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Fluostatins C~E, Novel Members of the Fluostatin Family Produced by *Streptomyces* Strain Acta 1383†

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Abstract Three new members of the fluostatin family, fluostatins C~E, were discovered in a culture filtrate extract of strain Acta 1383 during an HPLC screening program. The producing strain belongs to the genus *Streptomyces* and is closely related to type strains classified in the *Streptomyces lavendulae* 16S rRNA subclade. Fluostatins are named by their characteristic fluorenone chromophore. Fluostatin C shows moderate activity against selected human tumor cell lines.

Keywords *Streptomyces*, taxonomy, fermentation, novel fluostatins

Introduction

In the course of our ACTAPHARM project the analysis of DNA extracted from environmental samples from the community at various European habitats was compared with innovative selective isolation procedures based on specific pre-treatment regimes, novel media formulations and dereplication procedures. The aim of the project was making available high-quality and taxonomically diverse biological material for metabolite screening to detect novel antimicrobial agents for pharmaceutical applications.

Freshly isolated actinomycetes were grown in submerged culture in different media and extracts prepared from mycelia and culture filtrates at various fermentation times. The secondary metabolite profiles were evaluated by HPLC-diode array analysis in combination with an in-house developed HPLC-UV-Vis database [2]. Strain Acta 1383 was found to be of special interest because of the presence of a dominant metabolite in the culture filtrate extract; in addition two minor congeners belonging to the same family of metabolites were detected by their nearly congruent UV-visible spectra. The characteristic spectra of these compounds differed from those of 840 reference compounds held in the HPLC-UV-Vis database, but showed a relationship to anthraquinone-type compounds. HPLC-ESI-MS analysis of the raw extract revealed a molecular mass of 324.1 Da. for the main compound. A search in commercially available databases of natural products showed that the main metabolite was novel. High-resolution FTICR-MS of the main metabolite gave an exact mass of 324.05650 Da which corresponded to the molecular formula $C_{18}H_{12}O_6$ (**1**). Structural data on the isolated

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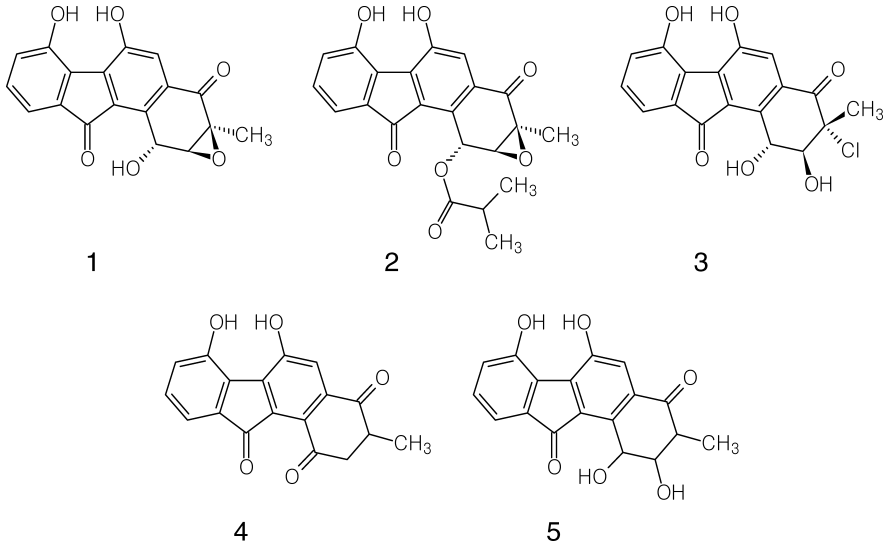

Fig. 1 Structures of novel fluostatins C (**1**), D (**2**) and E (**3**) produced by *Streptomyces* strain Acta 1383, and of fluostatins A (**4**) and B (**5**)

metabolites from strain Acta 1383 [3] showed a relationship to fluostatins A (**4**) and B (**5**); the latter are inhibitors of dipeptidyl peptidase III produced by a member of the genus *Streptomyces* [4, 5]. The structures of the fluostatins are shown in Fig. 1.

This report deals with the taxonomy of the producing strain, its fermentation, and the isolation, physico-chemical properties and biological activities of the novel fluostatins.

Materials and Methods

Microorganisms

Strain Acta 1383 was isolated from the rhizosphere of *Ebenus sibthorpii* (Fabaceae), an endemic plant found in low numbers in the Kaisariani area, a forest preserve site 4 km from the centre of Athens [6]. The soil at the sampling site is a sandy loam with a pH of 8.2. Analysis of particle sizes indicated the presence (percent, dry weight) of: sand 59, silt 30, clay 11 and organic carbon 2.7; the phosphorus content was 4 mg/liter dry soil. Mineralogy analysis showed the presence of: illite 76%, chlorite 11%, kaolinite 12%, calcite 1%; smectite and talc were not detected [7]. The strain was isolated following the procedures described by Katsifas *et al.* [7] and maintained on AGS medium [8].

Taxonomy of Strain Acta 1383

The organism, which was presumptively assigned to the genus *Streptomyces* on the basis of colonial characters, was examined for a range of phenotypic properties known to be of value in streptomycete systematics [9, 10]. The isomeric form of diaminopimelic acid was determined by TLC of a whole-organism hydrolysate [11] and fatty acid composition after Suzuki and Komagata [12]. 16S rRNA gene amplification and sequencing were carried out after Kim *et al.* [13] and the resultant sequence compared with corresponding sequences of available *Streptomyces* type strains using the neighbour-joining algorithm [14].

Fermentation and Isolation

Batch fermentations of strain Acta 1383 were carried out in a 20-liter fermentor equipped with a turbine impellor system in a medium consisting of soluble starch 20 g, KNO_3 5 g, K_2HPO_4 0.5 g, NaCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g and $FeSO_4 \cdot 7H_2O$ 0.01 g in 1.0 liter deionized water. The fermentor was inoculated with 5% by volume of a shake flask culture, grown at 27°C in 500 ml-Erlenmeyer flasks with one baffle for 48 hours on a rotary shaker at 120 rpm. The fermentation was carried out for 5 days with an aeration rate of 0.5 vvm and an agitation of 1000 rpm.

Hyphlo Super-cel (2%) was added to the fermentation broth, which was separated by multiple sheet filtration into culture filtrate and mycelium. The culture filtrate (17 liters) was applied to an Amberlite XAD-16 column (resin volume 2 liters); the resin was washed with water and water - MeOH (60+40). Fluostatins were eluted with MeOH, concentrated *in vacuo* to an aqueous residue; the latter was adjusted to pH 4 (1 M HCl) and extracted four times with EtOAc. The organic extracts were combined and concentrated *in vacuo* to dryness. The crude product was dissolved in CH_2Cl_2 and added to a silica gel column

(45×2.6 cm, silica gel SI 60, Merck), the separation was accomplished by a linear gradient from CH_2Cl_2 to MeOH within 4 hours at a flow rate of 5 ml/minute. Fractions containing the single fluostatin compounds were purified by Sephadex LH-20 and Toyopearl TSK HW-40 chromatography (each column 90×2.5 cm) using MeOH as the eluent. After concentration to dryness *in vacuo*, the fluostatins were obtained as dark red powders.

Fermentations with Amberlite XAD-16 Supplementation

400 ml (2 vol-%) of the polystyrene resin Amberlite XAD-16 was added to the medium (19 liters) prior to sterilization. The incubation conditions were the same as for the 20-liter standard fermentations. The mycelium and polystyrene resin were separated by filtration through a metal sieve, washed with water and transferred to a glass column. The separation procedure was the same as described above.

HPLC-DAD Analyses

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a diode-array detector and a HP Kayak XM 600 ChemStation (Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435 and 500 nm and UV-visible spectra measured from 200 to 600 nm. A 10-ml aliquot of the fermentation broth was centrifuged, and the supernatant adjusted to pH 4 and extracted with the same volume of EtOAc. After centrifugation, the organic layer was concentrated to dryness *in vacuo* and resuspended in 1 ml MeOH. A 10-μl aliquot of the sample was injected onto an HPLC column (125×4.6 mm) fitted with a guard-column (20×4.6 mm) filled with 5-μm Nucleosil-100 C-18 (Maisch). The samples were analysed by linear gradient elution using 0.1% *ortho*-phosphoric acid as solvent A and acetonitrile as solvent B at a flow rate of 2 ml/minute. The gradient was from 0% to 100% for solvent B in 15 minutes with a 2-minute hold at 100% for solvent B.

Biological Assays

An agar plate diffusion assay was used to determine the antibacterial and antifungal properties of fluostatins produced by strain Acta 1383 using *Arthrobacter aurescens* DSM 20166, *Bacillus subtilis* DSM 10, *Brevibacillus brevis* DSM 30, *Staphylococcus aureus* DSM 20231 and *Streptomyces viridochromogenes* Tü 57 (Gram-positive bacteria) and *Escherichia coli* K12, *Pseudomonas fluorescens* DSM 50090 and *Proteus mirabilis* ATCC 35501 (Gram-negative bacteria). The yeasts and filamentous fungi examined were *Candida albicans* Tü 164, *Saccharomyces cerevisiae* ATCC 9080, *Aspergillus viridi mutans* CBS 12756, *Botrytis cinerea* Tü 157, *Paecilomyces variotii* Tü 137 and *Penicillium notatum* Tü 136.

Aliquote of 10 μl of the samples were applied to filter paper disks (6 mm diameter). These were put on inoculated test plates that were incubated for 24 hours (bacteria) and 48 hours (fungi) at temperatures which permitted optimal growth of the test organisms.

The inhibitory activities of the fluostatins on the growth of tumor cells was tested according to NCI guidelines [15] using human cell lines from gastric adenocarcinoma (HMO2), breast carcinoma (MCF 7) and hepatocellular carcinoma (HepG2). Cells were grown in 96-well microtiter plates in RPMI 1640 with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 in air. The fluostatins (0.1～10 μl/ml) were added to the cells after incubation for 24 hours. Stock solutions were prepared in DMSO; the final DMSO concentration of the cultures was 0.1%. The cells were fixed and cell protein was assayed with sulforhodamine B after a 48-hours incubation.

Results and Discussion

Taxonomy of the Producing Strain

Strain Acta 1383 was one of 35 strains isolated from the rhizosphere of *Ebenus sibthorpii*. It produced an abundant white aerial spore mass and yellow brown substrate pigments but no diffusible pigments on oatmeal agar, contained LL-diaminopimelic acid and fatty acids rich in *iso*- and *anteiso*-components, properties consistent with its assignment to the genus *Streptomyces* [16]. 16S rRNA gene sequence data showed that it belonged to the *Streptomyces lavendulae* subclade which also encompasses the type strains of *S. avidinii*, *S. cirratus*, *S. nojiriensis*, *S. spororaveus*, *S. subrutilus*, *S. vinaceus* and *S. virginiae*. The isolate formed straight chains of smooth surfaced spores, melanin pigments on peptone yeast extract iron agar and used cellobiose, fructose, meso-inositol, inulin, raffinose, rhamnose and xylose as sole carbon sources but not adonitol or mannitol, a phenotypic profile that distinguishes it from the representatives of the species classified in the *S. lavendulae* 16S rRNA gene subclade [17].

Screening and Fermentation

A crude extract of the culture filtrate of strain Acta 1383 was screened using an HPLC-DAD method [2], the resultant elution profile was characterised by a family of peaks showing nearly identical UV-visible spectra (Fig. 2). Lack of spectral matches with approximately 840 reference

compounds stored in the HPLC-UV-Vis database prompted us to undertake the isolation and structure elucidation of the unknown metabolites.

Strain Acta 1383 was cultivated in 20-liter fermentors with and without supplementation of the polystyrene resin Amberlite XAD-16. In each fermentation fluostatin C (**1**) was found to be the dominant secondary metabolite. In conventional fermentations (without XAD-16 addition), fluostatin C was produced at a concentration of 15 mg/liter after an incubation period of 162 hours and fluostatin B (**5**) as a minor congener at a concentration of 3 mg/liter. The production of fluostatin C (**1**) increased during fermentations with the addition of the neutral polystyrene resin Amberlite XAD-16, reaching a maximal value of 20 mg/liter after 92 hours of incubation. Besides **1** as the main compound, a further fluostatin was produced, fluostatin D (**2**), which reached a maximal production after 44 hours at a concentration of 13 mg/liter. **2** decreased and the production of **1** increased in the course of further cultivation.

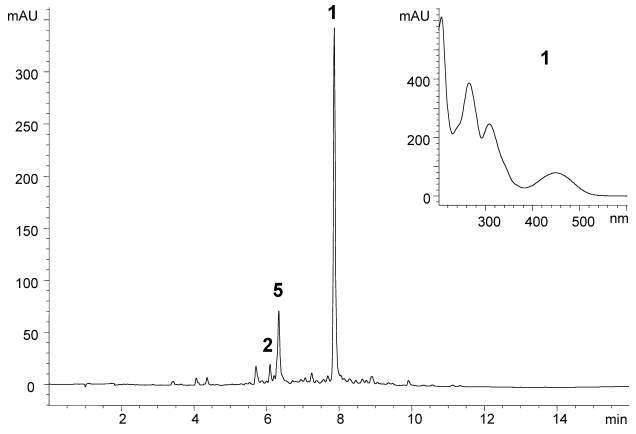

Fig. 2 HPLC analysis of a culture filtrate extract of *Streptomyces* strain Acta 1383 at a fermentation time of 162 hours, monitored at 260 nm, and UV-visible spectrum of fluostatin C (**1**)

Isolation and Physico-chemical Properties

The fluostatins isolated from the culture filtrate by Amberlite XAD-16 chromatography were separated and purified by a succession of chromatographic steps. Fluostatin E (**3**) was isolated as a minor congener during the purification procedure. All of the fluostatins were obtained as dark red powders after lyophilisation. Fluostatin A (**4**) was not observed in all of the fermentations of strain Acta 1383 or in all of the isolation trials. The structures of the novel fluostatins C∼E were elucidated as described in a previous report [3]. The physico-chemical properties of the compounds are summarized in Table 1. The chemical structures of **1**, **2** and **3** contained a characteristical fluorenone skeleton which revealed a strong relationship to fluostatins A (**4**) and B (**5**) produced by a *Streptomyces* strain [5] hence these metabolites were aligned within the fluostatin family.

Biological Properties

Fluostatins C (**1**), D (**2**) and E (**3**) did not show antibacterial or antifungal activity against any of the tested organisms.

Table 2 Activity (μg/ml) of fluostatin C (**1**) against selected human tumor cell lines

Cell line	GI_{50}[a]	TGI[b]
HMO2	3.2	>10*
HepG2	3.1	>10**
MCF 7	6.0	>10***

[a] Drug concentration causing 50% growth inhibition. [b] Drug concentration causing 100% growth inhibition.
* 81%, ** 89% and *** 57% growth inhibition at 10 μg/ml.

Table 1 Physico-chemical properties of fluostatins C∼E

	C (**1**)	D (**2**)	E (**3**)
Appearance	dark red powder	dark red powder	dark red powder
Molecular weight	324	394	360
Molecular formula	$C_{18}H_{12}O_6$	$C_{22}H_{18}O_7$	$C_{18}H_{13}ClO_6$
ESI-FT-ICR MS (m/z)			
Found	323.05650 $(M-H)^-$	395.11288 $(M+H)^+$	383.03021 $(M+Na)^+$
Calcd	323.05611	395.11252	383.02929
UV λ_{max}^{MeOH} nm (ε)	216 (10,238), 261 (10,028), 302 (5,330), 449 (2,130),	215 (24,320), 263 (23,404), 302 (12,470), 452 (4,551),	216 (10,269), 264 (8,928), 301 (4,707), 455 (1,593)

The cytostatic effects of **1** and **3** were tested against various human tumor cell lines. **1** displayed a moderate inhibitory potency on the growth of HMO2, HepG2 and MCF 7 cells (see Table 2), whereas **3** was inactive against all three tumor cell lines up to 10 μg/ml (data not shown).

4 and **5** were detected in the course of a screening for inhibitors of human placental dipeptidyl peptidase III. Both compounds showed very weak antimicrobial activities and a low toxicity by intraperitoneal injection in mice [4].

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