Cyclo (Tyrosyl-Prolyl) Produced by *Streptomyces* sp.: Bioactivity and Molecular Structure Elucidation

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Determination of bioactivity by minimum inhibitory concentration (MIC) methods and molecular structure identification of antibiotic produced by *Streptomyces* sp. have been carried out. The antibiotic was produced by liquid culture using *Streptomyces* sp. isolate. Purification of antibiotic was carried out by silica gel column chromatography and preparative HPLC. Molecular structure identification was carried out using ESI-MS, \(^1\)H NMR, \(^13\)C NMR, and \(^13\)C DEPT NMR. Pure antibiotic showed inhibition activity to Gram-negative and Gram-positive bacteria. MIC to *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, and *Bacillus subtilis* ATCC 66923 were 27.0, 68.7, 80.2, and 73.7 µg mL\(^{-1}\), respectively. Identification using ESI-MS showed that the molecular weight of this antibiotic was 260 g mol\(^{-1}\), and molecular formula was \(C_{30}H_{40}N_{7}O_{11}\). Elucidation of molecular structure using \(^1\)H NMR, \(^13\)C NMR, and \(^13\)C DEPT NMR showed that antibiotic was cyclo(tyrosyl-prolyl).

Key words: antibiotic, cyclo(tyrosyl-prolyl), inhibitory concentration, bioactivity

Increasing microbial resistance due to the use of antibiotics and the emergence of new pathogenic microbes has inspired the search of new antibiotics from microbes. This situation encourages the growing importance of business to get cheap antibiotic materials continuously available in large quantities and has all the elements needed for the manufacture of antibiotics.

Approximately 800 classes of peptide antibiotics have been discovered and studied by several researchers. Most of these peptide antibiotics have been included in clinical trials and has been used in several clinical drugs such as actinomycin, gramicidine, bacitracin, polymyxyn, tyrocidine, and many other peptide antibiotics (Dubin et al. 2005).

Developments in science and inventions peptide antibiotics initiated new drug developments, especially for the treatment of infections (Kesting et al. 2010). The discovery of peptide antibiotics produces broad-spectrum antimicrobial activity with the potential to make peptides as anti-cancer drugs (Lu and Chen 2010) and anti-virus (Lee et al. 2011) or parasite infections (Manfredini et al. 2010). One member of the class of the simplest peptide antibiotics with low molecular weight was cyclo dipeptide group. Although cyclo dipeptide antibiotic have a low molecular weight and simple structure, this antibiotic is known have antimicrobial activity with a broad spectrum (Rhee 2004). For example antibiotics cyclo (leu-pro) has a minimum inhibition concentration (MIC) 32 µg mL\(^{-1}\) against *Enterococcus faecalis* K-01-511, and the MIC µg mL\(^{-1}\) against *E. faecalis* K-01-511. Cyclo(phe-pro) has a minimum inhibition concentration µg mL\(^{-1}\) against *E. faecium* K-99-38. Combination of cyclo(leu-pro) with cyclo(phe-pro) was able to increase antimicrobial activity, where the MIC against *E. faecium* 99-38 K-1 was 1 µg mL\(^{-1}\) and MIC against *E. faecalis* K-01-511 was 0.5 µg mL\(^{-1}\) (Rhee 2004). The objective of this research was to obtain information on bioactivity of an antibiotic produced by *Streptomyces* sp. and elucidate molecular structure of antibiotic.

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MATERIALS AND METHODS

Microorganism. Streptomyces sp. was isolated from sediment of marine site in Banten West Java (Sunaryanto et al. 2010). The isolate was deposited in strain collection of the Biotechnology of Microbial Culture Collection (Bio-MCC) BPPTpuspipherk Serpong, Tangerang.

Liquid Culture and Extraction of Active Substance. An established slant of isolate was inoculated into a 250 mL flask containing 100 mL of vegetative medium (YEME medium) consisting of: bacto peptone 5 g L⁻¹, yeast extract 3 g L⁻¹, malt extract 3 g L⁻¹, glucose 3 g L⁻¹, demineral water 25 mL, and sea water 75 mL. The pH value of the medium was adjusted to 7.6 before sterilization. The flask was incubated at 30 °C for 2 days in an incubator-shaker. Fifty milliliters of the culture was transferred to 1000 mL of the fermentation medium. Fermentation medium consisted of bacto peptone 15 g L⁻¹, yeast extract 3 g L⁻¹, Fe (III) citrate hydrate 0.3 g L⁻¹, demineralized water 250 mL, and sea water 750 mL (Nedialkova and Mariana 2005). The pH value of the medium was adjusted to 7.6 before sterilization. The fermentation was carried out at 30 °C for 5 days in incubator-shaker.

For extraction of active substance, the culture broth was centrifuged at 14 000 x g for 15 min. Biomass was dried and weighed than extracted twice using methanol 500 mL. Methanol extract was concentrated by evaporation under vacuum to the least possible volume, after dehydration with anhydrous Na₂SO₄ and weighed. The broth supernatants were extracted using ethyl acetate. Supernatant and the organic solvent were mixed thoroughly by shaking them in 2 L capacity separating funnel and allowed to stand for 30 min. Two layers were separated; the aqueous layer and the organic layer, which contained the solvent and the antimicrobial agent. The organic layer was concentrated by evaporation under vacuum to the least possible volume, after dehydration with anhydrous Na₂SO₄ and weighed. The aqueous layer was re-extracted and the organic layer added to the above organic layer. The organic layer was concentrated by repeated cycle of evaporation under vacuum.

The dry extract of the supernatant was purified using silica gel column chromatography. Dry extract was injected onto the column and then eluted stepwise with chloroform-methanol solvent system as follows: first the column was eluted with 100% chloroform (fraction 1). Then repeated with reducing the chloroform by 10% in each fraction while the methanol was increased by 10% in each fraction, until the percentage of methanol was 100%. Thirty fractions were collected (each of 20 mL) and then concentrated and dried for testing their antimicrobial activities. The active fractions obtained from chromatography column were further purified by preparative HPLC.

Preparative HPLC. Purification by preparative HPLC was conducted using a Waters 2695 HPLC, photodiode array detector (PAD), and column puresil 5μ C18 4.6x150 mm. The sample volume was 100 L per injection under conditions of average pressure of 1267 psi, and the flow rate was 1 mL min⁻¹ where the mobile phase was 0-45% methanolwater and time period was 25 min.

Elucidation of Chemical Structure. Molecular weight and formula were determined using ESI-MS (LCT Premier-XE waters), molecular structure elucidation of active compound were determined using FTIR (Shimadzu 8300), ¹H NMR, ¹³C NMR, and DEPT ¹³C NMR (Buker AV-500 (500 Mhz)).

Antimicrobial Activity Assay. Antimicrobial activity was monitored by the agar diffusion paper-disc (6 mm) method. Discs were dripped with methanol solution of extract, dried, and then placed over the agar surface plates freshly inoculated with either E. coli ATCC 25922, Staphylococcus aureus ATCC25923, Bacillus subtilis ATCC 66923, and Pseudomonas aeruginosa ATCC27853 as test organisms. Suspensions of test organisms were adjusted to 10⁶ CFU mL⁻¹. Each experiment was run in duplicate, and the most potent isolates were noted for each test microorganism, based on the mean diameter of inhibition zones (Bonev et al. 2008). Rifampicin (500 ppm) was used as a control.

Minimum Inhibitory Concentration (MIC). MIC determinations were performed using the agar-dilution methods according to methods of Andrews (2001) and Bonev et al. (2008). Active purified compound was dissolved in methanol (6500 μg mL⁻¹) and taken as standard stock. A series of two fold dilutions each solution were dripped on paper disc 6 mm, dried, then placed over on agar surface plates freshly inoculated with either E. coli ATCC 25922, S. aureus ATCC25923, B. subtilis ATCC 66923, or P. aeruginosa ATCC27853. Minimum inhibitory concentration is defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a micro-organism and determined toward a standard curve of clearing zone diameter and the concentration of active compound. The MIC of tetracycline and streptomycin as positive controls were also determined and each experiment was run in duplicate.

HPLC Analysis. Analysis was performed using HPLC with an analytical Sunfire C18 column (4.6 x 250 mm, Shimcodo Co. Ltd., Tokyo, Japan). Mobile
phase used methanol-water (0-100% linear gradient for 25 min and then isocratic elution with 100% methanol over 10 min), at a flow rate of 1 mL min⁻¹, volume of injection 10 µL per injection, and detection was at a wavelength of 210 nm.

**RESULTS**

Liquid culture of isolate was carried out for 5 days by using yeast-peptone medium. From a 5 L volume of culture, 4.72 g of dry biomass was obtained and after extraction by methanol 2.72 g of extract was obtained. On the other hand, 0.33 g of ethyl acetate extract was obtained from supernatant. Antibacterial activity assay of the both extract against ATCC 25922, ATCC 25923, ATCC 27853, ATCC 66923 showed that the extract of supernatant was active, but no activity with the extract of biomass (Table 1).

Supernatant extract was further purified using column chromatography and preparative HPLC. Pure active fraction was collected and MIC was determined using four bacterial testes. The active fraction had a strong inhibition against Gram-positive and Gram-negative (Table 2).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Diameter of clear zone (mm)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Escherichia coli ATCC 25922</td>
</tr>
<tr>
<td>Extract of biomass</td>
<td>-</td>
</tr>
<tr>
<td>Extract of supernatant</td>
<td>9.55</td>
</tr>
<tr>
<td>Control (rifampicin 500 ppm)</td>
<td>10.12</td>
</tr>
<tr>
<td>Diameter of disc paper: 6 mm</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Antibacterial activity of biomass and supernatant extract from Streptomyces sp.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Minimum inhibitory concentration µg mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli ATCC 25922</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 25923</td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis ATCC 66923</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 27853</td>
<td></td>
</tr>
<tr>
<td>Active purified compound</td>
<td>27.0</td>
</tr>
<tr>
<td>Tetracycline (positive control)</td>
<td>64.0</td>
</tr>
<tr>
<td>Streptomycin (positive control)</td>
<td>50.0</td>
</tr>
</tbody>
</table>

Table 2 Minimum inhibitory concentration of active purified compound.

**ESI-MS, ¹H NMR, §C NMR (Fig 1), and FTIR respectively (Fig 4). 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⁻¹. 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 carbon, nitrogen, oxygen, and hydrogen atoms were confirmed by ¹H NMR, §C NMR, and FTIR. This chemical characteristics were indicated by ESI-MS at m/z 261 (M+H)⁺ (Fig 1A).

High-resolution ¹H NMR spectrum were obtained on a Bruker AV-500 (500 MHz) with tetramethylsilane (TMS) as internal standard in CDCl₃ and give

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Fig 1  Spectrum of bioactive compound produced by *Streptomyces* sp.: a, ESI-MS m/z 261 (M+H); b, ¹H NMR; c, ¹³C NMR.
following data: \( \delta \): 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 \( ^{13}C \) 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 \( \delta \): 1.7 - \( \delta \): 0.9 (Fig 1B). Based on information of ESI-MS, \( ^1H \) NMR, and \( ^{13}C \) NMR (Fig 3) spectrums the molecular structure of active compound can be predicted as Fig 2. Spectrum data of \( ^1H \) NMR and \( ^{13}C \) NMR were presented on Table 3. Structure elucidation of active compound was also conducted using DEPT \( ^{13}C \) NMR (Fig 3).

Two singlet carbons representing a ketone group were evident in the \( ^{13}C \) spectrum at \( \delta \): 170.795 (s) (C1) and \( \delta \): 166.935 (s) (C4) (Table 3, Fig 1C). Further analysis of the \( ^{13}C \) spectrum revealed two other non-substituted carbons [\( \delta \): 127.651 (C1'), 157.699 (C4')], six methine carbons [\( \delta \): 57.926 (C3), 60.082 (C6), 132.135 (C2'), 116.315 (C3'), 116.215 (C5'), 132.135 (C6')], and four methylene carbons [\( \delta \): 29.42 (C7), 45.942 (C9), 37.694 (C10)]. DEPT 45° spectrum on Fig 3 showed that there were 3 non-substituted carbon [\( \delta \): 127.651 (C1'), 157.699 (C4'), and 166.935 (C4')]. DEPT 135° and 90° showed that there were six methine carbons [\( \delta \): 57.926 (C3), 60.082 (C6), 132.135 (C2'), 116.315 (C3'), 116.215 (C5'), 132.135 (C6')] and four methylene carbons [\( \delta \): 29.42 (C7), 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\(^{-1}\) (methylene), 1232 cm\(^{-1}\) (phenol), 1116 cm\(^{-1}\) (C-O), 827 cm\(^{-1}\) (\( p \)-disubstituted benzene ring) (Fig 4).

**DISCUSSION**

The active compounds inhibited both Gram-positive and Gram-negative bacteria. According to Rhee (2004), most of antibiotic peptides especially cyclic dipeptide has antimicrobial properties with broad spectrum. Several cyclo peptides have broad-
spectrum activity and also able to act as an anti-viral and anti cancer. The same case were also presented by (Lu and Chen 2010; Lee et al. 2011). In addition to having antimicrobial activity with a broad spectrum, our compound also had lower MIC compared with tetracycline suggesting higher activity than tetracyclin and streptomycin. The same result is shown by Rhee (2004).

Activity of cyclo(tyrosyl-prolyl) on the P. aeruginosa is lower compared with tetracycline. It is suspected that the P. aeruginosa has a resistance to cyclo(tyrosyl-prolyl). According to Macfarlane et al. (2000) and Reig et al. (2009), P. aeruginosa has a resistance to type of peptide and aminoglycoside antibiotics. Cyclo(tyrosyl-prolyl) was peptide antibiotic and streptomycin was aminoglycoside antibiotic. P. aeruginosa has highly adaptable nature, including the ability to develop resistance. It can grow on a wide variety of substrates and alter its properties in response to changes in the environment (Lambert 2002). Thus, lower inhibition of cyclo(tyrosyl-prolyl) and streptomycin than that of tetracycline on P. aeruginosa might be caused by resistance.

This active compound was included in group cyclo dipeptide, namely was cyclo(tyrosyl-prolyl). This active compound has the same profile such as ¹H NMR, ¹³C NMR, Infra red spectrum, and molecular weight with cyclo(tyrosyl-prolyl) that found previously but from different origin (Stierle et al. 1988; Guo et al. 2007). Previously, this compound was produced by Alternaria alternate and can be used as a host-specific phytotoxin for spotted knapweed (Stierle et al. 1988), and produced by P. fluorescens GcM5-1A isolated from the pine wood nematode (PWN), and from Bursaphelenchus xylophilus (Guo et al. 2007).

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