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Purification and structure elucidation of a cyclodipeptide from *Streptomyces cirratus* IHBB 10198 isolated from a glacier near Kunzum Pass in the Indian Trans-Himalayas

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Abstract. *Streptomyces cirratus* IHBB 10198 was isolated from a glacier in Lahaul-Spiti, located in Himachal Pradesh, India on yeast malt extract agar (YMEA). The isolate was identified as *Streptomyces cirratus* NRRL B-3250T based on 16S rRNA gene sequencing. Crude extract from the cell-free culture supernatant showed antibacterial activity against Gram-positive bacteria including *Micrococcus luteus* MTCC 2470, *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS 16 MTCC 2940, *Staphylococcus aureus* MRSA ATCC 43300 and Gram-negative *Klebsiella pneumoniae* ATCC 43816. The crude extract also showed antibacterial activity against three out of thirty-three strains of *Staphylococcus aureus* from the clinical samples. The culture was subjected to twenty-five liters of large-volume culturing in tryptone soya broth (TSB). Cell-free supernatant was extracted twice with ethyl acetate, dried under vacuum, and then defatted. A cyclodipeptide, cyclo (L-Pro-L-Val) was purified from the culture crude extract by flash chromatography and preparative HPLC. Structure elucidation was done by mass spectrometry, FT-IR, and NMR. No antimicrobial activity was shown by purified cyclodipeptide like the crude extract. The lack of activity may be attributed to the presence of cis-trans conformers in the natural cyclodipeptide.

Keywords: Antimicrobial activity, *Streptomyces*, cyclodipeptide, glacier.

Classification numbers: 3.4, 3.5.

1. INTRODUCTION

The emergence of resistant bacteria has become a major threat as there are few effective antibiotics available against these organisms. The widespread use, misuse, and overuse of antibiotics are the major causes of the emergence of resistant microorganisms [1]. Among antibiotic producers, *Streptomyces* is the major source of bioactive compounds representing 70-80 % of the isolated compounds [2-4]. Microbial cyclic peptides having proline-rich residues are

mainly derived from marine environments. These peptides showed useful bioactivities including antiproliferative, cytotoxic effects, and antimicrobial activities [5, 6]. Small cyclopeptides gained more importance for their use as lead molecules due to the stability in their structure [7, 8]. These are more resistant to exoproteases as compared to linear peptides [9]. Cyclo-(isoleucyl-prolylleucyl-alanyl) has been purified from seaweed-associated marine bacteria [10]. A cyclic dipeptide derivative reported from *Streptomyces* sp. TN17 strain showed activity against *M. luteus* LB 14110, *S. aureus* ATCC 6538, and *Fusarium* sp. [11]. Cyclo (L-Pro-L-Val)$_2$ and related cyclodipeptides reported from *Streptomycesbarakatei* J-2 showed antimicrobial activity against *B. subtilis*, *M. luteus*, *S. scabies*, *Mucor rammaniamus,* and *C. albicans* [5]. Cyclo (L-Pro-L-Val)$_2$ isolated from marine ascidian Cystodytes dellechiajei (Didemnidae) showed antineoplastic activity. No antineoplastic was there when this cyclodipeptide was obtained through synthesis [12]. *Staphylococcus aureus* is a major bacterial pathogen causing human and animal disease. Multidrug resistance strains of this pathogen make treating it more difficult [13]. Therefore, in our study aimed at finding antimicrobial activity against clinical strains of *S. aureus,* cyclo (L-Pro-L-Val*)* has been isolated from a terrestrial *Streptomyces* species. Isolation, purification, and structure elucidation of cyclodipeptide through flash chromatography, HPLC, Mass spectrometry, FT-IR, and NMR have been discussed. Isolated cyclic dipeptide has been tested against a panel of test organisms as well as thirty-three clinical strains of *S. aureus*.

2. MATERIALS AND METHODS

2.1. Isolation of culture

Streptomyces cirratus IHBB 10198 was isolated using the serial dilution technique from a soil sample of a glacier in Lahaul-Spiti, located in Himachal Pradesh, India on yeast malt extract agar (YMEA) (HiMedia, India) after incubation at 28 °C for 7 days. A creamish-white-colored colony was purified by subculturing on YMEA. The pure culture was preserved in cryovials containing 20 % glycerol w/v and stored at -80 °C till further application.

2.2. Identification of Culture

Biochemical characteristics were determined using GEN III microplates and by the BIOLOG system (BIOLOG Microstation™ system, USA). Isolate was identified based on 16S rRNA gene sequencing as per the previously described procedure [14]. The sequence data was analyzed using Sequencher™ 4.10.1 software (Gene Codes Corporation, MI, USA). The isolate was identified by BLAST analysis of 16S rRNA sequence data using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.* [15]). The phylogenetic tree was constituted using the neighbor-joining (NJ) method on MEGA 5 software [16].

2.3. Large colume culturing and extraction of compound

Pre-inoculum was prepared by growing the culture in 150 mL of TSB media at 28 °C for 48 hours. The pre-inoculum was then inoculated into 25 liters of TSB media at 1 % v/v concentration. The culture was grown under shaking conditions at 200 rpm at 28 °C for 4 days. After incubation, the cells were separated from the supernatant by filtration, and the cell-free supernatant was extracted twice with ethyl acetate at a 50:50 ratio and dried in a Rotavapor apparatus (Buchi® Rotavapor R-210, Switzerland) to yield 18 g of crude extract.

2.4. Screening for antimicrobial activity

The antimicrobial screening was done as per the standard operating procedure for extract preparation and antimicrobial activity described by the CSIR-Institute of Microbial Technology [17]. Crude extract and fractions from different steps of purification were screened against *B. subtilis* MTCC 121, *C. albicans* MTCC 3017, *Escherichia coli* MTCC 739, *Klebsiella pneumoniae* ATCC 43816, *M. luteus* MTCC 2470, *Pseudomonas aeruginosa* MTCC 2453, *Raoultella planticola* MTCC 530, *S. aureus* (MRSA) ATCC 43300, *S. aureus* MLS 16 MTCC 2940 and *S. aureus* MTCC 96. The crude extract was also screened against thirty-three clinical strains of *S. aureus*. Minimum inhibitory concentration (MIC) was determined by the broth microdilution method as per previously described by Sharma *et al.* [18].

2.5. Purification of the Antimicrobial Compound

Dried ethyl acetate extract was mixed with 10 mL of 1 M NaCl, 10 mL of methanol, and 20 mL of hexane and mixed vigorously for 30 min with the help of a magnetic stirrer. The upper organic phase was removed in a separating funnel from the lower aqueous phase. The lower phase was again extracted with 10 mL of 1 M NaCl, 10 mL of methanol, and 20 mL of hexane. The resultant upper phase was removed from the lower one in a separating funnel and the lower phase was extracted five times with ethyl acetate. The ethyl acetate extracted phase was dried in Rotavapor, yielding 6 g of defatted extract. All the fractions were subjected to antimicrobial assay.

The defatted extract was purified using flash column chromatography and the Isolera One™ flash purification system (Biotage, Sweden). Solvent conditions for flash chromatography were standardized on TLC plates using combinations of different solvent systems. A solvent system containing hexane and ethyl acetate was found to be the best-suited solvent system and therefore applied in flash chromatography. Flash chromatography was performed in a Snap 50 g silica cartridge using a stepwise gradient from 50 % ethyl acetate to 100 % ethyl acetate and a flow rate of 15 mL/min. The purification process was monitored at two different wavelengths, 254 and 260 nm (Figure 1).

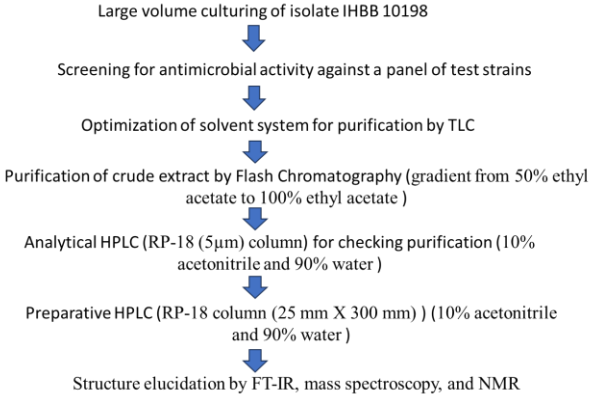

Figure 1. Diagram of purification and structure elucidation process of cyclodipeptide from isolate IHBB 10198.

2.6. Analytical and preparative HPLC

Analytical HPLC of fraction F6 was performed on an RP-18 (5 µm) column, Dionex system using an isocratic solvent system of 10 % acetonitrile and 90 % water and at a flow rate of

1 mL/min. Preparative HPLC was done on an Agilent system with an RP-18 column (25 mm X 300 mm) using isocratic separation (acetonitrile: 10 %, water: 90 %) at a flow rate of 15 mL/min.

2.7. Structure elucidation

Structure elucidation was performed by FT-IR, mass spectrometry, and NMR. The sample for the NMR experiments was prepared by dissolving 25 mg of the compound in 600 µL of CD_3OD. ^{1}H and ^{13}C experiments were recorded in a Bruker 300 spectrometer (chemical shift in δ, ppm). FT-IR spectrum was recorded between 4,000 and 450 cm^{-1} (Perkin-Elmer).

3. RESULTS AND DISCUSSION

3.1. Carbon source utilization

The IHBB 10198 isolate utilized the following carbon sources for growth: D-maltose, gentiobiose, D-melibiose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, α-D-Glucose, D-mannose, D-fructose, D-galactose, D-fucose, D-fructose- 6-PO_4, glycyl-L-proline, L-histidine, D-galacturonic acid, L-galactonic acid lactone, D-glucuronic acid, glucuronamide, citric acid, α-keto-glutaric acid and α-hydroxybutyric acid.

3.2. Identification of isolate IHBB 10198

The crude extract from the IHBB 10198 isolate showed promising antimicrobial activity against both gram-positive and gram-negative test strains including *Micrococcus luteus* MTCC 2470, *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MTCC 96, *Staphylococcus aureus* MLS 16 MTCC 2940, *Staphylococcus aureus* MRSA ATCC 43300, and *Klebsiella pneumoniae* ATCC 43816, indicating its importance for further identification of isolate and characterization of the bioactive compound. Based on phylogenetic and BLAST analysis of 16S rRNA gene sequence (Figure 2), the IHBB 10198 isolate showed relatedness with *Streptomyces cirratus* NRRL B-3250^{T} (99.6 %), *Streptomyces vinaceus* NBRC 13425^{T} (99.6 %), *Streptomyces subrutilus* DSM 40445 ^{T} (99.5 %) and *Streptomyces nojiriensis* LMG 20094^{T} (99.5 %).

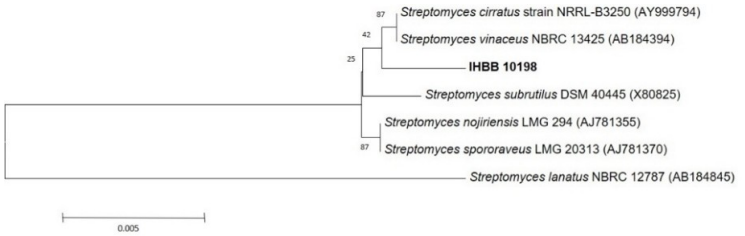

Figure 2. Evolutionary relationship of strain IHBB 10198 and its related taxa constructed using the neighbor-joining method. The percentage of replicated trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Thus, based on the carbon source utilization pattern, 16S rRNA sequencing and phylogenetic analysis data, isolate IHBB 10198 was identified as *Streptomyces cirratus.*

3.3. Fermentation and extraction of compound

Ethyl acetate and defatted extract from culture supernatant of *Streptomyces cirratus* IHBB 10198 showed antimicrobial activity against *B. subtilis* MTCC 121, *M. luteus* MTCC 2470, *S.*

aureus MTCC 96, *S. aureus* MLS 16 MTCC 2940, *S. aureus* MRSA ATCC 43300 and *K. pneumoniae* ATCC 43816 with the diameter of the inhibition zone ranging from 8 to 14 mm (Table 1). Out of thirty-three clinical strains screened the crude extract showed antimicrobial activity against only *S. aureus* 91, *S. aureus* 3466, and *S. aureus* 9296 with a diffused zone of inhibition having a diameter of 10 mm against *S. aureus* 91, 15 mm against *S. aureus* 3466 and *S. aureus* 9296. A solvent system of hexane and ethyl acetate in a 1:9 (v/v) ratio produced optimal separation of compounds from the crude extract in TLC (Figure S1).

Table 1. Screening of ethyl acetate and defatted extracts of *Streptomyces cirratus* IHBB 10198 for antimicrobial activity.

		Inhibition Zone (mm)					
		B. subtilis (MTCC 121)	*M. luteus* (MTCC 2470)	*S. aureus* (MTCC 96)	*S. aureus* MLS 16 (MTCC 2940)	*S. aureus* MRSA (ATCC 43300)	*K. pneumoniae* (ATCC 43816)
Ethyl acetate extraction	Organic phase	11	12	12	12	12	8
	Aqueous phase	-	-	-	-	-	-
Defatting	Upper phase	12	8	12	14	12	10
	Lower phase	-	-	-	-	-	-
Neomycin (30 µg/50 µl)		15*	25	15	9	7	9
Ampicillin (10 µg/50 µl)		27	40	40	8	-	-
Uninoculated medium		-	-	-	-	-	-
DMSO		-	-	-	-	-	-

*Zone of inhibition exclusive of well diameter.

3.4. Purification of the antimicrobial compound

Fraction F6 collected from flash chromatography (Figure S2) of defatted extract yielded 400 mg and was found to be the most active fraction that showed antimicrobial activity against *B. subtilis* MTCC 121, *M. luteus* MTCC 2470, *S. aureus* MTCC 96, *S. aureus* MLS 16 MTCC 2940, *S. aureus* MRSA ATCC 43300 and *K. pneumoniae* ATCC 43816 (Table 2). Analytical HPLC of fraction F6 indicated multiple peaks, so further purification of fraction F6 was performed by preparative HPLC (Figure S3, Figure S4) which yielded five fractions. A purified compound was obtained from fraction F4 at 16.8 min retention time and yielded about 41 mg. The purified compound showed only a diffused zone of inhibition of 5 mm against *S. aureus* MTCC 96 (Table 3). No antimicrobial activity like the crude extract has been found even after pooling all fractions collected from the Preparative HPLC. Fractions collected after the column washing with 50 % acetonitrile in water, 100 % acetonitrile, 50 % methanol in water, and 100 % methanol also showed no antimicrobial activity. Several authors reported the difference in activity between crude extracts and purified compounds. Rungprom *et al.* [10] reported antimicrobial activity in the methanolic extracts from the culture supernatant of *Pseudoalteromonas* against *S. aureus, M. luteus, B. subtilis, Escherichia coli,* and *Vibrio anguillarum*. However, no activity was reproduced in the fractions containing cyclopeptide and in the synthetic compound. They ascribed loss of activity to the multiple ring conformations linked to the presence of trans or cis amide bonds in

proline-rich cyclopeptides. Chen *et al.* [19] reported a decrease in antimicrobial activity in the purified protein than the ammonium sulfate extract and found that the decrease in activity was due to the loss of cofactors for antimicrobial activity during their purification process. Loss of antibacterial activity against Gram-negative bacteria was reported in 1-methyl ester-nigericin purified from *S. hygroscopicus* BRM10 [20]. Nwodo *et al.* [21] found a synergic effect of antimicrobial compounds and a loss of antimicrobial activity during the purification process in type and clinical strains of *Escherichia coli*. Eijsink *et al.* [22] also reported the synergic effect of antimicrobial compounds during the purification process of sakacin P.

Table 2. Screening of different fractions from flash chromatography of *Streptomyces cirratus* IHBB 10198 for antimicrobial activity.

	Inhibition zone (mm)					
	B. subtilis MTCC 121	*M. luteus* MTCC 2470	*S. aureus* MTCC 96	*S. aureus* MLS 16 MTCC 2940	*S. aureus* MRSAATCC 43300	*K. pneumoniae* ATCC 43816
F1	-	-	-	-	-	-
F2	-	-	-	-	-	-
F3	-	8	-	-	-	-
F4	-	14	-	-	-	-
F5	-	21	-	-	-	-
F6	13	31	10	8	9	10
F7	-	11	-	-	-	-
F8	-	7	-	-	-	-
Neomycin (30μg/50μL)	15 *	25	15	9	7	9
Ampicillin (10μg/50μL)	27	40	40	8	-	-
Uninoculated medium	-	-	-	-	-	-
DMSO	-	-	-	-	-	-

* Zone of inhibition exclusive of well diameter.

Table 3. Screening of different fractions from peparative HPLC of fraction F6 from flash chromatography of *Streptomyces cirratus* IHBB 10198 for antimicrobial activity.

	Inhibition zone (mm)				
	B. subtilis MTCC 121	*M. luteus* MTCC 2470	*S. aureus* MTCC 96	*S. aureus* MLS 16 MTCC 2940	*S. aureus* (MRSA) ATCC 43300
F1	-	-	-	-	-
F2	-	-	-	-	-
F3	-	-	-	-	-
F4	-	-	5(d)	-	-
F5	-	-	-	-	-
Neomycin (30 μg/50 μL)	15*	25	15	9	7
Ampicillin (10 μg/50 μL)	27	40	40	8	-

	Inhibition zone (mm)				
	B. subtilis MTCC 121	*M. luteus* MTCC 2470	*S. aureus* MTCC 96	*S. aureus* MLS 16 MTCC 2940	*S. aureus* (MRSA) ATCC 43300
Uninoculated medium	-	-	-	-	-
DMSO	-	-	-	-	-

*Zone of inhibition exclusive of well diameter, d - diffused, - no inhibition.

Table 4. Screening of purified cyclic dipeptide against clinical strains of *S. aureus* for antimicrobial activity.

	Inhibition zone (mm)						
	Clinical isolate No.						
	2	6	12	13	15	22	28
Cyclic dipeptide (30 µg/ 50µL)	8	12	9	6	8	7	6
Neomycin (30 µg/50 µL)	12*	15	10	8	9	9	8
Ampicillin (10 µg/50 µL)	15	12	10	-	10	10	-
Uninoculated medium	-	-	-	-	-	-	-
DMSO	-	-	-	-	-	-	-

*Zone of inhibition exclusive of well diameter, d - diffused, - no inhibition.

Table 5. MIC of purified cyclic dipeptide against clinical strains of *S. aureus* for antimicrobial activity.

	MIC (µg/mL)						
	Clinical isolate No.						
	2	6	12	13	15	22	28
Cyclic dipeptide	8	4	4	8	4	4	16
Neomycin	4	4	4	4	4	8	32
Ampicillin	4	4	4	R	4	4	R*

*R - resistance.

3.5. Structure elucidation of the antimicrobial compound

Mass data showed m/z = 98.32, m/z = 100.3, and m/z = 197.35 for $[M^{+1}]$ ion corresponding to Pro, Val, and Pro-Val, respectively (Figure S5). FT-IR spectra showed characteristic absorption peaks at 3434.40 cm^{-1} and 1644.93 cm^{-1} indicating the presence of amine stretch (N-H) and amide stretch (C=O), respectively.

^{1}H NMR (300 MHz) 4.21-4.16 (m, 2H), 4.02-4.00 (m, 1H), 3.56-3.47 (m, 5H), 3.30-3.28 (m, 2H), 2.49-2.44 (m, 1H), 2.32-2.28 (m, 2H), 2.02-1.89 (m, 7H), 1.07 (d, 6H, J = 7.3 Hz), 0.91 (d, 6H, J = 6.8 Hz).

In ^{13}C NMR two carbonyl signals at δC 171.25 and 166.2 were assigned to Pro and Val, respectively. ^{13}C NMR recorded in CD_3OD at 300 MHz (Table 6) showed similar chemical shifts

as recorded by Ahmed *et al*. [5]. The compound was found to be a cyclodipeptide Cyclo (L-Pro-L-Val) with molecular formula $C_{20}H_{32}N_4O_4$ (Figure 3; Figure S7) and 392.49 molecular weight which was previously isolated from the *Streptomyces barakatei* J-2 reported for antimicrobial activity against *B. subtilis*, *M. luteus*, *S. scabies*, *Mucor rammaniamus,* and *C. albicans* [5] and from marine ascidian *Cystodytes dellechiajei* (Didemnidae) reported for antineoplastic activity [12]. A lack of antineoplastic activity in cyclic peptides was also observed by Aracil *et al.* [12] after obtaining the purified compound through synthesis and the activity in natural peptides may be attributed to the existence of active conformation which arises due to cis-trans exchanging of proline residues at the X-Pro amide bond. A lack of bioactivity in other cyclic peptides has also been observed by some researchers, and they attributed it to multiple ring conformations in proline-richcyclic peptides [10, 23].

Table 6. ^{13}C NMR of cyclotetrapeptide showing chemical shift in δ, ppm

Position	δC
Pro-α	58.70
Pro-β	28.19
Pro-γ	21.92
Pro-δ	44.84
Pro-carbonyl	171.25
Val-α	60.17
Val-β	28.53
Val-γ	15.32; 17.52
Val-carbonyl	166.2

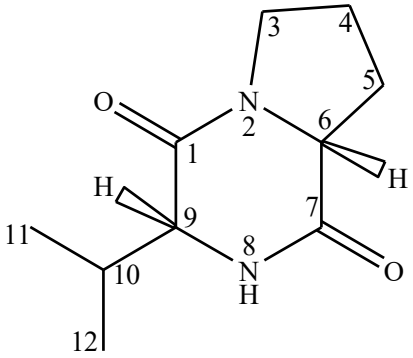

Figure 3. Structure of a cyclodipeptide from *Streptomyces cirratus* IHBB 10198, Cyclo (L-Pro-L-Val).

3.6. Screening of the purified compound against clinical isolates of *S. aureus* for antimicrobial activity and MIC determination

Purified cyclodipeptide showed potent antimicrobial activity against seven clinical isolates of *S. aureus* with the zone of inhibition ranging from 6 mm to 12 mm (Table 4). The purified compound showed MIC values between 4 and 16 μg/ml (Table 5) against the clinical isolates indicating its potential use against drug-resistant *Staphylococcus* infections [24]. Recent reports of cyclic peptides from various habitats including the marine habitats [25], Hawaiian way [26], and Kalahari Desert [27] highlight the importance of studying cyclic peptides in managing drug resistance bacteria, particularly *S. aureus*. The present study adds to the significance of

bioprospecting novel habitats for finding new cyclic dipeptides against drug-resistant *S. aureus*. Further, unexplored habitats like the glaciers of Indian trans-Himalayas explored in the present study highlight the importance of bioprospecting novel niches for new molecules against drug-resistant strains. To the best of our knowledge, this is the first report on a cyclic dipeptide of *Streptomyces* from the trans-Himalayan region exhibiting potent activity against clinical isolates of *S. aureus*.

4. CONCLUSIONS

In conclusion, the crude culture extract from *Streptomyces cirratus* IHBB 10198 showed prominent antimicrobial activity against Gram-positive bacteria including MRSA and Gram-negative *K. pneumoniae*. There was a loss of antimicrobial activity in purified cyclopeptides against some strains. However, purified cyclopeptides showed potent antimicrobial activity against clinical isolates of *S. aureus*. This indicated the potential of unexplored habitats in the Indian trans-Himalayas for bioprospecting microbes for novel peptides against drug-resistant pathogens. Thus, the present study employing methods for purifying and elucidating the structure of cyclic peptides from novel niches of cold habitats in the trans-Himalayan region opens a new frontier of bioprospecting microbes for novel peptides and/or new bioactivities against drug resistant pathogens.

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CRediT authorship contribution statement. M. Pal: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. A. Kumari: Investigation, Writing - review & editing. S. Chhibber: Supervision, Writing - review & editing. A. Gulati: Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of competing interest. The authors declare that they have no conflict or competing interests.

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