Production of Diketopiperazine Derivative Cyclo (L-Leu-L-Arg) by *Streptomyces* sp. TN262 After Exposure to Heat-Killed Fungus *Fusarium* sp.

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Summary: In a screening program for new active secondary metabolites producers, a strain of *Streptomyces* called TN262 was isolated from Tunisian soil and selected for its ability to produce eleven active compounds in pure culture conditions. In this work, the effect of different concentrations of heat-killed fungus *Fusarium* sp. on the production of active compounds by TN262 strain was studied. The ethyl acetate extract from the culture of *Streptomyces* sp. TN262 combined with heat-killed *Fusarium* sp. at 50 µg/ml inhibited the growth of the three used indicator microorganisms. In fact, an increase of 36%, 21% and 20% in inhibitory activity was obtained against *Micrococcus luteus* LB 14110, *Escherichia coli* ATCC 8739 and *Fusarium* sp. respectively. The HPLC chromatographic profiles of the ethyl acetate extracts from both culture conditions were different and an additional active compound was produced only under induced conditions. This active component was isolated and identified as Cyclo (L-Leu-L-Arg) (**1**), a diketopiperazine derivative, possessing antibacterial and antifungal activity. Consequently, this study showed that the addition of heat-killed fungus is a useful method for inducing the production of bioactive compounds.

Keywords: *Streptomyces*; Heat-killed fungus *Fusarium* sp.; Induction; Cyclo (L-Leu-L-Arg); Antimicrobial activity.

Introduction

The filamentous soil bacteria belonging to the genus *Streptomyces* are rich sources of a high number of bioactive natural products, which were extensively used as pharmaceuticals and agrochemicals [1, 2].

The sequencing of the complete genomes of some Streptomycete species revealed the presence of a large number of "cryptic" secondary metabolic gene clusters, and led to the realization that these organisms have the ability to produce many more natural products than had previously been recognized [3, 4]. To date, various methods have been used to activate genes synthesizing cryptic secondary metabolites [5-7]. It has been reported that the co-culture of *Streptomyces endus* S-522 with the bacterium *Tsukamurella pulmonis* TP-B0596 induced the production of a new active compound (Alchivemycin A) which was not produced in a pure culture [7]. Fourati-Ben Fguira et al. [6] reported that the addition of heat-killed fungi (*Verticillium dahliae*, *Fusarium* sp. and *Aspergillus oryzae*) to the culture media of the *Streptomyces* sp. US80, significantly increased the secretion of the same three antifungal molecules produced in a pure culture but without production of other active compounds.

We have previously reported the purification and characterization of eleven active compounds from *Streptomyces* sp. TN262 isolated from south Tunisian soil [8]. In this work and in order to improve the production of these eleven active compounds and/or to obtain new active biomolecule(s), the effect of the addition of different concentrations of heat-killed fungus *Fusarium* sp. to the culture of the S*treptomyces* sp. TN262 strain was studied.

Results and Discussion

Effect of Heat-Killed Fusarium sp. Addition on Active Compounds Production

In this work, we studied the effect of the addition of seven different heat-killed fungus *Fusarium* sp. concentrations to the culture of *Streptomyces* sp. TN262 on active antimicrobial compounds production. Eight cultures of TN262 strain were grown on Bennett medium under the optimized conditions [8] and one of them was used as control (pure culture).

According to our microbiological tests, the addition of the heat-killed fungus, at the seven studied concentrations, was able to enhance antimicrobial activity against the three tested microorganisms (Fig. 1). The highest antimicrobial activity was observed at concentration of 50 µg/ml of heat-killed *Fusarium* sp. At this concentration and in comparison to the control, an increase of 36%, 21%

and 20% in activity was obtained against *Micrococcus luteus* LB 14110, *Escherichia coli* ATCC 8739 and *Fusarium* sp. respectively. However, no significant difference has been observed concerning the growth of the *Streptomyces* sp. TN262 strain after the addition of the heat-killed cells (data not shown). These observations were in agreement with the results of Fourati-Ben Fguira et al. [6]. To determine whether the increase of the antimicrobial activity is due to the enhancement of biosynthesis of the same eleven active molecules obtained in a pure culture, or the biosynthesis of additional active compounds after exposure to the heat-killed fungus, HPLC chromatographic profiles of the cultures of *Streptomyces* sp. TN262 (without and with heat-killed *Fusarium* sp. at five different concentrations 10, 20, 30, 40 and 50 µg/ml) were compared. As shown in Fig. 2, all HPLC profiles were quite similar and the meaningful difference was due to the presence of a well defined peak (P) having a retention time of 39.567 min. Furthermore, we observed that the intensity of this peak depends on the concentration of heat-killed cells with an optimum at 50µg/ml.

Purification of the Compound **(1)** *having a Retention Time of 39.567 min*

A culture of two liters from *Streptomyces* sp. TN262 combined with heat-killed *Fusarium* sp. at 50 µg/ml was prepared and the corresponding active dry extract supernatant was applied to preparative HPLC to purify the compound (**1**). As shown in Table-1, this component possesses antibacterial (against Gram positive and Gram negative bacteria) and antifungal activity. In this context, Furtado et al. [9] reported that the HPLC chromatographic profiles of the chloroform extracts from cultures of the antimicrobial activities producer *Aspergillus fumigatus* were different in presence and in absence of autoclaved bacteria cell wall fragments. In addition to the active compounds secreted by this fungus under normal condition, two other active compounds (3,4-dimethoxyphenol and 1,3,5-trimethoxybenzene) were obtained after the addition of autoclaved bacteria. To explain our finding, we suggested that *Fusarium* sp. dead cells may have lysed during *Streptomyces* sp. TN262 cultivation and hence contributed Cyclo (L-Leu-L-Arg) stimulants or compounds that may act as precursors for the product.

Table-1: Antimicrobial activity of compound (1) against *Micrococcus luteus* LB 14110, *Escherichia coli* ATCC 8739 and *Fusarium* sp. at three different concentrations 25, 50 and 75 µg/ disk.

Compound (1)	Test microorganism (inhibition zones (mm))		
	M. luteus LB 14110	*E. coli* ATCC 8739	*Fusarium* sp.
(25µg/disk)	14	11	11
(50µg/disk)	19	18	16
(75µg/disk)	25	23	22

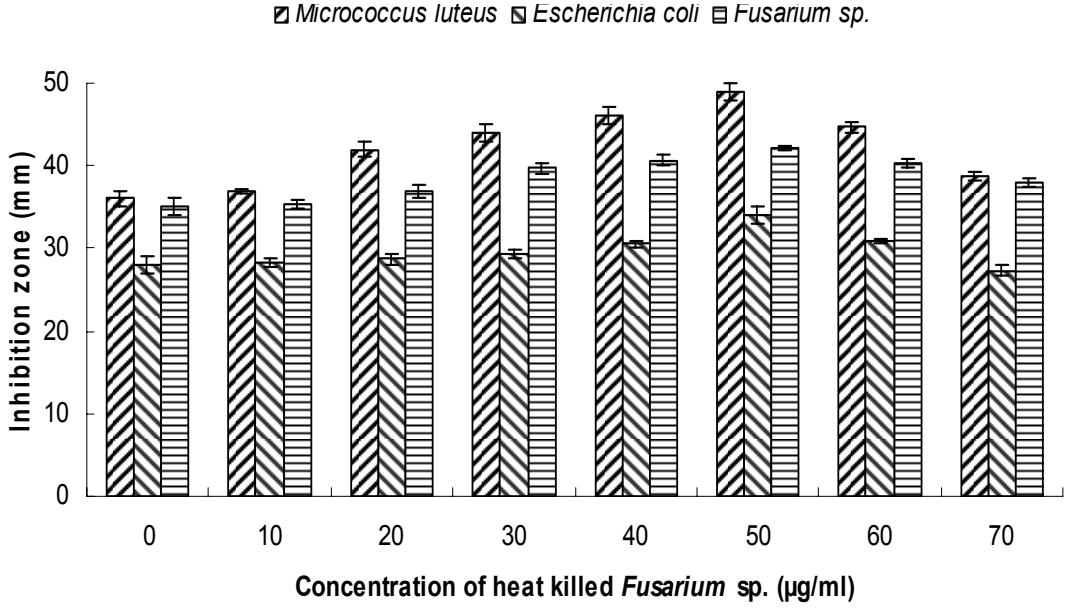

Fig. 1: Effect of heat-killed *Fusarium* sp. cells addition on antimicrobial activity production by the *Streptomyces* sp. TN262 strain. Data are means ± SD of three replicates.

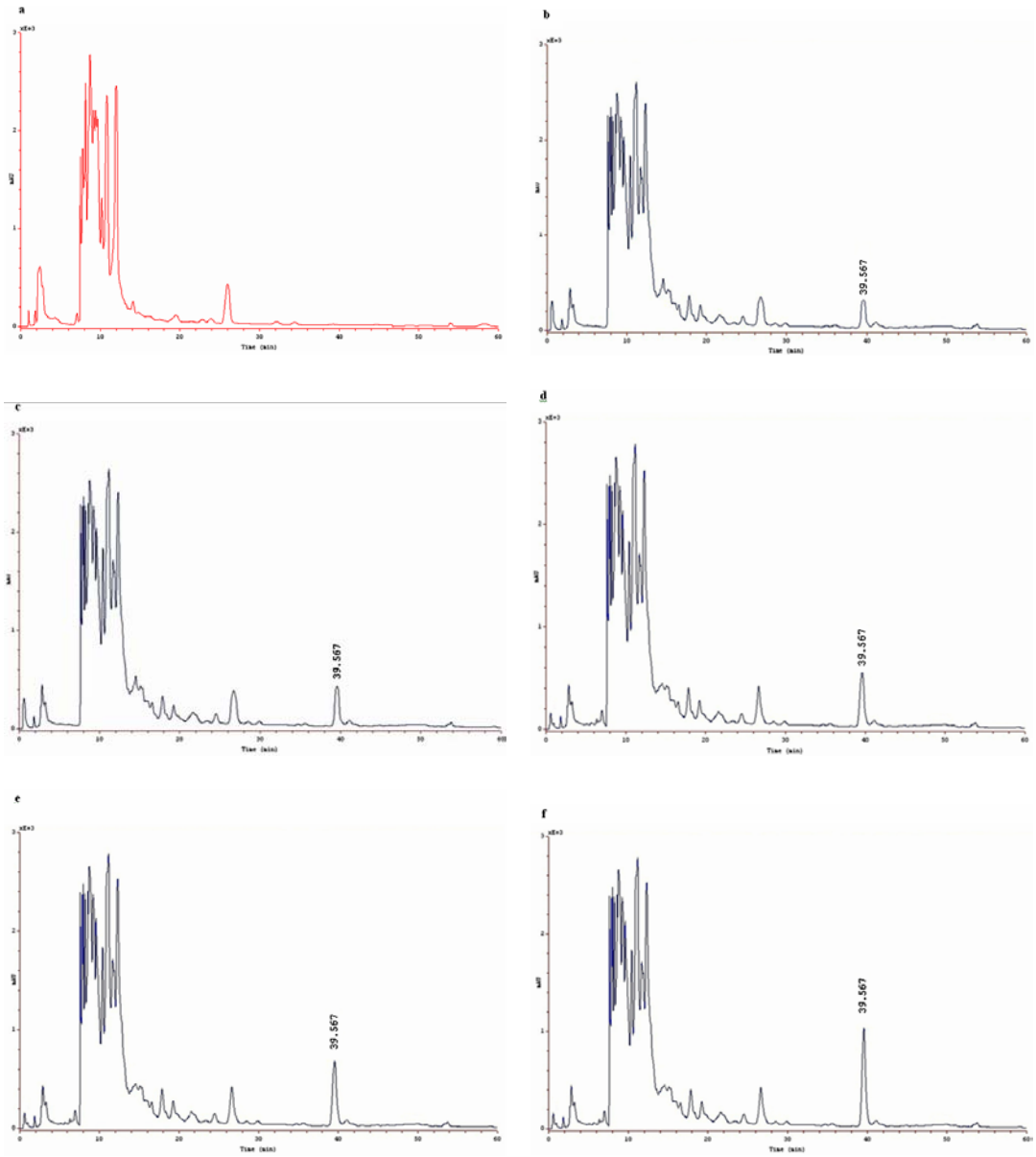

Fig. 2: HPLC chromatograms of crude extract supernatant cultures of the *Streptomyces* sp. TN262 strain without eat-killed fungus addition (a) and in presence of 10 (b), 20 (c), 30 (d), 40 (e) and 50 (f) µg/ml of heat-killed *Fusarium* sp. cells.

Structure Characterization of Compound (**1**)

Based on the LC/MS analysis, a molecular ion peak of (**1**) was shown at *m/z* 268 $[M-H]^-$, affording a molecular weight of 269 Dalton with a corresponding molecular formula $C_{12}H_{23}N_5O_2$ (Fig. 3). Based on the ^1H, ^{13}C NMR, and 2D (H,H COSY, HMQC, HMBC, and NOE) spectra and comparison with our recently published one [10], compound (**1**) was deduced as Cyclo (L-Leu- L-Arg), a diketopiperazine derivative (Fig. 4). Diketopiperazine molecules constitute a family of secondary metabolites with diverse and interesting biological activities such as antibacterial, fungicidal, herbicidal, immunosuppressor, antitumors and antiviral [11].

Experimental

Microorganisms

The *Streptomyces* sp. TN262 strain is a new bacterium isolated from south Tunisian soil and it produces eleven active compounds [8]. *Micrococcus*

luteus LB 14110 and *Escherichia coli* ATCC 8739 were used as indicator microorganisms for the antibacterial activity assays. Antifungal activity was determined against the filamentous fungus *Fusarium* sp.

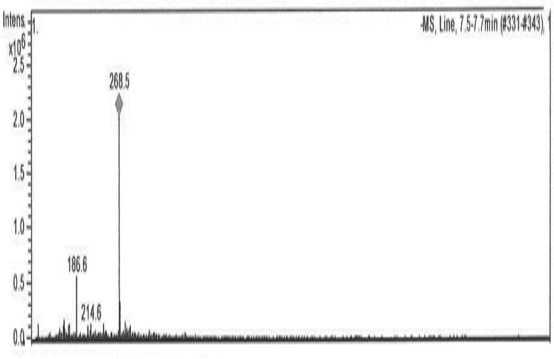

Fig. 3: LC/MS analysis of the compound (1) corresponding to the HPLC peak with a retention time of 39.567 min.

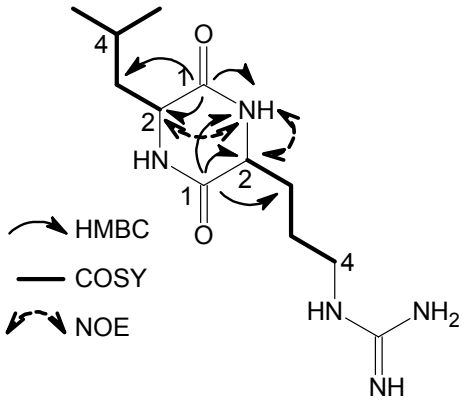

Fig. 4: Structure formula of the compound (**1**) corresponding to the HPLC peak with a retention time of 39.567 min.

Preparation of Heat-Killed Fungus Fusarium sp.

The fungus *Fusarium* sp. was grown in PDA medium at 30 °C for 7 to 10 days and collecting spores was harvested with sterile water, counted in a cell of Thoma and then adjusted to a spore density of approximately 10^7 spores/ml. Heat-killed *Fusarium* sp. was prepared as follows: spores at 10^7/ml of *Fusarium* sp. were used to inoculate 1000 ml Erlenmeyer flasks with four indents containing 200 ml of Sabouraud medium (10 g/l peptone, 10 g/l glucose and pH adjusted to 5.6). After incubation at 30 °C for 48 h in an orbital incubator with shaking at 200 rpm, the cultivation medium was filtered in a sterile environment, through non-absorbent cotton wool of a filter tube for spore suspension and hyphal fragments separation. Hyphal fragments retained in the cotton wool were washed twice with distilled water, mixed with 50 ml of distilled water and then autoclaved twice at 120 °C during 20 min. After cooling, the hyphal suspension was placed 2 h at -80 °C and then freeze-dried overnight. The obtained dry extract corresponds to a destroyed fungus hyphal. Agar PDA plates were inoculated with a sample of a suspension of this dry extract to confirm the absence of survivor hyphae.

Culture Conditions and Biological Assays

Eight cultures of the *Streptomyces* sp. TN262 strain, 400 ml each one, were performed according to Elleuch et al. [8] and one of them was used as control (without heat-killed *Fusarium* sp. addition). The influence of adding heat-killed fungus to the culture media on the production of active compounds was determined using seven different concentrations (10, 20, 30, 40, 50, 60 and 70 µg/ml) of heat-killed *Fusarium* sp. After 72 h of incubation at 30 °C with an agitation rate of 250 rpm, the culture broth of each one was filtered to separate mycelium and supernatant and this later was exhaustively extracted, two times, by ethyl acetate. The eight obtained organic phases were concentrated in *vacuo* to dryness and then dissolved, each one, in 400 µl dichloromethane–methanol (90% -10%). Mycelium biomass of the eight *Streptomyces* sp. TN262 strain cultures was determined by measurement by weighing to constant dry weight after drying at 105 °C.

Antimicrobial activities against *M. luteus* LB 14110, *E. coli* ATCC 8739 and the *Fusarium* sp. were determined by paper disc method according to Elleuch et al. [8].

Purification and Structure Elucidation of the Compound (**1**)

To purify the compound (**1**), two liters of the culture of *Streptomyces* sp. TN262 supplemented with heat-killed *Fusarium* sp. at 50 µg/ml, was prepared. The obtained supernatant was extracted by ethyl acetate (*v/v*, two times). The resulting organic phases were evaporated to dryness under vacuum to give a brown crude extract (300 mg). The extract was then dissolved in 2 ml dichloromethane–methanol (90% -10%) and applied to fractionation by HPLC (waters: controller 600, pump 600, dual λ absorption detector 2487, Linear Recorder); column C18 ("250 ×

7" 8mm UP ODS). Elution was at a flow rate of 1 ml/min with a linear gradient of two solutions A (water) and B (acetonitrile) from 100% buffer A to 50% buffer A and 50% buffer B over the first 35 min, followed by a linear gradient to 100% buffer B from 35 to 45 min. Detection was by wavelength 280 nm. The well developed peak having a retention time of 39.567 min during different injections was collected, pooled and concentrated to dryness under vacuum affording 60 mg of yellowish component (**1**). This component was dissolved in 500 µl of methanol, tested for its antimicrobial activity against the three used indicator microorganisms at 25, 50 and 75 µg/disk and then analysed by LC/MS. This analysis was performed using an LC/MSD Trap XCT – Electrospray (Agilent Technologies), equipped with an HPLC Agilent 100 DAD detector (C18 column Zorbax 300 2.1 x 150 mm). NMR sample was prepared by dissolving the pure compound in DMSO. 1D and 2D ^{1}H and ^{13}C experiments were recorded on a Bruker Avance 600 spectrometer equipped with a 5 mm triple resonance inverse Z-gradient probe (TBI ^{1}H, ^{31}P, BB).

Cyclo (L-Leu-L-Arg) (**1**), $C_{12}H_{23}N_5O_2$ (269): UV non absorbing or fluoresce during TLC, which was detected as reddish violet on spraying anisaldehyde/sulphuric acid, and latter to blue. **^{1}H NMR** (DMSO-d_6, 600 MHz): δ 7.96 (1H, brs, Arg-NH), 4.19 (1H, dd, *J* = 8.1, 8.0, Arg-H-2), 4.01 (1H, dd, *J* = 6.9, 5.9, Leu-H-2), 3.41 (1H, m, Arg-H_{2a}-5), 3.28 (1H, m, Arg-H_{2b}-5), 2.13 (1H, m, Arg-H_{2a}-3), 1.92 (1H, m, Arg-H_{2b}-3), 1.90 (2H, m, Leu-H_{2a}-4), 1.79 (2H, m, Arg-H_{2b}-4), 1.77 (1H, m, Leu-H_{2a}-3), 1.37 (1H, m, Leu-H_{2b}-3), 0.88 (3H, d, *J* = 7.0, Leu-H_3-5), 0.87 (3H, d, *J* = 7.0, Leu-H_3-6); **^{13}C NMR** (DMSO-d_6, 150 MHz): δ 170.8 (C, Arg-C_1), 167.0 (C, Leu-C_1), 59.0 (CH, Arg-C_2), 53.1 (CH, Leu-C_2), 45.3 (CH_2, Arg-C_5), 38.3 (CH_2, Leu-C_3), 27.9 (CH_2, Arg-C_3), 24.6 (CH, Leu-C_4), 23.3 (CH_3, Leu-C_5), 22.9 (CH_2, Arg-C_4), 22.4 (CH_3, Leu-$C_{5'}$).

Conclusion

Several active secondary metabolites have been isolated from different *Streptomyces* species. The production of these metabolites depends on the culture conditions. Therefore, the development of a new culture method can facilitate the discovery of new natural products. In this work, we found an interesting phenomenon that the culture of *Streptomyces* sp. TN262 strain with heat-killed fungus *Fusarium* sp. induced the production of secondary metabolite which was not produced in a pure culture. Under induced conditions, the obtained additional active compound is a diketopiperazine derivative Cyclo (L-Leu-L-Arg), which possesses antibacterial and antifungal activities.

To our knowledge, this is the first time that heat-killed fungus *Fusarium* sp. has been demonstrated to induce the production of secondary metabolites. Consequently, this finding will constitute a promising tool to discovery novel bioactive compounds from this bacterial genus.

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