Biological activity and chemical identification of ornithine lipid produced by *Burkholderia gladioli* pv. *agaricicola* ICMP 11096 using LC-MS and NMR analyses

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Abstract

Lipoamino acids (LAs) have been isolated from bacterial species and are included among the most important microbial secondary metabolites. Some synthetic LAs are being increasingly used in pharmaceutical applications such as ornithine lipid (OL) which is present in relatively large amounts in some G-ve bacteria. Many *Burkholderia* spp. produce *in vitro* secondary metabolites with lipodepsipeptide nature and have showed relevant biological activities and potential practical applications. The purposes of this research were i) to study the antibacterial activity of cell-free culture filtrate of *B. gladioli* pv. *agaricicola* strain ICMP 11096; ii) HPLC fractionation and antibacterial evaluation of isolated compounds; iii) Finally, the identification by LC-MS and NMR analysis of the principle bioactive compound produced by the bacterium. Results showed that the cell-free culture filtrate has a promising antibacterial activity against the two studied target microorganisms. In addition, HPLC fractionation demonstrated the presence of five single bioactive compounds produced by the bacterium and their antibacterial activity stated that peak no. 2 is the most bioactive one against *B. megaterium* and *E. coli*. Successively, the principal bioactive compound was identified by LC-MS and ^{1}H NMR as OL with mass spectrum (m/z) 719. This research is considered the first report of isolation and chemical identification of OL compound isolated from *B. gladioli* pv. *agaricicola* ICMP 11096.

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Introduction

In the past five decades, the need for new natural antimicrobial products and new natural toxins had largely met demands to replace the synthetic products and a lot of natural toxins were discovered in the middle of the 20th century.[1,2] More recently, advances in technology allowed to discover novel natural products from bacterial sources.

The members of genus *Burkholderia* show a wide host range.[3,4] Many *Burkholderia* spp. have the ability to produce antimicrobial compounds.[5,6] *B. gladioli* is a species that causes disease in mammals, plants and fungi.[7]

In particular, *Burkholderia gladioli* pv. *agaricicola* (*Bga*) Yabuuchi is considered an important pathogen in the mushroom industry.[8,9] It causes soft rot disease on a number of artificially cultivated and commercially important mushrooms such as *Lentinula aedodes* (Berk.) Pegler, *Pleurotus ostreatus* (Jacq.) P. Kumm., *Flammulina velutipes* (Curtis) Singer, *Pholiota nameko* (T. Itô) S. Ito & S. Imai, *Hypsizygus marmoreus* (Peck) H.E. Bigelow and *Grifola frondosa* (Dicks.) Gray in Japan and different cultivated *Agaricus* species in New Zealand and Europe.[10,11] Several studies have reported the antimicrobial activities of *Bga* strains against a variety of phytopathogens.[12–16] In particular, Elshafie *et al.*[15] have reported that the potential biological activity of four *Bga* strains could be correlated with their ability to produce secondary metabolites. Andolfi *et al.*[17] suggested a lipodepsipeptide nature of secondary metabolites produced by *Bga* strains.

Lipoamino acids (LAs) have been isolated from bacterial species and are included among the most important microbial secondary metabolites. The best known one is probably the zwitterionic *N*-acyl-ornithine derivative, which is widely distributed among prokaryotes, especially G-ve bacteria and other eubacteria located predominantly on the outer membrane.[18–20]

Generally, some synthetic LAs have being increasingly used in pharmaceutical applications. Among the synthetic LAs, ornithine lipid (OL) is present in relatively large amounts in some G-ve bacteria such as *Brucella abortus*, *B. melitensis* and *Pseudomonas fluorescens*[21] and has been also evidenced in some species of G+ve bacteria belonging to *Mycobacterium* and *Streptomyces* genera, but it seems to be absent in *Archaea* and Eukarya.[20] OL belongs to a class of fatty acylated amino acids that do not contain either glycerol or phosphate.[22]

OLs has been reported to show a broad spectrum of antimicrobial effect on a number of G-ve and G+ve bacteria such as *Alcaligenes faecalis*, *Bacillus subtilis*, *Escherichia coli* and *P. aeruginosa* and on a variety of yeasts and fungi such as *Candida albicans*, *Cryptococcus neoformas*, *Saccharomyces cerevisiae* and *Aspergillus niger*.[23]

Recently, Elshafie *et al.*[24] have studied *in vitro* biological activity of four strains of *Bga* (ICMP: 11096, 11097, 12220 and 12322) and identified chemically some of their bioactive metabolites using GC-MS. They concluded that some *Bga* strains were able to inhibit the growth of two tested pathogenic bacteria *B. megaterium* de Bary ITM100 and *E. coli* (Migula) Castellani & Chalmers ITM103 (Institute of Tropical Medicine in Antwerp). In addition, GC-MS analysis investigated that strain *Bga* ICMP 11096 produced two bioactive fatty acids which were identified as methyl stearate and ethanol 2-butoxy phosphate with mass spectrum (m/z) 298 and 398, respectively. The aims of the current study were i) to study the antibacterial activity of cell-free culture filtrate of *B. gladioli* pv. *agaricicola* strain ICMP 11096; ii) to fractionate by HPLC and analyze by LC-MS and NMR analysis the principle bioactive compounds produced by the studied bacterium.

Materials and Methods

Bacterial strain

The bacterial strain used in this study was *Bga* ICMP 11096. The target microorganisms, *i.e. B. megaterium* and *E. coli*, were obtained from stock cultures of the same prokaryotes kept freeze-dried in collection at the Laboratory of Mycology of School of Agricultural, Food-Forestry and Environmental Sciences of University of Basilicata (Potenza, Italy), recultured on King B (KB) media[25] and stored at 4°C.

Antibacterial assay of cell-free culture filtrate

Bga ICMP 11096 was grown at 25°C under shaking conditions at 200 rpm in 500 mL Erlenmeyer flasks filled with 150 mL of liquid Minimal Mineral (MM) nutrient medium inoculated with 1.5 mL of a bacterial suspension having an optical density (O.D.) of 0.2 at 590 nm and containing 10^8 CFU mL^{-1}. The antibacterial activity of the cell-free culture filtrate was determined against *B. megaterium* and *E. coli* following the method of Lavermicocca *et al.*[26] with the following minor modifications. The bacterial broth was centrifuged at 20.000 *g* for 15 min and filtrated by sterile 0.22 mm filter (Millipore, USA) and then 10 µL of serial dilutions (1:1) were placed on 14 mL KB solid medium. After complete dryness, about 4 mL of soft 0.7% agar containing 0.5 mL of bacterial target suspension 10^8 CFU mL^{-1} from each studied strain was poured over the plates surface. The plates were incubated at 24±2°C for 48 hours. The inhibition zones referred to unit active per milliliter (Ua mL^{-1}) were detected according to Sinden *et al.*[27] as indication of bioactivity of the produced secondary metabolites in filtrates.

Extraction and identification of diffusible secondary metabolites

The bioactive cell-free culture filtrate was lyophilized using CHRIST ALPHA 1-4 (B. Braun Biotech International, Germany) and stored at –20°C. Lyophilized aliquots of 300 mg have been dissolved in 10 mL sterile distilled water and then loaded on C18 (Strata C18-T) cartridge syringe pre-washed with 2 mL of methanol and 2 mL of distilled water. Then, each loaded cartridge was washed with 1 mL distilled water and the purified bioactive substances were recovered by 1 mL of methanol. The antibacterial activity of the eluted methanol was tested against *B. megaterium* and its bioactivity was expressed as Ua mL^{-1}.

HPLC fractionation

The methanol fraction, collected from the cartridge, was injected in the analytical High-performance liquid chromatograph (HPLC-Agilent) 1200 series austere. The used column was Agilent ECLIPSE XDB, C18 (4.6×150 mm, 5 µm) at λ 380 nm under the following analytical conditions: 20 µL injected volume, 25°C column temperature, 4°C autosampler chamber temperature, 1.0 mL min^{-1} flow rate. The used mobile phases were: A: 0.2% formic acid (FA) in H_2O and B: 0.1% FA in CH_3CN. The HPLC gradient initiated with 80% A and 20% B from 0 to 50 min; 60% A and 40% B from 50 to 60 min and terminated by 80% A and 20% B from 60 to 65 min. This gradient was the result of a series of experimental tentative aiming at the best chromatographic separation. The isolated compounds were evaluated for the antibacterial activity against *B. megaterium* and *E. coli* to determine the most bioactive compound for further chemical identifications.

LC-MS analysis

The mass (MS) of the single peaks obtained from HPLC analysis have been analyzed by Liquid Chromatography coupled with Fourier-transform ion cyclotron resonance MS (LC/FTICR-MS) using a Surveyor LC system coupled to a Finnigan LTQ FT, a 7 Tesla hybrid linear ion trap-FTICR mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a 20 W continuous CO_2-laser (Synrad, Mukilteo, WA, USA; 10.6 µm). LC separation was performed at ambient temperature on a Agilent ECLIPSE XDB, C18 (4.6×150 mm, 5 µm) in the following analytical conditions: injected volume 25 µL, column temperature 25°C, autosampler chamber temperature 4°C, flow 1.0 mL min^{-1} which was split to 4:1 after the analytical column to allow 200 mL min^{-1} to enter the ESI source. Positive ion ESI-MS was chosen for the detection of OLs. Mobile phases were: A: 0.2% FA in H_2O and B: 0.1% FA in CH_3CN. The instrument was tuned to facilitate the ionization process and to achieve the highest sensitivity. The spray voltage was set at 4.50 kV, while the temperature of the ion transfer tube was set at 300°C and the applied voltage at 7 V. The sheath gas (N_2) flow rate used was 80 arbitrary units (AU) and no auxiliary gas was used.

Full-scan experiments were performed in both linear trap as well as the ICR cell in the 50-1500 m/z (mass to charge) range. The m/z ratio signals were acquired as profile data at a resolution of 100,000 (FWHM) at m/z 400. The automatic gain control (AGC) ion population target in full scan MS was 5,000,000 for FTICR

MS. The maximum ion injection time was 200 ms for FTICR. The ESI-FTICR mass spectra obtained were used to characterize the metabolites ionization behavior. In addition, the ESI- CID MS/MS fragmentation performance of the different peaks was investigated. Data acquisition and analyses were accomplished using the Xcalibur software package (version 2.0 SR1 Thermo Electron).

^{1}H NMR analysis

NMR analysis were acquired on a Varian 400 spectrometer located at the Department of Sciences (University of Basilicata, Potenza, Italy). The single peaks separated by HPLC were dissolved in 0.60 mL of CD_3OD and placed in a 5 mm NMR tube and then analyzed by ^{1}H NMR. The spectrometer was equipped with a 5 mm direct detection pulsed field *z*-axis gradient probe, operating at 399.96 MHz for ^{1}H. Exact 128 scans were acquired for each experiment. The temperature during all experiments was kept at 25°C without sample rotation.

Antioxidant activity of purified extracted metabolites

In the present study, two different antioxidant assays [2,2-diphenyl-1- picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzthiazoline-6-acid) (ABTS)] were performed following the methodological procedures of Martysiak- Żurowska and Wenta.[28] The two assays were carried out to determine Radical Scavenging Activity (RSA%) of most bioactive substance isolated from *Bga* ICMP 11096 strain utilizing the following formula:

$$RSA\% = (1 - A_t / A_c) \times 100\%$$

Where A_t: is the absorbance of sample and A_c is the absorbance of colorimetric radical substance without sample.

The stock radical DPPH solution was prepared by dissolving 20 mg of DPPH in 15 mL of ethanol. Thereafter, 1 mL of DPPH solution was diluted with 29 mL of ethanol. A definite amount of each collected fraction (50 µL) was diluted (1:20) using 950 µL of DPPH solution and was incubated at darkness for 30 minutes at room temperature.

The stock radical ABTS solution was prepared by dissolving 38 mg of ABTS in 10 mL of aqueous sodium persulphate solution (2.45 mM), and the solution was conserved in darkness for 16 hours at room temperature. Then, 1 mL of stock ABTS solution was diluted with 29 mL of ethanol. Twenty µL of the tested peak was diluted (1:50) using 980 µL of radical ABTS solution and was incubated after that in darkness for 2 hours at room temperature.

Both samples were centrifuged at 8000 rpm for 5 min and the absorbance was measured at 515 and 734 nm for DPPH and ABTS, respectively, by using spectrophotometer UV-Vis (LKB Biochrom 4050 Ultrospec II) considering the value of reference sample (ethanol).

Statistical analysis

The experimental output were statistically analyzed using statistical Package for the Social Sciences SPSS (version 13.0, Prentice Hall: Chicago, IL, USA, 2004). Experimental data were expressed as means±SD and comparisons were employed by one way ANOVA followed by Tukey *post-hoc* test at $P<0.05$.

Results and Discussion

Antibacterial assay

The maximum significant antibacterial activity of *Bga* strain ICMP 11096 filtrate against *B. megaterium* was observed after 168 h of incubation as 1600 Ua mL^{-1} whereas against *E. coli* was 400 Ua mL^{-1} after 120 hours (Figure 1). On the basis of the obtained results it is conceivable to hypothesize that the production of bioactive secondary metabolites has been initiated during the logarithmic phase of bacterial growth and their bioactivity becomes effective during the stationary phase. These results are in agree with those of Demain[29] who observed that the biosynthesis of the microbial secondary metabolites is induced by the nutrition system components and the bioactivity of produced metabolites reaches the maximum value at the end of stationary phase.

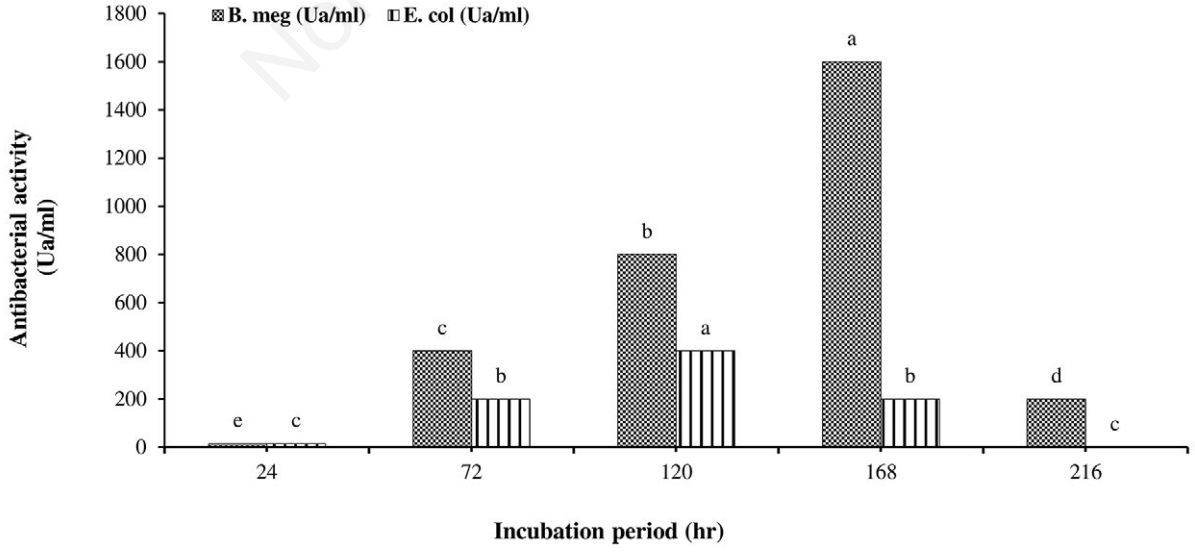

Figure 1. Antibacterial activity of cell-free culture filtrate of *Bga* ICMP 11096. Bars with different letters for each factor indicate means values significantly different at P<0.05 according to *Tukey* test. Data are expressed as mean of three replicates.

The production of secondary metabolites is regulated by signal molecules called N-acyl homoserine lactones (*N*.AHLs) which determine gene expression in response to the bacterial cell population size and nutrient media components.[30–34] The *N*-AHLs mediated quorum sensing have been already reported in regulation of several primary and secondary metabolisms in some G-ve bacteria such as *Pseudomonas*, *Burkholderia* and *Agrobacterium* spp.[35,36]

Purification and identification of secondary metabolites

As described before in the methodological procedures, the specific bioactivity of lyophilized filtrate after conservation at –20°C was estimated by 1600 Ua mg^{-1} against *B. megaterium*, then the eluted extracted methanol solution from the SPE C-18 cartridge was assessed by 1600 Ua mL^{-1} against the same bacterium. These results confirmed the recovery of the whole bioactive substances after the extraction.

HPLC analysis

From races conducted at λ=380 nm and relative chromatograms, it was noticed the presence of 5 single fractions (Figure 2) that were separated and evaluated for their antibacterial activity against *B. megaterium* and *E. coli*. The results shown in Table 1 revealed that all collected fractions have antibacterial activity towards the two target bacteria. In particular, the most bioactive fractions were 2 and 3. Their bioactivity was assessed by 12800 and 6400 Ua mL^{-1} against *B. megaterium* and, 6400 and 3200 Ua mL^{-1} against *E.* coli, respectively.

LC-MS-MS analysis

The MS, MS/MS and MS^3 spectra for peak 2 ion at m/z 719 in positive ion mode are shown in Figure 3. These spectra are compatible with the structure of an OL 3–OH 20:1/19:1. MS/MS of the protonated precursor at m/z 719 from *Bga* ICMP 11096 resulted in the dominant loss of H_2O (m/z 701) and NH_3 (m/z 702), along with two products corresponding to the loss of a 19:1 (m/z 423, 405) fatty acyl chain and ornithine (m/z 587), respectively. However, MS^3 of the $[M+ H- NH_3]^+$ product ion resulted in formation of the 18:2 product ion series (281, 263 and 235) obtained from fragmentation of 19:1 chain. Subsequent fragmentation of the m/z 423 product ion resulted in H_2O loss (m/z 405). Table 2 reports the relative abundance of the most abundant compound recognized in each single HPLC peak together with retention time and its molecular weight.

^{1}H NMR analysis

The ^{1}H NMR spectrum in CD_3OD of peak no. 2 shows the following signals: ϑ 1.3 (m, 9H, R'$_1$ 2 CH_2 in amino moiety). 2.1 (S, R_2, CH_2 adjacent to carbonyl group). 4.55 (S, CH_2 adjacent to amino group). 4.82 (S, methine proton adjacent to NH & methine proton adjacent to oxygen). 8.5 (broad signal due to NH proton) exchangeable by D_2O (Figure 4; Table 3). These results confirmed the hypothesis that the isolated bioactive compound is lipoamino acid (OL).

OL contains a non-hydroxy fatty acid with an estolide linkage to a 3-hydroxy acid (often but not always C16 or C18) and thence via an amide bond to the α-amino group of ornithine.[20] It may be

Table 1. Antibacterial activity of single HPLC peaks.

Peaks no.	Biological activity	
	B. megaterium Ua mL^{-1}	*E. coli* Ua mL^{-1}
1	3200c	1600c
2	12800a	6400a
3	6400b	3200b
4	1600d	800d
5	3200c	1600c

Ua mL^{-1} is unit active per millilitre. Values followed by different letters in each vertical column are significantly different according to Tukey test at P<0.05. Data are expressed as mean of three replicates.

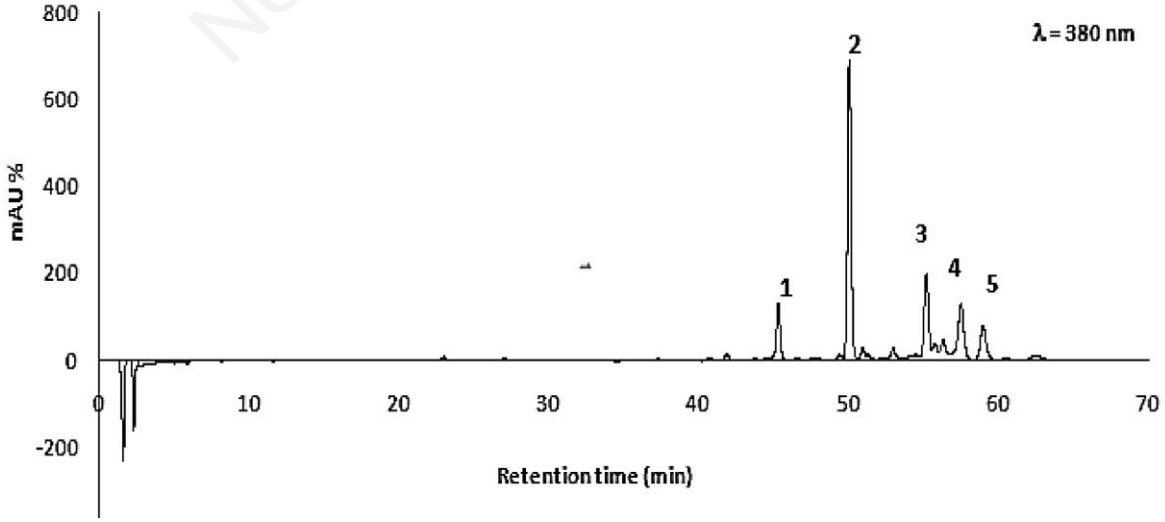

Figure 2. HPLC chromatogram of *Bga* strain ICMP 11096 at 380 nm.

relevant that such fatty acid linkages are also seen in the bacterial endotoxin lipid A.[22,37]

The biosynthesis of OL in *Bga* strain ICMP 11096 could be occurred in two steps via acyl-ACP-dependent acylation of ornithine by two different acyltransferases in agree with Vences-Guzmán *et al*.[37] The ester-linked fatty acid has a hydroxyl group in position 2 and analogous lipids in which the ornithine moiety is hydroxylated are known.[38,39]

OL has been reported to show a broad spectrum of antimicrobial effect on a number of G-ve and G+ve bacteria such as *Alcaligenes faecalis*, *B. subtilis*, *E. coli* and *P. aeruginosa* and a variety of yeasts and fungi such as *C. albicans*, *C. neoformas*, *S. cerevisiae* and *A. niger*.[23] OL demonstrated diverse biological effects in microorganisms and mammals.[18,40] It is of interest to know how OL plays a role in the outer membrane stability, whether or not it contributes in the resistance for the bactericidal peptides and how it displays a biological activity different from other recognized OLs.

Antioxidant activity

Both DPPH and ABTS assays evidenced the high antioxidant activity where the RSA of peak 2 was assessed by 93,94% (±2.5 SDs) and 98.74% (±1.87 SDs), respectively, without significant differences between both assays. The antioxidant activity is mainly due to the redox properties and chemical structure of bioactive OL substance, which may help in chelating transitional metals, inhibiting lipoxygenase and scavenging free radicals.[38,39]

In conclusion, the results of bioassays of *Bga* strain ICMP 11096 confirmed the production of some antimicrobial substances that are produced in broth culture. Results from LC-MS and NMR investigations demonstrated that the main isolated bioactive substance is an amino lipid compound identified as OL. This bioactive compound

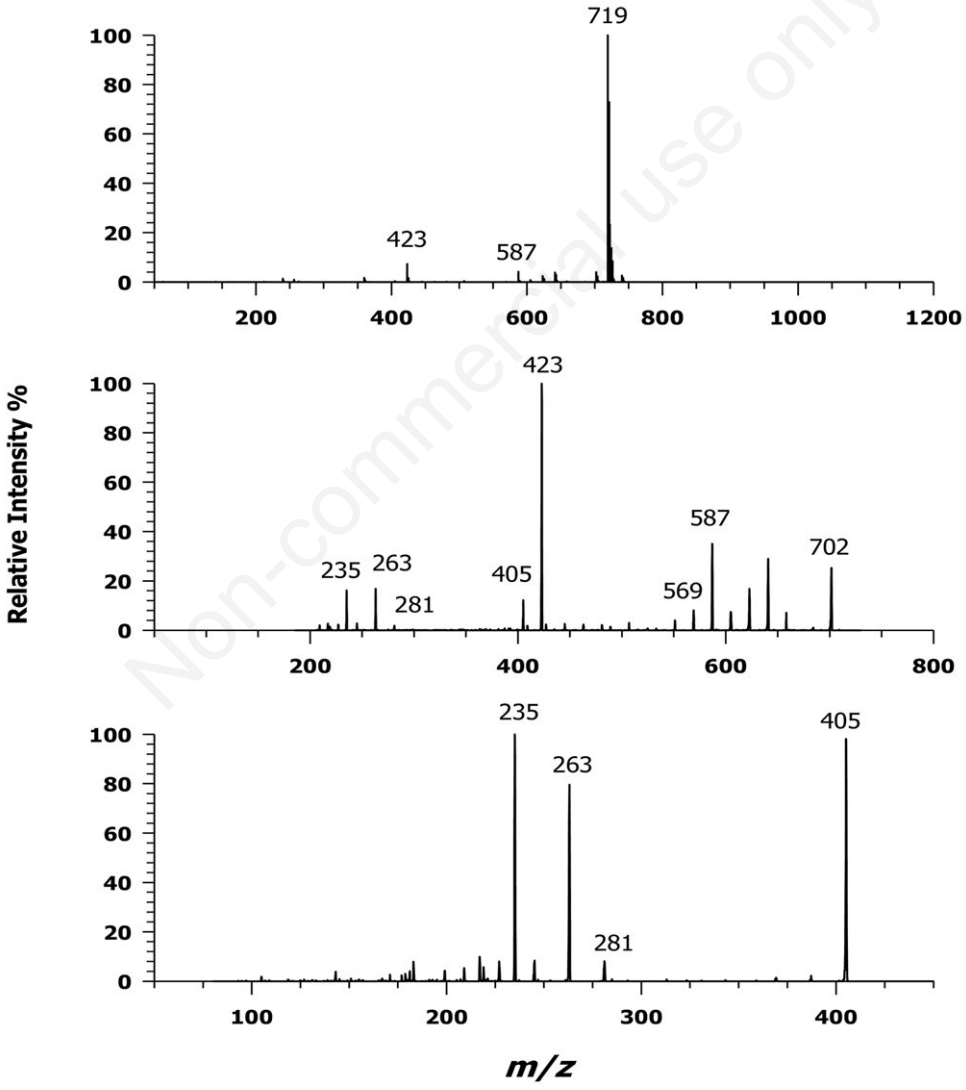

Figure 3. MS, MS/MS and MS^3 spectra for the ion at m/z 719 (peak 2) in positive ion mode. HPLC-Agilent 1200 series, column ECLIPSE XDB, C18 (4.6x150 mm, 5 µm) at λ=380 nm; gradient elution starting at 80 of 0.2% formic acid in water in 5 min and remain stable at 80% until 50 min, then gradually decreased until 60% at 60 min and terminated by 80% at 65 min; whereas 0.1% formic acid % in CH_3CN started at 20% in 5 min and remain stable until 50 min, then gradually increased until 40% at 60 min and terminated with 20% at 65 min; at a flow rate 1.0 mL min^{-1}.

Table 2. Retention time and molecular weight of the most abundant compound recognized in a single HPLC peak.

Peak no.	RT (min)	MW	mAU	Relative abundance (%)
1	43.86	607	320×10^3	45
2	48.34	719	540×10^3	100
3	52.85	641	400×10^3	78
4	54.52	607	300×10^3	49
5	56.18	641	280×10^3	55

RT, retention time; MW, molecular weight; mAU, milli absorbance unit.

Table 3. ^{1}H NMR Spectroscopic data for compound ornithine lipid in CD_3OD (δ, ppm, Hz)[a].

H/C	δ_H	H/N	δ_H	R group	δ_H
1	N/A	H_2N	8.5	R1	1.3
2	N/A			R2	2.1
3	1.3				
4	1.3				
5	4.9				
1° 2°	R1-N/A				
3°	4.5				

[a]Data obtained at 399.96 MHz for ^{1}H.

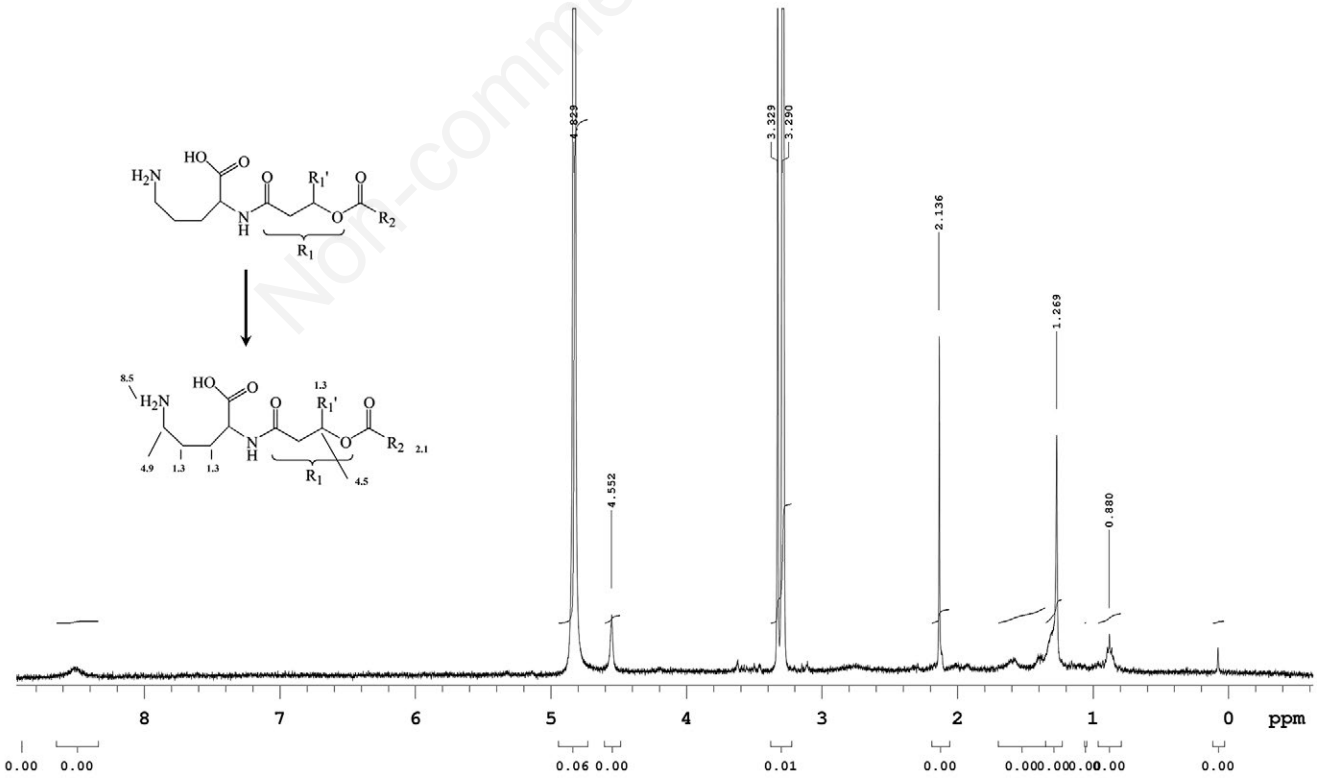

Figure 4. ^{1}H NMR analysis of the bioactive compound no. 2 fractionated from *Bga* ICMP 11096; ^{1}H NMR spectra was equipped with 5 mm direct detection pulsed field *z*-axis; at 399.96 MHz for ^{1}H; 128 scans were acquired without sample rotation.

has shown diverse biological effects in microorganisms and mammals. In particular, OL obtained from *Bga* ICMP 11096 represented a major polar lipid constituent of the whole bacterial cell.

The current research reports for the first time, the exact identification, chemical structure and biological characterization of OL produced by *Bga* strain ICMP 11096. The results presented here should encourage the use of isolated bioactive secondary metabolites of *Bga* strains in pharmaceutical industry, food preservatives and further nutraceuticals applications, due to their antioxidant and antibacterial properties. Future researches should be directed to determine the eventual production of other bioactive substances by the same bacterial strain, as well as to find out the action mode of OL in controlling human and plant pathogens.

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