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Simocyclinones, Novel Cytostatic Angucyclinone Antibiotics Produced by *Streptomyces antibioticus* Tü 6040

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

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Two novel angucyclinone-type antibiotics, simocyclinones D4 and D8, were detected in the mycelium extract of *Streptomyces antibioticus* Tü 6040 by HPLC-diode-array and HPLC-electrospray-mass-spectrometry screening. The compounds show antibiotic activities against Gram-positive bacteria and cytostatic effects on various tumor cell lines.

In the course of our search for novel microbial secondary metabolites using HPLC-DAD-MS screening, we investigated streptomycetes strain Tü 6040 which was isolated from a soil sample collected in Iguaçu, Argentina. Humic acid-vitamin agar[2] was used for the isolation. The culture filtrate, a 10-fold concentrated ethyl acetate extract thereof, and a methanol extract of the mycelium were analyzed by gradient reversed-phase HPLC coupled with a multi-wavelength diode array monitoring system (HPLC-DAD). The UV-visible spectra of the resulting peaks were compared with those of 580 reference compounds, mostly antibiotics, stored in our HPLC-UV-Vis-database[3]. Compounds presumed novel after the comparison were further analyzed by HPLC-ESI-MS to establish their molecular mass.

The HPLC-DAD analysis of mycelium extract of strain Tü 6040 revealed two compounds (retention time: 10.4 and 10.7 minutes) that have UV-visible spectral maxima at 366 nm and 365 nm, respectively (Fig. 1). They were found to be similar to a group of reference compounds stored in

[†] Art. No. 15 on 'Biosynthetic Capacities of Actinomycetes'. Art. No. 14: See ref. 1.

Table 1. Cultural characteristics of strain Tü 6040

Medium	Growth	Aerial spore mass colour	Reverse side colour of the colonies	Diffusible pigments
Yeast extract-malt extract-agar (ISP 2)	Good	Whitish with gray spots	Yellowish brown, grayish brown, dark brown	None
Oatmeal -agar (ISP 3)	Good	Grayish brown with pale gray and whitish spots	Yellowish brown, gray brown	None
Inorganic salts-starch agar (ISP 4)	Good	Grayish brown	Grayish brown, gray	None
Glycerol-asparagine agar (ISP 5)	Poor	Whitish, poorly developed	Yellowish brown, brown	None
Oatmeal-nitrate-agar *	Good	Grayish brown	Gray, yellowish gray	None

* Oatmeal-nitrate agar: oatmeal 3.0 g, KNO_3 0.2 g, K_2HPO_4 0.5 g, $MgSO_4 \times 7\ H_2O$ 0.2 g, agar 20 g, aqua deion. 1 litre.

the polyene sub-library of our database. Analysis by HPLC-ESI-MS revealed their molecular mass to be 897 and 931, respectively. A search in commercially available databases[4,5] found no positive match for their identity suggesting the novelty of these compounds. The compounds were named simocyclinones D4 (**1**) and D8 (**2**).

This report deals with the taxonomy of the producing strain, the fermentation and isolation, and biological activities of **1** and **2**. Investigations on their chemical structure will be reported in a forthcoming publication. As indicated by these results, the simocyclinones are structurally related to angucyclinone-type antibiotics[6,7] with additional structural feature of fumagillin[8] and novobiocin[9].

Taxonomy of the Producing Strain

Strain Tü 6040 was assigned to the genus *Streptomyces* because of its characteristic chemotaxonomic features: LL-diaminopimelic acid in the peptidoglycan and menaquinones MK-9(H_6), MK-9(H_4), MK-9(H_2), MK-9(H_8) in a ratio of 35 : 34 : 20 : 4 and because of its typical morphology. The sporulation of the aerial mycelium was different depending on the culture media and varied from pale gray to grayish brown. The spore chains were open loops, hooks and short extended spirals (retinaculumapertum or spiral type). The spore surface was smooth as revealed by electron microscopy. The substrate mycelium was of a yellowish, grayish and dark brown colour. Soluble pigments other than melanin were not observed. Melanoid pigments were produced on peptone-yeast extract iron agar (ISP 6) and tyrosine agar (ISP 7). The cultural characteristics of strain Tü 6040 are summarized in Table 1. The utilization pattern of selected carbon sources of strain Tü 6040 was compared with the type strains of *S. naganishii* DSM 40282^T, *S. antibioticus* IMET 40227^T and *S. violaceusniger* DSM 40563^T (Table 2).

Strain Tü 6040 exhibited highest similarity to *S. naganishii* with respect to taxonomic characteristics described above. However, the typical red pigmentation of the substrate mycelium on ISP 3 medium exhibited by *S. naganishii* was missing in Tü 6040. Tü 6040 thus could be considered either as a strain of *S. naganishii* or *S. antibioticus* because of their similarity. WILLIAMS *et al.*[11] assigned *S. naganishii* as an allied species to the heterogenous *S. antibioticus*. We therefore propose to place strain Tü 6040 in the species of *S. antibioticus*. Earlier, HÜTTER[12] considered *S. naganishii* to be a synonym of *S. violaceusniger*. The type strain of this species was therefore included in our studies. The observed morphological differences between strain Tü 6040 and the type strain of *S. violaceusniger* DSM 40563^T during our study led us to conclude that Tü 6040 is not affiliated with *S. violaceusniger*.

Table 2. Carbohydrate utilization by strain Tü 6040 and other streptomycetes according to the procedure of SHIRLING & GOTTLIEB[10]

	Tü 6040	*S. naganishii* DSM 40282^T	*S. antibioticus* IMET 40227^T	*S. violaceusniger* DSM 40563^T
D-Glucose	+	+	+	+
D-Fructose	+	+	+	+
Raffinose	+	+	-	+
Sucrose	-	-	-	+
D-Xylose	+	+	+	+
Cellulose	-	-	-	-
L-Arabinose	+	+	+	+
D-Mannitol	+	+	+	+
myo-Inositol	+	+	+	+
Rhamnose	+	+	+	+

Growth: +, good; -, none.

Fermentation and Isolation

Batch fermentations of *Streptomyces antibioticus* Tü 6040 were carried out in a 20-litre fermenter using a complex medium that consisted of mannitol 2% and soybean meal 2% (pH 7.5). Production of **1** and **2** started at about 25 hours and reached a maximum after 70 hours with a concentration of 30 and 26 mg/litre, respectively, in the mycelium. The beginning of production was correlated with the depletion of the carbon source at the end of the growth phase. The time course of a representative fermentation is shown in Fig. 3.

The simocyclinones were isolated from the mycelium by extraction with MeOH. The extract was concentrated, extracted at pH 4 with ethyl acetate, and subjected to a LiChroprep Diol column to separate **1** from **2** with CH_2Cl_2-MeOH gradient elution. Finally, pure simocyclinones were obtained after preparative reversed-phase HPLC using Nucleosil-100 C-18 material and 0.01% trifluoroacetic acid-acetonitrile gradient elution, resulting in yellow powders after concentration to dryness.

Characterization and Physico-chemical Properties

1 and **2** in the mycelium extract were characterized as polyene-type antibiotics by means of our HPLC-UV-Vis database. The molecular masses were determined by HPLC-ESI-MS as 897 and 931, respectively. The novelty of the compounds was confirmed during the investigations of structure elucidation. The physico-chemical properties of **1** and **2** are summerized in Table 3, and structures are shown in Fig. 2. In addition to these two major compounds further metabolites belonging to the simocyclinone group were also detected in the mycelium and culture filtrate extracts. The production of these compounds is dependent on fermentation conditions, especially on the medium composition[1]. HPLC-MS data and structure elucidation suggested, that these compounds are precursors of **1** and **2**.

Biological Properties

The antimicrobial spectra of **1** and **2** were determined by an agar plate diffusion assay (Table 4), and the minimal inhibition concentration by the broth dilution method

Fig. 1. HPLC analysis of a mycelium extract of *S. antibioticus* Tü 6040 monitored at 210 nm.

Insert: UV-visible spectrum of simocyclinone D8 (**2**; 10.7 minutes).

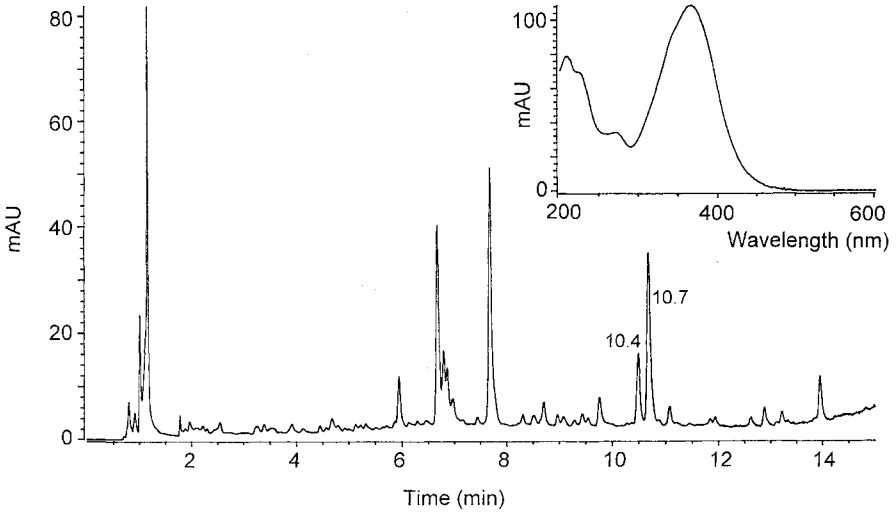
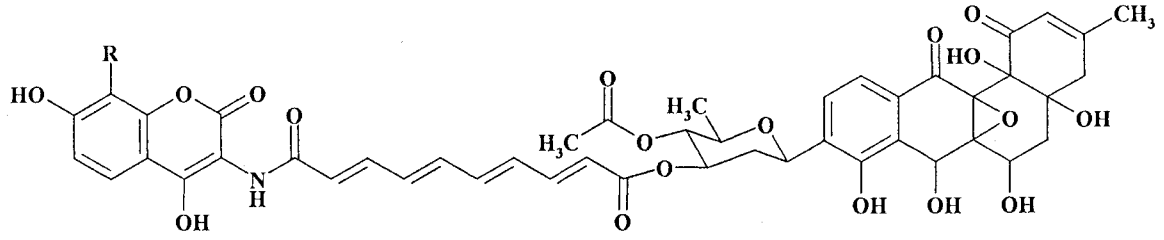


Fig. 2. Structures of simocyclinones D4 (**1**) and D8 (**2**), produced by *S. antibioticus* Tü 6040.



1 : R = H (Simocyclinone D4)

2 : R = Cl (Simocyclinone D8)

(Table 5). Both metabolites showed antibiotic activities against Gram-positive bacteria. Gram-negative bacteria, such as *Agrobacterium tumefaciens*, *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas fluorescens* were not inhibited. The filamentous fungi *Botrytis cinerea*, *Mucor hiemalis*, *Paecilomyces varioti* and *Penicillium notatum* were not sensitive to the simocyclinones, as well as the yeasts *Saccharomyces cerevisiae*, *Rhodotorula rubra* and *Candida albicans*. The cytostatic and cytotoxic activities of **1** and **2** are given in Table 6. Both simocyclinones exibited better growth inhibition against HMO 2 and MCF 7 cells than 5-fluorouracil, whereas both compounds were less active towards HEP G2 cells.

Discussion

The combined use of HPLC-DAD and HPLC-MS techniques in a chemical screening program for the detection of novel secondary metabolites also provides additional insight into the biosynthetic capacity of a microbial culture being examined. The metabolite pattern can be interpreted rapidly with the regard to the type of natural substances and their molecular mass. This, together

Table 3. Physico-chemical properties of simocyclinones D4 (**1**) and D8 (**2**).

	1	**2**
Appearence	yellow powder	yellow powder
$[\alpha]_D^{20}$ (MeOH)	+ 61.6° (c = 0.66)	+ 66.3° (c = 0.77)
Mp (°C)	> 200	> 200
Rf-values(silica gel)		
CH_3Cl/MeOH, 9:1	0.23	0.18
BuOH/HAc/H_2O, 4:1:5	0.89	0.86
Molecular formula	$C_{46}H_{43}NO_{18}$	$C_{46}H_{42}NO_{18}Cl$
Molecular weight	897.8	932.3
FAB-MS (m/z)	898.7 $[M+H]^+$	954.3 $[M+Na]^+$
UV (MeOH) λ_{max} nm (log ε)	215 (4.54),	225 (4.52)
	278 (4.17),	277 (4.09)
	340 (4.71)	339 (4.71)
IR (KBr) ν_{max} cm^{-1}	3352, 2935, 1695, 1633,	3427, 2934, 1698, 1619,
	1579, 1437, 1372, 1240,	1572, 1528, 1436, 1369,
	1124, 1089, 1050, 1010	1238, 1126, 1087,1048, 1012
Elemental analysis		
Calculated	n.d.	C 59.26, H 4.54, N 1.50
Found	n.d.	C 59.55, H 4.71, N 1.42

with searches in commercial databases permit quick identification and determination of compounds' novelty in the very early stage of investigation. In case of streptomycete strain Tü 6040, two metabolites were detected in the mycelium extract by HPLC-DAD-MS and determined to be novel on the basis of their UV-visible spectra, molecular masses and the data available from commercial databases.

The simocyclinones D4 (**1**) and D8 (**2**) are characterized by weak activity against Gram-positive bacteria with the exception of *S. viridochromogenes* Tü 57, which is in general sensitive to various antibiotics. The producing strain itself is not sensitive to its metabolites. Simocyclinones did not inhibit the growth of Gram-negative bacteria, yeasts or filamentous fungi at concentration of 100 μg/ml. However, **1** and **2** show distinct cytostatic activities against the human tumor cell lines HMO 2 and MCF 7 without significant cytotoxicity.

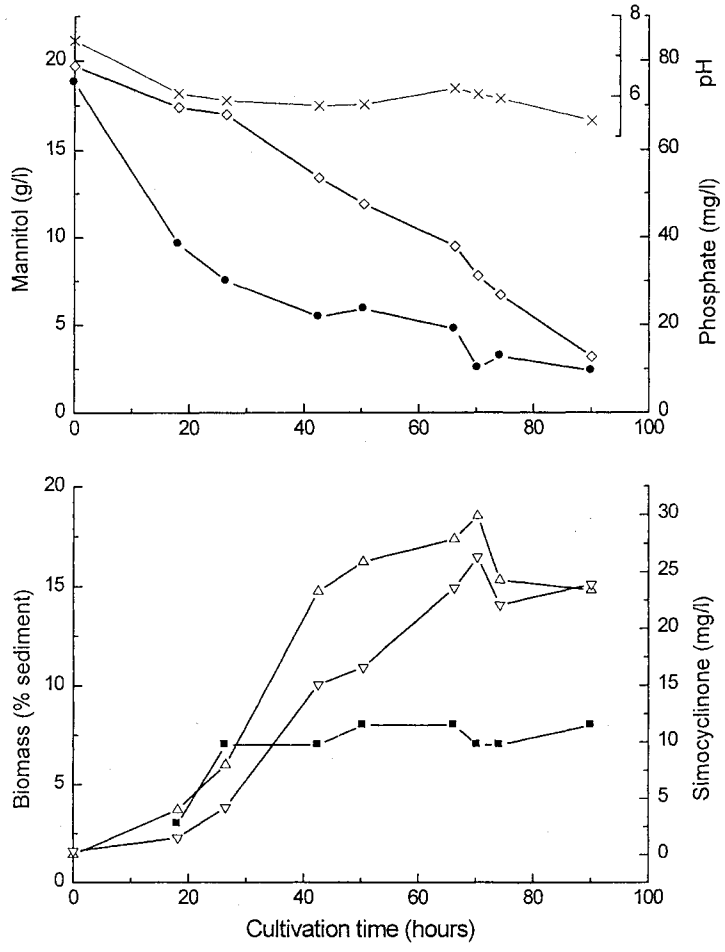
Experimental

Microorganisms

Strain Tü 6040 was isolated from a soil sample collected in Iguaçu, Argentina, and was identified according to HÜTTER[12] and SHIRLING & GOTTLIEB[10] as a strain of *S.*

Fig. 3. 20-litre batch-fermentation of *S. antibioticus* Tü 6040.

◇ mannitol; ● phosphate; × pH; ■ biomass △ simocyclinone D4 (**1**); ▽ simocyclinone D8 (**2**).



antibioticus. It is deposited in the culture collection of our institute.

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

Fermentation

S. antibioticus Tü 6040 was cultivated in a 20-litre fermenter (type b20, Giovanola) using a production medium consisted of mannitol 2% and soybean meal 2% in tap water (pH 7.5, adjusted with 5 M NaOH). 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 at 27°C in the same medium. The fermentation was carried out for 96 hours at 27°C with an aeration rate of 0.5 v/v/m and an agitation of 1000 rpm.

The production of **1** and **2** was measured by reversed-phase HPLC. Mannitol was determined by a HPLC method described by PLAGA *et al.*[13]. Phosphate was determined as described by COOPER[14].

Isolation

Hyphlo Super-cel (2%) was added to the fermentation broth which was separated by multiple sheet filtration into culture filtrate and mycelium. For the isolation of **1** and **2**, the mycelium cake was extracted three times with MeOH. After concentration *in vacuo*, the aqueous residue was adjusted to pH 4 (1 M HCl) and extracted four times with ethyl acetate. After concentration of the organic extract to dryness, it was dissolved in CH_2Cl_2 and applied to a diol-modified silica gel column (LiChroprep Diol, 40~63 μm, Merck; 460×26 mm). **1** and **2** were separated by linear

Table 4. Antimicrobial spectrum of simocyclinones D4 (**1**) and D8 (**2**) as determined by the agar plate diffusion assay at a concentration of 1 mg/ml.

Organism	Inhibition zone (mm)	
	1	**2**
Bacillus brevis DSM 30[a]	(13)	7 (12)
Bacillus brevis DSM 30[b]	23	18
Bacillus subtilis DSM 10[a]	9 (15)	(9)
Bacillus subtilis DSM 10[b]	21	12
Micrococcus luteus ATCC 381[a]	7	8 (11)
Staphylococcus aureus DSM 20231[a]	13	12
Streptomyces viridochromogenes Tü 57[a]	43	46

[a] Complex medium.

[b] Chemically defined medium (per litre): glucose 10 g, $(NH_4)_2SO_4$ 2 g, KCl 0.3 g, $MgSO_4 \times 7\ H_2O$ 0.15 g, $CaCl_2 \times 7\ H_2O$ 94 mg, $CuSO_4 \times 5\ H_2O$ 0.8 mg, $FeCl_3 \times 6\ H_2O$ 4.8 mg, $MnSO_4 \times 2\ H_2O$ 3.5 mg, biotin 0.01 mg, *myo*-inositol 20 mg, Ca-pantothenate 10 mg, thiamine hydrochloride 2 mg, pyridoxal phosphate 0.5 mg, Bacto agar 15 g (pH 6.8).

( ) Partial inhibition.

Table 5. Minimal inhibition concentrations of simocyclinones D4 (**1**) and D8 (**2**) as determined by the broth dilution method.

Organism	MIC (μg/ml)	
	1	**2**
Bacillus subtilis DSM 10	>100	>100
Bacillus brevis DSM 30	30	10
Staphylococcus aureus DSM 20231	100	100
Streptomyces viridochromogenes Tü 57	1	1
Streptomyces coelicolor MÜLLER DSM 3030	10	10
Streptomyces antibioticus Tü 6040	>100	>100

gradient elution from CH_2Cl_2 to CH_2Cl_2-MeOH (95 : 5) within 3 hours at a flow rate of 360 ml/hour (medium pressure pump Mod. 381, gradient controller Mod. 388, Büchi). Fractions containing **1** and **2**, respectively, were concentrated to dryness. Pure simocyclinones 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% trifluoracetic acid-ACN, starting from 50% ACN to 70% ACN within 30 minutes at a flow rate of 20 ml/minute. The preparative system consisted of two high-pressure pumps (Sepapress HPP-200/100; Kronwald), gradient unit

Table 6. Activities (μmol/litre) of simocyclinones D4 (**1**) and D8 (**2**) against selected human tumor cell lines.

	GI_{50}[a]			TGI[b]			LC_{50}[c]		
	HMO 2	MCF 7	HEP G2	HMO 2	MCF 7	HEP G2	HMO 2	MCF 7	HEP G2
1	0.30	5.6	8.5	> 50[d]	10	25	> 50	> 50	> 50
2	0.50	0.95	> 50	> 50[d]	1.0	> 50	> 50	> 50	> 50
5-Fluorouracil	1.2	50	0.1	18	> 50	25	> 50	> 50	> 50
Cis-Platinium	0.17	0.1	0.5	1.5	10	5.0	36	> 50	> 50

[a] Drug concentration causing 50% growth inhibition [b] Drug concentration causing 100% growth inhibition

[c] Drug concentraion causing 50% reduction of the cells present at time point zero, *i.e.* at 24 hours

[d] Note: **1** and **2** caused 90-95% growth inhibition at 1 μmol/litre, but failed to reach total (100%) growth inhibition

(Sepacon GCU-311) and a Valco preparative injection valve (Mod. 6UW; VICI) with a 5-ml sample loop. The UV absorbance of the eluate was monitored simultaneously at 232 and 360 nm by a Gilson spectrophotometer Mod. 116, equipped with a preparative cell (0.2 mm pathlength, 0.7 μl volume). After lyophilization **1** and **2** were obtained as yellow powders.

HPLC-DAD-Analysis

The chromatographic system consisted of a HP 1090M liquid chromatograph equipped with a built-in diode-array detector and HP 79994B workstation (Hewlett-Packard). Multiple wavelength monitoring was performed at 210, 230, 260, 280, 310, 360 and 435 nm. The spectrum range was from 200 to 600 nm with a 2-nm step and a sampling interval of 640 mseconds.

For analysing the mycelium, a 10 ml-sample of the fermentation broth was centrifuged (10 minutes at 13,000 *g*). The mycelium cake was extracted twice with MeOH, concentrated to dryness and dissolved in 1 ml MeOH. Ten μl of the samples were 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 (Grom). The samples were analysed by linear gradient elution using 0.1% 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 1-minute hold at 100% B, followed by a 5-minute post-time at initial conditions.

HPLC-ESI-MS-Analysis

The system consisted of an ABI 140A HPLC gradient pump (Applied Biosystems) and an API III Taga 6000 E mass spectrometer equipped with a nebulizer-assisted electrospray source (Sciex). 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 3-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 60 minutes at a flow rate of 200 μl/minute.

Biological Assays

An agar plate diffusion assay was used to determine the antimicrobial spectrum of the simocyclinones. Ten μl of the samples were applied to filter disks (6 mm diameter). The test plates were incubated for 24~48 hours at a temperature that permitted an optimal growth of the microorganisms.

A broth dilution method was used to determine the minimal inhibition concentrations of the simocyclinones. The antibiotics were dissolved in DMSO with final DMSO concentrations in the cultures not to exceed 5%. The bacteria were grown in a medium consisted of nutrient broth 0.8% and NaCl 0.5% prepared in tap water. Streptomycetes cultures were grown in malt extract 1%, glucose 0.4% and yeast extract 0.4% in tap water (pH 7.3). Bacterial cells and spores at 10^6 cells/ml were used as inocula and growth inhibition was evaluated after incubation for 24 and 96 hours at 27°C and 37°C,

respectively, on a rotary shaker.

The antitumor activity of **1** and **2** was tested according to NCI guidelines[15] with human cell lines from gastric adenocarcinoma (HMO 2), mamma carcinoma (MCF 7) and hepatocellular carcinoma (HEP G2). Cells were grown in 96-well microtiterplates in RPMI 1640 with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 in air. After 24 hours of incubation **1** and **2** (0.1~50 μmol/litre) were added to the cells. Stock solutions were prepared in DMSO. After 48 hour incubation period the cells were fixed, and cell protein was assayed with sulforhodamine B.

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