Kanchanamycins, New Polyol Macrolide Antibiotics Produced by *Streptomyces olivaceus* Tü 4018

I. Taxonomy, Fermentation, Isolation and Biological Activities†

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The kanchanamycins, a group of novel 36-membered polyol macrolide antibiotics were detected in the culture filtrate and mycelium of *Streptomyces olivaceus* Tü 4018 by HPLC-diode-array and HPLC-electrospray-mass-spectrometry screening. The compounds show antibacterial and antifungal activities, and are especially effective against *Pseudomonas fluorescens*. Besides the kanchanamycin complex, strain Tü 4018 produces the 42-membered macrolactones, oasomycin A and desertomycin A, as well as tryptophan-dehydrobutyrine diketopiperazine and daidzein.

In the course of our HPLC screening we investigated the metabolite diversity of *Streptomyces* strain Tü 4018 which was isolated from a soil sample collected in Kanchana Buri, Thailand. Our screening method is based on a two-step strategy. First, culture filtrates, culture filtrate extracts and mycelium extracts were analysed by reversed-phase HPLC and multiwavelength diode array monitoring (HPLC-DAD), followed by identification or characterisation of the resulting chromatographic peaks by our HPLC-UV-Vis database[2]. Second, samples of those strains which produced presumably novel compounds were analysed by HPLC-ESI-MS from which information on the molecular masses of the metabolites could be obtained. These data permit an efficient search in commercially available databases for the novelty of compounds.

Regarding the HPLC-UV-Vis database search results, the secondary metabolite pattern of strain Tü 4018 could be divided into four different types of compounds. Type I is composed of four compounds belonging to the group of 36-membered polyol macrolide antibiotics and were named the kanchanamycins. The minor congener kanchanamycin C is identical to the recently described macrolide antibiotic RS-22A[3,4]. Type II compounds were identified as the 42-membered macrolactones desertomycin A[5] and oasomycin A[6]. The type III compound was found to be tryptophan-dehydrobutyrine diketopiperazine[7], while type IV was identified as the isoflavone, daidzein. Furthermore, in order to examine the biosynthetic capacity and variability of strain Tü 4018, some investigations were made using modified cultivation conditions.

This paper deals with the taxonomy of the producing strain, diversity of its secondary metabolites, and the fermentation and isolation of the kanchanamycins. Investigations on structure elucidation are reported in the following paper[8].

Taxonomy

The kanchanamycin producing organism, strain Tü 4018, was isolated from a soil sample collected in Kanchana Buri, Thailand. The whole-cell hydrolysate contained LL-diaminopimelic acid as a diagnostic diamino acid of the peptidoglycan. The aerial mycelium was sparsely developed. Sporulation was poor and could be observed only on oatmeal agar (ISP 3) and inorganic

† Art. No. 6 on biosynthetic capacities of actinomycetes. Art. No. 5: See ref. 1.

Table 1. Cultural characteristics of strain *S. olivaceus* Tü 4018.

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP 2)	Well developed wrinkled colonies	Sparse, white	Greenish brown-dark brown	Greenish yellow
Oatmeal agar (ISP 3)	Very good	Sparse, white-gray	Yellowish green-brown-dark brown	Yellowish green
Inorganic salts-starch agar (ISP 4)	Good	Sparse, white-pale gray	Brown-dark brown	None
Glycerol-asparagine agar (ISP 5)	Good	Sparse, whitish	Beige-pale brown	None
Soil extract agar*	Good, flat, in close connection with the agar	Sparse, white	White-yellowish	None
HV-medium[12]	Moderate, flat, like a feather	Strongly reduced, white	Comparable with agar	None

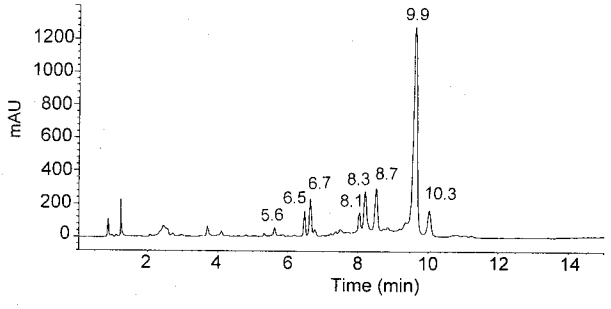
* Soil extract agar: soil extract 250 ml, yeast extract 1 g, tap water 750 ml, agar 20 g.

Table 2. Carbohydrate utilization of strain *S. olivaceus* Tü 4018, IMET 40350^T & IMET 40351.

	Tü 4018	IMET 40350^T (ISP 5072)	IMET 40351 (DSM 41536)
Glucose	+	+	+
Fructose	+	(+)	+
Raffinose	+	−	(+)
Sucrose	−	−	−
Xylose	(+)	(+)	+
Cellulose	−	−	−
Arabinose	+	+	+
Mannitol	+	+	+
Inositol	+	(+)	−
Rhamnose	−	+	+

Growth: +, good; (+), weak; −, none.

Fig. 1. HPLC analysis of the mycelium extract from *S. olivaceus* Tü 4018 monitored at 230 nm.



salts-starch agar (ISP 4). Because of the gray colour of the sporulating aerial mycelium, strain Tü 4018 was grouped into the *cinereus* series according to SCHIRLING & GOTTLIEB[9] and HÜTTER[10]. The aerial mycelium formed long straight flexible chains of spores of the rectiflexibiles (RF) type. Spores were ellipsoid and had a smooth surface as revealed by electron microscopy. More than ten spores per chain were formed. The colour of the substrate mycelium varied from yellowish-white to greenish or yellowish-brown to dark brown. Melanoid pigments were not produced on peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7). The cultural characteristics of strain Tü 4018 are summarised in Table 1. Utilisation of carbohydrates in comparison with two reference strains is demonstrated in Table 2. According to the presence of LL-diaminopimelic acid and morphological features, strain Tü 4018 belongs to the genus *Streptomyces*. Based on our morphological and physiological results described above and in comparison with two reference strains of *S. olivaceus* and the data in the literature, we propose to assign strain Tü 4018 to *S. olivaceus* (Waksman 1923) Waksman and Henrici 1984. LOCCI[11] considered *S. olivaceus* as a subjective synonym of *Streptomyces halstedii* (Waksman and Curtis 1916) Waksman and Henrici 1948.

Diversity of Metabolites

The HPLC chromatogram of the mycelium extract of *S. olivaceus* Tü 4018 is shown in Fig. 1. The metabolite pattern can be divided into four types of compounds by HPLC-DAD analysis of the culture filtrate and extracts according to their UV-visible spectra (Fig. 2).

Type I consisted of a complex of four compounds showing the same UV-visible spectrum but having different retention times of 8.3 minutes (kanchanamycin C), 8.7 minutes (kanchanamycin B), 9.9 minutes (kanchanamycin A) and 10.3 minutes (kanchanamycin D). This group of structurally related compounds was not

Fig. 2. Metabolite diversity of *S. olivaceus* Tü 4018 regarding their UV-visible spectra.

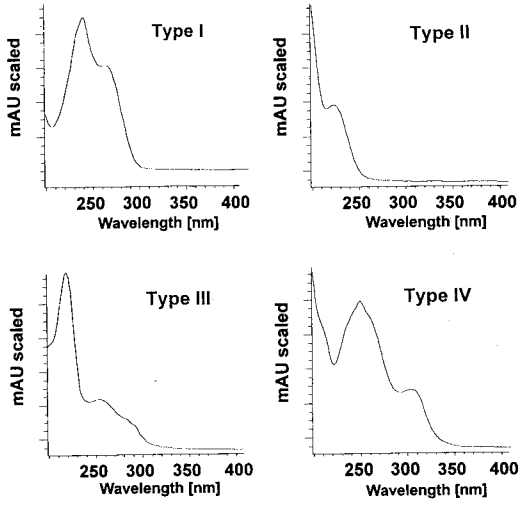
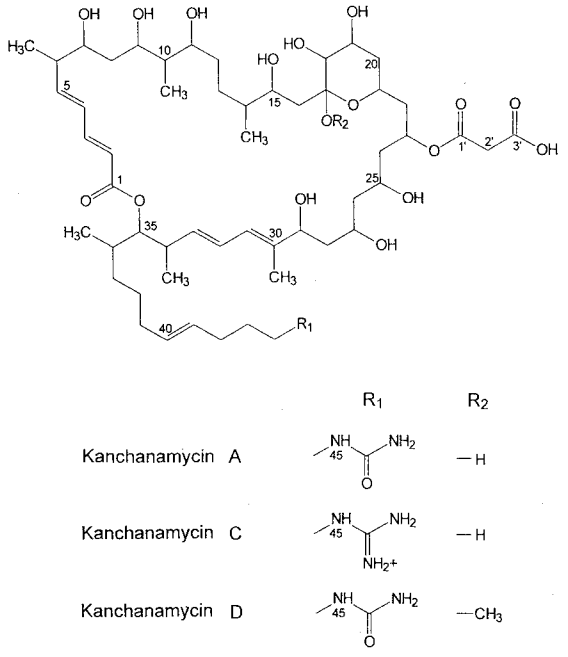


Fig. 3. Structural formulae of kanchanamycins.



identified but was classified by the HPLC-UV-Vis database as macrolide antibiotics due to their high degree of spectral matches in comparison to macrolide reference compounds. HPLC-MS analysis revealed molecular masses of 1055 (kanchanamycin A), 1054 (kanchanamycin C) and 1069 (kanachanmycin D). A search in commercially available databases indicated the novelty of compounds A and D which was confirmed by structure elucidation[6], whereas kanchanamycin C was found to be identical to RS-22[3,4]. The structures of these 36-membered polyol macrolides are shown in Fig. 3.

Type II consisted of two compounds with retention times of 6.5 minutes and 8.1 minutes. The second compound was identified as oasomycin A on the basis of a high degree of spectral matching and retention time with the reference compound. The first compound having a retention time of 6.5 minutes was not identified but characterised as an oasomycin-like compound because of its high conformity in spectral matching. HPLC-MS analysis revealed a molecular mass of 1193 which confirmed its identity as desertomycin A.

The type III compound with a retention time of 5.6 minutes could not be characterised by the HPLC-DAD technique, because of insufficient spectral matches compared with the 480 reference compounds stored in our HPLC-UV-Vis database. HPLC-MS analysis revealed a molecular mass of 283. A computer aided search of commercially available databases and NMR analysis lead to the identification of the type III compound as tryptophan-dehydrobutyrine diketopiperazine, which is also a metabolite from *Streptomyces spectabilis*[5].

The type IV compound having a retention time of 6.7 minutes was identified by the HPLC-UV-Vis database as the isoflavone, daidzein, showing maximal spectral identity with a match factor of 1000 and the same retention time as the reference compound.

Changes in cultivation conditions resulted in variation of the metabolite concentrations. This is exemplified by the replacement of the nitrogen source, soybean meal, by cornsteep powder or cotton seed, which resulted in a strong decrease in production of all secondary metabolites. Addition of the polystyrene resin Amberlite XAD-16, 36 hours subsequent to inoculation of the fermenter, lead to a threefold increase in desertomycin A. Moreover, production of kanchanamycin C increased from 130 mg/liter, without XAD, up to 240 mg/liter, and was the main component under these conditions. At the same time, the production of kanchanamycin A decreased from 385 mg/liter to 200 mg/liter.

Fermentation and Isolation

Batch fermentations of *S. olivaceus* Tü 4018 were carried out in 10-liter stirred tank fermenters using a complex medium that consisted of mannitol 2% and soybean meal 2% (pH 7.5). Production of kanchanamycins started at about 40 hours and reached a maximum after 90 hours with a concentration of 385 mg/liter of kanchanamycin A in the mycelium (Fig. 4). The produc-

Fig. 4. Batch-fermentation of *S. olivaceus* Tü 4018.

● Kanchanamycin A; □ DNA; ◇ pH; ▽ pO_2.

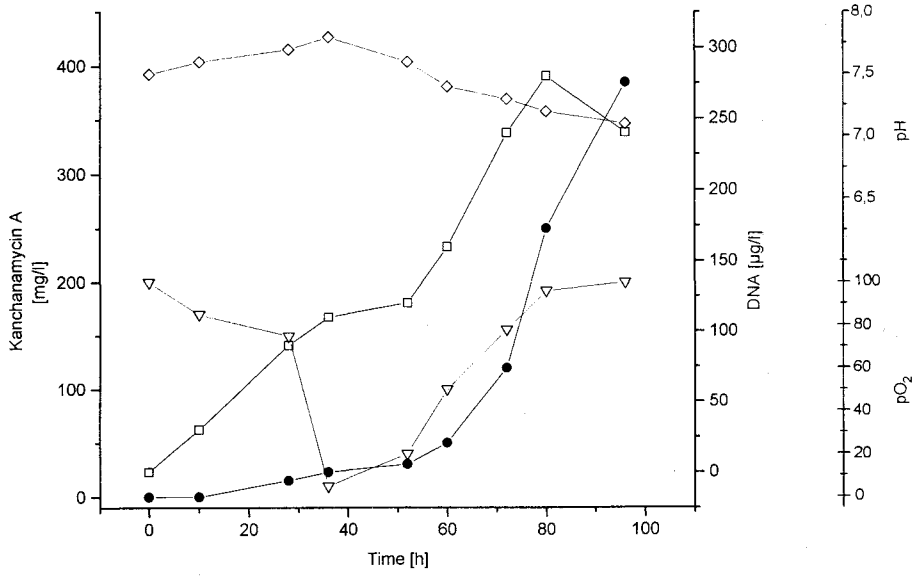


Table 3. Antimicrobial spectra of kanchanamycins A, C, D and azalomycin F, determined by the agar plate diffusion assay.

Test organisms	Kanchanamycin A	Kanchanamycin C	Kanchanamycin D	Azalomycin F
Arthrobacter aurescens ATCC 13344	–	10	–	14
Bacillus brevis ATCC 9999	–	10	–	9
Bacillus subtilis ATCC 6051	–	14	–	11
Staphylococcus aureus ATCC 11632	–	13	–	13
Streptomyces viridochromogenes Tü 57	–	–	–	10
Agrobacterium tumefaciens ATCC 15955	–	12	–	12
Escherichia coli K12	–	10	–	12
Proteus mirabilis ATCC 29906	–	13	–	14
Pseudomonas fluorescens ATCC 13525[a]	–	14	–	–
Pseudomonas fluorescens ATCC 13525[b]	17	27	n.d.	16
Candida albicans ATCC 10231	–	13	–	15
Rhodotorula rubra Tü 8093	–	15	–	18
Saccharomyces cerevisiae Tü 125	–	–	–	10
Aspergillus viridinutans CBS 12754	–	–	–	–
Botrytis cinerea Tü 157[c]	+	+ +	+	+ +
Mucor miehei Tü 284	9	16	–	–
Paecilomyces variotii Tü 137	–	14	–	10
Penicillium notatum Tü 136	18	20	–	–

Antibiotic concentrations 1 mg/ml, inhibition zones in mm.

[a] Complex medium.

[b] Chemically defined medium (per liter): glucose 5 g, tri-Na-citrate·2 H_2O 0.5 g, KH_2PO_4 3 g, K_2HPO_4 7 g, $MgSO_4$·7 H_2O 0.1 g, $(NH_4)_2SO_4$ 1 g, Bacto agar 15 g.

[c] Test on giant colony; + inhibition, + + strong inhibiton. n.d. not determined.

tion course was strongly correlated with the pH value which decreased from pH 8.5 to 5.9 at the production maximum. The addition of polystyrene resin during cultivation did not influence the fermentation course with the exception of the production of the various kanchanamycins.

The kanchanamycins were isolated from the biomass by extraction with ethanol. The ethanol layer was concentrated, extracted with 1-butanol and subjected to exclusion chromatography with Sephadex LH-20. Pure kanchanamycins were obtained after preparative reversed-phase HPLC using Nucleosil-100 C-18 material

Table 4. Minimal inhibition concentrations of kanchanamycins A, C, D and azalomycin F determined by the broth dilution method.

Test organisms	MIC (μg/ml)			
	Kanchanamycin A	Kanchanamycin C	Kanchanamycin D	Azalomycin F
Arthrobacter aurescens ATCC 13344	>100	3	>100	1
Bacillus brevis ATCC 9999	>100	>100	>100	100
Bacillus subtilis ATCC 6051	>100	10	>100	3
Staphylococcus aureus ATCC 11632	30	10	>100	3
Escherichia coli K12	>100	10	>100	3
Pseudomonas fluorescens ATCC 13525	3	0.1	3	1
Candida albicans ATCC 10231	>100	10	>100	10
Saccharomyces cerevisiae Tü 125	>100	10	>100	10
Aspergillus viridinutans CBS 12754	100	10	100	3
Paecilomyces variotii Tü 137	>100	30	>100	10
Penicillium notatum Tü 136	30	3	30	3

with 0.01% TFA-MeOH gradient elution, resulting in white powders after lyophilisation.

Biological Properties

Antimicrobial spectra of the kanchanamycins were examined by an agar plate diffusion assay (Table 3) and minimal inhibition concentrations were determined by a broth dilution method (Table 4). Kanchanamycins A and D showed only a weak activity against Gram-positive bacteria and filamentous fungi and no activity against yeasts, but were distinguished by high activity against *P. fluorescens*. Kanchanamycin C, on the contrary, demonstrated a very broad activity spectrum similar to azalomycin F. It was effective against Gram-positive and Gram-negative bacteria, especially against *P. fluorescens*, and against yeasts and filamentous fungi.

Discussion

The efficiency of the HPLC-DAD screening technique is increased greatly when complemented with HPLC-MS analysis. The identification of a compound *via* HPLC-DAD analysis and comparison of its retention time and UV-visible spectrum given in the HPLC-UV-Vis database can be ascertained with high reliability. If the reference compound is lacking for such a prognosis, additional information on the molecular mass of the respective fraction by HPLC-MS enables reliable identification using commercially available databases. The data required for this strategy are obtained from the culture filtrates or raw extracts; no time-consuming isolation procedures of the metabolites are necessary.

The prognosis of HPLC-DAD characterization was that *S. olivaceus* Tü 4018 may produce new macrolide antibiotics, which was confirmed by structure elucidation. A family of novel 36-membered polyol macrolides, the kanchanamycins, was isolated from the mycelium of strain Tü 4018. Kanchanamycin A and D are the only representatives of large-ring polyol macrolides known so far which have urea as terminal moiety. All other structurally related compounds, such as azalomycins[13], niphimycins (amycins)[14], copiamycin (niphithricin)[15], guanidylfungins[16], malolactomycin A[17], shurimycins[18] and RS22A-C[4] are characterised by a guanidino moiety. Kanchanamycin D represents a natural minor congener produced by *S. olivaceus*. It is not a side-product formed by methylation of the 17'OH-group during the isolation procedure. The compound can be detected also in ethanolic and butanolic extracts of the mycelium, and in small amounts in the culture filtrate (data not shown).

The biosynthetic capacity of strain Tü 4018 was strongly dependent on the cultivation conditions. Besides the 36-membered polyol macrolide antibiotics, relatively high amounts of the 42-membered macrolactone antibiotics, as well as tryptophan-dehydrobutyrine diketopiperazine and daidzeine were produced using the same fermentation conditions. Addition of the polystyrene resin, Amberlite XAD-16, during the fermentation process resulted in an alteration of the quantitative composition of oasomycin A and desertomycin A, as well as in the members of the kanchanamycin family. It is a well known phenomenon that addition of non-ionic polystyrene resins to growing streptomycete cultures may have an influence on the biosynthetic pathways of secondary metabolites[19,20]. Addition of high amounts of cobalt sulphate during the fermentation process resulted in the production of an antibiotically active fatty acid, (*E*)-4-oxonon-2-enoic acid, which will be reported in a separate publication[21]. Due to these results, *S. olivaceus* Tü 4018 can be designated as a biosynthetically talented and variable strain, since it produces different classes of secondary metabolites and shows a diversity in metabolite production depending on the cultivation conditions.

Kanchanamycins A and D are characterised by a weak antimicrobial activity spectrum in contrast to kanchanamycin C which was the most active compound. The biological activity is strongly influenced by the

terminal moiety which is urea in the cases of kanchanamycins A and D, and a guanidino residue in the cases of kanchanamycin C and the structural related azalomycin F. The highly basic guanidino moiety is responsible for potent antibiotic activity and broad spectrum, whereas a substitution by urea reduced both activity and antimicrobial spectrum.

Experimental

Microorganisms

Strain Tü 4018 was isolated from a soil sample collected in Kanchana Buri, Thailand, and identified according to HÜTTER[10] and SHIRLING & GOTTLIEB[22] as a strain of *S. olivaceus*. It is deposited in the culture collection of our institute.

The standard strains for testing the biological activity spectrum were obtained from the stock collection of our laboratory, ATCC and CBS.

Fermentation

S. olivaceus Tü 4018 was cultivated in a 10-liter fermenter (Biostat E; B. Braun, Melsungen) using a production medium consisting of: mannitol 2% and soybean meal 2% in tap water (pH 7.5, adjusted with 1 N HCl). The fermenter was inoculated with 5 vol-% of shaking cultures grown for 48 hours in 500-ml Erlenmeyer flasks with one buffle on a rotary shaker at 120 rpm and 27°C in the same medium. For production of kanchanamycin A, the fermentation was carried out at 27°C for 90 hours with an aeration rate of 0.5 v/v/m and an agitation rate of 500 rpm.

Kanchanamycin C production was selectively increased to become the main component when the 10-liter fermenter was supplemented after 36 hours of inoculation with 1 kg of Amberlite XAD-16 suspended in 1.2 liters water. The polystyrene resin was sterilised in an autoclave for 20 minutes at 120°C.

Isolation

Hyflo Super-cel (2%) was added to the fermentation broth which was separated by multiple sheet filtration into culture filtrate and mycelium cake. The kanchanamycins were located in both fractions. The culture filtrate was passed through an Amberlite XAD-16 column (10% resin volume relating to culture filtrate volume). Impurities were washed out with H_2O-MeOH (60+40) and the kanchanamycins were desorbed with H_2O-MeOH (20+80). The mycelium was extracted twice with EtOH, combined with the XAD eluate and concentrated *in vacuo*.

In case of XAD-resin fermentation, the culture filtrate was discarded and the XAD-mycelium cake was extracted three times with ethanol and concentrated *in vacuo*.

The aqueous residue was adjusted to pH 4.5 and extracted twice with 1-butanol. After concentration of the organic extract to dryness, the residue was dissolved in a small amount of MeOH and purified on a Sephadex LH 20 column using MeOH as eluent. The kanchanamycin containing fractions were combined and concentrated to dryness.

Pure kanchanamycins were obtained by preparative reversed-phase HPLC using a stainless steel column (250 × 16 mm) filled with 10-μm Nucleosil-100 C-18, and linear gradient elution with 0.01% TFA-MeOH, starting from 60% MeOH to 90% MeOH within 15 minutes at a flow rate of 20 ml/minute. The preparative HPLC system consisted of two high-pressure pumps (Sepapress HPP-200/100; Kronwald, Sinsheim), gradient unit (Sepacon GCU-311), and Valco preparative injection valve (6UW; VICI) with a 5 ml sample loop. The UV absorbance of the eluate was monitored simultaneously at 250 and 280 nm by a Gilson spectrophotometer Mod. 116 equipped with a preparative cell.

HPLC-DAD-Analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a built-in diode array detector, HP 79994B Pascal-workstation (200 MB hard disk) and HP 79988A software rev. 5.3 (Hewlett-Packard, Waldbronn). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360 and 435 nm without reference wavelength; the spectrum range was from 200 to 600 nm with a 2 nm step and a sampling interval of 640 msec.

For analysing the culture filtrate, a sample of the fermentation broth was centrifuged (10 minutes, 13000 × *g*). For analysing extracts, 5 ml culture filtrate were extracted with the same volume of ethyl acetate; the organic extract was concentrated to dryness and dissolved in 10 vol-% methanol. The biomass was extracted with methanol, filtered, concentrated to dryness and dissolved in 10 vol-% methanol.

10 μl of the samples were injected onto an 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 (Grom, Herrenberg). The samples were separated by linear gradient elution using solvent 0.1% phosphoric acid as solvent A and acetonitrile as solvent B. The gradient was from 0% to 100% solvent B in 15 minutes with a 1-minute hold at 100% B and a 5-minute post time at initial conditions, at a flow rate of 2 ml/minute.

HPLC-MS-Analysis

The system consisted of an ABI 140A HPLC gradient pump (Applied Biosystems, Weiterstadt), and an API III Taga 6000 E mass spectrometer equipped with a nebulizer-assisted electrospray source (Sciex, Thornhill, Canada). The HPLC column eluate was split by a T and introduced into the ion source at a constant flow rate of 40 μl/minute.

4 μl of the samples were injected onto an HPLC column (100 × 2 mm) packed with 5-μm Nucleosil-120 C-18 (Grom). Samples were separated by linear gradient

elution. Solvent A was 0.01% trifluoroacetic acid and solvent B was acetonitrile. The gradient was from 0% to 100% solvent B in 15 minutes at a flow rate of 200 μl/minute.

Biological Assays

An agar-plate diffusion assay was used to determine the antibacterial spectra of the kanchanamycins. The test solutions were applied to filter discs (6 mm diameter) and the test plates were incubated for 24 hours at 37°C.

A broth dilution method was used to determine the minimal inhibition concentrations of the kanchanamycins. The antibiotics were dissolved in DMSO, giving DMSO concentrations in the cultures of not more than 5%. The bacteria were grown in nutrient broth 0.8% and NaCl 0.5% prepared in tap water; yeasts and filamentous fungi were grown in malt extract 1%, glucose 0.4% and yeast extract 0.4% in tap water (pH 5.5). 10^6 Cells/ml and spores/ml, respectively, were used as inoculum of the complex media and growth inhibition was evaluated after incubation for 24 and 48 hours at 27°C and 37°C, respectively, on a rotary shaker.

Acknowledgments

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