ANTIBIOTICS FROM BASIDIOMYCETES XLI[†]

CLAVICORONIC ACID, A NOVEL INHIBITOR OF REVERSE TRANSCRIPTASES FROM *Clavicorona pyxidata* (PERS. EX FR.) DOTY

Gerhard Erkel and Timm Anke*

LB Biotechnologie der Universität,
D-6750 Kaiserslautern, Germany

Alberto Gimenez and Wolfgang Steglich

Institut für Organische Chemie und Biochemie der Universität,
D-5300 Bonn, Germany

(Received for publication July 19, 1991)

A novel inhibitor of RNA-directed DNA-polymerases was isolated from fermentations of *Clavicorona pyxidata*. Its structure was elucidated by spectroscopic methods. Clavicoronic acid (**1**) is a noncompetitive inhibitor of avian myeloblastosis virus (Ki 130 μM) and Moloney murine leukemia virus (Ki 68 μM) reverse transcriptases. In permeabilized cells and isolated nucleic DNA- and RNA-synthesis are not affected. Clavicoronic acid markedly inhibits the multiplication of vesicular stomatitis virus in baby hamster kidney cells by interfering with this virus's RNA-directed RNA-polymerase. **1** exhibits no cytotoxic and very weak antimicrobial activities.

The reverse transcriptase plays an important role in the life cycle of retroviruses and the stable inheritance of the viral genome[2]. Effective and selective inhibitors of this enzyme therefore are considered potential antiviral chemotherapeutics[3]. In the course of screening for fungal metabolites inhibiting avian myeloblastosis virus (AMV) reverse transcriptase a new compound, clavicoronic acid (**1**), was isolated from cultures of the basidiomycete *Clavicorona pyxidata* (= *Artomyces pyxidatus* (Pers. ex Fr.) Jülich). In this paper we wish to describe its formation by fermentation, isolation, structural elucidation and biological characterization.

Materials and Methods

General

Spectral data were recorded on the following instruments: ^{1}H and ^{13}C NMR, Bruker AM-400; MS, A.E.I. MS-50; IR, Perkin-Elmer 1420 spectrometer; UV, Hewlett Packard 8452A diode array spectrophotometer; CD, Jobin Yvon CNRS Roussel-Jouan Dichrographe III. The mp's were determined with a Reichert hot-plate microscope and are uncorrected. For TLC aluminium foils coated with silica gel Merck 60 F_{254} were used. PTLC was carried out on glass plates precoated with silica gel (Merck 60 F_{254}, 0.5 mm).

Clavicorona pyxidata (Pers. ex Fr.) Doty[4], Strain 8675

Mycelial cultures were obtained from spore prints of fruiting bodies collected from pine wood in the Smoky Mountains, U.S.A. The strain is deposited in the culture collection of the Lehrbereich Biotechnologie, University of Kaiserslautern.

[†] See ref 1.

Fermentation

For maintenance 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. The medium (YMPG2) used for the production of clavicoronic acid contained: maltose 20 g, glucose 10 g, yeast extract 0.8 g, peptone 2 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 1 g, $FeCl_3$ 10 mg, $ZnSO_4$ 2 mg and $CaCl_2$ 55 mg in 1 liter deionized water. A well grown seed culture of *Clavicorona pyxidata* (200 ml) in YMG was used to inoculate 20 liters of YMPG2 medium in a Biolafitte C6 fermentation apparatus. The fermenter was incubated at 22°C with an aeration rate of 3 liters air/minute and agitation (130 rpm). The production of clavicoronic acid 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 reverse transcriptase.

Isolation

During purification clavicoronic acid was detected using the standard assay for AMV reverse transcriptase. After removal of the mycelia by filtration, the pH of culture broth (20 liters) was adjusted to 3.0 and clavicoronic acid extracted with ethyl acetate (10 liters). Evaporation of the organic phase yielded a crude extract (15.8 g), which was applied to a column (2.5 × 25 cm) containing LiChroprep Diol. Elution with cyclohexane - *tert*-butyl methyl ether (40 : 60) resulted in 2.8 g of an enriched product. This was further purified by preparative HPLC (LiChrosorb Diol, column 2.5 × 25 cm, elution with cyclohexane - *tert*-butyl methyl ether (19 : 81)) to yield 187 mg of clavicoronic acid (1). Further elution with a gradient, starting from the previous conditions to pure *tert*-butyl methyl ether, in 45 minutes, afforded two biologically inactive compounds, the structure of which will be reported in due course.

Biological Assays

Antimicrobial spectra, cytotoxicity and macromolecular syntheses in whole L1210 cells (ATCC CCL 163) were measured as described previously[5]. The effect of clavicoronic 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 (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 benzylpenicillin 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 albumine (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 (Boehringer, Mannheim) were incubated at 37°C for 60 minutes. The reaction was terminated by adding 1 ml of cold 20% 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) 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 (Pharmacia, Uppsala). Unless otherwise specified the reaction mixture was incubated for 60 minutes at 37°C and the radioactivity of the acid insoluble fractions was determined as described above.

Nucleic Acid Syntheses and Multiplication of Vesicular Stomatitis Virus (VSV): Nucleic acid syntheses in permeabilized L1210 cells were performed according to BERGER[9]. RNA-syntheses in isolated nuclei were measured as described previously[10]. The effect of clavicoronic acid on multiplication of VSV (ATCC VR 158, Indiana strain) in BHK 21 cells was measured as described previously[11].

Assay of RNA-directed RNA-polymerase of VSV: Roller bottles containing confluent monolayer cultures of BHK 21 cells in 150 ml G-MEM medium containing 2% fetal calf serum were infected with 2 × 10^4 PFU of VSV and the cultures were incubated at 37°C for 36~40 hours at 1 rpm. The medium containing the released virions was clarified by centrifugation (2,000 × g, 10 minutes) and the supernatant was concentrated by ultrafiltration (Amicon, Diaflo XM300 membrane). The resulting suspension (15 ml)

was washed with three volumes of buffer A (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl$_2$, 4 mM DTT) and the virions were pelleted by ultracentrifugation (100,000 × g, 60 minutes) through a 50% glycerol pad of buffer A. The pellet was resuspended in buffer A containing 30% glycerol to 4~5 × 10^{10} PFU/ml and stored at −80°C until use.

The assay of RNA-directed RNA-polymerase was carried out in a reaction mixture (100 μl) containing: 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM MgCl$_2$, 4 mM DTT, 1 mM each of ATP, GTP, CTP, 15 μM [2-^{14}C]UTP (0.1 μCi), 0.05% Triton X-100 and 10 μl of 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 to the method of AMES *et al.*[12]. Mutants of *Salmonella thyphimurium* strain TA 98 and TA 100 were used in the pour plate assay as described by VENITT *et al.*[13].

Clavicoronic Acid (1)

White powder: MP 75~77°C; Rf 0.57 (butyl acetate - butanol - HOAc - H$_2$O, 4 : 4 : 1 : 1); [α]$_D^{22}$ +42.7° (c 0.33, MeCN); UV λ_{max}^{MeCN} nm (log ε) 210, 240 (sh), 278 (3.08); CD λ_{max}^{MeCN} nm 250 (+), 272 (0), 315 (−), 345 (−), 365 (−), 400 (0); IR (KBr) cm^{-1} 3420, 2940, 2920, 2860, 1730, 1700, 1680, 1600, 1530, 1330, 1220, 1116; ^{1}H and ^{13}C NMR, Table 1; EI-MS (direct inlet, 180°C) m/z (relative intensity %) 218.1273 (2, M$^+$ − CO$_2$, calcd for C$_{14}$H$_{18}$O$_2$ 218.1423), 200 (70), 187 (90), 44 (100).

Methyl Clavicoronate (2)

A solution of clavicoronic acid (20 mg) in CHCl$_3$ (2 ml) was stirred at 0°C for 1 minute with an excess of ethereal diazomethane. Evaporation of the solvent and purification of the residue by PTLC on SiO$_2$ using light petrol - ethyl acetate (90 : 10) afforded methyl clavicoronate (2) (16 mg, 76%) as a colorless oil [α]$_D^{22}$ +40.2° (c 0.55, MeCN); UV λ_{max}^{MeCN} nm (log ε) 210 (4.16), 240 (sh, 3.08), 278 (3.57); CD λ_{max}^{MeCN} nm (Δε) 255 (+9.03), 270 (0), 310 (−6.52), 350 (−1.53), 365 (−2.29), 400 (0); IR (CHCl$_3$) cm^{-1} 2960, 2940, 1730, 1705, 1675, 1310, 1165; ^{1}H and ^{13}C NMR, Table 1; EI-MS (direct inlet, 180°C) m/z (relative intensity %) 276.1364 (2, M$^+$, calcd for C$_{16}$H$_{20}$O$_4$ 276.1570), 218 (17), 217 (100), 161 (43), 147 (25), 105 (26).

Results and Discussion

Production of Clavicoronic Acid

A typical fermentation of *C. pyxidata* is shown in Fig. 1. The production of clavicoronic acid as measured by the inhibition of the AMV reverse transcriptase starts 25 days after inoculation. The highest content of the inhibitor is reached after 40 days.

Structural Elucidation

Clavicoronic acid exhibits ^{1}H NMR signals for an aldehyde group, three tertiary methyl groups, one olefinic proton, and six additional aliphatic protons in the aliphatic region (Table 1).

The spin-spin connectivities in the ^{1}H NMR spectrum of 1 were established by 2D ^{1}H-^{1}H COSY experiments. Thus, the olefinic proton at δ 6.01 shows a coupling with the methine proton at δ 2.68 which, in turn, is coupled with an AB system with components at δ 0.96 and 1.61 and with a second methine proton at δ 2.36. The latter is further coupled with an AB system at δ 0.95 and 1.48. Moreover, the pseudo-equatorial components of each AB system (δ 1.61 and 1.48) were found spin-connected *via* a W-type coupling ($J = 2$ Hz), consistent with partial structure **A**.

The ^{13}C NMR spectrum of clavicoronic acid shows 15 discrete carbon resonances (Table 1). At low field, carbonyl resonances for an α,β-unsaturated aldehyde, an unsaturated ketone and a carboxylic acid were observed.

Fig. 1. Fermentation of *Clavicorona pyxidata.*

○ Mycelial dry weight (g/liter), △ pH, ■ inhibition of AMV-reverse transcriptase (RT) (%).
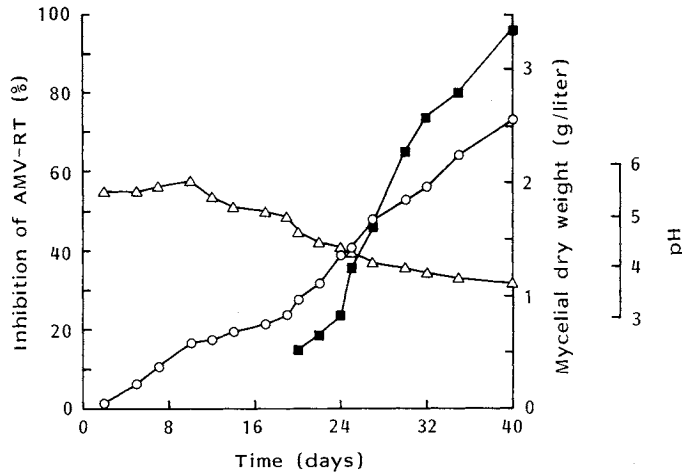
The production of clavicoronic acid was followed by estimating the inhibitory effect of 2.5 μl of a crude mycelial extract (concentrated 100-fold as compared to the culture fluid) on the incorporation of [^{14}C]TMP in the standard assay of AMV-RT.

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **1** and **2** in benzene-d_6.

	1	2		1	2
1-H$_\alpha$	0.95 t	0.95 t	C-1	49.9 tm	48.9
1-H$_\beta$	1.48 ddd	1.39 ddd			
2-H	2.36 td	2.23 td	C-2	44.3 dm	44.2
			C-3	147.6 m	145.6
			C-4	189.8 br s	186.5
			C-5	162.7 s	162.3
			C-6	123.5 m	124.2
			C-7	135.9 dd	135.2
8-H	6.01 d	5.69 d	C-8	151.8 dm	149.8
9-H	2.68 dddd	2.48 dddd	C-9	38.7 dm	37.9
10-H$_\alpha$	0.96 dd	0.97 dd	C-10	49.4 tm	48.4
10-H$_\beta$	1.61 ddd	1.46 ddd			
			C-11	35.6 m	35.1
12-CH$_3$	1.63 s	1.63 s	C-12	19.7 qd	19.0
13-H	8.95 s	8.94 s	C-13	193.3 dd	191.6
14-CH$_3$	0.72 s	0.65 s	C-14	27.5 qm	27.2
15-CH$_3$	0.83 s	0.78 s	C-15	29.4 qm	28.4
OCH$_3$	—	3.44 s	C-OCH$_3$		52.1

J (Hz): $1_\alpha,1_\beta=1_\alpha,2=10_\alpha,10_\beta=12$; $1_\beta,2=9,10_\beta=8$; $1_\beta,10_\beta=2$; $2,9=12$; $9,10_\alpha=9$; $8,9=3.5$; J_{CH} (Hz): C1 = C10 = 130; C2 = C9 = 132; C14 = C15 = 126; C12 = 128 and 3.5; C7 = 26 and 5.5; C8 = 160; C13 = 178 and 8.

A   B
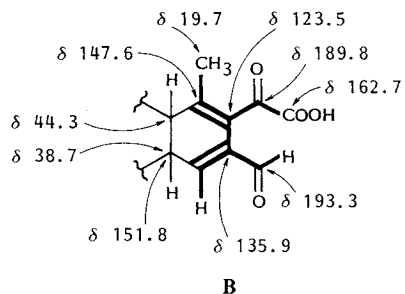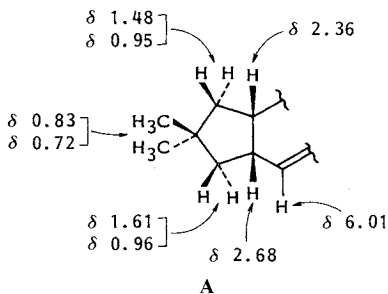
The location of these substituents was unequivocally assigned by the 2D ^{1}H-^{13}C COSY spectra. Thus, in the long range correlation spectrum, the carbonyl resonance of the aldehyde group (δ_C 193.3) is linked to the olefinic proton at δ_H 6.01 and, in the same way, the carbonyl resonance of the unsaturated ketone (δ_C 189.8) can be correlated with the protons of the methyl group at δ_H 1.63. The carbon resonance of this methyl group (δ_C 19.7) is correlated with the methine proton at δ_H 2.36. The presence of a substituted cyclohexadiene moiety was evident from the correlations observed for the carbon resonances at δ 123.5, 135.9 and 147.6. Thus, the first resonance is linked with the proton of the aldehyde group (δ_H 8.95), the olefinic proton (δ_H 6.01), one of the methine protons (δ_H 2.36), and the protons of the methyl group at δ_H 1.63. The second resonance showed correlations with the aldehyde proton and the proton at δ_H 2.68. Finally, the resonance at δ_C 147.6 can be connected with the protons of the methyl group at δ_H 1.63 and both methine protons (δ_H 2.68 and 2.36) which leads to partial structure **B**. The lack of correlations for the carboxylic acid resonance at δ_C 162.7 is consistent with its presence in an α-oxocarboxylic acid moiety.

Combinaion of the two part structures **A** and **B** and incorporation of a C(CH$_3$)$_2$ moiety which gives rise to signals at δ 35.6, 27.5, and 29.4, establishes structure **1** for clavicoronic acid.

In the EI-MS, **1** undergoes easy decarboxylation to a pseudo-molecular ion at m/z 218. Treatment of **1** with diazomethane afforded methyl clavicoronate (**2**), which gave the spectroscopic data of ester **2** (Table 1 and Materials and Methods) are in accord with the given structure.

The relative stereochemistry at the ring junction of **1** has been established to be *cis* by differential NOE experiments. Because the biogenetically closely related sesquiterpenoids stearylvelutinal and isovelleral of known absolute configuration[14] occur in the fruit bodies of the same fungus[15], we propose the absolute stereochemistry given in formula **1**.

Clavicoronic acid (**1**) can be considered to be derived biogenetically from a sterpurene precusor **3**[16] *via* oxidation to the cyclobutadione derivative **4** followed by fragmentative opening of the cyclobutane ring and consecutive oxidation of the methyl group as depicted in Scheme 1.

Biological Properties

The inhibitory effects of clavicoronic acid on the reverse transcriptases of AMV and MMuLV and the RNA-directed RNA-polymerase of VSV are shown in Fig. 2.

MMuLV reverse transcriptase activity was reduced to 50% at a concentration of 19~38 μM (IC$_{50}$), whereas the IC$_{50}$ was 114~133 μM for AMV reverse transcriptase and 190 μM for the RNA-directed RNA-polymerase of VSV. Preincubation of AMV reverse transcriptase with clavicoronic acid

1 R = H
2 R = CH$_3$
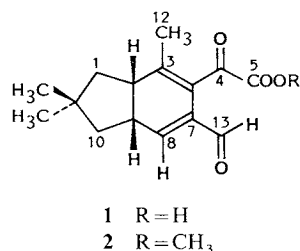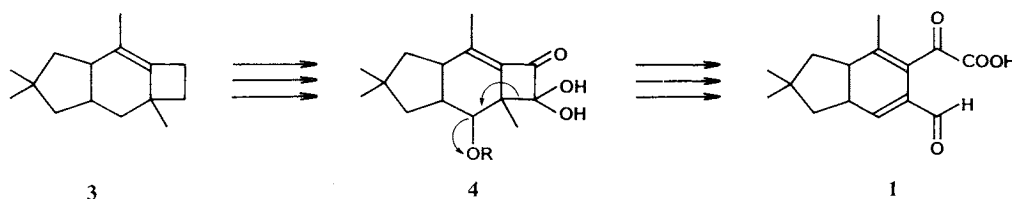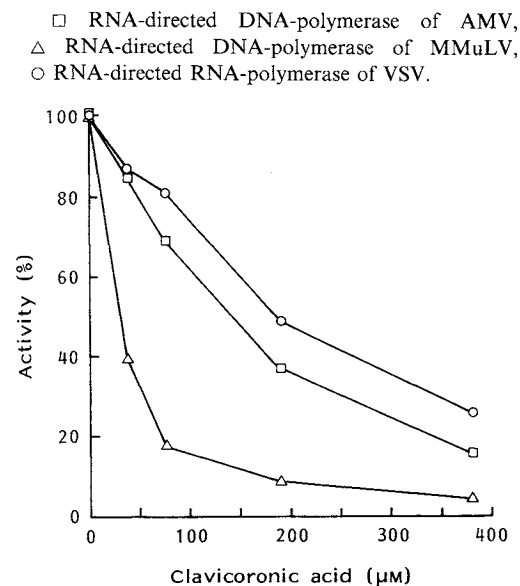
Scheme 1.

3   4   1

Fig. 2. Effect of clavicoronic acid on the reverse transcriptases (RT) of AMV and MMuLV and on the RNA-directed RNA-polymerase of VSV.

□ RNA-directed DNA-polymerase of AMV,
△ RNA-directed DNA-polymerase of MMuLV,
○ RNA-directed RNA-polymerase of VSV.

Controls without antibiotic (100%): AMV-RT, 90 pmol [^{14}C]TMP incorporation in 60 minutes; MMuLV-RT, 260 pmol [^{14}C]TMP incorporation in 60 minutes; RNA-directed RNA-polymerase of VSV, 240 pmol [^{14}C]UMP incorporation in 120 minutes.

Table 2. Effect of preincubation of reverse transcriptase (RT) with clavicoronic acid.

Clavicoronic acid (μM)	Inhibition (%)	
	AMV-RT	MMuLV-RT
No preincubation		
38	17	60
56	35	83
Preincubation with enzyme		
19	23	73
38	50	93
56	85	100

The enzymes (20 u/ml) were incubated in the absence or presence of clavicoronic acid at 37°C for 10 minutes without substrates. The other components were added to complete the reaction mixture (50 μl) which was then incubated at 37°C for 50 minutes.
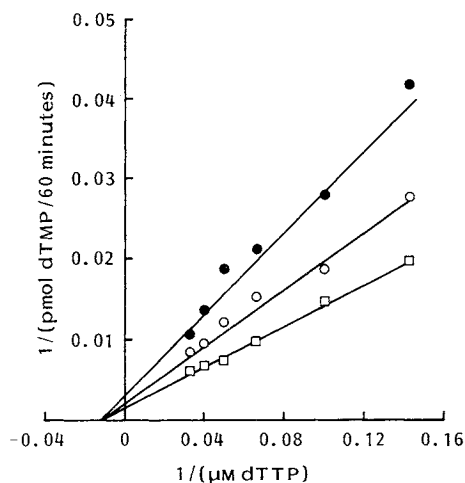
Table 3. Effect of template-primer concentration on the inhibitory effect of clavicoronic acid on AMV- and MMuLV-reverse transcriptases (RT).

AMV-RT

poly(A)-(dT)$_{15}$ (μg/ml)	dTMP incorporation (pmol)	
	Clavicoronic acid	
	0 μM	190 μM
5	70	32
20	65	33
50	59	25

MMuLV-RT

poly(A)-(dT)$_{15}$ (μg/ml)	dTMP incorporation (pmol)	
	Clavicoronic acid	
	0 μM	76 μM
5	330	61
20	453	125
50	477	129

Except for the template-primer concentrations the standard reaction mixtures were used.

for 10 minutes at 37°C increased its inhibitory effect on AMV reverse transcriptase (IC$_{50}$ 38 μM). Preincubation of MMuLV reverse transcriptase with 38 μM clavicoronic acid resulted in a complete inhibition of enzyme activity (Table 2).

Preincubation of the template-primer poly(A)-(dT)$_{15}$ did not affect the inhibitory action of clavicoronic acid on both reverse transcriptases (data not shown). A 10-fold increase in the concentration of the template-primer had almost no effect on the inhibition of AMV and MMuLV reverse transcriptase (Table 3).

The initial rates of incorporation of [^{14}C]dTTP were measured in the absence or the presence of increasing amounts of clavicoronic acid. The Lineweaver-Burk plots (Figs. 3 and 4) indicate a noncompetitive inhibition of both reverse transcriptases with respect to TTP indicating that the binding site for clavicoronic acid is different from those for substrate and template-primer. The Ki values for clavicoronic acid were calculated to 130 μM for AMV reverse transcriptase and 68 μM for MMuLV reverse transcriptase.

The activities of clavicoronic acid (1) and its methyl ester (2) on the reverse transcriptases of AMV and MMuLV are compared in Table 4. The methyl ester (2) inhibited MMuLV reverse transcriptase and the

Fig. 3.   Lineweaver-Burk plot of inhibition of AMV-reverse transcriptase by clavicoronic acid in the presence of no inhibitor, 76 μM clavicoronic acid, and 190 μM clavicoronic acid.

□ No inhibitor, ○ 76 μM clavicoronic acid, ● 190 μM clavicoronic acid.

The reactions were carried out as described in the Materials and Methods section.

Fig. 4.   Lineweaver-Burk plot of inhibition of MMuLV-reverse transcriptase by clavicoronic acid in the presence of no inhibitor, 19 μM clavicoronic acid, and 38 μM clavicoronic acid.

□ No inhibitor, ○ 19 μM clavicoronic acid, ● 38 μM clavicoronic acid.

The reactions were carried out for 20 minutes as described in the Materials and Methods section.

Table 4.   Effect of clavicoronic acid (1) and its methyl ester derivative (2) on reverse transcriptases (RT) of AMV and MMuLV.

Compound	IC_{50} (μM)	
	AMV-RT	MMuLV-RT
1	114 ~ 133	19 ~ 38
2	181	54

Table 5.   Cytotoxic properties of clavicoronic acid.

Cell line	IC_{50} (μM)
L1210	>380
ECA	>380
HUT 78	>380
BHK 21	>380
3T3/MMSV	>380
HeLa S3	>380

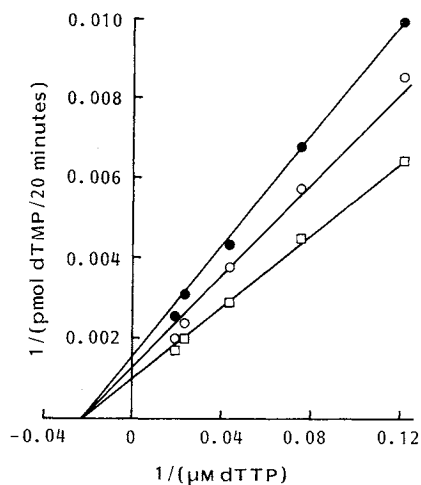Fig. 5.   Effect of clavicoronic acid on the multiplication of VSV in BHK 21 cells.

reverse transcriptase of AMV to a slightly lesser extent.

Clavicoronic acid exhibits no cytotoxic properties (Table 5). No inhibition of proliferation of the tested cell lines could be observed up to concentrations of 380 μM clavicoronic acid.

Fig. 5 shows the effect of clavicoronic acid on the propagation of VSV in baby hamster kidney (BHK) cells. Multiplication of the virus is significantly reduced by concentrations starting from 190 μM. At a
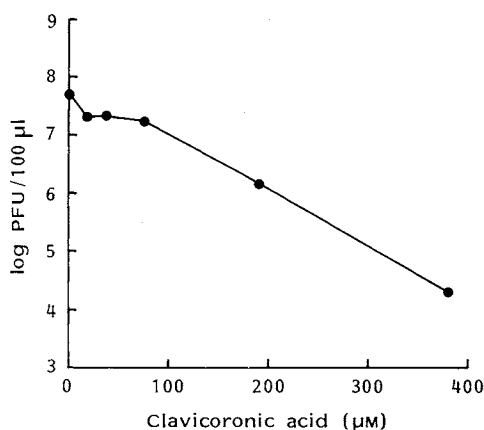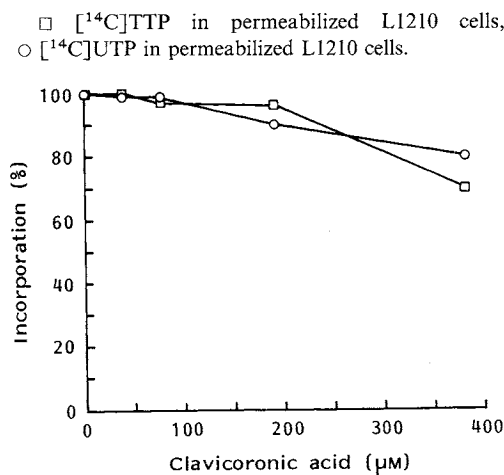
Fig. 6. Effect of clavicoronic acid on the incorporation of [^{14}C]TTP and [^{14}C]UTP in permeabilized L1210 cells.

□ [^{14}C]TTP in permeabilized L1210 cells,
○ [^{14}C]UTP in permeabilized L1210 cells.

Controls without antibiotic (100%): [^{14}C]TMP incorporation 2,450 cpm, [^{14}C]UMP incorporation 2,690 cpm.

Table 6. Effect of clavicoronic acid on RNA-syntheses in isolated nuclei of L1210 cells.

	Incorporation of [^{14}C]UMP (pmol)	
	Control	Clavicoronic acid (380 μM)
RNA-polymerase I	39.44	18
RNA-polymerase II	44.11	28.54
RNA-polymerase III	6.56	7

Control:

α-Amanitin	Incorporation of [^{14}C]UMP (pmol)
None[a]	90.11
0.5 μg/ml[b]	46
100 μg/ml[c]	39.44

[a]   RNA-polymerases I, II, III.
[b]   RNA-polymerases I, II.
[c]   RNA-polymerase.

concentration corresponding to the IC$_{50}$ of RNA-directed RNA-polymerase (190 μM) the plaque formation was reduced 97%.

Because reverse transcriptase shares many properties of cellular DNA- and RNA-polymerases, the inhibitory effect of clavicoronic acid on DNA and RNA syntheses was studied in permeabilized L1210 cells and isolated nuclei.

In permeabilized cells 380 μM of it inhibited the incorporation of TTP into DNA and UTP into RNA, 30% and 20% (Fig. 6), respectively.

In isolated nuclei of L1210 cells 380 μM clavicoronic acid inhibited the RNA-polymerase I by 55% and RNA-polymerase II by 35% (Table 6). At the same concentration no inhibition of RNA-polymerase III was observed. In contrast to the results obtained with permeabilized L1210 cells the RNA-syntheses in isolated nuclei (distinguished by the fungal toxin α-amanitin) were more sensitive to the inhibition by clavicoronic acid. This might be due to the loss of factors required for correct transcription during nuclear isolation as discussed by GILROY et al.[17].

Further Biological Activities

In the test for mutagenicity according to AMES et al.[12] and VENITT et al.[13] no induction of revertants of S. typhimurium TA 98 and TA 100 could be observed with 100 μg of clavicoronic acid/plate (pour plate assay with and without addition of rat liver microsomes).

Acknowledgments

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