Antiviral Activity of Cholesteryl Esters of Cinnamic Acid Derivatives

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Cholesteryl Esters of Cinnamic Acid Derivatives, *o*-Coumaroyl Ester of 3β-(2'-hydroxyethoxy)-cholest-5-en, Antiviral Activity, *in vitro* Screening

Cholesteryl 3'',4''-dimethoxycinnamate (**7**) and a new synthesized o-coumaroyl ester of 3β-(2'-hydroxyethoxy)-cholest-5-en (**13**) exhibited a marked activity against poliovirus type 1 (Mahoney). Compound **7** showed an approximately 20-fold greater selectivity in its antiviral activity than compound **13**. These compounds were selected from thirteen steryl esters of cinnamic acid derivatives through an *in vitro* antiviral screening against viruses belonging to taxonomic groups with causative agents of important human infectious diseases to which chemotherapy is indicated, i.e. Picornaviridae, Orthomyxoviridae, Paramyxoviridae and Herpesviridae.

Introduction

3-Phenylpropionyl esters of sterols and triterpenes are widely distributed in the plant kingdom. Various biological properties have been found for these compounds: antioxidant (Graf, 1992), cholesterol-lowering (Rogers *et al.,* 1993) and cytostatic (Kashiwada *et al.,* 1993). Data about their antiviral properties are very limited in the literature. Among a series of natural triterpene esters of cinnamic acid derivatives the caffeoyl ester only showed a scarce antiviral effect vs. human rhinovirus 1B (De Tommasi *et al.,* 1992). These compounds were inactive towards Sindbis virus and HIV-1.

Recently we elaborated a new method for a synthesis of cholesteryl esters of cinnamic acid derivatives, using the Wittig reaction under sonochemical conditions (Elenkov *et al.,* 1995). Here we present results of the testing for antiviral activity of a series of compounds belonging to this group.

Materials and Methods

Compounds

Compounds **1–12** (Table I) were synthesized according to the method described by Elenkov *et al.* (1995). Their ^{1}H-NMR spectra were recorded on Bruker 250 MHz for solutions in $CDCl_3$ or CD_3OD with TMS as internal standard. The UV spectra in EtOH solutions were measured with a Specord UV-VIS spectrophotometer.

The synthesis of a new compound **13**, *o*-coumaroyl ester of 3β-(2'-hydroxyethoxy)-choles-5-en is shown in Scheme 1.

Compound **13a** (oil). Cholesteryltosylate (305.4 mg, 0.57 mmol) was dissolved in 12 ml dry dioxane and ethyleneglycol (6500 mg, 85.5 mmol) was added. The reaction mixture was heated gently for about 2h and after that it was submitted to column chromatography. The yield – 77%. ^{1}HNMR (250 MHz, $CDCl_3$). 0.67 (3H, s, 18-CH_3), 0.86 (6H, d, J=6.5; 26,27-CH_3), 0.91 (3H, d, J=6.5; 21-CH_3), 1.00 (3H, s, 19-CH_3), 3.20 (1H, m, 3α-H), 3.56 (2H, t, J=4.4; 2'-H), 3.72 (2H, t, J=4.4; 1'-H), 5.35 (1H, m, 6-H).

Compound **13b**. **13a** (117.2 mg, 0.27 mmol), bromoacetic acid (49.3 mg, 0.36 mmol), DCC (74.2 mg, 0.36 mmol) and 4-DAP (4.9 mg, 0.04 mmol) were dissolved in 20 ml dry THF. The reaction mixture was stirred at room temperature for 2h. The residue of dicyclourea was filtered and washed with chloroform, and the combined solutions evaporated under vacuum. The residue was purified by column chromatography on silica. Yield – 71%. ^{1}HNMR (250 MHz, $CDCl_3$). 0.66 (3H, s, 18-CH_3), 0.87 (6H, d, J=7; 26,27-CH_3), 0.96 (3H, d, J=6; 21-CH_3), 1.00 (3H, s, 19-CH_3), 3.10

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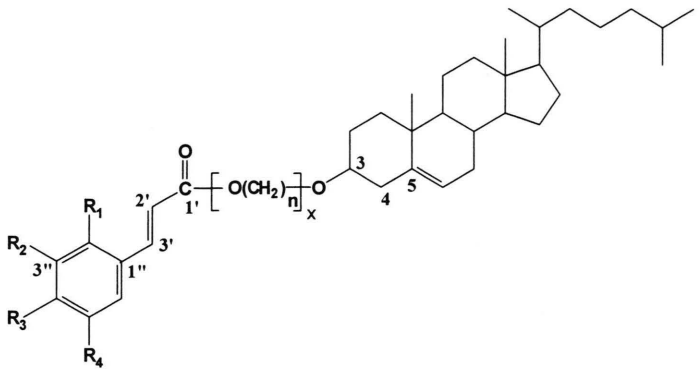
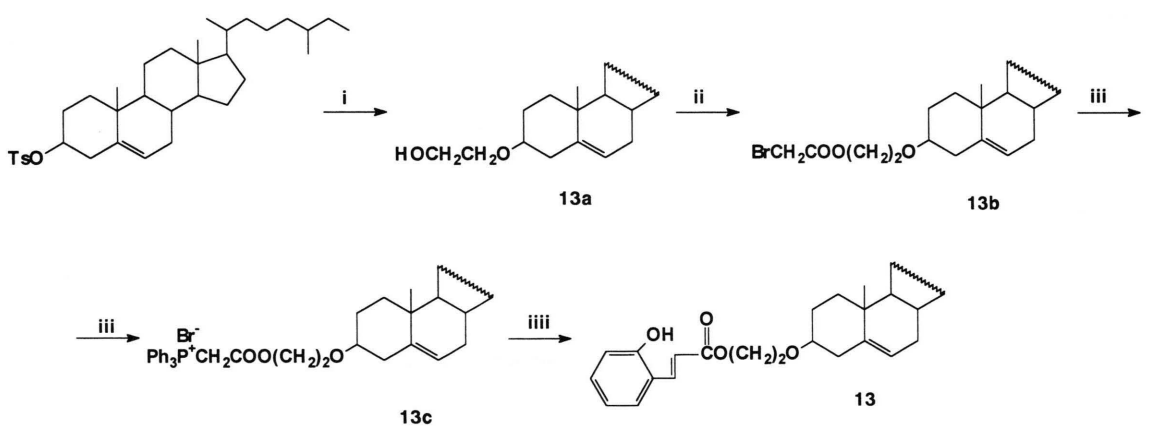


Table I. Synthesis of steryl esters of substituted cinnamic acids under sonochemical conditions.

Compound	x	n	R_1	R_2	R_3	R_4
1	0	0	H	H	H	H
2	0	0	H	H	OH	H
3	0	0	H	OH	OH	H
4	0	0	OH	H	OH	H
5	0	0	H	OCH_3	H	H
6	0	0	H	H	OCH_3	H
7	0	0	H	OCH_3	OCH_3	H
8	0	0	OH	OCH_3	H	H
9	0	0	H	OCH3	OH	H
10	0	0	H	-O-CH_2-O-		H
11	0	0	H	OCH_3	OH	OCH_3
12	0	0	OH	H	H	H
13	1	2	OH	H	H	H



Scheme 1. i, ethyleneglycol, 1,4-dioxane, t°; ii, tetrahydrofuran, N,N-dicyclohexylcarbodiimide, 4-dimethylaminopyridine; iii, tetrahydrofuran,triphenylphosphine; iiii, *o*-hydroxybenzaldehyde chloroform-1,4-dioxane (1:1), K_2CO_3, sonication.

(1H, m, 3α-H), 3.70(2H, t, *J*=4.5; 1'-H), 3.87 (2H, s, Br**C**$\mathbf{H}_2$COO), 4.32 (2H, t, *J*=4.5; 2'-H), 5.36 (1H, m, 6-H).

Compound **13c. 13b** (50.0 mg, 0.09 mmol) and Ph_3P (26.0 mg, 0.10 mmol) were dissolved in 0.4 ml anhydrous benzene. The reaction mixture stayed at room temperature for 22 h. The residue of phosphonium salt was filtered and washed with *n*-hexane. Yield - 86%. ^{1}HNMR (250 MHz, $CDCl_3$). 0.66 (3H, s, 18-CH_3), 0.80 (6H, d, *J*=6.5;

26,27-CH_3), 0.96 (3H, d, *J*=7; 21-CH_3), 1.00 (3H, s, 19-CH_3), 3.04 (1H, m, 3α-H), 3.50 (2H, t, *J*=4.5; 1'-H), 4.13 (2H, t, *J*=4.5; 2'-H), 5.32 (1H, m, 6-H), 5.56 (2H, d, *J*=16, PC**H**$_2$COO), 7.45- 8.00 (15H, m, aromatic).

Compound **13**. A solution of 0.14 mmol of **13c** in 0.5 ml $CHCl_3$ and a solution of aromatic aldehyde in 0.5 ml dioxane were mixed and added to 0.35 mmol K_2CO_3. The reaction mixture was sonicated in a Lechpan Type UM 0.5 ultrasonic bath at 25 °C. The reaction was monitored by TLC (Alufolien Kieselgel 60_{F254}, Merck, *n*-hexane:acetone 2:1 v/v). The reaction mixture was washed successively with 5% HCl and water. The organic phase was dried over Na_2SO_4, evaporated to dryness, and subjected to CC.

UV (EtOH) λmax 232, 278, 332 nm. ^{1}HNMR (250 MHz, $CDCl_3$). 0.74 (3H, s, 18-CH_3), 0.87 (6H, d, *J*=7; 26,27-CH_3), 0.96 (3H, d, *J*=6; 21-CH_3), 1.09 (3H, s, 19-CH_3), 3.22 (1H, m, 3α-H), 3.58 (2H, t, *J*=4.9; 1'-H), 3.70 (2H, m, 2'-H), 5.35 (1H, m, 6-H), 6.61 (1H, d, *J*=16; H-2"), 7.28- 7.59 (4H, m, aromatic), 7.84 (1H, d, *J*=16, H-3").

Viruses

Poliovirus type 1 (Mahoney strain) (PV1) (WHO Regional Reference Laboratory, Institute of Poliomyelitis and Viral Encephalitides, Vnukovo, Moscow District, Russia), influenza virus A/chicken/Germany/27/Weybridge (H7N7) (FPV) (Institute of Virology, Bratislava, Slovak Republic), Newcastle disease virus (Russeff strain) (NDV), (Central Veterinary Research Institute, Sofia) and pseudorabies virus (Aujeszki, A2 strain) (Central Veterinary Research Institute, Sofia) were used.

Cell cultures

FL cells were grown in a medium containing 10% heated calf serum in a mixture of equal parts of medium 199 (Difco) and Hanks' saline, supplemented with antibiotics (penicillin 100 IU/ml and streptomycin 100 μg/ml).

Primary chick embryo fibroblast cultures (CEC) were prepared according to Porterfield (1960) and cell suspension (1–1.5x10^6 cells/ml) was seeded in Eagle's MEM (Difco) growth medium supplemented with 10% calf serum and antibiotics.

Antiviral tests

The agar-diffusion plaque-inhibition test with cylinders (Rada and Zavada, 1962) was performed as described previously (Galabov *et al.*, 1996) and was used for the initial screening of antiviral activity. The compounds tested (0.1 ml of 0.5% w/v solutions in DMSO) were added dropwise in 6-mm glass cylinders fixed in the agar overlay. The antiviral effect (E) was recorded on the basis of the difference between the size of the zone of plaque inhibition (diameter Φ_i in mm) and zone of cytotoxicity (Φ_t) and was estimated as follows: "-", $\Delta\Phi \leq 5$ mm; "±", $\Delta\Phi$ 5–10 mm; "+", $\Delta\Phi$ = 11–20 mm; "++",$\Delta\Phi$ = 21–40 mm; "+++", $\Delta\Phi$ >40 mm.

Then the compounds which showed a marked antiviral effect ($\Delta\Phi$ >10 mm) were studied by the cytopathic effect (CPE) inhibition multicycle test on monolayer cell cultures in 96-well plastic microplates (Flow) following the setup described by Galabov *et al.* (1996). The minimal 50% inhibitory concentration (MIC_{50}) value was determined.

Cytotoxicity test

The compounds tested were added immediately before cell seeding to the growth medium in 24-well plastic microplates (three wells per sample). The cell growth curve followed until the stationary phase was reached and then the cell growth 50% inhibitory concentration ($CGIC_{50}$) was calculated as compared to the control (no compound in the growth medium).

Results and Discussion

The series of compounds was tested for activity against viruses belonging to taxonomic groups with causative agents of important human infectious diseases to which chemotherapy is indicated, namely Picornaviridae, Orthomyxoviridae, Paramyxoviridae and Herpesviridae. The screening results are summarized on Table II. Two of the compounds, **7** and **13**, manifested a marked activity against PV1. Another seven compounds (**1**, **2**, **3, 4, 6**, **9** and **10**) showed a borderline effect towards the same virus. None of the compounds showed effect against the representatives of the other taxonomic groups tested.

Table II. Screening for antiviral activity by agar-diffusion plaque-inhibition test.

Compound	Conc. [mM]	PV1			FPV			NDV			PsRV		
		Φ_i	Φ_t	E	Φ_i	Φ_t	E	Φ_i	Φ_t	E	Φ_i	Φ_t	E
1	10	20.6	15.0	±	11.3	8.2	–	0	16.2	–	0	22.2	–
2	10	18.0	11.5	±	0	0	–	0	22.0	–	17.1	11.1	±
3	10	15.2	9.0	±	0	7.3	–	0	14.4	–	0	19.0	–
4	10	15.0	7.2	±	15.5	6.5	±	11.2	8.5	–	0	14.0	–
5	10	0	12.0	–	0	0	–	0	18.0	–	0	21.6	–
6	10	15.0	8.2	±	12.8	7.7	±	0	19.2	–	0	18.5	–
7	10	31.1	17.0	+	0	7.2	–	0	25.0	–	0	18.2	–
8	10	15.0	12.5	–	0	0	–	0	18.0	–	0	18.2	–
9	10	19.0	11.5	±	11.5	8.2	–	0	15.5	–	0	14.5	–
10	10	15.5	8.2	±	0	13.2	–	0	25.0	–	0	17.5	–
11	10	0	11.0	–	9.0	7.2	–	15.6	11.2	–	0	12.0	–
12	10	0	10.8	–		n.d.			n.d.			n.d.	
13	10	31.5	20.8	+	0	7.2	–	0	29.5	–	0	12.5	–

Φ_i = diameter of inhibition zone (mm);
Φ_t = diameter of toxicity zone (mm);
E = antiviral effect;
PV1, poliovirus;
FPV, influenza virus A(H7N7);
NDV, Newcastle disease virus;
PsRV, pseudorabies virus;
n.d. – not done.

Table III. Cytotoxicity and antiviral effect *vs.* poliovirus 1 of compounds **7** and **13** in the cytopathic inhibition test.

Compound	Cytotoxicity	Antiviral activity	
	$CGIC_{50}$, μM*	MIC_{50}, μM	SI**
7	114.28	0.178	642.0
13	21.70	0.55	39.4

* Half-inhibition of cell growth (see Materials and Methods).
** Selectivity index = $CGIC_{50}/MIC_{50}$ ratio.

The antiviral effects of the selected active compounds were then studied in both the multicycle CPE inhibition setup and the cytotoxicity test (Table III). Compound **7** showed an approximately 16-fold greater selectivity in its antiviral activity than compound **13.**

The structure-activity analysis of the data obtained showed that increasing the chain length at C-3 position in the steroid ring transforms the inactive compound **12** to the active one, i.e. **13**. On the other hand, replacement of H in the inactive compound **5** by -OCH_3 group at p-position in the phenyl moiety leads to the active compound **7**.

It is worth mentioning that the so described compounds and the structurally related one described by De Tomassi *et al.* (1992) are effective against viruses belonging to the same family.

The selected two active compounds are selected for further experiments on their antipicornavirus action. The results of this pilot study could serve as a basis for future planned synthesis of new active derivatives.

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