# Isolation and Identification of *Streptomyces* sp. Act4Zk, a Good Producer of Staurosporine and Some Derivatives

**Zahra Khosravi Babadi[1,2,*†], Gholam Hossein Ebrahimipour[1], Joachim Wink[2], Abolfazl Narmani[3,4,] and Chandra Risdian[2,5]**

[1]**Department of Microbiology & Microbial Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti** University GC, Tehran, Iran.
[2] Microbial Strain Collection, Helmholtz Centre for Infection Research GmbH (HZI), Inhoffenstrasse 7, 38124 Braunschweig, Germany.
[3] Department of Plant Protection, Faculty of Agriculture, University of Tabriz, Tabriz, Iran;
[4] Department of Microbial Drugs, Helmholtz Centre for Infection Research and German Centre for Infection Research (DZIF), partner site Hannover/Braunschweig, Inhoffenstrasse 7, 38124 Braunschweig, Germany
[5] Research Unit for Clean Technology, Indonesian Institute of Sciences (LIPI), Bandung 40135, Indonesia

* Correspondence: zahrakhosravi1365@yahoo.com ; Tel.: +989372849677; Fax: +982122432706

**The GenBank accession number for the 16S rRNA gene sequence of strain Act4Zk is MK418597.**

**Significance and Impact of the Study:** Because of the importance of Actinobacteria, especially *Streptomyces* strains, as the prolific producers of bioactive natural compounds, it is crucially important to consider sources selected from distinctive habitats. In this manuscript, we present the secondary metabolites of *Streptomyces* sp. Act4Zk, with antibacterial, antifungal, and especially, anti-*Mycobacterium smegmatis* activity. The results showed that the interesting species of the genus *Streptomyces* being a good producer of staurosporine and some derivatives.

**Abstract:** In the present study, strain *Streptomyces* sp. Act4Zk was isolated based on a method developed for the isolation of myxobacteria. Due to the low efficiency of the majority of conventional DNA extraction techniques, for molecular identification of the strain *Streptomyces* sp. Act4Zk, a new technique for DNA extraction of Actinobacteria was developed. In order to explore potential bioactivities of the strain, extracts of the fermented broth culture were prepared by an organic solvent (i.e., ethyl acetate) extraction method using. These ethyl acetate extracts were subjected to HPLC fractionation against standard microorganisms, followed by LC/MS analysis. Based on morphological, physiological, biochemical, and 16S rRNA gene sequence data, strain *Streptomyces* sp. Act4Zk is likely to be a new species of *Streptomyces*, close to *Streptomyces genecies* and *Streptomyces roseolilacinus*. Antimicrobial assay indicated high antifungal activity as well as antibacterial activity against *Mycobacterium smegmatis* and Gram-positive bacteria for the new strain. HPLC and LC/MS analyses of the extracts led to the identification of three different compounds and confirmed our hypothesis that the interesting species of the genus *Streptomyces* being a good producer of staurosporine and some derivatives.
**Keywords:** *Streptomyces***,** DNA extraction method, Stausporine, Antifungal activity, Antibacterial activity

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**Introduction**

Soil microorganisms are known to produce a plethora of secondary metabolites with diverse structural and biological properties (Newman et al. 2003). Among them, Actinobacteria, especially *Streptomyces* strains, comprise a group of filamentous, Gram-positive bacteria that are considered one of the most important sources of bioactive natural products, including several clinically used antibiotics and anti-cancer drugs (Abd-Elnaby et al. 2016; Van der Meij et al. 2017); interestingly, about two-thirds of the antibiotics of natural origin are produced by members out of this genus (Baltz 1998; Weber et al. 2003). It has become increasingly difficult to isolate new actinobacterial strains that are able to produce novel bioactive substances with unique structures (Mohr 2016). Furthermore, the number of antibiotic-resistant strains such as *Staphylococcus aureus* ('golden staph' or MRSA (methicillin-resistant Staphylococcus aureus)) and *Neisseria gonorrhoeae* (the cause of gonorrhoea) and the rate of re-isolation of known compounds have increased (Fenical et al. 1999; Cooper and Shales 2011), since as Davies suggests (Davies 2006) "*resistance develops within two or three years after the introduction of a new antibiotic treatment*" (Mohr 2016). Thus, it is crucially important to consider sources selected from distinctive habitats, for isolation of new bacteria as potential sources of novel bioactive secondary metabolites. Among the different types of drugs available in the market, a small number of them are antifungal antibiotics, and they have an essential role in the management of mycotic diseases (Bevan et al. 1995).

Staurosporine is an indolocarbazole alkaloid with antifungal and oomycete activities that was isolated in 1977 from a culture of an actinomycete, *Streptomyces* strain AM-2282$^T$, during screening for microbial alkaloids using a TLC detection method (Zhou et al. 2006). The compound also shows activity against *Leishmania major* and *Trypanosoma brucei* (Gemperlin 2014).
The strain AM-2282 has been renamed through repeated revisions of the taxonomy of soil actinomyces as *Streptomyces staurosporeus* AM-2282$^T$ in 1977, *Saccharothrix aerocolonigenes* subsp. *staurosporea* AM-2282$^T$ in 1995 (Omura et al. 1977; Takahashi et al. 1995) and *Lentzea albida* in 2002 (Xie et al. 2002). Over the past 30 years, staurosporine and related natural indolocarbazole compounds, have been isolated from several bacteria, and cyanobacteria. Staurosporine derivatives have also been isolated from marine invertebrates, such as sponges, tunicates, bryozoans, and mollusks (Sanchez et al. 2006).
In 1986, 10 years after the discovery, staurosporine was found to be a nano molar inhibitor of protein kinases (Nakano et al. 1987). The structure and absolute configuration of staurosporine was revealed by X-ray crystallographic analysis (Funato et al. 1994). and confirmed by total synthesis by Link et al (1995) and an another group. The reports led many laboratories and pharmaceutical companies to find selective protein kinase inhibitors by chemical synthesis or screening of new natural products. In 1996, a bcr-abl tysorine kinase inhibitor by chemical synthesis, Gleevec (the trade name for the generic drug name Imatinib Mesylate), entered human clinical trial of chronic myelogenous leukaemia and was approved in 2001 in USA (Sanchez et al. 2006). Staurosporine is commercially available for biochemical research.
In a continuation of our screening program, a presumptively interesting *Streptomyces* strain, isolate *Streptomyces* sp. Act4Zk$^T$, was isolated from desert soil in Esfahan, Iran. A polyphasic study based on genotypic and phenotypic procedures showed that the isolate belongs to the genus *Streptomyces. Streptomyces roseolilacinus* was most closely related to *Streptomyces* sp. Act4Zk strain, but readily differentiated from it in a number of parameters. In this manuscript, we present the secondary metabolites of *Streptomyces* sp. Act4Zk, with antibacterial, antifungal, and especially, anti-*Mycobacterium smegmatis* activity. The results showed that the interesting species of the genus *Streptomyces* being a good producer of staurosporine and some derivatives. Because of the importance of Actinobacteria, especially

*Streptomyces* strains, as the prolific producers of bioactive natural compounds, the overall aims of this study are (i)): to optimize new techniques for extraction of Actinobacteria DNA (ii) to identify the species and (iii) to extract and identify the produced metabolites using HPLC and LC-MS and to evaluate their antimicrobial properties.

**Results and Discussion**

Characterization of strain *Streptomyces* sp. Act4Zk

Morphological and growth characteristics

Fragmentation of substrate mycelium and aerial mycelium was observed on the media ISP 2 and ISP 3. The color of the aerial mycelium is in the grey group. The aerial mycelium on ISP 4 medium was white to pale blue, and no aerial mycelium was formed on ISP 5, ISP 6, and ISP (Pimentel et al. 2010).
The color of the substrate mycelium from strain *Streptomyces* sp. Act4Zk was pretty different as for ISP 2- ISP 7, ochre yellow, daffodil yellow to maize yellow, saffron yellow to sandy yellow, ivory, olive-brown, and ivory were observed.
*Streptomyces* sp. Act4Zk produced no soluble pigment on ISP 2, ISP 3, ISP4, and ISP 7, while soluble light ivory to green- brown one was produced on medium ISP 5, and ISP 6. *Streptomyces* sp. Act4Zk grew well on ISP 2, ISP 3, and ISP 4 while it did not grow well on other media. The surface of the spores was smooth. No aerial mycelium was found for strain *Streptomyces* sp. Act4Zk as compared to the type strain (Figure 2).
Comparison of morphological and growth characteristics (listed in Table 2), showed that new isolate *Streptomyces* sp. Act4Zk is most probably different from the closest related type strain *S. roseolilacinus* NBRC 12815$^{T}$. The following differences were observed: the type strain produced no soluble pigment on ISP5 and ISP6; it grew well on ISP5, ISP6, and ISP7 with some differences in colony color and aerial mycelium (Table 2).

Phylogenetic analysis

The new method described here considerably increased DNA concentration and quality in comparison with other methods and was particularly useful for actinobacterial species [Khosravi et al. unpublished data].

Based on the 16S rRNA gene analysis, the new strain *Streptomyces* sp. Act4Zk$^{T}$ was found to be highly related to *S. roseolilacinus* NBRC 12815 $^{T}$ (98.8% similarity). The final sequence alignment of the 16S rRNA comprising of 31 internal taxa, had 1347 characters and 75 unique site patterns. *Nocardiopsis dassonvillei* Al H7A1$^{T}$ (KF384494) was used as the outgroup taxon. GTR+I+G was the best-fitting substitution model. Bayesian analyses resulted in 602 generations. After discarding the first 25% of generations as burn-in, the remaining 452 (75%) generations were used to calculate the consensus

Bayesian tree and posterior probabilities. Results indicated that the strain *Streptomyces* sp. Act4Zk$^{T}$ clustered in the same clade with *Streptomyces roseolilacinus* NBRC 12815 $^{T}$ (AB184167) and *Streptomyces sudanensis* SD 504 $^{T}$ (EF515876) with highly supported value (Figure 1).

Physiological characteristics and chemotaxonomy

Characterizations of the selected strains are described in Bergey's Manual of Systematic Bacteriology (Garrity and Halt 2001) and Compendium of Actinobacteria by Dr. Joachim M. Wink, University of Braunschweig, Germany (El-Naggar et al. 2002).

The carbohydrate utilization test played a prominent role in the taxonomic characterization of the strains. Studies on the requirement of carbon sources for growth showed that the tested strain could utilize glucose, arabinose, xylose, raffinose, fructose, and cellulose but not sucrose, inositol, mannose, and rhamnose. Meanwhile, the type strain *S. roseolilacinus* was able to utilize sucrose, xylose, rhamnose, and fructose.

The API Zym and API-Coryne systems offer a useful method for the detection of selected enzymes in *Streptomyces* species (Humble et al. 1977). According to the results, a positive reaction was determined for alkaline phosphatase, leucine arylamidase, phosphatase acid, valine arylamidase, beta-galactosidase, alpha-glucosidase, beta-glucosidase. In contrast, a weak positive reaction was observed for esterase-lipase, lipase, trypsin, naphthol-ASBI-phosphohydrolase, and N-acetyl glucoseamidase. *Streptomyces* sp. Act4Zk$^{T}$ had positive result for esculine (beta glucosidase), urease and gelatin (hydrolysis), while the carbohydrate fermentation tests were negative. According to Vitezova 2013 (Vitzova 2013), in all streptomycete strains, leucine arylamidase and acid phosphatase were found as common enzymes, and 89% of isolates showed valine aryl amidase activity. Consistent with our results, the least frequent enzyme was glucuronidase. The activity for esterase, cystine arylamidase, chymotrypsin, alpha-galactosidase, mannosidase, and fucosidase was very low.

The temperature range for growth was 25-37 °C, with the optimum temperature was 30 °C. The strain exhibited salt tolerance up to 5% with optimum growth at 2.5% NaCl; hence, the strain could be placed in an intermediate salt tolerance group according to Tresner *et al*. 1986 (Tresner et al. 1968). Nevertheless, for type strain *S. roseolilacinus*, the temperature range for growth was 20-45 °C and salt tolerance was up to 2.5%.

A characteristic fatty acid pattern was obtained for *Streptomyces* species with significant amounts of iso-C16:0 dominating. The major cellular fatty acids of the new strain *Streptomyces* sp. Act4Zk with nearly 5 % or more of the total were iso-$C_{14}$:0, iso-$C_{15}$:0, anteiso-$C_{15}$:0, iso-$C_{16}$:0, $C_{16}$:1, $C_{16}$:0, iso-$C_{17}$:0, iso-$C_{17}$:0, and anteiso-$C_{17}$:0. Table 4 presents fatty acid compounds as well as their quantities.

Overall, the molecular analysis confirmed the morphological and cultural, physiological, and biochemical test results for the new isolate. The identification of the strain *Streptomyces* sp. Act4Zk$^{T}$, using a polyphasic approach, indicated that the strain *Streptomyces* sp. Act4Zk represents possibly a new species of the genus *Streptomyces*. More molecular and biochemical tests should be conducted prior to introducing and registering Strain *Streptomyces* sp. Act4Zk as a new species.

Antimicrobial Activity and Bioactive Compounds of the Strain *Streptomyces* sp. Act4Zk

The MIC values for the antimicrobial activity of the tested strain are given in Table 1. The results revealed that the extract (obtained from 5254 medium) exhibited the strongest antimicrobial activity against yeast *Candida albicans* DSM 1665, *Mucor hiemalis* DSM 2656, *Pichia anomala* DSM 6766,

*Escherichia coli* TolC (1.67 µg $ml^{-1}$), *Bacillus substilis* DSM 10 (6.68 µg $ml^{-1}$), *Mycobacterium smegmatis* ATCC 700084 and *Staphylococcus aureus* Newman (26.75 µg $ml^{-1}$). The extract was subjected to HPLC fractionation against all mentioned strains and then, active compounds were analyzed using LC/MS. In HPLC chromatogram, at least ten peaks out of which 4 were interesting. The compound appeared at retention time 11.5-14 min was active against *C. albicans, P. anomala* and *M*. *hiemalis*, those appeared at retention time 12.5 and 26-27 min (Linoleic acid) were active against *S. aureus* and the one found at retention time 12-13.5 min was active against *E. coli Tol C* and *M*. *smegmatis* (Figure 3). The chromatographic separation and mass spectrophotometer detection provided a large number of fragmentation pattern. Analysis can use ESI positive and negative charges. The negative ESI mode is characterized by the formation of the [M-H]- ion, and the positive ESI mode is characterized by the formation of the [M+H]+ ion.

This experiment used positive ESI. HPLC coupled with LC/MS is one of the most powerful tools for detecting bioactive compounds from microorganisms (Jothy et al. 2011). Further analyses done by LC/MS confirmed the previous data showing three different compounds with the following characteristics. **Compound 1:** Rt(acid)=6.56 min; Rt(buffer)=12.4 min; M466; $C_{28}H_{26}N_4O_3$; staurosporin (Figure S1) (Omura et al. 1977). The dominant peak in the HPLC chromatogram, which reached a maximal intensity at 168 h of cultivation, was identified as staurosporine based on its characteristic UV–visible maxima at 206, 238, 292, 334, 354, and 372 nm, and the accurate mass [M+H]+ = 467.2133 that can be found in the Dictionary of Natural Products (DNP). **Compound 2:** Rt(acid)=8.60 min; Rt(buffer)=15.86 min; M469; $C_{27}H_{23}N_3O_5$; can be identified as antibiotic MLR 52 with accurate mass [M+H]+ = 470.1705; and UV–visible maxima at 224, 292, 336, 354, and 370 nm based on DNP; (Figure S2) (McAlpine et al. 1994) and **Compound 3:** Rt(acid)=8.22 min; Rt(buffer)=14.85 min; M457; $C_{26}H_{23}N_3O_5$; was determined as antibiotic K 252d ($N^{13}$–(α-L Rhamnopyranosyl) –Staurosporinone) with accurate mass [M+H]+ = 458.1705 and UV–visible maxima at 224, 290, 334, 348, and 364 nm according to DNP. However, antibiotic K252d was reported before from *Nocardiopsis sp*. (Figure S3) (Akanishi et al. 1986).

To make sure that biological activity results are different from data reported for the compound "staurosporine", antimicrobial activity test against the entire our group pathogens in our tests, was done (table 1). Staurosporine showed a weak antifungal activity (range of 2.11-4.05 µg $ml^{-1}$), but the extract had a strong antifungal activity (1.67 µg $ml^{-1}$) and were active against *M. smegmatis* and other Gram-positive bacteria, confirming our hypothesis that the new species is a good producer of staurosporine and some derivative of staurosporine with antifungal and antibacterial activity. For comparing our obtained results with previously published, Sturosporine standard maxis is shown in Figure S4.

In this study, strain *Streptomyces* sp. Act4Zk$^T$ and myxobacetria were isolated from soil samples collected in 2015 from the Jarghooye city. Extraction and concentrating DNA are the first critical steps in molecular analytical methodologies. The new method described here considerably increased the concentration of DNA in comparison with other methods and was particularly useful for actinomyces species. Based on morphological, physiological, and biochemical findings as well as 16S rRNA gene sequence, this strain was identified as a *Streptomyces* species and *S. roseolilacinus* was the closest type strain to our isolate. The antimicrobial assay indicated marked antifungal and, anti- *M. smegmatis* and other Gram-positive bacteria activities, for this strain. HPLC and LC/MS analysis of the extract led to the identification of 3 different compounds, and to introduce the novel species of the genus *Streptomyces* being a good producer of staurosporine and some derivatives. There is no description about our new

optimized method, new isolate strain *Streptomyces* sp. Act4Zk nor the new sorurce for antimicrobial compound, and this strain can be a promising candidate for further studies.

## Materials and Methods

### Characterization of strain *Streptomyces* sp. Act4Zk$^T$

#### Cultural and Micro-Morphological Characteristics

The strains grew on agar plates were analyzed for morphology and colony description, then, plated on different media as described by Shirling and Gottlieb (International Streptomyces Project ISP) (Shirling and Gottlieb 1966). Here, about 500 µl of a well-grown liquid culture was plated on the following agar plates: Streptomyces medium GYM, yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7). Furthermore, ISP 6 and ISP 7 as well as Suter medium (Suter 1978) with and without tyrosine, were used for detection of melanin production which is visible as a dark brown-to-black pigment in the agar (Starr et al. 2013). After 10-14-day incubation at 30 °C, morphological properties like growth, colony color (detected via RAL-code color cards), aerial mycelium and soluble pigments were observed and correlated with the closest related type strains.

For the light microscopic images, a well-grown agar plate with GYM was used for observation of spore chain morphology using a Zeiss Axio Scopie. A1 microscope. To determine the structure of mycelium and spores, electron microscopy was done. Therefore, the cultures were grown on agar plates on complex media like GYM or the ISP3 oatmeal medium. According to the description provided by Wink (Wink **1981), a well-covered piece was cut out and fixed in glutaraldehyde. After critical-point-dehydration and** gold-palladium-sputtering, a Zeiss Merlin field emission scanning electron microscope (SEM) was used to observe diverse spores and spore chains. The SEM harbour an Everhart-Thornley SE-detector and Inlens-SEM detector at 25: 75 % ratio; SEMSmart software version 5.05 was also applied. The electron microscopic analyses were done by Prof. Dr. Manfred Rohde, HZI Braunschweig. and the procedure was described by O'Donnell et al (1993).

#### Physiological and Biochemical Characteristics

Utilization of different carbon sources was assessed using a microplate technique with 12-well plates as described previously (Shirling and Gottlieb 1966).

Sodium chloride tolerance was tested on microtiter plates (6-well) using a previously described technique (Starr et al. 2013).

ApiZym® stripes were developed by BioMérieu for identification of microorganisms by determining a specific fingerprint of enzymatic activities (Humble et al. 1977). In 1978, Kilian demonstrated successful identification of Actinomycetales and related bacteria by using this method. The method was performed as described by the manufacturer.

Cellular fatty acid extraction was done by the fatty acid methyl ester (FAME) method (Garcia et al. 2011). The strain was cultivated in 50 ml of GYM medium at 30°C and 160 rpm for 4 days. The cultures were harvested by centrifugation. GC analysis and identification of fatty acids were performed according to

the methods of Gemperlein et al. (2014). The DNA G+C content of the novel bacterium was determined by HPLC after nuclease P1 digestion of the genomic DNA (Shimles and Giese 2011; Li et al. 2003).

Genomic DNA Extraction, PCR and Sequencing of 16S rRNA Gene

For molecular identification, the strain *Streptomyces* sp. Act4Zk was cultured in 100 ml of GYM medium and incubated for 3 days (at 160 rpm and 30 °C). Due to the low efficiency of most of the conventional DNA extraction techniques, such as commercial kits and other methods for DNA extraction from strain *Streptomyces* sp. Act4Zk which yield low concentration, low quality and low quantity of recovered DNA, for molecular analysis, new technique for DNA extraction from Actinobacteria were optimized; these techniques are explained below:

Cell mass preparation

The strains were cultured on GYM medium, and incubated at 30 °C under constant shaking at 160 rpm in darkness. To obtain cell mass, bacterial suspension (100 ml) was centrifuged at 7000 ×g for 15 min and the supernatant was discarded completely. For each strain, 150 mg of precipitated cell mass was measured and used for each DNA extraction method. The experiment was performed as three replicates.

DNA extraction by the new method

The precipitated cell mass was suspended in 5 ml SET buffer in 15 ml tube (75 mM NaCl, 25 mM EDTA, pH 8, 20 mM Tris HCL, pH 7.5) and 15 glass beads were added to each vial. The samples were homogenized using a crashing machine (6 m $s^{-1}$/ for 2*40 s). Samples were incubated at 100 ° C for 5 min, then frozen in liquid $N_2$ for 3 min. Tubes were incubated at 100 °C for 5 min and then, contents were transferred into new 15 ml falcon, and 300 µl SDS 20 %, 300 µl proteinase K (10 mg $ml^{-1}$ in 50 mM Tris HCL pH 8, 1 mM Cacl2) and 300 µl lysozyme (10 mg $ml^{-1}$) were added and incubated at 55 °C for 2 hours. The container was inverted at least every 15 min. Next, 50 ml phenol: chloroform: isoamyl alcohol (25:24:1) was added and the falcon was rotated for 1 hour. The mixture was centrifuged at 9000 ×g for 5 min at room temperature. The white layer was significantly reduced or disappeared. If this is not the case, the extraction step should be repeated once more and the tube should be rotated for 1 hour. After centrifugation, the upper phase was transferred into a new falcon. Then, 50 ml of chloroform/isoamyl alcohol (24/1) was added and the falcon was rotated for 30 min. The mixture was centrifuged at 9000 ×g for 5 min at room temperature. The upper phase was transferred into a new falcon. Then, 1/10 volume of 1 M NaOAC (pH 4.8) was added and mixed properly. Falcons were incubated in the freezer (-20 °C) for 10 min. Samples were centrifuged at 14000 ×g at 4 °C for 10 min. The upper phase was transferred into a new falcon and an equal volume of cold isopropanol was added. Samples were incubated in the freezer (-20 °C) for 10 min; then, they were centrifuged at 14000 ×g, 10 min, at 4 °C. The upper phase was discarded. The pellet was washed with cold ethanol 70 % and centrifuged at 14000 ×g, at 4 °C for 10 min (this step was repeated once more). Pellet was resuspended in deionized distilled water or TE buffer and stored at -20 °C.

The 16S rRNA gene region was amplified by PCR using two universal primers, the forward primer binds to the position F27 and the reverse primer attaches the position R1541 of the 16S rRNA gene sequence. For partial 16S rRNA sequencing, two universal primers, forward primers bind to the position F27 and reverse primers bind the position R518 and for the whole 16S rRNA sequencing, five primers were used: F27, R518, F1100, R1100, and R1541. Alignment of the 16S rRNA gene sequence of strain *Streptomyces* sp. Act4Zk was performed using the Cap contig assembly function of the BioEdit Sequence Alignment Editor Version 7.0.5 software (Stackbrandt et al. 1993). 16S rRNA sequence data for references strains were obtained from EzTaxon-e. Bayesian analyses were performed in PAUP v.4.0b10 and MrBayes v3.2.2 (Ronquist et al. 2003). The most suitable model of evolution was estimated by using Mrmodeltest v.2.2 (Nylander 2004).

DNA–DNA relatedness values between isolate *Streptomyces* sp. Act4Zk$^T$ and *Streptomyces roseolilacinus* NBRC
12815$^T$ were determined using the nitrocellulose filter hybridization method as described by Seldin & Dubnau (1985). Genomic DNA of the two strains was extracted by a DNA extraction method optimized for extraction of Actinobacteria species DNA.

Probe DNA samples were labeled using the non-radioactive digoxigenin (DIG) High Prime System (Roche), hybridized DNA was visualized using DIG luminescent detection kits (Roche) and DNA–DNA relatedness was quantified using a densitometer (Bio-Rad).

**Strain Origin and maintenance**

Strain *Streptomyces* sp. Act4Zk$^T$ was isolated from a soil sample collected in 2015 from the Jarghooye city (66 km south east of Esfahan, centeral Iran), besides the isolation of myxobacetria (Reichenbach and Dworkin 1992) by using St21 agar medium (solution A: $K_2HPO_4$ 0.1% (w $v^{-1}$); yeast extract (Difco) 0.002% (w/v); Agar 1% (w $v^{-1}$); solution B: $KNO_3$ 0.1% (w $v^{-1}$), $MgSO_4 \cdot 7H_2O$ 0.1% (w $v^{-1}$), $CaCl_2 \cdot 2H_2O$ 0.1% (w $v^{-1}$), $FeCl_3$ 0.02% (w $v^{-1}$), $MnSO_4 \cdot 7H_2O$ 0.01% (w $v^{-1}$) + 100 µg $mL^{-1}$ Cycloheximide). After isolation and subsequent reculturing, purification of the isolate was done on 5336 medium (containing 1% soluble starch, 0.1% casein, 0.05% $K_2HPO_4$, 0.5% $MgSO_4$ x 7 $H_2O$ 2%, Bacto agar, distilled water (pH 7.3)) and then, on GYM medium agar (glucose 4.0 g, yeast extract 4.0 g, malt extract 10.0 g, $CaCO_3$ 2.0 g, agar 12.0 g, distilled water 1000.0 ml). pH was adjusted to 7.2 before adding agar, and what were maintained as glycerol suspensions (50%, v $v^{-1}$) at -20 °C for long-term storage.

Production, Extraction and Isolation

The pre-culture of strain *Streptomyces* sp. Act4Zk was grown in a 250-ml flask which contained 100 ml of GYM medium (0.4% glucose, 0.4% yeast extract, 1% malt extract, 0.2% $CaCO_3$; pH 7.2; sterilized for 20 min at 121°C) and incubated on a rotary shaker (160 rpm) for 7 days at 30 °C. The resultant culture was transferred 1:10 in two 250-ml flasks, one filled with 100 ml of medium 5294 (1% soluble starch, 0.2% yeast extract, 1% glucose, 1% glycerol, 0.25% corn steep liquor, 0.2% peptone, 0.1% NaCl, and 0.3% CaCO3; pH 7.2) and the other filled with 100 ml of medium 5254 (1.5% glucose, 1.5% soy flour, 0.5% corn steep liquor, 0.2% $CaCO_3$, and 0.5% NaCl; pH 7.2), suspended in distilled water and sterilized for 20 min at 121 °C. The flasks were incubated on a rotary shaker (160 rpm) for 7 days at 30 °C. The extract of the isolate was screened against *Escherichia coli* (DSM 1116), *Escherichia coli* TolC, *Staphylococcus aureus* (Newman), *Candida albicans* (DSM 1665), *Pseudomonas aeruginosa* PA14 (DSM 19882), *Bacillus subtilis* (DSM 10), *Micrococcus luteus* (DSM 1790), *Mycobacterium smegmatis* (ATCC700084),

*Chromobacterium violaceum* (DSM 30191), *Mucor hiemalis* (DSM 2656) and *Pichia anomala* (DSM 6766). Tested strains were obtained from Microbial Strain Collection Group (MISG) of Helmholtz Centrum for Infection Research (HZI) in Braunschweig, Germany.

Next, 25 ml of a 7-day old culture was mixed with 25 ml of ethyl acetate (Sigma Aldrich, USA) in a 50-ml reaction tube. After a 2-min shaking step, the tubes were mixed for 10 min on a rotary shaker. Afterwards, the samples were centrifuged at 10000 ×g for 10 min and the upper phase was transferred into a 50-ml round bottom flask. At about 40 °C the ethyl acetate was evaporated in a rotary evaporator (Heidolph, Germany). Finally, the extract (6mg) was dissolved in 1 ml of methanol 95%. Determination of MIC values was carried out by preparing 4-6 h cultures of indicator bacteria followed by dilution with Mueller- Hinton (MH) broth (Merck, Germany) to obtain 0.01 and 4-6 h culture of yeast by dilution on Mycosel broth (Cazin et al. 1989) to obtain 0.05 with OD 600 nm. Minimal inhibition concentration (MIC) was determined by using the broth microdilution method19 in 96- well microplates (BRAND, Germany). The MIC value was defined as the lowest concentration of the tested extract that inhibited visible growth of test microorganisms. MIC determination was done using twofold serial dilutions in MH/MYC broth (Carl Roth GmbH + Co.KG, Germany/ 1% phytone peptone, 1% glucose, 50 mM HEPES (11.8 g $l^{-1}$), water). Strain *Streptomyces* sp. Act4Zk was investigated by HPLC-diode array analysis for the production of secondary metabolites. Staurosporin pure compound used in this study was prepared from Cayman chemical company (USA), a solution in ethyl acetate (100 µg 100 $\mu l^{-1}$).

Metabolite Identification

Ethyl acetate extract was fractionated using an Agilent 1100 HPLC system equipped with a diode-array UV detector (200-400 nm), and a fraction collector. HPLC conditions: XBridge C18 column 100×2.1 mm (Waters, Milford, USA), 3.5 µm, solvent A [H2O−acetonitrile (95/5), 5 mmol NH4Ac, 0.04 mL $L^{-1}$ CH3COOH]; solvent B [H2O−acetonitrile (5/95), 5 mmol NH4Ac, 0.04 mL $L^{-1}$ CH3COOH]; gradient system, 10% B increasing to 100% B in 30 min; flow rate 0.3 mL $min^{-1}$; 40 °C. Fractions from the HPLC column, were collected in 96-well plates every 0.5 min.

The fractions in the 96-well plate, were dried for 45-60 min at 40 ºC with heated nitrogen in MiniVap (Porvair Sciences, UK). Afterwards, each well was filled with 150 µl of the test organisms such *Candida albicans, Pichia anomala, Mycobacterium smegmatis* and *Mucor hiemalis* in MYC medium. The extract was subjected to LC/MS analysis; LC/MS consisted of an RP-HPLC system Agilent 1260 series with DAD detector (200-600nm) connected to a maXis ESI-TOF-MS spectrometer (Bruker Daltonics, USA) for recording high resolution electron spray ionization mass spectrometry (HRESIMS) data. Samples were analyzed using a Waters ACQUITY UPLC BEH C18 Column (2.1 x 50 mm, 1.7 µm). The LC/MS system had the following conditions: the mobile phase consisted of gradient elution using solvent A: 0.1% formic acid in H2O, B: 0.1% formic acid in acetonitrile; gradient system: 5% B for 0.5 min, in 19.5 min to 100% B and holding 5 min at 100% B; flow rate 0.6 mL $min^{-1}$; 40 °C; UV-detection at 200-600 nm. Molecular formulae were calculated using the SmartFormula algorithm including the isotopic pattern (Bruker) with mass range: 100-2500 Da.

Active compounds were identified by comparison of molecular weights, UV spectra, and retention times with authentic standards. The main software used for processing of results was Data Analysis included in the Compass-software from Bruker (USA).

**Author Contributions:** methodology, K.B.Z and N.A.; investigation and writing –original draft preparation, K.B.Z.; writing—review and editing, K.B.Z.; R.C. and W.J.; project administration, W.J. and E.G. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare that they have no conflict of interest.

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**Figures and Tables**

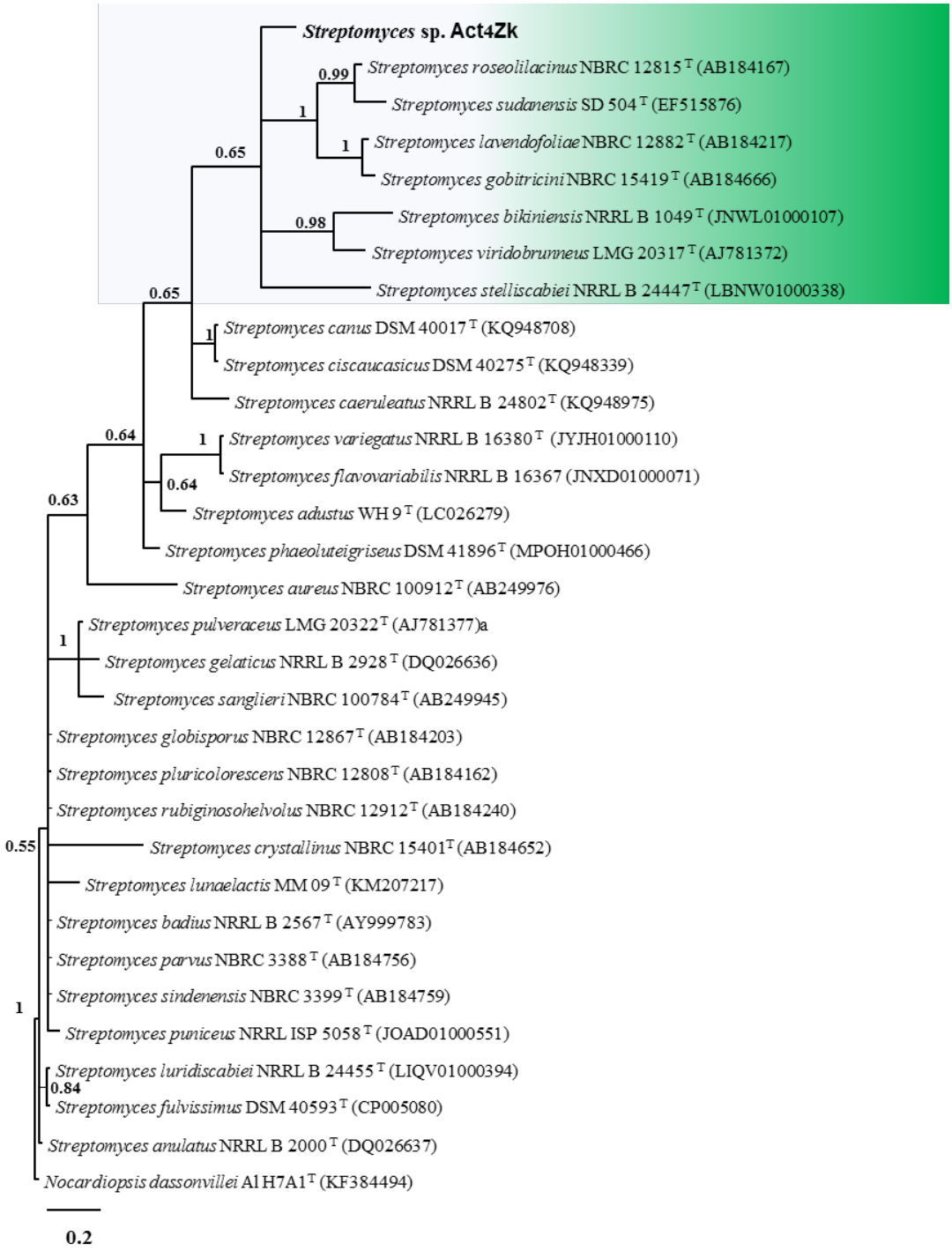


**Figure 1**. Consensus phylogram (75% majority rule) of 452 trees resulting from a Bayesian analysis of 16S rRNA gene sequence alignment using MrBayes v. 3.2.2 of various *Streptomyces* species. The scale bar indicates 0.2 expected changes per site. The tree was rooted to *Nocardiopsis dassonvillei* Al H7A1$^{T}$ (KF384494).

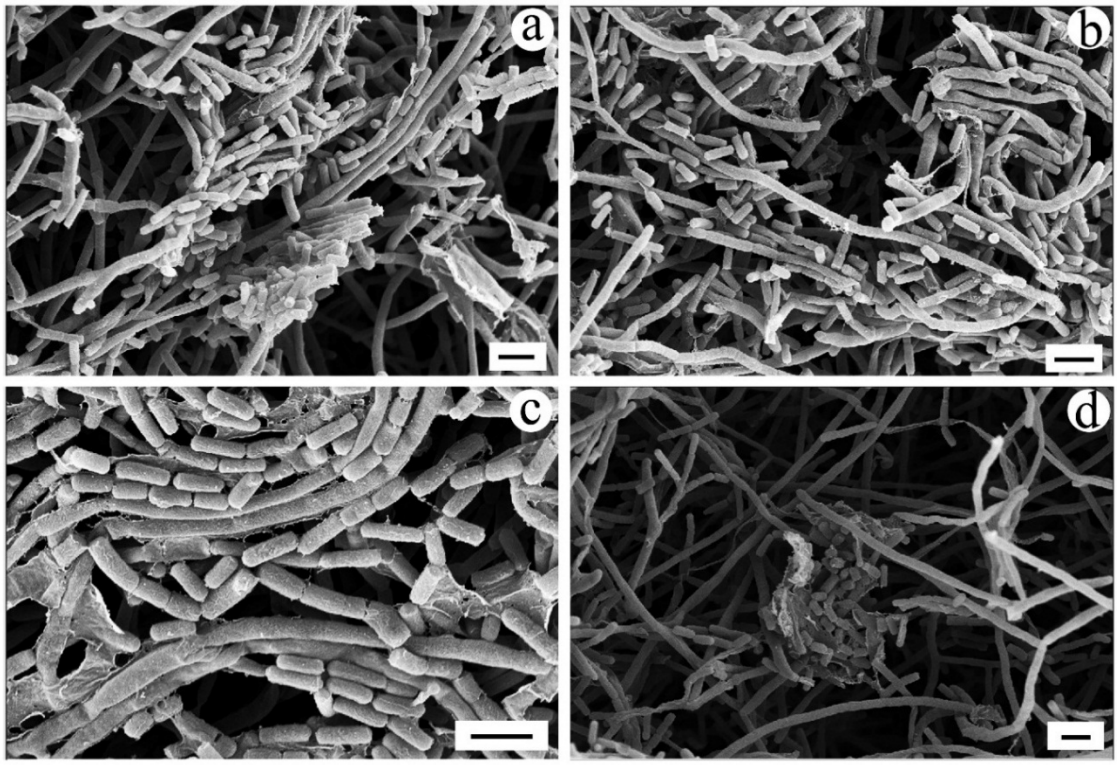


**Figure 2** a, b, c & d: Scanning electron micrograph of spore morphology of Actinomycete strain *Streptomyces* s p . Act4Zk incubated for 14 days at 30 ºC in oatmeal agar (ISP 3) Scale bar = 2 µm).

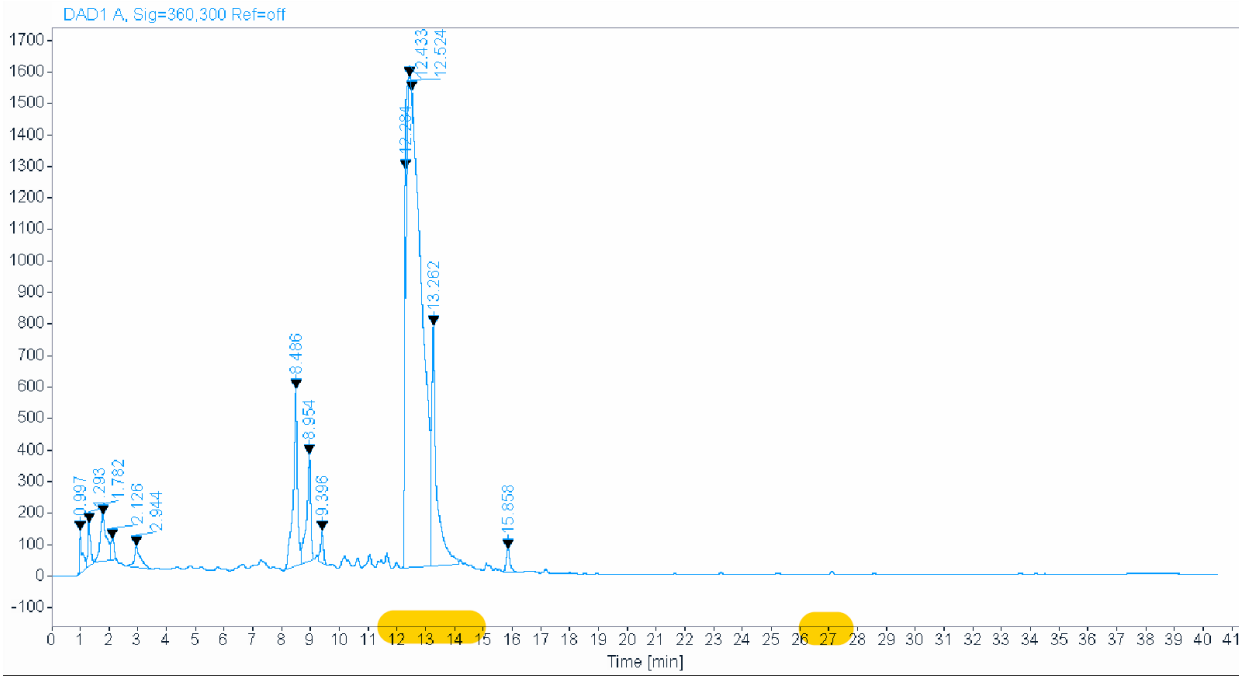


**Figure 3** Fractionation RP-HPLC chromatogram of methanol extract of *Streptomyces* s p . Act4Zk, UV 300 nm, highlighted retention times show the activation peaks (12-15 min).

| Production media | *E.coli* DSM1116 | *E.coli* TolC | *Chromobac. Violaceum* DSM30191 | | *Pseudomonas aeruginosa* PA14 | | *Staph. aureus* Newman | *Micrococ. luteus* DSM1790 | *Mycobact. smegmatis* ATCC700084 | *Mucor hiemalis* DSM2656 | *Pichia anomala* DSM6766 | *Candida albicans* DSM1665 | *Bacillus substilis* DSM10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | WH | FH | WH | FH | | | | | | | |
| **5254 (Crude Extract)** | - | 1.67 | - | - | - | - | 26.75 | - | 26.75 | 1.67 | 1.67 | 1.67 | 6.68 |
| **5294 (Crude Extract)** | - | 3.34 | 428 | 428 | - | - | 26.75 | - | 53.5 | 1.67 | 1.67 | 1.67 | 6.68 |
| **Staurosporine compound (Pure)** | - | 16.94 | - | - | - | - | - | - | - | 2.11 | 2.11 | 4.05 | - |
| **Positive control (Reference)** | 1.66 O | 0.41 O | 0.41 O | 0.41 O | 2.08 G | 2.08 G | 0.41 O | 0.41 O | 0.41 K | 16.66 N | 16.66 N | 33.33 N | 8.33 O |
| **Negative control ( Methanol)** | - | - | - | - | - | - | - | - | - | - | - | - | - |

**Table 1** Antimicrobial activity of strain *Streptomyces* sp. Act4Zk (5254 & 5294 media) and staurosporine compound (100 µg/100 µl in ethyl acetate) against human pathogens. MIC ($\mu g ml^{-1}$).
*N* nystatin, *O* oxytetracyclin, *K* kanamycin, *G* gentamycin

**Table 2** Growth characteristics of strain *Streptomyces* sp. Act4Zk$^T$
ISP, International Streptomyces Project (Shirling and Gottlieb 1966). Colors were taken from ISCC–NBS color charts (Kelly 1964).
Comparison of morphology in ISP2, ISP3, and ISP4.

| Strain | ISP2 | | | | ISP3 | | | | ISP4 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Growth/G** | **Colony color/R** | **Aerial mycelium / A** | **Soluble pigment/S** | **Growth/G** | **Colony color/R** | **Aerial mycelium /A** | **Soluble pigment/S** | **Growth/G** | **Colony color/R** | **Aerial mycelium /A** | **Soluble pigment/S** |
| *Streptomyces* sp. **Act4Zk** | +good | Ochre yellow | Sparse, telegray | - | +good | Daffodil yellow, maize yellow | Sparse, telegray | - | +good | Saffron yellow, sandy yellow | Pastel blue, signal white | - |
| ***S. roseolilacinus*** | + weak | Ochre yellow/ Sand yellow/Pure white | Sand yellow | - | + weak | Irovy light | Oyster white | - | + + | Ochre yellow/Grey white | Silk grey | - |

**Table 2** cont
Comparison of morphology in ISP5, ISP6, and ISP7.

| Strain | ISP5 | | | | ISP6 | | | | ISP7 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Growth/G** | **Colony color/R** | **Aerial mycelium / A** | **Soluble pigment/S** | **Growth/ G** | **Colony color/R** | **Aerial mycelium / A** | **Soluble pigment/S** | **Growth/G** | **Colony color/R** | **Aerial mycelium / A** | **Soluble pigment/S** |
| *Streptomyces* sp. **Act4Zk** | (+) poor | ivory | - | Light ivory | (+)poor | Olive brown | - | Green brown | (+)poor | Ivory | - | - |
| ***S. roseolilacinus*** | + | Light/very silk grey | Olive yellow | - | + | Ochre yellow | - | - | + | Sand yellow/Pure white | Ivory | - |

**Table 3** Metabolites identified from *S. roseolilacinus* actinobacterial strains positive.

| Source(s) | Compound (reference) | Chemical group | Activity | Dereplication analysis |
|---|---|---|---|---|
| Compound 1 | Staurosporin (Fiedler 1993) | indolocarbazole alkaloid | exhibiting anti-cancer activity, Active against fungi and yeast, Antifungal, platelet aggregation inhibitor, anti-parasitic, nematocide, cell cycle progression | MS, UV |
| Compound 2 | Antibiotic MLR 52 (McAlpine et al. 1994) Staurosporin derivative | Alkaloid | Inhibitor of protein kinase C | MS, UV |
| Compound 3 | Antibiotic K 252d (Akanishi et al. 1986) Staurosporin derivative | indolocarbazole alkaloid | Inhibitor of protein kinase C, calmodulin inhibitor and serotonin release inhibitor | MS, UV |

**Table 4** Cellular fatty acid profiles of strain *Streptomyces* sp. Act4ZkT and the type strain *S. roseolilacinus* (NBRC12815$^T$) in identical growth phases. Fatty acids representing more than 5 % of the total, are marked in bold type.

| **Fatty acid** | ***S. roseolilacinus* NBRC 12815$^T$** | *Streptomyces* sp. **Act4Zk$^T$** |
|---|---|---|
| iso-13:0 | 0.24 | 0.10 |

| | | |
|---|---|---|
| anteiso-13:0 | 0.23 | 0.05 |
| iso-14:0 | 3.64 | **5.81** |
| $C_{14}$:0 | 0.09 | 0.16 |
| iso-15:0 | **8.16** | **10.17** |
| anteiso-15:0 | **21.20** | **17.28** |
| $C_{15}$:0 | 0.23 | 0.30 |
| iso-16:1 | 2.19 | 3.88 |
| iso-16:0 | **23.86** | **27.95** |
| **$C_{16}$:1** | **5.51** | 0.99 |
| $C_{16}$:0 | **5.98** | **7.65** |
| iso-17:1 isomer 1 | 2.44 | 1.12 |
| iso-17:1 isomer 2 | 1.88 | 1.79 |
| iso-17:0 | **5.93** | **5.68** |
| anteiso-17:0 | **14.33** | **11.48** |
| $C_{17}$:1 | 0.00 | 3.11 |
| $C_{17}$:0 | 0.20 | 0.20 |
| $C_{18}$:0 | 3.89 | 2.29 |