J. Antibiot. 61(12): 736–746, 2008

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Electrospray Ionization Mass Spectra of Piperazimycins A and B and γ-Butyrolactones from a Marine-derived *Streptomyces* sp.[†]

Khaled A. Shaaban, Mohamed Shaaban, Petrea Facey, Serge Fotso, Holm Frauendorf, Elisabeth Helmke, Armin Maier, Heinz H. Fiebig, Hartmut Laatsch

Received: July 22, 2008 / Accepted: December 9, 2008

Abstract Chemical investigation of the marine-derived *Streptomyces* sp. Act8015 led to the isolation of two cyclic peptide antibiotics, piperazimycins A and B (**1a**, **1b**). Their structures were confirmed on the basis of a detailed HRESI-MS/MS analysis. Additionally, a new butanolide, 4,10-dihydroxy-10-methyl-dodecan-4-olide (**2**), and the respective acid, 4,10-dihydroxy-10-methyl-dodecanoic acid (**3a**) were identified. Further isolated compounds were staurosporin, adenine, indole-3-carboxylic acid, ferulic acid, tryptophol, and three γ-butyrolactones: virginiae butanolide E (**4e**) and Graefe's Factors I (**4f**) and III (**4g**). The structures of **2** and **3a** were confirmed by detailed 1D and 2D NMR studies and MS spectra and by comparison with related structures. A full signal assignment of virginiae butanolide E (**4e**) is reported here for the first time.

Keywords γ-butyrolactones, piperazimycins, marine-derived *Streptomyces* sp.

Introduction

Numerous bioactive cyclic peptides containing piperazic acid have been isolated from *Streptomyces* [1∼5] and *Actinomadura* spp. [6]. They are known for their antitumor [7∼9], tuberculostatic [10], anti-inflammatory [11], and anti-HIV activity [12, 13] and are chemically characterized by further rare amino acids, like diaminobutyric acid, dehydroamino acids, *p*-dimethylaminophenylalanine *etc.* [14].

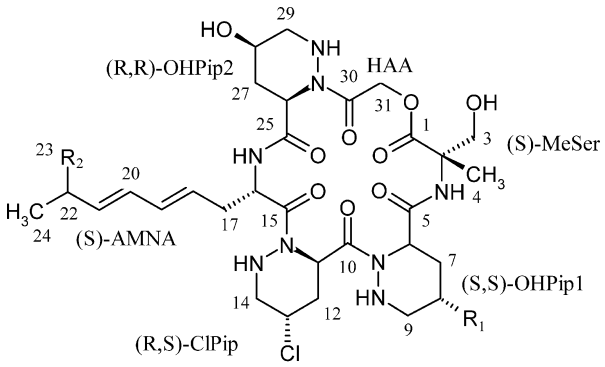
The extract from the marine-derived *Streptomyces* sp. Act8015 exhibited *in vitro* antitumor activities against a number of cancer cells at very low concentrations. TLC of the crude extract displayed several UV absorbing zones, which turned yellowish-brown and/or reddish-brown with anisaldehyde/sulfuric acid. Further zones without UV absorption appeared as violet-blue and/or greenish-blue spots with the same reagent.

A large-scale shaker culture was performed on M_2^+ medium [15] for 5 days at 28°C. Chromatography of the ethyl acetate extract afforded 12 compounds, the peptides

K. A. Shaaban, P. Facey, S. Fotso, H. Frauendorf, H. Laatsch (Corresponding author)**:** Department of Organic and Biomolecular Chemistry, University of Göttingen, Tammannstrasse 2, D-37077 Göttingen, Germany, E-mail: hlaatsc@gwdg.de
M. Shaaban: Department of Chemistry of Natural Compounds, Division of Pharmaceutical Industries, National Research Centre, El-Behoos st. 33, Dokki-Cairo 12622, Egypt
E. Helmke: Alfred-Wegener-Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany
A. Maier, H. H. Fiebig: Oncotest GmbH, Am Flughafen 12–14, D-79108 Freiburg, Germany

[†] Art. No. XXXIX on Marine Bacteria. Art. XXXVIII: Ding L., Pfoh R., Rühl S., Qin S., Laatsch H. T-Muurolol Sesquiterpenes from Marine *Streptomyces* sp. Qd491 and Revision of the Configuration of Previously Reported Amorphanes. J. Nat. Prod., 2008 in press.

1a and **1b**, 4,10-dihydroxy-10-methyl-dodecan-4-olide (**2**), and 4,10-dihydroxy-10-methyl-dodecanoic acid (**3a**). In addition, virginiae butanolide E (**4e**), Graefe's Factors I (**4f**) and III (**4g**), staurosporine, adenine, indole-3-carboxylic acid, ferulic acid, and tryptophol were identified by comparison with reference data [14]. In this paper, we focus on the mass-spectrometric fragmentation of the rare peptidolactones, piperazimycin A (**1a**) and B (**1b**), and on the structure elucidation of the new dodecanoic acid derivatives **2** and **3a**.



1a: $R_1 = OH$, $R_2 = CH_3$
1b: $R_1 = H$, $R_2 = CH_3$

Results and Discussion

CID-MS/MS Analysis of Piperazimycins A and B

The strong antibiotic and cytotoxic activity of the crude extract was in part due to staurosporine [16]. Two further highly cytotoxic antibiotics were identified as chlorine-containing peptides by their formulas $C_{31}H_{47}N_8O_{10}Cl$ and $C_{31}H_{47}N_8O_9Cl$ (by HRESI-MS) and the positive chlorine/tolidine reaction for amide bonds. According to their NMR data, they were found to be identical with the previously reported but incompletely defined sohbumycin [17] and the recently published antibiotics piperazimycin A (**1a**) and B (**1b**) [18]. As part of our ongoing investigation of the MS/MS fragmentation of cyclopeptides and cyclodepsipeptides [19], we report here their mass-spectrometric fragmentation pattern.

CID tandem mass spectrometry (MS/MS) combined with high resolution MS/MS of component **1a** showed a sequential loss of amino acids from the $[M+Na]^+$ ion at *m/z* 749, with six predominant peaks at *m/z* 648, 620, 602, 520, 492 and 328. According to their fragment compositions (Table 1), the ions at *m/z* 648 and 520 were assigned to a subsequent loss of Me-Ser and OHPip, respectively; while the ions of *m/z* 620 and 492 were due to the expulsion of CO from the precursor ions at *m/z* 648 and 520, respectively. The fragment ion at *m/z* 328 resulted from the loss of ClPip from *m/z* 492, followed by dehydration.

Table 1 HRCIDMS^2 of the key fragments from the pseudomolecular $[M+H]^+$ and $[M+Na]^+$ ions of piperazimycin A (**1a**)

Mass	Molecular formula	Amino acid sequence
328.16346	$C_{16}H_{23}N_3O_3Na$	HAA-OHPip-AMNA $-H_2O$
492.19934	$C_{21}H_{32}N_5O_5ClNa$	HAA-OHPip-AMNA-ClPip $-CO+Na^+$
520.19424	$C_{22}H_{32}N_5O_6ClNa$	HAA-OHPip-AMNA-ClPip$+Na^+$
602.24822	$C_{26}H_{38}N_7O_6ClNa$	HAA-OHPip-AMNA-ClPip-OH-Pip$-CO$, $-H_2O+Na^+$
620.25882	$C_{26}H_{40}N_7O_7ClNa$	HAA-OHPip-AMNA-ClPip-OH-Pip$-CO+Na^+$
648.25376	$C_{27}H_{40}N_7O_8ClNa$	HAA-OHPip-AMNA-ClPip-OH-Pip$+Na^+$
749.30262	$C_{31}H_{47}N_8O_{10}ClNa$	*cyclo*[HAA-OHPip-AMNA-ClPip-OH-Pip-MeSer]$+Na^+$
376.13892	$C_{14}H_{23}ClN_5O_5$	ClPip-OH-Pip-MeSer $-H_2O+H^+$
498.21242	$C_{22}H_{33}N_5O_6Cl$	HAA-OHPip-AMNA-ClPip$+H^+$
541.25498	$C_{24}H_{38}ClN_6O_6$	AMNA-ClPip-OH-Pip-MeSer $-H_2O+H^+$
559.26558	$C_{24}H_{40}ClN_6O_7$	AMNA-ClPip-OH-Pip-MeSer$+H^+$
709.30961	$C_{31}H_{46}N_8O_9Cl$	*cyclo*[HAA-OHPip-AMNA-ClPip-OH-Pip-MeSer]$-H_2O+H^+$
727.31763	$C_{31}H_{48}N_8O_{10}Cl$	*cyclo*[HAA-OHPip-AMNA-ClPip-OH-Pip-MeSer]$+H^+$

Fragmentation of the *pseudo*molecular ion at *m/z* 727 $[M+H]^+$ led mainly to four fragment ions at *m/z* 709, 559, 541 and 376. Tilvi and co-workers proposed [20] that the first dissociation step for protonated cyclic depsipeptides results in the cleavage of one of the amide bonds or the lactone bond. In the case of **1a**, however, the OHPip and HAA residues were initially removed to afford *m/z* 559 ([M−OHPip−HAA]+H), suggesting a ring opening at the lactone C–O. The fragment at *m/z* 559 showed a further expulsion of a water molecule (*m/z* 541), followed by splitting of the AMNA residue (*m/z* 376), which corresponded to the sequence of piperazimycin A (**1a**).

Similarly, MS/MS of the molecular ion peak at *m/z* 733.3 $[M+Na]^+$ of piperazimycin B (**1b**) delivered, beside others, six fragment ion peaks at *m/z* 705, 632, 604, 520, 492 and 328. As in piperazimycin A (**1a**), the fragment at *m/z* 632 was attributed to the delactonization and splitting of Me-Ser, followed by a loss of CO (*m/z* 604). The fragment ion at *m/z* 492 (same as in **1a**) was due to a splitting of 112 amu (for Pip) from the ion peak at *m/z* 604 corresponding to a different amino acid residue in **1b** with respect to **1a**. As in **1a**, the last fragment at *m/z* 328 was indicative for a loss of

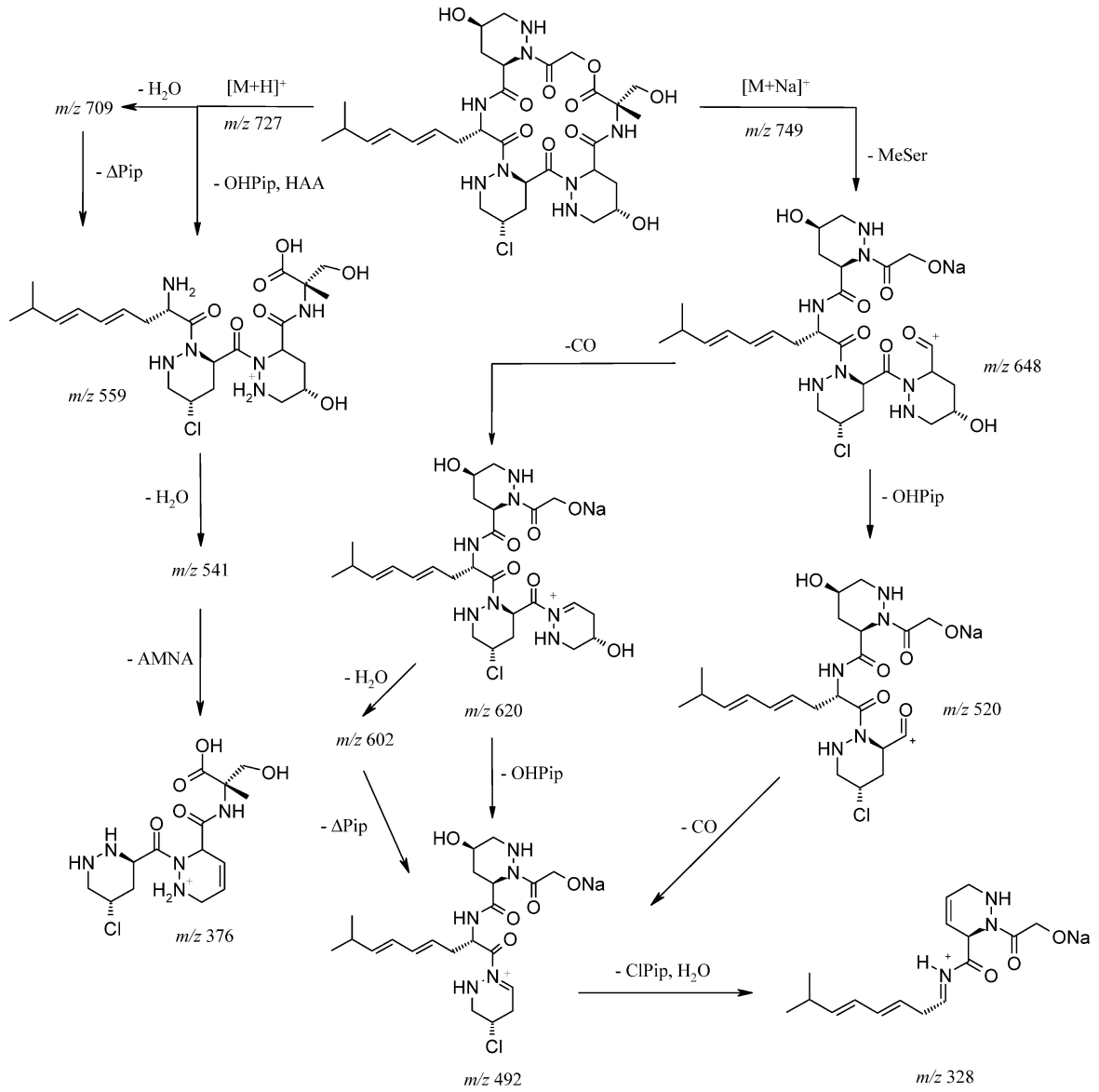


Fig. 1 Fragmentation of piperazimycin A (**1a**) by CID-MS^2 sequencing of the *pseudo*-molecular ions $[M+H]^+$ and $[M+Na]^+$.

The charge may be localized also on other positions.

ClPip from the ion at *m/z* 492.

The ^{13}C- and ^{1}H-NMR data of **1a** were identical with published values of sohbumycin within the experimental error [18]. As our 2D NMR data were also in agreement with the published structure of piperazimycin A (**1a**) [17], we state that the previously unidentified antibiotic sohbumycin is identical with piperazimycin A (**1a**), a cyclodepsipeptide containing the rare acids *S*-methylserine, γ-hydroxypiperazic acid, γ-chloropiperazic acid, 2-amino-8-methyl-4,6-nonadienoic acid and hydroxyacetic acid.

4,10-Dihydroxy-10-methyldodecan-4-olide

Compound **2** was obtained as colorless oil, which did not absorb UV light and turned violet and later greenish-blue on spraying with anisaldehyde/sulfuric acid. Based on the (+)-ESI and HRESI mass spectra, the molecular formula of **2** was established as $C_{13}H_{24}O_3$.

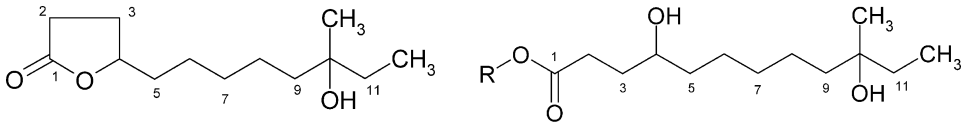
The ^{1}H-NMR spectrum of **2** indicated signals for 24 protons, all localized in the aliphatic region: Two of them were due to methyl groups (δ 0.85 t, 1.11 s); multiplets of 15 protons (δ 2.60 and 1.89～1.36) seemed to be due to an elongated methylene chain, and at δ 4.23, an oxymethine multiplet was visible. The ^{13}C/HSQC spectra indicated 13 signals, *i.e.* the carbonyl of an acid or ester (δ 175.9), two sp^3 oxy-methines, one at δ 72.8 and the other a quaternary carbon at δ 80.5. The remaining signals indicated eight methylenes (δ 40.9～19.9) and two methyls δ 26.3 and 8.2). Due to the number of double bond equivalents, the compound must be cyclic.

The HMBC experiment and the chemical shift of the methylene group CH_2-2 (δ_H 2.60, δ_C 34.9) proved the direct neighborhood to the carbonyl C-1 (δ 175.9) by ^{2}J couplings. H-H COSY correlations allowed the assignment of further six methylene groups, with the oxymethine group in γ-position to the carbonyl, which was further confirmed by HMBC correlations. As there was no reaction with diazomethane detectable, a free carboxylic acid was excluded and a γ-butyrolactone moiety was assumed, in spite of the missing ^{3}J coupling between H-4 and C-1.

The remaining fragment $C_4H_9O_4$ was found to contain two methyls; one of them appeared as the triplet (δ_H 0.85, δ_C 8.2) of a terminal ethyl group. The other methyl giving a singlet at δ_H 1.11 was attached to the quaternary oxycarbon C-10 (δ 72.8) due to a ^{2}J correlation, and showed a ^{3}J coupling to the methylene carbon C-11 (δ 34.3) of the terminal ethyl fragment. Hence, an isobutyl alcohol as further partial structure resulted. Based on the observed ^{3}J coupling from CH_2-8 (δ 1.62～1.36) to C-10 and those between the methyl singlet methyl and C-9 (δ 40.9), the two partial structures are connected between C_9-C_{10} (Fig. 2), resulting in the new 4,10-dihydroxy-10-methyl-dodecan-4-olide (**2**).

4,10-Dihydroxy-10-methyl-dodecanoic Acid

The colorless oily **3a** exhibited the same color reactions on TLC as **2**. The ESI HRMS-derived molecular formula $C_{13}H_{26}O_4$ contained 1 mol of H_2O more than the lactone **2** and one double bond equivalent less. As also the ^{1}H- and ^{13}C-NMR data were similar to those of **2** (Table 2), the most likely explanation was a ring opening of the γ-butyrolactone in **2** to afford the free acid **3a**. This conclusion was supported by methylation with diazomethane giving an ester **3b** with a molecular weight of 260 Dalton and a methyl singlet at δ_H 3.65. Accordingly, the oxy-methine multiplet of H-4 (δ 4.23) and methylene protons of H_2-2 (δ 2.60) in **2** were upfield shifted in **3a** to δ



2 **3a:** R = H **3b:** R = CH_3

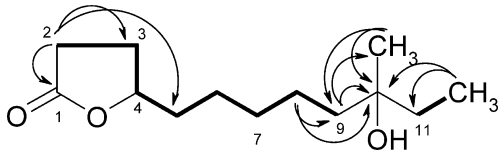


Fig. 2 Selected HMBC (→) and H-H COSY (—) correlations in 4,10-dihydroxy-10-methyldodecan-4-olide (**2**).

Table 2 ^{1}H- and ^{13}C-NMR assignments of 4,10-dihydroxy-10-methyldodecan-4-olide (**2**) and 4,10-dihydroxy-10-methyl-dodecanoic acid (**3a**)

Position	Butanolide **2**		Acid **3a**	
	δ_C[a]	δ_H (J in [Hz])[b]	δ_C[c]	δ_H (J in [Hz])[b]
1	175.9	—	178.0	—
2	34.9	2.60 (m)	38.3	2.20 (t, 7.0)
3	23.0	1.89 (m, H_a), 1.62∼1.36 (m, H_b)	27.4	1.60 (m)
4	80.5	4.23 (m)	72.3	3.55 (br, m)
5	28.3	1.89 (m, H_a), 1.62∼1.36 (m, H_b)	26.8	1.65∼1.30 (m)
6	34.6	1.62∼1.36 (m)	38.3	1.65∼1.30 (m)
7	36.9	1.62∼1.36 (m)	39.0	1.65∼1.30 (m)
8	19.9	1.62∼1.36 (m)	21.2	1.65∼1.30 (m)
9	40.9	1.62∼1.36 (m)	42.3	1.65∼1.30 (m)
10	72.8	—	73.5	—
10-CH_3	26.3	1.11 (s)	26.3	1.11 (s)
11	34.3	1.70 (m, H_a), 1.62∼1.36 (m, H_b)	34.9	1.65∼1.30 (m)
12	8.2	0.85 (t, 7.4)	8.5	0.85 (t, 7.4)

[a] 150 MHz; [b] 300 MHz; [c] 75 MHz.

3.55 and 2.20 (t), respectively. Structure **3a** was further confirmed by H-H COSY and HMBC experiments, exhibiting the same connectivities as **2** (Fig. 3). Hence, compound **3a** was deduced as the unreported 4,10-dihydroxy-10-methyl-dodecanoic acid.

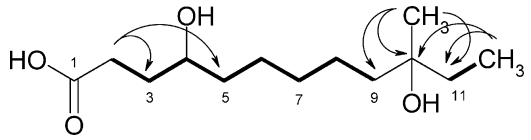


Fig. 3 (—) H-H COSY and selected HMBC couplings in 4,10-dihydroxy-10-methyl-dodecanoic acid (**3a**).

Virginiae Butanolide E

A further colorless oil with a similar chromatographic staining behavior as **2** had a molecular weight of 216 Dalton (ESI-MS) and a molecular formula of $C_{11}H_{20}O_4$ (HRESI-MS). By these data and the NMR spectra, it was identified as the previously described virginiae butanolide E (**4e**) [21]. As no complete set of NMR data has been published so far, **4e** was fully assigned now by a detailed interpretation of H-H COSY, HMQC, and HMBC experiments (Fig. 4, Table 3). Further butanolides were identified as Graefe's Factors I (**4f**) and III (**4g**) by means of AntiBase data [14].

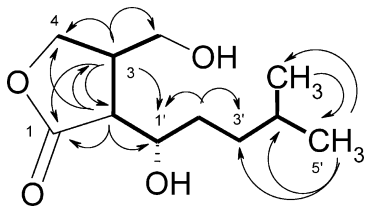
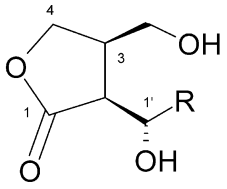


Fig. 4 (—) H-H COSY and (→) selected HMBC coupling in virginiae butanolide E (**4e**).

Biological Activity

Sohbumycin was reported as a potent cytotoxic agent against multiple tumor cell lines in the nanomolar range [17]. Recently, also the piperazimycins A and B (**1a**/**1b**) were found to be highly potent cytotoxins [18]. This agrees well with our own results, determined in a monolayer cell proliferation assay using a panel of 36 human tumor cell lines, comprising 14 different solid tumor types. Piperazimycin A (**1a**) showed cytotoxic activity against a range of human tumor cell lines with an overall potency of 0.13 μg/ml (mean IC_{50} value of 36 tumor cell lines tested), and a mean IC_{70} of 0.21 μg/ml. Cytotoxic activity was rather unselective with a steep concentration response between 0.03 and 0.3 μg/ml. IC_{50} values determined in 32 out of 36 tumor cell lines ranged between 0.09 and



4a: R = -$(CH_2)_3CH(CH_3)_2$ **4b:** R = -$(CH_2)_2CH(CH_3)CH_2CH_3$
4c: R = -$(CH_2)_4CH_3$ **4d:** R = -$(CH_2)_5CH_3$
4e: R = -$(CH_2)_2CH(CH_3)_2$ **4f:** R = -$(CH_2)_4CH(CH_3)_2$
4g: R = -$(CH_2)_5CH(CH_3)_2$

Table 3 ^{1}H- and ^{13}C-NMR assignments of virginiae butanolide E (**4e**)

C	δ_C[a]	δ_H (J in [Hz])[b]
1	178.7	—
2	48.1	2.54 (dd, 7.2, 3.7)
3	38.1	2.81 (m)
3-CH_2	63.4	3.70 (m)
4	69.5	4.40 (t, 8.8, H_b)
		4.08 (dd, 9.0, 6.5, H_a)
1′	71.1	4.03 (m)
2′	32.7	1.57 (m)
3′	34.9	1.17∼1.35 (m)
4′	27.9	1.57 (m)
4′-CH_3	22.6	0.88 (d, 6.6)
5′	22.5	0.88 (d, 6.6)
OH	—	2.40 (br s)

[a] 75 MHz; [b] 300 MHz.

0.13 μg/ml (Table 4). Three tumor cell lines were clearly less sensitive (head and neck cancer HNXF 635L, ovary cancer OVXF 899L, and renal cancer RXF 486L) with IC_{50} values between 0.82 and 1.13 μg/ml (Table 4).

In the brine shrimp microwell assay, piperazimycin A (**1a**) showed a mortality rate of 20% at 10 μg/ml. The antimicrobial activity of all compounds isolated from *Streptomyces* sp. Act8015 was determined using the agar diffusion method. At a concentration of 40 μg/disc, compounds **1a** and **1b** exhibited substantial activities against *Bacillus subtilis*, *Mucor miehei*, *Staphylococcus aureus*, *Streptomyces viridochromogenes* (Tü 57), *Escherichia coli*, *Candida albicans*, and *Mucor miehei* (Table 5).

At least 60% of *Streptomyces* species appear to produce certain low-molecular signaling compounds like γ-butyrolactones [22]: Besides an effect on sporulation of

Table 4 Cytotoxic activity of piperazimycin A (**1a**) against tumor cell lines in a monolayer proliferation assay

Tumor type	Cell line	IC_{50} [μg/ml]	IC_{70} [μg/ml]
Bladder	BXF 1218L	0.113	0.176
	BXF T24	0.098	0.159
Glioblastoma	CNXF 498NL	0.088	0.149
	CNXF SF268	0.097	0.156
Colon	CXF HCT116	0.105	0.163
	CXF HT29	0.098	0.154
Stomach	GXF 251L	0.123	0.201
Head & neck	HNXF 536L	0.871	1.405
Lung	LXF 1121L	0.105	0.167
	LXF 289L	0.117	0.187
	LXF 526L	0.123	0.190
	LXF 529L	0.103	0.167
	LXF 629L	0.102	0.166
	LXF H460	0.098	0.154
Breast	MAXF 401NL	0.110	0.177
	MAXF MCF7	0.103	0.165
Melanoma	MEXF 276L	0.127	0.207
	MEXF 394NL	0.098	0.158
	MEXF 462NL	0.107	0.175
	MEXF 514L	0.125	0.186
	MEXF 520L	0.111	0.182
Ovary	OVXF 1619L	0.127	0.202
	OVXF 899L	1.102	1.818
	OVXF OVCAR3	0.113	0.184
Pancreas	PAXF 1657L	0.125	0.212
	PAXF PANC1	0.100	0.163
Prostate	PRXF 22RV1	0.092	0.157
	PRXF DU145	0.105	0.163
	PRXF LNCAP	0.118	0.176
	PRXF PC3M	0.099	0.157
Mesothelioma	PXF 1752L	0.110	0.173
Kidney	RXF 1781L	0.143	0.252
	RXF 393NL	0.103	0.163
	RXF 486L	1.129	1.798
	RXF 944L	0.096	0.161
Uterus	UXF 1138L	0.101	0.162
Mean		0.130	0.210

Streptomyces griseus [23], the A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) is *e.g.* of importance for streptomycin production [24]. Virginiae butanolides A∼E (**4a**∼**4e**) were isolated from *Streptomyces virginiae* [25] as inducing agents for antibiotics biosynthesis. Butanolides seem also to play an important role as extracellular signaling molecules in the biology of *Streptomyces* species [26]. All butenolides isolated here were antibiotically

Table 5 Antibacterial, antifungal and anti-microalgal activities of compounds **1a**, **1b**, **2**, **3a**, and **4e** in the agar diffusion test at 40 μg/disk (ϕ9 mm); diameter of inhibition zones in [mm]

Compounds	BS[a]	SA[b]	SV[c]	EC[d]	CA[e]	MM[f]	CV[g]	CS[h]	SS[i]	RS[j]	PU[k]
1a	14	22	26	17	0	15	0	0	0	0	0
1b	14	21	25	16	14	24	16	14	0	++	+++
2	0	0	0	0	0	0	—	—	—	—	—
3a	0	0	0	0	0	0	0	0	0	0	0
4e	0	0	0	0	0	0	0	0	0	0	0

[a] *Bacillus subtilis*, [b] *Staphylococcus aureus*, [c] *Streptomyces viridochromogenes* (Tü 57), [d] *Escherichia coli*, [e] *Candida albicans*, [f] *Mucor miehei*, [g] *Chlorella vulgaris*, [h] *Chlorella sorokiniana*, [i] *Scenedesmus subspicatus*, [j] *Rhizoctonia solani*, [k] *Pythium ultimum*; ++=active, +++=highly active; —=not tested.

inactive and did not stimulate spore formation in *Streptomyces virido-chromogenes* or in the producing strain Act8015.

Experimental

^{1}H- and ^{13}C-NMR spectra were measured on Varian Unity 300 (300.145 and 75.036 MHz) and Varian Inova 600 spectrometers (at 600.7 and 150.175 MHz, respectively). Chemical shifts (δ) are given in ppm with respect to TMS as an internal standard. Electrospray ionization mass spectrometry (ESI-MS): Finnigan LCQ ion trap mass spectrometer. High-resolution mass spectra (HR-MS) were recorded by ESI-MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). Rf values were measured on Polygram SIL F/UV$_{254}$ (Macherey-Nagel, Düren; pre-coated sheets). Size exclusion chromatography was done on Sephadex LH-20 (Pharmacia). Optical rotations were measured on a Perkin-Elmer polarimeter (model 343).

Taxonomy

The strain Act8015 has been derived from sediment of the Laguna de Terminos at the Gulf of Mexico and was isolated on Oil No. 2 agar [27] containing 50% natural seawater. The almost complete 16S rRNA gene sequence of the strain is 99% identical to that of *Streptomyces humidus* ssp. *antitumoris*. The strain produces brown substrate mycelium and gray aerial mycelium on yeast extract-malt extract agar. The reference culture of Act8015 is kept on yeast extract-malt extract agar in the Collection of Marine Actinomycetes at the Alfred-Wegener-Institute for Polar and Marine Research in Bremerhaven.

Fermentation, Extraction and Isolation

The marine-derived *Streptomyces* sp. Act8015 was cultured on a linear shaker with 95 rpm at 28°C for 5 days in 80 of 1 liter Erlenmeyer flasks each containing 250 ml of M_2^+ medium. The brown culture broth was mixed with Celite and filtered under pressure. The filtrate was passed through a column of XAD-2. The column was washed with demineralized (25 liters) water, followed by elution with MeOH (15 liters). The MeOH extract was concentrated *in vacuo*, and the water residue was re-extracted with EtOAc. The Celite phase was extracted with EtOAc (4×) followed by Me_2CO (1×). The Me_2CO extract was concentrated and the aqueous residue was re-extracted with EtOAc. Based on similar chromatograms, all extracts were combined, and the residue (2.3 g) was chromatographed on silica gel (column 3×60 cm, 150 g). The column was eluted with a CH_2Cl_2/MeOH gradient: CH_2Cl_2, 0.5 liters; 3.0% MeOH, 1.5 liters; 6.0% MeOH, 0.5 liters; 10% MeOH, 0.3 liters; 20% MeOH, 0.5 liters; 50% MeOH, 0.4 liters; 100% MeOH, 0.2 liters to afford six fractions. Further purification of fraction II (0.1 g) on silica gel (CH_2Cl_2) followed by Sephadex LH-20 (CH_2Cl_2/40% MeOH) gave virginiae butanolide E (**4e**; 20.9 mg, colorless oil). Separation of fraction III (0.85 g) on Sephadex LH-20 (CH_2Cl_2/50% MeOH) delivered two sub-fractions IIA, IIB. On further chromatography of IIA and IIB, eight compounds were obtained: tryptophol (5.3 mg, white solid), ferulic acid (40 mg, colorless solid), a mixture of Graefe's Factors I and III (8.2 mg, colorless oil), piperazimycin A (**1a**; 29.8 mg, white crystals) and piperazimycin B (**1b**; 3.6 mg, white solid), 4,10-dihydroxy-10-methyldodecan-4-olide (**2**; 18.7 mg, colorless oil), and 4,10-dihydroxy-10-methyldodecanoic acid (**3a**; 5.7 mg, colorless oil). Purification of fractions IV (0.3 g), V (0.21 g) and VI (0.4 g) on Sephadex LH-20 resulted, respectively, in indole-3-carboxylic acid (2.3 mg, white solid), staurosporine

(3.1 mg, white powder) and adenine (25 mg, white powder) (Fig. 5).

Piperazimycin A (**1a**)

White crystals, on TLC blue chlorine/tolidine reaction; Rf 0.26 (CH_2Cl_2/5.0% MeOH), UV absorbing (254 nm). $[\alpha]_D^{20}$ −167° (*c* 0.38, $CHCl_3$). (+)-ESI-MS *m/z* (%) 1475 ($[2M+Na]^+$, 30), 749 ($[M+Na]^+$, 100). (−)-ESI-MS *m/z* 725 $[M-H]^-$. (+)-HRESI-MS: *m/z* 727.31763 ($[M+H]^+$, calcd. 727.31764 for $C_{31}H_{48}N_8O_{10}Cl$), 749.29956 ($[M+Na]^+$, calcd. 749.29958 for $C_{31}H_{47}N_8O_{10}ClNa$).

Piperazimycin B (**1b**)

White solid, blue chlorine/tolidine reaction; Rf 0.33 (CH_2Cl_2/5.0% MeOH), UV absorbing (254 nm). $[\alpha]_D^{20}$ −28° (*c* 0.08, $CHCl_3$). (+)-ESI-MS *m/z* (%) 1443 ($[2M+Na]^+$, 28), 733 ($[M+Na]^+$, 100). (−)-ESI-MS *m/z* (%) 755 ($[M+HCOO]^-$, 100), 709 ($[M-H]^-$, 60). HRESI-MS: *m/z* 711.32271 ($[M+H]^+$, calcd. 711.32272 for $C_{31}H_{48}N_8O_9Cl$), 709.30700 ($[M-H]^-$, calcd. 709.30815 for $C_{31}H_{46}N_8O_9Cl$).

4,10-Dihydroxy-10-methyldodecan-4-olide (**2**)

Colorless oil, Rf 0.47 (CH_2Cl_2/5.0% MeOH), not UV absorbing; initially violet, after 10 minutes greenish-blue with anisalde hyde/sulfuric acid. $[\alpha]_D^{20}$ −40° (*c* 1.0 mg/ml, MeOH). ^{1}H-NMR (300 MHz, $CDCl_3$) see Table 1. ^{13}C-NMR (150 MHz, $CDCl_3$) see Table 1. (+)-ESI-MS *m/z* (%) 479 ($[2M+Na]^+$ 100), 251 ($[M+Na]^+$ 95), 229 ($[M+H]^+$, 12). (+)-HRESI-MS *m/z* 229.17991 ($[M+H]^+$, calcd.

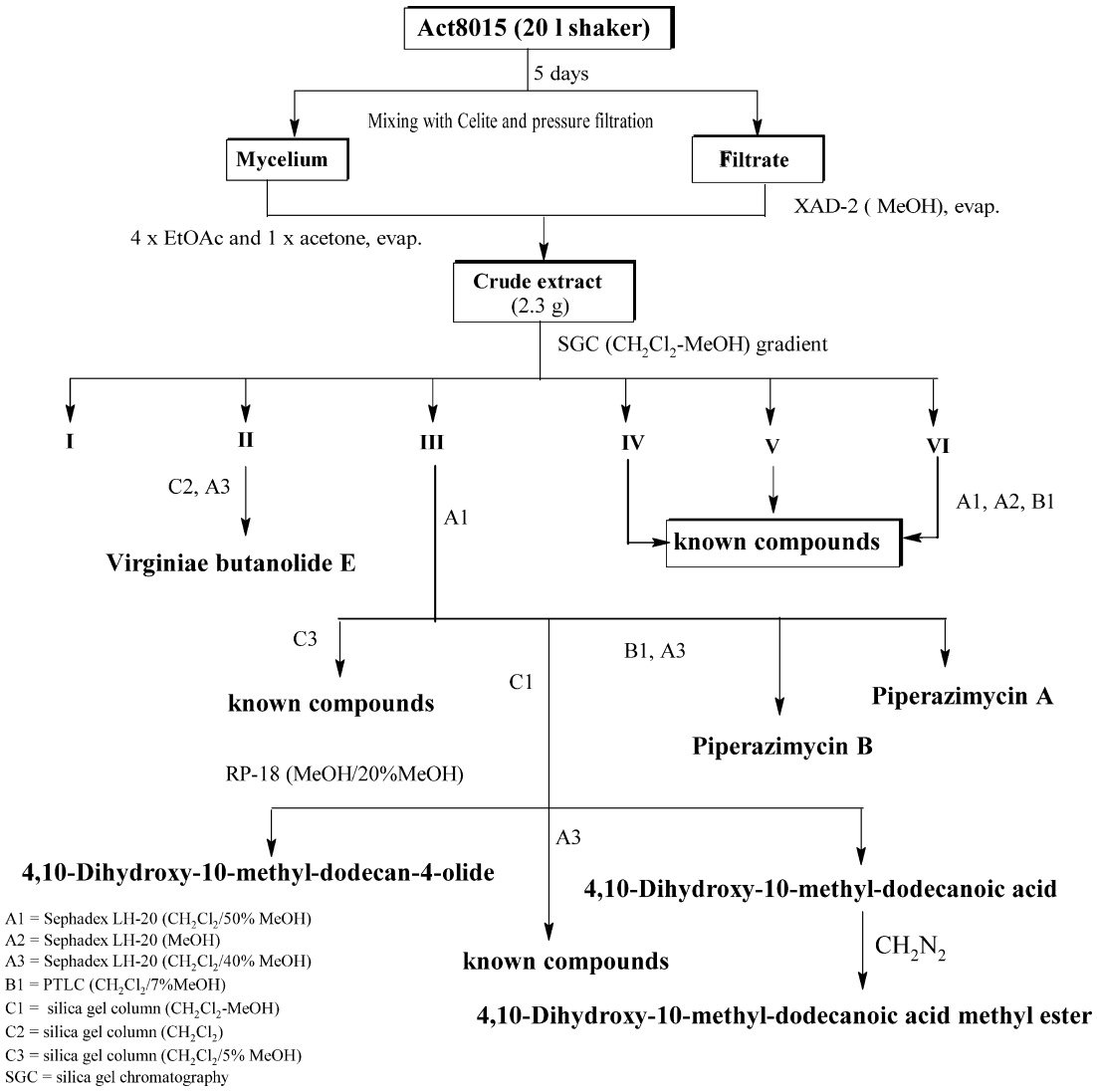


Fig. 5 Separation scheme for extracts of the marine *Streptomyces* sp. Act8015.

229.17982 for $C_{33}H_{25}O_3$), 251.16178 ($[M+Na]^+$, calcd. 251.16177 for $C_{13}H_{24}O_3Na$).

4,10-Dihydroxy-10-methyldodecanoic acid (**3a**)
Colorless oil with the staining behavior of **2**, Rf 0.10 (CH_2Cl_2/5.0% MeOH). $[\alpha]_D^{20}$ 0° (*c* 1.0 mg/ml, MeOH). ^{1}H-NMR (300 MHz, $CDCl_3$) see Table 1. ^{13}C-NMR (75 MHz, $CDCl_3$) see Table 1. (+)-ESI-MS *m/z* 269 $[M+Na]^+$. (−)-ESI-MS: *m/z* (%) 513 ($[2M-2H+Na]^-$, 50), 491 ($[2M-H]^-$, 30), 245 ($[M-H]^-$, 100). (+)-HRESI-MS *m/z* 269.17241 ($[M+Na]^+$, calcd. 269.17233 for $C_{13}H_{26}O_4Na$).

4,10-Dihydroxy-10-methyldodecanoic acid methyl ester (**3b**)
A solution of 2.5 mg of **2** in 1.0 ml diethyl ether was treated with an excess of an etherial solution of diazomethane and evaporated to dryness after 1 minunte: colorless oil (2.1 mg), Rf 0.25 (CH_2Cl_2/5.0% MeOH). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.65 (s, 3H, 1-OCH_3), 3.55 (br s, 1H, H-4), 2.35 (t, 7.0 Hz, 2H, CH_2-2), 1.60 (m, 2H, CH_2-3), 1.65∼1.30 (m, 12H, CH_2-5, 6, 7, 8, 9, 11), 1.11 (s, 3H, 10-CH_3), 0.85 (t, 7.4 Hz, 3H, CH_3-12).

Virginiae Butanolide E; VB-E (**4e**)
Colorless oil with the same color reactions as **2** and **3a**; Rf 0.33 (CH_2Cl_2/5.0% MeOH). $[\alpha]_D^{20}$ −74° (*c* 2.0 mg/ml, MeOH); ^{1}H-NMR (300 MHz, $CDCl_3$) see Table 2. ^{13}C-NMR (75 MHz, $CDCl_3$) see Table 2. (+)-ESI-MS *m/z* (%) 455 ($[2M+Na]^+$, 22), 239 ($[M+Na]^+$, 100), 217 ($[M+H]^+$, 8). (−)-ESI-MS *m/z* 261 ($[M+HCOO]^-$. (+)-HRESI-MS *m/z* 217.14356 ($[M+H]^+$, calcd. 217.14343 for $C_{11}H_{21}O_4$), 239.12552 ($[M+Na]^+$, calcd. 239.12537 for $C_{11}H_{20}O_4Na$).

MS/MS Studies
Samples of piperazimycins A (**1a**) and B (**1b**) were dissolved in methanol/water (3 : 1) containing 0.1% formic acid. Low resolution MS^2 and MS^3 measurements were performed on a LCQ quadrupole ion trap instrument (Finnigan, San Jose, USA) using electrospray ionization in the positive and negative ionization mode with an electrospray voltage of +/− 4.5 kV. Samples were introduced by means of a syringe pump with a flow rate of 3 μl/minunte. $[M+H]^+$, $[M+2H]^{2+}$, $[M+Na]^+$, and $[M-H]^-$ ions were isolated (isolation width 3.0 Dalton) and submitted to fragmentation by collision induced dissociation with helium as collision gas. For MS^3 investigations, fragment ions were isolated (isolation with 3.0 Dalton peak width) and fragmented under the same conditions.

Accompanying high-resolution MS/MS investigations were performed on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer. Samples were infused with a flow rate of 2 μl/minute. CID-MS/MS studies were carried out on the $[M+H]^+$ and $[M+Na]^+$ species inside the ICR cell with argon as collision gas. Conditions: electrospray voltage 4.2 kV, capillary exit voltage 100 V, nebulizing gas nitrogen (30 psi), drying gas nitrogen (250°C), hexapole accumulation 0.1 second, mass range *m/z* 100∼1400.

Antimicrobial Activity
The compounds were dissolved in CH_2Cl_2/10% MeOH at a concentration of 1.0 mg/ml. Aliquots of 40 μl were soaked on filter paper disks (9 mmϕ, no. 2668, Schleicher & Schüll, Germany) and dried for 1 hour at room temperature under sterilized conditions. The paper disks were placed on inoculated agar plats and incubated for 24 hour at 38°C in the dark (bacteria and fungi) or room temperature at sunlight (algae).

Cytotoxicity Testing
The test procedure has been described elsewhere [28]. Human tumor cell lines tested were derived from patient tumors engrafted as a subcutaneously growing tumor in NMRI nu/nu mice, or obtained from American Type Culture Collection, Rockville, MD, USA, National Cancer Institute, Bethesda, MD, USA, or Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. Piperazimycin A (**1a**) was tested at 5 concentrations ranging from 0.3 ng/ml to 3.0 μg/ml.

Brine Shrimp Microwell Cytotoxicity Assay
The cytotoxic assay was performed according to Takahashi *et al.* [29] at concentrations of 10 μg/ml with actinomycin D (1.0 μg/well) for comparison.

Acknowledgements We thank Mr. R. Machinek for NMR measurements, F. Lissy for biological activity tests and A. Kohl for technical assistance. P. Facey thanks the Alexander von Humboldt Foundation (AvH) for a research fellowship. Financial support from the BMBF (Project No. 03F0415A) is gratefully acknowledged.

References

1. Graefe U, Schlegel R, Ritzau M, Ihn W, Dornberger K, Stengel C, Fleck WF, Gutsche W, Haertl A, Paulus EF. Aurantimycins, new depsipeptide antibiotics from *Streptomyces aurantiacus* IMET 43917: production, isolation, structure elucidation, and biological activity. J

Antibiot 48: 119–125 (1995)
2. Maehr H, Liu CM, Palleroni NJ, Smallheer J, Todaro L, Williams TH, Blount JF. Microbial products. VIII. Azinothricin, a novel hexadepsipeptide antibiotic. J Antibiot 39: 17–25 (1986)
3. Umezawa K, Nakazawa K, Uemura T, Ikeda Y, Kondo S, Naganawa H, Kinoshita N, Hashizume H, Hamada M, Takeuchi T, Ohba S. Polyoxypeptin isolated from *Streptomyces*: a bioactive cyclic depsipeptide containing the novel amino acid 3-hydroxy-3-methylproline. Tetrahedron Lett 39: 1389–1392 (1998)
4. Leet JE, Schroeder DR, Golik J, Matson JA, Doyle TW, Lam KS, Hill SE, Lee MS, Whitney JL, Krishnan BS. Himastatin, a new antitumor antibiotic from *Streptomyces hygroscopicus* III. Structural elucidation. J Antibiot 49: 299–311 (1996)
5. Umezawa K, Ikeda Y, Uchihata Y, Naganawa H, Kondo S. Chloptosin, an apoptosis-inducing dimeric cyclohexapeptide produced by *Streptomyces*. J Org Chem 65: 459–463 (2000)
6. Ogita T, Sato A, Enokita R, Suzuki K, Ishii M, Negishi T, Okazaki T, Tamaki K, Tanzawa K. Matlystatins, new inhibitors of typeIV collagenases from *Actinomadura atramentaria*. I. Taxonomy, fermentation, isolation, and physico-chemical properties of matlystatin-group compounds. J Antibiot 45: 1723–1732 (1992)
7. Sakai Y, Yoshida T, Tsujita T, Ochiai K, Agatsuma T, Saitoh Y, Tanaka F, Akiyama T, Akinaga S, Mizukami T. GE3, a novel hexadepsipeptide antitumor antibiotic, produced by *Streptomyces* sp. I. Taxonomy, production, isolation, physico-chemical properties, and biological activities. J Antibiot 50: 659–664 (1997)
8. Konishi M, Ohkuma H, Sakai F, Tsuno T, Koshiyama H, Naito T, Kawaguchi H. Structures of BBM-928 A, B, and C. Novel antitumor antibiotics from *Actinomadura luzonensis*. J Am Chem Soc 103: 1241–1243 (1981)
9. Konishi M, Ohkuma H, Sakai F, Tsuno T, Koshiyama H, Naito T, Kawaguchi H. BBM-928, a new antitumor antibiotic complex. III. Structure determination of BBM-928 A, B and C. J Antibiot 34: 148–159 (1981)
10. Morimoto K, Shimada N, Naganawa H, Takita T, Umezawa H, Kambara H. Minor congeners of antrimycin: application of secondary ion mass spectrometry (SIMS) to structure determination. J Antibiot 35: 378–380 (1982)
11. Hensens OD, Borris RP, Koupal LR, Caldwell CG, Currie SA, Haidri AA, Homnick CF, Honeycutt SS, Lindenmayer SM, Schwartz C, Weissberger B, Woodruff H, Zink D, Zitano L, Fieldhouse J, Rollins T, Springer M, Springer J. L-156,602, a C5a antagonist with a novel cyclic hexadepsipeptide structure from *Streptomyces* sp. MA6348: fermentation, isolation and structure determination. J Antibiot 44: 249–254 (1991)
12. Inouye Y, Take Y, Nakamura S, Nakashima H, Yamamoto N, Kawaguchi H. Screening for inhibitors of avian myeloblastosis virus reverse transcriptase and effect on the replication of AIDS virus. J Antibiot 40: 100–104 (1987)
13. Lingham RB, Hsu AHM, O'Brien JA, Sigmund JM, Sanchez M, Gagliardi MM, Heimbuch BK, Genilloud O, Martin I, Diez M, Hirsch C, Zink D, Liesch J, Koch G, Gartner S, Garrity G, Tsuo N, Salituro G. Quinoxapeptins: novel chromodepsipeptide inhibitors of HIV-1 and HIV-2 reverse transcriptase. I. The producing organism and biological activity. J Antibiot 49: 253–259 (1996)
14. Laatsch H. AntiBase 2008, A Data Base for Rapid Structural Determination of Microbial Natural Products. Wiley-VCH, Weinheim, Germany; see http://wwwuser.gwdg.de/~ucoc/laatsch/AntiBase.htm
15. M_2^+ medium: 10 g malt extract, 4 g yeast extract, and 4 g glucose in 0.5 liters of artificial sea water and 0.5 liters of tap water was set to pH 7.8 with 2 N NaOH and sterilized for 30 minutes at 121°C
16. Meksuriyen D, Cordell GA. Biosynthesis of staurosporine, 1. Proton and carbon-13 NMR assignments. J Nat Prod 51: 884–892 (1988)
17. Umezawa I, Tronquet C, Funayama S, Okada K, Komiyama K. A novel antibiotic, sohbumycin. Taxonomy, fermentation, isolation and physicochemical and biological characteristics. J Antibiot 38: 967–971 (1985)
18. Miller ED, Kauffman CA, Jensen PR, Fenical W. Piperazimycins: cytotoxic hexadepsipeptides from a marine-derived bacterium of the genus *Streptomyces*. J Org Chem 72: 323–330 (2007)
19. Sajid I, Shaaban KA, Frauendorf H, Hasnain S, Laatsch H. Val-Geninthiocin: Structure Elucidation and MS^n Fragmentation of Thiopeptide Antibiotics Produced by *Streptomyces* sp. RSF18. Z Naturforsch B 63b: 1223–1230 (2008)
20. Tilvi S, Naik CG. Tandem mass spectrometry of kahalalides: identification of two new cyclic depsipeptides, kahalalide R and S from *Elysia grandifolia*. J Mass Spectrom 42: 70–80 (2007)
21. Kondo K, Higuchi Y, Sakuda S, Nihira T, Yamada Y. New Virginiae Butanolides from *Streptomyces virgniae*. J Antibiot 42: 1873–1876 (1989)
22. Ohashi H, Zheng YH, Nihira T, Yamada Y. Distribution of virginiae butanolides in antibiotic-producing actinomycetes, and identification of the inducing factor from *Streptomyces* antibioticus as virginiae butanolide A. J Antibiot 42: 1191–1195 (1989)
23. Horinouchi S, Beppu T. *In* Genetics and Biochemistry of Antibiotic Production *Eds.*, Vining LC, Stuttard C, pp. 103–119, Butterworth-Heinemann, Newton, MA (1994)
24. Ohnishi Y, Kameyama S, Onaka H, Horinouchi S. The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. Mol Microbiol 34: 102–111 (1999)
25. Yamada Y, Nihira T, Sakuda S. Bio/Technology of Antibiotics, pp. 63–79, Marcel Dekker, Inc., New York (1997)
26. Yamada Y. *In* Microbial Signaling and Communication *Eds.*,

England R, Hobbs G, Bainton N, Roberts D, pp. 177–196, Cambridge University Press, Cambridge, United Kingdom (1999)

27. Walker JD, Colwell RR. Factors affecting enumeration and isolation of actinomycetes from Chesapeake Bay and southeastern Atlantic Ocean sediments. Mar Biol 30: 193–201 (1975)
28. Dengler WA, Schulte J, Berger DP, Mertelsmann R, Fiebig HH. Development of a propidium iodide fluorescence assay for proliferation and cytotoxicity assays. Anticancer Drugs 6: 522–532 (1995)
29. Takahashi A, Kurasawa S, Ikeda D, Okami Y, Takeuchi T. Altemicidin, a new acaricidal and antitumor substance. I. Taxonomy, fermentation, isolation and physico-chemical and biological properties. J Antibiot 32: 1556–1561 (1989)