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# Purification, structural elucidation and bioactivity of tryptophan containing diketopiperazines, from *Comamonas testosteroni* associated with a rhabditid entomopathogenic nematode against major human-pathogenic bacteria

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ABSTRACT

The cell free culture filtrate of a *Comamonas testosteroni* associated with an Entomopathogenic nematode (EPN), *Rhabditis (Oscheius)* sp. exhibited promising antimicrobial activity. The ethyl acetate extract of the bacterial culture filtrate was purified by silica gel column chromatography to obtain five diketopiperazines or cyclic dipeptides (DKP 1–5). The structure and absolute stereochemistry of the compounds were determined based on extensive spectroscopic analyses (HR-MS, $^{1}$HNMR, $^{13}$CNMR, $^{1}$H–$^{1}$H COSY, $^{1}$H–$^{13}$C HMBC) and Marfey's method. Based on the spectral data the compounds were identified as Cyclo-(L-Trp-L-Pro) (1), Cyclo-(L-Trp-L-Tyr) (2), Cyclo-(L-Trp-L-Ile) (3), Cyclo-(L-Trp-L-Leu) (4) and Cyclo-(L-Trp-L-Phe) (5), respectively. Three diketopiperazines (DKP 2, 3 and 5) were active against all the ten bacteria tested. The highest activity of 0.5 μg/ml by Cyclo-(L-Trp-L-Phe) was recorded against *Vibrio cholerae* followed by *Salmonella typhi* (1 μg/ml) a human pathogen responsible for life threatening diseases like profuse watery diarrhea and typhoid or enteric fever. The activity of this compound against *V. cholerae* and *S. typhi* is more effective than ciprofloxacin and ampicillin, the standard antibiotics. Cyclo-(L-Trp-L-Phe) recorded significant antibacterial activity against all the test bacteria when compared to other compounds. Five diketopiperazines were active against all the test fungi and are more effective than bavistin the standard fungicide. Diketopiperazines recorded no cytotoxicity to FS normal fibroblast and VERO cells (African green monkey kidney) except DKP 3 and 4. To our best knowledge this is the first report of antimicrobial activity of the tryptophan containing diketopiperazines against the human pathogenic microbes. The production of cyclic dipeptides by *C. testosteroni* is also reported here for the first time. We conclude that the *C. testosteroni* is promising sources of natural bioactive secondary metabolites against human pathogenic bacteria which may receive great benefit in the field of human medicine in near future.


## 1. Introduction

Antibiotics are one of the pillars of modern medicine. Throughout the ages, natural products have been the most consistently successful sources of lead compounds that have found many applications in the fields of human medicine, pharmacy and agriculture [52,54]. Microbial natural products have been the source of most of the antibiotics in current use for the treatment of various infectious diseases. Many terrestrial born bacteria are reported to produce secondary metabolites having antimicrobial property [5]. However, bacterial resistance emerges when an antimicrobial agent is introduced into the market or just after its introduction due to misuse [4]. So there is a continuous need for new chemotherapeutants, especially novel antibiotics, to combat new diseases and drug-resistant pathogens that are becoming a significant threat to public health [56]. The discovery and development of new drugs from natural products (NPs) has played a significant role over the last few decades. Over 28% of the new chemical entities and 42% of the anticancer drugs introduced into the market can be traced back to NPs [42]. The majority of these have been isolated from terrestrial-borne microbes. Meanwhile, the emergence of severe resistance to antibiotics in microbial pathogens, such as methicillin and vancomycin-resistant *Staphylococcus aureus* (MRSA & VRSA) and vancomycin-resistant *Enterococci* (VRE), and the current

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increase in the number of new diseases/pathogens, e.g., Gram-negative New Delhi metallo-beta-lactmase (NMD-1) bacteria have caused a revival of interest in the discovery of Microbial based Natural Products (MNPs) with unique scaffolds to meet the urgent demand for new drugs [57]. Recent trends in drug discovery underline that terrestrial microorganisms are a potentially productive source of novel secondary metabolites and have great potential to increase the number of NPs in clinical trials.

Entomopathogenic nematodes carrying symbiotic bacteria represent one of the best non-chemical strategies supporting insect control [36,59]. In the soil, the infective juveniles of these nematodes (Heterorhabditis or Steinernema genera) actively seek the host, penetrate through insect's natural openings, travel to the hemocoel and release symbiotic bacterial cells (*Photorhabdus* and *Xenorhabdus* spp.). The bacteria multiply and release a number of virulence factors, including complexes of toxins, hydrolytic enzymes, hemolysins, and antimicrobial compounds that kill the insect host within 48 h [20], thus providing nutrients for the nematodes development and reproduction within the insect cadaver. By doing so, the bacterial symbionts are believed to prevent putrefaction of the insect cadaver and establish conditions that favor the development of both the nematode and bacterial symbionts. These metabolites have been used in biological pesticides and therapeutic agents such as antibiotic, antimycotic, insecticidal, nematicidal, antiulcer, antineoplastical and antiviral for many decades [55]. The antimicrobial nature of metabolites produced by *Xenorhabdus* and *Photorhabdus* spp. is known, and several of such compounds with antibiotic activity have been isolated and identified [21].

During our studies on EPN, a new entomopathogenic nematode belonging to the genus *Rhabditis* and subgenus *Oscheius* was isolated from soil samples collected from Central Tuber Crops Research Institute (CTCRI) farm, Thiruvananthapuram [41]. The nematodes can be cultured on laboratory reared *Galleria mellonella* larvae and maintained alive for several years. The specific symbiotic bacteria were found to be associated with the nematode [41] and can be isolated from the haemolymph of nematode infested *G. mellonella* larvae. Based on molecular characteristics, *nematode* resembles *Rhabditis (Oscheius)* at D2 and D3 (nucleotide sequence region) expansion segments of 28S rDNA [18]. The cell free culture filtrate of the bacteria was found to inhibit several pathogenic bacteria and fungi [41], suggesting that it could be a rich source of biologically active compounds. In the present study we reported the taxonomic study of the symbiotic bacteria, purification and structure elucidation of five tryptophan containing diketopiperazines, its antimicrobial activity with special reference to medically important bacteria. The present study also reported the antimicrobial tryptophan containing diketopiperazines for the first time from *Comamonas testosteroni.*

## 2. Materials and methods

### 2.1. Chemicals and media

All the chemicals used for extraction, column chromatography and high performance liquid chromatography (HPLC) were from Merck Limited, Mumbai, India. Silica gel (230–400 mesh) used for column chromatography and precoated silica gel 60 $GF_{254}$ plates used for Thin Layer Chromatography (TLC) were from Merck Limited, Germany. Microbiological media were from Hi-Media Laboratories Limited, Mumbai, India. All other reagents and chemicals used in this study were of the highest purity. The standard antibiotics ciprofloxacin, ampicillin and amphotericin B were purchased from Sigma Aldrich (USA). The software used for the chemical structure drawing was Chemsketch Ultra, Toronto, Canada.

### 2.2. Pathogen microbial targets

Gram positive bacteria: *Bacillus subtilis* MTCC 2756, *S. aureus* MTCC 902, *Staphylococcus epidermis* MTCC 435 and *Staphylococcus simulans* MTCC 3610; Gram negative bacteria: *Escherichia coli* MTCC 2622, *Klebsiella pneumoniae* MTCC 109, *Proteus mirabilis* MTCC 425, *Vibrio cholerae* MTCC 3905, *Pseudomonas aeruginosa* MTCC 2642, and *Salmonella typhi* MTCC 3216; medically important fungi: *Aspergillus flavus* MTCC 183, *Candida tropicalis* MTCC 184, *Candida albicans* MTCC 277, *Cryptococcus gastricus* MTCC 1715 and *Trichophyton rubrum* MTCC 296 and agriculturally important fungi: *Fusarium oxysporum* MTCC 284, *Rhizoctonia solani* MTCC 4634, and *Penicillium expansum* MTCC 2006. All the test microorganisms were purchased from Microbial Type Culture collection Centre, IMTECH, Chandigarh, India. The test bacteria were maintained on nutrient agar slants and test fungi were maintained on potato dextrose agar slants.

### 2.3. Bacterial isolation

The bacterium was isolated from the haemolymph of *G. mellonella* infected with IJs of *Rhabditis (Oscheius)*. Dead *G. mellonella* larvae were surface sterilized in 70% alcohol for 10 min, flamed and allowed to dry in a laminar airflow chamber for 10 min. Then larvae were opened with sterile needles and scissors, care being taken not to damage the gut, and a drop of the oozing haemolymph was streaked onto nutrient agar plates. After 24–48 h incubation at 30 °C, single colonies on the nutrient agar plates were selected and aseptically transferred to fresh nutrient agar medium in slant tubes.

### 2.4. 16S rRNA sequencing and phylogenetic analysis

Genomic DNA was extracted from bacterial cultures through enzymatic hydrolysis [43]. The complete 16S rRNA gene (1.4–1.5 kp) was amplified via PCR, using universal bacterial primers 27 F (5′-AGA GTT TGATCC TGG CTC AG-3′) and 1541R (5′-AAG GAG GTG ATC CAG CCG CA-3′). The amplification was carried out on a DNA thermal cycler (BIORAD, USA). The 50 μl PCR reactions contained 4 μl of 2.5 U/μl Taq DNA polymerase (Genei, Bangalore, India), 5 μl of 10× buffer (Genei), 1 μl of 20 mM dNTPs (Genei), 37 μl of SDW, 1 μl of 50 μM each primer, and 1 μl of template.

The PCR conditions were initial denaturation at 94 °C for 4 min, 30 amplification cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, and a final elongation at 72 °C for 10 min. The PCR product was purified using a QIAquick Gel extraction kit (QIAGEN, Tokyo, Japan) and sequenced in both directions using the same primers as for the PCR amplification. The nucleotide sequence obtained was processed to remove low quality reads, transformed into consensus sequences with Geneious Pro software version 5.6. The resulted high quality sequences were analyzed with BLASTn (NCBI) to confirm the authenticity of the bacterium. The sequences of related species and genus were downloaded from the Genbank database and a phylogenetic study was carried out with the program MEGA version 5 [51]. Sequences were aligned using the computer package ClustalW [53] and were analyzed to determine the relationships between isolates by the neighbor-joining method [44] using the Maximum Composite Likelihood model. Bootstrap values were generated using 2000 replicates.

### 2.5. Fermentation and extraction

The bacterial fermentation was carried out using modified Tryptic soya broth (TSB) (tryptone 17 g/l, soytone 3.0 g/l, glucose 2.5 g/l, NaCl 5.0 g/l, meat peptone 10 g/l, water 1000 ml) supplemented by 0.1% tryptophan. A single colony of *Comamonas testosteroni* from