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# Chalcones as Novel Non-peptidic μ-Calpain Inhibitors

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In order to extend the scaffold of non-peptidic calpain inhibitor, we have designed and synthesized 14 chalcone derivatives categorized into two groups based on their structures. Compounds **7** ($IC_{50}$ = 16.67 ± 0.42 μM) and **8** ($IC_{50}$ = 16.92 ± 0.14 μM) in group A were most selective μ-calpain inhibitor over cathepsins B and L. On the other hand, compound **14** possessing furan ring exhibited inhibitory activities for μ-calpain ($IC_{50}$ = 15.39 ± 1.34 μM) as well as cathepsin B ($IC_{50}$ = 20.59 ± 1.35 μM). The results discovered implicated that chalcone analogues possessing proper size and functional groups can be a potential lead core for selective non-peptidic μ-calpain inhibitor. Furthermore, dual inhibitors for μ-calpain and cathepsin B can also be developed from chalcones by elaborate structure manipulation.

**Key Words :** Chalcone, Calpain inhibitor, Cathepsin B & L

## Introduction

Calpains first discovered in the CNS[1] are a family of intracellular $Ca^{2+}$-dependent cysteine protease expressed in the various cells and tissues.[2,3] Calpains consist of at least 15 isoforms encoded by an independent genes, which are well conserved, ubiquitously expressed and tissue specific.[4] Among them, calpain 1 (μ-calpain) and calpain 2 (μ-calapin) are ubiquitously distributed and the most widely studied isoforms that require micromolar and millimolar $Ca^{2+}$ ion concentrations respectively, for the activation in vitro.[5,6] Calpains have regulatory roles in the diverse cell physiology and abnormally elevated activation of calpain has been related with numbers of human diseases,[7,8] such as brain and spinal cord injury, Alzheimer disease, cancer, and cataract formation, etc.

Due to the diverse implication in the diseases, μ-calpain has attracted much interest as potential therapeutic target and numbers of inhibitors have been developed. Most inhibitors to date are peptide analogues represented by MDL-28170 (**1a**, Figure 1).[4,6,9] Peptidic calpain inhibitors, however, have limitation of clinical uses because of their lack of selectivity for calpain over other cysteine proteases, such as cathepsines B and L, and poor pharmacokinetic profiles *in vivo.* There is another peptidomimetic calpain inhibitor, E64d (**1b**)[10] which has been most widely employed as calpain inhibitor for in vivo study due to its enhanced cell permeability. Although enhanced permeability and potency inside cell, E64d forms a covalent bond with cysteine thiolates in the active site of enzymes leading to no selectivity for calpain. Efforts to evade the drawbacks of the peptidic calpain inhibitors have led researchers to focus on non-peptidic inhibitors acting as noncompetitive manner. Quinolinecarboxamide derivative, **2**,[11] showed potent μ-calpain inhibitory activity ($IC_{50}$ = 0.50 μM) and 50-fold higher selectivity for calpain over cathepsines B and L. Flavone analogue kaempferol, **3**, also exhibited moderate calpain inhibitory activity with $IC_{50}$ value of ~50.9 μM.[12] Another potent synthetic μ-calpain

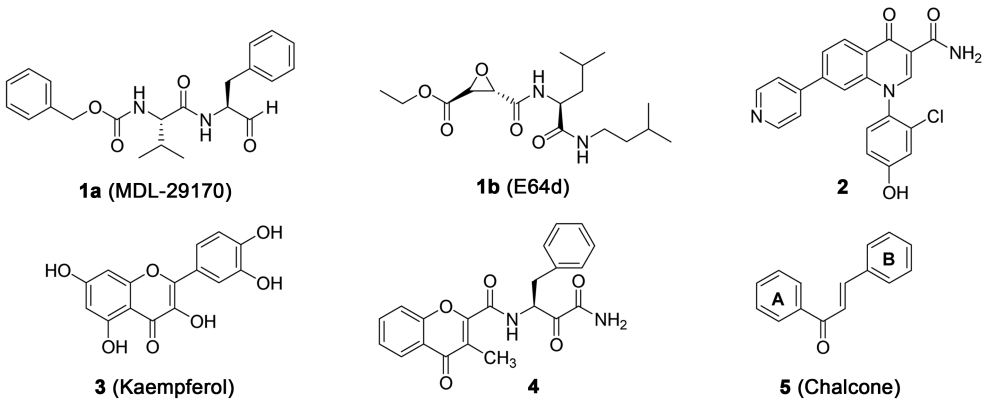



**Figure 1.** Representative calpain inhibitors.

inhibitor was chromone carboxamide analogue **4**. Compound **4** showed comparable inhibitory activity to MDL-28170 and even better selectivity over cathepsin B and L than MDL-28170.[13]

Natural and synthetic chalcones display a wide variety of pharmacological effects, including *anti*-proliferative, anti-cancer, antioxidant, *anti*-inflammatory, or *anti*-infective activities.[14] Chalcone derivatives have shown potential as lead compounds for the new drug discovery area due to their promising biological activity and safety profiles. Chalcones are also known as precursors of the flavonoids in higher plants. Flavones and chalcones are structurally very similar.

As an effort to extend the scaffold of non-peptidic calpain inhibitor we have synthesized a number of chalcone derivatives and tested their μ-calpain inhibitory activity versus cathepsins B and L.

## Experimental

The solvents and reagents were of the best commercial grade available and were used without further purification unless noted. TLC plates were Kieselgel 60 $F_{254}$ (Art A715, Merck) and silica gel for column chromatography was Silica gel 60 (0.040-0.063 mm ASTM, Merck). μ-calpain (human erythrocyte), cathepsin B and L were purchased from Calbiochem (Darmstadt, Germany). Pep2, a substrate of μ-calpain was synthesized by the Peptron Corp. (Daejeon, Korea). The cathepsin substrates, RR-AMC for cathepsin B and Z-FR-AMC for cathepsin L, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). MDL28170 (known as a μ-calpain inhibitor), E64d (known as a μ-calpain inhibitor), CA-074 (known as a cathepsin B inhibitor), and Z-FF-FMK (known as a cathepsin L inhibitor) were purchased from Sigma Chemical Co. $^1$H and $^{13}$C NMR spectra were taken on 400 MHz (Varian NMR AS) instruments. Chemical shifts (δ) are in parts per million (ppm) relative to tetramethylsilane as an internal standard, and coupling constants (*J* values) are in Hertz. The melting points were measured on a Barnstead International MEL-TEMP 1202D instrument or Gallenkamp Melting Point Apparatus without correction. Mass spectral investigations were run on a liquid chromatography-electrospray ionization-time of flight mass spectrometry (LC-ESI-TOF-MS, Agilent, USA) in a positive mode.

**General Procedure.** A mixture of acetophenone analogue (or acetyl 5-membered heteroaromatic analogue), benzaldehyde analogue and NaOH or $Ba(OH)_2$ in ethanol was stirred at room temperature. To the mixture was added 4 M-HCl and this mixture was stirred for more 20-40 min. 1) Water was added and solid formed was filtered and dried on the air. Solid was tritulated with mixed solvent, filtered and dried to give desired compound. or 2) After adding water, the reaction mixture was extracted with ethyl acetate and organic layer was washed with water. Organic layer was dried over anhydrous $Na_2SO_4$ and solvent was removed under reduced pressure. The residue was applied to silica gel column chromatography to give desired compound.

**(*E*)-3-(4-Hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (6).** A mixture of 1-(4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)ethanone (0.56 g, 2.55 mmol), 3-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)benzaldehyde (0.60 g, 2.55 mmol) and 10%-NaOH (1.01 mL, 2.5 mmol) in ethanol (15 mL) was stirred (16 h). Tritulation with mixed solvent (EtOAc:*n*-hexane) gave compound **6** (0.35 g, 50.2%) as an yellow solid. $R_f$: 0.07 (EtOAc/*n*-hexane = 1:3); mp 226-228 °C; $^1$H-NMR (400 MHz, $CDCl_3$+$CD_3OD$) δ 3.96 (s, 3H), 6.91 (d, *J* = 7.6 Hz, 1H), 6.92 (d, *J* = 8.0 Hz, 2H), 7.16 (s, 1H), 7.19 (d, *J* = 8.0 Hz, 1H), 7.41 (d, *J* = 15.6 Hz, 1H), 7.72 (d, *J* = 15.6 Hz, 1H), 7.96 (d, *J* = 8.0 Hz, 2H); $^{13}$C-NMR (100 MHz, $CDCl_3$+$CD_3OD$) 60.0, 114.7, 119.4, 119.5, 123.2, 127.4, 131.3, 134.2, 135.2, 148.9, 151.7, 153.0, 165.9, 194.0 ppm; HRMS (ESI) $[M-H]^-$ $C_{16}H_{13}O_4$ calcd 269.0819, found 269.0826.

**(*E*)-3-(5-Hydroxy-2-methoxyphenyl)-1-(4-hydroxyphenyl)prop-2-en-1-one (7).** A mixture of 1-(4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)ethanone (0.20 g, 0.89 mmol), 2-methoxy-5-(tetrahydro-2*H*-pyran-2-yloxy)benzaldehyde (0.21 g, 0.89 mmol) and NaOH (18.0 mg, 0.45 mmol) in ethanol (10 mL) was stirred (14 h). Tritulation with mixed solvent (EtOAc:*n*-hexane) gave compound **7** (85.0 mg, 35.6%) as an yellow solid. $R_f$: 0.38 (EtOAc/*n*-hexane = 1:3); mp 217-219 °C; $^1$H-NMR (400 MHz, $CD_3OD$) δ 3.83 (s, 3H), 6.83 (dd, *J* = 2.8, 8.8 Hz, 1H), 6.88 (d, *J* = 8.8 Hz, 2H), 6.89 (d, *J* = 8.8 Hz, 1H), 7.14 (d, *J* = 2.8 Hz, 1H), 7.64 (d, *J* = 15.8 Hz, 1H), 7.96 (d, *J* = 8.8 Hz, 2H), 8.01 (d, *J* = 15.8 Hz, 1H); $^{13}$C-NMR (100MHz, $CD_3OD$) 55.5, 112.8, 114.1, 115.2, 118.7, 121.7, 124.4, 129.9, 131.1, 139.3, 151.1, 152.8, 162.7, 190.1 ppm; HRMS (ESI) $[M-H]^-$ $C_{16}H_{13}O_4$ calcd 269.0819, found 269.0818.

**(*E*)-1-(4-Hydroxy-2-methoxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one (8).** A mixture of 1-(2-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)ethanone (0.23 g, 0.92 mmol), 4-(tetrahydro-2*H*-pyran-2-yloxy)benzaldehyde (0.19 g, 0.92 mmol) and NaOH (36.8 mg, 0.92 mmol) in ethanol (10 mL) was stirred (4 d). Tritulation with mixed solvent (EtOAc:*n*-hexane) gave compound **8** (0.18 g, 72.6%) as an yellow solid. $R_f$: 0.23 (EtOAc/*n*-hexane = 2:1); mp 223-225 °C; $^1$H-NMR (400 MHz, $CD_3OD$) δ 3.89 (s, 3H), 6.46 (dd, *J* = 2.4, 8.4 Hz, 1H), 6.52 (d, *J* = 2.4 Hz, 1H), 6.82 (d, *J* = 8.8 Hz, 2H), 7.41 (d, *J* = 15.6 Hz, 1H), 7.50 (d, *J* = 8.8 Hz, 2H), 7.56 (d, *J* = 15.6 Hz, 1H), 7.58 (d, *J* = 8.4 Hz, 1H); $^{13}$C-NMR (100 MHz, acetone-$d_6$) 55.4, 99.4, 108.1, 116.1, 121.4, 124.7, 127.3, 130.3, 132.8, 141.5, 159.8, 161.1, 162.9, 189.5 ppm; HRMS (ESI) $[M-H]^-$ $C_{16}H_{13}O_4$ calcd 269.0819, found 269.0819.

**(*E*)-1-(2,4-dihydroxyphenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (9).** A mixture of 1-(2-hydroxy-4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)ethanone (0.25 g, 1.1 mmol), 4-methoxybenzaldehyde (0.14 g, 1.1 mmol) and $Ba(OH)_2$ (0.34 g, 1.9 mmol) in ethanol (5 mL) was stirred (18 h). Tritulation with mixed solvent (EtOAc:*n*-hexane) gave compound **9** (93.3 mg, 37.3%) as an orange solid. $R_f$: 0.23 (EtOAc/*n*-hexane = 1:3); mp 184-185 °C; $^1$H-NMR (400 MHz, DMSO-$d_6$) δ 3.80 (s, 3H), 6.26 (d, *J* = 2.0 Hz, 1H),

6.39 (dd, $J$ = 1.6, 8.8 Hz, 1H), 7.00 (d, $J$ = 8.8 Hz, 2H), 7.75 (d, $J$ = 15.0 Hz, 1H), 7.82 (d, $J$ = 15.0 Hz, 1H), 7.84 (d, $J$ = 8.8 Hz, 2H), 8.17 (d, $J$ = 8.8 Hz, 1H); $^{13}$C-NMR (100 MHz, DMSO-$d_6$) 56.1, 103.2, 108.8, 113.6, 115.1, 119.2, 127.9, 131.7, 133.7, 144.4, 162.1, 165.8, 166.5, 192.2 ppm; HRMS (ESI) $[M-H]^-$ $C_{16}H_{13}O_4$ calcd 269.0819, found 269.0820.

**(*E*)-1-(3',4'-Methylenedixoyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one (10).** A mixture of 3',4'-(methylenedioxy)acetophenone (0.40 g, 2.42 mmol), 4-(tetrahydro-2*H*-pyran-2-yloxy)benzaldehyde (0.50 g, 2.42 mmol) and NaOH (24.2 mg, 0.6 mmol) in ethanol (10 mL) was stirred (16 h). Purification by silica gel column chromatography (eluent: EtOAc/*n*-hexane = 1:3) gave compound **10** (0.13 g, 20.0%) as an yellow solid. $R_f$: 0.18 (EtOAc/*n*-hexane = 1:3); mp 178-180 °C; $^1$H-NMR (400 MHz, $CDCl_3$+$CD_3OD$) δ 6.03 (s, 2H), 6.83 (d, $J$ = 8.8 Hz, 2H), 6.86 (d, $J$ = 8.4 Hz, 1H), 7.32 (d, $J$ = 15.6 Hz, 1H), 7.47 (d, $J$ = 2.0 Hz, 1H), 7.49 (d, $J$ = 8.4 Hz, 2H), 7.61 (dd, $J$ = 2.0, 8.4 Hz, 1H), 7.72 (d, $J$ = 15.6 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$+$CD_3OD$) 102.0, 108.1, 108.5, 116.1, 118.7, 124.7, 126.7, 130.6, 133.2, 145.2, 148.4, 151.8, 159.7, 189.3 ppm; HRMS (ESI) $[M-H]^-$ $C_{16}H_{11}O_4$ calcd 267.0663, found 267.0674.

**(*E*)-1-(4-Hydroxy-2-methoxyphenyl)-3-(pyridin-2-yl)-prop-2-en-1-one (11).** A mixture of 1-(2-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)ethanone (0.5 g, 2.0 mmol), 2-pyridinecarboxaldehyde (0.21 g, 2.2 mmol) and NaOH (80.0 mg, 2.0 mmol) in ethanol (15 mL) was stirred (15 h). Tritulation with mixed solvent (EtOAc:*n*-hexane) gave compound **11** (0.41 g, 80.4%) as a tan solid. $R_f$: 0.42 (EtOAc/*n*-hexane = 2:1); mp 170-172 °C; $^1$H-NMR (400 MHz, DMSO-$d_6$) δ 3.48 (s, 3H), 6.48 (dd, $J$ = 2.0, 8.4 Hz, 1H), 6.53 (d, $J$ = 2.0 Hz, 1H), 7.36-7.39 (m, 1H), 7.51 (d, $J$ = 15.6 Hz, 1H), 7.57 (d, $J$ = 8.4 Hz, 1H), 7.72 (d, $J$ = 7.6 Hz, 1H),7.84 (ddd, $J$ = 1.2, 7.6, 7.6 Hz, 1H), 7.91 (d, $J$ = 15.6 Hz, 1H), 7.64 (d, $J$ = 4.8 Hz, 1H), 10.46 (s, 1H); $^{13}$C-NMR (100 MHz, DMSO-$d_6$) 61.2, 104.7, 113.6, 125.1, 129.9, 135.8, 137.9, 142.6, 145.1, 155.4, 155.5, 158.6, 166.3, 168.7, 194.4 ppm; HRMS (ESI) $[M-H]^-$ $C_{15}H_{12}NO_3$ calcd 254.0823, found 254.0827.

**(*E*)-1-(4-Hydroxy-2-methoxyphenyl)-3-(pyridin-4-yl)-prop-2-en-1-one (12).** A mixture of 1-(2-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)phenyl)ethanone (0.5 g, 2.0 mmol), 4-pyridinecarboxaldehyde (0.24 g, 2.2 mmol) and 10%-NaOH (1.2 mL, 3.0 mmol) in ethanol (7 mL) was stirred (2 h). To the mixture was added 4 M-HCl (7 mL) and this mixture was stirred for more 30 min. After adding water, the reaction mixture was neutralized (pH = around 7) with sat-$NaHCO_3$, extracted with ethyl acetate and organic layer was washed with water. Purification by silica gel column chromatography (eluent: EtOAc/*n*-hexane = 1:1 → 5:1) gave compound **12** (92.0 mg, 12.9%) as an orange solid. $R_f$: 0.09 (EtOAc/*n*-hexane = 1:1); mp 245-247 °C; $^1$H-NMR (400 MHz, DMSO-$d_6$) δ 3.86 (s, 3H), 6.47 (d, $J$ = 8.8 Hz, 1H), 6.51 (s, 1H), 7.45 (d, $J$ = 15.6 Hz, 1H), 7.58 (d, $J$ = 8.4 Hz, 1H), 7.66 (d, $J$ = 4.8 Hz, 2H), 7.70 (d, $J$ = 15.6 Hz, 1H), 8.62 (d, $J$ = 4.8 Hz, 2H); $^{13}$C-NMR (100 MHz, DMSO-$d_6$) 56.4, 99.9, 108.9, 120.0, 122.8, 132.1, 133.3, 138.1, 143.0, 151.0, 161.7, 164.2, 189.1 ppm; HRMS (ESI) $[M-H]^-$ $C_{15}H_{12}NO_3$ calcd 254.0823, found 254.0827.

**(*E*)-3-(4-Hydroxy-3-methoxyphenyl)-1-(1*H*-pyrrol-2-yl)-prop-2-en-1-one (13).** A mixture of 2-acetylpyrrole (0.36 g, 3.3 mmol), 2-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)-benzaldehyde (0.78 g, 3.3 mmol) and 10%-NaOH (1.32 mL, 3.3 mmol) in ethanol (10 mL) was stirred (18 h). Purification by silica gel column chromatography (eluent: EtOAc/*n*-hexane = 1:1) gave compound **13** (40.0 mg, 5.3%) as an yellow solid. $R_f$: 0.47 (EtOAc/*n*-hexane = 1:1); mp 160-162 °C; $^1$H-NMR (400 MHz, $CDCl_3$) δ 3.75 (s, 3H), 6.33 (dd, $J$ = 1.2, 2.4 Hz, 1H), 6.48 (d, $J$ = 2.0 Hz, 1H), 6.53 (dd, $J$ = 2.0, 8.4 Hz, 1H), 7.08-7.10 (m, 2H), 7.36 (d, $J$ = 15.6 Hz, 1H), 7.47 (d, $J$ = 8.4 Hz, 1H), 8.10 (d, $J$ = 15.6 Hz, 1H), 8.18 (brs, 1H), 9.86 (s, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$) 55.6, 99.5, 108.5, 111.3, 116.4, 117.0, 119.4, 125.7, 131.0, 133.4, 139.2, 160.6, 160.8, 180.6 ppm; HRMS (ESI) $[M-H]^-$ $C_{14}H_{12}NO_3$ calcd 242.0823, found 242.0827.

**(*E*)-1-(Furan-2-yl)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-one (14).** A mixture of 2-acetylfuran (0.41 g, 3.7 mmol), 2-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)benzaldehyde (0.88 g, 3.7 mmol) and NaOH (0.15 g, 3.8 mmol) in ethanol (30 mL) was stirred (12 h). Purification by silica gel column chromatography (eluent: EtOAc/*n*-hexane = 1:3) gave compound **14** (0.42 g, 46.0%) as an yellow solid. $R_f$: 0.51 (EtOAc/*n*-hexane = 1:1); mp 162-164 °C; $^1$H-NMR (400 MHz, $CDCl_3$) δ 3.96 (s, 3H), 6.04 (s, 1H), 6.59 (dd, $J$ = 1.6, 4.0 Hz, 1H), 6.96 (d, $J$ = 8.4 Hz, 1H), 7.14 (s, 1H), 7.22 (d, $J$ = 8.4 Hz, 1H), 7.31 (d, $J$ = 15.6 Hz, 1H), 7.32 (d, $J$ = 4.0 Hz, 1H), 7.64 (s, 1H), 7.83 (d, $J$ = 15.6 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$) 56.2, 110.3, 112.7, 115.1, 117.3, 118.9, 123.7, 127.6, 144.5, 146.4, 147.0, 148.6, 154.1, 178.3 ppm; HRMS (ESI) $[M+H]^+$ $C_{14}H_{13}O_4$ calcd 245.0808, found 245.0804.

**(*E*)-3-(4-Hydroxy-3-methoxyphenyl)-1-(thiophen-2-yl)-prop-2-en-1-one (15).** A mixture of 2-acetylthiophene (0.29 g, 2.3 mmol), 3-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)-benzaldehyde (0.54 g, 2.3 mmol) and 10%-NaOH (0.9 mL, 2.3 mmol) in ethanol (10 mL) was stirred (15 h). Purification by silica gel column chromatography (eluent: EtOAc/*n*-hexane = 1:2) to give compound **15** (0.30 g, 50.4%) as a dark yellow solid. $R_f$: 0.28 (EtOAc/*n*-hexane = 1:3); mp 121-123 °C; $^1$H-NMR (400 MHz, $CDCl_3$) δ 3.97 (s, 3H), 6.00 (s, 1H), 6.97 (d, $J$ = 8.0 Hz, 1H), 7.12 (s, 1H), 7.18 (dd, $J$ = 4.2, 4.8 Hz, 1H), 7.24 (d, $J$ = 8.4 Hz, 1H), 7.28 (d, $J$ = 15.2 Hz, 1H), 7.67 (d, $J$ = 4.8 Hz, 1H), 7.80 (d, $J$ = 15.2 Hz, 1H), 7.87 (d, $J$ = 4.2 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$) 56.2, 110.5, 115.1, 119.4, 123.5, 127.5, 128.4, 131.7, 133.8, 144.6, 145.9, 147.0, 148.6, 182.3 ppm; HRMS (ESI) $[M-H]^-$ $C_{14}H_{11}O_3S$ calcd 259.0434, found 259.0432.

**(*E*)-1-(Furan-2-yl)-3-(4-hydroxy-2-methoxyphenyl)prop-2-en-1-one (16).** A mixture of 2-acetylfuran (0.17 g, 1.5 mmol), 2-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)benzaldehyde (0.36 g, 1.5 mmol) and 10%-NaOH (1.0 mL, 2.5 mmol) in ethanol (5 mL) was stirred (48 h). Solid was washed with water, *n*-hexane and mixed solvent ($CH_2Cl_2$/*n*-hexane), successively, to give compound **16** (0.12 g, 31.8%)

as an orange solid. $R_f$: 0.43 (EtOAc/*n*-hexane = 1:1); mp 168-170 °C; $^1$H-NMR (400 MHz, $CDCl_3$) δ 3.88 (s, 3H), 6.45 (d, *J* = 2.4 Hz, 1H), 6.48 (dd, *J* = 2.4, 8.0 Hz, 1H), 6.58 (dd, *J* = 1.6, 3.6 Hz, 1H), 7.30 (dd, *J* = 0.8, 3.6 Hz, 1H), 7.42 (d, *J* = 15.6 Hz, 1H), 7.50 (d, *J* = 8.4 Hz, 1H), 7.65 (dd, *J* = 0.8, 2.0 Hz, 1H), 8.13 (d, *J* = 15.6 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$) 55.6, 99.3, 108.3, 112.6, 115.8, 117.4, 118.4, 131.4, 140.7, 146.6, 154.1, 161.1, 161.5, 179.5 ppm; HRMS (ESI) $[M-H]^-$ $C_{14}H_{11}O_4$ calcd 243.0663, found 243.0657.

**(*E*)-3-(4-Hydroxy-2-methoxyphenyl)-1-(thiophen-2-yl)-prop-2-en-1-one (17).** A mixture of 2-acetylthiophene (0.27 g, 2.1 mmol), 2-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)-benzaldehyde (0.50 g, 2.1 mmol) and NaOH (84.7 mg, 2.1 mmol) in ethanol (20 mL) was stirred (12 h). Solid was washed with water, *n*-hexane and mixed solvent ($CH_2Cl_2$/*n*-hexane), successively, to give compound **17** (0.37 g, 67.1%) as an yellow solid. $R_f$: 0.18 (EtOAc/*n*-hexane = 1:2); mp 155-157 °C; $^1$H-NMR (400 MHz, $CDCl_3$+$CD_3OD$) δ 3.88 (s, 3H), 6.45 (s, 1H), 6.48 (d, *J* = 8.4 Hz, 1H), 7.18 (dd, *J* = 4.0, 4.8 Hz, 1H), 7.44 (d, *J* = 15.6 Hz, 1H), 7.49 (d, *J* = 8.4 Hz, 1H), 7.66 (d, *J* = 4.8 Hz, 1H), 7.84 (s, 1H), 8.07 (d, *J* = 15.6 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$+$CD_3OD$) 55.5, 99.2, 108.3, 115.6, 118.9, 128.3, 131.7, 133.4, 133.5, 140.9, 146.0, 161.1, 161.5, 183.6 ppm; HRMS (ESI) $[M-H]^-$ $C_{14}H_{11}O_3S$ calcd 259.0434, found 259.0428.

**(*E*)-3-(4-Hydroxyphenyl)-1-(thiophen-2-yl)prop-2-en-1-one (18).** A mixture of 2-acetylthiophene (0.36 g, 2.9 mmol), 4-(tetrahydro-2*H*-pyran-2-yloxy)benzaldehyde (0.59 g, 2.9 mmol) and 10%-NaOH (1.14 mL, 2.9 mmol) in ethanol (10 mL) was stirred (18 h). Solid was washed with water, *n*-hexane and mixed solvent ($CH_2Cl_2$/*n*-hexane), successively, to give compound **18** (0.46 g, 70.3%) as an yellow solid. $R_f$: 0.28 (EtOAc/*n*-hexane = 1:3); mp 177-179 °C; $^1$H-NMR (400 MHz, $CDCl_3$+$CD_3OD$) δ 6.88 (d, *J* = 8.6 Hz, 2H), 7.19 (dd, *J* = 4.0, 4.8 Hz, 1H), 7.29 (d, *J* = 15.2 Hz, 1H), 7.54 (d, *J* = 8.6 Hz, 2H), 7.69 (dd, *J* = 0.8, 4.8 Hz, 1H), 7.81 (d, *J* = 15.2 Hz, 1H), 7.87 (dd, *J* = 0.8, 4.0 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$+$CD_3OD$) 116.1, 118.5, 126.4, 128.4, 130.7, 131.9, 133.8, 144.9, 145.7, 160.0, 182.7 ppm; HRMS (ESI) $[M-H]^-$ $C_{13}H_9O_2S$ calcd 229.0329, found 229.0331.

**(*E*)-3-(4-Hydroxyphenyl)-1-(thiophen-2-yl)prop-2-en-1-one (19).** A mixture of 2-acetylthiophene (0.35 g, 2.7 mmol), 3-methoxy-4-(tetrahydro-2*H*-pyran-2-yloxy)benz-aldehyde (0.70 g, 2.6 mmol) and 10%-NaOH (4.4 mL, 11.0 mmol) in ethanol (10 mL) was stirred (15 h). Purification by silica gel column chromatography (eluent: EtOAc/*n*-hexane = 1:2) gave compound **19** (0.50 g, 62.8%) as an yellow solid. $R_f$: 0.56 (EtOAc/*n*-hexane = 1:1); mp 149-150 °C; $^1$H-NMR (400 MHz, $CDCl_3$) δ 3.97 (s, 6H), 5.86 (s, 1H), 6.89 (s, 2H), 7.19 (dd, *J* = 4.0, 4.4 Hz, 1H), 7.27 (d, *J* = 15.4 Hz, 1H), 7.68 (d, *J* = 4.8 Hz, 1H), 7.78 (d, *J* = 15.4 Hz, 1H), 7.88 (d, *J* = 4.0 Hz, 1H); $^{13}$C-NMR (100 MHz, $CDCl_3$) 56.6, 105.8, 119.7, 126.4, 128.4, 131.8, 133.9, 137.8, 144.9, 145.9, 147.5, 182.2 ppm; HRMS (ESI) $[M-H]^-$ $C_{15}H_{13}O_4S$ calcd 289.0540, found 289.0548.

**Fluorimetric μ-calpain Inhibition Assay.** The fluorimetric assay was performed in 96-well plates as described previously.[15] The substrate used was fluorescence-based probe and designed to possess the calpain-cleavage site in p35 which was [2-Abz]-Ser-Thr-Phe-Ala-Gln-Pro-[3-nitro-tyrosine]-$NH_2$ named pep2. The specific cleavage in pep2 by μ-calpain occurs between phenylalanine and alanine. It consists of a donor fluorescence group and an acceptor moiety that is capable of quenching intramolecularly the donor's fluorescence. The cleavage between phenylalanine and alanine results in the increase in fluorescence intensity and thus an indication of calpain activity. The assay was performed in a final volume of 100 μL. Stock solutions of pep2 and inhibitors were prepared in DMSO and stored at −20 °C. Calpain inhibition was assayed in 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 pep2, 2.5 mM $CaCl_2$ and 5.25 units/mL μ-calpain. The reaction was initiated by adding in the order of substrate, μ-calpain, compound, and $CaCl_2$ solution. Thereafter, incubated with shaking at room temperature for 30 min. Fluorescence intensities were measured using 360 nm excitation and 420 nm emission wavelength. The end-point fluorescence intensity in each well was measured in a Microplate Fluorescence Reader (FL600, Bio Tek), and the $IC_{50}$ values were obtained using the data graphing software TableCurve 2D (Systat software Inc.). Fluorescence intensity was indicated by Relative fluorescence unit (RFU). RFU was calculated by subtracting the RFU of the control from all other values. To determine percent inhibition, the percent change in RFU between the activity of the enzyme in the presence and absence of the inhibitor was calculated. Resulting RFU in the absence of inhibitor represents 100% enzyme activity.

**Fluorimetric Cathepsin B and L Assay.** The method for cathepsin B and L activity measurement was modified from the method of Dominic Cuerrier *et al.*[16] This method was performed in a final volume of 100 μL. Cathepsin B and L inhibitory activities were assayed in the reaction buffer (50 mM NaOAc-HCl, 2 mM dithiothreitor (DTT), 2 mM EDTA, adjusted to pH 5.5 for Cat-B and 0.1 M NaOAc-HCl, 1 mM EDTA, 0.1% β-mercaptoethanol, adjusted to pH 5.5 for Cat-L) with 20 μM each substrate, 1.5 nM cathepisn-B and 4 nM cathepsin L, respectively. The substrates used were RR-AMC for cathepsin B and Z-FR-AMC for cathepsin L. The cathepsins were activated by pre-incubation in assay buffer for 30 min prior to initiating the reaction by addition of substrate and inhibitor. Afterwards, shaking incubation was performed at room temperature for 30 min. Fluorescence intensities were determined using 360 nm excitation and 460 nm emission wavelength. The % inhibition of compounds was obtained as described above in fluorimetric μ-calpain inhibition assay

## Results and Discussion

**Chemistry.** General synthetic methods[17] for the chalcone compounds are described in Scheme 1. The phenolic OH group at 4-position on the acetyl aryl and aryl aldehyde was

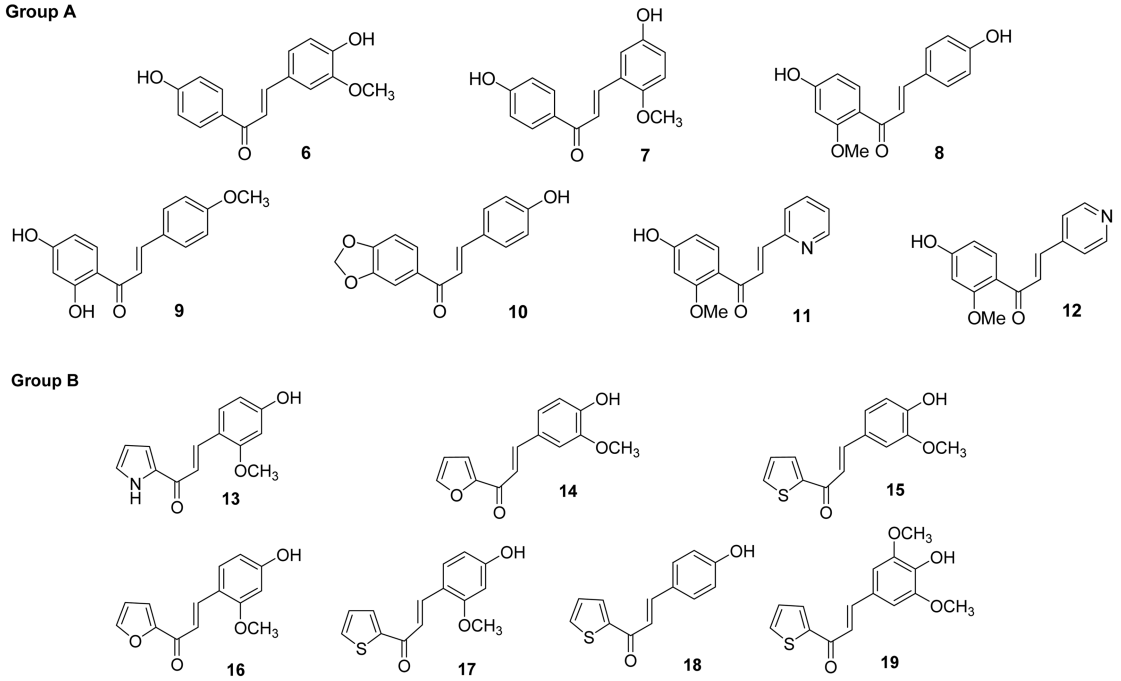
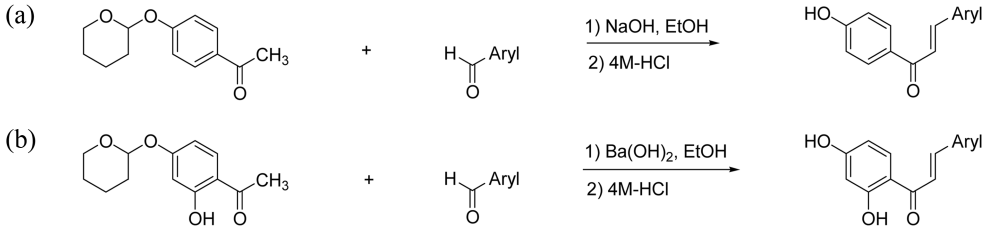



**Figure 2.** Structures of prepared chalcone derivatives.




**Scheme 1.** General methods for chalcone analogue synthesis.

protected with tetrahydropyranyl group. Without protection of these OH groups the reaction has not provided satisfactory results. All the reactions provided expected products in 5.3-80.4% reaction yields.

We have designed and synthesized two different groups, A and B (Figure 2). Group A compounds are conventional chalcone derivatives and possess 4-OH on A-ring of chalcone core except compound **10**, introduced methylenedioxy substituent. Group B compounds that phenyl ring was replaced by hetero aromatic rings were prepared. The purpose of comparison of these two groups is to understand and identify the skeleton of chalcone derivatives contributing to calpain inhibitory activities. A total of 14 chalcone derivatives were prepared.

**Pharmacological Test.** All the compounds were tested for the μ-calpain inhibitory activities over cathepsins B and L, respectively. MDL28170 (μ-calpain inhibitor), E64d (μ-calpain inhibitor), CA-074 (cathepsin B inhibitor), and Z-FF-FMK (cathepsin L inhibitor) were used as positive controls for each enzymes.[15] The results are listed in Table 1.

Most compounds showed from weak to moderate μ-calpain inhibitory activities regardless of structural modification in the conventional chalcones. In the group A, compounds **6-9**

**Table 1.** Inhibitory activity of compounds and positive controls against μ-calpain, cathepsin B and L

| Compounds | Inhibitory activity as $IC_{50}$ (μM)[a] | | |
|---|---|---|---|
| | μ-Calpain | Cathepsin B | Cathepsin L |
| MDL28170 | 0.0990 ± 0.0002 | 0.0142 ± 0.0017 | 0.0004 ± 0.0005 |
| CA-074 | >50 | 0.0037 ± 0.0003 | >50 |
| Z-FF-FMK | 0.3971 ± 0.0004 | 0.0656 ± 0.0029 | 0.0837 ± 0.0008 |
| E64d | 62.26 ± 7.73 | 14.39 ± 0.18 | 2.41 ± 0.09 |
| **6** | 17.03 ± 0.49 | >50 | 43.29 ± 2.92 |
| **7** | 16.67 ± 0.42 | >50 | >50 |
| **8** | 16.92 ± 0.14 | >50 | >50 |
| **9** | 14.69 ± 1.15 | >50 | 35.16 ± 2.84 |
| **10** | 21.06 ± 1.25 | >50 | >50 |
| **11** | 42.59 ± 6.77 | >50 | >50 |
| **12** | 43.90 ± 9.60 | >50 | >50 |
| **13** | 33.83 ± 2.10 | >50 | >50 |
| **14** | 15.39 ± 1.34 | 20.59 ± 1.35 | >50 |
| **15** | 22.51 ± 2.47 | 44.72 ± 1.20 | >50 |
| **16** | 13.38 ± 0.02 | 17.49 ± 3.85 | 44.52 ± 0.39 |
| **17** | 20.36 ± 1.77 | 20.66 ± 2.56 | 41.92 ± 1.54 |
| **18** | 15.95 ± 2.23 | >50 | 48.28 ± 0.31 |
| **19** | 18.53 ± 0.84 | 24.71 ± 2.38 | 48.24 ± 0.47 |

[a]Each data point represents mean ± S.D. from three different experiments performed in triplicate.

inhibited μ-calpain activity moderately with $IC_{50}$ values around 17 μM but compounds **11** and **12**, pyridyl group congeners in B ring of chalcone, showed decreased activities by almost half. All the compounds in group A were more selective inhibitors for μ-calpain than cathepsin B. Compounds in the group B have hetero aromatic ring in A part of chalcone and inhibited μ-calpain activity with moderate $IC_{50}$ values between 13.38 ± 0.02 μM (compound **16**) and 33.83 ± 2.10 μM (compound **13**). Furan coupled compounds (**14** and **16**) showed increased μ-calpain inhibitory activity than corresponding thiophene coupled ones (**15** and **17**). The selectivity of compounds in group B on μ-calpain was lower than that of group A compounds. All the compounds in group B except compound **13** showed comparable inhibitory activity on μ-calpain and cathepsins B and/or L.

In conclusion, we have designed and prepared 14 chalcone derivatives categorized into two different groups based on their structures and tested their μ-calpain inhibitory activity over cathepsins B and L. Most compounds inhibited μ-calpain activity with moderate $IC_{50}$ values. Compounds **7** and **8** in group A were most selective μ-calpain inhibitor over cathepsins B and L. Compounds **14**, **16**, **17** and **19** in group B, however, showed comparable inhibitory activity on both μ-calpain and cathepsins B. Our findings from the study provided that chalcone can be a potential lead core for the non-peptidic μ-calpain inhibitor and rational modification of the structures can derive selective μ-calpain inhibitor and dual inhibitor for μ-calpain and cathepsin B.

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