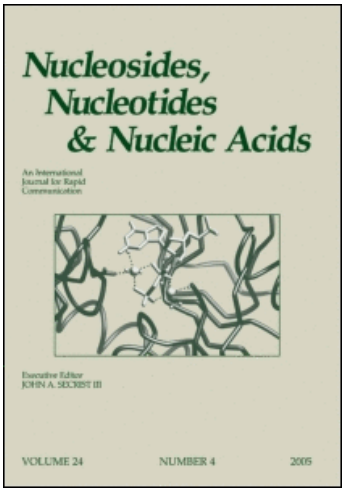


## Nucleosides, Nucleotides and Nucleic Acids

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# Cinnamic Esters of Acyclovir-Synthesis and Biological Activity

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# CINNAMIC ESTERS OF ACYCLOVIR-SYNTHESIS AND BIOLOGICAL ACTIVITY

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□ *In the present study we have synthesized esters of acyclovir* ***(2–4)*** *with cinnamic acids (*p-*coumaric, ferulic, and sinapic acids) and evaluated them for their antiviral and antioxidant potential. The antiviral activity of the newly synthesized compounds has been tested against human herpes virus 1 (HSV-1) in vitro. The results indicate that none of the synthesized compounds inhibits the tested virus strain. The antioxidant properties have been studied using 2,2-diphenyl-1-picrylhydrazyl (DPPH)* test.*

**Keywords** Acyclovir; cinnamic acids; HSV-1; antioxidant effect; DPPH* test



## INTRODUCTION

Following the discovery of the first effective antiviral compound (idoxuridine) in 1959, nucleoside analogues, especially acyclovir, 9-[(2-hydroxyethoxy)methyl] guanine (ACV), for the treatment of herpesvirus infections, have dominated antiviral therapy for several decades.[1,2] However, ACV and similar acyclic nucleosides suffer from low aqueous solubility and low bioavailability following oral administration. Derivatives of acyclic nucleosides, typically esters, were developed to overcome this problem, and valaciclovir (L-valine ester of ACV) was among the first of a new series of compounds that were readily metabolized upon oral administration to produce the antiviral nucleoside in vivo, thus increasing the bioavailility by several fold.[3,4]

Valacyclovir represents a prototype prodrug for enhancing the oral bioavailability of a poorly absorbed drug like acyclovir. The enhancement in

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oral absorption of this amino acid ester prodrug has since been attributed to carrier-mediated transport by intestinal oligopeptide transporters, followed by rapid bioconversion to acyclovir. The successful clinical utility of oral valacyclovir indicated the potential for amino acids as promoieties for other potent agents.

*Trans*-Cinnamic acids, originally isolated from plant sources,[5,6] have been reported.[7–9] This leads us to investigate a new pharmacological activity of trans-cinnamic acid and its derivatives. For example, *trans*-cinnamic acid induces cytostasis and a reversal of malignant properties of human tumour cells in vitro. Furthermore, molecular analysis have been shown that the antitumor activity of cinnamic acid may be due in part to the inhibition of protein isoprenylation in mitogenic signal transduction.[10] *p*-Coumaric acid or 4-hydroxy-*trans*-cinnamic acid has shown to possess antioxidant activity. It minimized the oxidation of low-density lipoprotein (LDL) involving direct scavenger of reactive oxygen species (ROS).[11] Moreover, the dehydrogenated polymers of *p*-coumaric acid inhibited HIV-1 protease activity.[12]

4-Methoxy-*trans*-cinnamic acid exhibited a potent hepatoprotective activity in rat hepatocytes from toxicity induced by carbon tetrachloride ($CCl_4$).[13,14] Consequently, a broad range of biological activities of cinnamic acids have been reported, this leads us to investigate a new pharmacological activity of *trans*-cinnamic acid and its derivatives.

The aims of this study include the synthesis and biological characterization of acyclovir analogues, derived from two compounds with proven pharmacological activity-cinnamic esters and acyclovir. The cytotoxic and antiherpetic activity, as well as the antioxidative effect of the sinapic-, p-coumaric-, ferulic- esters of acyclovir are reported.

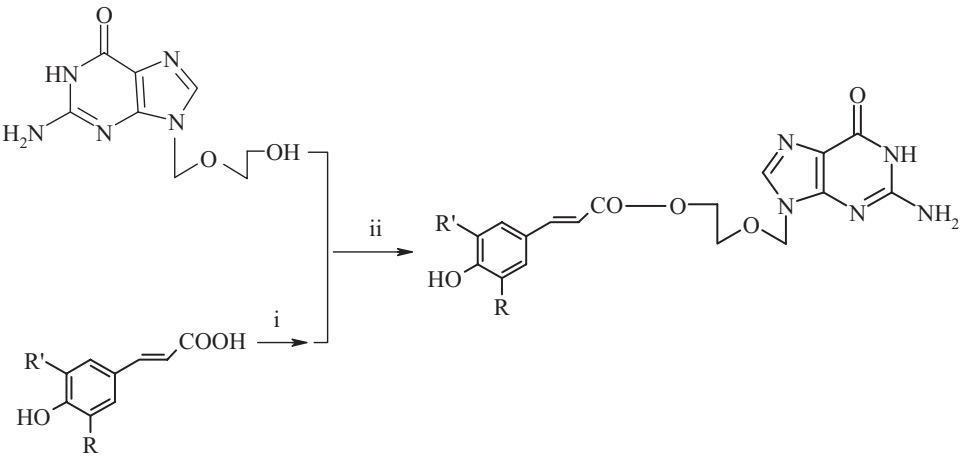


## RESULTS AND DISCUSSION

### Chemistry

Acyclovir derivatives modified with amino acids and peptides[15–18] and acyclovir containing peptidomimetics[19,20] have been reported but acyclovir containing cinnamic acids are not known. In order to obtain analogues with more desirable characteristics, we synthesized new esters of acyclovir containing *p*-coumaric, ferulic, and sinapic acids.

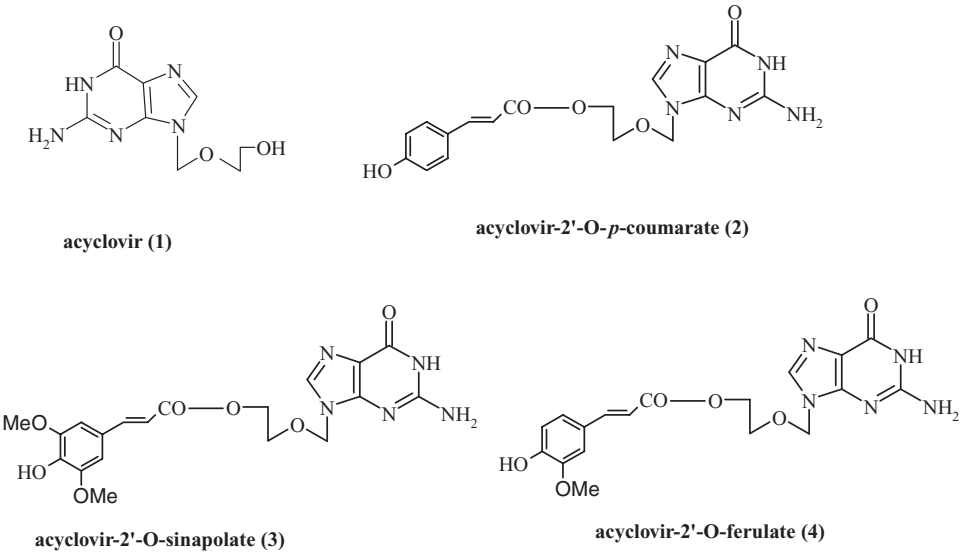
A mixture of cinnamic acid and *N,N'*-dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) was stirred for 1 hour at 0°C under nitrogen atmosphere. A solution of ACV (Scheme 1) and 4-*N*,*N*-(dimethylamino)-pyridine (DMAP) was added to the reaction mixture and stirred for 24 hours. The $^{1}H$, $^{13}C$NMR and mass spectra were consistent with the desired structure.




**SCHEME 1** Synthesis of the compounds **2–4. (i)** DCC, DMF, 0°C, 1 hour; **(ii)** DMAP, room temperature, 24 hours.



Nuclear magnetic resonance (NMR) data recorded in this research clearly indicated the structure of the esters (Figure 1). The cinnamic acids could react with ACV at either $CH_2$–OH or $NH_2$ sites. However, only the ester linkage was formed in this research, which was supported by the change of chemical shift of the $CH_2$–OR signal from 3.35 in ACV to 4.1 after the reaction. The values of the proton–proton vicinal coupling constants (3JH/H about 15.5 Hz) measured for the olefinic protons of feruloyl-, sinapoyl-, and p-coumaroyl residues define the E configuration of the double bond of all




**acyclovir (1)**

**acyclovir-2'-O-*p*-coumarate (2)**

**acyclovir-2'-O-sinapolate (3)**

**acyclovir-2'-O-ferulate (4)**

**FIGURE 1** Structures of acyclovir (ACV) and its cinnamic esters.

**TABLE 1** Cytotoxicity and antiherpetic activity of the compounds[21] **(2–4)** in GMK cells

| Compound | 50% cytotoxic concentration ($\mu$g/ml) | Antiherpetic activity |
|---|---|---|
| Acyclovir-p-coumarate (2) | 23.4 | not active |
| Acyclovir-sinaponate (3) | 41.5 | not active |
| Acyclovir-ferulate (4) | >50 | not active |

studied compounds. The chemical shift of the esters protons established the ester linkage of the acyclovir with cinnamic acid.

## Biological Evaluation and Discussion

Cytotoxicity and antiherpetic activity of the newly synthesized cinnamic esters of acyclovir (**2–4**) was tested in GMK cells.

To exclude nonspecific antiviral activities, the derivatives of acyclovir were tested for cytotoxicity on confluent monolayer's of host cells. Table 1 summarizes the 50% cytotoxic concentrations of the test compounds in GMK cells. The results demonstrate a good compatibility of the new acyclovir analogous for the tested cell lines. None of the compounds inhibited the HSV-1-induced cytopathic effect in GMK cells (Table 1).



## Antioxidant Activity

The antioxidative potential of the synthesized esters was studied against DPPH* (1,1-diphenyl-2-picrylhydrazyl radical). The obtained results are summarized in Table 2. The synthesized cinnamoyl esters of acyclovir were found

**TABLE 2** Antioxidative activity of esters of acyclovir with cinnamic acid by DPPH*

| | (%) RSA | | | | | |
|---|---|---|---|---|---|---|
| | 0.9 mM | | 1.8 mM | | 3.6 mM | |
| | | | Reaction time (min) | | | |
| Compound | 10′ | 20′ | 10′ | 20′ | 10′ | 20′ |
| Acyclovir **1** | 1.3 | 1.4 | 1.6 | 1.7 | 2.1 | 2.7 |
| p-Coumaric acid | 2.1 | 2.9 | 3.7 | 4.7 | 4.5 | 6.1. |
| Acyclovir-p-coumarate **2** | 1.2 | 1.4 | 1.7 | 1.9 | 2.0 | 2.3 |
| Sinapic acid | 16.1 | 17.2 | 26.5 | 31.9 | 69.0 | 69.6 |
| Acyclovir-sinaponate **3** | 4.1 | 5.3 | 5.7 | 7.1 | 8.5 | 10.1 |
| Ferulic acid | 12.0 | 13.8 | 21.0 | 25.1 | 36.7 | 44.3 |
| Acyclovir-ferulate **4** | 2.2 | 2.7 | 3.3 | 3.7 | 4.3 | 4.9 |

% RSA—percentage radical scavenging activity; sinapic-, ferulic-, p-coumaric, acids and acyclovir used as standards.

to be inefficient radical scavengers. The compounds (**2–4**) showed lower antioxidative effect than that of the standards: p-coumaric, ferulic, and sinapic acids. The ester of acyclovir with sinapic acid showed border line activity compared to the free cinnamic acids in the DPPH* test.

In conclusion, p-coumaroyl, sinapoyl-, feruloyl-, esters of acyclovir have been synthesized by the methods used in the peptide chemistry, and their antiherpetic activity, as well as their antioxidant potential, has been explored. The results show that modification of acyclovir with cinnamic acids reduced its antiviral activity. The new compounds **2–4** showed antioxidant activity, but lower to the standards, p-coumaric, ferulic, and sinapic acid.

## EXPERIMENTAL

### Chemistry

3-(4-Hydroxyphenyl)-2-propenoic acid (*p*-coumaric), 3-methoxy-4-hydroxy-cinnamic (ferulic) and 3,5-dimethoxy-4-hydroxy-cinnamic (sinapic) acids were purchased from Fluka (Buchs, Switzerland) and DMAP and *N, N*'-dicyclohexylcarbodiimide (DDC) were purchased from Merck (Darmstadt, Germany).

Thin layer chromatography (TLC) analysis was performed on aluminum silica gel sheets 60 $F_{254}$ plates (Merck) and spots were detected using an UV lamp at 254 nm.

The NMR spectra was obtained on a Bruker Avance DRX-500 spectrometer (Bruker, Germany), operating at 500.13 MHz. The ESI mass spectra was obtained on Finnigan MAT 8200 (Finnegan, MasCom, Bremen, Germany). "Agilent 8453" spectrophotometer was used for the measurement of the reduction of DPPH* (1,1-diphenyl-2-picrylhydrazyl radical) absorbance at 516 nm.

#### *General Synthetic Procedure for Preparation of Acyclovir Cinammic Esters 2–4*

The cinnamic acid (*p*-coumaric-, ferulic-, and sinapic-) (0.8 mM) and was dissolved in DMF and the solution was cooled at 0°C and *N,N*′-dicyclohexylcarbodiimide (DDC) (0.165 g, 0.8 mmol) was added. After 1 hour, ACV (0.100 g, 0.4 mmol) and DMAP (0.097 g, 0.8 mmol) were added. The reaction mixture was kept at 0°C for 1 hour and was stirred continuously for 24 hour at room temperature. The urea derivative was removed by filtration. After DMF was removed in vacuum, 30 ml of ethyl acetate was added to the residue. This solution was washed with 10 ml of saturated sodium bicarbonate solution and 20 ml of brine, respectively. The organic layer was collected and dried over MgSO4. After removal of most of the solvent, the residue was loaded on a silica gel column and eluted with a mixture of hexane/ethyl acetate = 1:6.

***2-(2-Amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methoxy)ethyl-p-coumarate 2***

Yield: 0.076 g (51%); $^1$H NMR (500 MHz; $CDCl_3$) $\delta = 3.69$ (t, 1H, CH), 3.73 (s, 6H, OCH3), 4.1 (t, 2H, $CH_2$), 5.28 (s, H), 5.42 (s, 2H,NH2), (6.03, (s, H), 6.5 (s, H), 6.59 (d, 1H, $J = 15.5$, CH=), 7.21, 7.68 (d, 1H, $J = 15.4$ Hz, CH=), 7.89 (s, 1H). $^{13}$C-NMR (500 MHz; $CDCl_3$): $\delta = 173.3$, 164.0, 166.1, 160.1, 147.4, 146.5, 137.2, 131.3, 126.8, 120.8, 119.4, 118.2, 115.3, 59.78. ESI-MS: 372 ($[M + H]^+$).

***2-(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methoxy)ethyl-sinaponate 3***

Yield: 0.104 g (60%); $^1$H NMR (500 MHz; $CDCl_3$) $\delta = 3.85$ (t, 1H, CH), 3.91 (s, 6H, OCH3), 4.10 (t, 2H, $CH_2$), 6.53 (d, 1H, $J = 15.5$, CH=), 6.67 (s, H), 6.74 (s, 2H, Ar–H), 7.57 (d, 1H, $J = 15.4$ Hz, CH=), 7.83 (s, 1H).$^{13}$C-NMR (500 MHz; $CDCl_3$): $\delta = 162.58$, 152.65, 114.22, 105.48, 104.64, 60.37, 56.36, 56.12, 55.59. ESI-MS: 432 ($[M + H]^+$).

***2-(2-amino-1,6-dihydro-6-oxo-9H-purin-9-yl)methoxy)ethyl-ferulate 4***

Yield: 0.094 g (58%); $^1$H NMR (500 MHz; $CDCl_3$) $\delta = 3.87$ (s, 6H, OCH3), 4.1 (t, 2H, $CH_2$), 5.86 (br. s, H), 5.42 (s, 2H,NH2), 6.3 (d, 1H, $J = 15.4$ Hz, CH=), 6.8 (s, H, NH2), 6.9 (d, 1H, $J = 8.9$, Ar-H (m)), 7.02 (d, 1H, J = 1.7 Hz, Ar–H(o)), 7.6 (d, 1H, J = 15.5 Hz, CH=), 7.8 (s, H), 9.89 (s, H). $^{13}$C-NMR (500 MHz; $CDCl_3$): $\delta = 206.79$, 167.23, 147.93, 146.79, 144.62, 122.99, 115.73, 114.72, 109.38, 60.31, 55.95. ESI-MS: 402 ($[M + H]^+$).

## Evaluation of the Biological Activity

***Estimation of Radical Scavenging Activity (RSA) by the DPPH* Test***

1,1-Diphenyl-2-picrylhydrazyl radical ($DPPH_*$) was used as stabile radical. For each compound and concentration tested (0.9, 1.8, and 3.6 mM), the reduction of DPPH* radical was followed by monitoring the decrease of absorbance at 516 nm. The absorption was monitored at the start and at 10 and 20 minutes. The results are expressed as% RSA = [$Abs_{516}$ nm ($t = 0$) − $Abs_{516}$ nm ($t = t'$) × 100/$Abs_{516}$ nm ($t = 0$)], as proposed by Pekkarien et al.[22]

***Estimation of Cytotoxic and Antiviral Activity***

*Cell Culture and Virus Propagation.* Green monkey kidney cells (GMK; Schaper and Bruemmer, Salzgitter, Germany) were grown in Dulbecco's modified MEM/E (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Greiner, Germany), 100 U/ml penicillin, and 100 mg/ml streptomycin (CCpro, Germany). The test medium used for the cytotoxic assays and anti-HSV-1 tests contained only 2% of the appropriate serum. All cells were proved to be free of mycoplasma contamination.

The acyclovir- and phosphonoformic acid-sensitive laboratory herpes simplex virus type 1 strain Kupka (HSV-1) was propagated in GMK cells and 50 $\mu$l aliquots of the virus stocks were stored at $-70^{\circ}$C until use.

*Cytotoxicity Test.* Cytotoxicity test was described previously.[21] Briefly, 2-day-old confluent GMK cell monolayer's grown in 96-well cell culture platesin an incubator with 5% $CO_2$, 37°C, and 95%, were incubated with different concentrations (100 $\mu$l/well, dilution factor of 2, 2 parallels/concentration, 0.2–50 $\mu$g/ml)) in culture medium. For the control value determination (six untreated cell controls) were used. 72 hours after substance application and incubation, staining of the cells with crystal violet/methanol was carried out. After extraction of the dye the optical density of individual wells was quantified spectrophotometrically at 5570/630 nm with Dynex Immuno Assay System (DIAS, Guernsey, GB). Cell viability was evaluated as the percentage of the mean value of optical density resulting from six mock-treated cell controls which was set 100%. The 50% cytotoxic concentration ($CC_{50}$) was defined as the compound concentration reducing the viability of untreated cell cultures by 50%.

*Antiviral Tests.* The cytopathic effect (CPE) inhibitory assay was performed as described previously.[21] HSV-1 replication results in the complete destruction of host cells (CPE) that can be selectively inhibited by the addition of active antiviral agents (50 $\mu$l/well, dilution factor 2, each concentration in triplicate). Compound dilutions were added immediately before inoculation of a virus dilution (50 $\mu$l/well, multiplicity of infection of 0.05) to 2-day-old confluent GMK. This virus dose leads to a complete CPE in the 6 untreated virus controls 48 hours after infection. At this time point, the still adherent cells were fixed and stained with a crystal violet/formalin solution. The inhibition of virus-induced CPE was photometrically determined by comparing the individual optical density (OD) of compound treated, virus-infected wells with that of the mean OD of 6 untreated, uninfected cell control that was set 100%.



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