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 (1H-NMR, 13C-NMR, FTIR and elemental analysis). Some of their biological properties were evaluated: antimicrobial and antioxidant (DPPH radical scavenging activity, Fe2+ 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: EC50 < 100 µM). The Fe3+ 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 agents1-3.

During the last decade, researchers oriented their work towards the discovery of new compounds, with an improved pharmacological profile4-6. 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 evaluated1,7. Some of these substances were found to be better antimicrobial and antioxidant agents than the corresponding free acids1.

4-aminoantipyrine is a biologically active compound and its analogues and other pyrazole derivatives have shown anti-inflammatory, analgesic, antiviral, antipyretic and antimicrobial properties8,9. 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 1H and 13C NMR spectra were recorded on a Bruker Avance 400 DRX spectrometer operating at 400 MHz for 1H and 100 MHz for 13C 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 0°C. 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 0°C for 30 minutes and then at room temperature for 2 hours. Methylenone
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 NaHCO₃ and water, dried over anhydrous sodium sulphate, filtered and evaporated. The residue was purified by recrystallization 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)-3-(4-hydroxyphenyl) acrylamide (1b):** Brown powder. Yield 85%. Mp 175-176 °C. Anal. Calcd. for C₂₀H₁₉N₂O₃: 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). ¹H NMR (400 MHz, CDCl₃, δ / ppm): 8.72 (1H, s, NH), 7.61 (1H, d, J₂, 3 = 8Hz), 7.48-7.30 (10H, overlapped peaks, H₆₂-H₆₇), 6.73-6.69 (1H, d, J₅, 6 = 15.6 Hz), 3.11 (3H, s, CH₃ attached to N₂), 2.32 (3H, s, CH₃ attached to C₂) ¹³C NMR (100 MHz, CDCl₃, δ / ppm): 161.5 (C₁), 161.63 (C₃), 149.34 (C₁₄), 141.66 (C₉), 134.94 (C₁₀), 134.71 (C₁₃), 129.57 (C₁₅), 129.34 (C₁₆+C₂₄), 128.69 (C₁₂+C₁₃), 128.02 (C₁₇+C₁₈), 127.25 (C₆a), 124.63 (C₆b+C₇), 108.76 (C₄), 35.92 (CH₂ attached to N₂), 12.59 (CH₃ attached to C₃).

**Microbiology:** The disk diffusion method 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, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10281. The bacterial and fungal strains were incubated over night at 30°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°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 37°C for bacteria and 48 hrs. at 24°C for *Candida albicans*), the plates were observed.

**Antioxidant capacity: DPPH radical scavenging assay:** The experimental procedure was adapted from literature, only slight modifications being made. Briefly, 1.5 ml solution of DPPH (2, 2-diphenyl-1-picyrylhydrazyl) 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:

\[
\text{DPPH radical scavenging activity (％) } = \frac{100 \times (\text{Astart} - \text{Aend})}{\text{Astart}}
\]
DPPH radical scavenging activity (EC50) was given as concentration (mM) of compound that inhibited 50% of total free DPPH radicals.

Fe(III) Reducing power assay (FRAP): The reducing power of Fe(III) to Fe(II) was evaluated14,15. 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 FeCl3 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 EC50 values, which represent the effective concentrations at which the absorbance read at 700 nm was 0.516.

Statistical Analysis: All assays were carried out in triplicate. Results are expressed as means ± SD. The CE50 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-aminoantipyrine. 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-oxyl-tris-(dimethylamino)-phosphoniumhexafluorophosphate) as coupling agent1 (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 active17,18.

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).

![Figure 1](https://example.com/figure1.png)

**Synthesis of compounds 1b-4b**
Table-1

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>S. aureus ATCC 25923</th>
<th>Sarcina lutea ATCC 9341</th>
<th>B. cereus ATCC 14579</th>
<th>B. subtilis ATCC 25922</th>
<th>E. coli ATCC 25921</th>
<th>C. albicans ATCC 10231</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a (cinnamic acid)</td>
<td>16</td>
<td>25</td>
<td>10</td>
<td>15</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>2a (p-coumaric acid)</td>
<td>14</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>3a (ferulic acid)</td>
<td>13</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>4a (caffeic acid)</td>
<td>10</td>
<td>26</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>1b</td>
<td>22</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2b</td>
<td>20</td>
<td>15</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3b</td>
<td>29</td>
<td>10</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4b</td>
<td>30</td>
<td>10</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ampicillin 25 μg</td>
<td>21</td>
<td>40</td>
<td>0</td>
<td>18</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol 30μg</td>
<td>21</td>
<td>30</td>
<td>20</td>
<td>20</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>Nystatin 100 μg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>26</td>
</tr>
</tbody>
</table>

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 Fe³⁺ to Fe²⁺ reducing power, determined by the electron donating capacity of the compounds. The results are summarized in table 2.

Table-2

Antioxidant activities of compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>DPPH Scavenging Activity EC₅₀ (mM)</th>
<th>Fe³⁺ Reducing Power EC₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>&gt;200</td>
<td>&gt; 200</td>
</tr>
<tr>
<td>2a</td>
<td>12.80±0.10</td>
<td>0.42±0.01</td>
</tr>
<tr>
<td>3a</td>
<td>(54.40±0.20) x 10⁻³</td>
<td>0.22±0.01</td>
</tr>
<tr>
<td>4a</td>
<td>(7.70±0.10) x 10⁻³</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>1b</td>
<td>1.70±0.10</td>
<td>1.65±0.16</td>
</tr>
<tr>
<td>2b</td>
<td>6.40±0.10</td>
<td>0.27±0.00</td>
</tr>
<tr>
<td>3b</td>
<td>(73.10±0.20) x 10⁻³</td>
<td>0.28±0.01</td>
</tr>
<tr>
<td>4b</td>
<td>(1.00±0.10) x 10⁻³</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>(16.90±0.10) x 10⁻³</td>
<td>0.28±0.00</td>
</tr>
</tbody>
</table>
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, presented remarkable antioxidant properties, similar (compound 3b, 4b (ferulic and caffeic acid amides) of superior to that of ascorbic acid. Compounds 1b, 2b 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.

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 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.

References