# Accepted Manuscript


Design, synthesis and antitumor evaluation of novel celastrol derivatives

Manyi Xu, Na Li, Zihao Zhao, Zhixian Shi, Jianbo Sun, Li Chen

PII: S0223-5234(19)30368-X

DOI: https://doi.org/10.1016/j.ejmech.2019.04.050

Reference: EJMECH 11285

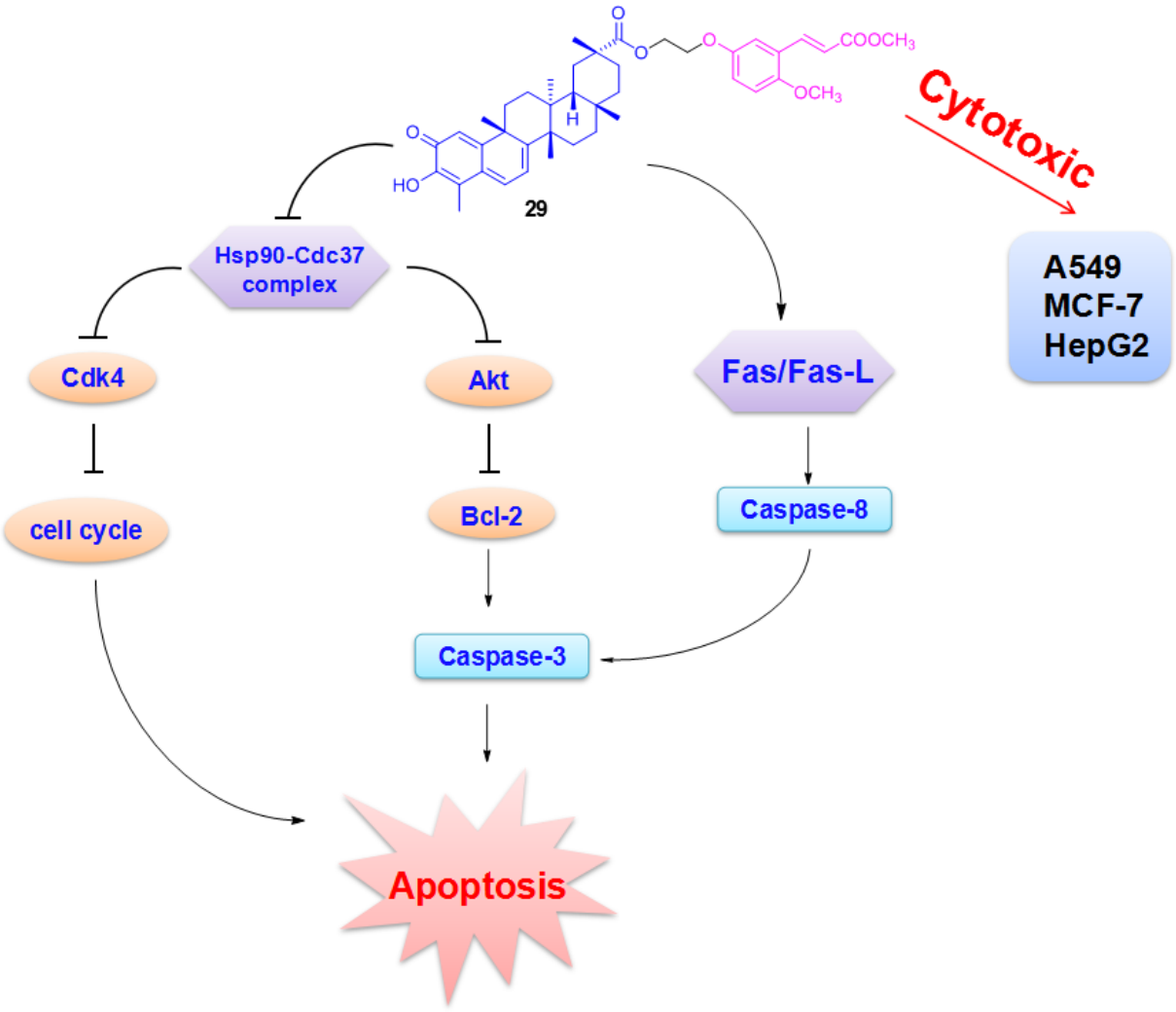
To appear in: *European Journal of Medicinal Chemistry*

Received Date: 27 December 2018

Revised Date: 13 April 2019

Accepted Date: 17 April 2019

Please cite this article as: M. Xu, N. Li, Z. Zhao, Z. Shi, J. Sun, L. Chen, Design, synthesis and antitumor evaluation of novel celastrol derivatives, *European Journal of Medicinal Chemistry* (2019), doi: https://doi.org/10.1016/j.ejmech.2019.04.050.


COOCH3
OCH3
O
HO
H
29
Cytotoxic
A549
MCF-7
HepG2
Hsp90-Cdc37 complex
Cdk4
Akt
Fas/Fas-L
cell cycle
Bcl-2
Caspase-8
Caspase-3
Apoptosis

# Design, synthesis and antitumor evaluation of novel celastrol derivatives

Manyi Xu, Na Li, Zihao Zhao, Zhixian Shi, Jianbo Sun*, and Li Chen**

*State Key Laboratory of Natural Medicines, Department of Natural Medicinal Chemistry, School of Traditional Chinese Pharmacy, China Pharmaceutical University, 24 Tong Jia Xiang, Nanjing 210009, People's Republic of China*

** Corresponding author: Tel: +86 83271415, E-mail addresses: sjbcpu@gmail.com (J. Sun)*

*** Corresponding author: Tel: +86 83271447, chenli627@cpu.edu.cn (L. Chen).*

***Keywords*:** *Celastrol, ferulic acid, apoptosis, anti-tumor, hybridization*

## ABSTRACT

On the basis of the hybridization strategy of natural products, a total of 32 novel celastrol hybrids were designed, synthesized and evaluated for their antitumor activities. Most of these derivatives exihibited significant antiproliferative activities compared to celastrol, among which compound **29** displayed the strongest inhibitory capability [$IC_{50}$ = 0.15 ± 0.03 μM (A549),0.17 ± 0.03 μM (MCF-7), 0.26 ± 0.02 μM (HepG2)], which exhibited equal or superior anti-cancer activities in comparison to 2-cyano-3,12-dioxoolean-1,9(11)-dien-28-oic acid methyl ester (CDDO-Me). The mechanism of pharmacological research indicated that **29** possessed the ability to disrupt Hsp90-Cdc37 complex which was similar to celastrol. Meanwhile, compound **29** could induce abnormal regulation of clients (p-Akt and Cdk4) of Hsp90 and cell cycle arrest at $G_0/G_1$ phase in a concentration-dependent manner. In addition, compound **29** could also induce cell apoptosis through the death receptor pathway on A549 cells. Taken together, our results demonstrated that **29** might be a promising novel candidate for further druggability research.

## 1. Introduction

Celastrol (**CE)** (Fig.1), a pentacyclic triterpenoid obtained from the root bark of *Tripterygium wilfordii* Hook F.[1-2], possesses an unique quinone methide moiety exhibiting multiple pharmacological activities, especially for its widely investigated anti-cancer activity[3]. Zhang *et al.*[4] pointed out that the anti-cancer property of celastrol mainly due to the disruption of the interaction between heat shock protein 90 (Hsp90) and its molecular co-chaperone, cell division cycle protein 37 (Cdc37), so as to prevent a variety of downstream client proteins from achieving stable conformations, cell transportation and transmembrane. Most of these client proteins are oncogenic proteins, such as protein kinase B (Akt), cyclin-dependent kinases 4 (Cdk4), Raf family proteins and MEK1/2[5].

Ferulic acids (FA, 4-hydroxy-3-methoxycinnamic acid), a class of phenolic acid widely existed in fruits, vegetables, and Chinese medicinal herbs such as *Angelica sinensis, Cimicifuga racemosa* and *Ligusticum chuanxiong*[6-8]. FA has been proved to possess potential therapeutic effects for the advantages of anti-oxidation, anti-diabetes, anti-inflammatory, neuroprotection, etc[9]. It is worth mentioning that FA and its derivatives also showed promising anticarcinogenic efficacy *in vivo* and *vitro*[10-15]. Silva and Batista[16] have reviewed hundreds of naturally occurring compounds containing feruloyl moieties with various biological activities. As a naturally oleanolic acid derivative bearing a trans-feruloyl unit at C-27 position, uncarinic acid A[17] (Fig.1) showed potent cytotoxicity against A549 (human lung cancer cell lines) and MCF-7 (human breast cancer cell lines) with $EC_{50}$ values 4.6 μg/ml and 7.7 μg/ml, respectively. Li *et al.*[18] also introduced various feruloyl moieties into glycyrrhetinic acid (GA) and obtained a series of glycyrrhetinic acid derivatives (Fig.1), which displayed enhanced anti-cancer activities. Therefore, feruloyl moieties can be used as a promising fragment in in anti-cancer agents design.

Molecular hybridization, a strategy which aims to fuse more than one pharmacophoric group to obtain new hybrids with improved bioactivities[19-20] is widely used in drug design. In order to develop novel anticarcinogenic agents, many

hybrids have been reported via this strategy based on natural products[21-24]. Thus, combined with the strategy and analysis above, we designed and synthesized thirty-two (**1-32**) novel celastrol hybrids in which 29-carboxyl group was modified by methyl ferulate and its derivatives with different linkers. All hybrids were assayed *in vitro* for their anti-proliferative activities against A549, MCF-7, HepG2 cancer cells. Among them, compound **29** showed strongest cytotoxicity on the above three cancer cells. Furthermore, disruption of Hsp90-Cdc37 complex, apoptosis, cell cycle arrest, regulation of Hsp90 clients of **29** were assessed. Additionally, related proteins of exogenous apoptotic pathway of **29** were preliminarily evaluated by western blot.

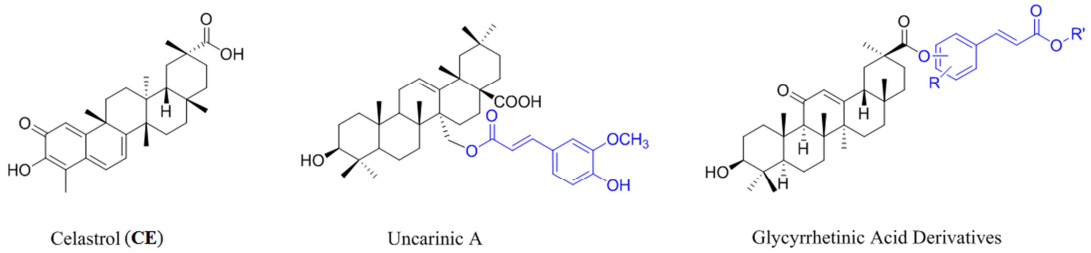
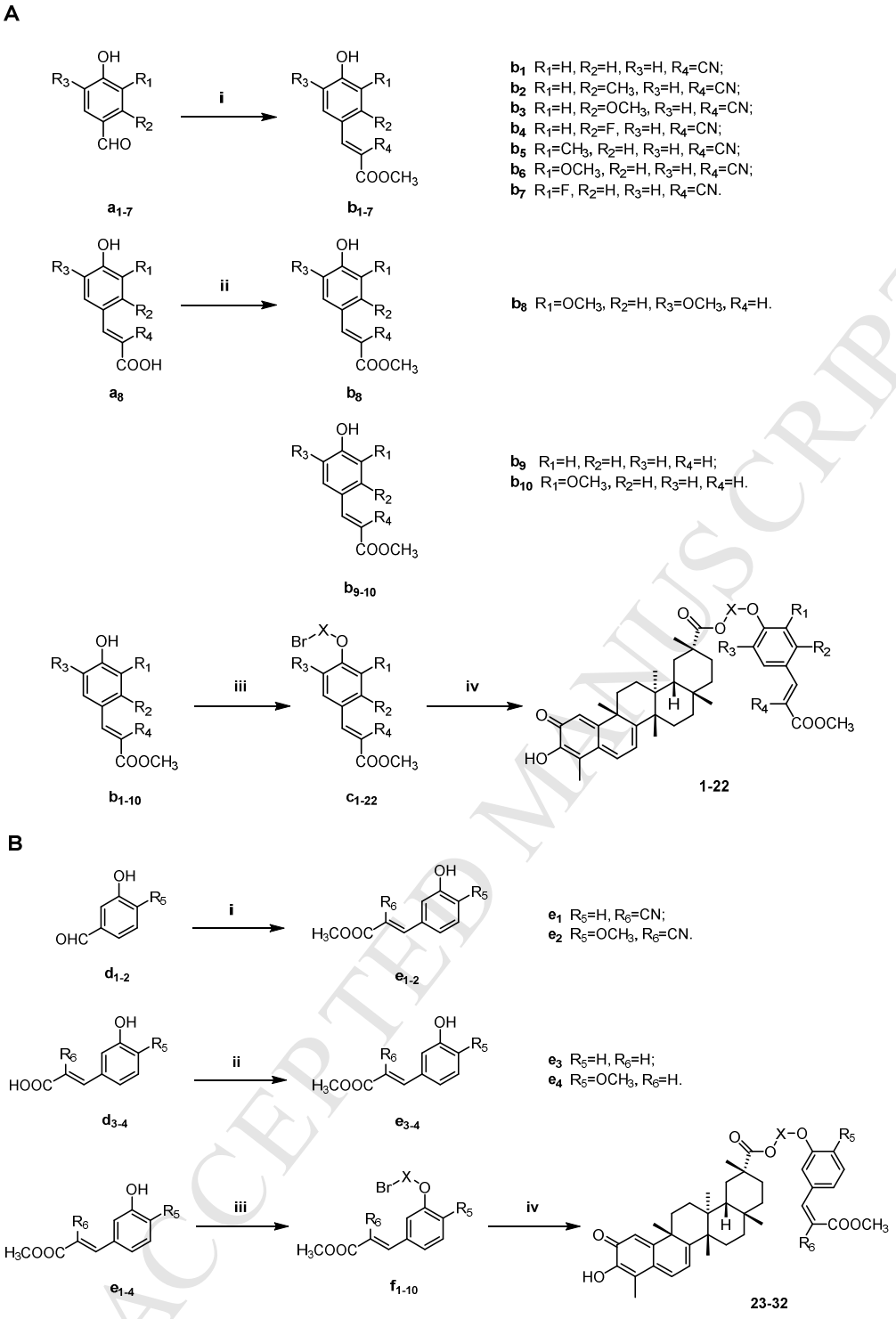



**Fig.1** The structure of celastrol, and several examples of feruloyl moiety-containing natural and synthetic antitumor agents.

## 2. Results and discussion

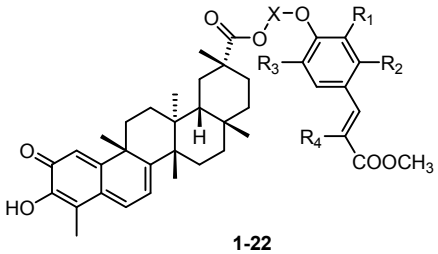
### *2.1. Chemistry*

The synthetic routes of 32 target compounds (**1-32**) are depicted in Scheme 1. The different hydroxy aryl aldehyde (**a**$_{1-7}$ and **d**$_{1-2}$) were treated with methyl cyanoacetate for 12 h at 100 ℃ to get the α-cyano substituted methyl cinnamate derivatives **b**$_{1-7}$ and **e**$_{1-2}$, respectively[25]**.** Different kinds of benzene acrylic acid derivatives **a**$_8$ or **d**$_{3-4}$ were under esterification reaction to get compounds **b**$_8$ and **e**$_{3-4}$. Then the intermediates were obtained via coupling **b**$_{1-10}$ or **e**$_{1-4}$ with trans-1,4-dibromo-2-butene catalyzed by $K_2CO_3$ and $Bu_4N^+Br^-$ or alkyl bromides catalyzed by $K_2CO_3$[26]. Subsequently, the target compounds **1-32** were synthesized by connecting intermediates (**c**$_{1-22}$ or **f**$_{1-10}$) to C-29 carboxyl group of celastrol with $NaHCO_3$ in DMF[27]. The substituent groups $R_1$-$R_6$ of **1-32** were listed in Table 1 & Table 2, respectively.




**Scheme 1**. Reagents and conditions: i) Methyl cyanoacetate, $NH_4OAc$, tolune, 100 □, reflux, 12 h; ii) $CH_3OH$, $H_2SO_4$, 70 □, reflux, 12 h; iii) alkyl bromides, $K_2CO_3$, acetone, 50 □, reflux, 7-8 h or trans-1,4-dibromo-2-butene, $K_2CO_3$, $Bu_4N^+Br^-$, acetone, 50 □, reflux,7 h; iv) celastrol, $NaHCO_3$, DMF, 60 □, reflux, 7 h.

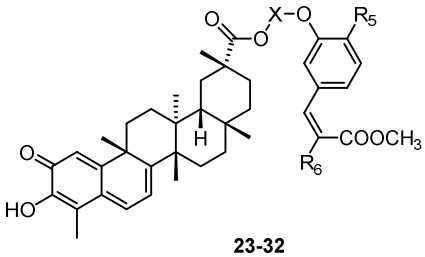
**Table 1** Structures of target compounds **1-22**.




**1-22**

| Cpd. | Intermediates | X | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|---|---|
| **1** | $b_1$, $c_1$ | $(CH_2)_3$ | H | H | H | CN |
| **2** | $b_2$, $c_2$ | $(CH_2)_3$ | H | $CH_3$ | H | CN |
| **3** | $b_3$, $c_3$ | $(CH_2)_3$ | H | $OCH_3$ | H | CN |
| **4** | $b_4$, $c_4$ | $(CH_2)_3$ | H | F | H | CN |
| **5** | $b_5$, $c_5$ | $(CH_2)_3$ | $CH_3$ | H | H | CN |
| **6** | $b_6$, $c_6$ | $(CH_2)_2$ | $OCH_3$ | H | H | CN |
| **7** | $b_6$, $c_7$ | $(CH_2)_3$ | $OCH_3$ | H | H | CN |
| **8** | $b_6$, $c_8$ | $(CH_2)_4$ | $OCH_3$ | H | H | CN |
| **9** | $b_6$, $c_9$ | $CH_2CH=CHCH_2$ | $OCH_3$ | H | H | CN |
| **10** | $b_7$, $c_{10}$ | $(CH_2)_3$ | F | H | H | CN |
| **11** | $b_8$, $c_{11}$ | $(CH_2)_2$ | $OCH_3$ | H | $OCH_3$ | H |
| **12** | $b_8$, $c_{12}$ | $(CH_2)_3$ | $OCH_3$ | H | $OCH_3$ | H |
| **13** | $b_8$, $c_{13}$ | $(CH_2)_4$ | $OCH_3$ | H | $OCH_3$ | H |
| **14** | $b_8$, $c_{14}$ | $CH_2CH=CHCH_2$ | $OCH_3$ | H | $OCH_3$ | H |
| **15** | $b_9$, $c_{15}$ | $(CH_2)_2$ | H | H | H | H |
| **16** | $b_9$, $c_{16}$ | $(CH_2)_3$ | H | H | H | H |
| **17** | $b_9$, $c_{17}$ | $(CH_2)_4$ | H | H | H | H |
| **18** | $b_9$, $c_{18}$ | $CH_2CH=CHCH_2$ | H | H | H | H |
| **19** | $b_{10}$,$c_{19}$ | $(CH_2)_2$ | $OCH_3$ | H | H | H |
| **20** | $b_{10}$, $c_{20}$ | $(CH_2)_3$ | $OCH_3$ | H | H | H |
| **21** | $b_{10}$, $c_{21}$ | $(CH_2)_4$ | $OCH_3$ | H | H | H |
| **22** | $b_{10}$, $c_{22}$ | $CH_2CH=CHCH_2$ | $OCH_3$ | H | H | H |

**Table 2** Structures of target compounds **23-32**.




**23-32**

| Cpd. | Intermediates | X | $R_5$ | $R_6$ |
|---|---|---|---|---|
| **23** | $e_1$, $f_1$ | $(CH_2)_3$ | H | CN |
| **24** | $e_2$, $f_2$ | $(CH_2)_2$ | $OCH_3$ | CN |
| **25** | $e_2$, $f_3$ | $(CH_2)_3$ | $OCH_3$ | CN |
| **26** | $e_2$, $f_4$ | $(CH_2)_4$ | $OCH_3$ | CN |
| **27** | $e_2$, $f_5$ | $CH_2CH=CHCH_2$ | $OCH_3$ | CN |
| **28** | $e_3$, $f_6$ | $(CH_2)_3$ | H | H |
| **29** | $e_4$, $f_7$ | $(CH_2)_2$ | $OCH_3$ | H |
| **30** | $e_4$, $f_8$ | $(CH_2)_3$ | $OCH_3$ | H |
| **31** | $e_4$, $f_9$ | $(CH_2)_4$ | $OCH_3$ | H |
| **32** | $e_4$, $f_{10}$ | $CH_2CH=CHCH_2$ | $OCH_3$ | H |

*2.2. Biological activities*

*2.2.1. In vitro cytotoxic activity*

The synthesized compounds (**1-5, 7, 10, 16, 20, 23, 25, 28, 30**) with linker bearing three carbon atoms were preliminarily evaluated their inhibitory effects *in vitro* against MCF-7 by MTT assay at 1.0 μM, adopting celastrol as positive control. According to the data listed in Table 3, we selected compounds whose inhibition rate exhibited over 50% for further research to discuss their inhibitory activities by replacing different linkers. Additionally, we found that derivatives containing benzene ring with a methoxy group showed best inhibitory ability. Thus, we synthesized four more compounds (**11-14**) to explore whether the number of methoxy group made any sense. As shown in Table 3, eight target compounds (**7, 11, 15, 20, 24-25, 29-30**) displayed superior inhibitory activity (≥ 60%) compared to celastrol.

The preliminary structure and activity relationships (SARs) could be concluded as follows: (1) Various linkers had certain influence on potency. Hybrids with unsaturated alkyl possessed higher anti-proliferative activity than those with saturated butyl linker, but lower than two or three carbons. Compounds with 2 carbons exhibited stronger anti-cancer capability than 3 carbons (**11** and **12, 15** and **16, 24** and **25, 29** and **30**), while **6** and **7, 19** and **20** showed reversed results. (2) Under the condition that the compounds had the same linker, methoxy group exhibited the best effect on the activities as the substituent group in benzene of ferulic acid derivatives compared with the $R_1$ analogs **1** (-H), **5** (-$CH_3$), **7** (-$OCH_3$), and **10** (-F). However, the $R_2$ analogs **1** (-H), **2** (-$CH_3$), **3** (-$OCH_3$), **4** (-F) possessed similar less cytotoxicity. (3) Compared with **11-14** and **15-18** and **19-22**, it was interesting to find out compounds with one methoxy group displayed the best cytotoxicity except for the situation that compound with one methoxy group(**19**) had the least cytotoxicity while those without (**15**) or with two (**11**) methoxy groups are equipotent. (4) The presence of CN group had little effect on the activity of the compounds, e.g. **1** and **16**, **6-9** and **19-22**, **23** and **28**, **24-27** and **29-32**. (The CN group was designed to investigate whether it had the function to increase the electrophilicity of the unsaturated methyl cinnamate group

like CDDO-Me. However, we only treated it as a common substituent group due to the experimental results.)

To comprehensively evaluate the cytotoxicity of synthesized compounds, the $IC_{50}$ values (concentration of compound required to reduce 50% of cell viability) of these compounds against three human cancer cells lines (A549, MCF-7, HepG2) were then measured using MTT assay (Table 4). We used CDDO-Me as a positive control, which was studied in phase III clinical trail[28-30]. Evidently, eight selected products were proved to show superior cytotoxicity against A549, MCF-7 and HepG2 cells than celastrol. Particularly, compound **29** displayed the strongest cytotoxcity with $IC_{50}$ values of 0.15 μM, 0.17 μM, and 0.26 μM on A549, MCF-7 and HepG2, respectively. Meanwhile, **29** showed equal or better anti-proliferative activity compared to CDDO-Me. Thus, **29** was selected for further investigation. Moreover, A549 cell lines were chosen for subsequent experiments as they were the most sensitive to **29**.

**Table 3** Preliminary inhibitory effects of the tested compounds on MCF-7 cells.

| **Cpd.** | Inhibition rate (%) at 1.0 μM[a] | **Cpd.** | Inhibition rate (%) at 1.0 μM[a] | **Cpd.** | Inhibition rate (%) at 1.0 μM[a] |
|---|---|---|---|---|---|
| **1** | 22.07 | **12** | 8. 61 | **23** | 46.97 |
| **2** | 19.83 | **13** | 7. 52 | **24** | 79.69 |
| **3** | 11.04 | **14** | 18.01 | **25** | 65.91 |
| **4** | 8.36 | **15** | 63.89 | **26** | 27.42 |
| **5** | 21.95 | **16** | 50.47 | **27** | 45.59 |
| **6** | 59.91 | **17** | 0.94 | **28** | 32.96 |
| **7** | 66.45 | **18** | 5.29 | **29** | 87.70 |
| **8** | 13.75 | **19** | 27.70 | **30** | 67.42 |
| **9** | 25.67 | **20** | 78.53 | **31** | 40.09 |
| **10** | 18.84 | **21** | 15.93 | **32** | 49.18 |
| **11** | 60.30 | **22** | 32.50 | **CE**[b] | 60.21 |

[a]MTT methods, cells were incubated with corresponding compounds at a concentration of 1.0 μM for 48 h. Values are mean of three independent experiments.

[b]Positive control.

**Table 4** $IC_{50}$ values of eight compounds on three human cancer cell lines(A549, MCF-7, HepG2)

| **Cpd.** | **Cytotoxicity $IC_{50}$ (μM)**[a] |
|---|---|

| | A549 | MCF-7 | HepG2 |
|---|---|---|---|
| **7** | 0.66 ± 0.09 | 0.65 ± 0.31 | 0.75 ± 0.15 |
| **11** | 1.07 ± 0.42 | 0.88 ± 0.21 | 0.89 ± 0.22 |
| **15** | 0.60 ± 0.13 | 0.67 ± 0.12 | 0.60 ± 0.13 |
| **20** | 0.56 ± 0.21 | 0.45 ± 0.11 | 0.53 ± 0.12 |
| **24** | 0.46 ± 0.02 | 0.30 ± 0.04 | 0.37 ± 0.03 |
| **25** | 0.78 ± 0.06 | 0.64 ± 0.11 | 0.71 ± 0.15 |
| **29** | 0.15 ± 0.03 | 0.17 ± 0.03 | 0.26 ± 0.02 |
| **30** | 0.70 ± 0.05 | 0.67 ± 0.15 | 0.65 ± 0.22 |
| **CE** | 1.28 ± 0.24 | 1.06 ± 0.17 | 1.33 ± 0.13 |
| **CDDO-Me**[b] | 0.36 ± 0.18 | 0.35 ± 0.07 | 0.26 ± 0.09 |

[a]MTT methods, cells were incubated with corresponding compounds for 48 h. $IC_{50}$ (μM) values (means ± SD, n = 3).

[b]Positive control.

*2.2.2. **29** disrupted the Hsp90-Cdc37 Interaction in vitro*

To make a thorough inquiry about the function of **29** on the interaction of Hsp90-Cdc37, immunoprecipitation experiment had been carried out with DMSO as negative control and **CE** as positive control. We treated A549 cells with **29** or **CE** at the concentration of 5.0 μM for 6 h, respectively, followed by the pull-down of Hsp90α with γ-phosphate-linked ATP-Sepharose. As shown in Fig. 2(A-a) and (A-b), there were no non-specific proteins that affected the results of the experiment. Furthermore, it was proved that when Hsp90α was pulled down, **29** could decrease more amount of Cdc37 distinctly compared to **CE** (Fig. 2B). Thus, these results indicated that **29** possessed stronger function to disrupt the interaction between Hsp90 and Cdc37 than **CE**.

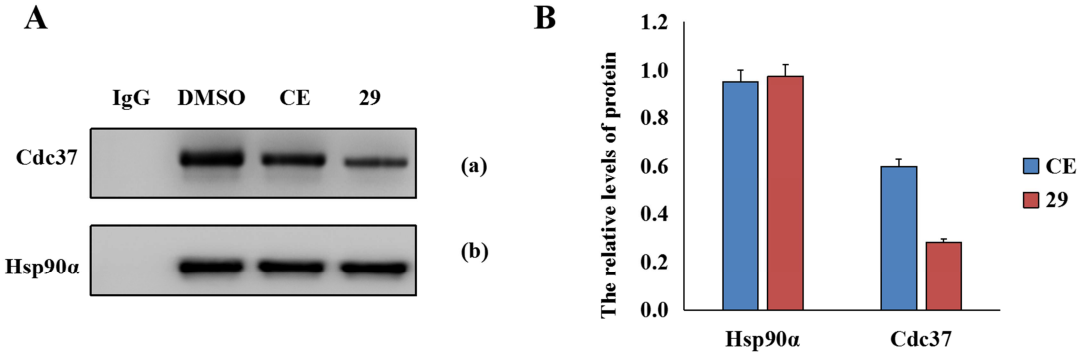



**Fig. 2 29** disrupted the Hsp90-Cdc37 interaction in A549 cells. A549 cells were

treated with 5.0 μM **29** or **CE** for 6 h, respectively. **29** decreased the amount of Cdc37 associated with Hsp90. A: (a) Expression of Cdc37 after Hsp90α was pulled down;(b) Expression of Hsp90α after Hsp90α was pulled down. IgG is to exclude interference from non-specific proteins. B: Grayscale analysis was presented by means of the density ratios of proteins treated with **29** or **CE** to those untreated. Data were expressed as the mean ± SD (n = 3).

*2.2.3. **29** regulated the Hsp90-Cdc37 client proteins and apoptosis-related proteins*

Immunoprecipitation assay has proved **29** as a Hsp90-Cdc37 disruptor, which could result in degradation of downstream client proteins. Once the biological behaviour of these clients playing a key role in the oncogenic process was hindered, they might trigger the apoptosis-related proteins to induce apoptosis. Thus, we detected these proteins with specific antibodies in **29**-treated A549 cells at different concentrations. As depicted in Fig. 3, it was observed that **29** could down-regulate p-Akt and Cdk4 in a concentration-dependent manner. Moreover, compared to **CE**, the expression level of the clients induced by **29** at the same concentration was much lower, which indicated that **29** exhibited stronger inhibitory activity. The levels of Bax, a pro-apoptotic protein of Bcl-2 family, were positively related to concentration. Instead, Bcl-2 displayed the opposite behavior as an anti-apoptotic protein. Additionally, the amount of activated ultimate apoptosis-executing protein caspase-3 got higher as the concentration increased. All these results were consistent with what we have designed and visually represented by grayscale analysis in Fig. 3B. Considering, **29** had inhibition on the clients of Hsp90 and regulate apoptosis-related proteins to induce cell apoptosis, whose effect was stronger than celastrol to some extent.

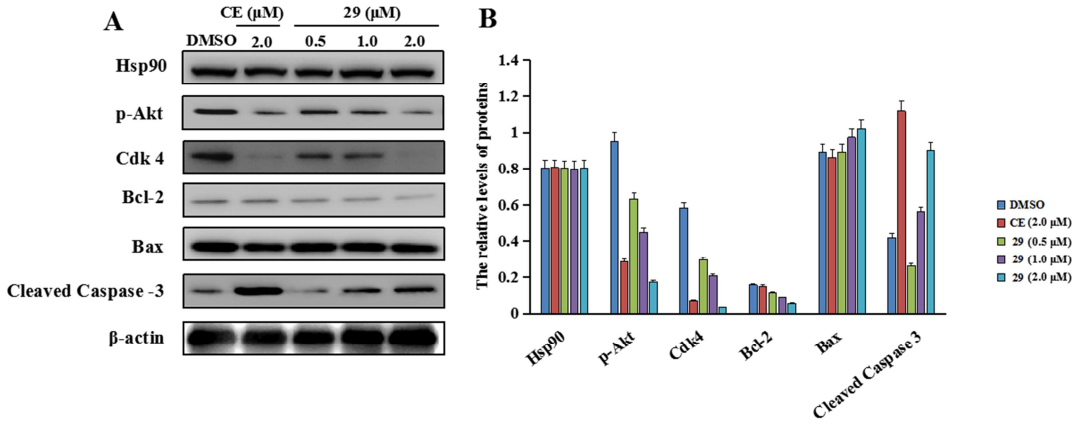



**Fig. 3** Effects of **29** on clients of Hsp90-Cdc37 and apoptosis-related proteins in A549 cells. (A) The western blot results revealed the expression levels of Hsp90, Cdk4, p-Akt, cleaved caspase-3, Bax, Bcl-2 in A549 cells untreated (control) and treated with **29** for 8 h at different concentrations (0.5, 1.0, 2.0 μM). **CE** (2.0 μM) was used as a positive control. β-actin was used as a loading control. (B) Grayscale analysis was presented by means of the density ratios of proteins to β-actin. Data were expressed as the mean ± SD (n = 3).

*2.2.4. Effects of **29** on cell-cycle distribution*

Cdk4 is a key regulator protein of cell cycle. As mentioned above, **29** could cause a decrease in the expression level of Cdk4 after destroying Hsp90-Cdc37 complex. To test whether the anti-proliferative activity was due to cell cycle arrest, A549 cells were treated with compound **29** at three different concentrations (0.06, 0.18, and 0.27 μM) for 12 and 24 h and untreated cells as the control. The analysis was performed by flow cytometry using a Fluorescence-Activated Cell Sorter (FACS) after labelling with propidium iodide (PI). As shown in Fig. 4, the results indicated that **29** arrested cell cycle at G0/G1 phase. Thus, cell cycle arrest may be one of the mechanisms of the anti-proliferation activity of **29**. The analysis lead to a concentration-dependent accumulation of cells in the $G_0/G_1$ phase with a concomitant increase after both 12 and 24 h of treatment. Moreover, from the results illustrated in Fig. 4E, the proportions of cells treated after 24h had a negligible accumulation than the proportions of cells treated after 12h at the same concentration. Thus, these results suggest that growth inhibition of the A549 cells proliferation by **29** may be

related to induction of $G_0/G_1$ phase arrest.

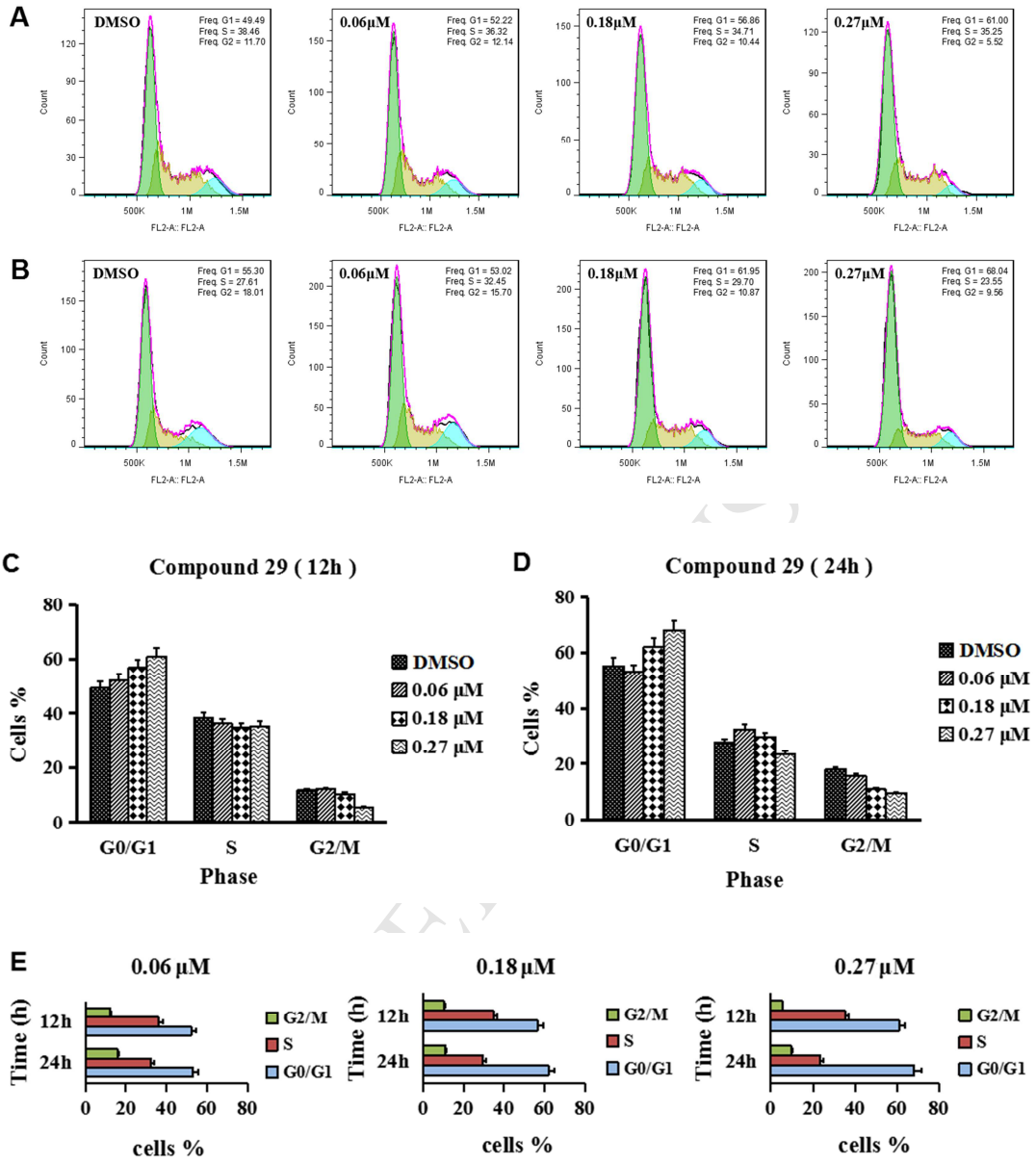



**Fig. 4** Cell cycle analysis of **29** in A549 cells. (A) and (C) Cells were treated with **29** at 0.06, 0.18 and 0.27 μM for 12 h, harvested, stained with PI, and then analyzed by flow cytometry. (B) and (D) Cells were treated with **29** at 0.06, 0.18 and 0.27 μM for 24 h, harvested, stained with PI, and then analyzed by flow cytometry. (E) Time-dependent effects of **29** on cell cycle were investigated at three different concentrations (0.06, 0.18, and 0.27 μM).

*2.2.5. **29** induced apoptosis by the extrinsic death receptor pathway*

To further explore the apoptotic mechanism induced by **29**, the levels of related

proteins in the extrinsic signaling pathway were analyzed. It is well-known that cell apoptosis is complex. One of its main mechanisms is extrinsic pathway, which is also called Fas/Fas-L death receptor pathway[31]. Caspase-8 is an initiating apoptotic protease, which is in response to extracellular apoptosis-inducing ligands after being activated[32]. PARP, a cleavage substrate for caspase, is another core member in cell apoptosis[33]. Consequently, we evaluated the effects of **29** on Fas, Fas-L, cleaved caspase-8 and cleaved PARP, adopting β-actin as an internal reference. As shown in Fig. 5, the expressions of all tested proteins were performed in a concentration-dependent manner. These findings proved that **29** could induce apoptosis in an extrinsic death receptor pathway. Moreover, it was worth mentioned that the band of cleaved PARP treated with **29** at 2.0 μM got unusually conspicuous while those which were induced by **CE** at the same condition or untreated were almost unobserved. The results could indicate to some extent that PARP was more sensitive to **29**, in other words, **29** might target mainly at PARP to cause cell death. However, the specific mechanism is intricate, which deserves our in-depth research.

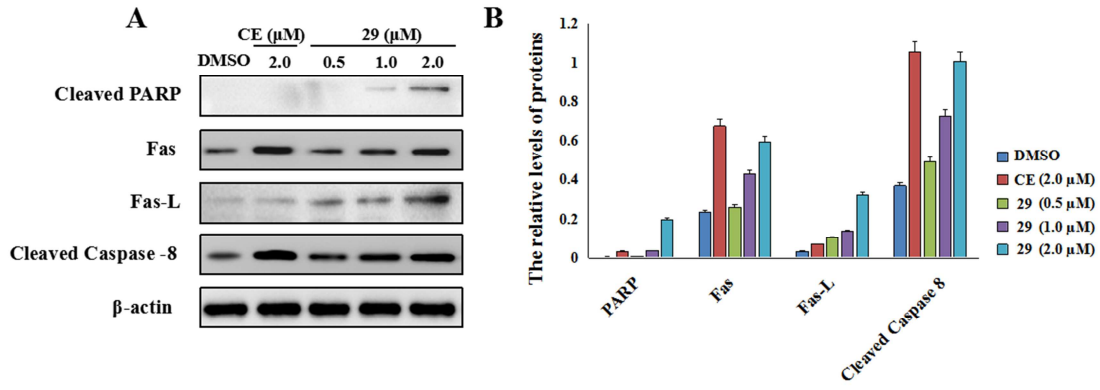



**Fig. 5** Effects of **29** on extrinsic death receptor pathway proteins in A549 cells. (A) The western blot results revealed the expression levels of Fas, Fas-L, cleaved caspase-8, cleaved PARP in A549 cells untreated (control) and treated with **29** for 8 h at different concentrations (0.5, 1.0, 2.0 μM). **CE** (2.0 μM) was used as a positive control. β-actin was used as a loading control. (B) Grayscale analysis was presented by means of the density ratios of proteins to β-actin. Data were expressed as the mean ± SD (n = 3).

*2.2.6. Effects of **29** on cell apoptosis*

On the basis of the experiments above, **29** was witnessed to induce cell apoptosis. In order to provide more convincing evidence, we analyzed A549 cells treated with different concentrations of **29** by flow cytometry after Annexin V-FITC/PI double staining. As observed in Fig. 6, the total numbers of apoptotic cells grew with the increasing consistency. Particularly, late apoptosis was conspicuous. The greatest consequent of **29** was at 1.2 μM with 11.3 % early and 52.2 % late apoptotic cells while those are 4.1 % and 4.2 % separately in the control condition. Interestingly, 0.4 μM **29**-incubated cells had similar effects on those dealt with 2.0 μM **CE**. In other words, the potency of anti-tumor of **29** was approximately five times stronger compared to the parent compound. Hence, our structural modification design for celastrol **CE** could promote the apoptosis of A549.

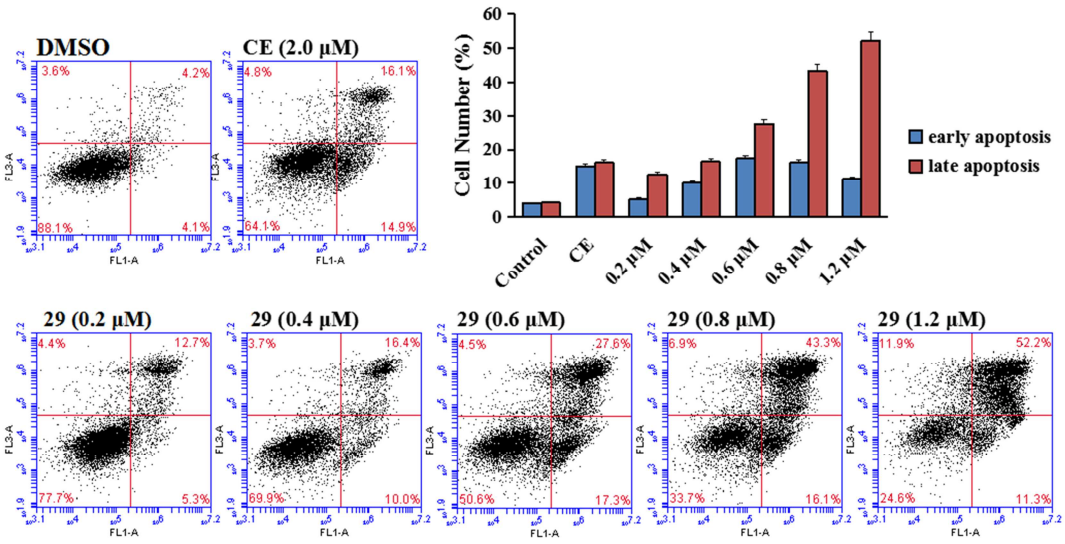



**Fig. 6** Apoptosis analysis of **29** and **CE** in A549 cells. Cells were treated with **CE** (2.0 μM) and compound **29** at distinct concentrations (0.2, 0.4, 0.6, 0.8, 1.2 μM) for 24 h, analyzed by flow cytometry. The results were presented in the form of a column chart. The figures were representative of three independent experiments and the values were expressed as means ± SD.

*2.2.7. Morphological analysis of A549 treated with **29***

It's reported that apoptotic cells acquire morphological characteristics, such as cell shrinkage, nuclear fragmentation, zeiosis and apoptotic bodies formation[34]. To corroborate the effect of compound **29** on induction of apoptosis, cells treated with

compound **29** for 48 h at different concentrations (0.06, 0.09, and 0.18 μM) were observed by fluorescence microscopy after stained with Hoechst 33342. As depicted in Fig. 7, with the concentration increased, A549 cells showed typical apoptotic features such as cell shrinkage and nuclear fragmentation. Moreover, the amount of cells was obviously decreased, which indicated that there was a concentration-dependent effect of **29** on cell apoptosis. These morphological observations further confirmed our hypothesis that compound **29** induces apoptosis in A549 cells.

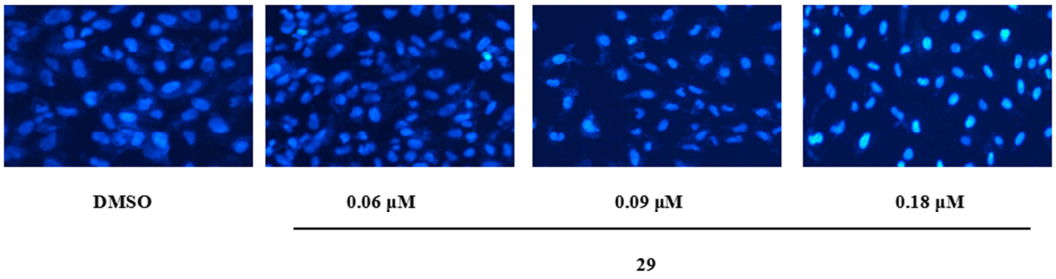



**Fig. 7** Fluorescence microscopy images of A549 cells stained by Hoechst 33342. A549 cells were treated with the **29** at indicated concentrations (0.06, 0.09, and 0.18 μM) for 48 h.

## 3. Conclusion

In summary, based on the hybridization strategy of developing novel anti-tumor drugs, a series of celastrol hybrids were designed and synthesized. Some derivatives performed promoted anti-proliferative activity on three human cancer cell lines (A549, MCF-7, HepG2) *in vitro*. Among them, compound **29** showed the strongest anti-tumor activity ($IC_{50}$ = 0.15-0.26 μM). The SAR of all target products was also discussed. Further bio-assay proved that **29** retained the ability to disrupt the interaction of Hsp90 and Cdc37 compared to celastrol. Meanwhile, it could down-regulate Hsp90 client proteins, followed by triggering cell apoptosis. Studies on mechanism suggested that **29** induced apoptosis in A549 cells by activating Fas/Fas-L, caspase-8 and PARP through extrinsic signaling pathway. Moreover, **29** arrested cell cycle at $G_0/G_1$ phase, which was considered as another main mechanism of cell death. Taken together, as a promising candidate for cancer therapy, compound

**29** is worth further research to obtain more details.

## 4. Experimental

*4.1.Chemistry*

All reagents were available from commercial suppliers and used directly unless otherwise stated. Routine thin-layer chromatography (TLC) was performed to monitor reactions on silica gel plates with an ultraviolet lamp. Column chromatography was carried out on silica gel (200-300 mesh). $^{1}H$ NMR and $^{13}C$ NMR spectra were all recorded on a Bruker AVANCE instrument at 25□. The molecular weights were detected on HP 1100LC/MSD spectrometer.

*4.1.1. Synthesis of intermediates and target compounds*

*4.1.1.1. General procedures for the preparation of Compound*

*4.1.1.1.1. General procedures for Synthesizing Compounds* ***b*****$_{1-7}$*****, e*****$_{1-2}$**

$NH_4OAc$ (0.5 eq, 4.0 mmol, 308.0 mg) and hydroxy aryl aldehyde $\mathbf{a}_{1-7}$ or $\mathbf{d}_{1-2}$ (0.9 eq, 7.2 mmol) were dissolved in a solution of methyl cyanoacetate (1.0 eq, 8.0 mmol, 706 μl) in tolune (45 ml).The mixture was refluxed at 100 □ for 12 h by stirring. The reaction mixture was filtered and then the crude product was purified by column chromatography using petroleum ether : ethyl acetate (EtOAc) = 2:1 (v/v).

*4.1.1.1.2. General procedures for Synthesizing Compounds* ***b*****$_{8}$*****, e*****$_{3-4}$**

Sinapic acid ($\mathbf{a}_8$) or 3-hydroxycinnamic acid ($\mathbf{d}_3$) or isoferulic acid ($\mathbf{d}_4$) (1.0 eq, 5.0 mmol) was dissolved in methanol (50 ml), stirred under the catalysis of $H_2SO_4$ (1.0-2.0 ml) at 70 □ for 12 h. On cooling, the solvent was removed and drops of $Na_2CO_3$ were then added to the residue until the pH was greater or equal to 7.0. The resulting solution was extracted with EtOAc (3×20 ml). The combined organic layer was washed with NaCl solution (2×20 ml), dried with $Na_2SO_4$, and subsequently solvent was evaporated to acquire the crude product $\mathbf{b}_8$ or $\mathbf{e}_{3-4}$.

*4.1.1.1.3. General procedures for Synthesizing Compounds* ***c*****$_{1-22}$*****, f*****$_{1-10}$**

*4.1.1.1.3.1. General procedures for Synthesizing Compounds* ***c*****$_{1-8}$*****, c*****$_{10}$*****, c*****$_{11-13}$*****, c*****$_{15-17}$*****, c*****$_{19-21}$*****, f*****$_{1-4}$*****, f*****$_{6-9}$**

Compound $\mathbf{b}_{1-7}$ or methyl sinapate ($\mathbf{b}_8$) or methyl 4-hydroxycinnamate ($\mathbf{b}_9$) or methyl

ferulate (**$b_{10}$**) or compound **$e_{1\text{-}2}$** or methyl 3-hydroxycinnamate (**$e_3$**) or isoferulic acid methyl ester (**$e_4$**) (1.0 eq, 1.2 mmol) and $K_2CO_3$ (3.0 eq, 3.6 mmol) were successively dissolved in anhydrous acetone (8 ml). A solution of different alkyl bromides (5.0 eq, 6.0 mmol) in anhydrous acetone was added and this reaction mixture was stirred at 50 □ for 7-8 h. The reaction mixture was concentrated under vaccum and the residue was dissolved in dichloromethane. The organic layer was washed with 5% aqueous $NaHCO_3$ solution (60 ml) and aqueous saturated NaCl solution (30 ml). After drying over anhydrous $Na_2SO_4$, the dichloromethane was removed in vaccum. The crude materials was purified by column chromatography on silica gel [petroleum ether /ethyl acetate=6:1 (v/v)] .

*4.1.1.1.3.2. General procedures for Synthesizing Compounds* ***$c_9$, $c_{14}$, $c_{18}$, $c_{22}$, $f_5$, $f_{10}$***

Methyl sinapate (**$b_8$**) or methyl 4-hydroxycinnamate (**$b_9$**) or methyl ferulate (**$b_{10}$**) or compound **$e_2$** or isoferulic acid methyl ester (**$e_4$**) (1.0 eq, 0.5 mmol) , $K_2CO_3$ (2.0 eq, 1.0 mmol, 138.0 mg), $Bu_4N^+Br^-$ (0.06 eq, 0.03 mmol, 9.67 mg) and trans-1,4-dibromo-2-butene (3.0 eq, 1.5 mmol, 321.0 mg) were added into anhydrous acetone. The reaction mixture was refluxed for 7 h by stirring. The acetone was removed under reduced pressure and the residue was extracted with ethyl acetate (60 ml). The combined organic layers were dried over sodium sulfate, filtered, and the solvent was evaporated. The crude materials was purified by column chromatography on silica gel [petroleum ether /ethyl acetate=10:1 (v/v)] to afford pure intermediates **$c_9$, $c_{14}$, $c_{18}$, $c_{22}$, $f_5$, $f_{10}$**.

*4.1.1.1.4. General procedures for Synthesizing Target Compounds* ***1-32***

A mixture of celastrol (1.0 eq, 0.1 mmol, 45.1 mg), **$c_{1\text{-}22}$** or **$f_{1\text{-}10}$** (3.0eq, 0.3 mmol) and $NaHCO_3$ (5.0 eq, 0.5 mmol, 42.0 mg) in anhydrous DMF (6 ml) was stirred at 60 ℃ under reflux for 6 h. The reaction solution was washed with DCM (100 ml) for four times. The combined organic layers were washed by aqueous saturated NaCl solution (30 ml), then dried over sodium sulfate, filtered, and the solvent was evaporated. The crude product was purified by column chromatography [petroleum ether /ethyl acetate=3:1 (v/v)].

*4.1.1.1.4.1.* Compound **1**. Obtained from celastrol and **$c_1$**. Orange red powder, 40.5% yield. Analytical data for **1**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.53 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.25 (3H, s), 1.44 (3H, s), 2.24 (3H, s), 3.94 (3H, s), 4.05-4.23 (4H, m), 6.30 (1H, d, *J* = 7.1 Hz), 6.50 (1H, s), 6.96 (2H, d, *J* = 8.9 Hz), 7.05 (1H, d, *J* = 7.0 Hz), 8.00 (2H, d, *J* = 8.8 Hz), 8.22 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 170.0, 164.7, 163.6, 163.0, 154.7, 146.0, 134.0, 133.8, 127.4 (C×2), 124.4, 119.5, 118.2, 117.2, 116.3, 115.1 (C×2), 98.9, 64.7, 60.9, 53.2, 45.0, 44.2, 42.9, 40.5, 39.4, 38.2, 36.3, 34.8, 33.4, 32.8, 31.6, 30.9, 30.5, 29.8, 29.7, 28.4, 28.1, 21.6, 18.6, 10.3; HRMS (ESI) calculated for $C_{43}H_{52}NO_7$ $[M + H]^+$ 694.3744, found 694.3753.

*4.1.1.1.4.2.* Compound **2**. Obtained from celastrol and **$c_2$**. Orange red powder, 38.8% yield. Analytical data for **2**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.54 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.25 (3H, s), 1.44 (3H, s), 2.23 (3H, s), 2.45 (3H, s), 3.94 (3H, s), 4.01-4.14 (3H, m), 4.17-4.23 (1H, m), 6.31 (1H, d, *J* = 7.2 Hz), 6.51 (1H, s), 6.78 (1H, s), 6.81 (1H, d, *J* = 8.8 Hz), 7.04 (1H, d, *J* = 7.1 Hz), 8.34 (1H, d, *J* = 8.8 Hz), 8.52 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 169.9, 164.7 (C×2), 162.3, 156.5, 152.1, 146.0, 134.1, 131.0, 127.4 (C×2), 123.3, 119.5, 118.2, 117.2 (C×2), 112.3, 99.9, 64.5, 60.9, 53.2, 45.0, 44.2, 42.9, 40.5, 39.3, 38.2, 36.3, 34.8, 33.4, 32.8, 31.6, 30.9, 30.5, 29.8, 29.7, 28.5, 28.2, 21.6, 20.1, 18.6, 10.3; HRMS (ESI) calculated for $C_{44}H_{54}NO_7$ $[M + H]^+$ 708.3900, found 708.3895.

*4.1.1.1.4.3.* Compound **3**. Obtained from celastrol and **$c_3$**. Orange red powder, 39.6% yield. Analytical data for **3**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.45 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.22 (3H, s), 1.42 (3H, s), 2.25 (3H, s), 3.75 (3H, s), 3.93 (3H, s), 4.01-4.06 (1H, m), 4.11-4.22 (3H, m), 6.20 (1H, d, *J* = 7.1 Hz), 6.36 (1H, d, *J* = 2.2 Hz), 6.50 (1H, s), 6.55 (1H, dd, *J* = 9.0 Hz, 2.2 Hz), 7.00 (1H, d, *J* = 7.0 Hz), 8.41 (1H, d, *J* = 8.9 Hz), 8.73 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 170.0, 165.0, 164.7, 164.1, 148.9, 146.0, 143.3, 134.3, 131.2, 127.3, 119.4, 118.1, 117.3, 116.9, 114.1, 105.6 (C×2), 98.8, 64.5, 60.7, 55.8, 45.0, 44.1, 42.9, 40.5, 39.2, 38.2, 36.3, 35.0, 33.2, 33.0, 31.6, 30.9, 30.5, 29.7, 29.6, 28.4, 28.0, 21.6, 20.1, 18.6,

10.3; HRMS (ESI) calculated for $C_{44}H_{54}NO_8$ $[M + H]^+$ 724.3849, found 724.3855.

*4.1.1.1.4.4.* Compound **4**. Obtained from celastrol and **$c_4$**. Orange red powder, 41.2% yield. Analytical data for **4**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.52 (3H, s), 1.10 (3H, s), 1.18 (3H, s), 1.24 (3H, s), 1.44 (3H, s), 2.23 (3H, s), 3.94 (3H, s), 4.03-4.18 (4H, m), 6.29 (1H, d, *J* = 7.4 Hz), 6.51 (1H, s), 6.73 (1H, d, *J* = 8.9 Hz), 6.78 (1H, d, *J* = 8.9 Hz), 7.03 (1H, d, *J* = 7.3 Hz), 7.06 (1H, s), 8.50 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 170.0, 164.7 (C×2), 158.3, 157.5, 153.1, 146.0, 134.1, 130.3, 127.4, 119.5, 118.2, 117.2, 116.9, 111.6, 106.6, 102.2, 98.9, 65.3, 60.8, 53.3, 45.0, 44.2, 42.9, 40.5, 39.4, 38.2, 36.3, 34.8, 33.5, 32.8, 31.6, 30.9, 30.5, 29.7, 29.6, 28.5, 28.0, 21.6, 18.6, 10.3; HRMS (ESI) calculated for $C_{43}H_{51}FNO_7$ $[M + H]^+$ 712.3650, found 712.3646.

*4.1.1.1.4.5.* Compound **5**. Obtained from celastrol and **$c_5$**. Orange red powder, 35.1% yield. Analytical data for **5**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.50 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.23 (3H, s), 1.43 (3H, s), 2.13 (3H, s), 2.26 (3H, s), 3.94 (3H, s), 4.07-4.21 (4H, m), 6.25 (1H, d, *J* = 7.1 Hz), 6.50 (1H, s), 6.90 (1H, d, *J* = 8.6 Hz), 7.07 (1H, d, *J* = 7.2 Hz), 7.79 (1H, s), 7.93 (1H, d, *J* = 8.6 Hz), 8.19 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 170.0, 164.6, 163.7, 153.6, 152.0, 146.0, 134.0, 130.9, 127.4 (C×2), 124.8, 119.5, 118.4, 118.2, 117.2, 115.7, 114.1, 100.6, 66.0, 60.8, 53.4, 45.0, 44.2, 42.9, 40.5, 39.4, 38.2, 36.3, 34.8, 33.5, 32.8, 31.6, 30.9, 30.5, 29.8, 29.7, 28.5, 28.2, 21.6, 20.1, 18.5, 10.3; HRMS (ESI) calculated for $C_{44}H_{54}NO_7$ $[M + H]^+$ 708.3900, found 708.3904.

*4.1.1.1.4.6.* Compound **6**. Obtained from celastrol and **$c_6$**. Orange red powder, 37.5% yield. Analytical data for **6**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.53 (3H, s), 1.11 (3H, s), 1.21 (3H, s), 1.25 (3H, s), 1.43 (3H, s), 2.21 (3H, s), 3.92 (3H, s), 3.95 (3H, s), 4.26-4.34 (3H, m), 4.38-4.51 (1H, m), 6.31 (1H, d, *J* = 7.2 Hz), 6.42 (1H, s), 6.93 (1H, d, *J* = 8.4 Hz), 7.00 (1H, d, *J* = 7.1 Hz), 7.41 (1H, dd, *J* = 8.5 Hz, 2.1 Hz), 7.80 (1H, d, *J* = 2.2 Hz), 8.18 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 169.7, 164.6, 163.6, 154.9, 152.7, 149.7, 146.0, 133.9, 127.6, 127.4, 125.1, 119.5, 118.1, 117.2 (C×2), 112.9, 112.3, 99.3, 66.9, 62.5, 56.0, 45.0, 44.2, 42.9, 40.6, 39.4,

38.2, 36.4, 34.8, 33.4, 32.8, 31.6, 30.8, 30.5, 29.8, 29.6, 28.5, 21.6, 18.6, 10.3; HRMS (ESI) calculated for $C_{43}H_{52}NO_8$ $[M + H]^+$ 710.3693, found 710.3688.

*4.1.1.1.4.7*. Compound **7**. Obtained from celastrol and **$c_7$**. Orange red powder, 36.8% yield. Analytical data for **7**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.50 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.24 (3H, s), 1.44 (3H, s), 2.24 (3H, s), 3.82 (3H, s), 3.95 (3H, s), 4.04-4.12 (1H, m), 4.17-4.21 (3H, m), 6.28 (1H, d, *J* = 7.2 Hz), 6.49 (1H, s), 6.92 (1H, d, *J* = 8.6 Hz), 7.07 (1H, d, *J* = 7.0 Hz), 7.49 (1H, d, *J* = 8.5 Hz), 7.75 (1H, s), 8.20 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 169.8, 164.6, 163.6, 155.0, 153.1, 149.5, 146.0, 134.1, 127.8, 127.4, 124.6, 119.5, 118.2, 117.2, 116.4, 112.1, 111.8, 98.8, 65.3, 60.9, 55.8, 45.0, 44.2, 42.9, 40.5, 39.3, 38.2, 36.3, 34.9, 33.3, 32.8, 31.6, 30.9, 30.5, 29.7, 29.6, 28.4, 28.0, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{44}H_{54}NO_8$ $[M + H]^+$ 724.3849, found 724.3852.

*4.1.1.1.4.8*. Compound **8**. Obtained from celastrol and **$c_8$**. Orange red powder, 39.2% yield. Analytical data for **8**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.49 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.23 (3H, s), 1.43 (3H, s), 2.23 (3H, s), 3.82 (3H, s), 3.95 (3H, s), 4.06-4.11 (1H, m), 4.17-4.19 (3H, m), 6.28 (1H, d, *J* = 6.8 Hz), 6.50 (1H, s), 6.92 (1H, d, *J* = 8.6 Hz), 7.08 (1H, d, *J* = 6.9 Hz), 7.49 (1H, d, *J* = 8.5 Hz), 7.76 (1H, s), 8.22 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 170.0, 164.7, 163.7, 155.0, 153.2, 149.5, 146.0, 134.2, 127.9, 127.1, 124.5, 119.5, 118.1, 117.2, 116.4, 112.0, 111.8, 98.8, 68.5, 64.0, 56.0, 53.2, 45.0, 44.3, 42.9, 40.4, 39.4, 38.3, 36.4, 34.9, 33.5, 32.8, 31.6, 30.9, 30.5, 29.8, 29.7, 25.7, 25.3, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{45}H_{56}NO_8$ $[M + H]^+$ 738.4006, found 738.4004.

*4.1.1.1.4.9*. Compound **9**. Obtained from celastrol and **$c_9$**. Orange red powder, 40.3% yield. Analytical data for **9**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.57 (3H, s), 1.12 (3H, s), 1.21 (3H, s), 1.28 (3H, s), 1.46 (3H, s), 2.21 (3H, s), 3.95 (6H, s), 4.46-4.51 (2H, m), 4.68-4.69 (2H, m), 5.99 (2H, m), 6.36 (1H, d, *J* = 7.2 Hz), 6.55 (1H, d, *J* = 1.4 Hz), 6.91 (1H, d, *J* = 8.5 Hz), 7.02 (1H, dd, *J* = 7.1 Hz, 1.4 Hz), 7.45 (1H, dd, *J* = 2.1 Hz, 8.6 Hz), 7.83 (1H, d, *J* = 2.1 Hz), 8.20 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$:177.8 (C×2), 170.0, 164.6, 163.6, 155.0, 152.6, 149.5, 146.0, 134.0,

128.5, 128.0, 127.7, 127.4, 124.8, 119.4, 118.1, 117.1, 116.4, 112.4, 111.8, 90.0, 68.5, 63.8, 56.1, 45.0, 44.2, 42.9, 40.5, 39.4, 38.3, 36.3, 34.8, 33.5, 32.8, 31.6, 30.8, 30.5, 29.8, 29.6, 28.7, 21.8, 18.7, 10.3; HRMS (ESI) calculated for $C_{45}H_{54}NO_8$ $[M + H]^+$ 736.3849, found 736.3853.

*4.1.1.1.4.10*. Compound **10**. Obtained from celastrol and **$c_{10}$**. Orange red powder, 38.1% yield. Analytical data for **10**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.55 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.26 (3H, s), 1.45 (3H, s), 2.23 (3H, s), 3.95 (3H, s), 4.07-4.15 (1H, m), 4.19-4.22 (3H, m), 6.32 (1H, d, *J* = 7.2 Hz), 6.49 (1H, s), 7.01 (1H, d, *J* = 8.8 Hz), 7.04 (1H, s), 7.05 (1H, d, *J* = 7.0 Hz), 7.77 (1H, d, *J* = 8.9 Hz), 8.17 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 170.0, 164.7, 163.2, 153.4, 152.1, 146.0, 134.1, 130.8, 127.4 (C×2), 123.3, 119.5, 118.1, 117.2 (C×2), 116.3, 112.4, 100.0, 64.5, 60.9, 53.2, 45.0, 44.2, 42.9, 40.5, 39.3, 38.2, 36.3, 34.8, 33.4, 32.8, 31.6, 30.9, 30.5, 29.8, 29.7, 28.5, 28.2, 21.6, 20.1, 18.6, 10.3; HRMS (ESI) calculated for $C_{43}H_{51}FNO_7$ $[M + H]^+$ 712.3650, found 712.3638.

*4.1.1.1.4.11*. Compound **11**. Obtained from celastrol and **$c_{11}$**. Orange red powder, 33.8% yield. Analytical data for **11**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.57 (3H, s), 1.11 (3H, s), 1.20 (3H, s), 1.26 (3H, s), 1.41 (3H, s), 2.22 (3H, s), 3.84 (6H, s), 4.10-4.17 (1H, m), 4.21-4.26 (3H, m), 6.34 (1H, d, *J* = 7.3 Hz), 6.35 (1H, s), 6.40 (1H, d, *J* = 16.1 Hz), 6.77 (2H, s), 7.01 (1H, d, *J* = 7.4 Hz), 7.65 (1H, d, *J* = 16.0 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 170.0, 167.5, 164.8, 153.5 (C×2), 146.0, 144.9, 139.0, 134.0, 130.1, 127.4, 119.5, 118.1, 117.2, 117.0, 105.2 (C×2), 70.0, 64.0, 56.0 (C×2), 51.7, 45.1, 44.3, 42.9, 40.4, 39.5, 38.2, 36.4, 34.7, 33.4, 32.6, 31.6, 30.9, 30.6, 29.7, 29.3, 28.6, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{43}H_{55}O_9$ $[M + H]^+$ 715.3846, found 715.3854.

*4.1.1.1.4.12*. Compound **12**. Obtained from celastrol and **$c_{12}$**. Orange red powder, 37.8% yield. Analytical data for **12**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.57 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.27 (3H, s), 1.46 (3H, s), 2.21 (3H, s), 3.80 (3H, s), 3.85 (6H, s), 3.90-3.95 (1H, m), 4.02-4.10 (2H, m), 6.35 (1H, d, *J* = 6.9 Hz), 6.36 (1H, d, *J* = 15.8 Hz), 6.51 (1H, s), 6.74 (2H, s), 7.00 (1H, d, *J* = 6.9 Hz), 7.59 (1H, d,

*J* = 15.8 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 170.0, 167.4, 164.6, 153.6 (C×2), 146.0, 144.9, 138.9, 134.0, 130.0, 127.4, 119.5, 118.1, 117.1, 117.0, 105.2 (C×2), 70.0, 61.5, 56.1 (C×2), 51.7, 45.0, 44.3, 42.9, 40.4, 39.4, 38.2, 36.4, 34.8, 33.4, 32.8, 31.6, 30.8, 30.5, 29.8, 29.6, 29.2, 28.6, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{44}H_{57}O_9$ $[M + H]^+$ 729.4003, found 729.3997.

*4.1.1.1.4.13*. Compound **13**. Obtained from celastrol and **$c_{13}$**. Orange red powder, 38.3% yield. Analytical data for **13**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), *δ* ppm: 0.57 (3H, s), 1.11 (3H, s), 1.19 (3H, s), 1.27 (3H, s), 1.45 (3H, s), 2.22 (3H, s), 3.82 (3H, s), 3.86 (6H, s), 3.92-3.96 (1H, m), 4.01-4.09 (2H, m), 6.35 (1H, d, *J* = 6.9 Hz), 6.36 (1H, d, *J* = 15.8 Hz), 6.51 (1H, s), 6.74 (2H, s), 7.02 (1H, d, *J* = 6.9 Hz), 7.62 (1H, d, *J* = 15.9 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 170.0, 167.4, 164.7, 153.6 (C×2), 146.0, 144.9, 139.2, 134.1, 129.8, 127.4, 119.5, 118.1, 117.1, 116.9, 105.2 (C×2), 72.7, 64.2, 56.1 (C×2), 51.7, 45.1, 44.3, 42.9, 40.4, 39.5, 38.2, 36.4, 34.8, 33.5, 32.8, 31.6, 30.8, 30.6, 29.8, 29.7, 28.6, 26.7, 25.0, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{45}H_{59}O_9$ $[M + H]^+$ 743.4159, found 743.4159.

*4.1.1.1.4.14*. Compound **14**. Obtained from celastrol and **$c_{14}$**. Orange red powder, 41.1% yield. Analytical data for **14**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), *δ* ppm: 0.56 (3H, s), 1.11 (3H, s), 1.19 (3H, s), 1.27 (3H, s), 1.45 (3H, s), 2.22 (3H, s), 3.83 (3H, s), 3.88 (6H, s), 4.36-4.48 (2H, m), 4.54-4.56 (2H, m), 5.80-5.87 (1H, m), 5.97-6.07 (1H, m), 6.35 (1H, d, *J* = 6.6 Hz), 6.37 (1H, d, *J* = 16.1 Hz), 6.50 (1H, s), 6.75 (2H, s), 7.02 (1H, d, *J* = 6.7 Hz), 7.63 (1H, d, *J* = 16.1 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 177.9, 170.0, 167.4, 164.7, 153.6 (C×2), 146.0, 144.9, 139.3, 134.0, 130.0, 129.8, 127.5, 127.4, 119.5, 118.1, 117.0, 116.9, 105.1 (C×2), 73.0, 64.1, 56.1 (C×2), 51.7, 45.1, 44.3, 42.9, 40.4, 39.4, 38.2, 36.4, 34.8, 33.4, 32.8, 31.6, 30.8, 30.5, 29.8, 29.6, 28.7, 21.7, 18.5, 10.3; HRMS (ESI) calculated for $C_{45}H_{57}O_9$ $[M + H]^+$ 741.4003, found 741.4004.

*4.1.1.1.4.15*. Compound **15**. Obtained from celastrol and **$c_{15}$**. Orange red powder, 38.4% yield. Analytical data for **15**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), *δ* ppm: 0.57 (3H, s), 1.10 (3H, s), 1.21 (3H, s), 1.26 (3H, s), 1.45 (3H, s), 2.23 (3H, s), 3.82 (3H,

s), 4.13-4.27 (3H, m), 4.31-4.38 (1H, m), 6.33 (1H, d, *J* = 6.7 Hz), 6.37 (1H, d, *J* = 15.9 Hz), 6.51 (1H, s), 6.88 (2H, d, *J* = 8.7 Hz), 7.02 (1H, d, *J* = 6.8 Hz), 7.50 (2H, d, *J* = 8.7 Hz), 7.66 (1H, d, *J* = 16.0 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 169.8, 167.8, 164.7, 160.2, 146.0, 144.4, 134.0, 129.8 (C×2), 127.6, 127.4, 119.6, 118.1, 117.1, 115.6, 114.9 (C×2), 65.8, 62.9, 51.6, 45.0, 44.2, 42.9, 40.5, 39.4, 38.2, 36.4, 34.8, 33.6, 32.8, 31.6, 30.7, 30.5, 29.8, 29.7, 28.6, 21.6, 18.6, 10.3; HRMS (ESI) calculated for $C_{41}H_{51}O_7$ $[M + H]^+$ 655.3635, found 655.3622.

*4.1.1.1.4.16*. Compound **16**. Obtained from celastrol and **$c_{16}$**. Orange red powder, 40.7% yield. Analytical data for **16**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), *δ* ppm: 0.52 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.25 (3H, s), 1.44 (3H, s), 2.24 (3H, s), 3.82 (3H, s), 4.01-4.09 (3H, m), 4.16-4.24 (1H, m), 6.28 (1H, d, *J* = 7.0 Hz), 6.35 (1H, d, *J* = 16.0 Hz), 6.51 (1H, s), 6.86 (2H, d, *J* = 8.7 Hz), 7.02 (1H, d, *J* = 6.9 Hz), 7.47 (2H, d, *J* = 8.7 Hz), 7.67 (1H, d, *J* = 16.0 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 169.8, 167.8, 164.7, 160.5, 146.0, 144.5, 134.0, 129.8 (C×2), 127.4, 127.2, 119.5, 118.1, 117.1, 115.3, 114.7 (C×2), 64.3, 61.0, 51.6, 45.0, 44.2, 42.9, 40.5, 39.4, 38.2, 36.3, 34.8, 33.4, 32.8, 31.6, 30.9, 30.5, 29.8, 29.7, 28.5, 28.3, 21.6, 18.6, 10.3; HRMS (ESI) calculated for $C_{42}H_{53}O_7$ $[M + H]^+$ 669.3791, found 669.3792.

*4.1.1.1.4.17*. Compound **17**. Obtained from celastrol and **$c_{17}$**. Orange red powder, 39.1% yield. Analytical data for **17**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), *δ* ppm: 0.57 (3H, s), 1.11 (3H, s), 1.20 (3H, s), 1.27 (3H, s), 1.46 (3H, s), 2.22 (3H, s), 3.81 (3H, s), 3.93-3.97 (3H, m), 4.00-4.09 (1H, m), 6.31 (1H, d, *J* = 7.0 Hz), 6.33 (1H, d, *J* = 16.0 Hz), 6.53 (1H, s), 6.87 (2H, d, *J* = 8.6 Hz), 7.03 (1H, d, *J* = 7.2 Hz), 7.46 (2H, d, *J* = 8.6 Hz), 7.66 (1H, d, *J* = 16.1 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 170.0, 167.8, 164.7, 160.7, 146.0, 144.6, 134.1, 129.7 (C×2), 127.4, 127.1, 119.5, 118.2, 117.1, 115.2, 114.8 (C×2), 67.5, 64.1, 51.6, 45.0, 44.3, 42.9, 40.4, 39.4, 38.2, 36.4, 34.8, 33.5, 32.8, 31.6, 30.9, 30.5, 29.8, 29.7, 28.6, 26.9, 25.9, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{43}H_{55}O_7$ $[M + H]^+$ 683.3948, found 683.3948.

*4.1.1.1.4.18*. Compound **18**. Obtained from celastrol and **$c_{18}$**. Orange red powder, 34.4% yield. Analytical data for **18**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), *δ* ppm: 0.57

(3H, s), 1.11 (3H, s), 1.21 (3H, s), 1.27 (3H, s), 1.45 (3H, s), 2.21 (3H, s), 3.81 (3H, s), 4.43-4.49 (2H, m), 4.56 (2H, m), 5.94 (2H, s), 6.34 (1H, d, *J* = 16.1 Hz), 6.35 (1H, d, *J* = 6.9 Hz), 6.52 (1H, s), 6.89 (2H, d, *J* = 8.6 Hz), 7.02 (1H, d, *J* = 7.2 Hz), 7.48 (2H, d, *J* = 8.6 Hz), 7.66 (1H, d, *J* = 16.0 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 177.9, 170.0, 167.8, 164.7, 160.2, 146.0, 144.5, 134.1, 129.8 (C×3), 128.5 (C×2), 127.5, 127.4, 119.4, 118.1, 115.4, 115.0 (C×2), 67.6, 64.0, 51.6, 45.0, 44.2, 42.9, 40.4, 39.4, 38.2, 36.3, 34.7, 33.5, 32.8, 31.6, 30.8, 30.5, 29.8, 29.6, 28.6, 21.6, 18.6, 10.3; HRMS (ESI) calculated for $C_{43}H_{53}O_7$ $[M + H]^+$ 681.3791, found 681.3791.

*4.1.1.1.4.19.* Compound **19.** Obtained from celastrol and **$c_{19}$**. Orange red powder, 38.3% yield. Analytical data for **19**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.53 (3H, s), 1.10 (3H, s), 1.21 (3H, s), 1.25 (3H, s), 1.43 (3H, s), 2.22 (3H, s), 3.83 (3H, s), 3.89 (3H, s), 4.22-4.30 (3H, m), 4.35-4.45 (1H, m), 6.30 (1H, d, *J* = 7.2 Hz), 6.36 (1H, d, *J* = 15.9 Hz), 6.46 (1H, s), 6.88 (1H, d, *J* = 8.6 Hz), 6.99 (1H, s), 7.00 (1H, d, *J* = 7.1 Hz), 7.08 (1H, d, *J* = 8.4 Hz), 7.64 (1H, d, *J* = 15.9 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 169.8, 167.8, 164.5, 150.4, 149.5, 146.0, 144.8, 134.0, 127.5, 127.4, 122.3, 119.5, 118.1, 117.1, 115.5, 112.2, 110.1, 65.2, 61.0, 51.7, 45.0, 44.2, 42.9, 40.4, 39.3, 38.2, 36.3, 34.9, 33.3, 32.9, 31.7, 30.9, 30.5, 29.8, 29.6, 28.3, 21.7, 18.5, 10.3; ESI/HRMS (m/z) HRMS (ESI) calculated for $C_{42}H_{53}O_8$ $[M + H]^+$ 685.3740, found 685.3744.

*4.1.1.1.4.20.* Compound **20**. Obtained from celastrol and **$c_{20}$**. Orange red powder, 37.4% yield. Analytical data for **20**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.49 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.24 (3H, s), 1.43 (3H, s), 2.25 (3H, s), 3.77 (3H, s), 3.83 (3H, s), 4.01-4.14 (3H, m), 4.19-4.27 (1H, m), 6.25 (1H, d, *J* = 7.2 Hz), 6.36 (1H, d, *J* = 15.9 Hz), 6.50 (1H, s), 6.85 (1H, d, *J* = 8.3 Hz), 7.02 (1H, s), 7.02 (1H, d, *J* = 7.2 Hz), 7.11 (1H, d, *J* = 8.3 Hz), 7.67 (1H, d, *J* = 15.9 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 169.8, 167.7, 164.6, 150.4, 149.5, 146.0, 144.8, 134.0, 127.5, 127.4, 122.4, 119.5, 118.1, 117.0, 115.5, 112.2, 110.2, 65.3, 61.1, 51.6, 45.0, 44.2, 42.9, 40.4, 39.3, 38.2, 36.3, 34.9, 33.3, 32.9, 31.6, 30.9, 30.5, 29.7, 29.6, 28.4, 28.2, 21.7, 18.5, 10.3; ESI/HRMS (m/z) HRMS (ESI) calculated for $C_{43}H_{55}O_8$ [M +

H]$^+$ 699.3897, found 699.3900.

*4.1.1.1.4.21*. Compound **21**. Obtained from celastrol and **$c_{21}$**. Orange red powder, 39.2% yield. Analytical data for **21**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.56 (3H, s), 1.11 (3H, s), 1.19 (3H, s), 1.27 (3H, s), 1.46 (3H, s), 2.23 (3H, s), 3.82 (3H, s), 3.89 (3H, s), 3.93-3.99 (3H, m), 4.02-4.10 (1H, m), 6.33 (1H, d, *J* = 16.0 Hz), 6.35 (1H, d, *J* = 7.3 Hz), 6.53 (1H, s), 6.85 (1H, d, *J* = 8.4 Hz), 7.03 (1H, d, *J* = 7.2 Hz), 7.04 (1H, s), 7.09 (1H, d, *J* = 8.3 Hz), 7.65 (1H, d, *J* = 15.9 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 169.9, 167.7, 164.7, 150.4, 149.5, 146.0, 144.8, 134.1, 127.5, 127.4, 122.5, 119.5, 118.1, 117.1, 115.5, 112.6, 110.1, 68.4, 64.1, 55.9, 51.6, 45.0, 44.3, 42.9, 40.4, 39.4, 38.2, 36.3, 34.8, 33.5, 32.8, 31.6, 30.9, 30.8, 30.5, 29.8, 28.5, 25.8, 25.2, 21.6, 18.5, 10.2; HRMS (ESI) calculated for $C_{44}H_{57}O_8$ [M + H]$^+$ 713.4053, found 713.4053.

*4.1.1.1.4.22*. Compound **22**. Obtained from celastrol and **$c_{22}$**. Orange red powder, 36.0% yield. Analytical data for **22**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.55 (3H, s), 1.11 (3H, s), 1.19 (3H, s), 1.26 (3H, s), 1.44 (3H, s), 2.21 (3H, s), 3.81 (3H, s), 3.91 (3H, s), 4.39-4.54 (2H, m), 4.62-4.63 (2H, m), 5.86-6.06 (2H, m), 6.32 (1H, d, *J* = 7.8 Hz), 6.33 (1H, d, *J* = 15.7 Hz), 6.51 (1H, s), 6.84 (1H, d, *J* = 8.0 Hz), 7.01 (1H, d, *J* = 8.5 Hz), 7.06 (1H, s), 7.08 (1H, d, *J* = 8.6 Hz), 7.64 (1H, d, *J* = 16.0 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 177.8, 170.0, 167.7, 164.7, 150.4, 149.9, 146.0, 144.8, 134.1, 128.4, 128.0, 127.7, 127.4, 122.4, 119.5, 118.1, 117.1, 115.6, 112.8, 110.0, 68.5, 63.9, 55.9, 51.7, 45.0, 44.2, 42.9, 40.4, 39.4, 38.2, 36.3, 34.7, 33.4, 32.8, 31.6, 30.8, 30.5, 29.8, 29.6, 28.6, 21.6, 18.6, 10.3; HRMS (ESI) calculated for $C_{44}H_{55}O_8$ [M + H]$^+$ 711.3897, found 711.3902.

*4.1.1.1.4.23*. Compound **23**. Obtained from celastrol and **$f_1$**. Orange red powder, 38.1% yield. Analytical data for **23**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.54 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.25 (3H, s), 1.44 (3H, s), 2.23 (3H, s), 3.89 (3H, s), 4.07 (3H, s), 4.17-4.23 (1H, m), 6.32 (1H, d, *J* = 6.9 Hz), 6.52 (1H, s), 6.91 (1H, d, *J* = 8.0 Hz), 7.03 (2H, s), 7.15 (1H, d, *J* = 7.9 Hz), 7.29 (1H, d, *J* = 6.9 Hz), 8.19 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 170.0, 164.7 (C×2), 159.1, 155.3,

146.0, 134.1, 132.5, 127.4, 120.8, 119.5, 118.1, 117.1, 116.9, 114.6, 113.4, 98.9, 64.4, 61.0, 53.5, 45.0, 44.2, 42.9, 40.5, 39.4, 38.2, 36.3, 34.8, 33.4, 32.9, 31.6, 30.9, 30.5, 29.8, 29.7, 28.5, 28.2, 21.6, 18.6, 10.3; HRMS (ESI) calculated for $C_{43}H_{52}NO_7$ $[M + H]^+$ 694.3744, found 694.3747.

*4.1.1.1.4.24.* Compound **24**. Obtained from celastrol and **$f_2$**. Orange red powder, 37.4% yield. Analytical data for **24**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.55 (3H, s), 1.10 (3H, s), 1.22 (3H, s), 1.25 (3H, s), 1.41 (3H, s), 2.22 (3H, s), 3.92 (3H, s), 3.94 (3H, s), 4.26-4.28 (2H, m), 4.32-4.37 (2H, m), 6.32 (1H, d, $J$ = 7.2 Hz), 6.34 (1H, s), 6.96 (1H, d, $J$ = 8.7 Hz), 7.00 (1H, d, $J$ = 7.4 Hz), 7.50 (1H, d, $J$ = 8.6 Hz), 7.77 (1H, s), 8.12 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.4, 177.9, 170.0, 164.7, 163.7, 154.9, 154.2, 148.6, 146.0, 134.0, 128.2, 127.4, 124.3, 119.5, 118.1, 117.0 (C×2), 112.5, 111.2, 98.8, 68.7, 64.0, 56.0, 51.6, 45.0, 44.2, 42.9, 40.4, 39.4, 38.2, 36.3, 34.8, 33.4, 32.8, 31.6, 30.8, 30.5, 29.8, 29.7, 28.6, 21.7, 18.6, 10.3; HRMS (ESI) calculated for $C_{43}H_{52}NO_8$ $[M + H]^+$ 710.3693, found 710.3692.

*4.1.1.1.4.25.* Compound **25**. Obtained from celastrol and **$f_3$**. Orange red powder, 36.8% yield. Analytical data for **25**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.46 (3H, s), 1.09 (3H, s), 1.18 (3H, s), 1.22 (3H, s), 1.41 (3H, s), 2.25 (3H, s), 3.79 (3H, s), 3.94 (3H, s), 4.00-4.08 (1H, m), 4.14-4.18 (2H, m), 4.23-4.31 (1H, m), 6.23 (1H, d, $J$ = 7.0 Hz), 6.45 (1H, s), 6.84 (1H, d, $J$ = 8.5 Hz), 7.02 (1H, d, $J$ = 7.0 Hz), 7.44 (1H, d, $J$ = 8.6 Hz), 7.79 (1H, s), 8.17 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.4, 178.3, 170.0, 164.7, 163.7, 154.9, 154.2, 148.6, 146.0, 134.1, 128.3, 127.3, 124.3, 119.5, 118.1, 117.0 (C×2), 112.5, 111.2, 98.7, 65.0, 60.9, 55.9, 53.2, 45.0, 44.2, 42.9, 40.5, 39.3, 38.2, 36.3, 34.9, 33.3, 32.9, 31.6, 30.9, 30.5, 29.7, 29.6, 28.5, 28.1, 21.7, 18.5, 10.3; HRMS (ESI) calculated for $C_{44}H_{54}NO_8$ $[M + H]^+$ 724.3849, found 724.3852.

*4.1.1.1.4.26.* Compound **26**. Obtained from celastrol and **$f_4$**. Orange red powder, 35.1% yield. Analytical data for **26**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.57 (3H, s), 1.11 (3H, s), 1.19 (3H, s), 1.27 (3H, s), 1.45 (3H, s), 2.22 (3H, s), 3.94 (6H, s), 4.04-4.15 (4H, m), 6.36 (1H, d, $J$ = 7.2 Hz), 6.51 (1H, s), 6.93 (1H, d, $J$ = 8.4 Hz),

7.02 (1H, d, *J* = 7.1 Hz), 7.47 (1H, d, *J* = 8.6 Hz), 7.80 (1H, s), 8.18 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 170.0, 164.7, 163.7, 155.1, 154.2, 148.6, 145.8, 134.1, 128.1, 127.4, 124.4, 119.5, 118.1, 117.0 (C×2), 113.0, 111.2, 98.7, 68.5, 64.2, 56.1, 53.2, 45.1, 44.3, 42.9, 40.4, 39.4, 38.2, 36.4, 34.8, 33.5, 32.8, 31.6, 30.8, 30.6, 29.8, 29.7, 28.6, 25.8, 25.3, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{45}H_{56}NO_8$ $[M + H]^+$ 738.4006, found 738.4004.

*4.1.1.1.4.27.* Compound **27**. Obtained from celastrol and **f$_5$**. Orange red powder, 38.1% yield. Analytical data for **27**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.55 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.26 (3H, s), 1.44 (3H, s), 2.20 (3H, s), 3.93 (3H, s), 3.96 (3H, s), 4.41-4.54 (2H, m), 4.68 (2H, s), 6.00 (2H, s), 6.35 (1H, d, *J* = 7.0 Hz), 6.49 (1H, s), 6.96 (1H, d, *J* = 8.5 Hz), 7.01 (1H, d, *J* = 6.9 Hz), 7.50 (1H, d, *J* = 8.2 Hz), 7.79 (1H, s), 8.18 (1H, s); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 177.9, 170.0, 164.7, 163.6, 155.0, 148.0, 146.0, 134.1, 128.6, 128.4, 128.0, 127.4, 124.4, 119.5, 118.1, 117.1, 116.3, 113.3, 111.2, 98.8, 68.6, 64.0, 56.2, 53.2, 45.0, 44.2, 42.9, 40.4, 39.4, 38.2, 36.3, 34.8, 33.4, 32.8, 31.6, 30.8, 30.5, 30.2, 29.8, 29.6, 28.6, 21.7, 18.6, 10.3; HRMS (ESI) calculated for $C_{45}H_{54}NO_8$ $[M + H]^+$ 736.3849, found 736.3842.

*4.1.1.1.4.28.* Compound **28**. Obtained from celastrol and **f$_6$**. Orange red powder, 41.6% yield. Analytical data for **28**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.53 (3H, s), 1.09 (3H, s), 1.19 (3H, s), 1.24 (3H, s), 1.43 (3H, s), 2.23 (3H, s), 3.82 (3H, s), 4.04-4.09 (3H, m), 4.15-4.23 (1H, m), 6.29 (1H, d, *J* = 7.1 Hz), 6.43 (1H, d, *J* = 16.0 Hz), 6.50 (1H, s), 6.89 (1H, d, *J* = 8.1 Hz), 7.01 (1H, s), 7.02 (1H, d, *J* = 8.0 Hz), 7.12 (1H, d, *J* = 7.3 Hz), 7.28 (1H, t, *J* = 7.9 Hz), 7.66 (1H, d, *J* = 16.0 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3, 178.2, 169.9, 167.4, 164.7, 160.5, 159.0, 146.0, 144.8, 135.7, 134.2, 130.0, 127.4, 120.9, 119.5, 118.1 (C×2), 117.2, 116.7, 113.3, 64.3, 61.0, 51.6, 45.0, 44.2, 42.9, 40.5, 39.4, 38.2, 36.3, 34.8, 33.4, 32.8, 31.6, 30.9, 30.5, 29.8, 29.7, 28.5, 28.3, 21.6, 18.6, 10.3; HRMS (ESI) calculated for $C_{42}H_{53}O_7$ $[M + H]^+$ 669.3791, found 669.3790.

*4.1.1.1.4.29.* Compound **29**. Obtained from celastrol and **f$_7$**. Orange red powder, 42.1% yield. Analytical data for **29**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.53

(3H, s), 1.10 (3H, s), 1.22 (3H, s), 1.24 (3H, s), 1.41 (3H, s), 2.21 (3H, s), 3.82 (3H, s), 3.88 (3H, s), 4.24 (2H, m), 4.28-4.29 (1H, m), 4.35 (1H, m), 6.27 (1H, d, *J* = 16.1 Hz), 6.31 (1H, d, *J* = 7.2 Hz), 6.38 (1H, s), 6.89 (1H, d, *J* = 8.2 Hz), 6.99 (2H, m), 7.13 (1H, d, *J* = 8.1 Hz), 7.59 (1H, d, *J* = 15.8 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 169.8, 167.6, 164.6, 152.0, 148.2, 146.0, 144.4, 133.9, 127.3 (C×2), 123.7, 119.5, 118.1, 117.0, 115.6, 113.5, 111.9, 67.6, 63.2, 56.0, 51.6, 45.0, 44.3, 42.9, 40.5, 39.5, 38.2, 36.4, 34.8, 33.5, 32.8, 31.6, 30.8, 30.5, 29.8, 29.6, 28.6, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{42}H_{53}O_8$ $[M + H]^+$ 685.3740, found 685.3745.

*4.1.1.1.4.30*. Compound **30**. Obtained from celastrol and **f$_8$**. Orange red powder, 36.9% yield. Analytical data for **30**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.49 (3H, s), 1.10 (3H, s), 1.19 (3H, s), 1.24 (3H, s), 1.42 (3H, s), 2.24 (3H, s), 3.78 (3H, s), 3.82 (3H, s), 4.01-4.13 (3H, m), 4.21-4.29 (1H, m), 6.27 (1H, d, *J* = 7.3 Hz), 6.31 (1H, d, *J* = 15.9 Hz), 6.47 (1H, s), 6.81 (1H, d, *J* = 8.3 Hz), 7.01 (1H, d, *J* = 7.0 Hz), 7.03 (1H, s), 7.10 (1H, d, *J* = 8.2 Hz), 7.64 (1H, d, *J* = 16.0 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$: 178.3 (C×2), 170.0, 167.7, 164.7, 151.2, 148.4, 146.0, 144.8, 134.1, 127.1 (C×2), 122.9, 119.5, 118.1 (C×2), 115.4, 111.6, 111.2, 65.2, 61.1, 55.8, 51.7, 45.0, 44.2, 42.9, 40.4, 39.3, 38.2, 36.3, 34.9, 33.3, 32.9, 31.6, 30.9, 30.5, 29.7, 29.6, 28.5, 28.3, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{43}H_{55}O_8$ $[M + H]^+$ 699.3897, found 699.3901.

*4.1.1.1.4.31*. Compound **31**. Obtained from celastrol and **f$_9$**. Orange red powder, 38.2% yield. Analytical data for **31**: $^1$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.57 (3H, s), 1.11 (3H, s), 1.19 (3H, s), 1.27 (3H, s), 1.45 (3H, s), 2.22 (3H, s), 3.81 (3H, s), 3.89 (3H, s), 3.93-4.00 (1H, m), 4.05-4.09 (3H, m), 6.31 (1H, d, *J* = 16.0 Hz), 6.35 (1H, d, *J* = 7.4 Hz), 6.52 (1H, s), 6.87 (1H, d, *J* = 8.3 Hz), 7.01 (1H, d, *J* = 7.3 Hz), 7.06 (1H, s), 7.11 (1H, d, *J* = 8.4 Hz), 7.64 (1H, d, *J* = 16.1 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$:178.3 (C×2), 170.0, 167.7, 164.7, 151.9, 148.5, 146.0, 144.8, 134.1, 127.4, 127.3, 122.8, 119.5, 118.1, 117.1, 115.4, 111.6, 111.4, 68.6, 64.2, 56.0, 51.6, 45.0, 44.2, 42.9, 40.4, 39.4, 38.2, 36.4, 34.8, 33.5, 32.8, 31.6, 30.8, 30.5, 29.8, 29.7, 28.6, 26.0, 25.2, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{44}H_{57}O_8$ $[M + H]^+$ 713.4053,

found 713.4059.

*4.1.1.1.4.32.* Compound **32**. Obtained from celastrol and **$f_{10}$**. Orange red powder, 40.6% yield. Analytical data for **32**: $^{1}$H-NMR (300 MHz, $CDCl_3$, TMS), $\delta$ ppm: 0.55 (3H, s), 1.11 (3H, s), 1.19 (3H, s), 1.26 (3H, s), 1.44 (3H, s), 2.21 (3H, s), 3.81 (3H, s), 3.91 (3H, s), 4.39-4.56 (2H, m), 4.63-4.64 (2H, m), 5.87-6.05 (2H, m), 6.30 (1H, d, *J* = 16.0 Hz), 6.34 (1H, d, *J* = 7.2 Hz), 6.50 (1H, s), 6.89 (1H, d, *J* = 8.3 Hz), 7.04 (1H, d, *J* = 7.4 Hz), 7.05 (1H, s), 7.13 (1H, d, *J* = 8.2 Hz), 7.64 (1H, d, *J* = 15.9 Hz); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta_C$:178., 177.9, 169.9, 167.7, 164.7, 151.6, 148.0, 146.0, 144.7 134.0, 128.7, 128.0, 127.9, 127.3, 123.0, 119.5, 118.1, 117.1, 115.5, 112.1, 111.4, 68.8, 64.0, 56.0, 51.6, 45.0, 44.2, 42.9, 40.4, 39.4, 38.2, 36.4, 34.8, 33.4, 32.8, 31.6, 30.8, 30.5, 29.8, 29.7, 28.7, 21.6, 18.5, 10.3; HRMS (ESI) calculated for $C_{44}H_{55}O_8$ $[M + H]^+$ 711.3897, found 711.3899.

*4.2.Biological evaluation*

*4.2.1. Cytotoxic assay in vitro*

The cytotoxic activities of all celastrol hybrids were evaluated against lung carcinoma cells (A549), breast carcinoma cells (MCF-7), hepatocellular carcinoma cells (HepG2) by MTT method. For this assay, 2.5 ml ($5\times10^4$/ml) cells per well were seeded in 24-well plates and allowed to incubate for 24 h. Then, the compounds with different concentrations were added. After 48 h of incubation, MTT solution (0.5 mg/ml) was added , and the plates were incubated again for another 4 h at 37□. The supernatant was then removed before adding 100 μl of DMSO to each well. The OD values at 550 nm was immediately read by an ELISA plate reader (POLARstar Omega, Offenburg, Germany). Subsequently, the $IC_{50}$ values were calculated by Graphpad Prism 5. Three independent experiments were performed. Data are presented as the mean ± SD (n = 3)

*4.2.2. Cell cycle assay*

Cell-cycle distribution was measured by flow cytometry using a Fluorescence-Activated Cell Sorter (FACS). A549 cells ($15\times10^4$ cells/well) were

seeded in 6-well plates, and preincubated at 37 □ for 24 h. Then cells were incubated for 12 h and 24 h with DMSO (blank control) or **29** at specified concentrations. For cell cycle analysis, both floating and adherent cells were harvested, washed with PBS and fixed in 70% ethanol at 4 °C overnight. After washing with PBS, cells were suspended in PBS containing 50 mg/ml propidium iodide (PI) (KeyGEN, China) and 100 mg/mL RNase A, incubated at 37 °C for 0.5 h and analyzed with flow cytometry.

*4.2.3. Cell apoptosis assay*

A549 cells ($15 \times 10^4$ cells/well) were seeded in 6-well plates, and preincubated at 37 □ for 24 h. Then cells were incubated for 24 h with DMSO (blank control) or **29** at specified concentrations. For apoptosis analysis, cells were harvested in cold PBS and collected by centrifugation for 5 min at 3000 rpm. Then cells were were added to 250 µl 1 × binding buffer and incubated with 2.5 µl Annexin V-FITC and 2.5 µl PI staining (KeyGEN, China) in the dark at room temperature for 15 min, and analyzed by flow cytometry (BD Accuri C6 flow cytometer, Becton & Dickinson Company, Franklin Lakes, NJ).

*4.2.4. Fluorescence microscopy imaging*

A549 cells ($3\times10^4$ cells/well) were seeded in 24-well plates, and incubated at 37 □ for 24 h. Then cells were treated with DMSO (control) or compound **29** at three different concentrations (0.06, 0.09, and 0.18 µM) for 48 h. After removing the culture medium and washing with 1mL PBS of each well, 1ml 1640 incomplete medium and 10 µl Hoechst 33342 (KeyGEN, China) were added to each well, and the plates were incubated for 5 min at 37 □protected from the light. The cells were then observed on a fluorescence microscope (Ts2R, NIKON CORPORATION, Japan) to detect the differences on the morphological features of A549 cells.

*4.2.5. Western blotting analysis*

A549 cells were seeded ($5\times10^5$ cells) in 6 cm medium and incubated for 24 h. Then **29** or celastrol with specific concentrations were added for 8 h incubation. The proteins were extracted with lysis buffer and quantified by BCA method to diluted to 3-5 mg/ml. Each sample (10 µl) was separated on 10% sodium dodecyl sulphate

(SDS)-polyacrylamide gels, followed by incubated with the primary specific antibodies overnight at 4 □ and β-actin for 1 h at room temperature. Then the bands were visualised using a developing approach.

*4.2.6. Immunoprecipitation*

A549 cells were seeded ($5\times10^5$ cells) in 6 cm growth medium and incubated for 24 h. **29** and celastrol were added to the dishes with 5 μM for another 6 h-incubation. The proteins were extracted with lysis buffer. Then Hsp90α was pulled down by γ-phosphate-linked ATP-sepharose and the Cdc37 was pulled down by Hsp90α-sepharose. Equal amounts of total protein were subjected to SDS-PAGE to detect the levels of the target proteins by monoclonal antibodies were purchased (Abcam, Cambridge, UK).

## Acknowledgements

This work was financially supported by the Academic Innovation Project of Jiangsu Department of Education (No. 1150021667) and by "Double First Class" Subject Innovation Team Construction Project of China Pharmaceutical University (CPU2018GY12).

## Appendix A. Supplementary data

Supplementary data related to this article can be found at.

## References

[1] A. Salminen, M. Lehtonen, T. Paimela, K. Kaarniranta. Biochem. Biophys. Res. Commun. 394 (2010) 439-442.

[2] A.M. Lourenco, L.M. Ferreira, P.S. Branco. Curr. Pharm. Des. 18 (2012) 3979-4046.

[3] P. Yadav, V. Jaswal, A. Sharma, D. Kashyap, H.S. Tuli, V.K. Garg, S.K. Das, R. Srinivas. Pharm. Pat. Anal. 7 (2018) 155-167.

[4] T. Zhang, Y.Y. Li, Y.K. Yu, P. Zou, Y.Q. Jiang, D.X. Sun. J. Bio. Chem. 284 (2009) 35381-35389.

[5] W. Wei, S. Wu, X.L. Wang, C.K.W. Sun, X.Y. Yang, X.R. Yan, M.S. Chua, S. So. Oncotarget.

5 (2014) 5819-5831.

[6] M. D'Archivio, C. Filesi, R. Di Benedetto, R. Gargiulo, C. Giovannini, R. Masella. Ann. Ist. Super. Sanita. 43 (2007) 348-361.

[7] A.R. Rechner, A.S. Pannala, C.A. Rice-Evans. Free Radic. Res. 35 (2011) 195-202.

[8] S.Y. Ou, K.C. Kwok. J. Sci. Food. Agric. 84 (2004) 1261-1269.

[9] M. Srinivasan, A.R. Sudheer, V.P. Menon. J. Clin. Biochem. Nutr. 40 (2007) 92-100.

[10] C.J. Chang, J.H. Chiu, L.M. Tseng, C.H. Chang, T.M. Chien, C.W. Wu, W.Y. Lui. Eur. J. Clin. Invest. 36 (2006) 588-596.

[11] K. Kawabata, T. Yamamoto, A. Hara, M. Shimizu, Y. Yamada, K. Matsunaga, T. Tanaka, H. Mori. Cancer Lett. 157 (2000) 15-21.

[12] G.W. Yang, J.S. Jiang, W.Q. Lu. Int. J. Mol. Sci. 16 (2015) 24011-24031.

[13] J. Zhao, A. Suyama, M. Tanaka, T. Matsui. J. Nutr. Biochem. 25 (2014) 807-814.

[14] T.L. Serafim, F.S. Carvalho, M.P.M. Marques, R. Calheiros, T. Silva, J. Garrido, N. Milhazes, F. Borges, F. Roleira, E.T. Silva, J. Holy, P. J. Oliveira. Chem. Res. Toxicol. 24 (2011) 763-774.

[15] C. Eroğlu, M. Seçme, G. Bağcı, Y. Dodurga. Tumor Biol. 36 (2015) 9437-9446.

[16] O.S. Eliane, B. Ronan. Compr. Rev. Food. Sci. F. 16 (2017) 580-616.

[17] P.L. Wu, T.S. Wu, C.X. He, C.H. Su, K.H. Lee. Chem. Pharm. Bull. 53 (2005) 56-59.

[18] Y. Li, L. Feng, Z.F. Song, H.B. Li, Q.Y. Huai. Molecules. 21 (2016) e199.

[19] C. Viegas-Junior, A. Danuello, V.S. Bolzani, E.J. Barreiro, C.A.M. Fraga. Curr Med Chem. 14 (2007) 1829-1852.

[20] M. Decker. Curr Med Chem. 18 (2011) 1464-1475.

[21] K. Nakagawa-Goto, S. Nakamura, K.F. Bastow, A. Nyarko, C.Y. Peng, F.Y. Lee, F.C. Lee, K.H. Lee. Bioorg. Med. Chem. Lett. 17 (2007) 2894-2898.

[22] J. Tatsuzaki, M. Taniguchi, K.F. Bastow, K. Nakagawa-Goto, S.L. Morris-Natschke, H. Itokawa, K. Baba, K.H. Lee. Bioorg. Med. Chem. 15 (2007) 6193-6199.

[23] L. Shen, X.C. Yang, B. Yang, Q.J. He, Y.Z. Hua. Eur. J. Med. Chem. 45 (2010) 11-18.

[24] S. Ahadi, M. Zolghadr, H.R. Khavasi, A. Bazgir. J. Med. Chem. 46 (2003) 2958-2972.

[25] S. Tambe, H. Blott, A. Fülöp, N. Spang, D. Flottmann, S. Bräse, C. Hopf, H.D. Junker. Anal. Bioanal. Chem. 409 (2017) 1569-1580.

[26] L. Chen, Y.H. Zhang, X.W. Kong, S.X. Peng, J. Tian. Bioorg. Med. Chem. Lett. 17 (2007) 2979–2982.

[27] F. Jiang, H.J. Wang, Q.C. Bao, L. Wang, Y.H. Jin, Q. Zhang, D. Jiang, Q.D. You, X.L. Xu. Bioorg. Med. Chem. 24 (2016) 5431-5439.

[28] D. de Zeeuw, T. Akizawa, P. Audhya, et al. N. Engl. J. Med., 369 (2013) 2492-2503.

[29] J. Himmelfarb, K.R. Tuttle, et al. N. Engl. J. Med. 369 (2013) 2549-2550.

[30] H.E. Abboud. Kidney Int. 83 (2013) 785-787.

[31] M. Li, J. Lin. J. Int. Obstet. Gynecol. 41 (2010) 103-107.

[32] N.A. Thornberry, Y. Laebnik. Science. 281 (1998) 1312-1316.

[33] A.H. Boulares, A.G. Yakovlev, V. Ivanova, B.A. Stoica, G. Wang, S. Iyer, M. Smulson. J. Biol. Chem. 274 (1999) 22932-22940.

[34] U. Ziegler, P. Groscurth. News Physiol. Sci. 19 (2004) 124-128.

- A series of novel celastrol hybrids were designed, synthesized and evaluated.
- **29** exhibited superior potency compared to the other compounds.
- **29** disrupted Hsp90-Cdc37 complex and induced apoptosis through extrinsic signaling pathway in A549 cells.
- **29** down-regulated the expression levels of the Hsp90 clients (Akt and Cdk4), and induced apoptosis and the cell cycle arrested at the $G_0/G_1$ phase in A549 cells.