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Bioassay-guided Isolation of Antibacterial and Cytotoxic Compounds from the Mesophilic Actinomycete M-33-5

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One hundred and twenty-six mesophilic Actinomycete cultures were isolated from the Aegean region of Turkey. The antimicrobial activities of pure isolates were tested using the agar-plaque method. Based on high antimicrobial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* O157-H7 (*E. coli*), the isolate M-33-5 was selected for bioactivity-guided isolation. Fermentation, followed by solvent partition (H_2O-EtOAc, H_2O-*n*-BuOH), showed that the highest activity was present in the EtOAc extract. By using chromatographic methods, two bioactive compounds were isolated and their structures were determined by spectral methods to be 4′-deacetyl griseusin A and griseusin A. The MIC values of griseusin A and 4′-deacetyl griseusin A against MRSA and *E. coli* were ≤ 1.0 μg/mL. The cytotoxicities of the EtOAc extract and 4′-deacetyl griseusin A were also evaluated against two cancer cell lines (human servical cancer: HeLa; murine fibroblastic cells: L-929). The EtOAc extract showed strong cytotoxic activity against HeLa and L-929 lines with IC_{50} values of 1.57 and 2.43 μg/mL, respectively, whereas 4′-deacetyl griseusin A was very potent with IC_{50} values of 0.43 versus HeLa, and 0.12 μg/mL against L-929. The active strain M-33-5 was identified as *Streptomyces griseus* by 16SrDNA sequence data.

Keywords: Actinomycete, *Streptomyces griseus*, antimicrobial activity, methicillin resistant *Staphylococcus aureus, Escherichia coli* 232, cytotoxic activity, griseusin A, 4′-deacetyl griseusin A.

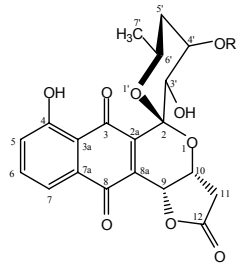
Antibiotics are natural chemical compounds produced by microorganisms as secondary metabolites to kill or inhibit other microorganisms. Since their first discovery in the middle of the 20th century, they have played an important role in the treatment of infectious diseases.

Unfortunately, infectious diseases have become a major health problem yet again due to a number of reasons, including the changing spectrum of pathogens, antibiotic resistance of pathogens [1-3], immunocompromising treatments, immunodeficiency diseases [4], and observation of new pathogens. These factors drive the need to search for novel antibiotics. In addition, the desire for safer cures with less mammalian toxicity is a major concern.

To meet the continued demand for new antibiotics against the resistant pathogens, screening efforts need to be continued [5]. Up to now, the pharmaceutical industry has primarily targeted drugs from soil organisms, and of the antibiotics in clinical use, most are of bacterial or fungal origin.

Among the bacteria, filamentous actinomycetales species produces over 10,000 bioactive compounds, 7,600 derived from *Streptomyces* and 2,500 from the so called rare actinomycetes (rare actino) species; these represent the largest group (45%) of bioactive microbial metabolites [6].

As part of our ongoing studies to discover new antimicrobial molecules active against resistant pathogens, we performed a screening study focusing on Actinomycetes living in unexplored areas of West Anatolia. In this study, 126 mesophilic Actinomycete cultures collected from 28 different locations were isolated. The antimicrobial activities of pure isolates were tested by using an agar-plaque method. Based on the high antimicrobial activity against methicillin resistant *Staphylococcus aureus* (ATTC 43300) (MRSA) and *Escherichia coli* O157-H7 (RSSK 232) (*E. coli*), the halotolerant isolate M-33-5 was selected for bioactivity-guided isolation. Fermentation, followed by solvent partition studies (H_2O-EtOAc, H_2O-*n*-BuOH) showed that the highest activity was present in the



R = H → (4'-Deacetyl griseusin A = **1**)
R = $COCH_3$ → (Griseusin A = **2**)

Figure 1: Structure of compounds **1** and **2**.

EtOAc extract. By using chromatographic methods, two bioactive compounds were isolated (**1** and **2**), which were determined to be 4'-deacetyl-(-)-griseusin A and (-) griseusin A, respectively, based on the comparison of their spectral data with literature values [7-10].

Compounds **1** and **2** were tested against MRSA and *E. coli*. Compound **1** showed remarkable antimicrobial activity with a MIC value against MRSA of 0.5 μg/mL, while compound **2** had promising activity with a value of 1.0 μg/mL. Against *E. coli*, both compounds displayed significant activity (MIC = 1.0 μg/mL for each). Interestingly, the EtOAc extract compared with the pure compounds (**1** and **2**) was almost equally active to pathogens, with MIC values of 0.75 μg/mL (MRSA), and 1.0 μg/mL (*E. coli*). Based on these results, we propose that the other constituents present in the EtOAc extract, which were missed during the bioactivity guided fractionation due to scarce quantities, might be more potent than the purified griseusin derivatives **1** and **2**.

Since quinone type antibiotics, such as daunorubicin and doxorubicin, are effective anticancer agents, the EtOAc extract and major compound **1** were evaluated for their cytotoxicity towards two cancer cell lines (human cervical cancer: HeLa; murine fibroblastic cells: L-929) by using the MTT assay.

The EtOAc extract showed strong cytotoxic activity against HeLa and L-929 lines with IC_{50} values of 1.57 and 2.43 μg/mL, respectively, whereas 4′-deacetyl griseusin A (**1**) was very potent, with IC_{50} values of 0.43 versus HeLa, and 0.12 μg/mL against L-929.

The isolate M-33-5 was identified according to its phenotypic characteristics (data not shown) and 16S rDNA gene sequence analysis to be *Streptomyces griseus*, a well known antibiotic producer. Although *S. griseus* is a common species, only a few griseusin derivatives have been identified and reported so far: 3'-*O*-α-D-forosaminyl-(+)-griseusin A from *S. griseus* [9], 4'-deacetyl-(-)-griseusins A and B from strain MJ932-SF3 [10], griseusin C from *Penicillium* sp., and griseusin D from *Nocardiopsis* sp. [11]. Among these compounds, only griseusin D (5'-one-4-hydroxy-12-methoxygriseusin) was evaluated for its cytotoxicity displaying strong activity against human leukemia cells (HL60) and modest cytotoxicity against human lung adenocarcinoma cell lines (AGZY) with IC_{50} values of 0.23 and 19.6 μg/mL, respectively.

To the best of our knowledge, the cytotoxicity of **1** was reported for the first time in this study. The results show that the 4′-deacetyl griseusin A (**1**) is a potent chemical entity for exploitation to cure cancer. Further studies are warranted to purify the minor constituents of M-33-5 isolate, and determine their potential as anti-infective and anticancer agents.

Experimental

General experimental procedures: NMR spectra were obtained on a Varian Mercury Plus 400 spectrometer at 30°C. The following equipment and conditions were also used: Christ-ALPHA 1-4 LD, freeze dryer; CAMAG UV Lamp; Welch 2511, vacuum pump; High Performance Flash Chromatography (HPFC) system (SP-2, Biotage, Inc., A Dyax Corp. Company); Heidolph Laborota 4001, rotavapor; pre-coated silica gel 60 F_{254} aluminum sheets (Merck) were used for TLC with $CHCl_3$-MeOH (9:1), $CHCl_3$-MeOH:H_20 (80:20:1, 80:20:2, 70:30:3); visualization, 30% H_2SO_4; column chromatography, silica gel 230-400 mesh (Merck); reverse-phase material (C-18, Sepralyte 40 μm) was used for vacuum liquid chromatography; Sephadex LH-20 was used for size exclusion chromatography; TLC plates were examined by UV fluorescence and after spraying with 1% vanillin in conc. H_2SO_4, followed by heating at 105°C for 1-2 min.

Isolation of microorganisms: One hundred and twenty-six mesophilic Actinomycete cultures were isolated from the Aegean Region of Turkey (West Anatolia). Soil samples collected from 10-15 cm depth with a sterile spoon were put into sterile plastic bags, and brought to the laboratory. After addition of 1% $CaCO_3$ to the samples, they were dried at room temperature. Samples were serially diluted and spread onto Actinomycetes Isolation Agar in duplicate. Plates were incubated at 25˚C for 5-7 days in a humidified atmosphere. After growth, Actinomycete isolates were purified and subcultured. Stock spore suspensions were prepared with 20% (v/v) sterile glycerol and stored at -20°C.

Based on the high antimicrobial activity towards methicillin resistant *Staphylococcus aureus* ATCC 43300 and *Escherichia coli* RSSK 232, the strain

M-33-5 was selected for bioactivity-guided isolation. This strain was collected from the vicinity of hot spring water located at Hisaralan village in Balıkesir province of West Anatolia.

Identification: Identification of the Actinomycetes strain M-33-5 was made according to phenotypic characteristics and 16Sr DNA sequence data. First, high molecular weight genomic DNA was isolated from the strain M-33-5 by using Pure Link™ Genomic DNA Mini Kit (Invitrogen™).

16S rDNA reactions were performed with primers F27 (5'- AGAGTTTGATCCTGGCTCAG-3) and R1492 (5'-TACGGCTACCTTGTTACGACTT-3'). The following internal primers were used to ensure overlapping sequences for analysis of 16S rDNA sequences: F514 (5' GTGCCAGCAGCCGCGGTAA-3') and F1114 (5'-GCAAC GAGCGCAACCC-3) and the reverse primers R530 (5'-CCGCGGCTGCTGGCACGTA-3') and R936 (5'-GTGCGGGCCCCCGTCAATT-3'). PCR reaction was performed with Gene Amp®PCR system (Applied Biosystems) in 50 μL mixtures containing 10xPCR buffer, 1.5 mM $MgCl_2$, 0.4 μM dNTP, 0,2 μM primer, 1.25 U of *Taq* polymerase (Amplitaq Gold, Applied Biosystems), and 50 to 500 ng of genomic DNA template. PCR conditions for 16S rDNA amplification were 1 cycle of denaturation for 5 min at 94°C; 30 amplification cycles consisted of denaturation (94°C for 30s), primer annealing (49°C for 30 s), primer extension (72°C for 90s), and a final extension of 7 min at 72°C.

16S rDNA amplicon was cleaned with Nucleospin Extract Kit (Takara Inc.) and bidirectionally sequenced on an ABI 3100 automated sequencer (Applied Biosystems, Foster City, Calif.). Cycle sequencing conditions for all reactions involved 40 to 60 ng of template DNA, 3.2 pmol of primer, 4 μL of Big Dye (Applied Biosystems), and water to a final volume of 20 μL.

Sequence files in abi format were transformed to fasta format by using ChromasPro v1.22 (Technelysium Pty Ltd). Nucleotide sequences of reference species were downloaded from NCBI Gene Bank. BLAST analysis was performed for isolates by using 16S rDNA sequence data. Bio Edit sequence alignment editor v7.0.1 (Isis Pharmaceutical Inc.) was used for Multiple Sequence Alignments. Maximum sequence differences were set as 0.75 and the neighbor joining tree method was used with Mega v2.1 for phylogenetic three constructions.

Fermentation: Strain M-33-5 was cultured in a medium consisting of (g/L): peptone from meat 5.0 g (Merck) and soluble starch 20 g (Merck). The pH of the medium was adjusted to 8.0 before steam sterilization and the culture was grown in 1000 mL Erlenmeyer flasks containing 250 mL medium. The flasks were inoculated with 5 μL stock spore suspension and incubated on a rotary incubator shaker at 28ºC with agitation (200 rpm) for 7 days. A total of 7 L cell-free supernatant was used for extraction studies.

Isolation of secondary metabolites: Fermentation broth (7 L) was extracted with EtOAc (4 x 2.5 L), and the combined extracts were evaporated under reduced pressure to dryness (656 mg). The dried EtOAc extract (461 mg) was suspended in $CHCl_3$ and then chromatographed on a Biotage HPFC system, eluting initially with $CHCl_3$ and then with $CHCl_3$:MeOH mixtures (5% - 40% MeOH). Fractions (505) were collected and monitored (Frs. 1 - 505). Based on TLC profiles, 21 fractions were pooled together as follows; A (Fr. 1 - 29), B (Fr. 30 - 70), C (Fr. 71 - 112), D (Fr. 113 - 115), E (Fr. 116 - 121), F (Fr. 122 - 128), G (Fr. 129 - 133), H (Fr. 134 - 140), I (Fr. 141 - 149), J (Fr. 150 - 158), K (Fr. 159 - 180), L (Fr.181 - 279), M (Fr. 280 - 287), N (Fr. 288 - 290), O (Fr. 291 - 367), P (Fr. 368 - 398), R (Fr. 399 - 425), S (Fr. 426 - 439), T (Fr. 440 - 460), U (Fr. 461 - 486), V (Fr. 487 - 505). Fr. B (52 mg) was chromatographed on a silica gel column (25 g). Elution was performed by using $CHCl_3$:MeOH mixtures (5% - 10% MeOH) to afford compound **2** (4.7 mg). Fr. D (40 mg) was subjected to open column chromatography (silica gel: 20 g) eluting with $CHCl_3$: MeOH mixtures (5% - 10% MeOH) to yield compound **1** (12.5 mg).

Determination of minimum inhibitory concentration: The minimum inhibitory concentration (MIC) was determined by means of the broth microdilution method described by the CLSI standards [13].

Cancer cell lines: The HeLa cell line was derived from cervical cancer cells, and, L929 transformed fibroblastic cells were derived from murines. They were purchased from the HÜKÜK (Cell Culture Collections) in the Foot-and-Mouth Disease Institute (Ankara) of the Ministry of Agriculture & Rural Affairs of Turkey. The cell lines were maintained in RPMI 1640 (Biochrom, Germany) medium supplemented with 10% heat–inactivated fetal bovine serum, 1% L-glutamine (Biochrom, Germany) and 1% gentamycin (Biochrom, Germany) in a humidified atmosphere with 5% CO_2, at 37^oC.

Cytotoxicity assay: Screening of crude extract and pure substances (4'-deasetil griseusin A) for cytotoxicity, based on metabolic cell viability, was carried out by using a modified MTT 3-(4, 5-dimethyl-2-thiazolyl)-2,

5-diphenyl-2H-tetrazolium bromide) assay [14], which effects the mitochondrial activity of viable cells. The survival of viable cells after treatment of extracts in monolayer culture was determined. HeLa and L-929 cell lines in 96 well microplates with $8x10^4$ cells/mL initial concentration were cultivated for 24 h. After that the cultures were treated with different dilutions of the extracts and incubated for 48 h. Growth inhibition was estimated as the 50% effective concentration (IC_{50}). Optical density of the dissolved material was measured at 570 nm (reference filter, 690 nm). Cytotoxicity was expressed as mean percentage increase relative to the unexposed control ± SD. Control values were set at 0% cytotoxicity. Cytotoxicity data (where appropriate) were fitted to a sigmoidal curve and a 4 parameter logistic model used to calculate the IC_{50}, which was the concentration of nanomaterial which caused a 50% inhibition in comparison with untreated controls. The mean IC_{50} is the concentration of + agent that reduces cell growth by 50% under the experimental conditions and is the average from at least 3 independent determinations that were reproducible and statistically significant. The IC_{50} values are reported ± 95% confidence intervals (± 95% CI). These analyses were performed with GraphPad Prism (San Diego, CA).

Supplementary data: Phylogenetic tree of the isolate M-33-5 designated as unknown based on the 16SrDNA sequence data

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