Cinnamic acid Derivatives and 4-Aminoantipyrine Amides – Synthesis and Evaluation of Biological Properties

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Abstract

Four new amides of cinnamic acid derivatives (cinnamic acid, p-coumaric acid, ferulic acid and caffeic acid) and 4-aminoantipyrine were synthesized and their structure was confirmed (^{1}H-NMR, ^{13}C-NMR, FTIR and elemental analysis). Some of their biological properties were evaluated: antimicrobial and antioxidant (DPPH radical scavenging activity, Fe^{3+} reducing power). The tested compounds were more effective against Staphylococcus aureus than the corresponding free acids, but presented no effect on Gram negative bacteria and Candida albicans. The amides of caffeic and ferulic acid were very efficient antioxidants (DPPH radical scavenging assay: $EC_{50} < 100 \mu M$). The Fe^{3+} reducing power for the synthesized substances was similar or superior to that of the positive control (ascorbic acid).

Keywords: Hydroxycinnamic acids, antioxidant, antimicrobial activity, DPPH, FRAP

Introduction

Many studies evaluated the biological properties of cinnamic acid derivatives (especially hydroxycinnamic acid derivatives) and concluded that some of these derivatives are potent antimicrobial, antiviral, antioxidant and anti-inflammatory agents[1-3].

During the last decade, researchers oriented their work towards the discovery of new compounds, with an improved pharmacological profile[4-6]. Although many hydroxycinnamic esters exhibit a broad spectrum of biological activities, their reduced chemical stability limits their use. That is one of the reasons why several amides of hydroxycinnamic acids (p-coumaric, ferulic, caffeic acid) were synthesized and evaluated [1,7]. Some of these substances were found to be better antimicrobial and antioxidant agents than the corresponding free acids[1].

4-aminoantipyrine is a biologically active compound and its analogues and other pyrazole derivatives have shown anti-inflammatory, analgesic, antiviral, antipyretic and antimicrobial properties[8,9]. Until now, it was not used as a coupling component for hydroxycinnamic acids.

The aim of this study was to synthesize amides of 4-aminoantipyrine and cinnamic or hydroxycinnamic acids (p-coumaric, ferulic and caffeic acid) and to evaluate their antimicrobial and antioxidant potentials. It can be expected that the coupling of cinnamoyl / hydroxycinnamoyl and 4-aminoantipyrine moieties could result in the enhancement of the antimicrobial and antioxidant activities of the newly synthesized compounds.

Material and Methods

All the reagents and solvents employed were of the best grade available and were used without further purification. Melting points were determined using an electro-thermal apparatus and were uncorrected. Elemental analyses were performed on an Elemental Exeter Analytical CE 440 Analyzer. The FTIR spectra were recorded on a FT-IR Shimadzu Prestige 8400s spectrometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 DRX spectrometer operating at 400 MHz for ^{1}H and 100 MHzfor ^{13}C nuclei.

Chemistry: General procedure for Synthesis of Cinnamic acid amide: The first step of this synthesis involved the transformation of cinnamic acid to cinnamoyl chloride, by heating 20 mmol cinnamic acid and 20 ml thionyl chloride under reflux and stirring for five hours. The excess thionyl chloride was removed. The resulting cinnamoyl chloride was diluted with 20 ml acetone and used in the next step without further purification. The cinnamoyl chloride (2.2 mmol) dissolved in acetone was added dropwise over a solution containing 2.8 mmol antipyrine and 2.6 mmol pyridine in 10 ml acetone, keeping the temperature at 0ºC. The mixture was stirred at room temperature for 20 hours, filtered and evaporated. The residue was purified by solubilization in ethyl acetate and co-precipitation with hexane.

General procedure for Synthesis of Hydroxycinnamic Acids Amides: The hydroxycinnamic acid (10 mmol) was dissolved in 20 ml DMF and 1.4 ml (10 mmol) triethylamine. The solution was cooled on an ice bath and 10 mmol of 4-aminoantipyrine were added, followed by a solution of 10 mmol BOP in 20 ml methylene chloride. The mixture was stirred at 0ºC for 30 minutes and then at room temperature for 2 hours. Methylene

chloride was removed under reduced pressure and the solution was diluted with 150 ml water. The product was extracted with ethyl acetate. The extract was washed successively with 1N HCl, water, 1M NaHCO$_3$ and water, dried over anhydrous sodium sulphate, filtered and evaporated. The residue was purified by recrystalization from ethyl acetate or by co-precipitation using ethyl acetate and hexane.

N-(2,3-dimethyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-4-yl) cinnamamide (**1b**): Brown powder. Yield 85%. Mp 175-178 ºC. Anal. Calcd. for C$_{20}$H$_{19}$N$_3$O$_2$: C, 72.05; H, 5.74; N, 12.60; O, 9.60 %. Found: C, 72.01; H, 5.79; N, 12.53; O, 9.64 %. IR (KBr, cm^{-1}): 3438 (N-H), 3024 (Ar-H), 1654 (C=O, amide). ^{1}H NMR (400 MHz, CDCl$_3$, δ / ppm): 8.72 (1H, *s*, NH), 7.61 (1H, *d*, H$_9$, $J_{9,8}$= 15.6 Hz), 7.48-7.30 (10H, overlapped peaks, H$_{2'}$-H$_{6'}$, H$_{11}$-H$_{15}$), 6.71 (4H, overlapped peaks, H$_{2'}$, H$_{4'}$, H$_{6'}$, H$_{13}$), 6.73-6.69 (1H, *d*, H$_8$, $J_{8,9}$= 15.6 Hz), 3.11 (3H, *s*, CH$_3$ attached to N$_2$), 2.32 (3H, *s*, CH$_3$ attached to C3); ^{13}C NMR (100 MHz, CDCl$_3$, δ / ppm): 165.11 (C$_7$), 161.63 (C$_5$), 149.34 (C$_3$), 141.66 (C$_9$), 134.94 (C$_{10}$), 134.37 (C$_{1'}$), 129.57 (C$_{13}$), 129.34 (C$_{3'}$+C$_{5'}$), 128.69 (C$_{12}$+C$_{14}$), 128.02 (C$_{11}$+C$_{15}$), 127.25 (C$_{4'}$), 124.63 (C$_{2'}$+C$_{6'}$), 120.50 (C$_8$), 108.76 (C$_4$), 35.92 (CH$_3$ attached to N$_2$), 12.59 (CH$_3$ attached to C$_3$).

(E)-N-(2,3-dimethyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-4-yl)-3-(4-hydroxyphenyl) acrylamide (**2b**): White powder. Yield 65%. Mp 171-174 ºC. Anal. Calcd. for C$_{20}$H$_{19}$N$_3$O$_3$: C, 68.75; H, 5.48; N, 12.03; O, 13.74 %. Found: C, 68.71; H, 5.43; N, 12.09; O, 13.68 %. IR (KBr, cm^{-1}): 3405 (broad peak, N-H and OH), 3060 (Ar-H), 1655 (C=O, amide). ^{1}H NMR (400 MHz, DMSO-*d$_6$*, δ / ppm): 9.90 (1H, *s*, OH), 9.20 (1H, *s*, NH), 7.37 (2H, *d*, H$_{2'}$+H$_{6'}$, $J_{2',3'}$ = $J_{6',5'}$=8Hz), 7.32 (1H, *t*, H$_{4'}$), 6.81 (2H, *d*, H$_{12}$+H$_{14}$, $J_{12,11}$= $J_{14,15}$= 8.4 Hz), 6.65 (1H, *d*, H$_8$, $J_{8,9}$=16 Hz), 3.06 (3H, *s*, CH$_3$ attached to N$_2$), 2.16 (3H, *s*, CH$_3$ attached to C$_3$); ^{13}C NMR (100 MHz, DMSO-*d$_6$*, δ / ppm): 164.49 (C$_7$), 161.70 (C$_5$), 159.07 (C$_{13}$), 151.87 (C$_3$), 140.04 (C$_9$), 135.01 (C$_{1'}$), 129.39 (C$_{11}$+C$_{15}$), 129.07 (C$_{3'}$+C$_{5'}$), 126.18 (C$_4$), 125.72 (C$_{10'}$), 123.44 (C$_{2'}$+C$_{6'}$), 117.78 (C$_8$), 115.78 (C$_{12}$+C$_{14}$), 107.79 (C$_4$), 36.05 (CH$_3$ attached to N$_2$), 11.52 (CH$_3$ attached to C$_3$).

(E)-3-(3,4-dihydroxyphenyl)-N-(2,3-dimethyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-4-yl)acrylamide (**3b**): White powder. Yield 65%. Mp 209-211 ºC. Anal. Calcd. for C$_{21}$H$_{21}$N$_3$O$_4$: C, 66.48; H, 5.58; N, 11.08; O, 16.87 %. Found: C, 66.41; H, 5.53; N, 11.09; O, 16.76 %. IR (KBr, cm^{-1}): 3429 (broad peak, N-H and OH), 3060 (Ar-H), 1654 (C=O, amide). ^{1}H NMR (400 MHz, DMSO-*d$_6$*, δ / ppm): 9.50 (1H, *s*, OH), 9.17 (1H, *s*, NH), 7.51 (2H, *t*, H$_{3'}$+H$_{5'}$), 7.44 (1H, *d*, H$_9$, $J_{9,8}$= 15.6 Hz), 7.37 (2H, *d*, H$_{2'}$+ H$_{6'}$, $J_{2',3'}$= $J_{6',5'}$=7.6 Hz), 7.32 (1H, *t*, H$_{4'}$), 7.17 (1H, *s*, H$_{11}$), 7.05 (1H, *d*, H$_{15}$, $J_{14,15}$= 8 Hz), 6.82 (1H, *d*, H$_{14}$, $J_{15,14}$= 8 Hz), 6.69 (1H, *d*, H$_8$, $J_{8,9}$= 15.6 Hz), 3.82 (3H, *s*, OCH$_3$), 3.06 (3H, *s*, CH$_3$ attached to N$_2$), 2.16 (3H, *s*, CH$_3$ attached to C$_3$); ^{13}C NMR (100 MHz, DMSO-*d$_6$*, δ / ppm): 164.50 (C$_7$), 161.70 (C$_5$), 151.84 (C$_3$) 148.52 (C$_{13}$), 147.81 (C$_{12}$), 140.33 (C$_9$), 135.02 (C$_{1'}$), 129.07 (C$_{3'}$+C$_{5'}$), 126.23 (C$_4$+C$_{10}$), 123.44 (C$_{2'}$+C$_{6'}$), 121.66 (C$_{15}$), 118.12 (C$_8$), 115.69 (C$_{14}$), 110.98 (C$_{11}$),

107.84 (C$_4$), 55.50 (OCH$_3$), 36.06 (CH$_3$ attached to N$_2$), 11.51 (CH$_3$ attached to C$_3$).

(E)-N-(2,3-dimethyl-5-oxo-1-phenyl-2,5-dihydro-1H-pyrazol-4-yl -3-(4-hydroxy-3-methoxyphenyl) acrylamide (**4b**): White powder. Yield 55%. Mp 168-171 ºC. Anal. Calcd. for C$_{20}$H$_{19}$N$_3$O$_4$: C, 65.74; H, 5.24; N, 11.50; O, 17.52 %. Found: C, 65.68; H, 5.23; N, 11.48; O, 17.55 %. IR (KBr, cm^{-1}): 3431 (broad peak, N-H and OH), 3056 (Ar-H), 1654 (C=O, amide). ^{1}H NMR (400 MHz, DMSO-*d$_6$*, δ / ppm): 9.43 (1H, *s*, OH attached to C$_{13}$), 9.21 (2H, overlapped peaks, *s*, OH attached to C$_{12}$, NH), 7.51 (2H, *t*, H$_{3'}$+H$_{5'}$), 7.38-7.32 (4H, overlapped peaks, H$_9$, H$_{2'}$, H$_{4'}$, H$_{6'}$), 7.01 (1H, *s*, H$_{11}$), 6.89 (1H, *d*, H$_{15}$, $J_{14,15}$= 8.4 Hz), 6.77 (1H, *d*, H$_{14}$, $J_{15,14}$= 8.4 Hz), 6.58 (1H, *d*, H$_8$, $J_{8,9}$= 15.6 Hz), 3.06 (3H, *s*, CH$_3$ attached to N$_2$), 2.16 (3H, *s*, CH$_3$ attached to C$_3$); ^{13}C NMR (100 MHz, DMSO-*d$_6$*, δ / ppm): 164.45 (C$_7$), 161.64 (C$_5$), 151.80 (C$_3$), 147.52 (C$_{13}$), 145.48 (C$_{12}$), 140.40 (C$_9$) 134.92 (C$_{1'}$), 128.99 (C$_{3'}$+C$_{5'}$), 126.11 (C$_{4'}$+C$_{10}$), 123.38 (C$_{2'}$+C$_{6'}$), 120.69 (C$_{15}$), 117.51 (C$_8$), 115.67 (C$_{14}$), 113.72 (C$_{11}$), 107.10 (C$_4$), 35.96 (CH$_3$ attached to N$_2$), 11.44 (CH$_3$ attached to C$_3$).

Microbiology: The disk diffusion method[10,11] was performed using Mueller Hinton (Oxoid) medium for bacteria and Sabouraud agar for fungal. The test microorganisms were *Staphylococcus aureus* ATCC 25923, *Sarcina lutea* ATCC 9341, *Bacillus cereus* ATCC 14579, *Bacillus subtilis*, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. The bacterial and fungal strains were incubated over night at 30^{0}C and from each microbial culture were prepared suspensions with the same density as the McFarland 0.5 turbidity standard. The suspensions of microorganisms were incorporated in Muller-Hinton medium, melted and cooled afterwards at 50^{0}C, in a 1/10 ratio. After homogenization, 25 mL of this mixture were placed in Petri plates with a diameter of 9 cm. On the surface of each plate, after solidification, absorbent disks impregnated with 10 µL of the DMSO solutions (10 mg/mL) of the tested compounds were placed (the quantity of active substance / disk = 100 µg). Positive control disks of Ampicillin 25 µg, Chloramphenicol 30 µg (for bacteria) and Nystatin 100 µg (for *Candida albicans*) were also used. After incubation (24 hrs. at 37ºC for bacteria and 48 hrs. at 24ºC for *Candida albicans*), the plates were observed.

Antioxidant capacity: DPPH radical scavenging assay: The experimental procedure was adapted from literature[12,13], only slight modifications being made. Briefly, 1.5 ml solution of DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical 0.1 mM in methanol were added over 1.5 ml of methanolic solution of the tested compound. The absorbance of DPPH radical solution at 517 nm was measured before (Astart) and 30 minutes after adding the solutions of the compounds (Aend). Ascorbic acid was used as positive control.

The ability to scavenge DPPH radical was calculated using the following formula:

DPPH radical scavenging activity (%) = 100 × (Astart − Aend) / (Astart)

DPPH radical scavenging activity (EC$_{50}$) was given as concentration (mM) of compound that inhibited 50% of total free DPPH radicals.

Fe^{3+} Reducing power assay (FRAP): The reducing power of Fe(III) to Fe(II) was evaluated[14,15]. 1 ml of sample was mixed with 2.5 ml of phosphate buffer (0.2 mol, pH 6.6) and 2.5 ml of potassium hexacyanoferrate solution (1% in water). After 30 minutes of incubation at 50ºC, 2.5 ml of trichloroacetic acid solution (10% in water) were added and the mixture was centrifuged. 5 ml of supernatant were mixed with 5 ml water and 1 ml FeCl$_3$ solution (0.1% in water), then, after 15 minutes, the absorbance was read at 700 nm. A calibration curve of ascorbic acid was used.

Within the FRAP assay, the reducing power was evaluated using the EC$_{50}$ values, which represent the effective concentrations at which the absorbance read at 700 nm was 0.5[16].

Statistical Analysis: All assays were carried out in triplicate. Results are expressed as means ± SD. The CE$_{50}$ values were calculated by linear interpolation between values above and below 50% activity.

Results and Discussion

The cinnamic acid amide was obtained in two steps: the first step involved the transformation of cinnamic acid into its acyl chloride form, this product being later condensed with 4-aminoantipine. In the case of the synthesis of hydroxycinnamic acids' amides, caffeic, ferulic and p-coumaric acid were condensed with 4-aminoantipyrine, using BOP (benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate) as coupling agent[1] (figure 1).

Antimicrobial screening: The presence of antibacterial or antifungal activity was indicated by the presence of an inhibition zone surrounding the absorbent disk impregnated with the solution of the tested compound. The zone of inhibition was measured and expressed in millimeters (table 1). Antibacterial/antifungal activity was recorded if the zone of inhibition was greater than 8 mm.

The antibacterial activity results were expressed in terms of the diameters of the zones of inhibition: <9 mm – compounds were considered inactive; 9-12 mm - partially active; 13-18 mm - active and >18 mm - very active[17,18].

All the tested compounds inhibited the growth of Gram positive bacteria. The newly synthesized amides were more active against *Staphylococcus aureus* ATCC 25923 than the corresponding free acids (figure 2). The highest activity was displayed by compounds **3b** and **4b**. None of the substances was active against Gram negative bacteria (*E. coli*).
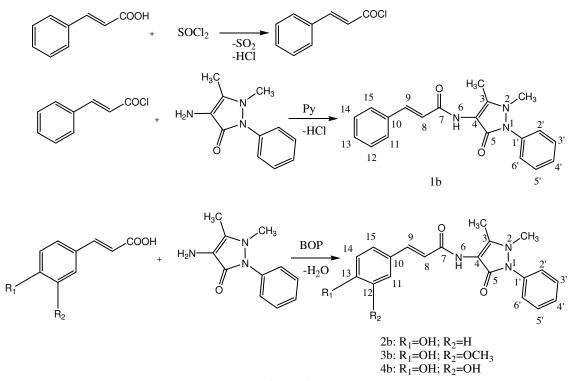

2b: R$_1$=OH; R$_2$=H
3b: R$_1$=OH; R$_2$=OCH$_3$
4b: R$_1$=OH; R$_2$=OH

Figure-1
Synthesis of compounds 1b-4b

Table-1
Antimicrobial activity - The diameters (mm) of the zones of inhibition for tested microorganisms

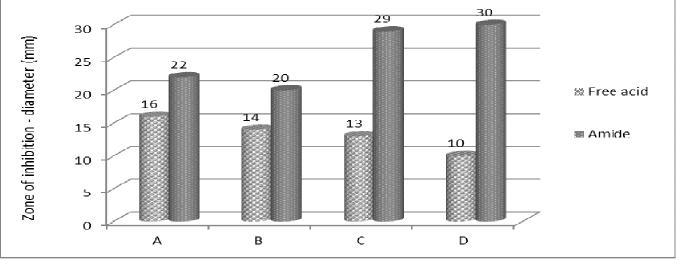| Compound	*S. aureus* ATCC 25923	*Sarcina lutea* ATCC 9341	*B.cereus* ATCC 14579	*B. subtilis*	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
1a (cinnamic acid)	16	25	10	15	0	30
2a (p-coumaric acid)	14	20	10	10	0	30
3a (ferulic acid)	13	20	10	10	0	30
4a (caffeic acid)	10	26	10	10	0	30
1b	22	12	12	0	0	0
2b	20	15	15	0	0	0
3b	29	10	18	0	0	0
4b	30	10	15	0	0	0
Ampicillin 25 µg	21	40	0	18	16	-
Chloramphenicol 30µg	21	30	20	20	22	-
Nystatin 100 µg	-	-	-	-	-	26

Figure-2
The activity against *S. aureus* ATCC 25923 – comparison between the newly synthesized amides and the corresponding free acids, Legend: A = 1a/1b; B = 2a / 2b; C = 3a / 3b; D = 4a /4b

Although the coupling of cinnamic and hydroxycinnamic acids with 4-aminoantipyrine had a positive effect on the antibacterial properties of the compounds, this tendency was not observed in the case of antifungal activity. The amides with 4-aminoantipyrine were inactive against *Candida albicans*, while the free acids were very potent antifungal agents, even superior to nystatin.

Infectious processes are usually accompanied by an increased production of free radicals; that is why substances presenting both antimicrobial and antioxidant properties are very useful. The antioxidant activity was evaluated using two *in vitro* tests: DPPH test, which intends to measure the ability of the compounds to scavenge the free stable radical 2.2-diphenyl-1-picrylhydrazyl, and FRAP (Ferric-reducing power) test, which estimates the Fe^{3+} to Fe^{2+} reducing power, determined by the electron donating capacity of the compounds. The results are summarized in table 2.

Table-2
Antioxidant activities of compounds

Compound	DPPH. Scavenging Activity EC_{50} (mM)	Fe^{3+} Reducing Power EC_{50} (mM)
1a	>200	> 200
2a	12.80±0.10	0.42±0.01
3a	$(54.40±0.20) \times 10^{-3}$	0.22±0.01
4a	$(7.70±0.10) \times 10^{-3}$	0.12±0.01
1b	1.70±0.10	1.65±0.16
2b	6.40±0.10	0.27±0.00
3b	$(73.10±0.20) \times 10^{-3}$	0.28±0.01
4b	$(1.00±0.10) \times 10^{-3}$	0.11±0.01
Ascorbic acid	$(16.90±0.10) \times 10^{-3}$	0.28±0.00

Substances 3b and 4b (ferulic and caffeic acid amides) presented remarkable antioxidant properties, similar (compound 3b) or superior (compound 4b) to those exhibited by ascorbic acid, used as positive control. Strangely and inexplicably considering their chemical structure, the DPPH· scavenging activity of compound 2b was lower than the scavenging activity of compound 1b, but this anomaly was not observed also in the FRAP test. The amides with 4-aminoantipyrine displayed a higher Fe^{3+} reducing power than the corresponding free acids.

Conclusion

Four new amides of 4-aminoantipyrine and cinnamic/hydroxycinnamic acids were synthesized. ^{1}H-NMR, ^{13}C-NMR, FTIR and elemental analysis confirmed the structure of the new compounds. The antimicrobial and antioxidant potentials of the substances were investigated, showing that all four new amides were highly active against *Staphylococcus aureus* and presented a significant Fe^{3+} reducing power, similar of superior to that of ascorbic acid. Compounds **3b** and **4b** showed a high ability to scavenge free radical DPPH. The coupling of cinnamic and hydroxycinnamic acids with 4-aminoantipyrine had a positive influence on the antibacterial and antioxidant potential of the compounds. These findings encourage the synthesis of new amides of 4-aminoantipyrine and other cinnamic acid derivatives.

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