GERMICIDIN, AN AUTOREGULATIVE GERMINATION INHIBITOR OF *Streptomyces viridochromogenes* NRRL B-1551

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(Received for publication December 7, 1992)

During germination spores of *Streptomyces viridochromogenes* NRRL B-1551 excrete a compound, germicidin, which has an inhibitory effect on the germination of its own arthrospores at a concentration as low as 200 pM (40 pg/ml). At higher concentrations germicidin inhibits porcine Na^+/K^+-activated ATPase and retards the germination of the cress *Lepidium sativum*. Germicidin is the first known autoregulative inhibitor of spore germination in the genus *Streptomyces* and was isolated from the supernatant of germinated spores, but also from the supernatant of the submerged culture.

Spectroscopic analysis and derivatization reactions revealed germicidin to be 6-(2-butyl)-3-ethyl-4-hydroxy-2-pyrone ($C_{11}H_{16}O_3$). Crude isolates of germicidin from the supernatant of submerged culture, but not from the spores, contained a second, structurally very similar compound ($C_{10}H_{14}O_3$), in which in contrast to germicidin a 2-propyl instead of the 2-butyl chain was bound to C-6 and which did not show any activity in the germination and ATPase assay. The germination assay was evaluated as a new screening model for specifically active compounds.

In the course of our screening program for new bioactive metabolites we are working with different cyto-differentiation assays because of their high specificity and sensitivity for autoregulative compounds. Since the selection pressure on such substances is estimated to be low, especially in this field, a large number of new natural metabolites could be expected. The genus *Streptomyces* belongs to a group of microorganisms, that are characterized by a complex change of their morphology. As a necessity of the permanently changing environmental and growth conditions, essential stages during their cell cycles *e.g.*, germination, vegetative cells and sporulation, are influenced by specific effectors[1].

Already in 1978 HIRSCH and ENSIGN described a low-molecular weight, acidic substance which is excreted by germinating spores of *Streptomyces viridochromogenes* NRRL B-1551 and which inhibits germination of another suspension of dormant arthrospores[2]. The inhibitor was shown to influence the sporal respiratory chain and to interact with the sporal Ca^{2+}-activated ATPase, preventing the support

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of energy required for germination[3,4]. Here we present results on the isolation, structure elucidation and biological activities of the autoregulative inhibitor, germicidin (**1**). For the detection of the germination inhibitory activity we used the germination assay described by HIRSCH and ENSIGN, which is based on the change of the optical density of the spore suspension[5].

Fermentation

An extensive optimization of the production medium of *Streptomyces viridochromogenes* NRRL B-1551 was carried out in order to enhance the titer of **1**. Whereas the variation of different carbon-sources (*e.g.*, glucose, succinic acid, glycerol, mannit, citric acid, starch, saccharose) had no significant effects, the presence of glutamic acid (Fig. 1), Na^+ (Fig. 2), and sulfate (Fig. 3) enhanced the production of the inhibitor. Best results were obtained with a fermentation medium consisting of 10.0 g pepton from casein, 0.5 g yeast extract, 0.1 g NaCl, 0.5 g K_2SO_4 and 1.5 g L-glutamic acid per liter. The diagram in Fig. 4 shows the inhibitory activity, pH, pO_2 and the dry weight during fermentation of a 25-liter batch.

Isolation and Purification

1 was isolated from the supernatant of submerged culture of *Streptomyces viridochromogenes* NRRL B-1551 by adsorption and size exclusion chromatography (isolation scheme see Fig. 5). Final purification was achieved by preparative RP-HPLC. During work-up individual fractions were tested for inhibitory activity by measuring the optical density (OD) of the spore suspension at 578 nm. The OD decreases by water up-take during sporulation. Therefore the decrease of absorbance is a direct measure for germinability of the spore[5].

After chromatography on TSK HW-40 the combined active fractions gave two peaks in reversed phase C-18 HPLC. The less polar and more

Fig. 1. Influence of different concentrations of glutamate on the production of **1** in the optimized medium.

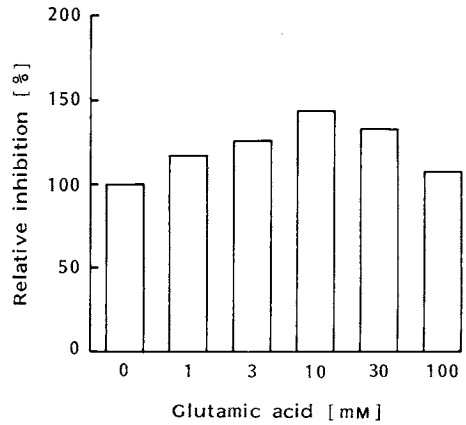


Fig. 2. Influence of different concentrations of sodium chloride on the production of **1** in the optimized medium.

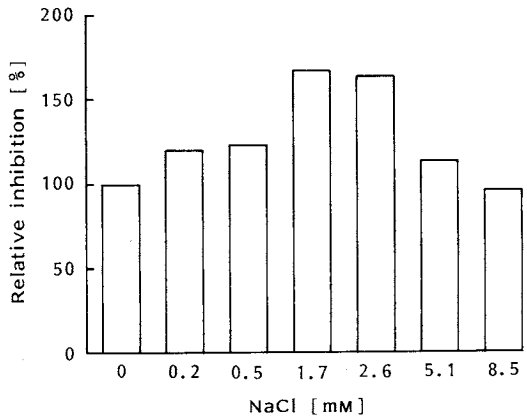


Fig. 3. Influence of different concentrations of potassium sulfate on the production of **1** in the optimized medium.

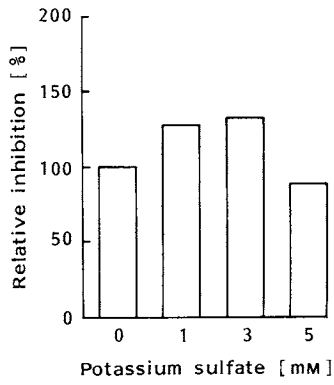


Fig. 4. Fermentation of *Streptomyces viridochromogenes* NRRL B-1551 in the optimized medium.
△ Inhibition, ○ pH, ■ pO_2, □ dry weight.

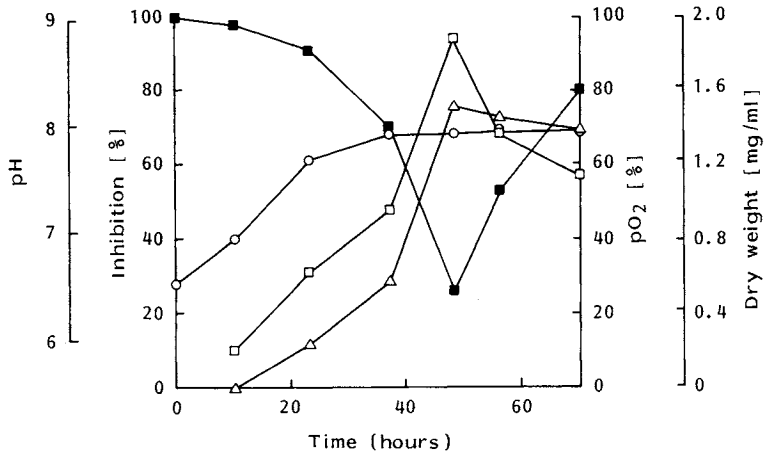


Fig. 5. Isolation of **1** and **2**.

Broth filtrated (25 liters)
- adsorbed to Amberlite XAD-4 resin
  eluted with 100% MeOH

Aqueous residue (pH 2.5)
- extracted with
  ethyl acetate - hexane (5 : 2)
- flash chromatography on silica gel
  eluted with ethyl acetate - hexane (5 : 2)
- chromatography on Sephadex LH-20
  eluted with MeOH
- chromatography on TSK HW-40
  eluted with MeOH
- preparative C-18 HPLC
  eluted with 30 to 70% MeOH in 0.2% aqueous acetic acid (linear gradient)

1 (germicidin) and **2** (1～2 mg each)

Table 1. Relative intensities (in %) of characteristic mass spectral fragments of **1** and **2** obtained by EI-MS and CID-MS of the respective *quasi*-molecular ions in positive (+) and negative (−) ion mode (—: intensity <1%).

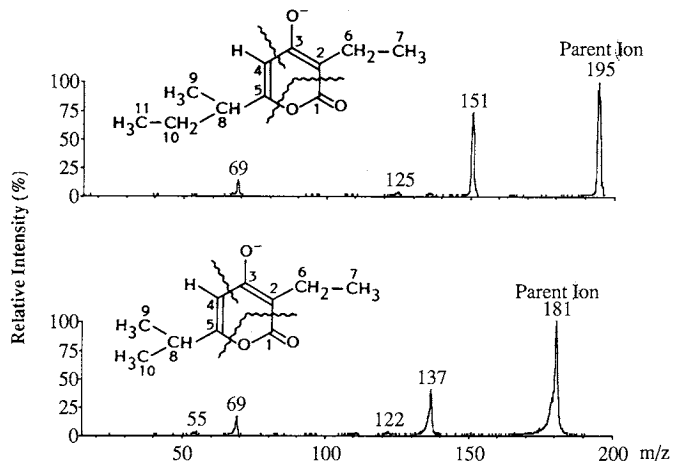
m/z	**1**			**2**	
	EI+	CID+	CID−	EI+	CID−
41	23	—	—	27	—
43	11	2.0	—	20	—
55	19	—	—	22	—
57	8	4.5	—	5	—
69	30	2.7	11.4	34	16.4
83	18	4.5	—	18	—
97	25	4.8	—	25	—
111	16	—	—	16	—
113	24	—	—	20	—
123	—	2.1	—	—	—
125	6	—	—	24	—
127	17	—	—	—	—
137	—	—	—	—	40.0
139	—	—	100		100
151	—	1.0	72.0	—	—
153	12	—	—	—	—
154	—	—	—	25	—
167	—	—	—	28	—
168	21	6.9	—	—	—
181	23	—	—	—	100.0
182	2	—	—	49	—
195	—	—	100.0	—	—
196	33	—	—	—	—
197	—	100	—	—	—

abundant component **1** was biologically active, whereas the second compound **2** eluting before **1**, did not show any germination inhibitory activity. Preparative HPLC afforded **1** and **2** in pure form.

Structure Elucidation

EI-MS of **1** and **2** showed an almost identical fragmentation pattern with a base ion peak at *m/z* 139 (Table 1). High resolution EI-MS of M^+ were recorded as *m/z* 196.1099 for **1** and *m/z* 182.0943 for **2**, corresponding to the formulae $C_{11}H_{16}O_3$ and $C_{10}H_{14}O_3$, respectively. A similar EI-MS fragmentation pattern as observed for **1** and **2** is found for 3-acyl-6-alkyl-4-hydroxy-2-pyrones, where the most

Fig. 6. Negative ion CID IS-MS of $(M-H)^-$ of **1** (parent ion *m/z* 195) and **2** (parent ion *m/z* 181).



abundant fragment ion in the spectrum is formed by cleavage of the 6-alkyl substituent[6]. Methanolic solutions of both **1** and **2** had a UV absorption maximum at 290 nm, which indicated their close structural relationship. Both compounds decolorized aqueous solutions of bromine.

In addition to EI-MS, **1** and **2** were investigated by ion spray mass spectrometry (IS-MS; pneumatically assisted electrospray)[7]. The positive ion IS-MS of **1** and **2** showed the *quasi*-molecular ions $(M+H)^+$ and $(M+Na)^+$ confirming the masses determined by EI-MS. In the negative ion mode the *quasi*-molecular ions were obtained as $(M-H)^-$, indicating the presence of an acidic functional group in both **1** and **2**. Additional structural information was obtained by IS tandem mass spectrometry (MS/MS), where the *quasi*-molecular ions were fragmented with argon in the collision region of the triple-quadrupole mass spectrometer (collision activated dissociation, CID). The positive ion CID spectra of $(M+H)^+$ of **1** and **2** showed a characteristic fragment with a mass differing by 29 amu from the mass of the parent ion (Table 1). In the negative ion CID spectra of **1** and **2** fragment ions at *m/z* 151 and 137, respectively, differing by 44 amu ($-CO_2$) from the $(M-H)^-$ were observed (Fig. 6). An ion at *m/z* 69 was common in both spectra. Derivatization of **1** with diazomethane afforded products which were 14 amu and 28 amu higher in mass than the educt. By on-line coupling of reversed-phase HPLC to the IS mass spectrometer (HPLC/MS), it could be shown that two different mono-methylated products **3a/b** (*m/z* 211) were formed, which under isocratic conditions (40% acetonitrile in 0.5% aqueous formic acid) eluted at 7.2 and 9.9 minutes, respectively (Fig. 7, upper part). In addition, two derivatives **3c/d** in lower amounts, both with masses of *m/z* 215, eluting at 12.1 and 16.9 minutes, could be detected. These derivatives showed abundant fragment ions at *m/z* 197 (Fig. 7, lower part). Reaction of **1** with acetic anhydride yielded an unstable mono-acetyl compound. IS-MS of the acetylation reaction mixture showed the $(M+H)^+$, $(M+Na)^+$ and $(M+K)^+$ of a mono-acetyl derivative **4**. Attempts to isolate **4** or to identify it by HPLC-MS failed. After addition of LiCl to the acetylation mixture, clustering of the molecular ion with H^+, Na^+ and K^+ was suppressed, and the formation of the *quasi*-molecular ion $(M+Li)^+$ **4** could be observed (spectrum not shown).

NMR Spectroscopy of **1**

2-Dimensional NMR spectroscopy allowed a complete assignment of all protons and carbon signals

Fig. 7. HPLC IS-MS of methylated **1** (nucleosil C-18, 5 μm; 100 × 2 mm i.d.; flow rate 200 μl/minute; isocratic 40% acetonitrile in 0.5% aqueous formic acid.

Reconstructed total ion current chromatogram (above) and IS mass spectra of **3a**~**3d** obtained in the apices of peaks 1~4 (below).

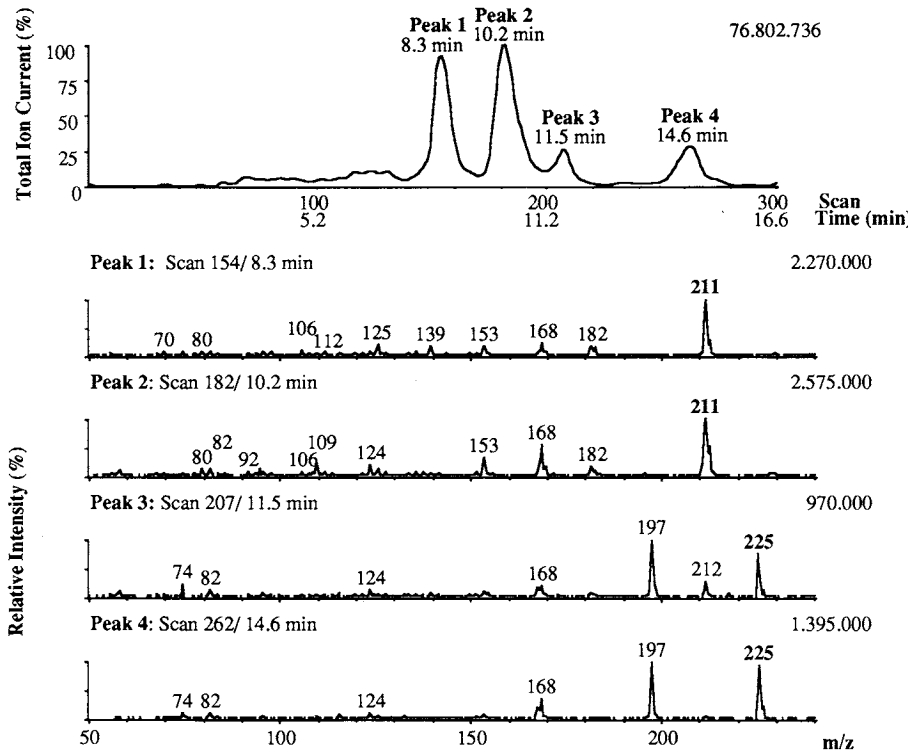


Table 2. ^{13}C NMR and ^{1}H NMR chemical shifts of **1** in $CDCl_3$ and C_6D_6.

	^{13}C (100 MHz) ppm		^{1}H (400 MHz) ppm (J, Hz)	
	$CDCl_3$	C_6D_6	$CDCl_3$	C_6D_6
C-1	167.5 s	168.6 s	—	—
C-2	104.7 s	105.0 s	—	—
C-3	167.2 s	167.8 s	—	—
C-4	99.6 d	100.4 d	6.08 s	6.32 s
C-5	165.9 s	167.3 s	—	—
C-6	16.5 t	17.0 t	2.46 q, J=7.4	2.79 q, J=7.3
C-7	12.5 q	12.9 q	1.08 t, J=7.4	1.30 t, J=7.3
C-8	39.7 d	39.7 d	2.41 m	2.00 m
C-9	17.7 q	17.6 q	1.16 d, J=7.0	0.90 d, J=6.9
C-10	27.4 t	27.5 t	a 1.49 m	a 1.23 m
			b 1.65 m	b 1.48 m
C-11	11.5 q	11.5 q	0.84 t, J=7.4	0.63 t, J=7.4
O-H	—	—	n.d.	(10.50 s, broad)

Numbering of carbon atoms see Fig. 6; n.d., not determined.

of **1** in $CDCl_3$ and C_6D_6 (Table 2). The H-H COSY spectrum of **1** revealed three spin systems: an isolated CH group, an ethyl and a 2-butyl group. The assignment of protons attached to carbon atoms was obtained by a proton detected heteronuclear multiple quantum coherence (HMQC)[8] spectrum. Quarternary carbons

Fig. 8. (a) ^{1}H-^{1}H NOESY and (b) ^{1}H-^{13}C long range coupling patterns of **1**.

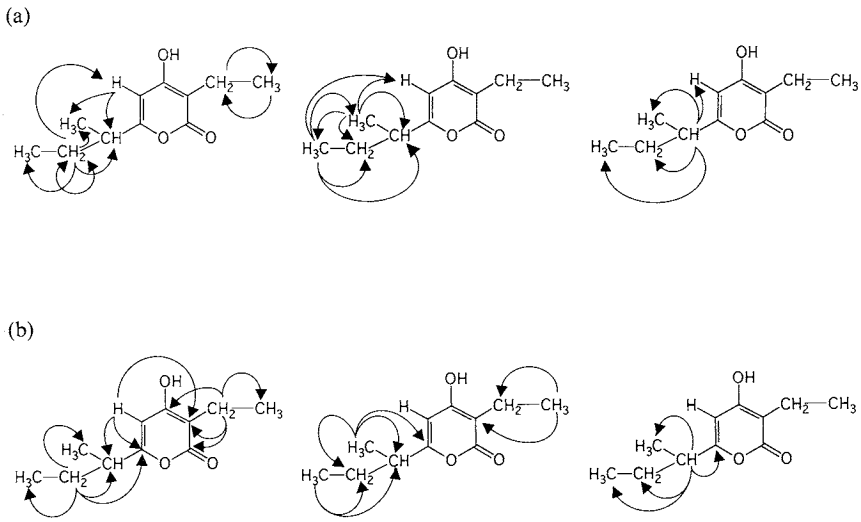
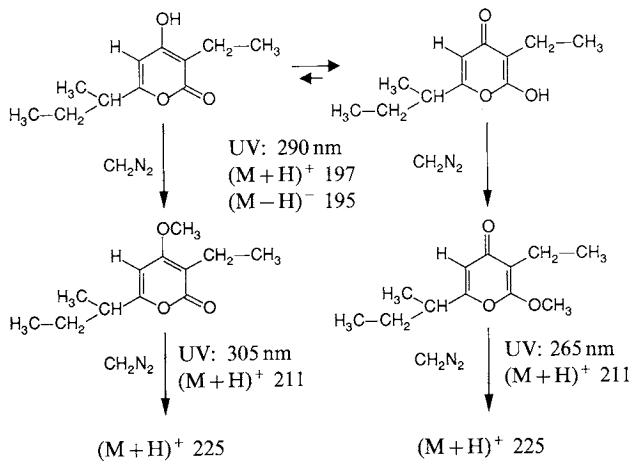


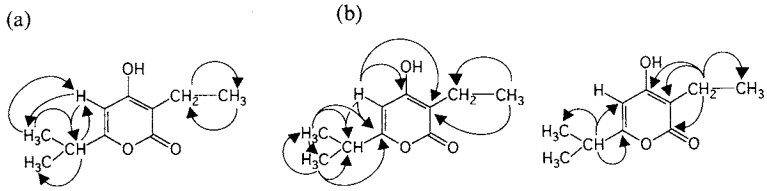
Fig. 9. Products obtained by methylation of **1** with diazomethane.



were determined in a heteronuclear multiple bond correlation (HMBC)[9] spectrum, which also established links between the fragments (Fig. 8b) obtained from homonuclear techniques. The position of the substituents was further confirmed by a NOESY spectrum (Fig. 8a). NMR (Table 2) as well as the UV and MS data were in agreement with the structure of 6-(2-butyl)-3-ethyl-4-hydroxy-2-pyrone. In the ^{1}H NMR spectrum of **1** in C_6D_6 at 300 K the enolic proton was observed as a very broad signal at 10.5 ppm. When $CDCl_3$ was used instead of C_6D_6 as solvent for NMR, a shift of signals (aromatic solvent induced shift, ASIS) due to an anisotropy effect was observed, which is characteristics of lactones possessing a dipole moment[10] (Table 2). As expected for this proposed structure, two methylation products were formed with diazomethane (Fig. 9). λ_{max}^{MeOH} of the 4-methoxy derivative was observed at 305 nm, whereas the 2-methoxy derivative had an absorption maximum at 265 nm.

The determination of the absolute configuration at the asymmetric carbon atom of the 2-butyl chain

Fig. 10. (a) ^{1}H-^{1}H NOESY and (b) ^{1}H-^{13}C long range coupling patterns of **2**.



of **1** will be carried out in connection with the total synthesis.

The structure of **2** was elucidated using the same NMR techniques as for **1**. Analysis of HMBC and NOESY spectra (coupling patterns see Fig. 10) proved the biologically inactive **2** to be 3-ethyl-4-hydroxy-6-(2-propyl)-2-pyrone.

Identification of 1 of the Autoregulative Inhibitor of Spore Germination

The amount of germination inhibitor in spores was expected to be low. Therefore, we first attempted to identify the germination inhibitory activity in the supernatant of submerged culture by RP HPLC and UV photodiode array detection. After such characterization, detection of the inhibitor in the germination supernatant of the spores was also attempted. A large amount of spores was obtained from slant cultures and germinated in the indicated medium (see Experimental). The active spore supernatant was subjected to several chromatographic steps (Fig. 5). After gel chromatography on Sephadex LH-20 the fractions with inhibitory activity were collected, concentrated and investigated by HPLC combined with photo diode array detection. It could be shown that the retention time as well as the UV spectrum of the main peak in the HPLC chromatogram was identical with the data of **1** isolated from submerged culture. Interestingly, **2** was not observed in the spore supernatant.

Biological Activity of 1

Minimal Detection Level

In a dilution series the minimal concentration of **1** was determined at which a retardation of spore germination was just detectable. The autoregulator had an effect on germination at a concentration of 200 pM (40 pg/ml), which is only about 2,400 molecules per spore (Fig. 11).

Anti-microbial and Anti-nematodal Activity

1 has a weak activity against strains of *Streptomyces viridochromogenes* Tü 57 and Tü 2580 and *Streptomyces griseus* Tü 2599 species. It does not inhibit the growth of various other Gram-positive and Gram-negative bacteria and various fungi. At a concentration of 1 mg/ml it neither reduced the mobility of *Caenorabditis elegans*, nor was it toxic for this species.

Inhibition of Porcine Na^+/K^+ ATPase

The activity of a porcine ATPase was inhibited by **1**. The ID_{50} was determined[4] to be at 100 μM (Fig. 12). **2** did not inhibit the enzyme. None of the two metabolites had an effect on the activity of a dog kidney ATPase.

Phytotoxicity Test

Pyrones have been described as natural germination inhibitors of plants[11]. Therefore **1** was tested

Fig. 11. Estimation of minimal inhibition concentration of **1** (1×10^{10} spores/ml; incubation time after addition of **1**: 1 hour).

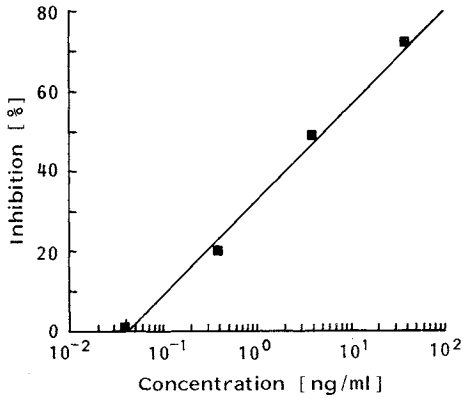


Fig. 12. Inhibition of a Na^+/K^+-dependent ATPase isolated from porcine brain by **1**.

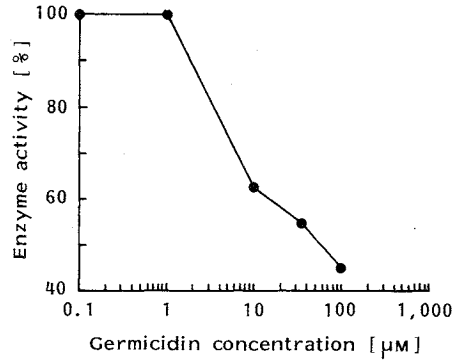
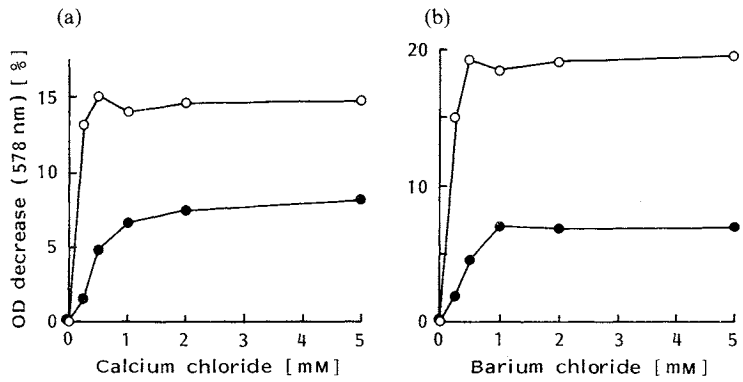


Fig. 13. Effect of (a) Ca^{2+}-ions and (b) Ba^{2+}-ions on germination (○ = in the absence of **1**; ● = in the presence of 50 ng **1** per ml).



for its effect on the tip growth of *Lepidium sativum* root. At a concentration of 1 mg/ml and an incubation time of 24 hours **1** inhibited the growth of the roots completely and caused the swelling of the seeds. After 48 hours the growth was significantly reduced. If 100 μg/ml of **1** were applied for 24 hours, the apical tip growth was reduced to about 30% compared to the control.

Influence of Ca^{2+}, Ba^{2+}, Mg^{2+}, Na^+, and K^+ on Germination Inhibition Induced by **1**

The presence of Ca^{2+} (Fig. 13a) and Ba^{2+} markedly reduced the inhibition of germination by **1** (Fig. 13b). The cations Mg^{2+}, Na^+ and K^+ did not effect the activity of **1**.

Structure/Activity Relationship

Substances involved in cytodifferentiation are active in extremely low concentrations and show a high degree of specificity. We therefore compared the inhibitory activity of the semisynthetic methylation products as well as **2** with the effect of **1** on spore germination. It could be shown that structural alterations as they occur in these derivatives were not tolerated and were connected with the reduction of inhibitory activity by a factor of $>30{,}000$.

Table 3. Germination inhibitory activity of various idiolites.

Idiolite	Minimal detection level (μg/ml)
Acetomycin	10.000
Chartreusin	>20.000
Erythromycin	0.080
Flavensomycin	>20.000
Germicidin	0.000040
Hormaomycin	>20.000
Lysolipin	>20.000
Novobiocin	12.000
Polymyxin	0.100
Rifampicin	2.000
Streptomycin	17.000
Vancomycin	6.000

Table 4. Germination inhibitory activity the supernatants of submerged cultures of various *Streptomyces* strains.

Strain	Inhibition (%)
Streptomyces sp. Tü 3556	0
Streptomyces sp. Tü 2484	13
S. griseoflavus Tü 2880	33
S. olivaceus Tü 2353	38
S. fradidae Tü 2717	48
S. gimbriatus Tü 2335	53
S. viridochromogenes Tü 2580	63
S. viridochromogenes Tü 57	68
S. viridochromogenes Tü 2974	68
S. viridochromogenes Tü 1678	80

Evaluation of the Germination Assay as a New Screening System

Sensitivity of Spore Germination to Various Antibiotics

Different idiolites were tested for their germination inhibitory activity to evaluate the specificity of the germination assay (Table 3). Erythromycin, an inhibitor of protein synthesis, showed the lowest detection level among the idiolites tested, however, it was still by a factor of >2,000 less active than **1**. These results show that in this screening system the probability of cross-reactivity with common antibiotics is low.

Screening for Inhibitory Activity in Supernatants of the Culture Broth of Various *Streptomyces* Strains

Ten *Streptomyces* strains were cultivated in our optimized production medium (see Experimental), and the formation of the activity was monitored in the course of four days (Table 4). All strains producing high inhibitory activity belong to the species of '*viridochromogenes*'. Nine out of ten supernatants were active in the spore assay. The supernatant of *Streptomyces viridochromogenes* Tü 1678 was partially purified. Comparison of the UV spectrum and the retention time with the data of **1** indicated that the inhibitory principle of this strain was identical with **1**.

Discussion

Several lactones and pyrones have been described as auto-inhibitors controlling the germination of plants and fungi[11~13], however, at substantially higher concentrations than the concentration at which the germination inhibitor **1** is active. Alternaric acid from *Alternaria solani* inhibits the germination of *Abscidia glauca*, *Myrothecium verrucaria* and *Stachybotrys atra*[12]. Cryptocaryalactone and its deacylated derivative from the plant *Cryptocarya moscata* suppressed germination of the fungus *Abutilon theophrasti*[13]. Cumarol and scopoletine are known as endogene germination inhibitors[11]. Also, several pyrones are described to be phytotoxic, *e.g.*, hyalopyrone of *Ascochyta hyalospora*[14], colletopyrone from *Colletotrichum nicotianae*[15], citreovirdin of *Penicillium citreoviridae*[16] and (±)-radicinin from *Stemphylium radicinum*[17]. In agreement with these findings, the 4-hydroxy-2-pyrone **1** is phytotoxic for *Lepidium sativum* seeds. Despite the close structural relationship of **1** with nectriapyrone from *Gyrostroma missouriense*[18] and also with synthetic 2,6-dialkyl-4-hydroxy-2-pyrones, which have been reported to be growth inhibitors of bacteria[19], **1** has only a weak and selective activity against few *Streptomyces* strains.

In general, even small alterations of the molecular structure of substances influencing cytodifferentiation lead to a drastic reduction or to the total loss of their bioactivity[1]. This is true also for **1**, for which the

2-butyl chain as well as the intact enolic OH-group and lactone moiety are obviously essential for inhibitory activity.

GRUND and ENSIGN suppose that the mechanism responsible for inhibition is the blocking of calcium-dependent ATPase located in the spore membrane[4]. Ion flux and electrolyte status are essential factors influencing the germination in genus *Bacillus* and *Streptomyces*[20,21]. Investigations of ion channel blockers showed that calcium, potassium and sodium ion fluxes are central factors that influence germination of *Bacillus megaterium* spores[21]. ENSIGN and coworkers showed that the calcium flux of the spores of *Streptomyces viridochromogenes* NRRL B-1551 during germination was blocked by dibucain and tetracain and that calcium channels were involved in the germination process[4]. We found that the ion channel blockers Bay K 8644 and nifedipine were able to retard spore germination, whereas the phenylalkylamine verapamil had no effect. The Na^+ antagonists amilorid hydrochloride, lidocaine, tetrathylammonium chloride and TMB-8 had no influence on spore germination either (data not shown). Since nifedipine and verapamil interact at different sites of the Ca^{2+} channel[22], a specific effect of the dihydropyridines Bay K 8644 and nifedipine must be presumed. The retarding effect of the dihydropyridine on spore germination was antagonized by Ca^{2+}. Interestingly, the inhibitory activity of **1** was also reduced in the presence of Ca^{2+} (see Fig. 13). Thus, it may be that **1** inhibits spore germination by direct or indirect interaction with Ca^{2+}. Since Ca^{2+} flux also plays an important role in the germination of plants[23,24], such an interaction could be also responsible for the inhibiting effect on the germination of cress seeds.

Nectriapyrone, a metabolite structurally related to **1**, is synthesized *via* the polyketide pathway[18]. The finding that ceruline, an inhibitor of the polyketide and fatty acid synthetase, was not able to affect the formation of **1**, makes the biosynthesis of **1** *via* the pentaketide pathway very unlikely.

The spore germination inhibition assay might be an interesting alternative screening system for natural products because it is fast, highly specific and shows low cross-reactivity with known antibiotics. By using this test system, it should be possible to find analogous inhibitors in other spore forming genera and species. For *Bacillus*[25] and *Streptomyces*[26] spore germination media have been already described. Since we showed that **1** is also produced by a second *Streptomyces viridochromogenes* strain, it may be advisable to screen species other than *viridochromogenes* in order to find new structures. However, the occurence of **1** in a second strain of the same species also indicates that it could be a more widespread compound and may represent a common regulatory principle, at least in *Streptomyces viridochromogenes*. Since investigations of this part of cell differentiation has been neglected so far, we expect that the germination inhibition test system allows access to further new natural products.

Experimental

General Methods

Mass Spectrometry

High resolution EI-MS were recorded on a MAT 711A mass spectrometer (*Varian* Bremen, Germany). IS-MS were recorded on a API III Taga 6000 E equipped with an ion spray source (Sciex, Thornhill, Canada).

The samples were analyzed by ion spray mass spectrometry and ion spray tandem mass spectrometry (MS-MS) either directly or on-line after separation on a C-18 reversed phase column (Synchropak C-18, 5 μm, 100 × 2 mm, i.d.; Grom, Herrenberg, Germany). An Applied Biosystem ABI 140A (Weiterstadt, Germany) HPLC-pump was used for on-line HPLC-MS. The samples were dissolved in 0.1% formic acid-methanol (1 : 1). Lithium chloride was added in form of a 10 mM aqueous solution in order to simplify identification of molecular peaks and to suppress fragmentation. For direct injection, the solution was introduced into the ion spray source at a constant flow rate of 5 μl/minute with a microliter syringe using a medical infusion pump (Harvard Apparatus, USA). Argon was used as collision gas for tandem mass spectrometry.

NMR Spectroscopy

High resolution NMR spectra were obtained on a Bruker AMX 360 spectrometer equipped with an inverse multinuclear probe (Bruker Analytische Meßtechnik, Karlsruhe, Germany). Five mM solutions in

C_6D_6 or $CDCl_3$ were used. Chemical shifts were referenced to the solvent peak (C_6D_6: $\delta_{^1H}=7.15$ ppm, $\delta_{^{13}C}=128.0$ ppm; $CDCl_3$: $\delta_{^1H}=7.25$ ppm, $\delta_{^{13}C}=77.0$ ppm). The two-dimensional data set consisted of COSY, NOESY, inverse detected HMQC and HMBC spectra with a sweep width in the F2 dimension of 3300 Hz. All two dimensional spectra were acquired as 256 experiments with 32 scans and a data size of 2 kilobyte at 300 K in the phase sensitive absorption mode with quadrature detection in both dimensions, using the time-proportional phase-increment method (TPPI). Standard pulse programs from the Bruker library were used. The mixing time in the NOESY experiment was set to 1 second. The inverse detected H-C COSY contained a BIRD pulse sequence[8] to suppress the signals of protons bound to ^{12}C. During acquisition, proton decoupling was achieved with a GARP composite pulse sequence. The sweep width in the F1 dimension was set to 135 ppm. The long range H-C correlated HMBC was acquired with 64 scans per increment. No decoupling was used during acquisition. Data processing consisted of zerofilling up to 2K (F2) in the F1 dimension. A squared sine bell weighting function shifted $\pi/2$ was used in both dimensions.

TLC and Column Chromatography

Rf values were determined on preformed plates $60F_{254}$, 5×10 cm (E. Merck, Darmstadt, Germany). Sephadex LH-20 (Pharmacia; Freiburg, Germany) and TSK HW-40 (F) were used for column chromatography. **1** and **2** were visualized by UV (254 nm).

Preparation of a Spore Suspension

The spore suspension was prepared in a defined germination medium as described previously[5].

Fermentation of *Streptomyces viridochromogenes* NRRL B-1551

The fermentor (Giovanola Freéres S.A., type b 20, Monthey, Switzerland) was inoculated with 24 liters of optimized medium (1 liter contains 10.0 g pepton from casein, 0.5 g yeast extract, 0.1 g NaCl, 0.5 g K_2SO_4 and 1.5 g L-glutamic acid; pH 7.2) and sterilized at 121°C for 30 minutes. After cooling to 27°C the fermentor was inocculated with 1 liter of a 18 hours pre-cultivate. Fermentation parameters: 27°C, 1,000 rpm, 0.25 vvm aeration, pH 7.2.

Test of Inhibitory Activity of **1**

For testing the inhibitory activity of **1** an assay described by GRUND and ENSIGN was used[5]. Since spores take up water during germination their optical density at 578 nm (OD_{578}) decreases during the swelling procedure. The decrease in optical density is a direct measure for the germinability of the spores, whereas reduced decrease in comparison to the control can be defined as germination inhibition.

Five ml of germination medium was adjusted to OD_{578} 0.3 with the spore suspension and incubated at 35°C. The optical density was determined (control). In order to determine the germination inhibitory activity 10, 100 μl or 1 ml of **1** were added prior to addition of the spores. The minimal inhibition concentration was determined in a dilution series test in DG medium[5] by extrapolation.

Preparation and Purification of **1** and **2**

The broth (25 liters) was adjusted to pH 2.5 with 5 N HCl, and the inactive mycelium cake separated by suction. The culture supernatant was adsorbed to Amberlite XAD-4 (bed volume 10% of the sample volume). The matrix was washed with 5 bed volumes of de-ionized water and eluted with 100% methanol p.a. Methanol was evaporated *in vacuo*, and the resultant aequeous solution extracted thrice with an equal volume of ethyl acetate-hexane (2.5:1, v/v). The organic layer was evaporated to dryness. The residue was dissolved in ethyl acetate-hexane (2.5:1, v/v) and applied onto a silica gel flash-column (size 61 cm × 3.7 cm; particle size 40 μm; J. T. Baker, Gross-Gerau, Germany). It was chromatographed with ethyl acetate-hexane (2.5:1, v/v) at a pressure of 0.3~0.5 bar. All fractions were evaporated to an oily residue and dissolved in methanol p.a. (10 ml). Five μl were used to determine the active fractions in the germination test. Active portions were collected, concentrated *in vacuo*, and re-dissolved in methanol (6 ml). Further purification was carried out by size exclusion chromatography on Sephadex LH-20 (Pharmacia, Uppsala, Sweden; column, 100 × 2.5 cm; eluent: methanol; 6 ml/minute; detection UV 290 nm) followed by gel filtration on TSK HW-40 (F) (column, 100 × 2 cm; eluent: methanol; 6 ml/minute; detection UV 290 nm).

Active portions (determined with 5 μl of the individual fractions) were combined and contained a mixture of **1** and **2**. Separation of these was achieved by preparative HPLC (Gradient Controller Sepacon GCU-311, high pressure pump Sepapress HPP-200, binary switching valve Sepacon PGM-318; Kronwald, Sinsheim, Germany) on a reversed-phase column (250 × 16 mm, i.d., Nucleosil C-18, 10 μm) by a linear gradient 30~70% B in 40 minutes (A = 0.2% aqueous acetic acid, B = methanol) at a flow rate of 20 ml/minute.

1 (2 mg): colorless oil; UV λ_{max}^{MeOH} nm 202, 290. Analytical HPLC (nucleosil C-18, 100% 0.1% H_3PO_4 to 100% MeOH in 15 minutes, linear): retention time 8.2 minutes. Negative $FeCl_3$ enol-reaction. NMR: see Table 2; coupling patterns see Fig. 9; MS and MS-MS see Table 1 and Fig. 6. HREI-MS *m/z* 196.1099 (M^+; $C_{11}H_{16}O_3$). Rf 0.85 (silica gel TLC; chloroform-methanol, 9:1); Rf 0.69 (ethyl acetate-hexane, 5:2). Soluble in water, DMSO, methanol, ethanol, acetone, ethyl acetate, benzene, dichloromethane, chloroform, pyridine; insoluble in hexane and light pertroleum (b.p. 30~50°C).

2 (1 mg): colorless oil; UV λ_{max}^{MeOH} nm 202, 290. Analytical HPLC (nucleosil C-18, 100% 0.1% H_3PO_4 to 100% MeOH in 15 minutes, linear): retention time 7.4 minutes. MS and MS-MS see Table 1 and Fig. 6. HREI-MS *m/z* 182.0943 (M^+; $C_{10}H_{14}O_3$). Negative $FeCl_3$ enol-reaction. Same solubility as **1**. ^1H NMR ($CDCl_3$; 100 MHz; ppm (*J*, Hz): 6.17 s; 2.52 q (*J* = 7.4 Hz); 1.14 t (*J* = 7.4 Hz); 2.73 m; 1.24 d (*J* = 7.0 Hz). ^{13}C NMR ($CDCl_3$; 100 MHz; numbering of carbon atoms see Fig. 6); ppm: 168.2 s (C-1); 104.7 s (C-2); 166.5 s (C-3); 98.6 d (C-4); 167.8 s (C-5); 16.4 t (C-6); 12.5 q (C-7); 32.4 d (C-8); 20.0 q (C-9,10). NMR coupling patterns: see Fig. 10.

Methylation of **1**

To a methanolic solution of **1** (100 μg, 0.5 μmol) excess diazomethane in ether solution was added at room temperature. After 1 hour the mixture was concentrated to dryness *in vacuo*. The residue was dissolved in methanol (0.5 ml) and directly used for HPLC/MS (see Fig. 7).

HPLC/IS-MS (nucleosil C-18, 5 μm; 100 × 2 mm i.d.; flow rate 200 μl/minute; isocratic 40% acetonitrile in 0.5% aqueous formic acid).

3a (Peak 1): Retention time 8.3 minutes; $(M+H)^+$ 211.5; UV λ_{max}^{MeOH} nm 210, 260. **3b** (Peak 2): Retention time 10.2 minutes; $(M+H)^+$ 211.5; UV λ_{max}^{MeOH} nm 207, 299. **3c** (Peak 3): Retention time 11.5 minute; $(M+H)^+$ 225.5. **3d** (Peak 4): Retention time 14.6 minutes; $(M+H)^+$ 225.5.

Acetylation of **1**

Germicidin (100 μg, 0.5 μmol) was dissolved in dry pyridine (0.5 ml) and acetic anhydride (0.05 ml). The reaction mixture was stirred at room temperature for 12 hours and evaporated to dryness *in vacuo*. The residue was dissolved in methanol (0.5 ml) and the resultant solution containing the mono-acylated derivative **4** directly investigated with IS-MS. $(M+H)^+$ 239.0, $(M+Na)^+$ 261.5, $(M+K)^+$ 277.5. After addition of lithium chloride: $(M+Li)^+$ 245.5.

Acknowledgments

This investigation was supported by the Deutsche Forschungsgemeinschaft (SFB 323). We thank Prof. Dr. J. C. ENSIGN, University of Wisconsin, Madison, for a gift of *Streptomyces viridochromogenes* NRRL B-1551. We wish to thank Prof. Dr. A ZEECK, University of Göttingen, for helpful discussions.

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