Synthesis and activity of $N$-$(o$-tolyl)$caffeamide and $N$-$(o$-tolyl)$-$p$-coumaramide against P388 leukemia murine cells

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Abstract. Ester derivatives of $p$-hydroxycinnamic compounds have high anticancer activity. However, the ester compounds usually easier to be decomposed than their amide derivates, so the ester compounds have a low potential to be applied as anticancer. In this research, the amide derivatives of caffeic and $p$-coumaric acids have been synthesized using $o$-tolylamine to give trans-$N$-$(o$-tolyl)$caffeamide (5a) and trans-$N$-$(o$-tolyl)$-$p$-coumaramide (5b), respectively. The products were characterized by FT-IR, $^{13}$C-NMR, and $^1$H-NMR methods. In the FT-IR spectrum, compound 5a showed absorption bands of N-H bond at 3236.55 cm$^{-1}$ and 1533.41 cm$^{-1}$ as stretching and bending vibrations, respectively; and compound 5b had absorption bands at 3267.41 cm$^{-1}$ and 1527.82 cm$^{-1}$. In the $^{13}$C-NMR spectrum, compound 5a gave 15 of peaks that representing 16 of carbons, and compound 5b gave 14 of peaks that were also representing 16 of carbons. In the $^1$H-NMR spectrum, the peak of N-H of compound 5a and compound 5b appeared at 8.57 ppm and 8.87 ppm, respectively. Activity assay results of both compounds against P388 leukemia murine cells indicated that both compounds have a high potential as anticancer, especially compound 5a. The compound 5a was more active than the analogous compounds which the previous synthetised.

1. Introduction
Caffeic and $p$-coumaric acids are hydroxycinnamate compounds which attract more attention of many researchers compared to the other members of the compound, especially their ester derivates. Their esters show activities as an antioxidant, antitumor, immune regulation [1], anti-inflammatory [1-4], and anticancer [4,5]. However, for applications to the field of medicine, the ester compounds are less important than the amide compounds. Generally, ester compounds are more reactive than amide compounds [6]. Therefore, the ester compounds will quickly decompose on their way to the treatment target in the body. Therefore, the compound should be converted into the amide compound in order to be used as a drug.

There are several methods in converting acid compounds to amides and ester compounds [7-13], but only an indirect method has been successful in converting hydroxycinnamic acid to their amides [14]. This method contains four steps of reactions namely, (1) protecting the phenolic group, (2) increasing reactivity of carboxyl group by chlorination and (3) amidating group by in situ method, and (4) deprotection the phenolic group (Figure 1). This method has been successful in converting ferulic acid to phenethyl trans-3-(4-hydroxy-3-methoxyphenyl) acrylate and trans-3-(4-hydroxy-3-methoxyphenyl)-N-phenethylacrylamide using phenethyl alcohol and phenethylamine [14]. By considering the success and simplicity of this method, synthesis of compound 5a from caffeic acid and
o-tollilamine, and compound 5b from p-coumaric acid and o-tollilamine were conducted using the method. Both of these compounds are new amides derived from cinnamic acid that have the potential to be used as anticancer.

As the initial objective of this study is to increase the anticancer activity of compounds, the potential of the two compounds was tested as anticancer. Potential assay for a compound as anticancer can be done by several methods, for example, HeLa cell testing for cervical cancer [15-23], MCF-7 cells for breast cancer [15, 23], and P388 murine leukemia cells for blood cancer [14, 24-27]. In this study, the anticancer test was carried out using P388 murine leukemia cells.

![Chemical structures and reaction schemes](image)

**Figure 1.** The pathway of synthesis reaction of compounds 5 and 6

The activity test results showed that both compounds were active, and the compounds 5a more active than the compound 5b. The data indicated that the presence of hydroxyl groups on the meta position of p-hydroxycinnamamide compounds increase their activity against P388 murine leukemia cells.

2. Methodology

2.1. Materials

Caffeic acid and p-coumaric acid purchased from Sigma Aldrich, and acetic anhydride, thionyl chloride, pyridine, o-toluidine, toluene, acetone, ethyl acetate, dichloromethane, benzene, methanol, pyrrolidine, triethylamine, n-hexane, ammonium chloride, hydrochloride acid, and sodium sulfate purchased from Merck, along with P388 leukemia murine cells. All chemical were used without purification.
2.2 Equipment
The FT-IR spectrums were obtained from Spectrophotometer Shimadzu Prestige 21, the NMR spectrums were obtained from Spectrometer Agilent operating at 500 MHz for 1H and 125 MHz for 13C nuclei, the melting points were determined using electro-thermal apparatus, and the activity as anticancer were tested by MTT method against P388 Leukemia murine cells in the Natural Product Laboratory, Institute Technology of Bandung (ITB).

2.3 Procedures
Synthesis procedure in this research was adopted from reference [14] with slight modification. The modification was conducted in step 1a where the moles of anhydride acetic was doubled from the mole of the original procedure.

2.3.1 Synthesis of compound 2a. This reaction step gave a white solid (57.68% yields), melted at 188-183°C. IR (KBr): v (cm⁻¹) 1764.87 (C=O, acetyl ester), 2987.74, 2941.44 & 2823.79 (sat. C-H), 1373.32 & 1431.18 (methyl), 1687.71 (C=O, conj. carboxyl), 1629.85 (C=C, olefin), 985.62 (trans-olefin), 3055.24 (unsat. C-H), 1581.63 & 1502.55 (C=C, Ar), 910.40 & 829.39 (1,2,4-trisubst. Ar).

2.3.2 Synthesis of compound 4a. The compound 4a was obtained through two steps of reaction, namely acetylation and in situ amidation, gave a white crystalline solid (42.46% yields), melted at 146-148°C. IR (KBr): v (cm⁻¹) 3236.55 (N-H sec. amide), 1338.60 (C-N), 1533.41 (C-N amide), 1660.71 (C=O amide), 1766.80 (C=O ester), 1622.13 (C=C olefin), 975.98 (trans olefin), 3030.17 & 3107.32 (C-H unsat.), 1581.63 & 1500.00 (C=C Ar), 754.17 (orto disubst. Ar), 840.96 & 900.76 (1,2,4-trisubst. Ar), 2856.58, 2924.09 & 2954.95 (C-H sat.), 1369.46 & 1425.40 (methyl).

2.3.3 Synthesis of compound 5a. This reaction step gave a white crystalline (63.51% yields), melted at 190-193°C. IR (KBr): v (cm⁻¹) 3363.86 (O-H), 3215.34 (N-H), 1533.41 (C-N amide), 1651.07 (C=O amide), 3026.31 (C-H unsat.), 1622.10 (C=C olefin), 979.84 (trans olefin), 131.13, 128.39, 126.92, 125.37, 124.22, 121.85, 119.78, 116.35, 115.01, and 18.17. 13C-NMR {(CD3)2CO} δ (ppm) 165.03, 148.02, 146.25, 141.95, 137.93, 131.13, 128.39, 126.92, 125.37, 124.22, 121.85, 119.78, 116.35, 115.01, and 18.17. 1H-NMR {(CD3)2CO} δ (ppm) 8.55 (s, 1H, N-H, amide), 8.24 (s, 2H, O-H, 3,4-dihydroxyphenyl), 7.88 & 6.86 (dd, 2H, J = 10 Hz, H6-Ar & H5-Ar, 3,4-dihydroxyphenyl), 7.12 (s, 1H, H2-Ar, 3,4-dihydroxyphenyl), 6.99 (d, 1H, J = 5 Hz, H6-Ar, o-tolyl), 7.20 (s, 1H, H2-Ar, 3,4-dihydroxyphenyl), 7.17 (d, 1H, J = 5 Hz, H5-Ar, o-tolyl), 7.05 (t, 1H, J = 5 Hz, H4-Ar, o-tolyl), and 2.31 (s, 3H, CH3, o-tolyl).

2.3.4 Synthesis of compound 2b. This reaction step produced a yellowish crystalline (70.08% yields), melted at 207-209°C. IR (KBr): v (cm⁻¹) 1747.51 (C=O, acetyl ester), 2831.50 & 2983.88 (sat. C-H), 1533.2 & 1427.32 (methyl), 1683.86 (C=O, conj. carboxyl), 1627.92 (C=C, olefin), 993.34 (trans-olefin), 1600.92 & 1506.33 (C=C Ar), 754.17 (orto disubst. Ar), 879.54 & 852.54 (1,2,4-trisubst. Ar), 2852.72 & 2922.16 (C-H sat.), 1365.60 & 1452.40 (methyl), 13C-NMR {(CD3)2CO}: δ (ppm) 165.03, 148.02, 146.25, 141.95, 137.93, 131.13, 128.39, 126.92, 125.37, 124.22, 121.85, 119.78, 116.35, 115.01, and 18.17. 1H-NMR {(CD3)2CO}: δ (ppm) 8.55 (s, 1H, N-H, amide), 8.24 (s, 2H, O-H, 3,4-dihydroxyphenyl), 7.88 & 6.86 (dd, 2H, J = 15 Hz, trans-olefin), 7.17 (d, 1H, J = 5 Hz, H3-Ar, o-tolyl), 6.99 (d, 1H, J = 5 Hz, H6-Ar, o-tolyl), 7.20 (t, 1H, J = 5 Hz, H5-Ar, o-tolyl), 7.05 (t, 1H, J = 5 Hz, H4-Ar, o-tolyl), and 2.31 (s, 3H, CH3, o-tolyl).

2.3.5 Synthesis of compound 4b. Similar to compound 4a, the compound 4b was obtained through two steps of reaction, namely acetylation and amidation in situ. This reaction gave a yellowish solid (51.28% yields), melted at 168-170°C. IR (KBr): v (cm⁻¹) 3275.13 (N-H sec. amide), 1332.81 (C-N alkyl), 1529.55 (C=N amide), 1654.92 (C=O amide), 1759.08 (C=O ester), 1622.13 (C=C olefin), 974.05 (trans olefin), 3034.03 (C-H unsat.), 1591.27 & 1500.00 (C=C Ar), 754.17 (orto disubst. Ar), 912.33 & 844.82 (1,2,4-trisubst. Ar), 2981.95, 2931.80 & 2856.58 (C-H sat.), 1369.46 & 1458.18 (methyl).

2.3.6 Synthesis of compound 5b. This reaction step gave a yellowish crystalline (74.05% yields), melted at 219-220°C. IR (KBr): v (cm⁻¹) 3315.63 (O-H), 3267.41 (N-H), 1581.63 (C=N amide), 1649.14 (C=O...
amide), 3020.53 (C-H unsat.), 1581.63 (C=C Ar), 750.31 (ortho disubst. Ar), 866.04 & 835.18 (1,2,4-trisubst. Ar), 977.91 (trans olefin), 2860.43 & 2922.00 (C-H sat.), 1348.24 & 1438.90 (methyl). 13C-NMR {(CD3)2CO}: δ (ppm) 165.14, 159.98, 141.66, 137.91, 131.13, 130.59, 130.39, 127.60, 126.90, 125.45, 124.46, 119.58, 116.62, 18.17. 1H-NMR {(CD3)2CO}:(ppm) 8.87 (s, 1H, N-H, amide), 8.65 (s, 1H, O-H, p-hydroxyphenyl), 7.62 & 6.83 (dd, 2H, J = 15 Hz, trans-olefin), 7.47 & 6.88 (dd, 4H, J = 10 Hz, H2-Ar & H6-Ar, H3-Ar & H5-Ar, p-hydroxyphenyl), 7.87 (d, 1H, J = 10, H6-Ar, o-tolyl), 7.21 (d, 1H, J = 10, H3-Ar, o-tolyl), 7.18 (t, 1H, J = 10, H5-Ar, o-tolyl), 7.05 (t, 1H, J = 10, H4-Ar, o-tolyl), 2.31 (s, 3H, CH3, o-tolyl).

2.3.7 Cytotoxic assay. Anticancer potential testing of compounds 5a and 5b were carried out using P388 murine leukemia cells. The testing procedure is following the method used in references [24] and [27]. By this method, the compound each 5a dan 5b gave IC50 values of 0.91 μg/mL and 16.97 μg/mL, respectively.

3. Discussion

3.1 Structure elucidation of compound 5a and 5b
Derivatization of cinnamic acid compounds to their amides can be conducted through four steps, namely acetylation, chlorination followed by in situ amidations, and deacetylation [14]. This method was adopted to synthesize the compounds 5a and 5b. In the synthesis, the products of each stage were controlled with FT-IR spectroscopy method. The success of the acetylation stage was indicated by the appearance of absorption of C=O acetyl groups at the wavenumber of 1764.87 cm-1 for the synthesis of compounds 2a and 1747.51 cm -1 for the synthesis of compound 2b. Both compounds 2a dan 2b was used as a precursor for further synthesis stage.

Before the amidation reaction was carried out, the reactivity of the carbonyl group against nucleophiles of each compound 2a and 2b were increased through the chlorination reaction which each resulted compounds 3a and 3b, then proceeding with an in situ amidation reaction. The amidation reaction is carried out in situ because the acidic halide product feared will be decomposed by water vapor in the atmosphere [14]. The success of the amidation reaction was indicated by the appearance of N-H at a wavenumber of 3236.55 cm -1 for the synthesis of compounds 4a and 3275.13 cm -1 for compound 4b.

The target compounds 5a and 5b were obtained after the compounds 4a and 4b were deacetylated. The success of this reaction was indicated by the absence of an absorption band of C = O esters in the spectrum of both compounds. Besides using FT-IR spectroscopy, both target compounds were characterized using 13C-NMR and 1H-NMR spectroscopy. The compound 5a gave fifteen peaks representing sixteen carbon atoms, whereas the compound 5b gave fourteen peaks representing sixteen carbon atoms, too.

In the 1H-NMR spectrum, the compound 5a gave four singlets, six doublets, and two triplets peaks. The singlets peaks are correlated to two of hydroxyl protons and one of aril proton in the dihydroxyphenyl group, one secondary amide proton, and three methyl protons in the o-tolyl group. The doublets peaks are correlated to two protons in the olefin group, two aromatic protons of the dihydroxyphenyl group, and two aromatic protons in the o-tolyl group. The two triplets peaks are correlated to two aromatic protons in the o-tolyl group. Similarly, the compound 5b gave three singlet peaks which correlated to hydroxyl proton, secondary amide proton, and methyl protons; six doublet peaks which correlated to two olefin protons, four aromatic protons in p-hydroxyphenyl group, and two aromatic protons in o-tolyl group; and two triplet peaks which correlated to two aromatic protons in o-tolyl group.

All FT-IR, 1H-NMR, and 13C-NMR data have been described according to the structure of each target compound 5a and 5b (data corresponding to the intended compound) as shown in Figure 1. Based on the results of the elucidation, the target compounds have been synthesized successfully.
3.2 Cytotoxic of compound 5a and 5b
Activity assay of compounds 5a and 5b against P388 murine leukemia cells gave IC\textsubscript{50} values of 0.91 and 16.97 μg/mL, respectively. Because of compound 5a more active than compound 5b, it can be stated that the presence of a hydroxyl group at meta position of the p-hydroxycinnamamide compounds increase their activity against P388 murine leukemia cells. Thus, the meta position of the p-hydroxycinnamamide compound is the active site of the compound.

There are two reasons for increasing the activity of the p-hydroxycinnamamide compound by the presence of the hydroxyl group in the meta position: first, the formation of intramolecular hydrogen bonds (Figure 2a) before the release of hydrogen radicals reduces the polarity of the compound so that it can easily penetrate the cell wall that is lipophilic. Secondly, the formation of a hydrogen bonding between the meta hydroxyl group and the para oxygen radical formed after the release of hydrogen radicals (Figure 2b) stabilize the radical species. Both of these corroborate each other in increasing their activity against P388 murine leukemia cells.

Comparing to analogous compounds which previous synthesized, the cytotoxic of compound 5a is stronger than methyl 2-cinnamamido-3-hydroxy propanoate, methyl 3-(4-nitrophenyl)acrylate, and methyl 3-[(2-nitrophenyl)acrylate with IC\textsubscript{50} values of 10.78 μg/mL, 7.98 μg/mL, and 27.78 μg/mL [28,29], respectively; and also to apigenin compounds isolated from Macaranga gigantifolia leaves with IC\textsubscript{50} 14.13 μg/mL [30].

4. Conclusion
Compounds 5a and 5b can be synthesized by the reaction of o-toluidine with caffeic and p-coumaric acids, respectively using an indirect method. By this method, the compound 5a was obtained as a white crystalline with a melting point of 190-193°C, and compound 5b was obtained as a yellowish crystalline with a melting point 219-220°C. The activity of compound 5a and 5b against murine leukemia P388 was in strong and moderate categories, respectively. The presence of the hydroxyl group at the meta position increases the activity of compound 5a.

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