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Article

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Synthesis, Evaluation, and Structure-Activity Relationship Study of Lanosterol Derivatives to Reverse Mutant Crystallins Induced Protein Aggregation

Xinglin Yang[1‡], Xiang-Jun Chen[2,3‡], Zimo Yang[1‡], Yi-Bo Xi[2,4], Liguo Wang[1], Yue Wu[1], Yong-Bin Yan[2*], Yu Rao[1*]

1 MOE Key Laboratory of Protein Sciences, School of Pharmaceutical Sciences, MOE Key Laboratory of Bioorganic Phosphorus Chemistry & Chemical Biology, Tsinghua University, Beijing 100084, P.R. China.
2 State Key Laboratory of Membrane Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China
3 Eye Center of the Second Affiliated Hospital, Institutes of Translational Medicine, Zhejiang University School of Medicine, Hangzhou 310058, China.
4 Tsinghua-Peking Joint Center for Life Sciences, Tsinghua University, Beijing 100084, China
‡ These authors contributed equally.

Abstract

We describe here the development of potent synthetic analogues of naturally occurring triterpenoid lanosterol to reverse protein aggregation in cataracts. Lanosterol showed superiority to other scaffolds in terms of efficacy and generality in previous studies. Various modified lanosterol derivatives were synthesized via modification of the side chain, ring A, ring B and ring C. Evaluation of these synthetic analogues draws a clear picture for SAR. In particular, hydroxylation of the 25-position in the side chain profoundly improved the potency, and 2-fluorination further enhanced the biological activity. This work also revealed that synthetic lanosterol analogues could reverse multiple types of mutant crystalline aggregates in cell models with excellent potency and efficacy. Notably, lanosterol analogues have no cytotoxicity but can improve the viability of the

HLE-B3 cell line. Furthermore, representative compound **6** successfully redissolved the aggregated crystallin proteins from the amyloid-like fibrils in a concentration-dependent manner.

Introduction

Cataracts, which are a clouding of the lens in the eyes, lead to degradation of visual acuity and even blindness. According to a recent published systematic analysis, cataracts are the leading cause of blindness worldwide, especially in developing countries[1]. Every year millions of patients undergo cataract surgery, which involves removing the opacified lens and replacing it with an artificial lens implant. While surgical treatment of cataract is effective, the risk of complications, such as posterior capsular opacification and retinal detachment is inevitable[2]. In addition, receiving cataract surgery is limited to those with accessible facilities, doctors, and funds in the developing world where cataract incidence is increasing[3]. Lenses are an avascular tissue, and maturation of lens fiber cells involves coordinated organelle degradation and high soluble crystallin protein expression. These anatomic characteristics contribute to lens transparency. Crystallin proteins, including α, β and γ crystallins, comprise 90% of the proteins in the mature lens fiber cells. Normally, these highly concentrated proteins can remain soluble throughout life, which is helped by α-crystallin, an ATP-independent chaperone. In cataractous lenses, aggregated lens proteins with high molecular weight are found, which are insoluble and account for light scattering[4, 5]. Mutations in crystallin proteins can cause protein misfolding and induce the formation of aggregates, which is typical pathogenesis and clinical characteristic of cataracts[6]. So, if a compound can dissolve various mutant crystallin aggregates, it may be developed as the anti-cataract agent in future. Other factors[7-9], such as oxidative damage, dysregulation of Ca^{2+} concentration and an increased glucose level in the lens, can also lead to different kinds of

cataracts. Based on the etiology of cataracts, different nonsurgical strategies[10-12] were developed, such as the inhibition of aldose reductase and the usage of antioxidants. However, currently, there are no widespread, efficient drugs to prevent or treat cataracts. Because cataracts are viewed as a protein aggregation disease, compounds that can inhibit or even reverse protein aggregation are promising drug candidates for cataract treatment. The screening assays was analysis of compounds' capability of dissolving various mutant crystallin aggregates.

In 2015, it was reported that lanosterol, an amphipathic molecule enriched in the lens, could reverse protein aggregation in cataracts[13]. Shortly afterwards, another study found that 25-hydroxycholesterol (C29) could serve as pharmacological chaperone for a-crystallin to partially restore transparency in cataract models[14]. Remarkably, these two works provide a brand-new strategy for the prevention and treatment of cataracts. However, from a perspective of drug-likeness properties, both lanosterol and 25-hydroxycholesterol have their respective limitations as potential candidates. For example, in terms of the efficacy against α-crystallin, 25-hydroxycholesterol is better than lanosterol. Additionally, it was found that lanosterol could reduce intracellular aggregation by various cataract-causing mutant crystallin proteins, including α, β and γ-crystallin. In contrast, 25-hydroxycholesterol is only effective towards α-crystallin. In addition to its relatively low efficacy (EC50 = 1.4 μM), lanosterol is an endogenous tetracyclic triterpenoid that can be transformed to different steroids with important bioactivities[15]. The uncontrolled transformation in vivo may decrease the half-time in vivo. Meanwhile, 25-hydroxycholesterol is also an endogenous oxysterol. It was reported that 25-hydroxycholesterol is an LXR agonist that will activate SREBP-1C in the liver leading to hepatic steatosis and hypertriglyceridemia[16].

Taken together, these facts imply that lanosterol may represent a suitable compound for further SAR study and we envisioned that an optimization of lanosterol may yield good lead candidates, which can remain active to all three subtypes of crystalline with improved efficacy to address the abovementioned limitations. Herein, we reported that through a comprehensive modification of lanosterol and biological evaluation of diverse synthetic analogues, a few new synthetic lanosterol analogues could reduce intracellular aggregation by various cataract-causing mutant crystalline proteins (α, β and γ) with excellent potency and efficacy. More importantly, the aggregated crystallin proteins from the amyloid-like fibrils can be readily redissolved by representative compound **6**.

Results and discussion

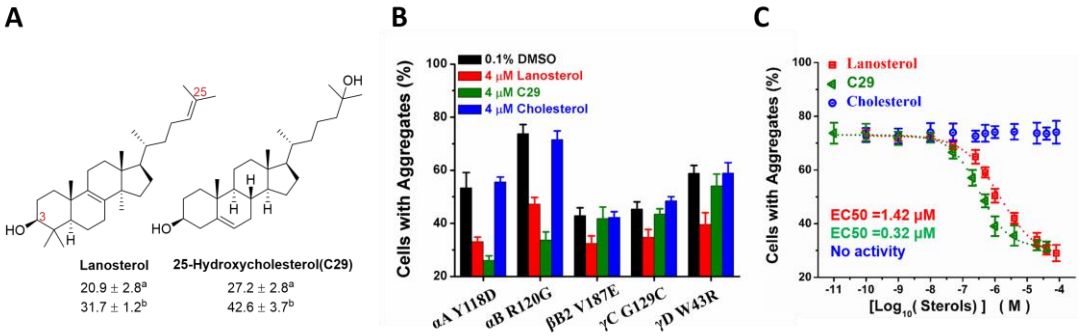


Figure 1. A. Chemical structures of lanosterol and 25-hydroxycholesterol and their efficacy to inhibit α-crystallinR120G induced aggregation in the cell model. [a]Aggregation inhibition (%) by 0.4 μM of compounds. [b]Aggregation inhibition (%) by 4 μM of compounds.. B, C. Activity analysis of lanosterol and C29, lanosterol could reduce intracellular aggregation by various cataract-causing mutant crystallin proteins, while C29 is only effective towards α-crystallin.

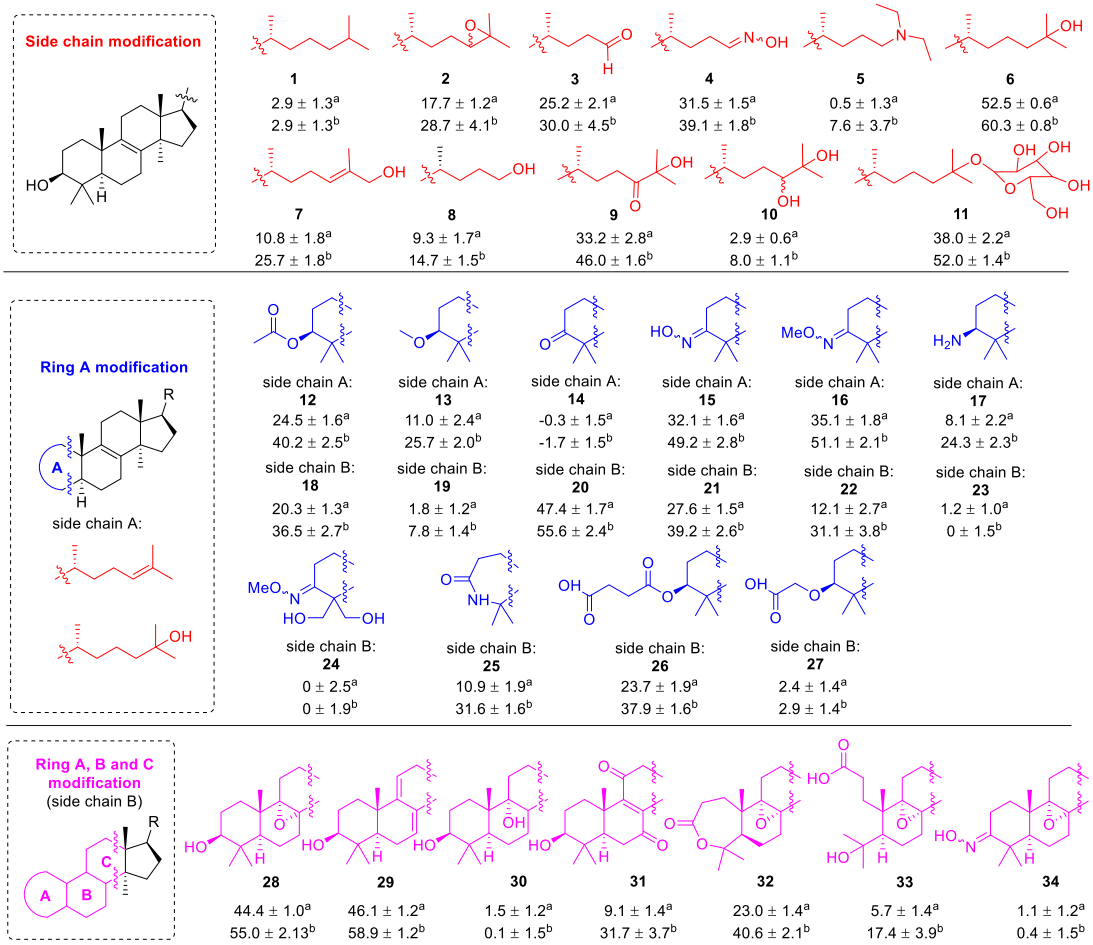


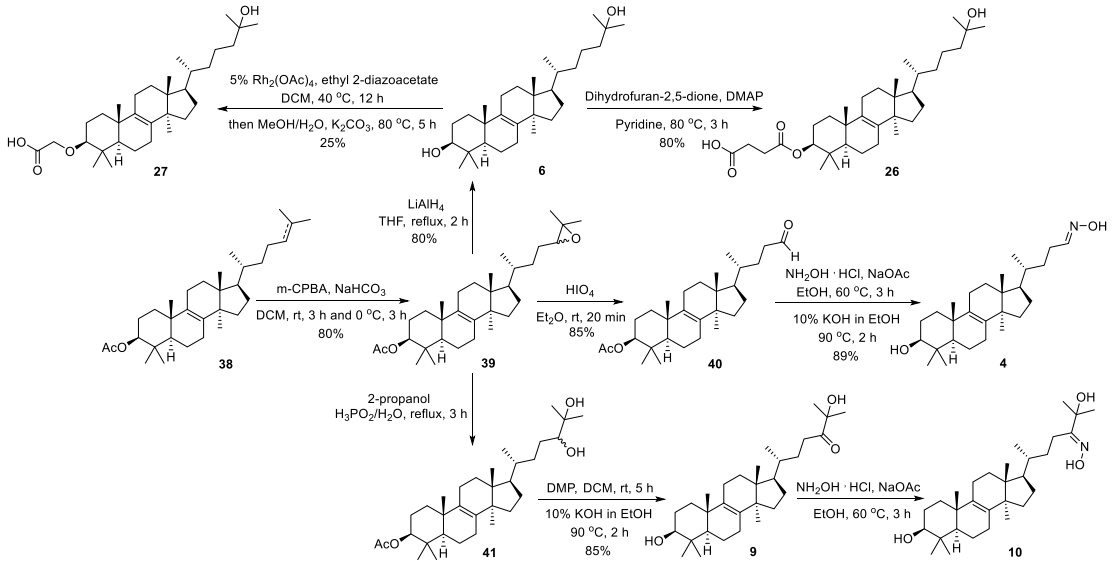
Figure 2. Chemical structure of lanosterol derivatives and their efficacy to inhibit α-crystallinR120G induced aggregation in the cell model. [a]Aggregation inhibition (%) by 0.4 μM of compounds. [b]Aggregation inhibition (%) by 4 μM of compounds.

Structure of lanosterol derivatives

As shown in figure 2, a highly diverse derivative of lanosterol was designed and synthesized, which included three major patterns of modification, namely, 1) side chain modification, 2) ring A modification, and 3) ring B and ring C modification. At the beginning of our investigation, the side chain was systematically modified. Different kinds of functional groups were introduced to the side chain, including alkyl (**1**), epoxide (**2**), aldehyde (**3**), hydroxyl oxime (**4**), amine (**5**), hydroxyl (**6**) and carbonyl (**9**) groups. In addition, glycoside (**11**) with improved aqueous solubility was also synthesized. Evaluation of these substituents, which can serve as both the hydrogen bond acceptor and donor, will offer a quite clear picture of the side chain requirement

for high efficiency. In parallel, the ring A of lanosterol was also modified diversely. 3-OH was acetylated (**12**), methylated (**13**), oxidized (**14**) or converted to corresponding hydroxyl oxime (**15**), methoxy oxime (**16**) and amine (**17**) groups. Due to the high efficacy of 25-hydroxylanosterol (figure 2), this compound was selected for further modification at rings A, B, and C. Same with ring A modification of compound **12**-**17**, the modification of compound 6 afforded compounds **18**-**23**. Ring A of compound **6** also underwent further chemical diversification. Hydroxyl (**24**), lactam (**25**) and carboxylic acid (**26, 27**) moieties were introduced into ring A. Additionally, ring A is further opened via hydroxylation of lactone (**32**), affording compound **33**. The double bond between ring B and ring C can be smoothly transformed to epoxy (**28**), dienes (**29**), and hydroxyl (**30**). Carbonyl group was introduced to position 7 and 11, affording compound **31**. Compounds **1-3, 5-8, 10, 12-18, 20,** and **31** were prepared according to the literature[1]. Synthesis of other derivatives is illustrated in schemes 1-3.

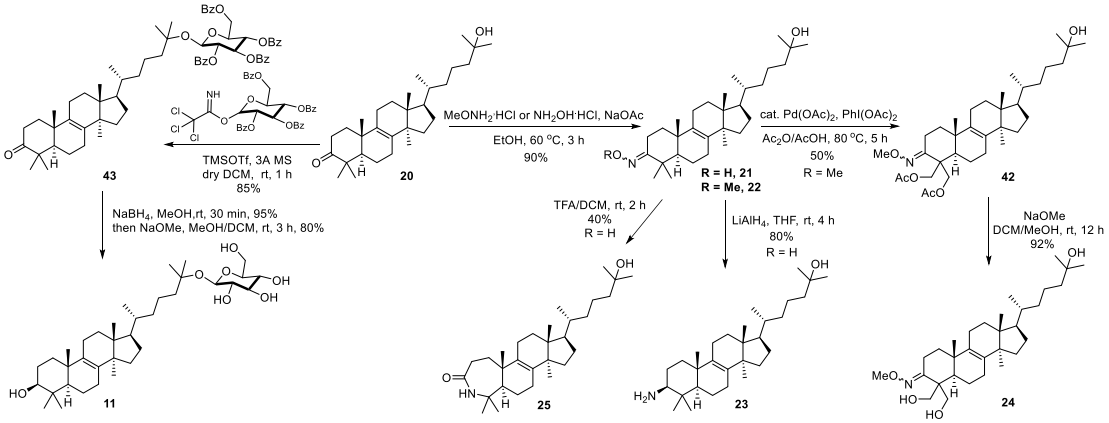
Scheme 1. Preparation of Compounds 4, 6, 9, 10, 26 and 27



The side chain double bond of compound **38** was selectively oxidized to epoxide (**39**) with

m-CPBA as the oxidant under mild conditions. The epoxide analogue (**39**) was further converted to the aldehyde analogue (**40**) when treated with HIO_4 and subsequently reacted with hydroxylamine to give oxime analogue (**4**). Compound **39** can be readily reduced to afford compound **6** using $LiAlH_4$ and hydrolyzed to compound **41** using H_3PO_2, respectively. The Rh-catalyzed O-H insertion reaction using ethyl 2-diazoacetate as a reaction partner and subsequent hydrolysis can transform compound **6** into compound **27**. Compound **6** reacted with succinic anhydride to provide compound **26**. The secondary alcohol in the side chain of compound **41** was selectively oxidized to ketone (**9**) using DMP as an oxidant. Later, through condensation with hydroxylamine hydrochloride, compound **9** was smoothly converted into oxime analogue **10**.

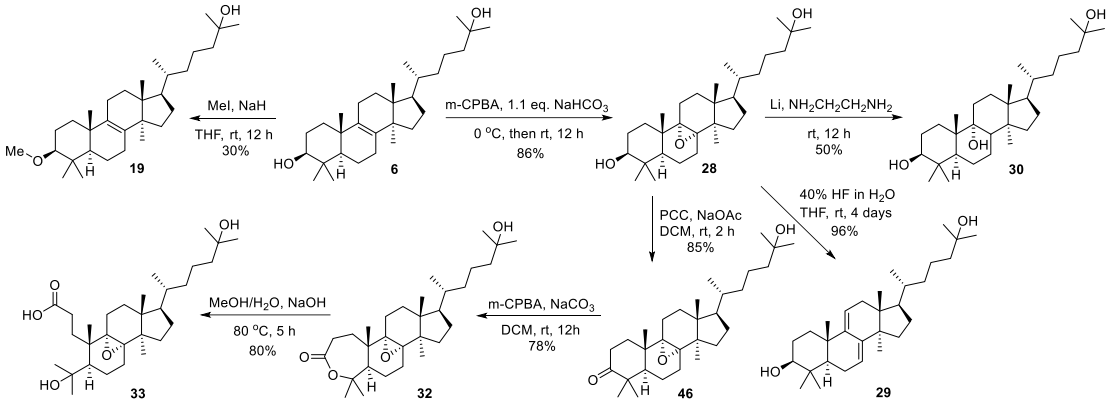
Scheme 2. Preparation of Compounds 11 and 21-25



As demonstrated in scheme 2, oxime analogues **21** and **22** were readily obtained when compound **20** reacted with hydroxylamine and methoxyamine, respectively. Oxime **21** was transformed into the 3-NH_2 analogue **23** and lactam analogue **25** via reduction and Beckman rearrangement. The Pd-catalyzed C-H activation strategy was used to prepare compound **24**. The two angular methyl groups were oxidized using O-methyl oxime as the directing group and $PhI(OAc)_2$ as the oxidant[22]. Subsequent hydrolysis gave the desired product **24**. Benzoyl-protected glycosyl dichloroacetamide[23] was employed as the donor for the glycosylation of compound **20**. Then, the

ketone group of compound **43** was reduced using $NaBH_4$ and the benzoyl protecting group was removed using NaOMe to afford compound **11**.

Scheme 3. Preparation of Compounds 19, 28-30, 32 and 33



As shown in scheme 3, the double bond of compound **6** was oxidized to epoxide **28** with m-CPBA as the oxidant[24]. Diene analogue **29** was obtained by treatment of epoxide (**28**) with 40% HF solution. The epoxide of compound **28** was reduced to a hydroxyl group with lithium as the reductant, affording compound **30**[25]. The 3-OH of compound **28** was oxidized to a carbonyl group (**46**), which was further transformed into a lactone via Baeyer–Villiger oxidation. Subsequently, ring A was opened by hydrolysis of the lactone under basic conditions, affording the ring A-opening derivative **33**.

Biological evaluation of synthetic lanosterol analogues and SAR discussion

We used mutant crystallin cell lines as screening system and the cells were transfected with plasmids containing the mutated crystallin genes. After transfection for 16 h, the mutant crystallins were overexpressed and formed the aggregates (figure S1), then the compounds were added into cell culture medium. After incubated for 6~8 h, we used the fluorescence microscopy to analyze compounds' capability of dissolving various mutant crystallin aggregates[13].The

inhibition of αB R120G crystallin aggregation with 0.4 μM and 4 μM compounds was calculated based on DMSO-treated cells. The results are summarized in figure 1 and 2. It was found that side chain modification greatly changed the activity of lanosterol. 2H-Lanosterol (**1**) showed nearly no activity at a concentration of 4 μM in our assay system. The transformation of the double bond to epoxide (**2**) slightly decreased the activity, which demonstrated that the double bond of the side chain was not essential for good efficacy. The activity of the aldehyde analogue (**3**) shows a similar activity with lanosterol. The oxime derivative (**4**), which can serve as not only a hydrogen bond donor but also a hydrogen bond acceptor, demonstrated increased activity with respect to lanosterol. Interestingly, introduction of the diethylamine group (5), which may serve as a hydrogen bond acceptor decreased the activity greatly. Notably, the introduction of the hydroxyl group (**6**) to the side chain significantly increases the activity, which is consistent with the positive activity of **C29**. 25-Hydroxylanosterol (**6**), with lanosterol skeleton and bearing the same side chain as **C29**, shows excellent activity to various mutant crystallin proteins. While cholesterol shows no activity to mutant crystallin proteins, and **C29** bearing cholesterol skeleton, only shows activity to mutant α-crystallin proteins (Figure 1 and 2). These results imply that the lanosterol skeleton has priority to cholesterol to be modified as possible therapeutic treatments for cataracts. Derivatives with a short or long primary alcohol side chain (**7**, **8**) show a loss of potency. When other functional groups, such as ketone and second hydroxyl, were introduced to the 24-position of 25-hydroxylanosterol, the activity of the corresponding analogues (**9**, **10**) was generally reduced in different degrees. Compounds bearing the glucose motif (**11**) exhibited moderate activity.

As illustrated in figure 2, ring A was also modified, and the corresponding derivatives were tested in the screening system. 3-OH was acetylated and the activity of the corresponding analogue **12**

slightly increased. Methylation (**13**) and oxidation (**14**) of 3-OH in lanosterol decreased the activity. Compounds **15** and **16** bearing hydroxyl oxime and methoxy oxime showed obvious activity improvements. Moderate activity was observed when the hydroxyl group was replaced with an amine group (**17**). Introduction of the oxime group in the 3-position improved the activity compared to that of lanosterol. However, compounds **21** and **22** with modifications of both 25-OH and 3-oxime showed a decreased activity compared to compound **6**. This result implied that there was no synergistic effect between the side chain and the ring skeleton modification of lanosterol. Similar with the ring A modification of lanosterol, several analogues of 25-hydroxylanosterol (**18**-**23**) were less potent compared to 25-hydroxylanosterol. Then, derivatives of compound **6** with ring A modification were further tested in this assay. Oxidation of two angular methyl groups leads to a total activity loss, which suggested that two angular methyl groups were essential for favorable activity. The activity of 7-membered lactam derivatives **25** remained partial. Compound **26** with the succinic acid motif in the 3-position exhibited a similar activity to the acetylated derivative **18**. However, compound **27** with the acetic acid motif showed nearly no activity. These results suggested that esterified derivatives (**18**, **26**) but not alkylated derivatives (**19**, **27**) could remain partly active.

It was found that the modification of the lanosterol skeleton body, ring B and ring C, generally decreased the activity of the corresponding derivatives to different degrees. The transformation of the double bond in compound **6** to the diene group makes rings B and C of compound **29** more flat, which results in a slight shift of ring A and 3-OH observed from the computer calculation (Maestro 11). The slight perturbation of conformation undoubtedly accounts for the activity loss. Compound **29**, with an epoxy group at the 8,9-position, had a reduced efficacy with respect to **6**.

Meanwhile, installation of a hydroxyl group at the 8-position dramatically decreased the activity of compound **30**. In addition, introduction of the carbonyl groups at positions 7 and 11 led to an activity loss as well. It was observed that lactone derivative **32** showed less potency than compound **28**. Opening ring A afforded compound **33,** which had a low efficacy. These results imply that the hydrophobic property and unique conformation of lanosterol skeleton are indispensable for the high efficacy to inhibit protein aggregation.

Based on the above testing results, compound **6** demonstrated the best activity among these synthetic analogues. SAR showed that the introduction of 25-hydroxyl greatly improved the potency. The conformation and hydrophobicity of ring B and ring C was essential for high potency. Although some modifications of 3-OH maintained the activity of the lanosterol derivatives, compound 6 with 3-OH demonstrated the superior activity over other analogues. As shown in scheme 4, the 2-position of compound **6** was further modified with F and OH functional groups. The silyl enol ether intermediate (**44**) was oxidized using selectfluor and m-CPBA, affording 2-fluorinated and 2-hydroxylated ketone derivatives. These two intermediates underwent sequential reduction and deprotection to furnish compounds **35**, **36**, and **37**, respectively. Then, compounds **6**, **20**, **35**, **36,** and **37** were tested to evaluate their activity regarding the effectivity on the aggregates of αB-R120G. As shown in table 1 and figure 3, to our delight, it was found that 2-fluorination of compound **6** improved the potency further (compound **35**, EC50 = 18 nM), which is possibly due to the special hydrophobic/hydrophilic balance of the fluorine atom[26]. Besides, introduction of fluorine might reduce the metabolism rate of corresponding compound in vivo[15]. To our delight, the CLogP value of compound 35 is also lower than that of lanosterol (CLogP of lanosterol is 11.077, CLogP of compound **35** is 8.713). Lower CLogP value means

better solubility which is important for drug formulation development. 3-Epi-**35** (**36**) showed a similar activity to compound **35**. However, introduction of the OH group in the 2-position (**37**) decreased the activity. All these compounds show a good dose-dependent activity. 25-Hydroxyllanosterol (**6,** EC50 = 57 nM) was approximately 25 times more potent than lanosterol (**1,** EC50 = 1420 nM), which implied that the 25-hydroxyl group played a crucial role in reversing protein aggregation. Compound **20**, with oxidation of the 3-hydroxyl group, shows a 2-fold lower potency than compound **6**, which suggested that the 3-hydroxyl group may serve as a hydrogen bond acceptor rather than a donor. Compound **29**, with a flatter conformation, showed a slightly decreased potency.

Scheme 4. Preparation of Compounds 35-37

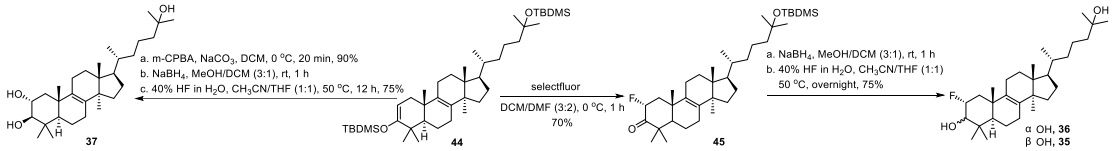
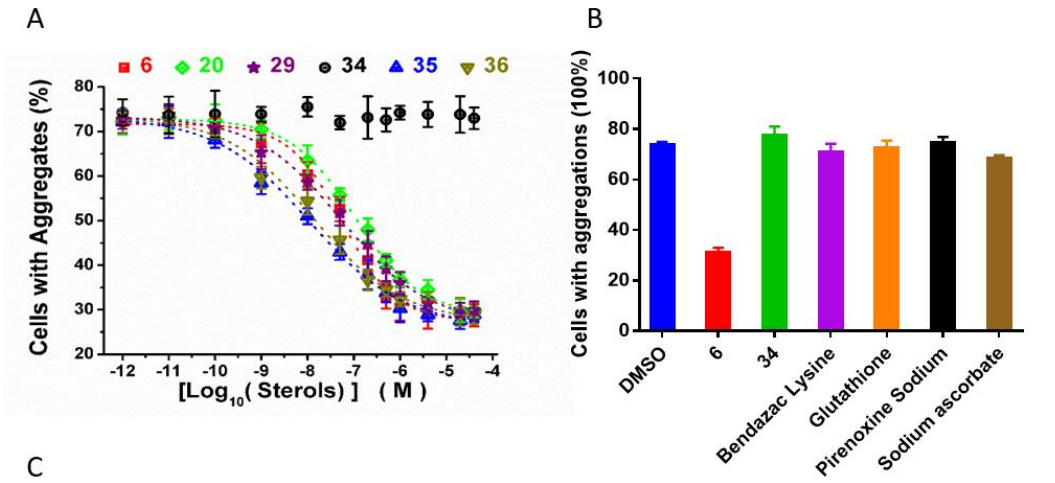


Table 1. The efficacy of lanosterol analogues to inhibit α-crystallinR120G induced aggregation in the cell model

Compound number	Aggregation inhibition (%)（αB R120G）		Compound number	Aggregation inhibition (%)（αB R120G）	
	0.4 μM	4 μM		0.4 μM	4 μM
6	52.5±0.6	60.3±0.8	**36**	56.1±1.9	60.6±2.6
35	56.7±1.7	62.1±1.4	**37**	33.4±1.7	51.1±0.9



C

Compounds	6	20	29	34	35	36
EC50 (nM)	57	106	64	No	18	26

Figure 3. The activity evaluation of lanosterol derivatives and reported compounds that could inhibit or postpone cataracts. A, C. EC 50 assay of lanosterol derivative B. The activity assay of the reported compounds that could inhibit or postpone cataracts.

Meanwhile, several reported compounds[27-30], which could inhibit or postpone cataracts were tested in the αB R120G HeLa cell lines, including Bendazac lysine (an aldose reductase inhibitor), pirenoxine sodium (a compound that can reduce selenite or calcium ions), glutathione and ascorbate (important antioxidant in the eye). As illustrated in figure 3B, no obvious effect could be observed with these compounds, which verifies the unique biological mechanism of our new synthetic compounds.

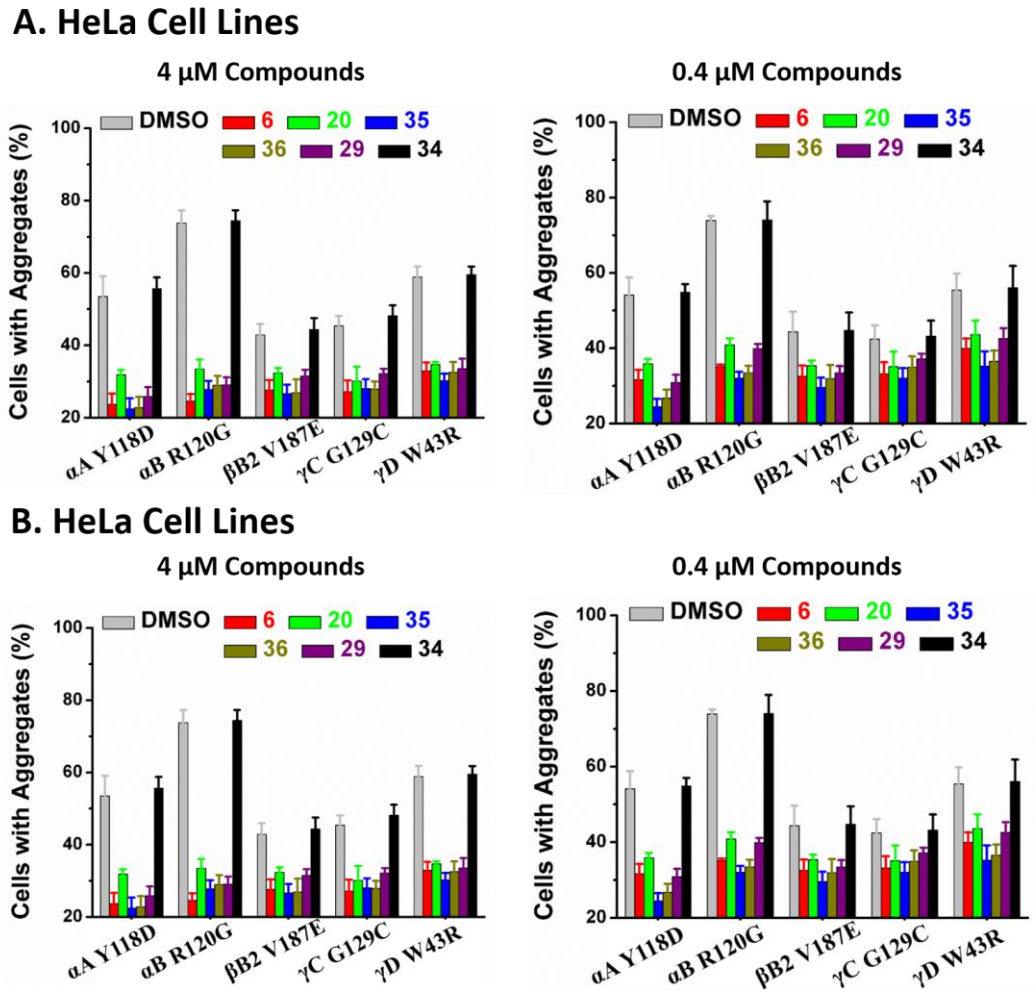


Figure 4. Activity evaluation of lanosterol derivatives towards different mutant crystallins. A, In HeLa cell lines, the effectivities of lanosterol analogues was general to the mutated crystallins B, In HLE-B3 cell lines, the lanosterol analogues also show significant activities, and the effectivities were also general to the mutated crystallins.

Furthermore, these potent lanosterol derivatives and negative control 4H were evaluated on aggresomes caused by other crystallin mutations, including αA Y118D, βB2 V187E, γC G129C and γD W43R[31]. As shown in figure 4A and 4B, these potent derivatives exhibited general efficiency towards multiple types of mutant crystallins in both HeLa and HLE-B3 cell lines. Meanwhile, compound **34**, as a negative control, showed no activity towards all the crystallins. These results demonstrated that lanosterol was a unique scaffold that can inhibit multiple types of

mutant crystallin induced aggregation. It was worth noting that the efficacy of lanosterol derivatives remain consistent among different kinds of mutant crystalline. For instance, compound **35,** the most potent compound in the αB R120G-containing cell line, is also the most potent compound in other mutant crystallin cell lines, which suggested that different mutant-crystallin aggregation was inhibited by lanosterol analogues via a same mechanism.

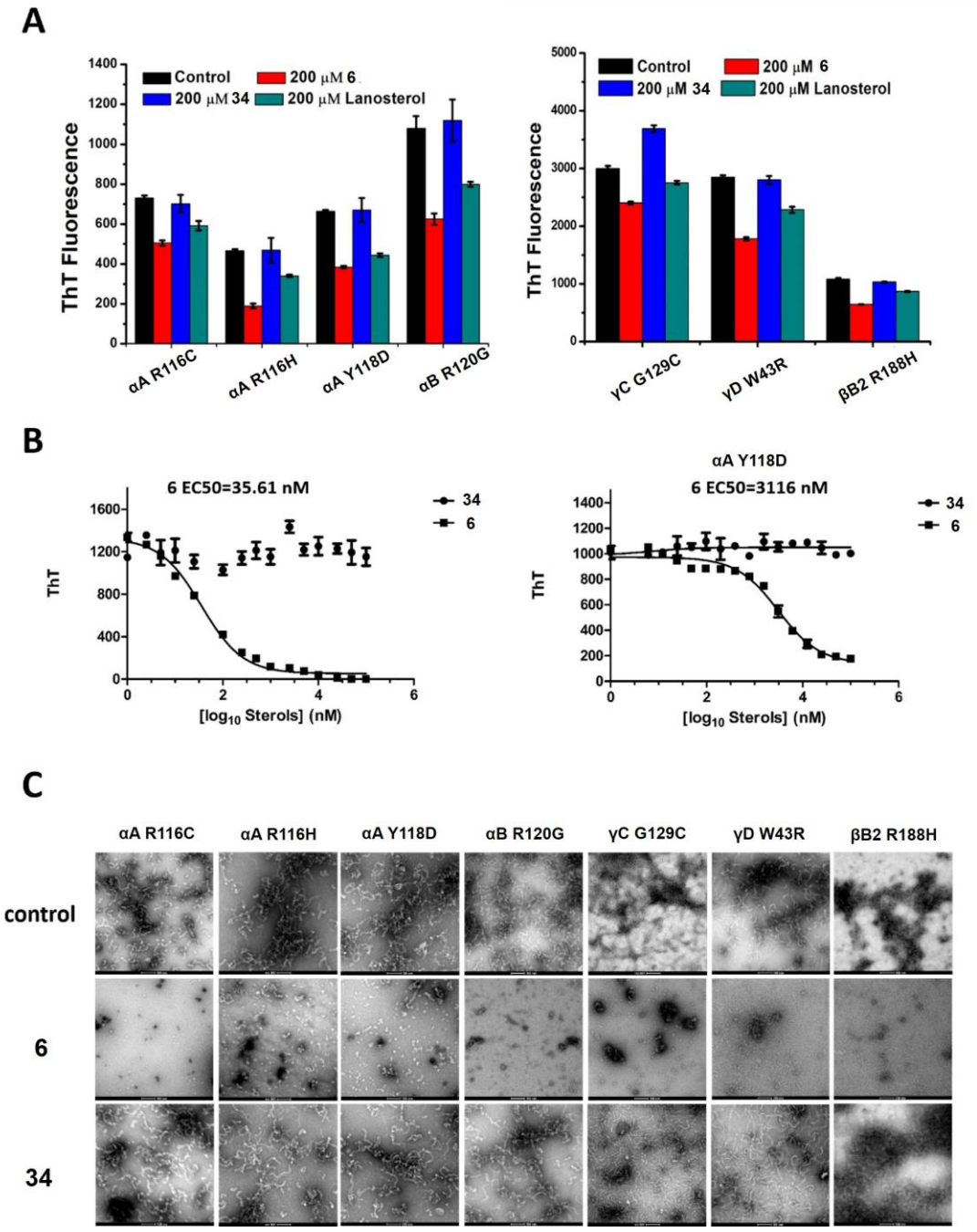


Figure 5. Compound **6** redissolved preformed amyloid-like fibrils of crystallin proteins. A. Effect of lanosterol and compound **6** on the redissolution of crystallin aggregates by ThT fluorescence (n = 3). Left, a-crystallin mutants; right, β/γ-crystallin mutants. B. EC50 assay of compound **6** on the redissolution of crystallin aggregates by ThT fluorescence. C. Negatively stained TEM photographs of aggregates of seven crystallin mutant proteins treated by a liposome vehicle, compounds **6** and **34** in liposomes.

To investigate whether lanosterol derivatives have a direct effect on the dissolution of aggregated proteins, the aggregates of seven mutant crystallins were obtained by heating crystallins in the presence of 1 M guanidine chloride as described before[13]. Under this condition, all crystallin proteins formed amyloid-like fibrils as revealed by the enhancement of thioflavin T (ThT) fluorescence and the fibrillary structures under negatively stained transmission electron microscopy (TEM). Compound **6**, with good efficacy towards different mutant crystallins in the cell model, was chosen for investigation of the dissolution effect in vitro with compound **34** as a negative control. Dipalmitoyl phosphatidylcholine was used to increase the solubility of lanosterol derivatives. As shown in figure 5A, compound **6,** but not **34,** could efficiently decrease the ThT fluorescence of all the seven mutant crystallin aggregates. Consistent with the cell model assay, compound **6** also showed a better efficacy than lanosterol. Then, the EC50 was tested based on thioflavin T (ThT) fluorescence of αA Y118D and αB R120G. As shown in figure 5B, a good dose activity relationship was observed and the EC50 of compound **6** is 35.6 nM for αA Y118D, 3116 nM for αB R120G. In addition, it was observed that all the fibrillar structures treated with compound **6,** but not **34,** were redissolved in the negatively stained TEM photographs (figure 5C).

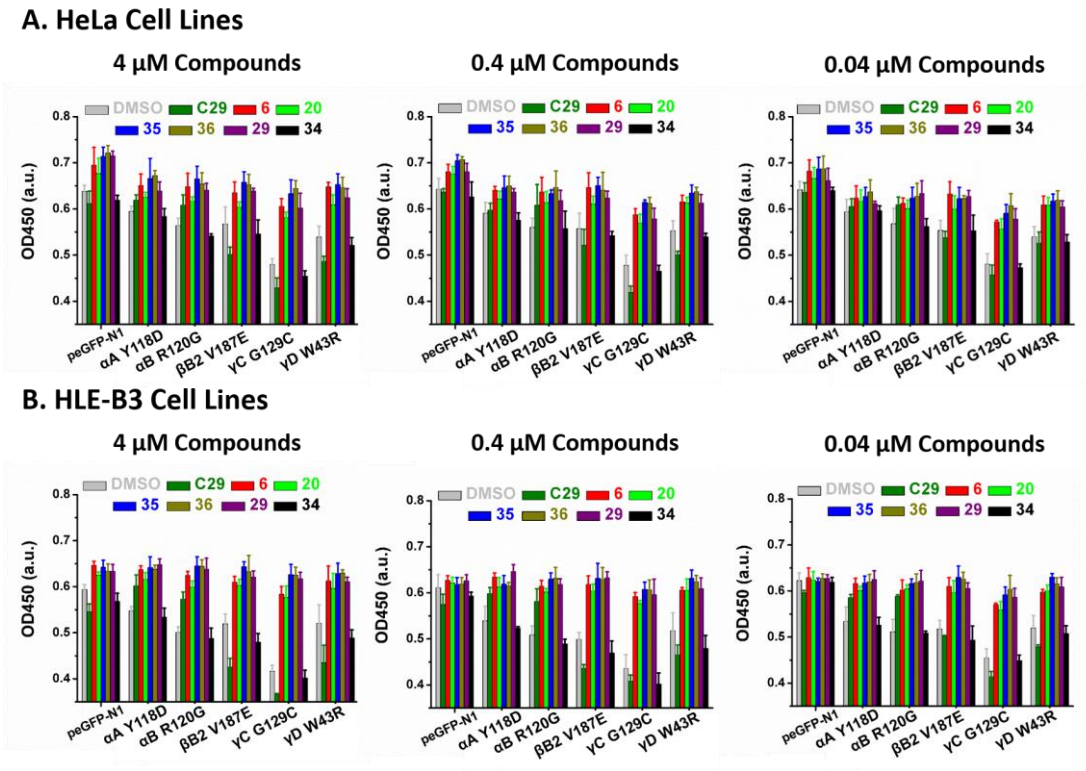


Figure 6. Toxicity evaluation of lanosterol derivatives towards different mutant crystallins in cells. A. HLE-B3 cell lines and B. HeLa cell lines

The toxicity of new prepared lanosterol derivatives and C29 were tested in HeLa or HLE-B3 cell lines with peGFP-N1 and multiple types of mutant crystallins. It was observed that lanosterol analogues could improve the cell viability towards both HeLa and HLE-B3 cell lines with peGFP-N1. However, a slight decrease in cell viability was observed for C29. These results demonstrated that lanosterol derivatives are promising compounds for cataract treatment and the toxicity of C29 may be due to multiple functions of endogenous oxysterol. Mutant crystallin proteins usually cause cytotoxicity. Both lanosterol derivatives and C29 can inhibit the aggregation of mutant a-crystallins. Consistently, these compounds could improve the cell viability of both HeLa and HLE-B3 cell lines, with α-crystallins obviously being even at a concentration of 40 nM. For cells with beta and gamma mutant crystallins, lanosterol derivatives could inhibit the corresponding protein aggregation; consequently, they can also obviously

improve the cell viability. In contrast, C29 is not efficient towards β and γ crystallins, which also show slight toxicity.

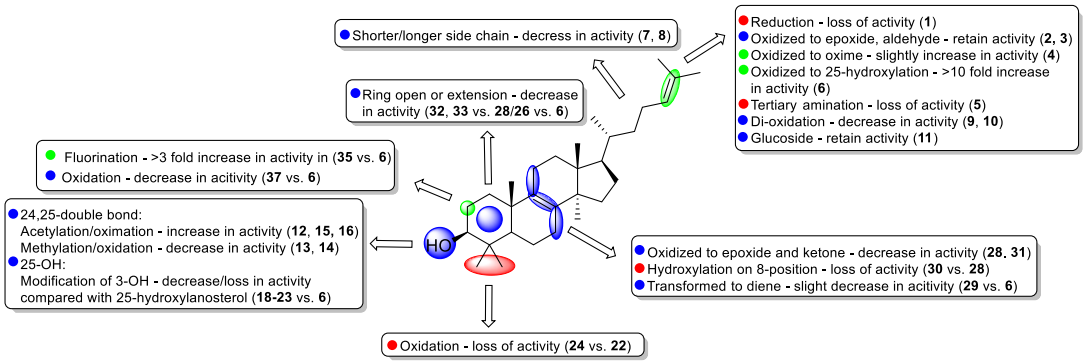


Figure 7. Summary of the structure–activity relationships of lanosterol to reverse mutant crystallins induced protein aggregation in HeLa cell.

To explore the structural features of lanosterol that are responsible for reversing mutant crystallin-induced protein aggregation and improve potency, we synthesized and evaluated 37 synthetic lanosterol analogues. As shown in figure 7, the SAR summary was depicted. In general, modification of structures in green could improve the potency significantly. For example, introduction of 25-hydroxyl and 2-fluorine greatly improved the potency (**35** EC50 = 18 nM vs lanosterol EC50 = 1.42 μM). In addition, structures in blue can be modified; however, modification may lead to decrease or loss of activity. Compound **20** with 3-carbonyl groups maintained the activity but had low potency (**20** EC50 = 106 nM vs **6** EC50 = 57 nM). Compound **30** with 8-hydroxyl lost the activity. Oxidation of gem-dimethyl in red also led to a loss of activity.

Conclusion

In summary, using mutant crystallin cell line as a screening system, we demonstrated lanosterol derivatives as efficient compounds to reverse different kinds of protein aggregation in cataracts

with good potency and efficacy (**6,** EC50 = 57 nM, **35**, EC50 = 18 nM). These potent compounds could not only inhibit the mutant crystalline aggregation in the cell model but also redissolve the mutant crystalline fiber in vitro. In addition, it was found that lanosterol analogues could also improve cell viability. Extensive modification of lanosterol provides a detailed SAR study, which lays a solid foundation for the development of drug candidates to treat cataracts and probes to elucidate the biological mechanism. A preliminary SAR is summarized based on our results. Further biological evaluation of the new synthetic potent molecule in different kinds of cataract animal models and detailed mechanism study is ongoing in our laboratory.

Experimental Section

Materials

All commercial materials (Alfa Aesar, Aladdin, J&K Chemical LTD.) were used without further purification. All solvents were analytical grade. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker 400 MHz spectrometer in $CDCl_3$ using TMS or solvent peak as a standard. The representative signals are shown for ^{1}H-NMR[32]. All ^{13}C-NMR spectra were recorded with complete proton decoupling. Low-resolution mass spectral analyses were performed with a Waters AQUITY UPLC^{TM}/MS. High-resolution mass spectral analyses were performed with a Bruker Impact HD/QTOF. Analytical TLC was performed on Yantai Chemical Industry Research Institute silica gel 60 F254 plates and flash column chromatography was performed on Qingdao Haiyang Chemical Co. Ltd silica gel 60 (200-300 mesh). The rotavapor was BUCHI's Rotavapor R-3.

High-purity chemical compounds, such as lanosterol, cholesterol, dimethyl sulfoxide (DMSO), paraformaldehyde and triton X-100 were purchased from Sigma. Compound 29 (5-cholesten-3β, 25-diol) reported by Leah N. Makley et al. was from Aladdin. Hoechst 33342 was obtained from

Invitrogen. The p62/SQSTM1 antibody was from Proteintech. Plasmid Maxipre kit was from Vigorous Biotechnology. The transfection reagent Lipofectamine™ 2000 and other cell culture materials were purchased from Invitrogen. Cell Counting Kit-8 was from Dojindo laboratories. All other chemicals were products of analytical grade. The purity of tested compounds, determined by HPLC, was >95%.

(R,E)-4-((3S,5R,10S,13R,14R,17R)-3-hydroxy-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)pentanal oxime (4). To a 50 ml round-bottom flask, were added 50 mg of compound **40** (0.11 mmol, 1.0 eq) and 10% KOH in EtOH, the resulting solution was stirred at 90 °C for 2 h. Then the mixture was washed with water and extracted with DCM. The organic layer was concentrated by evaporation to obtain crude intermediated which was used without further purification. To a 50 ml round-bottom flask, were added compound (1.0 eq) prepared above, 23.6 mg of $NH_2OH{\cdot}HCl$ (0.33 mmol, 3.0 eq), 36 mg of NaOAc (0.44 mmol, 4.0 eq), 5 ml of EtOH. After stirred at 60 °C for 3 h, the resulting solution was washed with saturated $NaHCO_3$ solution and extracted with EtOAc. The organic layer was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 5:1), yielding 41 mg pure product (I.Y. = 89%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 7.40 (t, 1H, *J* = 6 Hz), 3.24 (dd, 1H, *J* = 11.6 Hz, *J* = 4.4 Hz), 0.97 (d, 6H, 29-H3, 19-H), 0.92 (m, 3H, 21-H3), 0.86 (s, 3H, 30-H3), 0.79 (s, 3H, 28-H3), 0.67 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm)153.1, 134.6, 134.5, 79.2, 50.6, 50.5, 50.4, 50.0, 44.7, 39.1, 37.2, 36.5, 36.3, 35.8, 33.1, 32.6, 31.1, 31.0, 29.9, 28.4, 28.3, 28.1, 28.0, 26.7, 24.4, 21.2, 19.3, 18.54, 18.48, 18.4, 15.9, 15.6. LC-MS: calculated for $C_{27}H_{46}NO_2$ $[M+H]^+$: 416.35, found 416.65.

(10S,13R,14R,17R)-17-((R)-6-hydroxy-6-methyl-5-oxoheptan-2-yl)-4,4,10,13,14-pentamethyl -2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl acetate. (**9**) To a solution of 50 mg compound **41** (0.1 mmol, 1.0 eq) in DCM at room temperature was added 50 mg of DMP (0.12 mmol, 1.2 eq). After stirred at room temperature for 5 h, the resulting solution was washed with saturated $NaHCO_3$ solution. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed by evaporation, then the mixture was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc= 10:1), yielding 43 mg pure product (I.Y. = 85%). ^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 3.84 (s, 1H), 3.23 (d, 1H, *J* = 8.0 Hz), 2.57-2.48 (m, 2H), 1.38 (s, 6H, 26-H3, 27-H3), 0.98 (d, 6H, 29-H3, 19-H3), 0.90 (m, 6H, 21-H3, 30-H3), 0.80 (s, 3H, 28-H3), 0.69 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 215.1, 134.6, 134.5, 79.1, 76.4, 50.60, 50.57, 50.0, 44.7, 39.1, 37.2, 36.3, 35.8, 32.8, 32.1, 31.2, 31.0, 30.4, 29.9, 29.5, 28.3, 28.1, 26.72, 26.67, 24.4, 22.8, 21.2, 19.3, 18.6, 18.4, 15.9, 15.6, 14.3. MALDI-TOF-MS: calculated for $C_{30}H_{51}O_3Na$ $[M+Na]^+$: 481.31, found 481.380.

(6R)-2-hydroxy-6-((10S,13R,14R,17R)-3-hydroxy-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12, 13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-methylheptan-3-one (**9**). The compound prepared above was dissolved in the solution of KOH in EtOH (10%). After stirred at 90 °C for 2 h, the mixture was concentrated by evaporation, a moderate amount of water was added, and the suspension was extracted with DCM. The organic layer was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 5:1), yielding compound **9** as white powder (I.Y. = 90%). ^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 3.25-3.22 (m, 1H), 2.57-2.48 (m, 2H), 1.00 (s, 3H), 0.98 (s, 3H), 0.91-0.88 (m, 10H), 0.81 (s, 3H), 0.69 (s,

3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 215.1, 134.6, 134.5, 79.1, 76.4, 50.60, 50.57, 50.0, 44.7, 39.1, 37.2, 36.3, 35.8, 32.8, 32.1, 31.2, 31.0, 30.4, 29.9, 29.5, 28.3, 28.1, 26.72, 26.67, 24.4, 22.8, 21.2, 19.3, 18.6, 18.4, 15.9, 15.6, 14.3. MALDI-TOF-MS: calculated for $C_{30}H_{51}O_3Na$ $[M+Na]^+$: 481.31, found 481.380.

(2R,3S,5R,6S)-2-(((R)-6-((3S,5R,10S,13R,14R,17R)-3-hydroxy-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-methylheptan-2-yl)oxy)-6-(hydroxymethyl)tetrahydro-2H-pyran-3,4,5-triol (**11**) To a solution of 100mg compound **43** (0.1 mmol, 1.0 eq) in 5ml methanol at room temperature was added 10 mg of $NaBH_4$ (0.25 mmol, 2.5 eq). After stirred at room temperature for 30 min, the solvent was removed by evaporation, then the mixture was washed with saturated $NaHCO_3$ solution. The organic later was concentrated to obtain 95 mg of crude product. ^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 8.00 (d, *J* = 7.7 Hz, 2H), 7.94 (d, *J* = 7.7 Hz, 2H), 7.90 (d, *J* = 7.7 Hz, 2H), 7.82 (d, *J* = 7.7 Hz, 2H), 7.55-7.50 (m, 3H), 7.44-7.33 (m, 10H), 7.30-7.26 (m, 2H), 5.92 (t, *J* = 9.7 Hz, 1H), 5.58 (t, *J* = 9.7 Hz, 1H), 5.50 (t, *J* = 8.4 Hz, 1H), 5.00 (d, *J* = 7.9 Hz, 2H), 4.60-4.56 (m, 1H), 4.50-4.49 (m, 1H), 4.17-4.15 (m, 1H), 3.24-3.22 (m, 1H), 0.64 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 166.21, 165.9, 165.4, 165.0, 134.49, 133.52, 133.28, 133.18, 129.93, 129.87, 129.79, 129.73, 129.55, 129.00, 128.92, 128.52, 128.44, 128.41, 128.38, 95.91, 79.06, 78.92, 73.25, 72.14, 72.10, 70.32, 63.81, 50.61, 50.50, 49.86, 44.51, 42.84, 38.99, 37.10, 36.69, 36.50, 35.70, 31.05, 30.91, 28.28, 28.07, 27.95, 27.19, 26.59, 25.31, 24.37, 21.09, 20.70, 19.25, 18.64, 18.35, 15.85, 15.54.

To a solution of 58 mg compound (0.06 mmol, 1.0 eq) prepared above in DCM/methanol : 1 ml/3 ml) was added 6 mg of NaOMe (0.11 mmol, 1.9 eq). After stirred at room temperature for 3 h,

the solvent was concentrated by evaporation, then washed with saturated $NaHCO_3$ solution and extracted with DCM. The organic layer was dried over anhydrous Na_2SO_4 and purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 5:1), yielding 28 mg of compound **11** (I.Y. = 80%). ^{1}H-NMR(400 MHz, CD_3OD:$CDCl_3$ = 2:1, representative signals) δ (ppm) 4.43 (d, 1H, *J* = 7.7 Hz), 3.79-3.77 (m, 1H), 3.70-3.66 (m, 1H), 3.38-3.35 (m, 2H), 3.24-3.23 (m, 1H), 3.18-3.14 (m, 2H), 1.24 (s, 6H, 26-H3, 27-H3), 0.98 (s, 6H, 29-H3, 19-H3), 0.89 (m, 6H, 21-H3, 30-H3), 0.80 (s, 3H, 28-H3), 0.69 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, CD_3OD/$CDCl_3$ = 2/1) δ (ppm) 135.05, 134.91, 97.67, 79.10, 79.00, 77.22, 76.32, 74.25, 70.94, 62.41, 51.16, 51.06, 50.32, 49.63, 48.36, 45.00, 42.90, 39.34, 37.51, 37.35, 37.07, 36.24, 31.55, 31.32, 30.14, 28.74, 28.24, 27.82, 27.01, 26.72, 26.37, 24.55, 21.49, 19.44, 19.00, 18.78, 16.09, 15.80. MALDI-TOF-MS: calculated for $C_{36}H_{63}O_7Na$ $[M+Na]^+$: 629.44, found 629.429.

(3S,5R,10S,13R,14R,17R)-3-methoxy-4,4,10,13,14-pentamethyl-17-((R)-6-methylhept-5-en-2-yl)-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene (13)

To a 50 ml round-bottom flask were added 90 mg of lanosterol (0.2 mmol, 1.0 eq) and 10 ml dry THF, followed by 24.3 mg of NaH (1.0 mmol, 5.0 eq), 62 ul of MeI (1.0 mmol, 5.0 eq). After stirred at room temperature for 12 h, the reacting solution was quenched by water, and the extracted with DCM. The organic layer was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 30:1), yielding 90 mg of compound **13** (I.Y. = 90%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 5.10 (t, *J* = 6.2 Hz, 3H), 3.58 (s, 3H), 2.67 (dd, 1H, *J* = 11.6 Hz, *J* = 4.4 Hz), 0.98 (s, 6H, 29-H3, 19-H3), 0.90 (m, 6H, 21-H3, 30-H3), 0.79 (s, 3H, 28-H3), 0.68 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 134.7, 134.5, 131.1, 125.4,

88.8, 57.7, 51.1, 50.6, 50.0, 44.7, 39.0, 37.2, 36.5, 36.4, 35.7, 31.2, 31.0, 28.4, 28.1, 26.6, 25.9, 25.1, 24.4, 22.8, 21.2, 19.3, 18.8, 18.3, 17.8, 16.3, 15.9.

(5R,10S,13R,14R,17R)-4,4,10,13,14-pentamethyl-17-((R)-6-methylhept-5-en-2-yl)-1,2,4,5,6,7, 10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthren-3-one O-methyl oxime (16). Refer to the synthetic method of compound **22**. ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 5.09 (t, 1H, *J* = 6.8 Hz), 3.81 (s, 3H), 3.03-3.00 (m, 1H), 1.15 (s, 3H, 28-H), 1.06 (d, 6H, 29-H3, 19-H3), 0.91 (d, 3H, 21-H3), 0.85 (s, 3H, 30-H3), 0.69 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 166.2, 134.9, 133.9, 131.0, 125.4, 61.14, 51.61, 50.55, 50.02, 44.62, 40.18, 37.21, 36.50, 36.41, 35.83, 31.15, 31.01, 28.32, 27.22, 26.56, 25.87, 25.08, 24.38, 23.37, 21.21, 19.13, 18.83, 18.79, 18.25, 17.77, 15.96. MS (ESI): calculated for $C_{31}H_{52}NO$ $[M+H]^+$: 454.40, found 454.41.

(R)-6-((3S,5R,10S,13R,14R,17R)-3-methoxy-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13, 14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-methylheptan-2-ol (19). To a 50 ml round-bottom flask were added 90 mg of compound **6** (0.2 mmol, 1.0 eq) and 10 ml dry THF, followed by 24.3 mg of NaH (1.0 mmol, 5.0 eq), 62 ul of MeI (1.0 mmol, 5.0 eq). After stirred at room temperature for 12 h, the reacting solution was quenched by water, and the extracted with DCM. The organic layer was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 30:1), yielding 30 mg of compound **19** (I.Y. = 30%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 3.36 (s, 3H), 2.66 (dd, 1H, *J* = 11.6 Hz, *J* = 4.0 Hz), 2.57-2.48 (m, 2H), 1.20 (s, 6H, 26-H3, 27-H3), 0.97 (d, 6H, 29-H3, 19-H3), 0.89 (m, 6H, 21-H3, 30-H3), 0.78 (s, 3H, 28-H3), 0.68 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 134.7, 134.5, 88.8, 77.5, 77.2, 76.8, 74.8, 57.7, 51.1, 50.7, 50.0, 49.2, 44.6, 40.5, 39.0, 37.2, 37.0,

36.6, 35.6, 31.2, 31.0, 28.4, 28.1, 26.6, 25.2, 24.4, 22.8, 21.2, 20.7, 19.3, 18.9, 18.3, 16.3, 15.9. MS (ESI): calculated for $C_{31}H_{54}O_2Na$ $[M+Na]^+$: 481.40, found 481.40.

(5R,10S,13R,14R,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyl-1, 2,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthren-3-one oxime (21). To a 50 ml round-bottom flask, were added 100 mg of compound **20** (0.23 mmol, 1.0 eq), 47 mg of $NH_2OH{\cdot}HCl$ (0.68 mmol, 3.0 eq), 55.8 mg of dry NaOAc (0.68 mmol, 3.0 eq), 10 ml EtOH. After stirred at 60 °C for 3 h, the resulting solution was concentrated by evaporation. The mixture was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 10:1), yielding 95 mg of compound **21** (I.Y. = 90%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 9.11(s, br, 1H), 3.14-3.10(m, 1H), 0.88 (m, 6H, 21-H3, 30-H3), 0.68 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 167.2, 134.9, 133.9, 71.3, 51.5, 50.6, 50.0, 44.6, 44.5, 40.5, 37.3, 36.8, 36.6, 35.8, 31.1, 31.0, 29.5, 29.3, 28.3, 27.0, 26.5, 24.4, 23.2, 21.3, 21.2, 19.1, 18.9, 18.8, 17.7, 15.9. MS (ESI): calculated for $C_{30}H_{52}NO_2$ $[M+H]^+$: 458.40, found 458.40.

(5R,10S,13R,14R,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyl-1, 2,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthren-3-one O-methyl oxime (22). To a 50 ml round-bottom flask, were added 100 mg of compound **20** (0.23 mmol, 1.0 eq), 56.4 mg of $MeONH_2{\cdot}HCl$ (0.68 mmol, 3.0 eq), 55.8 mg of dry NaOAc (0.68 mmol, 3.0 eq), 10 ml EtOH. After stirred at 60 °C for 3 h, the resulting solution was concentrated by evaporation. The mixture was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 15:1), yielding 99 mg of compound **22** (I.Y. = 92%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 3.81 (s, 3H), 3.00 (d, 1H, J = 14.4 Hz,), 0.89 (m, 6H, 21-H3, 30-H3), 0.69 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 166.2, 134.9, 134.0,

77.5, 77.2, 76.8, 71.3, 61.2, 51.6, 50.7, 50.0, 44.62, 44.56, 40.2, 37.2, 36.9, 36.6, 35.8, 31.1, 31.0, 29.5, 29.4, 28.4, 27.2, 26.5, 24.4, 23.4, 21.3, 21.2, 19.1, 18.8, 18.3, 16.0. LC-MS: calculated for $C_{31}H_{54}O_2$ $[M+H]^+$: 472.41, found 472.99.

(R)-6-((3S,5R,10S,13R,14R,17R)-3-amino-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-methylheptan-2-ol (23). To a solution of 50 mg compound **21** (0.1 mmol, 1.0 eq) in THF was added 8.3 mg of $LiAlH_4$ (0.2 mmol, 2.0 eq). After stirred at room temperature for 4 h, the reacting system was quenched with water and then the mixture was extracted with DCM. The organic layer was purified by 200-300 mesh silica gel flash column chromatography (DCM:MeOH = 10:1), yielding 35 mg of compound **23** (I.Y. = 80%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 2.68 (s, 1H), 2.43 (d, 1H, *J* = 10.8 Hz), 0.67 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 134.6, 134.2, 70.1, 68.4, 59.7, 51.1, 50.9, 50.5, 49.7, 44.3, 43.9, 38.3, 37.04, 36.97, 36.9, 36.7, 36.4, 35.8, 35.6, 31.0, 30.9, 30.5, 27.9, 27.7, 27.0, 26.3, 25.4, 25.2, 23.3, 20.69, 20.65, 18.4, 18.13, 18.07. LC-MS: calculated for $C_{30}H_{54}NO$ $[M+H]^+$: 444.41, found 444.96.

(5R,10S,14R,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4-bis(hydroxymethyl)-10,14-dimethyl-1,2,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthren-3-one O-methyl oxime (24).

To a 10ml round bottom flask were added compound **22** (0.2 mmol, 94 mg), $Pd(OAc)_2$ (0.1 equiv.), $PhI(OAc)_2$ (3.0 equiv.) and $AcOH/Ac_2O$ (2 mL/2 mL). The reaction was stirred at 80 °C for 12 h and monitored by TLC. Water was added to dilute the reaction mixture and $NaHCO_3$ was added to neutralize the reaction mixture. Then reaction mixture was extracted by DCM for three times, the organic layer dried over anhydrous Na_2SO_4 and concentrated on rotavapor under

reduced pressure. Finally, the residue was purified by silica gel column chromatography to give the compound **42** (51 mg, 45%). Then compound 42 was added to a solution of DCM/MeOH (1 mL/3 mL) containing MeONa (2.0 eq), the reaction system was stirred at room temperature for 12 h. Then reaction mixture was extracted by DCM for three times, the organic layer dried over anhydrous Na_2SO_4 and concentrated on rotavapor under reduced pressure. Finally, the residue was purified by silica gel column chromatography to give the compound **24** (35 mg, 80%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 4.08-4.02 (m, 2H), 3.90-3.77 (m, 6H), 3.51 (t, 1H, *J* = 6.4 Hz), 3.00 (dd, 1H, *J* = 16 Hz, *J* = 2.4 Hz), 2.26-2.18 (m, 1H), 1.20 (s, 6H, 26-H3, 27-H3), 1.07 (s, 3H, 19-H3), 0.89 (m, 6H, 21-H3, 30-H3), 0.68 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 164.5, 135.2, 133.5, 71.2, 69.4, 65.4, 61.9, 50.6, 50.0, 49.0, 46.8, 44.53, 44.51, 37.0, 36.8, 36.6, 34.9, 31.0, 30.9, 29.5, 29.3, 28.3, 26.4, 24.4, 21.3, 21.2, 19.3, 19.1, 18.8, 15.9. MS (ESI): calculated for $C_{30}H_{51}NO_4Na$ $[M+Na]^+$: 526.39, found 526.38.

(5aS,7aR,8R,10aR,12aR)-8-((R)-6-hydroxy-6-methylheptan-2-yl)-1,1,5a,7a,10a-pentamethyl-1,4,5,5a,6,7,7a,8,9,10,10a,11,12,12a-tetradecahydrocyclopenta[5,6]naphtho[2,1-c]azepin-3(2H)-one (25). To a round-bottom flask were added 50 mg of compound **21** (0.1 mmol, 1.0 eq) and TFA/DCM : 1 ml/1 ml. After stirred at room temperature for 2 h, the resulting solution was washed with saturated $NaHCO_3$ and extracted with DCM. The organic layer was concentrated and purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 30:1), yielding 18 mg of compound **25** (I.Y. = 40%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 8.38 (s, br, 1H), 3.13-3.07 (m, 1H), 2.19-2.10 (m, 1H), 1.14 (s, 3H, 28-H3), 1.06 (s, 6H, 29-H3, 19-H3), 0.88-0.84 (m, 6H, 21-H3, 30-H3), 0.69 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 167.3, 135.0, 134.0, 89.5, 51.5, 50.6, 50.0, 44.6, 40.9, 40.5, 37.3, 36.43, 36.38,

35.8, 31.1, 31.0, 28.3, 27.1, 26.5, 25.8, 25.7, 24.4, 23.2, 21.2, 20.6, 19.1, 18.9, 18.7, 17.7, 16.0. LC-MS: calculated for $C_{30}H_{52}O_2$ $[M+H]^+$: 458.39, found 458.74.

4-(((3S,5R,10S,13R,14R,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)-4-oxobutanoic acid (26). To the solution of 40 mg compound **6** (0.1 mmol, 1.0 eq) in 2 ml pyridine were added 12 mg of succinic anhydride (0.11 mmol, 1.1 eq) and 11mg of DMAP (0.1 mmol, 1.0 eq). After stirred at 80 °C for 3 h, the resulting solution was washed with 10% HCl and extracted with EtOAc. The organic layer was concentrated and purified by 200-300 mesh silica gel flash column chromatography (DCM:MeOH = 20:1), yielding 44 mg of compound **26** (I.Y. = 80%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 4.52 (dd, *J* = 11.6 Hz, *J* = 4.8 Hz, 1H), 2.69-2.63(m, 4H), 1.20 (s, 6H, 26-H3, 27-H3), 0.99 (s, 3H, 28-H3), 0.90-0.86 (m, 12H, 21-H3, 30-H3, 29-H3, 19-H3), 0.68 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 172.0, 170.7, 134.6, 134.3, 81.6, 71.4, 60.6, 50.6, 49.9, 44.6, 44.5, 38.0, 37.0, 36.8, 36.6, 35.3, 31.1, 30.9, 29.5, 29.4, 29.3, 29.1, 28.5, 28.4, 28.0, 26.5, 24.4, 24.2, 21.3. 21.2, 21.1, 19.3, 18.8, 18.2, 16.7. MALDI-TOF-MS: calculated for $C_{34}H_{56}O_5Na$ $[M+Na]^+$: 567.40, found 567.441.

2-(((3S,5R,10S,13R,14R,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)acetic acid (27). To a sealed tube were added 224 mg of compound **6** (0.5 mmol, 1.0 eq), 8 mg of $Rh_2(OAc)_4$ (5%), ethyl 2-diazoacetate, 10 ml DCM. After stirred at 40 °C for 12 h, the resulting solution was concentrated by evaporation and purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 30:1). The obtained product was dissolved in MeOH/H_2O : 2 ml/1 ml, followed by the addition of 345.5 mg K_2CO_3 (2.5 mmol, 5.0 eq). After stirred at 80°C for

5 h, the mixture was concentrated by evaporation and the extracted by DCM. The organic layer was purified by 200-300 mesh silica gel flash column chromatography (DCM:MeOH = 50:1), yielding 62.8 mg of compound **27** (I.Y. = 25%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 4.07 (q, 2H), 2.90(d, 1H, *J* = 10 Hz), 1.15 (s, 6H, 26-H3, 27-H3), 0.97 (d, 6H, 29-H3, 19-H3), 0.83 (m, 9H, 21-H3, 30-H3, 28-H3), 0.66 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 134.76, 134.71, 88.87, 71.15, 51.15, 50.88, 50.10, 44.78, 44.51, 39.27, 37.23, 37.11, 36.81, 35.74, 31.31, 31.09, 28.94, 28.77, 28.51, 28.19, 26.74, 24.42, 23.52, 21.46, 21.32, 19.34, 18.84, 18.41, 16.35, 15.94.

(5S,8S,9S,10S,13R,14R,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyltetradecahydro-11H-8,9-epoxycyclopenta[a]phenanthren-3-ol (**28**). To a solution of 50 mg **6** (0.11 mmol, 1.0 eq) in DCM (30 ml) was added 22.4 mg of m-CPBA (0.13 mmol, 1.2 eq) and 10 mg of $NaHCO_3$ (0.12 mmol, 1.1 eq) at 0 ˚C. After stirred at room temperature for 12 h, the resulting solution was washed with saturated $NaHCO_3$. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed by evaporation, then the mixture was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 5:1), yielding 43 mg of compound **28** (I.Y. = 85%). ;^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 3.21-3.19(m, 1H), 1.23 (s, 6H, 26-H3, 27-H3), 1.13 (s, 3H), 0.96 (s, 3H), 0.88 (d, *J* = 6.3 Hz, 3H, 21-H3), 0.79 (s, 3H, 30-H3), 0.75 (s, 3H, 28-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 78.6, 71.2, 70.8, 68.3, 49.0, 48.5, 44.5, 43.7, 41.9, 38.6, 38.0, 36.7, 36.4, 33.0, 29.42, 29.36, 28.6, 28.4, 27.3, 27.0, 23.6, 21.6, 21.2, 20.1, 19.1, 17.1, 16.6, 16.4, 15.2. LC-MS: calculated for $C_{30}H_{52}O_3$ $[M-H]^-$:459.39, found 459.97.

(3S,5R,10S,13R,14R,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyl

-2,3,4,5,6,10,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol (29). To a solution of 30 mg compound **28** (0.06 mmol, 1.0 eq) in 3 ml THF was added 100ul of 40% HF aqueous solution. After stirred at room temperature for 4 days, the solution was washed with saturated NaHCO3 and extracted with DCM. The organic layer was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 3:1), yielding 25.5 mg of compound **29** (I.Y. = 96%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 5.47(s, br, 1H), 5.31-5.30(m, 1H), 3.24(dd, *J* = 11.2 Hz, *J* = 4.4 Hz, 1H), 1.21 (s, 6H, 26-H3, 27-H3), 1.00 (s, 3H, 29-H3), 0.97 (s, 3H, 19-H3), 0.88 (d, J = 6.3 Hz, 3H, 21-H3),0.87 (s, 6H), 0.55 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 146.0, 142.8, 120.3, 116.5, 79.1, 71.2, 51.2, 50.5, 49.2, 44.5, 43.9, 38.8, 38.0, 37.5, 36.8, 36.4, 35.8,31.6, 29.5, 29.4, 28.3, 28.1, 27.9, 25.7, 23.1, 22.9, 21.3, 18.6, 15.9, 15.8. MS (ESI): calculated for $C_{30}H_{51}O_2$ $[M+H]^+$: 443.39, found 443.37.

(5S,9R,10S,13R,14S,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyl hexadecahydro-9H-cyclopenta[a]phenanthrene-3,9-diol (30).

To a solution of 200 mg compound **28** in 4 ml diethylamine was added 100 mg of Li. After stirred at room temperature for 12 hours, methanol was added dropwise until no gas was generated. Then the solution was washed with saturated $NaHCO_3$ and extracted with DCM. The organic layer was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 5:1), yielding 50 mg of compound **30** (I.Y. = 25%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 3.19-3.16 (m, 1H), 1.19 (s, 3H), 1.01 (s, 3H), 0.98 (s, 3H), 0.91 (s, 3H), 0.89 (d, *J* = 6.3 Hz, 3H), 0.79 (s, 3H), 0.77 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 78.2, 77.3, 71.2, 50.6, 47.6, 45.8, 45.3, 44.5, 42.8, 40.6, 38.9, 36.9, 36.1, 33.9, 29.6, 29.4, 29.32, 29.26, 28.4, 28.1, 28.1, 28.06, 27.5, 23.9, 21.5, 21.2, 18.8, 18.4, 16.9, 15.5, 14.7. MS (ESI): calculated for $C_{30}H_{55}O_3$

$[M+H]^+$: 463.41, found 463.42.

(5aS,5bS,7aR,8R,10aR,10bS,12aR)-8-((R)-6-hydroxy-6-methylheptan-2-yl)-1,1,5a,7a,10a-pentamethyldodecahydro-6H-5b,10b-epoxycyclopenta[5,6]naphtho[2,1-c]oxepin-3(1H)-one (32).

To a 50 ml round-bottom flask were added 66 mg of compound **46** (0.14 mmol, 1.0 eq), 60 mg of m-CPBA (0.35 mmol, 2.5 eq), 18.5 mg of $NaHCO_3$ (0.22 mmol, 1.6 eq) and 5 ml DCM. After stirred at room temperature for 12 h, the resulting solution was washed with water. Organic layer was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 3:1), yielding 51.8 mg of compound **32** (I.Y. = 78%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 2.75-2.53 (m, 2H), 2.27 (dd, *J* = 12.2 Hz, *J* = 3.4 Hz, 1H), 1.22 (s, 8H), 0.87 (s, 6H), 0.74 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 175.0, 85.9, 71.5, 71.2, 69.8, 49.3, 48.4, 44.4, 44.1, 43.4, 40.5, 36.6, 36.3, 33.1, 32.4, 32.3, 31.8, 29.4, 29.3, 28.5, 26.9, 25.6, 24.9, 22.0, 21.6, 21.2, 20.1, 19.1, 19.0, 16.5.

3-((3R,3aR,5aS,6S,7R,9aS,9bR)-3-((R)-6-hydroxy-6-methylheptan-2-yl)-7-(2-hydroxypropan-2-yl)-3a,6,9b-trimethyldecahydro-1H-5a,9a-epoxycyclopenta[a]naphthalen-6-yl)propanoic acid (33).

To a 50 ml round-bottom flask were added 47 mg of compound **32** (0.1 mmol, 1.0 eq), 60 mg of m-CPBA (0.35 mmol, 2.5 eq), 18.5 mg of $NaHCO_3$ (0.22 mmol, 1.6 eq) and 5 ml DCM. After stirred at room temperature for 12 h, the resulting solution was washed with water. Organic layer was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 3:1), yielding 51.8 mg of compound 45 (I.Y. = 78%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 2.82-2.76 (m, 1H), 2.56-2.50 (m, 2H), 2.08-1.89 (m, 6H), 0.76 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 179.9, 75.7, 71.4, 70.2, 65.2, 49.1, 48.6, 44.4, 43.5, 42.1, 42.0, 36.7,

36.4, 34.3, 33.2, 32.4, 30.0, 29.8, 29.4, 29.3, 28.6, 27.3, 24.8, 22.8, 22.0, 21.2, 21.0, 20.8, 20.1, 19.0, 16.3, MALDI-TOF-MS: calculated for $C_{30}H_{52}O_5Na$ $[M+Na]^+$:515.37, found 515.407.

(5S,8S,9S,10S,13R,14R,17R,E)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyldodecahydro-11H-8,9-epoxycyclopenta[a]phenanthren-3(2H)-one oxime (34). Refer to the synthetic method of compound **21**. ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 3.14-3.10 (m, 1H), 2.20-2.12 (m, 1H), 1.23 (s, 3H), 1.19 (s, 6H), 1.08 (s, 3H), 1.01 (s, 3H), 0.87 (s, 6H), 0.76 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 166.4, 71.3, 70.7, 68.3, 49.0, 48.5, 44.5, 43.7, 43.1, 40.0, 38.1, 36.7, 36.4, 32.9, 32.1, 29.44, 29.38, 28.6, 27.6, 26.9, 23.9, 22.8, 21.7, 21.2, 20.1, 19.1, 17.4, 17.2, 17.0, 16.4. MS (ESI): calculated for C30H51NNaO3 $[M+Na]^+$: 496.37, found 496.33.

(2R,3R,5R,10S,13R,14R,17R)-2-fluoro-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol (**35**). To a solution of 57 mg **45** (0.1 mmol, 1.0 eq) in DCM/MeOH : 1 ml/3 ml was added 20 mg of $NaBH_4$ (0.5 mmol, 5.0 eq) at room temperature. After stirred at room temperature for 1 h, the resulting solution was washed with saturated $NaHCO_3$. The organic later was dried over anhydrous Na_2SO_4 and purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 50:1). The obtained products (ratio = 1：6) were dissolved respectively in MeCN/THF : 1 ml/1 ml followed by addition of 40% HF aqueous solution at room temperature. After the mixture was stirred at 50 ˚C for 16 h, utilized $CaCl_2$ solution was added to neutralize the reacting system, the solution was extracted with DCM. The organic layer was dried over anhydrous Na_2SO_4 and purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc= 10:1), yielding 42 mg compound **35** (I.Y. =75%) and 7 mg compound 36 (I.Y.

=12%). ^{1}H-NMR(400 MHz, $CDCl_3$) δ (ppm) ^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 4.66-4.49 (m, 1H), 3.28 (dd, *J* = 13.0 Hz, *J* = 9.6 Hz, 1H), 1.23 (s, 6H, 26-H3, 27-H3), 1.06 (s, 3H, 29-H3), 1.04 (s, 3H, 19-H3), 0.88 (d, *J* = 6.3 Hz, 3H, 21-H3), 0.87 (s, 3H, 30-H3), 0.86 (s, 3H, 28-H3), 0.68 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 134.9, 133.5, 93.4 (d, *J* = 165.6 Hz, 1C), 81.0 (d, *J* = 15.4 Hz, 1C), 71.1, 50.4, 50.2, 50.1, 49.8, 44.5, 44.4, 41.1, 40.9, 39.5, 39.4, 38.7, 38.5, 36.7, 36.5, 30.8, 30.76, 29.7, 29.3, 29.2, 28.4, 28.2, 26.2, 24.3, 21.3, 21.1, 20.2, 18.7, 18.0, 16.6, 15.7. MALDI-TOF-MS: calculated for $C_{30}H_{52}FO_2Na$ $[M+Na]^+$:485.38, found 485.401.

(2R,3S,5R,10S,13R,14R,17R)-2-fluoro-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol (36). ^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 5.01-4.85 (m, 1H), 3.67 (d, *J* = 7.7 Hz, 1H), 1.23 (s, 3H), 1.19 (s, 6H, 26-H3, 27-H3), 1.04 (s, 3H, 29-H3), 1.02 (s, 3H, 19-H3), 0.88 (d, J = 6.3 Hz, 3H, 21-H3), 0.87 (s, 3H, 30-H3), 0.67 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 134.7, 133.9, 91.5 (d, *J* = 167.1 Hz, 1C), 76.7, 71.2, 50.5, 50.0, 44.6, 44.5, 43.5, 38.8, 38.7, 38.6, 38.5, 36.8, 36.6, 35.8, 35.6, 31.0, 30.9, 29.8, 29.5, 29.4, 28.3, 28.2, 26.1, 24.4, 21.9, 21.3, 21.26, 20.3, 18.8, 17.9, 15.8. MALDI-TOF-MS: calculated for $C_{30}H_{52}FO_2Na$ $[M+Na]^+$:485.38, found 485.370.

(2R,3R,5R,10S,13R,14R,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyl-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene-2,3-diol (37). To a solution of 290 mg **44-1** (0.63 mmom, 1.0 eq) in MeOH/DCM was added 238.3 mg of $NaBH_4$ (10.0 eq) at room temperature slowly. After stirred at room temperature for 16 h, the solvent was removed by evaporation, then the mixture was purified by 200-300 mesh silica gel

flash column chromatography (Hexane:EtOAc = 40:1). The obtained intermediate was then dissolved in MeCN/DCM : 1 ml/1 ml, followed by addition of 40% HF (100 uL). After stirred at 50 ˚C for 16 h, the mixture was added $CaCl_2$ solution, and extracted with DCM. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed by evaporation, then the mixture was purified by 200-300 mesh silica gel flash column chromatography (DCM:MeOH = 50:1, representative signals), yielding 36 mg compound **37** (I.Y. = 15% for two steps). ^{1}H-NMR(400 MHz, CD_3OD) δ (ppm) 3.66-3.60 (m, 1H), 2.93-2.91 (d, *J* = 9.6Hz, 1H), 1.12 (s, 6H, 26-H3, 27-H3), 0.98 (s, 3H, 29-H3), 0.95 (s, 3H, 19-H3), 0.84 (d, J = 6.3 Hz, 3H, 21-H3), 0.81 (s, 3H, 30-H3), 0.76 (s, 3H, 28-H3), 0.62 (s, 3H, 18-H3); ^{13}C-NMR (100 MHz, CD_3OD) δ (ppm) 135.48, 135.14, 83.97, 71.26, 69.66, 51.49, 51.41, 50.61, 45.38, 44.98, 44.75, 40.02, 38.91, 37.70, 37.39, 31.88, 31.57, 29.11, 28.99, 28.95, 28.91, 27.18, 24.55, 21.97, 21.84, 20.58, 19.12, 19.09, 17.07, 16.18. MS (ESI): calculated for $C_{30}H_{52}O_3Na$ $[M+Na]^+$: 483.38, found 483.38.

(2S,3S,5S,6R)-2-((benzoyloxy)methyl)-6-(((R)-2-methyl-6-((5R,10S,13R,14R,17R)-4,4,10,13,14-pentamethyl-3-oxo-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)heptan-2-yl)oxy)tetrahydro-2H-pyran-3,4,5-triyl tribenzoate (43). To a 50 ml round-bottom flask, were added 60 mg (0.14 mmol, 1.0 eq) of compound **20**, 130 mg of trichloroacetonitrile carbohydrate donor (0.18 mmol, 1.3 eq), activated 3A molecular sieve and 25 ml anhydrous DCM, then the flask was charged with argon, TMSOTf (3uL, 10%) was added to the flask at 0 ˚C. After stirred at room temperature for 1h, the solvent was washed with saturated $NaHCO_3$ solution. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed by evaporation, then the mixture was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc= 10:1), yielding 118 mg compound **43** (I.Y. = 85%).

^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 8.00 (d, *J* = 7.7 Hz, 2H), 7.94 (d, *J* = 7.7 Hz, 2H), 7.90 (d, *J* = 7.7 Hz, 2H), 7.82 (d, *J* = 7.7 Hz, 2H), 7.55-7.50 (m, 3H), 7.44-7.33 (m, 10H), 7.30-7.26 (m, 2H), 5.92 (t, *J* = 9.7 Hz, 1H), 5.58 (t, *J* = 9.7 Hz, 1H), 5.50 (t, *J* = 8.4 Hz, 1H), 5.00 (d, *J* = 7.9 Hz, 2H), 4.60-4.56 (m, 1H), 4.50-4.45 (m, 1H), 4.19-4.15 (m, 1H), 2.62-2.54 (m, 1H), 2.42-2.37 (m, 1H), 1.20 (s, 6H), 1.12 (s, 3H), 1.09 (s, 3H), 1.06 (s, 3H), 0.84 (s, 3H), 0.72 (d, *J* = 6.1 Hz, 3H), 0.67 (s, 3H).

(2R,5R,10S,13R,14R,17R)-17-((R)-6-((tert-butyldimethylsilyl)oxy)-6-methylheptan-2-yl)-2-hydroxy-4,4,10,13,14-pentamethyl-1,2,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthren-3-one (44-1). To a solution of 380 mg **44** (0.6 mmol, 1.0 eq) in DCM were added 97.8 mg of m-CPBA (0.6 mmol, 1.0 eq) and 35.3 mg of $NaHCO_3$ (0.42 mmol, 0.7 eq) at 0 ˚C in portions. After stirred for 20 min, the resulting solution was washed with saturated $NaHCO_3$ and extracted with DCM. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed by evaporation, then the mixture was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 80:1), yielding 290 mg of intermediate **44-1** (I.Y. = 90%). ^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 4.65-4.60 (m, 1H), 1.17-1.16 (d, 6H), 1.11 (s, 3H), 1.08 (s, 3H), 0.90 (s, 6H), 0.71 (s, 3H), 0.14 (s, 3H), 0.02 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 214.08, 135.16, 133.69, 73.64, 71.62, 52.11, 50.45, 49.86, 48.19, 46.85, 45.62, 44.60, 37.87, 36.87, 36.52, 32.02, 30.91, 30.84, 30.18, 29.74, 29.45, 28.29, 26.21, 25.96, 25.94, 24.97, 24.34, 22.78, 21.84, 21.55, 20.92, 20.07, 19.31, 19.17, 18.81, 18.71, 18.19, 15.82, 14.50, -1.98, -4.50, -5.45.

Tert-butyl(((R)-6-((5R,10S,13R,14R,17R)-3-((tert-butyldimethylsilyl)oxy)-4,4,10,13,14-pentamethyl-4,5,6,7,10,11,12,13,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-

methylheptan-2-yl)oxy)dimethylsilane (44). To a solution of 140 mg compound **20** (0.32 mmol, 1.0 eq) in 20 ml anhydrous DCM was added 219 ul of TBDMSOTf (1.28 mmol, 4.0 eq), 187 ul of 2,6-lutidine (1.6 mmol, 5.0 eq) at 0 °C under argon. After stirred at room temperature for 2 h, the solvent was removed by evaporation, then the mixture was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 200:1), yielding 190 mg of compound **44** (I.Y. =90%). ^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 4.62-4.60 (m, 1H),1.19 (s, 3H), 1.18 (s, 3H), 1.05 (s, 3H), 1.00 (s, 3H), 0.95 (s, 12H), 0.86 (s, 12H), 0.73 (s, 3H), 0.17 (s, 3H), 0.15 (s, 3H), 0.07 (s, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 156.5, 156.5, 135.2, 133.1, 98.9, 73.7, 50.6, 50.2, 49.5, 45.7, 44.6, 38.5, 37.0, 36.7, 36.4, 31.3, 31.2, 30.3, 29.8, 28.6, 28.4, 27.1, 26.7, 26.1, 26.0, 24.4, 21.0, 20.8, 19.8, 19.6, 18.9, 18.6, 18.5, 18.3, 16.1, -1.88, -3.9, -4.5.

(2R,10S,13R,14R,17R)-17-((R)-6-((tert-butyldimethylsilyl)oxy)-6-methylheptan-2-yl)-2-fluoro-4,4,10,13,14-pentamethyl-1,2,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-3H-cyclopenta[a]phenanthren-3-one (45). To a solution of 100 mg 53 (0.15 mmol, 1.0 eq) in DCM/DMF : 3 ml/2 ml was added 50 mg of selectfluor (0.15mmol, 1.0 eq) at 0 ˚C. After stirred at room temperature for 1 h, the solution was washed with saturated $NaHCO_3$. The organic layer was dried over anhydrous Na_2SO_4 and purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc= 50:1), yielding compound **45** (I.Y. = 71%). ^{1}H-NMR(400 MHz, $CDCl_3$, representative signals) δ (ppm) 5.34 (ddd, *J*=48.0 Hz, *J* = 13.0 Hz, *J* = 6.2 Hz, 1H), 2.53-2.74 (m, 1H), 1.31 (s, 3H), 1.177 (s, 3H), 1.170 (s, 3H), 1.15 (s, 3H), 1.11 (s, 3H), 0.91 (d, *J* = 6.4 Hz, 3H), 0.89 (s, 3H), 0.87 (s, 9H), 0.71 (s, 3H), 0.05 (s, 6H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 210.2 (d, *J* = 11.8 Hz, 1C), 135.75, 133.0, 89.3 (d, *J* = 184.9 Hz, 1C), 73.6, 52.1, 50.4, 49.9, 48.5, 45.6, 44.6, 43.7, 43.6, 38.1, 38.0, 36.9, 36.5, 30.8, 30.2, 29.8, 28.3, 26.1, 26.0, 24.6, 24.4, 21.6, 21.4,

20.9, 20.1, 19.0, 18.8, 18.2, 15.8, -1.9.

(5R,8S,9S,10S,13R,14R,17R)-17-((R)-6-hydroxy-6-methylheptan-2-yl)-4,4,10,13,14-pentamethyldodecahydro-11H-8,9-epoxycyclopenta[a]phenanthren-3(2H)-one (46). To a 50 ml round-bottom flask were added 100 mg of compound **28** (0.22 mmol, 1.0 eq), 71.1 mg of PCC (0.33 mmol, 1.5 eq), 11 mg of dry NaOAc (0.13 mmol, 0.6 eq) and 10 ml DCM. After stirred at room temperature for 2 h, the resulting solution was washed with water. Organic layer was purified by 200-300 mesh silica gel flash column chromatography (Hexane:EtOAc = 20:1), yielding 86 mg of compound **46** (I.Y. = 85%). ^{1}H-NMR (400 MHz, $CDCl_3$, representative signals) δ (ppm) 2.56-2.40 (m, 2H), 1.24 (s, 3H), 1.20 (s, 6H), 1.02 (s, 3H), 1.00 (s, 3H), 0.89-0.89 (m, 6H), 0.77 (s, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm) 217.9, 135.47, 133.28, 71.17, 51.4, 50.6, 50.0, 47.5, 44.6, 44.5, 37.0, 36.9, 36.6, 36.2, 34.7, 31.1, 31.0, 29.5, 29.4, 28.3, 26.5, 26.3, 24.4, 21.4, 21.2, 19.6, 18.8, 16.0.

Plasmid constructs

αA-Y118D, αB-R120G, βB2-V187E, γC-G129C and γD-W43R are the mutants of αA-, αB-, βB2-, γC- and γD-crystallin, inserted into the pEGFP-N1 plasmid as described previously[2]. All recombinant plasmids were transformed into *E. coli* DH5α cells and the endotoxin-free plasmids were obtained using the Plasmid Maxiprep kit (Vigorous) and verified by DNA sequencing.

Cell culture

HeLa cells and human lens epithelial cells (B-3) were obtained from the China center of ATCC. The HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS), HLE-B3 cells were cultured in F12 medium with 20% FBS, all cells were cultured at 37°C in 5% CO_2 incubator. The cells for microscopy analysis were

seeded on glass coverslips pretreated with TC (Solarbio), after cultivated for 24 h to reach 90% confluency, the cells were transfected with plasmids containing the mutated crystallin genes. The transfection process was performed using Lipofectamine™ 2000 (Invitrogen) according to the instructions from the manufacturer. After transfection for 4 h, the cells were further cultivated with fresh DMEM medium containing 10% fetal bovine serum for 16 h. Then the transfection cells were cultivated with 4 μM lanosterol analogues for 8 h, 4 μM lanosterol was as the positive control, 4 μM cholesterol was as the negative control, the solvent 0.1% DMSO was used as control, and we also tested the activity of C29.

Fluorescence microscopy

The microscopy samples were prepared by washing the slips by phosphate buffered saline (PBS) three times. The cells were fixed with 4% paraformaldehyde for 40 min followed by three times washing with PBS. The cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked with 5% normal Goat serum in PBS for 1 h at 37 °C. Immunostaining was carried out by adding anti-p62 antibody (1:200) in PBS buffer containing 5% normal goat serum and cultivated for 1 h at 37 °C. Then the slips were washed three times with PBS, and further incubated with Alexa 647-conjugated goat anti-rabbit IgG (1:250) for 1 h at ambient temperature. The nuclei were counterstained with Hoechst 33342. The mounted cells were analyzed using a Carl Zeiss LSM 710 confocal microscope. The impact of lanosterol analogues on intracellular aggregation were evaluated in single-blinded observer studies. Experiments have been repeated at least three times. The image processing and data analysis were by ImageJ. P values were calculated using Student's t-tests. Each experiment had three replicates, and repeated at least three times.

Cell viability assay

The cells transfected with plasmids containing the mutated crystallin gene, the peGFP-N1 plasmid as control and the no-transfected cells were seeded in each pool of 96-well plates cultivated for 24 h to reach 90% confluency. All cells were cultivated with 4 μM, 0.4 μM or 40 nM lanosterol analogues for 12 h. After treatment, the cells were further cultivated in fresh DMEM medium with the CCK-8 reagent and incubated at 37 °C for 1 h. Then the absorbance at 450 nm was recorded using a micro-plate reader (Bio-Rad Model-680).

Protein aggregation and aggregate dissociation

The aggregates of αA-Y118D and αB-R120G proteins were obtained by heating the protein solutions containing 1M guanidine chloride (ultrapure, Sigma-Aldrich) at a concentration of 5mg/ml at 60 °C for 2 h. The aggregates of mutated β- and γ-crystallins were prepared by heating the protein solutions in pH 2 at 37 °C for 48 h. The formation of aggregates was confirmed by ThT fluorescence and transmission electron microscopy (TEM) observations. The preformed aggregates were re-suspended in 20 mM PBS with a final concentration of 0.2mg/ml (approximately 10 μM). The re-suspended aggregates were treated with 200 μM lanosterol, compound 6 and compound 34 in liposomes formed by 200 μM DPPC (Sigma-Aldrich) at 37 °C. Aggregates treated by 200 μM DPPC liposome were used as a negative control. After 24 h of treatment, the protein solutions were used for ThT fluorescence and negatively stained TEM observations. The TEM samples were prepared by depositing the protein solutions onto a freshly glow-discharged carboncoated copper grid. Negative-staining samples were obtained by staining the grid with 1.25% uranyl acetate for 30 s. The negatively stained TEM pictures were obtained on a Hitachi H-7650B transmission electron microscope with a voltage of 120kV and a magnification of 48,000.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Figure S1. Confocal images of crystallin protein aggregates in human lens progenitor cells; **Figure S2.** Number system for lanosterol; NMR sprectrum for compounds **4**, **6**, **9**, **11**, **13**, **16**, **19**, **21-30**, **32-37**, **44-46**. (PDF)

Molecular formula strings (CSV)

Corresponding Author

ybyan@tsinghua.edu.cn (Yong-Bin Yan)

yrao@tsinghua.edu.cn (Yu Rao)

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ABBREVIATIONS USED

SAR, structure activity relationships; DCM, dichloromethane; DMAP, 4-dimethylaminopyridine; m-CPBA, meta-chloroperoxybenzoic acid; DMP, Dess-Martin periodinane; TMSOTf,

trimethylsilyl trifluoromethanesulfonate; TFA, trifluoroacetic acid; PCC, pyridinium chlorochromate; NMR, nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

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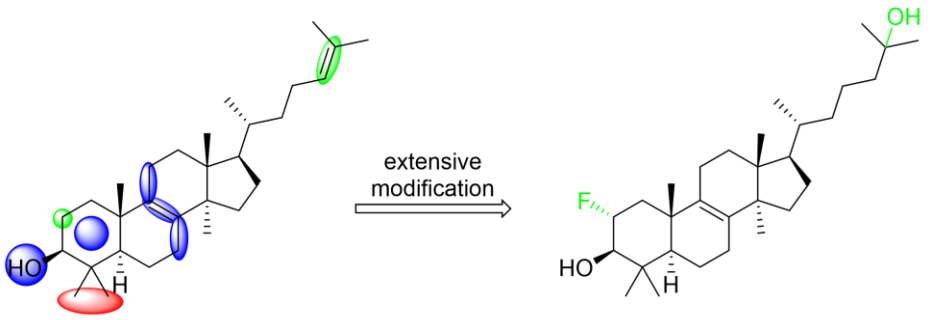
Table of Contents graphic



lanosterol
EC50(αB R120G) = 1.42 uM
effective for reversing
α, β and γ-crystallins aggregation

Compound 35
EC50(αB R120G) = 18 nM
effective for reversing
α, β and γ-crystallins aggregation