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Original article

Synthesis of chromone carboxamide derivatives with antioxidative and calpain inhibitory properties

Sang Hoon Kim [a,b], Young Hoon Lee [a,b], Seo Yun Jung [a], Hyoung Ja Kim [c], Changbae Jin [c], Yong Sup Lee [a,b,*]

[a] Department of Pharmaceutical Science, College of Pharmacy, Kyung Hee University, 1 Hoegi-dong Dongdaemoon-ku, Seoul 130-701, Republic of Korea
[b] Department of Life and Nanopharmaceutical Sciences, Kyung Hee University, Seoul 130-701, Republic of Korea
[c] Life and Health Division, Korea Institute of Science and Technology, PO Box 131 Cheongryang, Seoul 130-650, Republic of Korea

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ABSTRACT

The overactivation of μ-calpain can cause serious cell damage in several diseases. Furthermore, cell death in a number of neurodegenerative disorders is linked to the overproduction of reactive oxygen species. Therefore, antioxidants and μ-calpain inhibitors could have the therapeutic potentials to treat cell death related diseases. New chromone carboxamide derivatives **3** were synthesized to provide alternative μ-calpain inhibitors to compound **2**, a conformationally constrained structural variant of MDL 28,170. Compounds **3h** and **3l** exhibited the most potent μ-calpain inhibitory activities (IC_{50} = 0.09–0.10 μM), and were comparable to **2** in this respect (IC_{50} = 0.07 μM). Compound **3i** showed both potent μ-calpain inhibitory activity (IC_{50} = 0.28 μM) and antioxidant activities in DPPH scavenging and lipid peroxidation inhibition assays.

1. Introduction

The calpains (E.C. 3.4.22.17) are a family of cytosolic cysteine proteases, which are expressed ubiquitously in cells and tissues [1]. Two major classes of calpains have been identified, calpain I (μ-calpains) and calpain II (m-calpains), which need micromolar and millimolar concentrations of calcium ions, respectively, for activation [2]. Calpains are involved in numerous cellular processes, such as, signal transduction, cell migration, differentiation, and apoptosis [3]. However, in some neurological disorders such as stroke [4], Parkinson's disease [5] and Alzheimer's disease [6], calpains are overactivated and cause serious cell damage or even cell death. These enzymes have therefore attracted considerable interest as an important class of targets in neurodegenerative diseases [7]. Moreover, recent reports suggest that calpain inhibition may be beneficial for alleviating memory loss in Alzheimer's disease [8] and for preventing lissencephaly [9], heat stress-induced germ cell apoptosis [10], and TNF-α-induced inflammatory responses [11].

Most of the previously reported calpain inhibitors bind competitively to its catalytic site and are usually derived from small peptides, such as MDL 28,170 (**1**), which structurally mimic the cleavage sites of calpain substrates [12,13]. However, MDL 28,170 suffers from the disadvantages of non-selectivity, instability during storage, and a short half life *in vivo* due to its peptide character and the high reactivity of its aldehyde group [14]. Concerning our work to identify a new scaffold for μ-calpain inhibitors, we recently reported that chromone carboxamide or quinoline carboxamide, which are conformationally restricted cyclic analogs of MDL 28170, displayed the μ-calpain inhibitory activity [15–17]. However, the most potent chromone carboxamide (**2**, KYS 4516), which has a primary amide group at its keto-amide position, was unstable during storage and under acidic conditions, limiting our further research pursuits. Thus, extended derivatization of **2** was needed to produce alternative inhibitors with the same level of μ-calpain inhibitory activity as **2**. In this report, we describe the synthesis of new chromone carboxamide derivatives **3** and their biological evaluation for their μ-calpain inhibitory activities (Fig. 1). We modified the chromone ring (R^1, R^2) and amide region (R^3) of **2**, and introduced three different keto-amides, a primary amide, a benzyl, or 4-methoxyphenethyl amide to the warhead position (R^3) of the catalytic site of the enzyme and their μ-calpain inhibitory activities were later compared.

* Corresponding author. Kyung Hee East-West Pharmaceutical Research Institute, Department of Pharmaceutical Science, College of Pharmacy, Kyung Hee University, 1 Hoegi-dong Dongdaemoon-ku, Seoul 130-701, Republic of Korea. Tel.: +82 2 961 0370; fax: +82 2 966 3885.
E-mail address: kyslee@khu.ac.kr (Y.S. Lee).

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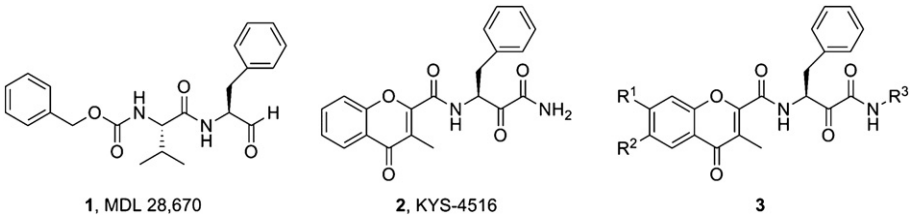


Fig. 1. Design of chromone derivatives as new μ-calpain inhibitors.

For substituents at R^1 and R^2, methoxy and hydroxyl groups were chosen, because both provide hydrogen-bonding donors or acceptors to promote binding at the active sites of enzymes. Cell death in a number of neurodegenerative disorders is also linked to the overproduction of reactive oxygen species [18]. Therefore, antioxidants that can scavenge oxygen free radicals have therapeutic potential for the treatment of neuronal cell death. The chromone ring has been used as an important scaffold for the synthesis of antitumor, insecticidal and pesticidal agents [19–21]. We also found that polyphenolic chromones can protect neuronal cells against H_2O_2-induced oxidative injuries [22]. Thus, it was expected that the substitution of hydroxyl groups on the chromone ring of **3** would lead to antioxidant and μ-calpain inhibitory activities. Accordingly, synthesized compounds were also assessed for their antioxidant activities using two assay systems.

2. Chemistry

The synthesis of chromone carboxamide derivatives **3** was accomplished using a general pathway, which involves a coupling reaction between a chromone carboxylic acid **7** and a hydroxylamide **12** using an EDC/HOBt system, and subsequent oxidation of the resulting hydroxylamides under Dess–Martin periodinane conditions, as illustrated in Scheme 3. For the synthesis of **3**, which contains a hydroxyl-substituent on its aromatic ring, a final deprotection step was required.

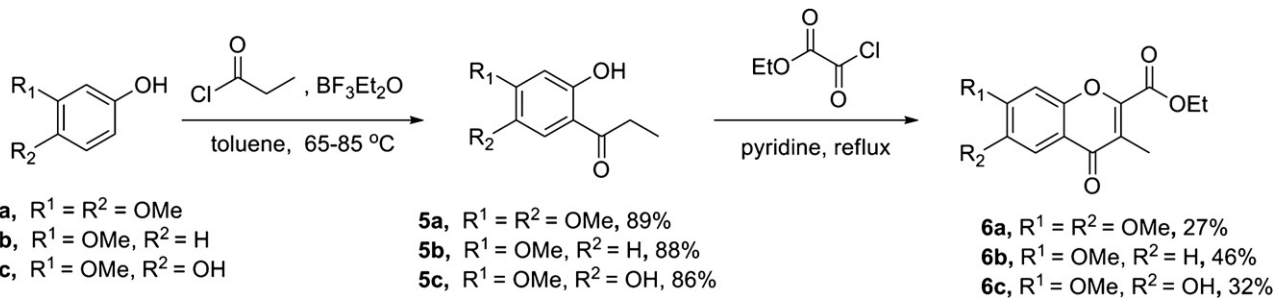
Phenols **4a** and **4c** were transformed to 2-hydroxypropiophenones **5a** and **5c** by Fries rearrangement by propionyl chloride treatment in the presence of $BF_3 \cdot Et_2O$ at reflux temperature [23]. For phenol **4b**, toluene was used as a co-solvent because of its low solubility in $BF_3 \cdot Et_2O$ [24]. A chromone ring was constructed by acylating **5a**–**c** with ethyl oxalyl chloride followed by *in situ* cyclization of the resulting ester in the presence of pyridine at reflux temperature to afford chromone carboxylic acid ethyl esters **6a**–**c** (Scheme 1).

The chromone carboxylic acids **7**, which have a methoxy or MOM-protected hydroxyl group on the phenyl ring were prepared from three chromone carboxylic acid ethyl esters **6** (Scheme 2). The methoxy-substituted chromone carboxylic acids **7a** and **b** were obtained directly by the hydrolysis of the corresponding ethyl esters **6a** and **b** using KOH in aqueous ethanol. To synthesize the hydroxyl-substituted chromone carboxamides **3g**–**l**, hydroxyl groups at the aromatic ring of chromone were protected with MOM before hydroxyl-amide **12** coupling. Compound **6c** was reacted with methoxymethyl chloride (MOM-Cl) and then hydrolyzed with KOH in aqueous ethanol to afford **7c**. To synthesize the 7-hydroxy- and 6,7-dihydroxychromone carboxamides **3i**–**l**, the required hydroxyl groups were generated by the hydrolysis of the methyl ether group in **6a** and **b**. Briefly, the compounds **6a** and **b** were treated with 48% HBr in the presence of glacial acetic acid [25] and then converted to esters (**10a** and **b**) using H_2SO_4 in MeOH. The hydroxyl esters **10a** and **b** were again transformed to MOM-protected chromone carboxylic acids (**7d** and **e**) after MOM-protection followed by ester hydrolysis.

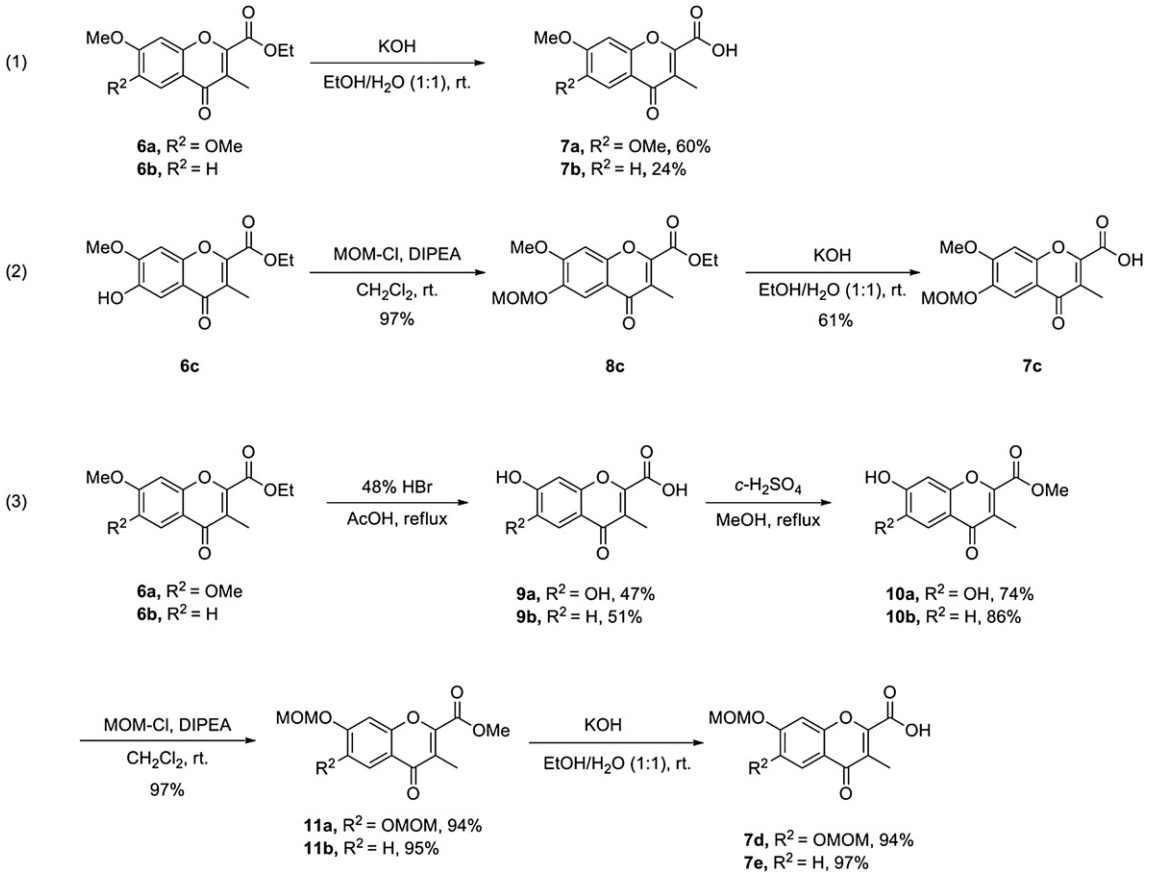
The synthesis of chromone carboxamide derivatives **3a**–**l** is illustrated in Scheme 3. The chromone carboxylic acids **7a** and **b** were coupled with three types of hydroxy-amides **12a**–**c** using an EDC/HOBt system followed by oxidation of the resulting hydroxylamides with Dess–Martin periodinane to afford chromone carboxamides **3a**–**f** [17]. Chromone carboxamides **3g**–**l**, which contain a hydroxyl-substituent on their aromatic rings, were obtained by removing the MOM group during the final stage with 1% HCl in MeOH under reflux. However, the hydroxy-substituted chromone carboxamides derived from hydroxyl-amide **12a**, which has a primary group, could not be obtained, confirming their instability under acidic MOM-deprotection conditions. The chemical yields at each step are summarized in Table 1.

3. Pharmacology

The μ-calpain inhibitory activities of the chromone carboxamide derivatives **3a**–**l** were evaluated using human calpain I isolated from erythrocytes, and Suc-Leu-Tyr-AMC as the fluorogenic substrate [16,26]. Parent chromone carboxamide **2** was tested for comparison purposes and assay results are summarized in Table 1. The chromone derivatives were also evaluated for antioxidant activities by examining their DPPH scavenging and lipid peroxidation inhibitory effects



Scheme 1. Synthesis of chromone carboxylic acid esters **6a**–**c**.



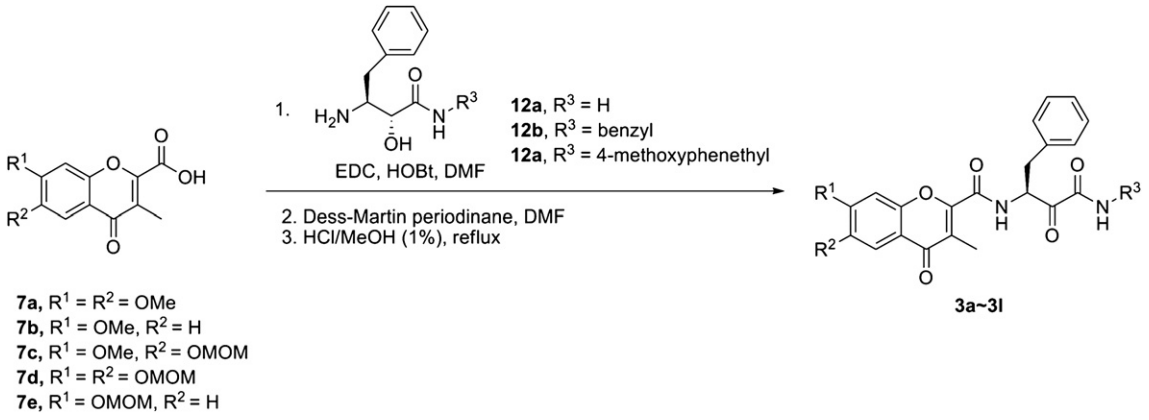
Scheme 2. Synthesis of chromone carboxylic acids **7a**–**e**.

using rat liver homogenate (Table 2); ascorbic acid and trolox were included as positive controls.

4. Results and discussion

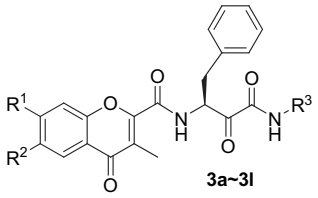
The calpains are promising targets for the treatment of cell death related diseases. During the course of our program to identify potent μ-calpain inhibitors, we recently found that chromone carboxamide **2** is a potent μ-calpain inhibitor. However, during our efforts to enhance its activities and improve its physicochemical characteristics, we found that further modification of its aromatic ring is difficult due to the instability of a primary amide moiety under acidic conditions. Accordingly, we attempted to synthesize alternative inhibitors that retained the inhibitory activity of **2** for μ-calpain by replacing the primary amide of **2** with another amide group.

First, we introduced substituents on the aromatic ring of chromone carboxamides to examine their influence on μ-calpain inhibitory activity. When methoxy groups were substituted at the C-6 and/or C-7 positions of the chromone ring, the primary amide-derived compounds **3a** and **d** were found to inhibit μ-calpain almost as well as **2**. However, replacing the primary amide group in the warhead region with benzyl or 4-methoxyphenethyl decreased inhibitory activity by 2–4 fold. Hydroxy-chromone carboxamides derived from the primary amide group were not available due to



Scheme 3. Synthesis of chromone carboxamide derivatives **3a**–**l**.

Table 1
The yields of the coupling, oxidation, and deprotection steps and the μ-calpain inhibitory activities of **3a**–**l** and that of the parent compound **2**.



Compds	R^1, R^2	R^3	Yields (%)			Caipaln Inhibition (IC_{50}, μM)[a]
			EDC coupling	Oxidation	Deprotection	
3a	$R^1 = R^2 = OCH_3$	H	63	30		0.09 ± 0.00
3b		Benzyl	75	39		0.19 ± 0.01
3c		4-Methoxyphenethyl	29	62		0.27 ± 0.01
3d	$R^1 = OCH_3$, $R^2 = H$	H	54	31		0.12 ± 0.02
3e		Benzyl	89	45		0.43 ± 0.07
3f		4-Methoxyphenethyl	31	71		0.23 ± 0.01
3g	$R^1 = OCH_3$, $R^2 = OH$	Benzyl	46	47	60	0.11 ± 0.00
3h		4-Methoxyphenethyl	82	51	44	0.10 ± 0.01
3i	$R^1 = R^2 = OH$	Benzyl	89	64	61	0.28 ± 0.00
3j		4-Methoxyphenethyl	86	44	47	1.29 ± 0.04
3k	$R^1 = OH$, $R^2 = H$	Benzyl	80	60	56	0.13 ± 0.00
3l		4-Methoxyphenethyl	92	69	51	0.09 ± 0.01
2	$R^1 = R^2 = H$	H				0.07 ± 0.00

[a] IC_{50} was defined as the concentration resulting in 50% inhibition. Data are presented as the means ±SDs of three independent experiments.

their instabilities. However, benzyl and 4-methoxyphenethyl derived hydroxyl-chromone carboxamides were readily obtained after removing the hydroxyl-protecting group under acidic conditions. Fortunately, the potencies of the monohydroxyl-substituted compounds **3g**–**h** and **3k**–**l** were not decreased even after benzyl or 4-methoxyphenethyl groups were incorporated. On the other hand, the potencies of the dihydroxyl-substituted compounds **3i** and **j** were 4–18 fold less than that of **2**. Of the synthesized compounds, **3h** and **l** showed the greatest inhibitory effects (IC_{50} = 0.09–0.10 μM) and almost matched compound **2** (IC_{50} = 0.07 μM), indicating that these compounds can be considered alternatives for **2** for further *in vitro* studies.

Reactive oxygen species are also involved in several cell death related neuronal diseases [18]. At the initiation of this study, we considered that compounds with μ-calpain inhibitory and antioxidant activities would synergistically protect cells from damage, and thus, synthesized compounds were also tested for their abilities to scavenge DPPH and inhibit lipid peroxidation. Methoxy or monohydroxyl-substituted compounds, like the parent compound **2**, showed no antioxidant activity at concentrations of <100 μM in either assay system. However, compounds **3i** and **j**, which both possess a catechol group at the C-6 and C-7 positions of the chromone ring, exhibited DPPH scavenging activity levels similar to that of ascorbic acid and more potent lipid peroxidation inhibitory activities than trolox. Although compound **3i** showed μ-calpain inhibitory activity 4-fold less than compound **2** (IC_{50} 0.28 versus 0.07 μM), its antioxidant activity was greater than those of ascorbic acid and trolox.

Table 2
The antioxidant activities of **3i**–**j** and **2**.

Compds	DPPH scavenging (IC_{50}, μM)	Lipid peroxidation inhibition (IC_{50}, μM)[a]
3i	26.51 ± 0.30	48.68 ± 4.57
3j	41.35 ± 1.14	52.46 ± 3.56
2	>100	>100
Ascorbic acid	30.81 ± 1.01	>100
Trolox	NT[b]	71.44 ± 5.54

[a] IC_{50} was defined as the concentration resulting in 50% inhibition.
[b] Not tested. Data are presented as the means ±SDs of three independent experiments.

5. Conclusions

Here, new chromone carboxamide derivatives **3** were synthesized as conformationally constrained structural variants of MDL 28,170 to provide alternative μ-calpain inhibitors, by replacing the primary amide group in the warhead position of **2** with other amide groups. Of the derivatives synthesized, compounds **3h** and **l**, which possess a 4-methoxyphenethyl group at the keto-amide position, most potently inhibited μ-calpain (IC_{50} = 0.09–0.10 μM) to levels comparable to that of the parent compound **2**. Interestingly, unlike **3h** and **l**, compound **3i** showed both potent μ-calpain inhibitory activity (IC_{50} = 0.28 μM) and DPPH scavenging and lipid peroxidation inhibitory effects, whereas MDL 28,170 and compound **2** exhibited no antioxidant activity at concentrations under 100 μM. These biological assay results support that the compounds **3h**, **i**, and **l** should be considered as alternatives of **2**, which can be used for further *in vitro* studies.

6. Experimental

6.1. Instrumentation and chemicals

The high resolution mass spectra were recorded using a Jeol AccuTOF (JMS-T100TD) equipped with a DART (direct analysis in real

time) ion source (from IonSense, Tokyo, Japan) in the positive modes. ^{1}H and ^{13}C NMR spectra were recorded on a Gemini Varian-300 (at 300 and 75 MHz, respectively). Analytical thin layer chromatography (TLC) was carried out using precoated silica gel (E. Merck Kiesegel $60F_{254}$ layer thickness 0.25 mm), and flash column chromatography was performed using Merck Kiesegel 60 Art 9385 (230–400 mesh). All solvents were purified using standard procedures.

6.2. Syntheses

6.2.1. General procedure for the preparation of 2-hydroxypropiophenones (**6a**–**c**)

A mixture of phenols **4** (1.0 eq.), propionyl chloride (1.5 eq.), and $BF_3 \cdot Et_2O$ (1.5 eq.) was heated at 80–85 °C for 2–6 h. In the reaction of phenol **4b**, toluene was used as a co-solvent. After reaction completion, mixtures were cooled to rt, poured into water, and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and then concentrated to afford **5**.

*6.2.1.1. 1-(2-Hydroxy-4,5-dimethoxyphenyl)propan-1-one (**5a**).* Yield 88%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (1H, s), 6.46 (1H, s), 3.91 (3H, s), 3.86 (3H, s), 2.98–2.92 (2H, m), 1.24 (3H, t, J = 7.3 Hz).

*6.2.1.2. 1-(2-Hydroxy-4-methoxyphenyl)propan-1-one (**5b**).* Yield 88%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.65 (1H, d, J = 9.3 Hz), 6.57 (1H, dd, J = 9.2, 2.4 Hz), 6.47 (1H, d, J = 2.36 Hz), 3.91 (3H, s), 3.11–3.06 (2H, m), 1.38 (3H, t, J = 7.4 Hz).

*6.2.1.3. 1-(2,5-Dihydroxy-4-methoxyphenyl)propan-1-one (**5c**).* Yield 86%, ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.15 (1H, s), 6.36 (1H, s), 3.84 (3H, s), 2.83 (2H, q, J = 7.2 Hz), 1.14 (3H, t, J = 7.2 Hz).

6.2.2. General procedure for the preparation of ethyl 4-oxo-4H-chromen-2-carboxylate derivatives (**6a**–**c**)

Ethyl oxalyl chloride (2.0 eq.) was added a solution of **5** (1.0 eq.) in pyridine and heated at reflux for 12 h. After reaction completion, the mixture was cooled to rt, poured into water, and extracted with EtOAc. The organic layer was dried over $MgSO_4$, concentrated, and purified by flash column chromatography (EtOAc/hexane = 1:5) to afford **6**.

*6.2.2.1. Ethyl 7-methoxy-3-methyl-4-oxo-4H-chromen-2-carboxylate (**6a**).* Yield 27%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (1H, s), 6.90 (1H, s), 4.47–4.42 (2H, m), 3.97 (2H, s), 3.96 (2H, s), 2.35 (3H, s), 1.43 (3H, t, J = 7.1 Hz).

*6.2.2.2. Ethyl 6,7-dimethoxy-3-methyl-4-oxo-4H-chromen-2-carboxylate (**6b**).* Yield 46%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.06 (1H, d, J = 8.9 Hz), 6.94 (1H, dd, J = 8.9, 2.4 Hz), 6.86 (1H, d, J = 2.4 Hz), 4.47–4.42 (2H, m), 3.88 (3H, s), 2.33 (3H, s), 1.43 (3H, t, J = 7.1 Hz).

*6.2.2.3. Ethyl 6-hydroxy-7-methoxy-3-methyl-4-oxo-4H-chromen-2-carboxylate (**6c**).* Yield 32%, ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.27 (1H, s), 7.09 (1H, s), 4.41–4.36 (2H, m), 3.92 (3H, s), 2.19 (3H, s), 1.35 (3H, t, J = 7.2 Hz).

6.2.3. General procedure for the preparation of 4-oxo-4H-chromen-2-carboxylic acid derivatives (**7a**–**b**)

KOH (2 eq.) was added to solutions of **6a** or **b** (1 eq.) in 50% aqueous ethanol, and stirred at rt for 1 h. Mixtures were treated with 3 N HCl to a pH of 2, and the resulting precipitates were filtered and dried to afford **7a** or **b**.

*6.2.3.1. 6,7-Dimethoxy-3-methyl-4-oxo-4H-chromen-2-carboxylic acid (**7a**).* Yield 60%, ^{1}H NMR (400 MHz, pyridine-d_5) δ 11.17 (1H, brs, *–OH*), 7.73 (1H, s), 7.10 (1H, s), 3.81 (1H, s), 3.74 (1H, s), 2.77 (3H, s).

*6.2.3.2. 7-Methoxy-3-methyl-4-oxo-4H-chromen-2-carboxylic acid (**7b**).* Yield 24%, ^{1}H NMR (400 MHz, pyridine-d_5) δ 8.50 (1H, brs, *–OH*), 8.32 (1H, d, J = 8.8 Hz), 7.50 (1H, dd, J = 8.4, 2.4 Hz), 7.02 (1H, d, J = 2.4 Hz), 3.72 (3H, s), 2.71 (3H, s).

6.2.4. Procedure for the preparation of 7-methoxy-6-(methoxymethoxy)-3-methyl-4-oxo-4H-chromen-2-carboxylic acid (**7c**)

Methoxymethyl chloride (633 μl, 8.41 mmol) was added slowly to a solution of **6c** (780 mg, 2.8 mmol) and diisopropylethylamine (2.44 μl, 14 mmol) in CH_2Cl_2 (20 ml) at 0 °C and stirred at rt for 2 h. The mixture was then diluted with EtOAc and washed successively with water and brine. The organic layer was dried over $MgSO_4$ and concentrated to afford **8c** (877 mg, 2.72 mmol, 97%), which was hydrolyzed with KOH using the procedure described for **7a** to afford **7c** in 61% yield. **8c**: ^{1}H NMR (400 MHz, pyridine-d_5) δ 8.07 (1H, s), 7.11 (1H, s), 5.29 (2H, s), 4.43–4.37 (2H, m), 3.79 (3H, s), 3.39 (3H, s), 2.49 (3H, s), 1.28 (3H, t, J = 7.2 Hz); **7c**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.53 (1H, s), 7.18 (1H, s) 5.26 (2H, s), 3.93 (3H, s), 3.41 (3H, s), 2.21 (3H, s).

6.2.5. Procedure for the preparation of 6,7-bis(methoxymethoxy)-3-methyl-4-oxo-4H-chromen-2-carboxylic acid (**7d**)

HBr (48%) in glacial acetic acid (11.64 ml) was added to a solution of **6a** (1.7 g, 5.82 mmol) in acetic acid (23 ml) and heated at reflux for 12 h. The reaction mixture was then diluted with EtOAc and washed successively with water and brine. The organic layer was concentrated to afford **9a** (644 mg, 2.73 mmol, 47%). H_2SO_4 (0.5 ml) was then added to a solution of **9a** (3.6 g, 15.24 mmol) in MeOH (60 ml) and heated at reflux for 12 h. The reaction mixture was then diluted with EtOAc and washed successively with water and brine. The organic layer was dried over $MgSO_4$ and concentrated to afford **10a** (2.8 g, 11.21 mmol, 74%). Compound **10a** was transformed to **11a** (94%) and then to **7d** (94%) using procedures similar to those described for **8c** and **7c**, respectively. **9a**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.59 (1H, s), 9.85 (2H, brs), 7.28 (1H, s), 6.85 (1H, s), 2.18 (3H, s); **10a**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.62 (1H, s, *–OH*), 9.91 (1H, s, *–OH*), 7.29 (1H, s), 6.85 (1H, s), 3.92 (3H, s), 2.20 (3H, s); **11a**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (1H, s), 7.28 (1H, s), 5.41 (2H, s), 5.30 (2H, s), 3.43 (3H, s), 3.42 (3H, s), 2.23 (3H, s); **7d**: ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.59 (1H, s), 7.29 (1H, s), 5.42 (2H, s), 5.31 (2H, s), 3.44 (3H, s), 3.43 (3H, s), 2.23 (3H, s).

6.2.6. Procedure for the preparation of 7-methoxymethoxy-3-methyl-4-oxo-4H-chromen-2-carboxylic acid (**7e**)

The compound **7e** was prepared from **6b** according to the procedure described for **7d**. **9b**: Yield 51%, ^{1}H NMR (400 MHz, DMSO) δ 7.88 (1H, d, J = 8.7 Hz), 6.92 (1H, dd, J = 8.7, 1.6 Hz), 6.81 (1H, d, J = 1.6 Hz), 2.19 (3H, s); **10b**: Yield 86%, ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.90 (1H, s, *–OH*), 7.86 (1H, d, J = 8.8 Hz), 7.03 (1H, dd, J = 8.8, 2.2 Hz), 6.77 (1H, d, J = 2.2 Hz), 3.91 (3H, s), 2.17 (3H, s); **11b**: Yield 95%, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (1H, d, J = 8.8 Hz), 7.04 (1H, d, J = 2.3 Hz), 6.98 (1H, dd, J = 8.8, 2.3 Hz), 5.19 (2H, s), 3.92 (3H, s), 3.43 (3H, s), 2.28 (3H, s); **7e**: Yield 97%, ^{1}H NMR (400 MHz, pyridine-d_5) δ 7.97 (1H, d, J = 8.8 Hz), 7.18 (1H, d, J = 2.0 Hz), 7.13 (1H, dd, J = 8.8, 2.0 Hz), 5.38 (2H, s), 3.43 (3H, s), 2.22 (3H, s).

6.2.7. Procedure for the preparation of (S)-N-(4-amino-3,4-dioxo-1-phenylbutan-2-yl)-6,7-dimethoxy-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3a**)

To a solution of **7a** (129 mg, 0.49 mmol) and 1-hydroxybenzotriazole (HOBt, 93.8 mg, 0.69 mmol) in DMF (5 ml) were added successively (2*R*, 3*S*)-3-amino-2-hydroxy-4-phenylbutanamide (**12a**, 95 mg, 0.49 mmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide·HCl (EDC, 133 mg, 0.69 mmol) at 0 °C. The mixture was then stirred at rt for 2 h, treated with water, and the resulting precipitate was filtered, washed with ether, and dried to afford

a coupling adduct, *N*-((2*S*, 3*R*)-4-amino-3-hydroxy-4-oxo-1-phenylbutan-2-yl)-6,7-dimethoxy-3-methyl-4-oxo-4*H*-chromen-2-carboxamide (134 mg, 63%). This compound (100 mg, 0.23 mmol) was then dissolved in DMF (5 ml), treated with Dess–Martin periodinane (193 mg, 0.46 mmol) at 0 °C, and stirred at rt for 2 h. 10% $Na_2S_2O_3$ aqueous solution (1 ml) was then added and stirred for 5 min. The resulting solid was filtered and purified by flash column chromatography (CH_2Cl_2:MeOH = 30:1) to afford **3a** (23 mg, 23%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.34 (1H, d, J = 7.6 Hz), 8.18 (1H, s, −*NH*), 7.92 (1H, s, −*NH*), 7.37–7.19 (6H, m), 7.18 (1H, s), 5.49–5.44 (1H, m), 3.93 (3H, s), 3.86 (3H, s), 3.28 (1H, dd, J = 14.0, 3.6 Hz), 2.90 (1H, dd, J = 14.0, 10.1 Hz), 1.95 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 196.1, 176.3, 162.5, 161.1, 154.6, 150.8, 147.6, 137.3, 129.6, 128.9, 128.4, 126.6, 118.2, 115.2, 103.5, 100.3, 100.2, 56.4, 55.8, 34.7, 9.6; DART-MS *m*/*z* 439.1492 (calcd for $C_{23}H_{22}N_2O_7$ [M + H]$^+$, 439.1500).

6.2.8. (S)-N-(4-Benzylamino-3,4-dioxo-1-phenylbutan-2-yl)-6,7-dimethoxy-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3b**)

Compound **3b** was prepared from **7a** (120 mg, 0.45 mmol) and (2*R*, 3*S*)-3-amino-*N*-benzyl-2-hydroxy-4-phenylbutanamide (**12b**, 129 mg, 0.45 mmol) using the procedure described for **3a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.41 (1H, d, J = 7.2 Hz), 9.38 (1H, t, J = 5.6 Hz), 7.35–7.14 (11H, m), 7.10 (1H, s), 5.49–5.44 (1H, m), 4.37 (2H, t, J = 5.6 Hz), 3.93 (3H, s), 3.86 (3H, s), 3.30 (1H, dd, J = 14.0, 4.4 Hz), 2.97 (1H, dd, J = 14.0, 9.6 Hz), 1.97 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.7, 176.3, 161.1, 160.6, 154.6, 151.4, 150.8, 147.6, 138.4, 137.2, 129.0, 128.4, 128.2, 127.3, 126.9, 126.6, 118.4, 115.2, 103.5, 100.1, 56.3, 55.9, 55.8, 42.1, 34.8, 9.5; DART-MS *m*/*z* 529.1961 (calcd for $C_{30}H_{28}N_2O_7$ [M + H]$^+$, 529.1969).

6.2.9. (S)-6,7-Dimethoxy-N-(4-(4-methoxyphenethyl amino)-3,4-dioxo-1-phenylbutan-2-yl)-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3c**)

Compound **3c** was prepared from **7a** (64 mg, 0.24 mmol) and (2*R*, 3*S*)-3-amino-2-hydroxy-*N*-(4-methoxyphenethyl)-4-phenylbutanamide (**12c**, 80 mg, 0.24 mmol) using the procedure described for **3a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.34 (1H, d, J = 7.3 Hz), 8.90 (1H, t, J = 5.3 Hz), 7.34–6.83 (11H, m), 5.49–5.43 (1H, m), 3.92 (3H, s), 3.86 (3H, s), 3.67 (3H, s), 3.18 (1H, dd, J = 13.7, 3.1 Hz), 2.86 (1H, dd, J = 13.7, 10.4 Hz), 1.95 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.7, 176.3, 161.1, 160.3, 157.7, 154.6, 151.6, 150.8, 147.5, 137.3, 130.8, 129.6, 128.9, 128.4, 126.7, 118.1, 115.2, 113.7, 103.4, 100.1, 56.3, 55.9, 55.7, 54.9, 40.4, 34.6, 33.6, 9.6; DART-MS *m*/*z* 573.2248 (calcd for $C_{32}H_{32}N_2O_8$ [M + H]$^+$, 573.2232).

6.2.10. (S)-N-(4-Amino-3,4-dioxo-1-phenylbutan-2-yl)-7-methoxy-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3d**)

Compound **3d** was prepared from **7b** (110 mg, 0.47 mmol) and **12a** (91.3 mg, 0.47 mmol) as described for **3a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.36 (1H, d, J = 7.6 Hz), 8.18 (1H, s, −NH_2), 7.33–7.15 (5H, m), 7.12 (1H, d, J = 2.4 Hz), 7.08 (1H, dd, J = 8.8, 2.4 Hz), 5.49–5.43 (1H, m), 3.92 (3H, s), 3.28 (1H, dd, J = 14.0, 4.0 Hz), 2.90 (1H, dd, J = 14.0, 10.0 Hz), 1.93 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 196.1, 176.6, 164.0, 162.4, 160.9, 156.5, 151.9, 137.3, 129.2, 129.0, 128.4, 128.1, 126.5, 118.6, 115.8, 100.3, 56.1, 55.7, 34.7, 9.4; DART-MS *m*/*z* 409.1377 (calcd for $C_{32}H_{32}N_2O_8$ [M + H]$^+$, 409.1394).

6.2.11. (S)-N-(4-Benzylamino-3,4-dioxo-1-phenylbutan-2-yl)-7-methoxy-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3e**)

Compound **3e** was prepared from **7b** (150 mg, 0.64 mmol) and **12b** (182 mg, 0.64 mmol) as described for **3a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.42 (1H, d, J = 7.2 Hz), 9.37 (1H, t, J = 6.4 Hz), 7.95 (1H, d, J = 8.8 Hz), 7.33–7.18 (10H, m), 7.10 (1H, dd, J = 7.2, 2.4 Hz), 7.07 (1H, d, J = 2.4 Hz), 5.48–5.43 (1H, m), 4.37 (2H, t, J = 5.6 Hz), 3.91 (3H, s), 3.29 (1H, dd, J = 14.4, 4.4 Hz), 2.96 (1H, dd, J = 14.4, 10.0 Hz), 1.94 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 196.1, 177.1, 164.5, 161.5, 161.1, 157.0, 152.3, 138.9, 137.7, 129.6, 128.9, 128.8, 127.8, 127.4, 127.2, 127.0, 119.3, 116.3, 115.7, 100.8, 56.6, 56.5, 42.6, 35.3, 9.9; DART-MS *m*/*z* 499.1870 (calcd for $C_{32}H_{32}N_2O_8$ [M + H]$^+$, 499.1864).

6.2.12. (S)-7-Methoxy-N-(4-(4-methoxyphenethylamino)-3,4-dioxo-1-phenylbutan-2-yl)-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3f**)

Compound **3f** was prepared from **7b** (57 mg, 0.24 mmol) and **12c** (80 mg, 0.24 mmol) as described for **3a**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.36 (1H, d, J = 7.3 Hz), 8.90 (1H, t, J = 5.4 Hz), 7.35–6.80 (12H, m), 5.48–5.44 (1H, m), 3.91 (3H, s), 3.67 (3H, s), 3.18 (1H, dd, J = 13.8, 3.0 Hz), 2.85 (1H, dd, J = 13.6, 10.3 Hz), 1.93 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.6, 176.6, 164.0,160.9, 160.3, 157.7, 156.5, 151.9, 137.2, 130.8, 129.6, 128.9, 128.4, 126.7, 126.5, 118.6, 115.8, 115.2, 113.7, 100.3, 56.1, 55.9, 54.8, 40.4, 34.6, 33.6, 9.4; DART-MS *m*/*z* 543.2134 (calcd for $C_{32}H_{32}N_2O_8$ [M + H]$^+$, 543.2126).

6.2.13. (S)-N-(4-Benzylamino-3,4-dioxo-1-phenylbutan-2-yl)-6-hydroxy-7-methoxy-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3g**)

(*S*)-*N*-(4-Benzylamino-3,4-dioxo-1-phenylbutan-2-yl)-7-methoxy-6-(methoxymethoxy)-3-methyl-4-oxo-4*H*-chromen-2-carboxamide was obtained by coupling **7c** (150 mg, 0.51 mmol) and **12b** (145 mg, 0.51 mmol) followed by Dess–Martin periodinane oxidation as described for **3a**. A portion of the above coupling compound (40 mg, 0.07 mmol) obtained was then dissolved in 1% methanolic HCl (10 mL) and heated at 70 °C for 2 h. This mixture was then diluted with EtOAc and washed successively with water and brine, and the organic layer was dried over $MgSO_4$, concentrated, and purified by column chromatography (CH_2Cl_2:MeOH = 30:1) to afford **3g** (22 mg, 60%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.86(1H, s, −*OH*), 9.39–9.35 (2H, m), 7.32–7.21 (11H, m), 7.11 (1H, s), 5.47–5.41 (1H, m), 4.36 (2H, t, J = 5.6 Hz), 3.92 (3H, s), 3.28 (1H, dd, J = 14.0, 4.4 Hz), 2.95 (1H, dd, J = 14.0, 10.0 Hz), 1.93 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.7, 176.3, 161.2, 160.6, 154.1, 151.4, 149.9, 145.5138.4, 137.2, 129.0, 128.4, 128.2, 127.3, 126.9, 126.6, 117.9, 115.6, 107.0, 100.1, 56.2, 55.9 42.1 34.8, 9.5; DART-MS *m*/*z* 515.1836 (calcd for $C_{32}H_{32}N_2O_8$ [M + H]$^+$, 515.1813).

6.2.14. (S)-N-(4-(4-Methoxyphenethylamino)-3,4-dioxo-1-phenylbutan-2-yl)-6-hydroxy-7-methoxy-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3h**)

Compound **3h** was prepared from **7c** (108 mg, 0.37 mmol) and **12c** (120 mg, 0.37 mmol) as described for **3g**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.33 (1H, d, J = 7.4 Hz, −*NH*), 8.92 (1H, t, J = 5.8 Hz, −*NH*), 7.35–6.80 (11H, m), 5.46 (1H, m), 3.91 (3H, s), 3.67 (3H, s), 3.17 (1H, dd, J = 13.9, 3.8 Hz), 2.85 (1H, dd, J = 13.9, 9.6 Hz), 2.72–2.74 (2H, m), 1.93 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.7, 176.3, 161.2, 160.3, 157.7, 154.1, 151.6, 149.9, 145.4, 137.3, 130.8, 129.6 (2C), 129.0 (2C), 128.5 (2C), 126.6, 117.8, 115.6, 113.76 (2C), 106.9, 100.1, 56.2, 55.9, 54.9, 40.4 34.6, 33.6, 9.5; DART-MS *m*/*z* 559.2080 (calcd for $C_{32}H_{32}N_2O_8$ [M + H]$^+$, 559.2075).

6.2.15. (S)-N-(4-Benzylamino-3,4-dioxo-1-phenylbutan-2-yl)-6,7-dihydroxy-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3i**)

Compound **3i** was prepared from **7d** (150 mg, 0.46 mmol) and **12b** (131.5 mg, 0.46 mmol) as described for **3g** above. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.38–9.33 (2H, m, two −*NH*), 7.33–7.21 (11H, m), 6.91 (1H, s), 5.38 (1H, m), 4.36 (2H, t, J = 6.4 Hz), 3.27 (1H, dd, J = 13.8, 3.6 Hz), 2.96 (1H, dd, J = 13.8, 9.6 Hz), 1.94 (3H, s); ^{13}C

NMR (100 MHz, DMSO-d_6) δ 195.8, 176.3, 161.3, 160.7, 152.9, 150.8, 149.9, 144.9,138.4, 137.3, 129.1 (2C), 128.4 (2C), 128.36 (2C), 127.36 (2C), 126 9, 126.6, 118.0, 114.96 (2C), 107.4, 102.7, 56.1, 42.1, 34.7, 9.4; DART-MS m/z 501.1677 (calcd for $C_{32}H_{32}N_2O_8$ $[M + H]^+$, 501.1656).

*6.2.16. (S)-6,7-Dihydroxy-N-(4-(4-methoxyphenethylamino)-3,4-dioxo-1-phenylbutan-2-yl)-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3j**)*

Compound **3j** was prepared from **7d** (119 mg, 0.37 mmol) and **12c** (120 mg, 0.37 mmol) as described for **3g**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.28 (1H, d, J = 7.3 Hz), 8.92 (1H, t, J = 5.6 Hz), 7.31–6.83 (11H, m), 5.42–5.37 (1H, m), 3.67 (3H, s), 3.25–3.21 (1H, m), 3.16 (1H, dd, J = 13.8, 3.7 Hz), 3.10–3.04 (1H, m), 2.86 (1H, dd, J = 13.8, 10.4 Hz), 2.75–2.71 (2H, m), 1.94 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.7, 176.2, 160.3, 157.6, 152.8, 149.8, 144.9, 137.3, 130.7, 130.4, 129.5, 128.9, 128.2, 127.5, 126.6, 117.8, 114.8, 113.6, 107.3, 102.7, 54.8, 54.7, 40.6 33.5, 33.4, 9.4; DART-MS m/z 545.1908 (calcd for $C_{32}H_{32}N_2O_8$ $[M + H]^+$, 545.1919).

*6.2.17. (S)-N-(4-Benzylamino-3,4-dioxo-1-phenylbutan-2-yl)-7-hydroxy-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3k**)*

Compound **3k** was prepared from **7e** (200 mg, 0.76 mmol) and **12b** (215 mg, 0.76 mmol) as described for **3g**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.43–9.38 (2H, m, *–NH*), 7.88 (1H, d, J = 8.8 Hz), 7.33–7.21 (10H, m), 6.97 (1H, d, J = 2.4 Hz), 6.95 (1H, s), 5.43–5.38 (1H, m), 4.37 (2H, t, J = 6.0 Hz), 3.28 (1H, dd, J = 14.0, 4.4 Hz), 2.97 (1H, dd, J = 14.0, 10.0 Hz), 1.94 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.7, 176.6, 163.1, 161.1, 160.8, 156.5, 151.3, 138.4, 137.3, 129.1, 128.3, 128.2, 127.3, 126.9, 126.8, 126.6, 118.7, 115.6, 114.8, 102.1, 56.1, 42.1 34.7, 9.4; DART-MS m/z 485.1735 (calcd for $C_{32}H_{32}N_2O_8$ $[M + H]^+$, 485.1707).

*6.2.18. (S)-7-Hydroxy-N-(4-(4-methoxyphenethylamino)-3,4-dioxo-1-phenylbutan-2-yl)-3-methyl-4-oxo-4H-chromen-2-carboxamide (**3l**)*

Compound **3l** was prepared from **7e** (64 mg, 0.24 mmol) and **12c** (80 mg, 0.30 mmol) as described for **3g**. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.9 (1H, brs, *–OH*), 9.31 (1H, d, J = 7.4 Hz, *–NH*), 8.90 (1H, t, J = 5.7 Hz, *–NH*), 7.89 (1H, d, J = 8.8 Hz), 7.33–6.83 (11H, m), 5.41 (1H, m), 3.68 (3H, s), 3.17 (1H, dd, J = 13.6, 3.6 Hz), 2.86 (1H, dd, J = 13.6, 9.6 Hz), 2.72–2.75 (2H, m), 1.94 (3H, s); ^{13}C NMR (100 MHz, DMSO-d_6) δ 195.7, 176.6, 162.9, 161.1, 160.3, 157.7, 156.5, 151.4, 137.3, 130.8, 129.6 (2C), 129.0 (2C), 128.4 (2C), 126.9, 126.6, 118.6, 115.5, 114.8, 113.7 (2C), 102.1, 56.1, 54.9, 40.4 34.5, 33.6, 9.4; DART-MS m/z 529.1934 (calcd for $C_{32}H_{32}N_2O_8$ $[M + H]^+$, 529.1969).

6.3. Biological activity testing

6.3.1. Calpain inhibitory activity

Calpain inhibitory activity was assayed using a slight modification of a previously reported procedure [26]. Briefly, calpain I (Calbiochem) from human erythrocytes and the fluorogenic calpain substrate (Suc-Leu-Tyr-7-amino-4-methylcoumarin, Calbiochem) were used as enzyme and substrate, respectively. In a typical experiment, 1 μl of a test compound solution (at different concentrations), 25 μl of 1 mM calpain substrate, 10 μl of 1 M NaCl, and 11 μl of 1 mM $CaCl_2$ were added to the wells of 96-well plates. All chemicals except for the test compounds were made up in 50 mM Tris–HCl (pH 7.5) buffer containing 1 mM dithiothreitol immediately before use. Test compounds were dissolved in DMSO, but DMSO concentrations in the final experimental solutions did not exceed 1%. Incubations were initiated by adding 53 μl of 34 nM calpain and mixtures were then incubated for 30 min at rt. After incubation, the fluorescence of the cleavage product, 7-amino-4-methylcoumarin, was measured using a spectrofluorimeter at λ_{ex} = 380 nm and λ_{em} = 460 nm against calpain free blank. IC_{50} values were calculated based on percent inhibitions of enzyme activity.

6.3.2. DPPH radical scavenging activity assay

The antioxidant activities of the synthesized compounds were assessed by examining their abilities to scavenge the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Reaction mixtures containing test samples (dissolved in EtOH) and 100 μM of ethanolic DPPH solution in 96-well plates were incubated at 37 °C for 30 min. Absorbances were measured at 515 nm. Percent inhibitions were calculated versus ethanol-treated controls. IC_{50} values denote the concentration required to scavenge 50% of DPPH radicals.

6.3.3. Inhibition of lipid peroxidation

The effects of the synthesized compounds on lipid peroxidation induced by an iron-ascorbic acid mix were determined in rat liver homogenate. In brief, rat liver homogenate (300 μl, 11 mg protein/ml) was incubated with 10 μM Fe_2SO_4, 0.4 mM ascorbic acid and various concentrations of the test compounds in 50 mM Tris–HCl (pH 7.5) in a total volume of 1 ml at 37 °C for 30 min. After incubation, lipid peroxidation levels were determined by measuring the formation of thiobarbituric acid-reactive substance (TBARS). Reactions were terminated by adding 2 ml of a solution of 0.375% thiobarbituric acid in 15% trichloroacetic acid containing 0.25 N HCl and 0.01% butylated hydroxytoluene (TBA–TCA reagent). Mixtures were then heated at 95 °C for 30 min, cooled, and centrifuged at 5000 × g for 10 min. The absorbances of supernatants were measured at 535 nm.

Protein contents in liver homogenates were determined using the Bradford method using bovine serum albumin as a standard.

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