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Gephyromycin, the first bridged angucyclinone, from *Streptomyces griseus* strain NTK 14 ☆

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Abstract

The new, highly oxygenated angucyclinone gephyromycin was isolated from an extract of a *Streptomyces griseus* strain. Its unprecedented ether-bridged structure was elucidated by NMR methods and substantiated by single crystal X-ray analysis. The absolute configuration was evidenced by quantum chemical CD calculations. Gephyromycin exhibits glutaminergic activity towards neuronal cells. Furthermore, the known compounds fridamycin E and dehydrorabelomycin were identified.

Keywords: *Streptomyces*; Angucyclinones; Natural products; Secondary metabolites; Glutaminergic activity; Circular dichroism (CD); Quantum chemical CD calculations; X-ray structure analysis

1. Introduction

Extremophilic actinomycetes constitute an increasingly important, highly productive source of bioactive secondary metabolites (Fiedler and Goodfellow, 2004). In the course of our HPLC-diode array screening (Fiedler, 1993) to discover novel compounds of such microorganisms, with a focus on alkaliphilic and psychrophilic strains (Fiedler and Goodfellow, 2004; Bruntner et al., 2005), we have investigated the *Streptomyces* strain NTK 14, which had been isolated from an antarctic soil sample. One of the typical classes of compounds produced by actinomycetes are the angucyclinones like dehydrorabelomycin (**1**, Liu et al., 1970, see Fig. 1). These are metabolites based on a tetracyclic ring structure with a characteristic angular array, and with a high number of oxygen functions, comprising more than 100 compounds (Rohr and Thiericke, 1992). Many of these substances exhibit strong biological activities, mainly of antibiotic and cytostatic nature (Rohr and Thiericke, 1992). In this paper, we report on the discovery of gephyromycin (**2**), the first representative of a novel-type angucyclinone with an unprecedented intramolecular

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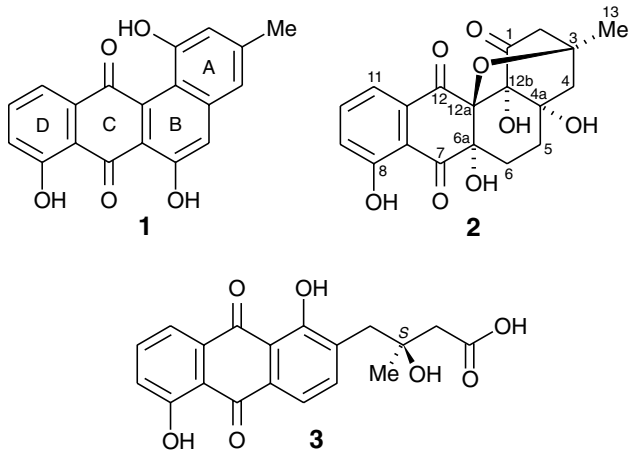

Fig. 1. Metabolites of the antarctic *Streptomyces griseus* strain NTK 14, among them the novel angucyclinone gephyromycin (**2**).

ether bridge, its isolation, structural elucidation, and its biological properties. From the same strain we isolated the known (Liu et al., 1970) dehydrorabelomycin (**1**) and identified the likewise known (Rohr and Thiericke, 1992) antibiotic fridamycin E (**3**) by means of our HPLC–UV–Vis database (Fiedler, 1993).

2. Results and discussion

The HPLC–UV chromatogram of the extract of the culture filtrate showed peaks of several metabolites, one of which was identified by search in our HPLC–UV–Vis database to be the known antibiotic fridamycin E (**3**) based on its identical retention time and UV–Vis spectrum. In addition, two presumptively new metabolites not found in the database were characterized, belonging to the anthraquinone group according to their UV–Vis properties. The two compounds were isolated by subsequent adsorption and size-exclusion chromatographic steps. After isolation, one of them was easily identified as the likewise known (Liu et al., 1970) angucyclinone dehydrorabelomycin (**1**) by NMR and mass spectrometry.

The second compound displayed a molecular ion in HREIMS at 374.1000 indicating a molecular formula of $C_{19}H_{18}O_8$ (calcd. 374.1002). This formula was substantiated by its ^{13}C NMR spectrum, which showed a total of 19 signals. Evaluation of the one- and two-dimensional NMR data in $CDCl_3$ (see Table 1) and, with in part complementary information, in dioxane-d_8 (see Table 2), particularly with respect to the long-range H,C-couplings, showed the compound to belong to the group of angucyclinones. The aromatic protons H-9, H-10, and H-11 were attributed to the D-ring of the angucyclinone skeleton. These protons showed long-range couplings to the remaining carbons of the D-ring, C-7a, C-8, and C-11a, and to the carbonyl carbons of the C-ring (C-7 and C-12). Starting from the proton signals of the methyl group H-13 and the methylene groups H-2 and H-4, all carbon signals of the A-ring were assigned, with its keto group C-1 and the quaternary carbons C-4a and C-12b, which provide the connection to the B-ring. The high $^2J_{HH}$-coupling constant (19.0 Hz)

Table 1
NMR data of gephyromycin (**2**) in $CDCl_3$

Position	^{13}C (ppm)	^1H (ppm)	HMBC	ROESY	COSY (J_{HH} (Hz))
1	205.2				
2	48.0	α 2.55 *d*	1, 3, 4, 12b, 13	11	2β (19.0)
		β 2.62 *dd*	3, 4, 12b, 13	11	2α, 4β (2.5)
3	75.9				
4	46.9	α 1.97 *d*	2, 3, 4a, 5, 12b		4β (14.8)
		β 2.35 *dd*	2, 3, 5, 12b, 13	5α, 13	4α, 2β
4a	71.7				
5	30.1	α 1.83 *ddd*	4, 4a, 6, 6a, 12b	4β	6α, 6β, 5β (13.5)
		β 2.07 *ddd*	4, 4a, 6		6α, 6α (∼14), 5α
6	25.6	α 2.43 *ddd*	5, 6a, 7		6β (15.0), 5α (∼5), 5β
		β 2.32 *ddd*	4a, 5, 6a, 7, 12a		6α, 5α (∼2.5), 5β (4.0)
6a	77.4				
7	198.6				
7a	115.6				
8	162.4				
8-OH		11.50 *bs*			
9	125.0	7.30 *dd*	7, 7a, 8, 11	13	10 (8.3), 11 (1.1)
10	136.7	7.63 *dd*	8, 11a	13	9, 11 (7.4)
11	119.6	7.58 *dd*	7, 7a, 9, 12	2α, 2β, 13	9, 10
11a	133.2				
12	192.5				
12a	79.9				
12b	78.5				
13	25.4	1.24 *s*	1, 2, 3, 4, 4a, 12a	4β, 9, 10,11	

Table 2
NMR data of gephyromycin (**2**) in dioxane-d_8

Position	^{13}C (ppm)	^{1}H (ppm)	HMBC	ROESY	COSY
1	204.2				
2	48.2	2.45 *s*	1, 3, 4, 12b, 13	4α, 13	4β
3	76.3				
4	47.3	α 1.80 *d*	2, 4a, 5	2, 4a-OH, 13	4β
		β 2.30 *d*	2, 3, 5, 12b, 13	4a-OH, 5α, 13	2, 4α
4a	71.5				
4a-OH		4.05 *s*	4, 4a	4α, 5α, 5β, 6β	
5	30.0	α 1.60 *m*	4a, 6, 6a, 12b	4a-OH, 4β	6α, 5β
		β 2.05 *ddd*	4, 4a, 6, 6a	4a-OH, 6a-OH, 12b-OH	6α, 5α
6	26.0	α 2.45 *m*	5, 6a	6a-OH	5α, 5β, 6β
		β 2.10 *m*	4a, 5, 6a, 12a		6α
6a	77.6				
6a-OH		5.10 *s*	6a, 7, 12a	5β, 6α	
7	199.7				
7a	116.0				
8	162.3				
8-OH		11.25 *s*	7a, 8, 9, 10	6β, 9	
9	124.8	7.30 *dd*	7a, 8, 11	8-OH	10, 11
10	136.9	7.66 *dd*	8, 11a		9, 11
11	119.3	7.55 *dd*	7a, 9, 12		9, 10
11a	134.1				
12	192.8				
12a	80.1				
12b	78.9				
12b-OH		5.95 *s*	1, 4a, 12a, 12b	5β	
13	24.7	1.15 *s*	2, 3, 4		

of the diastereotopic protons H_α-2 and H_β-2 provided further evidence for the position of this methylene group next to the keto group C-1. The two methylene groups C-5 and C-6 with their likewise diastereotopic protons were consequently part of the B-ring. Their long-range H,C-couplings allowed for the assignment of the remaining quaternary carbons C-6a and C-12a, which constitute the connection between the B- and C-rings.

The chemical shifts of the five quaternary carbons (C-3, C-4a, C-6a, C-12a, and C-12b) ranged between 71.7 and 78.5 ppm. This indicated that, different from all other angucyclinones, none of these carbons was part of a double bond, but rather suggested the presence of tertiary alcohol, ether, or epoxide functions. From the molecular formula $C_{19}H_{18}O_8$, it was deduced that three of these quaternary carbons should carry hydroxyl groups while two were linked by an ether bridge.

To identify the positions of the free hydroxyl groups, HMBC and ROESY spectra were acquired in dioxane-d_8 (see Table 2); in this solvent, aliphatic alcohols often provide sharp signals. As expected, the proton spectrum contained three additional singlets, attributable to hydroxyl groups (the slowly exchanging, since hydrogen-bonded, aromatic hydroxyl group at C-8 were also observed in $CDCl_3$). The HMBC spectrum showed these hydroxyl groups to be attached to C-4a, C-6a, and C-12b. As a consequence, an – as yet unprecedented – ether bridge between C-3 and C-12a was expected, thus leading to the constitution corresponding to structure **2** (see Fig. 2). Due to the unique bridged structure, the compound was henceforth named gephyromycin (greek: *gephyro* = bridge). In support of this novel constitution, a four-bond H,C-coupling between the methyl group H-13 and C-12a was observed. The rigidity of the

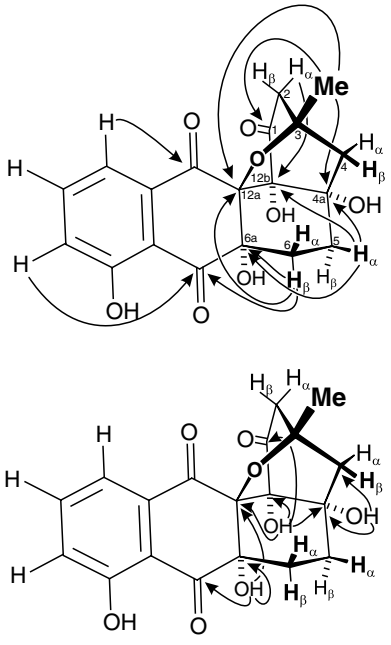

Fig. 2. Long-range H,C-couplings relevant for the structure elucidation of **2**.

2-oxa[2.2.2]bicyclooctane system in the 'eastern' half of gephyromycin (**2**) and the resulting 'W'-shape conformation of bonds between H_β-2 and H_β-4 also accounted for the unusually large $^4J_{HH}$-coupling constant (2.5 Hz, in $CDCl_3$) between these equatorial protons. Accordingly, the axial proton H_α-4 showed dipolar coupling to one of the protons at C-2. The likewise large $^3J_{HH}$-coupling constant (~14 Hz) between H_β-5 and H_α-6 indicated *anti*-periplanar conformation of these protons and thus chair conformation of the B-ring.

The rigid structure of gephyromycin (**2**) permitted only one possible relative configuration of the four stereocenters C-3, C-4a, C-12a, and C-12b, while for C-6a both configurations would be sterically possible. The ROESY spectra acquired in dioxane-d_8 (see Table 2, Fig. 2) served to clarify this remaining stereochemical question. All protons of the hydroxyl groups at the B-ring showed dipolar couplings to the same axial proton H_β-5 revealing that they are located at the same side of this ring.

Thus the configuration of gephyromycin was either (3*S*, 4a*S*, 6a*S*, 12a*R*, 12b*R*) or (3*R*, 4a*R*, 6a*R*, 12a*S*, 12b*S*). This structure, with its bridged constitution and the relative configuration of **2**, was further corroborated by an X-ray diffraction analysis (see Fig. 3).

In order to assess the absolute configuration of gephyromycin (**2**) the theoretical CD spectra for both possible enantiomers were predicted by using quantum chemical calculations (Bringmann et al., 1998, 2003, 2004). To define the conformational species of **2**, the molecule was submitted to a conformational analysis using the semiempirical AM1 approach (Dewar et al., 1985). The calculations arbitrarily started with the (3*S*, 4a*S*, 6a*S*, 12a*R*, 12b*R*)-enantiomer of **2**. Due to the rigid bridged angucyclinone framework of the molecule, only 11 conformers regarding the flexibility of the hydroxy functions were found within a range of 3 kcal/mol above the global minimum. For each conformer, the respective single CD spectrum was calculated using the semiempirical OM2 method (Weber and Thiel, 2000). The single spectra were added up Boltzmann-weighted, i.e., according to the heats of formation of the corresponding conformers to give the calculated overall CD spectrum for (3*S*, 4a*S*, 6a*S*, 12a*R*, 12b*R*)-**2**. Due to known problems of the AM1 approach in correctly reproducing strong hydrogen bonds (Jensen, 1999) as occurring in gephyromycin (**2**, e.g., between OH-6a and OH-12b, OH-4a and OH-12b, OH-12b and the keto group C-12, and between OH-8 and the keto group C-7), ambiguous results were obtained for **2**. Therefore, the respective tetra-*O*-acetylated compound, **4**, was used as a more appropriate 'substrate' for the calculations. The CD spectra thus predicted for both enantiomers of this tetraacetate were then compared with the experimental one of the authentic, non-acetylated natural product **2**, since it failed to give a fully acetylated product experimentally, also because of the small quantities of **2** available after biological testing. Still, the comparison seems feasible because of the rigidity of the main chiral chromophore of the molecular core. The experimental CD spectrum of the natural product was in a good agreement with the one predicted for the (3*S*, 4a*S*, 6a*S*, 12a*R*, 12b*R*)-isomer of **4**, while the CD spectrum calculated for the (3*R*, 4a*R*, 6a*R*, 12a*S*, 12b*S*)-enantiomer proved to be virtually opposite to the measured one (Fig. 4), clearly showing the natural product to possess the (3*S*, 4a*S*, 6a*S*, 12a*R*, 12b*R*)-configuration.

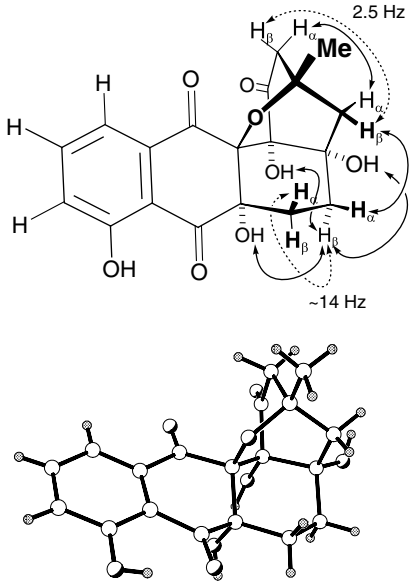

Fig. 3. The three-dimensional structure of gephyromycin (**2**), as verified by ROESY (full arrows) and COSY (dotted arrows) couplings, compared to the structure obtained from single-crystal X-ray diffraction.

Gephyromycin (**2**) is the first angucyclinone with an intramolecular ether bridge (not counting epoxides). From known angucyclinones it differs also in the unusually high degree of hydroxylation and, hence, sterogenic centers. While several angucyclines are known with oxygen functions at C-3, C-4a, C-12a, or C-12b (e.g. urdamycin I, Drautz et al., 1986), gephyromycin (**2**) is the first to be oxygenated at *all* of these positions and, additionally, at C-6a. Another striking difference between **2** and other angucyclins is the *S*-configuration at C-3; for the urdamycins A and B, the *R*-configuration at this position has been proven by X-ray diffraction and total synthesis (Liu et al., 1970; Künzel et al., 1999). The formation of gephyromycin from a hypothetical precursor **5a** or **b** by intramolecular attack of the 3-OH group to an epoxide at C-6a/C-12a or C-12a/C-12b, respectively, necessitates the unusual 3*S*-configuration as present only in gephyromycin (**2**); for steric reasons, it would not be possible in angucyclins with the conventional 3*R*-configuration. From an evolutionary point of view, it is interesting to note how this at first sight small

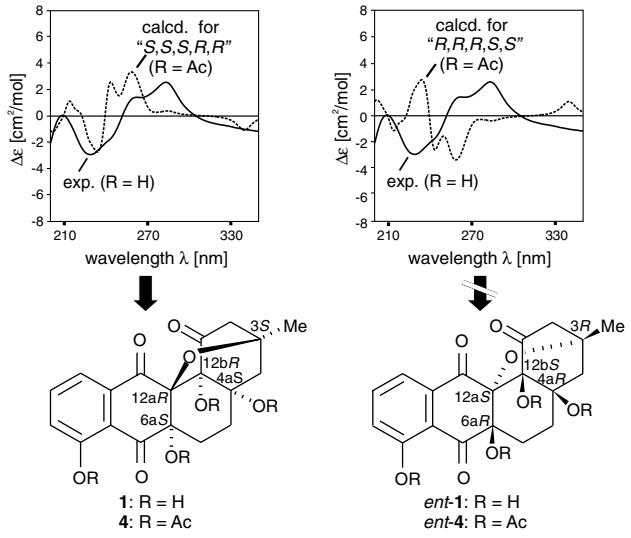

Fig. 4. Determination of the absolute configuration of gephyromycin (**2**) by comparison of the experimental CD spectrum of **2** with the CD curves predicted for the two possible enantiomers of its tetraacetate, **4**.

structural change – the *S*-configuration at C-3 – can lead to the formation of a novel, here cage-like, type of metabolites (see Fig. 5).

Besides its unique structural framework, gephyromycin (**2**) likewise displays intriguing biological activities: Incubation of neurons with 3 μg/ml of **2** caused a significant increase in the intracellular Ca^{2+} concentration after 5 min from 0.811 ± 0.027 (ratio units) to 1.730 ± 0.071 (Fig. 6). In the control sample no changes of the calcium level had been detected. The concentration found to be active for gephyromycin (**2**) is in the range of, e.g., the glycin derivative DCG-IV, which had been considered to be the most potent glutamate agonist (Uyama et al., 1997).

The mode of action of gephyromycin (**2**) is still unknwon. The results obtained so far suggest a glutaminergic activity (agonist) of **2** on neurons. Upon binding of gephyromicin (**2**), the ion channels open and allow an entry of ions (e.g., Na^+, K^+, and Ca^{2+}). This effect is well established, also for other compounds; thus, if glutamate or aspartate are released into the extracellular space of the brain, a hyperactivation of the glutamate receptors occurs; this effect has been termed "excitotoxicity" (Arundine and Tymianski, 2003; Stys, 2004). The effect is comparable to that of the known ASP (amnesic shellfish poisoning) toxins kainic acid and domoic acid (Nijjar and Nijjar, 2000). An excessive raise in $[Ca^{2+}]_i$ accounts for multiple cytotoxic damage to the neurons such as perturbation of cytoskeletal proteins and activation of proteases and phospholipases (Arundine and Tymianski, 2003). Further studies are necessary to prove the effect of gephyromycin (**2**) also on the NMDA receptor. At present, we suggest that **2** represents as new potent glutamate agonist.

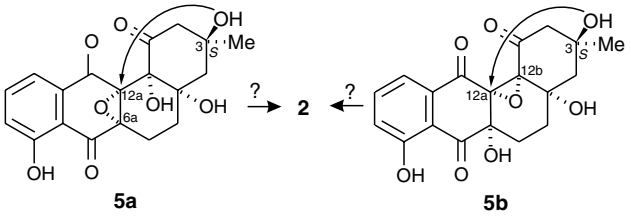

Fig. 5. Formation of gephyromycin (**2**) from a hypothetical precursor **5a** or **5b**.

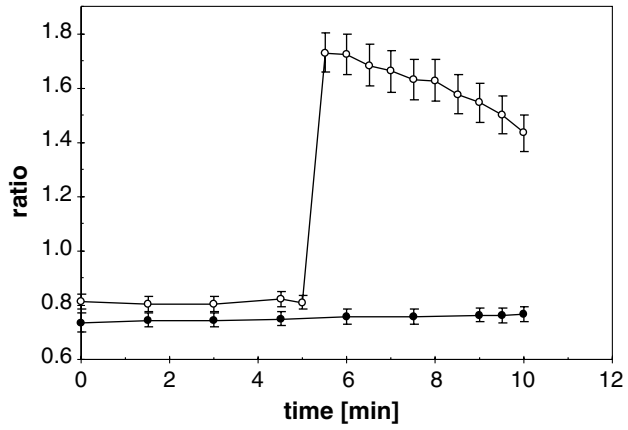

Fig. 6. The effect of gephyromycin (**2**) on the intracellular Ca^{2+} level $[Ca^{2+}]_i$ in neurons. Neurons were incubated for 5 min in the presence (○) or absence (●) of 3 μg/ml of **2**. The results are expressed as mean value (±SD; $n = 35–50$).

On the other hand, possibly as a consequence of the loss of the usually flat molecular shape, gephyromycin (**2**) exhibits no acute cytostatic or antibacterial activities, in contrast to the other, conventional angucyclinones. In addition, the data show that **2** causes a modulatory activity on neuronal receptor function. The lack of cytotoxicity of **2** makes its above mentioned neuroprotective properties even more valuable.

3. Experimental

3.1. General

Melting points were measured on a Reichert-Jung Thermovar hot-plate and are uncorrected. IR spectra were taken on a Jasco FT/IR-410 spectrometer, optical rotations on a Jasco P-1020 polarimeter (25 °C, 10 cm cell), and CD spectra (20 °C, MeOH, 0.05 cm cell) on a Jasco J-715 spectropolarimeter. ^1H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were acquired on a Bruker DMX 600 instrument, using $CDCl_3$ (δ 7.25 and 77.01) and dioxane-d_8 (δ 3.53 and 66.66) as the solvents and internal ^1H and ^{13}C standards. Proton-detected, heteronuclear correlations were measured using HMQC (optimized for $^1J_{HC} = 145$ Hz) and

HMBC (optimized for $^{n}J_{HC}$ = 7 Hz or $^{n}J_{HC}$ = 3.2 Hz) pulse sequences. ROE effects were measured using a standard pulse sequence from the standard Bruker pulse program library. EIMS (70 eV) and HREIMS (70 eV) were determined on Finnigan MAT 8200 and Finnigan MAT 90 instruments.

3.2. Computational methods

The conformational analysis of natural gephyromycin (**2**) was performed on Silicon Graphics OCTANE R10000 workstations by means of the semiempirical AM1 (Dewar et al., 1985) method as implemented in the program package VAMP6.5 (Rauhut et al., 1996) starting from preoptimized geometries generated by the TRIPOS force field (SYBYL 6.9, 2002). The respective geometries of the tetra-*O*-acetylated compound **4** were created with Molden 4.2 (Schaftenaar and Noordik, 2000) by replacing the hydrogen atoms of the hydroxy functions of **2** by acetyl groups.

For both compounds, the wave functions required for the calculation of the rotational strengths for the electronic transitions from the ground state to excited states were obtained by OM2 (Weber and Thiel, 2000) calculations. These calculations were carried out with Linux Pentium III workstations by the use of the MNDO99 (Thiel, 1999) program package. The single theoretical CD spectra were weighted following to the Boltzmann statistics, according to the respective heats of formation. For a better visualization, the rotational strengths were transformed into $\Delta\varepsilon$ values and superimposed with a Gaussian band shape function.

3.3. Taxonomy of the producing strain and its fermentation

Strain NTK 14 was isolated from a soil sample collected at Edmundson Point, Terra Nova Bay, Antarctica. Comparison of the almost complete 16S rRNA gene sequence of strain NTK 14 with the sequences of 92 *Streptomyces* type strains placed the strain within the *Streptomyces griseus* radiation.

The strain was cultivated in a 20-l turbine impeller fermentor in a medium consisting of 1% glucose, 1% starch, 1% glycerol, 0.25% corn steep powder, 0.5% peptone, 0.2% yeast extract, 0.1% NaCl, and 0.3% $CaCO_3$ in tap water; pH was adjusted to 7.3 prior to sterilization. The fermentation was carried out for 86 h at 27 °C with an aeration rate of 0.5 vvm and an agitation of 1000 rpm.

3.4. HPLC-DAD analyses and isolation of 1 and 2

The chromatographic system consisted of an HP 1090M liquid chromatograph equipped with a diode-array detector and an HP Kayak XM 600 ChemStation (Agilent Technologies). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360, 435, and 500 nm. The UV–Vis spectra were measured from 200 to 600 nm.

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. A 10-μl aliquot of the samples was 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 (Maisch). The samples were analyzed by linear gradient elution using 0.1% aqueous *ortho*-phosphoric acid as the solvent A and MeCN as the solvent B at a flow rate of 2 ml/min. The gradient was from 0% to 100% solvent B in 15 min with a 2-min hold at 100% solvent B.

For isolation of the two unidentified compounds, Hyplo Super-cel (2%) was added to the fermentation broth, which was separated by multiple sheet filtration into culture filtrate and mycelium. The culture filtrate (17 l) containing 8.6 mg/l of **1** and 4.9 mg/l of **2** was applied to an Amberlite XAD-16 column (80x5 cm). The resin was washed with water and eluted with increasing ratios of MeOH. The fraction containing **1** and **2** (80% MeOH) was concentrated under reduced pressure and partitioned against EtOAc. The EtOAc phase was dried in vacuo and the residue was subjected to a LiChroprep-Diol column (40 × 2.6 cm; Merck) using a linear gradient from CH_2Cl_2 to CH_2Cl_2/MeOH (70:30) within 3 h at a flow rate of 5 ml/min. After size-exclusion chromatography on Sephadex LH-20 and Fractogel TSK HW-40 (each column 90 × 2.5 cm; flow rate: 0.5 ml/min;), 26 mg of dehydrorabelomycin (**1**) and 10 mg of gephyromycin (**2**) were obtained.

Dehydrorabelomycin (***1***). Dark green crystals, m.p. 202 °C (CH_2Cl_2). ^{1}H and ^{13}C NMR as well as EIMS data were in good agreement with those reported in the literature (Yamaguchi et al., 1992).

Gephyromycin (***2***). White crystalline solid (MeOH), m.p. 212 °C. $[\alpha]_D^{20}$ −51 (MeOH, *c* 0.05. CD: $\Delta\varepsilon_{192}$ −3.7, $\Delta\varepsilon_{210}$ 0.0, $\Delta\varepsilon_{227}$ −3.2, Δ_{261} +1.6, $\Delta\varepsilon_{283}$ +2.8, $\Delta\varepsilon_{351}$ −1.4 (MeOH, *c* 0.1). IR ν_{max}^{KBr} cm^{-1}: 3422, 2930, 1742, 1706, 1655, 1458, 1329, 1279, 1232, 1185, 1161, 1098, 1055. For ^{1}H and ^{13}C NMR spectral data, see Tables 1 and 2. EIMS *m*/*z* (rel.int.): 374 M^+ (100), 356 (11), 274 (12), 253 (13), 145 (18), 233 (13), 217 (12), 189 (17), 121 (21), 43 (37). HREIMS *m*/*z*: 374.1000 M^+ ($C_{19}H_{18}O_8$ requires 374.1002).

3.5. Effect of gephyromycin (2) on the intracellular calcium concentration in primary neurons

Primary cortical cell culture was prepared from 17 to 18 days old rat embryos following a modified procedure (Freshney, 1987; Perovic et al., 1994, 1998).

Gephyromycin (**2**) was dissolved in 100% (v/v) DMSO (stock solution 10 mg/ml) and finally diluted with 100% (v/v) fetal calf serum (FCS) to the concentration of 100 μg/ml (1% DMSO in FCS). The 100 μg/ml stock solution of **2** was incubated for 3 days at 37 °C. After incubation, the solution was stored at −20 °C. Prior to incubation (0–5 min) the base line of the calcium level in the neurons was determined. Then, the compound was added for a period of 5–10 min to the neurons; the final concentration was 3 μg/ml of gephyromycin (**2**); DMSO in FCS served as a control. In all sets of experiments, Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose and 10 mM Hepes; pH 7.4; without Ca^{2+} and Mg^{2+}) was used as a buffer. The intracellular calcium concentration $[Ca^{2+}]_i$ level was monitored for 10 min.

3.6. Effect of gephyromycin (2) on the viability of L5178y cells

L5178y cells were grown in RPMI1640 (Sigma–Aldrich, Taufkirchen, Germany) supplemented with 10 mM Hepes (Sigma), 10% fetal calf serum (FCS, Gibco, Berlin, Germany), and 0.1% gentamycin (Sigma). The cells were routinely passaged twice a week. They were maintained in culture no longer than 20 passages. The stock solution of **2** was prepared as described before (see 3.6). L5178y cells were plated in 96-well flat-bottomed microtiter plates at a cell density of 6000 cells/ml. Gephyromycin (**2**) was added to the cells at four concentrations in half-log increments (1, 3, 10, and 30 μg/ml). The cell viability was determined using the MTT assay (Scudiero et al., 1988) following 3 days of continuous drug exposure. The evaluation was performed at 595 nm using an ELISA plate reader (BioRad 3550).

3.7. Crystal data of 2

The crystal data of gephyromycin (**2**) were collected from shock-cooled oil coated crystals on a BRUKER SMART-APEX diffractometer with D8 goniometer (graphite-monochromated Mo Kα radiation, λ = 0.71073 Å) equipped with a low-temperature device in omega-scan mode at 100(2) K (Stalke, 1998). The data were integrated with SAINT (Bruker-AXS, 2000) and a semi-empirical absorption correction was applied (Sheldrick, 2000). The structures were solved by direct methods (SHELXS-97, Sheldrick, 1990) and refined by full-matrix least squares methods against F^2 (SHELXL-97, Sheldrick, 1997) All non-hydrogen atoms were refined with anisotropic displacement parameters. All hydrogen atoms in **2** were located by difference Fourier syntheses and refined without any distance or thermal motion constraints.

Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary Publication No. CCDC-265298. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: (internat.) + 44(1223)336-033; e-mail: deposit@ccdc.cam.ac.uk].

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