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ORIGINAL ARTICLE

Elaiomycins K and L, new azoxy antibiotics from *Streptomyces* sp. Tü 6399*

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Elaiomycins K and L, two new azoxy-type antibiotics, were detected by HPLC-diode array screening in the culture filtrate extract of *Streptomyces* sp. Tü 6399. The structures were determined by high-resolution MS and 2-dimensional ^{1}H and ^{13}C correlated NMR spectroscopy including ^{15}N-NMR experiments and established these compounds as new members of the elaiomycin family. Both metabolites show a weak antibacterial activity against *Bacillus subtilis* and *Staphylococcus lentus* as well as against the phytophathogenic strain *Xanthomonas campestris*.
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INTRODUCTION

The rhizosphere is a highly diverse community of plant roots, symbiotic fungi, pathogenic fungi and bacteria including numerous members of the order *Actinomycetales*, an order which has been shown to produce a wide variety of natural products.[2,3] Freshly isolated actinomycetes from a rhizospheric soil collected in a spruce stand near Tübingen, Germany, were included in our HPLC screening program to detect novel secondary metabolites useful for pharmaceutical applications. The strains were grown in various complex media as shake flask cultures, samples were taken at various incubation times, and metabolic profiles of extracts were prepared by HPLC-diode array detection (DAD) analysis followed by HPLC–UV–Vis database evaluation and HPLC–ESI–MS analysis.[4] Strain Tü 6399 was of interest because of the presence of two characteristic peaks in the HPLC chromatogram with retention times of 8.6 and 9.9 min, and nearly identical UV-spectra, which differed from those of 960 reference compounds stored in our in-house HPLC–UV–Vis database. HPLC–ESI–MS analysis revealed molecular masses of 246 and 260 Da, respectively. In this report we describe the taxonomy and fermentation of the producing strain, the isolation, structure determination and the biological activities of the two compounds, which were identified as novel members of the family of elaiomycin antibiotics[5–7] with an azoxy chromophore, and were named elaiomycin K and L. The structures of elaiomycins K (**1**) and L (**2**) are shown in Figure 1 together with elaiomycin (**3**).

RESULTS

Taxonomy of the producing strain

When strain Tü 6399 was grown on ISP-2 agar[8] it produced a white aerial spore mass and a yellow-beige pigmented substrate mycelium. Whole-cell hydrolysates of strain Tü 6399 showed the presence of LL-diaminopimelic acid in the peptidoglycan, and *iso*- and *anteiso*-branched fatty acids with *iso*-$C_{16:0}$ and $C_{16:0}$ as major components. The predominant menaquinones found were MK-9(H_2), MK-9(H_4) and MK-9(H_6) in a ratio of 25:40:35. Sequencing of the almost complete 16 S ribosomal RNA gene confirmed the affilitation to the genus *Streptomyces*. The phylogenetic analysis showed a close relationship of strain Tü 6399 (JQ028670) to *Streptomyces* sp. BK 190 (FR692115). Both strains shared a 16 S ribosomal RNA similarity of 99.9% with a difference in one base at position 652 (Figure 2). Strain BK 190 was isolated from a hay meadow soil taken from Cockle Park Experimental Farm in Northumberland, UK, from which we have reported in a previous communication to produce the azoxy antibiotic elaiomycin (**3**) and the novel alkylhydrazide antibiotics elaiomycin B and C.[6,7]

Screening, fermentation and isolation

Strain Tü 6399 was in the course of our HPLC-diode array screening of special interest because of the presence of two characteristic peaks in the HPLC elution profile of the culture filtrate extract with retention times of 8.6 min (**1**) and 9.9 min (**2**), respectively. Both peaks were not identified by means of our HPLC-UV–Vis database

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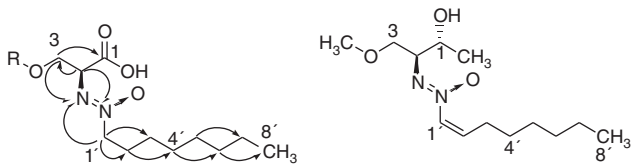

1 R = H
2 R = CH3

3

Figure 1 Structures of elaiomycin K (**1**) and L (**2**), and elaiomycin (**3**). Selected ^{1}H-^{13}C-HMBC correlations are shown by '→' and ^{1}H-^{15}N-HMBC correlations by '⇀'.

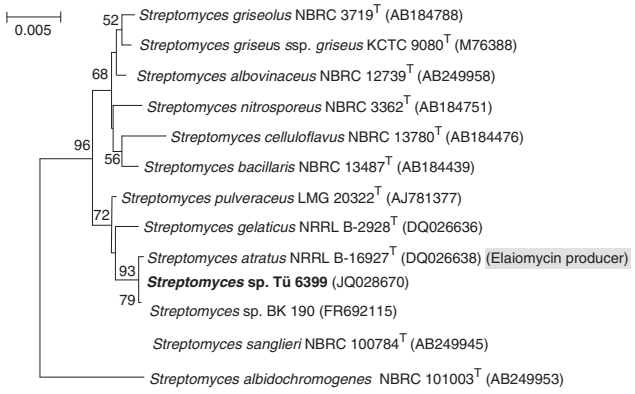

Figure 2 Neighbour-joining tree based on nearly complete 16S RNA gene sequences showing relationships between strain Streptomyces sp. Tü 6399 and representatives of closely related Streptomyces species.[7] The numbers at the nodes indicate the level of bootstrap support (%) based on the analysis of 1000 resampled data sets; scale bar: substitutions per nucleotide position.

considering retention times and UV–Vis spectra of the reference compounds. However, **1** and **2** showed nearly congruent UV spectra to elaiomycin (**3**) with a hypochromic shift of 10-nm from 220 nm, which is the UV maximum of **3**, to 210 nm, the UV maxima of **1** and **2**. A shift to the lower UV range suggested saturation of a double bond in the elaiomycin molecule. Further information on structural details was obtained by HPLC–ESI–MS analysis, which revealed molecular masses of 246 Da for elaiomycin K (**1**) and 260 Da for elaiomycin L (**2**). The mass difference of 14 Da between **1** and **2** suggested either a methylation or an extended alkyl chain of **2**. Furthermore, the assumption for the presence of novel elaiomycin-type antibiotics was strengthened by the taxonomic characterization of strain Tü 6399, which showed a 99.9% identity with the elaiomycin producing strain *Streptomyces* sp. BK 190.

Batch fermentations of strain Tü 6399 were carried out in a 10-l stirred-tank fermenter, reaching a maximal production of 31 and 33 mg l^{-1} for elaiomycin K (**1**) and L (**2**), respectively, after a cultivation time of 8 days. **1** and **2** were isolated from the culture filtrate by Amberlite XAD-16 chromatography, ethyl acetate extraction and subsequent chromatographic separation steps on diol-modified silicia gel, Sephadex LH-20 and preparative reversed-phase HPLC. Compounds were obtained in yields of 95 mg of **1** and 78 mg of **2** as yellowish oils.

Structure determination

In previous contributions of our groups,[6,7] we described the isolation and structure elucidation of two novel alkylhydrazides, elaiomycins B and C, together with the known azoxy-antibiotic elaiomycin (**3**). The NMR spectroscopic data of the herein presented secondary metabolites isolated from strain Tü 6399 showed strong similarities to those of elaiomycin (**3**). Therefore we expected **1** and **2** to be new members of the elaiomycin family.

The molecular formula of compound **1** was established as $C_{11}H_{22}N_2O_4$ from the exact molecular mass (*m/z* 247.16455,

Table 1 NMR spectroscopic data for elaiomycins K (1) and L (2) (500 MHz)

	1[a]				***2***[b]			
Position	δ_H (J *in Hz*)	$\delta_C\delta_N$	*COSY*	*HMBC*	δ_H (J *in Hz*)	$\delta_C\delta_N$	*COSY*	*HMBC*
1	—	168.9, C	—	—	—	172.2, C	—	—
2	4.23[c] (t, 5.8)	65.9, CH	3	1, 3, N2[d]	4.63 (t, 4.9)	63.4, CH	3	N2[d]
3	3.77, 3.82 (dd, dd, 4.6, 10.9, 6.4, 10.7)	60.8, OCH_2	2	1, 2, N1[d]	3.81, 3.91 (dd, dd, 4.3, 10.1, 5.8, 10.1)	70.2, OCH_2	2	1, 4, N1[d]
4	—	—	—	—	3.40 (s)	59.7, OCH_3	—	3
1′	4.22[c] (m)	68.9, NCH_2	2′	2′, 3′, N1[d]	4.25 (m)	71.2, NCH_2	2′	2′, 3′, N1[d]
2′	1.83 (m)	27.3, CH_2	1′, 3′	1′, 3′, 4′	1.95 (m)	28.1, CH_2	1′, 3′	1′, 3′, 4′
3′	1.33[c] (m)	25.5, CH_2	2′, 4′	1′, 5′	1.35[c] (m)	26.4, CH_2	2′, 4′	1′, 5′
4′	1.31[c] (m)	28.4, CH_2	3′, 4′	2′, 3′, 6′	1.32[c] (m)	29.1, CH_2	3′, 5′	2′, 6′
5′	1.27[c] (m)	28.5, CH_2	4′, 6′	7′	1.29[c] (m)	29.2, CH_2	4′, 6′	7′
6′	1.25[c] (m)	31.1, CH_2	5′, 7′	4′, 8′	1.26[c] (m)	31.9, CH_2	5′, 7′	8′
7′	1.24[c] (m)	22.1, CH_2	6′, 8′	5′	1.24[c] (m)	22.8, CH_2	6′, 8′	6′, 8′
8′	0.85 (t, 6.7)	14.9, CH_2	7′	6′, 7′	0.85 (t, 6.7)	14.3, CH_3	7′	6′, 7′
N1	—	344.0, N	—	—	—	344.6, N	—	—
N2	—	345.6, N(O)	—	—	—	343.5, N(O)	—	—

[a]In dimethyl sulfoxide-*d6*.
[b]In $CDCl_3$.
[c]Overlapping signals.
[d]^{1}H-^{15}N-HMBC correlation, δ in p.p.m.

$[M+H]^+$) derived from the high-resolution Orbitrap-ESI mass spectrum (*m/z* calcd 247.16578, Δm 2.7 p.p.m.). The ^{1}H-NMR spectrum of compound **1** lacks two signals assigned to the olefinic double bond (C1′/C2′) of elaiomycin (**3**). Instead two multiplet signals appear at δ_H 4.22 and δ_H 1.83, which were assigned to two methylene groups, corroborating the lack of a double bond in **1**. These findings are accompanied by data from the ^{13}C-NMR spectrum of compound **1**, where two methylene signals at δ_C 68.9 and δ_C 27.3 assigned to C1′ and C2′, respectively, replace the olefinic signals at δ_C 136.4 and δ_C 132.8 (C1′ and C2′) of compound **3**. Furthermore, ^{1}H-NMR and ^{13}C-NMR spectra of compound **1** lack the signals of the secondary methyl group (C1, δ_C 20.2 and δ_H 1.02) found in the 1D NMR spectrum of elaiomycin (**3**). Likewise the signals of a previously found methoxy group (δ_H 3.19 and δ_C 58.5) of **1** are absent. In contrast, a signal characteristic for a quaternary carbon at δ_C 168.9 in the ^{13}C-NMR spectrum of **1** together with a broadened signal at δ_H 12.56 in the ^{1}H-NMR spectrum reveal the presence of a carboxylic function in compound **1**. The ^{1}H $-^1$H-COSY spectrum of **1** shows a correlation between H2 (δ_H 4.23, t, $J=5.8$, 1H) and H3 (δ_H 3.77, 3.82, dd, dd, $J=4.6$, 10.9, 6.4, 10.7, 2H). These data, together with HMBC correlations from H2/H3 to C1 (δ_C 168.9, C), establish a 3-hydroxypropanoic acid moiety (C1–C3). Likewise, COSY correlations between H1′ and H2′, H2′ and H3′, overlapping correlations between H4′/H5′/H6′/H7′, and a COSY correlation between H7′ and the methyl group H8′ support the presence of a saturated alkyl chain in compound **1**. Additionally, HMBC correlations from H1′ to C2′/C3′, H2′ to C1′/C3′/C4′, H4′ to C6′ and from the methyl group (H8′) to C6′/C7′ establish the presence of an octyl chain in compound **1** (Figure 1). Furthermore, ^{15}N-NMR experiments were performed to establish the presence of two nitrogen atoms as implied from the molecular formula. ^{1}H-^{15}N-HMBC experiments revealed correlations from H2 to N2 (δ_N 345.6, N(O)). ^{1}H-^{15}N-HMBC correlations from H3/H1′ to N1 (δ_N 344.0, N) established an azoxy group in compound **1** linked to C2 and C1′. Consequently interpretation of 1D and 2D NMR (Table 1) data identified compound **1** as a novel member of the family of elaiomycin antibiotics[6,7] with an azoxy chromophore, named elaiomycin K.

The molecular formula of compound **2** was determined as $C_{12}H_{24}N_2O_4$ by the analysis of the high-resolution Orbitrap-ESI–MS data of **2** (*m/z* 261.1801, $[M+H]^+$; calcd 261.18143, Δm 3.0 p.p.m.). As mentioned above, the molecular formula of **2** showed a difference of 14 Da compared with compound **1**. This suggested the presence of an additional methyl or methylene group in compound **2**. In general, the ^{1}H- and ^{13}C-NMR spectra of compound **2** showed a high similarity to those of compound **1**. ^{1}H-NMR of **1** revealed an additional signal at δ_H 3.40, as well as an additional signal in the ^{13}C-NMR spectrum at δ_C 59.7 assigned to a methoxy group. This finding suggested the presence of an additional methoxy group in compound **2**. The upfield regions of the ^{1}H, ^{13}C spectra of compound **2** are highly similar to those of compound **1**, while the downfield regions showed some differences. Besides the additional methoxy signals, the downfield shift of the oxygen bearing methylene C3 from δ_C 60.8 (**1**) to δ_C 70.2 (**2**) and the downfield shift of the carboxylic carbon from δ_C 168.9 (**1**) to δ_C 172.2 (**2**) suggested the attachment of the methoxy group to the structure moiety C1-C3 of compound **2**. Finally, a HMBC correlation from the oxygenated methylene (H2, δ_H 3.81, 3.91, dd, dd, $J=4.3$, 10.1, 5.8, 10.1 Hz) to the methoxy carbon (C4, δ_C 59.7) established the structure of compound **2** as the methoxy derivative of **1**. Correlations from H2 to N2 (δ_N 343.5, N(O)) and from H3/H1′ to N1 (δ_N 344.6, N) derived from ^{1}H-^{15}N-HMBC data of compound **2** were in accordance with those of compound **1** and revealed an azoxy group in compound **2** as well. Consequently, 1D and 2D NMR identified compound **2** as a new member of elaiomycin antibiotics[6,7] with an azoxy chromophore, named elaiomycin L.

Biological activity

Elaiomycin K (**1**) and L (**2**) were weakly active against the Gram-positive bacteria *B. subtilis* and *S. lentus*, as well as against the phytophathogenic strain *X. campestris*. The IC_{50} values ranged between 23 and 51 μM: 30.05 (±2.45) and 22.9 (±1.2) μM for *B. subtilis*, 54.15 (±0.75) and 41.7 (±1.9) μM for *S. lentus*, as well as 47.5 (±1.5) and 51.3 μM (±8.3) for *X. campestris*, repectively.

DISCUSSION

Our initial studies indicated the presence of two novel elaiomycin-type metabolites in the extract of *Streptomyces* sp. Tü 6399 supported by the similarity of their UV spectra with those of elaiomycin. Furthermore, they displayed a similar molecular mass range and also the phylogenetic analysis of the producing strain, which showed a very close relationship of the almost complete 16S ribosomal RNA gene sequence with the elaiomycin-producing strain *Streptomyces* sp. BK 190 was in favour of this assumption. The presence of two novel members of the elaiomycin family was confirmed by structure determination using MS and NMR experiments. These structures strongly suggest that the biosynthesis may involve the amino acid, serine. Elaiomycin (**3**) was described as an antibiotic with a chemically unique aliphatic α,β-unsaturated azoxy group produced by *Streptomyces gelaticus*.[5] Interestingly, azoxy-type antibiotics are not commonly found among natural products listed in public databases. Besides elaiomycin the antibiotic LL-BH872α,[9] maniwamycins A and B,[10] azoxybacilin,[11] valanimycin[12] and jietacins A and B[13] are members of the azoxy group of natural products. Recently new members of the elaiomycin family, elaiomycins D-H, were published.[14]

Elaiomycin showed a strong inhibitory activity *in vitro* against the Gram-positive pathogen *Mycobacterium tuberculosis* in concentrations of 0.24–1.25 μg ml^{-1}, but it was ineffective in the treatment of experimental tuberculosis in mice.[15] Elaiomycins K (**1**) and L (**2**) weakly inhibited the growth of *B. subtilis, S. lentus* and *X. campestris* with an average IC_{50} value of 41 μM. In contrast, only *S. lentus* was slightly inhibited by elaiomycin B and elaiomycin C using a concentration of 100 μM. Moreover, elaiomycin was toxic to mice and guinea-pigs, and carcinogenic action was demonstrated in rats.[16] As we reported previously,[6,7] elaiomycin showed a moderate inhibition of the human tumour cell line HepG2 ($IC_{50}=16.3$ μM), whereas elaiomycins B and C and the herein described elaiomycins K (**1**) and L (**2**) have not shown cytotoxic activity towards tumour cell lines. Only **2** weakly inhibited the enzyme acetylcholinesterase (60%) at a concentration of 50 μM. This finding is in contrast to elaiomycin B and C, which showed an inhibitory activity against acetycholinesterase with IC_{50} values of 1.0 and 2.0 μM, respectively, and phosphodiesterase with IC_{50} values of 6.5 and 8 μM, respectively.[7]

METHODS

Producing strain

Strain Tü 6399 was isolated on humic acid-vitamin agar[17] from a rhizospheric soil collected in a spruce stand located in the Rammert Forest near Tübingen, Germany. The strain is deposited in our strain collection at the Mikrobiologisches Institut, Universität Tübingen, Germany. Strain Tü 6399 was examined for morphological and chemotaxonomic characteristics and 16 S ribosomal RNA gene sequencing, which are commonly used in the systematics of the genus *Streptomyces*.[18]

HPLC screening

The HPLC-DAD system consisted of a HP 1090M liquid chromatograph equipped with a diode array detector, HP Kayak XM 600 ChemStation and HPLC software revision A.08.03 (Agilent Technologies, Waldbronn, Germany). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 435 and 500 nm, and UV–Vis spectra were measured from 200 to 600 nm. Sample preparation and chromatographic conditions were performed as described earlier.[19] Evaluation of the chromatograms was done by means of an in-house HPLC–UV–Vis database that contained about 960 entries, mostly antibiotics.[4]

HPLC–ESI–MS analysis was done with an Agilent 1200 HPLC series equipped with a diode array detector and LC/MSD Ultra Trap System XCT 6330 (Agilent Technologies). About 2.5 µl of the samples was injected onto an HPLC column (Nucleosil-100 C-18, 3 µm, 100 mm × 2 mm) and separated by 0.1% aqueous HCOOH as solvent A and 0.06% HCOOH in CH_3CN as solvent B by a linear gradient from 10 to 100% B over 15 min at a flow rate of 400 µl min^{-1}.

Fermentation and isolation

Batch fermentations of strain Tü 6399 were carried out in a 10-l stirred tank fermenter (FS-314, New Brunswick; New Brunswick, NJ, USA) in a medium consisting of glucose 1%, glycerol 1%, soluble starch 1%, corn steep powder 0.25%, Bacto peptone 0.5%, yeast extract 0.2%, NaCl 0.1% and $CaCO_3$ 0.3% in tap water; the pH was adjusted to 7.3 (5 M NaOH) prior to sterilization. The fermenter was inoculated with 5% per volume of a shake flask culture grown in the same medium at 27 °C in 500-ml Erlenmeyer flasks with a single baffle for 72 h on a rotary shaker at 120 r.p.m. The fermentation was carried out for 8 days with an aeration rate of 0.5 volume air per volume per minute and agitation at 250 r.p.m.

Hyphlo Super-cel (4%) was added to the fermentation broth (8.5 l), which was separated by multiple sheet filtration into culture filtrate and mycelium. The mycelium was discarded. The culture filtrate (7.5 l), containing 31 mg l^{-1} **1** and 33 mg l^{-1} **2**, was applied to an Amberlite XAD-16 column (80 cm × 4 cm; Rohm and Haas, Frankfurt, Germany) with a flow rate of 5 l h^{-1}. The resin was washed with H_2O and H_2O-MeOH (4:1), compounds **1** and **2** were eluted with H_2O-MeOH (1:4). The eluate was concentrated *in vacuo* to an aqueous residue, adjusted to pH 4.0 and extracted four times with EtOAc (each 250 ml). The organic extracts were combined, concentrated *in vacuo* to dryness and applied to a diol-modified silica gel column (40 cm × 2.6 cm, LiChroprep Diol; E. Merck, Darmstadt, Germany). Separation of **1** and **2** was accomplished by a linear gradient from CH_2Cl_2 to CH_2Cl_2-MeOH (9:1) within 4 h at a flow rate of 5 ml min^{-1}. Fractions containing **1** and **2**, respectively, were concentrated to dryness and purified by chromatography on Sephadex LH-20 (90 cm × 2.5 cm; Amersham, Freiburg, Germany) with MeOH as eluent. Pure compounds were obtained by preparative reversed-phase HPLC on 10 µm Nucleosil-100 C-18 (250 mm × 16 mm; Maisch, Ammerbuch, Germany) and isocratic elution with 0.1% HCOOH-MeOH (3:7) at a flow rate of 20 ml min^{-1}. After concentration to dryness *in vacuo*, **1** and **2** were obtained as yellowish oils.

Structure determination

HR–ESI–MS experiments were recorded using an LTQ-Orbitrap XL Exactive-MS (Thermo Scientific, Bremen, Germany) coupled to an Agilent 1260 1200 HPLC system including a UV detector (Agilent Technologies, Waldbronn, Germany). One-dimensional (1D) and 2D NMR experiments were performed on a DRX 500 NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a broadband inverse detection probe head with *z* gradients. Dimethyl sulfoxide-d_6 and $CDCl_3$ were used as solvents for NMR experiments and chemical shifts were referenced to the solvent peaks. ^{15}N-NMR and ^{15}N, ^{1}H-correlations were obtained on an Avance III 500 MHz NMR spectrometer (Bruker) equipped with a BBFO^{plus} smartprobe probehead.

Biological assays

The antimicrobial activity of compounds **1** and **2** against *Bacillus subtilis* DSM 347, *Staphylococcus lentus* DSM 6672 and *Xanthomonas campestris* DSM 2405 was determined according to Schulz *et al.*[20] The cytotoxic activity against the cell lines NIH-3T3, HepG2 and HT29, as well as the determination of the acetylcholinesterase and phosphodiesterase (PDE4-4B2) inhibitory activity of compounds **1** and **2,** was performed according to Kim *et al.*[7]

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