Antiviral Activity of Some Hydroxycoumarin Derivatives

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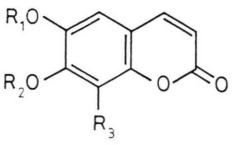
Esculetin (6,7-dihydroxycoumarin) and its diacetate exhibited a marked inhibitory effect on Newcastle disease virus replication in cell cultures at concentrations of 36 μM and 62 μM, respectively. These compounds were selected from ten hydroxycoumarin derivatives through an *in vitro* antiviral screen involving viruses of the picorna-, orthomyxo-, paramyxo-, and herpes virus families.

Introduction

The 6,7-dihydroxy- and 6,7,8-trihydroxycoumarin derivatives are widely distributed in the plant kingdom and occur freely or as glycosides. Various biological actions have been found for these compounds; e.g., antiinflamatory, antimicrobial, immunomodulatory, liver-protecting, and vitamin P-like activities; and inhibition of cAMP phosphodiesterase, lipoxygenase and cyclooxygenase (Yamagami *et al.*, 1968; Jurd *et al.*, 1974; Kimura and Okuda, 1985; Tsukamoto *et al.*, 1985; Handa *et al.*, 1986; Nishibe *et al.*, 1986; Tubaro *et al.*, 1988). However, studies on their antiviral effect are very limited. Only esculin has been tested and it failed to show effects on herpes simplex virus type 1 replication and on human immunodeficiency virus type 1 reverse transcriptase (Alarcon *et al.*, 1984; Tan *et al.*, 1991). This prompted us to study the antiviral properties of the structurally related compounds **1–10.** This work is connected with our interest in the chemistry and biological activity of the hydroxycoumarin components of *Fraxinus* spp. (Kostova, 1992; Kostova *et al.*, 1993; Lazarova *et al.*, 1993).



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1: R_1=R_2=R_3=H
2: R_1=R_2=Ac, R_3=H
3: R_1=H, R_2=Me, R_3=H
4: R_1=R_2=Me, R_3=H
5: R_1=Glu, R_2=R_3=H
6: R_1=Glu.4Ac, R_2=Ac, R_3=H
7: R_1=Glu, R_2=Me, R_3=H
8: R_1=Me, R_2=H, R_3=OH
9: R_1=Me, R_2=Ac, R_3=OAc
10: R_1=R_2=Me, R_3=OMe

Materials and Methods

Plant material

Stem bark from mature trees of *Fraxinus ornus* L. (*Oleaceae* family) collected from the region of Kresna, South Bulgaria, was authenticated, and a voucher specimen deposited in the herbarium of the Institute of Botany, Bulgarian Academy of Sciences, Sofia.

Compounds

Dried and powdered bark (100 g) was extracted (3×) with 300 ml EtOH under reflux for 90 min. Esculin (**5**), 2 g, was isolated from the combined EtOH extracts after concentration to a small volume (200 ml) under reduced pressure, and crystallization (3×) of the solid from EtOH–H_2O (1:1 v/v). Esculetin (**1**) was prepared by acid hydrolysis (10% aqueous H_2SO_4, 4 h) of pure esculin, followed by crystallization from EtOH–H_2O (1:1 v/v). Fraxetin (**8**) was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, U.S.A.). Samples

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of 6,7-dimethoxycoumarin (**4**), 7-methylesculin (**7**) and 6,7,8-trimethoxycoumarin (**10**) were prepared by CH_2N_2-methylation of **1, 5** and **8,** respectively, using standard procedures. Acetylation of **1, 5** and **8** by Ac_2O/Pyr (room temperature, 24 h) afforded the corresponding acetates **2, 6** and **9.** Isoscopoletin (**3**) was prepared by acid hydrolysis (10% aqueous H_2SO_4, 4 h) of 7-methylesculin (**9**). The identity of compounds **1–7, 9** and **10** was proven on the basis of their physical and spectral data (Table I), compared with literature data (Kuznetsova, 1967; Tsukamoto *et al.,* 1984, 1985) and by direct comparison (co-TLC) with authentic samples (Kostova, 1992). The purity (>97%) of all compounds used in this study was confirmed by TLC and RP-HPLC (Nykolov *et al.,* 1993). Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was supplied by ICN Nucleic Acid Research Institute (Irvine, CA).

Viruses

Poliovirus 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 (Aujeszky, A2 strain) (PsRV) (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 penicillin (100 IU/ml and streptomycin (100 μg/ml).

Primary chick embryo fibroblasts cultures (CEC) were prepared after Porterfield (1960) by seeding suspension, $1-1.5\times10^6$ cells/ml, in a growth medium Eagle's MEM (Difco) supplemented with 10% calf serum.

Agar-diffusion plaque-inhibition test

Monolayer cell cultures of FL cells for PV1 and CEC for FPV, NDV and PsRV in 90 mm-petri dishes were inoculated (60 min adsorption at 20 °C) with a virus dose giving semiconfluent

Table I. IR and ^{1}H NMR ($CDCl_3$* and MeOD**) data[a] of compounds **1–10.**

Comp.	IR [cm^{-1}]	^{1}H NMR – δ H (*J* in Hz)				
		3 - H	4 - H	5 - H	8 - H	Other protons
1*	3300, 1680, 1625, 1580, 1400	6.13 d (9.4)	7.63 d (9.4)	6.91 s	6.79 s	
2*	1728, 1625, 1560, 1500, 1440	6.43 d (9.5)	7.64 d (9.5)	7.34 s	7.22 s	2.32, 2.33 (each 3H, s, 2 Ac)
3*	3320, 1715, 1620, 1555, 1510	6.16 d (9.4)	7.55 d (9.4)	6.89 s	6.75 s	3.91 (3H, s, OMe)
4*	1715, 1614, 1554, 1515	6.35 d (9.4)	7.62 d (9.4)	6.84 s	6.51 s	4.01, 3.90 (each 3H, s, 2OMe)
5**	3270, 1660, 1645, 1600, 1550, 1500	6.23 d (9.4)	7.85 d (9.4)	6.81 s	7.44 s	3.95 (1H, d (12.0), 6′-Ha), 3.73 (1H, dd (12.0, 5.3), 6′-Hb), 3.57–3.36 (4H, m, 2′,3′,4′,5′-H)
6*	1740, 1690, 1620, 1570, 1505	6.43 d (9.7)	7.62 d (9.7)	7.12 s	7.08 s	5.33–5.12 (4H, m, 1′,2′,3′,4′-H), 4.29–4.22 (2 H, m, 6′-H_2), 3.95 (1H, m, 5′-H), 2.31 (3H, OAc-ar), 2.09, 2.08, 2.06, 2.04 (each 3H, s, 4OAc)
7**	3350, 1690, 1620, 1570, 1510	6.26 d (9.5)	7.87 d (9.5)	7.01 s	7.39 s	4.92 (1H, d (7.2), 1′-H), 3.89 (1H, dd (12.3, 1.9), 6′-Ha), 3.68 (1H, dd (12.1, 5.4), 6′-Hb), 3.58–3.37 (4H, m, 2′,3′,4′,5′-H), 3.93 (3H, s, OMe)
8*	3200, 1665, 1650, 1595, 1550	6.28 d (9.5)	7.60 d (9.5)	6.53 s		3.94 (3H, s, OMe)
9*	1725, 1690sh, 1575, 1490	6.42 d (9.6)	7.64 d (9.6)	6.87 s		3.88 (3H, s, OMe), 2.40, 2.34 (each 3H, s, 2OAc)
10*	1720, 1600, 1568, 1429	6.31 d (10.0)	7.87 d (10.0)	7.03 s		3.88, 3.84, 3.81 (each 3H, s, 3OMe)

[a] IR: Bruker IFS 113V, KBr; ^{1}H NMR (δ, ppm), *J* (Hz) were obtained at 250 MHz using TMS as internal standard.

plaques after incubation at 37° (48 h with PV1 and FPV, 72 h with NDV and PsRV). Test compounds (0.1 ml of 0.5% w/v solutions in DMSO) were added dropwise within 6-mm glass cylinders fixed in the agar overlay [(1% Bactoagar (Difco) in Eagle's MEM (Flow) medium with heated calf serum, 1.65 mg/ml sodium bicarbonate and antibiotics, 100 U/ml each of penicillin and streptomycin]. A second overlay containing 1.5% w/v agar and 0.02% w/v neutral red in physiological saline was added following incubation. The antiviral effect (E) of a given compound was recorded on the basis of the size [diameter (φ), in mm] of the zone of plaque inhibition (φ_i) and the zone of cytotoxycity (φ_t) (four cylinders per compound, situated in a separate petri dish each) and designated as follows: –, $\Delta\varphi \leq 5$ mm; ±, $\Delta\varphi = 6-10$ mm; +, $\Delta\varphi = 11-20$ mm; ++, $\Delta\varphi = 21-40$ mm; +++, $\Delta\varphi > 40$ mm.

Cytopathic effect (CPE) inhibition multicycle test

Monolayer cell cultures of CEC grown in 96-well plastic microplates were used. Compounds (at subsequent 0.5 $\log_{10}$ dilutions within the 0.1–320 μM concentrations range) were applied in the maintenance medium [Eagle's MEM (Flow) with 2% calf serum and antibiotics] immediately after virus inoculation at three different viral doses (10, 100 and 1000 $CCID_{50}$/well). Three wells per test sample were used. CPE were scored on a 0–4 basis with 4 representing total cell destruction. These data were used to obtain dose-response curves for each compound at a given viral dose. From these graphs the minimal concentration causing a 50% reduction of CPE as compared to the untreated controls (MIC_{50} value) was determined.

Cytotoxicity test

Compounds tested were 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 cell count per well was determined every 24 h and the dose-response curve was drawn for each compound. 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.

Results and Discussion

Primary screening for antiviral activity was carried out using the agar-diffusion plaque-inhibition method with cylinders (Rada and Závada, 1962; Galabov *et al.*, 1980). It involved testing of com-

Table II. Screening of hydroxycoumarin derivatives **1–10** for antiviral activity by the agar-diffusion plaque-inhibition test[a].

Compound	Dose mM[b]	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	2.8	0	10.0	–	0	10.0	–	27.1	7.5	+	0	8.5	–
2	1.9	0	14.0	–	0	8.3	–	29.8	11.6	+	0	10.0	–
3	2.6	0	9.5	–	0	11.0	–	0	9.7	–	0	10.0	–
4	2.4	0	11.2	–	11.1	10.3	–	0	10.2	–	11.0	8.5	–
5	1.4	0	0	–	9.3	7.8	–	0	10.0	–	0	10.0	–
6	0.9	0	14.0	–	0	6.8	–	0	7.7	–	0	7.0	–
7	1.3	9.7	7.5	–	0	8.0	–	11.3	9.5	–	0	8.5	–
8	2.4	0	10.0	–	0	12.7	–	0	14.5	–	0	13.3	–
9	1.7	0	10.7	–	0	9.0	–	0	14.5	–	0	8.7	–
10	2.1	0	8.2	–	0	8.5	–	0	8.5	–	0	7.0	–
Ribavirin													
	0.4							10.0	0	–			
	2.0							34.5	0	++			
	4.1							48.7	0	+++			

[a] φ_i = diameter of inhibition zone (mm); φ_t = diameter of toxicity zone (mm); $\varphi_i - \varphi_t = \Delta\varphi$; E = antiviral effect (cf. Materials and Methods); [b] Per cylinder (dissolved in 0.1 ml DMSO).

pounds against one representative in each of the four taxonomic viral groups; namely, picorna-, orthomyxo-, paramyxo- and herpes viruses, which represent a few of the most important families of human pathogens. The viruses used were PV1, FPV, NDV and PsRV, respectively. Compounds showing a marked antiviral effect were then studied in the CPE inhibition multicycle system. A compound is considered to have a marked antiviral effect in the agar-diffusion plaque-inhibition assay if the difference between the diameter of the zone of inhibition (φ_i) and the diameter of the zone of toxicity (φ_t) is greater than 10 mm.

As seen in Table II, two of the ten hydroxycoumarin derivatives tested showed activity against NDV, namely esculetin (**1**) and its diacetate (**2**), when applied in cylinders at a dose of 2.8 and 1.9 mM/0.1 ml, respectively. Their activity was significant, although inferior when compared to that of ribavirin (used as a reference paramyxovirus inhibitor) at a dose of 2.0 mM/0.1 ml. **1** and **2** showed a marked toxicity area in this test, lacking in ribavirin even when applied at a twice higher dose (4.1 mM/0.1 ml). The remaining compounds tested were without effect on the replication of the four viruses studied. Evidently, no definitive conclusions could be drawn regarding structure-activity correlations. Nevertheless, it could be noticed that methylation and glucosylation of esculetin (**1**) lead to a loss of activity.

Table III summarizes the results of a more detailed analysis of **1** and **2** effect on NDV replication in CEC. As far as cytotoxicity is concerned **1** is less toxic to CEC than **2,** and a slight stimulation of cell growth (a cell count 21–28% higher than that observed in the negative control) was founds with **1** at a concentration of 56.2–112.4 μM (not documented).

In contrast to lack of toxicity of ribavirin in the agar-diffusion test, study of the influence of ribavirin on CEC growth curve revealed toxicity approximately twice higher than that of **1** and significantly higher than **2** (Table III). Evidently, ribavirin possesses a strong effect on cell growth.

A marked dependence of antiviral effect on viral dose value was found for **2.** As seen in Table III, a 12-fold decrease in antiviral activity (MIC_{50} values) was observed when the viral dose was increased from 10 to 100 ID_{50}/well. Compound **1** exhibited marked activity over a broader range of viral dose values.

Table III. Antiviral effect of compounds **1** and **2** on NDV replication in CEC (CPE inhibition test)[a].

Parameters	$CCID_{50}$ per well	**1**	**2**	Ribavirin
$CGIC_{50}$ (μM)	0	290.4	190.8	155.7
MIC_{50} (μM)	10	30.3	5.1	3.5
	100	36.0	62.2	3.9
	1000	56.2	>127.1	>307.4
SI	10	9.6	37.4	44.5
	100	8.1	3.1	39.9
	1000	5.2	<1.5	<0.5

[a] CEC, primary cultures of chick embryo fibroblasts; CPE, cytopathic effect; $CCID_{50}$, cell culture 50% infectious dose; $CGIC_{50}$, cell growth 50% inhibitory concentration; MIC_{50}; minimal 50% inhibitory concentration; SI, selectivity index ($CGIC_{50}/MIC_{50}$ value for given compound).

Ribavirin manifested a higher activity than **1** at 10 and 100 $CCID_{50}$/well (MIC_{50} and SI values 9 and 5 times lower, respectively). As compared to **2,** ribavirin was also more active, clearly seen at 100 $CCID_{50}$/well (15 and 13 times lower MIC_{50} and SI values, respectively). However, at 1000 $CCID_{50}$/well ribavirin was inactive, while **1** still showed a marked antiviral activity.

The results obtained provide impetus for the investigation of more structural analogues of the hydroxycoumarins. Except for ribavirin which is used only topically in respiratory syncytial virus infection in children because of its high toxicity, no other selective inhibitors of paramyxovirus replication are known.

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B. Alarcon, I. C. Lacal, J. M. Fernandez-Sousa, and L. Carrasco (1984), Screening for new compounds with antiherpes activity. Antiviral Res. **4,** 231–244.

A. S. Galabov, B. S. Galabov, and N. Neykova (1980), Structure-activity relationship of diphenylthiourea antivirals. J. Med. Chem. **23,** 1048–1051.

S. S. Handa, A. Sharma, and K. K. Chakraborti (1986), Natural products and plants as liver protecting drug. Fitoterapia **7,** 307–351.

L. Jurd, J. Corse, A. D. King Jr., H. Bayne, and K. Mihara (1974), Antimicrobial properties of 6,7-dihydroxy-7,8-dihydroxy-6-hydroxy- and 8-hydroxycoumarins. Phytochemistry **10,** 2971–2974.

Y. Kimura and H. Okuda (1985), Inhibition of formation of 5-hydroxy-6,8,11,14-eicosatetraenoic acid from arachidonic acid in polymorphonuclear leukocytes by various coumarins. Biochim. Biophys. Acta **834,** 224–229.

I. Kostova (1992), Hydroxycoumarins from *Fraxinus ornus* bark. Planta Med. **58,** 484.

I. Kostova, N. Nikolov, and L. Chipilska (1993), Antimicrobial properties of some hydroxycoumarins and *Fraxinus ornus* bark extracts. J. Ethnopharmacol. **39,** 205–208.

G. A. Kuznetsova (1967), Natural Coumarins and Furocoumarins (in Russian), Nauka, Leningrad, pp. 72–76.

G. Lazarova, I. Kostova, and H. Neychev (1993), Photodynamic damage prevention by some hydroxycoumarins. Fitoterapia **64,** 134–136.

S. Nishibe, H. Tsukamoto, S. Hisada, T. Nikaido, T. Omoto, and U. Sankawa (1986), Inhibition of cAMP-PDA by lignans and coumarins of *Olea* and *Fraxinus* bark (in English). Shoyakugaku Zasshi **40,** 89–94.

N. Nykolov, T. Iossifova, E. Vassileva, I. Kostova, and G. Stoev (1993), Reverse phase high pressure liquid chromatographic analysis of hydroxycoumarins in plant extracts. Phytochem. Analysis **4,** 86–88.

J. S. Porterfield (1960), A simple plaque-inhibiton test for arthropod-borne viruses. Bull. WHO **22,** 373–380.

B. Rada and J. Závada (1962), Screening test for cytostatic and virostatic substances. Neoplasma **9,** 57–60.

G. T. Tan, J. M. Pezzuto, and A. D. Kinghorn (1991), Evalution of natural products as inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. J. Nat. Prod. **54,** 143–154.

H. Tsukamoto, S. Hisada, and S. Nishibe (1985), Coumarins from bark of *Fraxinus japonica* and *Fraxinus mandshurica.* Chem. Pharm. Bull. **33,** 4069–4073.

H. Tsukamoto, S. Hisada, S. Nishibe, D. G. Roux, J. P. Rourke (1984), Coumarins from *Olea africana* and *Olea capensis.* Phytochemistry **23,** 699–700.

A. Tubaro, P. D. Negro, E. Ragazzi, S. Zampiron, and R. D. Loggia (1988), Anti-inflammatory and peripherical analgesic activity of esculin *in vivo.* Pharmacol. Res. Comm. **20,** 83–85.

I. Yamagami, Y. Suzuki, and K. Ito (1968), Pharmacologic studies on the components of *Fraxinus japonica* (in Japanese). Nippon Yakurigaki Zasshi **64,** 714–729.