NOTE

Elaiomycins B and C, novel alkylhydrazides produced by *Streptomyces* sp. BK 190*

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Dereplicated novel actinomycetes from neglected habitats provide high quality biological material for screening programs designed to detect novel bioactive secondary metabolites.² Such organisms include neutrotolerant acidophilic streptomycetes which grow from pH 4.5 to 7.5, with an optimum between pH 5.0 and 5.5.³ Members of this group were isolated from a hay meadow soil taken from Cockle Park Experimental Farm in Northumberland, UK. The isolates were grown as submerged cultures in complex media, and extracts from the culture filtrates and mycelia included in our HPLC-diode array screening program to detect novel secondary metabolites by means of an in-house HPLC-UV-Vis database, which contains approximately 950 natural products, mainly antibiotics.⁴ Strain BK 190 was of interest because of the presence of three prominent peaks in the HPLC profile of a culture filtrate extract. The strain was assigned to the genus *Streptomyces* by its morphological and chemotaxonomic properties.⁵ Strain BK 190 formed an extensively branched substrate mycelium, a grey aerial spore mass and aerial hyphae, which differentiated into spiral chains of smooth-surfaced spores on oatmeal agar, contained LL-diaminopimelic acid, galactose, glucose and xylose as major sugars, *N*-acetylated muramic acid, predominant amounts of hexa- and octa-hydrogenated menaquinones with nine isoprene units, and produced iso- and anteiso-branched fatty acids with C_{15:0} and iso-C_{16:0} as major components. The temperature and pH ranges for growth were 10–35 °C and pH 4.0–8.0, respectively. Phylogenetic analyses showed that the organism was most closely related to the type strain of *Streptomyces sanglieri*; the two organisms shared a 16S rRNA similarity of 99.9%, a value that corresponds to a single nucleotide difference at 1434 locations.

The metabolite with retention time of 12.3 min in our standardized reversed-phase gradient elution profile was identified by HPLC-diode array and HPLC-ESI-MS analysis to be elaiomycin (1), an azoxy antibiotic that was first isolated from *Streptomyces hepaticus* and found

to strongly inhibit the growth of *Mycobacterium tuberculosis*.⁶ Two further prominent peaks, elaiomycin B (2) with a retention time of 16.4 min, and elaiomycin C (3) with a retention time of 17.2 min showed characteristic UV-vis spectra that differed from those of all of the reference compounds stored in the database. A significant production of 1–3 was observed in oatmeal medium in the 100 ml shake flask scale, which was reproducible in a scale up to a 20-liter fermentor. Strain BK 190 reached a maximal biomass of 12 volume (%) at a cultivation time of 96 h and this correlated with the highest production of elaiomycins, which reached yields of 24 mg l⁻¹ elaiomycin (1), 15 mg l⁻¹ elaiomycin B (2) and 9.5 mg l⁻¹ elaiomycin C (3) in the culture filtrate, and 15 mg l⁻¹ of 2 and 12 mg l⁻¹ of 3 in the mycelium, respectively. Compounds 1–3 were isolated from the culture filtrate by separation on an Amberlite XAD-16 column (Rohm and Haas Deutschland, Frankfurt, Germany), followed by extraction with ethyl acetate. Compounds 2 and 3 were also isolated in remarkable amounts from the mycelium by extraction. The crude product was purified by subsequent chromatography on diol-modified silica gel and Sephadex LH-20 (Amersham, Freiburg, Germany). Pure compounds 1, 2 and 3 were obtained by preparative reversed-phase HPLC, 1 resulted as a colorless oil, 2 and 3 as white powders. The physico-chemical properties of compounds 1–3 are summarized in Table 1. They are soluble in methanol, ethylacetate, dichloromethane and cyclohexane. Detailed informations on structure determination were reported by Helaly *et al.*⁷ Compounds 2 and 3 contain a hydrazide chromophore as the characteristic structural element, as shown in Figure 1.

Antimicrobial assays were performed as recently described by Schneemann *et al.*⁸ Compounds 2 and 3 were slightly active against *Staphylococcus lentus* DSM 6672. Growth inhibition of 23% and 24%, respectively, was observed at a concentration of 100 µM. No antibiotic activity was found against *Bacillus subtilis* DSM 347, *Propionibacterium acnes* DSM 1897T or *Xanthomonas campestris* DSM 2405.

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Table 1 Physico-chemical properties of elaiomycin (1), elaiomycin B (2) and elaiomycin C (3)

	1	2	3
Appearance	Colorless oil	White powder	White powder
Molecular mass (g mol ⁻¹)	258.1	478.5	476.5
Molecular formula	C ₁₃ H ₂₆ N ₂ O ₃	C ₂₉ H ₅₄ N ₂ O ₃	C ₂₉ H ₅₂ N ₂ O ₃
<i>High-resolution orbitrap-ESI-MS (m/z⁻¹)</i>			
Found	259.20077 [M+H] ⁺	479.42053 [M+H] ⁺	477.40483 [M+H] ⁺
Calcd	259.20217, δm -3.31 p.p.m.	479.41344, δm -0.35 p.p.m.	477.39779, δm -0.50 p.p.m.
UV $\lambda_{\max}^{\text{MeOH}}$ (nm) (ϵ (l mol ⁻¹ cm ⁻¹))	233 (7827.7)	214 (65581.2)	217 (133971.3)
[α] _D ²⁵ (c, 2.8, EtOH)	+33.2	—	—
IR $\nu_{\max}$ (cm ⁻¹)	3450, 2957, 2926, 2857, 1467, 1373, 1303, 1117, 786	3267, 2954, 2925, 2855, 1692, 1466, 1379, 1117, 723	3273, 2953, 2924, 2854, 1693, 1466, 1387, 1117, 723

Figure 1 Structures of elaiomycin (1), elaiomycin B (2) and elaiomycin C (3).

Compound 1 did not show an antibacterial activity. Enzyme inhibition assays showed an inhibitory activity of compounds 2 and 3 against acetylcholinesterase (IC₅₀=1.0 μ M and IC₅₀=2.0 μ M, respectively) and phosphodiesterase (PDE-4B2; IC₅₀=6.5 μ M and IC₅₀=8.0 μ M, respectively) in contrast to elaiomycin (1), which was not active in both assays. Compounds 2 (50 μ M) and 3 (50 μ M) did not show cytotoxic activity towards tumor cell lines HepG2 and HT29, whereas a moderate inhibition of the cell line HepG2 was observed for elaiomycin (1) (IC₅₀=16.3 μ M).

Besides elaiomycin, only a few antibiotics containing an azoxy chromophore have been described; these include: antibiotic LL-BH872 α ,⁹ maniomyces A and B¹⁰ and azoxybacillin¹¹ (all show strong antifungal activity); valanimycin, which shows antitumor and antibacterial activity;¹² and jietacins A and B, which exhibit a pronounced nematocidal and weak antifungal activity.¹³ In contrast to azoxy-containing compounds, naturally occurring hydrazines and hydrazides are rarely described. Only two fungal metabolites have been reported, agaritine from the cultivated mushroom *Agaricus bisporus*¹⁴ and gyromitrin from the false morel *Gyromitra esculenta*.¹⁵ As far as we know, elaiomyces B (2) and C (3) are the first natural occurring

alkylhydrazides with therapeutic potential as shown by their inhibitory activity against clinically relevant enzymes.

EXPERIMENTAL PROCEDURE

Producing organism and classification

Strain BK190 was isolated from a soil sample collected from Palace Leas hay meadow plot 6 at Cockle Park Experimental Farm, Northumberland, UK (National Grid Reference NZ 200913). The microorganism was examined for chemotaxonomic and morphological properties known to be useful in the systematics of *Streptomyces*.⁵ The 16S rRNA gene amplification and sequencing were carried out using an established method¹⁶ and the resultant, almost complete sequence, compared with corresponding sequences of related *Streptomyces* using the MEGA 4.0 software.¹⁷ Phylogenetic trees were inferred by using the neighbour-joining tree-making algorithm.¹⁸

Fermentation and isolation

Batch fermentations of strain BK 190 were carried out in a 20-l fermentor, equipped with a turbine impeller system (b20; B. Braun, Melsungen, Germany) in a complex medium which consisted of (per liter tap water) oatmeal (Holo Hafergold, Neuform, Germany) 20 g and 5 ml of a trace element solution composed of (per liter deionized water) CaCl₂·2H₂O 3 g, iron (III) citrate 1 g, MnSO₄·H₂O 200 mg, ZnCl₂ 100 mg, CuSO₄·5H₂O 25 mg, Na₂B₄O₇·10H₂O 20 mg, CoCl₂·6H₂O 4 mg and Na₂MoO₄·2H₂O 10 mg; the pH was adjusted to 7.3 (5 M HCl) prior to sterilization. The fermentation was carried out for 96 h with an aeration rate of 0.5-volume air per volume per min and agitation at 1000 r.p.m.

Compounds 1–3 were isolated from the culture filtrate by Amberlite XAD-16 column chromatography (resin vol 2 l) and were eluted with MeOH. The concentration was extracted three times with EtOAc and concentrated *in vacuo* to dryness (325 mg). The mycelium, which contained 2 and 3, was extracted twice with MeOH-Me₂CO (1:1); the extracts were combined, concentrated *in vacuo* to an aqueous residue and re-extracted three times with EtOAc. The crude product (7.36 g) was dissolved in CH₂Cl₂ and applied to a diol-modified silica gel column (E. Merck, Darmstadt, Germany). Compounds 1–3 were separated by a linear gradient from CH₂Cl₂ to CH₂Cl₂-MeOH (9:1). Further purification was achieved by chromatography on Sephadex LH-20 (Amersham) with MeOH as eluent. Fractions containing 2 and 3 were separated to pure compounds by prep RP-HPLC (Nucleosil-100 C-18, 20×250 mm; Maisch, Ammerbuch, Germany) and elution with MeOH-H₂O (95:5). After concentrating to dryness *in vacuo*, elaiomycin (1) was obtained as colourless oil, whereas elaiomyces B (2) and C (3) were obtained as white powders.

Cytotoxic assays

The sensitivity of the cell lines NIH-3T3, HepG2 and HT-29 to the isolated compounds was evaluated by monitoring of the metabolic activity using the CellTiter-Blue Cell Viability Assay (Promega, Mannheim, Germany).

The cultivation of the cell lines and the bioassays were performed as described by Schneemann *et al.*⁸

Enzyme inhibition assays

The determination of the acetylcholinesterase inhibitory activity of compounds 1–3 was performed according to Ellmann *et al.*¹⁹ and adapted for a microplate test system, using a volume of 200 µl per well. The following solutions were used: sodium phosphate buffer (100 mM, pH 7.4), substrate (acetylthiocholine iodide 7.5 mM in sodium phosphate buffer), reagent (5,5-dithio-bis-2-nitrobenzoic acid, 10 mM in sodium phosphate buffer) and enzyme (human acetylcholinesterase (Sigma C1682, Taufkirchen, Germany) 200 U in 2 ml aqua bidest.). First, the absorbance of the suspension containing compound (10 µM), buffer (100 mM), enzyme (10 mU) and reagent (150 µM) was determined at 412 nm (blank). The reaction was started by the addition of the substrate (140 µM). After an incubation period of 5 min at 30 °C, the absorbance was measured at 412 nm (final value). The activity was calculated by the difference of the final value and the blank. (–)-Huperazine A (1 µM and 0.1 µM) was used as the positive control.²⁰

Analysis of the effect of compounds 1–3 on human recombinant cAMP-specific PDE-4B2 was carried out by adding 10 µM of the compound in 50 mM Tris-HCl buffer (pH 7.5), containing 8.3 mM MgCl₂, 1.7 mM EGTA and 5 U recombinant human cAMP-specific PDE-4B2 (Biotac 60042-BPS, Heidelberg, Germany) in a volume of 30 µl per well. The reaction was started with 10 µl of 12 µM cAMP (Sigma A9501) dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 8.3 mM MgCl₂ and 1.7 mM EGTA. After an incubation period of 30 min at 30 °C, the reaction was stopped and the AMP concentration quantified using a PDELIGHT HTS cAMP Phosphodiesterase Assay Kit (Lonza LT07-600, Wuppertal, Germany) according to the instructions of the supplier. The luminescence was measured using the microtiter plate reader Infinite M200 (Tecan, Crailsheim, Germany) with 0.1 s integration time. For inhibition of the PDE-4B2, 1 and 10 µM of rolipram (4-(3-(cyclopentyloxy)-4-methoxyphenyl)-2-pyrrolidinone; Merck Bioscience Cat. No. 557330, Darmstadt, Germany) were used as positive controls.

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