Isolation and identification of *Streptomyces* sp. Act4Zk, a good producer of Staurosporine and some derivatives

Z. Khosravi Babadi\(^1,2\), G. Ebrahimipour\(^1\), J. Wink\(^2\), A. Narmani\(^3,4\) and C. 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), 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), Braunschweig, Germany
5 Research Unit for Clean Technology, Indonesian Institute of Sciences (LIPI), Bandung, Indonesia

**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.

**Keywords**
- antibacterial activity
- antifungal activity
- DNA extraction method
- Staurosporine
- *Streptomyces*

**Abstract**

In this 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 micro-organisms, 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.

**Introduction**

Soil micro-organisms 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 S. aureus)) and Neisseria gonorrhoeae (the cause of gonorrhea) 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-2282², 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 (Gemperlein et al. 2014).

The strain AM-2282 has been renamed through repeated revisions of the taxonomy of soil actinomycetes as Streptomyces staurosporeus AM-2282³ in 1977, Saccharothrix aerocolonigenes subsp. staurospoream AM-2282⁴ 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 elucidated by X-ray crystallographic analysis (Funato et al. 1994). The total synthesis of staurosporine has been reported by many groups. The first total synthesis was reported by Link et al. (1995). 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 tyrosine 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 programme, a presumptively interesting Streptomyces strain, isolate Streptomyces sp. Act4Zk⁵, 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 roseolilacinas 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 colour 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 7 (Pimentel-Elardo et al. 2010).

The colour 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, ISP 4 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 (Fig. 1).

Comparison of morphological and growth characteristics (listed in Table 1), showed that new isolate Streptomyces sp. Act4Zk is most probably different from the closest related type strain S. roseolilacinas NBRC 12815⁶. 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 colour and aerial mycelium (Table 1).
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. Act4ZkT was found to be highly related to *S. roseolilacinus* NBRC 12815T (98.8% similarity). The final sequence alignment of the 16S rRNA comprising of 31 internal taxa, had 1347 characters and 75 unique site patterns. *Nocardiosis dassonvillei* Al H7A1T (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. Act4ZkT clustered in the same clade with *S. roseolilacinus* NBRC 12815T (AB184167) and *Streptomyces sudanensis* SD 504T (EF515876) with highly supported value (Fig. 2).

Physiological characteristics and chemotaxonomy

Characterizations of the selected strains are described in Bergey’s Manual of Systematic Bacteriology (Garrity and Holt 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 had positive result for esculine (beta glucosidase), urease and gelatin (hydrolysis) while the carbohydrate fermentation tests were negative. According to Vitezova (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%
### Table 1 Growth characteristics of strain *Streptomyces* sp. Act4ZkT

| Strain              | ISP2          | ISP3          | ISP4          |
|---------------------|--------------|--------------|--------------|
|                     | Growth/G     | Colony colour/R | Aerial mycelium/A | Soluble pigment/S | Growth/G | Colony colour/R | Aerial mycelium/A | Soluble pigment/S | Growth/G | Colony colour/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 | – |
|                      | – Weak        | Ochre yellow/ Sand yellow/ Pure white | – | + Weak Ivory light | Oyster white | – | –                      | –                      | – |

**Comparison of morphology in ISP2, ISP3, and ISP4**

- *Streptomyces* sp. Act4Zk
  - + Good Ochre yellow
  - Sparse, telegray
- *Streptomyces roseolilacinus*
  - + Weak Ochre yellow/ Sand yellow/ Pure white
  - + Weak Olive brown
  - Sandy yellow/Pure white

**Comparison of morphology in ISP5, ISP6, and ISP7**

| Strain              | ISP5          | ISP6          | ISP7          |
|---------------------|--------------|--------------|--------------|
|                     | Growth/G     | Colony colour/R | Aerial mycelium/A | Soluble pigment/S | Growth/G | Colony colour/R | Aerial mycelium/A | Soluble pigment/S | Growth/G | Colony colour/R | Aerial mycelium/A | Soluble pigment/S |
| *Streptomyces* sp. Act4Zk | (+) poor Ivory | (+) poor Olive brown | (+) poor Ivory | – | – |
|                      | – Light ivory silk grey | – | – | – | – |
| *Streptomyces roseolilacinus* | + Light/Very olive yellow | + Ochr yellow | + Sand yellow/Pure ivory | – | – |

**ISP, International Streptomyces Project (Shirling and Gottlieb 1966). Colours were taken from ISCC–NBS colour charts (Kelly 1964).**
Figure 2 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 H7A1T (KF384494). [Colour figure can be viewed at wileyonlinelibrary.com]
Table 2 Cellular fatty acid profiles of strain *Streptomyces* sp. Act4ZkT and the type strain *Streptomyces roseolilacinus* (NBRC12815T) in identical growth phases. Fatty acids representing more than 5% of the total, are marked in bold type.

| Fatty acid      | *Streptomyces roseolilacinus* NBRC 12815T | *Streptomyces* sp. Act4ZkT |
|-----------------|------------------------------------------|----------------------------|
| iso-13:0        | 0.24                                     | 0.10                       |
| anteiso-13:0    | 0.23                                     | 0.05                       |
| iso-14:0        | 3.64                                     | **5.81**                   |
| C16:0           | 0.09                                     | 0.16                       |
| iso-15:0        | **8.16**                                 | **10.17**                  |
| anteiso-15:0    | **21.20**                                | **17.28**                  |
| C17:0           | 0.23                                     | 0.30                       |
| iso-16:1        | 2.19                                     | 3.88                       |
| iso-16:0        | **23.86**                                | **27.95**                  |
| C14:0           | 5.51                                     | 0.99                       |
| C15:0           | **5.98**                                 | **7.65**                   |
| iso-17:1 isomer | 2.44                                     | 1.12                       |
| 1               | 1.88                                     | 1.79                       |
| iso-17:1 isomer | 2                                       |                            |
| 2               | **5.93**                                 | **5.68**                   |
| anteiso-17:0    | **14.33**                                | **11.48**                  |
| C12:1           | 0.00                                     | 3.11                       |
| C17:0           | 0.20                                     | 0.20                       |
| C18:0           | **3.89**                                 | **2.29**                   |

Table 2 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. Act4ZkT, using a polyphasic approach, indicated that the strain *Streptomyces* sp. Act4ZkT 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 (Table 3).

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 4. The results revealed that the extract (obtained from 5254 medium) exhibited the strongest antimicrobial activity against yeast *Candida albi- cans* DSM 1665, *Mucor hiemalis* DSM 2656, *Pichia anom- ala* DSM 6766, *Escherichia coli* TolC (1.67 µg ml⁻¹), *Bacillus subtilis* DSM 10 (6.68 µg ml⁻¹), *M. smegmatis* ATCC 700084 and *S. aureus* Newman (26.75 µg ml⁻¹). The extract was subjected to HPLC fractionation against all mentioned strains and then, active compounds were analysed using LC/MS. In HPLC chromatogram, at least 10 peaks out of which four 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* (Fig. 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.

Table 3 Metabolites identified from *Streptomyces roseolilacinus* actinobacterial strains positive

| Source(s) | Compound (reference) | Chemical group | Activity | Deregulation 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 Inhibitor of protein kinase C | MS, UV |
| Compound 2 | Antibiotic MLR 52 (McAlpine et al. 1994)Stauroporin derivative | Alkaloid | Inhibitor of protein kinase C | MS, UV |
| Compound 3 | Antibiotic K 252d (Akanishi et al. 1986)Stauroporin derivative | Indolocarbazole alkaloid | Inhibitor of protein kinase C, calmodulin inhibitor and serotonin release inhibitor | MS, UV |
This experiment used positive ESI. HPLC coupled with LC/MS is one of the most powerful tools for detecting bioactive compounds from micro-organisms (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; C28H26N4O3; staurosporin (Fig. 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, 336, 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; C27H23N3O5; 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 (Fig. S2) (McAlpine et al. 1994) and **Compound 3**: Rt(acid) = 8.22 min; Rt(buffer)=14.85 min; M457; C26H23N3O5; was determined as antibiotic K 252d (N13-[α-L-Rhamnopyranosyl]-Stauroporinone) 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 Nocardiosis sp. (Fig. 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 4). 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, Stauroropin standard maxis is shown in Fig. S4.

In this study, strain *Streptomyces* sp. Act4ZkT and myxobacteria 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 actinomycetes 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

### Table 4: Antimicrobial activity of strain *Streptomyces* sp. Act4Zk (5254 & 5294 media) and staurosporine compound (100 µg/100 µl in ethyl acetate) against human pathogens. MIC (µg l⁻¹)

| Production media | Pseudomonas aeruginosa PA14 | Staphylococcus aureus Newman | Microcococcus smegmatis ATCC 700864 | Mycobacterium smegmatis DSM1790 | Staphylococcus luteus DSM1116 | Escherichia coli DSM10901 |
|------------------|-----------------------------|-----------------------------|------------------------------------|-------------------------------|-------------------------------|---------------------------|
| 5254 (crude extract) | 16.66 N | 3333 N | 8333 O | – | – | – |
| 5294 (crude extract) | – | – | – | – | – | – |
| Staurosporine compound | – | – | – | – | – | – |
| Positive control (reference) | 16.66 N | 3333 N | 8333 O | – | – | – |
| Negative control (methanol) | – | – | – | – | – | – |

**N** = nystatin, **O** = oxytetracyclin, **K** = kanamycin, **G** = gentamycin.
bacterial activities, for this strain. HPLC and LC/MS analysis of the extract led to the identification of three 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 source for antimi-
crobial compound, and this strain can be a promising candidate for further studies.

**Materials and Methods**

**Characterization of strain *Streptomyces* sp. Act4Zk**

*Cultural and micro-morphological characteristics*

The strains grew on agar plates were analysed 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- to 14-day incubation at 30°C, morphological properties like growth, colony colour (detected via RAL-code colour 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 (2003), 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 ver. 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 microtitre plates (six-well) using a previously described technique (Starr et al. 2013).

ApiZym® stripes were developed by BioMérieux 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 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 rev min⁻¹ for 4 days. The cultures were harvested by centrifugation. GC analysis and identification of fatty acids were performed according to the methods of Gumperlein et al. (2014). The DNA G + C content of the novel bacterium was determined by HPLC after nuclease P1 digestion of the genomic DNA (Li et al. 2003; Shimelis and Giese 2006).

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 rev min⁻¹ 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 rev min⁻¹ 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 mmol l⁻¹ NaCl, 25 mmol l⁻¹ EDTA, pH 8, 20 mmol l⁻¹ 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 x 40 s). Samples were incubated at 100°C for 5 min, then frozen in liquid N₂ 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 mmol l⁻¹ Tris HCl pH 8, 1 mmol l⁻¹ Cacl2) and 300 µl lysozyme (10 mg ml⁻¹) were added and incubated at 55°C for 2 h. The container was inverted at least every 15 min. Next, 50 ml phenol: chloroform: isoamyl alcohol (24:1) was added and the falcon was rotated for 1 h. 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 h. 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 mol l⁻¹ NaOAC (pH 4-8) was added and mixed properly. Falcons were incubated in the freezer (−20°C) for 10 min. Samples were centrifuged at 14 000 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 14 000 g, 10 min, at 4°C. The upper phase was discarded. The pellet was washed with cold ethanol 70% and centrifuged at 14 000 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 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 ver. 7.0.5 software (Stackebrandt 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 and Huelsenbeck 2003). The most suitable model of evolution was estimated using Mrmodeltest v.2.2 (Nylander 2004).

DNA–DNA relatedness values between isolate Streptomyces sp. Act4Zk and S. roseolilacinus NBRC

12815T were determined using the nitrocellulose filter hybridization method as described by Seldin and 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 labelled using the non-radioactive digoxigenin (DIG) High Prime System (Roche, Mannheim, Germany), hybridized DNA was visualized using DIG luminescent detection kits (Roche) and DNA–DNA relatedness was quantified using a densitometer (Bio-Rad Laboratories GmbH, Feldkirchen, Germany).

Strain origin and maintenance

Strain *Streptomyces* sp. Act4ZkT was isolated from a soil sample collected in 2015 from the Jarghooye city (66 km south east of Esfahan, central Iran), besides the isolation of myxobacteria (Reichenbach and Dworkin 1992) using St21 agar medium (solution A: K2HPO4 0.1% (w/v); yeast extract (Difco laboratories GmbH, Heidelberg, Germany) 0.002% (w/v); Agar 1% (w/v); solution B: KNO3 0.1% (w/v), MgSO4 7H2O 0.1% (w/v), CaCl2: 2H2O 0.1% (w/v), FeCl3 0.02% (w/v−1), MnSO4 7H2O 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% K2HPO4, 0.5% MgSO4 × 7 H2O 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, CaCO3 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% CaCO3; pH 7.2; sterilized for 20 min at 121°C) and incubated on a rotary shaker (160 rev min−1) 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% CaCO3, 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 rev min−1) for 7 days at 30°C. The extract of the isolate was screened against *E. coli* (DSM 1116), *E. coli* TolC, *S. aureus* (Newman), *C. albicans* (DSM 1665), *Pseudomonas aeruginosa* PA14 (DSM 19882), *B. subtilis* (DSM 10), *Micrococcus luteus* (DSM 1790), *M. smegmatis* (ATCC 700084), *Chromobacterium violaceum* (DSM 30191), *M. 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) 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 10 000 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, Schwabach, Germany). Finally, the extract (6 mg) 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, Darmstadt, 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 using the broth microdilution method19 in 96-well microplates (BRAND, Darmstadt, Germany). The MIC value was defined as the lowest concentration of the tested extract that inhibited visible growth of test micro-organisms. MIC determination was done using twofold serial dilutions in MH/MYC broth (Carl Roth GmbH + Co.KG, Germany)/1% phytone peptone, 1% glucose, 50 mmol l−1 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 µl−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, MA), 3.5 µm, solvent A [H2O – acetonitrile (95/5), 5 mmol NH4Ac, 0.04 mol l−1 CH3COOH]; solvent B [H2O – acetonitrile (5/95), 5 mmol NH4Ac, 0.04 mol 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°C with heated nitrogen in MiniVap (Porvair Sciences, UK). Afterwards, each well was filled with...
150 µl of the test organisms such as C. albicans, P. anomala, M. smegmatis and M. 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 (200–600 nm) connected to a mXis ESI-TOF-MS spectrometer (Bruker Daltonics, Hamburg, Germany) for recording high resolution electron spray ionization mass spectrometry (HRESIMS) data. Samples were analysed using a Waters ACQUITY UPLC BEH C18 Column (2.1 × 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 Optik GmbH (Leipzig, Germany).

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

Funding
The authors received no specific grant from any funding agency.

Acknowledgement
The authors thank Klaus Konrad, Birte Trunkwalter, Stephani Schulz and Vanessa Stiller for expert technical assistance.

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

References
Abd-Elnaby, H., Abo-Elala, G., Abdel-Raouf, U., Abd-elwahab, A. and Hamed, M. (2016) Antibacterial and anticancer activity of marine Streptomyces parvus: optimization and application. Biotechnol Biotechnol Equip 30, 180–191.

Akanishi, S., Matsuda, Y., Ishahashi, K. and Kase, H. (1986) K-252b, c and d, potent inhibitors of protein kinase C from microbial origin. J Antimicrobes Chemother 39, 1066–1071.

Baltz, R.H. (1998) Genetic manipulation of antibiotic producing Streptomyces. Trends Microbiol 6, 76–83.

Bevan, P., Ryder, H. and Shaw, I. (1995) Identifying small-molecule lead compounds: the screening approach to drug discovery. Trends Biotechnol 13, 115–121.

Cazin, J., Wiemer, D.F. and Howard, J.J. (1989) Isolation, growth characteristics, and long-term storage of fungi cultivated by antiseptic ants. Appl Environ Microbiol 55, 1346–1350.

Cooper, M.A. and Shlaes, D. (2011) Fix the antibiotics pipeline. Nature 472, 32.

Davies, J. (2006) Where have all the antibiotics gone? Can J Infect Dis Med 17, 287–289.

El-Naggar, N.E.A., Abdel-Fattah, M.G., Abo-Hamed, N.A., Adamek, V., Kralova, B., Suchova, M., Valentova, O., Demmerova, B. et al. (2002) Compendium of actinobacteria from Dr. Joachim M. Wink, University of Braunschweig, an electronic manual including the important bacterial group of the actinomycetes. Int J Pharmacol 11, 445–454.

Fenical, W., Baden, D., Burg, M., De Goyet, C.V., Grimes, J.D., Katz, M., Marcus, N.H., Pomponi, S. et al. (1999) Marine derived pharmaceuticals and related bioactive compounds. From Monsoons to Microbes: Understanding the Ocean’s Role in Human Health, ed. Fenical W., (pp. 71–86). Washington, DC: National Academies Press

Fiedler, H.P. (1993) Biosynthetic capacities of actinomycetes. Screening for secondary metabolites by HPLC and UV-visible absorbance spectral libraries. Nat Prod Lett 2, 119–128.

Funato, N., Takayanagi, H., Konda, Y., Toda, Y., Harigaya, Y., Iwai, Y. and Ømura, S. (1994) Absolute configuration of staurosporine by X-ray analysis. Tetrahedron Lett 35, 1251–1254.

Garcia, R., Pistorius, D., Stadler, M. and Müller, R. (2011) Fatty acid-related phylogeny of myxobacteria as an approach to discover polysaturated omega-3 fatty acids. J Bacteriol Res 193, 1930–1942.

Garrity, G.M. and Holt, J.G. (2001) The road map to the manual. In Bergey’s Manual® of Systematic Bacteriology eds. Garrity, G., Boone, D.R., and Castenholz, R.W., (pp. 119–166). New York, NY: Springer.

Gemperlein, K., Rachid, S., Garcia, R.O., Wenzel, S.C. and Müller, R. (2014) Polysaturated fatty acid biosynthesis in myxobacteria: different PUFA syntheses and their product diversity. Chem Sci 5, 1733–1741.

Humble, M.W., King, A. and Phillips, I. (1977) API ZYM: a simple rapid system for the detection of bacterial enzymes. J Clin Pathol 30, 275–277.

Jothy, S.L., Zakaria, Z., Chen, Y., Lau, Y.L., Latha, L.Y., Shin, L.N. and Saisidharan, S. (2011) Bioassay-directed isolation of active compounds with antifeedant activity from a Cassia fistula seed extract. Molecules 16, 7583–7592.
Kelly, K.L. (1964) *Color-Name Charts Illustrated with Centroid Colors*. Chicago, IL: Inter-Society Color Council-National Bureau of Standards.

Li, G., Shimelis, O., Zhou, X. and Giese, R.W. (2003) Scaled-down nuclease P1 for scaled-up DNA digestion. *Biotechniques* **34**, 908–909.

Link, J.T., Raghavan, S. and Danishefsky, S.J. (1995) First total synthesis of staurosporine and ent-staurosporine. *J Am Chem Soc* **117**, 552–553.

McAlpine, J.B., Karwowsky, J.P., Jackson, M., Mullally, M.M., Hochlowski, J.E., Premachandran, U. and Burres, N.S. (1994) MLR-52, (4’-demethylaminO4’5’-dihydroxystaurosporine), A new inhibitor of protein kinase C with immunosuppressive activity. *J Antibiot* **47**, 281–288.

Mohr, K.I. (2016) History of antibiotics research. In: *How to Overcome the Antibiotic Crisis – Facts, Challenges, Technologies & Future Perspective*, ed. Stadler, M. and Dersch, P. (pp. 237–272). Curr top Microbiol Immunol, Springer. 303–338.

Nakano, H., Kobayashi, E., Takahashi, I., Tamaoki, T., Kuzzu, Y. and Iba, H. (1987) Staurosporine inhibits tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein. *J Antibiotics* **40**, 706–708.

Newman, D.J., Cragg, G.M. and Snader, K.M. (2003) Natural products as sources of new drugs over the period 1981–2002. *J Nat Prod* **66**, 22–1037.

Nylander, J.A. (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

O’Donnell, A.G., Falconer, C., Goodfellow, M., Ward, A.C. and Williams, E. (1993) Biosystematics and diversity amongst novel carboxydotrophic actinomycetes. *Anton Leeuw Int J G* **64**, 325–340.

Omura, S., Iwai, Y., Hirano, A., Nakagawa, A., Awaysa, J., Tsuchiya, H., Takahashi, Y. and Asuma, R.A. (1977) New alkaloid AM-2282 of Streptomyces origin taxonomy, fermentation, isolation and preliminary characterization. *J Antibiot* Res **30**, 275–282.

Pimentel-Elardo, S.M., Kozyska, S., Bugni, T.S., Ireland, C.M., Moll, H. and Hentschel, U. (2010) Anti-parasitic compounds from Streptomyces sp. strains isolated from Mediterranean sponges. *Mar Drugs* **8**, 373–380.

Reichenbach, H. and Dworkin, M. (1992) The myxobacteria. *The Prokaryotes* eds. Balows, A., Truper, H.G., Dworkin, M., Harder, W., and Schleifer, K.H., pp. 3416–3487. New York, NY: Springer.

Ronquist, F. and Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572–1574.

Sánchez, C., Méndez, C. and Salas, J.A. (2006) Indolocarbazole natural products: occurrence, biosynthesis, and biological activity. *Nat Prod Rep* **23**, 1007–1045.

Seldin, L. and Dubnau, D. (1985) Deoxyribonucleic acid homology among Bacillus polymyxa, Bacillus macerans, Bacillus azotofixans, and other nitrogen-fixing Bacillus strains. *Int J Syst Evol Microbiol* **35**, 151–154.

Shimelis, O. and Giese, R.W. (2006) Nuclease P1 digestion/ high-performance liquid chromatography, a practical method for DNA quantitation. *J Chromatogr A* **1117**, 132–136.

Shirling, E.T. and Gottlieb, D. (1966) Methods for characterization of Streptomyces species1. *Int J Syst Evol Microbiol* **16**, 313–340.

Stackebrandt, E., Liesack, W. and Goebel, B.M. (1993) Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FEMS Microbiol Lett* **7**, 232–236.

Starr, M.P., Stolp, H., Trüper, H.G., Balows, A. and Schlegel, H.G. (Eds.) (2013) *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria*. Heidelberg, Germany: Springer-Berlin Heidelberg.

Suter, M.A. (1978) Isolierung und Charakterisierung von Melanin-negativen Mutanten aus Streptomyces glaucescens. Doctoral dissertation, ETH Zurich. https://www.research-collection.ethz.ch/handle/20.500.11850/136235

Takahashi, Y., Shinose, M., Seino, A., Iwai, Y. and Omura, S. (1995) Transfer of Staurosporine-producing strain *Streptomyces staurosporeus* AM-2282 to the Genus Saccharothrix as *Saccharothrix aerocolonigenes* (Labeled 1986) subsp. *staurosporeus* subsp. nov. *Actinomycetologica* **9**, 19–26.

Tresner, H.D., Hayes, J.A. and Backus, E.J. (1968) Differential tolerance of streptomycetes to sodium chloride as a taxonomic aid. *Appl Microbiol* **16**, 1134–1136.

Van der Meij, A., Worsley, S.F., Hutchings, M.I. and van Wezel, G.P. (2017) Chemical ecology of antibiotic production by actinomycetes. *FEMS Microbiol Rev* **41**, 392–416.

Vitezova, M. (2013) Characterisation of actinomycetes community from the heavy metals-pollute soil. *Acta Univ Agric et Silvic Mende Brun* **61**, 1471–1478.

Weber, T., Welzel, K., Pelzer, S., Vente, A. and Wohlleben, W. (2003) Exploiting the genetic potential of polyketide producing streptomycetes. *J Biotechnol* **106**, 221–232.

Wink, J. (2003) Polyphasic taxonomy and antibiotic formation in some closely related genera of the family pseudonocardiae. In: *Recent Research Developments in Antibiotics*, ed. Pandalai, S.G. (pp. 97–140). Kerala, India: Transworld Research Network 1981.

Xie, Q., Wang, Y., Huang, Y., Wu, Y., Ba, F. and Liu, Z. (2002) Description of *Lentzea flaviverucosa* sp. nov. and transfer of the type strain of *Saccharothrix aerocolonigenes* subsp. *staurosporeae* to *Lentzea albidia*. *Int J Syst Evol Microbiol* **52**, 1815–1820.

Zhou, L., Hopkins, A.A., Huhman, D.V. and Sumner, L.W. (2006) Efficient and sensitive method for quantitative analysis of alkaloids in hardinggrass (*Phalaris aquatica* L.). *J Agric Food Chem* **54**, 9287–9291.
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. (a) LC/MS chromatogram for compound 1, t = 6.56 min; Rt(buffer) = 12.4 min; Staurosporin (compound 1) (b) UV spectrum of compound 1; (c) ESI-HRMS spectrum, showing the prominent ion clusters.

Fig S2. (a) LC/MS chromatogram for compound 2, t = 8.60 min; Rt(buffer) = 15.86 min; Antibiotic MLR 52 (compound 2). (b) UV spectrum of compound 2. (c) ESI-HRMS spectrum, showing the prominent ion clusters.

Fig S3. (a) LC/MS chromatogram for compound 3, t = 8.22 min; Rt(buffer) = 14.85 min; Antibiotic K 252d Staurosporin-derivative (compound 3). (b) UV spectrum of compound 3; (c) ESI-HRMS spectrum, showing the prominent ion clusters.

Fig S4. Staurosporine standard Maxis. (a) UV spectrum of Staurosporine; (b) ESI-HRMS spectrum.