ABSTRACT

Streptomyces avidinii strain SB9 was isolated from permafrost soil samples in Spitsbergen, Arctic Ocean. The strain is psychrotolerant with optimal growth temperature between 20°C and 28°C. During our continuous search for unique secondary metabolites, we investigated an arctic isolate of Streptomyces avidinii SB9 and found for the first time, that Streptomyces avidinii strain SB9 produces 2-amino-3-dodecanol [1], norophthalmic acid (2S)-2-amino-4-[(1S)-1-(carboxymethylcarbamoyl)ethyl]carbamoyl]butanoic acid [2] and phthalic acid ester [3]. The compounds were purified by solvent extraction, silica gel column chromatography and preparative TLC consecutively. The structures of the compounds were elucidated by NMR experiments and mass spectrometric investigations. These compounds showed antibacterial activity against important Gram-positive bacteria and fungi. Our studies are motivated by an interest in the functional role played by natural products in the ecological interactions of the strain with other members of the microbial community.

Keywords: psychrotolerant actinomycete, Arctic habitats, Streptomyces avidinii SB9, 2-amino-3-dodecanol, norophthalmic acid, phthalic acid ester

Introduction

During the course of our screening program for bioactive secondary metabolites, we investigated an arctic isolate SB9 of Streptomyces avidinii and obtained a natural products as 2-amino-3-dodecanol [1], norophthalmic acid [2] and di-(2-ethylhexyl) phthalate (DEHP) [3]. Our studies are motivated by an interest in the functional role played by natural products in the ecological interactions of the strain with other members of the microbial community. 2-amino-alkanols and their unsaturated derivatives have been isolated from the ascidian Clavelina oblonga, collected in Brazil and the Mediterranean ascidian Clavelina phlegraea (1, 6). Unique glutathione analogues, γ-l-glutamyl-l-alanylglutamine (norophthalmic acid) and γ-l-glutamyl-l-a-amino-n-butyrylglycine (ophthalmic acid), were identified for the first time from plants, the brown alga, Undaria pinnatifida. Positive fast atom bombardment tandem mass spectrometry (FAB-MS/MS) was applied for peptide sequencing, particulary for determining the gamma glutamyl linkage (5, 8, 9). Di-(2-ethylhexyl) phthalate (DEHP), isolated from Streptomyces sp. is known to be a cell aggregation factor (10). DEHP, isolated from Penicillium olsonii (3) and Streptomyces bangladeshensis sp. nov. (2) shows activity against Gram-positive, Gram-negative bacteria and some fungi. Compared with mesophilic or thermophilic taxa, psychrophilic and psychrotolerant Streptomyces have been relatively poorly studied. The Arctic region is a unique area among Earth’s ecosystems with its cold winters and cool summers. Survival of microorganisms in these cold and extreme conditions requires a special kind of adaptation and hardiness against stress factors as substrate limitation, UV irradiations, all-year low temperatures and short-time intensive heating during the Arctic summer. So, screening of the strains isolated from the regions with temperatures under 0°C attains a special significance regarding isolation and identification of secondary metabolites which could be used in pharmacy. This work describes the isolation, characterization and structure elucidation of a bioactive metabolites produced by Streptomyces avidinii SB9 strain, isolated from permafrost soil in Spitsbergen, Arctic.

Materials and Methods

Description of the producing strain

Streptomyces avidinii SB9 strain was isolated from a soil samples, collected on Spitsbergen, Arctic Ocean (78° North, 15° East) and deposited in the collection of the Institute of Microbiology, Bulgarian Academy of Sciences. According to a commonly accepted definition for cold-adapted bacteria, the strain is psychrotolerant with growth at temperatures between 4 and 30°C and optimal growth temperature between 20 and 28°C.

Fermentation

Culture broth for analysis of bioactive compounds was obtained by cultivation in fermentation medium as follows. The mature slant culture of strain SB9 was inoculated into a 50 ml Erlenmeyer shake flask, containing 25 ml of a seed medium consisting of: glucose 10, soybean meal 15, KH₂PO₄ 0.5, MgSO₄ 5, KCl 1 and CaCO₃ 5 (pH 7.0) [in g l⁻¹]. The inoculated flask was shaken at 250 rpm 28°C for 44-48 hours.
The seed culture (3 ml) was transferred to a 100 ml Erlenmeyer flask containing 50 ml of fermentation medium consisting [in g l⁻¹]: soybean meal 30, casein hydrolysate 5, sodium glutamate 3, MgSO₄ 5, CaCO₃ 4, NH₄Cl 3, NH₄NO₃ 1 and CoCl₂ 10 (pH 7.0). The cultivation was carried out for 7 days at 28°C on a rotary shaker (250 rpm).

**Extraction and separation**

At the end of the fermentation period, the culture broth (3 litres) of *Streptomyces avidinii* SB9 was centrifuged. The mycelium was extracted three times with methanol. The combined solvent extracts were concentrated *in vacuo* to a small amount of MeOH, filtered and precipitated with acetone. The obtained crude product was dissolved in a methanol and re-precipitated with Me₃CO-ether (2.0:0.5 (v/v)). An amount of 200 mg crude product was obtained. The complete separation and purification of compounds 1 and 2 could be achieved only by preparative TLC (Merck Silica gel 60 F 254), developed with chloroform-methanol (7:3, v/v) for compound 1 and ethylmethylketone-ethanol-ammonia (1:1:1, v/v/v) for compound 2. The compounds 1 and 2 were eluted from the silica gel plates with methanol. After evaporation of the solvent *in vacuo* 10 and 12 mg of pure compounds 1 and 2 were obtained. The chromatographic spots were visualized by spraying with 1% solution of ninhydrine and heating at 120°C for 1-3 minutes. The supernatant fluid of *Streptomyces avidinii* SB9 was extracted 2 times each with 2.0 l of ethyl acetate. After washing of the organic layer with water and drying at reduced pressure, yielding 210 mg of crude product, which was dissolved in chloroform and chromatographed on a silica gel column (70-230 mesh) with chloroform-methanol (2:1, v/v). The fractions obtained after liquid phase chromatography were concentrated and spotted on preparative TLC (Merck Silica gel 60 F 254), developed with n-hexane-ether (4:1, v/v); chloroform; CH₃Cl-MeOH (95:5, v/v) and detected with UV lamp at 254 nm. The compound 3 was eluted from the silica gel plates with chloroform. The fraction was concentrated to oil liquid and was delivered the pure compound 3.

**General experimental procedures**

Electrospray mass spectra (ESI-MS) were recorded on a LCQ Finnigan Mass Spectrometer. ESI-HR mass spectra were recorded on an APEX IV, 7T, FT-ICR MS Bruker Daltonik. ¹H (300 and 600 MHz) and ¹³C (75.5 and 125.7 MHz) NMR spectra were measured on a Bruker AMX 300 and on a Varian Inova 600 (599.740 MHz) spectrometer in CD₃OD and CDCl₃. The chemical shifts are expressed in δ values with TMS as an internal standard. UV-vis spectroscopy was recorded on a Spectro 2000 instrument (Analytik Jena, Germany). IR spectra were recorded on a Beckman DU 601 and Shimadzu IR scanning spectrophotometers.

**Antimicrobial activity**

Antimicrobial activity was determined by agar diffusion test according to European Pharmacopoeia (4). Test organisms were suspended in the melted nutrient agar (Serva) and poured into petri dishes. Holes of 8 mm diameter were cut in the agar and filled with 100 μl of a 100 mg l⁻¹ solution of the compound. Inhibition zones were read after incubation for 18 h at 37°C for bacterial strains and 30°C for yeasts.

**Results and Discussion**

**Identification of compounds 1, 2 and 3**

Compound 1 was obtained as an optically active yellow solid. It is soluble in methanol, ethanol, n-butanol, but insoluble in ethyl acetate, chloroform and n-hexane. Compound 1 gave positive reaction (red color) with ninhydrine. The (+)-ESI mass spectrum of 1 displayed *pseudo*-molecular ions at *m/z* 202.0 (M+H)⁺, 224.0 (M+Na)⁺ and 403.0 (2M+Na)⁺. ESI-MS measurements on the (M+H)⁺ ion (*m/z*=202.21651) indicated the molecular formula C₁₂H₂₅NO for 1. The chemical formula of 1 suggested absence of double bond equivalents. The ¹H NMR spectrum in CD₃OD contained two methine resonances at δH 3.18 and 3.62, consistent with protons next to nitrogen and oxygen atoms, respectively, as supported by corresponding ¹³C NMR signals at δC 51.50 and 71.09. The remaining signals of the proton spectrum included a large signals (δH 1.30-1.33) due to a number of overlapping methylene signals as well as a methyl doublet at δH 1.02 (δC 11.90) and a methyl triplet at δH 0.90 (δC 13.50) (see Table 1). From these results, the structure of 1 was determined to be 2-amino-3-dodecanol (Fig. 1) (1, 6). The structures of these molecules are generally related to the widely distributed amphiphilic sphingosine, the central structural element of sphingolipids; their carbon-chain length varies from C₁₂ to C₃₀. The antimicrobial activity of the 2-amino-3-dodecanol is presented in Table 2.

**TABLE 1**

<table>
<thead>
<tr>
<th>Assignment</th>
<th>δH (Hz)</th>
<th>δC</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.02 (3H, d, 5.4 Hz)</td>
<td>11.9 q</td>
<td>CH₃</td>
</tr>
<tr>
<td>2</td>
<td>3.18 (1H, dq, 2.93, 5.3 Hz)</td>
<td>51.5 d</td>
<td>CH</td>
</tr>
<tr>
<td>3</td>
<td>3.62 (1H, m)</td>
<td>71.1 d</td>
<td>CH</td>
</tr>
<tr>
<td>4</td>
<td>1.39 (2H, m)</td>
<td>32.6 t</td>
<td>CH₂</td>
</tr>
<tr>
<td>5</td>
<td>1.68/1.36 (2H, m)</td>
<td>25.5 t</td>
<td>CH₂</td>
</tr>
<tr>
<td>6</td>
<td>1.30-1.33 (2H, m)</td>
<td>29.3 t</td>
<td>CH₂</td>
</tr>
<tr>
<td>7</td>
<td>1.30-1.33 (2H, m)</td>
<td>29.3 t</td>
<td>CH₂</td>
</tr>
<tr>
<td>8</td>
<td>1.30-1.33 (2H, m)</td>
<td>29.3 t</td>
<td>CH₂</td>
</tr>
<tr>
<td>9</td>
<td>1.30-1.33 (2H, m)</td>
<td>29.3 t</td>
<td>CH₂</td>
</tr>
<tr>
<td>10</td>
<td>1.29 (2H, m)</td>
<td>31.6 t</td>
<td>CH₂</td>
</tr>
<tr>
<td>11</td>
<td>1.30 (2H, m)</td>
<td>22.3 t</td>
<td>CH₂</td>
</tr>
<tr>
<td>12</td>
<td>0.90 (3H, t, 6.7 Hz)</td>
<td>13.5 q</td>
<td>CH₃</td>
</tr>
</tbody>
</table>

s: singlet; d: doublet; t: triplet; q: quartet
TABLE 2

Antimicrobial activity in vitro of 2-amino-3-dodecanol in the agar diffusion assay

<table>
<thead>
<tr>
<th>Test organisms</th>
<th>Diameter of inhibition zones (mm)</th>
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</thead>
<tbody>
<tr>
<td>Bacillus mycoides</td>
<td>17.6</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>18.2</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>17.1</td>
</tr>
<tr>
<td>Staphylococcus viridochromogenes</td>
<td>15.5</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>19.0</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>17.4</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>12.2</td>
</tr>
<tr>
<td>Mucor miehei</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Compound 2 is a colorless solid, soluble in methanol, ethanol, n-butanol, but insoluble in ethyl acetate, chloroform and n-hexane. Compound 2 gave positive reaction (red color) with ninhydrine and Dragendorff’s reagent. The UV-vis spectrum showed absorption maxima at $\lambda_{max}$ 225.0 and 260.0 nm. The identification of 2 was settled on the basis of electrospray (ESI-MS) and high-resolution electrospray mass spectrometry (ESI-HRMS), and NMR spectroscopy. The (+)-ESI mass spectrum of 2 displayed pseudo-molecular ions at $m/z$ 276.2 (M+H)$^+$, 298.1 (M+Na)$^+$ and 573.0 (2M+Na)$^+$. The molecular weight (275.2 Dalton) and the chemical formula $C_{10}H_{17}N_3O_6$ were determined by ESI-HRMS. The chemical formula of 2 suggested the presence of 4 double bond equivalents. The chemical shifts of all proton and carbon atoms of 2 are summarized in Table 3.

On the basis of the data (Table 3 and ESI-HRMS), it was established that compound 2 was identified as norophthalmic acid (Fig. 2) (5, 8). Norophthalmic acid is a tripeptide, also known as norophthalmate and chemically is (γ-l-glutamyl-l-alanylglycine). Positive electrospray mass spectrometry (ESI-MS/MS) was applied for peptide sequencing, particularly for determining the gamma glutamyl-containing peptides (Fig. 2). Norophthalmic acid is an analogue of glutathione (L-cysteine replaced by L-alanine) and of ophthalmic acid (L-2-aminobutyrate replaced by L-alanine). Norophthalmic acid like glutathione and ophthalmic acid is an antioxidant, protects cells from toxins such as free radicals.

The compound 3 was obtained from the culture filtrate of Streptomyces avidinii strain SB9 as a colorless oily liquid. It is soluble in ethanol, ethyl acetate, chloroform and n-hexane, but insoluble in water. Compound 3 gave positive reactions (purple color) with conc. $\text{H}_2\text{SO}_4$ and 0.5% solution of vanillin in methanol/sulphuric acid/acetic acid, but not with ninhydrine, $\text{FeCl}_3$ and $\text{SbCl}_3$, reagents on TLC plates. The UV-vis spectrum showed absorption maxima at $\lambda_{max}$ 250 and 283 sh (in n-hexane. The (+)-ESI mass spectrum of 3 displayed pseudo-molecular ions at $m/z$ 413.3 (M+Na)$^+$ and 803.2 (2M+Na)$^+$. The molecular weight (390 Dalton) and the chemical formula $C_{24}H_{38}O_4$ were readily determined by ESI-HRMS due to $m/z$ 390.276461. Compound 3, isolated from Streptomyces avidinii SB9 was determined to be di-(2-ethylhexyl) phthalate (7).

$^1$H and $^13$C NMR chemical shifts of norophthalmic acid (2) in CD$_3$OD (500 MHz and 125 MHz, TMS as internal standard, chemical shifts in $\delta$ values)

<table>
<thead>
<tr>
<th>Assignment</th>
<th>$\delta_H$ (Hz)</th>
<th>$\delta_C$ (Hz)</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.50</td>
<td>-</td>
<td>OH</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>172.9 s</td>
<td>C=O</td>
</tr>
<tr>
<td>3</td>
<td>3.80 (1H, m)</td>
<td>56.3 d</td>
<td>CH</td>
</tr>
<tr>
<td>4</td>
<td>8.47</td>
<td>-</td>
<td>NH$_2$</td>
</tr>
<tr>
<td>5</td>
<td>2.00 (2H m)</td>
<td>29.6 t</td>
<td>CH$_2$</td>
</tr>
<tr>
<td>6</td>
<td>2.42 (2H, m)</td>
<td>31.3 t</td>
<td>CH$_2$</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>171.1 s</td>
<td>C=O</td>
</tr>
<tr>
<td>8</td>
<td>8.61</td>
<td>-</td>
<td>NH</td>
</tr>
<tr>
<td>9</td>
<td>4.86 (1H, d)</td>
<td>52.3 d</td>
<td>CH</td>
</tr>
<tr>
<td>10</td>
<td>1.32 (3H, d, 5.3 Hz)</td>
<td>17.4 q</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>170.4 s</td>
<td>C=O</td>
</tr>
<tr>
<td>12</td>
<td>8.61</td>
<td>-</td>
<td>NH</td>
</tr>
<tr>
<td>13</td>
<td>4.21 (2H, m)</td>
<td>43.3 t</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>175.7 s</td>
<td>C=O</td>
</tr>
<tr>
<td>15</td>
<td>8.50</td>
<td>-</td>
<td>OH</td>
</tr>
</tbody>
</table>

s: singlet; d: doublet; t: triplet; q: quartet

Fig. 2. HPLC-DAD/ESI-MS/MS analysis of quasi-molecular ion (M+H)$^+$ 276 and peptide sequencing of norophthalmic acid (2)

Conclusions

A streptomycete strain SB9, isolated from a permafrost Arctic region was found to be closely identical to strain Streptomyces avidinii. No previous reports were found on location of this strain in Arctic regions or growing at low temperatures. In

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present work we found for the first time that *S. avidinii* strain SB9 produces 2-amino-3-dodecanol, norophthalmic acid and di-(2-ethylhexyl) phthalate. Cold-adapted organisms and their products have potential applications in a broad range of industrial, agricultural and medical processes.

**Acknowledgments**

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**REFERENCES**