Kuehneromycins A and B, Two New Biological Active Compounds from a Tasmanian *Kuehneromyces* sp. (Strophariaceae, Basidiomycetes)

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Kuehneromyces sp., Basidiomycetes, Sesquiterpenoids, Drimanes, Nordrimanes

In a search for new inhibitors of RNA-directed DNA-polymerases kuehneromycin A (**1**) was isolated from fermentations of a Tasmanian *Kuehneromyces* species. Its structure was elucidated by spectroscopic methods. Kuehneromycin A (**1**) is a non-competitive inhibitor of avian myeloblastosis virus (K_i 200 μM) and moloney murine leukemia virus (K_i 40 μM) reverse transcriptases. The second compound, kuehneromycin B (**2**) is a strong inhibitor of platelet aggregation stimulated with different inducers. In addition, both compounds exhibit cytotoxic and antimicrobial activities.

Introduction

Basidiomycetes provide an interesting source of novel secondary metabolites with a variety of biological activities. A screening of some 400 strains for the production of inhibitors of avian myeloblastosis virus (AMV)- reverse transcriptase so far resulted in the isolation of podoscyphic acid (Erkel *et al.*, 1991) from a Tasmanian *Podoscypha* species, clavicoronic acid (Erkel *et al.*, 1992) from *Clavicorona pyxidata* and hyphodontal (Erkel *et al.*, 1994) from a *Hyphodontia* species.

In the following we describe the fermentation, isolation, structural elucidation, and biological characterization of two new inhibitors from *Kuehneromyces* sp. strain 8758.

Materials and Methods

General

Spectral data were recorded on the following instruments: ^1H and ^{13}C NMR, Bruker AM-400 and AMX-600; EI-MS, Finnigan MAT 90 and 95 Q; FT-IR, Bruker IFS 48; UV, Perkin-Elmer Lambda 16; CD, Jobin Yvon CD 6. Optical rotations were recorded with a Dichrograph Mark IV polarimeter. For TLC aluminum foils coated with silica gel Merck 60 F_{254} were used. All solvents were distilled prior to use.

Kuehneromyces sp. strain 8758

Mycelial cultures were obtained from tissue plugs of fruiting bodies growing on buried wood in the Hartz Mountains, Tasmania. The specimen show the characteristics of the genus as described by Singer (1986). The species, however, could not be identified. Voucher specimen and cultures are deposited in the collection of the Lehrbereich Biotechnologie, University of Kaiserslautern.

Fermentation

For maintenance and production of kuehneromycins A (**1**) and B (**2**) the fungus was cultivated in YMG medium composed of: yeast extract 0.4%, malt extract 1%, glucose 0.4% and agar 1.5% for solid media. A well grown seed culture of *Kuehneromyces* sp. 8758 (200 ml) in YMG was used to inoculate 20 l of YMG medium in a Biolafitte C6 fermentation apparatus. The fermenter was incubated at 22 °C with an aeration rate of 3 l air/min and agitation (130 rpm). Larger batches were grown in a 150-liter tank (Deutsche Metrohm,

Abbreviations: AMV, avian myeloblastosis virus; MMuLV, Moloney murine leukemia virus; RT, reverse transcriptase.

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Stuttgart) containing 100 l of YMG medium (inoculum 10%, 100 rpm, 15 l air/min, 22 °C). The production of kuehneromycin A was followed by estimating the inhibitory effect of 2.5 µl of a crude extract (concentrated 100 times as compared to the culture fluid) in the standard assay of AMV RT.

Isolation of kuehneromycins A (**1**) *and B* (**2**)

During purification, kuehneromycin A (**1**) was detected using the standard assay for AMV RT. After removal of the mycelia by filtration, kuehneromycin A was extracted from the culture filtrate (90 l) with EtOAc (two times 15 l). Evaporation of the organic phase yielded a crude extract (7.5 g), which was purified by repeated chromatography on silica gel (Merck 60; elution with cyclohexane - EtOAc 75 : 15) resulting in 170 mg of an enriched product. This was further purified by preparative HPLC (LiChrosorb Si60, column 2,5 x 25 cm, elution with cyclohexane - 2-propanol (78 : 22) to yield 120 mg of kuehneromycin A (**1**). In some batches rechromatography of the product obtained after this step on LiChrosorb CN (column 2,5 x 25 cm, elution with cyclohexane - *tert*-butyl methyl ether 22 : 78) resulted in the separation of a second compound, kuehneromycin B (**2**). Yield: 40 mg from a 100 l fermentation.

Kuehneromycin A (**1**)

Colorless oil, R_f 0.75 (toluene/acetone/acetic acid = 70:30:1); $[\alpha]_D^{21}$ -55 (*c* 0.20, EtOH); UV (MeOH) λ_{max} 229 nm (log ε 4.01); CD (EtOH) λ_{max} 223 nm (Δε -4.96), 247 (0), 257 (+0.23, sh), 296 (+1.16), 380 (0); IR (KBr) cm^{-1} 3440, 2957, 2930, 1712, 1683, 1653, 1370, 1157; ^1H and ^{13}C NMR spectra see Table I; HREI-MS (70 eV; DI 180 °C) *m/z* (relative intensity %) 278.1163 (0.2, M^+, cald for $C_{15}H_{18}O_5$ 278.1154), 234 (1, $C_{14}H_{18}O_3$), 206 (51, $C_{13}H_{18}O_2$), 132 (80), 107 (25), 77 (41), 55 (100).

Kuehneromycin B (**2**)

Colorless oil, R_f 0.75 (toluene/acetone/acetic acid = 70:30:1); $[\alpha]_D^{21}$ -108 (*c* 0.25, EtOH); UV (MeOH) λ_{max} 229 nm (log ε 3.79); CD (MeCN) λ_{max} 216 nm (Δε -5.50), 231 (0), 243 (+3.04), 266 (+0.80), 287 (+1.18), 380 (0); IR (KBr) cm^{-1} 3420, 2958, 2931, 1706, 1678, 1646, 1371, 1166; ^1H and ^{13}C NMR spectra in benzene-d_6 see Table I; ^1H NMR (600 MHz, $CDCl_3$) δ 1.04 (3H, s, 13-H), 1.14 (3H, s, 14-H), 1.63 (1H, ddd, *J* = 12.8, 11.5 and 4.5 Hz, 5-H), 1.64 (1H, ddd, *J* = 14.8, 13.5 and 4.5 Hz, 3α-H), 1.77 (1H, ddd, *J* = 13.5, 6.0 and 2.5 Hz, 3β-H), 2.32 (1H, dddd, *J* = 19.5, 11.5, 3.8 and 2.5 Hz, 6β-H), 2.38 (1H, ddd, *J* = 14.8, 4.5 and 2.5 Hz, 2α-H), 2.50 (1H, dddd, *J* = 19.5, 6.0, 4.5 and 2.0 Hz, 6α-H), 2.51 (1H, ddd, *J* = 14.8, 14.8 and 6.0 Hz, 2β-H), 2.85 (1H, dd, *J* = 12.8 and 10.0 Hz, 10-H), 3.80 (1H, ddddd, *J* = 10.0, 3.8, 2.5, 2.0 and 2.0 Hz, 9-H), 6.98 (1H, ddd, *J* = 6.0, 2.5 and 2.5 Hz, 7-H), 9.33 (1H, s, 12-H), 10.32 (1H, d, *J* = 2.0 Hz, 11-H); HREI-MS (70 eV; DI 85 °C) *m/z* (relative intensity %) 234.1227 (0.4, M^+, cald for $C_{14}H_{18}O_3$ 234.1256), 206 (45, $C_{13}H_{18}O_2$), 173 (4, $C_{12}H_{13}O$), 150 (15, $C_9H_{10}O_2$), 137 (16, $C_8H_9O_2$), 132 (85, C_9H_8O), 108 (23, C_7H_8O), 107 (30, C_7H_7O), 91 (28, C_7H_7), 79 (43, C_6H_7), 77 (44, C_6H_5), 56 (47), 55 (100), 53 (47).

Conversion of kuehneromycin B (**2**) *into panudial* (**4**) *and* vice versa

To a stirred solution of **2** in THF (3 ml) was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). After stirring for 2 h at 20 °C, the reaction mixture was diluted with Et_2O (15 ml) and washed successively with 0.1 N HCl (3 x 10 ml), saturated aqueous $NaHCO_3$ (10 ml) and brine (10 ml). Evaporation of the dried organic layer (Na_2SO_4) *in vacuo* yielded a residue, which consisted of a 4 : 1 mixture of **2** and **4** according to the ^1H NMR spectrum (600 MHz; $CDCl_3$). Diagnostic aldehyde signals appeared at δ 9.33 (4/5H, s, 12-H of **2**), 9.53 (1/5H, s, 12-H of **4**), 9.62 (1/5H, s, 11-H of **4**), and 10.32 (4/5H, d, *J* = 2 Hz, 11-H of **2**).

Under the same conditions panudial (**4**) yielded a 1 : 10 mixture of **2** and **4**. Longer reaction times led to decomposition in both cases.

Biological assays

Antimicrobial spectra, cytotoxicity and macromolecular syntheses in whole L 1210 cells (lymphocytic leukemia, mouse ATCC CCL 219) were measured as described previously (Weber *et al.*, 1990). HeLa cells (ATCC CCL 2.2) and Ehrlich ascites carcinoma cells (H. Probst, University of

Tübingen) were grown in Ham's F 12 medium, BHK 21 (ATCC CCL 10) in G-MEM, 3T3/MMSV cells (Moloney murine sarcoma virus transformed) and HUT 78 cells, (ATCC TIB-161) in RPMI 1640 supplemented with 10% fetal calf serum and 65 µg/ml penicillin G and 100 µg/ml streptomycin sulfate in a humidified atmosphere containing 5% of CO_2 at 37 °C.

Nucleic acid syntheses in permeabilized L 1210 cells were performed according to Berger (1978).

Assay for avian myeloblastosis virus (AMV) RT: The method reported by Hanajima *et al.* (1985) was modified: a reaction mixture (50 µl) consisting of 80 mM Tris-HCl (pH 8.3), 6 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 60 mM KCl, 200 µg/ml bovine serum albumin (BSA), 10 µM dTTP containing 0.01 µCi [2-^{14}C]-dTTP (44 cpm/pmol), 5 µg/ml poly(A)-$(dT)_{15}$ and 20 U/ml AMV RT (Boehringer, Mannheim) were incubated at 37 °C for 60 min. The reaction was terminated by adding 1 ml of cold 20% trichloroacetic acid (TCA) containing 20 mM pyrophosphate. The acid insoluble fractions were collected on cellulose nitrate filters presoaked with 20 mM pyrophosphate solution. The filter papers were washed three times with cold 5% TCA solution and the remaining radioactivity was measured in a liquid scintillation counter.

Assay for Moloney murine leukemia virus (MMuLV) RT: The reaction mixture (50 µl) contained 80 mM Tris-HCl (pH 8.3), 10 mM DTT, 8 mM $MgCl_2$, 30 mM KCl, 200 µg/ml BSA, 5 µg/ml poly(A)-$(dT)_{15}$, 14 µM dTTP containing 0.01 µCi [2-^{14}C]-dTTP (40 cpm/pmol) and 20 U/ml MMuLV RT (Pharmacia, Uppsala). Unless otherwise specified the reaction mixture was incubated for 60 min at 37 °C and the radioactivity of the acid insoluble fractions was determined as described above.

Assay for human immunodeficiency virus (HIV-1) RT: HIV-1 RT (United States Biochemicals) activity (20 U/ml) with poly(A)-$(dT)_{15}$ as template-primer was assayed using the reaction conditions described for MMuLV RT.

The RT assay with a 1080 bp LTR-template (kindly provided by Dr. S. Weiss (Weiss *et al.*, 1992), Boehringer, Mannheim) and a 18mer complementary primer was performed as follows: 2.8 µM LTR-template and 20 µM 18mer primer were combined after annealing at 66 °C and slowly cooling to 20 °C with 80 mM Tris-HCl (pH 8.3), 8 mM $MgCl_2$, 30 mM KCl, 10 mM DTT, 200 µg/ml BSA, 10 µM dTTP, dATP, dGTP, dCTP, 1 µCi [^{3}H]-dTTP (3.34 pmol), and 20 U/ml HIV RT. The reaction mixture (50 µl) was incubated for 60 min at 37 °C and the radioactivity in the acid-insoluble fractions were determined as described above.

Platelet aggregation assay: 9 volumes of fresh bovine slaugther blood were mixed with 1 volume of citrate buffer (93 mM sodium citrate, 140 mM glucose, pH adjusted to 7.4 with 1 M citric acid). To obtain platelet rich plasma (PRP), the anticoagulated blood was centrifuged at 150 x *g* for 15 min at 22 °C. In general the PRP contained 3–4 x 10^5 platelets/µl.

Platelet poor plasma (PPP) was obtained by centrifugation of the anticoagulated blood at 1000×*g* for 10 min.

To test the effect of thrombin, bovine platelets were washed two times with citrate buffer pH 7.4 and stored at a density of 1 x 10^6 platelets/µl in PBS buffer (g/l: NaCl 8; KCl 0.2; Na_2HPO_4 x 2 H_2O 1.44; KH_2PO_4 0.2; glucose 1; pH 7.4).

The aggregation assay was carried out in a spectrophotometer (Hitachi, model 100–60) with temperated (37 °C) and stirred plastic cuvettes. After preincubation for 10 min with the inhibitors the aggregation was stimulated with different inducers. The change of transmittance was monitored at 600 nm with PPP or PBS buffer as blanks, respectivly.

The aggregation of human platelets was measured in a platelet aggregation profiler (BIO DATA Corporation) after 10 min of preincubation.

To compare different inducers and compounds, the concentration of inhibitor that caused a 30% reduction of aggregation (IC_{30}), was determined.

Test for mutagenicity: Mutagenicity was tested according to the method of Ames *et al.* (1975). Mutants of *Salmonella typhimurium* strain TA 98 and TA 100 were used in the pour plate assay as described by Venitt *et al.* (1984).

Results and Discussion

Production of kuehneromycin A (**1**)

A typical fermentation of *Kuehneromyces* sp. 8758 is shown in Fig. 1. The production of kuehneromycin A (**1**), as measured by the inhibition of AMV RT, starts approximately 200 h

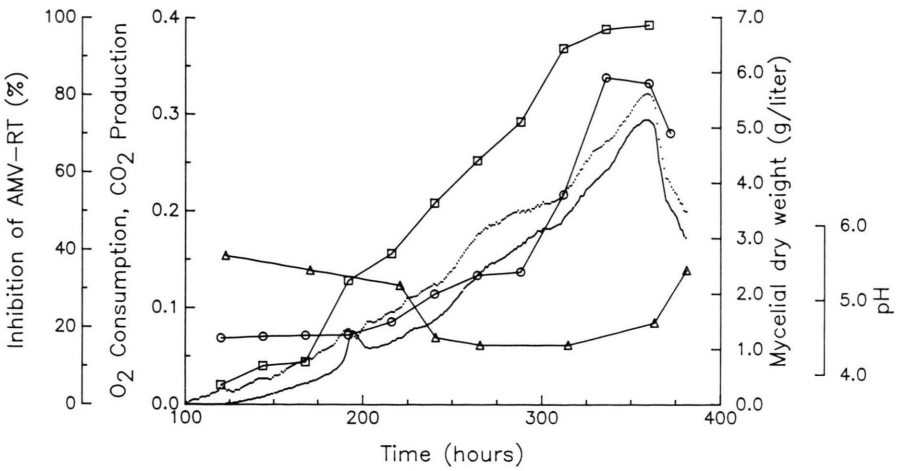
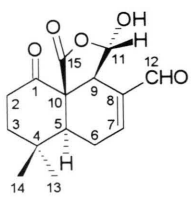


Fig. 1. Fermentation of *Kuehneromyces* sp. Dry weight (○), pH (△), activity (□), determined as described in the experimental section, O_2-consumption (——), CO_2-production (···).



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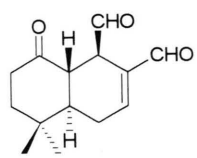

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after inoculation. The highest concentration of the inhibitor is reached after 340 h.

Structural elucidation

Kuehneromycin A exhibits a molecular ion at *m/z* 278 in the EI mass spectrum which corre-

Table I. ^1H and ^{13}C NMR data of kuehneromycin A (**1**) and B (**2**) (400 and 100.6 MHz, respectively; δ-values; in benzene-d_6)[a].

	1	**2**		**1**	**2**
			C-1	209.5 (m)	207.6
2α-H	1.91 (ddd)	2.02 (ddd)	C-2	35.7 (Tt, 130&4)	37.3
2β-H	2.42 (ddd)	1.89 (ddd)			
3α-H	1.05 (ddd)	0.98 (ddd)	C-3	37.2 (Tm, 130)	40.4
3β-H	1.50 (ddd)	1.06 (ddd)			
			C-4	31.3 (m)	32.1
5-H	0.94 (dd)	0.82 (ddd)	C-5	45.8 (Dm, 130)	45.2
6α-H	1.58 (dddd)	1.51 (m)	C-6	24.5 (Tm, 124)	27.4
6β-H	1.83 (dddd)	1.53 (m)			
7-H	5.90 (dt)	5.97 (dt)	C-7	151.7 (Dm, 160)	150.4
			C-8	137.1 (dm, 26)	140.9
9-H	3.35 (m)	3.71 (dddd)	C-9	47.3 (Dm, 140)	46.8
10-H	–	2.68 (dd)	C-10	61.7 (m)	49.8
11-H	5.48 (d)	10.78 (d)	C-11	101,2 (D, 184)	201.1
12-H	8.96 (s)	9.01 (s)	C-12	191.4 (Dd, 176&9)	191.9
13-H	0.58 (s)	0.48 (s)	C-13	30.7 (Qm, 124)	28.5
14-H	1.19 (s)	0.48 (s)	C-14	22.6 (Qm, 124)	18.5
			C-15	170.2 (dt, 10&6)	–
11-OH	6.50 (d)[b]	–			

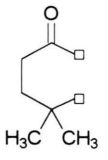
1: *J* (Hz): 2α,2β = 16; 2α,3α = 4; 2α,3β = 7; 2β,3α = 12; 2β,3β = 5; 3α,3β = 14; 5,6α = 4; 5,6β = 12.5; 6α,6β = 19; 6α,7 = 7; 6α,9 = 1.5; 6β,7 = 1.5; 6βb,9 = 3; 7,9 = 1.5; 11,11-OH = 13. – **2:** *J* (Hz): 2α,2β = 14; 2α,3α = 4; 2α,3β = 3; 2β,3α = 13; 2β,3β = 7; 3α,3β = 13; 5,6α = 6; 5,6β = 10; 5,10 = 13; 6α,7 = 5; 6β,7 = 3; 6β,9 = 4; 7,9 = 3; 9,10 = 10; 9,11 = 2.

[a] ^1H (600 MHz) NMR data of **2** in $CDCl_3$ see Materials and Methods.
[b] Exchanges on addition of deuterium oxide, and a singulet for δ 5.48.

sponds to the molecular formula $C_{15}H_{18}O_5$. The compound shows a UV maximum (MeOH) at λ_{max} 232 nm and intense IR absorptions (KBr) at 1770 (γ-lactone), 1711 (ketone) and 1692 cm^{-1} (α,β-unsaturated carbonyl).

The ^1H NMR spectrum in C_6D_6 (Table I) reveals the presence of eight aliphatic protons, two tertiary methyl groups (δ 0.58, 1.19), two protons in the olefinic region (δ 5.48, 5.90) and one aldehyde proton (δ 8.96). A doublet at δ 6.50 disappears on addition of D_2O and can thus be assigned to a hydroxy proton. From inspection of the coupling patterns given in Table I and 2D ^1H-^1H-COSY experiments the aliphatic protons can be ascribed to an isolated $-CH_2CH_2-$ moiety (A) and partial structure >CH-CH_2-CH=C-CH< (B). In unit B the methylene protons centered at δ 1.58 and 1.83 are coupled with both the methine proton at δ 0.94 and the olefinic proton at δ 5.90 and are connected *via* homoallylic coupling with the second methine proton at δ 3.35.

The substituents attached to partial structures A and B were detected by the ^1H coupled ^{13}C NMR spectrum (Table I) and 2D ^1H-^{13}C COSY experiments. Thus, in the long range correlation spectrum of kuehneromycin A the carbonyl resonance at δ_C 209.5 can be correlated with both methylene groups of A. The same applies to the quaternary carbon resonance at δ_C 31.3 which in addition exhibits two cross peaks with the methyl singlets at δ_H 0.58 and 1.19. This allows the extension of partial structure A to C.



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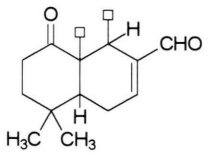


D

Moreover, in partial structure B the olefinic quaternary carbon resonance at δ_C 137.1 is connected to the protons of the allylic methylene group, the vicinal methine proton at δ_H 3.35, and an aldehyde proton at δ_H 8.96 which confirms the presence of an α,β-unsaturated aldehyde moiety (δ_C 191.4). Partial structures B and C can be extended to decalin system D due to the occurrence of cross peaks between the methine proton at δ_H 0.94 and the quaternary carbon at δ_C 31.3. A further connectivity is observed between the methine proton at δ_H 3.35 and the carbonyl resonance at δ_C 209.5.

Finally, the carbon resonance at δ_C 101.2 assigned to a hemiacetal moiety correlates to the methine proton at δ_H 3.35 while the lactone carbonyl resonance at δ_C 170.2 is connected to the methine protons at δ_H 0.94 and 3.35 and the hemiacetal proton at δ_H 5.48. This leads to the drimane structure **1** for kuehneromycin A.

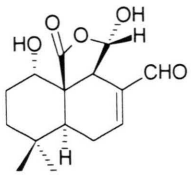
The lack of coupling between the adjacent methine protons at C-9 and C-11 is typical for drimane derivatives with β-configuration of the lactol hydrogen 11-H (Ayer and Craw, 1989).

According to its molecular formula $C_{14}H_{18}O_3$, kuehneromycin B is a decarboxylation product of **1**. In the ^1H NMR spectrum (C_6D_6) two aldehyde resonances at δ 9.01 (s) and 10.78 (d, J = 2 Hz) and a methine multiplet at δ 2.68 instead of the hemiacetal signal are the major differences to the spectrum of **1**. In the ^{13}C NMR spectrum (Table I) the presence of two aldehyde groups is confirmed by resonances at δ_C 201.1 and 191.9. The absence of signals for the lactone and hemiacetal moieties and the appearance of a methine signal at δ_C 49.8 immediately suggests formula **2** for kuehneromycin B. The substitution pattern in the decalin system was unequivocally established by ^1H-^{13}C COSY experiments. Thus, in the long range correlation spectrum the aldehyde carbonyl at δ_C 201.1 correlates with the methine proton 9-H, while the second aldehyde group at δ_C 191.9 is connected with the olefinic proton 7-H. In the same way the signal of C-10 gives cross peaks with the methylene protons at C-2 and C-6. Furthermore, in the ^1H NMR spectrum of **2** the methine proton at C-10 experiences a J = 10 Hz coupling with the angular proton at C-5 in agreement with a *trans* stereochemistry at the ring junction (Ayer and Craw, 1989).

The *trans*-decalin structure of **2** was confirmed by NOE experiments. Thus, irradiation at the signal of the angular 10-H enhances the signal of the pseudoaxial 2β-H, whereas irradiation of 9-H leads only to signal enhancement of the angular proton at the 5-position.

The close correspondence of the CD curve of **1** (Fig. 2a) with those of related drimane lactones, e.g. mniopetal F (**3**) (Kuschel *et al.*, 1994, Velten

et al., 1994), allows the assignment of the absolute configuration given in the formulas.



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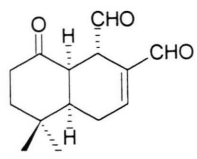

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Interestingly, panudial (**4**), a stereoisomer of kuehneromycin B (**2**) with *cis* annulated AB rings and an α-oriented aldehyde group has been obtained from cultures of a *Panus* sp. (Lorenzen *et al.*, 1994). According to NMR experiments, treatment of either **2** or **4** with 1,8-diazabicyclo[5.4.0]undec-7-ene in THF at room temperature leads to equilibration. However, due to concurrent decomposition the proportion of **2** and **4** at thermodynamic equilibrium could not be determined. Similarly, attempts to correlate the absolute stereochemistry of **4** with **2** by means of this equilibration reaction were unsuccessful. The CD spectra of **2** and **4** are depicted in Fig. 2b.

2 seems to arise biosynthetically from kuehneromycin A (**1**) by decarboxylation of its open chain β-oxo acid form. A series of closely related drimane and nordrimane derivatives have recently been isolated by Ayer and Craw (1989) and Ayer *et al.* (1989) from cultures of *Marasmius oreades.*

Biological properties

The inhibitory effect of kuehneromycin A on the RTs of AMV and MMuLV are shown in Fig. 3.

The activity of MMuLV RT was reduced to 50% at a concentration of 36 μM (10 μg/ml; IC_{50}) whereas the IC_{50} for AMV RT was 270 μM (75 μg/ml). Preincubation of AMV and MMuLV RTs with kuehneromycin A but without substrates for 10 min at 37 °C resulted in a reduction of the IC_{50} to 125 μM (35 μg/ml) for AMV- and 18 μM (5 μg/

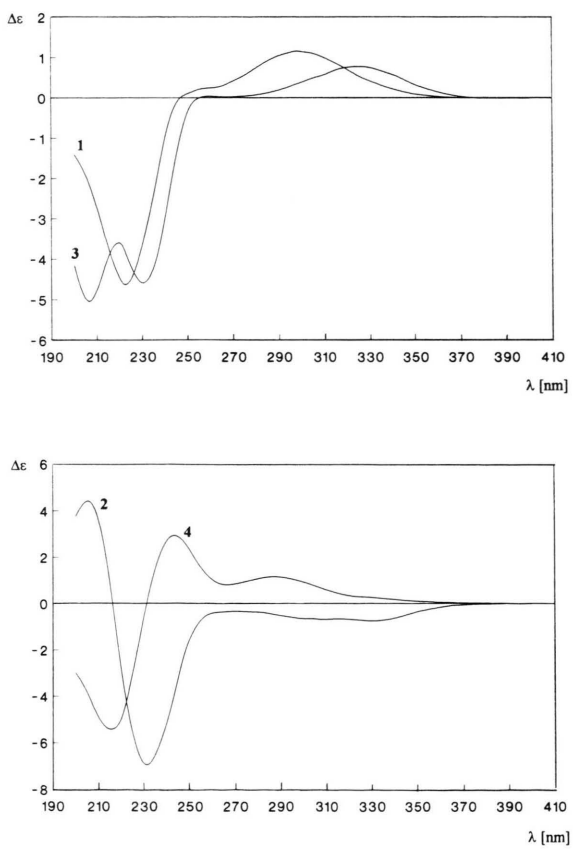

Fig. 2. a) CD spectra of kuehneromycin A (**1**) (2 mg/ml EtOH) and mniopetal F (**3**) (0.15 mg/ml MeCN, 200 - 245 nm, 0.25 mg/ml MeCN, 245 - 400 nm). b) CD spectra of kuehneromycin B (**2**) (2.5 mg/ml EtOH) and panudial (**4**) (0.07 mg/ml MeCN, 200 - 245 nm, 0.81 mg/ml MeCN, 245 - 400 nm).

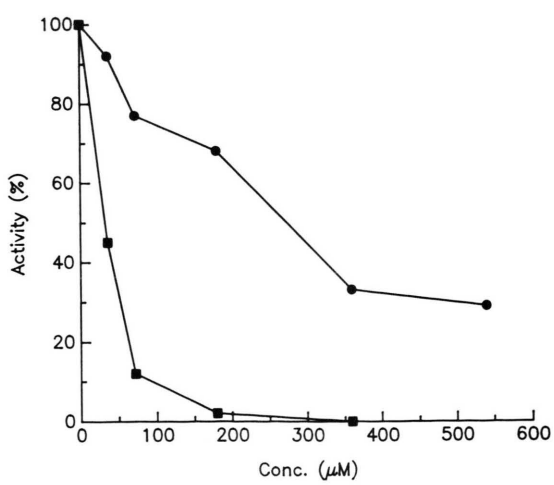

Fig. 3. Effect of kuehneromycin A on the reverse transcriptases of AMV and MMuLV. (●) AMV reverse transcriptase, (■) MMuLV reverse transcriptase. Control without antibiotic (100%): 91 pmol [^{14}C]TMP incorporation for AMV reverse transcriptase and 300 pmol [^{14}C]TMP incorporation for MMuLV reverse transcriptase.

ml) for MMuLV RT (data not shown). The initial rates of incorporation of ^{14}C-dTTP were measured in the absence or presence of increasing amounts of kuehneromycin A. As shown in Figs. 4 and 5 the Lineweaver-Burk plots indicate a noncompetitive inhibition of both RTs with respect to dTTP. The K_i values for kuehneromycin A were calculated to 200 µM for the AMV and 40 µM for the MMuLV RT.

A ten fold increase of the concentration of primer-template poly(A)-$(dT)_{15}$ did not affect the IC_{50} of kuehneromycin A for both the AMV and the MMuLV RTs (data not shown).

The inhibitory effect of kuehneromycin B on RTs of AMV and MMuLV are shown in Fig. 6.

For kuehneromycin B an IC_{50} of 43 µM (10 µg/ml) for AMV RT and 21.5 µM (5 µg/ml) for MMuLV RT was determined.

Compared with the activities on RTs of AMV and MMuLV, the kuehneromycins A and B exhibit only weak inhibitory activity on HIV-1 RT with poly (A)-$(dT)_{15}$ as template-primer as shown in Fig. 7.

The activity of HIV-1 RT was reduced to 50% at a concentration of 667 µM (185 µg/ml) of kuehneromycin A. The IC_{50} for kuehneromycin B on HIV-1 RT with the synthetic primer-template was calculated to 407 µM (95 µg/ml).

With the natural primer-template the kuehneromycins A and B reduced the activity of HIV-1 RT to 50% at a concentration of 54 µM and 64.5 µM (15 µg/ml).

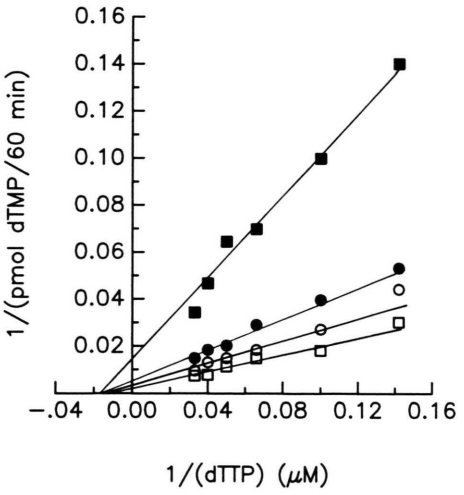

Fig. 4. Lineweaver-Burk plot of inhibition of AMV-RT by kuehneromycin A in the presence of no inhibitor (□), 20 µg/ml kuehneromycin A (○), 50 µg/ml kuehneromycin A (●), 80 µg/ml kuehneromycin A (■). The reactions were carried out as described in the Material and Methods section.

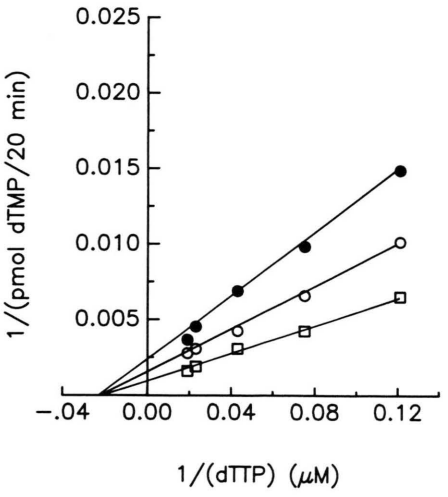

Fig. 5. Lineweaver-Burk plot of inhibition of MMuLV-RT by kuehneromycin A in the presence of no inhibitor (□), 10 µg/ml kuehneromycin A (○), 20 µg/ml kuehneromycin A (●). The reactions were carried out for 20 min as described in the Material and Methods section.

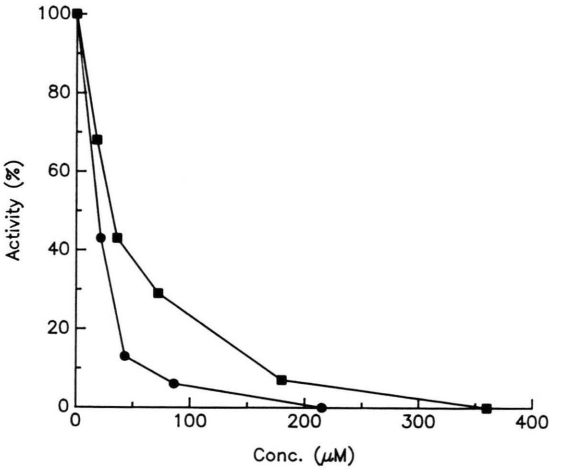

Fig. 6. Effect of kuehneromycin B on the reverse transciptases of AMV and MMuLV. (■) AMV reverse transcriptase, (●) MMuLV reverse transcriptase. Control without antibiotic (100%): 78 pmol [^{14}C]TMP incorporation for AMV reverse transcriptase and 203 pmol [^{14}C]TMP incorporation for MMuLV reverse transcriptase.

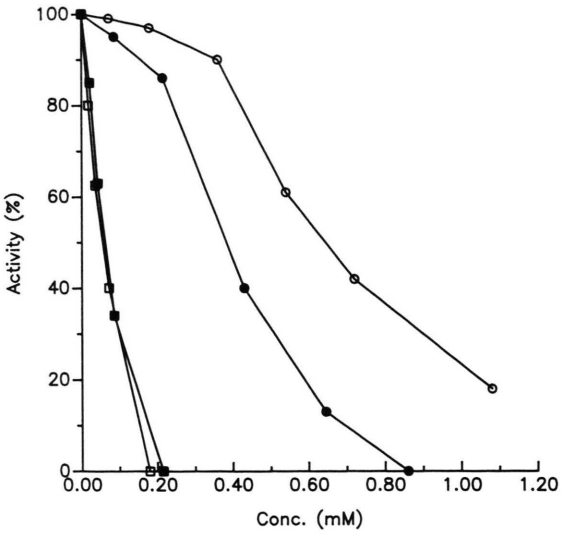

Fig. 7. Effect of kuehneromycins A and B on the HIV-1 reverse transcriptase. (○) kuehneromycin A with poly(A)-$(dT)_{15}$; (●) kuehneromycin B with poly(A)-$(dT)_{15}$. Control without antibiotic (100%): 385 pmol [^{14}C]TMP incorporation; (□) kuehneromycin A with natural primer-template; (■) kuehneromycin B with natural primer-template; control without antibiotic (100%): 3.34 pmol [^{3}H]TMP incorporation.

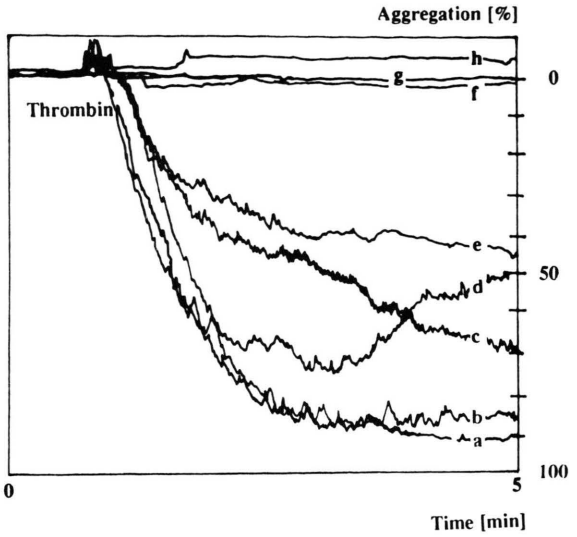

Fig. 8. Inhibition of thrombin induced human platelets aggregation by kuehneromycin B. a: control with 0.1 U/ml thrombin; b: 0.5 μg/ml; c: 0.75 μg/ml; d: 1.0 μg/ml; e: 2.5 μg/ml kuehneromycin B; f: 5.0 μg/ml; g: 10 μg/ml; h: 30 μg/ml.

Because reverse transcriptases share many properties of celluar DNA- and RNA polymerases, the inhibitory effect of kuehneromycin A on DNA and RNA syntheses was studied in permeabilized L1210 cells. RNA- and DNA-syntheses starting from the corresponding nucleoside triphosphates were only weakly inhibited at comparatively high concentrations of 100 μg/ml (360 μM) of kuehneromycin A (data not shown).

As shown in Table II, kuehneromycin B is a potent inhibitor of bovine and human platelet aggregation stimulated with different inducers. All IC_{30} values, except for the thrombin induced aggregation, varied between 1–10 μg/ml (4–43 μM). The tenfold lower IC_{30} value for the inhibition of the thrombin induced aggregation indicates a preferential inhibition of the thrombin receptor, glycoprotein Ib (Fig. 8). No inhibition of platelet aggregation was observed up to 430 μM (100 μg/ml) of kuehneromycin A.

Table II. IC_{30} (70% aggregation) values for the inhibition of platelet aggregation by kuehneromycin B.

Inducer	IC_{30} value [μM]
A) Human platelets	
ADP (4.45 μM)	43
Collagen (0.3 mg/ml)	21
U46619 (0.45 μM)*	21
Ristocetin (0.4 mg/ml)	43
Arachidonic acid (0.6 μg/ml)	43
Thrombin (0.1 U/ml)	3
B) Bovine platelets	
ADP (2.5 μM)	6
Collagen (0.3 mg/ml)	24
Thrombin (0.1 U/ml)	3

* Thromboxane A2 analogue, UpJohn.

Table III. Cytotoxic properties of kuehneromycins A and B.

Cell line	Kuehneromycin A IC_{50} [μM]	Kuehneromycin B
L 1210	>360	22
ECA	180	n.t.
HUT 78	>360	n.t.
HeLa S3	18	22
3T3/MMSV	20	n.t.
BHK	54	86

n.t., not tested.

Table IV. Antibacterial and antifungal activity of kuehneromycins A and B in the agar diffusion assay.

Organism	Diameter of inhibition zone [mm] 100 µg/disc	
	Kuehneromycin A	Kuehneromycin B
Bacteria		
Acinetobacter calcoaceticus	17	n.t.
Bacillus brevis	15	12
Bacillus subtilis	14	n.t.
Bacillus licheniformis	16	n.t.
Micrococcus luteus	16	–
Mycobacterium phlei	13	n.t.
Enterobacter disolvens	8	8
Fungi		
Aspergillus ochraceus	12	n.t.
Mucor miehei	18	–
Nematospora coryli	–	15
Paecilomyces varioti	12	–
Penicillium islandicum	10	n.t.
Penicillium notatum	12	12

–, No inhibition zone; n.t., not tested.

Panudial (**4**) shows similar effects on platelet aggregation. In this case, however, no selectivity with respect to the inducers was observed.

Kuehneromycin A exhibits cytotoxic properties on HeLa S3, 3T3/MMSV, BHK, and ECA cells (Table III). No cytotoxic (lytic) effects were observed on L 1210 and HUT 78 cells. Pronounced cytotoxic effects of kuehneromycin B could be observed on all tested cell lines in the range of 22 µM to 86 µM (Table III).

In the agar diffusion assay the kuehneromycins A and B exhibit very weak antibacterial and antifungal activities (Table IV).

In the test for mutagenicity according to Ames *et al.* (1975) and Venitt *et al.* (1984) no induction of revertants of *S. typhimurium* TA 98 and TA 100 could be observed with 100 µg/plate kuehneromycin A (pour plate assay with and without addition of rat liver microsomes).

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