



Isolation and characterization of violacein from an Antarctic *Iodobacter*: a non-pathogenic psychrotolerant microorganism

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Abstract

Violacein is an intensely purple pigment synthesized by various genera of bacteria that has been discovered to have a wide range of interesting biological activities which range from anticarcinogenic to antibacterial. One of the hindrances for its real-life application is that the first microorganisms found to produce the compound may act as opportunistic pathogens. Here, we report the isolation and characterization of violacein from a non-pathogenic Antarctic *Iodobacter* strain. Its anti-microbial properties were also tested. The method proposed here for the purification of violacein shows high yields, indicating that this Antarctic microorganism could be a valuable source for this important pigment. This is the first characterization of violacein from an Antarctic *Iodobacter* strain and here we also present a viable method to obtain this pigment for potential biotechnological applications.

Keywords Violacein · *Iodobacter* · Antarctic microbiology · Pigment · Natural compounds

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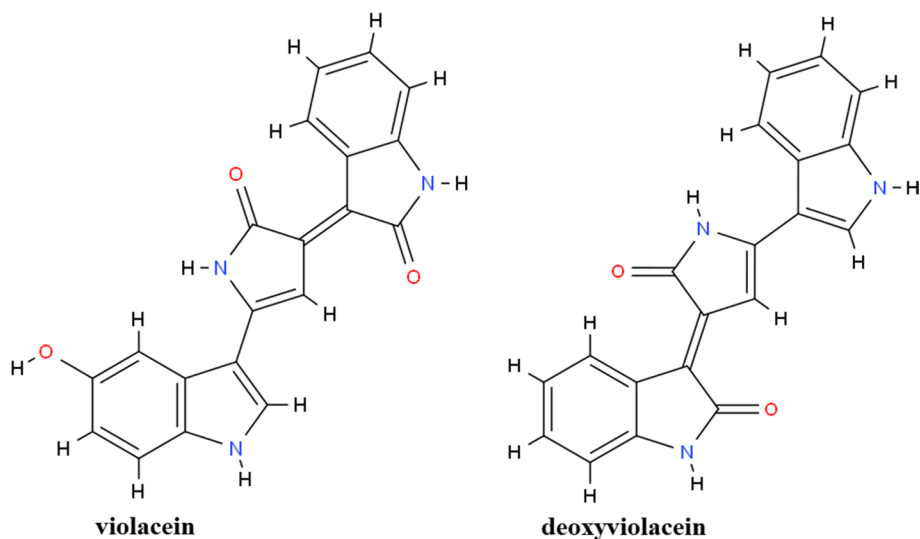
Introduction

Violacein (Viol) is a natural indole derivative, intensely purple in color. It is biosynthesized by various bacterial genera from the condensation of two tryptophan molecules in response to quorum sensing signals (Gómez-Gómez et al. 2019). This pigment was first isolated from *Chromobacterium violaceum*, and its chemical structure was determined in early studies (Reilly and Pyne 1927).

Besides its role as a quorum-sensing metabolite, it has been found to possess a broad range of biological activities, including anti-tumoral (Bromberg et al. 2010), bacteriostatic and antibiotic potential (Nakamura et al. 2002; Shankar et al. 2014), as well as antifungal activity (Sasidharan et al. 2015). Viol not only works itself as an antibiotic but has also been demonstrated to potentiate the effects of other antibiotics acting synergistically.

The related pigment deoxyviolacein (dViol), which is a by-product of the biosynthesis of Viol, has also been found to have similar, but slightly differing antibacterial and antifungal properties. Studies showed that it acts as a less efficient anti-microbial agent, but it is a stronger antifungal (Wang et al. 2009). Structurally, dViol differs from Viol in a single oxygen atom located in the indole ring (Fig. 1).

Fig. 1 Violacein and deoxyviolacein chemical structures



Compared to Viol, it is produced in considerably smaller amounts in wild-type strains. The relative difficulty in separating both pigments and producing them in sufficient quantities for laboratory analysis has remained a significant challenge.

Despite its medically interesting biological activities, their potential as drugs remains unexploited. To date, no human trials have been performed, although it has been shown to be harmless in animal models (Bromberg et al. 2010). Part of the hindrances in the development of these pigments as commercially available pharmaceuticals stems from the fact that the bacterial genus first reported to produce these pigments has also been found to act as an opportunistic pathogen (Batista and da Silva Neto 2017). Human infections with *C. violaceum*, while uncommon, show high mortality rates and life-threatening sepsis; the bacterium rapidly spreads to several organs, including lungs and liver (Durán and Menck 2001). This limits the real-life application of Viol and dViol obtained from such bacterium, because it could not apply for the FDA “Generally Regarded as Safe” approval.

An unexplored area of application for Viol is the functionalization of surfaces for the production of bioactive functionalized materials. Its relatively small size makes it a good candidate for this kind of application. One interesting example of this would be the production of metallic alloys functionalized with Viol to repel bacteria inhibiting the formation of biofilms on their surface. Biocorrosion, a bacteriogenic oxidation of metallic surfaces, is a major problem worldwide, and Viol holds promise as an interesting candidate for solving it.

With the increasing prominence of multidrug-resistant bacteria, a renewed effort has been put towards finding, isolating and characterizing novel bacterial strains capable of producing Viol. Various bacteria from diverse genera have

been found to produce Viol, including *Chromobacterium* (Hoshino 2011), *Collimonas* (Hakvåg et al. 2009), *Pseudalteromonas* (Timmermans et al. 2019), *Janthinobacterium* (Lu et al. 2009), *Iodobacter* (Doing et al. 2018) and *Duganella* (Wang et al. 2009).

This work focuses on the isolation and the development of a viable purification procedure for Viol from a non-pathogenic *Iodobacter* strain isolated recently from the Antarctic territory. We were able to successfully purify the compound, as determined by spectroscopic analysis. In contrast to pigments obtained from pathogenic bacteria, this finding could be relevant to the pharmaceutical industry for potential applications. It is also the first characterization of Viol production from an Antarctic *Iodobacter*.

Materials and methods

Bacterial strain culture

Iodobacter sp. 7MAnt was originally isolated from King George island near Eduardo Frei Montalva, Chilean Air Base (62°12'0"S58°57'51"W), Antarctic Peninsula.

This microorganism was grown in TSA media consisting of 15 g/L peptone, 5 g/L tryptone and 5 g/L NaCl supplemented with 0.03% tryptophan, adjusted to pH 7 and then inoculated at 10% with *Iodobacter* sp. 7MAnt. Inoculated media was incubated with agitation at 220 rpm for 16 h at 23 °C.

Identification of 7MAnt bacterial strain

Total DNA was isolated from *Iodobacter* sp. 7MAnt cultures, and the 16S rRNA gene was amplified using 515F, a specific primer for bacteria. We also used the universal

primer 1492R. Reaction mix consisted of 0.25 μL of Taq DNA polymerase (500 U/mL, Sigma), 5 μL of each deoxynucleotide (2 mM) (dATP, dCTP, dGTP and dTTP), 5 μL of buffer, 4 μL of MgCl_2 (0.75 mM) and 0.5 μL of each primer (100 mM). The following thermal conditions were applied: 95 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s. Each cycle was repeated thirty times. Final elongation step was done at 72 °C for 10 min. Amplification reactions were carried on a Palm Gradient Cycler (Corbett).

Sequencing of the amplified DNA was performed by the Georgia Genomics Facility (University of Georgia, USA), using an Illumina MiSeq platform. Sequences obtained were then assembled, analyzed, and edited using Chromas Pro software (Technelysium Pty Ltd.). A nucleotide BLAST against the NCBI database was performed to identify the specie. For further insight, a Phylogenetic tree was constructed using Phylogeny.fr online server (Dereeper et al. 2008). 16S rRNA gene sequences from other Viol producing bacteria were used as input for the multiple sequence alignment. *E. coli* was used as an outgroup. Sequences were obtained from NCBI database.

Screening of enzyme activities

Activity for several enzymes was studied using a semi-quantitative method for detection of enzymatic activities. Metabolic profiling using an analytical profile index kits (API) was performed for further identification of the bacterial strain. API 20E and API 20Ne systems (BioMerieux) were used in parallel and results were in agreement with each other.

Violacein purification

Eight grams of *Iodobacter* sp. 7MAnt were harvested after centrifugation of bacterial culture at $36,389\times g$ for 30 min in a Hitachi CP80WX preparative centrifuge. Cells were lysed by sonication using a VWR Branson 450 sonifier, and pigment was extracted using 99% ethanol. The lysate obtained was then centrifuged ($36,389\times g$ for 20 min) to remove cellular debris and the supernatant containing the pigment was collected. The pellet obtained from the centrifugation was resuspended in ethanol and the same centrifugation procedure previously described was repeated, until the pellet showed minimal color.

The total supernatant obtained was stored overnight at $-20\text{ }^\circ\text{C}$ and then centrifuged ($36,389\times g\times 20\text{ min}$) to further precipitate impurities. The liquid phase was recovered and loaded into a PF-30C18AQ-F0-220 column (Interchim, France) using a FPLC system for chromatographic separation. The mobile phase used during the loading and chromatographic separation was 70% ethanol. Each fraction obtained from the chromatography

was spectrophotometrically analyzed to find the fractions with the characteristic absorption wavelength for Viol. The UV–VIS spectrum for each fraction was obtained in the range between 700 nm and 300 nm using a Shimadzu spectrophotometer. All fractions with an absorbance peak between 574 and 577 nm were pooled and then solvent was removed in a rotatory evaporator (IKA RV10/HB10). Viol remaining in the aqueous phase was lyophilized (Liotop L101). The lyophilized fraction containing the partially purified Viol was dissolved in acetone and kept at $-20\text{ }^\circ\text{C}$ overnight and then it was centrifuged at ($36,389\times g\times 20\text{ min}$) to further precipitate the remaining impurities. Acetone was then removed using a second cycle of rotatory evaporation and lyophilization. Purity of Viol was determined by Mass Spectroscopy using a triple quadrupole mass spectrometer (Agilent 6400) equipped with an Agilent LC 1200 series.

Determination of the pigment concentration

Pigment concentration was determined with two independent methods, a colorimetric method measuring absorbance at 575 nm and using HPLC profile and the area of peaks obtained at a given retention time in comparison with the HPLC profile of the standard solution prepared with commercial Viol (Sigma V9389). Both methods gave similar results. For colorimetric determination, a calibration curve using commercial Viol was prepared.

Identification of the pigment

The chemical structure of the purified pigment was determined by ^1H NMR. Pigment was dissolved in deuterated dimethyl sulphoxide (DMSO) and deuterated ethanol and then analyzed (600 MHz, Varian Innova-600). For corroboration of the identity of the compound a Viol standard solution (Sigma V9389) was also used.

Antibacterial assay

Solid culture plates were grown using bacterial lawns of a pathogenic strain of *Pseudomonas aeruginosa*. For this test, TSA media supplemented with 1% agar was used. 34 μg , 54 μg and 72 μg of purified Viol were dropped before incubation of the bacterial strain for 20 h at 37 °C. The inhibition of bacterial growth was visually monitored.

Results

Characterization of the bacterial strain

A sample of meltwater stream sediments taken during Chilean Antarctic expedition ECA 46 from a small lagoon

located in King George Island, located at South Shetland, Antarctica. (coordinates of sampling site: 62°12'02.5"S and 58°58'18.9"W) was used to inoculate TSA media. Using serial dilution, a gram-negative, rod-shaped, motile, psychrotolerant bacterium was isolated from the inoculated enriched media (Fig. 2). This bacterium was found to grow at pH 7 in the temperature range between 8 °C and 25 °C.

Bacterial colonies formed in solid culture were observed to develop a strong purple color after 24 h of incubation (Fig. 3). In liquid media, the purple pigmentation reached its maximum after 16 h (Fig. 4). When culture media was supplemented with 0.03% tryptophan, the color became more intense, suggesting the indolic nature of the compound.

Total DNA was extracted from bacterial cultures and the 16S rRNA gene was amplified for sequencing. The sequence obtained (Fig. 5) was used to identify the species in a BLAST analysis, showing that the microorganism found and cultured is closely related to the *Iodobacter* genus. To better establish the identity of the isolate, a phylogenetic tree was constructed using 16S rRNA sequences from other Viol producing bacteria (Fig. 6). This once again placed the sequence in close proximity with other *Iodobacter* species and it is most closely related to *Iodobacter arcticus*. The isolate was, therefore, classified as *Iodobacter* sp 7MAnt.

A broad metabolic characterization of the bacteria was performed using API kits (Table 1). Results obtained indicate that this bacterium is a nitrate reducing

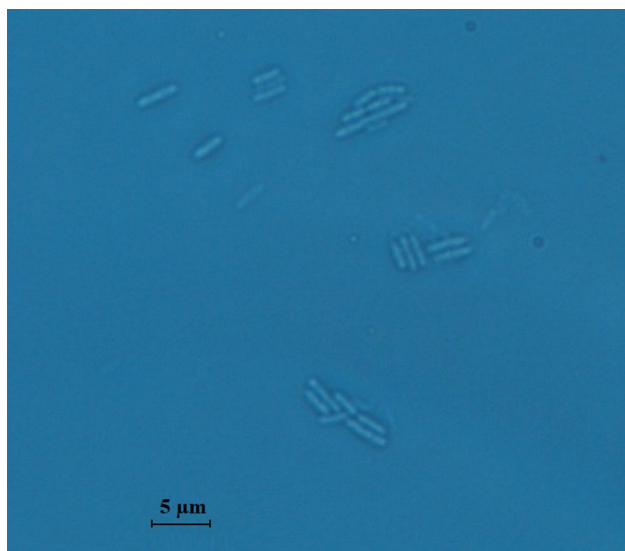


Fig. 2 Microscopy showing the isolated *Iodobacter* sp. 7MAnt bacterium. The isolate was found to be gram-negative, rod-shaped, motile, psychrotolerant bacterium isolated from the inoculated enriched TSA media

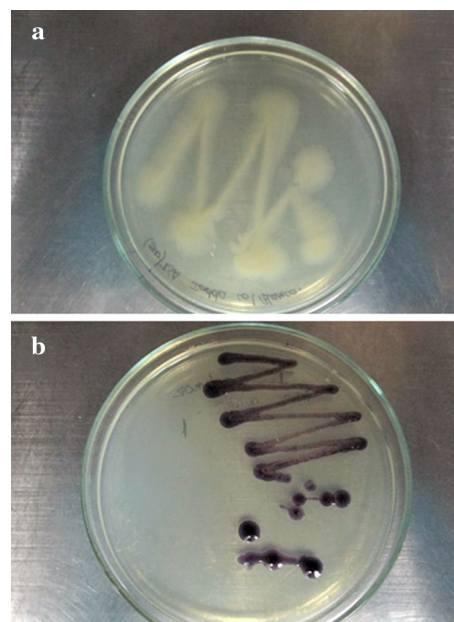


Fig. 3 Colonies of *Iodobacter* sp. 7MAnt in culture plates with solid TSA media. The upper image shows the initial growth stage, with no purple coloring. Bottom image shows growth after 24 h of incubation, with the apparition of the pigment

microorganism capable of assimilating various carbon sources.

Purification of the pigment

After incubating *Iodobacter* sp. 7MAnt for 16 h, the strongly violet pigment reached maximum on culture media and in cells. The cells were harvested by centrifugation and then lysed by sonication to purify the pigment using a combination of organic solvent extraction and liquid chromatography. Rotatory evaporation was used to remove the ethanol, followed by a lyophilization step. After this step, insoluble contaminants were still present upon resuspension, so an acetone wash was applied to the lyophilizate.

To remove acetone, three independent methods were assayed: room temperature evaporation, overnight incubation at 40 °C, and rotatory evaporation followed by a second lyophilization step. Based on HPLC/MS analysis, the best method used was rotary evaporation and lyophilization, as the purity obtained for the three methods was 87, 91 and 97%, respectively.

Following this procedure, 8 grams of bacteria yielded 1.089 mg of pigment, giving a yield of 0.01% of pure pigment. The scalability of this procedure needs to be developed.

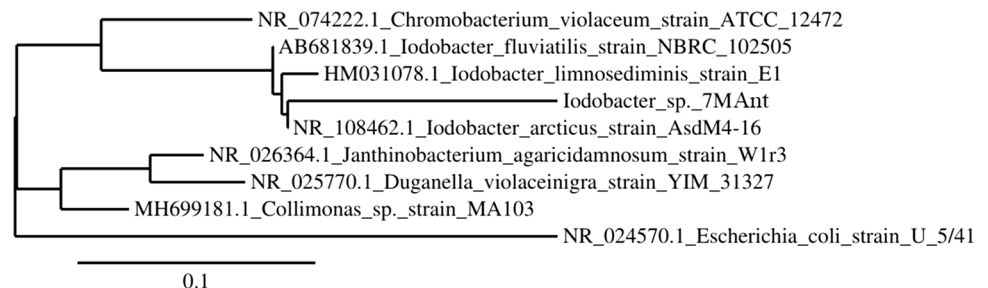
Fig. 4 Liquid culture of *Iodobacter* sp. 7MAnt. **a** Shows a bioreactor containing culture media recently inoculated with 7MAnt. **b** Liquid culture of 7MAnt after 16 h of incubation in the bioreactor



Fig. 5 16S rRNA sequence obtained for the bacterial isolate using Illumina sequencing technology

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>TACATGCACCAACCCCAAAGCATGCATTTCGNNCCCCACAGCGCTGCTTGCACCNATAACCAC
TAAATGTTTGGGTTGAAGGTAATATATCGGAACCTACCCTAGTAATGGGGGCATAACTTATCCG
AAAGGTTATCTAATACCGCATACGCCCTGAGGAGGAAAGAGGGGGATCGCAAGACCTCTCGTT
ATTAGAGCGCCGATATCAGATTAGCTAGTTGGTGAGGCAAAGGCTCACCAAGGCCACGATCTG
TAGCGGGTCTTAGAGGACGATCCGCCACTGGAAGTGGACACGGTCCAGACTCCTACGGCAG
GCAGCAGTGGGGAATCTTGNACAATGGGNGCAAGCCTGATCCAGCGATGCCGCGTGCCTGAAG
CAGGCTTCGGGTTGTAAAGCCTTTTGTTTCGAGAGCAAATCCTAGGGGCTAATANCTTTTGGGG
ATGACAGTACCGGAAGAATAAGGACNGGCTAACTACGTGCCAGCAGCCGCGTAATACGNAGG
GCCAAGCGTAAATCGGAATTACTGGGCGTAAAGGGTGCGCAGGTGGTTGATTCCCGTGTNATG
TGAAAGCCCCGGTCTCAACTTGGGAATTGCATTGCAAAGTGTCAACTAGAGTATGGCANAGGG
GGGTTGGAATTCCGCGTGTAGCANTGAAATGCGTAGAGATNCGGAGGAAACACCAATGGCGGA
AGGCAAACCCCTGGGCTAATACTGACNCTCATGCACAAAAAGCTTGGGGNGCCAAACGGGGA
TTAGATACCCCGGTAAGTCCACCCCTAAAACAATGTCTACTAGTTTTTGGGGGAATTCGTC
CCTTTAAAAAAACNAAAGCTAAANGNGGGGAAGGTAACCNGCCTGGGGGGGTACGGGCCCC
CCNAAGGGTAAAAAACTNN<
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Fig. 6 Phylogenetic analysis of *Iodobacter* sp. 7MAnt. *E. coli* strain U_5/14 was used as an outgroup. These results show that *Iodobacter* sp. 7MAnt is most closely related to *Iodobacter arcticus*



Identification of violacein

The pigment was identified on the basis of its absorption spectrum and its retention time on HPLC when

compared to a standard (Fig. 7). The characteristic absorption peak of Viol was obtained at 575 nm. A small peak was also evident at a slightly longer retention time in the chromatogram.

Table 1 Metabolic profiling of *Iodobacter* sp. 7MAnt using an API kit

TEST	Active components	Reaction	Results
NO3	Potassium nitrate	Nitrite reduction into nitrites.	+
		Nitrate reduction into N ₂	+
TRP	L-tryptophane	Indole formation	–
GLU	D-glucose	Fermentation	–
ADH	L-arginine	Arginine hydrolysis	–
URE	Urea	Urease reaction	–
ESC	Esculin ferric citrate	Hydrolysis	+
GEL	Gelatine	Proteolytic hydrolysis	+
PNPG	4-nitrophenyl-β-D-galactopyranoside	B-galactosidase reaction	–
GLU	D-glucose	Assimilation	+
ARA	L-arabinose	Assimilation	–
MNE	D-mannose	Assimilation	–
MAN	D-mannitol	Assimilation	–
NAG	N-acetyl-glucosamine	Assimilation	+
MAL	D-maltose	Assimilation	+
GNT	Potassium gluconate	Assimilation	+
CAP	Capric acid	Assimilation	–
ADI	Adipic acid	Assimilation	–
MLT	Malic acid	Assimilation	+
CIT	Trisodium citrate	Assimilation	+
PAC	Phenylacetic acid	Assimilation	–

The structure of the molecule was confirmed by analyzing the purified pigment by ¹H-NMR (Fig. 8). NMR spectra were obtained in DMSO and deuterated ethanol. Signals obtained were consistent with those obtained with a Viol standard solution. Chemical shifts obtained were also in agreement with the ones reported in a work by Aruldass et al. (2015). Although the resolution was low in the spectrum obtained due to the concentration of the sample, all the relevant peaks were visible and identifiable. The spectrum obtained using deuterated ethanol showed no peaks in the area between 10 and 12 ppm, in contrast the one obtained when DMSO was the solvent for the sample. A combined analysis of spectra under both experimental conditions mentioned above, allowed us to confirm the structure of the molecule.

Antibacterial activity assays

The bioactivity of the purified pigment towards biofilm-forming bacteria was tested in agar plates containing a culture lawn of the opportunistic pathogen *Pseudomonas aeruginosa*. Three different quantities of Viol were cast dropped on the agar plate and the effect was observed visually as an inhibition halo in the bacterial lawn (Fig. 9). 54 μg of the pigment was sufficient to inhibit bacterial growth. The

antibacterial effect appears to achieve saturation when the amount of Viol was 72 μg. The inhibition halo shows no apparent difference between the two highest amounts of pigment used during the test.

Discussion

Iodobacter sp. 7MAnt showed to be a producer of violet pigment when media was supplemented with tryptophan. The highest absorption peak of the pigment was obtained at 575 nm, which is consistent with what is reported for Viol in the literature (Jiang et al. 2012; Kothari et al. 2017). NMR spectrum of the purified pigment coincides with the spectrum obtained for a commercial standard Viol, confirming the identity of the pigment purified from *Iodobacter* sp. 7MAnt. NMR spectra were obtained using deuterated DMSO and ethanol as solvents for the spectroscopic measurements. The spectrum obtained using deuterated ethanol did not show the peaks that are characteristic of the indole ring. Thus, DMSO was preferred in our analysis for the identification of the molecule.

The yield of Viol biosynthesis is approximately 0.01%, obtaining 1.1 mg of Viol per 1 L of bacterial cells. Although with this yield *Iodobacter* sp. 7MAnt is not as proficient in producing the pigment as other bacteria, like *Duganella violaceinigr*a which is reported to produce 18.6 mg per culture liter, or *Chromobacter piscinae*, with approximately 15 mg per liter (Choi et al. 2015a), this is nevertheless an important discovery because it is the only report of an Antarctic *Iodobacter* producing Viol. Improvement on the procedure for induction of Viol produce by the microorganism is required.

To the best of our knowledge, the only studies on Viol production using Antarctic bacteria corresponds to a microorganism of the *Janthinobacterium* genera (Mojib et al. 2011; Smith et al. 2016). This is the first characterization of Viol production from an Antarctic *Iodobacter*. As opposed to *Janthinobacterium*, *Iodobacter* is described as a non-pathogenic genus, opening the way for potential biotechnological application of its pigment. The fact that this microorganism is also psychrotolerant, gives better opportunities to manipulate the microorganism in an industrial setting, for their cultivation at large scale avoids the additional cost of incubation at higher temperatures. The use of psychrophilic bacteria is also desirable because their adaptation to cold environments diminishes the possibility that they could act as human pathogens. For the eventual pharmaceutical production of violacein this is a key point.

One of the difficulties in the purification of Viol is the separation from the structurally related compound dViol. Retention time between both compounds is similar enough that most protocols reported end up with a mixture of both compounds. As dViol is a common byproduct

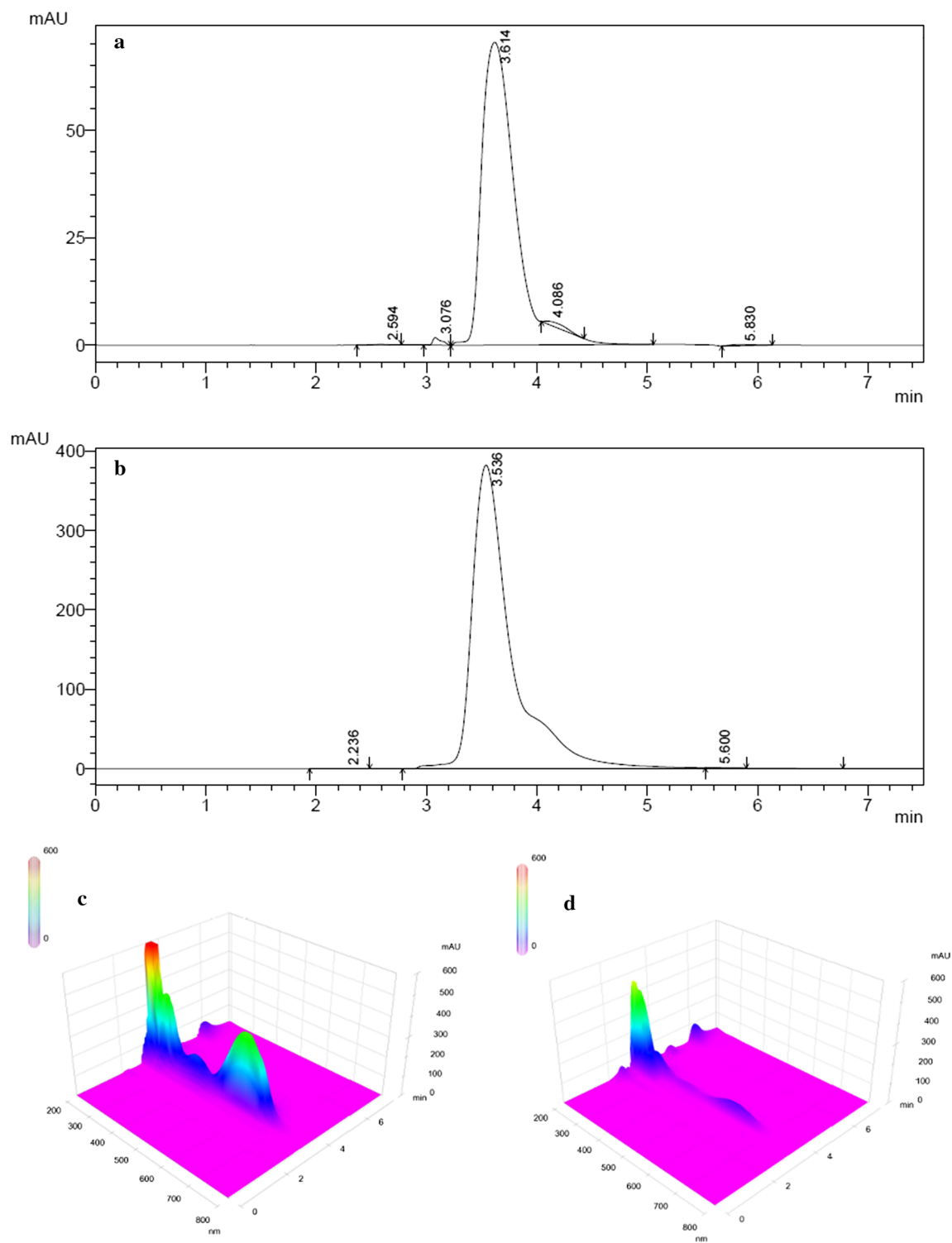


Fig. 7 HPLC Chromatograms and 3-D graphs. **a** Shows the chromatogram of the purified pigment, while **b** corresponds to the chromatogram of the violacein standard. **c** Shows a 3-D graph relating absorp-

tion spectra of the Viol standard solution in relation to its retention time. **d** Shows the absorption spectra and retention time of the purified violacein

in the biosynthesis of Viol (Rodrigues et al. 2012), it is likely that it would be found as an impurity in the isolated pigment. We observed a small, additional peak in the

chromatogram of our purified compound, which is speculated on the basis of its absorption peak at 560 nm, to be dViol. To completely confirm the identity of the compound

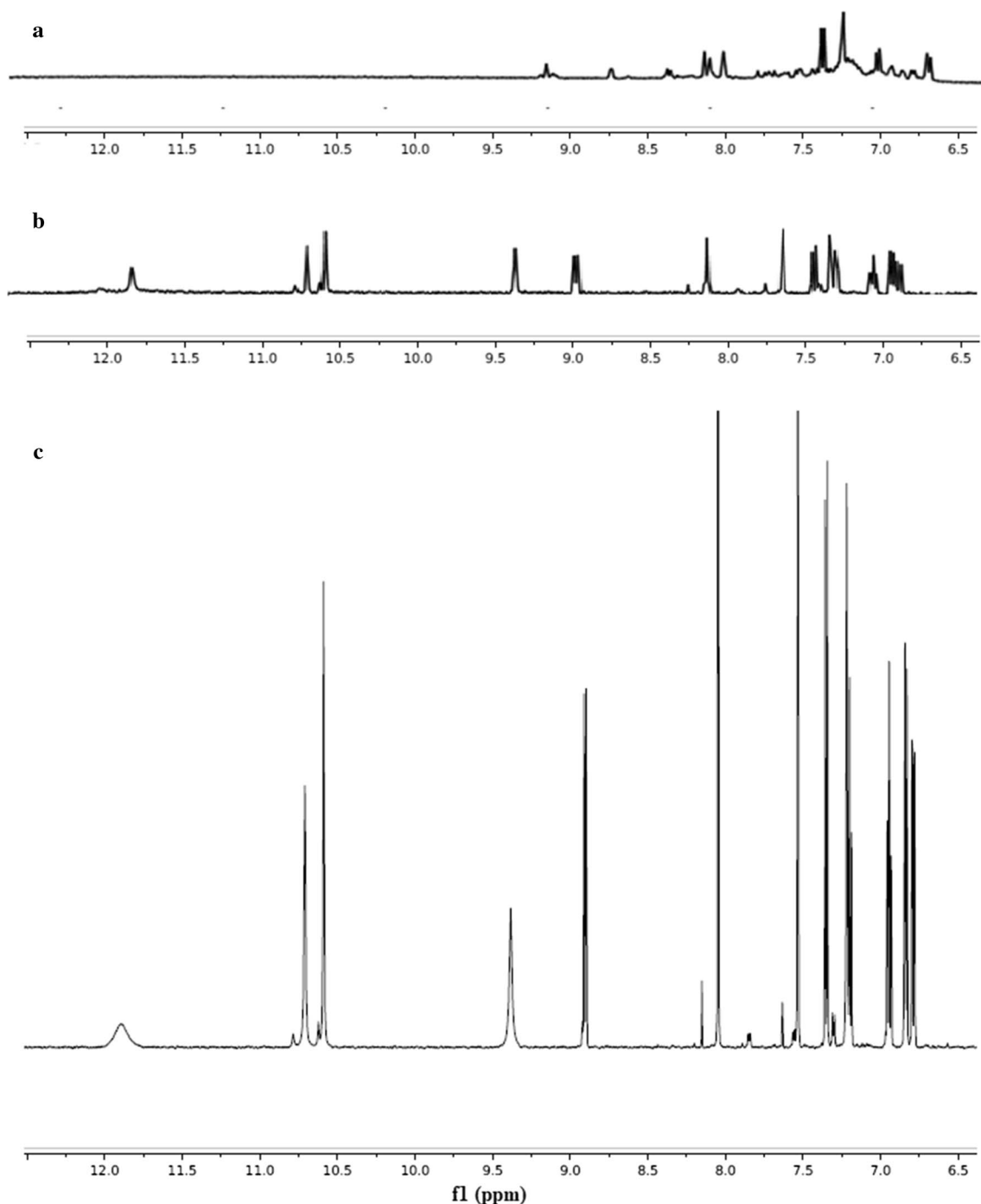


Fig. 8 ^1H -NMR spectra of the purified violacein obtained in deuterated ethanol (a), in DMSO (b) and ^1H -NMR spectra of a commercial violacein standard sample obtained in DMSO (c)

causing this additional peak, it would be necessary to purify it and analyze it using NMR spectroscopy.

Nonetheless, the method described here for the purification of Viol allowed us to obtain the pigment at remarkably high purity. Using mass spectroscopy, the purity of the pigment was estimated to be at 97%, which is higher

than the commercially available Viol Sigma V9389, which has 85% purity according to the distributor.

The pigment purified performed as an inhibitor of bacterial growth on solid media as demonstrated with the experiment using *Pseudomonas aeruginosa*. The presence of an antibiotic compound from an Antarctic microorganism hints

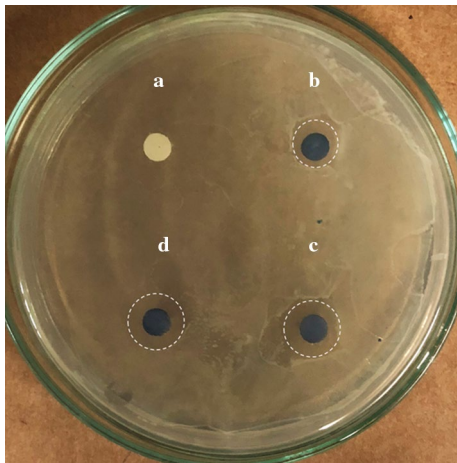


Fig. 9 Anti-bacterial activity assay. A bacterial lawn of *P. aeruginosa* was grown, and three different amounts of Viol were applied to investigate its antibacterial properties. **a** Is a control, with no Viol added. **b–d** Show the effect of adding 54 ug, 72 ug and 108 ug, respectively, to the plate. The dotted line circles were added to help visualizing the inhibition halos

at the necessity to eliminate any possible competitors in an environment with such harsh conditions for life as is the Antarctic soil. As violacein is a wide-spectrum abiotic, effective against bacteria and fungi, both of which can be found in Antarctic soil, it is an efficient resource for survival in a competitive and extreme environment.

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Compliance with ethical standards

Conflict of interest We have no competing interests to declare.

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