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Synthesis of Methyl 2-Cinnamamido-3-Hydroxy Propanoate Having Activity against P388 Leukemia Cells

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ABSTRACT

Cinnamic acid derivative compound was investigated for the anti-cancer inhibitory activity. To be able to obtain compounds that have bioactivity as above, it is needed to study quantitative structure-activity relationship (QSAR) which is the process by which the chemical structure is quantitatively correlated with biological activity/chemical reactivity. Chemical methods used in synthesizing the chemical of methyl *trans*-cinnamate derivatives are tailored to match their targeted bioactivities. Here, we investigated the anti-cancer inhibitor compound with the method amidation of cinnamic acid derivative compounds. In this reaction we use two steps to get the target product. Firstly, we hydrolize of methyl trans-cinnamate to cinnamic acid. That reaction has a yield 85.5 % and secondly, we amidate of cinnamic acid to metil 2-cinnamamido-3-hydroxy propanoate has a yield of 51.5%. The compound of metil 2-cinnamamido-3-hydroxy propanoate showed against P388 leukemia cells inhibitory activity with IC_{50} = 10.78 µg/mL.

INTRODUCTION

Methyl *trans* cinnamate has been use as a component of plant-derived scents, flavorings and fragrance (Hoskins *et al.*, 1984). It was isolated from galangal rhizome (*Alpinia malaccensis*). Hydrolysis of methyl *trans*- cinnamate with strong base produce cinnamic acid. Cinnamic acid possesses an α,β-unsaturated carbonyl moiety, which can be considered as a Michael acceptor, an active moiety often employed in design of anti-cancer drugs (Zhou *et al.*, 2006). α,β-unsaturated amides are important products (Davies *et al.*, 2005, Williams *et al.*, 2007 and Park Choo *et al.*, 2000).

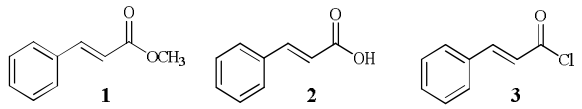

Fig. 1: (1) Methyl trans-cinnamate, (2) cinnamic acid, (3) cinamoyl chloride.

α,β-unsaturated ketones have demonstrated preferential activity toward thiol. Alkylation with thiol may also occur with cinnamates leading to adducts at the β-position. Hence, α,β- unsaturated carbonyl containing compounds may be free from problems of mutagenicity (Lee *et al.*, 1971). Chlorination of carboxylic acids can be done by using thionyl chloride (White *et al.*, 1997). The cinnamoyl functionality is also present in a variety of secondary metabolites of phenylpropanoid biosynthetic. A lot of naturally occurring and biologically active alcohols have been linked with cinnamoyl through the ester linkage to convert their anti-cancer efficacy. Cinnamic acid amides and related compounds, like cinnamic acid ester, form a class of anticancer agents. Recently, a lot of cinnamamide compounds were also synthesized and their anti-cancer abilities were evaluated. 2-Methyl cinnamide (figure 2) isolated from a fermentation beer of *Streptomyces griceoluteus*, showed significant anti-invasive or anti-metastatic effects (Welch *et al.*, 1993). It also 2-methyl cinnamide was pretreatment of maglinant melanoma cells (C8161 and A375 M *in vitro*) caused a dose and time-dependent reversible reduction (IC_{50}= 12.5µg/mL) of invasion. Similarly, lung colonization was significantly inhibited when tumor cells were pretreated *in vitro* with the same prior to intravenous injection (P<0.05) (Baltas *et al.*, 2011). In common, the formation of carboxamides from carboxylic acid involves activation can be achieved by conversion of the carboxylic group to more reactive functional group for example acyl halide, anhydride,

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acyl azide, active ester and most common in situ activation by coupling reagent with *N,N*-dicyclohexylcarbodiimide (DCC) (Sheehan, 1955). Additional coupling reagents that have been used $TiCl_4$ (Ali khalafi *et al*. 2005), activated phosphate (Madeleine M *et al.*, 2010) $Sn[N(TMS)_2]_2$ (Burnell-Curty *et al.*, 1993), triphenylphosphine and trichloroacetonitrile (Jang *et al.*, 1999) $ArB(OH)_2$ (Ishihara *et al.*, 1996).

Here, we focus to synthesize of the methyl 2-cinnamamido-3-hydroxy propanoate compound and also evaluated for in vitro inhibitory activity against P388 leukemia cells.

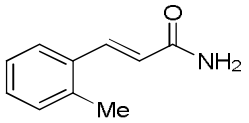


Fig. 2: 2-Methyl Cinnamide.

MATERIALS AND METHOD

Materials

All solvents were dried and distilled according to standard procedure. Analytical thin layer chromatography (TLC) was performed on Merck silica gel plates (Kiesel gel $60F_{254}$ 0.25 mm) and preparative TLC was carried out on Merck silica gel plates (Kiesel gel $60F_{254}$ 0.5 mm). Silica gel column chromatography was carried out on Daisogel IR-60. Cinnamic acid from hydrolisis of methyl *trans*-cinnamate was used as starting material for the synthesis of metil 2-cinnamamido-3-hydroxy propanoate. L-serine methyl ester was used as reagent. 1,3 Dicyclohexylcarbodiimide and 4-Dimethylamino pyridine was used as catalyst and pyridine was used as solvent.

Instruments

^1H and ^{13}C NMR spectra were recorded on JEOL 1NM–LA for 500 MHz in deuterio chloroform unless otherwise specified. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (δ 0.00) or $CDCl_3$ (δ 7.26) for ^1H NMR and δ 77.0 for ^{13}C NMR as internal standard, and coupling constant are reported in Hertz.

Methods

Synthesis of Cinnamic acid

To a 250 mL round-bottomed flak equipped were charged methyl *trans*-cinnamates, 2M NaOH and 95% ethanol. The reaction mixture was heated at of 50-60 ° C for 3 hours. After completion (monitored by TLC), the reaction was neutralized with HCl 1M, the reaction mixtured was extracted with butanol. The organic phase was washed with water, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to afford the product 85 % yield.

Methyl 2-cinnamamido-3-hydroxy propanoate

To a 100 mL round-bottomed flask equipped were charged cinnamic acid (0.005 mol), L-serin methyl ester (0.01 mmol) in pyridine 20 mL was added 1,3 dicyclohexylcarbodiimide (0.011 mol) and 4-dimethylamino pyridine (0.002 mol). The reaction mixture was heated to 60 ^oC for 4h. After completion (monitored by TLC), the reaction mixture was cooled and quenched with water (50 mL) and extracted with ethyl acetate (3x50 mL). The organic phase was washed with water, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure to afford the crude product, which was purified by column chromatography over silica gel using hexane and ethyl acetate as eluent.

Bioassay

Hatching the brine shrimp

Brine shrimp eggs (*Artemia salina)* were hatched in artificial sea water prepared from commercial sea salt. The hatching process was done under light regime condition. After 48 hours incubation at room temperature (25-29°C), nauplii (larvae) were collected by pipette from the lighted side whereas their shells were left in another side.

BSLT method

The procedure for BSLT was modified from the assay described by Solis *et al.* (1993). Ten milligrams of the sample were made up to 2 mg/ml in artificial sea water except for water insoluble compounds which were dissolved in DMSO. 50 µl prior to adding sea water. Serial dilutions were made in the wells of 96-well microplates in triplicate in 120 µl sea water. Control wells with DMSO were included in each experiment. A suspension of nauplii containing 10 organisms (100 µl) was added to each well. The plates were covered and incubated at room temperature (25-29°C) for 24 hours. Plates were then examined under the binocular steromicroscope and the numbers of dead (non-motile) nauplii in each well were counted. One hundred microlitrs of methanol were then added to each well to immobilize the nauplii and after 15 minutes the total numbers of brine shrimp in each well were counted. Analysis of the data was performed by probit analysis on a Finney computer program to determine the lethal concentration to half of the test organisms (LC_{50}).

Anti-Cancer Bioassay

MTT Method

Toxicity test was done by using MTT method (3 - (4,5-dimethyltiazol-2-yl) -2.5-difeniltetrazolium bromide] (Mosman, 1983). Cytotoxicity test performed using P388 leukemia cells.

The growth of cell cultures

Stock cell cultures in medium RPMI-1640 serum (PBS) was incubated at 37 °C with a flow of 5% CO_2 for 24 hours and observed under an inverted microscope. If there were cells attached to the walls of flasks, the medium were removed and replaced with new serum medium, then incubated at 37 °C primarily to the flow of 5% CO_2 until confluent growth. Refurbishing the medium continues until the number of cells obtained sufficient for testing.

Preparation of cells to be tested

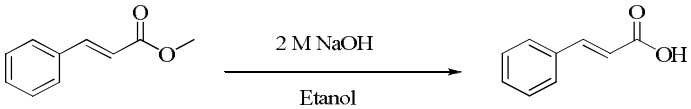
Once the number of cells to be tested was sufficiently adequate, the cell medium was discarded, then cells were washed and added to RPMI medium. Cells were transferred into a sterile tube and then centrifuged at 1200 rpm for 5 minutes. Supernatant was discarded, the cell sediment was added into RPMI 1640 (Sigma Chemical Co.) containing 10% FBS (Sigma Chemical Co.). Cell suspension was taken, then the cell density was calculated using hemocymeter under a microscope. The number of cells was calculated by counting: (the number of cells in four chambers x 103 cells / ml)/4. The number of cells that had been known then was made as dilutions of the cells with medium RPMI-serum to obtain cell 2 x 104 cells/ml cell suspension.

Cytotoxicity test

Cells with the number 1 - 2x104 cells/wells in RPMI 1640 culture medium was distributed in 96 wells plate and incubated in a CO_2 incubator (5%) at 37^{o}C for 24 hours to adapt and stuck to the bottom wells. The next day the media was taken, washed FBS and then added 100 ml of culture medium containing DMSO or sample and incubate for 48 hours. At the end of incubation, culture media containing the sample was removed, washed with 100 ml of FBS. Into each of the wells was added 100 ml of culture medium containing MTT 5 mg/ml. The culture medium was incubated for 4 hours at 37^{o}C. Living cells will interact with MTT to form purple formazan crystals. After 4 hours the media containing MTT was removed, washed with FBS and then added a solution of 200 ml isopropanoate to dissolve the formazan crystals. In order to dissolve formazan then incubated at room temperature for 12 hours and then read with ELISA reader at a wavelength of 550 nm (Surendra *et al.*., 1998).

$$\% \text{ live cells} = \frac{\text{A 550 of treated cells sel} - \text{A550 of control medium}}{\text{A 550 control cells} - \text{A550 control medium}} \times 100$$

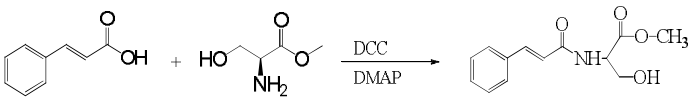
Then proceed to determine the equation of the regression line and determine its IC_{50} (concentration that causes the death of 50% of the population of cells).



Scheme. 1: Hydrolysis of methyl *trans* cinnamate to cinnamic acid.

RESULT AND DISCUSSION

Methyl 2-cinnamamido-3-hydroxy propanoate synthesized from simple and readily available chemicals using cinnamic acid . Cinnamic acid obtained from hydrolysis of methyl *trans* cinnamate (Scheme 1), in this reaction was produce a product of cinnamic acid in 85 % yield, respectively. Esterification of cinnamic acid and L-serin methyl ester with 1,3 dicyclohexylcarbodiimide and 4-dimethylamino resulted methyl 2-cinnamamido-3-hydroxy propanoate in pyridine at 60 ^{o}C for 4 hours (Scheme 2). The esterification reaction was produce methyl 2-cinnamamido-3-hydroxy propanoate in 51.5 % yield.



Scheme. 2: Synthesis of Methyl 2-cinnamamido-3-hydroxy propanoat

Mass Spectrometry, ^{1}H NMR and ^{13}C NMR methods have been applied to elucidate the chemical structure of methyl 2-cinnamamido-3-hydroxy propanoate. The Molecular weight of methyl 2-cinnamamido-3-hydroxy propanoate was determined to be 249.26 for $C_{13}H_{15}O_4$ by mass spectroscopy. The high-resolution LC-MS value for the positive ion MS $[M+H]^+$ was 250.2285. The theoretical value for the methyl 2-cinnamamido-3-hydroxy propanoate positive ion for $C_{13}H_{15}O_4$ is 250.2285. The ^{1}H NMR and ^{13}C NMR data are presented in table 1 below. In the ^{1}H NMR spectra data of methyl 2-cinnamamido-3-hydroxy propanoate, one methoxy signal δH 3.71 (3H, s) and one protons δH 3.93(1H, t) were detected along with –NH groups, two protons δH 4.02, 4.04 (2H,d, J= 3.9 Hz), one proton was detected along olefin δH 6.54 (1H, d, J= 15.6 Hz), olefin proton δH 7.54 (1H, d, J=15.6 Hz) and aromatic proton δH 7.41 (1H, d, J=3.25 Hz), δH 7.27 (1H, t), δH 7.28 (1H, t). In the ^{13}C NMR spectra data of methyl 2-cinnamamido-3-hydroxy propanoate, one methoxy signal 52.74, one ester signal 171.33, carbon was bonded to-NH group 55.06, carbon was bonded to –OH group 62.94, one amide signal 166.55, olefin signals 120.16, 141.86, and aromatic signals 134.66, 127.99, 128.85 and 129.89.

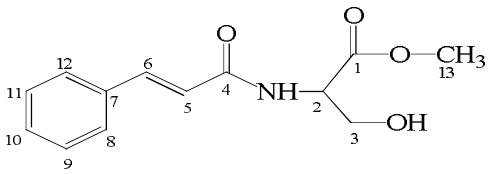


Figure 3. Structure of Methyl 2-cinnamamido-3-hydroxy propanoate

Table. 1: The ^{1}H NMR (500 MHz) and ^{13}C NMR Data for Methyl 2-cinnamamido-3-hydroxy propanoate in CDl_3

Position	Chemical Shift (δ, J in Hz)	
	^{1}H-NMR	^{13}C-NMR
1		171.33
2	3.93 (1H, *t*)	55.06
3	4.02, 4.04 (2H, *d*, J= 3.9 Hz)	62.94
4		166.55
5	6.54 (1H, *d*, J= 15.6 Hz)	120.16
6	7.54 (1H, *d*, J=15.6 Hz)	141.86
7		134.66
8	7.41(1H, *d*, J= 3.25 Hz)	127.99
9	7.27 (1H, t)	128.85
10	7.28 (1H, t)	129.89
11	7.27 (1H, t)	128.85
12	7.41 (1H, *d*, J= 3.25 Hz)	127.99
13	3.71 (3H, *s*)	52.74

Bioactivity of Methyl 2-cinnamamido-3-hydroxy propanoate

Cytotoxity test used to find out potential anticancer properties of methyl 2-cinnamamido-3-hydroxy propanoate.

Cytotoxity tests of methyl 2-cinnamamido-3-hydroxy propanoate conducted with BSLT and MTT method. Brine Shrimp Lethality test (BSLT) assay showed that methyl 2-cinnamamido-3-hydroxy propanoate compound have LC_{50}= 91.20 µg/mL, respectively. Compound resulting in LC_{50} values less than 250 µg/mL were considered significantly active and had potential for further investigation. In this research, cytotoxity test also was performed by MTT method. The assay was based on the capacity of mitochondrial dehydrogenase to convert the tallow water-soluble substrate MTT into dark blue formazan product which was insoluble in water. Cell mortality data could be used to determine the IC_{50}. Methyl 2-cinnamamido-3-hydroxy propanoate was initially tested in vitro for inhibitory activity against P388 leukemia cells. MTT assay showed that methyl 2-cinnamamido-3-hydroxy propanoate compound have IC_{50} values of 10.78 µg/mL. The value of this activity is one indication that the methyl 2-cinnamamido-3-hydroxy propanoate compound has potential as an anti-cancer agent.

CONCLUSION

Synthesis amide of cinnamic acid was done because it lesser known attributes towards anti-cancer activity. Herein, we synthesize of methyl 2-cinnamamido-3-hydroxy propanoate from cinnamic acid, respectively. In this reaction we use two steps to get the target product. Firstly, we hydrolize of methyl trans-cinnamate to cinnamic acid. That reaction has a yield 85.5 % and secondly, we amidate of cinnamic acid to metil 2-cinnamamido-3-hydroxy propanoate has a yield of 51.5%. The compound of methyl 2-cinnamamido-3-hydroxy propanoate showed a candidate anti-cancer inhibitor with BSLT (Brine Shrimp Lethality Test) assay is LC_{50} = 91.20 µg/mL. And the compound of methyl 2-cinnamamido-3-hydroxy propanoate showed against P388 leukemia cells inhibitory activity with IC_{50} = 10.78 µg/mL.

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