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SHORT COMMUNICATION

Analytical pyrolysis evidences the presence of granaticins in the violet stains of a Roman tomb

Marta Diaz-Herraiz[1], Leonila Laiz[1], Valme Jurado[1], Ana Z. Miller[1], Jose Antonio Gonzalez-Perez[1], Juan Luis Santos[2], Esteban Alonso[2] and Cesareo Saiz-Jimenez[1]*

[1]*Instituto de Recursos Naturales y Agrobiologia, IRNAS-CSIC, Avenida Reina Mercedes 10, 41012 Sevilla, Spain*

[2]*Escuela Politecnica Superior, Universidad de Sevilla, Virgen de Africa 7, 41011 Sevilla, Spain*

*Corresponding author:
Cesareo Saiz-Jimenez
Instituto de Recursos Naturales y Agrobiologia, IRNAS-CSIC, Avenida Reina Mercedes 10, 41012 Sevilla, Spain. E-mail: saiz@irnase.csic.es

Running title: Granaticins in a Roman tomb

Abstract

The walls of the Circular Mausoleum tomb (Roman Necropolis of Carmona, Spain) exhibit an important number of violet stains of unknown origin. Analytical pyrolysis revealed granaticin A in the tomb wall samples, a violet pigment with an isobenzochromanequinone structure. This and other related pigments were identified in the extracts of two bacterial strains isolated from the walls. The strains were identified as members of the *Streptomyces griseus* clade. High performance liquid chromatography confirmed that these *Streptomyces* synthesized dihydrogranaticin A, granaticin A and granaticin B as major pigments.

Keywords: Granaticins, *Streptomyces*, Roman tomb, mortar, pyrolysis, high performance liquid chromatography

1. Introduction

Colored stains in cultural heritage sites and monuments are caused by chlorophylls, carotenoid pigments, anthraquinones, melanins, etc., synthesized by bacteria, fungi, algae and lichens [1-4]. Actinobacterial growth on building materials produces a variety of colorations usually related to the excretion of secondary metabolites, including antibiotics and pigments [5].

Violet staining on paintings and mortars are rare in cultural heritage properties. This phenomenon was only previously reported in two Etruscan tombs. However, no data on the bacterial species producing the pigments and their chemical structure were reported [6].

The Circular Mausoleum tomb, at the Roman Necropolis of Carmona, Spain, dates back from the 1st century AD and is characterized by the abundant presence of violet stains on the walls (Fig. 1). Sampling campaigns allowed us to isolate and characterize two *Streptomyces* strains involved in the production of a diffusible violet pigment on the tomb walls, which originates a noticeable biodeterioration. The isolation of strains that produce the violet pigments, either in nature or in the laboratory, together with the identification of the violet pigment by analytical pyrolysis and reinforced by high performance liquid chromatography-mass spectrometry allowed disclosing the nature of the violet stains and related pigments from the Circular Mausoleum tomb.

2. Experimental

2.1. Sampling of violet stains

Two sampling campaigns were conducted in the Circular Mausoleum tomb, Roman Necropolis of Carmona, Spain, in the years 1997 and 2005. The samplings were focused on the isolation of bacteria producing violet stains on the tomb walls. The violet stains were gently scrapped from the mortar using a sterilized scalpel, and placed into sterile tubes.

Samples for bacterial isolation were processed the day of sampling and inoculated on Petri plates containing tryptone soy agar (TSA) medium with 0.2% glycerol. Cultures were incubated at 30°C for several weeks and the species producing violet pigments were isolated. The bacterium *Streptomyces*

67 *vietnamensis* DSM 41927, reported to produce violet pigments [7], was used for
68 comparative studies.

69 *2.2. 16S rDNA identification of isolates*

70 Bacterial isolates producing violet pigments were identified by direct PCR from
71 colonies. DNA was extracted by dispersing a bacterial colony in 100 µl of 10mM TE
72 buffer, and freeze-thawing at -80°C and 65°C, respectively. Oligonucleotides
73 primers targeting the 16S rDNA of bacteria were used for PCR. The nucleotide
74 sequences of the primers were 5´-AGAGTTTGATYMTGGCTCAG-3´ (*Escherichia*
75 *coli* 16S rDNA positions 8 to 27) [8] and 5'-GGCTACCTTGTTACGACTT-3'
76 (positions 1510 to 1492) [9]. PCR reactions and amplifications were performed in a
77 Bio-Rad iCycler thermal cycler as described in [10]. The PCR products were run on
78 a 1% (w/v) agarose gels and purified using the JetQuick PCR Purification Spin Kit
79 (Genomed, Löhne, Germany). DNA sequencing of the PCR products was carried
80 out by Macrogen Europe Sequencing Services (Amsterdam, The Netherlands).
81 Sequences were phylogenetically classified and their nearest relative was
82 identified using the BLASTN algorithm. The isolates were identified using the
83 EzTaxon server (http://www.ezbiocloud.net/eztaxon) [11] on the basis of 16S rRNA
84 sequence data.

85 *2.3. Pigment extraction and analysis*

86 The liquid medium used for pigment production was composed of sucrose,
87 glucose, peptone, yeast extract, malt extract and $MgCl_2$, as described by Hopwood

et al. [12]. The pigments were extracted with ethyl acetate and analyzed by thin layer chromatography (TLC) on silica gel plates (HPTLC silica gel 60 Merck, Damstadt, Germany), using the solvents chloroform/acetic acid, 4:1. All reagents and solvents used were of analytical grade.

The chemical nature of the pigments was investigated using analytical pyrolysis and liquid chromatography–mass spectrometry (HPLC-MS). For analytical pyrolysis the materials containing pigments i.e. small pieces of violet stained mortar were deposited on small crucible capsules while liquid extracts were directly deposited on a stainless steel pyrolysis wire (Eco-cup and Ecos-stick; Frontier Labs., Fukushima, Japan). Pyrolysis-gas chromatography/mass spectrometry was performed using a double-shot pyrolyzer (Frontier Labs. model 2020i) attached to a GC/MS system Agilent 6890N, as described by González-Pérez et al. [13]. Briefly, the samples were introduced into a preheated micro-furnace set at temperatures of 100 to 250°C for thermal desorption and/or at 350 to 500°C for pyrolysis. The analytical protocol was set at 150°C for thermal desorption and 350°C for pyrolysis, which provided the best results. The pyrolysate was then directly injected into the GC/MS for analysis. The gas chromatograph was equipped with a HP-5ms-UI capillary column. The detector consisted of an Agilent 5973 mass selective detector, and mass spectra were acquired at 70 eV ionizing energy. Compound assignments were achieved by single-ion monitoring (SIM) and by comparison with mass spectra [14].

HPLC-MS analyses were performed as described by Martín et al. [15]. Briefly, an Agilent 1200 series HPLC equipped with a Zorbax Eclipse XDB–C18 Rapid Resolution HT (4.6x50 mm i.d.; 1.8 µm) column was used. Elution was carried out with acetonitrile (containing formic acid 0.1%, v/v) (solvent A) and aqueous 10 mM ammonium formate solution (containing formic acid 0.1%, v/v) (solvent B), at a flow rate of 0.6 mL/min with the column thermostated at 25 °C. The elution programme used was: 0-7.5 min: linear gradient from 10% to 35% of solvent A, 7.5-14.5 min: isocratic 35% solvent A, 14.5-14.6 min: linear gradient from 35% to 100% of solvent A, 14.6-18.5 min: isocratic 100% solvent A. Mass spectrometry analyses were performed on an Agilent 6410 QqQ instrument equipped with an electrospray ionization source. Ionization of analytes was carried out using the following settings: MS capillary voltage of 3000 V, drying-gas flow rate of 9 L/min, drying-gas temperature of 350 ºC and nebulizer pressure of 40 psi.

Each extract was measured using scan and product ion modes. First, scan mode was applied to the determination of the retention time of each of the studied compounds. Scan was carried out using positive polarity and applying a voltage to the exit end of the capillary (fragmentor) of 50 V. Mass spectra were registered from *m/z* 80 to 950.

Product ion mode was carried out using positive polarity for the monitoring of the ions of *m/z* 445, 447 and 559, corresponding to ions [M+1] of granaticin A, dihydrogranaticin A and granaticin B, respectively. The applied fragmentor was 50

V and the voltage applied to the ions in the collision cell (collision energy) was 12 V. MS/MS spectra from *m/z* 50 to 570 were obtained using a scan time of 300 ms.

3. Results and Discussion

3.1. Bacterial isolation

Two bacterial strains MC48 and MC05 (accession numbers LN615177 and LN615178, respectively) producing a diffusible violet pigment under laboratory conditions were isolated from the violet stains collected in 1997 and 2005, respectively. Both bacteria produced the same pigments.

The sequences were phylogenetically classified and both strains were included in the *Streptomyces griseus* clade. It is difficult to identify the different *Streptomyces* species included in this clade due to the fact that their sequences showed 100 % similarity when compared to each other.

Using the EzTaxon database [11], *Streptomyces* MC48 showed 98% similarity with *Streptomyces badius, Streptomyces parvus, Streptomyces pluricolorescens* and *Streptomyces rubiginosohelvolus* type strains. MC05 strain showed 100% similarity with *Streptomyces badius, Streptomyces parvus, Streptomyces pluricolorescens, Streptomyces rubiginosohelvolus*, *Streptomyces sindedensis* and *Streptomyces globisporus* type strains, therefore we only can identify our strains as members of the *S. griseus* clade.

3.2. Pigment production

Two *Streptomyces* strains isolated from the violet stains of the Circular Mausoleum tomb were able to synthesize violet compounds in a medium used for pigment production [12]. The pigment, violet in alkaline media, turned out red in acid media which denoted a quinone structure.

Few *Streptomyces* species have been reported to produce violet or blue pigments. We compared the two isolated strains with *Streptomyces vietnamensis* type strain [7], which was selected as control for violet pigment production.

Thin layer chromatography (TLC) of the pigments extracted from *S. vietnamensis* and *Streptomyces* strain MC05 showed similar R_f for the main pigments (Fig. 2). MC48 showed identical TLC pattern. Chromatographic data suggested that the pigments synthesized by *Streptomyces* strain MC05 could be related to the granaticins produced by *S. vietnamensis* [16].

The chemical nature of the violet pigments from *Streptomyces* MC05 strain and *S. vietnamensis* was also disclosed by analytical pyrolysis. For this approach, the bacterial culture extracts and pieces of the tomb mortars were subjected to several thermal desorptions (from 100 till 250°C) and pyrolysis conditions (from 350°C till 500°C). The most suitable analytical protocol for the samples was a double shot consisting on a first thermal desorption stage at 150°C, releasing lipids and other volatile compounds, followed by a thermal desorption/mild pyrolysis at 350°C, which allow obtaining high molecular weight products.

Analytical pyrolysis of the violet extract produced by *S. vietnamensis* (Fig. 3A) showed a conspicuous peak at the end of the chromatogram (30.6 min), both

under thermal desorption at 150°C and mild pyrolysis conditions at 350°C, with a mass spectrum characterized by the molecular ion at *m/z* 444 and $-H^+$ ion at *m/z* 443, which is characteristic of granaticin A (Fig. 3A). The same peak and mass spectrum was obtained for *Streptomyces* MC05 strain (data not shown). The peak corresponding to granaticin A was also obtained from the direct analysis of a small piece of violet-colored mortar from the tomb (Fig. 3B).

Analytical pyrolysis confirmed the presence of granaticin A in the culture extracts of the two strains of *Streptomyces* isolated from the tomb, in the extract of *S. vietnamensis*, and in the violet-stained mortar collected from the walls. Thus, analytical pyrolysis revealed to be a useful tool for the direct detection of granaticin A in organic and inorganic matrices. The characteristic ions for the peak found −under our experimental conditions− at the end of the chromatogram (Fig. 3) are of diagnostic value for the direct confirmation and molecular characterization of granaticin A.

HPLC-MS of the extracts from *S. vietnamensis* (Fig. 4A) and from *Streptomyces* strain MC05 (Fig. 4B) confirmed the production of dihydrogranaticin A, granaticin A and granaticin B, although with different relative abundances for each pigment. Mass spectra of the three pigments are shown in Figure 4C-D and their structures in Figure 5. Granaticin A and B exhibited antibacterial activity against Gram-positive bacteria and inhibitory activity against several cancer cell lines [16]. The production of dihydrogranaticin A by *S. vietnamensis* was not previously reported by Deng et al. [16].

To conclude, the origin of the violet stains causing biodeterioration on the walls of a Roman tomb from the first century AD was the excretion of granaticins. These are soluble pigments with aromatic polyketide structure produced by *Streptomyces* strains, which were active in the tomb, as they were isolated in different periods of time.

The major violet pigment, granaticin A, is soluble in alkaline water but the solubility of the pigment decreased with pH value. The pH of carbonated lime mortars is near 9, which allowed the solubilization of pigments in aqueous medium. It can be assumed that in the Circular Mausoleum tomb and during growing periods the *Streptomyces* synthesizes the violet pigments which diffuse onto the mortar. In raining and/or wetting (water condensation) periods, the pigments were solubilized by alkaline water and diffused from the starting position to the surrounding mortar, enlarging the pigmented area as denoted by an increasing intensity on the stain edges and a lower intensity in the stain centre, as can be observed in Figure 1D.

In cultural heritage research we rarely have the opportunity to isolate a microorganism which reproduces in the laboratory the synthesis of pigments causing the deterioration phenomena, as shown here. Similar violet stains were also reported on the mural paintings from the Tomb of the Chase and Fishing and Tomb of the Bulls, Tarquinia, Italy. These Etruscan tombs were sampled in July 2009, but no pigment-producing bacteria could be isolated at that time, probably due to a recent cleaning and restoration intervention. Sampling on the violet-

219 colored walls was not allowed by the Italian cultural authorities, therefore, the
220 nature of the violet stains in Etruscan tombs still remain unknown.
221
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227
228 **References**
229 [1] C. Saiz-Jimenez, Microbial melanins in stone monuments, Sci. Total Environ.
230 167 (1995) 273–286.

231 [2] C. Saiz-Jimenez, J.J. Ortega-Calvo, J.W. de Leeuw, The chemical structure of
232 fungal melanins and their possible contribution to black stains in stone
233 monuments, Sci. Total Environ. 167 (1995) 305–314.

234 [3] G. Caneva, M.P. Nugari, O. Salvadori, La Biologia Vegetale per i Beni Culturali,
235 Vol. I, Nardini Editore, Firenze, 2005.

236 [4] J.D. Ettenauer, V. Jurado, G. Piñar, A.Z. Miller, M. Santner, C. Saiz-Jimenez, et
237 al., Halophilic microorganisms are responsible for the rosy discolouration of
238 saline environments in three historical buildings with mural paintings, PLoS
239 ONE 9(8) (2014) e103844, doi: 10.1371/journal.pone.013844.

[5] J. Bérdy, Bioactive microbial metabolites, J. Antibiot. 58 (2005) 1−26.

[6] G. Agarossi, Biodeterioramento in ambienti ipogei: esperienze e considerazioni, in: F. Guidobaldi (Ed.), Studi e Ricerche sulla Conservazione delle Opere d'Arte Dedicati alla Memoria di Marcello Paribeni, CNR, Roma, 1994; pp. 1−11.

[7] H-H. Zhu, J. Guo, Q. Yao, S-Z. Yang, M-R. Deng, L.T.B. Phuong, et al., *Streptomyces vietnamensis* sp. nov., a streptomycete with violet–blue diffusible pigment isolated from soil in Vietnam, Int. J. Syst. Evol. Microbiol. 57 (2007) 1770−1774.

[8] S. Juretschko, G. Timmermann, M. Schmid, K-H. Schleifer, A. Pommerening-Röser, H-P. Koops, et al., Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations, Appl. Environ. Microbiol. 64 (1998) 3042−3051.

[9] A. Echigo, M. Hino, T. Fukushima, T. Mizuki, M., Kamekura, R. Usami, Endospores of halophilic bacteria of the family *Bacillaceae* isolated from non-saline Japanese soil may be transported by Kosa event (Asian dust storm), Saline Syst. 1 (2005) 8, doi: 10.1186/1746-1448-1-8.

[10] M. Diaz-Herraiz, V. Jurado, S. Cuezva, L. Laiz, P. Pallecchi, P. Tiano, et al., The actinobacterial colonization of Etruscan paintings, Sci. Rep. 3 (2013) 1440, doi: 10.1038/srep01440.

[11] O.S. Kim, Y.J. Cho, K. Lee, S.H. Yoon, M. Kim, H. Na, et al., Introducing EzTaxon: a prokaryotic 16S rRNA Gene sequence database with phylotypes

that represent uncultured species, Int. J. Syst. Evol Microbiol. 62 (2012) 716−721.

[12] D.A. Hopwood, M.J. Bibb, K.F. Chater, T. Kieser, C.J. Bruton, H.M. Kieser, et al., Genetic Manipulation of *Streptomyces*: A Laboratory Manual, John Innes Foundation, Norwich, 1985.

[13] J.A. González-Pérez, N.T. Jiménez-Morillo, J.M. de la Rosa, G. Almendros, F.J. González-Vila, Pyrolysis-gas chromatography–isotope ratio mass spectrometry of polyethylene, J. Chromatogr. A 1388 (2015) 236−243.

[14] M. Junack, E. Kormann, A. Eick, W. Sichtermann, A. Benninghoven, H. Pape, Detection of bacterial products of granaticin by secondary ion mass spectrometry (SIMS), Fresenius Z. Anal. Chem. 311 (1982) 411.

[15] J. Martín, J.L. Santos, I. Aparicio, E. Alonso, Pharmaceutically active compounds in sludge stabilization treatments: anaerobic and aerobic digestion, wastewater stabilization ponds and composting, Sci. Total Environ. 503-504 (2015) 97−104.

[16] M-R. Deng, J. Guo, X. Li, C-H. Zhu, H-H. Zhu, Granaticins and their biosynthetic gene cluster from *Streptomyces vietnamensis*: evidence of horizontal gene transfer, Anton. Leeuw. Int. J. G. 100 (2011) 607−617.

Figure legends

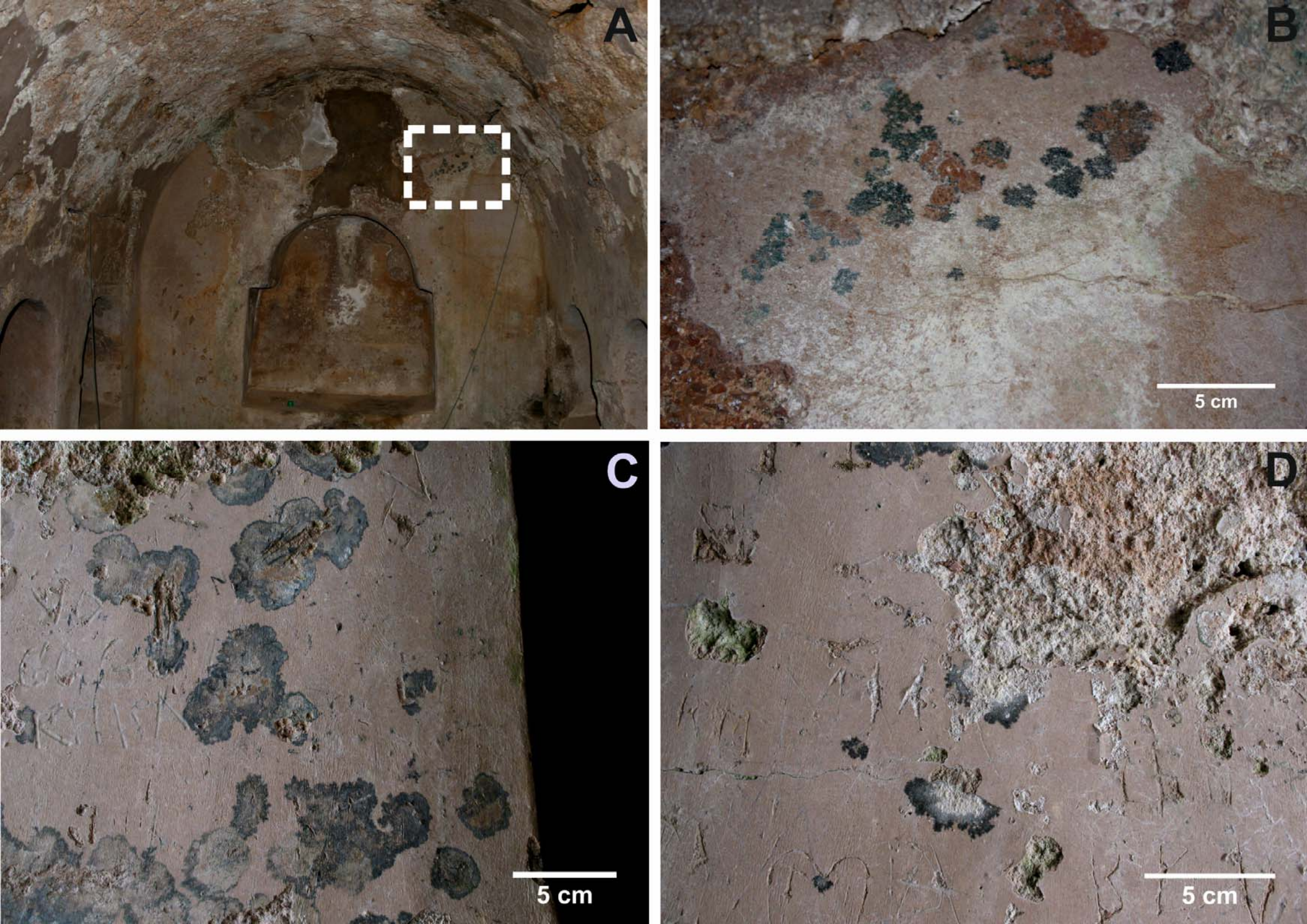
Figure 1. Violet stains in the Circular Mausoleum, Roman Necropolis of Carmona, Spain. A: Back wall of the tomb. B: Details of violet stains from A. C: Violet stains in the right wall. D: Violet stains in the left wall.

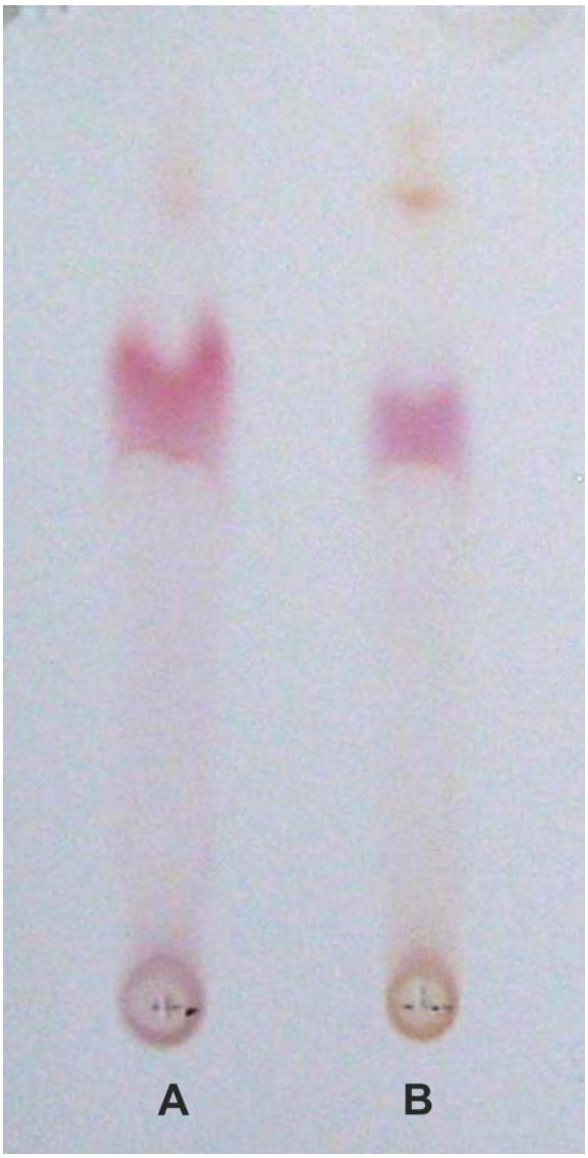
Figure 2. Thin layer chromatography of violet extracts. A: *Streptomyces vietnamensis*. B: *Streptomyces* strain MC05.

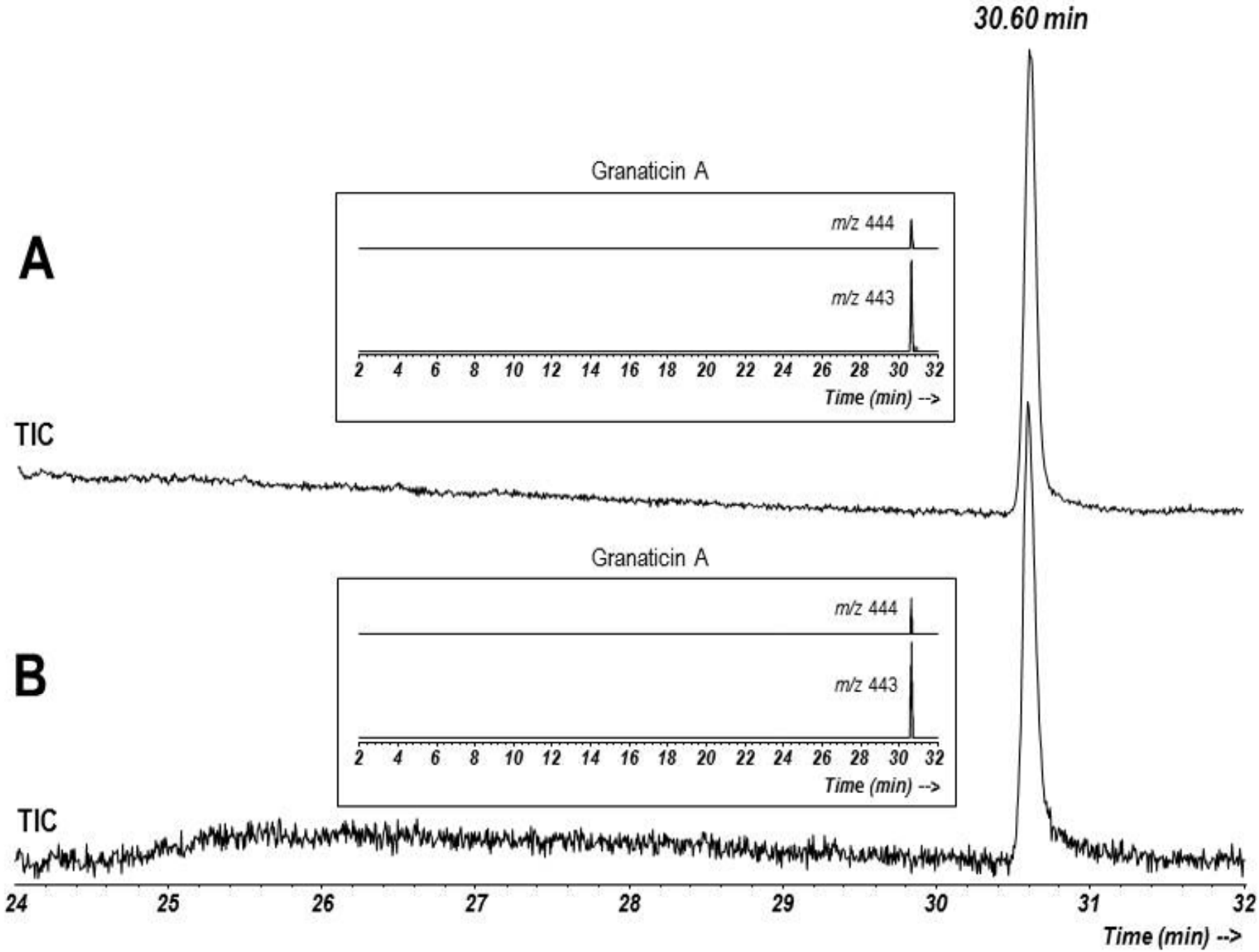
Figure 3. Analytical pyrolysis of violet pigments. A: Total ion chromatogram and mass ion chromatogram (window) of *S. vietnamensis* culture extract. B: Total ion chromatogram and mass ion chromatogram (window) of a small piece of violet-colored mortar.

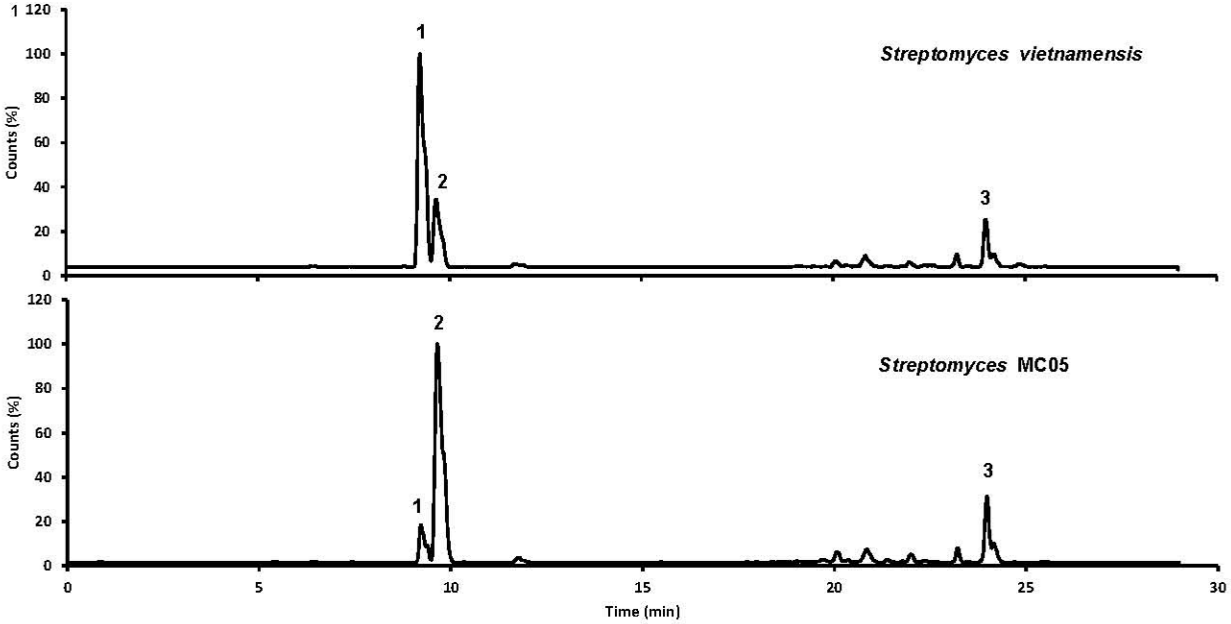
Figure 4. High performance liquid chromatography-mass spectrometry of extracts from *Streptomyces vietnamensis* and *Streptomyces* strain MC05. 1: Mass spectrum of dihydrogranaticin A (R_t 9.2 min), 2: Mass spectrum of granaticin A (R_t 9.6 min), 3: Mass spectrum of granaticin B (R_t 23.9 min).

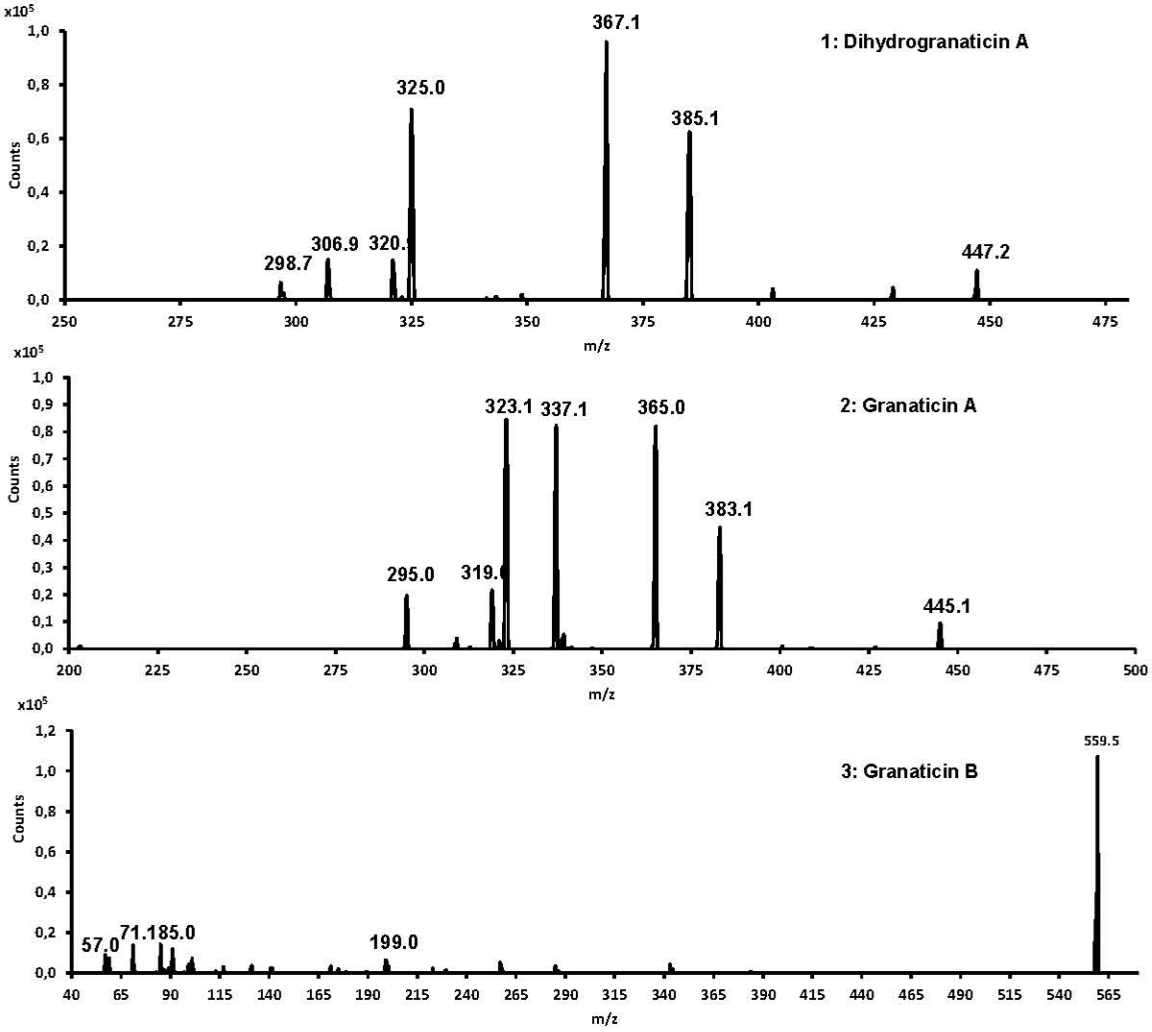
Figure 5. Structures of granaticin A, dihydrogranaticin A and granaticin B.


A
B
5 cm
C
5 cm
D
5 cm


A
B


30.60 min
Granaticin A
A
m/z 444
m/z 443
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Time (min) -->
TIC
Granaticin A
B
m/z 444
m/z 443
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Time (min) -->
TIC
24 25 26 27 28 29 30 31 32
Time (min) -->


Streptomyces vietnamensis
Streptomyces MC05
Counts (%)
Time (min)
1
2
3


1: Dihydrogranaticin A
2: Granaticin A
3: Granaticin B
Counts
m/z
x10^5
367.1
325.0
385.1
306.9
298.7
447.2
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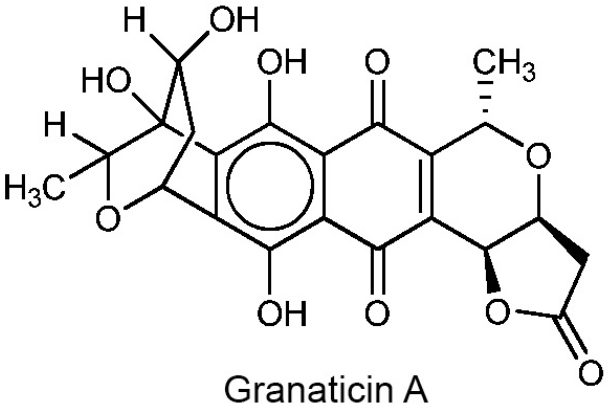
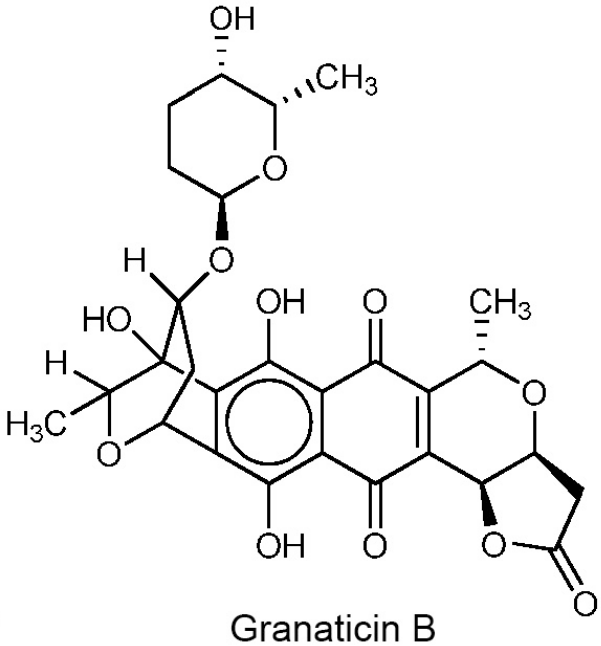
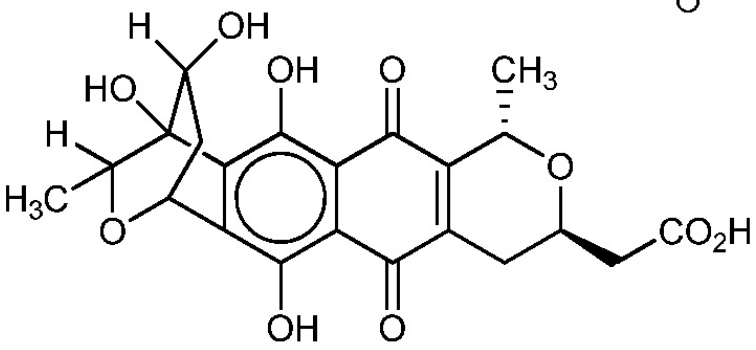
445.1
559.5
57.0
71.1
85.0
199.0



Granaticin A



Granaticin B



Dihydrogranaticin A