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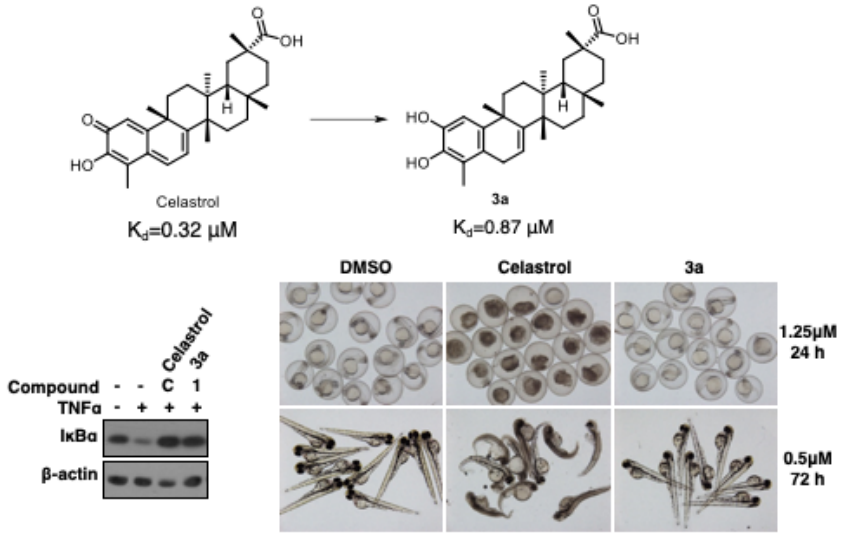
Celastrol
K_d=0.32 μM
3a
K_d=0.87 μM
Compound - - C 1
TNFα - + + +
Celastrol
3a
IκBα
β-actin
DMSO
Celastrol
3a
1.25μM
24 h
0.5μM
72 h

SAR study of celastrol analogs targeting Nur77-mediated inflammatory pathway

Zi-Wen Chen [a, 1], Duo Zhang [a, 1], Si-Wei Yan [a, 1], Chao-Chao Hu [a], Zhen-Fei Huang [a], Zhuo-Er Li [a], Shuang-Zhou Peng [a], Xiao-Tong Li [a], Yi Zhu [a], Hong-Yu Yu [a], Bao-Huan Lian [a], Qi Kang [a], Mingyu Li [a], Zhi-Ping Zeng [a], Xiao-Kun Zhang [a, *] and Ying Su [a, b, *]

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Abstract

Nur77, an orphan member of the nuclear receptor superfamily, plays an important role in the regulation of inflammatory processes. Our previous work found that celastrol, a pentacyclic triterpene, bound to Nur77 to inhibit inflammation in a Nur77-dependent manner. Celastrol binding to Nur77 promotes Nur77 translocation from nucleus to cytoplasm, resulting in clearance of inflamed mitochondria and then alleviation of inflammation. Here, we report the design, synthesis, SAR study and biological evaluation of a series of celastrol analogues. A total of 24 celastrol derivatives were made. Compound **3a** with a K_d of 0.87 μM was found to be less toxic than celastrol and could be a hit molecule for further optimization.

Key words：Nur77, celastrol, anti-inflammatory, celastrol derivatives, SAR

1. Introduction

Nur77 (also known as TR3, NGFI-B, and NR4A1), an orphan member of the nuclear receptor superfamily, is known to play an integral role in many different cellular processes especially the inflammatory responses [1-3]. Though, Nur77 possesses common structural features shared by the NR family: an activation function 1 (AF-1), a DNA binding domain (DBD), a ligand binding domain (LBD), and a ligand-dependent transcriptional activation domain (AF-2), from N-terminal to C-terminal [4], its binding pocket in the LBD is shown to be incapable of binding ligands [5]. Consistently not endogenous ligands have been identified for Nur77 and Nur77 remains to be an orphan receptor. However, in recent years, ligands that bind to the surface regions of the LBD of Nur77 have been found and reported to mediate the biological functions of Nur77 [6-13].

Our lab recently reported the identification of celastrol as a submicromolar ligand of Nur77 [12]. Celastrol is a potent anti-inflammatory agent isolated from the root of *Tripterygium wilfordii*, a traditional Chinese medicine also known as “Thunder God Vine”. Traditionally, “Thunder God Vine” has been used to treat rheumatoid arthritis, lupus and other auto-immune diseases [13-17]. As the key bioactive molecule of Thunder God Vine, celastrol has been studied widely and recognized to be involved in multiple pathways and to have the potential for therapeutic applications [18-20]. We found that celastrol binds to Nur77 with an affinity of 292 nM and demonstrated *in vitro* and in animal models that the anti-inflammatory effects of celastrol requires its binding to Nur77, which therefore identifies Nur77 as a direct intracellular target of celastrol. Furthermore, using multiple approaches we demonstrated and

confirmed that celastrol binds to Nur77 via a reversible covalent bond resulted from Michael addition reaction at C551 in Nur77. In addition, we showed the formation of this covalent bond requires specific noncovalent interactions with Nur77 to position celastrol in close proximity to the -SH group of C551. Such specific noncovalent interactions render celastrol's binding selectivity, providing opportunity to develop celastrol-derived agents that can selectively bind Nur77 to modulate its anti-inflammatory effect [21].

Here we report our design, synthesis, SAR study and biological evaluation of 24 celastrol analogs. Binding activities of the synthesized compounds were studied to understand the structure-activity relationship. Compounds were also evaluated for their anti-inflammatory effects. Compounds **3a** and **1e** displayed similar anti-inflammatory effects as celastrol and were selected for further biological evaluation. **3a** was found to be less toxic when its toxicity was assessed in comparison to celastrol in Zebrafish model. It is worth noting that celastrol and analogs have been widely studied for their anti-cancer potentials [22-24], but less studied for their anti-inflammatory applications. This study will provide insights into the optimization of celastrol derivatives selectively targeting Nur77 for anti-inflammatory therapy.

2. Chemistry

The synthetic routes of the proposed celastrol derivatives of formula I and II (Fig. 1) were outlined in Schemes 1-3. First, derivatives with modifications to the carboxylic acid or hydroxyl groups were introduced (Scheme 1, **1a** - **1g**, **2a** - **2c**). Treating celastrol with DAST (Diethylaminosulfurtrifluoride, $(CH_3CH_2)_2NSF_3$) in dichloromethane at -78 °C for 1 h, gave the product **1a** in moderate yield 58%. Esterification of celastrol with various alkyl iodides at room temperature catalyzed by $NaHCO_3$ yielded corresponding derivatives in moderate yield (87% for **1b**, 78% for **1d** and 79% for **1f**). Under the catalysis of PyBOP (Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) and DIPEA (N,N-diisopropylethylamine), treatment of celastrol with three different primary amines (methylamine, ethylamine and propylamine) generated the products **1c** (72% yield), **1e** (48% yield) and **1g** (31% yield). **2a** - **2c** were obtained by treating **1b** with methyl iodide, bromoethane and 1,1-Difluoro-2-iodoethane (F_2CHCH_2I) respectively, catalyzed by K_2CO_3 (**2c**, 57% yield) or by both K_2CO_3 and TBAB (Tetrabutylammonium bromide) (**2a**, 54% yield and **2b**, 85% yield).

3a was obtained in 99% yield by treating celastrol with $NaBH_4$ in all deuterated methanol. Conjugation of nitromethane to celastrol with the assistance of TBAF (Tetrabutylammonium fluoride) formed **3b** in 87% yield. Michael addition of celastrol with acetone in the present of catalytic amount hydrochloric acid (12 mol/L) resulted in product **3c** (51% yield, Scheme 1, step ix). As indicated in

Scheme 1, catalyzed by MeONa celastrol could react with heterocyclic reagent pyrrole to afford product **3d** (82% yield, Scheme 1, step x). Catalyzed by 5 mol% $AlCl_3 \cdot 6H_2O$ dimethylphosphonate group was introduced to C-6 position of celastrol, producing compound **3e** with a moderate yield 55% (Scheme 1, step xi). Products **4a** and **4b** were prepared as described in Scheme 1, steps xii and xiii: first, celastrol was protected by benzyl group $PhCH_2Br$ with the help of K_2CO_3 and triethylamine (TEA) in DMF solution, giving the intermediate compound; after purification using flash column chromatography, the intermediate compound continues to react with MeMgBr (1 mol/L) in THF at 0 °C for 1 h to obtain compound **4a** in 56% yield. The two benzyl protective groups were removed from **4a** by hydrogen gas and Pd/C at room temperature for 12 h, yielding the product **4b** in 87% yield. **5c** was obtained according to Scheme 2: the two hydroxyl groups of **3b** were protected by 2,2-dimethoxypropane to afford compound **5a** in 63% yield. Subsequently, the nitro group of **5a** was reduced by Pd/C and $H_2NNH_2 \cdot H_2O$ in EtOH to give compound **5b** in 36% yield. Removing the protecting group from compound **5b** in the presence of catalyst $In(OTf)_3$ and under the microwave condition (120 °C for 3 h) afforded **5c** in 77% yield [25]. Treatment of compound **3a** with 2,2-dimethoxypropane for 12 h in the presence of catalytic acid *p*-$TsOH \cdot H_2O$ afforded **6a** in moderate yield (57%). **6b** was obtained by oxidizing **6a** using PDC and dimethylpyrazole in DCM for 12 h at room temperature. Using $In(OTf)_3$ as a catalyst, the protective group acetonides were removed from compound **6b** efficiently in CH_3CN/H_2O (10:1 v/v) under the microwave irradiation, and **6c** was obtained (37%). Compounds **7a** and **7f** were synthesized by reducing compounds **1b** and **2a** with 10 equiv. $NaBH_4$ in deuterated MeOH (step vii in Scheme 1), respectively (78% yield for **7f** and 99% for **7a**). **7a** was methylated by dimethyl sulfate to afford compound **7e** (24% yield, step xiv). Compound **7g** was obtained in 66% yield by using another methylating reagent - CH_3I under the same condition (step xv). Subsequently, compound **7e**, **7f** and **7g** were hydrolyzed by KOH under 100 °C in mixed dioxane/water (1:1 v/v) solvent to obtain **7b**, **7c** and **7d** in high yields (step xvi in Scheme 1). All synthesized compounds were structurally elucidated by different spectrometric methods, including ^1H NMR (600 MHz), ^{13}C NMR (151 MHz), and high-resolution mass spectra (HR-MS) (see the spectra in the supporting information).

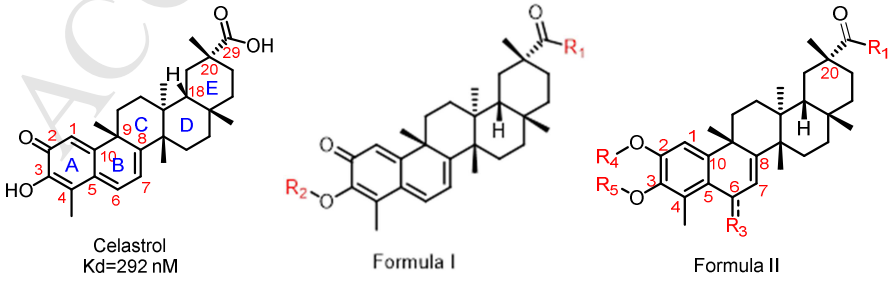
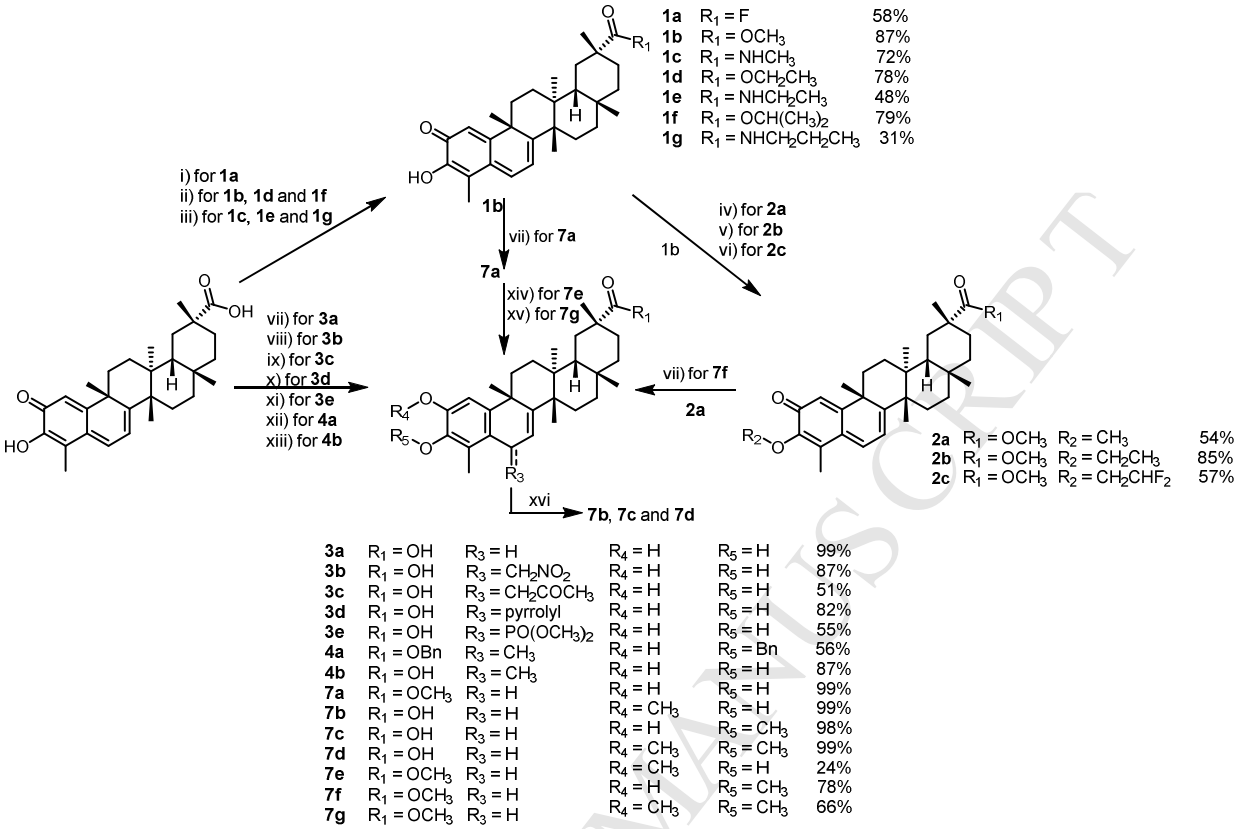
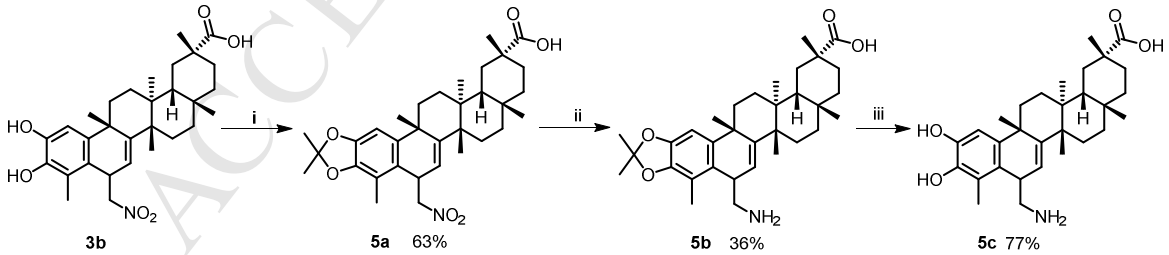


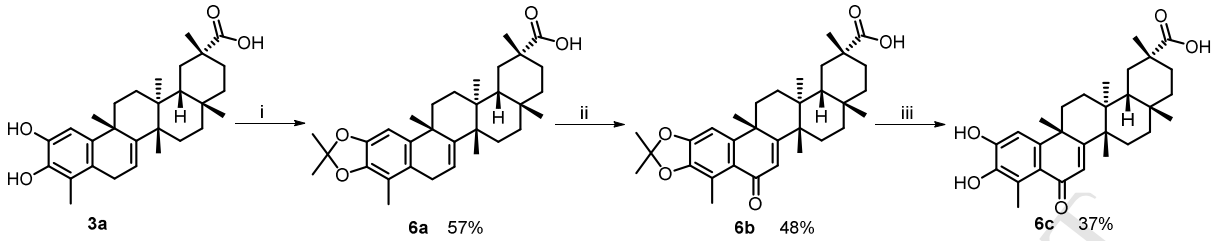
Fig. 1. Chemical structures of celastrol and its analogs



Scheme 1. Reagents and conditions: i) DAST, DCM, -78 □, 1 h; ii) Alkyl iodides, $NaHCO_3$, DMF, r. t., 12 h; iii) Various amines, DMF, PyBOP, DIPEA, 0 □ 30 min and then r. t. for 2 h; iv) CH_3I, K_2CO_3, TBAB, THF, 20 mol% Dioxane, 70 □, 12 h; v) CH_3CH_2Br, K_2CO_3, TBAB, THF, 20 mol% Dioxane, 70 □, 12 h; vi) F_2CHCH_2I, K_2CO_3, DMF, 50 □, 24 h. vii) $NaBH_4$, CD_3OD, r. t., 30 min; viii) CH_3NO_2, TBAF, THF, r. t., 1 h; ix) CH_3COCH_3, 1 drop HCl (12 mol/L), r. t., 12 h; x) MeONa, pyrrole, r. t., 5 min; xi) $AlCl_3{\cdot}6H_2O$, $HPO(OCH_3)_2$, DCM, r. t., 6 h; xii) $PhCH_2Br$, DMF, K_2CO_3, Et_3N, 80 □, 2.5 h, 68% ; Then MeMgBr, THF, 0 □, 1 h, 83% yield, (Two Steps: 56%); xiii) Pd/C, H_2, MeOH, r. t., 12 h; xiv) $(CH_3O)_2SO_2$, K_2CO_3, acetone, 70 □, 12 h; xv) MeI, K_2CO_3, acetone, 70 □, 12 h; xvi) KOH, dioxane/H_2O = 1:1 (v/v), 100 □, 4 h, then, naturally cool to r. t. for additional 10 min.



Scheme 2. Reagents and conditions: i) $(MeO)_2CMe_2$, *p*-TsOH, DCM, r. t., overnight, 63% yield; ii) Pd/C, $H_2NNH_2{\cdot}H_2O$, EtOH, 75 □, overnight, 36% yield; iii) $In(OTf)_3$, MeCN/water = 10:1 (v/v), microwave, 120 □, 3 h, 77% yield.



Scheme 3. Reagents and conditions: i) $(MeO)_2CMe_2$, *p*-TsOH, DCM, r. t., overnight, 57% yield; ii) PDC, 3,5-dimethylpyrazole, DCM, r. t., overnight, 48% yield; iii) $In(OTf)_3$, MeCN/water = 10:1 (v/v), microwave. 120 □, 1 h, 37% yield.

3. Results and Discussion

3.1 Design of celastrol derivatives

We recently reported the binding characterization of celastrol to Nur77 [21]. We demonstrated that celastrol binds to Nur77-LBD via specific noncovalent interactions and a reversible covalent bond. We also showed that covalent bond is formed through Michael addition reaction between celastrol and C551 in Nur77. Furthermore, we developed a binding model of celastrol to Nur77 by using molecular modeling and mutagenesis approaches. In this predicted model (Fig. 2A), celastrol fits well to a groove formed by loop H7-H8, helices 8, 9, 10 and loop H9-H10; the hydroxyl group on ring A and the carboxyl on ring E form H-bonds with D499 and Q547 respectively; the central portion of the celastrol molecule is hydrophobic, making Van Der Waals interactions with many hydrophobic residues; C-6 atom where the nucleophilic addition occurs is positioned next to the -SH group of C551. These modeling results prompted us to investigate the effects of different substituents on rings A, B and E. First, we designed and synthesized derivatives **1a-g** and **2a-c** to explore the importance of the hydroxyl and the carboxyl groups.

Our previous studies revealed that celastrol binding to Nur77 requires its interaction with Nur77 first via specific noncovalent interactions. We found that due to these specific noncovalent interactions, compound **3a** of which the C-6 atom is reduced and is not capable of nucleophilic addition can still bind to Nur77. Therefore, secondly, we designed and synthesized compounds **3b-e** and **7b-g** to study the role of substituents at C-6 and the importance of the hydroxyls on ring A in the noncovalent interactions between celastrol derivatives and Nur77.

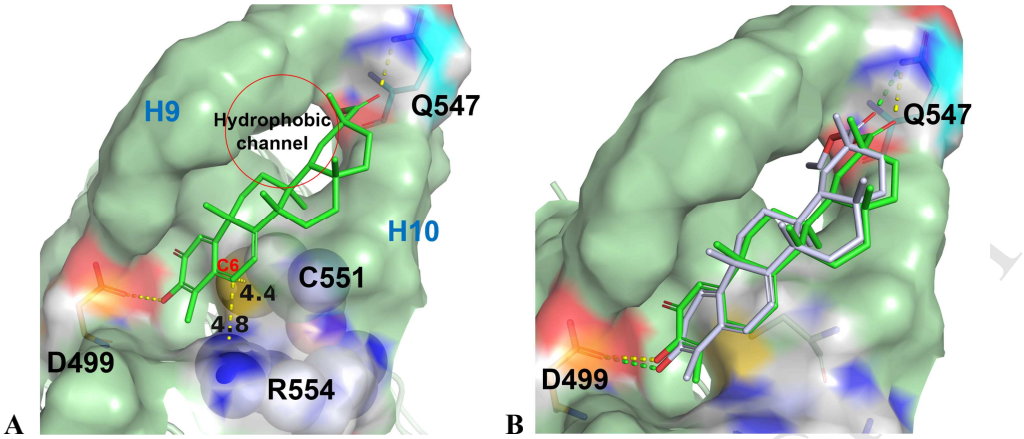


Fig. 2. Modeling studies**.** (A) Modeling study of celastrol binding to Nur77-LBD. The protein is displayed as surface representation and celastrol is shown as green sticks. The contributing hydrophobic residues are colored in pale green. The polar residues Q547 and D499 are colored in blue and in orange, respectively. (B) Binding comparison between compound **1d** (gray sticks) and celastrol.

3.2 Fluorescence binding studies

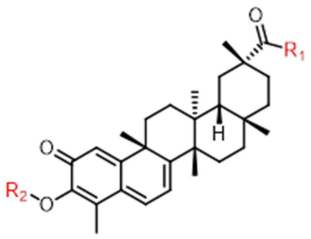
The binding affinities of the synthesized celastrol derivatives to Nur77 were evaluated by a fluorescence quenching assay. The compounds were titrated into the cuvette filled with a 3 mL Nur77-LBD protein solution (1 μmol/L) with an increasing concentration from 0.1 μmol/L to 5 μmol/L. The fluorescence intensity excited by Nur77-LBD was quenched due to the interaction between the protein and compounds. Using the fluorescence quenching assay, the dissociation constant of celastrol was evaluated as 0.32 μM [21], which is close to the K_d of 0.29 μM reported previously by the Surface Plasmon Resonance (SPR) binding analysis [12]. Thus, Celastrol was used as the positive control.

Binding affinity results (Fig. S1) for compounds **1b-g** made from the esterification and amidation of the -COOH at position 20 (Fig. 1) are summarized in Table 1. Overall these compounds' binding activities are not significantly different from celastrol. Replacing the -OH of carboxylic acid with $-OCH_3$ or $-NHCH_3$ slightly weakened the binding. However, when larger groups (R_1 = **$OCH(CH_3)_2$ or $NHCH_2CH_2CH_3$**) were used the weakening effects were reduced and some improvement was observed for compound **1d** of which R_1 = **OCH_2CH_3**. These observations suggested that when substituting the -OH group in the carboxyl group, small groups such as methyl amide and methoxy are not the ideal ones for binding improvement, which is supported by the result of **1a** and is consistent with information gathered from our previous modeling study. Our predicted model shows there is a small hydrophobic channel below the -OH group and alkyl chains with moderate length (**1d)** would fit well (Fig. 2B).

In the predicted model, the hydroxyl group at position 3 forms a H-bond with D499 (see Fig. 2A). It is anticipated that disruption of this H-bond would drastically weaken the binding to Nur77. To evaluate

the contribution of this H-bond, compounds **2a-2c**, of that the ability of H-bond formation was disabled at position 3 were synthesized and indeed the binding results (Table 1) showed several-fold reduction in their binding affinities.

Table 1. Binding and biological results of **1a-g** and **2a-c**.



Formula I

Compounds	R_1	R_2	Binding affinity (μM)	Inhibition of TNFα-induced IκB-α degradation
Celastrol	**OH**	**H**	**0.32**	***[c]
1a	**F**	**H**	**0.72 ± 0.01**	***[c]
1b	**OCH_3**	**H**	**0.96 ± 0.03**	***[c]
1c	**$NHCH_3$**	**H**	**0.80 ± 0.31**	***[c]
1d	**OCH_2CH_3**	**H**	**0.22 ± 0.08**	***[c]
1e	**$NHCH_2CH_3$**	**H**	**0.83 ± 0.09**	***[c]
1f	**$OCH(CH_3)_2$**	**H**	**0.52 ± 0.05**	***[c]
1g	**$NHCH_2CH_2CH_3$**	**H**	**0.27 ± 0.12**	***[c]
2a	**OCH_3**	**Methyl**	**2.94 ± 0.79**	**NS[a]**
2b	**OCH_3**	**Ethyl**	**4.13 ± 0.39**	**NS[a]**
2c	**OCH_3**	**CH_2CHF_2**	**3.30 ± 0.21**	**NS[a]**

[a] NS: no significant;
[c]: *P<0.05, **P<0.01, ***P<0.001

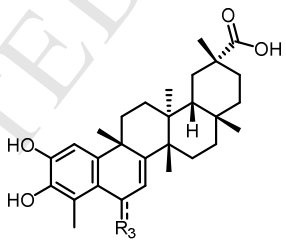
Reduction of the quinone methide motif in celastrol affords a hydrolyzed celastrol incapable of undergoing Michael addition. Though losing the ability to make covalent bond interaction with Nur77, the reduced celastrol can still bind to Nur77 via noncovalent bond interaction [21]. For example, compound **3a** can bind to Nur77 with a K_d of 0.87 μM, indicating strong noncovalent bond interaction with the protein. This inspired us to synthesize compounds listed in Table 2 to study the impact of different substituents at C-6 position on the noncovalent interactions between compounds and Nur77. Binding affinity results (Fig. S2) shows that any substituents at C-6 position weakened the interactions between the reduced celastrol (**3a**) and Nur77. Replacing H with substituents (**4b**, **5c**, **3d** and **3e**) ranging from small group such as methyl to sterically bulky dimethyl phosphonate group demolished the binding

although carbonyl (**6c**) and medium-sized substituents (**3b** and **3c**) did not show the same impact. **6c**, **3b** and **3c** displayed binding affinity towards Nur77 though weaker in comparison to **3a** (Table 2). These results implied that size was not the only factor playing roles in the interactions with Nur77 protein. Our model showed that R554 is about 4.8 Å away from the C-6 (Fig. 2) and would form H-bonds with the H-bond donors, the carbonyl and nitro groups in **6c**, **3b** and **3c**, which explained why **6c** and the medium-sized substituents **3b** and **3c** were different from **4b** and **5c**.

Compound **3a** possesses 2 hydroxyl groups on the A ring and our model showed that there was not much empty space around the hydroxyl groups (Fig. 3A) and the hydroxyl group at C-3 position could form a H-bond with D599 similar to celastrol (Fig. 3A). Therefore, we decided to design compounds **7b-d** to evaluate the importance of these 2 hydroxyl groups for the noncovalent interactions. Binding results (Fig. S3) showed that methylation of one or both of the hydroxyl groups (**7b-d)** led to loss of the binding activity. Consistently, the same results were observed for compounds **7e-g.**

In summary, studies on the binding affinities of the aforementioned 24 celastrol derivatives helped us gain some understanding of the SAR, which is summarized in Fig. 4. Binding affinity is not very sensitive to the modification to the -COOH at C-20. However, modification to C-6 with groups larger than H is in general not favorable. In addition, the hydroxyl groups on the A-ring of the reduced celastrol or the hydroxyl group on the A-ring of celastrol are important to the binding.

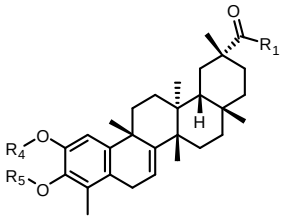
Table 2. Binding and biological results of **3b-e**, **4b, 5c** and **6c**.



Compounds	R_3	Bond type between R_3 and C-6 atom	Binding affinity (μM)	Inhibition of TNFα-induced IκB-α degradation
3a	**H**	**Single Bond**	**0.87**	***[c]
3b	**CH_2NO_2**	**Single Bond**	**5.56 ± 0.15**	**NS[a]**
3c	**CH_2COCH_3**	**Single Bond**	**3.80 ± 0.31**	**NS[a]**
3d	**Pyrrolyl**	**Single Bond**	**NB[b]**	**NS[a]**
3e	**$PO(OCH_3)_2$**	**Single Bond**	**NB[b]**	**NS[a]**
4b	**CH_3**	**Single Bond**	**NB[b]**	**NS[a]**
5c	**CH_2NH_2**	**Single Bond**	**NB[b]**	**NS[a]**
6c	**O**	**Double Bond**	**2.69 ± 0.07**	**NS[a]**

[a]NS: no significant;
[b]NB: no binding;
[c]:*P<0.05, **P<0.01, ***P<0.001

Table 3. Binding and biological results of **7b-7g**.



Compounds	R_1	R_4	R_5	Binding affinity (μM)	Inhibition of TNFα-induced IκB-α degradation
3a	**OH**	**H**	**H**	**0.87**	*** [c]
7b	**OH**	**CH_3**	**H**	**NB[b]**	**NS[a]**
7c	**OH**	**H**	**CH_3**	**NB[b]**	**NS[a]**
7d	**OH**	**CH_3**	**CH_3**	**NB[b]**	**NS[a]**
7e	**OCH_3**	**CH_3**	**H**	**NB[b]**	**NS[a]**
7f	**OCH_3**	**H**	**CH_3**	**NB[b]**	* [c]
7g	**OCH_3**	**CH_3**	**CH_3**	**NB[b]**	**NS[a]**

[a]NS: no significant;
[b]NB: no binding;
[c]:*P<0.05, **P<0.01, ***P<0.001

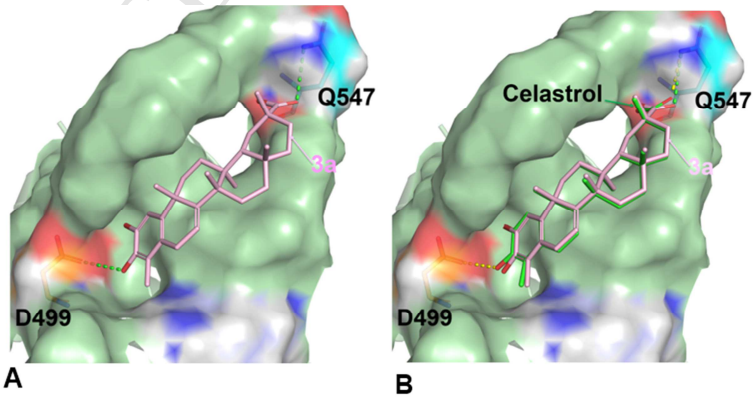


Fig. 3. Modeling studies of **3a**. (A) Docking mode of compound **3a**, compound was shown as sticks while binding site in surface presentation; (B) Superimpose compound **3a** (pink sticks) with celastrol (green sticks) at the binding site.

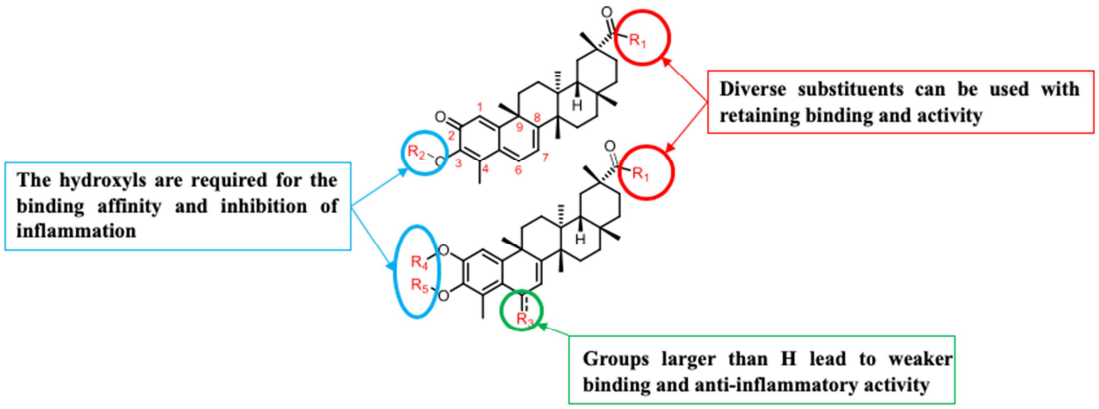


Fig. 4. Summary of the structure-activity relationship

3.3 Evaluation of the anti-inflammatory effects of celastrol derivatives

Celastrol could antagonize the effects of inflammatory cytokine TNFα on inducing IκBα degradation in a Nur77-dependent manner [12, 26]. Therefore, we asked if the synthesized celastrol derivatives possessed such an anti-inflammatory effect. HepG2 cells treated with TNFα in the presence or absence of celastrol or analogs were analyzed by Western blot for the level of IκBα (Fig. 5). Consistent with previous observation [12, 26], TNFα-induced downregulation of IκBα level was largely prevented when cells were cotreated with celastrol. Analysis of the effect of celastrol analogs revealed a close correlation between their anti-inflammatory effects and binding affinities. Compounds (**2a-c**, **3b-e** and **4b**, **5c**, **6c**) that either failed to bind or bound weakly to Nur77 exhibited weak anti-inflammatory effect in comparison to celastrol (also see Tables 1–3), whereas compounds (**1a-g** and **3a**) that bind Nur77 with a K_d of less than 1 μM could strongly antagonize the effect of TNFα on inducing IκBα degradation (also see Tables 1–2).

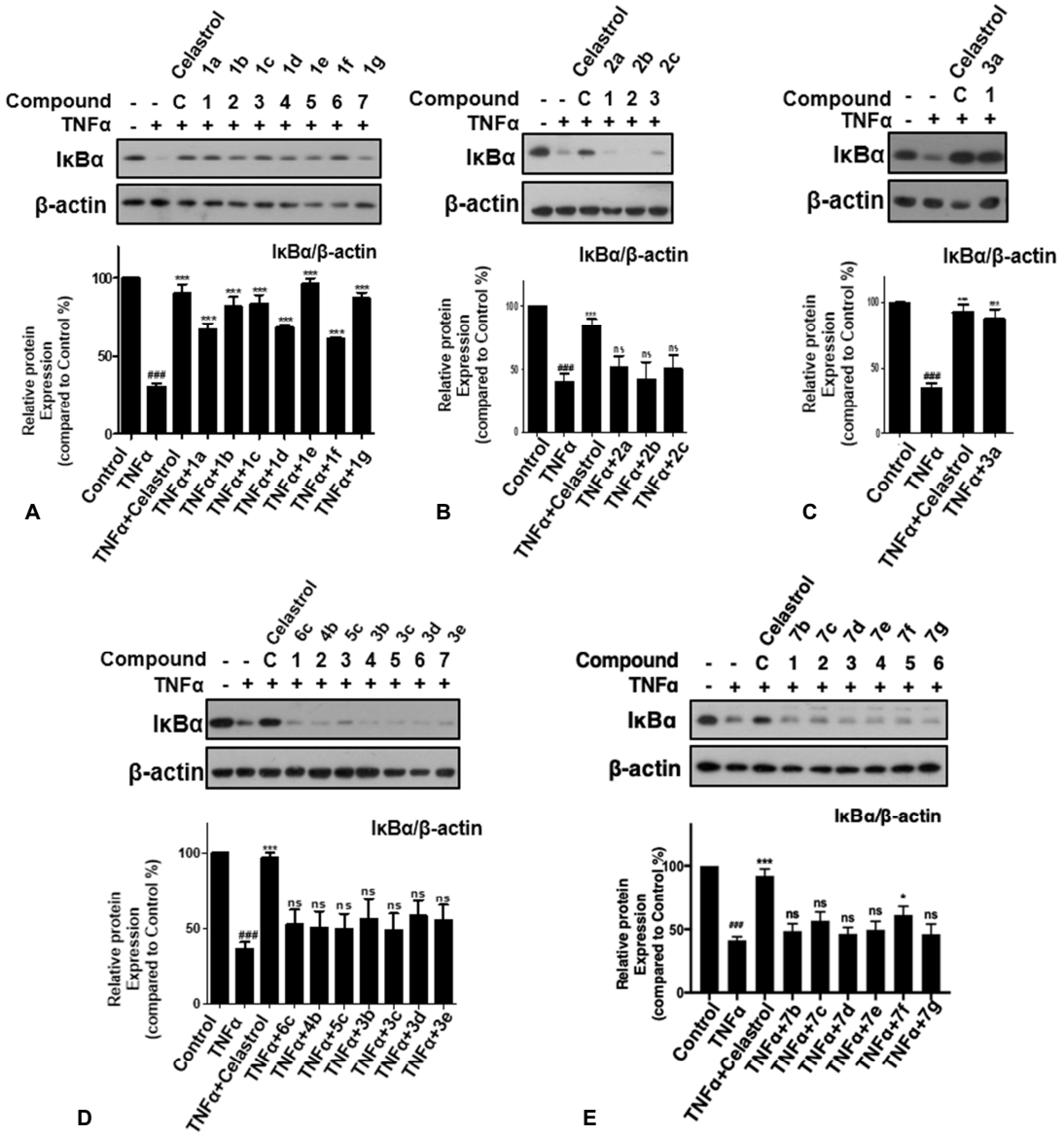


Fig. 5. Western blot analysis of the anti-inflammatory effect of celastrol derivatives. Lysates from HepG2 cells treated with compound (2 μM) for 1 hr and TNFα (20 ng/mL) for 30 min were analyzed by Western blotting. The grey processing was carried out using imageJ. All the bar graphs represent mean ± SEM of three independent experiments. Ns, not significant, *P<0.05, **P<0.01, ***P<0.001 vs the TNFα group, ###P<0.001 vs the control group (Student's test).

3.4 Compound binding promotes Nur77 interactions with TRAF2 and p62/SQSTM1

We previously showed that Nur77 mediates the anti-inflammatory effect of celastrol by translocating to mitochondria where it interacts with tumor necrosis factor receptor-associated factor 2 (TRAF2) and the autophagic adaptor p62/SQSTM1, a process that results in the elimination of damaged mitochondria

via autophagy. Thus, we asked if aforementioned celastrol analogs that displayed both good binding affinity and anti-inflammatory effect could act through a similar mechanism. Compounds **3a** and **1e** were selected for such an evaluation. To examine whether they promoted the interaction between Nur77 and p62/SQSTM1, HepG2 cells transfected with GFP-Nur77 and mCherry-p62/SQSTM1 were treated with TNFα together with celastrol or compound **3a** or **1e**. Confocal microscopy analysis showed that GFP-Nur77 resided exclusively in the nucleus in control cells. However, when cells were treated with TNFα and celastrol, GFP-Nur77 could be found in the cytoplasm, colocalizing extensively with p62/SQSTM1 (Fig. 6A). Similar results were obtained when cells were treated with TNFα and compound 3a or 1e. To study the effect on Nur77 interaction with TRAF2, HepG2 cells transfected with Myc-Nur77 and Flag-TRAF2 were treated TNFα and celastrol or compounds **3a** or **1e** and analyzed by co-immunoprecipitation (coIP) assay (Fig. 6B). Our results showed that treatment of cells with compound **3a** or **1e** could promote the interaction between Nur77 and TRAF2, similar to the effect of celastrol. Thus, like celastrol **3a** and **1e** could promote the interactions between Nur77 and TRAF2 and p62/SQSTM1, respectively (Fig. 6B). Collectively, compounds **3a** and **1e** can inhibit inflammatory response by promoting the interactions of Nur77 with TRAF2 and p62/SQSTM1.

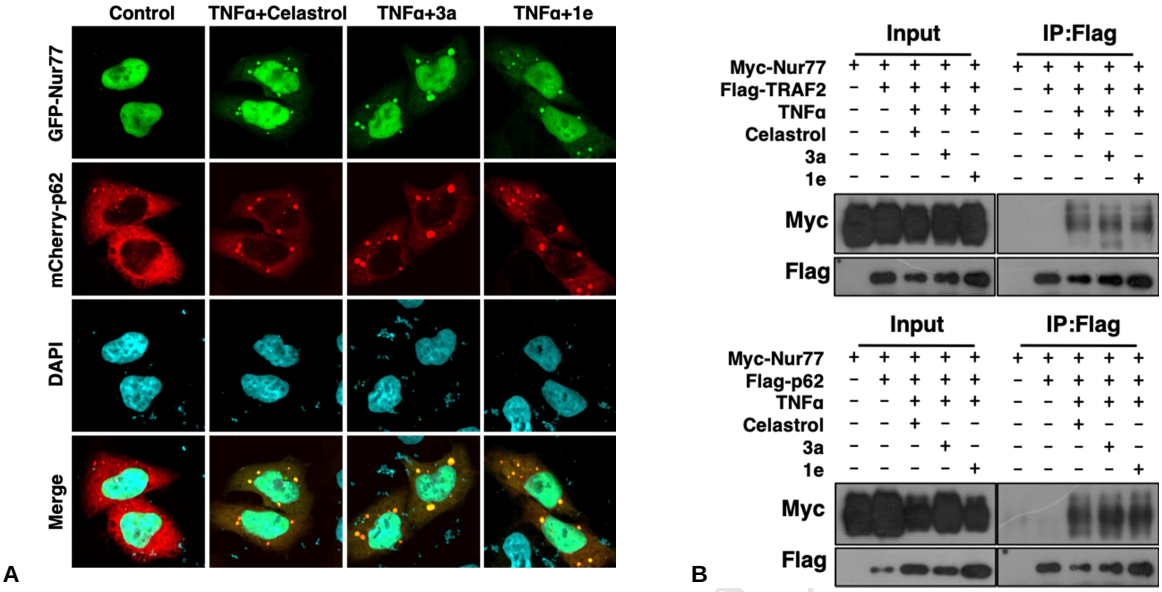


Fig. 6. Compounds **3a** and **1e** promote Nur77 interaction with TRAF2 and p62. (A) Representative images show colocalization of transfected GFP-Nur77 with mCherry-p62 in HepG2 cells after treatment with celastrol, **3a**, **1e** and TNFα, examined by immunostaining; (B) HepG2 cells transfected with Myc-Nur77 and Flag-TRAF2 or Flag-p62 were treated with celastrol, **3a** or **1e** (2 μM) and TNFα (20 ng/mL) for 1 hr and analyzed by coIP.

3.5 Toxicity assessment of compounds **3a** and **1e** using *in vitro* assays and Zebrafish model

Celastrol is well known for its toxicity, which is one of the key factors limiting its clinical translation[27]. For anti-inflammatory agents, their apoptotic actions can lead to unwanted side effects. Thus, first we performed *in vitro* assays to assess the apoptotic effects of **1e** and **3a**. In the PARP cleavage assay, **3a** was less active, however **1e** showed stronger PARP cleavage effect than celastrol (Fig. 7A). The effect of **1e** and **3a** on cell death was also assessed using flow cytometry-based Annexin V/ Propidium iodide (PI) apoptosis assay. **3a** consistently showed to induce less cell death than **1e** and celastrol (Fig. 7B). Fig.7B showed that more than 10% of HepG2 cells were apoptotic when treated with 2 μM of celastrol or **1e** for 10 hours, while only 3.12% of cells were apoptotic when treated with 2 μM of **3a**.

We then evaluated the toxicity of both **3a** and celastrol in zebrafish model (Fig. 7C-E). Fig. 7C showed that celastrol killed zebrafish embryo as much as 3 times more than **3a** when zebrafish embryo was treated with 0.5 μM of celastrol or **3a** for 24 hours. When the treatment time was prolonged to 72 hours, the death rate of zebrafish embryo caused by celastrol increased to near 100%, whereas the effect of **3a** did not change significantly. When the compound concentration was increased to 1.25 μM, their difference in toxicity was more pronounced with 90% of the zebrafish embryo killed by celastrol in 24 hours, whereas, the toxicity of **3a** at 1.25 μM stayed comparable to its corresponding one at 0.5 μM. In

addition, the impact of **3a** on the malformation of zebrafish was studied. Fig. 7D showed that celastrol had a much higher impact on the malformation of zebrafish than **3a**. Fig. 7E showed visually under the same conditions, **3a** had less effect than celastrol on the death rate and malformation of zebrafish either at a concentration of 1.25 μM for 24 hours or at a concentration of 0.5 μM for 72 hours. In summary, our data showed that **3a** was less toxic than celastrol and could be a better anti-inflammatory agent as a probe molecule or a hit molecule for further optimization.

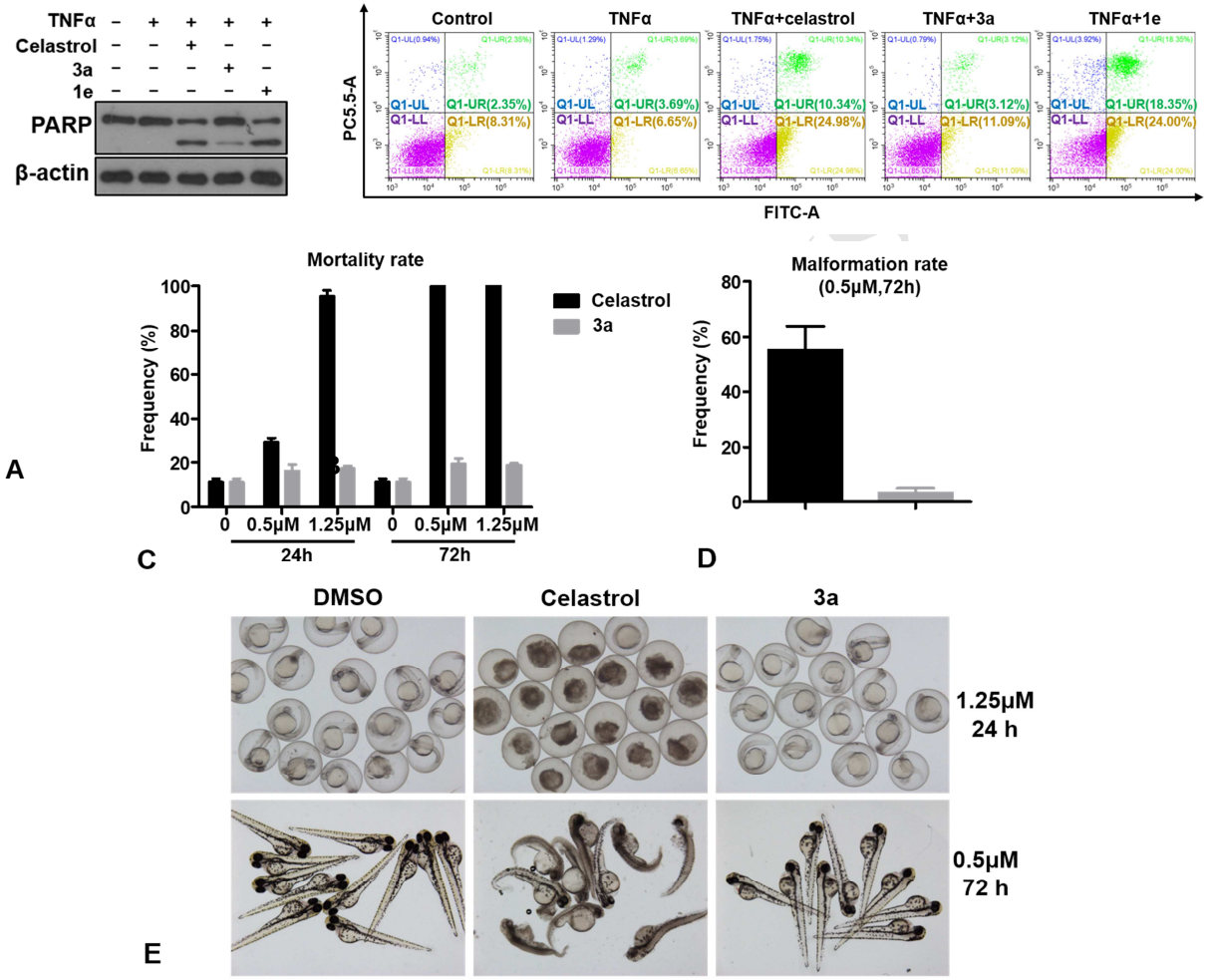


Fig. 7. Toxicity studies. (A) Western blot analysis for apoptosis effect of **3a**, **1e** and celastrol, HepG2 cells treated with celastrol, **3a**, **1e** (2 μM) and TNFα (20 ng/mL) for 10 hr were analyzed by WB, the apoptosis effect was detected through cleaved-PARP; (B) Flow cytometry analysis of **3a** and **1e**, Q1-UL: necrotic cells, Q1-UR: viable apoptotic cells, Q1-LR: non-viable apoptotic cells, Q1-LL: normal cells; HepG2 cells treated with celastrol，**3a** or **1e** (2 μM) and TNFα (20 ng/mL) for 10 hr were stained by FITC Annexin V and Propidium Iodide Staining Solution, and then analyzed by CytoFLEX; (C-E) Embryotoxicity and developmental toxicity assay, 20 zebrafish embryos per condition at 2 hpf were exposed to celastrol or **3a** at the concentrations of 0.5 μM, 1 μM and 1.25 μM and 0.1% DMSO served as control. C) The statistics of

24 hpf and 72 hpf mortality rate; D) The statistics of 72 hpf malformation rate in 0.5 μM group; E) Morphology of 24 hpf embryo or 72 hpf zebrafish larvae treated with celastrol or **3a**.

4. Conclusion

In conclusion, we described the synthesis and SAR studies and biological evaluation of 24 celastrol analogs as anti-inflammatory agents by targeting the orphan nuclear receptor Nur77. Our results validated a proposed binding model of celastrol and provide insight into the design of improved celastrol analogs. Compound **3a**, a reduction product of celastrol, having comparable binding affinity and biological effects to celastrol, displayed remarkable improvement in cell toxicity, offering a hit molecule for further optimization for anti-inflammatory application.

5. Experimental section

5.1 Chemistry

5.1.1. General methods

Celastrol was purchased from Chengdu Pufei De Biotech Co., Ltd. All commercially available reagents and solvent were purchased from Energy Chemical (Shanghai, China) and used without further purification unless otherwise stated. ^{1}H and ^{13}C spectra were recorded in $CDCl_3$ or DMSO-d_6 (dimethyl sulfoxide-d_6) on a Bruker AV600 spectrometer (Switzerland) with tetramethylsilane (TMS) as an internal standard. Chemical shifts are expressed in δ (ppm) units downfield from TMS. All coupling constants (*J* -values) were reported in Hertz (*Hz*). Signals are described as follow: s, singlet; br. s., broad signal; d, doublet; t, triplet; q, quartet; m, multiplet. Chemical shifts of common trace ^{1}H-NMR impurities (ppm): H_2O: 3.29-3.4 in DMSO-d_6, DMSO-d_6: 2.50, $CDCl_3$: 7.27. Mass spectra were recorded on a Q-Exactive-LC/MS/MS ThermoFisher instrument (America) with a HESI, APCI or nanoscale ESI ion source and high-resolution MS (HRMS) on Orbitrap. The samples for HR-MS analysis were prepared as described: Compounds were dissolved in HPLC purity methyl alcohol individually, to obtain a 10 μg/mL solution of compound, and then 10 μL of 10 μg/mL sample was injected to Q-Exactive LC-MS apparatus for analysis.

Flash column chromatography was carried out with 300-400 mesh silica gel using Biotage Isolera Prime (Sweden). The melting points (Mp.) were determined using a SGW® X-4 Micro Melting Point apparatus (Shanghai INESA Physico-Optical Instrument Co.,Ltd., China). All tested compounds have a purity ≥ 95%.

5.1.2. (9β,13α,14β,20α)-3-Hydroxy-9,13-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oyl fluoride (**1a**) **CAS: 2172373-26-9**

Compound **1a** was synthesized according to procedure i in **Scheme 1**. 50 mg (0.11 mmol) celastrol was dissolved in 2 mL DCM and stirred in a closed 25-mL tube. After 150 μL (10 equiv) DAST was added, the solution was kept to react in -78 □ for 1 h. Cold water (10 mL) was added to quench the reaction, and subsequently the aqueous phase was separated and extracted with DCM (15 mL * 3). The organic layers were combined and dried with anhydrous sodium sulfate. Finally, compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:4). Orange red solid, 29.2 mg, yield: 58%. Mp: 169.0 - 171.0 □. $[\alpha]_D^{24}$ = -168.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.73 (s, 1H), 7.06 (dd, *J* = 1.28, 6.97 Hz, 1H), 6.38 (d, *J* = 1.28 Hz, 1H), 6.35 (d, *J* = 7.34 Hz, 1H), 2.23 (d, *J* = 12.65 Hz, 1H), 2.17 - 2.21 (m, 1H), 2.09 (s, 3H), 1.97 (br. s., 1H), 1.88 - 1.94 (m, 1H), 1.81 - 1.87 (m, 1H), 1.77 - 1.81 (m, 1H), 1.69 - 1.74 (m, 1H), 1.65 - 1.69 (m, 1H), 1.60 - 1.65 (m, 1H), 1.58 (d, *J* = 7.89 Hz, 1H), 1.56 (d, *J* = 4.95 Hz, 1H), 1.49 - 1.54 (m, 1H), 1.46 - 1.49 (m, 1H), 1.41 - 1.46 (m, 1H), 1.38 (s, 3H), 1.29 (s, 3H), 1.22 (s, 3H), 1.08 (s, 3H), 0.94 - 1.01 (m, 1H), 0.56 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 178.0, 167.1, 167.6 (d, *J* = 369.7 Hz, 1 C), 162.8, 146.4, 132.9, 127.0, 120.2, 118.1, 117.2, 44.3, 43.3, 41.9, 40.2, 39.2, 38.7, 37.9, 35.8, 33.8, 32.7, 31.2, 29.9, 29.5, 29.3, 29.3, 27.9, 21.4, 18.9, 10.1 ppm; HRMS (ESI): *m/z* calcd for $C_{29}H_{37}FNaO_3^+$ $[M+Na]^+$: 475.2619, found: 475.2626.

5.1.3. (9β,13α,14β,20α)-3-Hydroxy-9,13-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid methyl ester (**1b**) **CAS: 1258-84-0**

Compound **1b** was synthesized according to procedure ii in **Scheme 1**. 50 mg (0.11 mmol) celastrol was dissolved in 3 mL DMF and stirred in a 25-mL round-bottom flask, and then 94 mg (0.66 mmol) CH_3I and 28 mg (0.33 mmol) $NaHCO_3$ were added in the solution. After reacting for 12 h at room temperature, 0.1 mol/L HCl (5 mL) and distilled water (5 mL) were added to quench the reaction. Subsequently, the aqueous phase was separated and extracted with ethyl acetate (15 mL) three times. The organic layers were combined and dried with anhydrous sodium sulfate. Compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8). Orange solid, 44.9 mg, yield: 87%. Mp: 198.8-200.2 □, $[\alpha]_D^{24}$ = -266.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.72 (s, 1 H), 7.07 (dd, *J* = 7.15, 1.10 Hz, 1 H), 6.39 (d, *J* = 1.28 Hz, 1 H), 6.35 (d, *J* = 7.15 Hz, 1 H), 3.48 (s, 3 H), 2.31 (d, *J* = 15.77 Hz, 1 H), 2.17 - 2.22 (m, 1 H), 2.09 (s, 3 H), 2.06 (d, *J* = 14.12 Hz, 1 H), 1.95 (td, *J* = 13.98, 3.76 Hz, 1 H), 1.78 - 1.86 (m, 1 H), 1.61 - 1.72 (m, 4 H), 1.50 - 1.59 (m, 3 H), 1.41 - 1.46 (m, 1 H), 1.38 (s, 3 H), 1.30 - 1.35 (m, 1 H), 1.21 (s, 3 H), 1.12 (s, 3 H), 1.07 (s, 3 H), 0.91 (d, *J* = 14.31 Hz, 1 H), 0.44 (s, 3 H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 178.0, 177.9, 167.8, 162.9, 146.4, 133.1, 126.9, 120.2, 118.1, 117.3, 51.4, 44.5, 43.6, 42.0, 39.8, 38.8, 37.8, 36.0, 34.4, 32.9, 32.2, 31.3, 30.3, 30.1, 29.4, 29.2, 28.1, 21.4, 18.0, 10.1 ppm; HRMS (ESI): *m/z* calcd for $C_{30}H_{40}NaO_4^+$ $[M+Na]^+$: 487.2819; found: 487.2816.

5.1.4. (9β,13α,14β,20α)-3-Hydroxy-N,9,13-trimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-amide (**1c**) **CAS: 1105067-36-4**

Compound **1c** was synthesized according to procedure iii in **Scheme 1**. To a solution of celastrol (50 mg, 0.11 mmol) in 2 mL DMF, PyBOP (68.7 mg, 0.132 mmol) and DIPEA (28.4 mg, 0.22 mmol) were added. The reaction was cooled down to 0 □ for 30 min, and then transferred to room temperature. After that, the reactant CH_3NH_2 (33 wt% in absolute ethanol, 55 μL, 0.33 mmol) was added and the solution was stirred for another 2 h. Finally, the mixture was quenched with water (15 mL) and extracted with ethyl acetate (3 * 15 mL). After washed by saturated brine (3 * 10 mL). The organic layers were combined and dried with anhydrous sodium sulfate. Compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:4). Red solid, 36.0 mg, yield: 72%. Mp: 218.7-220.9 □. $[\alpha]_D^{24}$ = -156.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.71 (s, 1H), 7.55 (q, *J* = 3.97 Hz, 1H), 7.07 (d, *J* = 7.15 Hz, 1H), 6.39 (s, 1H), 6.34 (d, *J* = 7.34 Hz, 1H), 2.43 (d, *J* = 4.22 Hz, 3H), 2.38 (d, *J* = 15.41 Hz, 1H), 2.18 (d, *J* = 9.90 Hz, 1H), 2.09 (s, 3H), 2.05 (d, *J* = 14.12 Hz, 1H), 1.94 - 2.01 (m, 1H), 1.79 - 1.85 (m, 1H), 1.73 - 1.79 (m, 1H), 1.61 - 1.67 (m, 2H), 1.56 - 1.60 (m, 1H), 1.54 (s, 2H), 1.50 (d, *J* = 7.89 Hz, 1H), 1.41 (d, *J* = 13.94 Hz, 1H), 1.37 (s, 3H), 1.26 - 1.33 (m, 1H), 1.20 (s, 3H), 1.08 (s, 3H), 1.02 (s, 3H), 0.86 (d, *J* = 12.65 Hz, 1H), 0.49 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 177.9, 177.5, 168.3, 163.0, 146.4, 133.2, 126.8, 120.1, 117.9, 117.2, 44.5, 44.0, 42.0, 38.8, 37.7, 36.1, 34.8, 33.2, 33.0, 31.4, 30.8, 30.2, 29.4, 28.9, 28.2, 26.0, 21.4, 17.4, 10.1 (One was buried in solvent peaks) ppm; HRMS (ESI): *m/z* calcd for $C_{30}H_{41}NNaO_3^+$ $[M+Na]^+$:486.2979, found: 486.2982.

5.1.5. (9β,13α,14β,20α)-3-Hydroxy-9,13-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid ethyl ester (**1d**) **CAS: 1105067-39-7**

Compound **1d** was synthesized according to procedure ii in **Scheme 1** with the steps mentioned above (e.g.: **1b**) and purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8). Starting with 50 mg celastrol, 41.4 mg product was obtained. Orange solid, yield: 78%. Mp: 133.7-136.1 □. $[\alpha]_D^{24}$ = -81.34 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.72 (s, 1H), 7.07 (dd, *J* = 1.01, 7.06 Hz, 1H), 6.38 (d, *J* = 1.10 Hz, 1H), 6.34 (d, *J* = 7.15 Hz, 1H), 3.86 - 3.95 (m, 2H), 2.33 (d, *J* = 15.59 Hz, 1H), 2.20 (d, *J* = 11.19 Hz, 1H), 2.08 (s, 3H), 2.03 - 2.07 (m, 1H), 1.89 - 1.97 (m, 1H), 1.78 - 1.86 (m, 1H), 1.61 - 1.71 (m, 4H), 1.50 - 1.57 (m, *J* = 6.60, 6.60 Hz, 3H), 1.40 - 1.45 (m, 1H), 1.37 (s, 3H), 1.30 - 1.35 (m, 1H), 1.21 (s, 3H), 1.13 (t, *J* = 7.20 Hz, 3H), 1.11 (s, 3H), 1.06 (s, 3H), 0.90 (d, *J* = 14.12 Hz, 1H), 0.47 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 178.0, 177.4, 167.9, 163.0, 146.5, 133.3, 126.9, 120.2, 118.1, 117.4, 59.9, 44.5, 43.7, 42.0, 40.0, 39.7, 38.9, 37.8, 36.0, 34.4, 32.9, 32.4, 31.4, 30.2, 29.4, 29.2, 28.2, 21.4, 18.1, 13.9, 10.1 ppm; HRMS (ESI): *m/z* calcd for $C_{31}H_{42}NaO_4^+$ $[M+Na]^+$: 501.2975; found: 501.2966.

5.1.6. (9β,13α,14β,20α)-N-ethyl-3-Hydroxy-9,13-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-amide (**1e**) **CAS: 1810867-89-0**

Compound **1e** was synthesized according to procedure iii in **Scheme 1** with the same steps as described for **1b** and purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8). Starting with 50 mg celastrol, compound (25.2 mg) was obtained. Red solid, yield: 48%. Mp: 134.6-136.5 □. $[\alpha]_D^{24}$ = -262.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.71 (s, 1H), 7.55 (t, *J* = 5.32 Hz, 1H), 7.07 (dd, *J* = 1.10, 6.97 Hz, 1H), 6.39 (d, *J* = 1.10 Hz, 1H), 6.33 (d, *J* = 7.15 Hz, 1H), 2.89 - 3.02 (m, 2H), 2.40 (d, *J* = 15.59 Hz, 1H), 2.19 (d, *J* = 8.62 Hz, 1H), 2.09 (s, 3H), 2.07 (dd, *J* = 1.00, 6.79 Hz, 1H), 1.93 - 1.99 (m, 1H), 1.76 - 1.86 (m, 2H), 1.63 (d, *J* = 7.70 Hz, 2H), 1.55 - 1.58 (m, 1H), 1.54 (br. s., 1H), 1.48 - 1.52 (m, 1H), 1.42 (d, *J* = 14.31 Hz, 1H), 1.37 (s, 3H), 1.28 (dt, *J* = 3.94, 13.98 Hz, 1H), 1.22 (br. s., 1H), 1.20 (s, 3H), 1.04 - 1.06 (s, 3H), 1.02 (s, 3H), 0.91 (t, *J* = 7.24 Hz, 3H), 0.82 - 0.87 (m, 1H), 0.54 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 177.9, 176.7, 168.3, 163.0, 146.4, 133.2, 126.8, 120.0, 117.9, 117.2, 44.5, 44.0, 42.0, 39.3, 38.8, 37.7, 36.1, 34.9, 33.6, 33.3, 33.0, 31.4, 30.5, 30.2, 29.4, 28.8, 28.2, 21.4, 17.8, 14.5, 10.1 ppm; HRMS (ESI): *m/z* calcd for $C_{31}H_{43}NNaO_3^+$ $[M+Na]^+$:500.3135, found: 500.3138.

5.1.7. (9β,13α,14β,20α)-3-Hydroxy-9,13-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid, 1-methylethyl ester (**1f**) **CAS: 1105067-40-0**

Compound **1f** was synthesized according to procedure ii in **Scheme** and purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8). Starting with 50 mg celastrol, compound (43.3 mg) was obtained, Orange solid, yield: 79%. Mp: 105.1-107.8 □. $[\alpha]_D^{24}$ = -246.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.73 (s, 1H), 7.07 (d, *J* = 6.60 Hz, 1H), 6.38 (s, 1H), 6.35 (d, *J* = 6.97 Hz, 1H), 4.70 (td, *J* = 6.17, 12.43 Hz, 1H), 2.33 (d, *J* = 15.59 Hz, 1H), 2.22 (d, *J* = 12.47 Hz, 1H), 2.09 (s, 3H), 2.05 (d, *J* = 12.10 Hz, 1H), 1.94 (dt, *J* = 3.12, 15.59 Hz, 1H), 1.78 - 1.86 (m, 1H), 1.60 - 1.71 (m, 4H), 1.50 - 1.58 (m, 3H), 1.43 (d, *J* = 13.94 Hz, 1H), 1.38 (s, 3H), 1.29 - 1.36 (m, 1H), 1.21 (br. s., 3H), 1.14 (d, *J* = 6.24 Hz, 3H), 1.09 (br. s., 3H), 1.08 (d, *J* = 6.24 Hz, 3H), 1.07 (br. s., 3H), 0.90 (d, *J* = 13.57 Hz, 1H), 0.51 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 178.0, 176.8, 167.8, 163.0, 146.5, 133.3, 126.9, 120.1, 118.1, 117.3, 66.9, 44.5, 43.6, 42.0, 39.7, 38.9, 37.7, 36.0, 34.3, 33.0, 32.3, 31.3, 30.2, 29.9, 29.3, 29.0, 28.1, 21.4, 21.3, 21.3, 18.2, 10.1 ppm; HRMS (ESI): *m/z* calcd for $C_{32}H_{45}O_4^+$ $[M+H]^+$: 493.3312, found: 493.3315.

5.1.8. (9β,13α,14β,20α)-N-n-propyl-3-Hydroxy-9,13-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-amide (**1g**) **CAS: 2027514-22-1**

Compound **1g** was synthesized according to procedure iii in **Scheme 1** like compound **1c**. It was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8). Red solid, 17.2 mg, yield: 31%. Mp: 122.3-124.4 □. $[\alpha]_D^{24}$ = -231.4 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.72 (s, 1H), 7.56 (t, *J* = 5.50 Hz, 1H), 7.08 (dd, *J* = 1.01, 7.06 Hz, 1H), 6.40 (d, *J* = 0.92 Hz, 1H), 6.35 (d, *J* = 7.34 Hz, 1H), 2.89 - 2.97 (m, 1H), 2.78 - 2.85 (m, 1H), 2.42 (d, *J* = 15.41 Hz, 1H), 2.19 (d, *J* = 9.17 Hz, 1H), 2.11 (br. s., 1H), 2.09 (s, 3H), 1.93 - 1.99 (m, 1H), 1.76 - 1.86 (m, 2H), 1.62 - 1.65 (m, *J* = 7.30 Hz, 2H), 1.57 - 1.60 (m, 1H), 1.53 - 1.56 (m, 2H), 1.49 - 1.53 (m, 1H), 1.43 (d, *J* = 13.75 Hz, 1H), 1.38 (s, 3H), 1.31 - 1.36 (m, 2H), 1.28 - 1.30 (m, 1H), 1.21 (s, 3H), 1.06 (s, 3H), 1.04 (s, 3H), 0.85 - 0.88 (m, 1H), 0.77 (t, *J* = 7.43 Hz, 3H), 0.54 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 177.9, 176.9, 168.3, 163.0, 146.4, 133.2, 126.8, 120.0, 117.9, 117.2, 44.5, 44.0, 42.0, 40.8, 39.4, 38.9, 37.7, 36.1, 34.9, 33.4, 33.0, 31.5, 30.5, 30.2, 29.5, 28.8, 28.2, 21.9, 21.5, 17.7, 11.5, 10.1 ppm; HRMS (ESI): *m/z* calcd for $C_{32}H_{45}NNaO_3^+$ $[M+Na]^+$: 514.3292, found: 514.3297.

5.1.9. (9β,13α,14β,20α)-3-Methoxy-9,13-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid methyl ester (**2a**) **CAS: 1365906-42-8**

Compound **2a** was obtained according to procedure iv in **Scheme 1**. (50 mg, 0.108 mmol) **1b** and CH_3I (153.4 mg, 1.08 mmol) were dissolved in 2 mL THF, then 10 mol% TBAB (Tert-butyl ammonium bromide) (3.2 mg, 0.011 mmol), 20 mol% Dioxane (400 μL) and K_2CO_3 (60 mg, 0.43 mmol) were added to the solution. The mixture was stirred at 70 □ for 12 h. The mixture was quenched with 1 mol/L HCl a.q. (5 mL), extracted with ethyl acetate (3 * 15 mL). The organic layers were combined and dried with anhydrous sodium sulfate. Compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8). Yellow solid, 27.7 mg, yield: 54%. Mp: 204.7-207.0 □. $[\alpha]_D^{24}$ = -69.4 (c 0.1, MeOH). ^{1}H NMR (600 MHz, CHLOROFORM-d) δ 6.99 (d, *J* = 6.60 Hz, 1H), 6.45 (s, 1H), 6.31 (d, *J* = 6.97 Hz, 1H), 3.85 (s, 3H), 3.56 (s, 3H), 2.42 (d, *J* = 15.77 Hz, 1H), 2.22 (s, 3H), 2.17 - 2.21 (m, 1H), 2.10 - 2.16 (m, 1H), 2.05 (dt, *J* = 3.67, 14.03 Hz, 1H), 1.86 - 1.91 (m, 1H), 1.81 - 1.86 (m, 1H), 1.78 (dd, *J* = 3.76, 13.48 Hz, 1H), 1.68 - 1.73 (m, 1H), 1.66 - 1.68 (m, 1H), 1.61 - 1.66 (m, 1H), 1.58 (d, *J* = 8.62 Hz, 1H), 1.53 - 1.57 (m, 1H), 1.50 (dd, *J* = 3.85, 14.67 Hz, 1H), 1.46 (s, 3H), 1.38 (dt, *J* = 4.40, 14.03 Hz, 1H), 1.26 (s, 3H), 1.18 (s, 3H), 1.10 (s, 3H), 0.97 (d, *J* = 14.12 Hz, 1H), 0.54 (s, 3H) ppm; ^{13}C NMR (151 MHz, CHLOROFORM-d) δ 180.7, 178.7, 170.3, 162.5, 150.3, 134.3, 132.1, 127.2, 123.5, 117.9, 60.1, 51.6, 45.1, 44.3, 42.4, 40.4, 39.2, 38.2, 36.4, 34.8, 33.6, 32.7, 31.6, 30.8, 30.5, 29.9, 29.6, 28.6, 21.9, 18.4, 10.8 ppm; HRMS (ESI): *m/z* calcd for $C_{31}H_{43}O_4^+$ $[M+H]^+$: 479.3156; found: 479.3155.

5.1.10. (9β,13α,14β,20α)-3-Ethoxy-9,13-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid methyl ester (**2b**)

Compound **2b** was obtained according to procedure v in **Scheme 1**. (50 mg, 0.108 mmol) **1b** and CH_3CH_2Br (117.7 mg, 1.08 mmol) were dissolved in 2 mL THF, and then 10 mol% TBAB (Tert-butyl ammonium bromide) (3.2 mg, 0.011 mmol), 20 mol% Dioxane (400 μL) and K_2CO_3 (60 mg, 0.43 mmol) were added to the solution. After stirred at 70 □ for 10 h, the mixture was quenched with 1 mol/L HCl a.q. (5 mL), and then extracted with ethyl acetate (3 * 15 mL). The organic layers were combined and dried with anhydrous sodium sulfate. Compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8). Yellow solid, 44.8 mg, yield: 85%. Mp: 141.3-142.9 □. $[\alpha]_D^{24}$ = -62.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, CHLOROFORM-d) δ 6.95 (d, *J* = 6.79 Hz, 1H), 6.41 (s, 1H), 6.29 (d, *J* = 6.97 Hz, 1H), 4.02 - 4.21 (m, 2H), 3.56 (s, 3H), 2.42 (d, *J* = 15.96 Hz, 1H), 2.22 (s, 3H), 2.19 (d, *J* = 11.19 Hz, 1H), 2.12 (dd, *J* = 2.20, 13.75 Hz, 1H), 2.05 (dt, *J* = 3.67, 14.03 Hz, 1H), 1.86 - 1.92 (m, 1H), 1.82 - 1.86 (m, 1H), 1.78 (dd, *J* = 3.48, 13.39 Hz, 1H), 1.68 - 1.72 (m, 1H), 1.65 - 1.68 (m, 1H), 1.62 - 1.65 (m, 1H), 1.58 (d, *J* = 8.44 Hz, 1H), 1.53 - 1.57 (m, 1H), 1.49 (dd, *J* = 4.68, 14.58 Hz, 1H), 1.45 (s, 3H), 1.37 - 1.41 (m, 1H), 1.34 (t, *J* = 7.06 Hz, 3H), 1.26 (s, 3H), 1.18 (s, 3H), 1.10 (s, 3H), 0.97 (d, *J* = 13.94 Hz, 1H), 0.55 (s, 3H) ppm; ^{13}C NMR (151 MHz, CHLOROFORM-d) δ 181.0, 178.7, 169.5, 162.3, 149.4, 133.7, 132.0, 127.4, 123.6, 117.9, 68.1, 51.6, 45.0, 44.3, 42.2, 40.4, 39.2, 38.2, 36.4, 34.8, 33.5, 32.7, 31.6, 30.8, 30.5, 29.9, 29.6, 28.6, 21.9, 18.4, 15.8, 11.0 ppm; HRMS (ESI): *m/z* calcd for $C_{32}H_{45}O_4^+$ $[M+H]^+$: 493.3312; found: 493.3311.

5.1.11. (9β,13α,14β,20α)-3-(2,2-difluoroethoxy)-9,13-dimethyl-2-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid methyl ester (**2c**)

Compound **2c** was obtained according to procedure vi in **Scheme 1**. (100 mg, 0.215 mmol) **1b** was dissolved in 2 mL DMF, and then 1,1-difluoro-2-iodoethane (F_2CHCH_2I, 413 mg, 195 μL, 2.15 mmol) and K_2CO_3 (59.34 mg, 0.43 mmol) were added to the solution. The mixture was stirred at 50 □ for 24 h (1 day). The mixture was diluted in 50 mL ethyl acetate, and then washed with saturated lithium chloride a.q. (3 * 25 mL) and saturated brine (50 mL). The organic layers were combined and dried with anhydrous sodium sulfate. Compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8). Yellow solid, 64.5 mg, yield: 57%. Mp: 189.3-191.5 □. $[\alpha]_D^{24}$ = -23.4 (c 0.1, MeOH). ^{1}H NMR (600 MHz, CHLOROFORM-d) δ 7.03 (dd, *J* = 1.01, 7.06 Hz, 1H), 6.42 (d, *J* = 1.10 Hz, 1H), 6.32 (d, *J* = 7.15 Hz, 1H), 6.00 - 6.20 (m, 1H), 4.26 - 4.35 (m, 2H), 3.56 (s, 3H), 2.42 (d, *J* = 15.77 Hz, 1H), 2.24 (s, 3H), 2.18 - 2.23 (m, 1H), 2.14 (dd, *J* = 2.57, 13.75 Hz, 1H), 2.03 - 2.09 (m, 1H), 1.87 - 1.92 (m, 1H), 1.82 - 1.87 (m, 1H), 1.79 (dd, *J* = 5.41, 13.66 Hz, 1H), 1.69 - 1.73 (m, 1H), 1.66 - 1.69 (m, 1H), 1.62 - 1.66 (m, 1H), 1.58 (d, *J* = 8.44 Hz, 1H), 1.55 (dd, *J* = 4.13, 6.51 Hz, 1H), 1.50 (dd, *J* = 4.31, 15.13 Hz, 1H), 1.46 (s, 3H), 1.35 - 1.41 (m, 1H), 1.27 (s, 3H), 1.18 (s, 3H), 1.10 (s, 3H), 0.98 (d, *J* = 14.12 Hz, 1H), 0.55 (s, 3H) ppm; ^{13}C NMR (151 MHz, CHLOROFORM-d) δ 180.0, 178.7, 170.8, 162.8,

148.5, 134.9, 131.8, 126.9, 123.4, 118.0, 114.4 (t, *J* = 239.90 Hz), 70.6 (t, *J* = 28.61 Hz), 51.6, 45.1, 44.3, 42.5, 40.4, 39.3, 38.2, 36.4, 34.8, 33.6, 32.7, 31.6, 30.9, 30.5, 29.9, 29.6, 28.6, 21.9, 18.4, 10.7 ppm; HRMS (ESI): *m/z* calcd for $C_{32}H_{43}F_2O_4^+$ $[M+H]^+$: 529.3124; found: 529.3124.

5.1.12. (9β,13α,14β,20α)-2,3-Dihydroxy-9,13-dimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**3a**) **CAS: 193957-88-9**

Compound **3a** was obtained as white solid according to procedure vii in **Scheme 1**. 50 mg (0.11 mmol) celastrol was dissolved in 2 mL CD_3OD and stirred in a 25-mL flask, and then 44 mg (1.1 mmol, 10 equiv) $NaBH_4$ was added. The mixture was kept at room temperature for 30 min. After that, 0.1 mol/L HCl (3 mL) was added to quench the reaction, and subsequently the aqueous phase was separated and extracted with DCM (15 mL) three times. The organic layers were combined and dried with anhydrous sodium sulfate. Compound was purified by removing the solvent under vacuum conditions. White solid, 49.7 mg, yield: 99%. Mp: 178.3-180.1 □. $[\alpha]_D^{24}$ = -4.0 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.05 (br. s., 1H), 8.80 (s, 1H), 7.82 (s, 1H), 6.61 (s, 1H), 5.72 (dd, *J* = 1.74, 6.14 Hz, 1H), 3.18 (dd, *J* = 6.24, 20.54 Hz, 1H), 2.91 (dd, *J* = 1.47, 19.99 Hz, 1H), 2.34 (d, *J* = 15.59 Hz, 1H), 2.04 (d, *J* = 13.57 Hz, 1H), 2.01 (s, 3H), 1.94 - 2.00 (m, 2H), 1.86 (dt, *J* = 5.00, 13.80 Hz, 1H), 1.79 (dt, *J* = 6.50, 13.80 Hz, 1H), 1.63 - 1.69 (m, 1H), 1.59 - 1.63 (m, 1H), 1.53 - 1.59 (m, 1H), 1.43 - 1.51 (m, 3H), 1.36 - 1.41 (m, 1H), 1.29 (dt, *J* = 4.40, 13.70 Hz, 1H), 1.22 (s, 3H), 1.17 (s, 3H), 1.11 (s, 3H), 1.05 (s, 3H), 0.85 (d, *J* = 13.94 Hz, 1H), 0.66 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 179.5, 149.2, 143.1, 140.6, 139.4, 123.1, 120.1, 117.7, 108.2, 43.8, 43.3, 39.4, 37.1, 36.6, 36.1, 34.4, 34.1, 34.1, 32.4, 31.4, 30.2, 30.1, 29.8, 29.5, 28.4, 27.3, 22.7, 18.1, 11.5 ppm; HRMS (ESI): *m/z* calcd for $C_{29}H_{40}NaO_4^+$ $[M+Na]^+$: 475.2819, found: 475.2823.

5.1.13. (6β,9β,13α,14β,20α)-2,3-Dihydroxy-9,13-dimethyl-6-(nitromethyl)-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**3b**) **CAS: 1350630-32-8**

Compound **3b** was obtained according to procedure viii in **Scheme 1**. Nitromethane (14.04 mg, 0.23 mmol) and 51.6 mg (0.115 mmol) celastrol were dissolved in 1 mL THF, and then 1 M solution of TBAF in THF (600 μL, 0.6 mmol) was added. The mixture was stirred at room temperature for 1 h. After quenched with water (20 mL), the solution was extracted with ethyl acetate (3 * 20 mL). The organic layers were combined and dried with anhydrous sodium sulfate. Compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:2). Light brown solid, 49.0 mg, yield: 87%. Mp: 199.3-202.4 □. $[\alpha]_D^{24}$ = -80.0 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.08 (br. s., 1H), 9.14 (br. s., 1H), 8.11 (br. s., 1H), 6.69 (s, 1H), 5.62 (d, *J* = 6.05 Hz, 1H), 4.77 (dd, *J* = 3.94, 11.28 Hz, 1H), 4.10 - 4.15 (m, 1H), 4.07 (d, *J* = 11.19 Hz, 1H), 2.33 (d, *J* = 15.59 Hz, 1H), 2.15 (s, 3H), 2.00 - 2.08 (m, 2H),

1.92 - 1.99 (m, 1H), 1.79 (dt, *J* = 6.14, 13.98 Hz, 1H), 1.63 - 1.71 (m, 2H), 1.60 (dd, *J* = 8.25, 15.77 Hz, 2H), 1.46 - 1.53 (m, 2H), 1.40 (s, 3H), 1.30 - 1.36 (m, 1H), 1.22 - 1.30 (m, 2H), 1.17 (s, 3H), 1.10 (s, 3H), 1.05 (s, 3H), 0.82 - 0.89 (m, 1H), 0.62 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 179.5, 153.3, 144.4, 141.4, 140.6, 120.6, 120.5, 117.6, 109.0, 80.9, 43.9, 43.6, 39.4, 37.4, 37.0, 36.3, 36.2, 35.8, 35.3, 34.5, 32.4, 31.4, 30.2, 30.0, 29.8, 29.4, 28.5, 22.1, 18.0, 11.1 ppm; HRMS (ESI): m/z calcd for $C_{30}H_{40}NO_6^-$ $[M]^-$: 510.2861; found: 510.2856.

5.1.14. (6β,9β,13α,14β,20α)-2,3-Dihydroxy-9,13-dimethyl-6-(2-oxopropyl)-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**3c**) **CAS: 1643802-69-0**

Compound **3c** was obtained according to procedure ix in **Scheme 1**. 50 mg (0.11 mmol) celastrol was dissolved in 4 mL acetone and then stirred in a 25-mL flask. After that, 1 drop concentrated HCl (12 mol/L) was added as catalyst and the mixture was kept at room temperature for 12 h. After the reaction finished, the solvent was removed under vacuum conditions. Compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:4). White solid, 28.8 mg, yield: 51%. Mp: 121.6-122.7 □. $[\alpha]_D^{24}$ = -72.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.06 (br. s., 1H), 8.91 (s, 1H), 7.91 (s, 1H), 6.63 (s, 1H), 5.72 (d, *J* = 6.24 Hz, 1H), 3.75 (ddd, *J* = 2.84, 6.10, 10.87 Hz, 1H), 2.67 (dd, *J* = 2.75, 16.14 Hz, 1H), 2.31 - 2.36 (m, 1H), 2.29 (d, *J* = 11.00 Hz, 1H), 2.10 (s, 3H), 2.03 - 2.05 (m, 1H), 2.03 (s, 3H), 2.00 (d, *J* = 13.76 Hz, 1H), 1.93 - 1.98 (m, 1H), 1.74 - 1.82 (m, 1H), 1.66 - 1.72 (m, 1H), 1.61 - 1.65 (m, 1H), 1.58 - 1.61 (m, 1H), 1.52 - 1.58 (m, 1H), 1.48 (d, *J* = 8.44 Hz, 1H), 1.42 - 1.47 (m, 1H), 1.39 (s, 3H), 1.33 - 1.38 (m, 1H), 1.28 - 1.33 (m, 1H), 1.22 - 1.28 (m, 1H), 1.15 (s, 3H), 1.10 (s, 3H), 1.04 (s, 3H), 0.79 - 0.90 (m, 1H), 0.62 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 207.5, 179.5, 149.4, 143.4, 141.2, 140.0, 126.1, 121.6, 119.6, 108.6, 59.8, 51.3, 43.9, 43.3, 39.4, 37.3, 36.5, 36.4, 36.3, 35.1, 34.5, 32.5, 32.4, 31.4, 30.3, 30.2, 30.0, 29.9, 29.4, 28.6, 18.0, 11.2 ppm; HRMS (ESI): *m/z* calcd for $C_{32}H_{43}O_5^-$ $[M]^-$: 507.3116; found: 507.3109.

5.1.15. (6β,9β,13α,14β,20α)-2,3-Dihydroxy-9,13-dimethyl-6-(1H-pyrrol-3-yl)-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**3d**)

Compound **3d** was obtained according to procedure x in **Scheme 1**. 50 mg (0.11 mmol) celastrol was dissolved in 1 mL pyrrole in a 10-mL closed tube, and then 20 μL sodium methoxide was added. The mixture was stirred at room temperature for 5 minutes. When the reaction was finished, 1 mol/L HCl a. q. (5 mL) was added to stop the reaction and then subsequently the aqueous phase was separated and extracted with ethyl acetate (20 mL) three times. The organic layers were combined and dried with anhydrous sodium sulfate. Then the solvent was removed *in vacuo*, giving a crude mixture, which was purified by flash chromatography on silica column (Hexane/Ethyl acetate/AcOH = 4:1:0.005) to give the

pure product. Wine red solid, yield: 82%. Mp: >300 □. $[\alpha]_D^{24}$ = -58.0 (c 0.1, MeOH). ^{1}H-NMR (600 MHz, DMSO-d_6) δ 12.06 (br. s., 1H), 10.38 (d, *J* = 1.83 Hz, 1H), 8.89 (s, 1H), 7.83 (s, 1H), 6.66 (s, 1H), 6.51 (d, *J* = 1.65 Hz, 1H), 6.00 (d, *J* = 6.60 Hz, 1H), 5.75 (q, *J* = 2.63 Hz, 1H), 5.12 (d, *J* = 0.55 Hz, 1H), 4.52 (d, *J* = 6.24 Hz, 1H), 2.33 (d, *J* = 15.41 Hz, 1H), 2.04 (d, *J* = 15.59 Hz, 1H), 1.99 - 2.01 (m, 1H), 1.95 - 1.99 (m, 1H), 1.87 (s, 3H), 1.77 - 1.82 (m, 1H), 1.73 - 1.77 (m, 1H), 1.61 - 1.67 (m, 1H), 1.55 - 1.61 (m, 1H), 1.52 - 1.55 (m, 1H), 1.48 - 1.52 (m, 1H), 1.47 (d, *J* = 8.07 Hz, 1H), 1.39 (dd, *J* = 3.39, 13.85 Hz, 1H), 1.28 - 1.33 (m, 1H), 1.25 - 1.28 (m, 1H), 1.24 (s, 3H), 1.10 (s, 3H), 1.06 (s, 3H), 1.04 (s, 3H), 0.86 (d, *J* = 11.37 Hz, 1H), 0.67 (s, 3H) ppm; ^{13}C-NMR (151 MHz, DMSO-d_6) δ 179.5, 147.6, 143.5, 140.8, 140.3, 134.5, 124.8, 121.5, 121.1, 115.8, 108.3, 106.7, 103.4, 43.8, 43.2, 39.4, 39.2, 37.2, 36.5, 36.4, 35.1, 34.9, 34.5, 32.4, 31.4, 30.2, 30.1, 29.8, 29.5, 28.4, 22.0, 18.1, 11.4 ppm; HRMS (ESI): *m/z* calcd for $C_{33}H_{42}NO_4^-$ $[M]^-$: 516.3119; found: 516.3118.

5.1.16. (6β,9β,13α,14β,20α)-2,3-Dihydroxy-9,13-dimethyl-6-(dimethoxyphosphoryl)-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**3e**)

Compound **3e** was obtained as white solid according to procedure xi in **Scheme 1**. 50 mg (0.11 mmol) celastrol, and 123 mg dimethyl phosphonate (1.1 mmol) were dissolved in 2 mL dichloromethane and stirred in a 50-mL closed tube, and then 2.7 mg (0.1 equiv, 5 mol%) $AlCl_3 \cdot 6H_2O$ was added as a catalyst. The mixture was kept at room temperature for 6 h. After the reaction finished, saturated sodium chloride solution (15 mL) was added to quench the reaction, and then subsequently the aqueous phase was separated and extracted with ethyl acetate (15 mL) three times. The organic layers were combined and dried with anhydrous sodium sulfate. Compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:2). White solid, 34.2 mg, yield: 55%. Mp: 199.0-201.4 □. $[\alpha]_D^{24}$ = -91.34 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.04 (br. s., 1H), 9.04 (br. s., 1H), 7.97 (s, 1H), 6.66 (s, 1H), 5.64 (dd, *J* = 3.21, 6.33 Hz, 1H), 4.19 (dd, *J* = 6.05, 29.34 Hz, 1H), 3.51 (d, *J* = 8.99 Hz, 3H), 3.49 (d, *J* = 8.80 Hz, 3H), 2.32 (d, *J* = 15.59 Hz, 1H), 2.13 (d, *J* = 0.92 Hz, 3H), 2.04 (d, *J* = 13.20 Hz, 1H), 1.98 - 2.01 (m, 1H), 1.92 - 1.98 (m, 1H), 1.81 (dt, *J* = 5.87, 13.85 Hz, 1H), 1.60 - 1.64 (m, 1H), 1.58 - 1.60 (m, *J* = 7.90 Hz, 2H), 1.57 (s, 3H), 1.54 - 1.56 (m, 1H), 1.51 - 1.54 (m, 1H), 1.49 (d, *J* = 8.07 Hz, 1H), 1.43 (d, *J* = 5.32 Hz, 1H), 1.40 (d, *J* = 4.95 Hz, 1H), 1.28 (dt, *J* = 4.40, 13.66 Hz, 1H), 1.18 (s, 3H), 1.10 (s, 3H), 1.06 (s, 3H), 0.86 (d, *J* = 12.84 Hz, 1H), 0.61 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 179.5, 150.6 (d, *J* = 12.10 Hz, 1 C), 144.1, 141.1 (d, *J* = 3.30 Hz, 1 C), 140.5 (d, *J* = 6.60 Hz, 1 C), 121.3 (d, *J* = 4.40 Hz, 1 C), 117.9 (d, *J* = 7.70 Hz, 1 C), 114.6 (d, *J* = 12.10 Hz, 1 C), 109.3, 52.8 (d, *J* = 6.60 Hz, 1 C), 52.3 (d, *J* = 6.60 Hz, 1 C), 43.9, 38.8, 37.9, 37.4, 36.4, 34.6, 33.6, 33.5, 32.4, 31.5, 31.0, 30.2, 30.0 (d, *J* = 13.20 Hz, 1 C), 29.5, 28.8, 22.1, 21.6 (d, *J* = 6.60 Hz, 1 C), 18.0, 14.0, 12.6 ppm; ^{31}P

NMR (243 MHz, DMSO-d_6) δ 27.3 ppm; HRMS (ESI): *m/z* calcd for $C_{31}H_{45}NaO_7P^+$ $[M+Na]^+$: 583.2795, found: 583.2794.

5.1.17. (6β,9β,13α,14β,20α)-2-Hydroxy-3-(phenylmethoxy)-6,9,13-trimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid benzyl ester (**4a**) **CAS: 2095711-55-8**

Compound **4a** was obtained according to procedure xii in **Scheme 1** (two steps). To a solution of celastrol (300 mg, 0.67 mmol) in DMF (6 mL) K_2CO_3 (184 mg, 1.33 mmol), $PhCH_2Br$ (350 μL, 2.94 mmol) and Et_3N (200 μL) were added sequentially. The reaction was heated at 80 □ for 2.5 hours. After quenched by ice-water (30 mL), the mixture was exacted by ethyl acetate (50 mL) and then washed by pure-water (30 mL) and brine (30 mL). The organic layers were combined and dried with anhydrous sodium sulfate and then concentrated *in vacuo*. The intermediate product was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:4). Yellow solid, 285.6 mg, yield: 68%.

To a solution of intermediate product (300 mg, 0.45 mmol) in THF (10 mL), 3 M MeMgBr in THF solution (3.3 mL, 10 mmol) was added at 0 □. The reaction was stirred at 0 □ for 1 hour. The reaction was quenched by addition of pure-water (30 mL) and extracted with ethyl acetate (20 mL) three times. The organic layers were combined and dried with anhydrous sodium sulfate and then concentrated *in vacuo*. Our compound was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8) to get the product **4a**. Pale yellow solid, 254.6 mg, yield: 83%. Mp: 103.7-105.0 □. $[\alpha]_D^{24}$ = -30.0 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.00 (s, 1H), 7.50 (d, *J* = 1.10 Hz, 1H), 7.47 - 7.49 (m, 1H), 7.38 - 7.40 (m, 1H), 7.36 - 7.38 (m, 1H), 7.34 - 7.36 (m, 1H), 7.33 - 7.34 (m, *J* = 1.10, 1.10 Hz, 1H), 7.32 (d, *J* = 6.97 Hz, 1H), 7.28 - 7.30 (m, 2H), 6.73 (s, 1H), 5.73 (d, *J* = 6.24 Hz, 1H), 4.97 (d, *J* = 12.47 Hz, 1H), 4.91 (s, 1H), 4.89 (s, 1H), 4.84 (d, *J* = 11.00 Hz, 1H), 3.35 - 3.40 (m, 1H), 2.36 (d, *J* = 15.59 Hz, 1H), 2.12 (s, 3H), 2.09 (d, *J* = 13.75 Hz, 1H), 1.98 (dt, *J* = 3.85, 13.94 Hz, 1H), 1.92 (d, *J* = 12.84 Hz, 1H), 1.75 - 1.84 (m, 1H), 1.66 (dd, *J* = 8.07, 15.96 Hz, 1H), 1.57 - 1.63 (m, 1H), 1.55 (d, *J* = 2.75 Hz, 1H), 1.50 - 1.53 (m, 1H), 1.49 (d, *J* = 8.07 Hz, 1H), 1.46 (br. s., 1H), 1.45 (d, *J* = 2.93 Hz, 1H), 1.40 (s, 3H), 1.38 (d, *J* = 9.54 Hz, 1H), 1.33 - 1.37 (m, 1H), 1.25 - 1.30 (m, 1H), 1.16 (s, 3H), 1.14 (s, 3H), 1.08 (d, *J* = 6.97 Hz, 3H), 1.06 (s, 3H), 0.89 (d, *J* = 13.94 Hz, 1H), 0.49 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 177.1, 148.0, 147.4, 144.5, 142.6, 138.2, 135.9, 128.4 (2 C), 128.3, 128.2 (2 C), 128.0, 127.9 (2 C), 127.8 (2 C), 127.6, 126.8, 124.6, 110.3, 73.2, 65.6, 43.8, 43.1, 39.8, 37.2, 36.9, 36.8, 36.4, 34.8, 34.3, 32.4, 31.5, 31.3, 30.1, 30.0, 29.9, 29.4, 28.5, 22.2, 22.0, 17.8, 11.5 ppm; HRMS (ESI): *m/z* calcd for $C_{44}H_{54}NaO_4^+$ $[M+Na]^+$: 669.3914; found: 669.3911.

5.1.18. (6β,9β,13α,14β,20α)-2,3-Dihydroxy-6,9,13-trimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**4b**) **CAS: 2095711-56-9**

Compound **4b** was obtained according to procedure xiii in **Scheme 1**. To a solution of **4a** (130 mg, 0.20 mmol) in MeOH (6 mL), 20% Pd/C (26 mg) was added. The solution was hydrogenated with a balloon of hydrogen, and then kept at room temperature for 12 hours. After filtered by celite, the filterate was concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:4). Gray solid, 78.6 mg, yield: 87%. Mp: 139.3-140.8 □. $[\alpha]_D^{24}$ = -79.4 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.06 (br. s., 1H), 8.82 (br. s., 1H), 7.83 (br. s., 1H), 6.61 (s, 1H), 5.73 (d, *J* = 5.87 Hz, 1H), 3.35 - 3.39 (m, 1H), 2.33 (d, *J* = 15.59 Hz, 1H), 2.06 (s, 3H), 2.02 (d, *J* = 17.06 Hz, 1H), 1.94 - 1.99 (m, 1H), 1.76 - 1.86 (m, 1H), 1.65 - 1.73 (m, 1H), 1.60 - 1.65 (m, 1H), 1.59 (d, *J* = 7.52 Hz, 1H), 1.52 - 1.57 (m, 1H), 1.48 - 1.51 (m, 1H), 1.47 - 1.48 (m, 1H), 1.44 - 1.47 (m, 1H), 1.40 (br. s., 1H), 1.39 (s, 3H), 1.28 - 1.32 (m, 1H), 1.25 (d, *J* = 10.09 Hz, 1H), 1.17 (s, 3H), 1.10 (s, 3H), 1.07 (d, *J* = 6.79 Hz, 3H), 1.05 (s, 3H), 0.86 (d, *J* = 6.42 Hz, 1H), 0.64 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 179.5, 147.7, 143.0, 141.0, 139.5, 128.1, 124.5, 119.7, 108.6, 43.9, 43.1, 37.3, 36.9, 36.5, 36.5, 35.1, 34.5, 32.4, 31.4, 31.4, 30.9, 30.1, 30.0, 29.9, 29.4, 28.5, 22.3, 22.1, 17.9, 11.3 ppm; HRMS (ESI): m/z calcd for $C_{30}H_{41}O_4^-$ $[M]^-$: 465.3010; found: 465.3010.

5.1.19. (6β,9β,13α,14β,20α)-2,3-[(1-methylethylidene)bis(oxy)]-9,13-dimethyl-6-(nitromethyl)-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**5a**) **CAS: 2095710-94-2**

Compound **5a** was obtained according to procedure i in **Scheme 2**. To a solution of **3b** (337 mg, 0.66 mmol) in DCM (4 mL) was added $(MeO)_2CMe_2$ (3.24 mL, 2.75 g, 26.4 mmol) followed by *p*-TsOH·H_2O (18.9 mg, 0.132 mmol). The reaction was stirred at 35 □ overnight. The solution was diluted with DCM (50 mL), and then washed with sat. $NaHCO_3$ a. q. (50 mL) followed by brine (50 mL). The final solution was dried with anhydrous sodium sulfate and concentrated *in vacuo*. The crude product was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:4). White solid, 228.0 mg, yield: 63%. Mp: 174.5-176.2 □. $[\alpha]_D^{24}$ = -92.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, CHLOROFORM-d) δ 6.67 (s, 1H), 5.72 (d, *J* = 6.24 Hz, 1H), 4.54 (dd, *J* = 4.03, 11.37 Hz, 1H), 4.21 - 4.27 (m, 1H), 4.11 (t, *J* = 11.19 Hz, 1H), 2.39 (d, *J* = 15.77 Hz, 1H), 2.24 (s, 3H), 2.08 - 2.14 (m, 1H), 2.02 - 2.08 (m, 1H), 1.95 (dt, *J* = 3.94, 14.17 Hz, 1H), 1.82 - 1.89 (m, 1H), 1.76 - 1.81 (m, 1H), 1.70 - 1.74 (m, 1H), 1.69 (s, 3H), 1.65 (s, 3H), 1.60 - 1.64 (m, 1H), 1.55 - 1.60 (m, 1H), 1.51 (d, *J* = 7.89 Hz, 1H), 1.47 (s, 3H), 1.40 - 1.44 (m, 1H), 1.38 (d, *J* = 16.32 Hz, 1H), 1.34 (d, *J* = 4.58 Hz, 1H), 1.28 - 1.32 (m, 1H), 1.21 (s, 3H), 1.12 (s, 3H), 1.04 (s, 3H), 0.84 - 0.89 (m, 1H), 0.61 (s, 3H) ppm; ^{13}C NMR (151 MHz, CHLOROFORM-d) δ 183.9, 154.4, 146.5, 144.4, 143.5, 122.4, 117.4, 117.4, 114.2, 103.0, 80.8, 44.2, 44.1, 40.2, 38.1, 37.6, 36.5, 36.3, 36.0, 35.8, 34.5, 32.7, 31.5, 30.5, 30.5, 30.1, 29.5, 28.7, 26.1, 26.0, 22.1, 18.8, 11.1 ppm; HRMS (ESI): *m/z* calcd for $C_{33}H_{44}NO_6^-$ $[M]^-$: 550.3174; found: 550.3174.

5.1.20. (6β,9β,13α,14β,20α)-2,3-[(1-methylethylidene)bis(oxy)]-9,13-dimethyl-6-(aminomethyl)-24,25, 26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**5b**) **CAS: 2095710-95-3**

Compound **5b** was obtained according to procedure ii in **Scheme 2**. To a solution of **5a** (300 mg, 0.54 mmol) in EtOH (10 mL) was added Pd/C (54 mg) followed by $H_2NNH_2 \cdot H_2O$ (270 mg, 5.4 mmol). The reaction was stirred at 75 □ overnight. The solution was filtered, and the filtrate was concentrated *in vacuo*. Compound was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH = 15:1). White solid, 102.1 mg, yield: 36%. Mp: 209.2-211.7 □. $[\alpha]_D^{24}$ = -83.3 (c 0.1, DCM). ^{1}H NMR (600 MHz, DMSO-d_6) δ 6.68 (br. s., 1H), 5.91 (d, *J* = 5.87 Hz, 1H), 3.45 (br. s., 2H), 2.85 (d, *J* = 11.55 Hz, 1H), 2.34 (br. s., 1H), 2.32 (br. s., 1H), 2.10 (s, 3H), 2.04 - 2.09 (m, 1H), 2.02 (d, *J* = 9.54 Hz, 1H), 1.94 - 2.00 (m, 1H), 1.74 - 1.86 (m, 1H), 1.63 - 1.68 (m, 1H), 1.62 (s, 3H), 1.59 (br. s., 3H), 1.56 (br. s., 1H), 1.49 (d, *J* = 7.15 Hz, 1H), 1.43 - 1.47 (m, 1H), 1.39 - 1.43 (m, 1H), 1.38 (s, 3H), 1.29 - 1.36 (m, 1H), 1.25 - 1.29 (m, 1H), 1.22 - 1.25 (m, 1H), 1.20 (s, 3H), 1.09 (s, 3H), 1.06 (s, 3H), 0.86 (d, *J* = 7.89 Hz, 1H), 0.63 (s, 3H) ppm; ^{13}C NMR (151 MHz, METHANOL-d_4) δ 185.8, 155.7, 147.8, 145.8, 144.5, 131.0, 125.5, 119.0, 118.5, 115.3, 104.0, 47.5, 46.3, 45.5, 42.4, 39.5, 39.4, 38.4, 37.5, 37.3, 37.0, 36.5, 32.3, 32.3, 32.0, 31.9, 31.6, 30.3, 26.5, 26.3, 22.5, 19.3, 11.3; HRMS (ESI): *m/z* calcd for $C_{33}H_{48}NO_4^+$ $[M+H]^+$: 522.3578; found: 522.3582.

5.1.21. (6β,9β,13α,14β,20α)-2,3-Hydroxy-9,13-dimethyl-6-(aminomethyl)-24,25,26--trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**5c**)

Compound **5c** was obtained according to procedure iii in **Scheme 2**. To a solution of **5b** (70 mg, 0.14 mmol) in CH_3CN/Water mixed solvent (3.3 mL, 10:1 v/v) was added $In(OTf)_3$ (78 mg, 0.14 mmol). The reaction was stirred under microwave conditions at 120 □ for 3 h. After quenched with water (5 mL), the mixture was extracted with ethyl acetate (3 * 10 mL), and then washed with 5 mL brine. The organic layers were combined and dried with anhydrous sodium sulfate. Compound was purified by flash column chromatography on silica gel (CH_2Cl_2/MeOH = 10:1). Pale yellow solid, 49.7 mg, yield: 77%. Mp: 229.1-233.4 □. $[\alpha]_D^{24}$ = -66.0 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.90 (br. s., 1H), 9.03 (br. s, 1H), 8.06 (br. s., 1H), 7.68 (br. s 2H), 6.66 (s, 1H), 5.87 (d, *J* = 6.24 Hz, 1H), 3.59 (ddd, *J* = 3.58, 5.91, 11.23 Hz, 1H), 2.95 (dd, *J* = 3.39, 12.20 Hz, 1H), 2.40 (t, *J* = 11.83 Hz, 1H), 2.33 (d, *J* = 15.59 Hz, 1H), 2.10 (s, 3H), 2.03 - 2.07 (m, 1H), 2.02 (d, *J* = 3.85 Hz, 1H), 1.94 - 2.00 (m, 1H), 1.77 - 1.86 (m, 1H), 1.71 (dd, *J* = 5.78, 12.20 Hz, 1H), 1.64 - 1.68 (m, 1H), 1.63 (d, *J* = 5.32 Hz, 1H), 1.60 - 1.62 (m, 1H), 1.55 - 1.59 (m, 1H), 1.50 (d, *J* = 8.07 Hz, 1H), 1.44 - 1.49 (m, 1H), 1.40 - 1.44 (m, 1H), 1.39 (s, 3H), 1.29 (dt, *J* = 4.58, 13.66 Hz, 1H), 1.23 (s, 3H), 1.11 (s, 3H), 1.07 (s, 3H), 0.85 - 0.90 (m, 1H), 0.62 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 179.5, 152.5, 144.1, 141.4, 140.3, 122.4, 120.2, 118.3, 108.9, 45.3, 43.9,

43.8, 39.4, 37.3, 36.8, 36.6, 36.4, 35.3, 35.1, 34.5, 32.4, 31.4, 30.2, 30.0, 29.8, 29.4, 27.8, 21.4, 18.0, 11.5 ppm; HRMS (ESI): *m/z* calcd for $C_{30}H_{44}NO_4^+$ $[M+H]^+$: 482.3265; found: 482.3251.

5.1.22. (9β,13α,14β,20α)-2,3-[(1-methylethylidene)bis(oxy)]-9,13-dimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**6a**) **CAS: 2095710-88-4**

Compound **6a** was obtained according to procedure i in **Scheme 3**. To a solution of **3a** (150 mg, 0.33 mmol) in DCM (2 mL) was added $(MeO)_2CMe_2$ (407 μL, 345.1 mg, 3.3 mmol) followed by *p*-TsOH·H_2O (6.3 mg, 0.033 mmol). The reaction was stirred at room temperature overnight. The solution was diluted with DCM (40 mL), washed with saturated sodium bicarbonate (2 * 20 mL) and brine (50 mL). After that, the organic phase was dried with anhydrous sodium sulfate and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:10). White solid, 93.6 mg, yield: 57%. Mp: 164.2-166.4 □. $[\alpha]_D^{24}$ = -20.6 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 11.98 (br. s., 1H), 6.63 (s, 1H), 5.72 (dd, *J* = 1.47, 6.79 Hz, 1H), 3.21 (dd, *J* = 6.42, 20.36 Hz, 1H), 2.94 (d, *J* = 19.62 Hz, 1H), 2.34 (d, *J* = 15.59 Hz, 1H), 2.05 - 2.08 (m, 1H), 2.02 - 2.05 (m, 1H), 2.02 (s, 3H), 1.95 - 2.00 (m, 1H), 1.80 - 1.85 (m, 1H), 1.75 - 1.80 (m, 1H), 1.63 - 1.69 (m, 1H), 1.60 (s, 3H), 1.59 (s, 3H), 1.58 (d, *J* = 8.25 Hz, 1H), 1.52 - 1.57 (m, 1H), 1.46 - 1.51 (m, 3H), 1.36 - 1.42 (m, 1H), 1.25 - 1.32 (m, 1H), 1.24 (s, 3H), 1.18 (s, 3H), 1.10 (s, 3H), 1.04 (s, 3H), 0.87 (d, *J* = 12.65 Hz, 1H), 0.67 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 179.4, 149.0, 144.8, 142.9, 141.2, 124.8, 117.1, 116.5, 113.9, 101.6, 43.7, 43.2, 39.1, 37.0, 36.7, 36.5, 34.4, 34.0, 33.7, 32.4, 31.3, 30.1, 30.0, 29.7, 29.4, 28.4, 26.9, 25.7 (2 C), 22.6, 18.0, 11.4 ppm; HRMS (ESI): *m/z* calcd for $C_{32}H_{43}O_4^-$ $[M]^-$: 491.3167; found: 491.3172.

5.1.23. (9β,13α,14β,20α)-2,3-[(1-methylethylidene)bis(oxy)]-9,13-dimethyl-6-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**6b**) **CAS: 2095710-97-5**

Compound **6b** was obtained according to procedure ii in **Scheme 3**. To a solution of **6a** (80 mg, 0.16 mmol) in DCM (2 mL) was added 3, 5-dimethylpyrazole (30.7 mg, 0.32 mmol) followed by PDC (60.2 mg, 0.16 mmol). The reaction was stirred at room temperature overnight. The solution was filtered through celite and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:10). White solid, 39.7 mg, yield: 48%. Mp: 223.1-225.8 □. $[\alpha]_D^{24}$ = -77.4 (c 0.1, MeOH). ^{1}H NMR (600 MHz, CHLOROFORM-d) δ 6.71 (s, 1H), 6.24 (s, 1H), 2.55 (s, 3H), 2.38 - 2.42 (m, 1H), 2.15 - 2.19 (m, 1H), 2.11 - 2.15 (m, 1H), 1.96 - 2.05 (m, 1H), 1.88 - 1.94 (m, 1H), 1.83 - 1.87 (m, 1H), 1.77 - 1.83 (m, 1H), 1.70 (s, 3H), 1.67 (s, 3H), 1.65 (br. s., 1H), 1.60 - 1.64 (m, 1H), 1.52 - 1.60 (m, 3H), 1.51 (s, 3H), 1.45 (d, *J* = 14.86 Hz, 1H), 1.34 (dt, *J* = 4.13, 14.26 Hz, 1H), 1.26 (s, 3H), 1.14 (s, 3H), 1.08 (s, 3H), 0.90 - 0.94 (m, 1H), 0.64 (s, 3H) ppm; ^{13}C NMR (151 MHz, CHLOROFORM-d) δ 187.5, 183.5, 171.8, 153.3, 150.0, 145.2, 125.6, 123.1, 119.6, 118.2, 102.3, 44.7,

44.2, 40.8, 40.2, 39.0, 37.2, 36.3, 34.7, 34.1, 32.6, 31.6, 30.7, 30.4, 29.8, 29.5, 28.6, 26.1, 26.1, 20.7, 18.7, 14.3 ppm; HRMS (ESI): *m/z* calcd for $C_{32}H_{41}O_5^-$ $[M]^-$: 505.2959; found: 505.2959.

5.1.24. (9β,13α,14β,20α)-2,3-Dihydroxy-9,13-dimethyl-6-oxo-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**6c**) **CAS: 167882-66-8**

Compound **6c** was obtained according to procedure iii in **Scheme 3**. To a solution of **6b** (70 mg, 0.14 mmol) in CH_3CN/Water mixed solvent (3.3 mL, 10:1 v/v) was added $In(OTf)_3$ (15.6 mg, 0.028 mmol). The reaction was stirred under microwave conditions at 120 □ for 1 h. The mixture was quenched with water (5 mL), and extracted with ethyl acetate (3 * 10 mL). The organic layers were combined and dried with anhydrous sodium sulfate. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane/Acetic acid = 1:2:0.5%). Pale yellow solid, 23.8 mg, yield: 37%. Mp: >300 □. $[\alpha]_D^{24}$ = -113.4 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.11 (br. s., 1H), 10.11 (s, 1H), 8.38 (s, 1H), 6.83 (s, 1H), 6.02 (s, 1H), 2.46 (s, 3H), 2.33 (d, *J* = 15.59 Hz, 1H), 2.19 (d, *J* = 13.39 Hz, 1H), 2.04 (d, *J* = 13.76 Hz, 1H), 1.93 - 2.01 (m, 1H), 1.80 - 1.89 (m, 1H), 1.73 - 1.79 (m, 1H), 1.71 (br. s., 1H), 1.69 (d, *J* = 3.48 Hz, 1H), 1.63 (dd, *J* = 8.07, 15.59 Hz, 1H), 1.56 - 1.60 (m, 1H), 1.55 (br. s., 1H), 1.48 - 1.52 (m, 1H), 1.45 (s, 3H), 1.41 - 1.44 (m, *J* = 5.00 Hz, 1H), 1.27 - 1.33 (m, 1H), 1.25 (s, 3H), 1.11 (s, 3H), 1.07 (s, 3H), 0.88 (d, *J* = 13.75 Hz, 1H), 0.65 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 186.1, 179.5, 170.4, 149.4, 149.2, 141.9, 127.7, 125.9, 125.4, 124.8, 121.1, 108.5, 44.0, 43.7, 39.4, 38.5, 37.5, 36.1, 34.5, 33.7, 32.3, 31.4, 30.1, 29.4, 29.3, 28.1, 20.7, 18.1, 13.7 ppm; HRMS (ESI): *m/z* calcd for $C_{29}H_{37}O_5^-$ $[M]^-$: 465.2646; found: 465.2646.

5.1.25. (9β,13α,14β,20α)-2,3-Dihydroxy-9,13-dimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid methyl ester (**7a**) **CAS: 31654-72-5**

Compound **7a** was obtained as white solid according to procedure vii in **Scheme 1**. 50 mg (0.108 mmol) **1b** was dissolved in 2 mL CD_3OD and stirred in a 25-mL flask, and then 44 mg (1.1 mmol, 10 equiv) $NaBH_4$ was added. The mixture was kept at room temperature for 30 min. 0.1 mol/L HCl (3 mL) was added to quench the reaction, and subsequently the aqueous phase was separated and extracted with DCM (15 mL) three times. The organic layers were combined and dried with anhydrous sodium sulfate. The residue was purified by removing the solvent under vacuum conditions. White solid, 50.1 mg, yield: 99.8%. Mp: 206.8-208.9 □. $[\alpha]_D^{24}$ = -21.3 (c 0.25, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.78 (s, 1H), 7.82 (s, 1H), 6.60 (s, 1H), 5.72 (dd, *J* = 1.47, 6.79 Hz, 1H), 3.47 (s, 3H), 3.18 (dd, *J* = 6.24, 20.54 Hz, 1H), 2.91 (dd, *J* = 1.28, 20.36 Hz, 1H), 2.33 (d, *J* = 15.77 Hz, 1H), 2.06 (d, *J* = 13.94 Hz, 1H), 2.01 (s, 3H), 1.98 (d, *J* = 3.12 Hz, 1H), 1.93 - 1.97 (m, 1H), 1.84 (dt, *J* = 5.78, 13.07 Hz, 1H), 1.75 - 1.82 (m, 1H), 1.65 (dd, *J* = 8.07, 15.77 Hz, 1H), 1.59 (d, *J* = 3.67 Hz, 1H), 1.54 - 1.58 (m, 1H), 1.48 - 1.52 (m, 2H), 1.44 -

1.48 (m, 1H), 1.39 (dd, *J* = 3.48, 13.02 Hz, 1H), 1.31 - 1.37 (m, 1H), 1.24 (s, 3H), 1.17 (s, 3H), 1.12 (s, 3H), 1.06 (s, 3H), 0.88 (d, *J* = 13.57 Hz, 1H), 0.49 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 178.0, 148.9, 143.1, 140.6, 139.3, 123.0, 120.1, 117.7, 108.3, 51.4, 43.8, 43.2, 39.8, 37.1, 36.5, 36.0, 34.3, 34.2, 34.1, 32.3, 31.3, 30.2, 30.1, 29.9, 29.4, 28.4, 27.3, 22.5, 17.7, 11.5 ppm; HRMS (ESI): *m/z* calcd for $C_{30}H_{42}NaO_4^+$ $[M+Na]^+$: 489.2975, found: 489.2975.

5.1.26. (9β,13α,14β,20α)-2-Methoxy-3-hydroxy-9,13-dimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**7b**)

Compound **7b** was obtained according to steps xvi in **Scheme 1**. 20 mg (0.04 mmol) **7e** was dissolved in 3 mL mixed solvent of Dioxane/Water (1:1 v/v), and KOH solid (50 mg, 0.8 mmol) was added. The mixture was heated to 100 □ and stirred for 4 h. Then the mixture was cooled down to room temperature and acidized by 1 mol/L HCl to pH = 2.0, and extracted with ethyl acetate (3 * 10 mL). The organic layers were combined and dried with anhydrous sodium sulfate. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:10). White solid, 19.2 mg, yield: 99%. Mp: 225.8-227.8 □. $[\alpha]_D^{24}$ = -32.7 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.04 (br. s., 1H), 8.11 (s, 1H), 6.72 (s, 1H), 5.72 (dd, *J* = 1.83, 6.05 Hz, 1H), 3.76 (s, 3H), 3.22 (dd, *J* = 5.87, 20.72 Hz, 1H), 2.95 (dd, *J* = 1.10, 21.46 Hz, 1H), 2.34 (d, *J* = 15.59 Hz, 1H), 2.13 - 2.17 (m, 1H), 2.03 - 2.07 (m, 1H), 2.02 (s, 3H), 1.95 - 2.00 (m, 1H), 1.84 - 1.91 (m, 1H), 1.76 - 1.83 (m, 1H), 1.65 - 1.70 (m, 1H), 1.60 - 1.63 (m, 1H), 1.55 - 1.60 (m, 1H), 1.46 - 1.53 (m, 3H), 1.39 (dd, *J* = 2.66, 13.11 Hz, 1H), 1.28 - 1.33 (m, 1H), 1.26 (s, 3H), 1.19 (s, 3H), 1.11 (s, 3H), 1.05 (s, 3H), 0.83 - 0.89 (m, 1H), 0.68 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 179.5, 149.0, 145.8, 141.5, 139.1, 124.7, 119.9, 117.4, 105.1, 55.9, 43.9, 43.3, 39.5, 37.2, 36.6, 36.5, 34.5, 34.1, 34.0, 32.5, 31.4, 30.2, 30.1, 29.8, 29.5, 28.5, 27.3, 22.6, 18.1, 11.4 ppm; HRMS (ESI): *m/z* calcd for $C_{30}H_{42}NaO_4^+$ $[M+Na]^+$: 489.2975, found: 489.2979.

5.1.27. (9β,13α,14β,20α)-2-Hydroxy-3-methoxy-9,13-dimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**7c**)

Compound **7c** was obtained by according to steps xvi in **Scheme 1**. 100 mg (0.2 mmol) **7f** was dissolved in 3 mL mixed solvent of Dioxane/Water (1:1 v/v), and KOH solid (350 mg, 6 mmol) was added. The mixture was heated to 100 □ and stirred for 4 h. Then the mixture was cooled down to room temperature and acidized by 1 mol/L HCl to pH = 2.0, and extracted with ethyl acetate (3 * 10 mL). The organic layers were combined and dried with anhydrous sodium sulfate. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:10). White solid, 95.2 mg, yield: 98%. Mp: 157.9-162.6 □. $[\alpha]_D^{24}$ = -18.0 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.06 (br. s., 1H), 8.79 (s, 1H), 6.68 (s, 1H), 5.73 (dd, *J* = 1.83, 6.24 Hz, 1H), 3.62 (s, 3H), 3.19 (dd, *J* = 6.24, 19.07 Hz, 1H), 2.93

(dd, *J* = 1.47, 19.44 Hz, 1H), 2.34 (d, *J* = 15.41 Hz, 1H), 2.06 (s, 3H), 2.03 (d, *J* = 15.41 Hz, 1H), 2.00 (dd, *J* = 3.67, 9.72 Hz, 1H), 1.94 - 1.98 (m, 1H), 1.87 (dt, *J* = 5.10, 13.90 Hz, 1H), 1.77 (dt, *J* = 5.90, 14.30 Hz, 1H), 1.64 - 1.69 (m, 1H), 1.59 - 1.63 (m, 1H), 1.54 - 1.59 (m, 1H), 1.50 - 1.53 (m, 1H), 1.49 (d, *J* = 7.89 Hz, 1H), 1.44 - 1.48 (m, *J* = 5.00 Hz, 1H), 1.36 - 1.41 (m, 1H), 1.29 (dt, *J* = 4.59, 13.75 Hz, 1H), 1.23 (s, 3H), 1.18 (s, 3H), 1.11 (s, 3H), 1.05 (s, 3H), 0.82 - 0.87 (m, 1H), 0.66 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 179.5, 149.1, 147.9, 144.3, 143.3, 126.8, 123.3, 117.7, 109.5, 59.5, 43.8, 43.3, 39.5, 37.1, 36.5, 36.4, 34.4, 33.9, 33.8, 32.4, 31.4, 30.2, 30.1, 29.7, 29.4, 28.5, 27.2, 22.8, 18.1, 11.7 ppm; HRMS (ESI): *m/z* calcd for $C_{30}H_{42}NaO_4^+$ $[M+Na]^+$: 489.2975, found: 489.2974.

5.1.28. (9β,13α,14β,20α)-2,3-Dimethoxy-9,13-dimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid (**7d**) **CAS: 106407-53-8**

Compound **7d** was obtained according to step xvi in **Scheme 1**. 10 mg (0.02 mmol) **7g** was dissolved in 3 mL mixed solvent of Dioxane/Water (1:1 v/v), and KOH solid (33.6 mg, 0.6 mmol) was added. The mixture was heated to 100 □ and stirred for 4 h. Then the mixture was cooled down to room temperature and acidized by 1 mol/L HCl to pH = 2.0, and extracted with ethyl acetate (3 * 10 mL). The organic layers were combined and dried with anhydrous sodium sulfate. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:10). White solid, 9.7 mg, yield: 99%. Mp: 228.7-231.3 □. $[\alpha]_D^{24}$ = -24.0 (c 0.1, MeCN). ^{1}H NMR (600 MHz, DMSO-d_6) δ 12.03 (br. s., 1H), 6.80 (s, 1H), 5.74 (dd, *J* = 1.93, 6.14 Hz, 1H), 3.77 (s, 3H), 3.63 (s, 3H), 3.23 (dd, *J* = 6.24, 21.09 Hz, 1H), 2.97 (dd, *J* = 1.28, 21.46 Hz, 1H), 2.35 (d, *J* = 15.59 Hz, 1H), 2.15 - 2.21 (m, 1H), 2.08 (s, 3H), 2.04 (d, *J* = 13.94 Hz, 1H), 1.95 - 2.01 (m, 1H), 1.89 (ddd, *J* = 5.14, 14.12, 28.24 Hz, 1H), 1.80 (ddd, *J* = 6.24, 13.57, 27.33 Hz, 1H), 1.66 - 1.71 (m, 1H), 1.57 - 1.64 (m, 2H), 1.51 (d, *J* = 7.70 Hz, 2H), 1.44 - 1.49 (m, 1H), 1.40 (dd, *J* = 2.57, 12.65 Hz, 1H), 1.29 - 1.33 (m, 1H), 1.28 (s, 3H), 1.20 (s, 3H), 1.11 (s, 3H), 1.06 (s, 3H), 0.86 (d, *J* = 12.84 Hz, 1H), 0.68 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 179.5, 150.6, 148.8, 144.3, 144.1, 127.0, 124.8, 117.4, 106.2, 59.7, 55.5, 43.8, 43.3, 39.4, 37.1, 36.8, 36.5, 34.4, 33.9, 33.8, 32.5, 31.4, 30.1, 30.0, 29.7, 29.4, 28.4, 27.3, 22.7, 18.1, 11.6 ppm. HRMS (ESI): m/z calcd for $C_{31}H_{43}O_4^-$ $[M]^-$: 479.3167, found: 479.3167.

5.1.29. (9β,13α,14β,20α)-2-Methoxy-3-hydroxy-9,13-dimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid methyl ester (**7e**)

Compound **7e** was obtained by according to step xiv in **Scheme 1**. 170 mg (0.365 mmol) **7a** was dissolved in 4 mL acetone. K_2CO_3 (200 mg, 1.3 mmol) and dimethyl sulfate (100 μL, 1.02 mmol) were added, The mixture was heated to 70 □ and stirred for 12 h. The mixture was quenched with 1 mol/L HCl (10 mL), and extracted with ethyl acetate (3 * 25 mL). The organic layers were combined and dried with

anhydrous sodium sulfate. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:10). White solid, 37.5 mg, yield: 24.3%. Mp: 206.1-208.8 □. $[\alpha]_D^{24}$ = -34.0 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.10 (s, 1H), 6.72 (s, 1H), 5.72 (dd, *J* = 1.28, 6.42 Hz, 1H), 3.77 (s, 3H), 3.47 (s, 3H), 3.21 (dd, *J* = 6.24, 20.72 Hz, 1H), 2.95 (dd, *J* = 1.40, 20.17 Hz, 1H), 2.33 (d, *J* = 15.77 Hz, 1H), 2.12 - 2.18 (m, 1H), 2.06 (d, *J* = 13.57 Hz, 1H), 2.02 (s, 3H), 1.97 (dt, *J* = 4.03, 13.75 Hz, 1H), 1.84 - 1.90 (m, 1H), 1.80 (dt, *J* = 7.24, 13.43 Hz, 1H), 1.65 (dd, *J* = 8.16, 15.68 Hz, 1H), 1.55 - 1.61 (m, 2H), 1.47 - 1.52 (m, 3H), 1.37 - 1.42 (m, 1H), 1.32 - 1.37 (m, 1H), 1.27 (s, 3H), 1.18 (s, 3H), 1.12 (s, 3H), 1.06 (s, 3H), 0.87 - 0.92 (m, 1H), 0.51 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 178.0, 148.7, 145.8, 141.5, 139.0, 124.6, 119.8, 117.5, 105.3, 55.8, 51.4, 43.8, 43.2, 39.9, 37.1, 36.5, 36.4, 34.4, 34.2, 34.0, 32.4, 31.4, 30.3, 30.1, 29.9, 29.4, 28.4, 27.3, 22.4, 17.7, 11.4 ppm; HRMS (ESI): *m/z* calcd for $C_{31}H_{43}O_4^-$ $[M]^-$: 479.3167, found: 479.3161.

5.1.30. (9β,13α,14β,20α)-2-Hydroxy-3-methoxy-9,13-dimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid methyl ester (**7f**) **CAS: 125543-17-1**

Compound **7f** was obtained according to steps vii in **Scheme 1**. To a solution of **1b** (50 mg, 0.108 mmol), K_2CO_3 (60 mg, 0.43 mmol) and TBAB (3.2 mg, 0.011 mmol) dissolved in 2 mL THF, Dioxane (400 μL, 10 mol%) and CH_3I (150 μL, 1.08 mmol) were added, The mixture was heated to 70 □ and stirred for 12 h. The mixture was quenched with 1 mol/L HCl (10 mL), and extracted with ethyl acetate (3 * 25 mL). The organic layers were combined and dried with anhydrous sodium sulfate. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:8). Yellow solid, yield: 47.9%. And quickly the yellow product - **2a** (200 mg, 0.418 mmol) was treated with $NaBH_4$ (10 equiv, 170 mg, 4.2 mmol) in CD_4O (2 mL) according to procedure vii. The product was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:10). Light yellow solid, 158 mg, yield: 78%. Mp: 126.6-128.5 □. $[\alpha]_D^{24}$ = -16.0 (c 0.1, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.78 (s, 1H), 6.67 (s, 1H), 5.73 (dd, *J* = 1.83, 7.15 Hz, 1H), 3.62 (s, 3H), 3.47 (s, 3H), 3.18 (dd, *J* = 6.24, 22.92 Hz, 1H), 2.93 (ddd, *J* = 1.28, 19.62, 20.36 Hz, 1H), 2.33 (d, *J* = 15.77 Hz, 1H), 2.06 - 2.11 (m, 1H), 2.06 (s, 3H), 2.02 (d, *J* = 2.93 Hz, 1H), 1.97 - 2.01 (m, 1H), 1.92 - 1.97 (m, 1H), 1.81 - 1.86 (m, 1H), 1.78 (dd, *J* = 6.14, 13.85 Hz, 1H), 1.65 (dd, *J* = 8.16, 15.86 Hz, 1H), 1.58 - 1.61 (m, 1H), 1.50 - 1.53 (m, 1H), 1.46 - 1.50 (m, 1H), 1.42 - 1.46 (m, 1H), 1.37 - 1.41 (m, 1H), 1.32 - 1.36 (m, 1H), 1.24 (s, 3H), 1.17 (s, 3H), 1.12 (s, 3H), 1.05 (s, 3H), 0.88 (d, *J* = 11.92 Hz, 1H), 0.49 (s, 3H) ppm; ^{13}C NMR (151 MHz, DMSO-d_6) δ 178.0, 148.7, 147.9, 144.2, 143.3, 126.8, 123.1, 117.7, 109.7, 59.5, 51.4, 43.8, 43.3, 39.8, 37.0, 36.5, 36.3, 34.3, 34.0, 34.0, 32.3, 31.4, 30.2, 30.1, 29.9, 29.5, 28.4, 27.2, 22.6, 17.7, 11.7 ppm; HRMS (ESI): *m/z* calcd for $C_{31}H_{45}O_4^+$ $[M+H]^+$: 481.3312, found: 481.3316.

5.1.31. (9β,13α,14β,20α)-2,3-Dimethoxy-9,13-dimethyl-24,25,26-trinoroleana-1(10),3,5,7-tetraen-29-oic acid methyl ester (**7g**) **CAS: 1906-19-0**

Compound **7g** was obtained according to step xv in **Scheme 1**. 170 mg (0.365 mmol) **7a** was dissolved in 4 mL acetone. K_2CO_3 (200 mg, 1.3 mmol) and CH_3I (100 μL, 1.02 mmol) were added, The mixture was heated to 70 □ and stirred for 12 h. The mixture was quenched with 1 mol/L HCl (10 mL), and extracted with ethyl acetate (3 * 25 mL). The organic layers were combined and dried with anhydrous sodium sulfate. The residue was purified by flash column chromatography on silica gel (Ethyl acetate/Hexane = 1:10). White solid, 126.6 mg, yield: 66%. Mp: 208.1-209.1 □. $[\alpha]_D^{24}$ = -19.3 (c 0.1, DCM). ^{1}H NMR (600 MHz, CHLOROFORM-d) δ 6.78 (s, 1H), 5.77 (d, *J* = 4.58 Hz, 1H), 3.86 (s, 3H), 3.77 (s, 3H), 3.54 (s, 3H), 3.28 (dd, *J* = 5.87, 20.00 Hz, 1H), 3.02 (d, *J* = 19.99 Hz, 1H), 2.44 (d, *J* = 15.59 Hz, 1H), 2.18 - 2.23 (m, 1H), 2.17 (s, 3H), 2.07 - 2.13 (m, 2H), 2.01 - 2.07 (m, 1H), 1.85 (dt, *J* = 6.33, 13.71 Hz, 1H), 1.73 (d, *J* = 11.37 Hz, 1H), 1.63 - 1.69 (m, 2H), 1.57 - 1.63 (m, 1H), 1.51 - 1.57 (m, 2H), 1.44 (dd, *J* = 2.84, 14.40 Hz, 1H), 1.40 (dd, *J* = 4.22, 14.12 Hz, 1H), 1.34 (s, 3H), 1.22 (s, 3H), 1.17 (s, 3H), 1.08 (s, 3H), 0.94 (d, *J* = 13.75 Hz, 1H), 0.61 (s, 3H) ppm; ^{13}C NMR (151 MHz, CHLOROFORM-d) δ 179.0, 150.9, 149.1, 144.8, 144.6, 127.8, 125.5, 117.6, 106.3, 60.3, 55.9, 51.5, 44.3, 43.7, 40.4, 37.5, 37.2, 36.8, 34.8, 34.5, 34.2, 32.9, 31.6, 30.9, 30.5, 30.3, 29.9, 28.8, 27.8, 22.7, 18.3, 11.8 ppm; HRMS (ESI): *m/z* calcd for $C_{32}H_{46}NaO_4^+$ $[M+Na]^+$: 517.3288, found: 517.3281.

5.2 Biological assay

5.2.1 Cell culture

Human hepatocellular carcinoma cell lines HepG2 was maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (FBS).

5.2.2 Plasmids

Plasmids pcmv-myc-Nur77, pFlag-cmv-2-p62, pFlag-cmv-2-TRAF2, pEGFP-C1-Nur77, mCherry-p62 were described previously [12, 26].

5.2.3 Protein Expression and Purification

The human Nur77-LBD (367-598) was cloned as an N-terminal histidine-tagged fusion protein in pET15b expression vector and overproduced in Escherichia coli BL21 DE3 strain. Briefly, cells were harvested and sonicated, and the extract was incubated with the His60 Ni Superflow resin.

5.2.4 Fluorescence Titration

Wild-type Nur77-LBD protein or mutant protein (1 μM in 3 mL phosphate buffer) was measured with Agilent Technologies Cary Eclipse Fluorescence Spectrophotometer, and the fluorescence spectra were obtained from 300 nm to 500 nm. Compounds (from 100 nM to 1 μM with an increment of 100 nM, from 1 μM to 2 μM with an increment of 250 nM, from 2 μM to 5 μM with an increment of 500 nM) were added to the protein. After incubation for 30 s at RT, the incubation buffer was measured with the spectrophotometer. Data were processed and fitted to obtain the binding affinities using Origin 2016.

5.2.5 Western blotting

Cell lysates were boiled in sodium dodecyl sulfate (SDS) sample loading buffer, resolved by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose. The membranes were blocked in 5% milk in Tris-buffered saline and Tween 20 (TBST; 10 mM Tris– HCl [pH 8.0], 150 mM NaCl, and 0.05% Tween 20) for 1 hr at room temperature. After washing twice with TBST, the membranes were incubated with appropriate primary antibodies in TBST for 1 hr and then washed twice, probed with horseradish peroxide-linked anti-immunoglobulin (1:5000 dilution) for 1 hr at room temperature. After three washes with TBST, immunoreactive products were visualized using enhanced chemiluminescence reagents and autoradiography.

5.2.6 CoIP assay

Cells were harvested in lysis buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, and 5 mM ethylenediaminetetraacetic acid, and containing protease inhibitors). Lysate was incubated with 1 μg antibody at 4 □ for 2 hr. Immunocomplexes were then precipitated with 30 μL of protein A/Gsepharose. After an extensive washing with lysis buffer, the beads were boiled in SDS sample loading buffer and assessed by Western blotting (WB).

5.2.7 Confocal Microscopy

HepG2 cells transfected GFP-Nur77 and mCherry-p62 were mounted on glass slides. After fixated by paraformaldehyde, Cells were costained with 4′,6-diamidino-2-phenylindole (DAPI) (1:10000 dilution) to visualize nuclei. The images were taken under a fluorescent microscope (CarlZeiss) or an LSM-510 confocal laser scanning microscope system (CarlZeiss).

5.2.8 Flow Cytometry

Cells induced apoptosis were collected in Binding Buffer. After staining by FITC Annexin V and Propidium Iodide Staining Solution at room temperature, cells were detected by CytoFLEX (Beckman

Coulter). Binding Buffer, FITC Annexin V and Propidium Iodide Staining Solution are all from FITC Annexin V Apoptosis Detected Kit I (BD 556547).

5.2.9 Zebrafish embryo toxicity study

Wild-type (AB strain) zebrafish (*Danio rerio*) were maintained in flow-through tanks with fish water (0.2% Instant Ocean salt in deionized water, pH = 6.9–7.2, conductivity 480–510 μS/cm, and hardness 53.7–71.6 mg/L $CaCO_3$) with a photoperiod of 14/10 h light/dark. Embryos were obtained from spawning adults placed in groups of two males and one female in one spawning box overnight. Embryos were collected within 0.5 h of spawning and rinsed in fish water. 20 zebrafish embryos per condition at 2 hpf were exposed to celastrol or **3a** at the concentrations of 0.5 μM, 1 μM and 1.25 μM and 0.1% DMSO served as control. Zebrafish were selected from each group for visual observation and image acquisition in every 24 h.

5.3 Molecular docking

Schrodinger's (Porland, OR) Glide [28-30], a grid-based docking module of schrodinger suites, was employed to study how celastrol ligand to the Nur77 LBD. The crystal structure of Nur77 LBD in complex with DPDO (PDB ID: 4KZI) was used, the missing residues were filled by the prime module [31, 32], center of grid box is set at C551 and the length of grid is limitted within 15 angstrom. Standard Precision mode is used, ligand sampling in flexsible. Schrodinger's Maestro 11.0.

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Highlights:

- 24 celastrol derivatives targeting Nur77 were designed, synthesized and evaluated for their Nur77-mediated anti-inflammatory properties.
- The reduced celastrol, compound **3a**, having comparable binding affinity and biological effects to celastrol, and was significantly less toxic than celastrol both in vitro and in Zebrafish model.
- **3a** is a potential hit molecule for further optimization for anti-inflammatory application.