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Synthesis and biological activity of hydroxycinnamoyl-containing antiviral drugs

MAYA G. CHOCHKOVA[1]*, ASSYA P. GEORGIEVA[1], GALYA I. IVANOVA[2], NADYA NIKOLOVA[3], LUCHIA MUKOVA[3], LUBOMIRA NIKOLAEVA-GLOMB[3] and TSENKA S. MILKOVA[1]

[1]South-West University ''Neofit Rilski", Blagoevgrad, Bulgaria, [2]Departamento de Química, Faculdade de Ciências, Universidade do Porto, Porto, Portugal and [3]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

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Abstract: Seven *N*-hydroxycinnamoyl amides were synthesized by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide/1-hydroxybenzotriazole (EDC/HOBt) coupling of the corresponding substituted cinnamic acids (*p*-coumaric-, ferulic-, sinapic- and caffeic acids) with influenza antivirals (amantadine, rimantadine and oseltamivir). The DPPH (1,1-diphenyl-2-picrylhydrazyl) scavenging abilities and the inhibitory effect on mushroom tyrosinase activity (using L-tyrosine as the substrate) were investigated *in vitro*. Amongst the synthesized compounds, *N*-[(*E*)-3-(3,4-dihydroxyphenyl)-2-propenoyl]oseltamivir (**1**) and *N*-[(*E*)-3-(3,4-dihydroxyphenyl)-2-propenoyl]rimantadine (**4**), containing a catechol moiety, exhibited the most potent DPPH radical-scavenging activity. Amide (**1**) also displayed tyrosinase inhibitory effect toward L-tyrosine as the substrate (≈50 %). The synthesized compounds were also investigated for their *in vitro* inhibitory activity against the replication of influenza virus A (H3N2).

Keywords: *N*-hydroxycinnamoylamides; influenza antivirals; mushroom tyrosinase; monophenolase activity; antioxidant activity; anti-influenza activity.

INTRODUCTION

Phenolics are secondary plant metabolites, important not only for the functional aspects of plant life, but also they have a potential impact on public health. Amongst the main families of phenolic compounds, hydroxycinnamic acids (*e.g.*, ferulic, *p*-coumaric, caffeic acids, *etc.*) and their derivatives (esters, amides and glycosides) have become an emergent topic of many research groups and hence the subject of our interest.[1–4]

* Corresponding author. E-mail: mayabg2002@yahoo.com
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Hydroxycinnamic acid amides, both natural and synthetic, possess diverse physiological activities, such as being potent antioxidants,[5–12] antimicrobials,[3,4,6,13,14] inhibitors of hepatotoxic and proliferative activity,[15–18] acting as inhibitors of HIV integrase, [19–22] inhibitors of α-glucosidase,[23,24] and tyrosinase,[1,25,26] *etc*.

A huge amount of data on various polyhydroxy tyrosinase inhibitors exists in the literature. Tyrosinase inhibition is an important strategy in many medicinal, food and agricultural areas. Hyperpigmentation of the human skin and enzymatic browning in fruits and mushrooms are the results of two distinct reactions of tyrosinase (EC 1.14.18.1) – the hydroxylation of tyrosine by monophenolase action to 3,4-dihydroxyphenylalanine (L-DOPA), and further L-DOPA oxidation to *o*-dopaquinone (diphenolase activity). This copper-containing enzyme is widely present in bacteria, fungi and higher plants (mushrooms, bananas, apples, pears, potatoes, *etc*.).[27,28]

In order to overcome the instability and low degree of percutaneous absorption of natural polyphenols in cosmetic compositions, synthesized *N*-hydroxycinnamoyl amantadine amides, which manifested increased antioxidant, melanin inhibitory and procollagen inducing effects, were synthesized.[29]

The incorporation of the lipophilic adamantyl moiety in another class of small molecules, such as *N*-benzylbenzamide, appeared to be also an important element for enhanced depigmentation activity.[30]

Since 1960s, the aminoadamantanes (amantadine, rimantadine) have been representatives of the first class of antivirals, approved for prophylaxis and treatment of influenza. They are known to inhibit virus replication by blocking the proton channel of the M2 structural protein of the virion (M2 blockers).[31] As only the influenza A viruses possess this structural M2 ion channel protein, aminoadamantanes are not effective against influenza B viruses, since the latter do not encode for M2 protein.[32,33]

In the quest for therapeutic agents with a broader spectrum that are active against both types (A and B) of influenza viruses, neuraminidase inhibitors (oseltamivir and zanamivir) were discovered as the second class of anti-influenza antivirals.[34,35] They interfere with viral neuraminidase, which is essential for the release of newly synthesized infective virus progeny.

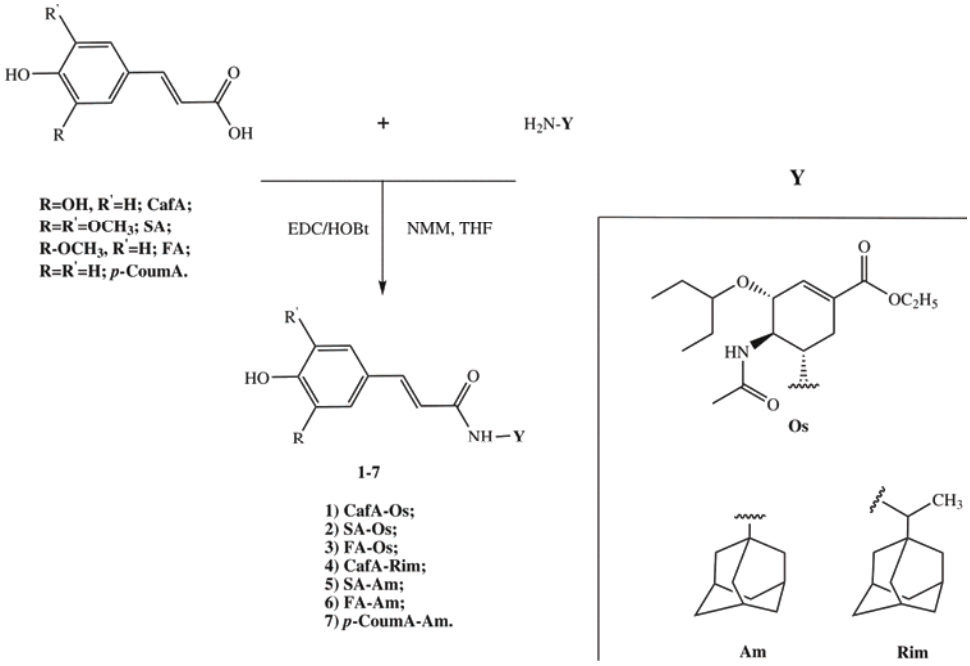
A set of polyphenol compounds (*e.g.*, resveratrol; curcumin and isoquercetin) was reported to inhibit both influenza viruses type A and B *in vitro*.[36] However one of the latest investigations on the matter revealed that the mentioned polyphenols possess an antiviral activity only against influenza A virus replication.[37]

It was found that the reception of a combination of antioxidants (*e.g.*, polyphenolics) with influenza drugs synergized on the reduction of viral replication.[38,39]

The above findings encouraged us to link chemically antioxidant-functioning hydroxycinnamic acids with anti-influenza drugs (amantadine, rimantadine and oseltamivir) and to study some of their biological activities.

RESULTS AND DISCUSSION

Seven *N*-hydroxycinnamoylamides were synthesized from hydroxycinnamic acids (ferulic, caffeic, sinapic and *p*-coumaric acids) and influenza antivirals (amantadine, rimantadine and oseltamivir) by a previously described procedure.[7] Unlike the three-step (reactions of the hydroxycinnamoyl amide synthesis, provided previously,[29] the present synthetic route (Scheme 1) was performed by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide/1-hydroxybenzotriazole (EDC/ /HOBt) coupling, without preliminary protection and further deprotection steps of the phenolic hydroxyl groups.



Scheme 1. EDC/HOBt-coupling in preparation of *N*-hydroxycinnamoylamides.

After purification by column or preparative thin-layer chromatography, the compounds were obtained in moderate to good yields. The geometry of the double bond in the cinnamoyl residue was confirmed by ^{1}H-NMR spectroscopy, where the resonance of olefinic protons in all *N*-hydroxycinnamoylamides was observed at 6.19–6.51 ppm and at 7.19–7.55 ppm as two doublets with $J \approx 15.5$ Hz. These signals are evidence that all of the synthesized amides present were *E*-diasteroisomers.

The yields of the compounds, and the analytic and complete spectral data for the synthesized compounds are given in the Supplementary material to this paper.

Scavenging effect on DPPH radicals

Since it is well known[40] that the DPPH test gives a rough, primary index of antioxidant activity, by measuring the ability of DPPH to scavenge an electron or hydrogen radical of the tested compounds (scavengers) *in vitro*, a myriad of articles have been published.

DPPH scavenging activity of the synthesized *N*-hydroxycinnamoylamides, as well as the used standards caffeic, ferulic, sinapic and *p*-coumaric acids, displayed an activity at 12, 24 and 48 μM in a concentration dependent manner (Table I). As shown in Table I (data for 12 and 24 μM concentration are not presented), the evaluated scavenging activity of the compounds decreased in the following order: CafA ≈ CafA-Os (**1**) > CafA-Rim (**4**) > SA > SA-Os (**2**) > FA > FA-Os (**3**) ≈ SA-Am (**5**) > FA-Am (**6**) > *p*-CoumA > *p*-CoumA-Am (**7**) ≈ Os > > Rim > Am.

TABLE I. DPPH radical scavenging activity of *N*-hydroxycinnamoylamides, antivirals and the tested references at a concentration of 48 μM. The given values are the means ± confidence interval, calculated at the level of significance 0.05 (n = 3)

Compound	DPPH radical scavenging activity, %
1	91.83±5.08
2	48.10±1.23
3	31.75±3.77
4	72.58±8.26
5	30.37±2.58
6	20.28±1.04
7	3.00±0.60
Caffeic acid	92.65±2.90
Sinapic acid	60.73±0.62
Ferulic acid	44.30±0.10
p-Coumaric acid	6.10±0.04
Oseltamivir	3.51±0.90
Amantadine hydrochloride	1.75±0.21
Rimantadine hydrochloride	2.48±0.30

Amongst the tested newly synthesized amides, *N*-caffeoylamides (**1** and **4**) revealed the best activity, commensurable with that of the free caffeic acid at 3.6 mM concentration. It is not surprising that the latter compounds are the most active ones. The presence of a catechol moiety is the structural feature, responsible for their better radical quenching abilities.[41] All the newly synthesized compounds demonstrated a higher radical scavenging activity than amantadine, rimantadine and oseltamivir, which do not possess DPPH scavenging ability at all. The reported results are in agreement with earlier findings for the disability of

rimantadine to scavenge superoxide radicals,[42] although its antioxidant effect has been observed *in vivo*. Moreover, the existence of the more polar substituted nucleus of ethyl cyclohex-1-ene-1-carboxylate (*i.e.*, the skeleton of oseltamivir) in the *N*-hydroxycinnamoylamides seems to be more efficient in radical scavenging than the corresponding amides of the hydrophobic aminoadamantanes.

Effect of N-*hydroxycinnamoylamides on mushroom tyrosinase*

Considering substituted cinnamic acids and their derivatives as potential tyrosinase inhibitors,[25,43–46] the effects of the obtained *N*-hydroxycinnamoylamides on the monophenolase activity of mushroom tyrosinase (using L-tyrosine as substrate) at 23 μM concentration was studied herein.

The results of the evaluated activity of the amides, presented as percentage (%) of mushroom tyrosinase inhibition, are outlined in Table II. Hydroquinone was used as a reference compound.

TABLE II. Tyrosinase inhibitory activity of *N*-hydroxycinnamoylamides at a concentration of 23 μM. The given values are the means ± confidence interval, calculated at the level of significance 0.05 (n = 3); NA –not analyzed; N.I. – no inhibition

Compound	Tyrosinase inhibition, %
Hydroquinone	98.61±3.45
CafA-Os (**1**)	49.32±9.37
SA-Os (**2**)	N.I.
FA-Os (**3**)	N.I.
CafA-Rim (**4**)	N.I.
SA-Am (**5**)	N.I.
FA-Am (**6**)	N.I.
p-CoumA-Am(**7**)	NA

Amongst the tested compounds, the amide CafA-Os (**1**) was found to inhibit the enzyme with half the activity of the used standard tyrosinase inhibitor hydroquinone.

Despite the fact that both amides (CafA-Os (**1**) and CafA-Rim (**4**)) contain the same caffeoyl moiety (catechol entity) in their structure, the inhibitory activity *in vitro* toward mushroom tyrosinase was lost in amide **4**, which could be attributed to the sterically hindered adamantane rest.

The obtained results confirmed the finding that the inhibitory activity of *p*-coumaric acid varies from weaker to stronger in comparison with kojic acid and arbutin, depending on the tyrosinase source (mushroom, human or murine).[47]

The data of the present investigation do not correspond to the developmental work on a study of tyrosinase inhibition by *N*-feruloylamantadine amide and related analogues, which possessed similar or greater melanin inhibitory effect than the positive controls – kojic acid and hydroquinone, in cell-based environments.[29]

Effect of the chemically connected hydroxycinnamic acids with rimantadine, amantadine and oseltamivir against the influenza virus A/Aichi/2/68 (H3N2)

Earlier studies showed that antioxidant therapy could be successfully used as a potential approach to influenza-associated complications.[39,48–50] The existence of these findings is connected with the synergistic combination of antioxidants with approved influenza antivirals, but antioxdiants and antivirals have never been combined chemically until now.

Therefore, the newly synthesized *N*-hydroxycinnamoylamides, were evaluated for their antiviral activity against influenza virus A/Aichi/2/68 (H3N2). The newly synthesized compounds did not reveal a significant inhibitory effect against the *in vitro* replication of influenza A virus H3N2.

EXPERIMENTAL

Materials and methods

1-Adamantylamine hydrochloride (amantadine hydrochloride), 1-(1-adamantyl)ethylamine hydrochloride (rimantadine hydrochloride), (*E*)-4-hydroxy-3-methoxycinnamic (ferulic, FA), (*E*)-3,4-dihydroxycinnamic (caffeic, CafA), (*E*)-4-hydroxy-3,5-dimethoxycinnamic (sinapic, SA), (*E*)-4-hydroxycinnamic (*p*-coumaric, *p*-CoumA) acids, 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide hydrochloride (EDC), *N*-methylmorpholine (NMM), mushroom tyrosinase (EC 1.14.18.1) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were from Sigma-Aldrich. Hydroquinone was obtained from Ferak (Germany) and L-tyrosine was obtained from Merck (Germany). Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)--1-cyclohexene-1-carboxylate (oseltamivir) was purchased from Aopharm (China). All solvents were of reagent grade and used without further purification and the water used was deionized.

Apparatus

The structures and purity of all newly synthesized amides were confirmed by spectral methods, *i.e.*, UV, ^{1}H- and ^{13}C-NMR spectroscopy and ESI-MS. The ^{1}H- and ^{13}C-NMR spectra were acquired on a Bruker Avance III 400 spectrometer, operating at 400.15 MHz for protons and 100.62 MHz for carbons. The UV spectra of the amides were measured with an Agilent 8453 UV–Vis spectrophotometer. Elemental analyses for C, H and N were realized on a Perkin Elmer 2400 analyzer. The ESI mass spectra were obtained on an Esquire3000 plus instrument.

General procedure for the preparation of N*-hydroxycinnamoylamides*

In a typical preparation, to a solution of substituted cinnamic acid (3.2 mmol), EDC (0.61 g, 3.2 mmol) and 1-hydroxybenzotriazole (0.43 g, 3.2 mmol) in 10 mL THF, after 10 min stirring at 0 °C, the antiviral compound (3.2 mmol) and NMM (0.35 mL, 3.2 mmol), dissolved in 7 mL THF were added. The resultant reaction mixture was stirred for 1 h at 0 °C and then for 24 h at room temperature, under a nitrogen atmosphere. After completion of the reaction (TLC control – CH_2Cl_2/CH_3OH (3:0.2; 3:0.3); CH_2Cl_2/EtOAc/CH_3OH (3:0.1:0.1)), the THF was evaporated *in vacuo*, and the residue was diluted with EtOAc and then was successively washed with 5 % $NaHSO_4$, $NaHCO_3$ and brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was purified by column chromatography or preparative TLC on silica gel (CH_2Cl_2/CH_3OH) to give the desired compound.

DPPH radical scavenging assay

The free DPPH scavenging assay was performed according to the Nenadis method.[51]

The vanishing of the deep violet color of the DPPH radical was accomplished by mixing 2.96 mL 0.1 mM ethanolic DPPH solution with 40 μL of a free radical scavenger (antioxidant) at the reaction mixture concentrations: 12, 24 and 48 μM. The decrease in absorbance at 516 nm was measured after 20 min incubation in the dark at room temperature.

The measurements were performed in triplicate and results are presented as the percentage of radical scavenging activity, calculated as follows:

$$\text{Free radical scavenging activity (\%)} = 100(A_{\text{DPPH}} - A_{\text{sample}})/A_{\text{DPPH}}$$

Mushroom tyrosinase inhibition assay

Inhibition of tyrosinase activity was determined spectrophotometrically by a modified dopachrome method[25] using L-tyrosine as the substrate.

The reaction media (3 mL) contained: phosphate buffer (1.0 mL, 0.1 M, pH 6.8); L-tyrosine (1.0 mL, 1.5 mM) dissolved in deionized water; inhibitor (0.350 mL, 0.2 mM) dissolved in DMSO; deionized water (0.350 mL); an aqueous mushroom tyrosinase solution (0.300 mL, 192 U mL^{-1}). After adding the mushroom tyrosinase solution, the reaction mixture was incubated at 37 °C for 20 min, and thereafter, the UV absorbance of the reaction mixture was measured at 475 nm. Results were compared with a reference solution consisting of 0.350 mL of DMSO instead of a sample (inhibitor).

The percentage of mushroom tyrosinase inhibitory activity was calculated using the following equation:

$$\text{Inhibition (\%)} = 100(A_{\text{refs}} - A_{\text{sample}})/A_{\text{refs}}$$

where, A_{refs} is the absorbance of the reference solution and A_{sample} is the absorbance of the test sample solution).

Each experiment was performed in triplicate and averaged. Results were evaluated at a level of significance 0.05. Hydroquinone was used as the positive control.

Antiviral activity assay

Cells and viruses. MDCK cells for the propagation of influenza virus A originated from the collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria, and were grown in a growth medium containing Dulbecco modified Eagles' medium (DMEM) (Gibko BRL, USA), supplemented with 10 % fetal bovine serum, 10 mM HEPES buffer (Merck, Germany) and antibiotics (penicillin 100 IU mL^{-1} and streptomycin 100 μg mL^{-1}). The cells were cultured as confluent monolayers in a humidified atmosphere containing 5 % CO_2 at 37 °C.

Influenza virus A/Aichi/2/68 (H3N2) from the collection of the Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, was grown in MDCK cells in a maintenance medium of Dulbecco modified Eagles' medium (DMEM) (Gibko BRL, USA), containing 0.5 % fetal bovine serum, 10 mM HEPES buffer and antibiotics, as well as 3 mg mL^{-1} trypsin (Gibco BRL).

Cytopathic effect (CPE) inhibition test. Monolayer MDCK cells in 96-well microplates (Costar, USA) were inoculated, following the removal of the growth medium, with 0.1 mL virus suspension containing 100 $CCID_{50}$ (cell culture infectious dose 50 %). After 1 h at 37 °C for virus adsorption, the innoculum was washed out and replaced by 0.1 mL of non-cytotoxic 0.5 $\log_{10}$ dilutions in the maintenance medium of the newly synthesized compounds. Each dilution was applied in quadruplicate. Cells that were not inoculated with virus were left for

cell controls (with only maintenance medium) and toxicity controls (with respective dilution of the compound in the maintenance medium). Cells inoculated with virus but not treated with a compound were left for virus controls. Then cells were incubated for 48 h in a humidified atmosphere with 5 % CO_2 at 37 °C or until the virus specific cytopathic effect had destroyed 100 % of the cells in the virus control wells. Then cells were stained according to the neutral red uptake procedure and the percentage of CPE inhibition, if present, was calculated using the following formula: % CPE = $(OD_{\text{test sample}} - OD_{\text{virus control}})/(OD_{\text{toxicity control}} - OD_{\text{virus control}}) - 100$.

CONCLUSIONS

Seven *N*-(hydroxycinnamoyl)amides were synthesized and identified, six of which were new. The compounds were evaluated for their anti-influenza, DPPH scavenging and mushroom tyrosinase inhibitory activities. The results indicated that amides **1** and **4**, containing the catechol moiety, were the most active as DPPH radical-scavengers. In addition, CafA-Os (**1**) was found to be the most active as a tyrosinase inhibitor (≈50 %) *in vitro*.

SUPPLEMENTARY MATERIAL

Analytic and spectral data for the synthesised compounds are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

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ABBREVIATIONS

DPPH – 1,1-diphenyl-2-picrylhydrazyl radical;
ROS – reactive oxygen species;
DMSO – dimethyl sulfoxide;
EDC – 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide;
HOBt – 1-hydroxybenzotriazole;
p-CoumA – *p*-coumaric acid;
FA – ferulic acid;
SA – sinapic acid;
CafA – caffeic acid;
Os – oseltamivir;
Rim – rimantadine;
Am – amantadine;
% RSA – percentage of radical scavenging activity.

И З В О Д

СИНТЕЗА И БИОЛОШКА АКТИВНОСТ ХИДРОКСИЦИНАМОИЛ АНТИВИРУСНИХ ЛЕКОВА

MAYA G. CHOCHKOVA[1], ASSYA P. GEORGIEVA[1], GALYA I. IVANOVA[2], NADYA NIKOLOVA[3], LUCHIA MUKOVA[3], LUBOMIRA NIKOLAEVA-GLOMB[3] И TSENKA S. MILKOVA[1]

[1]South-West University "Neofit Rilski", Blagoevgrad, Bulgaria, [2]Departamento de Química, Faculdade de Ciências, Universidade do Porto, Porto, Portugal and [3]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

Синтетисано је седам *N*-хидроксицинамоил-амида EDC/HOBt катализованим куповањем одговарајућих супституисаних циметних киселина (*p*-кумаринска, ферулинска,

синапинска и кафена киселина) са једињењима која показују антивиралну активност (амантадин, римантидин и оселтамивир). Испитивана је *in vitro* способност пресретања 1,1-дифенил-2-пикрилхидразил (DPPH) радикала и инхибиција активности тирозиназе гљива (употребом L-тирозина као супстрата). Од синтетисаних једињења, деривати *N*-[(*E*)-3-(3,4-дихидроксифенил)-2-пропеноил]оселтамивир (**1**) и *N*-[(*E*)-3-(3,4-дихидроксифенил)-2-пропеноил]римантидин (**4**), који садрже катехолски фрагмент, показују најбољу способност пресретања DPPH радикала. Такође, амид **1** показује инхибиторни ефекат према тирозинази (око 50 %). Испитивана је *in vitro* инхибиторна активност синтетисаних једињења према вирусу грипа А (H3N2).

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