Chapter- 7

Purification and Characterization of Bioactive Molecules
7. Purification and Characterization of bioactive molecules

7.1. Introduction

Microorganisms produce primary metabolites (amino acids, proteins, carbohydrates, vitamins, acetone, ethanol, organic acids etc) and secondary metabolites (antibiotics, toxins, alkaloids, gibberellins etc) during active cell growth or near the onset of stationary phase. There are seven known classes of antimicrobial substances including anthraquinones, bacteriocins (xenorhabdacin), indoles, phage, stilbenes, xenocoumacins and xenorhabdins isolated from various strains of *Xenorhabdus* and *Photorhabdus*. Xenocoumacins are produced by *X. nematophilus* and an unidentified *Xenorhabdus* (McInerney et al., 1991b), indoles and xenorhabdins by *X. bovienii*, and stilbenes and anthraquinones by *P. luminescens* (Akhurst and Dumphy, 1993). Little is known about the spectrum of antimicrobial substances produced by any one strain of *Xenorhabdus* or *Photorhabdus*, or about the efficacy and production of these known antimicrobial agents. These symbiotic bacteria have not only a wide-spectrum of activity but also their activity varies with bacterial growth, strain and species.

7.2. Materials and methods

Materials

The culture media used are listed in appendix (II). Chemicals used for the study are listed in appendix (III). Sterile nuclease-free and protease free glass wares and plastic wares are used for the preparation and storage of reagents and to carry out experimental procedures. The bacterial isolate used in the study is isolate 532. The bacterial and fungal strains used are given in the methods of antibacterial and antifungal activity test.

Methods

The bacterium was isolated from the infective juvenile nematodes of the *Rhabditis sp. (isolate 532)* maintained alive by sub culturing them on *Galleria mellonella* once in a month, inoculated into trypticase soy broth (TSB) and incubated at 28°C for 72 hr. The culture was centrifuged at 10,000 × g for 15 min at 4°C and the
cell free culture filtrate was separated into aqueous and organic fractions by extracting with ethyl acetate thrice. The organic fraction was evaporated to dryness by a Buchi rotary evaporator. The above isolation process was repeated several times to yield about 7 g of the oily organic residue from 15 L of culture broth.

**Bacterial fermentation**

To purify antimicrobial agents from the bacterial isolate 532, bacterial fermentation was carried out. The mother culture was prepared by inoculating the bacteria into 100 ml of trypticase soy broth (TSB)) and kept at 28°C at 150 × g for 24 hr incubation. This 24 hr mother culture (with an optical density of 1.5) was used to inoculate 400 ml of TSB and incubated at 28°C for 72 hr. The culture was centrifuged at 10,000 × g for 15 min at 4°C to get cell free culture filtrate which was concentrated with Buchi rotary evaporator.

**Separation of bioactive molecules in the organic fraction**

The active ethyl acetate fractions were pooled (7 gm) and subjected to silica gel column chromatography [60-120 mesh, 44 cm long and 2.5 cm in diameter]. The column was eluted with a gradient of solvent mixtures of increasing polarity starting from a non polar solvent such as hexane, followed by mixtures of dichloromethane in hexane, ethyl acetate in dichloromethane and finally with methanol in ethyl acetate. About 200 ml each solvent was passed and each 25 ml was collected separately, which were concentrated using a Buchi rotary evaporator. The different fractions were subjected to TLC; similar fractions were pooled and assayed for antimicrobial activity. Solvent systems for TLC were standardised to obtain optimum separation. The fractions which contain traces of impurities were subjected to preparative TLC in order to isolate the pure compound, followed by crystallization with various solvents. The pure compounds obtained were subjected to NMR (H1,13C), LCMS, IR, for the elucidation of the structure of the compound.

**Purification of the organic compounds**

Purification of organic fraction was done using column chromatography, TLC and HPLC.
**Column chromatography**

The ethyl acetate extracts were chromatographed on a silica gel column (silica gel 60, 45 x 2.5 cm). Two active subfractions were collected from column, through solvent systems such as dichloromethane/hexane, and methanol/ethyl acetate. These were mixture of compounds and purification was done by preparative TLC and crystallization process. The single compounds obtained from column were further purified by crystallization process.

**Thin layer chromatography**

The separation of the organic fraction was carried out by thin layer chromatography (TLC). The solvents used for the separation of subfractions were Benzene-hexane, acetone-benzene and methanol-chloroform combinations (Table 7.1). These solvent systems were found to be the most suitable ones.

Each subfraction was spotted (2 µl) on TLC plates in order to check the separation of the compounds. The subfractions were found to have mixture of compounds. Hence number of bands and Rf values were calculated. After standardization, following solvents were used for thin layer chromatography.

**Table 7.1. Sub fractions and solvents used for TLC**

<table>
<thead>
<tr>
<th>Sl.no.</th>
<th>Sub fractions</th>
<th>Solvents used for TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DCM/hexane</td>
<td>benzene/hexane</td>
</tr>
<tr>
<td>2</td>
<td>ethyl acetate/DCM</td>
<td>acetone/benzene</td>
</tr>
<tr>
<td>3</td>
<td>methanol/ethyl acetate</td>
<td>methanol/chloroform</td>
</tr>
</tbody>
</table>

**High Performance Liquid Chromatography (HPLC)**

After performing the TLC, only the pure compounds were subjected to HPLC using methanol as mobile phase.
Elucidation of the structure of the compound

The pure compounds obtained were subjected to NMR (H\textsuperscript{1,13}C) (Brucker AMX-500 at National Institute for Interdisciplinary science and Technology (CSIR), Thiruvananthapuram), FTIR (Perkin Elmer at Sree Chitra Thirunal Institute of Science and Technology, Thiruvananthapuram) and optical rotation (Rudolph Research analytical Autopol N polarimeter at Tropical Botanical Garden and Research Institute, Thiruvananthapuram) were carried out. The mass spectrum was recorded by JEOL-JMS mass spectrometer at National Institute for Interdisciplinary science and Technology (CSIR), Thiruvananthapuram) for the elucidation of the structure of the compound.

Antibacterial activity

Antibacterial activity of the purified compounds was measured using disc diffusion assays against the test organism \textit{B. subtilis}. The three identified compounds were tested against \textit{Bacillus subtilis} MTCC 2756, \textit{Escherichia coli} MTCC 2622 and \textit{Staphylococcus aureus} MTCC 902. Samples to be tested were filtered through 0.22 µm micro filters. The level of activity was measured by the diameter (mm) of the zone of inhibition.

Disc diffusion method

The disc diffusion method (Bauer \textit{et al.}, 1966) was used to screen the antimicrobial activity. In vitro antimicrobial activity was screened by using Mueller Hinton Agar (MHA) obtained from Himedia. The MHA plates were prepared by pouring 15 ml of molten media into sterile Petri plates. The plates were allowed to solidify for 5 min and 0.1% inoculum suspension was swabbed uniformly and the inoculum was allowed to dry for 5 min. The concentration of 50 µl of sample was loaded on 6 mm sterile disc. The loaded disc was placed on the surface of medium and the compound was allowed to diffuse for 5 min and the plates were kept for incubation at 37°C for 24 hr. Antibacterial activity was evaluated by measuring the diameter of the inhibition zone.
**Antifungal activity**

Antifungal activity was determined using well diffusion method (Perez et al., 1990). The prepared potato dextrose agar plates were swabbed with the test fungal culture *Aspergillus flavus* MTCC 183, *Fusarium oxysporum* MTCC 284 and *Rhizoctonia solani* MTCC 2644 by using plate method. Wells were made on the agar surface with 6 mm cork borer. The sample of 50 µl was poured into the well using sterile syringe. The plates were incubated at 37°C for 48 hr. The plates were observed for the zone formation around the wells. The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm).

**7.3. Results**

**Bacterial fermentation**

Fermentation of bacteria in tryptic soy broth was carried out under controlled conditions in order to obtain about 15 litres of cell free culture filtrate which was used for the isolation and purification of bioactive molecules. The 72 hr cell free culture filtrate was separated into organic and aqueous fractions using ethyl acetate. The ethyl acetate fraction on concentration yielded about 7 gm crude residues which were subjected to further purification.

**Separation of bioactive molecules in the organic fraction**

The crude ethyl acetate fraction was subjected to silica gel column chromatography and the solvent system was standardized for the best separation. Elution was carried out using a gradient of solvent mixtures of increasing polarity starting from a non polar solvent such as hexane followed by mixtures of dichloromethane in hexane (5%-100%), ethyl acetate in dichloromethane (1%-100%) and finally with methanol in ethyl acetate (1%-100%). The sub fractions collected in methanol/ethyl acetate solvent system were found to have mixtures of compounds. The inclusion of chloroform/ethyl acetate after ethyl acetate/dichloromethane improved the separation. The various fractions, after concentration were tested for antibacterial and antifungal activity. Two sub fractions eluted with dichloromethane/hexane and methanol/ethyl acetate possessed strong antimicrobial activity.
Purification of the organic compounds

The dichloromethane/hexane, ethyl acetate/dichloromethane, methanol/ethyl acetate fraction of TSB obtained after column chromatography were dissolved in 300 µl methanol and stored at -20°C for further purification and assay of bioactivity. TLC profile indicated a single spot which indicate the purity of the compounds. From the three sub fractions only one pure compound was obtained.

Fraction 1. Dichloromethane/hexane (50:70)

TLC analysis of fraction 1 was showed in Fig.7.1. It was a mixture of organic acids-acetic acid and butyric acid along with an unknown acid. This fraction gave positive result for the test for organic acid by dipping the chromatogram in a solution of 5 mg bromo cresol green in 25 ml ethanol and 0.25 ml of 0.1M NaOH). A yellow spot was obtained on a blue background (Fig.7.2). The HPLC elution profile of fraction 1 was displayed in Fig.7.3. This fraction possesses antibacterial and antifungal properties (Table 7.2 and Plate IV Fig.7.4-7.8).

Fig.7.1. TLC profile of fraction 1

Fig.7.2. Positive test for organic acid
Figure 7.3. HPLC elution profile of fraction 1 on Aminex HPX-87H column (biorad)

Table 7.2. Antimicrobial activity of organic acid mixture against the test organisms

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis</td>
<td>25</td>
</tr>
<tr>
<td>S. aureus</td>
<td>15</td>
</tr>
<tr>
<td>A. flavus</td>
<td>22</td>
</tr>
<tr>
<td>F. oxysporum</td>
<td>18</td>
</tr>
<tr>
<td>R. solani</td>
<td>14</td>
</tr>
</tbody>
</table>
Fig. 7.4. Antibacterial activity of organic acid mixture against *B. subtilis*

Fig. 7.5. Antibacterial activity of organic acid mixture against *S. aureus*

Fig. 7.6. Antifungal activity of organic acid mixture against *A. flavus*

Fig. 7.7. Antifungal activity of organic acid mixture against *F. oxysporum*

Fig. 7.8. Antifungal activity of organic acid mixture against *R. solani*
Fraction 2. Ethyl acetate/dichloromethane

One of the pure compounds was obtained in powder form from ethyl acetate fractions (16-19%) collected from column with Rf value of 0.58 (TLC profile on Fig.7.9) and the yield of the compound was 157 mg. The pure compound on HPLC indicated a single peak (Fig.7.10) and the retention time of the compound was 5.46.

Figure.7.9. TLC profile of fraction 2

Figure.7.10. HPLC elution profile of Cyclo (pro-leu). C -18 reverse phase column, 50% methanol –water as the mobile phase
Identification of compounds

NMR (C$^{13}$ and H$^1$) was recorded (Fig. 7.11 and 7.12). Mass was determined as 210 Da. It had a melting point of 169ºC (Fig. 7.13) and optical rotation of 139.7. The IR was also taken for the compound (Fig. 7.14). The compound was identified as Cyclo (pro-leu) (Fig. 7.15) based on the above spectral analysis.

Figure. 7.11. C13 NMR of Cyclo (pro-leu)
Figure 7.12. $^1$H NMR of Cyclo (pro-leu)

Fig. 7.13. Melting point of Cyclo (pro-leu)
Fraction 3. Methanol/ethyl acetate fraction

After passing 100% ethyl acetate, the column was eluted with 100% methanol. This was loaded on to a small column filled with LH-20 packed with 100% water. The column was eluted with increasing concentrations of methanol (1%, 2%...25%).

The fractions eluted from the column were evaporated with Buchi rotary evaporator. The solvent system used for TLC (Fig.7.16) was methanol-acetone (8:2). The HPLC elution profile of fraction 3 is on Fig.7.17. The fractions 1-9 and 19-28 were combined and this showed good antibacterial and antifungal activity (Table 7.3 and Plate V & VI Fig.7.18-7.26).
Table 7.3. Antimicrobial activity of methanol/ethyl acetate fraction against test organisms

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis</td>
<td>23</td>
</tr>
<tr>
<td>S. aureus</td>
<td>19</td>
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<tr>
<td>A. flavus</td>
<td>20</td>
</tr>
<tr>
<td>F. oxysporum</td>
<td>16</td>
</tr>
<tr>
<td>R. solani</td>
<td>13</td>
</tr>
</tbody>
</table>

Fig. 7.16. TLC profile of fraction 3

Fig. 7.17. HPLC elution profile of fraction 3 on C-18 reverse phase column, 100% methanol as the mobile phase
Fig. 7.18. Antibacterial activity of methanol/ethyl acetate fraction against *B. subtilis*

Fig. 7.19. Antibacterial activity of methanol/ethyl acetate fraction against *S. aureus*

Fig. 7.20. Antifungal activity of methanol/ethyl acetate against *A. flavus*

Fig. 7.21. Antifungal activity of methanol/ethyl acetate fraction against *F. oxysporum*

Fig. 7.22. Antifungal activity of methanol/ethyl acetate fraction against *R. solani*
PLATE VI

Fig. 7.23. Common control

Fig. 7.24. Common control

Fig. 7.25. Common control

Fig. 7.26. Common control
7.4. Discussion

Among the three fractions, two sub fractions eluted with dichloromethane/hexane and methanol/ethyl acetate possessed strong antimicrobial activity. The Fraction 2 i.e. ethyl acetate/dichloromethane was obtained in pure form. Based on NMR, FTIR and mass spectra the structure was elucidated and it was identified as a derivative of diketopiperazine (DKPs). The compound is identified as Cyclo (Pro-leu).

DKPs have received continuous interest because of their spectrum of pharmacological activities and are used as antibiotics, synthetic vaccines and in cancer chemotherapy (Funabashi et al., 1994).

DKPs are in themselves intriguing bioactive molecules. Several DKPs, cyclo (Delta Ala-L-Val), cyclo (L-Pro-L-Tyr) and cyclo (L-Phe-L-Pro), isolated from cell free supernatants of various Gram-negative bacteria, including Enterobacter agglomerans, Citrobacter freundii and various Pseudomonas species, have been implicated in quorum sensing. All three of these DKPs are capable of activating a recombinant LuxR based N-acylhomoserine lactone (AHL) biosensor in Escherichia coli as well as antagonizing the effect of the natural AHL activator, 3-oxo-C6-HSL. Additionally, cyclo (Delta Ala-L-Val) and cyclo (L-Pro-L-Tyr) are capable of inhibiting the AHL-mediated swarming motility of Serratia liquefaciens, and cyclo (L-Pro-L-Tyr) is capable of activating another AHL biosensor in Agrobacterium tumefaciens (Holden et al., 1999). Vibrio spp. also produce cyclo (Phe-Pro), which affects the expression of the Tox-R-dependent genes ompU, ctxA and ctxB, the latter two encoding subunits of cholera toxin which is involved in virulence of Vibrio cholera (Park et al., 2006). DKPs can also act as antifungal compounds. Cyclo (L-Phe-L-Pro) and cyclo- (L-Phe-trans -4-OH-L-Pro) isolated from Lactobacillus plantarum have been shown to be active against Fusarium sporotrichioides, Aspergillus fumigatus and Kluy Veromyces marxianus (Strom et al., 2002). Finally, DKPs have been shown to be bactericidal as two marine bacterial strains associated with cultures of Pecten maximus produce a series of five DD-DKPs that are active against Vibrio anguillarum (Fdhila et al., 2003).
It is known that cyclic dipeptides have many potential biological functions. The investigation of the preferred conformations of cyclic dipeptides is very important to explore the functionary mechanism and discover the biological characteristics of cyclic dipeptides. Cyclic peptides are more bioavailable and more stable against degradative peptidases than linear peptides, the relevant research is fundamental to many aspects of peptide chemistry. Stierle et al. (1988) first discovered DKPs in a Micrococcus sp. isolated from sponge *Tedania ignis*, which was the first demonstration that a bacterium associated with sponge produces secondary metabolites ascribed to the sponge host. In the case of cyclo (L-Trp-L-Phe), it was first isolated from an unidentified *Penicillium* sp. and proved to be with the biological function of regulating the growth of plant by Kimura et al. (1996). In 2008 cyclo (L-Trp-L-Phe) was isolated from fungal EF8 which was isolated from the conchocelis of *Porphyra yezoensis* by Ding et al. (2008) and proved to exhibit a moderate cytotoxicity against 37 human tumor cell lines with the average IC50 3.3 lg ml-1. But till now, few reports on the production of DKPs by microbial cultivation have been found (Sedlock et al., 1994). This study was the first to find the cyclo (Pro-leu) from the bacteria symbiotically associated with the nematode Rhabditis sp.

Diketopiperazines, the smallest cyclic peptides known, are very important in biology and drug discovery as it is possible to obtain amino acids from them, rendering them a possible substitute for common peptides. DKPs can be used to stereoselectively incorporate an amino acid functionality into a molecule and be selectively deprotected under mild condition using cerium ammonium nitrate (CAN) (Davies et al., 2007; Balducci et al., 2009; O’ Reilly et al., 2009). These compounds have been found in both microorganisms (Boeck et al., 1971) and plants (Musetti et al., 2007). Like many products of microbial secondary metabolism they show antimicrobial activity against some organisms (Musetti et al., 2007; Hirano et al., 2008) and in nature they are usually synthesised from a small library of primary metabolites.

DKPs can generate amino acids or peptides through hydrolysis in appropriated conditions (Davies et al., 2007). They are also important building blocks for other chiral compounds and they have potential applications in industry and medicine (Bartels et al., 1995; Gellerman et al., 2008; Davies et al., 2007). Research in this area is ongoing (O’ Reilly et al., 2009; Borthwick et al., 2006).
7.5. Conclusion

This is the first time that the Diketopiperazine derivative i.e. Cyclo (pro-leu) has been isolated, purified and identified from a nematode-bacterium system. Both the TLC and HPLC methods were applied to the analysis of the bioactive compounds obtained from the symbiotic bacterial isolate 532. Two sub fractions eluted with dichloromethane/hexane and methanol/ethyl acetate possessed strong antimicrobial activity. This is effective against human pathogenic bacteria and fungi and agriculturally important *F. oxysporum* and *R. solani*. The metabolites of the antagonistic bacteria may be developed to an effective antibiotic.