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Retymicin, Galtamycin B, Saquayamycin Z and Ribofuranosyllumichrome, Novel Secondary Metabolites from *Micromonospora* sp. Tü 6368

I. Taxonomy, Fermentation, Isolation and Biological Activities[†]

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Abstract A new xanthone compound named retymicin (**1**) was isolated together with galtamycin B (**2**) and saquayamycin Z (**3**), new members of the galtamycin and saquayamycin families, respectively, and the new lumichrome derivative 1-(α-ribofuranosyl)-lumichrome (**4**) from *Micromonospora* strain Tü 6368, isolated from a soil sample collected in Romania. Retymicin, galtamycin B and saquayamycin Z show cytostatic effects to various human tumor cell lines whereas saquayamycin Z is also active against Gram-positive bacteria.

Keywords new substance, antitumor, screening, taxonomy, fermentation, isolation

Introduction

Freshly isolated actinomycete strains from soils collected at various sites in Romania were included in our screening program to detect novel secondary metabolites by HPLC-diode array analysis (HPLC-DAD). Strain Tü 6368 was isolated from a soil collected at Rety, Romania, after applying dry heat to the soil. Chemotaxonomic methods and partial 16S rDNA analysis indicated that the strain belongs to the genus *Micromonospora*. HPLC-DAD analysis of the extracts from the culture filtrate and mycelium revealed a pattern of peaks belonging to different natural product groups based on their UV-visible spectra. By comparing UV-visible spectra and retention times with reference compounds stored in our HPLC-UV-Vis-Database [2] that contains about 750 entries, mostly antibiotics, five different natural product groups were identified, (i) three members of the rabelomycin family, (ii) two members of a not identified anthraquinone family, (iii) one member of the saquayamycin family, (iv) three members of the lumichrome group, and (v) a further unknown metabolite. Addition of the polystyrene resin Amberlite XAD-16 during fermentation increased the quantity and number of secondary metabolites produced by strain Tü 6368.

This report deals with the taxonomy of the producing strain, its fermentation and the isolation and biological

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We dedicate this publication to Emeritus Professor Hans Zähner in honour of his 75th birthday.

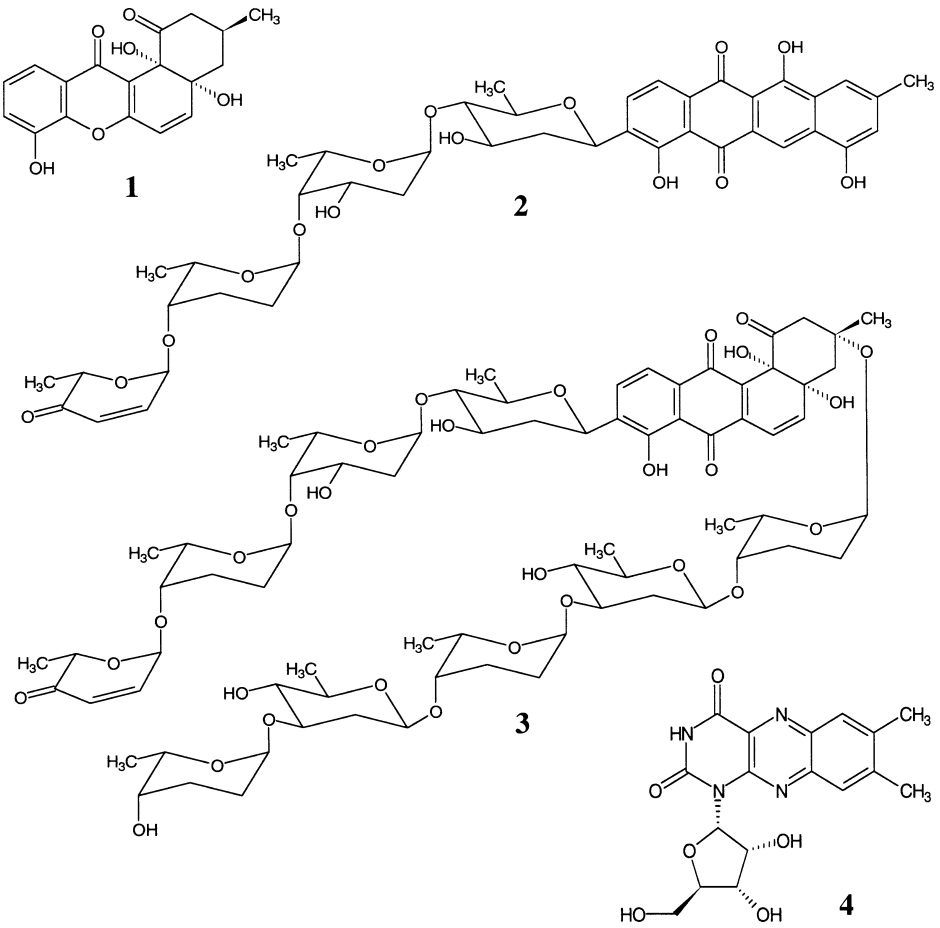

Fig. 1 Structural formulae of retymicin (**1**), galtamycin B (**2**), saquayamycin Z (**3**) and 1-(α-ribofuranosyl)-lumichrome (**4**).

properties of the secondary metabolites produced by Tü 6368. The following secondary metabolites were isolated from strain Tü 6368 and their structures elucidated, (i) rabelomycin [3], 3-deoxyrabelomycin [4] and dehydrorabelomycin [5], (ii) galtamycinone [6] and galtamycin B (**2**), (iii) saquayamycin Z (**3**), (iv) lumichrome and 1-methyllumichrome [7], and 1-(α-ribofuranosyl)-lumichrome (**4**), and (v) retymicin (**1**). The structures of the new metabolites are shown in Fig. 1. The results on structure elucidation are reported in the subsequent publication [8].

Materials and Methods

Microorganism

The producing strain Tü 6368 was isolated from a sandy soil collected at a birch reservation near Rety, Romania. Five grams of soil were incubated for 40 minutes at 60°C and resuspended in 5 ml of saline. The suspension was diluted with 45 ml saline containing 1.5% phenol (w/v) and shaken with glass beads for 30 minutes at 30°C. After filtration through sterile glass wool and dilution with saline (10^{-1}, 10^{-2} and 10^{-3}, respectively), 0.1 ml aliquots were spread onto starch - casein agar [9], containing cycloheximide (50 μg/ml), nystatin (50 μg/ml), nalidixic acid (20 μg/ml) and novobiocin (20 μg/ml). The plates were incubated 14 days at 27°C, and the resulting colonies were transferred to starch - casein agar and ISP-2 agar [10]. The strain Tü 6368 was deposited in our laboratory's culture collection at the University of Tübingen.

Taxonomy of the Producing Strain Tü 6368

The organism was inoculated onto ISP-2 agar, incubated at 27°C for 2 weeks, and examined visually to determine substrate mycelium pigmentation and spore colour. Diaminopimelic acid [11] and the composition of cell-wall sugars [12] was identified by TLC of the whole-organism hydrolysate. Fatty acids were determined by GC-MS [13, 14], and menaquinones by reversed-phase HPLC [15, 16]. 16S rRNA gene amplification and determination of the almost complete sequence (1495 bp) were carried out

according to Rainey *et al*. (1996) [17].

Fermentation

Batch fermentations of strain Tü 6368 were carried out in 10-liter stirred tank fermenters with and without addition of Amberlite XAD-16 (Rohm and Haas) to a medium consisting of 1% glucose, 2% soluble starch 0.5% yeast extract (Ohly Kat G, Deutsche Hefewerke), 0.5% Bacto casitone, and 0.1% $CaCO_3$ in tap water; pH was adjusted to 7.6 prior to sterilization. The fermenter was inoculated with 5% by volume with shake flask cultures, grown at 27°C in 500-ml Erlenmeyer flasks with one baffle for 72 hours on a rotary shaker at 120 rpm. The fermentation was carried out for 168 hours with an aeration rate of 0.5 vvm and an agitation rate of 250 rpm.

In the case of polystyrene resin supplemented fermentations, three liters of an aqueous suspension of Amberlite XAD-16 were added to the medium prior to sterilization.

Isolation and Physico-chemical Characterization

In fermentations without Amberlite XAD-16 supplementation, Hyflo Super-cel (2%) was added to the fermentation broth, just prior to separation by multiple sheet filtration into culture filtrate and mycelium. The culture filtrate (6 liters) was applied to an Amberlite XAD-16 column (650 ml). Impurities were washed out with water, and metabolite **4** was eluted with H_2O - MeOH (20+80) and concentrated *in vacuo* to an aqueous residue, which was adjusted to pH 5 (1 M HCl) and extracted three times with EtOAc. The organic extracts were combined and concentrated *in vacuo* to dryness. The crude product was dissolved in CH_2Cl_2 and subjected to a silica gel column (42×2.6 cm, silica gel SI 60, Merck). The separation was accomplished by a linear gradient using CH_2Cl_2 - MeOH, starting with CH_2Cl_2 and transitioning to 10% MeOH within 3 hours at a flow rate of 5.6 ml/minute. Pure **4** was obtained by Sephadex LH-20 chromatography (90×2.5 cm) using MeOH as the eluent. After concentration *in vacuo* to dryness, metabolite **4** was obtained as a yellow powder.

In fermentations with Amberlite XAD-16 supplementation, the mixture of mycelium and polystyrene resin was extracted three times with acetone. The extracts were combined, concentrated *in vacuo* to an aqueous residue, re-extracted three times with EtOAc and concentrated *in vacuo* to dryness. One part of the raw product was dissolved in CH_2Cl_2, purified through a silica gel Si 60 column and eluted with CH_2Cl_2 - MeOH, using a linear gradient from 0% to 10% MeOH within 3 hours at a flow rate of 5.6 ml/minute. After concentration *in vacuo* to dryness the crude product was dissolved in MeOH, subjected to a Sephadex LH-20 column using MeOH as the eluent. After concentration *in vacuo* to dryness, metabolite **1** was obtained as a yellow powder soluble in MeOH.

To avoid formation of MeOH adducts of **2** and **3**, MeOH-free separation steps were performed for the isolation of **2** and **3**. Therefore, the crude product was dissolved in acetone and subjected directly to a Sephadex LH-20 column using acetone as the eluent. The resulting fractions containing **2** and **3**, respectively, were finally purified by preparative reversed-phase HPLC using a LiChrospher RP-Select B column (250×16 mm, 10 μm; Maisch) and a linear gradient with H_2O - acetonitrile, starting from 35% to 90% acetonitrile within 25 minutes at a flow rate of 20 ml/minute. After concentration *in vacuo* to dryness, metabolites **2** and **3** were obtained as red and orange powders, respectively.

The pure compounds were characterised by UV at different pH values, by infrared spectroscopy (KBr), mass spectrometry, optical rotation, and thin-layer chromatography (Table 1).

HPLC-DAD Analyses

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a diode-array detector and HP Kayak XM 600 ChemStation and HPLC-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 measured 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. The supernatant was adjusted to pH 5 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. The mycelium pellet was extracted with 10 ml MeOH, the extract was concentrated to dryness and the residue was resuspended 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 was packed 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% solvent B in 15 minutes with a 2-minute hold at 100% solvent B, followed by a 5-minute post-time at initial conditions.

Biological Assays

An agar-plate diffusion assay was used to determine the antibacterial and antifungal spectrum of the metabolites

Table 1 Physico-chemical properties of retymicin (**1**), galtamycin B (**2**), saquayamycin Z (**3**) and 1-(α-ribofuranosyl)-lumichrome (**4**)

	1	**2**	**3**	**4**
Appearance	yellow solid	red solid	orange solid	yellow solid
$[\alpha]_D^{20}$	+54° (*c* 0.1, MeOH)	n.a.	−20° (*c* 0.05, AcCN)	+319° (*c* 0.1, MeOH)
HRESI-MS	329.10259 $[M+H]^+$ (found as calculated)	803.29204 $[M-H]^-$ (found as calculated)	1465.63952 $[M+Na]^+$ (found as calculated)	375.12972 $[M+H]^+$ (found as calculated)
Molecular formula	$C_{18}H_{16}O_6$	$C_{43}H_{48}O_{15}$	$C_{73}H_{102}O_{29}$	$C_{17}H_{18}N_4O_6$
MW	328.32	804.84	1443.59	374.35
IR (KBr) ν_{max} (cm^{-1})	3408, 1717, 1640, 1616, 1593, 1560, 1430, 1360, 1294, 1223, 1180, 1043	3447, 1700, 1636, 1560, 1458, 1437, 1384, 1288, 1261, 1087, 1040, 1014	3443, 1700, 1637, 1564, 1441, 1375, 1285, 1079, 1013	3425, 1768, 1755, 1682, 1558, 1430, 1401, 1355, 1228, 1129, 1039
UV λ_{max} nm (log ε)	215 (4.18), 268 (4.29), 303 sh, 328 sh[a]	249 sh, 264 (4.57), 294 sh, 485 (4.05)[b]	217 (4.45), 316 (3.64), 429 (3.74)[b]	214 (4.45), 254 (4.55), 340 (3.85)[a]
Rf[c]	0.54	0.62	0.52	0.22

[a] measured in MeOH, [b] measured in acetonitrile, [c] TLC, silica gel, $CHCl_3$-MeOH (9 : 1), n.a.=not available

produced by strain Tü 6368. 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.

To determine the minimal inhibition concentrations of the metabolites a 96-well microtiter plate assay was used. The antibiotics were dissolved in DMSO and the final DMSO concentration in the cultures was 5%. The test organisms were grown on a rotary shaker (120 rpm) in a medium consisting of nutrient broth 0.8% and NaCl 0.5% in deionized water. 10^6 cells/ml were used as inoculum, and growth inhibition was evaluated after incubation for 24 and 48 hours.

The inhibitory activity of **1**, **2** and **3** on the growth of tumor cells was tested according to NCI guidelines [18] with 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. After 24 hours incubation, **1**, **2** and **3** (0.1∼10 μl/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.

Results

Taxonomy of the Producing Strain

Strain Tü 6368 produced black spores within orange substrate mycelium on ISP-2 agar plates (Fig. 2). Whole organism hydrolysates showed *meso*-diaminopimelic acid in the peptidoglycan, galactose, arabinose and a small amount of xylose as cell-wall sugars, major menaquinone MK9 (H_4, H_6, H_8), and a fatty acid pattern consisting of unsaturated fatty acids and saturated *iso*- and *anteiso*-branched fatty acids. The morphological and chemotaxonomical characteristic indicated that the strain belongs to the genus *Micromonospora*. Almost complete 16S rRNA gene sequence analysis confirmed this taxonomic classification. The nearest phylogenetic neighbours of strain Tü 6368 were *M. aurantinigra* strain TT1-11 (AB159779) and *M. coerulea* DSM 43143T (X92598), showing sequence similarity values of 99.1%

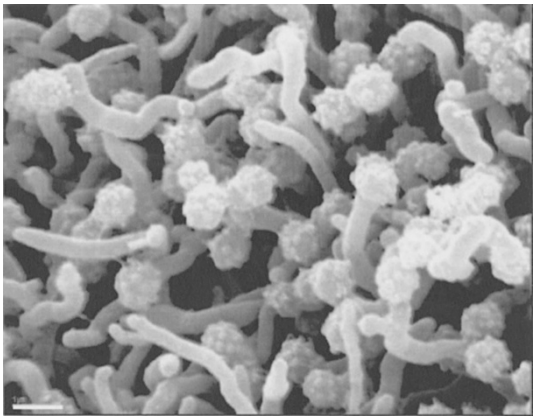

Fig. 2 Scanning electron micrograph of *Micromonospora* sp. Tü 6368. Bar: 1 μm

and 98.6%, respectively. All other *Micromonospora* type strains were less closely related (<98.2%).

Fermentation and Isolation

Strain Tü 6368 was cultivated in 10-liter stirred tank fermenters with and without supplementation of the polystyrene resin Amberlite XAD-16, resulting in different patterns of metabolites. In conventional fermentations (without XAD-16 addition), retymicin (**1**) was produced in an amount of 49 mg/litre together with various lumichrome compounds, for which ribofuranosyllumichrome (**4**) reached a maximal value of 4.1 mg/litre after an incubation period of 168 hours.

The production of retymicin (**1**) increased dramatically during fermentations with addition of the neutral polystyrene resin Amberlite XAD-16, reaching a maximal value of 2.4 g/litre after 168 hours of incubation. Besides **1** as main compound, remarkable amounts of galtamycinone and saquayamycin Z (**3**) were produced, yielding 105 mg/litre and 74 mg/litre, respectively, whereas galtamycin B (**2**), yielding 23 mg/litre, rabelomycin, 3-deoxyrabelomycin and dehydrorabelomycin were produced as minor congeners. The HPLC analysis of a mycelium/XAD-16 extact is shown in Fig. 3.

1, **2** and **3** were isolated from the mycelium extract and purified by various consecutive chromatographic steps. **4** was isolated from the culture filtrate by Amberlite XAD-16 chromatography and purified by extraction and silica gel chromatography.

Biological Properties

The antimicrobial spectra of the new secondary metabolites isolated from strain Tü 6368 were determined by agar plate diffusion assays. Only saquayamycin Z (**3**) exhibited a growth inhibition of Gram-positive bacteria (Table 2), whereas Gram-negative bacteria, such as *Escherichia coli* K12, *Pseudomonas fluorescens* DSM 50090, *Proteus mirabilis* ATCC 35501, yeast, such as *Saccharomyces cerevisiae* ATCC 9080, *Candida albicans* Tü 164, and filamentous fungi, such as *Botrytis cinerea* Tü 157, *Aspergillus viridi nutans* CBS 12756, *Penicillium notatum* Tü 136, *Paecilomyces variotii* Tü 137, were not sensitive against the metabolites. The minimal inhibition concentration of **3** was determined in a microtiter plate assay, and its activity was compared with saquayamycins A and B (Table 3).

The cytostatic effects of retymicin (**1**), galtamycin B (**2**) and saquayamycin Z (**3**) were tested in different tumor cell lines. All compounds showed significant growth inhibitory activity towards HM02 and HepG2 tumor cells (see Table

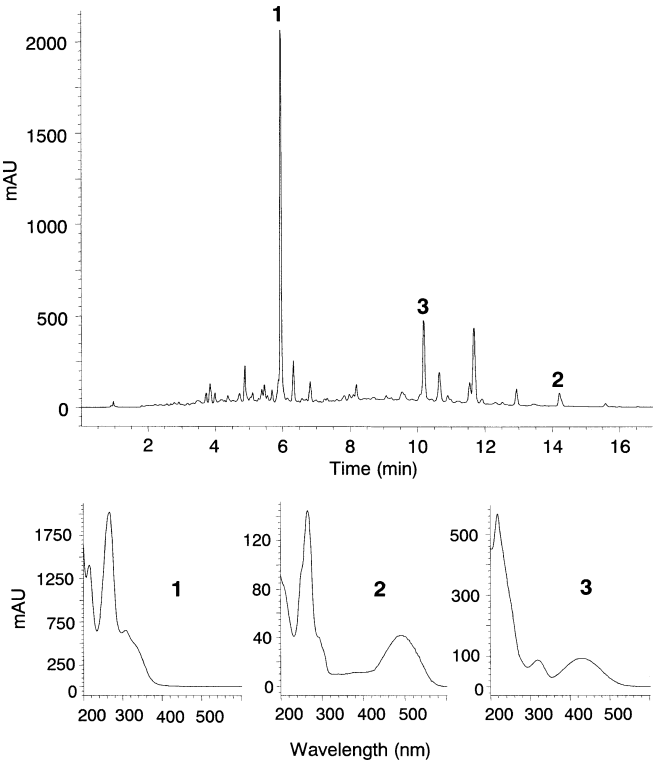

Fig. 3 HPLC-analysis of mycelium/XAD-16 extract from *Micromonospora* sp. Tü 6368, fermentation time 168 hours, monitored at 210 nm, and UV-visible spectra of retymicin (**1**, 5.9 minutes), galtamycin B (**2**, 14.2 minutes) and saquayamycin Z (**3**, 10.2 minutes).

Table 2 Antibacterial spectrum of saquayamycin Z (**3**) determined by the agar plate diffusion assay at various concentrations

Test organism	Inhibition zone (mm)		
	1 mg/ml	0.3 mg/ml	0.1 mg/ml
Arthrobacter aurescens DSM 20166	13	11	—
Bacillus subtilis DSM 10	16	13	11
Staphylococcus aureus DSM 20231	10	—	—

4); retymicin (**1**) was found to be inactive toward MCF7 cells (GI_{50} value $>10\,\mu g/ml$). Saquayamycin Z (**3**) possesses the highest cytostatic activity with GI_{50} value $<1\,\mu g/ml$.

Discussion

Strain Tü 6368 shows the morphological, chemotaxonomical and phylogenetic properties as defined for members of the genus *Micromonospora*, class *Actinobacteria*. 16S rRNA gene sequence analysis indicates close relationship to two other species but the values are not higher than those found for some other pairs of species, *e.g. M. aurantiaca* and *M. chalceae*, *M. aurantiaca* and *M. halophytica*, *M. echinospora* and *M. sagamiensis* [19]. It is likely that strain Tü 6368 represents a novel species, to be described when additional cultural and metabolic properties become available.

Applying the OSMAC approach [20], increasing chemical diversity by altering the cultivation conditions, *Micromonospora* sp. Tü 6368 produced two major groups of metabolites, three members of riboflavin derived lumichromes, and various members of type-2 polyketide compounds belonging to tetracenequinones, rabelomycins, saquayamycins and xanthones. Lumichrome was repeatedly detected by our HPLC-diode array screening in extracts of various actinomycetes strains (H.-P. F., unpublished data). The common breakdown product of riboflavin was identified as a signal molecule produced by the rhizosphere bacterium *Sinorhizobium meliloti* that increases root respiration and shoot growth in alfalfa (*Medicago sativa* L.) and also triggers a compensatory increase in whole-plant net carbon assimilation [21]. The production of lumichrome and lumichrome derivatives by rhizosphereous actinomycetes may play a similar role in the stimulation of plant growth.

The amount and the pattern of secondary metabolites produced by strain Tü 6368 changed remarkably in the presence of the neutral polystyrene resin Amberlite XAD-16, when the resin was added directly into the production fermentor. Retymicin (**1**) productivity increased from 49 to 2,400 mg/litre, further polyketide metabolites galtamycinone and saquayamycine Z (**3**) were produced in remarkable amounts. In addition the minor congeners galtamycin B (**2**), rabelomycin, 3-deoxyrabelomycin and dehydrorabelomycin were detected in fermentations with resin addition. Such a positive effect of polystyrene resins has been described in various fermentations with fungi and bacteria [22～24], however it seems not to be predictable. One reason for the stimulating effect may be the binding of a secondary metabolite to the resin, preventing its further metabolism and degradation [25, 26]. Another stimulating effect of a resin is due to the binding of metabolites to reduce end product inhibition or toxicity, *e.g.* in the fermentation processes of epidermin [27] and teicoplanin [28].

Saquayamycin Z (**3**), the largest angucycline compound identified thus far, is assembled by the aquayamycin aglycon and eight additional deoxy sugar moieties was the only potent antibacterial active compound within the new

Table 3 Minimal inhibition concentrations ($\mu g/ml$) saquayamycins A, B and Z (**3**) as determined by a microtiter plate assay.

Test organism	Saquayamycin		
	A	B	Z
Arthrobacter aurescens DSM 20166	2.7	2.7	1.4
Bacillus subtilis DSM 10	2.7	2.7	4.8
Staphylococcus aureus DSM 20231	0.8	0.8	14.4

Table 4 Cytostatic activities ($\mu g/ml$) of retymicin (**1**), galtamycin B (**2**) and saquayamycin Z (**3**) against selected human tumor cell lines.

	GI_{50}			TGI		
	HM02	MCF7	HepG2	HM02	MCF7	HepG2
1	6.5	>10[a]	4.7	>10	>10	>10[a]
2	8.1	4.4	4.8	>10[b]	>10[c]	>10[d]
3	0.94	0.94	0.76	2.1	7.0	1.7

GI50: 50% growth inhibition; TGI: 100% growth inhibition

[a] 78% inhibition at 10 $\mu g/ml$; [b] 65% inhibition at 10 $\mu g/ml$; [c] 76% inhibition at 10 $\mu g/ml$; [d] 60% inhibition at 10 $\mu g/ml$

metabolites produced by *Micromonospora* sp. Tü 6368. The cytostatic effects of retymicin (**1**) and saquayamycin Z (**3**) against HM02, HepG2 and MCF 7 cell lines were in the range of 4.4 to 8.1 μg/ml. This range is in the same range or partially better than that of 5-fluorouracil (GI_{50} value towards HM02, HepG2 and MCF 7 cells: 4.2, 15 and 45 μg/ml, respectively). **3** showed a cytostatic activity which was in the range of cisplatin (GI_{50} value towards HM02, HepG2 and MCF 7 cells: 0.8, 0.65 and 0.095 μg/ml, respectively). Overall, the cytostatic effect of **3** must be judged as moderate compared to doxorubicin (GI_{50} value towards HM02, HepG2 and MCF 7 cells: 0.006, 0.15 and 0.08 μg/ml, respectively).

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