

ANTIBIOTIC COMPOUND FROM MARINE ACTINOMYCETES (*Streptomyces sp A11*): ISOLATION AND STRUCTURE ELUCIDATION

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ABSTRACT

Purification and structure elucidation of antibiotic produced by marine actinomycetes (*Streptomyces sp A11*) was conducted. Production of antibiotic was carried out by liquid fermentation using yeast and peptone medium for 5 days fermentation. Purification of antibiotic was carried out by silica gel 60 (Merck, 0.063-0.200 mm) column chromatography and preparative HPLC. Structure elucidation was carried out using ESI-MS, ¹H NMR, ¹³C NMR, DEPT ¹³C NMR, and FTIR. This antibiotic was identified as cyclo (tyrosyl-prolyl) / (C₁₄H₁₆N₂O₃). This antibiotic had biological activity to *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 66923, *Pseudomonas aeruginosa* ATCC27853, and produced by extracellular secretion.

Keywords: antibiotic, actinomycetes, purification, structure elucidation

INTRODUCTION

Actinomycetes especially streptomyces are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites [1], notably antibiotics [1-2], antitumor agents [3], immunosuppressive agents [4] and enzymes [5-6].

Although the exploitation of marine actinomycetes as a source for discovery of novel secondary metabolites is at an early stage, numerous novel metabolites have been isolated in the past few years. For example; Abyssomicin C is a novel polycyclic polyketide antibiotic produced by a marine *Verrucosipora* strain [7], Diazepinomicin is a unique farnesylated dibenzodiazepinone produced by a *Micromonospora* strain. It possesses antibacterial, anti-inflammatory and antitumor activity [8], Salinosporamide A is a novel β-lactone-γ-lactam isolated from a fermentation broth of a new obligate marine actinomycete, *Salinispora tropica*. This compound is an orally active proteasome inhibitor [9].

We have previously reported the isolation of indigenous marine actinomycetes strain called *Streptomyces sp A11* producing biological activities [10]. We report here the purification and elucidation of chemical structure active compounds produced by *Streptomyces sp A11*. The objective of this research was production, purification and structure elucidation of antibiotic produced by marine actinomycetes (*Streptomyces sp A11*).

EXPERIMENTAL SECTION

Materials

Materials used for research as follows: bacto peptone (Oxoid), yeast extract (Oxoid), malt extract (Oxoid), glucose (Merck), iron(III) citrate hydrate (Merck), ethyl acetate (JT Baker), marine agar (Difco), demineral water, sea water, methanol (JT Baker), methanol for HPLC (Merck), silica gel 60 (Merck, 0.063-0.200 mm).

Instrumentation

HPLC Waters 2695 with analytical column Water Symmetry C18 (4.6 x 250 mm, Part No.WAT054275) and analytical column Millipore Water Puresil 5μ C18 (4.6x150 mm, Part No.N21017), ESI-MS (LCT Premier-XE waters), FTIR (Shimadzu 8300), ¹H NMR, ¹³C NMR, and DEPT ¹³C NMR (Bruker AV-500).

Procedure

Fermentation

The isolate *Streptomyces sp A11* was inoculated into a 250 mL flask containing 100 mL of the vegetative medium consisting of (g/L): bacto peptone 5 g/L (Oxoid), yeast extract 3 g/L (Oxoid), malt extract 3 g/L (Oxoid), glucose 3 g/L (Merck), demineral water 250 mL, and sea water 750 mL. pH value of the medium was adjusted at 7.6 before sterilization. The flask was incubated at 30 °C for 2 days in incubator shaker.

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50 mL of this culture was transferred to 1,000 mL of the fermentative medium [11]. Fermentative medium consisted of bacto peptone 15 g/L (Oxoid), yeast extract 3 g/L (Oxoid), iron(III) citrate hydrate 0.3 g/L (Merck), demineral water 250 mL, and sea water 750 mL. pH value of the medium was adjusted at 7.6 before sterilization. The fermentation was carried out at 30 °C for 5 days in incubator shaker [12]. The total of working volume for fermentation process was 5,000 mL.

Extraction and purification

Five thousands milliliter of culture broth was centrifuged at 14,000 x g for 15 min. The dark filtrate (supernatant) was divided from biomass and extracted using 5,000 mL ethyl acetate twice (2 x 5,000 mL). Biomass was dried and weighed and extracted using 500 mL of methanol twice. Ethyl acetate phase was concentrated by evaporation under vacuum to the least volume, after the dehydration using anhydrous Na₂SO₄. At the same time biomass extract (in methanol) was concentrated by evaporation under vacuum to the least volume. Extract of biomass and supernatant were tested by analytical HPLC.

Dry extract of supernatant and extract of biomass were purified using column chromatography (φ25 x 500 mm). Stationary phase was used silica gel 60 (Merck, 0.063-0.200 mm). Dry extract was injected on column chromatography then eluted stepwise with chloroform-methanol solvent system as follows: First, the column was eluted with 100% chloroform. This repeated by reducing the chloroform 10% and the methanol was increased 10% until percentage of methanol 100%. Thirty fractions were collected and tested for their biological activities. The active fractions obtained from column chromatography were further purified using preparative HPLC. Each fraction from preparative HPLC was collected and test to biological activity. The active fraction was collected and dried using vacuum evaporation. Active compound was elucidated by ESI-MS, ¹H NMR, ¹³C NMR, DEPT ¹³C NMR, and FTIR.

Biological activity assay

Biological activity was monitored by the agar diffusion paper-disc (6 mm), which are dripped by extract solution, dried and placed over the agar surface plates freshly inoculated with the *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 66923, *Pseudomonas aeruginosa* ATCC27853, *Candida albican* BIOMCC00122 and *Aspergillus niger* BIOMCC 00134 as test organisms. Suspensions of test organisms were adjusted to 10⁶ cfu/mL. The most potent isolates were noted for each test organism, based on the mean of diameter of inhibition zones.

Analysis HPLC

HPLC analysis was performed using HPLC Waters 2695, analytical column Water Symmetry C18 (4.6 x 250 mm, Part No.WAT054275), photodiode array detector, elution was used methanol-water (0-100% linear gradient for 35 min and then isocratic elution with 100% methanol until 10 min), and flow rate of 1.0 mL/min.

Preparative HPLC

Preparative HPLC was conducted using HPLC Waters 2695, photodiode array detector, and analytical column Millipore Water Puresil 5μ C18 (4.6 x 150 mm, Part No.N21017). Volume of injection was 50 uL per injection under condition of pressure average 1,267 psi, flow rate 1 mL/min where mobile phase 0-45% methanol – water for 25 min.

Elucidation of chemical structure

Molecular weight and formula were determined using ESI-MS (LCT Premier-XE waters), molecule structure elucidation of active compound were determined using FTIR (Shimadzu 8300), ¹H NMR, ¹³C NMR, and DEPT ¹³C NMR (Bruker AV-500 (500 MHz)).

RESULT AND DISCUSSION

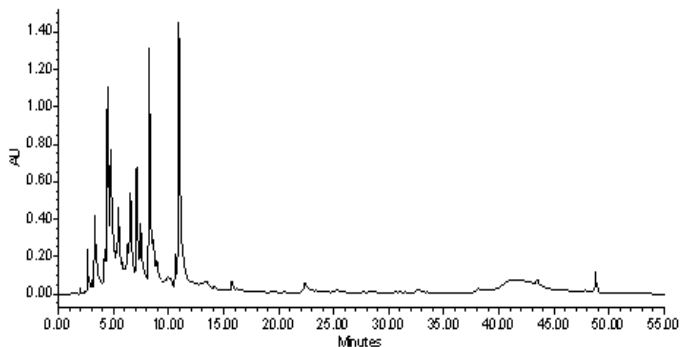
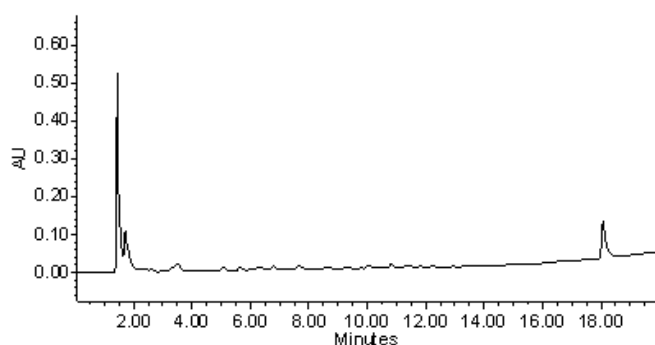
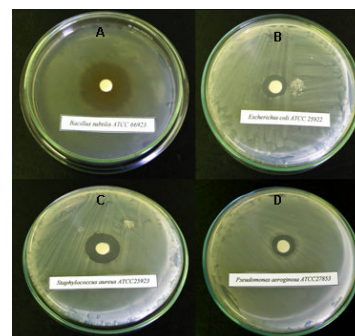
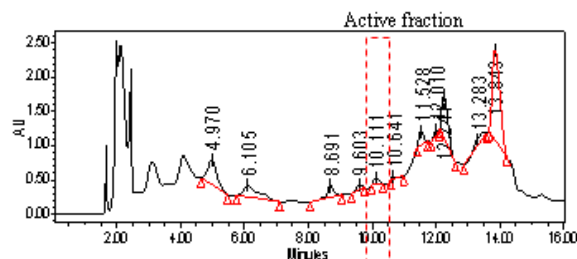
Fermentation isolate *Streptomyces sp A11* was carried out for 5 days with yeast-peptone medium. Five liters volume of fermentation produced 4.72 g dry biomass, and after extracted by methanol was obtained 1.72 g extract and extract of supernatant (ethyl acetate phase) was obtained 0.83 g. The concentrated syrup was tested the biological activity using *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 66923, *Pseudomonas aeruginosa* ATCC27853, *Candida albican* BIOMCC00122 and *Aspergillus niger* BIOMCC 00134 as test organism. Biological activity of supernatant and biomass extract showed in Table 1.

Table 1 showed that supernatant extract have strong antibacterial activity to 4 bacteria (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 66923, *Pseudomonas aeruginosa* ATCC27853) and there was no activity to yeast and fungi (*Candida albican* BIOMCC00122 and *Aspergillus niger* BIOMCC 00134). The strongest activity was showed to *Bacillus subtilis* ATCC 66923. Biomass extract didn't show biological activity to all test organisms. The same thing was showed on HPLC chromatogram. Pattern for HPLC chromatogram of supernatant and biomass extract indicated different pattern. Both chromatograms are presented in Fig. 1 and 2. Fig. 1 showed many peak was emerge, indicating that many compounds were produced by isolate

Table 1. The data of biological activity extract fermentation produced *Streptomyces sp A11*

Sample	Diameter of clear zone (mm)					
	<i>Staphylococcus aureus</i>	<i>Bacillus.s</i>	<i>Pseudomonas aeruginosa</i>	<i>E.coli</i>	<i>Candida albican</i>	<i>Aspergillus niger</i>
Extract of biomass	negative	negative	negative	negative	negative	negative
Extract of supernatant	10.39	24.43	9.64	9.55	negative	negative
Control (rifampicin 500 ppm)	21.27	44.57	10.08	10.12	negative	negative
control (nystatin 500 ppm)	negative	negative	negative	negative	7.34	7.3

Diameter of disk paper: 6 mm

**Fig 1.** Analysis HPLC chromatogram of supernatant extract**Fig 2.** Analysis HPLC chromatogram of biomass extract**Fig 3.** Inhibition of active compound to (A) *Bacillus subtilis* ATCC 66923, (B) *Escherichia coli* ATCC 25922, (C) *Staphylococcus aureus* ATCC 25923, (D) *Pseudomonas aeruginosa* ATCC 27853**Fig 4.** Preparative HPLC chromatogram of purification process

A11 and extractable from the supernatant, but the other side, there was not any compound showed on extract of biomass (Fig. 2). It is mean that this active compound was produced by extracellular secretion.

Pattern of HPLC chromatogram of supernatant and biomass extract were showed on Fig. 1 and Fig. 2. Inhibition of active compound to 4 test organisms was showed on Fig. 3.

Further purification of the antibiotic has been carried out by column chromatography. Supernatant extract was injected on column chromatography, and then eluted stepwise with methanol-chloroform solvent system. Thirty fractions were collected and each fraction was tested for their biological activities.

Further purification of active fractions was purified by preparative HPLC. Forty fractions were collected and test to biological activity. Fractions that showed biological activity were combined and dried under vacuum. Dried active compound showed white powder

and easy dissolved in methanol:chloroform (1:1). Preparative HPLC chromatogram of purification process was presented at Fig. 4.

Fig.4 showed that many compounds have not been eliminated before purification using preparative HPLC. However, after purification using preparative HPLC, a single active compound can be obtained (Fig. 5). Result from biologically activity assay showed that fraction 10.1 min of preparative HPLC chromatogram (Fig. 4) showed active fraction. Identification of pure active compound using analysis HPLC showed that this active compound have UV visible maximum absorption at 210 and 274.5 nm in methanol. It is reported that the most of peptide antibiotic exhibit maximum absorbance at 210-230 nm and 270-280 nm [13-15]. An absorbance at 220-230 nm is corresponding to characteristic absorption of peptide bonds [13]. Analysis HPLC Chromatogram and UV visible spectrum of active compound after purification using

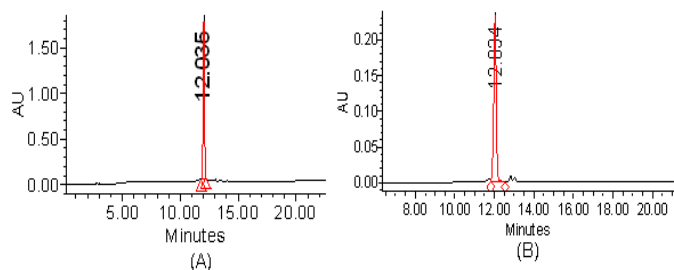


Fig 5. Analysis HPLC chromatogram of active compound after purification using preparative HPLC. Measurement at (A) λ 210 nm and (B) λ 274 nm

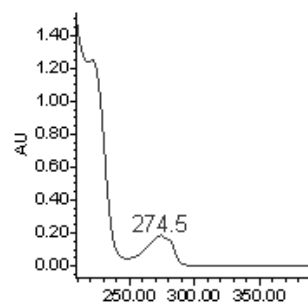


Fig 6. UV visible spectrum^{nm} absorbance of active compound after purification using preparative HPLC

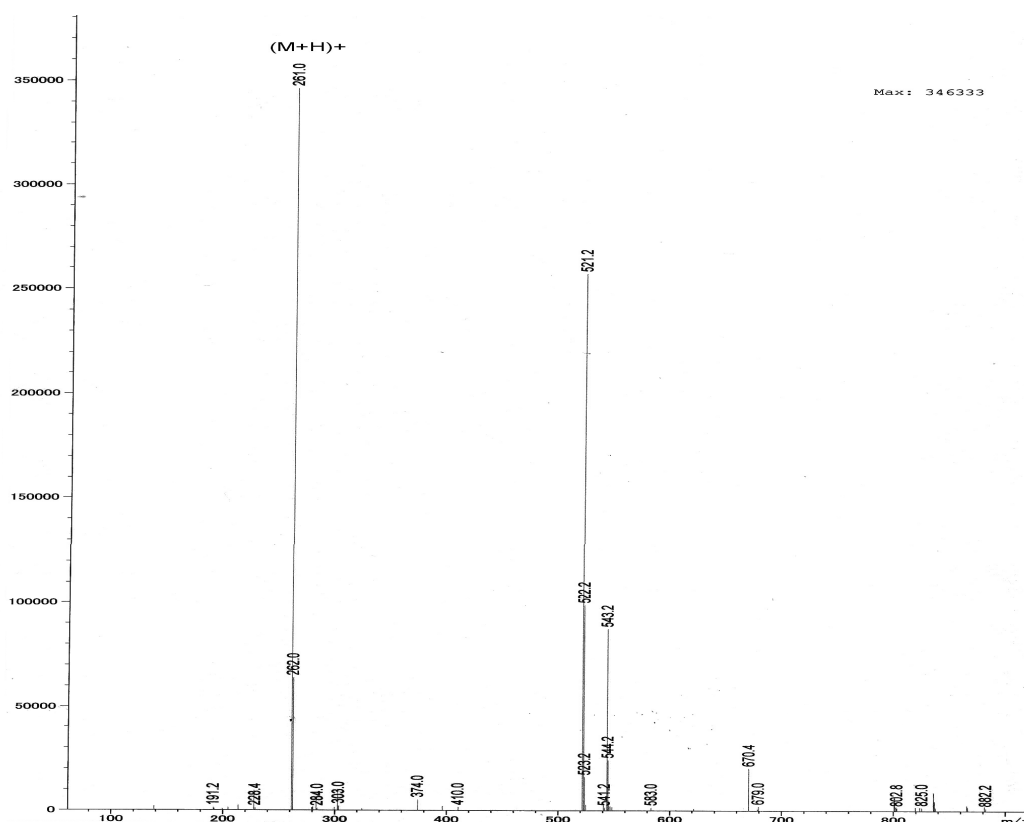


Fig 7. Spectrum ESI-MS m/z 261 (M+H)⁺ of active compound

preparative HPLC was presented at Fig. 5 and 6.

Determination of molecular weight, formula and structure elucidation of active compound was determined using ESI-MS, ¹H NMR, ¹³C NMR, and FTIR, respectively. ESI-MS spectra were obtained on LCT Premier-XE waters. ESI-MS spectra showed that this active compound has molecular weight of 260.0 g/mol. Data base from LCT Premier-XE Waters Program showed that this molecule had 14 carbon, 16 hydrogen, 2 nitrogen, and 3 oxygen. The most possible of molecular formula was C₁₄H₁₆N₂O₃. The position each atom carbon, nitrogen, oxygen, and hydrogen were confirmed by ¹H NMR, ¹³C NMR, and FTIR. This

chemical characteristics were indicated by ESI-MS at m/z 261 (M+H)⁺ as showed on Fig. 7.

High-resolution ¹H NMR spectrum were obtained on a Bruker AV-500 (500 MHz) with tetramethylsilane (TMS) as internal standard in CDCl₃ and give following datas: δ_H : 4.358 (t, 1H), 4.048(1H, dd), 2.088 (2H, m), 1.801 (2H, m), 3.518 (2H, dd), 3.066 (2H, dd), 7.031 (2H, d), 6.690 (2H, d), and ¹³C spectrum : 170.795 (s), 57.926 (d), 166.935 (s), 60.082 (d), 29.421 (t), 22.477 (t), 45.942 (t), 37.694 (t), 127.651 (s), 132.135 (d), 116.315 (d), 157.699 (s). The impurities of active compound was also showed at ¹H NMR δ_H 1.7 - δ_H 0.9. Spectrum of ¹H NMR and ¹³C NMR was showed on Fig. 8 and 9.

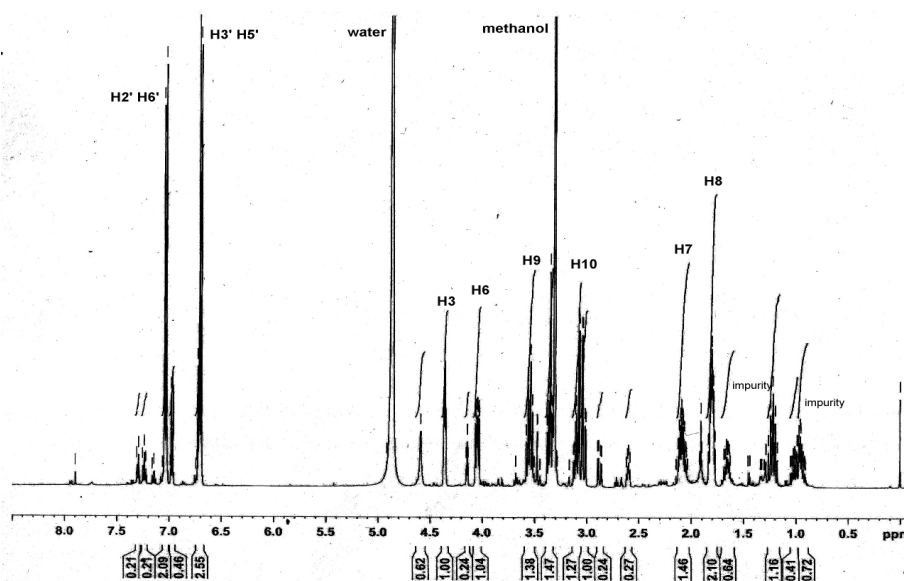


Fig 8. Spectrum of ^1H NMR active compound produced by *Streptomyces sp A11*

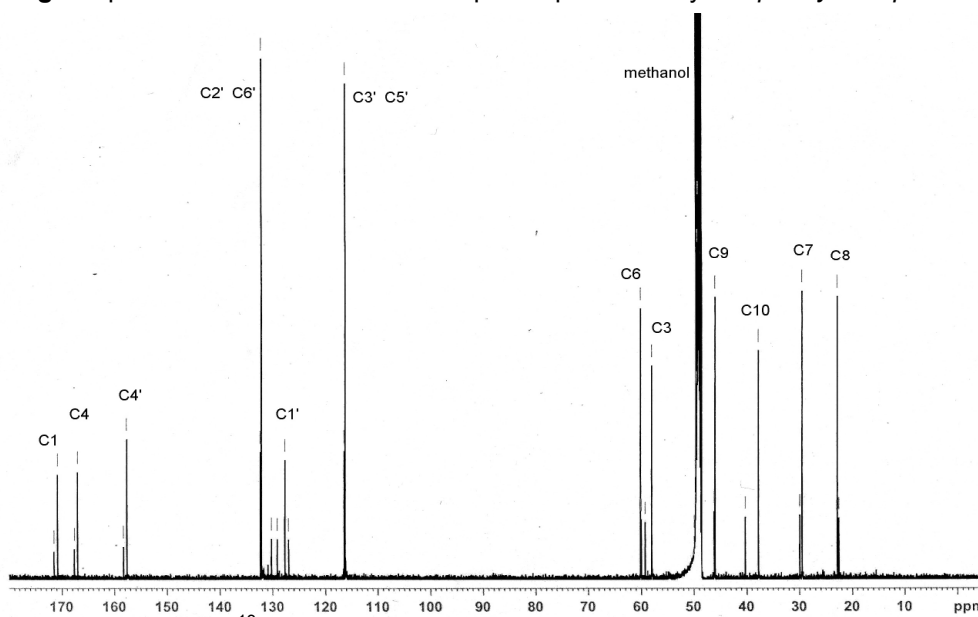


Fig 9. Spectrum of ^{13}C NMR active compound produced by *Streptomyces sp A11*

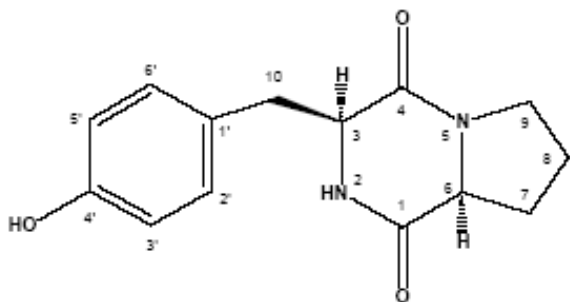


Fig 10. Molecule structure prediction of active compound produced by *Streptomyces sp A11*

Base on information of ESI-MS Spectrum and ^1H NMR, ^{13}C NMR spectrum can be predicted the molecule structure of active compound as Fig. 10.

Spectrum data of ^1H NMR and ^{13}C NMR were presented on Table 2.

Structure elucidation of compound was also conducted using DEPT ^{13}C NMR. DEPT ^{13}C NMR spectrum of active compound was presented on Fig. 11.

Two singlet carbons representing a ketone group were evident in the ^{13}C spectrum at δ 170.795 (s) (C1) and δ 166.935 (s) (C4) (see Table 2, Fig. 9). Further analysis of the ^{13}C spectrum revealed two other unsubstituted

carbons [δ 127.651 (C1'), 157.699 (C4')], six methine carbons [δ 57.926 (C3), 60.082 (C6), 132.135 (C2'), 116.315 (C3'), 116.215 (C5'), 132.135 (C6')], and four methylene carbons [δ 29.42 (C7), 22.477 (C8), 45.942 (C9), 37.694 (C10)]. DEPT 45° spectrum on Fig. 9 showed that there were 3 nonsubstituted carbon [δ 127.651 (C1'), 157.699 (C4'), and 166.935 (C4)]. DEPT 135° and 90° showed that there were six methine carbons [δ 57.926 (C3), 60.082 (C6), 132.135 (C2'), 116.315 (C3'), 116.215 (C5'), 132.135 (C6')] and four methylene carbons [δ 29.42 (C7), 22.477 (C8), 45.942 (C9)].

Carbons at position 3' and 5' appeared more upfield than C2' and C6'. This was due to the shielding effect of the hydroxyl group at C4' toward its ortho coupled carbon (C3' and C5'). A similar phenomenon occurred on C1' (para coupled with C4') which shifted more upfield than C2' and C6'.

The infra red spectrum in a KBr pellet showed characteristic bands at 3383 cm^{-1} (N-H), 3227 cm^{-1} (O-H), 2959 cm^{-1} (saturated C-H), 1660 cm^{-1} (C=O), 1515 cm^{-1} (benzene ring), 1456 cm^{-1} (methine), 1344 cm^{-1}

Table 2. Spectrum data of ^1H NMR and ^{13}C NMR of active compound produced by *Streptomyces sp A11*

No	δ ^{13}C (ppm)	δ ^1H (ppm) (in MeOD)	Functional group
1	170.795 (s)		$\begin{array}{c} \text{N}-\text{C}-\text{R} \\ \\ \text{O} \end{array}$
2			
3	57.926 (d)	4.358 (t)	$-\text{CH}-$
4	166.935 (s)		$\begin{array}{c} \text{N}-\text{C}-\text{R} \\ \\ \text{O} \end{array}$
5			
6	60.082 (d)	4.048 (dd)	$-\text{CH}-$
7	29.421 (t)	2.088 (m)	$-\text{CH}_2-$
8	22.477 (t)	1.801 (m)	$-\text{CH}_2-$
9	45.942 (t)	3.518 (dd)	$-\text{CH}_2-\text{N}-$
10	37.694 (t)	3.066 (dd)	$-\text{CH}_2-$
1'	127.651 (s)		$=\text{C}-\text{R}$
2'	132.135 (d)	7.031 (d)	$=\text{CH}-$
3'	116.315 (d)	6.690 (d)	$=\text{CH}-$
4'	157.699 (s)		$=\text{C}-\text{OH}$
5'	116.315 (d)	6.690 (d)	$=\text{CH}-$
6'	132.135 (d)	7.031 (d)	$=\text{CH}-$

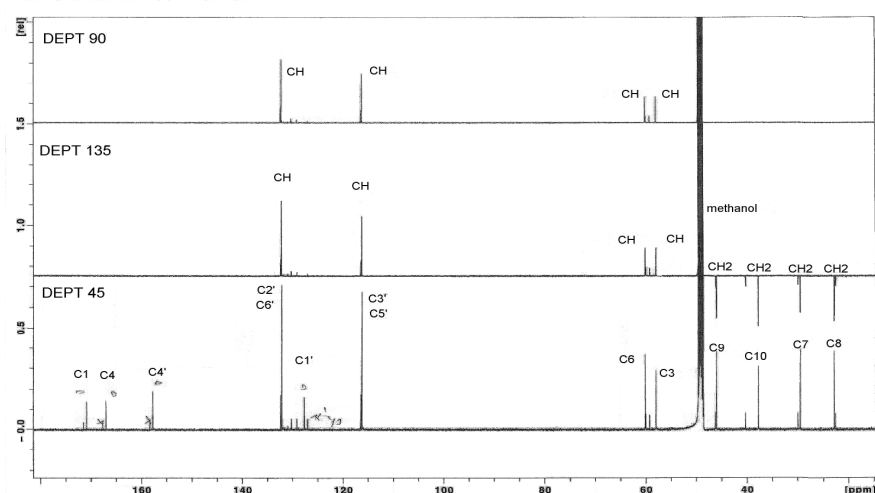


Fig 11. Spectrum of DEPT ^{13}C NMR active compound produced by *Streptomyces sp A11*

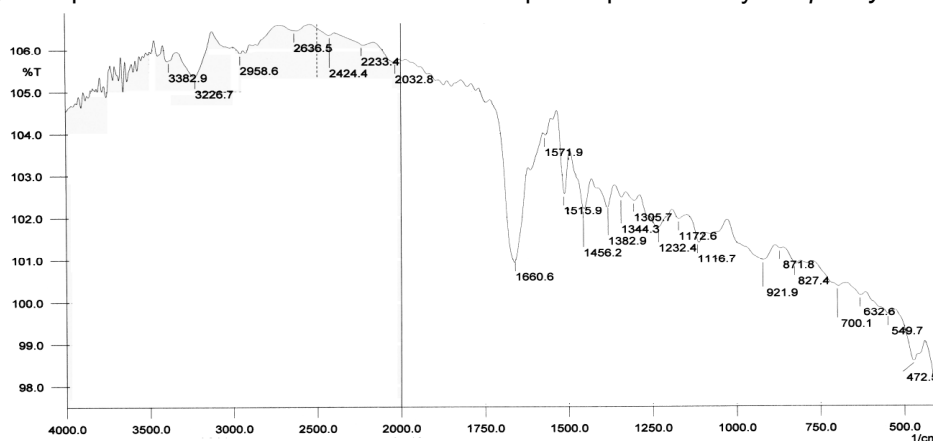


Fig 12. Infra red spectrum of active compound produced by *Streptomyces sp A11*

(methylene), 1232 cm^{-1} (phenol), 1116 cm^{-1} (C-O), 827 cm^{-1} (*p*-disubstituted benzene ring). This infra red spectrum was closely with previous published spectra for Cyclo (Tyr-Pro) [16-17]. Infra Red spectrum of active compound produced by *Streptomyces sp A11* was presented at Fig. 12.

This active compound was included in group cyclo-dipeptide, namely was cyclo (tyrosyl-prolyl). This active compound have the same profile such as ^1H NMR, ^{13}C NMR, UVmax absorption, Infra red spectrum, and molecular weight with cyclo(tyrosyl-prolyl) that found previous researcher but different origin [18-19]. Previously, this compound was produced by *Alternaria alternate* and can be used as a host-specific phytotoxin for spotted knapweed [18], and produced by *Pseudomonas fluorescens* GcM5-1A isolated from the pine wood nematode (PWN), *Bursaphelenchus xylophilus* [19].

CONCLUSION

Active compound produced by *Streptomyces sp A11* was detected as cyclopeptide antibiotic namely cyclo(tyrosyl-propyl)/(C₁₄H₁₆N₂O₃), molecular weight 260 g/mol with UV visible maximum absorbance 210 and 274.5 nm. This active compound was produced by extracellular secretion and active to *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 66923, *Pseudomonas aeruginosa* ATCC27853

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