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ORIGINAL ARTICLE

Synthesis, antioxidative and antiviral activity of hydroxycinnamic acid amides of thiazole containing amino acid

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Abstract The synthesis and the biological (antioxidant and antiviral) activities of novel hydroxycinnamic acid amides of a thiazole containing TFA.valine-4-carboxylic acid ethyl ester are reported. The amides have been synthesized from *p*-coumaric, ferulic and sinapic acids with the corresponding TFA.valine-thiazole-4-carboxylic acid ethyl ester using the coupling reagent *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 4-(dimethylamino) pyridine (DMAP) as a catalyst. The antioxidant properties of the newly synthesized amides have been studied for then antioxidative activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH)* test. The newly synthesized compounds have been tested against the replication in vitro of influenza virus A (H3N2) and human herpes virus 1 and 2 (HSV-1 and HSV-2).

Keywords Hydroxycinnamides · Thiazole containing valine · Peptidomimetics · Antioxidant effect · DPPH test · Antiviral activity

Abbreviations

BOC	*tert*-Butoxycarbonyl
DMAP	4-(dimethylamino)Pyridine
DMF	Dimethylformamide
DPPH	1,1-Diphenyl-2-picryl-hydrazyl radical
OEt	Ethyl ester
TFA	Trifluoroacetic acid
Thz	Thiazole
Val	Valine

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Introduction

In the last two decades, unprecedented biologically active natural products containing directly linked azoles, have been isolated from natural sources (Fusetani et al. 1993; Michael et al. 1993a, b). Many of these compounds are candidates for drug development. In particular, thiazole, oxazole and imidazole amino acids that may play a key role in biological activities of unusual peptides are also important intermediates for natural product synthesis and peptidomimetics (Videnov et al. 1996a, c; Bagley et al. 2005).

Cinnamic acids and their natural and synthetic esters, amides and glycosides exhibit a wide range of biological activities, including the antioxidative effect on low-density lipoprotein (LDL) (Moon and Terao 1998), the peroxyl radical scavenging effect (Castelluccio et al. 1996), the hepatoprotective (Perez-Alvarez et al. 2001), anti-inflammatory (Sudina et al. 1993), and antimutagenic effect (Namiki 1990), as well as the inhibitory effect on HIV-1 integrase (Burke 1995).

The cholesterol lowering effect of several hydroxylated cinnamic acid derivatives of amino acids has been evaluated in mice fed high cholesterol diets. The presence of the double bond in hydroxylated cinnamide derivatives decreases the cholesterol-lowering activities and the number of free phenolic hydroxy groups greatly affects the

biological activity. Amides of caffeic acids with hydrophobic side chain containing amino acids (alanine, valine, phenylalanine, and isoleucine) exhibit a potent cholesterol-lowering effect (Sangku Lee et al. 2007).

Two amides of the same hydroxycinnamic acid with an aliphatic and an aromatic amino acid residue have been used for the functional investigation on the potential activity in cell physiology (Hensel et al. 2007). It has been found that *N*-(*E*)-caffeic acid L-aspartic acid amide and *N*-(*E*)-caffeic acid L-tryptophan amide at 10 μg/mL have significantly enhanced the mitochondrial activity and the proliferation rate of human liver cells (HepG2). By monitoring the influence on selected phase I and II metabolizing enzymes, it was established that both compounds do not influence CYP3A4 gene expression, but they stimulated CYP1A2 gene expression and inhibit GST expression. Also the proliferation of human keratinocytes (NHK), that increased up to 150% by both amides and this stimulation, is also detectable on the gene expression level by an up-regulation of the transcription factor STAT6. *N*-(*E*)-Caffeic acid L-tryptophan amide have been found to possess significant antiadhesive properties as regards the adhesion of *Helicobacter pylori* to human stomach tissue.

On the other hand, the antioxidant activity in the bulk phase lipid autoxidation of cinnamic and hydroxycinnamic acid amides of 15 synthetic C-protected amino acids was also studied (Kancheva et al. 2006; Spasova et al. 2006). The highest antioxidant activity is found for *N*-feruloyl- and *N*-sinapoyl-amides containing the same phenylalanine rest.

Recently new cinnamoylamide and hydroxycinnamoylamide of aliphatic monoamines (Spasova et al. 2007) and alkaloid glaucine (Spasova et al. 2008) have been synthesized and their radical scavenging activity against DPPH* tests has been evaluated.

Considering all these facts, we have been interested in the search for new cinnamic acids analogues. Here, the synthesis of *p*-coumaroyl-, feruloyl- and sinapoyl-amides of valine containing thiazole ring is reported as well as the results for evaluation of the antioxidative and antiviral activities of the newly synthesized compounds.

Materials and methods

Chemicals

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 used without preliminary purification.

The amino acid derivatives were purchased from Sigma, DMAP and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride were purchased from Merck. TLC analysis were performed on aluminum sheets silica gel 60 F_{254} plates (Merck) and detection was performed using an UV lamp at 254 nm; solvent systems: Hexan/EtOAc:4:5; CHCI3/MeOH:9:1. The NMR spectra was obtained on a Bruker Avance DRX-600 spectrometer, operating at 600.13 MHz for ^{1}H. The ESI mass spectra was obtained on Esquire 3000 plus instrument. Optical rotation was measured with a Perkin–Elmer 141 polarimeter.

The UV spectra EtOH solutions were measured with a Specord UV–VIS spectrophotometer. "Agilent 8453" spectrophotometer was used for the measurement of the reduction of DPPH* (1,1-diphenyl-2-picrylhydrazyl radical) absorbance at 516 nm.

Synthetic procedures of amides

*p-Coumaroyl-2-valyl-thiazole-4-carboxylic acid ethyl ester (**2a**)*

p-Coumaric acid **(1a)** (0.250 g, 1.5 mmol) was dissolved in DMF and the solution was cooled at 0°C and *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (0.280 g, 1.5 mmol) was added. After 8 min TFA.valine-thiazole-4-carboxylic acid ethyl ester (0.480 g, 1.5 mmol), triethylamine (0.21 ml, 1.5 mmol), and DMAP (0.183 g, 1.5 mmol) were added. Reaction mixture was stirred for 18 h at room temperature. The mixture was poured into 5% $NaHCO_3$, extracted with CH_2Cl_2 (5 times), washed with brine, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by TLC on Kieselgel 60 F_{254} (Merck) using solvent system Hexan : EtOAc:4:5. Yield: 0.445 g (85%); $[\alpha]^{20}_{D}$ -0.19 (c 1.00, CH_3OH); UV (EtOH) $\lambda_{max} = 206$, 225, 315 nm; ^{1}H-NMR (600 MHz; $CDCl_3$) $\delta = 0.92$ (d, 3H, $J = 6.7$ Hz, CH_3), 0.92 (d, 3H, $J = 6.7$ Hz CH_3), 1.36 (3H, t, $J = 7.7$ Hz, OCH2CH3), 2.92 (m, 1H, CH), 4.23 (2H, q, $J = 7.7$ Hz, OCH2CH3), 4.73 (dd, 1H, $J = 7.7$, 7.7 Hz, CH), 5.25 (d, 1H, $J = 7.7$ Hz, NH), 5.97 (d, 1H, $J = 15.6$ Hz, C=), 6.75 (d, 2H, J=8.2 Hz, Ar–H), 7.31 (d, 2H, J=8.0 Hz, Ar–H), 7.41 (d, J=15.6 Hz, C=), 8.06 (CH_{Thz}). ^{13}C-NMR (600 MHz; CDCl3): $\delta = 173.3$, 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, 29.62, 18.29, 17.24. ESI-MS: 350 ($[M + H]^{+}$).

*Feruloyl-2-valyl-thiazole-4 carboxylic acid ethyl ester (**2b**)*

The feruloyl-2-valyl-thiazole-4 carboxylic acid ethyl ester (**2b**) was prepared from **1b** (0.296 g, 1.5 mmol) as described for compound **2a**. Yield: 0.479 g (82%); $[\alpha]^{20}_{D}$ -0.21 (c 1.00, CH_3OH); UV (EtOH) $\lambda_{max} = 203$, 236, 237, 326 nm; ^{1}H-NMR (600 MHz; $CDCl_3$) $\delta = 0.89$-0.99

(dd, $J = 7$ Hz, $J = 7$ Hz, 2 γCH_3) 2.65 (m, 1H, C**H**), 3.79 (s, 3H, O**CH$_3$**), 4.37–4.45 (dd, J(H, H) = 7 Hz, J'(H, H) = 7 Hz, 2H, CH_2 in Et), 4.73 (dd, 1H, $J = 8.8$, 4.9 C**H**), 5.28 (br d, $J = 9$ Hz, 1H, NH), 5.80 (br.s, 1H, O**H**), 6.3 (d, 1H, $J = 15.5$ Hz, C**H**=), 6.92 (d, 1H, $J = 8.1$ Hz Ar–**H**), 7.02 (d, 1H, $J = 1.8$ Hz Ar–**H**), 7.07 (dd, 1H, $J = 8.1$, 1.8 Hz), 7.60 (d, 1H, $J = 15.5$ Hz, C**H**=), 8.02 (s, 1H, CH_{Thz}). ^{13}C-NMR (600 MHz;$CDCl_3$): $\delta = 174.0$, 169.8, 161.4, 155.80, 148.3, 147.7, 147.0, 129.1, 126.6, 123.8, 116.1, 113.7, 111.7, 59.78, 56.30, 29.62, 19.5, 17.3. ESI-MS: 380 ($[M + H]^+$).

*Sinapoyl-2-vayl-thiazole-4 carboxylic acid ethyl ester (**2c**)*

The sinapoyl-2-valyl-thiazole-4 carboxylic acid ethyl ester (**2c**) was prepared from **(1c)** (0.345 g, 1.5 mmol) as described for compound **2a.** Yield: 0.504 g (80%); $[\alpha]^{20}_D$ -0.27 (c 1.00, CH_3OH); UV (EtOH) $\lambda_{max} = 204$, 232, 329 nm; ^{1}H-NMR (600 MHz; $CDCl_3$) $\delta = 0.95$ (d, 3H, $J = 6.9$ Hz, CH_3), 0.95 (d, 3H, $J = 6.9$ Hz, CH_3), 2.83 (m, 1H, CH), 3.91 (s, 6H, OCH3), 4.37–4.45 (dd, ^3J(H, H) = 7 Hz, $^3J'$(H, H) = 7 Hz, 2H, CH_2 in Et), 4.91 (dt, 1H, $J = 7.7$, 5.7 Hz, CH), 5.71 (s, 1H, OH), 6.11 (d, 1H, $J = 7.7$ Hz, NH), 6.27 (d, 1H, $J = 15.5$, CH=), 6.75 (s, 2H, Ar–H), 7.53 (d, 1H, $J = 15.5$ Hz, CH=), 8.02 (s, 1H, CH_{Thz}). ^{13}C-NMR (600 MHz; $CDCl_3$): $\delta = 173.3$, 166.6 (C-9), 161.41, 148.0, 147.4, 138.4, 126.8, 124.3, 114.9, 106.2, 60.01, 56.1, 29.90, 18.88, 18.29. ESI-MS: 410 ($[M + H]^+$).

Biological activity

Estimation of radical scavenging activity (RSA) by the DPPH* test

DPPH* (1,1-Diphenyl-2-picrylhydrazyl) is a compound that has a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Yamaguchi et al. 1998). It is well accepted that the DPPH* radical scavenging by antioxidants is attributable to their hydrogen donating ability (Chen and Ho 1995).

The radical scavenging activity of the newly synthesized compounds was based on the method of the Pekkarien et al. (1999). 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 after 10 and 20 min. The results were expressed as % RSA = $[Abs_{516nm(t=0)} - Abs_{516nm(t=t')} \times 100/Abs_{516nm(t=0)}]$, as proposed by Pekkarien et al. (1999).

Antiviral activity

Compounds

Cinnamic acid amides were initially dissolved in dimethylsulfoxide (DMSO) and then in cell culture maintenance medium without fetal bovine serum. Rimantadine hydrochloride, kindly supplied by Joint-Stock Co. Olainfarm (Riga, Latvia), M.W. 216, white powder, soluble in water, was used as a reference anti-flu antiviral.

Cell culture

Monolayer cell cultures of Madin–Darby Canine Kidney (MDCK) (NBL-2, ATCC No. CCL-34, USA) were used for the experiments with influenzavirus A (H3N2). Madin–Darby Bovine Kidney (MDBK) (ATCC No CCL-22, USA) cell line was used for the experiments with human herpes viruses. Both cell lines were grown in a medium containing 10% fetal calf serum in DMEM Gibco BRL, USA, supplemented with 10 mmol/L HEPES buffer (Gibco BRL, USA) and antibiotics (penicillin, 100 UI/mL, streptomycin, 100 μg/mL).

Viruses

Influenza A virus [Aichi/2/68 (H3N2)] [IAV] from the collection of the Stephan Angeloff Institute of Microbiology, BAS (Sofia, Bulgaria). The stock virus constituted the allantoic liquid of virus-inoculated 10-day-chick embryos, cultivated at 37°C. The infectious virus titer was $10^{7.0}$ $CCID_{50}$/mL.

Herpes simplex virus type 1, strain Da, (HSV-1) with infectious virus titer $10^{5.5}$ $CCID_{50}$/mL.and herpes simplex virus type 2, strain Bja, (HSV-2) with infectious titer $10^{6.0}$ $CCID_{50}$/mL.

Cytotoxicity test

The effect of the test compounds on uninfected confluent cell monolayer and cellular morphology was traced for overt signs of cytotoxicity during 96–120 h and the maximum tolerated (nontoxic) concentration (MTC) value was determined. The study has been carried out in parallel with the CPE inhibition test in 96-well plastic plates.

Cytopathic effect (CPE) inhibition test in viral multicycle growth setup

Monolayer cell cultutes grown in 96-well plastic microplates (Costar, USA) were used. Compounds (at subsequent 0.5 $\log_{10}$ dilution concentration range) were applied in the maintenance medium (0.2 mL/well), composed of DMEM (Gibco BRL, USA), supplemented with

10 mmol/L HEPES buffer, 3 μg/mL trypsin Gibco BRL, USA, and antibiotics immediately after virus inoculation at different viral doses. Virus inoculation (100 and 10 $CCID_{50}$ in 0.1 mL/well for influenza virus and 320 $CCID_{50}$ in 0.1 mL/well for herpes viruses)was followed by a 60-min adsorption at room temperature. The plates (four wells per test sample) were incubated at 37°C and on the 72nd–96th hour post-infection CPE was scored by inverted light microscope (Olympus, Japan) at 125× magnification on a 0–4 basis with 4 representing the total cell destruction. The obtained data were used to chart out the dose–response curves for each compound at the given viral dose. The minimal concentration causing a 50% reduction of CPE as compared to the untreated controls (MIC_{50}) was determined from the charts. The selectivity was determined as the ratio between the compound cytotoxicity (maximal tolerated concentration, MTC) and MIC_{50}.

Cytopathic effect (CPE) inhibition test

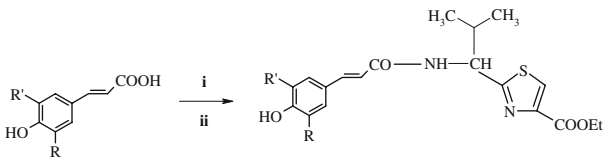
Cells were grown in 96-well microplates to a confluent monolayer after 24 h post-seeding and following the removal of the growth medium, virus was inoculated at 320 $CCID_{50}$/0.1 mL. After 1 h adsorption in room temperature, the investigated compounds, in respective dilutions, were added to the monolayer. Every dilution was applied in threefold repetitions. The viral cytopathic effect was determined by the four-cross system when a full destruction of the cell monolayer in the viral control was observed. The average value from three wells for every dilution was calculated and presented as a percentage of the viral control. On the basis of the so obtained results, dose–response curves were built and ED_{50} values were determined.

Results and discussion

Hydroxycinnamic acids modified with amino acids are found in nature, but hydroxycinnamic acids containing peptidomimetics are not known at all. In order to obtain hydroxycinnamic acid analogues with more favorable characteristics, three new compounds have been synthesized: *p*-coumaroyl-2-valyl-thiazole-4 carboxylic acid ethyl ester (**2a**), feruloyl-2-valyl-thiazole-4 carboxylic acid ethyl ester (**2b**) and sinapoyl-2-valyl-thiazole-4 carboxylic acid ethyl ester (**2c**) by the used in the peptide chemistry way shown in Fig. 1.

Synthesis of TFA.valine-thiazole-4-carboxylic acid ethyl ester

Synthesis of thiazole containing valine was prepared according to (Videnov et al. 1996b; Stanchev et al. 1998; Bagley et al. 2007).



1 a-c **2 a-c**

1 a) p-coumaric acid;
1 b) ferulic acid; **R**=OCH_3;
1 c) sinapic acid; **R, R'**=OCH_3.

(**i**) TFA.valine-thiazole-4-carboxylic acid ethyl ester; (**ii**) EDC/DMF/Et_3N/0°C;DMAP.

Fig. 1 Synthesis of hydroxycinnamic acid amides

Synthesis of hydroxycinnamic acid amides

A solution of hydroxycinnamic acid in dimethylformamide (DMF) was treated with the thiazole containing valine using the coupling agent *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 4-(dimethylamino)-pyridine (DMAP) as a catalyst, to produce the amides. The structure of the newly synthesized amides **2a**–**b** was confirmed by UV, ^1H, ^{13}C-NMR, and ESI-MS spectroscopy.

Biological activity

The results obtained for the antioxidative potential of the synthesized amides against DPPH* test are shown in Table 1.

The tested hydroxycinnamoyl amides demonstrated higher radical scavenging activity than TFA.valine-thiazole-4-carboxylic acid ethyl ester as seen in Table 1. The amide of ferulic acid exhibited a borderline activity. The amides of sinapic and *p*-coumaric acid showed lower antioxidative effect than the free hydroxicinnamic acids against DPPH* test.

Antiviral activity

Effect of the compounds (**2a**–**c**) on the replication of influenza virus A/Aichi/2/68 (H3N2)

The newly synthesized compounds, *p*-coumaroyl-2-valyl-thiazole-4 carboxylic acid ethyl ester (**2a**), feruloyl-2-valyl-thiazole-4 carboxylic acid ethyl ester (**2b**) and sinapoyl-2-valyl-thiazole-4 carboxylic acid ethyl ester (**2c**) were evaluated for their antiviral activity against *influenza virus A/Aichi/2/68 (H3N2)*. Results on the antiviral effect of compounds (**2a**–**c**) are shown in Table 2.

The obtained results show that compounds **2a**–**c** are ineffective against influenza virus A/Aichi/2/68 (H3N2) replication in MDBK cells when the applied virus inoculation dose is 100 $CCID_{50}$ per well (the customary virus dose

Table 1 Antioxidative activity of cinnamic acid amides of thiazole containing amino acid by DPPH*

AH	(%) RSA					
	0.9 mM		1.8 mM		3.6 mM	
	10 min	20 min	10 min	20 min	10 min	20 min
TFA.valine-thiazole-4-carboxylic acid ethyl ester	1.3	1.6	1.4	1.9	2.1	2.4
p-Coumaric acid	2.1	2.9	3.7	4.7	4.5	6.1
Compound **2a**	1.2	1.6	3.1	3.6	3.4	3.9
Ferulic acid	12.0	13.8	21.0	25.1	36.7	44.3
Compound **2b**	10.7	12.9	16.6	20.1	28.9	33.2
Sinapic acid	16.1	17.2	26.5	31.9	69.0	69.6
Compound **2c**	6.5	7.4	9.9	11.1	17.9	19.4

Table 2 Testing of *p*-coumaroyl, feruloyl and sinapoyl-2-valyl-thiazole-4 carboxylic acid ethyl esters (**2a–c**) against influenza virus A/Aichi/2/68 (H3N2)

Compound tested	Compound concentration (μM)[a]	CPE score on a 0–4 basis			
		100 $CCID_{50}$/well	CPE inhibition %	10 $CCID_{50}$/well	CPE inhibition %
2a	10	3.0	0	0.2	94.7
	3.2	3.0	0	0.7	76.7
	1.0	3.0	0	0.3	89.0
	0.32	3.0	0	0.3	89.0
	0	3.0	–	3.0	–
2b	64	4.0	0	0.5	83.3
	32	4.0	0	0.5	83.3
	10	4.0	0	1.1	63.3
	3.2	4.0	0	1.1	63.3
	1.0	4.0	0	1.5	50.0
	0.32	4.0	0	1.7	44.7
	0	4.0	–	3.0	–
2c	64	3.0	25.0	1.3	66.8
	32	3.0	25.0	2.0	50.0
	10	4.0	0	3.0	25.0
	3.2	4.0	0	3.0	25.0
	1.0	4.0	0	3.0	25.0
	0.32	4.0	0	3.0	25.0
	0	4.0	–	4.0	–
Rimantadine hydrochloride					
	3.2	0.5	93.8	0	100.0
	1.0	0.5	83.3	0	100.0
	1.0	1.0	75.0	0	100.0

[a] MTC values of compounds tested were as follows: 10 μM for **2a**, 200 μM for **2b** and **2c**

used in the CPE inhibition test). Evidently, the three amides tested possess a borderline activity against this virus as they inhibit its replication at 10 $CCID_{50}$ per well.

Effect of the compounds (**2a–c**) on the replication of HSV-1 and HSV-2

The hydroxycinnamic acid amides were explored against HSV-1, strain Da, and HSV-2, strain Bja. The compounds were applied in concentrations 100, 80, 40, 20, 10, 5, 1 and 0.5 μg/mL. Compounds **2a** and **2b** showed similar cytotoxicity, after 96 h of cultivation MTC were determined to be 20 μg/ml; the other compound, **2c**, showed higher cytotoxicity, 10 μg/mL.

The three investigated amides were applied in twofold concentrations. The compounds had no effect on the replication of HSV-1 and HSV-2 during the multicycle virus growth. Intermolecular reactions of the acids with some cell metabolites not involved in the viral replication were suggested.

In conclusion, three new compounds (*p*-coumaroyl-2-valyl-thiazole-4-carboxylic acid ethyl ester **2a**, feruloyl-2-valyl-thiazole-4 carboxylic acid ethyl ester **2b**, sinapoyl-2-valyl-thiazole-4 carboxylic acid ethyl ester **2c**) have been synthesized by the methods used in the peptide chemistry. All of the tested amides demonstrated higher radical scavenging activity than the valine containing thiazole. The amide of ferulic acid showed border line activity than the free cinnamic acid against DPPH* test. All of the tested amides do not demonstrate antiviral effect on the replication of influenza virus A, HSV-1 and HSV-2 in comparison to these modifications with natural and unnatural C-protected amino acids. Further, systematic researches are needed for investigating the role of other peptidomimetics containing compounds such as oxazole and thiazolyl-thiazole rings.

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