Secondary Metabolites of an of *Streptomyces griseorubens* Isolate Are Predominantly Pyrrole- and Linoleic-acid like Compounds

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Abstract: The study involved the isolation and identification of a member of *Streptomyces griseorubens* and the identification of its secondary metabolite content. Two extract samples were prepared by using butanol and chloroform. In the analyses of the extracts TLC, FT-IR, and GC-MS were employed. Butanol extract appeared to be dominated by three different pyrrole compounds (43.59%), while two fatty acids, linoleic- and erucic acids, were the most abundant secondary metabolites in the chloroform extract, 27.57% and 12.34%, respectively. Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-compound was represented by a single and distinct band on the thin layer chromatography plate. In GC-MS spectra, it also constituted 13.50% of the butanol extract.

Key words: erucic acid, linoleic acid, pyrrolo[1,2-a]pyrazine-1,4-dione hexahydro, *Streptomyces griseorubens*

1 Introduction

Actinomycetes are bona fide producers of new antibiotic agents⁴. Most of the commercialised antibiotics have been discovered from Actinomycetes by screening natural soil and water habitats⁵⁻¹⁰. A wide taxonomic range of Actinomycetes also has the ability to produce many other secondary metabolites with biological activities including anticancer, antiviral, and immunosuppressants⁶⁻¹⁰. Despite the increase in resistance to commonly used antibiotics, new antimicrobial agents are being constantly searched from novel Actinomycetes isolates⁴,¹ⁱ⁻¹⁵.

Many new types of Actinomycetes have been characterised and named, and their secondary metabolites have been extracted using various techniques⁴,¹⁶. Most Actinomycetes are free-living organisms that are widely distributed in nature. They are found in both aquatic and terrestrial habitats. The survival rate of these bacteria in unsuitable environmental conditions is high¹⁴.

Actinomycetes are grouped phylogenetically as Gram-positive bacteria with high guanine and cytosine content in their genome. They provide approximately 75% of the current bioactive compounds. Species of *Streptomyces* alone produce two-thirds of naturally occurring antibiotics worldwide¹⁵.

*Streptomyces* are filamentous Gram-positive bacteria. Its members make up a large and important group of the microflora in most of the natural environments. They can be found in ordinary soil, freshwater, lakes, river basins, composts, and in manure. In the past few decades, a large number of *Streptomyces* have been isolated from soil¹⁶. Members of this genus especially living in the humid, white soil provide an excellent source for the therapeutically important products. Currently faced drug resistance problems require the discovery of new antimicrobial compounds that would be effective against resistant pathogenic bacteria and fungi. To overcome the above-mentioned resistance problem, fruitful results are expected from novel *Streptomyces* isolates¹⁶.

In this study, the isolate K5 was found in the samples of humid white soil. This soil has traditionally been used as artisanal cosmetic by the local coiffeurs. The isolate was identified to be *Streptomyces griseorubens* by mass spectrometry and 16S rRNA gene sequencing. Two secondary metabolite extracts, of butanol and chloroform, were prepared from this isolate and analysed by gas chromatography-mass spectrometry (GC-MS). Butanol extract was dominated by pyrrole compounds, 43.59%, and contained mainly three pyrrole derivatives (pyrrolo[1,2-a]pyr-
azcline-1,4-dione, hexahydro-, 13.50%; pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, 18.85%; and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenethyl), 8.24%). These compounds have been reported to harbour antifungal properties and produced by other species of Streptomyces\textsuperscript{27, 28}, that is, no report have been available for the Streptomyces griseorubens. Chloroform extract appeared to be rich mainly in two fatty acids: 9,12-octadecadienoic acid, also known as linoleic acid (27.57%), and erucic acid, 12.77%. These fatty acids have generally been known to be the products of plant sources. Only in one study it has been shown that Streptomyces griseoincarnatus produced erucic acid\textsuperscript{19}.

### 2 Materials and Methods

#### 2.1 Soil sample collection

*Streptomyces* isolates were obtained from white soil samples collected from Gemerek (Sivas, Turkey). This soil has been exploited by the local coiffeurs as artisanal cosmetic.

#### 2.2 Isolation of Streptomyces

Ten grams of soil sample was suspended in 90 mL NaCl solution (0.85%) for 2h at room temperature. To obtain single colonies, 10\(^6\) cfu/mL dilution was spread onto ISP-2-agar plates. Incubation was performed for 48 h at 30\(^\circ\)C. Morphologically different colonies were picked and purified by three sequential passages. The purity of the isolates was checked under the light microscopy after Gram staining. Liquid cultures were prepared in 100 mL LB broth and the organic solvents to separate. The upper methanol phase was discarded and the chloroform phase was evaporated at 70\(^\circ\)C and 120 mg of dry extract was obtained. The dry sample was kept at −80\(^\circ\)C in 20% glycerol\textsuperscript{20}.

#### 2.3 Typing

##### 2.3.1 Identification by mass spectrometry

Partial protein homology was employed for the initial identification in a MALDI-TOFF MS instrument (Bruker IVD MALDI Biotyper, Sivas Cumhuriyet University Hospital). This analysis allowed the typing of the isolates at genus level and they all belonged to *Streptomyces*.  

##### 2.3.2 Identification by 16S rRNA gene sequencing

Genomic DNA was prepared by following the method described in Yavuz et al.\textsuperscript{20}. DNA sequencing was performed by (MG Bioinformatics). Approximately 1,400 bases were sequenced using the oligo-primers 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492R (5’-TACGGYTACCTTGTGACTT-3’)\textsuperscript{21}. Sequencing data were scanned in the BLAST database and the results were recorded\textsuperscript{21}. Isolates belonged to *Streptomyces griseorubens* were multiple aligned using Mafft program\textsuperscript{22}. Similarity percentage values (% identity) between samples were calculated using the aligned sequence data. In addition, phylogenetic trees were created by using the Kimura-2 genetic distance model and Neighbour-joining (NG) method to determine the relationship between the samples using aligned data\textsuperscript{23, 24}. 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: MT525003.

#### 2.4 Screening for antibacterial activity

Ten microliter supernatant of a three-day culture in LB was used in agar-diffusion experiments at 37\(^\circ\)C. The organisms tested were mostly Gram positive, including *Bacillus subtilis*, *Bacillus cereus*, *Bacillus pumilus*, *Exiguobacterium aurantiacum*, *Staphylococcus epidermidis*, and *Staphylococcus xylosus*.

#### 2.5 Secondary metabolite preparation

An overnight culture from the glycerol stocks was prepared in 50 mL LB in a 250 mL flask at 120 rpm at 37\(^\circ\)C. An aliquot of this culture, 100 \(\mu\)L, was added into 100 mL LB agar and incubated until the agar completely assumed red colour. This took around 7d. The agar was then meshed in 100 mL n-butanol. Organic phase was filtered three times through a filter paper. Eighty millilitres of the extract were evaporated at 70\(^\circ\)C and 120 mg of dry extract was obtained. The dry sample was kept at −20\(^\circ\)C. Remaining 20 mL was divided into two aliquots and evaporated at 100\(^\circ\)C. One of them was directly used as the pigment sample. The other one was used for the preparation of the chloroform extract: its pellet was suspended in 10 mL of chloroform/methanol mixture (4:6, v/v). The suspension was vortexed vigorously and was then left on the bench for the two organic solvents to separate. The upper methanol phase was discarded and the chloroform phase was evaporated at 65\(^\circ\)C. This sample was used as the chloroform extract\textsuperscript{25}.

#### 2.6 Thin layer chromatography

Qualitative analysis of the secondary metabolites was carried out by thin layer chromatography (silica gel 60, Merck). At the bottom of the gel, one centimetre margin was marked. Samples were run through a working fluid mixture, hexane: ethyl acetate (1:9, v/v). The plate was dried and the retention factor\((R_f)\) values of the red bands were calculated\textsuperscript{26}.

#### 2.7 Identification of secondary metabolite contents

Molecular content of the extracts was identified by Fourier Transform Infrared Spectroscopy (FTIR, Bruker, Tensor II) and gas chromatography and mass spectrometry (GC-MS, Shimadzu, Model: GCMS-QP 2010 ULTRA). GC-MS analyses were performed at Research Centre Laboratories of Kastamonu University, Turkey).
3 Results and Discussion

3.1 Identification of K5

Typing of bacteria by mass spectrometry (MALDI-TOF) relies on the partial proteome homology. The instrument has mainly been set for the identification of pathogens. Thus its software has been specifically designed for this purpose. Hence, it understandably identified the isolate K5 at genus level, *Streptomyces*. Homology studies with 16S rRNA gene sequence database essentially yielded a *Streptomyces griseorubens* dendrogram including the K5 isolate (Fig. 1). On the basis of this latter information the isolate was identified as *Streptomyces griseorubens* (Accession number MT525003, Gen Bank). This organism appeared to grow optimally at a temperature range between 30 and 40°C at pH7. It formed reddish colonies on agar media (Fig. 2) and it also turned liquid culture into dark red after a certain incubation period (Fig. 3). This red colouring was attributed to the presence of pyrrole-containing metabolites.

3.2 Antibacterial activity

Test strains used for the antibacterial activity were the members of Gram positive bacteria (Fig. 4). These were the bacteria available in the laboratory and they all appeared to be susceptible.
3.3 Thin layer chromatography
To be compatible with the literature ethyl-acetate extracts were analysed by thin layer chromatography, and one sharp- and three rather fuzzy coloured areas visualised on the chromatograph on the UV box (Biosafety Cabinet, Class II A2). It could be likely that the sharp band indicated the presence of pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- compound, as it was a unique molecule in the GC-MS spectra (peak 11 with 13.50% abundance). Similarly the following two broad areas probably represented pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- (peaks 16, 21, 23, 25, 26, and 43, Table 1) and pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- compounds (peaks 47 and 49, Table 1), respectively. The retention factor value of the first band was 0.81, and of the second band was 0.75 (Fig. 5). Streptomyces isolates have been reported to have Rf values of 0.5<sup>16</sup>. All of the coloured areas were scraped off and used for antimicrobial activity. All three of the areas displayed antibacterial activity (Fig. 5).

3.4 Identification of the secondary metabolite content
FTIR spectrometry of the ethyl-acetate extract produced peaks at 3029, 2956/2919/2872, 1649, and 1450 cm<sup>-1</sup>, and these indicated the presence of O-H bond in carboxyl groups, C-H stretches of alkane or alkyls, C=O carbonyl group, C-H bonds of alkenes, and carboxylic acid groups, respectively (Fig. 6). These data could be seen as the summary of the bonding patterns existed in the metabolite compounds. Further discussion, however, might lead to overinterpretation.

The original GC-MS Figures and Tables indicating the spectra and metabolite content, respectively, were presented in the Supplements section. The prominent peaks representing the pyrrole-compounds and fatty acids were presented separately in the text (Table 1 and Table 2, respectively). In the GC-MS analyses it could readily be noticed that each of the extract was dominated by a specif-
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Fig. 4  Antibiogram results; A: Bacillus clausii; B: Bacillus pumilus; C: Bacillus pumilus; D: Bacillus cereus; E: Streptococcus pyogenes; F: Staphylococcus sciuri; G: Exiguobacterium aurantiacum; H: Staphylococcus xylosus; I: Bacillus cereus.

Table 1  Pyrrole compounds of the butanol extract (GC-MS analysis).

| Peak | Retention time | Name of the compound                                      | Peak (%) |
|------|---------------|-----------------------------------------------------------|----------|
| 11   | 37.538        | Pyrrole[1,2-alpyrazine-1,4-dione, hexahydro-              | 13.50    |
|      |               |                                                           |          |
| 16   | 38.987        | Pyrrole[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- | 3.14     |
|      |               |                                                           |          |
| 21   | 41.502        | Pyrrole[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- | 1.82     |
|      |               |                                                           |          |
| 23   | 41.995        | Pyrrole[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- | 5.26     |
|      |               |                                                           |          |
| 25   | 42.319        | Pyrrole[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- | 7.49     |
|      |               |                                                           |          |
| 26   | 42.393        | Pyrrole[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- | 2.99     |
|      |               |                                                           |          |
| 43   | 48.152        | Pyrrole[1,2-alpyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- | 1.15     |
|      |               |                                                           |          |
| 47   | 51.399        | Pyrrole[1,2-alpyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- | 4.43     |
|      |               |                                                           |          |
| 49   | 52.351        | Pyrrole[1,2-alpyrazine-1,4-dione, hexahydro 3-(phenylmethyl)- | 3.81     |
|      |               |                                                           |          |
ic group of compounds. Butanol extract appeared to be rich in three pyrrole compounds (43.59%, Table 1; Suppl.), while the chloroform extract was rich in two plant fatty acids (39.91%, Table 2; Suppl.), namely, linoleic- and erucic acids.

Linoleic acid, also known as 9,12-octadecadienoic acid, is a polyunsaturated essential fatty acid for humans and also has many other health benefits, including a broad range of antibiotic functions.

Erucic acid is essentially a seed oil with favourable physical properties for industrial use. It is an excellent lubricant and it is widely used by steel, and plastic and rubber manufacturers. Furthermore, it is specifically used in extreme-condition lubricants and hydraulic fluids, in plastic films and paints, as well as in many other protective-coating materials and cooling-insulating fluids. Bacteria, *Streptomyces griseoincarnatus*, also appears to produce erucic acid.

Pyrrole compounds are produced as the characteristic secondary metabolite by the species of *Streptomyces*, *S. lateritius* and *S. mangrovisoli* sp. nov. The presence of the pyrrole ring has been reported to render these compounds their red colour as well as their outstandingly wide pharmaceutical range of use, spanning from anti-cancer agents to anti-coagulant drugs. Pyrrole compounds have also been identified as the inhibitors of several important enzymes, including cell division cycle 7 (Cdc7) kinase, cyclooxygenase-2, epidermal growth factor receptor (EGFR) tyrosine kinase, histone deacetylase, protein kinase G, and reverse transcriptase. This inhibitor property has been suggested to be a virtue of the nitrogen atom present in the pyrrole ring.

An outstanding feature of the study was that migration of the pigment molecules seemed to be inversely proportional to their molecular weights. The smallest compound...
pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, with a molecular weight of 196.25 g/mol (peak 11, in GC-MS spectra), displayed the lowest migration rate in the TLC plate. Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- compounds, having molecular weights around 210.27 g/mol, were clustered in the middle. The fastest pigment compounds appeared to be of pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-, with an approximate molecular weight of 244.28 g/mol. Comparable results were searched in the relevant literature available, but could not be found. A likely explanation could be that either the solvents, hexane: ethyl acetate (1:9, v/v), rendered an anomaly in the sense of the migration or the pigment compounds having a molecular weight higher than 200 g/mol, could not have entered the TLC plate (silica gel 60, Merck) used and thus run faster.

| Peak | Retention time | Name of the compound | Peak (%) |
|------|----------------|-----------------------|----------|
| 33   | 50.036         | Linoleic acid         | 27.57    |
| 34   | 50.480         | Erucic acid           | 12.34    |

Table 2 Two predominant fatty acids of the chloroform extract (GC-MS analysis).

4 Conclusion

The pyrrole compound, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, appeared as peak 11 in the GC-MS spectra, was represented by a single band on the thin layer chromatography plate. It was also the predominating pyrrolo- compound with 13.50% abundance (Table 1). These findings implied that this compound could be further enriched by successive chloroform extractions and/or by batch chromatography in the near future.

The results of the study also seemed to have some genetic implications. Actinomycetes, including Streptomyces, have a linear chromosome the ends of which appear to display high instability. This vulnerable genome might, on the other hand, have positive implications in terms of horizontal gene transfer, especially between Actinomycetes and plant root cells. This idea was prompted by the presence of plant fatty acids, synthesized by the species of Streptomyces as secondary metabolites in significant amounts. Another reason was that Streptomyces members could also be found among the plant root flora. It might thus seem to be feasible to initiate horizontal gene transfer in the laboratory environment, using germinating olive tree seeds and/or growing young olive trees in Streptomyces-enriched soil or in semi-liquid media. Horizontal transfer experiments can also be envisaged for other dicotyledons with agricultural importance by using genes that are responsible for the production of the colour molecules.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Supporting Information

This material is available free of charge via the Internet at http://dx.doi.org/10.5650/jos.ess20161

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