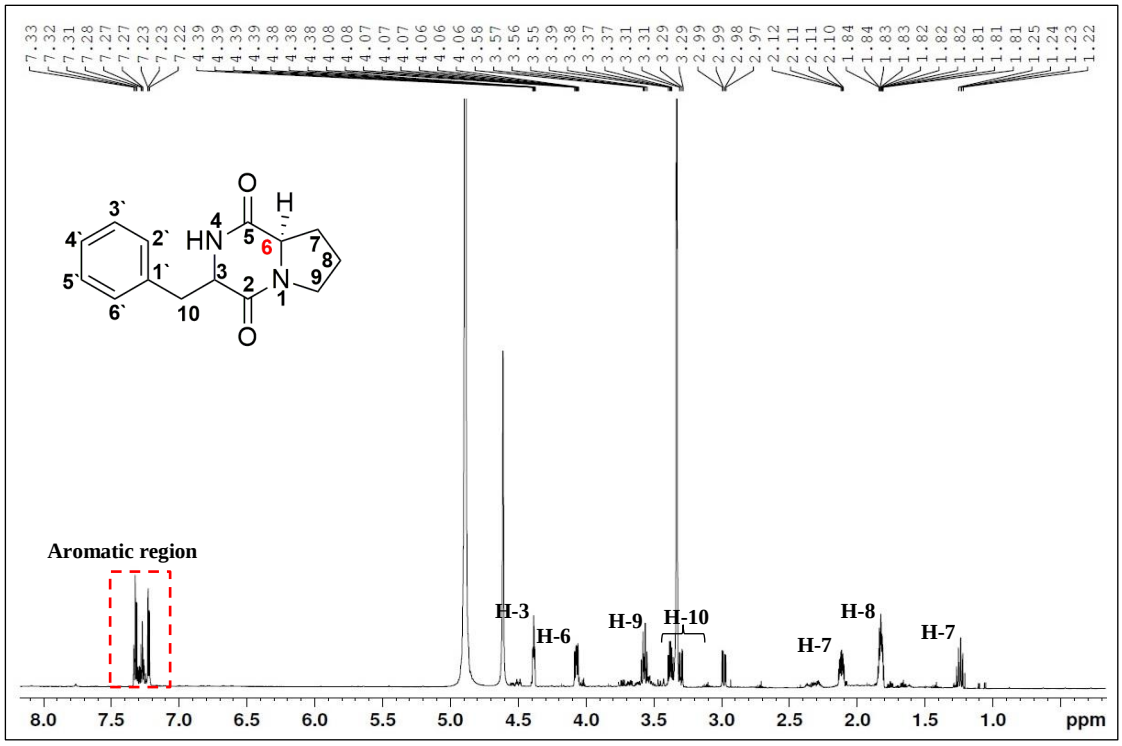

Figure 7. The ^{1}H-NMR (CD_3OD, 700 MHz) Spectrum of Cyclo(-Tyr-Phe) (**3**)

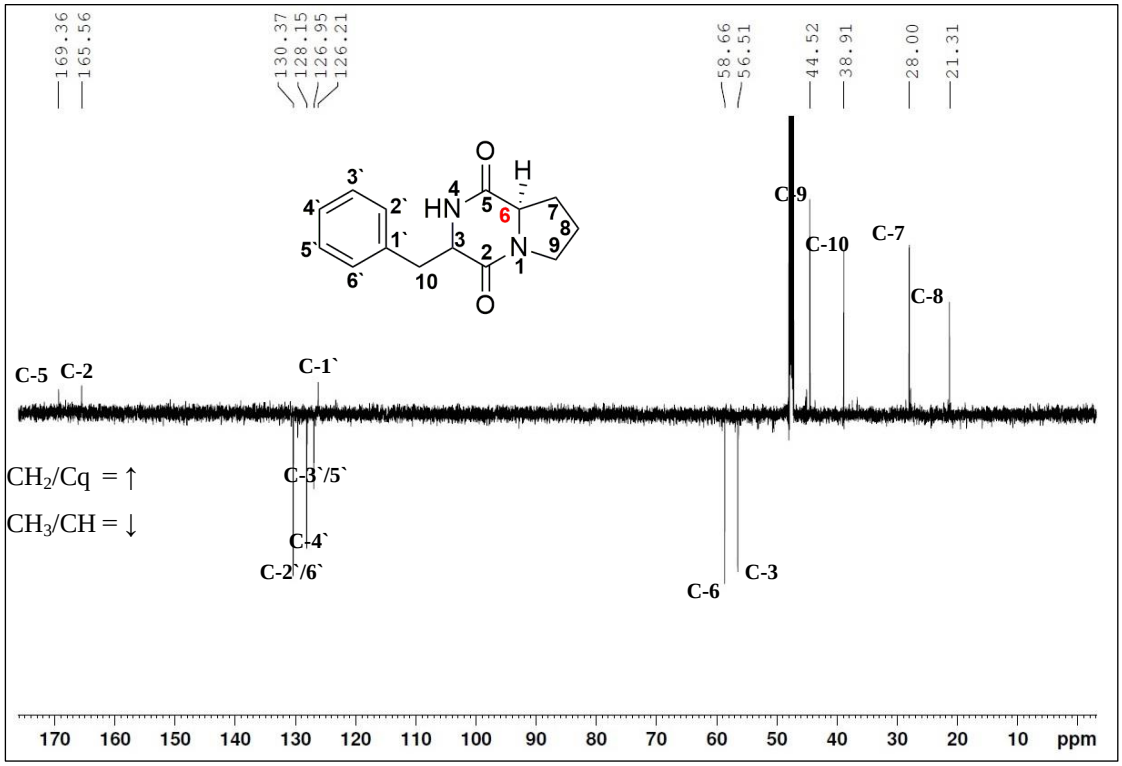

Figure 8. The NMR-APT (CD_3OD, 175 MHz) Spectrum of Cyclo(-Tyr-Asn) (**3**)

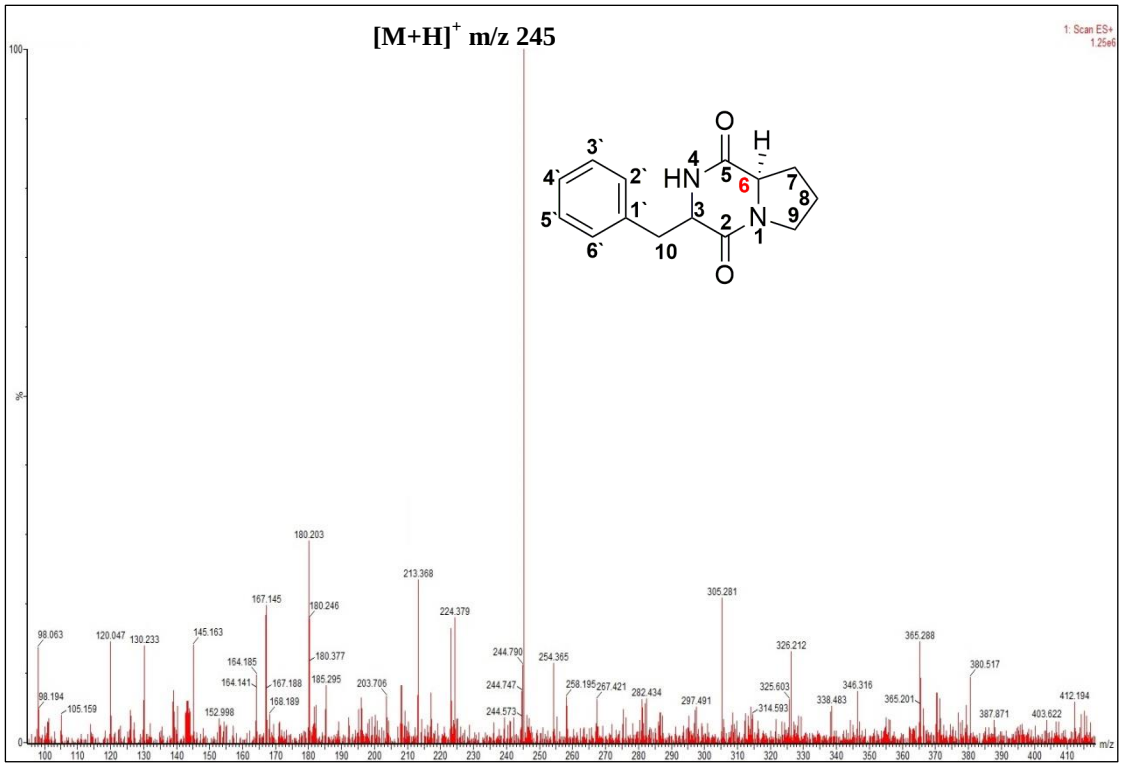

Figure 9. The LC-MS Spectrum of Cyclo(-Tyr-Phe) (**3**)

(+)-Cyclo(-Pro-Leu) (**4**), $[\alpha]_D^{20}$ +28.1 (*c* 0.032, ethanol), was isolated as a white amorphous material. The IR spectrum showed bands attributed to the aliphatic carbons (2958, 2930, 2872 cm^{-1}) and amide (1686, 1676 cm^{-1}) functional groups. ^{1}H NMR spectrum (Figure 10) 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-equivalents dimethyl at H-12 and H-13. NMR APT spectrum (Figure 11) 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 with molecular formula $C_{11}H_{18}N_2O_2$, supported by ESI–MS (Figure 12) $[M + H]^+$ *m/z* 211. The. (+)-Cyclo(Pro-Leu) previously isolated from the Gram-negative Proteobacteria of *Burkholderia cenocepacia* and *Serratia marcescens* [20], *Alternaria alternata* [12], the bacteria *Pseudoalteromonas* sp. [21] and *Vibrio alginolyticus* [22].

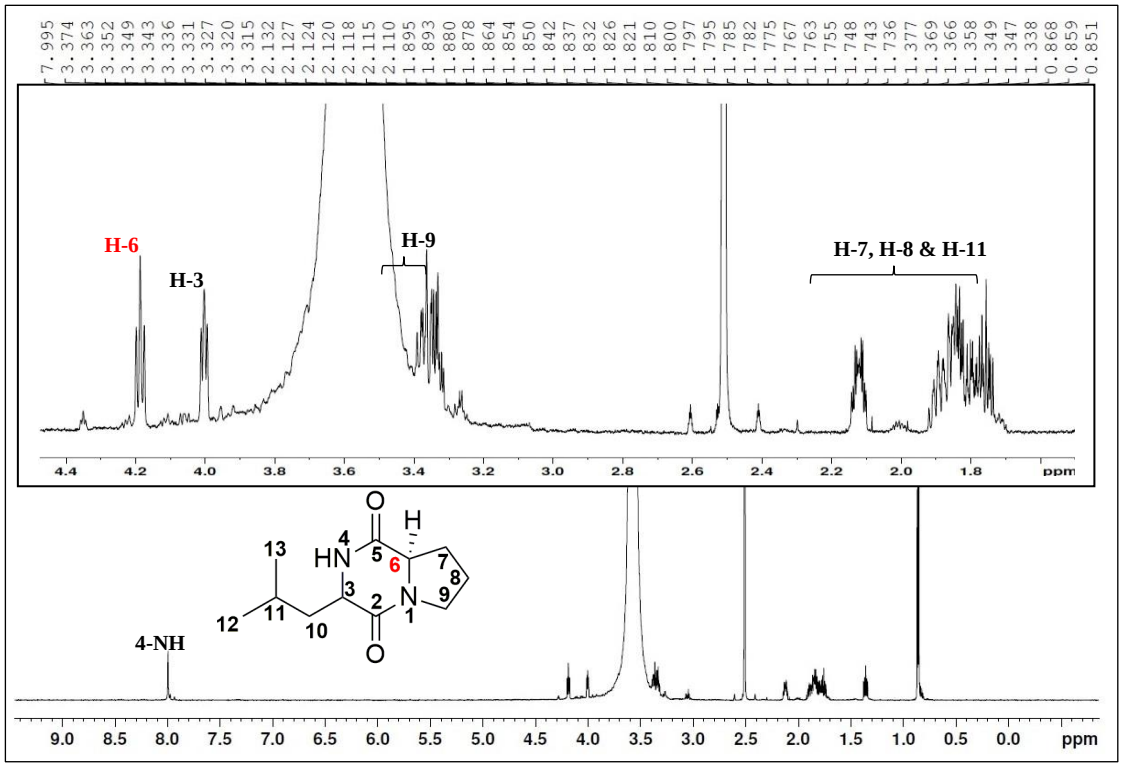

Figure 10. The ^{1}H-NMR (DMSO, 700 MHz) Spectrum of (+)Cyclo(-Tyr-Leu) (**4**)

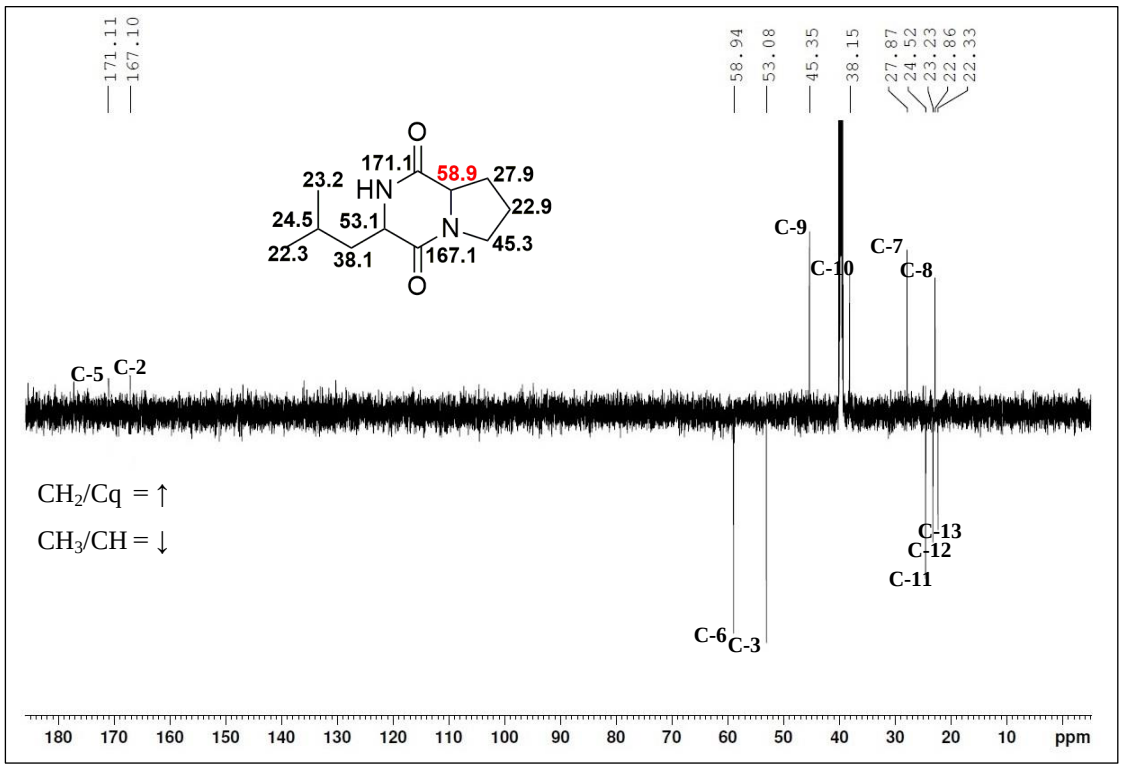

Figure 11. The NMR-APT (DMSO, 175 MHz) Spectrum of (+)Cyclo(-Tyr-Leu) (**4**)

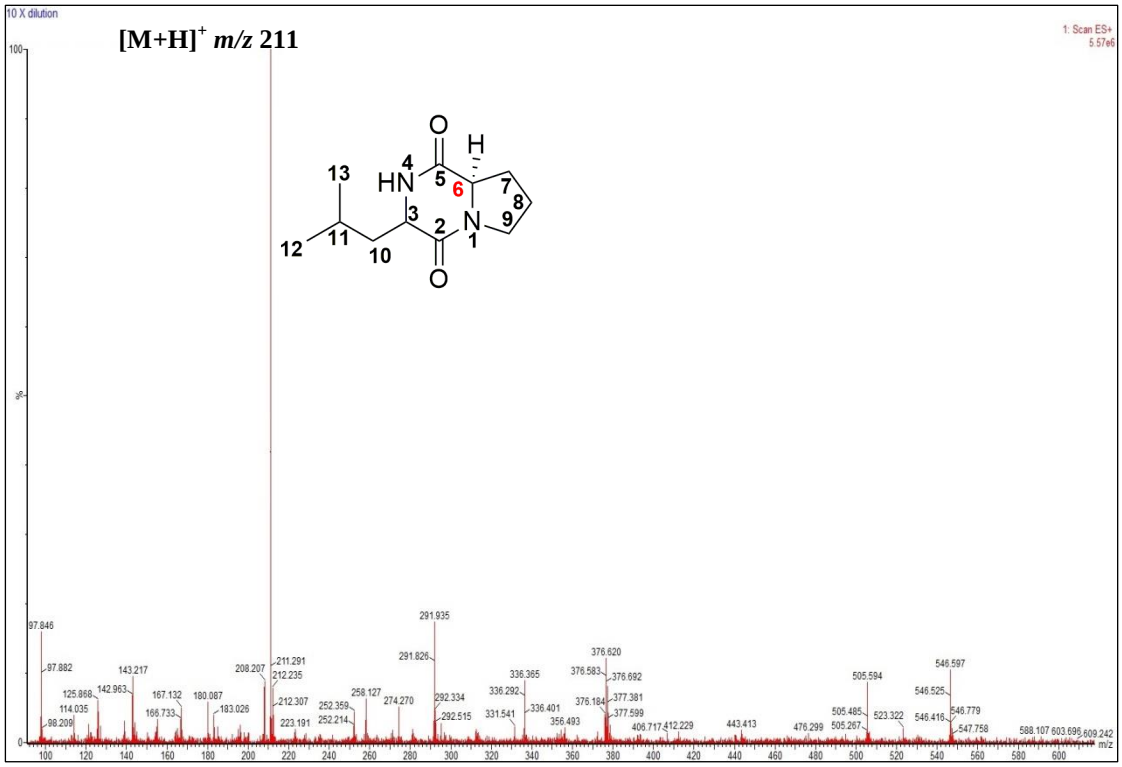

Figure 12. The LC-MS Spectrum of (+)Cyclo(-Tyr-Leu) (**4**)

Compound **5** was isolated as white amorphous from a UV absorbing zone in fraction III, which turned to violet with anisaldehyde reagent. The ^{1}H NMR spectrum (Figure 13) of compound **5** showed two *ortho*-coupled signals at δ_H 6.84 (H-2, 6) and 6.68 (H-3, 5), which pointed to an AA'-BB' system of a 1,4-disubstituted aromatic ring. In aliphatic region there was a methylene group at δ_H 2.11 and a methine at δ_H 3.85 ppm in the amino acid group. The NMR APT spectrum (Figure 14) exhibited seven carbon signals, among them the carbonyl signal of an amino acid derivative at δ_C 166.7 ppm and a methylene (C-7) as a link between amino acid with phenol. The ESI mass spectrum (Figure 15) indicated the peak at *m/z* 182 $[M + H]^+$ established the molecular formula as $C_9H_{11}NO_3$. A search in Anti-Base supported by ^{1}H, ^{13}C NMR, and MS spectroscopic data led to L-tyrosine (**5**) [23]. This compound previously isolated from bacteria *B. flavus* AJ-11955 [24] and *Klebsiella* sp. [25].

Out of five compounds, four are dipeptides with proline as one of its amino acid, and this is probably due to the nature of this microbe that can survive very low temperature. Some microbes were reported to produce higher amounts of L-proline at very low temperature as a cold adaptation mechanism [26, 27]. L-proline is a protectant stress agent that can moderate stability of cell membrane and control cell damage due to freeze-thaw mechanism during extreme low temperature [28, 29].

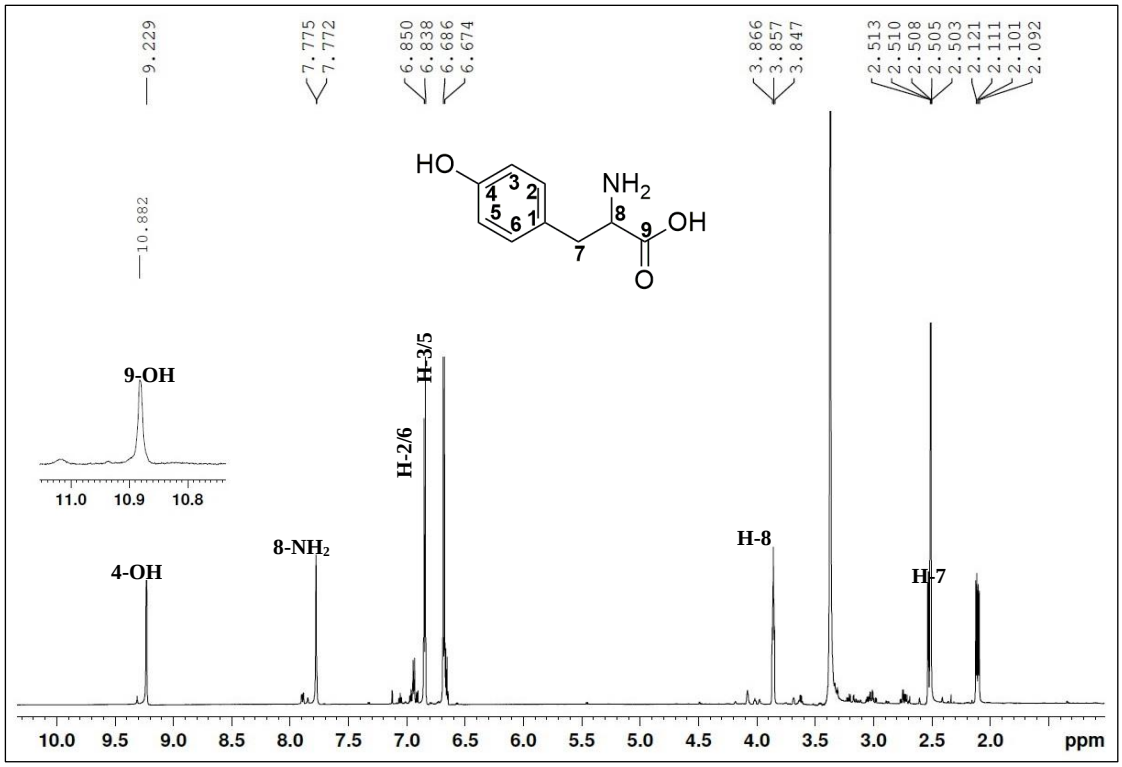

Figure 13. The ^{1}H-NMR (DMSO, 700 MHz) Spectrum of L-Tyrosine (**5**)

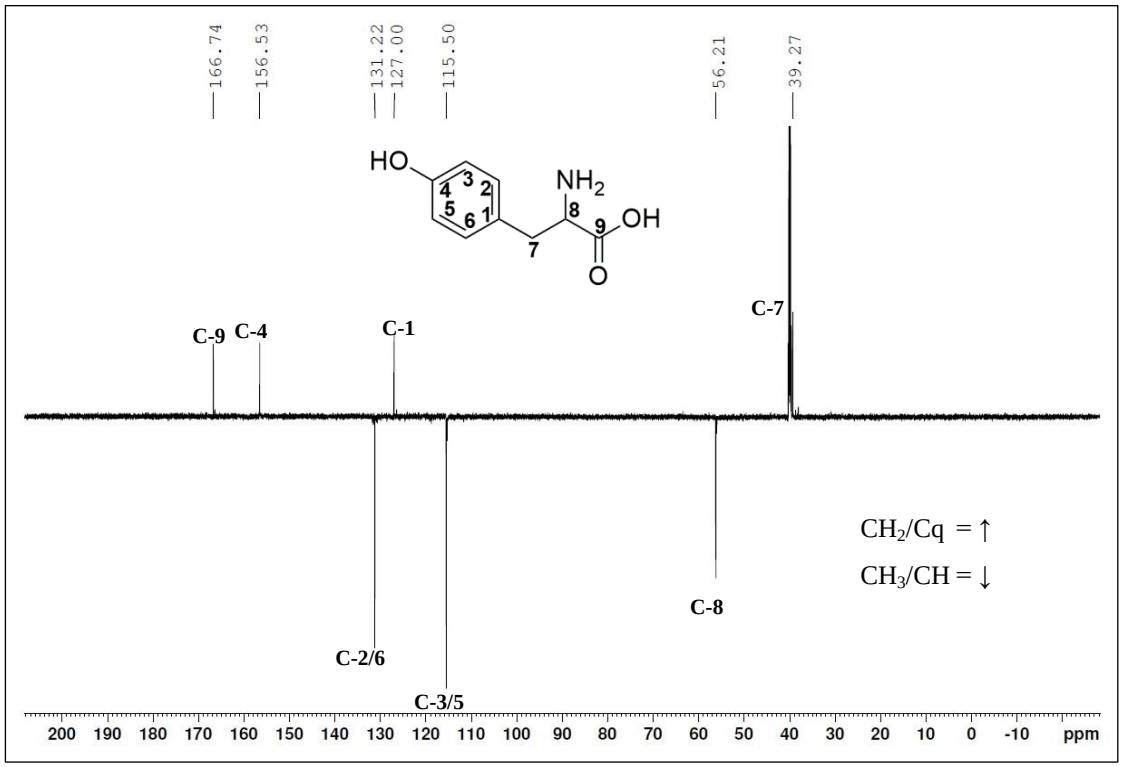

Figure 14. The NMR-APT (DMSO, 175MHz) Spectrum of L-Tyrosine (**5**)

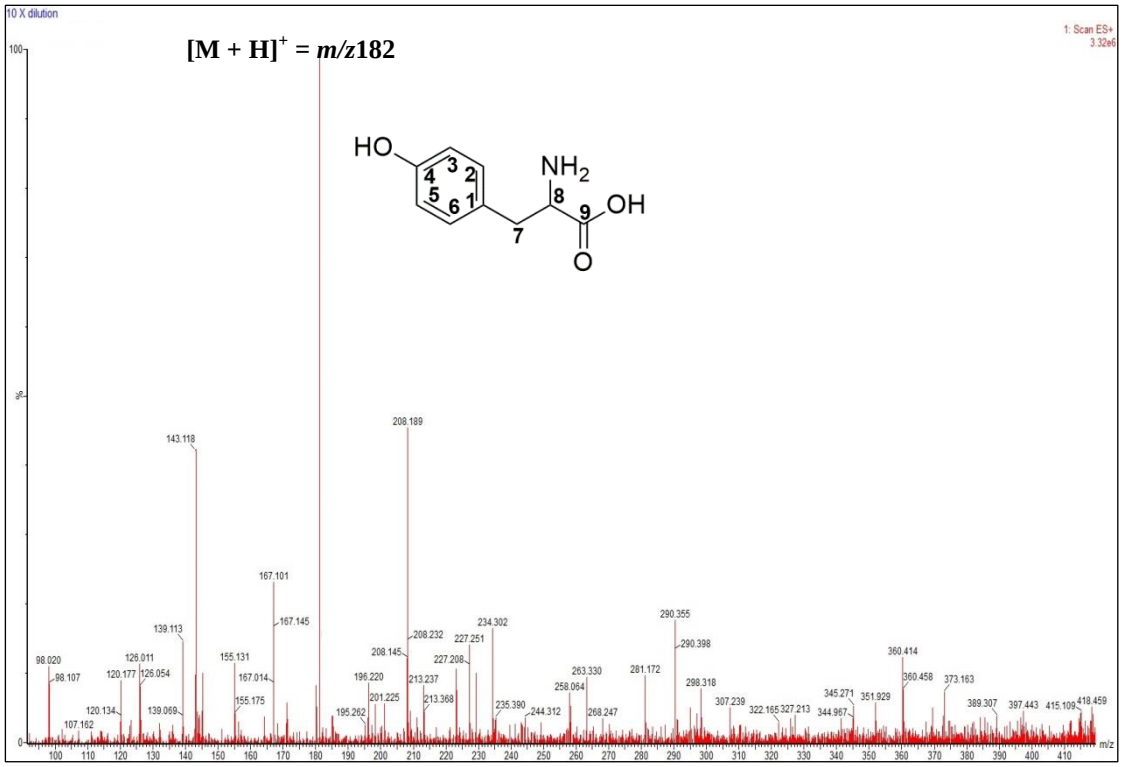

Figure 15. The LC-MS Spectrum of L-Tyrosine (**5**)

Conclusion

Five diketopiperazine derivatives namely cyclo(-Pro-Val) (**1**), (-)-cyclo(-Pro-Tyr) (**2**), (-)-cyclo(-Pro-Phe) (**3**), (+)-cyclo(-Pro-Leu) (**4**), and L-tyrosine (**5**) were isolated and reported for the first time from psychrotolerant actinobacteria *Barrientosiimonas* 39^T. These proline-based diketopiperazines probably act as modulators or molecules that play a part in cold-adaptation of psychrotolerant. All the compounds reported in the present study were not subjected to further bioactivity studies due to insufficient amounts.

Acknowledgement

We would like to thank the School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM); Malaysia Genome Institute (MGI) for providing research facilities. We would also like to express our gratitude to the Indonesia Endowment Fund for Education (LPDP), and Yayasan Penyelidikan Antartika Sultan Mizan for the financial support through the following research grant ST-2017-017.

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