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ORIGINAL ARTICLE

Identification of a Sorbicillinoid-Producing *Aspergillus* Strain with Antimicrobial Activity Against *Staphylococcus aureus*: a New Polyextremophilic Marine Fungus from Barents Sea

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Abstract

The exploration of poorly studied areas of Earth can highly increase the possibility to discover novel bioactive compounds. In this study, the cultivable fraction of fungi and bacteria from Barents Sea sediments has been studied to mine new bioactive molecules with antibacterial activity against a panel of human pathogens. We isolated diverse strains of psychrophilic and halophilic bacteria and fungi from a collection of nine samples from sea sediment. Following a full bioassay-guided approach, we isolated a new promising polyextremophilic marine fungus strain 8Na, identified as *Aspergillus protuberus* MUT 3638, possessing the potential to produce antimicrobial agents. This fungus, isolated from cold seawater, was able to grow in a wide range of salinity, pH and temperatures. The growth conditions were optimised and scaled to fermentation, and its produced extract was subjected to chemical analysis. The active component was identified as bisvertinolone, a member of sorbicillonoid family that was found to display significant activity against *Staphylococcus aureus* with a minimum inhibitory concentration (MIC) of 30 μg/mL.

Keywords Sediments · Marine fungi · *Aspergillus protuberus* · Bisvertinolone · Antimicrobial activity · MDR

Introduction

Antimicrobial resistance has spread dramatically in the last 30 years, leading to an increase in the number of deaths due to infectious diseases. The excessive and often inappropriate use of antimicrobial drugs has affected the development of a new group of microorganisms, the multidrug-resistant (MDR) bacteria, which show resistance towards the most common antibiotics (Muller et al. 2017). Currently, there is a pressing need to discover novel and effective antimicrobial drugs to counteract this dramatic emergence of MDR infections. Nature has always been the main source of new molecular scaffolds; a prime example was the discovery of penicillin. Henceforth, bioprospecting of marine macro- and microorganisms for new natural drug candidates still represents the best opportunity for the discovery of new bioactive compounds. In particular, extreme environments constitute an unexplored reservoir of biodiversity (Poli et al. 2017). The use of different strategies of adaptation by organisms and microorganisms allowed the colonisation of extreme habitats, which are characterised by low nutrient concentration, extremes of pH, low temperature, high pressure and salinity. Some of the most extreme marine habitats known like Mediterranean deep hypersaline anoxic basins (DHABs; water depth ~ 3500 m) are nearly saturated with salt, from these sediments were retrieved different halotolerant/halophilic fungal groups that might have adapted to different local environments

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(Bernhard et al. 2014). Currently, particular attention has been focused on marine fungi (Zhao et al. 2016; Saleem et al. 2007), since they are considered an interesting untapped resource of biodiversity and biotechnological potential for production of secondary metabolites (Ebada and Proksch 2015).

The ability to grow in different ranges of salt concentration is probably one of the most remarkable features of some species of marine fungi that could confer the potential to synthesise new bioactive metabolites (Gostincar et al. 2010). Particularly, under hypersaline conditions, the cell keeps its stability and integrity accumulating compatible solutes in its interior to overcome osmotic stress and to prevent lysis (Delgado-Jarana et al. 2006). This condition induces changes in the membrane composition that effectively increases the sterol to phospholipid ratio, and the fatty acid unsaturation in turn increases the membrane fluidity (Turk et al. 2004). Among all marine fungi, *Penicillium* and *Aspergillus* are a very powerful source of new bioactive compounds (Blunt et al. 2015). The genus *Aspergillus* is widespread all over the marine ecosystem. *Aspergillus* sp. fungi are heterotroph and polyextremophiles. This significant nutritional and physiological versatility is complemented by the metabolic capacity to produce numerous secondary metabolites, which is believed to be important in ecological signalling (Baker and Bennett 2007). Genomic studies are revealing putative biosynthetic genes of *Aspergillus* strains; however, the biocomposites described in the literature are still limited. The prediction of genes exceeds the number of new molecules obtained so far, leaving a large number of compounds yet to be discovered (Rateb and Ebel 2011).

Despite the fact that natural products from some fungal genera have been often and intensively studied, in particular, *Aspergillus* and *Penicillium*, there is still a great potential of secondary metabolites produced by these fungi, which have not as yet been fully and adequately explored.

Barents Sea is part of Arctic Continental Shelf, and it is also the deepest of the Arctic shelf seas. The most distinctive oceanographic feature of the Barents Sea is the influx of the salty Atlantic waters from the southwest that meets and mixes with Arctic water masses along the Polar Front (Loeng 1991). This condition makes the Barents Sea warmer in comparison with other areas of the same latitude, but the oceanographic conditions are highly variable. Barents Sea is a poorly explored environment, and therefore it is an appealing prospect within the biodiscovery pipeline.

Herein, we report the isolation of 14 fungi from marine sediments collected in the Barents Sea and their taxonomic characterisation. Furthermore, we also report the identification of a sorbicillonoid-producing strain of *Aspergillus protuberus* MUT 3638, a species recently described belonging to the *Aspergillus* section *Versicolores* (Jurjevic et al. 2012), and the isolation of bisvertinolone as an active antimicrobial component.

Materials and Methods

Sediment Collection

The strains used in this work were isolated from sub-sea sediments collected from the Barents Sea (Fig. 1), during the Barkut expedition in June 2015, in collaboration with Prof. Bjarne Landfald of the University of Tromsø. Sediments were collected from nine different sampling sites and depths (Table 1) using a multicorer drill device. Several layers were cut and then aseptically placed into 50-mL conical tubes and kept at 4 °C during the shipments and stored at − 80 °C after their arrival to the laboratory.

Culture Media

Specific culture media were designed for the isolation of different groups of psychrophilic and halotolerant/halophilic microorganisms present in extreme marine environments. Each medium was prepared with natural seawater and artificial seawater in a range of six salinities [3, 5, 10, 15, 20 and 25% (*w*/*v*) of total salts] and three different pH conditions. Natural seawater was used to prepare media with a basal salt concentration in order to mimic the marine environment (3–3.5% of total salinity) while a stock solution of artificial seawater SW30 (Subov's salt solution 30% (*w*/*v*)) was used to prepare other media with higher salt concentrations.

All media except LB were adjusted to three different pH values (4.0, 7.0 and 9.0) and sterilised by autoclaving at 121 °C for 15 min. The names and formula of each medium are given in grams per litre, and for solid media, 20 g/L of agar was added.

Artificial seawater (SW30): 234.0 g NaCl, 39.0 g $MgCl_2 \cdot 6H_2O$, 61.0 g $MgSO_4 \cdot 7H_2O$, 1.0 g $^*CaCl_2$; 6.0 g KCl, 0.2 g $NaHCO_3$, 0.7 g NaBr. This medium was prepared with distilled water up to 1000 mL. $^*CaCl_2$ was dissolved separately in 20 mL of distilled water to prevent the formation of insoluble complexes of $CaCO_3$.

The following media were prepared dissolving the compounds in natural seawater. For media with higher salinities, the artificial seawater SW30 diluted in distilled water was used to reach the salt concentration required.

Seawater minimal (SWM): 1.0 g casamino acids, 1.0 g yeast extract, 1.0 g peptone, 1.0 g glucose.

Casein yeast chitin (CYC): 5.0 g casein, 0.5 g yeast extract, 5.0 g chitin, 0.5 g K_2HPO_4, 5.0 g malt extract, 0.1 g sodium pyruvate salt, 500 μL glycerol.

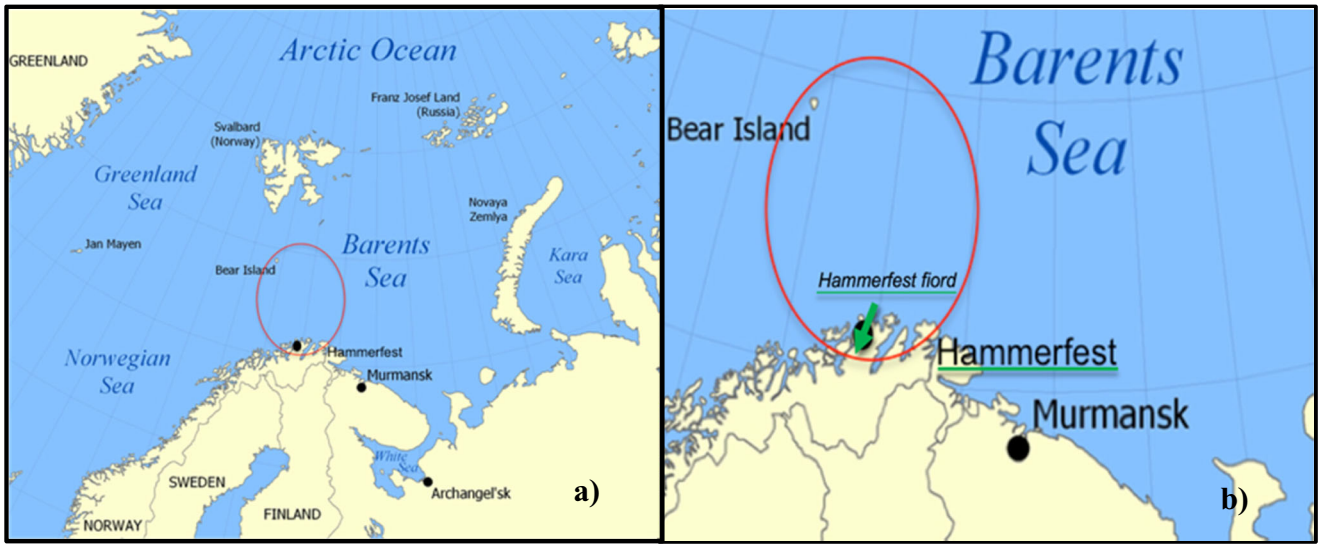

Fig. 1 Geographic localisation of the Barents Sea. **a** The area where sampling was performed is circled. **b** The city and the fiord of Hammerfest and final leg of the mission are highlighted.

Tryptone yeast starch (TYS): 10.0 g tryptone, 5.0 g yeast extract, 0.5 g starch, 10.0 g NaCl, 0.5 g malt extract, 0.1 g sucrose, 500 μL glycerol.
Malt yeast extract (MY): 10.0 g malt extract, 4.0 g yeast extract, 4.0 g glucose.
Luria Bertani (LB): 10.0 g tryptone, 5.0 g yeast extract, 10.0 g NaCl, pH 7 (20.0 g agar for solid media). This medium was prepared with distilled water up to 1000 mL.

Isolation of Microorganisms

Standard protocols for air quality controls were performed to discriminate contaminations. The bacterial and mould tests consisted of leaving opened for 1 h a Petri dish with LB agar for bacteria and Sabouraud agar for moulds, then closed and room temperature incubated for 7 to 14 days. Besides bacteria and mould testing, controls were always included with all media used in this work and incubated with the rest of samples at the temperature and time programmed.

Further of standard disinfection, all microbiological procedures: culture, isolation and screenings were rigorously performed under a biosafety cabinet class II with a laminar flow to reduce contamination by airborne spores.

For the isolation of microorganisms, two different growth media were used: SWM for bacteria and CYC for actinobacteria and fungi. Both media with increasing salt concentrations ranging from 3% to 25% (*w*/*v*) for the isolation of halotolerant and moderate to halophilic microorganisms.

In order to isolate microorganisms, 1.0 g of sediment was mixed with 9 mL of filtered and sterilised natural seawater (NSW) in a 15-mL conical tube and gently mixed. The supernatant was then serially diluted tenfold until 10^{-6} dilution (i.e. 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}), and 100 μL of the serial dilution was spread plated on sterile SWM and CYC Petri dishes.

The inoculated plates and controls were incubated for 5 to 20 days at two different temperatures, 4 and 15 °C. Then, several colonies were picked using as criteria the morphology, size and pigments and were then incubated for 5–6 days at 20 °C with agitation in liquid medium SWM and CYC, respectively. All the microorganisms isolated were suspended in glycerol (10% (*v*/*v*) for fungal strains, and 20% for the bacterial strains, of the final volume) and stored at − 80 °C in cryogenic vials.

Table 1 Coordinates and depth of several sampling sites from Barents Sea

Sampling location	Latitude and longitude	Depth (m)
Bjørnøyenna	72° 20′ 07″ N; 18° 09′ 50″ E	364
Salina	72° 00′ 97″ N; 20° 03′ 46″ E	345
Well 7119	71° 12′ 87″ N; 20° 03′ 46″ E	204
Well 7120-1	71° 07′ 65″ N; 20° 48′ 97″ E	163
Well 7120-2	71° 12′ 87″ N; 19° 56′ 59″ E	165
Bønna	70° 50′ 05″ N; 16° 33′ 20″ E	1365
Goliat	71° 17′ 09″ N; 22° 19′ 38″ E	380
Repparfjord	70° 27′ 59″ N; 24° 17′ 17″ E	47
HammerFest	70° 39′ 69″ N; 23° 40′ 12″ E	46

Identification of Fungal Strains

The isolated fungi were identified with a polyphasic approach, which couples morpho-physiological features with molecular studies. After determination of the genera according to the macroscopic and microscopic features (Domsch et al. 1980),

the fungal strains were transferred to the media recommended by the authors of a selected genus monographs for species identification. For molecular identification, genomic DNA of all strains was extracted from approximately 100 mg of mycelium, scraped from MY Petri dishes, using a NucleoSpin kit (Macherey Nagel GmbH, Duren, DE, USA), according to the manufacturer instructions. The quality and quantity of DNA samples were measured with the ND-1000 Spectrophotometer NanoDropH (Thermo Scientific, Wilmington, Germany). DNA extracts were stored at − 20 °C. The ITS sequences were amplified using the primer pair ITS1/ITS4 (White et al. 1990). For those strains morphologically identified as *Aspergillus* spp. and *Penicillium* spp., amplification of the β-tubulin gene was performed using primers Bt2a/Bt2b (Glass and Donaldson 1995; Geiser et al. 1998). Reaction mixtures consisted of 30 ng genomic DNA, 1 μM each primer, 1 U Taq DNA Polymerase (Qiagen, Chatsworth, CA, USA), 1× reaction buffer and 200 μM of dNTPs. DNA amplifications were performed using a T-Gradient thermal cycler (Biometra, Göttingen, Germany) with the following profile: 95 °C for 5 min; 35 cycles: 95 °C for 40 s, 55 °C (58 °C for Bt2a/Bt2b) for 45 s, 72 °C for 50 s; 72 °C for 8 min. PCR products were purified and sequenced at Macrogen Europe Laboratory (Amsterdam, The Netherlands).

All strains were taxonomically identified at the species level. The partial sequences obtained were verified and analysed by querying with the Blastn algorithm (default setting; mismatch 1/−2; gap cost linear) hosted at NCBI, the specific sequences against the GenBank (http://www.ncbi.nlm.nih.gov). Pairwise alignments were also performed using the sequence database of the Westerdijk Fungal Biodiversity Institute (http://www.westerdijkinstitute.nl/).

Finally, dereplication of the isolates belonging to the same species was performed applying the microsatellite screening by using the core sequence of the microsatellite M-13 as a primer (Abd-Elsalam et al. 2010). Amplicons were separated on 1.5% (*w*/*v*) agarose gels stained with 5 μL 100 mL^{-1} ethidium bromide and electrophoresed alongside a GelPilot 1 kb plus DNA Ladder; images were acquired with a Gel Doc 1000 System (Bio-Rad, Hercules, CA, USA), and fingerprints were analysed using Bionumerics 7.1 software.

Pathogenic Strains

The following human pathogens were used in this work: *Pseudomonas aeruginosa* PA01 (Alonso et al. 1999), *Staphylococcus aureus* 6538P (Lima et al. 2014), *Klebsiella pneumoniae* DF12SA (Shahi et al. 2013), *Acinetobacter baumannii* Ab13 (Poirel et al. 2008), *Burkholderia cenocepacia* LMG 16656 and *Burkholderia metallica* LMG 24068 (Soriano et al. 2009). The strains were routinely grown at 37 °C in LB medium.

Primary Screening for Antagonistic Activity

Preliminary screening for antimicrobial activity was performed by the cross-streaking method against a panel of human pathogens ("Pathogenic Strains"). This method allows the observation of antagonistic interactions among a tester strain and several target strains. To overcome the limitation of the assay that the pathogen and the tested strain are not able to grow on the same medium, a dual media Petri dish was developed. From one side of the plate, the culture medium was poured to allow the growth of marine bacterial or fungal isolates (SWM or TYS), and the other half of the plate was filled with LB medium, suitable for the growing of pathogenic bacteria. The tester strains were selected on the basis of different morphologies, pigments and shapes and were streaked onto one side of the plate with their appropriate medium and then incubated at 20 °C for 10 days. Thereafter, the pathogenic strains were streaked perpendicularly to the initial streak and incubated at 37 °C for 24 h. A control plate using the pathogens was also maintained without inoculating the marine fungal strains, to assess the normal growth of pathogenic bacteria.

Preparation of Crude Extracts

A pre-inoculum was prepared from the active fungal strains. A small piece of mycelium was inoculated in 3 mL of liquid medium TYS and incubated for 5 days at 20 °C. This pre-inoculum was used to inoculate 250 mL of the same medium in 500-mL flasks and incubated at different temperatures and days in a static condition. Thereafter, exhausted culture broths were centrifuged at 7000 rpm for 30 min at 4 °C. For the extracellular extraction, the supernatant was mixed and vigorously shaken five times with three volumes of ethyl acetate using a separator funnel. This mixing facilitated the transfer of a dissolved compound from one solvent layer to another. Finally, the solvent was dried using a Laborota 4000 Rotary Evaporator (Heidolph, Schwabach, Germany). The extract was weighed and dissolved in DMSO (100%) at a final concentration of 100 mg/mL.

Liquid Inhibition Assay

The produced crude extracts were checked for their ability to inhibit the growth of a selected panel of human pathogens, using a liquid inhibition assay in 96-well plates. The different extracts were placed into each well at an initial concentration of 2 mg/mL and were serially diluted twofold using the appropriate medium. Wells that contained no compounds represented the negative control. DMSO (2% (*v*/*v*)) was also used as control to determine the effect of the solvent on bacterial growth. A single colony of each pathogenic strain was used to inoculate 3 mL of LB broth in sterile bacteriological tube. After 5–8 h of incubation, the growth was measured by

monitoring the absorbance at 600 nm. The cells were then diluted using OD/CFU correlation growth curves, and about 40,000 CFU were dispensed into each well containing the dilutions of the compound. Wells not containing any compounds were used as negative control. Plates were incubated at 37 °C overnight, and growth was measured using a VICTOR X Multilabel Plate Reader (PerkinElmer, Waltham, MA) by monitoring the absorbance at 600 nm.

Purification of Active Compounds from the Newly Isolated *A. protuberus* MUT 3638

The crude extract obtained as described ("Compound Identification") was subjected to fractionation using Chromabond SPE C18 column cartridges (Macherey-Nagel, Duren, Germany) and selectively eluted with different percentages of a methanol-water system. The fractions obtained were subsequently screened again to identify the activity. The active fraction was further purified by reverse-phase high-performance liquid chromatography (HPLC) using a C18 column. HPLC separations were carried out using a 5-μm Nucleodur reversed-phase HTec (C18, 250/10 mm, L × i.d.) column connected to an UltiMate 3000 series pump with an UltiMate photodiode array detector and in-line degasser (Dionex, Sunnyvale, CA). Detection was carried out at 220, 254, 280 and 320 nm. Elution was carried out at 2.00 mL/min with a 46-min linear gradient of acetonitrile:water:trifluoroacetic acid. The peaks were manually collected, dried and tested for bioactivity.

Liquid Chromatography High-Resolution Mass Spectrometry

The crude extract from the fungus MUT 3638 was subjected to preliminary chemical profiling.

The analyses were carried out on a Dionex Ultimate 3000 quaternary system coupled to a hybrid linear ion trap LTQ Orbitrap XLTM Fourier Transform MS (FTMS) equipped with an ESI ION MAXTM source (Thermo-Fisher, San José, CA, USA). Chromatographic separation was accomplished by using a Kinetex C18 column, 2.6 μm, 2.10 × 100 mm (Phenomenex, USA) maintained at room temperature and eluted at 0.2 mL min^{-1} with water (eluent A) and acetonitrile (eluent B), both containing 0.1% (*v*/*v*) formic acid. The following gradient elution was used: 20% B at $t = 0$, 95% B at $t = 30$ min and hold for 5 min; re-equilibration time was 9 min. Injection volume was 5 μL.

Nuclear Magnetic Resonance Method

Spectral data for bisvertinolone ^{1}H NMR spectrum of bisvertinolone was obtained on Varian Inova 500 NMR and recorded in $CDCl_3$ ($\delta_H = 7.26$ and $\delta_C = 77.0$ ppm) and DMSO ($\delta_H = 3.50$). High-resolution full MS experiments (positive ions) were acquired in the m/z 100–1000 range at a resolving power of 30,000. The following source settings were used: spray voltage = 4.5 kV, capillary temperature = 400 °C, capillary voltage = 29 V, sheath gas flow = 34 and auxiliary gas flow = 0 (arbitrary units) and tube lens voltage = 115 V. Thermo Scientific software Xcalibur has been used to obtain molecular formula. The calibration procedure was carried out using Thermo Scientific positive calibration solution composed by caffeine, Met-Arg-Phe-Ala (MRFA) and Ultramark.

Bisvertonolone Light yellow amorphous powder; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm): 7.58 (1H, dd, $J = 14.8$, 9.7 Hz, H-12), 7.42 (1H, d, $J = 14.8$ Hz, H-11), 7.32 (1H, dd, $J = 14.6$, 11.6 Hz, H-21), 6.40 (1H, d, $J = 14.6$ Hz, H-20), 6.37 (3H, ovl, H-13, H-14 and H-22), 6.14 (1H, m, H-23), 3.75 (1H, s, H-9a), 1.93 (3H, d, $J = 4.8$ Hz, H_3–15), 1.89 (3H, d, $J = 6.5$ Hz, H_3–24), 1.49 (3H, s, H_3-C7), 1.47 (3H, s, H_3-C4), 1.45 (3H, s, H_3-C5a), 1.38 (3H, s, H_3-C9b) (see Fig. S4 supplemental material). HRESIMS: calculated for $C_{28}H_{33}O_9$ 513.2119, found 513.2124 $[M + H]^+$ (see Fig. S3 supplemental material). HRESI MSMS (major fragmentation peaks) 495.2012 ($C_{28}H_{31}O_8$), 477.1905 ($C_{28}H_{29}O_7$), 265.1070 ($C_{14}H_{17}O_5$), 249.1121 ($C_{14}H_{17}O_4$), 207.0651 ($C_{11}H_{11}O_4$), 95.0489 (C_6H_7O) (see Fig. S5 supplemental material).

Results and Discussion

Isolation of Marine Microorganisms

From a total of 9 sea sediment samples, 85 microorganisms were isolated; specifically, 75 of them were identified as bacteria and the remaining 14 were identified as fungi. No bacteria or Achaea were isolated using media from 15 to 25% (*w*/*v*) of total salts, while the total population of fungi was isolated by diluting the sediments in growth media at varying salt concentrations.

Identification of Fungal Strains

All the isolated fungi were identified using a polyphasic approach, according to their macroscopic and microscopic features, as well as by molecular analysis. The 14 isolated strains belong to the Ascomycota phylum: *Aspergillus protuberus* (seven strains), *Penicillium rubens* (three strains), *Penicillium chrysogenum* (two strains), *Aspergillus sydowii* and *Microascus trigonosporus* (one strain). Sequences related to the fungi isolated in this study were deposited at the NCBI database. GenBank accession numbers are reported in Table 2. The dereplication analysis showed that only two strains were isogenic within the same species (see Fig. S1, Supplementary materials), demonstrating that the isolation procedure using

Table 2 Identification of the isolated fungi preserved in MUT culture collection

Isolated strain	Sampling site	Final identification (taxa)	Isolation media	MUT code	GenBank acc. #
1Na	Goliat	*Penicillium rubens*	CYC 15%	3588	MF574999
1Nb	Goliat	*Penicillium chrysogenum*	CYC 20%	3589	MF575000
2Aa	Repparfjord	*Penicillium chrysogenum*	CYC 20%	3596	MF575001
3Na	HammerFest	*Penicillium rubens*	CYC 20%	3597	MF575002
4Na	Salina	*Aspergillus sydowii*	CYC 20%	3610	MF575003
4Nb	Salina	*Microascus trigonosporus*	CYC 5%	3612	MF509599
5Ea	Bjørnøyenna	*Aspergillus protuberus*	CYC 20%	3613	MF575004
5Na	Bjørnøyenna	*Aspergillus protuberus*	CYC 5%	3614	MF575005
5Nb* 5Nc*	Bjørnøyenna	*Aspergillus protuberus*	CYC 15%	3615	MF575006
6Na	Bønna	*Penicillium rubens*	CYC 3%	3616	MF575007
7Na	Bønna	*Aspergillus protuberus*	CYC 10%	3617	MF575008
7Nb	Bønna	*Aspergillus protuberus*	CYC 10%	3618	MF575009
7Nc	Bønna	*Aspergillus protuberus*	CYC 10%	3632	MF575010
8Na	Bønna	*Aspergillus protuberus*	CYC 15%	3638	MF150910

*Strains 5Nb and 5Nc were considered isogenic because of genetic dereplication; for this reason, only one strain was used for the following experiments

different media and different salinity is a powerful tool to retrieve more strains. All identified isolates were stored and preserved in the fungal collection bank, Mycotheca Universitatis Taurinensis (MUT) from the University of Turin. The 14 strains deposited were assigned with the culture collection code MUT. The main features of identification are reported in Table 2.

Antimicrobial Screening

A preliminary screening by cross-streaking (CT) revealed that only the fungus MUT 3638, identified as *Aspergillus protuberus*, was able to inhibit completely the growth of four pathogens from the panel tested: *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinectocater baumanii* and *Burkholderia metallica* with the exception of *Pseudomonas aeruginosa* that was not inhibited by the fungal strains. The qualitative interpretation is shown in Fig. S2.

As a fact, the anticancer, antimicrobial and antiviral potentials of marine fungi especially of *Aspergillus* and *Penicillium* are well known (Xu et al. 2015). Among these candidates, fungal strain MUT 3638 was selected, since it showed the best inhibitory activity compared to the other tested strains. The antimicrobial activity of all fungal strains is summarised in Table 3. Therefore, MUT 3638 was selected for up-scaled fermentation, growth optimisation and purification of the molecule involved in the antimicrobial activity.

Growth Optimisation for Antimicrobial Production

MUT 3638 was initially isolated at 15% (*w*/*v*) of salinity in media CYC. In order to optimise the growth conditions for the

Table 3 Growth analysis of fungal strains in cross-streaking experiments carried out using Petri dishes

Target strains	Fungal strains													
	Culture collection codes MUT													
	3588	3589	3596	3597	3610	3612	3613	3614	3615	3616	3617	3618	3632	3638
Staphylococcus aureus 6538P	±	+	+	±	+	+	+	±	±	+	±	+	+	−
Pseudomonas aeruginosa PA01	+	+	±	±	+	±	+	+	+	±	+	+	±	+
Klebsiella pneumoniae DF12SA	+	+	±	+	±	+	±	+	±	±	±	±	±	−
Acinetobacter baumannii Ab13	±	±	+	+	+	±	+	+	+	+	+	+	±	−
Burkholderia metallica LMG 24068	±	+	+	+	±	+	+	±	±	±	+	±	+	−

Symbols: +, non-inhibition, ±, partial inhibition, −, full inhibition

Table 4 Crude extract yield and antimicrobial activity against *S. aureus* of *A. protuberus* MUT 3638 species by optimising growth conditions

Conditions tested			Yield (mg) of crude extract from 500 mL exhausted broth	MIC (mg/mL) against *S. aureus* crude extract
pH	Incubation Time	Temperature (°C)		
pH 4.0	10 days	10	7	1
		25	10	1
		37	12	1
	25 days	10	15	0.5
		25	25	0.06
		37	34	0.06
pH 9.0	10 days	10	7	1
		25	9	1
		37	12	1
	25 days	10	14	0.25
		25	30	0.06
		37	38	0.06

secondary metabolite production, a series of parameters was changed.

The growth medium used for the first isolation (CYC) was replaced with one richer medium (TYS) maintaining the same concentration of salt (15%), in static conditions. In order to evaluate the antimicrobial activity of crude extracts, liquid inhibition assays were carried out against selected pathogenic bacteria. Despite that MUT 3638 was able to inhibit the vast major of pathogens (except *P. aeruginosa*) tested in the initial screening, after the liquid screening of crude extracts, the highest antimicrobial effect was detected towards *Staphylococcus aureus* 6538P with a minimal inhibitory concentration (MIC) of 0.06 mg/mL.

MUT 3638 extract showed antimicrobial activity towards all the tested strains with the exception of *P. aeruginosa* PA01. MIC values were 0.12 mg/mL against both *B. cenocepacia* strains and 0.5 mg/mL against *K. pneumoniae* and *A. baumannii Ab13*.

The impact of NaCl concentration on the growth and antimicrobial activity was then evaluated. The strain MUT 3638 was inoculated in TYS medium with varying NaCl concentrations from 3 to 25% (*w*/*v*), and the inoculum was incubated in the flask at 20 °C for 10 days. Interestingly, considering that the sediments do not come from waters with high salinities, MUT 3638 was able to grow from natural seawater (about 3% of salinity) until 25% (*w*/*v*) of NaCl. Concerning the production of metabolites, the best yield was obtained by adding NaCl at a concentration of 10–15% (*w*/*v*). In fact, from 250 mL of culture yielded 17 mg of crude extracts in presence of 10 and 15% of NaCl after 20 days of incubation. However, the activity of extracts was identical for all conditions tested (i.e. 60 μg/mL as per MIC results); therefore, the concentration of 10% NaCl was selected for growth optimisation.

The effects of incubation time, pH and temperature were subsequently evaluated. To this aim, the fungus was inoculated in TYS medium with 10% (*w*/*v*) NaCl, varying pH (from 4.0 to 9.0) and temperature (10, 25 and 37 °C). The length of incubation was 10 days and a second set for 25 days. After the incubation, the antimicrobial activity was evaluated and results are summarised in Table 4.

Most fungi are mesophilic; however, few fungal species can grow at low or high temperature (Deacon 2006). The *A. protuberus* strain MUT 3638 has shown higher versatility, with the ability to grow at low and high temperatures, and in a wide range of salt concentrations and diverse pH range. This particular behaviour makes this fungus a promising candidate for biotechnological applications.

Extracts from growth at pH 4.0 and pH 9.0 displayed identical antimicrobial activities, while temperature proved to be a key factor. The increase of temperature (25 and 37 °C) resulted in a higher yield and activity of the extract compared to the one obtained from the growth at 10 °C. Moreover, prolonged time of incubation also significantly affected the yield of the extracts, but not an increase of its biomass. Extracts from 25 days of incubation weighed up to three times more than their 10-day counterparts. This finding might suggest that the production of the antimicrobial compounds is triggered by the

Table 5 The inhibitory activity of fractions produced from the active extract of *A. protuberus* MUT 3638 reported as percentage of inhibition of target *S. aureus* 6538P

% Solvent	Concentration [mg/mL]						
	1	0.5	0.25	0.12	0.06	0.03	0.015
25% MeOH	20	20	10	10	10	10	10
50% MeOH	30	30	20	20	20	10	10
100% MeOH	100	100	100	90	70	40	20
100% MetOH + 0.1% TFA	30	30	20	10	10	10	10

Table 6 Metabolite profiling for sorbicillonoid components in the culture broth of *A. protuberus* MUT 3638 by LC-HRMS analysis of its crude extract

Compound	Formula	$[M+H]^+$ (calculated)	Retention time (RT) (min)
Sorbicillin	$C_{14}H_{16}O_3$	233.1165	15.07
Sorbicillinol	$C_{14}H_{16}O_4$	249.1113	21.9
Dihydrosorbicillinol	$C_{14}H_{18}O_4$	251.1278	19.43
Oxosorbicillinol	$C_{14}H_{16}O_5$	265.1071	21.31
Sorbicillactone A	$C_{21}H_{23}NO_8$	418.1502	10.60
Bisvertinol	$C_{28}H_{34}O_8$	499.2319	17.86
Bisvertinolone	$C_{28}H_{32}O_9$	513.2119	21.26
Dihydrobisvertinolone	$C_{28}H_{34}O_9$	515.2276	21.26

activation of the organism's secondary metabolism, in stressed conditions, such as starvation.

Bioactivity-Guided Purification of Aspergillus Bioactive Compounds

Once the best conditions for the production of antimicrobials by *A. protuberus* MUT 3638 to counteract *S. aureus* 6538P were established, an up-scaled fermentation was set up to 1.5 L. After 25 days of incubation, the exhausted broth was extracted, yielding 250 mg of crude extract. The crude extract was subsequently re-suspended in methanol and fractionated using solid-phase extraction (SPE) C-18 column cartridge, by using a water-methanol system as described in "Materials and Methods" ("Purification of Active Compounds from the Newly Isolated *A. protuberus* MUT 3638").

Four fractions were collected, dried and used to perform MIC assays against the pathogens. As reported in Table 5, the fraction eluted with 100% MeOH displayed a strong antimicrobial activity, compared to the other samples with a MIC between 0.25 and 0.112 mg/mL.

Subsequently, the 100% methanol fraction (88 mg) was subjected to purification by reverse phase HPLC by using a C-18 with an ACN/water linear gradient (as specified in "Materials and Methods," "Purification of Active Compounds from the Newly Isolated *A. protuberus* MUT 3638"). The assay against *S. aureus* showed that the peak # 9 is responsible of the activity. The peak # 9 has a retention time (RT) at minute = 26.534 and possesses a MIC of 30 μg/mL and a yield of 3.6 mg.

Preliminary Metabolic Profiling by LC-HRMS Analysis

The metabolic profile of the crude 100% methanol fraction by MUT 3638 strain was analysed by LC/HRMS analysis. The molecular formulas were deduced by HRESI analysis, and the corresponding structures were tentatively assigned by comparison with literature data (Harned and Volp 2011; Meng et al. 2016). The results of this analysis were reported in Table 6. The crude extract contained a complex mixture of monomeric and dimeric members of sorbicillinoid family This growing family of polyketide natural products is comprised of 90 members that have been mainly isolated from fungi of terrestrial origin (*Acremonium*, *Aspergillus*, *Clonostachys*, *Emericella*, *Penicillium*, *Phaeoacremonium*, *Scytalidium*, *Trichoderma* and *Verticillium* genera) and in few marine species (*Paecilomyces*, *Penicillium*, *Phialocephala*, *Trichoderma* and *Trichothecium* genera).

Compound Identification

Bioassay-guided HPLC fractionation of the crude 100% methanol extract revealed the antimicrobial activity exclusively associated to peak # 9. Combined HRESI MS, MS^n and ^1H NMR analysis indicated that peak 9 is a pure compound. An $[M+H]^+$ quasi-molecular ion detected in the HRESIMS spectrum at *m/z* 513.2124 indicated the molecular formula $C_{28}H_{32}O_9$ (calculated for $C_{28}H_{33}O_9$ 513.2119) (see Fig. S3 supplemental material). The presence of a Na adducts at *m/z* 535.1939 was also observed.

The ^1H NMR spectrum (see Fig. S4 supplemental material and "Material and Methods") of peak 9 showed signals superimposable to those reported for bisvertinolone (Trifonov et al. 1986). Indeed, the HRESI MS^2 (see Fig. S5 supplemental material) fragmentation pattern of peak 9 (precursor ion at *m/z* 513.2) matched with the backbone of bisvertinolone and furthermore was in good agreement with $EIMS^2$ data—acquired at unit resolution—reported by Trifonov et al. for

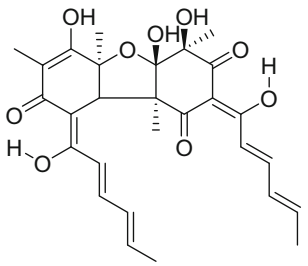

Fig. 2 Structure of the bisvertinolone compound isolated from *A. protuberus* MUT 3638

bisvertinolone. These data led to confirm the identity of peak 9 as bivertinolone.

Bisvertinolone was obtained as a light yellow amorphous powder. An $[M + H]^+$ HRESI MS quasi-molecular ion detected at m/z 513.2124 suggested the molecular $C_{28}H_{32}O_9$ (calculated for $C_{28}H_{33}O_9$ 513.2119) (see Fig. S3 supplemental material).

The compound was identified as bisvertinolone (Fig. 2) by comparison of its optical and NMR data.

Bisvertinolone was firstly detected from the culture medium of *Verticillium intertextum* (Trifonov et al. 1986) and later described as the first β-1,6-glucan biosynthesis inhibitor from the fungus *Acremonium strictum* (Kontani et al. 1994). Several biological activities have been ascribed to members of this family, including radical scavenger activity and antiproliferative activity. Although the data relative to the antiproliferative activity of the sorbicillinoids are fragmentary (Liu et al. 2005), bisvertinolone represents one of the most active compounds. Preliminary results seem to suggest the importance of two unsaturated sorbyl side chain in a correct relative orientation for the observed cytostatic activity. As concerning the antimicrobial activity, there are very few reports in the literature dealing with the investigation of the antibacterial activity of members of sorbicillinoid family. Some monomeric or dimeric sorbicillinoids demonstrated to possess weak activity against *Staphylococcus aureus* and *Bacillus subtilis* (Maskey et al. 2005). Recently, two dimeric sorbicillinoids, dihydrotrichodimerol and tetrahydrotrichodimerol, were reported as potent antibacterial agents against *Bacillus megaterium* with MIC values of 25 μg and 12.5 μg/mL, respectively (Zhai et al. 2016).

Conclusions

By exploiting a bioassay-driven purification approach, the bioactive compound bisvertinolone with antimicrobial activity against *Staphyloccoccus aureus* 6538P was isolated from a new polyextremophilic fungus *Aspergillus protuberus* MUT 3638.

To the best of our knowledge, the antibacterial activity of the bisvertinolone was demonstrated for the first time. Future investigation will be focused on unveiling the mechanisms of action and the scale-up production of the active compound.

Furthermore, this fungus was isolated from cold seawater, but showed a great versatility to different environmental conditions. In particular, it was able to grow in a wide salinity range, as well as in varying ranges of pH and temperature. This capability makes the new strain *A. protuberus* MUT 3638 an ideal candidate for biotechnological applications.

Author Contributions P.C., F.P.E. and P.T. equally contributed to the work. P.C., F.P.E, A.F. and D.d.P. conceived and designed the experiments; P.C., F.P.E., P.T., A.F., E.T., L.T., M.V.D.A., G.G. and G.C.V. performed the experiments; P.C., F.P.E, P.T., A.F. and D.d.P. analysed the data; G.C.V., L.T., M.V.D.A. and D.d.P. contributed reagent/material/analysis tools; P.C., F.P.E., P.T. and D.d.P. wrote the paper.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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