Ripromycin and Other Polycyclic Macrolactams from *Streptomyces* sp. Tü 6239:
Taxonomy, Fermentation, Isolation and Biological Properties

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Strain Tü 6239 was isolated from a soil sample collected in Brazil and determined as a new species of the genus *Streptomyces*. In the course of our HPLC-diode array screening program three metabolites were detected in the culture filtrate and mycelium extracts of strain Tü 6239. They were characterised as members of the macrolactam group, the new compound ripromycin (1), the previously described ikarugamycin (2) and a new derivative of it, ikarugamycin epoxide (3). They show antibiotic activities against Gram-positive bacteria and cytostatic effects to various human tumor cell lines.

Strain Tü 6239 was isolated from a soil sample collected in São José do Rio Preto, Brazil, during a program that was designed to the isolation of rare actinomycetes by heat and phenol treatment of the soils). Chemotaxonomic methods and 16S rRNA analysis indicated that strain Tü 6239 may represent a new species of the genus *Streptomyces*. Strain Tü 6239 was included in our HPLC-diode array (HPLC-DAD) screening program to investigate the chemical diversity of secondary metabolites in culture filtrate and mycelium extracts by means of our HPLC-UV-Visible Database). The UV-visible spectra and retention times of the resulting peaks were compared with those of more than 700 reference compounds, mostly antibiotics, stored in the database. Three dominant metabolites with retention times of 10.1, 11.8 and 14.6 minutes were produced by strain Tü 6239 (Fig. 1) showing a high conformity in their UV-visible spectra to maltophilin, a macrolactam antibiotic produced by *Stenotrophomonas maltophilia*, but differed significantly in their retention times.

This report deals with the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, and biological activities of the isolated metabolites. Investigations on their chemical structures and biosynthesis are reported in a separate paper). These results indicated that all three metabolites belong to the macrolactam group. The metabolite with a retention time of 10.1 minutes was characterised as the new macrolactam ripromycin (1), a further metabolite with a retention time of 14.6 minutes was identified as the previously described antiprotozoic macrolactam ikarugamycin (2), and the third metabolite with a retention time of 11.8 minutes was found to be ikarugamycin epoxide (3), a not as yet described derivative of 2. Their stuctures are shown in Fig. 2.

Materials and Methods

Microorganisms

Strain Tü 6239 was isolated from a soil sample collected at São José do Rio Preto, S. P., Brazil. The soil sample was

1 Art. No. 28 in 'Biosynthetic Capacities of Actinomycetes'. Art. No. 27: see ref. 1. We dedicate this article to Professor Dr. UDO GRAFE.

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Fig. 1. HPLC analysis of culture filtrate extract from *Streptomyces* sp. Tü 6239 monitored at 280 nm, and overlayed UV spectrum of ripromycin (1).

Fig. 2. Structures of ripromycin (1), ikarugamycin (2) and ikarugamycin epoxide (3), produced by *Streptomyces* sp. Tü 6239.
air-dried at room temperature and preserved in a freezer at 
\(-20^\circ\text{C}\). Two grams were incubated for 40 minutes at 60°C 
and resuspended in 5 ml saline. 0.5 ml of the suspension 
was added to 4.5 ml of saline containing 1.5% phenol 
(w/v). The solution was stirred with glass beads for 30 
minutes at 30°C, filtered through sterile glasswool and 
diluted with saline \((10^{-1}, 10^{-2} \text{ and } 10^{-3})\). 0.2 ml aliquots 
were spread onto HV agar plates\(^7\), containing nalidixic acid 
\((20\mu\text{g/ml})\), cycloheximide \((50\mu\text{g/ml})\), nystatin \((50\mu\text{g/ml})\). 
The plates were incubated 14 days at 27°C and the 
appearing colonies were collected and further inoculated on 
ISP-2 agar plates\(^8\) and HV agar plates. The strain is 
deposited as DSM 41778 in the strain collection of DSMZ, 
Braunschweig, Germany.

Strains for testing the biological activity spectrum and 
minimal inhibition concentrations were obtained from 
DSMZ, ATCC and the culture collection of our laboratory 
in Tübingen.

**Taxonomy**

Strain Tü 6239 was characterised by morphological\(^9\) and 
chemotaxonomic methods\(^{10,11} \) as a member of the genus 
*Streptomyces*. For molecular characterisation, fresh 
bacterial cells were ground using a sterile micro pestle 
(Eppendorf) to get a uniform suspension. Preparation of 
geomic DNA from the pure culture was performed 
following the protocol given by PUKALL et al.\(^{12}\). The 
amplification of the 16S rDNA was done as described 
by RAINEY et al.\(^{13}\) using the primer pair 27f 
\((5' \text{AGAGTTTGATCCTGCTCAG} 3')\) and 1500r 
\((5' \text{AGAAAGGAGGTGATCCAGCC} 3')\). Sequencing was 
done by BioLux DNA-Analytik using the four primers 
25f \((5' \text{CTACGGGRSGCAGCAG} 3')\), \(650f \) 
\((5' \text{AATTCCTGGTGTAGCGGT} 3')\) and \(926f \) 
\((5' \text{AAACTCAAAGGAATTGACGG} 3')\). Sequence of the 16S 
rDNA was manually aligned and compared to the 
sequences published previously. These were stored in 
DSMZ-internal database consisting of more than 6000 16S 
rDNA sequence entries, including those from the 
Ribosomal Database Project and EMBL. All analyses were 
done on SUN SparcII workstation.

**Fermentation**

Strain Tü 6239 was cultivated in a 10-liter stirred tank 
fermenter (New Brunswick) using a production medium 
composed of glucose 1%, glycerol 1%, starch 1%, 
cornsteep powder (Marcor) 0.25%, peptone 0.5%, yeast 
extract 0.2%, NaCl 0.1% and CaCO\(_3\) 0.3% in tap water 
(pH 7.2, adjusted with 1 N NaOH). The fermenter was 
inoculated with 5 vol-% of shaking cultures grown at 27°C 
in the same medium for 72 hours in 500 ml-Erlenmeyer 
flasks with one baffle on a rotary shaker at 120 rpm. The 
fermentation was carried out at 27°C for 165 hours with an 
aeration rate of 0.4 v/v/m and an agitation rate of 250 rpm. 
The production of 1, 2 and 3 was monitored by reversed-
phase HPLC.

**Isolation and Physico-chemical Characterisation**

Hyflo Super-cel (3%) was added to the fermentation 
broth which was separated by multiple sheet filtration into 
culture filtrate and mycelium. The culture filtrate was 
adjusted to pH 5 and extracted three times with EtOAc. The 
mycelium was extracted three times with MeOH. The 
extracts were combined, concentrated in vacuo to the 
aqueous residue, re-extracted three times with EtOAc and 
concentrated in vacuo to dryness. The raw product was 
dissolved in MeOH, subjected to a Sephadex LH-20 
column \((90 \times 2.5 \text{ cm})\) and separated using MeOH as eluent. 
Finally, pure 1, 2 and 3 were obtained by preparative 
reversed-phase HPLC using a stainless steel column 
\((250 \times 16 \text{ mm}; \text{Maisch})\) packed with 10-μm Nucleosil-100 
C-18 and a linear gradient with 0.5% aqueous HCOOH- 
MeOH starting at 65% MeOH to 100% MeOH within 20 
minutes at a flow rate of 20 ml/minute. The preparative 
HPLC system consisted of two high-pressure pumps 
(Sepapress HPP-200/100; Kronwald), a gradient unit 
(Sepacon GCU-311), and a Valco preparative injection 
valve \((6UW; \text{VICI})\) with a 5-ml sample loop. The UV 
absorbance of the eluate was monitored simultaneously at 
260 nm and 330 nm by a Gilson spectrophotometer Mod. 
116 equipped with a preparative cell.

The pure compounds were characterised by UV at 
different pH values, by infrared spectroscopy (KBr), mass 
spectrometry, optical rotation, and thin layer 
chromatography.

**Biological Assay**

An agar-plate diffusion assay was used to determine the 
antibacterial and antifungal spectrum of 1, 2 and 3. Ten μl 
of the samples were applied to filter disks (6 mm diameter). 
The test plates were incubated for 24 hours at a temperature 
that permitted optimal growth of the test organisms. 
A broth dilution method was used to determine the 
minimal inhibition concentrations of 1, 2 and 3. The 
antibiotics were dissolved in DMSO at the final 
fractionations of less than 5%. Bacteria were grown in the 
medium consisted of 0.8% nutrient broth and 0.5% NaCl in 
tap water. Streptomyces were grown in malt extract 1%, 
glucose 0.4% and yeast extract 0.4% in tap water (pH 7.3). 
Bacterial cells and spores \((10^5\text{ml})\) were used as inoculum,
and growth inhibition was evaluated after incubation at 37°C for 24 and 48 hours, respectively, on a rotary shaker.

The antitumor activity of 1, 2 and 3 was tested according to NCI guidelines with human cell lines from gastric adenocarcinoma (HMO2), mamma carcinoma (MCF 7), hepatocellular carcinoma (Hep G2) and hepatoma cells with mutated p53 (Huh 7). Cells were grown in 96-well microtiterplates in RPMI 1640 with 10% fetal calf serum in a humidified atmosphere of 5% CO₂ in air. After 24 hours of incubation 1, 2 and 3 (0.1–10 μg/ml) were added to the cells. Stock solutions were prepared in DMSO. The final DMSO concentration in the cultures was 0.1%. After a 48 hours incubation period the cells were fixed, and cell protein was assayed with sulforhodamine B.

HPLC-DAD-Analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a diode-array detector and a HP Kayak XM 600 ChemStation using software revision A.08.03 (Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435 and 500 nm; the spectrum range was from 200 to 600 nm with a 2-nm step and a sampling rate of 640 milliseconds.

A 10-ml aliquot of the fermentation broth was centrifuged (10 minutes, 13,000 g). The supernatant was adjusted to pH 5 and extracted with the same volume of EtOAc. The organic layer was concentrated to dryness and resuspended in 1 ml MeOH. The mycelium pellet was extracted with 10 ml MeOH, the extract was concentrated in 1 ml MeOH. A 10-μl aliquot of the samples was injected onto a HPLC column (125×4.6 mm) fitted with a guard-column (20×4.6 mm) which were packed with 5-μm Nucleosil-100 C-18 (Maisch). The samples were analysed by a linear gradient elution using 0.1% aqueous o-phosphoric acid-ACN starting at 0% ACN to 100% ACN within 15 minutes at a flow rate of 2 ml/minute with a 1-minute hold at 100% ACN, followed by a 5-minute post-time under initial conditions.

Results

Taxonomy

Strain Tü 6239 was assigned to the genus Streptomyces because of its morphological appearance and characteristic chemotaxonomic features, such as L-l-diaminopimelic acid in the peptidoglycan together with the cell-wall sugars galactose, glucose and ribose, and the typical pattern of saturated iso- and anteiso-branched fatty acids. The spore mass colour of the aerial mycelium was gray and the spore chains were spiral with more than ten spores per chain. (Fig. 3).

The complete 16S rDNA sequence of strain Tü 6239 was compared to sequences of other strains belonging to the family Streptomycetaceae, due to the presence of the family characteristic 16S rDNA signature nucleotides as described by Stackebrandt et al. The nearest phylogenetic neighbours of strain Tü 6239 were found to be Streptomyces carpaticus and Streptomyces kasugaensis, showing sequence similarity values of 98.1% and 96.3%, respectively. The moderate 16S rRNA gene sequence similarity with other members of Streptomyces indicates strain Tü 6239 to represent a new species of the genus.

Fermentation, Isolation and Physico-chemical Characterisation

Production of ripromycin (1) reached a maximal value of 125 mg/liter in batch fermentations after incubation of 165 hours. Production of ikarugamycin (2) and ikarugamycin epoxide (3) commenced 24 hours later and reached maximal values of 118 and 60 mg/liter, respectively. The time course of a representative fermentation is shown in Fig. 4. The compounds were isolated from the culture filtrate and mycelium extracts by Sephadex LH-20 chromatography. Pure compounds were obtained after preparative reversed-phase HPLC as yellow-beige powders. The physico-chemical properties of 1, 2 and 3 are summarized in Table 1.
Fig. 4. 10-Liter batch fermentation of *Streptomyces* sp. Tü 6239.

- □ biomass (dry weight); △ pH; ○ ripromycin (1); ■ ikarugamycin (2); □ ikarugamycin epoxide (3).

Table 1. Physico-chemical properties of ripromycin (1) and ikarugamycin epoxide (3).

<table>
<thead>
<tr>
<th></th>
<th>Ripromycin (1)</th>
<th>Ikarugamycin epoxide (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>yellow-beige powder</td>
<td>yellow-beige powder</td>
</tr>
<tr>
<td>MP</td>
<td>$190^\circ$C</td>
<td>$240^\circ$C</td>
</tr>
<tr>
<td>$[\alpha]_D^{20}$</td>
<td>$+114^\circ$ (c 0.77, MeOH)</td>
<td>$+126^\circ$ (c 0.38, MeOH)</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>$547 [M + Na]^+$</td>
<td>$517 [M + Na]^+$</td>
</tr>
<tr>
<td></td>
<td>$523 [M - H]^{-}$</td>
<td>n.a.</td>
</tr>
<tr>
<td>HREI-MS</td>
<td>$525.29512 [M + H]^+$</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>(found as calcld.)</td>
<td></td>
</tr>
<tr>
<td>Molecular formula</td>
<td>$C_{30}H_{49}N_2O_6$</td>
<td>$C_{29}H_{38}N_2O_3$</td>
</tr>
<tr>
<td>MW</td>
<td>524.66</td>
<td>494.63</td>
</tr>
<tr>
<td>IR (KBr) $\nu_{\text{max}}$ (cm$^{-1}$)</td>
<td>3445, 1690 sh, 1650, 1582, 1458, 1371, 1087</td>
<td>3421, 1650, 1587, 1550, 1448, 1372, 1250, 1101</td>
</tr>
<tr>
<td>UV $\lambda_{\text{max}}$ nm (lg $\varepsilon$)</td>
<td>314 (4.05), 290 sh, 238 sh, 204 (4.40)</td>
<td>319 (3.57), 240 (4.02), 203 (4.18)</td>
</tr>
<tr>
<td>TLC (Rf) CHCl$_3$–MeOH (9:1)</td>
<td>0.33</td>
<td>0.35</td>
</tr>
<tr>
<td>Colour reaction on TLC</td>
<td>Anisaldehyde–H$_2$SO$_4$ orange</td>
<td>orange</td>
</tr>
<tr>
<td></td>
<td>Orcin–FeCl$_3$–H$_2$SO$_4$ red-brown</td>
<td>yellow-brown</td>
</tr>
</tbody>
</table>

n.a. = not available
Biological Properties

The antimicrobial spectra of the ripromycin (1), ikarugamycin (2) and ikarugamycin epoxide (3) were examined by an agar plate diffusion assay (Table 2), and minimal inhibition concentrations was determined by the broth dilution method (Table 3). Gram-negative bacteria were not sensitive to the compounds which exhibited moderate activities against Gram-positive bacteria and some fungi. 1, 2 and 3 showed no inhibitory activity against the green algae Chlorella fusca and Lemna minor (duckweed).

The compounds strongly inhibited the proliferation of HMO2 and MCF 7 cells (IC\textsubscript{50} values: 0.22–2.0 μg/ml), and displayed cytotoxic activity in the cell line HMO2. The effect on hepatoma cell lines (Hep G2 and Huh 7) was less pronounced. Ikarugamycin (2) inhibited the proliferation of Hep G2 and Huh 7 cells by 50% at 5.8 and 2.7 μg/ml, respectively. Data are shown in Table 4.

Discussion

Three dominant metabolites were produced by Streptomyces sp. Tü 6239 and characterised by HPLC-DAD analysis to be similar to maltophilin\textsuperscript{4)}, a member of the group of macrolactam antibiotics, by comparing their UV-visible spectra. They were identified as the new metabolite ripromycin (1), the known antibiotic ikarugamycin\textsuperscript{6)} (2) and its yet unknown epoxide (3). The characteristic structural element of these metabolites is the polycyclic macrolactam consisting of a 16-membered ring in which a tetramic acid is integrated. Further members of this type of antibiotics are discodermide, which is produced by the marine sponge Discodermia dissoluta\textsuperscript{18)}, alteramid A produced by a marine Alteromonas species\textsuperscript{19)}, and the Streptomyces metabolites maltophilin\textsuperscript{4)}, dihydromaltophilin\textsuperscript{20)} and capsimycin\textsuperscript{21)}.

Ikarugamycin (2) was the most potent antibacterial and antitumor active compound within the macrolactam group isolated from Streptomyces sp. Tü 6239. All three
metabolites exhibited cytostatic and cytotoxic activities towards the human tumor cell lines HMO 2 and MCF 7. Ikarugamycin was reported to exhibit additional biological activities, such as antiprotozoic action and inhibition of the uptake of oxidized low-density lipoprotein in macrophages.

Acknowledgments

M.B. thanks the DAAD for a doctoral scholarship, and Prof. Kroppenstedt from the DSMZ Braunschweig for an introduction to taxonomic methods. The authors wish to thank Mr. G. Grewe for technical assistance in fermentations, Mr. H. Schoppmann, Zoologisches Institut, Universität Tübingen, for assistance in scanning electron microscopy, and Agilent Technologies Germany for HPLC software support.

References


Table 3. Minimal inhibition concentrations (µg/ml) of ripromycin (1), ikarugamycin (2) and ikarugamycin epoxide (3) as determined by the broth dilution method.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arthrobacter aurescens DSM 20166</td>
<td>10</td>
<td>0.3</td>
<td>10</td>
</tr>
<tr>
<td>Arthrobacter oxydans DSM 6612</td>
<td>&gt;100</td>
<td>10</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Arthrobacter pasculus DSM 20545</td>
<td>10</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>Arthrobacter globiformis DSM 20124</td>
<td>30</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>Bacillus subtilis DSM 10</td>
<td>100</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Staphylococcus aureus DSM 20231</td>
<td>100</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Streptomyces viridochromogenes Tü 57</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 4. Activities (µg/ml) of ripromycin (1), ikarugamycin (2) and ikarugamycin epoxide (3) against selected human tumor cell lines.

<table>
<thead>
<tr>
<th></th>
<th>Gl50a</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>HMO2</td>
<td>MCF 7</td>
<td>Hep G2</td>
<td>Huh 7</td>
<td>HMO2</td>
<td>MCF 7</td>
<td>Hep G2</td>
<td>Huh 7</td>
<td>HMO2</td>
</tr>
<tr>
<td>1</td>
<td>1.6</td>
<td>2.0</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>2.7</td>
<td>3.6</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>4.6</td>
</tr>
<tr>
<td>2</td>
<td>0.28</td>
<td>0.22</td>
<td>5.8</td>
<td>2.7</td>
<td>0.68</td>
<td>0.7</td>
<td>9.8</td>
<td>&gt;10</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>0.34</td>
<td>0.24</td>
<td>&gt;10</td>
<td>7.6</td>
<td>0.65</td>
<td>1.0</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>1.4</td>
</tr>
</tbody>
</table>

a Drug concentration causing 50% growth inhibition
b Drug concentration causing 100% growth inhibition
c Drug concentration causing 50% reduction of the cells present at time point zero, i.e. at 24 hours
1 75% inhibition at 10 µg/ml
2 78% inhibition at 10 µg/ml


