Environmental and metabolomic study of antibiotic production by actinomycetes

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CHAPTER 7

Antimicrobial Effect of Volatile Organic Compounds Released by Metabolically Diverse Actinomycetes

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ABSTRACT

A selection of 44 *Streptomyces* producing antibiotics with efficacy against multi-drug resistant pathogens were classified on the basis of metabolites produced in Trypone Soya Broth medium. NMR in combination with hierarchical cluster analysis was performed for discrimination and resulted in three main groups. Distinct metabolites were abundant in the different groups, including sucrose, benzoic acid and coumarin in group I, II and III, respectively. Moreover, many species released volatile organic compounds (VOCs) that not only had antimicrobial effects but also synergistically increased susceptibility of indicator bacteria to known antibiotics. Further investigation of two active *Streptomyces*, MBT3 and MBT39, using GC-MS analysis led to the identification of various VOCs, among which 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-naphthalene and two isomers of undecene, 10-methyl-1-undecene and 2-methyl-undecane, were identified as the most abundant VOCs excreted by these two species, respectively. Consequently, antibacterial activities of the identified compounds 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-naphthalene, 1-ethylideneoctahydro-7a-methyl -,(1Z,3aα,7aβ)-1H-indene and geosmin were assessed but none of them exhibited antibacterial properties against *Escherichia coli* ET8. Thus, discovering the active VOCs and the possible application of VOCs as antibacterial agents requires further investigation.

INTRODUCTION

Different microorganisms have their own unique metabolite profile, therefore NMR-based metabolomics can be employed for microbial species differentiation and classification (Chauton *et al.*, 2003; Himmelreich *et al.*, 2003). Metabolomic analysis is based on the comprehensive analysis of the low-molecular-weight metabolites that are present in sufficient concentrations, which are downstream of both gene transcription and enzyme activities. Metabolomics therefore provides an altogether different and in some ways also more accurate picture of microbial physiology (Griffin, 2003), and distinguishes metabolism of gene deletions from closely related areas (Allen *et al.*, 2003; Raamsdonk *et al.*, 2001).

Besides the diffusible natural products, volatile organic compounds (VOCs) excreted from microorganisms play an important role in structuring life and fulfilling diverse functions in both natural and artificial systems. VOCs are low in molecular weight (<300 Da) and of low polarity, but high in vapor pressure (Vespermann *et al.*, 2007). A wide variety of VOCs can be produced by bacteria (Chen *et al.*, 2008; Fernando *et al.*, 2005; Gu *et al.*, 2007; Kai *et al.*, 2007; Ryu *et al.*, 2003; Ryu *et al.*, 2004), fungi (Koitabashi *et al.*, 2004; Stinson *et al.*, 2003; Strobel *et al.*, 2001), *Streptomyces* spp. and other species of actinomycetes (Collins & Gaines, 1964; Dickschat et al., 2005; Gerber & Lecheval, 1965; Schöller et al., 2002; Wan et al., 2008). But most studies conducted have focused on metabolites excreted into medium, which are of considerable interest because of their pharmacological properties. Much less effort has been devoted to the analysis of volatiles, despite their often obvious odors and potential that these compounds might act as chemical signals in bacterial ecology (Harris *et al.*, 1986; Schöller *et al.*, 2002). Only bouquets of up to 80 different components has been reported from *Streptomyces* (Dickschat *et al.*, 2005). Although the purpose of volatiles remains unclear, they have been suggested to play a role in communication, growth and defense (Hockelmann *et al.*, 2004; Kai *et al.*, 2007; Schulz & Dickschat, 2007). Furthermore, they can induce resistance against bacterial pathogens in plants (Ryu *et al.*, 2004) or promote their growth (Ping & Boland, 2004). It also has been observed that the appearance of odorous compounds from actinomycetes has coincided with the development of aerial mycelium and spores (Bentley & Meganathan, 1981). Such findings imply that these VOCs may be useful as indicators of morphological differentiation and/or the sporulation process (Schöller *et al.*, 2002).

A number of reports describe volatiles with anti-fungal activity (Vespermann *et al.*, 2007; Wan *et al.*, 2008), however, antibacterial capabilities have been rarely descried. Only

albaflavenone, produced by *Streptomyces*, which inhibits growth of *Bacillus subtilis*, has so far been documented, although the mode of action is still unknown (Gürtler *et al.*, 1994). The objective of this study was to evaluate the potential of VOCs as antibacterial agents using a large collection of actinomycetes, which can produce a diverse range of metabolites and have high bioactivity against MDR pathogens on agar plates.

MATERIALS AND METHODS

Bacterial strains and growth media

The 44 actinomycetes employed in this study (Table 1) were selected from work presented in Chapter 3 (Table 2) and Chapter 4 (Table 3). Gram-positive bacteria *Bacillus subtilis* 168, *Micrococcus luteus* and Gram-negative *Escherichia coli* DH5*α*, and multi-drug resistant *Escherichia coli* ET8, which carries resistance cassettes against tetracyclin, chloramphenicol, streptomycin, kanamycin, neomycin, apramycin and β-lactam antibiotics, were used as indicator strains for antimicrobial activity tests. Fungal indicator strain *Rhizoctonia solani* was also used. Tryptone Soya Broth (TSB medium) was employed for hierarchical cluster analysis. For the volatile assays LB agar was used for the growth of bacteria, glucose casein agar (GCA), soy flour mannitol medium (SFM) and minimal medium (MM) (Kieser *et al.*, 2000) for Streptomyces, and potato dextrose agar (PDA) for fungus.

NMR based metabolomics study of metabolite diversity

A selection of 44 species (Table 1) was grown in liquid Tryptone Soy Broth (TSB) for five days at 30 °C for NMR-based metabolomics studies. Bacterial cultures were harvested by centrifugation and supernatants extracted twice with an equal volume of ethyl acetate (40 ml in total). Two grams of Na_2SO_4 was then added into the organic phase in order to remove remaining water and evaporated under vacuum at 38 °C. Samples were then re-dissolved in Methanol-*d*4 for NMR measurement. ^{1}H NMR spectra were measured as previously described (Kim *et al.*, 2010) and manually phased, baseline corrected and calibrated. ^{1}H NMR spectra were further automatically converted to ASCII files using AMIX software (v. 3.7, Biospin, Bruker). Spectral intensities were scaled to total intensity and the region of δ 0.3-10.0 was reduced to integrated regions of width (0.04 ppm). The regions δ 4.7 - 5.0 and δ 3.30 - 3.34 were excluded from the analysis because of the residual signal of H_2O and methanol-*d4*, respectively. Hierarchical cluster analysis (HCA) was performed with the SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden).

Volatile activity tests

Petri dishes centrally divided by a plastic barrier were used for the analysis of volatile organic compounds (VOCs) on bacterial or fungal growth. One compartment contained GCA agar and the other compartment contained either LB or PDA agar. *Streptomyces* were inoculated on GCA agar and incubated at 30 °C for four days, after which either four dilutions of the

bacterial indicators (10^8, 10^6, 10^4 and 10^2 cfu) or a small cube of agar containing *R. solani* were inoculated in the other compartment. The plates were then sealed and incubated at 30 °C overnight for the bacterial assays or for three days for fungal indicator *R. solani*. The antimicrobial activity was determined by comparing the growth of indicator strains between plates containing *streptomyces* and control plates without volatile-producing *streptomyces*.

The effect of VOCs on the sensitivity of indicator strains to known antibiotics was performed in similar way as described above. A selection of eight of the best volatiles producers were grown on different media, namely GCA, SFM or MM with peptone (0.8% w/v) (MM-P), and following four days incubation the other compartment was overlaid with a lawn of the indicator strains as described above and sterile paper disks containing antibiotics applied on the lawn. Ampicillin, apramycin, kanamycin or chloramphenicol was spotted onto filter discs at 1 mg/ml for *B. subtilis*, while 5 mg/ml and 10 mg/ml of ampicillin and apramycin were used for *E. coli* ET8. Inhibition zones were measured after incubation of sealed plates O/N at 37 °C.

Trapping and GC-MS analysis of bacterial volatiles

For the collection of bacterial volatiles Petri dishes glass lids were designed with an exit to which a steel trap with 150mg Tenax TA and 150mg Carbopack B (Markes International Ltd., Llantrisant, UK) could be fixed (Figure 1). Volatiles were collected at 48 hours and 96 hours, traps were removed, capped and stored at 4°C until analysis. Volatiles were desorbed from the traps using an automated thermo-desorption unit (model Unity, Markes International Ltd., Llantrisant, UK) at 200 °C for 12 min (flow 30ml/min). The trapped volatiles were introduced into the GC-MS (model Trace, ThermoFinnigan, Austin, TX, USA) by heating the cold trap for 3 min to 270°C (Pierre *et al.*, 2011). Split ratio was set to 1:4, and the column used was a 30 mm × 0.32 mm ID RTX-5 Silms, film thickness 0.33 µm (Restek, Bellefonte, PA, USA). Temperature program used was as follows: from 40°C to 95°C at 3°C/min, then to 165°C at 2°C/min, and finally to 250°C at 15°C/min. The VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (33–300 AMU, 0.4 scan/sec).

Identification of VOCs

Compounds were identified by their mass spectra using de-convolution software (AMDIS) and/or Thermo Xcalibur software in combination with NIST 2008 (National Institute of Standards and Technology, USA, http://www.nist.gov) and Wiley 7th edition spectral

libraries and by their linear retention indexes (lri). The lri values were compared with those found in the NIST and the local Iri database of the Netherlands Institute of Ecology (NIOO) in Wageningen. Mass spectra and lri values for identification were also collected from analysis of the pure compounds applied in this study.

RESULTS

Metabolite diversity of 44 selected antibiotic-producing actinomycetes

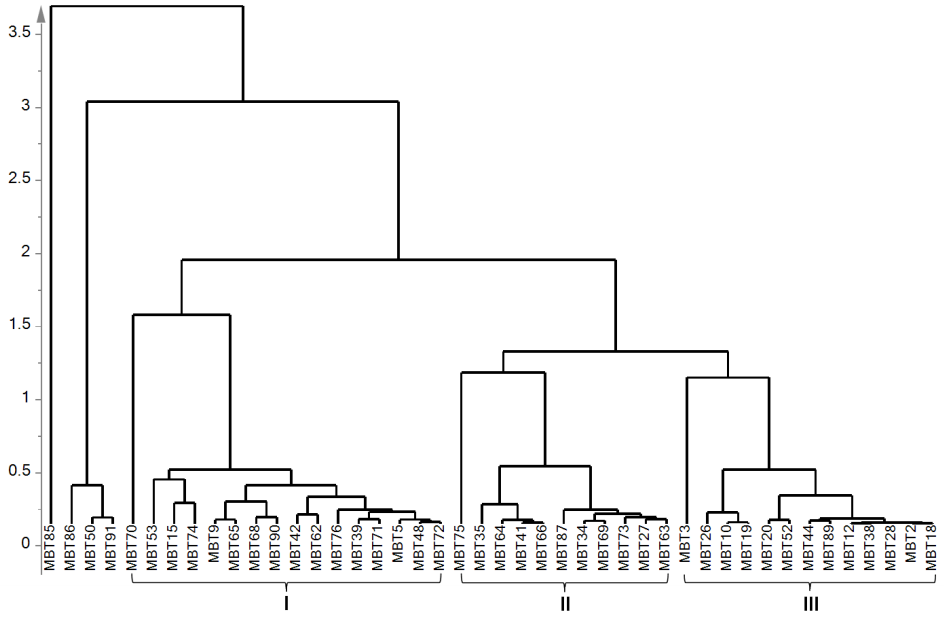

Figure 1. Metabolite Hierarchical cluster of the 44 promising antibiotics producer strains. The hierarchical analysis was calculated with ward and sorted by size.

A collection of 44 actinomycetes from the strain collection (Table 1) (see Table 3 in Chapter 4) was selected for their ability to produce antibiotics that inhibit growth of the multi-drug resistant ESKAPE pathogens (Rice, 2008). Many of these strains only produced bioactive compounds under specific growth conditions, suggesting the possible production of cryptic antibiotics. To compare the metabolic diversity of the strains, NMR-based metabolomics was performed and subjected to multivariate data analysis methods so as to reduce the complexity of the ^{1}H-NMR data. The various multivariate data analysis methods are explained in more detail in Chapters 5 and 6. Principal Component Analysis (PCA) did not provide sufficient information to discriminate between the different species (not shown). Therefore, Hierarchical Cluster Analysis (HCA) was applied. The hierarchical analysis was calculated based on the principal components (PCs) generated by PCA and was sorted by size. According to the similarity of the metabolites produced, the 44 species were clustered in HCA dendrograms and three main groups were formed as shown in Figure 1. Group I

Table 1. Antimicrobial activities of the 44 selected promising antimicrobial producing actinomycetes against six MDR pathogens under four different growth conditions.

Assessed conditions including: 1, MM agar plates with 0.5% mannitol and 1% glycerol as carbon source as control; 2, pH adjusted to 10; 3, with starch (1% w/v); 4, with peptone (0.8% w/v).

	E. faecium				*P. aeruginosa*				*S. aureus*				*K. pneumoniae*				*E. cloacae*				*A. baumannii*			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
MBT2	-	-	-	+	+	-	-	+	++	++	+	++	++	+	+	++	++	+	+	++	+	++	+	++
MBT3	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MBT5	-	+	+	+	-	-	-	+	+	+	+	+	++	+	++	-	+	+	+	++	+	++	++	++
MBT9	-	-	-	-	-	-	-	-	++	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
MBT10	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MBT12	-	+	+	+	-	-	-	+	+	+	+	+	++	+	++	++	+	+	-	+	+	++	++	+++
MBT15	-	-	+	+	+	-	-	-	+	+	+	+	+	+	+	++	+	++	+	++	+	+++	+	++
MBT18	-	+	+	+	-	-	-	+	+	+	+	+	+	+	+	++	+		+	+	+	++	+	+++
MBT19	-	-	-	+	-	-	-	-	+++	+++	++	+++	-	-	-	-	-	-	-	-	-	-	-	-
MBT20	+	-	++	-	+	+	-	-	-	+	+	-	+	+	-	-	+	+	+		+	++	-	-
MBT26	+	-	-	+	-	+	-	-	+	+	+	+	-	++	-	-	-	++	-	+	+	++	-	+
MBT27	+	+	+	-	+	-	+	+	+	+	+	+	++	++	++	-	+	-	+	-	+	+	+	-
MBT28	-	+	+++	+	-	+	-	+	+	+	+	-	+++	+	++	+	++	+	+	+	++	++	++	+++
MBT34	+	+	-	+	-	-	-	+	+	+	-	+	+++	++	-	-	++	++	-	+	++	+++	-	++
MBT35	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+
MBT38	+	+	+	+	-	+	-	+	+	+	+	+	++	+	++	+	+	+	+	+	+++	+++	++	+++
MBT39	+	+	+	+	-	-	-	+	+	+	++	+	+	+	++	+	++	+	+	+	++	+++	++	++
MBT41	-	-	-	-	-	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MBT42	+	+	++	+	-	-	-	-	+	+	++	++	+	+	+	-	+	+	+	-	-	-	-	+
MBT44	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
MBT48	+	-	+++	-	+	-	+	-	+++	-	+++	-	++	-	+	-	++	+	+	-	+	+	+	-
MBT50	-	-	-	+	-	+	-	+	+	+	+	+	++	+++	-	+	+	++	-	+	+	++	-	++
MBT52	-	-	++	-	-	-	-	-	+	+	+	-	+	++	+	+	+	+	+	+	+	+	++	-

MBT53	+	+	+	+	-	+	-	+	+	+	+	+	++	+	+	+	+	+	+	-	++	-	++	++
MBT62	+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+	++	+	+	+	++	+	+
MBT63	-	+	-	-	-	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	++	-	-
MBT64	-	-	-	-	-	-	-	-	+	+++	+	-	-	-	-	-	-	-	-	-	-	-	-	-
MBT65	++	-	-	+	-	-	-	+	+	-	-	-	-	+	-	+	-	+	-	+	-	+	-	++
MBT66	+	-	+++	+++	-	-	-	-	+	+++	+++	+++	-	++	-	-	-	-	-	-	-	++	-	-
MBT68	-	+	-	-	-	+	-	-	++	+	+	-	-	++	-	-	-	++	-	-	-	++	-	-
MBT69	-	+	-	+	+	+	-	+	+	+	+	-	++	+++	+	+	++	++	+	-	++	+++	+	+
MBT70	+++	+	+++	++	+	+	+	-	+++	+++	+++	+++	+	+	+		+	++	+	-	++	+	++	-
MBT71	+	+	++	+	-	+	-	+	+	+	+	+	+	++	+	+	+	+	-	+	+	++	+	++
MBT72	+	+	+	-	-	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
MBT73	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	++
MBT74	+	+	+	+	-	+	-	+	+	-	+	-	-	+	-	-	-	+	-	-	++	+	-	-
MBT75	+	+	+	-	-	+	-	-	+	+	+	+	-	+	+	-	-	+	+	-	-	+	++	+
MBT76	+	+++	+	++	-	-	-	-	+	++	+	+	-	-	-	++	-	-	-	+	-	-	-	+
MBT85	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
MBT86	-	+	+	-	-	+	-	+	-	+	+	-	+	+	-	+	+	+	-	+	+	++	-	+
MBT87	-	+	+	-	-	+	-	+	++	+	+	+	+	+	+	+	+	+	+	+	++	+	++	++
MBT89	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	++	++	+	+
MBT90	+	++	+	-	-	-	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	+	+
MBT91	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

'-': no activity; '+': have activity and diameter of the inhibition zone is < 20mm; '++': diameter of the inhibition zone is ≥20mm but < 30mm; '+++': diameter of the inhibition zone is ≥30mm.

commonly contained sucrose and uracil as well as intensive signals in the low magnetic field, where aromatic compounds are expected. In particular, complex signals of phenazine were observed in the metabolic profiling of MBT70, which separated it from the other species in group I. Metabolite profiles of the species assigned in group II all had compounds related to benzoic acid and lipids in the high magnetic field. Moreover, detection of phenylacetic acid and mannosucrose in MBT75 separated this species from all other strains, thus resulting in an independent branch in group II (Figure 1). The species clustered in group III were distinguished from the others groups because of the specific production of coumarins. High intensity of peaks related to phenylpropanoid was observed in MBT3. Besides, the metabolite profile of MBT85, which produced large amounts of sucrose, and MBT86, which produced large amounts of phenylacetic acid, the oxidation product of phenylalanine, was distinct from that of the other species. The comparison and evident difference among the metabolic profiles of the 44 strains confirmed the production of a diverse range of secondary metabolites, and - as suggested by the particularly high bioactivity against MDR pathogens - most likely many antibiotics.

Antimicrobial activity of volatile compounds

Having established the metabolic diversity of the 44 prolific antibiotic producers, we set out to further analyse the antimicrobials produced by these species. Detailed analysis of MBT70 and MBT76 are provided in Chapters 5 and 6, respectively. In this Chapter, rather than on diffusible compounds, the focus lies on an initial analysis of the volatile compounds (VOCs) produced by these strains. Little is known of the bioactivity of VOCs, despite that they have already been shown to have antifungal activity (Vespermann *et al.*, 2007; Wan *et al.*, 2008). We therefore analysed the VOCs produced by these 44 actinomycetes to establish if the VOCs also have antimicrobial activity. For this, actinomycetes were grown for four days on one half of an agar plate centrally divided by a plastic barrier to prevent diffusion of compounds through the agar; after four days, the indicator strain was then plated in dilutions on the other half (see Materials and Methods for further details on set-up and dilutions). Surprisingly, 17 inhibited growth of *M. luteus*, four of *B. subtilis*, eight of *E. coli* DH5α and 23 of *E. coli* ET8. The strains that produced VOCs only can inhibit growth of *B. subtilis* at the lowest density (10^2 colony forming units or cfu), while 16 out of 44 inhibited growth of *M. luteus*, with stronger inhibition of growth (Table 2, Figure 2). Furthermore, eight actinomycetes produced volatiles that suppressed growth of the Gram-negative *E. coli* DH5*α*, one of which suppressed growth even at the highest concentration of cells (10^8 cfu). No fewer than 24 strains produced volatiles that inhibited growth of *E. coli* ET8, which

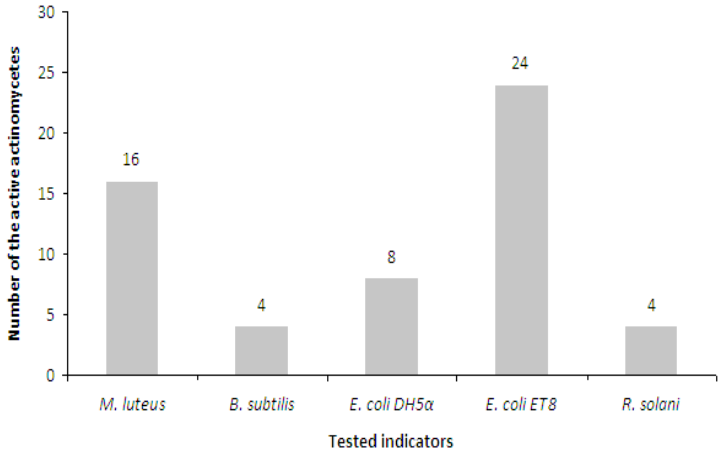

Figure 2. The number of the 44 actinomycetes producing volatiles active against different indicator strains. The horizontal axis represents the indicator strains tested including *Micrococcus luteus*, *Bacillus subtilis*, *Escherichia coli* DH5α, *Escherichia coli* ET8 and fungus *Rhizoctonia solani*, and the numbers on the vertical axis represent the amount of strains active against the indicators.

Table 2. Antibacterial activity of the volatile compounds produced by the 44 selected actinomycetes. The assayed bacterial indicators include *Micrococcus luteus*, *Bacillus subtilis*, *Escherichia coli* DH5 α and *Escherichia coli* ET12567 and the series of concentration are 10^8, 10^6, 10^4 and 10^2 cfu respectively.

Species NO.	*M. luteus*	*B. subtilis*	*E. coli* DH5α	*E. coli* ET8
		Group I		
MBT5	O O O O	O O O O	O O O O	O O O O
MBT9	O O	O O O O	O O O O	O O O O
MBT15	O O O O	O O O O	O O O O	O O O O
MBT39	O O	O O O	O	O
MBT42	O O O O	O O O O	O O O O	O O O O
MBT48	O O O O	O O O O	O O O O	O O O O
MBT53	O O O O	O O O O	O O O O	O O O O
MBT62	O O	O O O	O O O O	O O O O
MBT65	O O O O	O O O O	O	O O O O
MBT68	O O	O O O O	O O O O	O O
MBT70	O O	O O O O	O	O O
MBT71	O O O O	O O O O	O O O O	O O O O
MBT72	O O	O O O O	O O O O	O O O O
MBT74	O O O O	O O O O	O O O O	O O O O
MBT76	O O	O O O O	O O O O	O
MBT90	O O O O	O O O O	O O O O	O O O O

Group II				
MBT27	O O O O	O O O O	O	O O O O
MBT34	O O O O	O O O O	O	O O
MBT35	O O O O	O O O O	O O O O	O O O O
MBT41	O O O O	O O O O	O O O O	O O
MBT63		O O O O	O O O O	O O O O
MBT64	O O	O O O O	O O O O	O O
MBT66	O O O O	O O O O	O O O O	O O
MBT69	O	O O O O	O O O O	O O
MBT73	O O	O O O	O O O O	O O
MBT75	O O O O	O O O O	O O O O	O O O
MBT87	O O O O	O O O O	O O O O	O O
Group III				
MBT2	O O O O	O O O O	O O O O	O O O O
MBT3	O O	O O O O		O O
MBT10	O O O O	O O O O	O O O O	O O O O
MBT12	O O O O	O O O O	O O O O	O O
MBT18	O O O O	O O O O	O O O O	O O O
MBT19	O O	O O O O	O O O O	O O O O
MBT20	O O O O	O O O O	O O O O	O O
MBT26	O O O O	O O O O	O O O O	O O O O
MBT28	O O	O O O O	O	O O
MBT38	O O O O	O O O O	O O O O	O O
MBT44	O O O O	O O O O	O O O O	O O
MBT52	O O O O	O O O O	O O O O	O O
MBT89	O O	O O O	O O O O	O O
Others				
MBT50	O O O O	O O O O	O O O O	O O O O
MBT85	O O O O	O O O O	O	O O
MBT86	O O O	O O O O	O O O O	O O
MBT91	O O O O	O O O O	O O O O	O O

Note: the circles from left to right represent the concentration of tested indicators, which are 10^8, 10^6, 10^4 and 10^2 cfu, respectively. Four circles indicate no growth inhibition was observed; three circles indicate the assessed actinomycete could inhibit growth of the bacteria at a concentration of 10^2 cfu, two circles at 10^4 and 10^2 cfu, one circle at 10^6, 10^4 and 10^2 cfu, zero circles indicate the inhibition was detected at all the tested concentrations.

carries multiple resistant cassettes to the diffusible known antibiotics. 20 strains produced volatiles that suppressed growth of *E. coli* ET8 at a density of 10^4 cfu, two at 10^6 cfu and two at 10^2 cfu (Table 2, Figure 3). Some species produced VOCs that inhibited multiple indicator strains (Table 2, Figure 3). VOCs produced by MBT3 suppressed growth of *E. coli* DH5*α* at 10^8 cfu, *M. luteus* and *E. coli* ET8 at 10^4 cfu, respectively; VOCs produced by MBT76 inhibited growth of *E. coli* ET8 at 10^6 cfu and *M. luteus* at 10^4 cfu, while those produced by MBT70 inhibited growth of *E. coli* DH5*α* at 10^6 cfu, *M. luteus* and *E. coli* ET8 at 10^4 cfu,

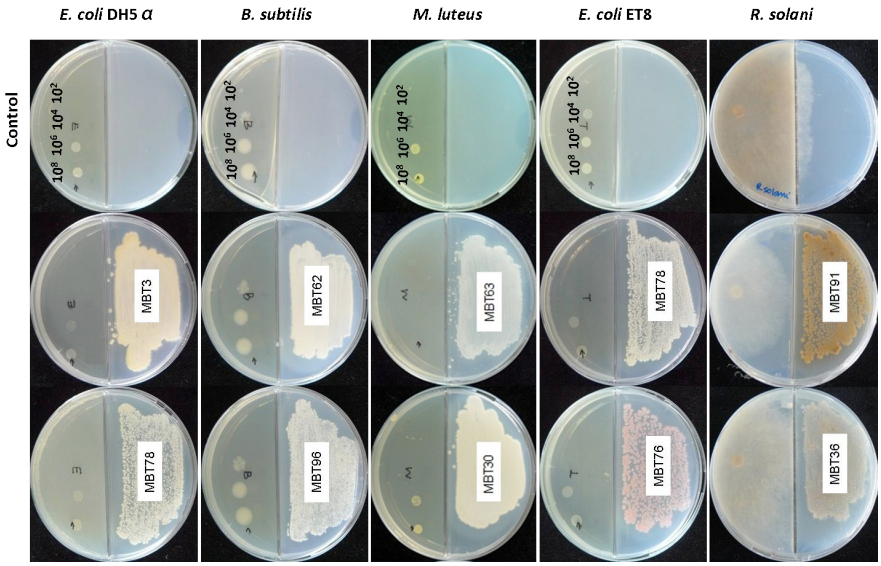

Figure 3. Activity of volatiles produced by the collected strains against *E. coli* DH5α, *B. subtilis*, *M. luteus*, *E. coli* ET8 and *R. solani*. The activity was assessed by comparison of growth of the indicator strains on control plates (without pre-grown actinomycetes in the other compartment) and in plates with actinomycetes. The indicator strains were spotted as dilutions of 10^8, 10^6, 10^4 or 10^2 cfu.

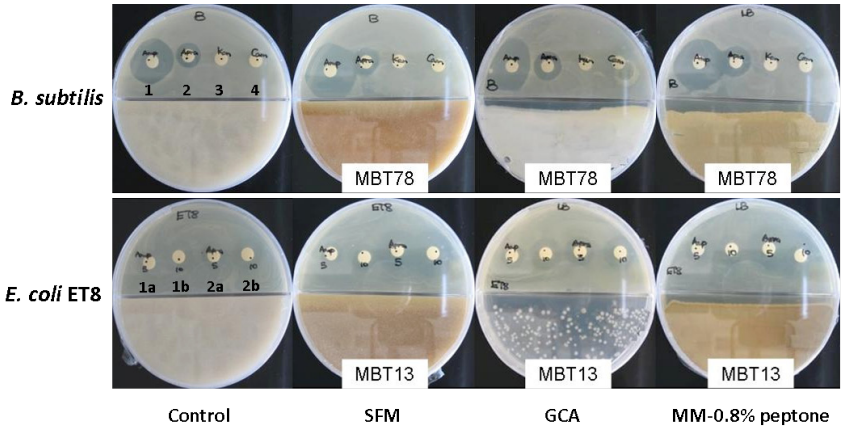

Figure 4. The effect of volatiles on the susceptibility of *B. subtilis* and *E. coli* ET8 to known antibiotics. Tested antibiotics include ampicillin, apramycin, kanamycin and chloramphenicol. The concentration of 1mg/ml of ampicillin (**1**), apramycin (**2**), kanamycin (**3**) and chloramphenicol (**4**) were used for test against *B. subtilis*, while 5mg/ml and 10ml/ml of ampicillin (**1a** and **1b**) and apramycin (**2a** and **2b**)were used for test against *E. coli* ET8.

respectively. *M. luteus* failed to grow entirely when inoculated on a plate with pre-grown MBT 63. The volatile test with *B. subtilis* as indicator strain was not successful as poor growth was observed in the control experiment.

In terms of bioactivity against fungi, despite that it was previously shown that VOCs inhibit fungal growth, only four of the 44 actinomycetes produced VOCs that suppressed growth of the fungus *R. solani* (Figure 2). Of these four, MBT 91 was the strongest suppressor of *R. solani*.

Table 3. The effect of VOCs produced by the eight selected *Streptomyces* on the susceptibility of the *B. subtilis* and *E. coli* ET8 to known antibiotics

Strains	Media	*B. subtilis*				*E. coli* ET8			
		Amp	Apra	Kan	Cam	Amp		Apra	
		1mg/ml				5mg/ml	10mg/ml	5mg/ml	10mg/ml
Control	MM-P[b]	19	11	-	-	-	-	12	14
	GCA	19	11	-	-	-	-	12	14
	SFM	19	11	-	-	-	-	12	14
MBT13	MM-P	22	14	-	-	10V[a]	13V	19	21
	GCA	21	15	-	-	9V	13V	14	16
	SFM	22	14	-	-	10V	12V	19	21
MBT61	MM-P	22	15	-	-	9V	13V	13	15
	GCA	23	14	-	-	10V	13V	13	14
	SFM	23	14	-	-	11V	14V	12	15
MBT29	MM-P	22	15	-	-	9V	13V	13	15
	GCA	22	12	-	-	8V	13V	13	15
	SFM	19	14	-	-	9V	14V	13	14
MBT74	MM-P	20	15	-	-	8V	13V	16	19
	GCA	20	15	-	-	9V	13V	16	18
	SFM	20	13	-	-	8V	13V	16	19
MBT76	MM-P	19	14	-	-	9V	12V	14	17
	GCA	20	14	-	-	10V	13V	13	15
	SFM	19	15	-	-	10V	13V	15	16
MBT78	MM-P	24	14	-	-	9V	12V	19	20
	GCA	23	14	-	-	11V	14V	13	15
	SFM	26	13	-	-	10V	13V	12	14
MBT85	MM-P	21	14	-	-	-	-	15	16
	GCA	20	14	-	-	-	-	15	15
	SFM	22	14	-	-	-	-	15	16
MBT95	MM-P	23	15	-	-	11V	15V	13	14
	GCA	23	15	-	-	12V	14V	14	15
	SFM	22	14	-	-	10V	14V	12	14

Note: “Amp” is ampicillin, “Apra” is apramycin, “Kan” is kanamycin and “Cam” is chloramphenicol .

[a]: vague inhibition zones; [b]: Minimal medium supplimented with 0.8% peptone.

Interestingly, besides their antimicrobial activity, VOCs also enhanced the susceptibility of the indicator bacteria to known antibiotics (Table 3). As such a synergistic effect was observed Figure 4, For example, VOCs produced by MBT78 greatly increased the sensitivity of *B. subtilis* to ampicillin, while VOCs produced by MBT13 improved the susceptibility of *E. coli* ET8 to both ampicillin and apramycin. However, no major differences were observed in terms of the activity of the VOCs produced on the different media. SFM and MM with peptone (0.8% w/v) generally gave slightly bigger inhibition zones than GCA, most likely because they supported better growth. Generally speaking, it was difficult to assess the precise amount of biomass produced - which varied per *Streptomyces* strain - and this influenced the amount of VOCs produced.

Analysis of VOCs by GC-MS

The identification of volatiles is usually accomplished using gas chromatography coupled with mass spectrometry (GC-MS) in electron ionization mode (EI), but sometimes other approaches such as membrane-inlet mass spectrometry (Petersen *et al.*, 2004) or isolation followed by NMR spectroscopy are used (Beck *et al.*, 2003; Gürtler *et al.*, 1994). Several large general mass spectral libraries such as the Wiley and the NITS library (McLafferty, 2005) are available, but more specialized, critically evaluated libraries for volatile compounds, for example Mass finder (König *et al.*, 2005), are sometimes more useful. To investigate which VOCs were responsible for the observed antibacterial activity, the isolates MBT3 and MBT39 that were shown to produce VOCs with significant antimicrobial activity were analyzed further. The culture medium is itself a source of volatiles, particularly as the autoclaving process forms several volatiles (Demilo *et al.*, 1996). Thus, MBT2 that did not produce antimicrobial VOCs was included as a negative control to nullify the inactive compounds. As a blank, the media alone was also processed to distinguish the VOCs of the medium from those of the *Streptomyces*.

GC-MS analyses showed that eight potential volatile agents released from cultures of MBT3 grown on autoclaved GCA were tentatively identified on the basis of their mass spectral properties as compared with those in the NIST database (McLafferty, 2005) and the local database of the Netherlands Institute of Ecology (NIOO) (Table 4). The most abundant VOC produced by MBT3 that was not produced by any of the controls was 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-naphthalene, followed by 1α-Hydroxy-12-methoxy-19-nor-5β-podocarpa-3,8,11,13-tetraen-2-one and trans-1,10-dimethyl-trans-9-decalol (geosmin) (Figure 5 and Table 4). Chemicals of lower intensity included 1,1,4,4-tetramethyl-2,6-bis(methylene)-cyclohexane, 1H-Benzocycloheptene,4,4a,5,6,7,8,9,9a-octahydro-4a-

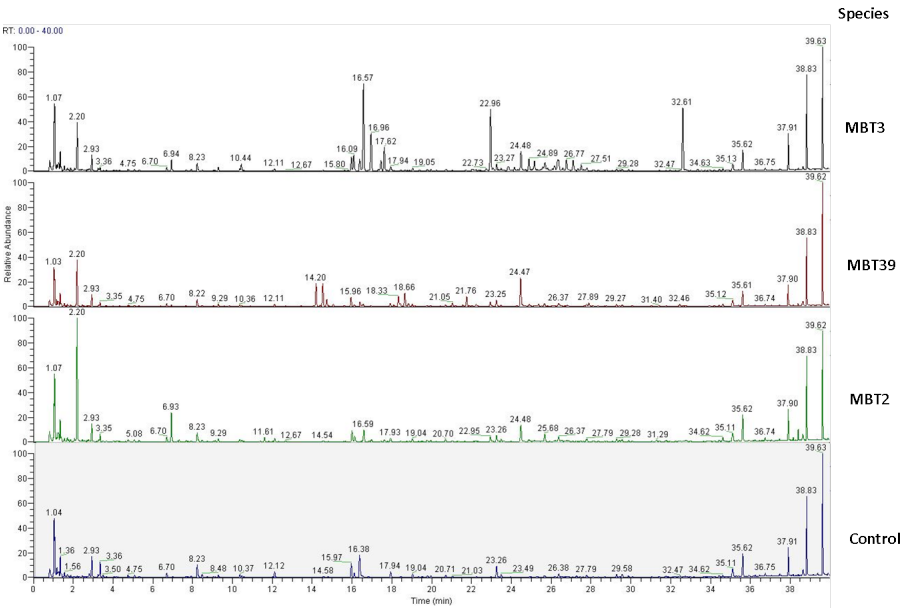

Figure 5. Chromatograms of volatiles collected from *Streptomyces* sp. MBT3, MBT39, MBT2 and empty medium following four days incubation.

Table 4. GC-MS analysis of VOCs produced by *Streptomyces* sp. MBT3 and MBT39

Species	Iri[a]	RT[b]	Possible VOCs	Formula	Exact Mass
MBT3	1193	16.00	Dodecane	$C_{12}H_{26}$	170.2034
	1212	16.09	1H-Benzocycloheptene, 4,4a,5,6,7,8,9,9a-octahydro-4a-methyl	$C_{12}H_{20}$	164.1565
	1217	16.57	1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-naphthalene	$C_{12}H_{20}$	164.1565
	1222	16.96	1,1,4,4-tetramethyl-2,6-bis(methylene)-cyclohexane	$C_{12}H_{20}$	164.1565
	1239	17.45	1-ethylideneoctahydro-7a-methyl-, (1Z,3aα,7aβ)-1H-indene	$C_{12}H_{20}$	164.1565
	1395	22.96	Geosmin	$C_{12}H_{22}O$	182.1670
	1432	24.89	2-methylene-5-(1-methylvinyl)-8-methyl- bicyclodecane	$C_{15}H_{24}$	204.1878
	2242	32.61	1α-Hydroxy-12-methoxy-19-nor-5β-podocarpa-3,8,11,13-tetraen-2-one	$C_{17}H_{20}O_3$	272.1412
MBT39	1154	14.20	10-methyl-1-undecene	$C_{12}H_{24}$	168.1878
	1185	14.56	2-methyl-undecane	$C_{12}H_{26}$	170.2034
	1193	15.96	Dodecane	$C_{12}H_{26}$	170.2034
	1161	18.33	9-methyl-1-undecene	$C_{12}H_{24}$	168.1878
	1271	18.66	3-methyl-dodecane	$C_{13}H_{28}$	184.2191
	1372	21.76	2-Dodecanone	$C_{12}H_{24}O$	184.1827
	1395	22.93	Geosmin	$C_{12}H_{22}O$	182.1670
	1400	23.25	Tetradecane	$C_{14}H_{30}$	198.2347

Note: the minor peaks and the peaks presenting in the controls were omitted from the total nalysis. Compounds detected in the control medium are not included in this table.

[a] Iri , linear retention indexes; [b] RT, retention time on the GC-MS.

methyl, dodecane, 1-ethylideneoctahydro-7a-methyl-(1Z,3aα,7aβ)-1H-indene and 2-methylene-5-(1-methylvinyl)-8-methyl- bicyclodecane. In contrast, MBT39 had a distinct GC profile from MBT3 and fewer signals were observed. The most abundant of these were compounds with retention times of 14.20 and 14.56 min, which were identified as two isomers, 10-methyl-1-undecene and 2-methyl-undecane (Figure 5 and Table 4). Much lower intensity of geosmin was detected in MBT39 than in MBT3. Dodecane, 9-methyl-1-undecene, 3-methyl-dodecane and 2-dodecanone are potentially interesting as they were absent in MBT2 and in the media control.

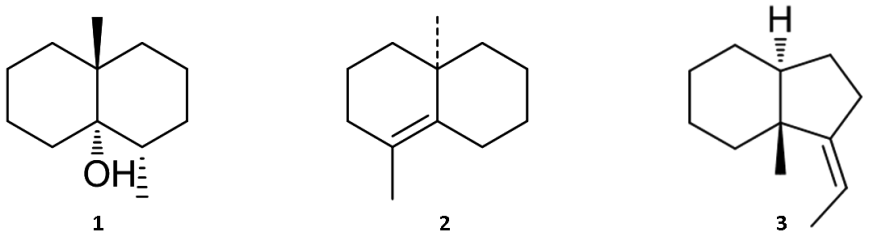

Figure 6. Structures of the volatiles identified and assessed for antibacterial activity. Compounds: **1**: 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2- naphthalene, **2**: geosmin, **3**: 1-ethylideneoctahydro-7a-methyl-(1z, 3aα,7aβ)-1H-indene .

The antibacterial activity of several of the major VOCs that were produced by MBT3 and not by any of the controls, were tested as pure compounds. These were geosmin, 1-ethylideneoctahydro-7a-methyl-(1Z,3aα,7aβ)-1H-indene and 1,2,3,4,4a,5,6,8a-octahydro -4a,8-dimethyl-naphthalene, of which the structures were showed in figure 6. However, no clear inhibition was seen in a test with *E. coli* ET8 as the indicator strain, neither separately or in combination, indicating that other VOCs were responsible for the observed antibiotic activity.

DISCUSSION

Virtually all of the research carried out on actinomycetes regarding their secondary metabolites is based on compounds isolated from the culture fluid of liquid-grown cultures. Volatile compounds (VOCs) are typically produced at low levels when the producers are under stress due to the presence of competitors, and are generally regarded as signaling molecules (Kai *et al.*, 2007; Schulz & Dickschat, 2007). Several studies described that VOCs can inhibit the growth of fungi (Dorman & Deans, 2000; Inouye *et al.*, 2000; Inouye *et al.*, 2001; Vespermann *et al.*, 2007), however, albaflavenone is so far the only example of a VOC that inhibits the growth of bacteria, in this case *B. subtilis* (Gürtler *et al.*, 1994). The current study demonstrates that there are probably multiple VOCs produced by actinomycetes displaying antimicrobial activity.

In this study we used a subset of 44 strains from the culture collection presented in Chapter 3. These were identified as very good antibiotic producers, and all of these strains inhibited growth of MDR clinical isolates. The evident differences between the metabolic profiles of these strains that suggested that rather distinct antibiotics may be responsible for those activities. HCA analysis of these 44 actinomycetes allowed the strains to be clustered by the families of compounds they produced. HCA analysis is a fast dereplication tool for selecting the most promising producers for further investigation as it can provide structural information of the metabolites produced, and is in principle applicable to identify the strains in a collection that are the most promising isolates to focus on for drug discovery efforts. This resulted in three main subgroups, which included 40 of the 44 strains. For subgroups II and III, ten strains produced VOCs that inhibit growth of other microbes, while eight in subgroup I. VOCs produced by the strains assigned to subgroup II exhibited relatively high antimicrobial activity, inhibiting growth of one or more indicator bacteria at higher cfu counts. We did not observe a particular correlation between bioactivity and the metabolome profiles.

In general, of the 44 strains tested, 17 inhibited growth of *M. luteus*, four of *B. subtilis*, eight of *E. coli* DH5α and 23 of *E. coli* ET8. This is a surprising as compared to those typically obtained for soluble antibiotics, where many more antibiotics are active against the Gram-positive bacterium *B. subtilis* than against the Gram-negative *E. coli*. Among the four indicator bacteria, *E. coli* ET8 was more sensitive to VOCs than the other bacteria. *E. coli* ET8 carries several mutations reduce growth rate, and while it harbours many drug resistance cassettes, these do not confer resistance to VOCs. The slow growth of *E. coli* ET8

may explain the increased susceptibility to VOCs. The trend shown in these experiments suggests that that VOCs are perhaps more active against Gram-negative than against Gram-positive bacteria, which is promising from the perspective of drug discovery, as infections with Gram-negative bacteria are a particularly large problem in the clinic.

Some noteworthy strains are highlighted here. VOCs released by MBT3 and MBT63 had strong antibacterial activity, and completely inhibited growth of *E. coli* DH5α and *M. luteus*, respectively. Many species produced VOCs with a broad host range in terms of growth inhibition, or produced multiple VOCs with antimicrobial activity. As an example, VOCs produced by MBT39 repressed the growth of all bacterial indicator strains, while phenazine producer MBT70 restrained growth of *M. luteus* and *E. coli* ET8 at 10^4 cfu, *E. coli* DH5α at 10^6 cfu, as well as the fungus *R. solani*. *R. solani* can cause seedling damping-off or root rot of numerous plants (Gill *et al.*, 2001). The inhibition effects of VOCs produced by MBT48, MBT53, MBT64 and MBT70 on *R. solani* observed in this study indicates the possibility of further exploitation as a bio-fumigant for control of this economically-important plant pathogen.

In contrast to the strong influence of the culturing conditions on the production of soluble antibiotics, as shown for example in Chapters 4-6, no significant effect of media were seen in terms of VOCs activity. This conflicts with previous reports that the culture media had a strong influence on the compounds or compound classes produced, although in these experiments related to VOC production by a fungus (*Chondromyces crocatus*) and not by a streptomycete (Schulz *et al.*, 2004; Schulz & Dickschat, 2007)

It is important to note that besides the direct effects of VOCs, *i.e.* as antibiotic compounds, our experiments also showed that VOCs can synergistically improve the susceptibility of bacteria to more traditional (soluble) antibiotics. This could be applied by inhalation of VOCs by patients suffering from for example lung infections in combination with treatment with more traditionally applied antibiotics.

One important question to answer is in how far VOCs with antimicrobial activity have the potential to be developed as antimicrobial agents for medical and/or commercial applications. To get more insight into the compounds that are produced, MBT3 and MBT39 were analysed by GC-MS, and compared to control samples from strains that did not inhibit growth of any of the indicator bacteria. This revealed the production of 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-naphthalene by MBT3 and 10-methyl-1-undecene and 2-methyl-undecane by MBT39, which were not observed in the control samples. Geosmin (trans-1,10-dimethyl-trans-9-decalol), its name reflecting the characteristic earthy smell it lends to the soil, was abundantly produced by MBT3 but could hardly be detected in MBT39

or in the negative control MBT2. Besides by *Streptomyces*, this volatile compound is also produced by numerous other microorganisms, including *Penicillium* spp., *Aspergillus* spp., non-cyanobacteria and cyanobacteria (Carpenterboggs et al., 1995; Gerber & Lecheval, 1965; Gerber, 1967; Juttner & Watson, 2007; Schöller et al., 2002). 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-naphthalene is a component of antimicrobial volatiles in essential oils of wood or volatile constituents of propolis, the resinous mixture that bees collect from botanical sources (Cavaleiro *et al.*, 2006; Laouer *et al.*, 2009; Pyun & Shin, 2006; Vukovic *et al.*, 2008). Tests with pure compounds revealed no bioactivity for 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-naphthalene, geosmin or 1-ethylideneoctahydro-7a-methyl-(1Z,3aα,7aβ)-1H-indene, neither as single compounds nor in combination. This suggests that other, less abundant, VOCs may cause the antibiotic activity.

In conclusion, our work provides a first proof of principle for the use of volatile compounds as antimicrobial agents. Thus, in conjunction with searching for more traditional antibiotics, an effort should also be made to incorporate searches for VOCs in drug-discovery approaches, whereby the synergistic effect of VOCs with other antibiotics is a particularly promising line of research. Very little is known of the effect and applicability of VOCs, and factors such as efficacy, spectrum of applications and cytotoxicity should be investigated. Above all, however, the major initial challenge clearly lies in determining the precise components that cause the antimicrobial activity observed in the experiments presented in this work. This is currently under investigation.