Production of vineomycin A1 and chaetoglobosin A by *Streptomyces* sp. PAL114

Adel Aouiche · Atika Meklat · Christian Bijani · Abdelghani Zitouni · Nasserdine Sabaou · Florence Mathieu

Abstract An actinobacteria strain PAL114, isolated from a Saharan soil in Algeria, produces bioactive compounds. Morphological and chemical studies indicated that this strain belongs to the genus *Streptomyces*. Analysis of the 16S rRNA gene sequence showed a similarity level of 99.8 % with *S. griseoflavus* LMG 19344^T, the most closely related species. Two bioactive compounds, named P44 and P40, were extracted by dichloromethane from the cell-free supernatant broth and were purified by HPLC. Minimum inhibitory concentrations (MIC) of the compounds were determined against pathogenic and toxigenic microorganisms, most of which are multiresistant to antibiotics. The P40 fraction showed a strong activity especially against *Candida albicans*, *Bacillus subtilis*, and *Staphylococcus aureus* and has lower MIC values than those of P44 against most microorganisms tested. Chemical structures of compounds were determined based on spectroscopic and spectrometric analyses (UV-visible, mass, ^{1}H, and ^{13}C NMR spectra). The compounds P44 and P40 were identified as vineomycin A1 and chaetoglobosin A, respectively. Vineomycin A1 is known to be produced by some *Streptomyces* species. However, chaetoglobosin A is known to be produced only by fungi belonging to the genera *Chaetomium*, *Penicillium,* and *Calonectria*. This is the first time that chaetoglobosin A, known for its antimicrobial, anticancer, and cytotoxic effects, is reported in prokaryotes.

Keywords *Streptomyces* · Vineomycin A1 · Chaetoglobosin A · Chemical structure · Bioactive compounds

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Introduction

Actinobacteria are Gram-positive bacteria with a percentage of guanine-cytosine higher than 55 %, and most of them produce mycelia. They are particularly interesting for their high capacity to produce secondary metabolites with diverse chemical structures (Watve et al. 2001). Among these compounds, there are, for example, antivirals, antiparasitics, immunostimulants, and immunosuppressants. However, actinobacteria are especially known for the production of antibiotics (Takahashi and Omura 2003; Solanki et al. 2008). Indeed, over 45 % of bioactive molecules of microbial origin are produced by actinobacteria (Solecka et al. 2012). Among these molecules, about 80 % are produced by species of the genus *Streptomyces*, the most common genus in the environment (Demain 2006; Jose et al. 2011). The antibiotics secreted by this genus may have antibacterial or antifungal activities, or cytostatic and antitumor properties, such as adriamycin and anthramycin (Butler 2004). Many of these molecules have found an important therapeutic application (Jose and Jebakumar 2013).

Our previous works showed the richness of Algeria Saharan soil with actinobacteria producers of bioactive compounds (Sabaou et al. 1998). Therefore, many strains proved to be new species producing new bioactive molecules (Lamari et al. 2002a, b; Zitouni et al. 2004a, b, 2005; Badji et al. 2006, 2007; Merrouche et al. 2010; Boubetra et al. 2012).

A previous work performed on a Saharan actinobacteria strain named PAL114 revealed the production of two bioactive molecules identified as saquayamycins A and C, known as anticancer agents (Aouiche et al. 2014). In the present article, which is the continuation of a previously published work, the taxonomy of actinomycete strain and the production, purification, structure elucidation, and bioactivity of two other bioactive molecules produced by the same strain are described.

Materials and methods

Identification of actinobacteria strain PAL114

The actinobacteria strain PAL114 was isolated from a Saharan soil collected from Ghardaïa province, south Algeria (Aouiche et al. 2014).

The morphological and cultural characteristics were observed by naked-eye examination of 14 day-old cultures grown on various ISP (International *Streptomyces* Project) media: yeast extract–malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts–starch agar (ISP4), and glycerol–asparagine agar (ISP5) (Shirling and Gottlieb 1966), and also on Bennett medium (Waksman 1961). The micromorphology and sporulation were observed by light microscopy.

For chemotaxonomic analyses, biomass was obtained from a culture grown in shaken ISP2 medium (Shirling and Gottlieb 1966) and incubated at 30 °C for 4 days. Analysis of diaminopimelic acid isomers and whole-cell sugar pattern were carried out using the method of Becker et al. (1964) and Lechevalier and Lechevalier (1970). Phospholipids were analysed according to the procedures developed by Minnikin et al. (1977).

For the physiological study, 18 tests were used (Locci 1989), including the production of melanoid pigments on ISP6 and ISP7 media, the assimilation of nine carbohydrates as sole carbon source, the degradation of xanthine, the production of nitrate reductase, the sensitivities to sodium chloride (7 % w/v), sodium azide (0.01 % w/v), phenol (0.1 % w/v), and penicillin (10 UI), and the growth at 45 °C.

For molecular analysis, DNA was extracted using the procedure recommended by Liu et al. (2000). PCR amplification of the 16S rRNA gene sequence of strain PAL114 was performed using two primers: 27f (5′-AGAGTTTGATCCTGGC TCAG-3′) and 1492r (5′- GGTTACCTTGTTACGACTT-3′). The 16S rRNA gene sequence was amplified by PCR using an Invitrogen Kit and sequenced. The sequences obtained were compared with sequences present in the public sequence databases and with the EzTaxon-e server (Kim et al. 2012).

Phylogenetic analyses were conducted using MEGA version 5.0 (Tamura et al. 2011). The 16S rRNA gene sequence of strain PAL114 was aligned using the CLUSTAL W program (Thompson et al. 1994) against corresponding nucleotide sequences of representatives of the *Streptomyces* genus retrieved from GenBank. Evolutionary distance matrices were generated as described by Jukes and Cantor (1969) and a phylogenetic tree was established using the neighbor-joining method of Saitou and Nei (1987). The topology of the tree was evaluated by bootstrap analysis (Felsenstein 1985) using 1,000 resamplings.

Production and purification of antimicrobial compounds

Production of bioactive compounds were conducted in ISP2 broth (malt extract 10 g, yeast extract 4 g, glucose 4 g, distilled water 1,000 mL, pH 7.2). A seed culture was prepared with the same medium and used to inoculate 80 Erlenmeyer flasks of 500 mL, each containing 100 mL of medium. The cultures were incubated on a rotary shaker (250 rpm) at 30 °C. The extraction of active compounds was performed on the fifth day, previously determined as being the day of optimum production. The ISP2 culture broth was centrifuged to eliminate cells. The cell-free supernatant was extracted with an equal volume of dichloromethane. The organic extract was concentrated to dryness by rotary evaporator under a vacuum at a temperature lower than 40 °C. The resulting dry extract was recuperated in 10 mL of methanol and bioassayed against *Candida albicans* M3, *Aspergillus carbonarius* M333, and *Bacillus subtilis* ATCC 6633 by paper disc diffusion method.

Preparative chromatography with silica gel plates (Merck Art. 5735, Kiesselgel 60HF 254–366; 20×20 cm) was employed for the partial purification of bioactive product. TLC plates were developed in the solvent system ethyl acetate–methanol (100:15 v/v). The developed plates were air dried overnight to remove all traces of solvents. The separated compounds were visualized under UV at 254 nm (absorbance) and 365 nm (fluorescence), and the active spot was detected by bioautography (Betina 1973). The retention factor (Rf) of the active spot was measured.

The final purification of bioactive compound was carried out by Waters reverse phase HPLC using an XBridge C18 (5 μm) column (200×10 mm, Waters) with a continuous linear gradient solvent system from 20 to 100 % methanol in water, a flow rate of 2 mL/min, and UV detection at 220 and 254 nm. All peak fractions were collected and tested against *Candida albicans* M3, *Aspergillus carbonarius* M333, and *Bacillus subtilis* ATCC 6633. The active fractions were re-injected into the HPLC system under the same conditions as previously, until their final purification.

Identification of bioactive compounds

These analyses were made with the pure bioactive compounds. The UV spectra were determined with a

Table 1 Resistance pattern of target fungi and bacteria to antibiotics

Target microorganisms	Resistant to:	Sensitivity to:
Yeasts and filamentous fungi		
Saccharomyces cerevisiae ATCC 4226	N, I, Th, T	C, A
Candida albicans M1, M3, IPA200 and IPA988	N, I, Th, T, C	A
Penicillium glabrum PG1	C	N, I, Th, T, A
Aspergillus carbonarius M333	C, N	I, Th, T, A
A. flavus AF3	C	N, I, Th, T, A
A. ochraceus AO1	C, N, T	I, Th, A
Fusarium culmorum FC200	C, N, T, I, A	Th
Bacteria		
Bacillus subtilis ATCC 6633	NEO	CH, CAR, CHL, ERY, K, SPI, SSS, VAN
Staphylococcus aureus S1	CAR, K, NEO, OLE, SPI, VAN	CH
Escherichia coli E195	AMC, AMX, CAZ, CF, CTX, CXM, FOX, TCC, TIC	CH, CXC

A, amphotericin B; C, cycloheximide; I, itraconazole; N, nystatin; T, thioconazole; Th, terbinafine; AMX, amoxicillin; AMC, amoxicillin + clavulanic acid; CH, chloramphenicol; CAR, carbenicillin; CAZ, ceftazidime; CF, cefalotin; CTX, cefotaxime; CXC, cefotaxime + clavulanic acid; CXM, cefuroxime; ERY, erythromycin; FOX, cefoxitin; K, kanamycin; NEO, neomycin; OLE, oleandomycin; SPI, spiramycin; SSS, sulfamide; TCC, ticarcillin + clavulanic acid; TIC, ticarcillin; VAN, vancomycin

Shimadzu UV1605 spectrophotometer. The mass spectra were recorded on an LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) with nanospray ion electro-spray ionization (ESI) source (positive and negative ion modes). ^{1}H and ^{13}C NMR spectroscopy were used for the characterization of two bioactive molecules. NMR sample was prepared by dissolving 3 mg of each compound in 600 μL of CD_3OD. All spectra were recorded on a Bruker Avance 500 spectrometer equipped with a cryoprobe. All chemical shifts

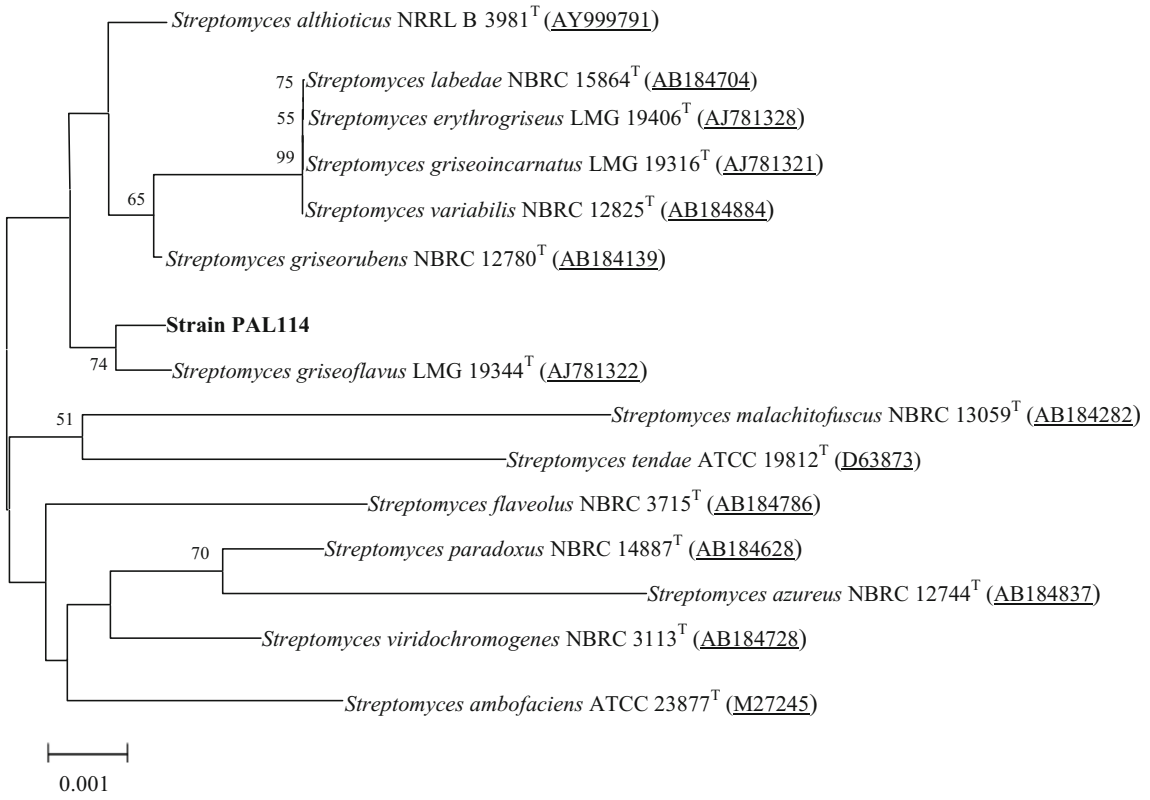

Fig. 1 Neighbor joining tree based on 16S rRNA gene sequences showing the relation between strain PAL114 and type species of the genus *Streptomyces*. The *numbers* at the nodes indicate the levels of bootstrap support (≥50 %) based on neighbour joining analyses of 1,000 resampled data sets. Bar, 0.001 nt substitution per nt position

Table 2 ^{1}H and ^{13}C NMR data assignments of P44 compound in CD_3OD at 298K. See Fig. 2b for numbering of hydrogen and carbon atoms

^{1}H and ^{13}C number	^{1}H chemical shift, ppm	^{13}C chemical shift, ppm
1	1.33	14.04
2	4.61	70.21
3		197.28
4	7.04	144.19
5	6.08	126.13
6	5.34	95.13
7	1.25	16.10
8	4.24	66.33
9	3.71	76.40
10	1.51 1.69	24.40
11	1.97	23.93
12	5.25	92.10
13	1.39	24.90
14		81.95
15	1.99 2.32	42.61
16		79.80
17		79.50
18		205.28
19	2.73 2.93	50.18
20	6.90	116.65
21	6.41	145.40
22		139.03
23		138.50
24		182.30
25		137.68
26		130.97
27		182.30
28		157.40
29		114.00
30	7.89	132.84
31	7.62	118.63
32	1.38	17.39
33	3.56	75.18
34	3.15	86.45
35	3.82	71.13
36	1.41 2.49	39.28
37	4.92	70.98
38	1.23	15.72
39	4.37	67.34
40	3.76	76.28
41	1.99 2.11	23.93
42	1.51 1.69	24.40
43	5.01	98.92
44	1.33	14.04
45	4.61	70.21
46		197.34
47	7.02	144.09
48	6.09	126.18
49	5.35	95.13

for ^{1}H and ^{13}C are relative to TMS using ^{1}H (residual) or ^{13}C chemical shifts of the solvent as a secondary standard. The temperature was set at 298 K. All the ^{1}H and ^{13}C signals were assigned on the basis of chemical shifts, spin-spin coupling constants, splitting patterns and signal intensities, and by using ^{1}H-^{1}H COSY45, ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC experiments. Gradient-enhanced ^{1}H COSY45 was realised including 36 scans for per increment. The mixing time for TOCSY was 60 ms and 96 scans per increment were accumulated. ^{1}H-^{13}C correlation spectra using a gradient-enhanced HSQC sequence (delay was optimised for $^{1}J_{CH}$ of 145 Hz) was obtained with 200 scans per increment. Gradient-enhanced HMBC experiment was performed allowing 62.5 ms for long-range coupling evolution (340 scans were accumulated). Typically, 2,048 t2 data points were collected for 256 t1 increments.

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of pure bioactive molecules were carried out using conventional agar dilution (Oki et al. 1990). Thirteen target microorganisms, the majority of which are pathogenic or toxigenic to humans and multiresistant to antibiotics (Table 1), were used. Three bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* S1, and *Escherichia coli* E195), five filamentous fungi (*Aspergillus carbonarius* M333, *A. flavus* AF3, *A. ochraceus* AO1, *Fusarium culmorum* FC200, and *Penicillium glabrum* PG1), and five yeasts (*Candida albicans* M1, M3, IPA200, and IPA988, and *Saccharomyces cerevisiae* ATCC 4226) were inoculated onto Mueller Hinton medium for bacteria and Sabouraud medium for fungi, containing different concentrations of active compounds (1, 2, 5, 10, 20, 30, 50, 75, and 100 μg/mL). After a growth period of 24–48 h at 37 °C for bacteria and 48–72 h at 28 °C for fungi, the plates were examined for growth and the lowest bioactive compound concentration that inhibited the growth of each organism was determined. Mueller Hinton and Sabouraud media, without active compound and inoculated with target organisms, were used as control treatments.

Results and discussion

Identification of strain PAL114

The strain PAL114 showed good growth on ISP2, ISP3, ISP4, ISP5, and Bennett media. The aerial and substrate mycelia were light to medium grey and light brown, respectively. A diffusible pigment with light brown color was produced on ISP2, ISP3, and Bennett media. The strain PAL114 formed a very well-developed aerial mycelium with long spiraled chains containing between 10 and 50 spores per chain. The spores were carried by sporophores and were oval and 1–1.5× 0.6–1 μm in size. The substrate mycelium was nonfragmented.

The chemotaxonomic study of strain PAL114 showed the presence of LL-diaminopimelic acid isomer and glycin in the cell wall. The whole-cell hydrolysates contained non-characteristic sugars (ribose, glucose, and galactose), typical of cell wall type IC (Lechevalier and Lechevalier 1970). The phospholipid profile contained phosphatidylethanolamine, corresponding to phospholipid type PII (Lechevalier et al. 1977). Based on the morphological and chemical characteristics, strain PAL114 was identified to the genus *Streptomyces* (Holt et al. 1994).

The strain PAL114 used arabinose, fructose, inositol, mannitol, melibiose, rhamnose, and xylose as carbon source, but not sucrose and raffinose. Xanthine was degraded and nitrate reduced. The strain grew at 45 °C in the presence of penicillin (10 UI), phenol (0.1 % *w/v*), sodium azide (0.01 % *w/v*), and NaCl (7 % *w/v*). The melanoid pigments were not produced on ISP6 and ISP7 media.

The alignment of the 16S rRNA gene sequence (1471 nucleotides) of strain PAL114 with those of *Streptomyces* reference species available in the GenBank database, can be seen in the neighbor-joining dendrogram (Fig. 1). The similarity level was 99.8 % with *S. griseoflavus* LMG 19344^T

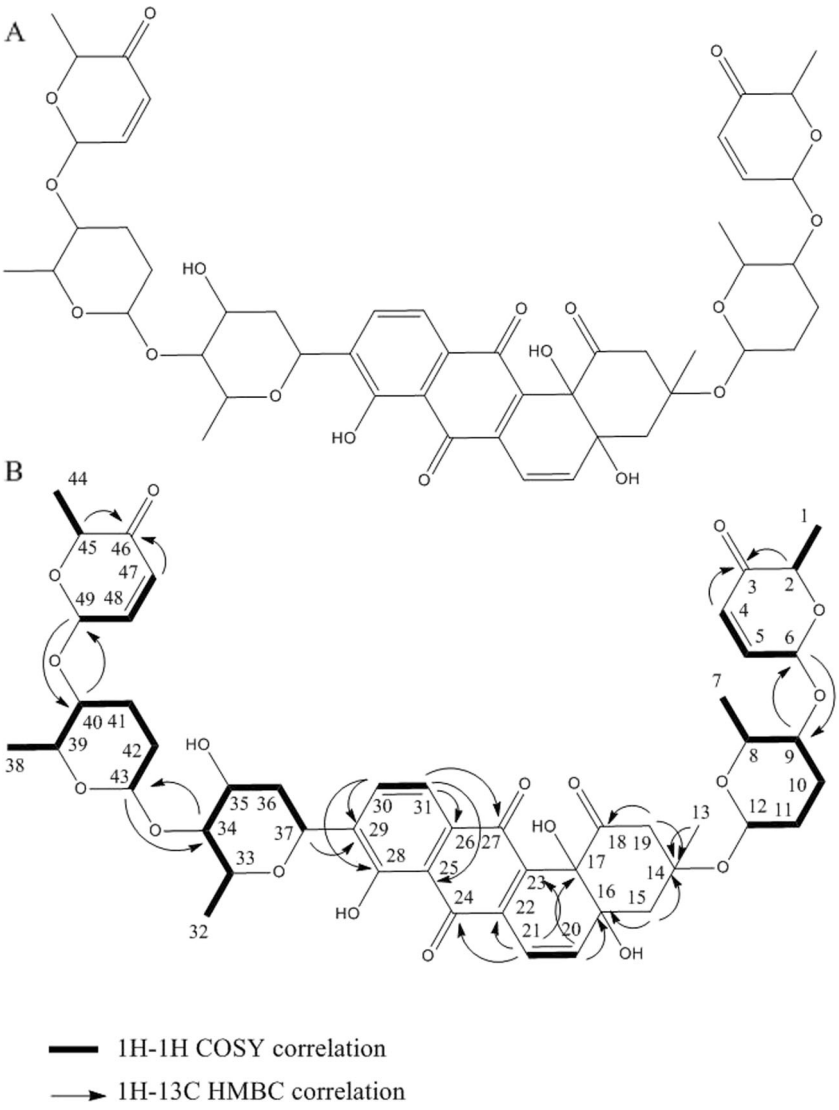

Fig. 2 Structure of bioactive compound P44 (**a**) and HMBC and COSY correlations (**b**)

(Holt et al. 1994), the most closely related species. The morphological and physiological properties of strain PAL114 are similar to those of the type strain of *S. griseoflavus* except for the tests of nitrate reduction and growth at 45 °C.

Purification of bioactive compounds

The dichloromethane extract was chromatographed by TLC and developed in an ethyl acetate–methanol system (100–15 *v/v*). After migration, we observed one active bioautographic compound, which showed a strong activity against *Candida albicans* M3 and *Bacillus subtilis* ATCC 6633. This compound (Rf=0.9), was selected and analyzed by HPLC. Two active fractions (P44 and P40) were purified with 80 % methanol in water. The fraction P40 was eluted at a retention time of 39.68 min and P44 at 44.08 min. A quantity of 5 mg was obtained for each molecule from 8 L of culture filtrate.

Identification of bioactive compounds

The UV-VIS spectrum of the bioactive compound P44 showed maxima at 218, 318, and 438 nm.

The mass spectrum of the compound was obtained in negative mode. It yielded a pseudo-molecular ion [M - H] = 933. Thus, the molecular weight of antimicrobial compound is *M*=934.

The ^{1}H and ^{13}C chemical shifts of P44 compound are given in Table 2 and structure in Fig. 2. The HSQC and HMBC spectra show 49 carbon signals for P44 molecule. It was possible to discern five ketone groups (δ_c 182.30 to 205.28), four hydroxyl groups (δ_c 71.13 to 157.40), nine ether functions (δ_c 67.34 to 95.13), 13 sp^2–hybridized carbons (δ_c from 114.00 to 144.09), and 13 sp^3-hybridized carbons (δ_c 14.04 to 42.61) for the P44 molecule. The hydrogens of the hydroxyl group are not observed due to rapid exchange with MeOD. The 2D ^{1}H-^{1}H and ^{1}H-^{13}C experiments, and especially the long range ^{1}H-^{13}C couplings observed in the HMBC spectrum (see Fig. 2b), permitted us to established the connectivity between all the groups of the molecule.

The structure of the P44 molecule was determined by NMR and mass spectrometry to be vineomycin A1. It is an antibiotic belonging to the aquayamycin group, class of angucycline, family of anthracycline (Maskey et al. 2003). It is known to be active against Gram-positive bacteria and sarcoma-180 solid tumors in mice (Imamura et al. 1982; Omura 2011). Also, vineomycin A1 is a suitable treatment for hypertrophic scar tissue and keloid disease. It is known to be a potent inhibitor of prolyl 4-hydroxylase, an essential enzyme involved in the post translational modification of collagen causing several diseases such as fibrosis, arteriosclerosis, and scleroderma (Cunliffe and Franklin 1986; Chen 2007). Furthermore, cytostatic activity in vivo has been reported for vineomycins (Rohr and Thiericke 1992). Vineomycins are known to be produced by actinobacteria, especially *Streptomyces* species such as *S. matensis* and *S. albogriseolus* (Ono et al. 1974; Chen 2007; Omura 2011), but have never been reported in *S. griseoflavus*, to which our strain was closely related.

For the P40 compound, the UV-VIS spectrum showed maxima at 220 and 276 nm.

The mass spectrum of the compound was obtained in negative mode. It yielded a pseudo-molecular ion [M - H] = 527. Thus, the molecular weight of antimicrobial compound is *M*=528. The ^{1}H and ^{13}C chemical shifts of the P40 compound are given in Table 3 and structure in Fig. 3. The HSQC and HMBC spectra show 32 carbon signals for P40. From these data, it was possible to discern two ketone groups

Table 3 ^{1}H and ^{13}C NMR data assignments of the P40 compound in CD_3OD at 298 K. See Fig. 3b for numbering of hydrogen and carbon atoms

^{1}H and ^{13}C number	^{1}H chemical shift, ppm	^{13}C chemical shift, ppm
1	6.95	124.2
2		108.0
3		127.5
4		136.6
5	7.26	111.3
6	7.49	118.1
7	7.00	118.4
8	7.03	120.7
9	2.84	32.1
10	3.91	57.7
11	2.89	46.4
12		63.1
13		174.3
14	1.73	36.3
15		58.0
16	2.78	62.1
17	2.05	48.9
18	1.08	12.1
19	1.30	18.4
20	6.04	128.3
21	5.13	132.7
22	2.00 2.29	41.3
23	2.47	31.8
24	5.53	139.2
25		132.3
26	5.00	81.5
27		200.5
28	6.13	131.7
29	7.27	134.4
30		197.3
31	1.00	19.8
32	1 33	9.5

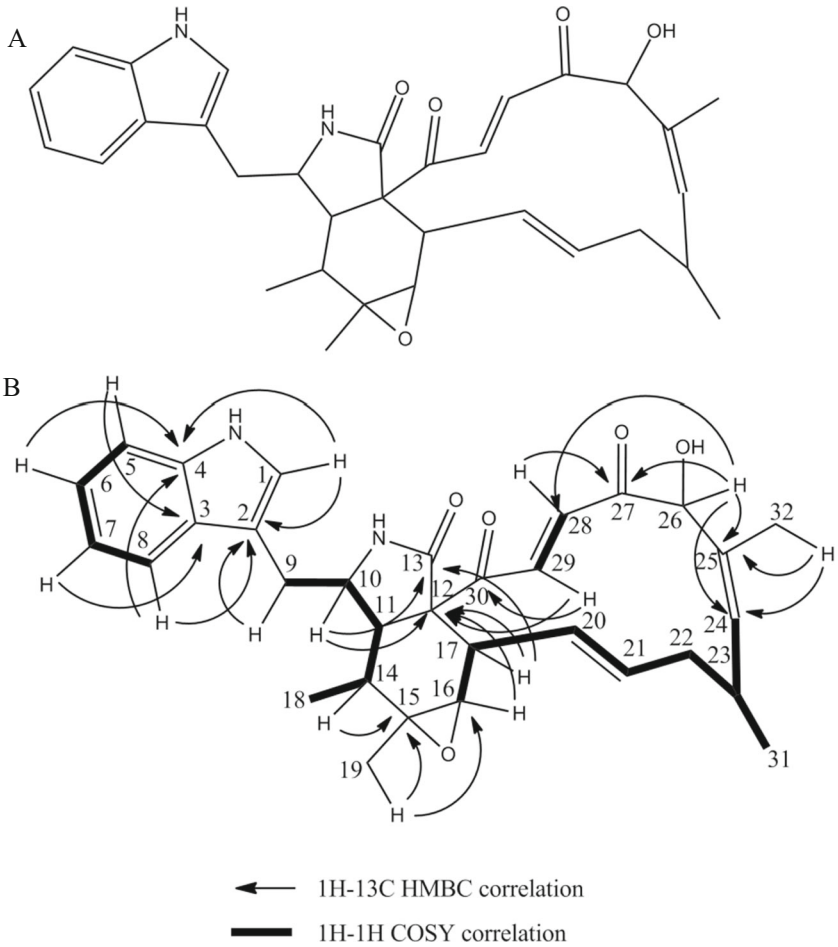

Fig. 3 Structure of bioactive compound P40 (**a**) and HMBC and COSY correlations (**b**)

(δ_c 197.3 and 200.5), one amide group (δ_c 174.3), one hydroxyl group (δ_c 81.5), one epoxy group (δ_c 58.0 and 62.1), 14 sp^2–hybridized carbons (δ_c from 108.0 to 136.6), and 14 sp^3-hybridized carbons (δ_c 12.1 to 81.5). The hydrogens of the hydroxyl group are not observed due to rapid exchange with MeOD. The 2D ^{1}H-^{1}H and ^{1}H-^{13}C experiments, and especially the long range ^{1}H-^{13}C couplings observed in the HMBC spectrum (see Fig. 3b), permitted us to establish the connectivity between all the groups of the P40 molecule.

The structure of the P40 compound was determined by NMR and mass spectrometry to be chaetoglobosin A. This compound is known for its antibacterial, antifungal, phytotoxic (June et al. 1998; Larsen et al. 2005; Zhang et al. 2013), anticancer, and cytotoxic effects towards mammal cells (Fisvad et al. 2004; Fogle et al. 2008), and nematicidal effects (Hu et al. 2012). It has also been reported that this compound increases fibrinolytic activity in bovine animals (Shinohara et al. 2000). This compound is a cytochalasin derivative (Ohtsubo et al. 1978; June et al. 1998). Furthermore, it is considered a mycotoxin, and it is known to be produced only by fungi such as *Chaetomium globosum* (Hu et al. 2012), *Penicillium discolor*, *P. expansum*, *P. marinum* (Fisvad et al.

Table 4 Minimum inhibitory concentrations (MIC) of the bioactive compounds P44 and P40 secreted by the strain PAL114 against several fungi and bacteria

Target microorganism	MIC (μg/mL) [a]	
	P40	P44
Saccharomyces cerevisiae ATCC 4226	50	50
Candida albicans M1	50	75
C. albicans M3	30	75
C. albicans IPA200	50	75
C. albicans IPA988	50	75
Aspergillus carbonarius M333	50	100
A. flavus AF3	75	100
A. ochraceus AO1	50	75
Fusarium culmorum FC200	75	100
Penicillium glabrum PG1	75	75
Bacillus subtilis ATCC 6633	20	50
Staphylococcus aureus S1	30	>100
Escherichia coli E195	>100	>100

[a] MIC values represent the average of two replicates

2004), and *Calonectria morganii* (Von Wallbrunn et al. 2001). It has never been described in prokaryotes. This is the first time that this compound is reported in the genus *Streptomyces*, belonging to the class of *Actinobacteria*. All experiments were made three times at different cultivation times to confirm fully the production of chaetoglobosin A by strain PAL114.

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) of bioactive molecules purified by HPLC are summarized in Table 4.

For vineomycin A1 (P44), MIC values were between 50 and 75 μg/mL for yeasts, and 75 and 100 μg/mL for filamentous fungi. For bacteria, *Bacillus subtilis* ATCC 6633 was the most sensitive (50 μg/mL). All other bacteria tested were resistant.

For chaetoglobosin A (P40), MIC values were between 30 and 75 μg/mL for yeasts, 50 and 75 μg/mL for filamentous fungi, and 20 and 30 μg/mL for Gram-positive bacteria. All Gram-negative bacteria tested were resistant. The most sensitive microorganisms were *Bacillus subtilis* ATCC 6633 (20 μg/mL), *Staphylococcus aureus* S1 (30 μg/mL), and *Candida albicans* M3 (30 μg/mL).

In conclusion, the strain PAL114, closely related to *S. griseoflavus*, showed an antibacterial and antifungal activity against pathogenic and toxigenic microorganisms, most of which are resistant to several antibiotics. The bioactive compounds produced by the strain proved to be the vineomycin A1 and chaetoglobosin A.

Vineomycin A1 belongs to the same group, class, and family as saquayamycins (Aouiche et al. 2014); it differs from these compounds only by the presence of a saccharide derivative bonded to the carbon 40. Chaetoglobosin A is known to be produced only by a small number of fungal species. This is the first time that chaetoglobosin A, which, in addition to its antimicrobial activity, is also considered a mycotoxin in the scientific literature, is produced by prokaryotes such as *Actinobacteria*.

Moreover, strain PAL114 represents a sample which confirms the potential of actinobacteria to produce a large variety of bioactive molecules.

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