Podoscyphic Acid, a New Inhibitor of Avian Myeloblastosis Virus and Moloney Murine Leukemia Virus Reverse Transcriptase from a *Podoscypha* Species [1]

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AMV, MMLV-Reverse Transcriptases, Inhibitor, Antibiotic, Basidiomycetes

A novel enzyme inhibitor of RNA-directed DNA-polymerases of avian myeloblastosis and murine leukemia virus was isolated from fermentations of an tasmanian *Podoscypha* species. Its structure was elucidated by spectroscopic methods and oxidative degradation as (*E*)-4,5-dioxo-2-hexadecenoic acid (**1**). The enzyme inhibitor, which was named podoscyphic acid, did not inhibit DNA and RNA synthesis in permeabilized L 1210 cells nor did it affect RNA synthesis in isolated nuclei of L 1210 cells. **1** inhibits protein synthesis in whole L 1210 cells and rabbit reticulocyte lysate and shows very weak antimicrobial and cytotoxic properties. The testing of ethyl (*E*)-4,5-dioxo-2-hexadecenoate (**2**) and (*E*)-4-oxo-2-tetradecenoic acid (**11**) revealed the importance of the free γ-oxoacrylic acid unit for the biological activities of **1**.

Introduction

Reverse transcription, a reaction catalyzed by RNA-directed DNA polymerase, is an essential step in the replication of retroviruses and the stable inheritance of the viral genome [2]. Effective and selective inhibitors of reverse transcriptases are therefore considered potential antiviral chemotherapeutics [3]. In the course of a screening for new fungal metabolites inhibiting avian myeloblastosis virus (AMV) reverse transcriptase podoscyphic acid (**1**) was isolated from the mycelium of the basidiomycete *Podoscypha* spec. strain 874. In the following we describe the fermentation, isolation, structural elucidation, and biological characterization of podoscyphic acid (**1**), the first compound with a γ,δ-dioxoacrylate moiety from a basidiomycete.

Materials and Methods

AMV reverse transcriptase, poly(A)-$(dT)_{15}$ and rabbit reticulocyte lysate type I were purchased from Boehringer, Mannheim, F.R.G. Moloney murine leukemia virus (MMuLV) reverse transcriptase was obtained from Pharmacia Fine Chemicals, Sweden. [2-^{14}C]dTTP (56 mCi/mmol), [2^{14}C]UTP (52 mCi/mmol), L-[4,5-^{3}H]leucine (133 Ci/mmol) and 2-amino[1-^{14}C]isobutyric acid (59 mCi/mmol) were purchased from Amersham Buchler, F.R.G. [1-^{14}C]leucine (54 mCi/mmol), [2-^{14}C]thymidine (55 mCi/mmol) and [2-^{14}C]uridine (50 mCi/mmol) were obtained from New England Nuclear, F.R.G. ^{1}H NMR spectra were run with a Bruker AC-200 instrument at 200 MHz. HPLC was performed with a Waters apparatus with diode array detection; Nucleosil 5C18 column (25 cm × 4 mm, 5 µm particle size); mobile phase A (H_2O/CH_3CN 9:1 + 0.5% CF_3CO_2H), mobile phase B (H_2O/CH_3CN 1:9); linear gradient within 45 min from 100% A to 100% B, flow rate 1 ml min^{-1}. GC-MS: Hewlett-Packard GC 5890 combined with a 5970 Series Mass-Selective-Detector.

Podoscypha spec. strain TA 874

The producing strain was obtained from a fruiting body collected in Hobart, Tasmania. The specimen shows all characteristic features of the genus [4], the species, however, could not be identified. Voucher specimen and strain 874 are deposited in the culture collection of the Lehrbereich Biotechnologie of the University of Kaiserslautern.

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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. The medium (YMPG) used for the production of podoscyphic acid contained (g/l): maltose 20, glucose 10, yeast extract 0.8, peptone 2, KH_2PO_4 0.5, $MgSO_4 \cdot 7\,H_2O$ 1, $FeCl_3$ 0.01, $ZnSO_4$ 0.002, and $CaCl_2$ 0.055. A well grown seed culture of *Podoscypha* spec. (200 ml) in YMG-Medium was used to inoculate 20 l of YMPG-Medium in a Biolafitte C 6 fermentation apparatus. The fermenter was incubated at 22 °C with an aeration rate of 3 l air/min and agitation (130 rpm). The production of podoscyphic acid was followed by estimating the inhibitory effect of 2.5 µl of a crude mycelial extract (concentrated 100 times as compared to the culture fluid) in the standard assay against AMV reverse transcriptase.

Isolation

After two weeks of fermentation, the mycelia (35 g) were separated from the culture fluid and extracted with 4 l of acetone. After evaporation of the organic phase 4 g of an enriched product was precipitated. Purified podoscyphic acid (3.5 g) was obtained by washing the precipitate several times with H_2O/MeOH (99:1).

*Physicochemical properties of podoscyphic acid [(E)-4,5-dioxo-2-hexadecenoic acid] (**1**)*

Light yellow solid, m.p. 105 °C, R_f 0.45 [silica gel; toluene–Me_2CO–AcOH (70:30:1)]; UV (MeOH) nm λ_{max} (log ε) 210 (3.75); IR ($CHCl_3$) cm^{-1} 3150 (br), 2960 (st), 2920 (sst), 2850 (sst), 1725 (sst), 1690 (sst), 1650 (m), 1630 (m), 1470 (m), 1460 (st), 1410 (st), 1390 (m), 1375 (m), 1300 (m), 1290 (st), 1260 (st), 1235 (m), 1220 (m), 1200 (w), 1160 (w), 1125 (w), 1095 (w), 1085 (w), 1010 (w), 975 (st), 955 (m), 890 (w), 870 (w), 730 (m), 720 (m), 650 (m), 630 (m), 620 (w); ^1H NMR ($CDCl_3$): δ 0.87 (t, J = 7.5 Hz, 3H), 1.16–1.42 (m, 16H), 1.63 (m, 2H), 2.81 (t, J = 7.5 Hz, 2H), 6.92 (d, J = 16.4 Hz, 1H), 7.78 (d, J = 16.4 Hz, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 14.1, 22.7, 22.9, 29.1, 29.3, 29.4, 29.5, 29.6, 29.7, 31.9, 36.4, 133.7, 134.3, 167.4, 186.8, 199.3; HR-MS (70 eV, DI 180 °C): m/z (relative intensity, %) 283.1904 (2.1, M + 1, calcd for $C_{16}H_{27}O_4$ 283.1909), 282.1822 (1.7, M^+, calcd for $C_{16}H_{26}O_4$ 282.1831), 264 (6), 254 (2), 238 (3), 227 (15), 200 (20), 183 (48), 181 (14), 174 (65), 156 (86), 129 (21), 128 (25), 111 (39), 101 (100), 97 (22), 95 (25), 85 (27), 83 (31), 73 (45), 71 (37), 69 (26), 57 (70), 55 (33).

*Ethyl (E)-4,5-dioxo-2-hexadecenoate (**2**)*

To a stirred solution of **1** (28 mg) in EtOH (2 ml) was added 2 ml of 3% ethanolic H_2SO_4 and the mixture was left for 24 h at 25 °C. Usual work-up yielded an oil which was purified by chromatography on Sephadex LH-20 (eluent MeOH) to afford **2** as a light-yellow oil (12 mg), R_f 0.60 [silica gel; hexane–EtOAc (10:1)]; UV (MeOH) nm λ_{max} 235; IR ($CHCl_3$) cm^{-1} 2925 (sst), 2855 (st), 1720 (sst), 1695 (st, sh), 1650 (w), 1620 (w); ^1H NMR ($CDCl_3$): δ 0.89 (m, 3H), 1.15–1.40 (m, 19H), 1.62 (m, 2H), 2.82 (t, J = 7.2 Hz, 2H), 4.28 (q, J = 7.0 Hz, 2H), 6.90 (d, J = 15.6 Hz, 1H), 7.66 (d, J = 15.6 Hz, 1H); HR-MS (70 eV, DI 180 °C): m/z (relative intensity, %) 310.2132 (0.8, M^+, calcd for $C_{18}H_{30}O_4$ 310.2144), 237 (6), 184 (10), 156 (14), 129 (100), 101 (63), 69 (8), 57 (8), 55 (10).

*Oxidative cleavage of **1***

To a solution of **1** (14.1 mg) in MeOH (0.8 ml) was added 0.1 ml of 1.8% aqueous H_2O_2 and the mixture was treated dropwise at 25 °C with 0.001 M ethylenediaminetetra-acetic acid (EDTA) in 2 N NaOH (0.1 ml). The mixture was stirred for 12 h, acidified with 2 N HCl and extracted with EtOAc (5 × 2 ml). The solvent was removed *in vacuo* and the residue dissolved in MeOH. Fumaric acid was detected by HPLC analysis (diode array detection) and co-chromatography with an authentic sample. After treatment of the MeOH solution with diazomethane, methyl dodecanoate was identified by GC-MS [m/z (relative intensity, %) 214 (5, M^+), 183 (6, $M-OCH_3$), 171 (7), 143 (10), 87 (53), 74 (100), 69 (9), 59 (12), 57 (10), 55 (22)].

*Reaction of **1** with 2,4-dinitrophenylhydrazine*

To a solution of **1** (28 mg) in dimethylformamide (DMF, 0.5 ml) was added 2,4-dinitrophenylhydrazine (0.2 mg) in DMF (2 ml) and a drop of conc. hydrochloric acid. After 12 h at room temperature, the mixture was diluted with 2 N HCl and the resulting red crystals were filtered off.

They were washed with 2 N HCl and water and chromatographed on Sephadex LH-20. Elution with MeOH yielded bishydrazone (**6**) (19.2 mg) and addition product **7** (3.1 mg).

6: orange-red crystals, m.p. 210 °C, R_f 0.44 [silica gel; toluene–Me_2CO–AcOH (70:30:1)]; UV (MeOH) nm λ_{max} (log ε) 430 (sh, 4.35), 395 (4.37), 250 (sh, 4.24), 210 (4.40); ^{1}H NMR (CD_3OD): δ 0.88 (m, 3H), 1.15–1.60 (m, 16H), 1.75 (m, 2H), 3.01 (t, J = 7.5 Hz, 2H), 6.57 (d, J = 16.5 Hz, 1H), 7.16 (d, J = 16.5 Hz, 1H), 8.11 (d, J = 9.5 Hz, 1H), 8.13 (d, J = 9.5 Hz, 1H), 8.42 (dd, J = 9.5 and 2.7 Hz, 1H), 8.48 (dd, J = 9.5 and 2.7 Hz, 1H), 9.07 (d, J = 2.7 Hz, 2H); MS (70 eV, DI 200 °C): m/z (relative intensity, %) 606 (1, M–CO_2), 530 (23), 500 (12), 470 (6), 403 (8), 391 (9), 390 (41), 183 (12), 129 (9), 97 (12), 85 (10), 83 (15), 75 (20), 73 (18), 71 (23), 60 (20), 57 (38), 55 (33), 44 (100), 43 (42), 41 (27).

7: orange-red crystals, m.p. 132 °C, R_f 0.37; UV (MeOH) nm λ_{max} (log ε) 425 (4.31, sh), 388 (4.33), 257 (4.11, sh), 202 (4.44); ^{1}H NMR (CD_3OD): δ 0.88 (m, 3H), 1.15–1.60 (m, 18H), 2.90–3.07 (m, 2H), 3.58 (m, 1H), 3.68 (m, 1H), 4.05 (dd, J = 10.5 and 6.0 Hz, 1H), 7.55 (d, J = 9.9 Hz, 1H), 7.86 (dd, J = 9.9 and 3.0 Hz, 1H), 7.96 (d, J = 9.0 Hz, 1H), 8.24 (d, J = 9.0 Hz, 1H), 8.33–8.45 (m, 2H), 8.70 (d, J = 3.0 Hz, 1H), 8.90 (d, J = 3.0 Hz, 1H), 9.03 (d, J = 3.0 Hz, 1H).

*Reaction of **1** with 1,2-diaminobenzene*

A solution of **1** (28 mg, 0.1 mmol) and 1,2-diaminobenzene (21.6 mg, 0.2 mmol) in EtOH (10 ml) was maintained for 5 h at 70 °C. The mixture was then stirred for 3 days at 25 °C without exclusion of oxygen. Purification of the resulting mixture by chromatography on a silica gel column [eluant: $CHCl_3$–Me_2CO (100:1)] yielded **9** as red crystals (4.1 mg): m.p. 253 °C, R_f 0.53 [silica gel; $CHCl_3$–Me_2O (50:1)]; UV (dioxan) nm λ_{max} (log ε) 468 (3.55), 450 (3.52, sh), 320 (3.95), 310 (3.91, sh), 275 (3.86), 233 (4.43), 201 (4.48); IR (KBr) cm^{-1} 3440 (br), 2920 (st), 2850 (st), 1680 (sst), 1670 (st), 1630 (st); ^{1}H NMR (CD_3OD): δ 0.89 (m, 3H), 1.16–1.63 (m, 16H), 1.89 (m, 2H), 3.14 (t, J = 6.0 Hz, 2H), 6.79 (s, 1H), 6.92–7.18 (m, 4H), 7.50–7.70 (m, 2H), 7.85–7.98 (m, 2H), 9.17 (s, 1H), 13.98 (s, 1H); MS (70 eV, DI 180 °C): m/z (relative intensity, %) 459 (19, M + 17), 458.2681 (51, M + 16, calcd for $C_{28}H_{34}N_4O_2$ 458.2681), 443 (35), 442.2733 (100, calcd for $C_{28}H_{34}N_4O$ 442.2733), 318 (22), 317 (12), 316 (34), 303 (15), 302 (37), 301 (28), 289 (14), 288 (39), 287 (23), 285 (13), 284 (33), 283 (77), 259 (12), 185 (10), 161 (14), 159 (15), 147 (17), 145 (18), 144 (29).

*1,1-Diethoxy-2-tetradecynoic acid (**10**)*

To a cold (–78 °C), stirred solution of 3,3-diethoxy-1-propyne (0.29 ml, 2 mmol) in 6 ml of dry THF (argon atmosphere) was slowly added *n*-butyllithium (1.6 molar solution in *n*-hexane, 1.25 ml, 2 mmol). After the solution had been stirred at –78 °C for 30 min, undecanal (0.41 ml, 2 mmol) was added and stirring was continued at –78 °C for 30 min. The mixture was slowly allowed to warm up to room temperature. Saturated ammonium chloride solution (10 ml), ice (4 g) and Et_2O (20 ml) were added and the mixture was stirred. The phases were separated and the aqueous layer was extracted with two 25 ml portions Et_2O. The combined organic phases were washed (sat. aqueous NaCl), dried ($MgSO_4$), and concentrated.

The remaining oil was dissolved in 6 ml of CH_2Cl_2 and at 0 °C slowly added to a stirred suspension of activated MnO_2 (5.2 g) in 10 ml of CH_2Cl_2. After 1 h at 0 °C the stirred mixture was allowed to warm up to room temperature within 2 h. Concentration of the filtered solution *in vacuo* yielded an oil which was chromatographed on a silica gel column. Elution with petroleum ether (40–60 °C)/EtOAc (50:1) afforded **10** as a colourless liquid (0.35 g, 59%); ^{1}H NMR ($CDCl_3$): δ 0.83 (t, J = 6.0 Hz, 3H), 1.10–1.40 (m, 20H), 1.42–1.92 (m, 2H), 2.56 (t, J = 7.5 Hz, 2H), 3.45–3.90 (m, 4H), 5.35 (s, 1H); MS (70 eV, DI 180 °C): m/z (relative intensity, %) 296.2326 (0.4, M$^+$, calcd for $C_{18}H_{32}O_3$ 296.2351), 295 (2.4), 252 (24), 251 (70), 125 (25), 111 (100), 103 (35), 83 (65).

*(E)-4-Oxo-2-tetradecenoic acid (**11**)*

To a stirred solution of 1,1-diethoxy-2-tetradecyn-4-one (0.32 g, 1.07 mmol) in 3.2 ml dioxan was added dropwise 1 ml of 2 N HBr. The mixture was heated at 80 °C for 15 h, cooled to 0 °C, and stirred with saturated aqueous $NaHCO_3$ (11 ml) and Et_2O (10 ml). The white precipitate was filtered off and the organic phase extracted with saturated $NaHCO_3$ (2 × 10 ml). The precipitate and

the combined aqueous phases were carefully acidified to pH 2 with 4 N HCl and extracted with EtOAc (4 × 20 ml). After drying ($MgSO_4$) and concentration of the organic phase the resulting residue was recrystallized from EtOAc–hexane to yield **11** in form of colourless crystals (0.17 g, 65%), m.p. 110 °C; IR (KBr) cm^{-1} 3510 (w), 3100 (br), 3065 (m), 2950 (st), 2910 (sst), 2850 (sst), 1740 (m), 1710 (st), 1680 (sst), 1660 (sst), 1620 (m); ^1H NMR ($CDCl_3$): δ 0.83 (m, 3H), 1.15–1.37 (m, 14H), 1.44–1.80 (m, 2H), 2.62 (t, J = 7.5 Hz, 2H), 6.62 (d, J = 16.0 Hz, 1H), 7.13 (d, J = 16.0 Hz, 1H), 7.70 (br, 1H); ^{13}C NMR (CD_3OD) δ 14.4, 23.7, 24.8, 30.1, 30.2, 30.4, 30.5, 30.6, 33.0, 42.0, 132.3, 140.7, 168.6, 202.1; MS (70 eV, DI 180 °C): m/z (relative intensity, %) 240.1729 (0.5, M^+, calcd for $C_{14}H_{24}O_3$ 240.1726), 195 (21), 137 (827), 123 (19), 115 (21), 114 (100), 99 (31), 96 (29), 57 (30), 55 (23), 43 (32), 41 (28).

Biological assays

Antimicrobial spectra, cytotoxicity and macromolecular synthesis in whole L 1210 cells (ATCC CCL 163) were measured as described previously [5]. The effect of podoscyphic acid on cell growth was measured according to the method of Mirabelli *et al.* [6] with slight modifications [7]. 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 cells (ATCC CCL 10) in G-MEM, 3T3/MMSV cells (Moloney murine sarcoma virus transformed Balb/3T3 mouse embryo cells, ATCC CCL 163.2) in D-MEM medium 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.

Assay for AMV reverse transcriptase

The method reported by Hanajima *et al.* [8] 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 reverse transcriptase were incubated at 37 °C for 60 min. The reaction was terminated by adding 1 ml of cold 20% trichloroacetic acid 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 MMuLV reverse transcriptase

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 reverse transcriptase. Unless otherwise specified, the reaction mixture was incubated for 60 min at 37 °C and the radioactivity of the acid insoluble fractions determined as described above.

Nucleic acid syntheses

Nucleic acid syntheses in permeabilized L 1210 cells were performed according to Berger [9]. RNA synthesis in isolated nuclei was measured as described previously [10].

Cell-free translation system

Rabbit reticulocyte lysate was incubated with 40 µg/ml β-globin mRNA using standard published methods [11].

Test for mutagenicity

Mutagenicity was tested according to the method of Ames *et al.* [12]. Mutants of *Salmonella typhimurium* strain TA 98 and TA 100 were used in the pour plate assay as described by Venitt *et al.* [13].

Results and Discussion

Production and isolation of podoscyphic acid

A typical fermentation of *Podoscypha* spec. 874 is shown in Fig. 1. The production of podoscyphic acid starts six to eight days after inoculation. The highest content of the inhibitor is reached after 14 days when the glucose in the medium is completely used up and growth stops.

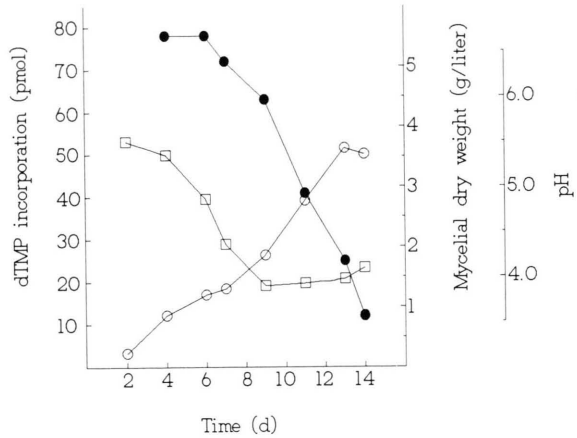
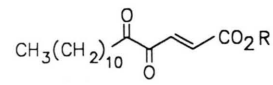


Fig. 1. Fermentation of *Podoscypha* spec. Dry weight (○), pH (□), activity (●), determined as described in the experimental section.

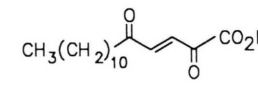
Structural elucidation and chemistry

Podoscyphic acid (**1**) forms an ethyl ester **2** on acid-catalyzed esterification and displays two carbonyl absorptions at 1720 and 1690 cm^{-1} in the IR spectrum ($CHCl_3$). The ^{13}C NMR spectrum shows a carboxyl signal at δ 167.4 and two additional signals for conjugated carbonyl groups at δ 186.8 and 199.3. The presence of an isolated (*E*)-double bond is indicated by an AB-system at δ 6.92 and 7.78 (J = 16.4 Hz) in the ^1H NMR spectrum and signals at δ 133.7 and 134.3 in the ^{13}C NMR spectrum. From an analysis of the ^1H and ^{13}C NMR data two possible structures **1** and **3** can be deduced for podoscyphic acid.

A decision in favour of structure **1** was achieved by oxidative degradation with alkaline H_2O_2 [14] which yielded fumaric acid (**4**) and dodecanoic acid (**5**). Podoscyphic acid is therefore (*E*)-4,5-dioxo-2-hexadecenoic acid (**1**).

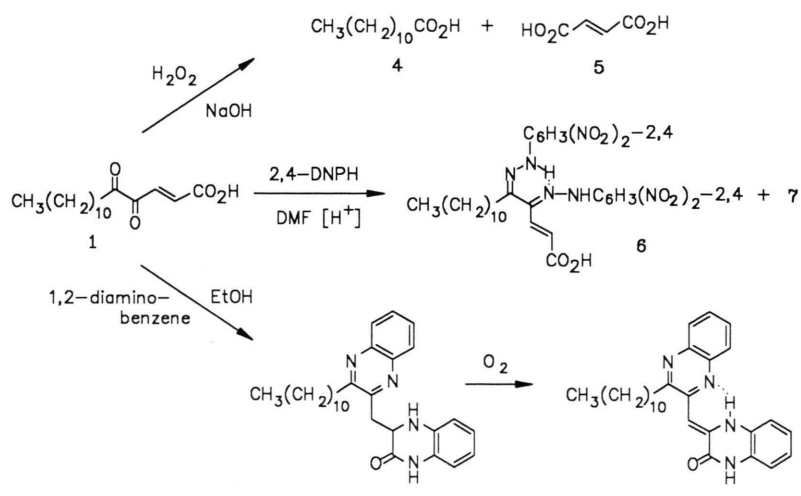


1, R = H
2, R = C_2H_5

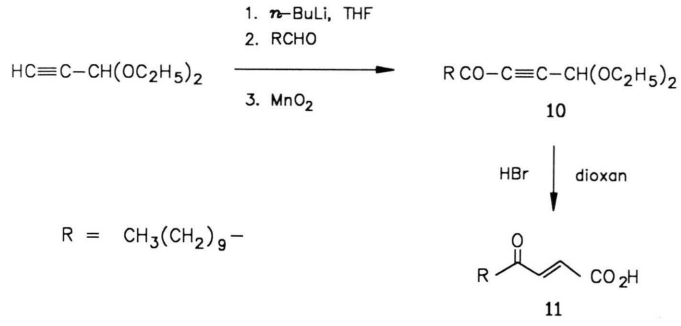


3

On reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH) in DMF [15], **1** yields the expected bishydrazone **6** and in addition a derivative **7** in which a third molecule of 2,4-DNPH has been added to the double bond. The regiochemistry of this addition could not be determined. A bright red bisquinoxalinomethene derivative **9** is formed by reaction of **1** with an excess of 1,2-diaminobenzene. Obviously, the primary condensation product **8** undergoes air oxidation to yield **9.** The presence of two singlets in the ^1H NMR spectrum at δ 6.97 (olefinic proton) and 13.98 (chelated NH) is in accord with structure **9.** Interestingly, an (M + 16)-ion in the mass spectrum indicates that **9** is contaminated with small amounts of an oxidation product.



Scheme 1



Scheme 2

For comparison of its biological activity, (*E*)-4-oxo-2-tetradecenoic acid (**11**) was synthesized according to Obrecht [16] *via* acid-catalyzed isomerization of acetal **10.** The latter compound was prepared by addition of the lithium salt of 3,3-diethoxy-1-propyne to undecanal followed by oxidation of the resulting carbinol with manganese dioxide.

Biological properties

The inhibitory effect of podoscyphic acid (**1**) on the reverse transcriptases of AMV and MMuLV is shown in Fig. 2. MMuLV reverse transcriptase activity was reduced to 50% at a concentration of 10–20 μg/ml (IC_{50}), whereas the IC_{50} was 100 μg/ml for the AMV reverse transcriptase. Preincubation of AMV reverse transcriptase with **1** for 10 min at 37 °C resulted in a slight increase of the inhibitory effect on AMV reverse transcriptase and a more pronounced effect on MMuLV reverse transcriptase (Table I). Preincubation of the template-primer poly(A)-$(dT)_{15}$ did not affect the inhibitory action of **1** on both reverse transcriptases (data not shown). A tenfold increase in the concentration of the template-primer had no effect on the inhibition of AMV reverse transcriptase while a reduction of the inhibitory effects of **1** against MMuLV reverse transcriptase could be observed (Table II).

The initial rate of incorporation of [^{14}C]dTTP was measured in the absence or the presence of podoscyphic acid. The Lineweaver-Burk plots (Fig. 3, 4) indicate a non-competitive inhibition of both reverse transcriptases with respect to TTP indicating that the binding site for **1** is different from those for substrate and template-primer. The K_i values for **1** were calculated to 390 μM for AMV reverse transcriptase and 113 μM for MMuLV reverse transcriptase. The biological activity of

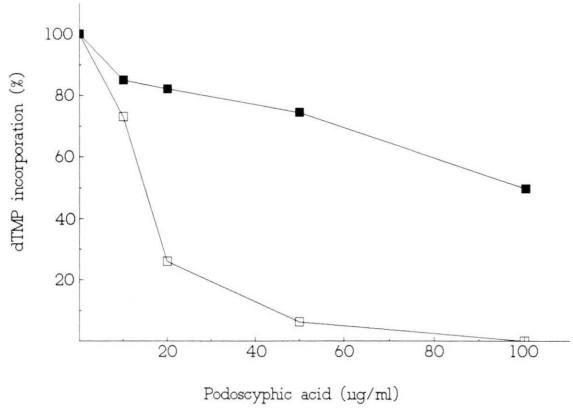


Fig. 2. Effect of podoscyphic acid on reverse transcriptases of AMV and MMuLV. ■, RNA-directed DNA polymerase of AMV; □, RNA-directed DNA polymerase of MMuLV. Controls without antibiotic (100%): 90 pmol [^{14}C]TMP incorporation in 60 min for AMV-RT and 260 pmol in the case of MMuLV-RT.

Table I. Effect of preincubation of reverse transcriptase with podoscyphic acid. The enzymes (20 U/ml) were incubated in the absence or presence of podoscyphic acid at 37 °C for 10 min without substrates. The other components were added to complete the reaction mixture (50 μl) which was then incubated at 37 °C for 50 min.

Podoscyphic acid [μg/ml]	% Inhibition AMV-RT	MMuLV-RT
No preincubation		
10	15	27
20	20	73
Preincubation with enzyme		
10	17	86
20	22	100

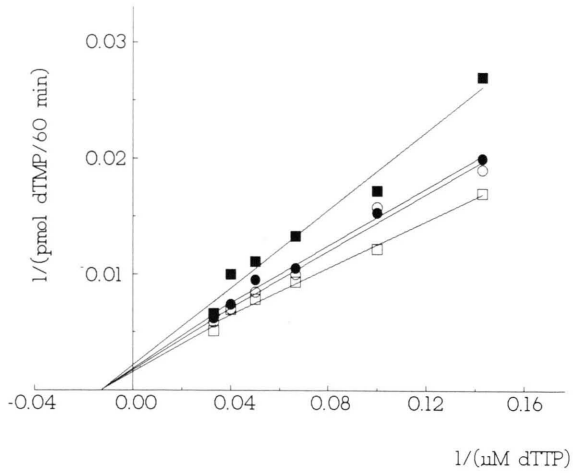


Fig. 3. Inhibition of AMV-RT by podoscyphic acid. Double reciprocal plot of TTP-dependent reaction velocities in the presence of no inhibitor (□); 20 μg/ml podoscyphic acid (○); 50 μg/ml podoscyphic acid (●); 100 μg/ml podoscyphic acid (■).

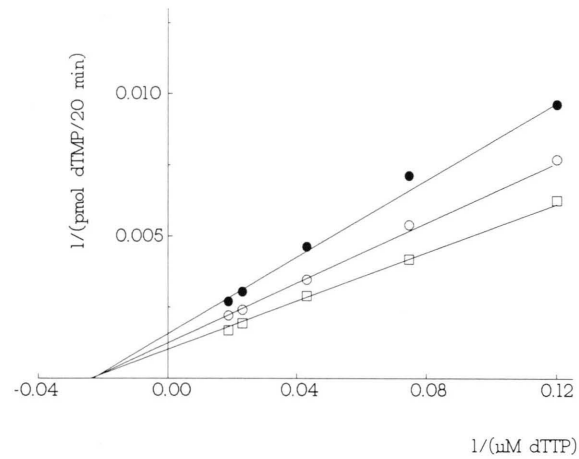


Fig. 4. Inhibition of MMuLV-RT by podoscyphic acid. Double reciprocal plot of TTP-dependent reaction velocities in the presence of no inhibitor (□); 10 μg/ml podoscyphic acid (○); 20 μg/ml podoscyphic acid (●). Reactions were carried out for 20 min.

podoscyphic acid, its ethyl ester **2** and synthetic 4-oxo-2-tetradecenoic acid (**11**) against the reverse transcriptases of AMV and MMuLV are shown in Table III. The IC_{50} values of 4-oxo-2-tetradecenoic acid (**11**) were comparable to those of podoscyphic acid, whereas the ester **2** was devoid of any inhibitory activity. We therefore assume that the free γ-oxoacrylic acid unit is essential for the inhibitory activity. **1** exhibits only weak antibacterial (Table IV) and cytotoxic (Table V) properties. At 20–50 μg/ml of **1** lysis of 50% of ECA cells was observed after 48 h of incubation. The IC_{50} against HeLa S3 cells was determined to 20 μg/ml. Reduced proliferation of L 1210 cells was observed at 50–100 μg/ml and no inhibition of growth of HUT 78, BHK 21 and 3T3/MMSV cells was detected up to concentrations of 100 μg/ml of **1.**

The effects of podoscyphic acid on the incorporation of leucine, uridine, and thymidine in L 1210 cells are shown in Fig. 5. At 10 μg/ml protein synthesis was almost completely inhibited, whereas DNA and RNA syntheses were affected to a much lesser extent. The influence of **1** on protein synthesis was further investigated in a cell-free trans-

Table II. Effect of template-primer concentration on the inhibitory effect of podoscyphic acid on AMV- and MMuLV-reverse transcriptases. Except for the template-primer concentrations the standard reaction mixtures were used.

poly(A)-$(dT)_{15}$ [μg/ml]	AMV-RT dTMP incorporation [pmol] Podoscyphic acid [μg/ml]	
	0	100
5	76	32
20	78	35
50	72	29
	MMuLV-RT dTMP incorporation [pmol] Podoscyphic acid [μg/ml]	
	0	100
5	330	34
20	453	83
50	477	133

Table III. Activities of podoscyphic acid and some of its derivatives against the reverse transcriptases of AMV and MMuLV.

Compound	IC_{50} [μg/ml]	
	AMV-RT	MMuLV-RT
1 (Podoscyphic acid)	50–70	10–20
11	100	10–20
2	>100	100

Table IV. Antibacterial and antifungal activity of podoscyphic acid in the agar diffusion assay.

Organism	Diameter of inhibition zone [mm] 100 µg/disc
Bacteria:	
Acinetobacter calcoaceticus	+
Bacillus brevis	10
Bacillus subtilis	10
Micrococcus luteus	+
Streptomyces spec.	+
Streptomyces viridi-chromogenes	−
Fungi:	
Aspergillus ochraceus	−
Candida albicans	−
Fusarium fujikuroij	−
Fusarium oxysporum	−
Mucor miehei	−
Nadsonia fulvescens	+
Nematospora corylii	+
Paecilomyces varioti	−
Penicillium notatum	−
Rhodotorula glutinis	−
Saccharomyces cerevisiae is 1[a]	−
Ustilago nuda	−

[a] Strain obtained from Prof. F. Lacroute, Straßbourg.
+: Inhibition zone <10 mm.
−: No inhibition zone.

Table V. Cytotoxic properties of podoscyphic acid.

Cell line	IC_{50} [µg/ml]
L 1210	50−100*
ECA	20− 50
HUT 78	>100
BHK 21	>100
3T3/MMSV	>100
HeLa S3	20

* Inhibition of proliferation.

Table VI. Effect of podoscyphic acid on cell-free protein synthesis.

Antibiotic added [µg/ml]		Incorporation of [^{3}H]leucine [cpm]	% of control
None		29,200	100
Verrucarin A	1	15,812	54
Podoscyphic acid	100	17,520	60

lation system. As shown in Table VI 100 µg/ml **1** inhibited the β-globin mRNA-dependend incorporation of ^{3}H-leucine into proteins 40%. The inhibitory effect of **1** on DNA and RNA synthesis was further studied in permeabilized L 1210 cells and isolated nuclei. In permeabilized cells no influence on the incorporation of dTTP into DNA was detected by concentrations of up to 100 µg/ml. At the same concentration of **1** the incorporation of UTP was reduced only 20% (Fig. 6). In isolated nuclei of L 1210 cells no inhibitory activity against one of the RNA polymerases (distinguished by their sensitivity to α-amanitin) was observed.

In the test for mutagenicity according to Ames *et al.* [12] and Venitt *et al.* [13] no induction of

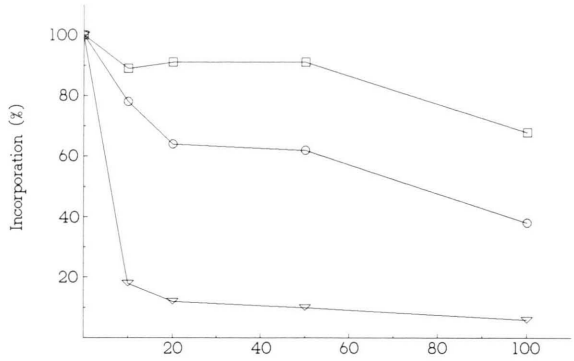


Fig. 5. Effect of podoscyphic acid on the incorporation of [^{14}C]thymidine (○), [^{14}C]uridine (□), and [^{14}C]leucine (▽) in L 1210 cells. Controls without antibiotic (100%): [^{14}C]thymidine, 4527 cpm, [^{14}C]uridine, 9151 cpm, [^{14}C]leucine, 27,448 cpm.

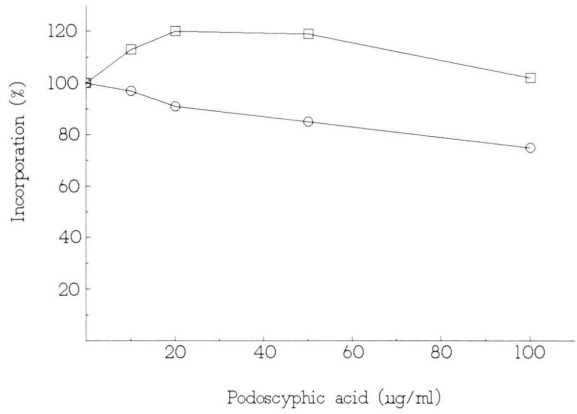


Fig. 6. Effect of podoscyphic acid on the incorporation of [^{14}C]TTP (□) and [^{14}C]UTP (○) in permeabilized L 1210 cells. Controls without antibiotic (100%): [^{14}C]TMP incorporation 2100 cpm, [^{14}C]UMP incorporation 2500 cpm.

revertants of *S. typhimurium* TA 98 and TA 100 could be observed with 100 µg of **1**/plate (pour plate assay with and without addition of rat liver microsomes).

Acknowledgements

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