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ORIGINAL PAPER

Tromethamine and dodecanol appear to be the major secondary metabolites of *Streptomyces decoyicus* M*

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Abstract

An isolate of *Streptomyces decoyicus* M* (code of the isolate) was identified by the sequencing of 16S rRNA gene. It was grown on solid media and secondary metabolites were extracted with *n*-butanol. The extract was dried and run in a sodium dodecyl sulphate–polyacrylamide gel (SDS–PAGE, 10%). Two main bands obtained were sliced and the metabolites were regained in *n*-butanol. These two samples were then identified by gas-chromatography–mass spectrometry (GC–MS), and Fourier-transform infrared spectroscopy (FT-IR). The results demonstrated that tromethamine- and 1-dodecanol were the main constituents (band 1: 61% and 17.7%; band 2: 41% and 54%, respectively). This finding maintained that the isolate of *Streptomyces decoyicus* produced high amounts tromethamine- and 1-dodecanol under the conditions investigated.

Keywords 1-Dodecanol · SDS-PAGE · Secondary metabolite · *Streptomyces decoyicus* · Tromethamine

Introduction

Emerging infectious agents as well as the problem of resistance necessitates the discovery and development of novel antimicrobial compounds (Berdy 2012; Manivasagan et al. 2013). *Actinomycetes* have been one of the most versatile microorganisms in this field as they constitute a rich reservoir of diverse secondary metabolites (Berdy 2012) that are not primarily required for the survival of the producing organism. Richness in diversity of *Actinomycetes* also continue to feed this inexhaustible reservoir (Mohan 2018; Elmallah et al. 2020) for a half century (Mincer et al. 2002; Dewi et al. 2017; Dror et al. 2020; Selim et al. 2021). Beside antibiotics, their secondary metabolites harbour many medically important biological compounds, spanning from antitumoral agents to immunosuppressants (Mincer et al. 2002).

Actinomycetes can be found in both aquatic and dry habitats (Pathalam et al. 2017). They reside in the transition zone between prokaryotes and eukaryotes. This is why they resemble fungi in morphology (Chaudhary et al. 2013). Their chromosome is linear and their DNA is rich in guanidine and cytosine nucleotides (Nayaka et al. 2020).

More than 20,000 microbial bioactive secondary metabolites have been isolated and nearly one third of these come from the species of a genus of *Actinomycetes*, namely *Streptomyces* (Vimal et al. 2009). Many of these compounds are potent antibiotics and this renders *Streptomyces* the main workhorse in antibiotic-producing efforts (Jensen et al. 2007; Ramesh et al. 2009; Valli et al. 2012). As most of the soil *Actinomycetes* are *Streptomyces* (Goodfellow and Simpson 1987), their compounds have also been exploited in the diverse fields of agriculture (Berdy 1995; Dewi et al. 2017).

Synthetic compounds still constitute the main body of the pharmacological inventory as they are well suited the needs of mass production, and high-throughput efforts for the discovery of their natural alternatives obstinately stagger behind such a gigantic global industry, because the core skeletons of most natural products, such as the antiparasitic drug Ivermectin, the antihyperlipidemic drug Pravastatin, and the anticancer agent Eribulin, have unique chemical structures found only in nature. Thus, screening natural products is important to enrich such versatile chemical structures.

This study showed that an electrophoresis system (SDS-PAGE) frequently used in molecular biology can be a versatile tool for the resolution of chemical compounds. The approach can be improved by adjusting the system

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parameters to the chemistry of the compounds, and its capacity can be improved by increasing the size of the instrumentation.

In this study, an isolate of *Streptomyces decoyicus* was used to produce tromethamine- and 1-dodecanol. These two metabolites were the predominant secondary metabolites that could be resolved in an SDS–polyacrylamide gel and separated into two distinct bands. Elution from the gel was achieved using *n*-butanol. After the elution the samples were subsequently identified by Gas Chromatography-Mass Spectrometry (GC–MS) and confirmed by Fourier-transform infrared spectroscopy (FT-IR).

Materials and methods

Isolation of bacteria

Twenty-five *Streptomyces* membres were isolated from red soil samples (Çamlıbel, Tokat, Turkey). Çamlıbel is located between latitude 40.087505 and longitude 36.475414. Samples were collected in August 2020. The soil was excavated approximately 10 cm and the soil samples were taken into sterile falcons and stored at + 4 °C.

Soil sample, 10 g, was homogenized in 90 ml of NaCl (0.85%) for 2 h at room temperature. Dilutions, up to 10^{-5}, were spread, to obtain single colonies, on LB-agar plates (g/L^{-1} 10 g peptone, 5 g yeast extract, 10 g NaCl, and 15 g agar, pH 7) and incubation took place at 37ºC for 48 h. Morphological characterisation involved Gram staining, light microscope and scanning electron microscopy (SEM). Pure isolates were then stored in 20% glycerol at -80 °C (UshaNandhini et al. 2018).

Species identification by 16S rRNA gene sequencing

Genomic DNA was prepared by utilizing the HotSHOT DNA extraction method (Lunt 2017). The V3-V4 variable region of 16S rRNA were amplified using the primers from Klindworth et al. (2013). DNA sequencing was performed in an Illumina MiSeq instrument in paired-end mode with 2 × 250 nucleotide read length at Sivas Cumhuriyet University Advanced Technology Research and Application Center (CUTAM). Sequencing data were queried in the BLAST database and the results were recorded (Camacho et al. 2009). Isolates belonged to *Streptomyces decoyicus* were Multiple Alignment using Fast Fourier Transform program (MAFFT) (Katoh et al. 2009). In addition, phylogenetic trees were created using the Kimura-2 genetic distance model and Neighbour-joining (NG) method to determine the relationship between the samples using aligned data (Kimura 1980; Tamuraet al. 2013). Bootstrap method was used to test the tree topology and it was repeated for 500 times. An accession number for these sequences has also been obtained from GenBank: MZ159946.

Secondary metabolite production

Streptomyces decoyicus cells in glycerol stocks were first activated by growing overnight at 37 °C in 50 ml LB broth. Secondary metabolites were produced in solid medium (250 ml of LB with 0.15% agar in 25 cm diameter glass pots) by spreading 100 µl of 72 h culture, and then incubating until the colour of the agar darkened. The metabolites were then eluted into 100 ml of n-butanol overnight at room temperature. The extract was clarified by filtration and the organic solvent was evaporated at 70 °C. The dry extract was stored at − 20 °C (Çetinkaya 2021; Çetinkaya et al. 2021).

Thin layer chromatography

A portion of the total extract was resolved by thin layer chromatography (silica gel 60, Merck) using chloroform: methanol (10:1, v/v). Bands were visualized under ultraviolet light at 200 400 nm (Thirumurugan et al. 2018). Bands were showed peaks in the range of 190 nm. Retention factors (Rf) of the bands were then calculated (Bundale et al. 2018).

Purification of secondary metabolites

Electrophoresis is a method used to separate charged molecules in an electric field. Gel electrophoresis, on the other hand, is based on the technique of separating molecules according to charge, size and conformation differences using a porous support medium. Using this versatile system, the secondary metabolite extracts could be partially purified.

The secondary metabolite extract was resolved in 10% master gel [30% acrylamide: bisacrylamide, 1.5 M Tris–HCl, pH 8.8, 10% SDS, 10% ammonium persulfate (APS)]. A stacking gel (5%) was loaded onto the main gel (30% acrylamide: bisacrylamide, 1.5 M Tris–HCl, pH 6.8; 10% SDS, 10% APS). Electrophoresis was performed for 2 h at 70 V. Bands visible to the naked eye were sliced out and metabolites were separated in *n*-butanol overnight at 4 °C. Organic phase was removed by evaporation.

Identification of secondary metabolite

Molecular determination was made using GC–MS analysis to identify the substances in the total extract. In addition, FT-IR spectroscopy analysis (CUTAM Central Laboratory facilities, Sivas Cumhuriyet University, Turkey), which is based on the absorption of infrared rays falling on the intramolecular bonds of these molecules by vibration and rotational movements of the bonds, was also used.

The samples eluted were analysed by gas-chromatography-mass spectrometry (GC–MS, Shimadzu, Model: GCMS–QP 2010 ULTRA, Research Centre Laboratories, Kastamonu University) and Fourier Transform Infrared Spectroscopy (FT-IR, Bruker, Tensor II). G C-MS conditions were as follows: column RTX-5MS (30 m × 0.25 mm × 0.25 µm); carrier gas, helium; oven column temperature, 40 °C; injection temperature, 250 °C; pressure, 100 kPa; split injection; split ratio, 10; injection volume 1µ; oven temperature setting: 3 min at 40 °C, temperature increase from 40 to 240 °C at 4°C/min; interphase temperature, 250 °C; and ion source temperature, 200 °C.

Results

Identification of the Streptomyces isolate

16S rRNA gene was sequenced and the sequence data was stored in GenBank (accession number: MZ159946). Homology search identified the isolate to be *Streptomyces decoyicus* (Fig. 1). This organism optimally grew at pH7 at 37 °C. It formed yellowish colonies on agar medium (Fig. S1). Gram staining was used for morphological identification under the light microscope (Fig. S2). The surface morphology of *Streptomyces decoyicus* isolate was visualized by scanning electron microscopy (SEM) at Sivas Cumhuriyet University CÜTAM Central Laboratory (Fig. 2).

In two previous studies, two other *Streptomyces* members have also been identified at species level by the sequencing of 16S rRNA genes: *Streptomyces griseorubens* (Çetinkaya

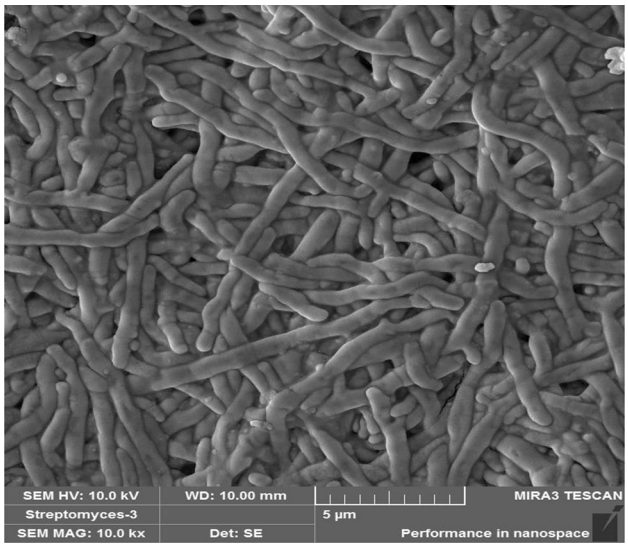

Fig. 2 SEM image of *Streptomyces decoyicus*

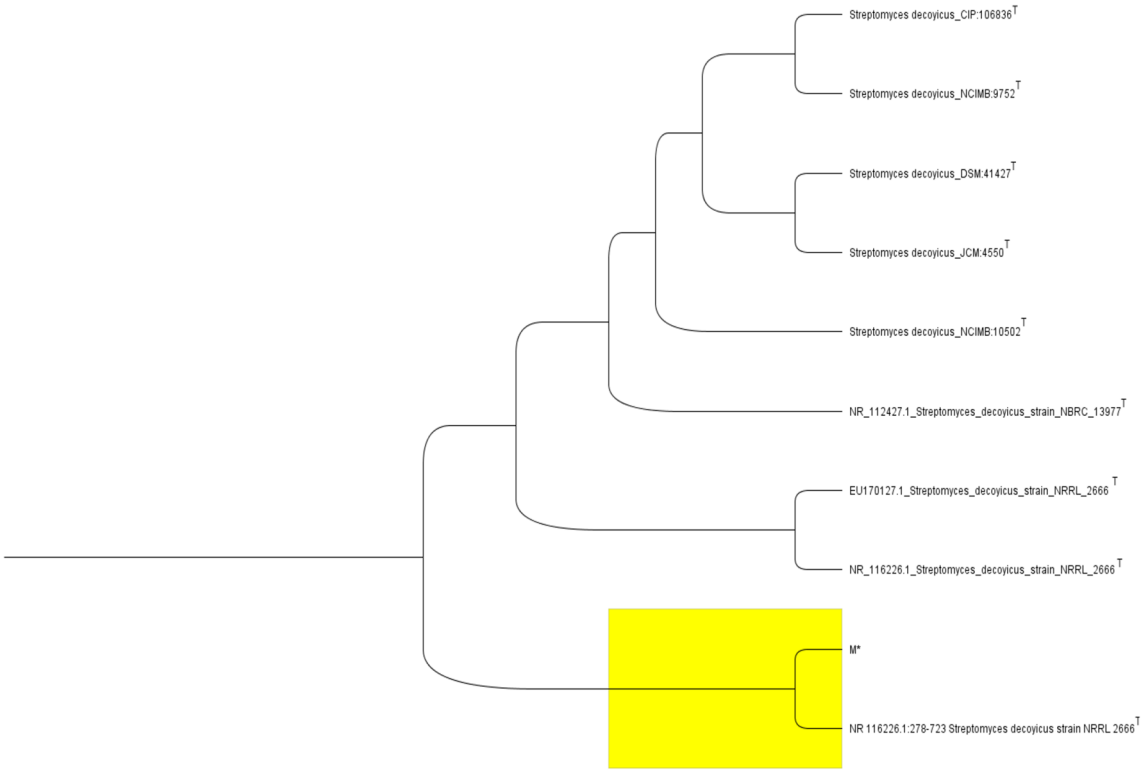

Fig. 1 Dendrogram of *Streptomyces decoyicus* type strains and M* isolate

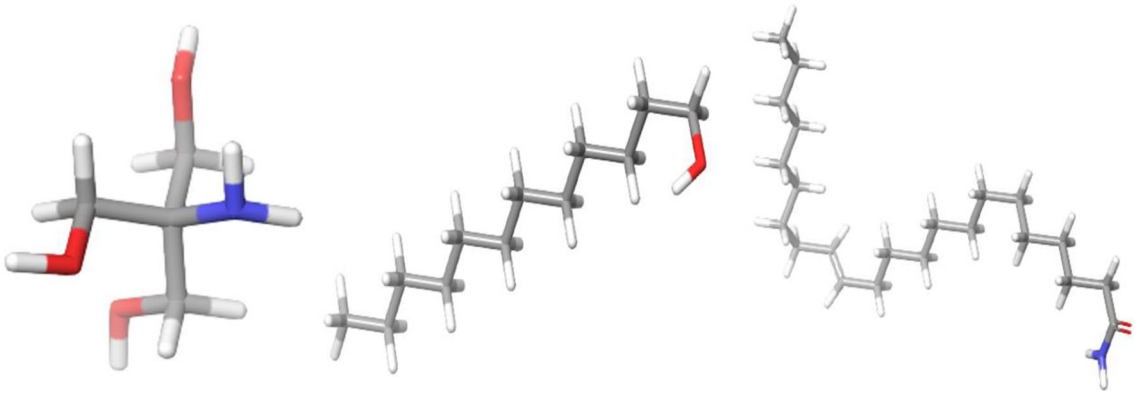

Fig. 3 Structure of Molecules; **A** Tromethamine; **B** 1-Dodecanol; **C** 13-Docosenamide

Table 1 Three predominant compounds of the M1 band (GC–MS analysis)

Peak	Retention time	Name of the compound	Peak (%)
1	26.140	Tromethamine	61.12
2	29.065	1-Dodecanol	17.77
3	46.220	13-Docosenamide	7.71

Table 2 Two predominant compounds of the M2 band (GC–MS analysis)

Peak	Retention time	Name of the compound	Peak (%)
1	29.107	1-Dodecanol	54.06
2	25.650	Tromethamine	41.16

2021, Çetinkaya et al. 2021), and *S. griseobrunneus* (Çetinkaya 2021).

Resolution of the crude extract

Crude *n*-butanol extract was first run in thin layer chromatography sheets and two visible bands were obtained. The Rf values of these bands were 0.30 and the 0.23 (Fig. S3). These two bands were also shown in SDS-PAGE gels (Fig. S4). The bands were cut out from the gel and their contents were eluted overnight in *n*-butanol. Dry extracts were obtained by evaporating the organic solvent and were then analysed by FT-IR and GC–MS.

Metabolite identification and characterization

The elution samples were identified by GC–MS analysis (Fig. 3). GC–MS chromatograms of the bands are included in the supplementary (Figs. S5, S6). One of the bands, M1, contained 61.12% tromethamine, 17.77% 1-dodecanol, 7.71% 13-docosenamide (Table 1). The band M2 had 54.06% 1-dodecanol and 41.16% tromethamine (Table 2). The presence of these compounds was also verified by FT-IR (Fig. 4): ν max. (cm^{-1}): 3347, 3327 (aliphatic NH-stretch bonds—3288 shoulder bond), 3182 (OH-stretch bond), 2920 (aliphatic CH-asymmetric stretch bond), 2850 (aliphatic CH-symmetric stretch bond), 1586 (NH-bending), 1287 (CN-stretch bond), 1206 (C–O stretch bond), 1034 cm^{-1} (aliphatic CN-stretch aliphatic amines), 800 cm^{-1} (CCl-stretch alkyl halides), and 628 cm^{-1} (CBr-stretch alkyl halides). N–H asymmetric and symmetric tensile bonds expected around 3550–3420 cm^{-1} and 3450–3320 cm^{-1} of the aliphatic NH_2 group (Erdik 2008) were observed in the 3347–3327 cm^{-1} region. Since the primary amine showed the characteristic doublet structure, it was thought that the metabolites mostly contained the primary NH_2 group. O–H stretch band at the end of aliphatic chains was observed in the region of 3183 cm^{-1}. In addition, the specific diffuse band of the O–H stretch band (Besson et al. 1997) was also observed. This result was compared with the similar results of (Nayaka et al. 2020).

The results of the GC–MS analysis suggested that mostly tromethamine and 1-dodecanol, and some 13-docosenamide were present in the metabolite samples. Accordingly, the asymmetric and symmetric tension and bending vibration bands of CH_2 and CH aliphatic groups, 2900 cm^{-1} and 2850 cm^{-1}, common to the three compounds, were observed in the FT-IR spectra.

In general, when the C=O group is attached to an atom carrying an unshared electron or is conjugated with a double bond, the effect of unshared electron pairs and double bonds in a structure creates the mesomeric effect, while the electron-withdrawing or electron-donating creates the inductive effect. Any factor that increases the dipole character of the C=O group, decreases the value of the force constant, and thus the absorption shifts to the lower frequency (Erdik 2008). Although the amide C=O stretch band in the 13-docosenamide compound was expected

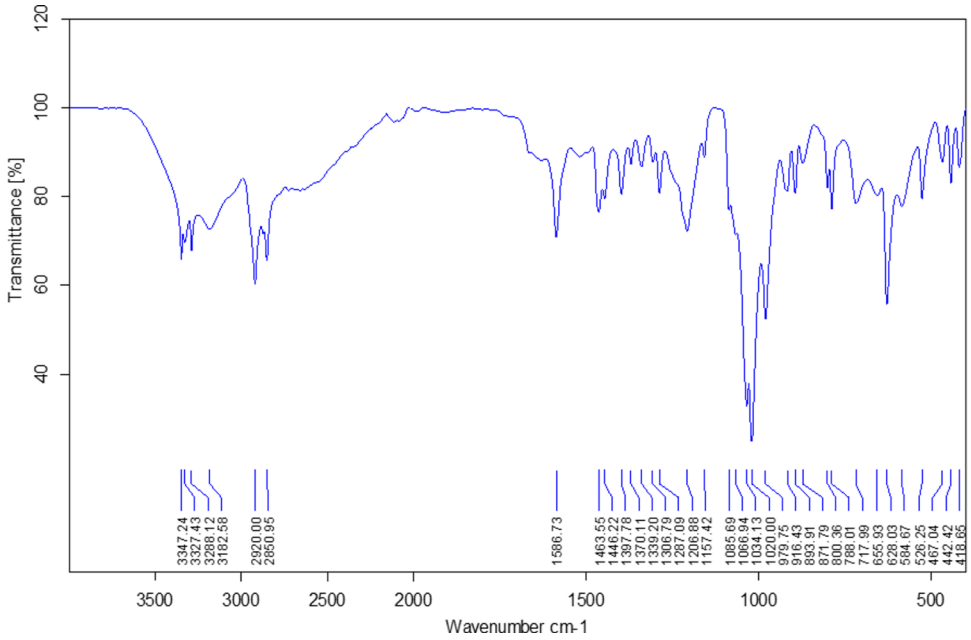

Fig. 4 Fourier-transform infrared spectra of M1 band content

to be observed within the 1650–1700 cm^{-1} region, no band was obvious in this region. In addition, there was also no C=C alkene bands available. These two findings might argue against the presence of 13-docosenamide in the metabolite samples, and it could be considered as the GC–MS artefact, resulting from the compendium of compounds specified by its software. Hence, the FT-IR results confirmed the abundance of two compounds, tromethamine and 1-dodecanol.

The FT-IR spectra of the M2 sample almost overlapped with the M1, except for the CO-stretch bond at 1206 (Fig. 5): ν max. (cm^{-1}): 3347, 3326 (aliphatic NH-stretch bonds—3288 shoulder bond), 3186 (OH-stretch bond), 2920 (aliphatic CH-asymmetric stretch bond), 2851(aliphatic CH-symmetric stretch bond), 1587 (C=O stretch bond), 1535 (NH-bending), 1287 (CN-stretch bond), 1020 cm^{-1} (aliphatic CN-stretch aliphatic amines), 800 cm^{-1} (CCl- stretch alkyl halides), and 627 cm^{-1} (CBr-stretch alkyl halides).

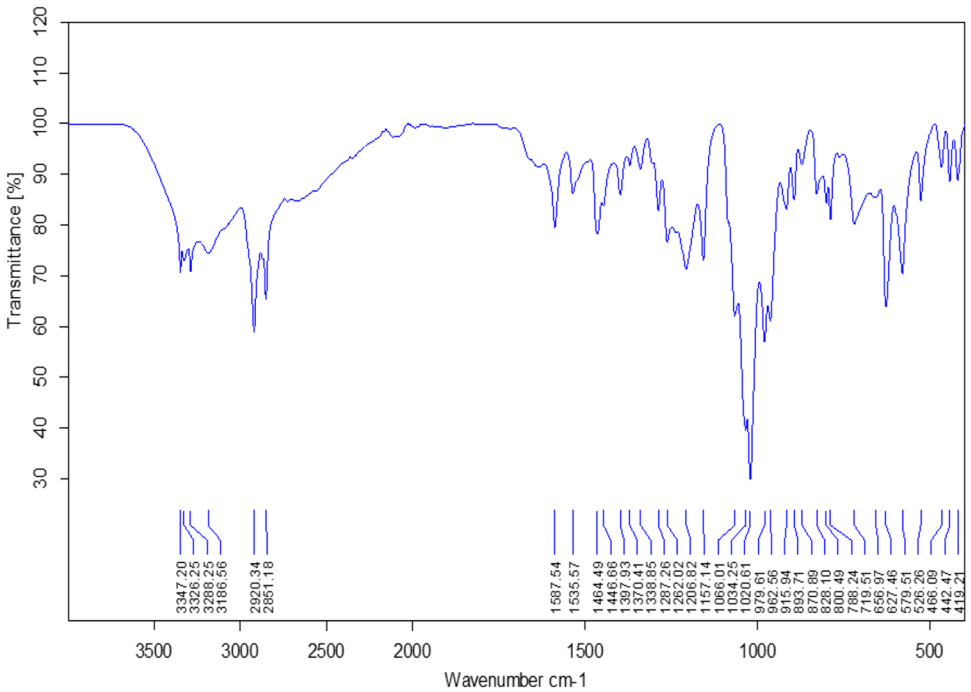

Fig. 5 Fourier-transform infrared spectra of M2 band content

Here, theoretically, the NH_2 group (Erdik 2008), which is expected to be observed around 3550–3420 cm^{-1} and 3450–3320 cm^{-1}, was observed in the 3347–3326 cm^{-1} region with N–H asymmetric and symmetric tension bands. The characteristic doublet structure of the primary amine suggested that the metabolites mostly contain the primary NH_2 group. From these data, it could be said that the metabolite content consisted mostly of tromethamine and 1-dodecanol (Besson et al. 1997).

Discussion

Isolation of rare and uncommon *Actinomycetes* has become an increasingly important part of natural product discovery efforts (Usha Nandhini et al. 2018). Although members of *Streptomyces* have long been extensively screened for bioactive compounds, rare species of this genus are expected to contain undiscovered metabolic products (Usha Nandhini et al. 2018; Khattab et al. 2016).

This work constituted our third attempt to demonstrate that different *Streptomyces* species could produce 1-dodecanol as the predominant secondary metabolite under the conditions employed. The presence of tromethamine was, however, unique to this study. Further studies involving the purification of these compounds by column chromatography and the analysis by NMR spectrometry are required for further characterisations.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) appeared to be a robust and relatively straight forward technique that yielded enough material for analytical purposes. And it was suggested that *n*-butanol could be the organic solvent of choice for the extraction of 1-dodecanol- and tromethamine.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00203-022-03076-5.

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Declarations

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

Research involving human and animal rights This article does not contain any studies involving animals performed by any of the authors.

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