Transformation of Cholanic Acid Derivatives into Pharmacologically Active Esters of Phenolic Acids by Heterogeneous Wittig Reaction

Antoaneta Ivanova[a], Tsenka Milkova[a], Angel S. Galabov[b], Lubomira Nikolaeva[b] and Emilia Voynova[c]

[a] Institute of Organic Chemistry with Center of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchevstr., Bl. 9, 1113 Sofia, Bulgaria
[b] Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[c] National Oncological Center, 1156 Sofia, Bulgaria

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Steroid esters of cynnamic acid derivatives have been synthesized by a heterogeneous Wittig reaction under sonochemical conditions from the corresponding triphenylphosphonium bromides and unprotected phenolic aldehyds using K_2CO_3 as a base. 5β-Cholan-3α, 7α, 12α, 24-*E*-ferulate (**11'**) exhibited a marked inhibitory effect on influenza virus A. The synthetic 3α, 24-*E*-diferulates of 5β-cholan-3α, 24- diol, 5β-cholan-3α, 12α, 24-triol and 5β-cholan-3α, 7α, 12α, 24-tetrol (**8, 9** and **12**) showed antitumor activity on leukemia P-388 in mice.

Introduction

In the course of the continuing search for biologically active agents from plants many new triterpene monoesters of phenylpropanoic acids were found (Tommasi *et al.,* 1992; Pan *et al.,* 1994). Recently the isolation and characterization of two new cytotoxic di-*p*-coumaroyl triterpenes from *Ilex asprella* was reported (Kashiwada *et al.,* 1993). The study of the general pharmacological activities of these natural compounds has been seriously hampered by their limited amount available from natural sources.

Recently in our laboratory a new synthesis of cholesteryl esters of cynnamic acids derivatives was elaborated, using the Wittig reaction in solid-liquid heterogeneous medium under sonochemical conditions (Elenkov *et al.,* 1995). The results of work prompted us to develop this method for preparation of compounds with more than one ester group starting from polyhydroxylated steroids and to test their biological activity.

This paper describes our work on the sonochemical Wittig reaction between different phenolic aldehydes and phosphonium salts of the corresponding halides of 5β-cholanol derivatives as well as the antiviral and antitumor study on the synthesized compounds.

Reprint requests to Dr. Milkova.
Fax: 0035 92-700-225.

Experimental

^{1}H-NMR spectra were recorded on Brucker 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.

General procedure for preparation of 5β-cholanols

14.2 mmol of Me ester of cholanic acid (cholic, deoxycholic, lithocholic) were dissolved in 100 ml dry THF and added under argon to 42 mmol $LiAlH_4$ in 20 ml THF. The reaction mixture was stirred 10 h at room temperature. At the end of reaction 20 ml 10% NH_4Cl were added. The residue was filtered and washed with EtOAc. The organic phase was washed with water, dried over Na_2SO_4, evaporated and subjected to CC or recrystallization from acetone.

5β-cholan-3α,24-diol (**1**), 80% yield, m.p. 177–176 °C

^{1}H-NMR ($CDCl_3$) 0.65 (3H, s, Me-18), 0.91 (3H, s, Me-19), 0.93 (3H, d, J = 6.4, Me-21), 3.61 (3H, m, H-3β, 24-CH_2-O).

5β-cholan-3α, 12α, 24-triol (**2**). 80% yield, m. p. 199–201 °C

^{1}H-NMR ($CDCl_3$) 0.68 (3H, s, Me-18), 0.91 (3H, s, Me-19), 0.99 (3H, d, J = 6.4, Me-21), 3.61 (3H, m, H-3β, -CH_2-OH-24), 3.99 (1H, m, H-7β)

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5β-cholan-3α, 7α, 12α, 24-tetrol (**3**). 89% yield, m.p. 206–208 °C

^{1}H-NMR ($CDCl_3$: CD_3OD = 1:1) 0.71 (3H, s, Me-18) 0.98 (3H, s, Me-19), 1.015 (3H, d, J = 6.6, Me-21), 3.45 (1H, m, H-3β), 3,54 (2H, t, J = 6.8, 24-CH_2-), 3.83 (1H, bs, H-7β), 3.98 (1H, bs, H-12β).

General procedure for esterification of 5β-cholanols with $BrCH_2COOH$

0.28 mmol 5β-cholanol, 0.75 mmol bromoacetic acid, 0.75 mmol DCC and 0.0112 mmol 4-DMAP were dissolved in 20 ml dry THF. The reaction mixture was stirred under argon at room temperature for 1 h. The residue of dicyclo hexylurea was filtered and washed with $CHCl_3$ and the combined solutions evaporated under vacuum. The residue was purified by CC on silica.

5β-cholan-3α,24-diol-3α,24-dibromoacetate (**4**), oil, 85% yield

^{1}H-NMR ($CDCl_3$) 0.65 (3H, s, Me-18), 0.91 (3H, s, Me-19), 0.93 (3H, d, J = 6.2, Me-21), 3.80 (2H, s, -$OCOCH_2Br$), 3.82 (2H, s, -$OCOCH_2Br$), 4.15 (2H, t, -CH_2-24), 4.75 (1H, m, H-3β).

5β-cholan-3α,12α,24-triol-3α,24-dibromoacetate (**5**), oil, 50% yield

^{1}H-NMR ($CDCl_3$) 0.69 (3H, s, Me-18), 0.93 (3H, s, Me-19), 0.99 (3H, d, J = 6.4, Me-21), 3.79 (2H, s, -$OCOCH_2Br$), 3.83 (2H, s, -$OCOCH_2Br$), 4.00 (1H, bs, H-12α), 4.15 (2H, t, -CH_2-24), 4.78 (1H, m, H-3β).

5β-cholan-3α,7α,12α,24-tetrol-24-monobromoacetate (**6**), oil, 22% yield

^{1}H-NMR ($CDCl_3$:CD_3OD 1:1) 0.71 (3H, s, Me-18), 0.91 (3H, s, Me-19), 3.43 (1H, m, H-3β), 3.83 (1H, bs, H-7β), 3.88 (2H, s, -$OCOCH_2Br$), 3.97 (1H, bs, H-12β), 4.16 (2H, t, J = 6.2, -CH_2-24).

5β-cholan-3α,7α,12α,24-tetrol-3α,24-dibromoacetate (**7**), oil, 55% yield

^{1}H-NMR ($CDCl_3$:CD_3OD 1:1) 0.71 (3H, s, Me-18), 0.92 (3H, s, Me-19), 1.00 (3H, d, J = 6.4, Me-21), 3.79 (2H, s, $OCOCH_2Br$), 3.83 (2H, s, -$OCOCH_2Br$), 3.86 (1H, bs, H-7β), 4.01 (1H, bs, H-12β), 4.13 (2H, t, J = 6.7, -CH_2-24), 4.65 (1H, m, H-3β).

Preparation of phosphonium salts

0.25 mmol dibromide and 0.56 mmol triphenylphosphine were dissolved in 30 ml dry THF. After 72 h at room temperature the residue was filtered, dissolved in MeOH and extracted twice with *n*-hexane. The lower layer was evaporated to dryness. Yields: 92%.

General procedure for synthesis of diesters

A solution of 0.234 mmol of phosphonium salt in 0.5 ml $CHCl_3$ and a solution of aromatic aldehyde in 0.5 ml 1,4-dioxane were mixed and added to 0.35 mmol of 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). The reaction mixture was washed successively with 5% HCl and H_2O. The organic phase was dried over Na_2SO_4, evaporated to dryness and subjected to CC (silica). All products were characterized by their UV- and ^{1}H-NMR spectra.

5β-cholan-3,24-diol-3α,24-di-*E*-ferulate (**8**)

UV (MeOH) λ_{max} 233.5, 300, 314.2 nm.

^{1}H-NMR ($CDCl_3$) 0.66 (3H, s, Me-18), 0.94 (3H, s, Me-19), 3.90 (3H, s, -OMe), 3.92 (3H, s, -OMe), 4.14 (2H, m, CH_2-24), 4.80 (1H, m, H-3β), 5.95 (1H, bs, Ar-OH), 6.26 (1H, d, J = 15.9, H-2'), 6.28 (1H, d, J=15.9, H-2'), 6.91 (2H, m, H-6"), 7.05 (4H, m, H-2", H-3"), 7.56 (1H, d, J = 15.9, H-3'), 7.60 (1H, d, J = 15.9, H-3').

5β-cholan-3α,12α,24-triol-3α,24-di-*E*-ferulate (**9**)

UV (MeOH) λ_{max} 234.8, 300, 326.2 nm.

^{1}H-NMR ($CDCl_3$) 0.70 (3H, s, Me-18), 0.94 (3H, s, Me-19), 1.01 (3H, d, J = 6.3, Me-21), 3.92 (3H, s, -OMe), 3.93 (3H, s, -OMe), 4.02 (1H, bs, H-12β), 4.17 (2H, m, -CH_2-24), 4.8 (1H, m, H-3β), 5.86 (1H, s, Ar-OH), 6.25 (1H,d, J = 15.9, H-2'), 6.28 (1H, d, J = 15.9, H-2'), 6.90 (2H, m, H-6"), 7.14 (4H, m, H-2", H-3"), 7.58 (1H, d, J = 15.9, H-3'), 7.60 (1H, d, J = 15.9, H-3').

5β-cholan-3α,7α,12α,24-tetrol-3α,24-di-*E*-cynnamate (**10**)

UV (MeOH) λ_{max} 280.

^{1}H-NMR ($CDCl_3$) 0.72 (3H, s, Me-18), 0.93 (3H, s, Me-19), 3.87 (1H, bs, H-7β), 4.03 (1H, bs, H-12β), 4.2 (2H, m, -CH_2-24), 4.72 (1H, m, H-3β), 6.39 (1H, d, J = 16.0, H-2'), 6,44 (1H, d, J = 16.0, H-2'), 7.38 (5H, m), 7.53 (5H, m), 7.65 (1H, d, J = 16.0, H-3'), 7.68 (1H, d, J = 16.0, H-3').

5β-cholan-3α,7α,12α,24-tetrol-3α,24-di-*E*-ferulate (**11**), oil

UV (EtOH) λ_{max} 227.5, 290, 342 nm.

^{1}H-NMR ($CDCL_3$) 0.73 (3H, s, Me-18), 0.96 (3H, s, Me-19), 1.05 (3H, d, *J* = 6.3, Me-21), 3.82 (1H, bs, H-7β), 3.88 (6H, s, -OMe), 3.98 (1H, bs, H—12β), 4.16 (2H, t, -CH_2-24), 4.66 (1H, m, H-3β), 6.50 (1H, d, *J* = 16.0, H-2'), 6.55 (1H, d, *J* = 16.0, H-2'), 6.77–6.83 (2H, m), 6.93–6.94 (2H, m), 6.97–7.20 (2H, m), 7.95 (1H, d, *J* = 16.0, H-3'), 7.98 (1H, d, *J* = 16.0, H-3').

5β-cholan-3α,7α,12α,24-tetrol-24-*E*-ferulate (**11'**), oil

UV (EtOH) λ_{max} 227.5, 287.5, 333.3 nm.

^{1}H-NMR ($CDCl_3$:CD_3OD 1:1) 0.70 (3H, s, Me-18), 0.90 (3H, s, Me-19), 1.01 (3H, d, *J* = 6.5, Me-21), 3.46 (1H, m, 1H-3β), 3.85 (1H, bs, H-7β), 3.92 (3H, s, -OMe), 4.00 (1H, s, H-12β), 4.16 (2H, m, -CH_2-24), 6.71 (1H, d, *J* = 16.2, H-2'), 6.86 (2H, m, H-5", H-6"), 7.10 (1H, m, H-2"), 7.94 (1H, d, *J* = 16.2, H-3').

5β-cholan-3α,7α,12α,24-tetrol-3α,24-di-*E*-ferulate (**12**), oil

UV (MeOH) λ_{max} 235.2, 299, 325 nm.

^{1}H-NMR ($CDCl_3$) 0.72 (3H, s, Me-18), 0.93 (3H, s, Me-19), 1.024 (3H, d, *J* = 6.4, Me-21), 3.87 (1H, bs, H-7β), 3.92 (3H, s, -OMe), 3.93 (3H, s, -OMe), 4.02 (1H, bs, H-12β), 4.18 (2H, m, -CH_2-24), 4.73 (1H, m, H-3β), 5.88 (1H, s, Ar-OH), 6.24 (1H, d, *J* = 15.9, H-2'), 6.28 (1H, d, *J* = 15.9,H-2'), 6.91 (1H, m, H-6"), 7.06 (2H, m, H-2", H-3"), 7.56 (1H, d, *J* = 15.9, H-3'), 7.63 (1H, d, *J* = 15.9, H-3').

Bioassays

Viruses

Poliovirus (Mahoney strain) (PV1) (WHO Regional Reference Laboratory, Institute of Poliomyelitis and Viral Encephalitis, 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 (PsRV, Aujszky, 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's saline, supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml).

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

Antiviral tests

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

The compounds showing a marked antiviral effect ($\Delta\phi$ > 10 mm) were then studied in 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 being determined.

Cytotoxicity test

The compound tested was added to the growth medium (10% calf serum and antibiotics in Eagle's MEM (Flow) for CEC) immediately before cell seeding in 24-well plastic microplates (three wells per sample). The compound cytotoxicity was assessed by following the course of cell growth until the stationary phase, when the cell growth 50% inhibitory concentration ($CGIC_{50}$) was evaluated.

Antitumor test

Tumor passages and therapeutic experiments in vivo were carried out according to the standard methods (Sofina *et al.*, 1980). Leukemia P-388 was used as transplantable tumor model. Leukemia P-388 was propagated in a peritoneal cavity of male DBA_2 mice with 10^6 cells/mouse on day 0. For the experiments transplanted BDF_1 mice of both sexes were treated i.p. with different doses of compounds to determine the maximum active one.

The animals were divided in experimental groups of six mice each and a control one. The

treatment was performed on the 24th hour after transplantation. The mice had been treated for 5 successive days. The samples were dissolved in saline to which 1–2 drops of Tween-80 were added to increase their solubility. The biological activity of the compounds was assessed by the increasing life span (ILS) of the mice at 125% minimum criteria for activity.

Results and Discussion

5β-Cholanols obtained by $LiAlH_4$ reduction of methyl esters of easily available cholanic acids (cholic, deoxycholic and lithocholic) were used as starting steroids. Since the reactivity order of the hydroxyl groups of the obtained 5β-cholanols (Fig. 1) is 24 > 3α > 7α > 12 α, the conditions have to be found to direct the acylation of **2** and **3** to an optimal quantity of the desired 5β-cholan-3α,12α, 24-triol-3α,24-dibromoacetate and 5β-cholan-3α,7α, 12α,24-tetrol-3α,24-dibromoacetate (Scheme 1). After much experimentation it was found that acylation of 5β-cholan-3α, 7α,12α,24-tetrol gave the desired 3α,24-dibromoacetate in 55% yield (22% 24-monobromoacetate and 13% tribromoacetate) by optimal ratio of tetrol: $BrCH_2COOH$:DCC:4-DMAP = 0.16:0.42:0.42:0.006 and reaction time 1h. By running of the reaction in an absence of 4-DMAP from the reaction mixture were isolated the starting tetrol, significant amount of 24-monobromoacetate and traces 3α,24-dibromoacetate.

The Wittig reaction of the phosphonium salts of the corresponding dibromides and different phenolic aldehydes (Scheme 1) was carried out under sonochemical condition in heterogeneous solid-liquid medium with K_2CO_3 as a base. The results obtained are shown in Table I. In the ^{1}H-NMR spectra of the synthesized diesters two one proton doublets at $\delta \approx 6.25-6.55$ with $J \approx 16$ Hz and two another one proton doublets with the same *J* const. at $\delta \approx 7.56-7.98$ were observed, characteristic for two -CH=CH- *trans* groups. The content of the *Z*-isomer in the products obtained is less than 5%, as estimated by ^{1}H-NMR spectroscopy. A monoester at C-24 of 5β-cholan-3α,7α,12α,24-tetrol with 2-hydroxy-3-methoxycynnamic acid **11'** was synthesized under the same conditions.

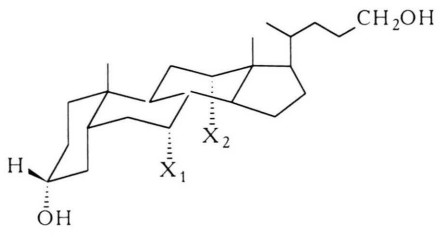
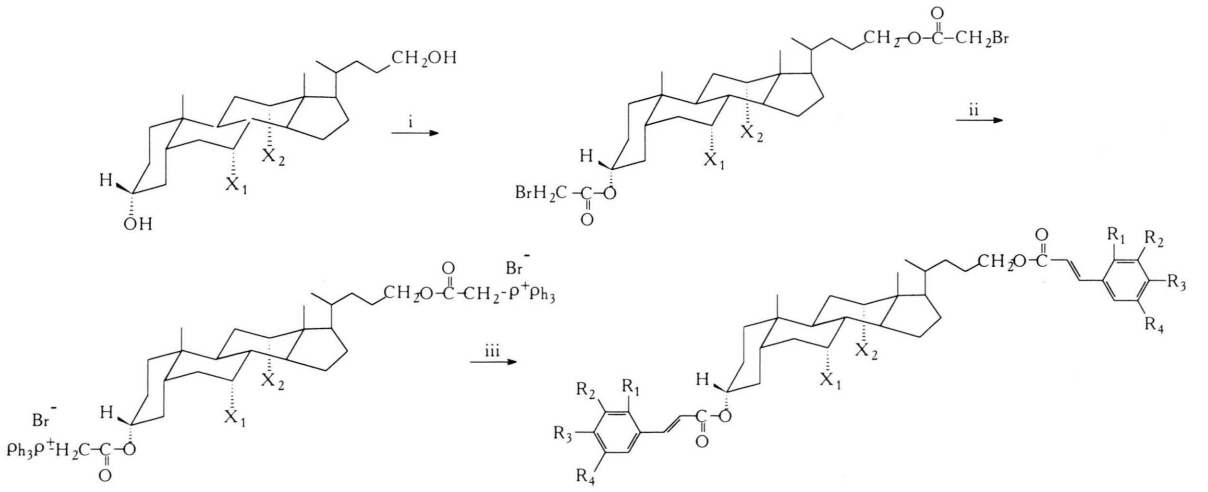
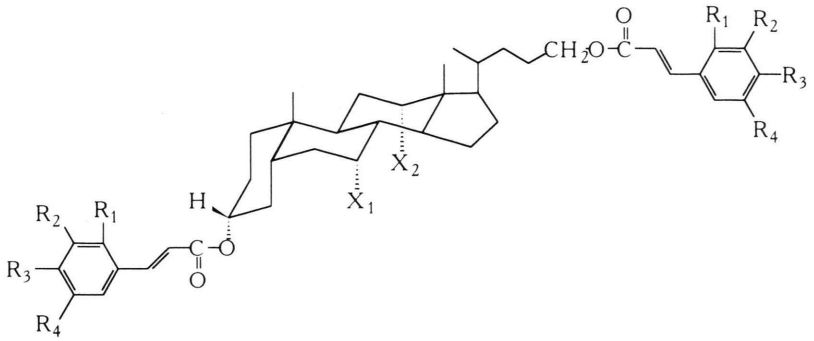


Fig. 1. 1. $X_1 = X_2 = H$
2. $X_1 = H$, $X_2 = OH$
3. $X_1 = X_2 = OH$



Scheme 1. i, tetrahydrofuran, N,N – dicyclohexylcarbodiimide, 4 – dimethylaminopyridine; ii, tetrahydrofuran, triphenylphosphine; iii, aromatic aldehyde, chloroform – 1,4 – dioxane (1:1), K_2CO_3, sonication.

Table I. Synthesis of steroid diesters of submitted cynnamic acid under sonochemical conditions.



Compound	X_1	X_2	R_1	R_2	R_3	R_4	Yields, %	Reaction time [h]
8	H	H	H	OCH_3	OH	H	45	12
9	H	OH	H	OCH_3	OH	H	38	12
10	OH	OH	H	H	H	H	50	4
11	OH	OH	OH	OCH_3	H	H	91	24
12	OH	OH	H	OCH_3	OH	H	51	12

Table II. Screening of synthetic esters for antiviral activity by agar-diffusion plaque-inhibition test[a].

Compd.	Dose	PV1			FPV			NDV			PsRV		
	[mM][b]	ϕ_i	ϕ_t	E	ϕ_i	ϕ_t	E	ϕ_i	ϕ_t	E	ϕ_i	ϕ_t	E
11'	8.8	0	6.8	-	22.7	11.2	+	20.2	10.4	+	25.0	17.5	+
11	6.7	0	9.2	-	18.7	10.0	±	18.0	10.1	+	21.9	15.5	+
12	6.7	0	7.5	-	0	7.0	-	0	8.5	-	10.2	9.7	-
9	6.8	0	6.7	-	0	8.0	-	0	7.7	-	0	9.2	-
10	7.6	0	7.3	-	0	7.7	-	0	8.0	-	0	9.2	-

[a] Φ_i = diameter of inhibition zone (mm); Φ_t = diameter of toxicity zone (mm); E = antiviral effect.
[b] Per cylinder (dissolved in 0.1 ml DMSO).
PVI, poliovirus; FPV, influenza virus; NDV, Newcastle disease virus; PsV, pseudorabies virus.

The method reported in this paper proceed without protection of the phenolic groups of the aromatic aldehydes. In most cases the yields were 40%- 91% with reaction time of 4–24 h.

Antiviral activity

The compounds **9**, **10**, **11**, **11'** and **12** were tested for antiviral activity in vitro against representatives of four taxonomic viral groups, namely, picorna-, orthomyxo-, paramyxo- and herpes viruses. The viruses used were PV1, FPV, NDV and PsRV, respectively.

As seen in Table II, compound **11'** demonstrated a marked activity against FPV in the agar-diffusion plaque-inhibition test. This effect was well reproducible also in the CPE inhibition test. MIC_{50} value of 3.2μg/ml was determined and selectivity ratio ($CGIC_{50}/MIC_{50}$) of 10.9 been evaluated. Compound **11** showed a borderline effect towards FPV and both compounds showed limited effects against NDV and PsRV in the screening test. The rest of the compounds was not active.

Antitumor activity

The synthetic esters **8**, **9** and **12** were investigated for antitumor activity on leukemia P-388 in mice. The inhibitory action of the compounds was evaluated by percentage of ILS (increasing life span) of the animals. Criteria for antitumor activity of the substances is ≥125%. The results were

Table III. Antitumor action of synthetic esters on leukemia P-388.

Compound	Dose [mg/kg]	MST[days]* Control	ILS** %
12	2.0	11.8/10.5	112.4
	3.0	10.3/9.0	114.0
	4.0	11.8/10.5	115.1
	6.0	10.8/9.0	120.0
	9.0	11.8/9.0	131.0
	10.0	11.4/9.3	122.6
	12.0	10.9/9.0	121.0
	14.0	10.6/9.2	116.0
8	2.0	11.0/10.5	104.8
	4.0	9.5/9.0	105.5
	5.0	11.2/10.5	106.6
	6.0	10.6/9.0	117.7
	8.0	11.5/9.0	127.8
	10.0	10.2/9.0	113.4
	12.0	10.3/9.2	111.9
	14.0	10.2/9.3	110.0
9	2.0	11.8/10.5	112.4
	4.0	10.5/9.0	116.6
	5.0	16.4/10.5	156.2
	6.0	11.3/9.2	122.8
	8.0	10.8/9.0	120.0
	10.0	10.2/9.0	113.3

* MST – mean survival time.
** ILS – increased life span.

shown in Table III. As seen in the table all three compounds possessed antitumor activity as follow: **12** at a dose of 9 mg/kg ILS of the mice 130.33%; **8** at a dose of 8 mg/kg ILS – 127.77% and **9** at a dose of 5 mg/kg ILS – 156.19%.

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