THE MNIOPETALS, NEW INHIBITORS OF REVERSE TRANSCRIPTASES FROM A *Mniopetalum* species (*Basidiomycetes*)

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

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Six novel enzyme inhibitors of RNA-directed DNA-polymerases of human immunodeficiency-, avian myeloblastosis and murine leukemia viruses were isolated from fermentations of a canadian *Mniopetalum* species. They were named mniopetals A, B, C, D, E and F. Their structures were elucidated by spectroscopic methods. The compounds, in addition to their inhibitory activities on reverse transcriptases, exhibit antimicrobial and cytotoxic properties.

The RNA-directed DNA-polymerases (reverse transcriptases (RT)) of the human immunodeficiency viruses (HIV)-1 and HIV-2 are among the prime targets for selective chemotherapy of AIDS. A world-wide search for inhibitors derived either by chemical synthesis or from natural sources has revealed that a variety of chemical classes of compounds exhibit inhibitory activity against the RT[1,2]. In our ongoing search for new biologically active compounds from basidiomycetes several inhibitors of avian myeloblastosis virus (AMV)-, moloney murine leukemia virus (MMuLV)-, as well as HIV-1- RT have been isolated previously[3~5].

In the following we describe the isolation and biological characterization of six new compounds, the mniopetals A~F, from fermentations of *Mniopetalum* sp. 87256. The elucidation of their structures will be described in a separate paper[6].

Materials and Methods

Mniopetalum sp. 87256

The producing strain was obtained from spore prints of fruiting bodies collected in Canada. The specimen show all characteristic features of the genus[7], the species however, could not be identified. Strain 87256 and voucher specimen are deposited in the culture collection of the LB Biotechnology of the University of Kaiserslautern.

Fermentation

For maintenance the fungus was grown 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 *Mniopetalum* sp. 87256 (400 ml) in YMG-medium was used to inoculate 20 liters of YMG-medium in a Biolafitte C6 fermentation apparatus (temperature: 22°C, aeration: 2.5 liters/minute, stirrer speed: 120 rpm). The production of mniopetals was followed by estimating the inhibitory effect of 2.5 μl of a crude ethyl acetate-extract (concentrated 100 times as compared to the culture fluid) in the assay of AMV RT.

Isolation

During purification the mniopetals were detected using the assay for AMV RT. After 12 days of fermentation, the culture fluid (17 liters) was separated from the mycelia, adsorbed to Mitsubishi Diaion HP 21 resin and eluted with 2 liters methanol and 2 liters acetone. Evaporation of the organic phase yielded a crude extract (2.6 g), which was applied to a column containing silica gel 60 (Merck, Darmstadt). Elution of the column with cyclohexane - ethyl acetate (3 : 7, 1 : 1, and 1 : 9) resulted in three fractions (I, 710 mg; II, 170 mg; III, 450 mg). These were further purified by preparative HPLC (Merck LiChrosorb Diol, column 2.5 × 25 cm, elution with cyclohexane - *tert*-butylmethyl ether) as shown in Fig. 2. Yields: Mniopetal A (**1**) 23.4 mg, B (**2**) 19.3 mg, C (**3**) 4.0 mg, D (**4**) 2.7 mg, E (**5**) 5.6 mg and F (**6**) 5.2 mg. During purification of the mniopetals, 1α,15-dihydroxymarasmene (**7**) and 11,12-dihydroxydrimene (**8**), two compounds exhibiting no activity towards HIV-1 and AMV RT, were also isolated. 1α,15-Dihydroxymarasmene had been previously described from *Marasmius oreades* by AYER *et al.*[8] and 11,12-dihydroxydrimene had been synthesized by HOLLINSHEAD *et al.*[9].

Biological Assays

Antimicrobial spectra and cytotoxic activities on L 1210 cells (ATCC CCL 163) and HL 60 cells (ATCC CCL 240) were measured as described previously[10]. The effect of the mniopetals on BHK 21 cells (ATCC CCL 10) and HeLa S3 cells (ATCC CCL 2.2) was determined according to the method of MIRABELLI *et al.*[11] with slight modifications[12]. HeLa cells were grown in HAM's F 12 medium, BHK 21 cells in G-MEM and HL 60 cells in RPMI-1640 supplemented with 10% fetal calf serum and 65 μg/ml penicillin G and 100 μg/ml streptomycin sulphate in a humidified atmosphere containing 5% of CO_2 at 37°C.

Assay for HIV-1 RT: With poly(A)-$(dT)_{15}$ (tp 1) as template-primer the method of KOPP *et al.*[13] was modified: The reaction mixture (50 μl) contained 80 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol (DTT), 8 mM $MgCl_2$, 30 mM KCl, 200 μg/ml bovine serum albumin (BSA), 10 μM (2-^{14}C)-dTTP (44 cpm/pmol), 5 μg/ml tp 1 and 20 U/ml HIV-1 RT (United States Biochemical, Cleveland, U.S.A.). With a 1080 bp LTR-template and a 18 mer complementary DNA primer (tp 2) (kindly provided by S. WEISS[14]) the HIV-1 reverse transcriptase assay contained: 80 mM Tris-HCl (pH 8.3), 10 mM DTT, 8 mM $MgCl_2$, 30 mM KCl, 200 μg/ml BSA, 10 μM each of dATP, dGTP, dCTP, (^{3}H-)dTTP (4,400 cpm/pmol) and 20 U/ml HIV-1 RT (United States Biochemical, Cleveland, U.S.A.). The LTR-template (2.8 μM) and 18 mer primer (20 μM) were annealed at 66°C, slowly cooled to room temperature and added to the reaction mixture. The reaction mixtures were incubated at 37°C for 60 minutes, then 1 ml of cold 20% trichloroacetic acid containing 20 mM pyrophosphate was added and the acid insoluble fractions were collected on cellulose nitrate filters presoaked with 20 mM pyrophosphate solution. These were washed three times with cold 5% TCA solution and the remaining radioactivity was measured in a liquid scintillation counter.

The assay for AMV RT was performed as described by ERKEL *et al.*[3,4].

Assay for MMuLV RT: The method described by ERKEL *et al.*[3,4] was modified: The reaction mixture (50 μl) contained 250 mM Tris-HCl (pH 8.3), 30 mM $MgCl_2$, 200 mM KCl, 50 mM DTT, 100 μg/ml BSA, 10 mM (2-^{14}C)-dTTP (44 cpm/pmol), 5 μg/ml tp 1 and 20 U/ml MMuLV reverse transcriptase (Boehringer, Mannheim). The reaction mixture was incubated at 37°C for 60 minutes and the radioactivity of the acid insoluble fractions was determined as described above.

Assay of RNA-directed RNA-polymerase of Vesicular stomatitis virus (VSV; ATCC VR 158, Indiana strain): Roller bottles containing confluent monolayer cultures of BHK 21 cells (ATTCC CCL 10) in 200 ml G-MEM medium containing 10% fetal calf serum infected with 2×10^4 plaque forming units (PFU) of VSV were incubated in 125 ml G-MEM medium (2% fetal calf serum) at 37°C for 24 hours at 2 rpm. The medium containing the released virions was clarified by centrifugation (10 minutes at 3,000 × *g*, 4°C).

After addition of 3.75 M ammonium acetate, 20% polyethylenglycol-8000, the supernatant was kept at 4°C overnight. After removal of cell debris by centrifugation (120 minutes at 25,000 × *g*), the virions were pelleted from the supernatant by centrifugation in TMSD-buffer (50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 100 mM NaCl, 4 mM DTT) through a sucrose-gradient (containing 0~40% sucrose in TMSD-buffer) at 20,000 × *g* for 90 minutes. The virions were resuspended in TMSD-buffer containing 30% glycerol and stored at −80°C.

Assay of RNA-directed RNA-polymerase in lysed VSV: The method reported by TALIB *et al.*[15] was modified. The reaction mixture (100 μl) contained: 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM $MgCl_2$, 4 mM DTT, 0.05% Triton X-100, 1 mM each of ATP, GTP, CTP, 0.2 μCi (2-^{14}C)-uridine (1,106 cpm/pmol) and 5 μl VSV-suspension (5 × 10^8 pfu). Incubation was carried out for 2 hours at 30°C and the radioactivity in the acid insoluble fraction was determined as described above.

Test for mutagenicity: Mutagenicity was tested according the method of AMES *et al.*[16]. Mutants of *Salmonella typhimurium* strain TA 98 and TA 100 were used in the "spot test" as described by VENITT *et al.*[17].

Test of hemolytic activity: 9 volumes of fresh bovine slaughter blood were mixed with 1 volume of sodium citrate buffer (93 mM sodium citrate, 140 mM glucose, pH 7.4) and centrifugated at 1,000 × *g* for 10 minutes. 20 μl of this suspension (1.2 × 10^9 erythrocytes/ml) were added to 2 ml phosphate buffered saline (g/liter: 8.0 NaCl, 0.2 KCl, 1.44 $Na_2HPO_4 \times 2H_2O$, 0.2 KH_2PO_4, pH 7.4) and incubated at 37°C for 30 minutes with or without the substances. After centrifugation for 10 minutes at 1,000 × *g* the extinction was measured at 452 and 577 nm: Complete lysis (100%) was achieved by adding 50 μl of Brij 58 (5%).

Results and Discussion

Production of Mniopetals

A typical fermentation of *Mniopetalum* spec. is shown in Fig. 1. The production of the mniopetals was measured by the inhibition of the AMV RT as described in the experimental section. The highest content of the inhibitors was reached after 260 hours. Culture extracts that inhibited AMV RT also inhibited HIV-1 RT in the assay with template-primer 2. Fig. 2 shows the isolation of the mniopetals A~F from the culture broth of a 20 liters fermentation. Their structures (Fig. 3) were elucidated by spectroscopic methods[6]. All compounds are substituted drimane-type sesquiterpenoids. It is interesting that the mniopetals can also be isolated from the closely related *Cyphellostereum laeve* (FR.) Reid.

Fig. 1. Fermentation of *Mniopetalum* sp.

□ Mycelial dry weight [g/liter], △ glucose [g/liter], ▽ inhibition of AMV reverse transcriptase (RT) with tp 1 [%], ○ inhibition of HIV-1 RT with tp 2 [%], --- CO_2-production [vol%], ------ O_2-consumption [vol%], —— oxygen saturation [vol%].

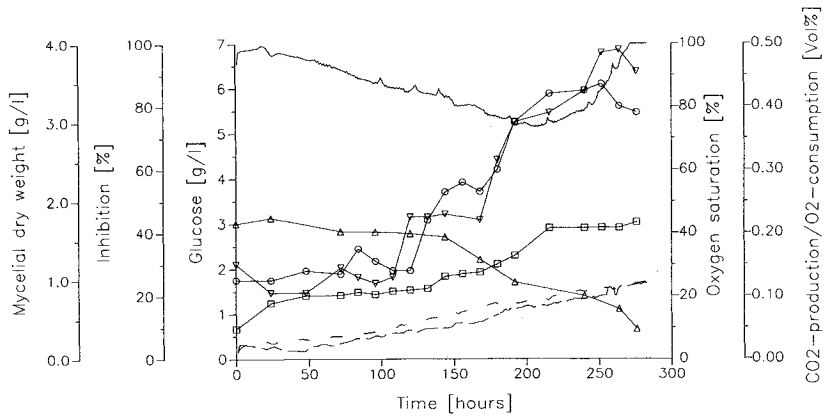
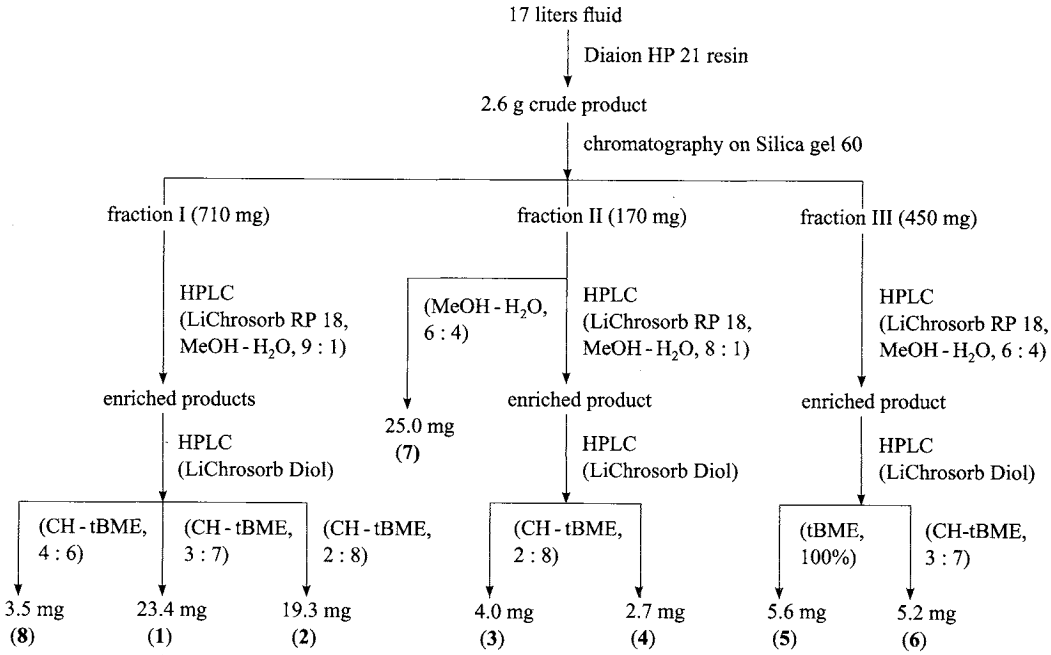


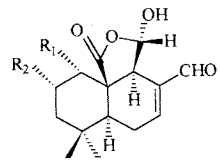
Fig. 2. Isolation of mniopetals from the culture filtrate of *Mniopetalum* sp.

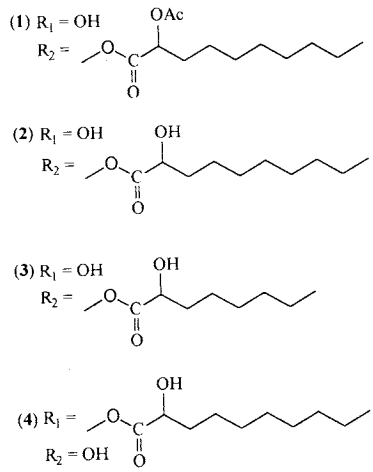


Abbreviations: CH=cyclohexane, MeOH=methanol, tBME=*tert*-buthylmethyl ether.

Fig. 3. Structures of mniopetals.

(**1**) mniopetal A, (**2**) mniopetal B, (**3**) mniopetal C, (**4**) mniopetal D, (**5**) mniopetal E, (**6**) mniopetal F, (**7**) 1α,15-dihydroxymarasmene, (**8**) 11,12-dihydroxydrimene.





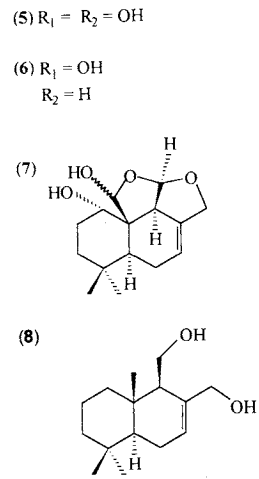


Table 1. Effects of mniopetals on the reverse transcriptases (RT) of HIV-1, AMV and MMuLV.

Compound	IC_{50} (μM)			
	AMV RT tp 1[a]	MMuLV RT tp 1[a]	HIV-1 RT tp 1[a]	HIV-1 RT tp 2[b]
Mniopetal A	41	4	>197	>197
Mniopetal B	42	1.7	>223	91
Mniopetal C	93	7	>238	190
Mniopetal D	77	6	>223	54
Mniopetal E	>338	59	>338	140
Mniopetal F	>320	30	>320	30

[a] poly(A)-$(dT)_{15}$.
[b] LTR-template + 18 mer primer.

Table 2. Effects of mniopetals on the RNA-directed RNA-polymerase of VSV.

Compound	IC_{50} (μM)
Mniopetal A	59~62
Mniopetal B	51
Mniopetal C	50
Mniopetal D	47~49
Mniopetal E	>338
Mniopetal F	61~64

Table 3. Cytotoxic activities of mniopetals towards different mammalian cell lines.

Compound	IC_{50} (μM)			
	Cell lines			
	L 1210	HL 60	BHK	HeLa S3
Mniopetal A	98~197	49~98	>197[a]	39~59
Mniopetal B	56~11	11~22	89	45~67
Mniopetal C	119~238	12~24	24~48	48
Mniopetal D	56~111	2~11	89	45
Mniopetal E	338[a]	68~169	68	169
Mniopetal F	18	18~36	107~143	18~36

[a] Growth inhibition.

Table 4. Hemolytic activities of mniopetals. The concentrations causing 100% lysis of erythrocytes are shown.

Compound	(μM)
Mniopetal A	10
Mniopetal B	11
Mniopetal C	95
Mniopetal D	11
Mniopetal E	338
Mniopetal F	—[a]

[a] No hemolytic activity.

Biological Properties

The effects of mniopetals A, B, C, D, E and F on the reverse transcriptases of HIV-1, AMV and MMuLV are shown in Table 1. The mniopetals inhibit the MMuLV RT most strongly while their IC_{50} values towards AMV RT are much higher. It is interesting that HIV-1 RT is inhibited only when the natural heteropolymeric template (tp 2) is used in the assay. Preincubation of HIV-1 RT with the mniopetals for 10 minutes at 37°C without substrates resulted in a change of the IC_{50} for mniopetals B (70 μM), C (48 μM), D (39 μM), E (198 μM), and F (18 μm). A 10-fold increase in the concentration of template-primer 2 had no effect on the inhibition of HIV-1 RT.

In addition to the RNA-directed DNA-polymerase the mniopetals, with the exception of mniopetal E, inhibit the RNA-directed RNA-polymerase of VSV at IC_{50} values of 50~64 μM (Table 2).

As shown in Table 3 the mniopetals exhibit cytotoxic activities, the most sensitive cell line being HL-60. This is at least partly due to a lytic action on the cytoplasmic at least partly due to a lytic action on the cytoplasmic membranes as can be also inferred from the hemolytic activities (Table 4).

The antibacterial and antifungal activities of the mniopetals are quite weak with the exception of *Streptomyces* sp. ATCC 23836 (Table 5).

Table 5. Antimicrobial activities of mniopetals in the serial dilution assay.

Organism	MIC (μM)					
	(1)	(2)	(3)	(4)	(5)	(6)
Bacteria						
Acinetobacter calcoaceticus	98	45	119	111	>338	357
Escherichia coli K12	>197	111	>238	>223	>338	>357
Salmonella typhimurium TA 98	>197	22	48	223	338	357
Arthrobacter citreus	40	111	119	111	338	>357
Bacillus brevis	197	223	119	>223	169	>357
Bacillus subtilis	20	11	24	223	169	>357
Corynebacterium insiduosum	98	11	24	45	>338	357
Micrococcus luteus	>197	>223	>238	>223	>338	>357
Mycobacterium phlei	10	11	24	223	169	>357
Streptomyces sp. ATCC 23836	2	0.22	2.4	1.1	169	71
Fungi						
Nadsonia fulvescens	98	45	119	111	>338	>357
Nematospora coryli	10	11	48	45	>338	>357
Saccharomyces cerevisiae S 288	197	>223	238	111	>338	>357
Saccharomyces cerevisiae is 1	40	11	238	45	338	18
Fusarium oxysporum	40	11	238	11	>338	357
Paecilomyces variotii	98	111	>238	>223	>338	>357
Penicillium notatum	98	111	>238	>223	>338	>357
Mucor miehei	98	45	>238	>223	>338	>357
Rhodotorula glutinis	40	45	238	111	338	357
Ustilago nuda	40	45	>238	223	>338	357

In the test for mutagenicity according to AMES *et al.*[16] and VENITT *et al.*[17] no induction of revertants of *S. typhimurium* TA 98 and TA 100 could be observed with 100 μg of the mniopetals/disc.

Acknowledgments

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