



Synthesis and investigation of dihydroxychalcones as calpain and cathepsin inhibitors



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ARTICLE INFO

Article history:

Received 9 May 2013

Available online 16 September 2013

Keywords:

Dihydroxychalcone

Calpain inhibitor

Cathepsin B

L Inhibitor

ABSTRACT

In order to identify potential calpain and cathepsin inhibitors we prepared 12 dihydroxychalcone analogues and tested their ability to inhibit μ -calpain, m -calpain, cathepsins B and L. In the calpain inhibition test, compound **10** exhibited the most active inhibitory activity against m -calpain with an IC₅₀ value of $25.25 \pm 0.901 \mu\text{M}$. With respect to inhibition of cathepsins B and L, compound **13** exhibited the most potent inhibitory activity on cathepsin L and moderate inhibitory activity on cathepsin B with IC₅₀ values of 2.80 ± 0.100 and $11.47 \pm 0.087 \mu\text{M}$, respectively. Our results suggest the possibility of developing dual calpain and cathepsin inhibitors by properly modulating structures and/or combining the essential aspects of the functional group effective for specific calpain and cathepsin inhibition.

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1. Introduction

Cathepsins belong to a family of cysteine proteases and a class of proteolytic enzymes. A total of 11 human cathepsins have been reported to date, several of which are highly expressed in malignant cells [1]. Cathepsins function in a diverse set of roles after being released into the extracellular system during tumorigenic process [2–4]. Especially, cathepsins B and L are involved in the progression of human pancreatic cancer [5,6]. Studies performed using the ripi-tag2 mouse model also showed that cell proliferation and tumor growth were reduced in cathepsins B and L knockout mice, indicating that cathepsins B and L may be potential therapeutic targets for anti-cancer drugs [5,7]. Cathepsins have also been implicated in the pathogenesis of Alzheimer's disease (AD) [8,9]. Specifically, neurotoxic insoluble β -amyloids are generated through sequential cleavage of amyloid precursor protein (APP) by β -site APP-cleaving enzyme (BACE1) and γ -secretase [9,10]. Cathepsins S, B and L cleave the wild type β -site of APP faster than BACE1 [9]. In addition, cathepsin B has been identified as a BACE1 in secretory vesicles [8]. Treatment with the cathepsin B specific inhibitor CA074 results in improved memory ability and reduction of β -amyloid formation in an AD animal model expressing the wild type β -site of APP, which is observed in the most sporadic AD patients [11]. The most well-studied synthetic cathepsin B inhibitors

reported to date are CA074 derivatives, namely epoxysuccinyl inhibitors, and vinyl sulfone inhibitors [12,13]. Both series of cathepsin B specific inhibitors are peptide-based inhibitors and derived based on the tertiary structure of substrate binding pocket of cathepsin B. Cathepsin L specific inhibitors are also developed based on its tertiary structure due to the structural similarity between cathepsins B and L. However, the binding cleft of cathepsin L has no bulky residues compared to that of cathepsin B [12]. Therefore, known cathepsin L inhibitors possessing peptide-like characteristics are in general more flexible and adopt bulky side chains such as benzyl and biphenyl groups compared to cathepsin B inhibitors [12,14].

Calpains are a family of intracellular Ca²⁺-dependent cysteine proteases that were first discovered in the CNS [15]. Since their initial discovery, expression of calpains has been observed in various cells and tissues [16,17]. Calpains comprises 15 isoforms that are expressed either ubiquitously or in a tissue-specific manner, depending on the isoform [18]. Calpain 1 (μ -calpain) and calpain 2 (m -calpain) require micromolar and millimolar Ca²⁺ ion concentrations to reach full activation *in vitro*, respectively, and are the most widely studied isoforms [19,20]. It has been known that the abnormal over-activation of calpains is related to a number of human diseases such as brain and spinal cord injury, Alzheimer disease, cancer and cataract formation [21,22]. The human body is consistently regulating intracellular calcium levels, and if a cell receives a stimulus such as oxidative stress, calcium homeostasis is perturbed and the corresponding levels of intracellular calcium are up-regulated [23]. Such calcium influx activates calpains,

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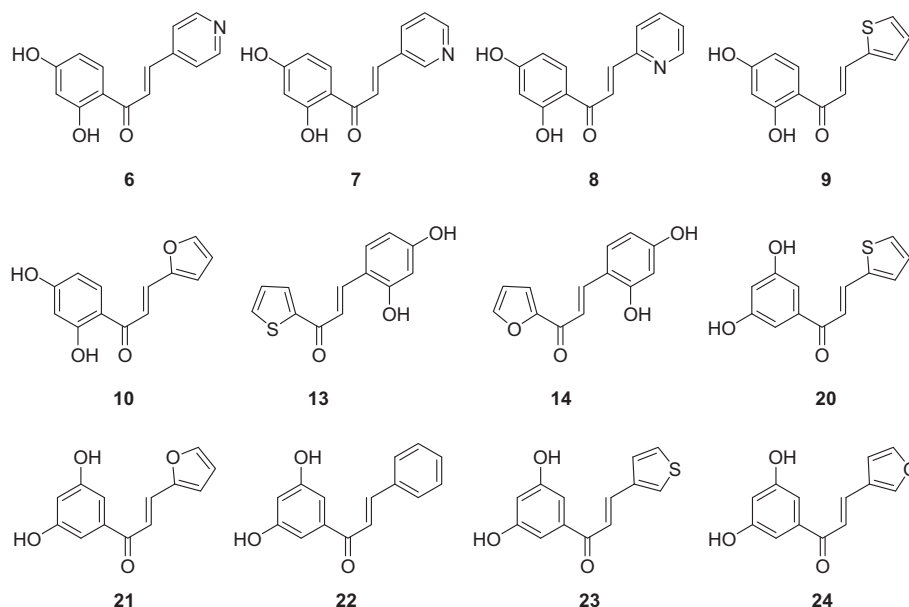
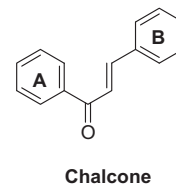


Fig. 1. Structure of synthesized compounds.

which in turn cleave many cytosolic proteins including α -spectrin, MAP-2 and tau, resulting in cytoskeletal rearrangements [17,24]. Heat shock protein (Hsp) 70.1, which is known as a protector for lysosomal membrane integrity from oxidative stress, is also cleaved by activated μ -calpain and carbonylated Hsp 70.1 in hippocampal cornu Ammonis 1 (CA1) tissue induced by oxidative stress is even more susceptible to μ -calpain-mediated cleavage leading to lysosomal rupture [25,26]. Lysosomal rupture is accompanied by the release of cathepsins, including cathepsin B, L and D, into the cytoplasm. These released cathepsins may in turn damage cellular constitutive proteins and cytoskeletons [27]. Especially, the released cathepsin B causes apoptotic neuronal cell death via activation of proapoptotic Bax and Bak and subsequent caspase activation pathway [28,29]. The released cathepsin L induces proteolytic activation of Bid, which in turn activates caspase-9 and caspase-3 [27]. The proteolytic activation of caspase-3 can be mediated by calpains besides cathepsin B and L and cleaved caspase-3 degrades calpastatin resulting in its inactivation, which consequently make worse pathological over-activation of calpain and cathepsins-mediated neuronal cell apoptosis [30,31]. Calpastatin, a well-known endogenous calpain inhibitor, has been reported to be involved in cell survival, apoptosis, and cognitive function in human brain by regulating calpain activation [30]. In this regard, the simultaneous regulation of μ -calpain and cathepsin B and L may be beneficial. Synthetic calpain inhibitors can be categorized into peptide-base inhibitors and non-peptide inhibitors [32]. Peptide-based calpain inhibitors are developed on the basis of calpain substrate. In general, they contain L-Val or L-Leu and are easily degraded by several proteases in cells due to their peptide-like characteristics and presence of an aldehyde moiety [33,34]. Conversely, several non-peptide calpain inhibitors have been reported, including chromone carboxamides, benzothiazine-1,1-dioxide, carboxamides, and aminoquinolinones [32,35–38]. Non-peptide calpain inhibitors may serve as better scaffolds for developing anti-neurodegenerative drugs because of their superior pharmacokinetic profiles compared with peptide-based calpain inhibitors [32].

Chalcones obtained by synthesis and from natural resources exhibit a diverse range of pharmacological effects, including anti-proliferative, anticancer, antioxidant, anti-inflammatory, and

antibiotic activities [39]. We previously reported that certain synthetic chalcones act as inhibitors of μ -calpain and cathepsins B and L [40]. As a continuation of this work to identify efficient calpain and cathepsins B and L inhibitors, we prepared several new dihydroxychalcone analogues and tested their biological activity using calpains and cathepsins B and L.

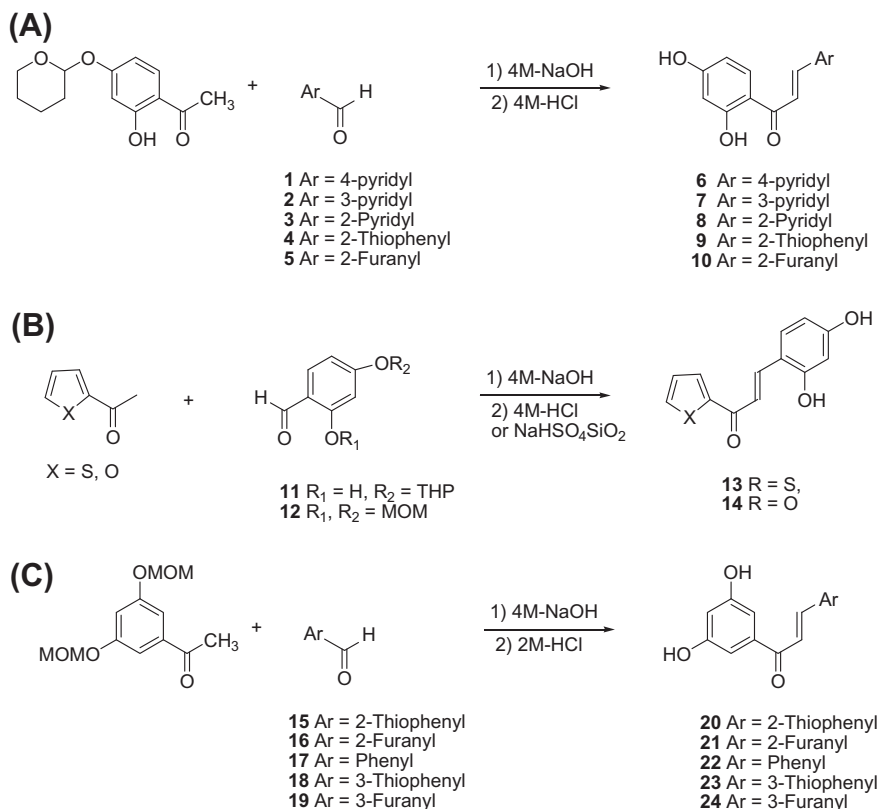


2. Results and discussion

2.1. Synthesis

All of the dihydroxylated chalcones were synthesized by a base-catalyzed *Claisen–Schmidt* condensation reaction using an appropriate aldehyde and ketone as previously described in the literature method with modification [39]. The general synthetic procedure is shown in Scheme 1. Briefly, aldehyde and ketone reagents containing hydroxyl groups were protected using either 2H-3,4-dihydropyran [41] or methyl chloromethyl ether (MOMCl) [42,43]. Overall the reaction proceeded smoothly with reaction yields of 64.5–80.2%; however, compounds **6–8**, **13** and **14** exhibited low reaction yields of 2.3–10.0%. We found that these compounds are relatively hydrophilic in nature which made the usual workup process difficult and may explain the low reaction yields of these compounds.

Activated silica-supported sodium hydrogen sulfate ($\text{NaHSO}_4 \cdot \text{SiO}_2$) [44,45] was used for the deprotection of compound **14**, since acid hydrolysis with aqueous HCl did not yield the desired product. All of the compounds prepared showed consistent spectroscopic data corresponding to the desired structures (see Fig. 1).



Scheme 1. Synthetic methods for the prepared compounds.

Table 1
Inhibitory activity of compounds against μ - and m -calpain.

Compound	Inhibitory activity as IC ₅₀ (μ M) ^a	
	μ -Calpain	m -Calpain
MDL28170	0.1000 \pm 0.0009	0.1500 \pm 0.0008
CA074	>100	>100
Z-FF-FMK	0.3971 \pm 0.0004	0.418 \pm 0.0046
6	>100	>100
7	>100	>100
8	>100	>100
9	56.17 \pm 0.048	39.89 \pm 1.054
10	86.74 \pm 0.751	25.35 \pm 0.901
13	>100	60.24 \pm 1.949
14	>100	>100
20	>100	>100
21	>100	>100
22	>100	>100
23	>100	97.29 \pm 2.357
24	>100	>100

^a Each data point represents the mean \pm S.D. of three independent experiments performed in triplicate. MDL28170 was used as a positive control for μ - and m -calpain inhibition.

2.2. Inhibitory activity against μ - and m -calpain

μ -Calpain and m -calpain inhibitory activities of compounds were determined according to the method previously reported [37]. Briefly, fluorometric assays were performed in 96-well plates and fluorescence intensity in each well was measured using a Microplate Fluorescence Reader (SpectraMAX GEMINI EM, Molecular Devices); the IC₅₀ values were obtained using the data graphing software, TableCurve 2D (Systat software Inc.). In the assay, only compounds **9**, **10** and **13** inhibited m -calpain activity with moderate efficiency (see Table 1). All of the other compounds were inactive. This result implied that 2,4-dihydroxy group on the A ring

Table 2
Inhibitory activity of compounds against cathepsin B and cathepsin L.

Compound	Inhibitory activity as IC ₅₀ (μ M) ^a	
	Cathepsin B	Cathepsin L
MDL28170	0.0958 \pm 0.0045	0.0031 \pm 0.0003
CA074	0.0044 \pm 0.00004	>100
Z-FF-FMK	0.0656 \pm 0.0029	0.0057 \pm 0.00002
6	50.25 \pm 0.004	44.40 \pm 0.216
7	>100	>100
8	>100	>100
9	>100	53.01 \pm 0.178
10	60.63 \pm 0.981	90.53 \pm 0.234
13	11.47 \pm 0.087	2.80 \pm 0.100
14	49.47 \pm 0.164	2.87 \pm 0.052
20	>100	>100
21	84.59 \pm 0.375	>100
22	>100	>100
23	>100	>100
24	>100	>100

^a Each data point represents the mean \pm S.D. of three independent experiments performed in triplicate. CA074 and Z-FF-FMK were used as positive controls for inhibition of cathepsin B and L, respectively.

of the chalcone structure (comparison of **9** vs **20**) was effective while presence of pyridine on the B ring in the chalcone structure hampered m -calpain inhibitory activity (comparison of **6–8** vs **9** and **10**). Thus, it is possible that the electronic effects (thiophene and furan vs pyridine) of the B ring site in the chalcone structure influence m -calpain inhibitory activity, although concrete evidence of this possibility was not obtained in the present study.

2.3. Inhibitory activity against cathepsin B and L

To determine the ability of compounds to inhibit cathepsins B and L, two substrates were used, namely, RR-AMC (Sigma, USA)

for cathepsin B and Z-FR-AMC (Sigma, USA) for cathepsin L. Compound **13** exhibited effective inhibitory activity against both cathepsin B and L with IC_{50} values of 11.47 ± 0.087 and 2.80 ± 0.100 μ M, respectively (see Table 2). Compound **14** was also a strong inhibitor of cathepsin L with an IC_{50} value of 2.87 ± 0.052 μ M. The other compounds either mildly inhibited cathepsin function or were inactive. Together, these results suggest that the 2,4-dihydroxy group on the B ring of the chalcone structure is important for cathepsin inhibition; however, we were unable to conclude a clear structure–activity relationship due to the limited numbers of compounds employed in this study. Regardless of position, the results of our study indicate that dihydroxy group in the A ring was not proper substituent for cathepsin B and L inhibitory activity.

3. Conclusion

In the present study we designed and synthesized 12 dihydroxychalcone analogues. Among the compounds that were prepared and tested, compound **10** exhibited the most active inhibitory activity against *m*-calpain with an IC_{50} value of 25.25 ± 0.901 μ M. With respect to inhibition of cathepsins B and L, compound **13** potently inhibited cathepsin L and moderately inhibited cathepsin B with IC_{50} values of 2.80 ± 0.100 and 11.47 ± 0.087 μ M, respectively. Based on our observations, the inhibitory activity of chalcone analogues on calpains and cathepsins appeared to require different functional groups. Specifically, a 2,4-dihydroxy group on the A ring was important for *m*-calpain inhibition while a 2,4-dihydroxy group on the B ring was needed for the inhibition of cathepsin B and L. Our results suggest the possibility of developing calpain and cathepsin dual inhibitors by properly modulating structures and/or combining the essential aspects of the functional group effective for specific calpain and cathepsin inhibition.

4. Experimental

4.1. General remarks

Compounds used as starting materials and reagents were obtained from Aldrich Chemical Co., Junsei or other chemical companies, and were utilized without further purification. HPLC grade acetonitrile (ACN) and methanol were purchased from Burdick and Jackson, USA. Thin-layer chromatography (TLC) and column chromatography (CC) were performed with Kieselgel 60 F_{254} (Merck) and silica gel (Kieselgel 60, 230–400 mesh, Merck), respectively. Because all of the prepared compounds contained aromatic rings, they were visualized and detected on TLC plates with UV light (short wave, long wave or both). NMR spectra were recorded on a Bruker AMX 250 or 300 (250 or 300 MHz, FT) for 1H NMR and 62.5 or 75.5 MHz for ^{13}C NMR, and chemical shifts were calibrated according to TMS. Chemical shifts (δ) were recorded in ppm and coupling constants (*J*) in hertz (Hz). Melting points were determined in open capillary tubes on electrothermal 1A 9100 digital melting point apparatus and were uncorrected.

HPLC analyses were performed using two Shimadzu LC-10AT pumps gradient-controlled HPLC system equipped with Shimadzu system controller (SCL-10A VP) and photo diode array detector (SPD-M10A VP) utilizing Shimadzu Class VP program. A sample volume of 10 μ L was injected in a Waters X-Terra[®] 5 μ M reverse-phase C_{18} column (4.6×250 mm) with a gradient elution of 40–100% of B in A for 10 min followed by 100–40% of B in A for 10 min at a flow rate of 1.0 mL/min at 254 nm UV detection. The mobile phase A consisted of double distilled water with 20 mM ammonium formate (AF) and B consisted of 90% ACN in

water with 20 mM AF. The purity of each compound was described as a percentage (%).

4.2. General synthetic method for protecting starting materials

Aldehyde or ketone containing hydroxyl groups were protected using either 2H-3,4-dihydropyran or methyl chloromethyl ether (MOMCl).

4.2.1. Synthesis of 1-(2-hydroxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl)ethanone

A solution of 2',4'-dihydroxyacetophenone (7.60 g, 50.00 mmol, 1.00 equiv.), pyridinium *p*-toluene sulfonate (0.50 g, 2.00 mmol, 0.04 equiv.) and 3,4-dihydro-2H-pyran (11.40 mL, 125.00 mmol, 2.50 equiv.) in methylene chloride was stirred for 4 h at room temperature. Then the reaction mixture was then extracted with methylene chloride (100 mL) and washed with water (30 mL \times 2) and a 1 M solution of Na_2CO_3 (30 mL \times 2). The organic layer was separated and dried over anhydrous Na_2SO_4 , and concentrated. When necessary, the residue was purified by silica gel column chromatography to yield a light orange solid compound (11.61 g, 98.3%). R_f = 0.38, E:H = 1:5, v/v; 1H NMR (250 MHz, $CDCl_3$) δ 12.58 (s, 1H, phenyl 2-OH), 7.65 (d, *J* = 8.8 Hz, 1H, phenyl H-6), 6.62 (d, *J* = 2.3 Hz, 1H, phenyl H-3), 6.57 (dd, *J* = 8.8, 2.4 Hz, 1H, phenyl H-5), 5.48 (t, *J* = 3.1 Hz, 1H, pyran H-2), 3.88–3.79 (m, 1H, pyran H-6), 3.64–3.59 (m, 1H, pyran H-6), 2.55 (s, 3H, CH_3), 2.02–1.56 (m, 6H, pyran H-3, H-4, H-5).

4.2.2. Synthesis of (2-hydroxy-4-((tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde (11)

A solution of 2,4-dihydroxybenzaldehyde (2.76 g, 20.00 mmol, 1.00 equiv.), pyridinium *p*-toluene sulfonate (0.20 g, 0.80 mmol, 0.04 equiv.) and 3,4-dihydro-2H-pyran (4.01 mL, 44.00 mmol, 2.20 equiv.) in methylene chloride (20 mL) was stirred for 4 h at room temperature. Then the reaction mixture was extracted then with methylene chloride (70 mL) and washed with water (25 mL \times 2) and a 1 M solution of Na_2CO_3 (20 mL \times 2). The organic layer was separated and dried over anhydrous Na_2SO_4 , and concentrated. When necessary, the residue was purified by silica gel column chromatography to yield an off-white viscous compound (4.01 g, 90%). R_f = 0.40, E:H = 1:5, v/v; 1H NMR (250 MHz, CD_3OD-d_4) δ 9.77 (s, 1H, CHO), 7.56 (d, *J* = 7.5 Hz, 1H, phenyl H-6), 6.67 (dd, *J* = 10.0, 2.5 Hz, 1H, phenyl H-5), 6.55 (dd, *J* = 2.5 Hz, 1H, phenyl H-3), 5.51 (t, *J* = 2.5 Hz, 1H, pyran H-2), 3.85–3.75 (m, 1H, pyran H-6), 3.64–3.56 (m, 1H, pyran H-6), 1.90–1.55 (m, 6H, pyran H-3, H-4, H-5).

4.2.3. Synthesis of 2,4-bis(methoxymethoxy)benzaldehyde (12)

To a solution of 2,4-dihydroxybenzaldehyde (2.00 g, 14.48 mmol, 1.00 equiv.) in DMF (43 mL), diisopropylethylamine (10.08 mL, 57.92 mmol, 4.00 equiv.) was added followed by dropwise addition of MOMCl (4.39 mL, 57.92 mmol, 4.00 equiv.) at room temperature. The reaction mixture was then stirred for 5 h at room temperature. Next, the reaction mixture was extracted with ethyl acetate (70 mL) and washed sequentially with water (15 mL \times 2), a 5% NaOH solution (10 mL \times 2) and a sat. NaCl solution (10 mL \times 2). The organic layer was separated, dried over anhydrous Na_2SO_4 , and concentrated to give a viscous compound. The final residue was separated by silica gel column chromatography using ethyl acetate and hexane as eluents to yield an off-white viscous compound (3.13 g, 95.7%). R_f = 0.48, E:H = 1:2, v/v; 1H NMR (250 MHz, $CDCl_3$) δ 10.34 (s, 1H, CHO), 7.82 (d, *J* = 8.7 Hz, 1H, phenyl H-6), 6.84 (d, *J* = 2.1 Hz, 1H, phenyl H-3), 6.77 (dd, *J* = 8.7, 2.1 Hz, 1H, phenyl H-5), 5.28 (s, 2H, OCH_2), 5.22 (s, 2H, OCH_2), 3.52 (s, 3H, OCH_3), 3.48 (s, 3H, OCH_3).

4.2.4. Synthesis of 1-(3,5-bis(methoxymethoxy)phenyl)ethanone

To a solution of 3',5'-dihydroxyacetophenone (1.00 g, 6.57 mmol, 1.00 equiv.) in ACN (25 mL), diisopropylethylamine (4.57 mL, 26.28 mmol, 4.00 equiv.) was added followed by dropwise addition of MOMCl (2.00 mL, 26.28 mmol, 4.00 equiv.) at room temperature. The reaction mixture was then stirred for 5 h at room temperature. The reaction mixture was then extracted with ethyl acetate (60 mL) and washed sequentially with water (10 mL \times 2), a 5% NaOH solution (10 mL \times 2) and a sat. NaCl solution (10 mL). The organic layer was then separated, dried over anhydrous Na_2SO_4 , and concentrated to give a brown viscous compound (1.30 g, 82.8%). R_f = 0.41, E:H = 1:2, v/v; ^1H NMR (250 MHz, CDCl_3) δ 7.27 (d, J = 2.2 Hz, 2H, phenyl H-2, H-6), 6.93 (t, J = 2.2 Hz, 1H, phenyl H-4), 5.19 (s, 4H, OCH_2), 3.48 (s, 6H, OCH_3), 2.55 (s, 3H, CH_3).

4.3. General synthetic method for hydroxylated chalcones

Method A: To an equimolar amount of the appropriate aldehyde and ketone in methanol/methylene chloride was added 4 M NaOH (2 equiv.) and left to react for 24 h at room temperature. The protecting group was subsequently removed by acid hydrolysis using 4 M HCl (3 equiv.) and reacted for 4 h at room temperature. Water was added to the reaction mixture (for compounds containing pyridine ring, neutralization with sat. NaHCO_3 after deprotection). The solid obtained was filtered and recrystallized in ethanol to yield desired chalcones. If solid was not formed, the mixture was extracted with methylene chloride and washed with water and brine. It was then dried over Na_2SO_4 and concentrated. The residue was then purified by silica gel column chromatography using ethyl acetate and hexane to obtain desired product.

Method B: To the equimolar amount of appropriate aldehyde and ketone in methylene chloride was added 4 M NaOH (2 equiv.) dropwise and the reaction was stirred for 3–5 h at 0 °C. Water was added to the reaction mixture. The solid obtained was filtered and washed with cold MeOH. The residue was then purified by silica gel column chromatography using ethyl acetate and hexane. The compound was characterized by ^1H NMR. The protecting groups were subsequently removed by acid hydrolysis using activated $\text{NaHSO}_4\cdot\text{SiO}_2$ (2.5 equiv.) and left to react for 3 h at room temperature. Then the catalyst was filtered off and washed with methanol. The filtrate was concentrated and dried. The final residue was separated by silica gel column chromatography to obtain the desired product.

Method C: To the equimolar amount of appropriate aldehyde and ketone in ethanol was added 4 M NaOH (2 equiv.) and the reaction was stirred for 20–24 h at room temperature. Then the reaction mixture was extracted with ethyl acetate and washed with water and brine. It was then dried over Na_2SO_4 and concentrated. The residue was then purified by silica gel column chromatography using ethyl acetate and hexane to obtain desired protected chalcone. The compound was characterized by ^1H NMR. The protected chalcone was dissolved in methanol followed by the addition of 2 M HCl (3 equiv.) at 60 °C and left to react for 1 h. The reaction mixture was submitted to the usual workup with ethyl acetate to give crude product, which was purified by chromatography on a silica gel column to give the desired product.

4.3.1. 1-(2,4-Dihydroxyphenyl)-3-(pyridin-4-yl)propenone (6)

Method A was employed with 1-(2-hydroxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl) ethanone and 4-pyridine carboxaldehyde to obtain yellow solid compound (0.10 g, 2.4%). mp 238–240 °C; R_f = 0.31, E:H = 3:1, v/v; purity by HPLC: 99%; ^1H NMR (250 MHz, $\text{DMSO}-d_6$) δ 12.94 (s, 1H, 1-phenyl 2-OH), 11.07 (br, 1H, 1-phenyl 4-OH), 8.95 (d, J = 7.5 Hz, 2H, 3-pyridine H-2, H-6), 8.44 (d, J = 15.0 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 8.42 (d, J = 7.5 Hz, 2H, 3-pyridine H-

3, H-5), 8.23 (d, J = 7.5 Hz, 1H, 1-phenyl H-6), 7.85 (d, J = 15.0 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 6.51 (dd, J = 10.0, 2.5 Hz, 1H, 1-phenyl H-5), 6.38 (d, J = 2.5 Hz, 1-phenyl H-3); ^{13}C NMR (62.5 MHz, $\text{DMSO}-d_6$) δ 190.46, 166.37, 166.00, 149.75, 143.54, 137.78, 133.79, 131.07, 125.36, 113.40, 108.96, 102.86.

4.3.2. 1-(2,4-Dihydroxyphenyl)-3-(pyridin-3-yl)propenone (7)

Method A was employed with 1-(2-hydroxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl) ethanone and 3-pyridine carboxaldehyde to obtain yellow solid compound (0.22 g, 5.3%). mp 247–249 °C; R_f = 0.33, E:H = 3:1, v/v; purity by HPLC: 98%; ^1H NMR (250 MHz, $\text{DMSO}-d_6$) δ 13.11 (s, 1H, 1-phenyl 2-OH), 10.91 (br, 1H, 1-phenyl 4-OH), 9.32 (s, 1H, 3-pyridine H-2), 8.88–8.81 (m, 2H, 3-pyridine H-6, H-4), 8.29 (d, J = 16.0 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 8.23 (d, J = 9.3 Hz, 1H, 1-phenyl H-6), 7.97 (dd, J = 8.0, 5.4 Hz, 1H, 3-pyridine H-5), 7.88 (d, J = 15.6 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 6.48 (dd, J = 8.8, 2.3 Hz, 1H, 1-phenyl H-5), 6.35 (d, J = 2.3 Hz, 1-phenyl H-3); ^{13}C NMR (62.5 MHz, $\text{DMSO}-d_6$) δ 190.85, 166.05, 144.82, 144.51, 141.76, 137.58, 133.57, 133.29, 126.41, 126.36, 113.18, 108.76, 102.84.

4.3.3. 1-(2,4-Dihydroxyphenyl)-3-(pyridin-2-yl)propenone (8)

Method A was employed with 1-(2-hydroxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl) ethanone and 2-pyridine carboxaldehyde to obtain yellow solid compound (0.10 g, 4.5%). mp 207–209 °C; R_f = 0.22, E:H = 1:2, v/v; purity by HPLC: 100%; ^1H NMR (250 MHz, $\text{DMSO}-d_6$) δ 12.99 (s, 1H, 1-phenyl 2-OH), 10.87 (br, 1H, 1-phenyl 4-OH), 8.80 (d, J = 5.0 Hz, 1H, 3-pyridine H-6), 8.46 (d, J = 15.4 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 8.26–8.24 (m, 2H, 3-pyridine H-4, H-3), 8.14 (d, J = 8.9 Hz, 1H, 1-phenyl H-6), 7.85 (d, J = 15.4 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 7.74 (dd, J = 8.6, 4.9 Hz, 1H, 3-pyridine H-5), 6.48 (d, J = 8.9 Hz, 1H, 1-phenyl H-5), 6.35 (d, J = 2.0 Hz, 1-phenyl H-3); ^{13}C NMR (62.5 MHz, $\text{DMSO}-d_6$) δ 190.61, 166.09, 165.92, 150.34, 146.69, 141.56, 137.86, 133.43, 128.20, 126.12, 125.93, 113.36, 108.95, 102.86.

4.3.4. 1-(2,4-Dihydroxyphenyl)-3-(thiophen-2-yl)propenone (9)

Method A was employed with 1-(2-hydroxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl) ethanone and 2-thiophene carboxaldehyde to obtain yellow solid compound (1.20 g, 64.5%). mp 140–142 °C; R_f = 0.40, E:H = 1:2, v/v; purity by HPLC: 98%; ^1H NMR (250 MHz, $\text{CD}_3\text{OD}-d_4$) δ 8.06 (d, J = 15.1 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 7.96 (d, J = 8.9 Hz, 1H, 1-phenyl H-6), 7.66 (d, J = 5.0 Hz, 1H, 3-thiophene H-5), 7.59 (d, J = 14.9 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 7.55 (d, J = 5.0 Hz, 1H, 3-thiophene H-3), 7.21 (dd, J = 5.0, 3.6 Hz, 1H, 3-thiophene H-4), 6.50 (dd, J = 8.9, 2.4 Hz, 1H, 1-phenyl H-5), 6.36 (d, J = 2.4 Hz, 1-phenyl H-3); ^{13}C NMR (62.5 MHz, $\text{MeOD}-d_4$) δ 192.75, 167.56, 166.64, 141.61, 137.56, 133.26, 133.16, 130.50, 129.52, 120.44, 114.59, 109.31, 103.88.

4.3.5. 1-(2,4-Dihydroxyphenyl)-3-(furan-2-yl)propenone (10)

Method A was employed with 1-(2-hydroxy-4-((tetrahydro-2H-pyran-2-yl)oxy)phenyl) ethanone and 2-furaldehyde to obtain orange solid compound (1.80 g, 78.2%). mp 123–125 °C; R_f = 0.22, E:H = 1:3, v/v; purity by HPLC: 100%; ^1H NMR (250 MHz, $\text{CD}_3\text{OD}-d_4$) δ 7.95 (d, J = 8.9 Hz, 1H, 1-phenyl H-6), 7.73 (s, 1H, 3-furan H-5), 7.71 (d, J = 15.2 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 7.62 (d, J = 15.2 Hz, 1H, $\text{CO}-\text{CH}=\text{CH}$), 6.93 (d, J = 3.4 Hz, 1H, 3-furan H-3), 6.65 (dd, J = 3.4, 1.8 Hz, 1H, 3-furan H-4), 6.49 (dd, J = 8.9, 2.4 Hz, 1H, 1-phenyl H-5), 6.36 (d, J = 2.4 Hz, 1-phenyl H-3); ^{13}C NMR (62.5 MHz, $\text{MeOD}-d_4$) δ 192.83, 167.54, 166.63, 153.18, 146.78, 133.23, 131.14, 119.13, 117.31, 114.66, 113.78, 109.30, 103.88.

4.3.6. 3-(2,4-Dihydroxyphenyl)-1-(thiophen-2-yl)propenone (13)

Method A was employed with 2-acetyl thiophene and (2-hydroxy-4-((tetrahydro-2H-pyran-2-yl)oxy)benzaldehyde to obtain red

viscous compound (0.05 g, 2.3%). R_f = 0.29, E:H = 1:1, v/v; purity by HPLC: 96%; ^1H NMR (250 MHz, $\text{CD}_3\text{OD}-d_4$) δ 8.09 (d, J = 15.5 Hz, 1H, CO—CH=CH), 7.96 (d, J = 3.7 Hz, 1H, 1-thiophene H-3), 7.80 (d, J = 4.9 Hz, 1H, 1-thiophene H-5), 7.59 (d, J = 15.6 Hz, 1H, CO—CH=CH), 7.51 (d, J = 8.1 Hz, 1H, 3-phenyl H-6), 7.21 (t, J = 4.4 Hz, 1H, 3-thiophene H-4), 6.36 (dd, J = 7.8, 2.0 Hz, 1H, 3-phenyl H-5), 6.32 (s, 1H, 3-phenyl H-3); ^{13}C NMR (62.5 MHz, MeOD- d_4) δ 185.06, 163.09, 161.01, 147.30, 142.46, 134.96, 133.19, 132.32, 129.51, 118.59, 115.31, 109.14, 103.54.

4.3.7. 3-(2,4-Dihydroxyphenyl)-1-(furan-2-yl)propenone (14)

Method B was employed with 2-acetyl furan and 2,4-bis(methoxymethoxy)benzaldehyde to obtain red viscous compound (0.02 g, 10%). R_f = 0.20, E:H = 1:1, v/v; purity by HPLC: 97%

^1H NMR (250 MHz, $\text{CD}_3\text{OD}-d_4$) δ 8.22 (d, J = 15.7 Hz, 1H, CO—CH=CH), 7.88 (dd, J = 1.6, 0.7 Hz, 1H, 1-furan H-5), 7.62 (d, J = 15.7 Hz, 1H, CO—CH=CH), 7.59 (d, J = 9.1 Hz, 1H, 3-phenyl H-6), 7.49 (dd, J = 3.6, 0.7 Hz, 1H, 1-furan H-3), 6.75 (dd, J = 3.6, 1.7 Hz, 1H, 1-furan H-4), 6.46 (dd, J = 8.7, 2.3 Hz, 1H, 3-phenyl H-5), 6.43 (d, J = 1.1 Hz, 1H, 3-phenyl H-3); ^{13}C NMR (62.5 MHz, MeOD- d_4) δ 180.82, 163.14, 161.03, 155.30, 148.37, 142.44, 132.24, 118.71, 118.27, 115.37, 113.55, 109.18, 103.58.

4.3.8. 1-(3,5-Dihydroxyphenyl)-3-(thiophen-2-yl)propenone (20)

Method C was employed with 1-(3,5-bis(methoxymethoxy)phenyl)ethanone and 2-thiophene carboxaldehyde to obtain yellow solid compound (0.58 g, 78.5%). mp 177–178 °C; R_f = 0.52, E:H = 1:1, v/v; purity by HPLC: 96%; ^1H NMR (300 MHz, $\text{CD}_3\text{OD}-d_4$) δ 7.85 (d, J = 15.3 Hz, 1H, CO—CH=CH), 7.54 (d, J = 5.1 Hz, 1H, 3-thiophene H-5), 7.42 (d, J = 3.3 Hz, 3-thiophene H-3), 7.27 (d, J = 15.6 Hz, 1H, CO—CH=CH), 7.08 (dd, J = 5.1, 3.9 Hz, 1H, 3-thiophene H-4), 6.85 (d, J = 2.1 Hz, 2H, 1-phenyl H-2, H-6), 6.45 (t, J = 2.1 Hz, 1H, 1-phenyl H-4); ^{13}C NMR (75.5 MHz, MeOD- d_4) δ 191.77, 160.14, 141.47, 141.25, 138.48, 133.63, 130.53, 129.57, 121.74, 108.27, 107.70.

4.3.9. 1-(3,5-Dihydroxyphenyl)-3-(furan-2-yl)propenone (21)

Method C was employed with 1-(3,5-bis(methoxymethoxy)phenyl)ethanone and 2-furaldehyde to obtain yellow solid compound (0.55 g, 80.0%). mp 189–190 °C; R_f = 0.45, E:H = 1:1, v/v; purity by HPLC: 100%; ^1H NMR (250 MHz, $\text{CD}_3\text{OD}-d_4$) δ 7.65 (d, J = 1.6 Hz, 1H, 3-furan H-5), 7.54 (d, J = 15.4 Hz, 1H, CO—CH=CH), 7.36 (d, J = 15.4 Hz, 1H, CO—CH=CH), 6.88 (d, J = 2.2 Hz, 2H, 1-phenyl H-2, H-6), 6.85 (d, J = 3.4 Hz, 3-furan H-3), 6.57 (dd, J = 3.4, 1.8 Hz, 1H, 3-furan H-4), 6.47 (t, J = 2.2 Hz, 1H, 1-phenyl H-4); ^{13}C NMR (62.5 MHz, MeOD- d_4) δ 191.86, 160.14, 152.96, 146.87, 141.32, 131.98, 120.30, 117.72, 113.85, 108.29, 107.72.

4.3.10. 1-(3,5-Dihydroxyphenyl)-3-phenylpropenone (22)

Method C was employed with 1-(3,5-bis(methoxymethoxy)phenyl)ethanone and benzaldehyde to obtain light yellow solid compound (0.25 g, 69.0%). mp 160–161 °C; R_f = 0.44, E:H = 1:1, v/v; purity by HPLC: 98%; ^1H NMR (250 MHz, $\text{CD}_3\text{OD}-d_4$) δ 7.83 (d, J = 15.7 Hz, 1H, CO—CH=CH), 7.78–7.75 (m, 2H, 3-phenyl H-2, H-6), 7.65 (d, J = 15.7 Hz, 1H, CO—CH=CH), 7.50–7.47 (m, 3H, 3-phenyl H-3, H-4, H-5), 7.01 (d, J = 2.1 Hz, 2H, 1-phenyl H-2, H-6), 6.59 (t, J = 2.1 Hz, 1H, 1-phenyl H-4); ^{13}C NMR (62.5 MHz, MeOD- d_4) δ 192.59, 160.11, 146.02, 141.39, 136.28, 131.70, 130.08, 129.59, 123.44, 108.36, 107.95.

4.3.11. 1-(3,5-Dihydroxyphenyl)-3-(thiophen-3-yl)propenone (23)

Method C was employed with 1-(3,5-bis(methoxymethoxy)phenyl)ethanone and 3-thiophene carboxaldehyde to obtain yellow solid compound (0.37 g, 77.5%). mp 127–128 °C; R_f = 0.44, E:H = 1:1, v/v; purity by HPLC: 100%; ^1H NMR (250 MHz, $\text{CD}_3\text{OD}-d_4$) δ 7.88 (dd, J = 2.5, 1.2 Hz, 1H, 3-thiophene H-2), 7.84 (d,

J = 15.6 Hz, 1H, CO—CH=CH), 7.61 (dd, J = 5.2, 1.2 Hz, 1H, 3-thiophene H-4), 7.56 (dd, J = 5.3, 2.8 Hz, 1H, 3-thiophene H-5), 7.47 (d, J = 15.6 Hz, 1H, CO—CH=CH), 6.99 (d, J = 2.1 Hz, 2H, 1-phenyl H-2, H-6), 6.58 (t, J = 2.1 Hz, 1H, 1-phenyl H-4); ^{13}C NMR (62.5 MHz, MeOD- d_4) δ 192.96, 160.07, 141.48, 139.64, 139.60, 130.76, 128.23, 126.46, 123.03, 108.25, 107.88.

4.3.12. 1-(3,5-Dihydroxyphenyl)-3-(furan-3-yl)propenone (24)

Method C was employed with 1-(3,5-bis(methoxymethoxy)phenyl)ethanone and 3-furaldehyde to obtain yellow solid compound (0.24 g, 80.2%). R_f = 0.38, E:H = 1:1, v/v, mp = 167–168 °C; purity by HPLC: 99%; ^1H NMR (250 MHz, $\text{CD}_3\text{OD}-d_4$) δ 7.97 (s, 1H, 3-furan H-2), 7.76 (d, J = 15.5 Hz, 1H, CO—CH=CH), 7.63 (dd, J = 1.9, 1.3 Hz, 1H, 3-furan H-5), 7.36 (d, J = 15.5 Hz, 1H, CO—CH=CH), 6.97 (d, J = 2.2 Hz, 2H, 1-phenyl H-2, H-6), 6.93 (dd, J = 1.9, 0.7 Hz, 1H, 3-furan H-4), 6.56 (t, J = 2.2 Hz, 1H, 1-phenyl H-4); ^{13}C NMR (62.5 MHz, MeOD- d_4) δ 192.63, 160.06, 147.49, 146.03, 141.39, 136.36, 124.75, 123.20, 108.49, 108.22, 107.87.

4.4. Measurement of μ - and m -calpain inhibitory activities of compounds

Fluorescence intensity was expressed as relative fluorescence units (RFUs). RFUs were calculated by subtracting the RFU of the no-enzyme control from all other values. To determine percent inhibition, the percent change in RFUs between the activity of the enzyme in the presence and absence of compounds was calculated. The value of RFU calculated in the absence of compound represents 100% enzyme activity.

Inhibition of μ -calpain (Calbiochem, Germany) was assayed in the reaction buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA and 5 mM β -mercaptoethanol, pH 7.5) with 100 μM pep1, 2.5 mM CaCl_2 , and 5.25 units/mL μ -calpain. The reaction was initiated by sequential addition of substrate A, μ -calpain, each compound, and finally a CaCl_2 solution. Thereafter, the reaction mixture of 100 μL was incubated at room temperature for 30 min with shaking. Fluorescence intensities were measured at 320 nm excitation and 420 nm emission wavelengths. The substrate A of μ -calpain was synthesized by Pepton Corp. (Daejeon, Korea) which is derived from the p35 cleavage site ([2-Abz]-Ser-Thr-Phe-Ala-Gln-Pro-[3-nitrotyrosine]-NH $_2$). Inhibition of m -calpain by the compounds was examined in the same way as for μ -calpain with minor modifications [46]. m -Calpain (recombinant, High Purity, *E. coli*) was obtained from Calbiochem (Darmstadt, Germany). Substrate B, originated from the calpain cleavage site in α -spectrin ([2-Abz]-Glu-Val-Tyr-Gly-Met-Met-[3-nitrotyrosine]-NH $_2$), was used as a substrate of m -calpain and was synthesized by the Pepton Corp. (Daejeon, Korea). The assay was performed in a final volume of 100 μL . Stock solution of substrate A, B and compounds were prepared in dimethylsulfoxide (DMSO) and stored at -20°C until needed. m -Calpain inhibition was assayed in reaction buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM β -mercaptoethanol, and 1 mM EDTA, pH 7.5) with 50 μM substrate B, 5 mM CaCl_2 and 2.88 U/mL m -calpain. The reaction was initiated by sequential addition of substrate B, m -calpain, the test compound, and the CaCl_2 solution. Thereafter, the mixture was incubated with shaking at room temperature for 30 min. Fluorescence intensities were determined at 320 nm for excitation and 420 nm for emission wavelengths. MDL28170 (Sigma, USA) was used as a positive control for μ - and m -calpain inhibition.

4.5. Measurement of cathepsin B and L inhibitory activities of compounds

Cathepsin B and L inhibitory activities were evaluated in reaction buffer (50 mM NaOAc-HCl, 2 mM dithiothreitol (DTT), 2 mM

EDTA, pH 5.5 for cathepsin B and 0.1 M NaOAc-HCl, 1 mM EDTA, 0.1% β -mercaptoethanol, pH 5.5 for cathepsin L) containing 20 μ M substrate and 1.5 nM cathepsin B or 4 nM cathepsin L. Cathepsins B and L were obtained from Calbiochem (Germany). RR-AMC (Sigma, USA) and Z-FR-AMC (Sigma, USA) were used as substrates for cathepsin B and L, respectively. Cathepsins were activated by incubation in assay buffer at 37 °C for 30 min prior to initiation of the reaction by addition of the substrate and test compound. The reaction mixtures were then incubated at room temperature for 30 min with shaking. Fluorescence intensities were determined at 360 nm excitation and 450 nm emission wavelengths. CA074 and Z-FF-FMK (Sigma, USA) were used as positive controls as for inhibition of cathepsin B and L, respectively.

Acknowledgements

This work was financially supported by CHA University, Pocheon, and the Korea Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0011007).

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