Two Inhibitors of Platelet Aggregation from a *Panus* Species (Basidiomycetes)

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Dedicated to Prof. Wolfgang Steglich on the occasion of his 60th birthday

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Basidiomycetes, Lentineae, *Panus* Species, Platelet Aggregation, Antibiotics, Drimanes

Two inhibitors of platelet aggregation were isolated from fermentations of *Panus* sp. 9096. One inhibitor proved to be identical to naematolon (**2**), an antibiotic previously isolated by S. Backens *et al.* from several *Hypholoma* species. The other metabolite, panudial (**1**), is a new nordrimane (*cis*-annelation of the bicyclus) lacking the carbon atom in position 10 of the drimane skeleton. Panudial is a potent inhibitor of bovine and human platelet aggregation stimulated by different inducers.

Introduction

Platelets are multiresponding cell fragments playing an important role in the development of vascular disorders and arterial thrombosis. Their complex structured signal transmission and their high number of specific receptors for different agonists and antagonists [1] offer a number of targets for selective pharmacological interventions.

In the course of screening fungal metabolites as specific aggregation inhibitors of collagen-induced bovine platelets, several hundred extracts derived from the submerged cultures of basidiomycetes and ascomycetes were tested. Previously, 2-methoxy-5-methyl-1,4-benzoquinone, a specific inhibitor of thromboxane A_2-induced aggregation of human platelets was isolated from *Lentinus adhaerens* [2] and cochlioquinone B was identified as a potent but less specific inhibitor from *Neobulgaria pura* [3].

In this publication we describe the fermentation, isolation, structure elucidation and biological properties of panudial (**1**) from *Panus* sp. 9096 [4].

Materials and Methods

General

IR and UV spectra were measured with a Bruker ISF 48 and a Perkin-Elmer Lambda 16 UV/VIS spectrometer respectively. $[\alpha]_D$-Value was measured with a Carr apparatus. For analytical HPLC a Hewlett-Packard 1090 series II instrument was used.

All NMR spectra were recorded on a Bruker AMX 500 spectrometer working at 500.14 MHz for ^1H and at 125.77 MHz for ^{13}C. The samples were solved in $CDCl_3$ and measured at 305 K. The solution of panudial (**1**) was 171 mM, that of kueh-neromycin B (**4**) was 13 mM.

ID spectral simulations were done with the Bruker NMRSIM program and visualized by Bruker WINNMR package on a 486 PC.

Mass spectra were measured with a Varian MAT 312 spectrometer.

Panus sp. strain 9096

Panus sp. strain 9096 was isolated from a fruiting body collected in Innisfail, Queensland, Australia. The strain is deposited in the culture collection of the LB Biotechnologie, University of Kaiserslautern. For maintenance on agar slants the fungus was grown on YMG medium (g/l: 4 yeast extract, 10 malt extract, 4 glucose, pH 5.5).

Fermentation and isolation of panudial (**1**) *and naematolon* (**2**)

Fermentations were carried out in a Biostat U fermentor equipped with a MFCS system (B. Braun Biotech) containing 20 l of MGP medium (g/l: 20 maltose, 10 glucose, 2 peptone, 1 yeast extract, 0.5 KH_2PO_4, 1.0 $MgSO_4$, 0.01 $FeCl_3$, 0.00178 $ZnSO_4$, 0.055 $CaCl_2$, pH 5.5). The content of oxygen in the

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medium and CO_2 and oxygen in the exhaust were determined on-line. The mycelial dry weight, pH, glucose concentration (hexokinase method) and the content of **1** and **2** were measured daily. Analytical HPLC of **1** and **2:** LiChrosorb RP-18, 5 µm, 4 × 125 mm; 1.5 ml/min, 40 °C, H_2O : MeOH → 100% in 20 min.

After 150 h of fermentation the mycelia were separated from the culture fluid by filtration. Panudial and naematolon were removed from the culture fluid (18 l) by adsorption to HP 21 resin (Mitsubishi) and eluted with acetone. The crude extract (2.66 g) was applied to a column (25 × 2.5 cm) of silica gel (0.063–0.2 mesh, Merck 60) and eluted with cyclohexane : ethyl acetate 1 : 1. Further purification was achieved by preparative HPLC (LiChrosorb CN, 7 µm, column 250 × 25 mm, Merck). Naematolon was eluted with cyclohexane : *tert*-butyl methylether (1 : 1) yielding 17.9 mg of pure **2.** The panudial containing fractions obtained by elution with cyclohexane : *tert*-butyl methylether (1 : 4) were applied to a column (250 × 25 mm) containing Merck LiChrospher PS 1, 7 µm, and eluted with isopropanol. Yield: 10.4 mg **1.** The purification of **1** and **2** was monitored by the platelet aggregation assay as described below.

Panudial (**1**)

Slightly yellow oil, soluble in methanol, acetone, chloroform, R_f 0.64 [toluene–acetone (7 : 3), silica gel], UV (MeOH) λ_{max}: 229 nm, ε = 11,573, $[\alpha]_D^{20}$ = −0.52 (c = 1, MeOH), IR (KBr) υ_{max}: 3421, 2927, 2857, 1707, 1680, 1646, 1603, 1371, 1166 cm^{-1}, MS, HR-EI-MS (70 eV, 250 °C, 3 kV), *m/z* (relative intensity, %) 234.1233 (2.3, M+, calcd for $C_{14}H_{18}O_3$ 234.1256), 206 (100.0, $C_{12}H_{14}O_3$), 173 (14.4, $C_{13}H_{18}O_2$), 150 (30.4, $C_9H_{10}O_2$), 145 (22.0, $C_{10}H_9O_1$), 132 (96.4, $C_9H_8O_1$). The molecular ion was confirmed by chemical ionization (MH+ = 235).

Table I summarizes the spectral data from the ^1H and ^{13}C NMR spectroscopy of panudial.

Platelet aggregation assay

Platelet-rich plasma (PRP) was obtained by mixing 9 volumes of fresh bovine slaughter blood with 1 volume of temperated citrate buffer (93 mM glucose, pH adjusted to 7.4 by 1 M citric acid) and centrifugation at 150 × g for 15 min at 22 °C. In general the supernatant contained 3–4 × 10^5 platelets/µl.

Table I. Spectral data from the ^1H and ^{13}C NMR spectroscopy of panudial (**1**).

Number	^{13}C [ppm]	^1H [ppm]	J_{hh} [Hz]
1	208.80	–	
2a	37.69	2.29*	$^3J_{H2a,H3a}$ = 2.0; $^3J_{H2a,H3b}$ = 5.3
2b		2.54$^\Delta$	$^3J_{H2b,H3b}$ = 14.1; $^2J_{H2a,H2b}$ = 14.6
3a	35.31	1.70*	$^2J_{H3a,H3b}$ = 14.1; $^3J_{H2b,H3a}$ = 7.1
3b		1.89$^\Delta$	$^4J_{H3a,H5}$ = 2.0
4	33.05	–	
5	43.83	1.92	$^3J_{H5,H6a}$ = 11.8; $^3J_{H5,H6b}$ = 5.3
6a	26.18	2.01$^\Delta$	$^3J_{H6a,H7}$ = 2.5; $^3J_{H6a,H6b}$ = 20.0
6b		2.50*	$^3J_{H6b,H7}$ = 5.3
7	151.22	6.92	$^5J_{H7,H10}$ < 1.6
8	135.58	–	
9	44.84	4.12	$^3J_{H9,H10}$ = 1.0
10	42.76	3.67	$^3J_{H5,H10}$ = 5.3
11	26.43	1.42	
12	27.74	1.02	
13	192.44	9.55	$^1J_{H,C}$ = 176.2
14	199.17	9.63	$^1J_{H,C}$ = 180.4; $^3J_{H14,H9} \ll 1.0$!

* Pro R.
$^\Delta$ Pro S.

Platelet poor plasma (PPP) was prepared by centrifugation at 1000 × g for 10 min at 22 °C.

To test the effect of thrombine, bovine PRP was washed two times with temperated citrate buffer and stored at a density of 1 × 10^6 platelets/ml in PBS buffer (g/l: 8 NaCl, 0.2 KCl, 1.44 $Na_2HPO_4 \times 2\,H_2O$, 0.2 KH_2PO_4, 1 glucose, 1 l H_2O, pH 7.4).

The aggregation assay was carried out in a spectrophotometer (Hitachi, model 100-60) with temperated (37 °C) and stirred cuvettes. After preincubation of 1.5 ml PRP for 10 min with the substances, the aggregation was stimulated by different inducers *e.g.* collagen, ADP or others. The change of transmittance was monitored at 600 nm with PPP or PBS as blanks, respectively.

The aggregation of human platelets was measured in a platelet aggregation profiler (BIO DATA Co.).

For screening purposes the assay was scaled down to 150 µl in 96-well microtiter plates (Greiner) and the change of transmittance was measured at 600 nm by using a BIO RAD EIA Reader.

Further biological tests

The tests for cytotoxicity, hemolytic activity and the serial dilution assays were carried out as described previously [5].

To determine the effects of **1** and **2** to the PL A_2 from *Naja mosambique* a assay according to Nieuwenhuizen [6] was used.

The influence of **1** and **2** on the biosynthesis of proteins, RNA and DNA was determined with appropriate ^{13}C-labeled precursors and HL 60 cells (10^7 cells/ml) in PBS buffer for 30 min at 37 °C and 100 rpm. After adding 0.1 μCi of the ^{14}C-labeled precursors leucine (58 mCi/mM), uridine (58.5 mCi/mM) or thymidine (52 mCi/mM) to the assays (1 ml each) the incubation was continued for 30 min. The incorporation was stopped by addition of 1 ml 10% TCA, the precipitates collected on nitrocellulose filters (0.45 μm, Sartorius), washed with 5 ml 5% TCA, dried, and the radioactivity measured by means of a liquid scintillation counter.

To determine the mutagenic properties of panudial and naematolon a pour plate test according to Ames *et al.* [7] was used. Four strains of *Salmonella typhimurium,* TA 97, TA 98, TA 100 and TA 102 were incubated with the compounds (50 μg/ml of medium) and the number of revertants counted after 24 and 48 h.

Results and Discussion

The genus *Panus* belongs to the order *Polyporales,* family Polyporaceae, and tribus Lentineae. Very few bioactive metabolites *e.g.* the cytotoxic panepoxydon [8, 9] have been reported from this genus.

Isolation and structure elucidation

Fig. 1 shows a typical fermentation of *Panus* sp. 9096. The production of **1** started immediately after the onset of growth and reached a maximum after 140 h. The naematolon content in the culture fluid reached a maximum after 130 h.

Panudial (**1**) and naematolon (**2**) were isolated as described in the experimental section. The identification of naematolon and the structure elucidation of panudial was achieved by spectroscopical methods.

Several interesting structural fragments are identified even in the 1 D, ^1H and ^{13}C spectra. In the DQF-H, H-COSY [10] spectrum, the protons can be assigned to two basic spin systems. The HMQC-TOCSY [11, 12] spectrum is very helpful as well.

Starting from the protons of the two methyl groups (C-11 and C-12), a central, quaternary C (C-4), a CH_2 (C-3) and a CH group (C-5) is found in the HMBC [13] spectrum. The CH_2 group (C-3) has another CH_2 group (C-2) as neighbour. Both of these show correlations to the keto-carbonyl signal at 208.80 ppm (C-1). From the above CH (C-5) group the system divides into a CH_2–CH= and another CH group (C-10). In the HMBC spectrum unambiguous cross-peaks show up between the double bond proton and the central CH (C-5), to the aldehyde carbon at 192.44 ppm and to the last CH group (C-9). Protons H-10 and H-9 show correlations to keto-carbonyl C-1 in the HMBC spectrum.

From these results the annelation of two six-membered rings follows. The rather small coupling between the bridgehead protons of 5.3 Hz is due to the *cis* connection of the rings which induces some peculiarities into the structure. In the *trans*-annelated kuehneromycin B (**4**) that coupling constant is 12.9 Hz. It is noteworthy that the torsional angle between protons H-9 and H-10 is about 75° leading to a coupling of only 1 Hz, *i.e.* the H-9 is rather equatorial and the aldehyde group is rather axial with respect to H-10 (*cf.* Fig. 2). As expected, in kuehneromycin B (**4**), this is not the case ($^3J_{H9,H10}$ = 10.0 Hz). Additionally, aldehyde proton H-14 does exhibit only a very small coupling constant with H-9. Again, in kuehneromycin B (**4**) this coupling is more usual (2.1 Hz). For further detail see [15].

In *cis*-decalin systems two conformations may be adopted. The elucidated structure and its counterpart were compared by molecular dynamics and energy minimization. The structural relevant NMR parameters (coupling constants and distances from ROESY [14]) do fit the shown structure well. Therefore, the other possible conformation can only be weakly populated.

Fig. 2 shows the structure of **1, 2** and other related fungal metabolites. The stereoisomer of **1,** kuehneromycin B (**4**), was first described by Erkel *et al.* [15]. Only relative configurations are given for **1** and **4. 4,** together with kuehneromycin A, were isolated from a tasmanian *Kuehneromyces* species as potent inhibitors of reverse transcriptases. Naematolon (**2**) together with naematolin (**3**), was first isolated from several *Hypholoma* species by

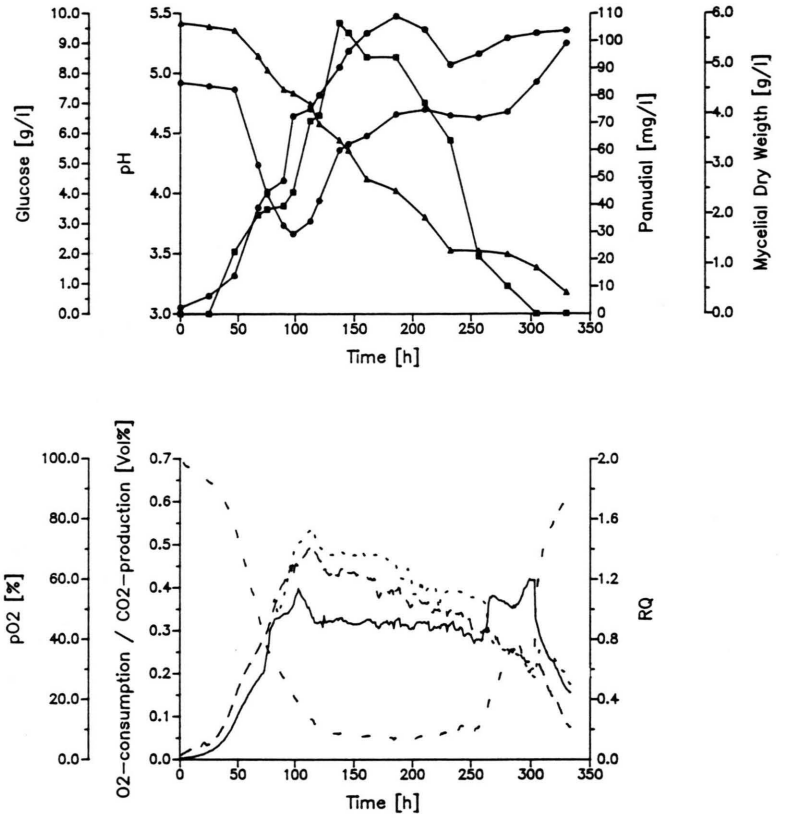
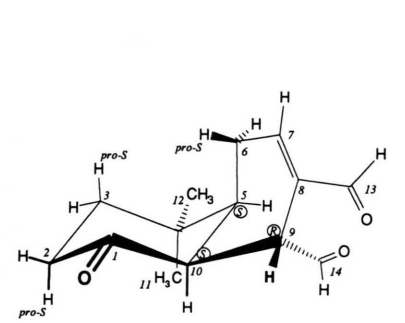
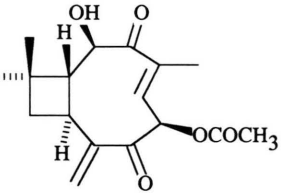


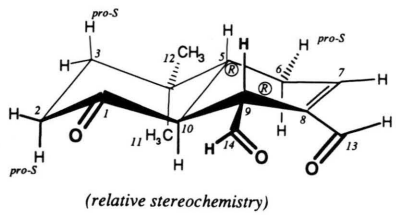
Fig. 1. Fermentation of *Panus* sp. in 20 l of MGP medium. ○—○ pH; □—□ panudial [mg/l]; △—△ glucose [g/l]; ●—● mycelial dry weigth [g/l]; —— O_2 consumption [vol%]; — — CO_2 production [vol%]; - - - - RQ; - - - pO_2 [%].



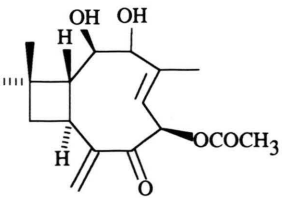
(relative stereochemistry)
Panudial [1]



Naematolon [2]



(relative stereochemistry)
Kuehneromycin B [4]



Naematolin [3]

Fig. 2. Structure of **1, 2** and related fungal metabolites. [1] Panudial; [2] naematolon; [3] naematolin; [4] kuehneromycin B.

Backens *et al.* [16]. Naematolon is the first caryophyllane isolated from a *Panus* species.

Biological activities

The inhibitory effect of **1** on the ADP-induced aggregation of bovine platelets is shown in Fig. 3. A complete inhibition of aggregation was obtained with 427 μM panudial with an IC_{30} value (30% inhibition) of 8.5 μM. In concentrations higher than 8.5 μM the second irreversible phase of aggregation was inhibited in a concentration-dependent manner while the first, reversible phase was hardly affected.

Fig. 4 shows the influence of **1** on the ADP-induced aggregation of human platelets. The complete aggregation was achieved within 5 min. As in the case of bovine platelets the primary phase of the ADP-induced aggregation was not affected by concentrations of up to 85 μM panudial. The IC_{30} value was determined to 25.5 μM and complete inhibition was observed at 512 μM.

The effects of panudial (**1**) and naematolon (**2**) on the aggregation of human and bovine platelets stimulated with different inducers are compared in Table II. **2** interfered only weakly with platelet aggregation, while **1** exhibited quite strong effects with the inducers ADP, collagen, U 46619, ristocetin, arachidonic acid, and thrombine. Kuehneromycin B and panudial exhibited similar inhibitory effects with the exception of the thrombine-induced

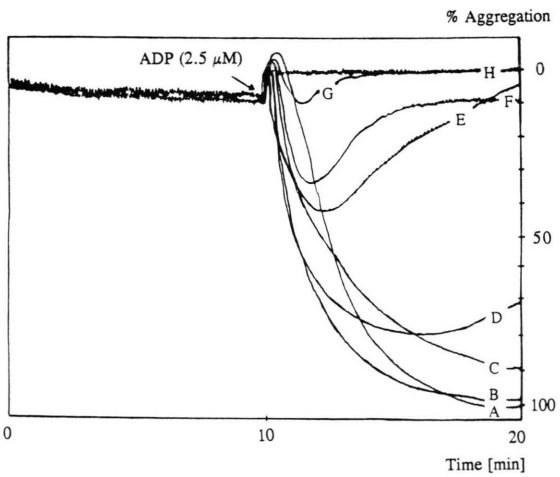


Fig. 3. Influence of **1** on the ADP-induced aggregation of bovine platelets. A) control; B) 1 μg/ml; C) 1.5 μg/ml; D) 2 μg/ml; E) 5 μg/ml; F) 7 μg/ml; G) 10 μg/ml; H) 100 μg/ml.

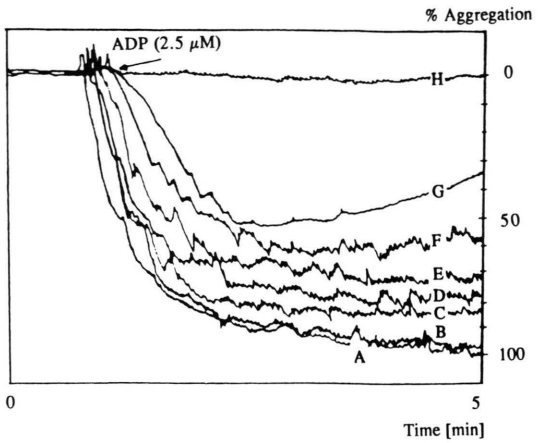


Fig. 4. Influence of **1** on the ADP-induced aggregation of human platelets. A) control; B) 1 μg/ml; C) 2.5 μg/ml; D) 5 μg/ml; E) 7.5 μg/ml; F) 10 μg/ml; G) 20 μg/ml; H) 120 μg/ml.

aggregation which was preferently inhibited by **4** [15]. This marked difference in the biological activities of **1** and **4** is also evident in the effects of both compounds on HIV-1 reverse transcriptase. While **4** is a strong inhibitor of reverse transcriptases, **1** is much less active [15].

The IC_{30} values of **1** for all inducers, except for thrombine, varied between 5 and 35 μM. A specific interference of **1** with one of the receptors for the inducers seems unlikely. This lack of specificity with regard to the different signal pathways may be due to an interference with some basic metabolic proc-

Table II. IC_{30} (70% aggregation) values for the inhibition of platelet aggregation by panudial (**1**) and naematolon (**2**).

Inducer	Compound [μg/ml] (μM) **1**	**2**
A) Human platelets		
ADP (4.45 μM)	6 (25.5)	50 (162)
Collagen (0.3 mg/ml)	5 (21)	10 (32)
U 46619 (0.45 μM)*	2.5 (11)	12.5 (40)
Ristocetin (0.4 mg/ml)	30 (128)	50 (162)
AA (0.6 μg/ml)	6 (25.5)	40 (130)
Thrombine (0.1 U/ml)	15 (64)	75 (244)
B) Bovine platelets		
ADP (2.5 μM)	2.5 (8.5)	10 (42)
Collagen (0.3 mg/ml)	4 (17)	12.5 (41)
Thrombine (0.1 U/ml)	60 (256)	15 (49)

* Thromboxane A 2 analogue, UpJohn.

ess, a view which is supported by the inhibition of at least one central enzyme, phospholipase A_2, by panudial (IC_{50} = 23 μg/ml for the type I PLA_2 from *Naja mosambique*). The effect of drimane-dialdehydes in several *in vitro* and *in vivo* assays has been described and is related to the stereochemistry of the dialdehyde groups [17].

The cytotoxic activities of **1** and **2** against BHK-, HeLa S3-, L 1210-, HL 60- and U 937-cell cultures were tested as described previously [6]. **1** and **2** lyzed HL 60 cells at concentrations above 5 μg/ml (21 μM), while the other cell lines were only affected by concentrations starting at 50 μg/ml (Table III).

The incorporation of ^{14}C-labeled thymidine, uridine and leucine into DNA, RNA and proteins of HL 60 cells was tested as described previously [5]. Fig. 5 shows a preferential inhibition of protein and RNA syntheses by panudial at concentrations from 4–8 μg/ml. A direct lytic action on the cytoplasmatic membrane as the primary cause of the cytotoxicity seems unlikely. This result is supported by a complete lack of hemolytic activity at concentrations up to 100 μg/ml of **1.**

The antifungal and antibacterial profile of panudial was determined in the serial dilution assay (Table IV). The organisms most sensitive to panudial were the yeast *N. coryli* and the smut fungus *U. nuda,* whereas bacteria and other fungi were less sensitive.

In the "pour plate test" for mutagenicity (without microsomes) with four strains of *S. typhimurium* (TA 97, TA 98, TA 100 and TA 102) no increase of the number of revertans could be observed at concentrations from 1–50 μg/ml (85 μM) of panudial.

Table III. Cytotoxic effects of **1** and **2** against different cell cultures.

	Compound [μg/ml]									
	1					**2**				
Cells	1	5	20	50	100	1	5	20	50	100
BHK	–	–	–	–	+	–	–	–	–	+
HeLa S3	–	–	–	+	+	–	–	–	–	–
L 1210	–	–	–	–	–	–	–	–	–	–
HL 60	–	+	+	+	+	–	+	+	+	+
U 937	–	–	–	–	–	–	–	–	–	–

– No effects after 48 h.
+ Lysis of cells after 48 h.

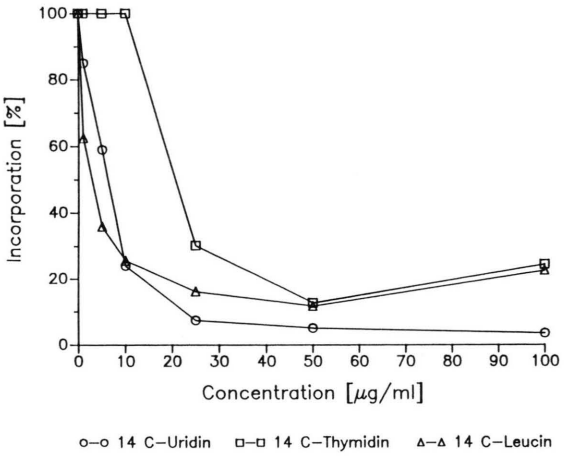


Fig. 5. Inhibition of protein, RNA, and DNA syntheses by panudial. ○—○ [^{14}C]Uridine; □—□ [^{14}C]thymidine; △—△ [^{14}C]leucine.

Table IV. Minimal inhibitory concentrations (MIC) of **1** in the serial diffusion assay.

Organism	MIC [μg/ml] of **1**
Acinetobacter calcoaceticus	100
Athrobacter citreus	100
Bacillus brevis	100
Bacillus subtilis	100
Corynebacterium insidiosum	100
Escherichia coli	100
Micrococcus luteus	50
Mycobacterium phlei	50
Proteus vulgaris	100
Salmonella typhimurium	100
Staphylococcus aureus	>100
Streptomyces sp.	10
Candida albicans	100
Fusarium oxysporum	10
Mucor miehei	>100
Nadsonia fulvescens	5
Nematospora coryli	5
Paecilomyces variotii	100
Penicillium notatum	100
Rhodotorula glutinis	>100
Saccharomyces cerevisiae is 1	1
Saccharomyces cerevisiae S 288 c	10
Ustilago nuda	5

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says with human blood. For a direct comparison of spectral data a sample of kuehneromycin B was provided by Dr. G. Erkel, University of Kaiserslautern.

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[1] H. Holmsen, Platelet secretion, in: Hemostasis and Thrombosis, Ch. **24** (R. W. Colman, ed.), pp. 390–403, J. B. Lippincott Company, Philadelphia 1982.
[2] U. Lauer, T. Anke, and F. Hansske, J. Antibiot. **44,** 1, 59–65 (1991).
[3] K. Lorenzen, T. Anke, U. Anders, H. Hindermayr, and F. Hansske, Z. Naturforsch. submitted (1994).
[4] R. Singer , The Agaricales in Modern Taxonomy, pp. 179–182, Koeltz Scientific Books, Königstein 1986.
[5] K. Leonhardt, T. Anke, E. Hillen-Maske, and W. Steglich, Z. Naturforsch. **42c,** 420–429 (1987), and K. Lorenzen, Dissertation, Universität Kaiserslautern (1993).
[6] W. Nieuwenhuizen, H. Kunze, and G. H. de Haas, in: Methods in Enzymology, **Vol. 32** (S. Fleischer and L. Packer, eds.), Academic Press, New York 1974.
[7] B. N. Ames, J. McCann, and E. Yamasaki, Mutation Research **31,** 347 (1975).
[8] H. Kresse and E. Buddecke, Physiol. Chem. **349,** 1507 (1968).
[9] Z. Kis, A. Closse, H. P. Sigg, L. Hruban, and G. Snatzke, Helv. Chim. Acta **53,** 1577 (1970).
[10] a) W. P. Aue, E. Bartholdi, and R. R. Ernst, J. Chem. Phys. **64,** 2229–2246 (1976);
b) D. Marion and K. Wüthrich, Biochem. Biophys. Res. Commun. **113,** 967 (1983).
[11] A. Bax and S. Subramanian, J. Magn. Reson. **67,** 565–569 (1986).
[12] L. Lerner and A. Bax, J. Magn. Reson. **69,** 375–380 (1986).
[13] A. Bax and M. F. Summers, J. Am. Chem. Soc. **108,** 2093–2094 (1986).
[14] a) A. A. Bothner-By, R. L. Stephens, J. Lee, C. D. Warren, and R. W. Jeanloz, J. Am. Chem. Soc. **106,** 811–813 (1984).
b) H. Kessler, C. Griesinger, R. Kerssebaum, K. Wagner, and R. R. Ernst, J. Am. Chem. Soc. **109,** 607–609 (1987).
[15] G. Erkel, K. Lorenzen, T. Anke, A. Gimenez, and W. Steglich, in preparation, and G. Erkel, Dissertation, Universität Kaiserslautern (1990).
[16] S. Backens, B. Stefan, W. Steglich, L. Zechlin, and T. Anke, Liebigs Ann. Chem. **1984,** 1332.
[17] B. J. M. Jansen and A. de Groot, Nat. Prod. Rep. **1991,** 309–318.