A novel hydroxamic acid-containing antibiotic produced by a Saharan soil-living *Streptomyces* strain

A. Yekkour[1,2], A. Meklat[1], C. Bijani[3], O. Toumatia[1], R. Errakhi[4], A. Lebrihi[4], F. Mathieu[5], A. Zitouni[1] and N. Sabaou[1]

1 Laboratoire de Biologie des Systèmes Microbiens (LBSM), Ecole Normale Supérieure de Kouba, Alger, Algeria
2 Centre de Recherche Polyvalent, Institut National de la Recherche Agronomique d'Algérie, Alger, Algeria
3 Laboratoire de Chimie de Coordination (LCC), CNRS, Université de Toulouse, UPS, INPT, Toulouse, France
4 Université Moulay Ismail, Meknès, Morocco
5 Université de Toulouse, Laboratoire de Génie Chimique UMR 5503 (CNRS/INPT/UPS), INP de Toulouse/ENSAT, Castanet-Tolosan Cedex, France

Significance and Impact of the Study: This study presents the isolation of a *Streptomyces* strain, named WAB9, from a Saharan soil in Algeria. This strain was found to produce a new hydroxamic acid-containing molecule with interesting antimicrobial activities towards various multidrug-resistant micro-organisms. Although hydroxamic acid-containing molecules are known to exhibit low toxicities in general, only real evaluations of the toxicity levels could decide on the applications for which this new molecule is potentially most appropriate. Thus, this article provides a new framework of research.

Keywords
antimicrobial activity, hydroxamic acid, *Streptomyces*, structure elucidation, taxonomy.

Correspondence
Nasserdine Sabaou, Laboratoire de Biologie des Systèmes Microbiens, Ecole Normale Supérieure de Kouba, Alger 16050, Algeria.
E-mail: sabaou@yahoo.fr

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Abstract

During screening for potentially antimicrobial actinobacteria, a highly antagonistic strain, designated WAB9, was isolated from a Saharan soil of Algeria. A polyphasic approach characterized the strain taxonomically as a member of the genus *Streptomyces*. The strain WAB9 exhibited a broad spectrum of antimicrobial activity toward various multidrug-resistant micro-organisms. A PCR-based assay of genomic potential for producing bioactive metabolites revealed the presence of PKS-II gene. After 6 days of strain fermentation, one bioactive compound was extracted from the remaining aqueous phase and then purified by HPLC. The chemical structure of the compound was determined by spectroscopic (UV–visible, and ^{1}H and ^{13}C NMR) and spectrometric analysis. The compound was identified to be 2-amino-*N*-(2-amino-3-phenylpropanoyl)-*N*-hydroxy-3-phenylpropanamide, a novel hydroxamic acid-containing molecule. The pure molecule showed appreciable minimum inhibitory concentration values against a selection of drug-resistant bacteria, filamentous fungi and yeasts.

Introduction

Actinobacteria are a group of ubiquitous Gram-positive bacteria with a percentage of guanine–cytosine higher than 55%, and most of them produce mycelia. Because of their ability to provide a broad range of bioactive compounds, these micro-organisms have attracted much interest. Members of this group, in particular those belonging to the genus *Streptomyces*, are considered as the most important producers of bioactive molecules. Therefore, *Streptomyces* have been thoroughly investigated with respect to their effects against pathogenic strains and have led to the discovery of a large number of novel active compounds of considerable value (Solecka *et al.* 2012). However, the present context of both a decline in the discovery of new antibiotics and an increase in drug-resistant pathogens makes the screening for new antibiotic-producing *Streptomyces* essential.

One of the strategies for enhancing the likelihood of obtaining particularly interesting isolates and secondary metabolites is to analyse extreme habitats (Santhanam *et al.* 2012), such as arid Saharan soils. Previous surveys

on the ecological distribution of Actinomycetes in Algerian Saharan soils have already demonstrated their appreciable biodiversity (Sabaou *et al.* 1998) and permitted new species and new antibiotics to be found (Boubetra *et al.* 2013; Meklat *et al.* 2013).

Hydroxamic acids (bearing the functional group *N*-hydroxy amide) are one such interesting class of molecule produced by many *Streptomyces* species (Dimkpa *et al.* 2008; Kodani *et al.* 2013). Their low toxicities in general, their weak acid properties and their ability for complexing transition metal ions have resulted in these molecules receiving considerable attention for broad therapeutic and industrial applications (Vanjari and Pande 2003; Rho *et al.* 2006; Jahangirian *et al.* 2011). Among their numerous properties, hydroxamic acids and derivatives have been largely reported as effective antibacterial and antifungal agents (Li *et al.* 2003; Pepeljnjak *et al.* 2005). These activities are connected with the ability of hydroxamic acids to potently and selectively inhibit a range of enzymes that are vital for the survival of bacteria, such as various metalloproteinases, urease, cyclooxygenases and peroxidase (Muri *et al.* 2004).

Recently, exploration of microbial antibiotic potential has focused on the screening of polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) genes. This exploration approach seems applicable for hydroxamic acid-containing molecules, since these molecules are already known to be synthesized through NRPS-dependent or independent pathways (Barry and Challis 2009; Oves-Costales *et al.* 2009).

Here, we describe the isolation of a highly antagonistic *Streptomyces* strain, designated WAB9, from a Saharan soil sample and its identification by conventional and molecular methods, together with production and structure elucidation of the corresponding bioactive compound, which was found to be a new hydroxamic acid-containing antibiotic. The minimum inhibitory concentrations (MICs) of this antibiotic were also evaluated on a set of various multidrug-resistant bacteria, filamentous fungi and yeasts.

Results and discussion

The strain WAB9 was isolated by plating a 2 : 10 serial dilution of the soil sample of Bechar on chitin-vitamin agar medium supplemented with selective antibiotics. This strain was found in only a single small colony, which shows it rarity.

Antagonistic properties and detection of PKS and NRPS sequences

The strain WAB9 exhibited a broad spectrum of antimicrobial activity (Table 1), since it was active against all the targeted bacteria, filamentous fungi and yeasts (distance of inhibition ranged between 15 and 35 mm). *Salmonella enterica* and *Candida albicans* were the least sensitive (distance of inhibition <20 mm). The use of antibiotics as selective agents in the isolation of actinobacteria has already been mentioned as a successful method for the isolation of interesting strains originating

Table 1 Antagonistic properties of the strain WAB9 against various multidrug-resistant micro-organisms

Target micro-organism (antibiotic resistance pattern*)	Activity† (mm)
Bacillus subtilis ATCC 6633 (NEO)	39
Staphylococcus aureus S1 (CAR, GEN, K, NEO, OLE, SPI, VAN)	28
Enterobacter cloacae E13 (AMC, ATM, CEP, CTX, FEP, FOX, GEN, PIP, TCC, TIC, TOB)	29
Escherichia coli E52 (ATM, CAZ, CEP, CTX, FEP, GEN, PIP, TIC, TOB)	35
Klebsiella pneumoniae K44 (AMX, AN, CF, CRO, CTX, CXM, GEN, K, MZ, SSS, TIC)	29
Pseudomonas aeruginosa IPA1 (AMX, CAR, GEN, NEO, SPI, SSS, VAN)	29
Salmonella enterica E32 (ATM, CAZ, CEP, CTX, FEP, GEM, PIP, TIC, TOB)	16
Fusarium culmorum (CHX, ITR, NYS, TIZ)	39
Fusarium moniliforme (AMB, CHX, NYS, TIZ)	29
Fusarium sporotrichoides (AMB, CHX, ITR, NYS, TIZ, TER)	20
Fusarium graminearum (CHX, NYS, TIZ)	25
Fusarium oxysporum f. sp. *lini* (ITR,TIZ)	25
F. oxysporum f. sp. *albedinis* (AMB, CHX, ITR, NYS, TIZ, TER)	26
Fusarium proliferatum (CHX, ITR, NYS, TIZ)	22
Fusarium equiseti (CHX, TIZ)	30
Aspergillus carbonarius (CHX, NYS)	33
Aspergillus niger (AMB, CHX, NYS, TIZ)	25
Aspergillus flavus (TIZ)	26
Aspergillus parasiticus (AMB, CHX, ITR, NYS, TIZ)	20
Penicillium glabrum (CHX)	38
Penicillium expansum	37
Umbelopsis ramanniana (CHX, ITR, TIZ, TER)	36
Candida albicans IPA 200 (NYS, ITR, TIZ, CHX, TER)	15
Saccharomyces cerevisiae ATCC 4226	34

AMC, amoxicillin + clavulanic acid; AMX, amoxicillin; AN, amikacin; ATM, aztreonam; C, chloramphenicol; CAR, carbenicillin; CAZ, ceftazidime; CFP, cefpirome; CRO, ceftriaxone; CTX, cefotaxime; CXM, cefuroxime; FEP, cefepime; FOX, cefoxitin; GEN, gentamicin; K, kanamycin; MZ, mezlocillin; NEO, neomycin; OLE, oleandomycin PIP, piperacillin; SPI; spiramycin; SSS, sulfamide; TCC, ticarcillin + clavulanic acid; TIC, ticarcillin; TOB, tobramycin; VAN, vancomycin; AMB, amphotericin B; CHX, cycloheximide; ITR, itraconazole; NYS, nystatin; TIZ, thioconazole; TER, terbinafine.

*The antibiotic resistance patterns were evaluated in house as described by Touati *et al.* (2006).

†Activity estimated by measuring the length of inhibition between Actinomycetes and target micro-organisms.

from Saharan soils (Sabaou *et al.* 1998) and has permitted novel species and antibiotics to be discovered (Boubetra *et al.* 2013; Meklat *et al.* 2013).

Only the PKS-II gene was detected through the PCR-based screening of genomic potential for producing bioactive metabolites (data not shown). This result suggests the possible involvement of a pathway mediated by PKS for the production of the antibiotic related to the activity expressed by the strain WAB9. Nevertheless, the implication of other genes in the biosynthesis of the bioactive product cannot be excluded since the absence of PKS and NRPS amplification products from some active actinobacterial strains has already been observed (Meklat *et al.* 2012).

Taxonomy of the strain WAB9

The strain grew well on all media. It formed nonfragmented, colourless substrate mycelium. The aerial mycelium was greenish grey and produced numerous spiralled chains of nonmobile spores on sporophores. Diffusible pigments were not observed. The strain contained LL-diaminopimelic acid isomer and glycine in the cell wall. The whole-cell hydrolysates contained galactose and glucose. This indicates that the strain WAB9 has cell wall chemotype IC (Lechevalier and Lechevalier 1970). The phospholipid pattern was type PII (Lechevalier *et al.* 1977) characterized by the presence of phosphatidylethanolamine. Based on the morphological and chemical characterization, the strain WAB9 was classified as a member of the genus *Streptomyces* (Holt *et al.* 1994). Physiologically, the strain utilized arabinose, fructose, galactose, inositol, mannitol, rhamnose, salicine, xylose, histidine, phenylalanine, proline, starch and xanthine, but not melibiose, raffinose or sucrose. The strain was able to grow in the presence of phenol (0·1% w/v), penicillin (10 UI) and rifampicin (50 μg ml^{-1}), but not at 45°C or in the presence of sodium azide (0·01% w/v) or NaCl (10% w/v). Furthermore, strain WAB9 could not reduce nitrate.

The 16S rRNA gene sequence (1378 bp) of the strain was determined and deposited in the GenBank data library under the accession number KF313072. The sequence was aligned with those of *Streptomyces* reference species available in the GenBank database, which confirmed the identification of the strain at the genus level. The phylogenic relationship between strain WAB9 and the other *Streptomyces* species can be seen in the neighbour-joining dendrogram (Fig. 1). The similarity level was 99·85% with *Streptomyces ambofaciens* ATCC 23877^T (Kämpfer 2012), the most closely related species. Phenotypically, the strain WAB9 was found to resemble *S. ambofaciens* except for the resistance to penicillin (10 UI), which is absent in *S. ambofaciens*. Furthermore, the strain WAB9 exhibited both antibacterial and antifungal activities, while *S. ambofaciens* was only known to produce antibacterial antibiotics, namely spiramycin and netropsin (Pang *et al.* 2004).

Detection and purification of the bioactive compound

The aqueous phase remaining after *n*-butanol extraction of the culture filtrate (resulting from 6-day-old fermentation) was found to provide the optimal conditions for both production and extraction (Fig. S1). The HPLC

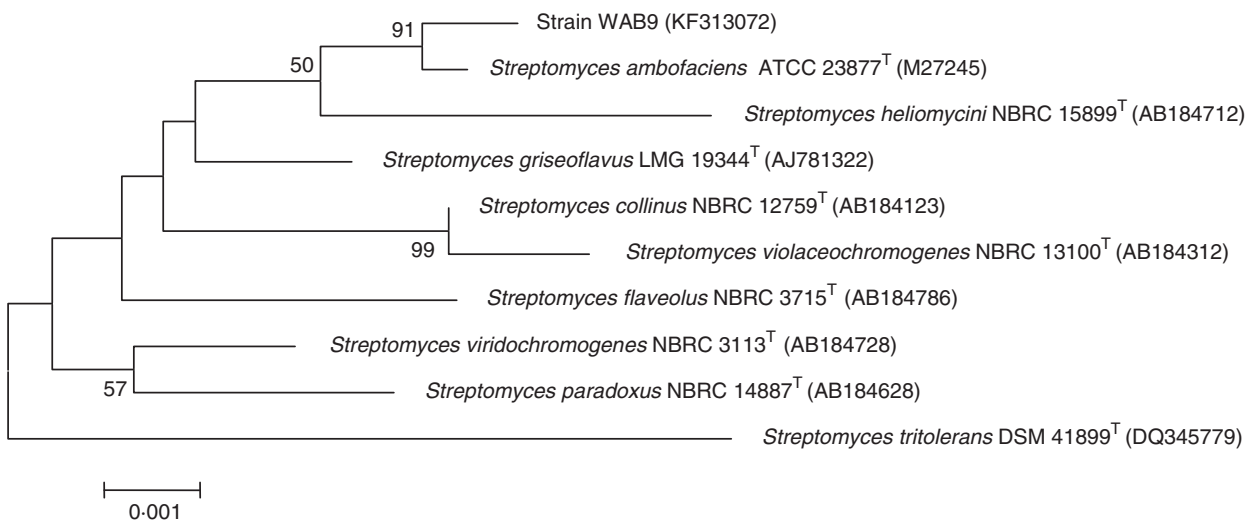

Figure 1 Neighbour-joining tree (Saitou and Nei 1987) based on 16S rDNA sequences showing the relationship between strain WAB9 and the other *Streptomyces* species. The numbers at the nodes indicate the levels of bootstrap support based on Neighbour-joining analyses of 1000 re-sampled data sets. Bar, 0·001 nt substitution per nt position. *Streptomyces tritolerans* DSM 41899^T has been used as outgroup.

profile of this phase showed one active peak (named W9) at a retention time of 25·02 min and UV detection at 220 nm. The final purification was achieved after the second re-injection in the HPLC system (Fig. S2).

Elucidation of the structure of the active compound

The UV–visible spectrum of the active compound W9 exhibited maximum absorptions at 257 and 265 nm, which putatively indicated the presence of unsaturated aromatic complexes. The ESI-MS spectrum contained an ion peak at *m/z* 326·0 $[M\text{-}H]^-$ (Fig. S3). Thus, the molecular weight of this compound was $M = 327$.

The ^1H and ^{13}C NMR chemical shifts of the compound are given in Table 2 and the structure in Fig. 2. The ^{13}C, HSQC and HMBC spectra show seven carbon signals for the W9 molecule. It was possible to discern one amide group (δ_c 174·29), four sp^2–hybridized carbons (δ_c from 127·41 to 134·80) and two sp^3-hybridized carbons (δ_c 36·45–55·93). The hydrogens of the hydroxyl group were not observed due to rapid exchange with MeOD. The 2D ^1H-^1H and ^1H-^{13}C experiments and especially the long range ^1H-^{13}C couplings observed in the HMBC spectrum (Fig. 2B) established the connectivity between all the groups of the molecule. The structure of the compound W9 was determined by NMR and mass spectrometry to be 2-amino-*N*-(2-amino-3-phenylpropanoyl)-*N*-hydroxy-3-phenylpropanamide, which thus corresponded to a hydroxamic acid-containing molecule with two phenylalanine residues. No resemblance was noted when the structure of compound W9 was compared with structures reported in the literature, notably in the www.sciencefinder.com and www.chemspider.com databases, or antibiotics described in The Dictionary of Natural Products (Buckingham 1997), or in Berdy's review of bioactive microbial metabolites (Berdy 2005).

Table 2 ^1H and ^{13}C NMR data assignments of WAB9 compound in CD_3OD at 298 K

^1H and ^{13}C number*	^1H chemical shift (ppm)	^{13}C chemical shift (ppm)
1-10	–	174·29
2-11	3·68	55·93
3-12	2·92–2·98	36·45
4-13	–	134·80
5-9-14-18	7·03	129·10
6-8-15-17	7·13	128·84
7-16	7·08	127·41

*See Fig. 2 for numbering of hydrogen and carbon atoms.

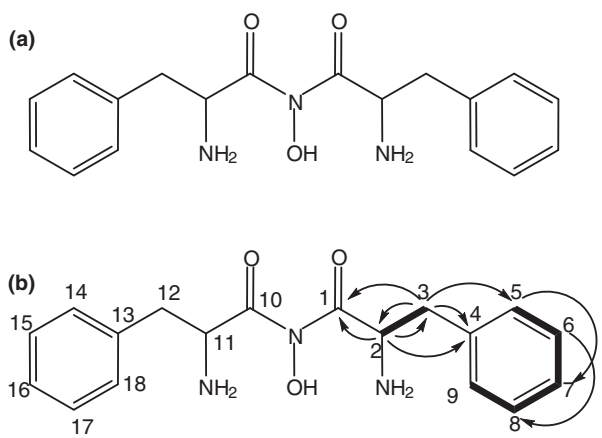

Figure 2 Structure of the bioactive compound (a) and HMBC and COSY correlations (b). (←—) 1H-13C HMBC correlation and (——) 1H-1H COSY correlation.

Numerous *Streptomyces* species have been reported to produce active acid-containing molecules (Dimkpa *et al.* 2008; Kodani *et al.* 2013). For example, alahopcin and actinonin are well-known antibiotic agents functionalized by hydroxamic acids, both of which were first isolated from a culture of *Streptomyces* sp. and showed a broad antimicrobial spectrum, especially against some previously antibiotic-resistant types of micro-organisms such as *Staphylococcus aureus* (Gordon *et al.* 1962; Higashide *et al.* 1985).

Furthermore, hydroxamic acids are also used for their metal-chelating properties. Desferrioxamine, which was first isolated from a *Streptomyces* species by Yang and Leong (1982), is routinely used for treating iron intoxication and overload in human patients (Franchini and Veneri 2004) and metal-chelating hydroxamic acids, such as those produced by *Streptomyces acidiscabies* E13, can be effective bioremediators in heavy-metal contaminated fields (Dimkpa *et al.* 2008).

Given their significant ability to scavenge reactive oxygen species, hydroxamic acids have also drawn much attention in oncology and food safety as protective agents against free radical-mediated disorders (Taira *et al.* 2002; Garcia-Manero *et al.* 2008).

Minimum inhibitory concentrations

As summarized in Table 3, the pure bioactive compound exhibited appreciable MIC values towards a broad range of the multidrug-resistant pathogens tested, including Gram-positive bacteria, Gram-negative bacteria, filamentous fungi and yeasts. Among these micro-organisms, the most sensitive were *Pseudomonas aeruginosa* IPA1 (10 $\mu g\ ml^{-1}$), *Escherichia coli* E52 (20 $\mu g\ ml^{-1}$) and all of the targeted *Fusarium* species (8–40 $\mu g\ ml^{-1}$). The strains of *Aspergillus* and *Penicillium* and the yeast *C. albicans* IPA 200 were the most resistant (40–80 ml^{-1}).

Table 3 Minimum inhibitory concentrations (MICs) of the antimicrobial compound produced by the strain WAB9 against several target micro-organisms

Target micro-organisms	MICs (μg ml^{-1})*
Bacillus subtilis ATCC 6633	10
Pseudomonas aeruginosa IPA1	10
Escherichia coli E52	20
Staphylococcus aureus S1	50
Saccharomyces cerevisiae ATCC 4226	20
Candida albicans IPA 200	60
Fusarium proliferatum	08
Fusarium oxysporum f. sp. *lini*	15
Fusarium culmorum	25
Fusarium graminearum	30
Fusarium equiseti	40
Umbelopsis ramanniana	20
Aspergillus flavus	40
Aspergillus ochraceus	50
Aspergillus carbonarius	80
Penicillium glabrum	50

*MIC values represent the mean of two replicates.

The broad antibacterial and antifungal capacities of hydroxamic acids have been extensively reported (Li *et al.* 2003; Pepeljnjak *et al.* 2005) and are attributed to their potent and selective inhibition of a range of enzymes. These include zinc, nickel and iron metalloproteases, ureases, cyclooxygenases and peptide deformylases (Muri *et al.* 2004). Such is the case of actinonin, which is regarded as a potential inhibitor of peptide deformylases (Chen *et al.* 2000).

However, the mode of action at the molecular level is still not well understood and has been speculated to be due to the electrophilic character of the hydroxamic moiety. In this hypothesis, the electrophilicity allows the molecule to react with nucleophilic centres present in the enzymes involved in fundamental processes (Bravo and Lazo 1996).

In conclusion, from the results presented in this paper, we found that the strain *Streptomyces* sp. WAB 9 produced a new, small, hydroxamic acid-containing molecule, displaying a broad antimicrobial spectrum with interesting activities against multidrug-resistant bacteria, filamentous fungi and yeasts. Regarding the nature and activity of the molecule, numerous possible therapeutic (human, veterinary) and nontherapeutic (food processing, waste management, biofungicide, etc.) applications could be considered. However, only *in vivo* evaluation studies could really prove the effectiveness of the compound in the applications in question.

Materials and methods

The Actinomycete strain, designated WAB9, was isolated in our laboratory by the dilution agar plating method, from a Saharan soil collected in Bechar (31°34′N; 2°16′W). Briefly, 1 g of the soil sample was aseptically added to 9 ml sterile distilled water. The suspension was vortexed and subjected to a 10-fold serial dilution. Aliquots (0·2 ml) of each dilution were spread on the surface of chitin-vitamin agar medium (Hayakawa and Nonomura 1987) supplemented with cycloheximide (50 mg l^{-1}) and rifampicin (10 mg l^{-1}). The plates were incubated at 30°C for 2 weeks and then observed by light microscopy. After isolation, the strain WAB9 was purified and maintained at 4°C on slants ISP-2 medium (Shirling and Gottlieb 1966).

Antagonistic properties and detection of PKS and NRPS sequences

The antagonistic properties of the strain WAB9 against several bacteria, filamentous fungi and yeasts (listed in Table 1) were determined by the streak method as described previously by Boubetra *et al.* (2013). The antibiotic resistance patterns of the target micro-organisms were evaluated in house according to the method described by Touati *et al.* (2006).

The genomic potential for producing bioactive metabolites of the isolate was assessed. Polyketide synthase I and II (PKS-I, PKS-II) and NRPS genes were amplified from genomic DNA with the method cited by Meklat *et al.* (2012).

Phenotypic, chemical and phylogenetic characterization

The strain WAB9 was identified at the genus level on the basis of both phenotypic and chemical features according to criteria described by Kämpfer (2012). The morphological characteristics of the strain were studied on International *Streptomyces* Project (ISP) medium 2, ISP medium 3, ISP medium 4 and ISP medium 5 at 30°C for 14 days.

Twenty-three physiological tests were used to characterize the strain. Degradation of different organic substrates and resistance to several chemical and physical agents were determined as described by Locci (1989).

For the chemical study of cell constituents, analyses of diaminopimelic acid and whole-cell sugars were carried out using the methods of Becker *et al.* (1964), and Lechevalier and Lechevalier (1970). Phospholipids were analysed according to the procedure developed by Minnikin *et al.* (1977).

For phylogenetic analysis, genomic DNA of the strain was extracted for 16S rRNA gene analysis according to the method of Liu *et al.* (2000). The 16S rRNA gene was PCR amplified using the forward FC27 (5′-AGAGTTT GATCCTGGCTCAG-3′) and reverse RC1492 (5′-GGTTA CCTTGTTACGACTT-3′) primers. Phylogenetic analysis was performed as described by Meklat *et al.* (2013).

Production, isolation and purification of the active compound

The fermentations were carried out in ISP-2 broth. This medium was chosen among several others because of its very good results in our preliminary work. A seed culture of strain WAB9 was prepared with the same medium and used to inoculate 500-ml Erlenmeyer flasks, each containing 100 ml of medium. The cultures were incubated on a rotary shaker (250 rev min^{-1}) at 30 °C. The extraction of the active compound was carried out on the day of fermentation exhibiting the optimal production (concomitantly determined by a kinetic assay) after centrifugation (5000 *g*, 20 min) of the culture broth to eliminate cells. The cell-free supernatant was extracted with an equal volume of *n*-butanol. The active aqueous phase was concentrated to dryness by a rotary evaporator under vacuum at a temperature lower than 40°C.

The resulting dry extract was recovered in 0·5 ml of ultrapure water for HPLC purification using an amphiphilic Atlantis® Prep T3 (5 μm) semi-preparative column (250 mm × 10 mm; Waters, Milford, MA) with a linear gradient of methanol–water (0–100% for 40 min), a flow rate of 1·5 ml min^{-1} and UV detection at 220 nm. All the fractions were collected and tested (6 mm paper disk diffusion method) against *Bacillus subtilis* ATCC 6633 and *Fusarium culmorum* to detect the active fraction and distinguish it from the nonactive fractions. These two target micro-organisms were selected as representative of bacteria and filamentous fungi with regard to their high sensitivity to the WAB9-produced compound.

Final purification of the active fraction was achieved after the second re-injection in the HPLC in the same conditions, except for the gradient (50–100% for 30 min).

Determination of the active compound structure

This analysis was carried out with the HPLC-purified compound. The UV spectrum was determined with a Shimadzu UV1605 spectrophotometer. The mass spectra were recorded on an LCQ ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) 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 the bioactive molecule. The NMR sample was prepared by dissolving 3 mg of the compound in 600 μl of CD_3OD. All spectra were recorded on a Bruker Avance 500 spectrometer equipped with a 5-mm triple resonance inverse Z-gradient probe (TBI ^{1}H, ^{31}P, BB). All chemical shifts for ^{1}H and ^{13}C were 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 using ^{1}H-^{1}H COSY45, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC experiments. Gradient-enhanced ^{1}H COSY45 was performed and included 36 scans per increment. ^{1}H-^{13}C correlation spectra using a gradient-enhanced HSQC sequence (delay optimized for $^{1}J_{CH}$ of 145 Hz) was obtained with 200 scans per increment. A gradient-enhanced HMBC experiment was performed allowing 62·5 ms for long-range coupling evolution (340 scans were accumulated). Typically, 2048 t2 data points were collected for 256 t1 increments.

Determination of MICs

MICs of pure bioactive compound were investigated using the conventional agar dilution method of Oki *et al.* (1990) toward a selection of 16 target micro-organisms.

These micro-organisms were inoculated onto nutrient agar medium containing different concentrations of active compounds (1, 2, 3, 5, 8, 10, 15, 20, 25, 30, 40, 50, 60, 80 and 100 mg ml^{-1}). 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 antibiotic concentration that inhibited the growth of each micro-organism was determined. Medium without active compound and inoculated with target micro-organisms was used as control.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Determination of the optimal conditions for production and extraction of antibiotic W9.

Figure S2. HPLC purification process for the antibiotic W9 secreted by the strain WAB9.

Figure S3. Nanospray ion electron-spray ionization–mass spectrum of the antibiotic W9 in negative mode.