European Journal of Medicinal Chemistry xxx (xxxx) xxx

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Discovery of lysosome-targeted covalent anticancer agents based on isosteviol skeleton

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ARTICLE INFO

Article history:
Received 26 July 2020
Received in revised form
25 September 2020
Accepted 27 September 2020
Available online xxx

Keywords:
Covalent inhibitors
Isosteviol
α
β-Unsaturated ketone
Five-membered unsaturated lactone
Lysosomes

ABSTRACT

Covalent drugs play corresponding bioactivities by forming covalent bonds with the target, which possess many significant pharmacological advantages including high potency, ligand efficiency, and long-lasting effects. However, development of covalent inhibitors is a challenge due to their presumed indiscriminate reactivity. Here, we report the discovery of series of lysosome-targeting covalent anticancer agents by introducing nitrogenous bases to the modified isosteviol skeleton in order to minimize the toxicity and increase the selectivity. By introducing the electrophilic α, β-unsaturated ketones into the A- and D-rings of isosteviol, the cytotoxicity of the obtained compounds were greatly increased. Further nitrogen-containing modifications to the D-ring led to the discovery of novel molecules that targeted lysosomes, and of which, compound **30** was the most potent and selective antiproliferative one to kill A549 cells in vitro and in vivo. Mechanism investigation revealed that compound **30** was trapped into lysosomes and damaged lysosomes to cause cell death.

1. Introduction

Covalent inhibitors that form a covalent attachment to their target have engendered anxiety concerning their potential for off-target reactivity and have led to them being disfavoured as a drug class [1]. However, covalent modifications have several properties that are beneficial for clinical use, for example, potent binding affinity with targets and less susceptible to drug resistance, etc. [2]. And, exploration of covalent inhibitors into clinical drugs has never been stopped. Many covalent drugs had been approved for clinical use by the FDA in recent years, such as afatinib (2013), ibrutinib (2013), osimertinib (2015) and acalabrutinib (2017) (Fig. 1) [3]. On the development of covalent inhibitors, researchers found out that a suitable reactivity window of electrophiles ($t_{1/2}$ with GSH: 25−400 min) could minimize toxicities and achieve selective target inhibition [4,5]. The findings provide a guidance to surmount challenges for the development of covalent agents.

The poor selected intracellular distribution and multitargeting of covalent inhibitors would also increase the probability of off-target reactivity and restrict their development into drugs. Lysosomes are key monolayer-membrane subcellular organelles that are involved in several cellular processes, including receptor degradation, autophagy, apoptosis, post-translational protein maturation, and the extracellular release of active enzymes [6]. They are more numerous, larger in volume, less stable, and exhibit greater cathepsin activity in cancer cells than those in normal cells [7]. They hold promise as drug targets for improved and selective drug therapy [8]. And weakly basic drugs have been found to accumulate specifically in lysosomes because of the acidic lysosomal conditions and eventually trigger cancer cell death [9,10]. Therefore, design of alkalescent covalent inhibitors for targeting lysosomes is a good strategy to deliver the inhibitors into these

Abbreviations: *m*-CPBA, 3-chloroperbenzoic acid; PCC, pyridinium chlorochromate; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; MsCl, methylsufonyl chloride; n-BuLi, n-butyllithium; *t*-BuOOH, tert-butyl hydroperoxide; LiHMDS, lithium bis(trimethylsilyl)amide; THF, tetrahydrofuran; DMF, N,N-dimethylformamide; DCM, dichloromethane; MeOH, methanol; LAMP1, lysosome associated membrane protein 1; LMP, lysosome membrane permeabilization; CTSB, cathepsin B.

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https://doi.org/10.1016/j.ejmech.2020.112896
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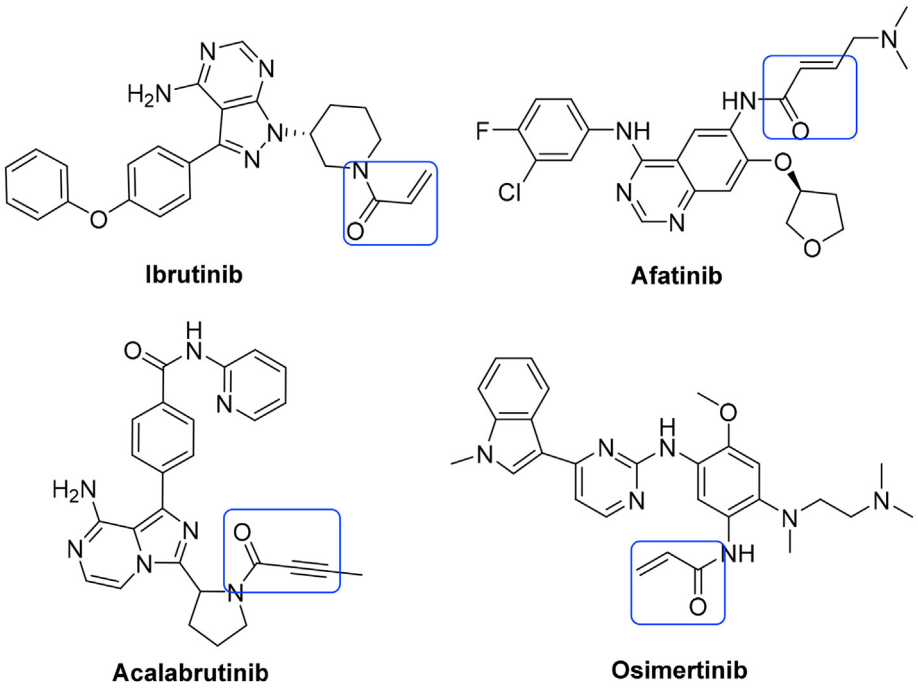


Fig. 1. Examples of US FDA-approved covalent inhibitors. The covalent warheads are highlighted in blue boxes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

specific subcellular organelles in order to prevent the off-target effects.

The scaffolds of natural products are biological relevance that is beneficial for drug development [11,12]. Tetracyclic diterpenes are a large class of natural products and many of these compounds have been clinically applied as cancer therapeutic agents, including oridonin [13]. Isosteviol, which has a typical tetracyclic diterpenoid skeleton, has attracted considerable attention due to its extensive pharmacological profiles, for example, anti-inflammation, anti-tumor and antimicrobial activity [14,15]. Using the scaffold of isosteviol could provide access to templates representative of the large and diverse family of diterpenes [16]. Consequently, modification of the scaffold of isosteviol is an attractive strategy to create natural product-like compounds with better pharmacological activities. Currently, most approaches were designed by coupling ester appendages to the carboxyl group [15,17]. However, little effort has been directed toward chemical modifications of the skeleton of isosteviol A-ring, possibly because of the synthetic challenges caused by the lack of reactive functionalities.

isosteviol, lbcellular organellTwo covalent warheads, α, β-unsaturated ketone [18] and five-membered unsaturated lactone [19], are frequently found in natural products and are essential for the potent cytotoxicity observed in many natural and synthetic molecules. They could covalently bind to proteins by reacting with the thiol groups of proteins resulting in protein modification and leading to cell death, and destruction of these functional groups would result in a loss of bioactivity [20,21].

Herein, these two warheads were first constructed in the skeleton of isosteviol A-ring to improve the cytotoxic activity (Fig. 2), and the covalent reactivity with glutathione (GSH) was also detected to assess the drug-like property. Meanwhile, weakly basic groups, especially groups that contain nitrogen atoms, were also introduced to the isosteviol skeleton for targeting lysosomes to further improve the selectivity.

2. Results and discussion

2.1. Construction of α, β-unsaturated ketones

Our synthesis commenced with the naturally abundant and commercially available stevioside (Scheme 1). Hydrolysis of stevioside with 10% H_2SO_4 gave isosteviol in high yield. Isosteviol was then treated with oxalyl chloride to afford an intermediate acyl chloride **3**, which was converted into pyridyl sulfide **4** by treatment with sodium omadine. Subsequent oxidation of pyridyl sulfide **4** with *m*-CPBA at −78 °C in CH_2Cl_2 gave an intermediate sulfoxide, which was eliminated in refluxing toluene to give C4-alkene **5** in 57% yield for the three steps [22]. Allyl position of compound **5** was oxidized with selenium dioxide and *tert*-butyl hydroperoxide to give compound **7** stereoselectively. In this step, the seleninic acid intermediate **6** was only formed from the β-face of the A-ring because of steric effects, eventually leading to the installation of hydroxyl group at 3β-position, as determined by HMBC and NOESY experiments (Fig. S1). The hydroxyl group of **7** was oxidized by PCC to give ketone **8**, which was then treated with DDQ for further oxidative dehydrogenation to produce compound **9**.

Hence, the α, β-unsaturated ketone was installed in the A-ring of isosteviol successfully. As a part of our initial efforts to discover more active new agents by targeting lysosomes, a Mannich base was formed on the D-ring by one-pot Mannich reaction, which is one of the most important C—C bond-forming reactions for the construction of nitrogen-containing compounds [23], to give compound **10**. Mannich base can be easily transformed to Michael acceptor via elimination of amine moiety and can be used as a prodrug [24]. To evaluate directly antiproliferative activity of the elimination product of Mannich base **10**, it was treated with methyl iodide followed by basic Al_2O_3 to generate product **11** with an *exo*-methylene cyclopentanone in the D-ring.

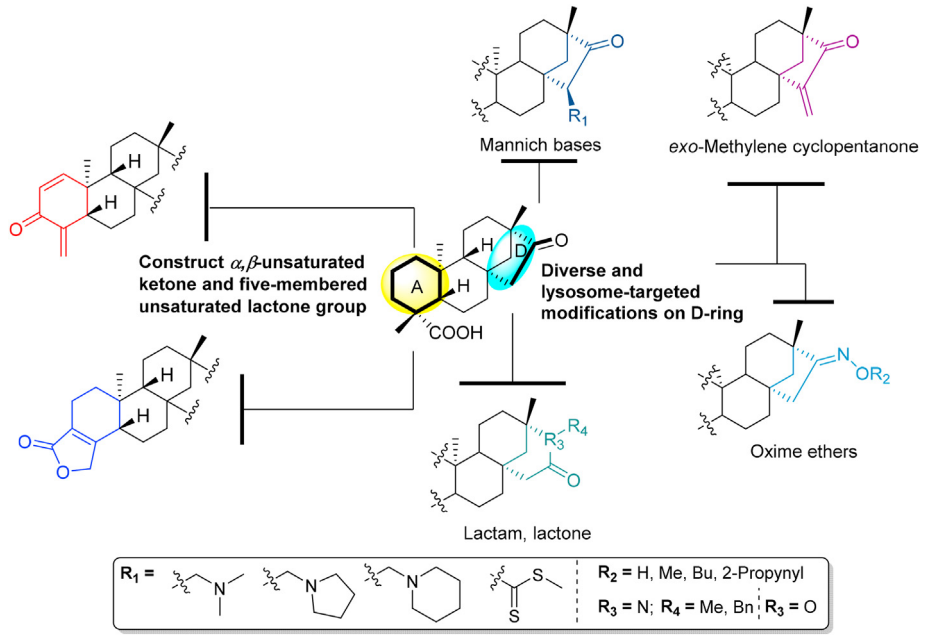
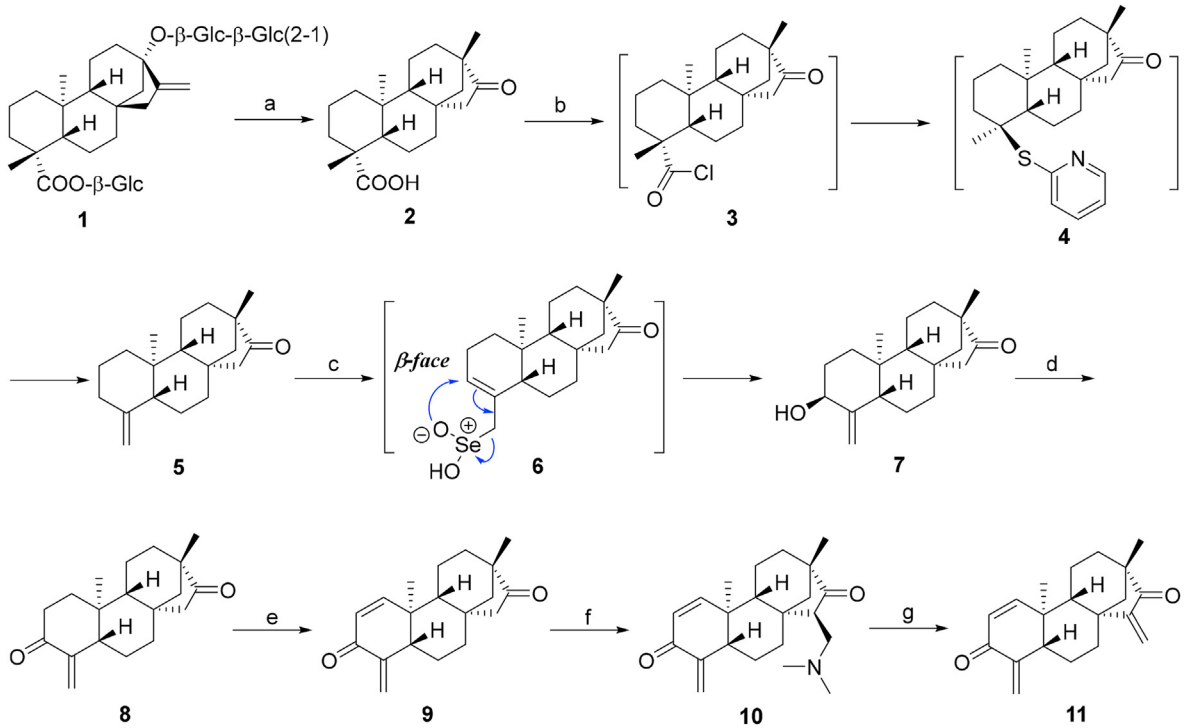


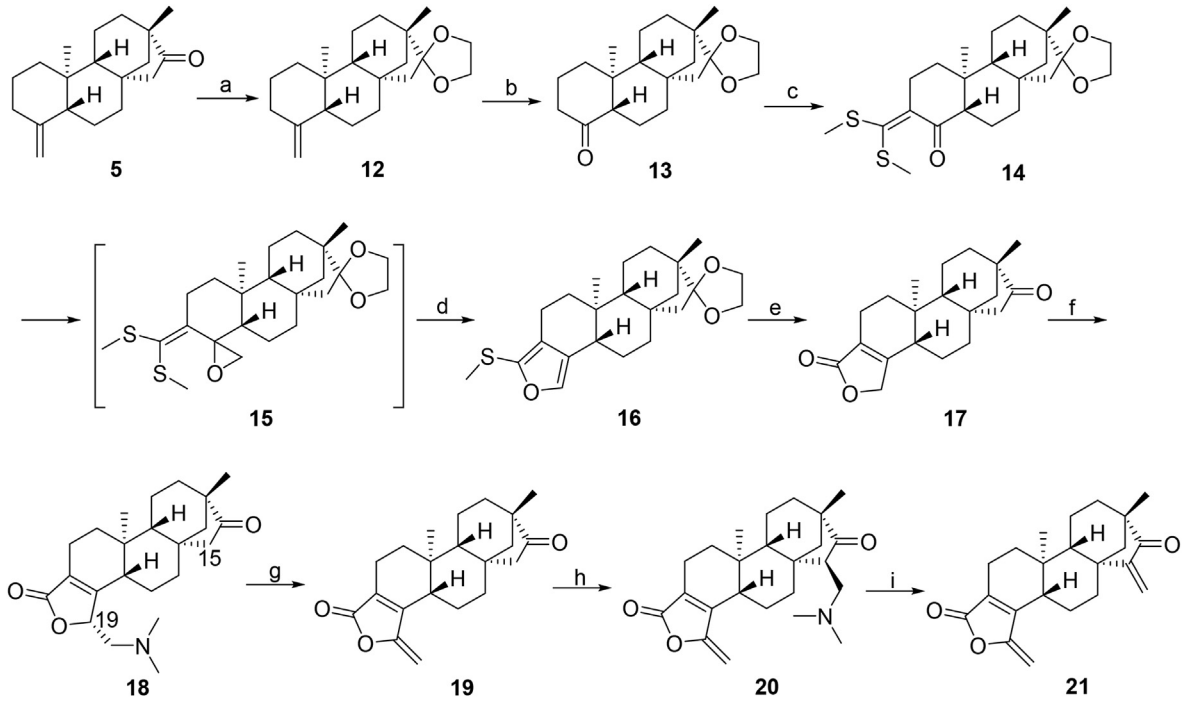
Fig. 2. Design strategy of diverse isosteviol derivatives.



Scheme 1. Introduction of an α, β-unsaturated ketone in the A-ring of isosteviol. *Reagents and conditions:* (a) 10% H_2SO_4, 90%; (b) 1) $(COCl)_2$, DMF, DCM; 2) sodium omadine, DMAP, toluene; 3) *m*-CPBA, toluene; 57%; (c) SeO_2, t-BuOOH, DCM, 90%; (d) PCC, DCM, 97%; (e) DDQ, dioxane, 59%; (f) LiHMDS, eschenmoser's salt, dry THF, 85%; (g) CH_3I, Al_2O_3, diethyl ether-dichloromethane (3:1), 60%.

2.2. Construction of five-membered unsaturated lactones

After introduction of the α, β-unsaturated ketone in the isosteviol skeleton, we turned our attention to another important class of five-membered unsaturated lactones. The synthetic strategy was showed in Scheme 2. The target products were synthesized starting from compound **5**, the carbonyl group of which was protected by ethylene glycol to give ketal **12**. Ozonolysis of the alkenyl group of **12** afforded ketone **13**. Compound **13** was reacted with carbon disulfide in the presence of lithium bis(trimethylsilyl)amide followed by addition of methyl iodide to afford the ketene dithioacetal **14**. Then, compound **14** was reacted with trimethylsulfonium iodide and *n*-butyllithium to yield an intermediate epoxide **15**, which was further treated with 0.5 M H_2SO_4/MeOH at room temperature



Scheme 2. Introduction of a five-membered unsaturated lactone in the A-ring of isosteviol and derivatives. *Reagents and conditions:* (a) 1,2-ethanediol, camphorsulfonic acid, cyclohexane, 96%; (b) O_3, dimethyl sulfide, DCM-MeOH (1:1), 72%; (c) 1) LiHMDS, CS_2, dry THF; 2) CH_3I, 88%; (d) $(CH_3)_3SI$, n-BuLi, dry THF, 89%; (e) 0.5 M H_2SO_4, MeOH, quantitative; (f) LiHMDS, eschenmoser's salt, dry THF, 65%; (g) CH_3I, Al_2O_3, diethyl ether-dichloromethane (3:1), 55%; (h) LiHMDS, eschenmoser's salt, dry THF, 43%; (i) CH_3I, Al_2O_3, diethyl ether-dichloromethane (3:1), 37%.

without isolation. However, this reaction failed to give the anticipated compound **17**, and resulted in an intermediate **16**, which was relatively stable. To our delight, increasing the reaction temperature to 60 °C produced the unsaturated lactone **17**. Then, we intended to prepare the Mannich base at C-15 using procedures similar to the preparation of compound **10** described above. Unfortunately, the reaction produced a complex mixture and the anticipated product was not found. Decreasing the temperature to −78 °C, unexpectedly resulted in only compound **18**, instead of the Mannich base at C-15, being obtained stereoselectively in 65% yield; the stereostructure was determined by HMBC and NOESY experiments (Fig. S2). These results suggested that the hydrogen atom at C-19 was extremely active under organic alkali conditions. Similarly, elimination of dimethyl amine of **18** provided compound **19**. Then, Mannich base of **19** at C-15 was formed through a further Mannich reaction similar to the preparation of **10**. Elimination of the dimethyl amine of compound **20** also formed an *exo*-methylene cyclopentanone in the D-ring.

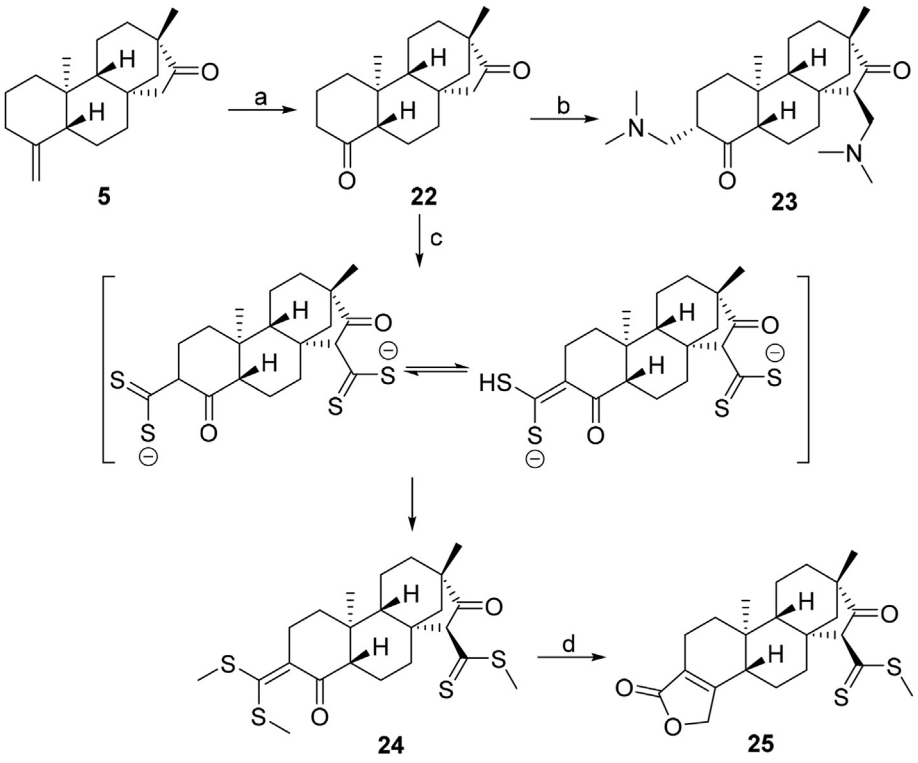
2.3. Construction of a mannich base or a five-membered unsaturated lactone in both the A- and D-rings simultaneously

As shown in Scheme 3, we attempted to introduce a Mannich base or a five-membered unsaturated lactone in both the A- and D-rings simultaneously, using the skeleton of isosteviol. This synthesis also started with compound **5**, which was oxidized by ozone to give the diketone **22**. A dimethyl amine was introduced into A- and D-rings by the Mannich reaction to produce the Mannich base **23**, stereoselectively. In this step, one dimethylaminomethyl was located at the equatorial position of C-3 and the other located on C-15 was positioned away from the skeleton to ensure the lowest energy; the stereostructure was determined by HMBC and ROESY experiments (Fig. S3). The results just explained the configuration of the Mannich bases mentioned above. Treatment of compound **22** using procedures similar to the preparation of **14**, however, failed to convert the thioketones to thioenols in the D-ring, presumably because the pentacyclic tension made the thioketones more stable. The stereostructure of compound **24** was determined by HMBC and NOESY experiments (Fig. S4). The ketene dithioacetal in the A-ring could be further converted to the five-membered unsaturated lactone **25**.

2.4. Detection of antiproliferative activity of representative compounds and structure-activity relationship (SAR) analysis

The antiproliferative effects of representative compounds from those obtained above against various human cancer cell lines were evaluated and summarized in Table 1. A normal human umbilical vein endothelial cell line (HUVEC) was included to compare the selectivity of these compounds. The results indicated that the compounds differentially exhibited antiproliferative activity. Compounds **9**, **10**, **11**, **20** and **21** were the most active antiproliferative agents among the tested derivatives. In the analysis of each individual cell line, K562 cells were shown to be potently inhibited by compounds **10**, **11**, **20** and **21**, with IC_{50} values of 0.80, 0.40, 2.1 and 1.2 μM, respectively, compared with the positive control (adriamycin IC_{50} = 0.74 μM). And compounds **9**, **10**, **11**, **20** and **21** could effectively inhibit A549 cells, with IC_{50} values of 3.2, 2.0, 1.4, 2.8 and 2.0 μM, respectively, compared with adriamycin (IC_{50} = 1.7 μM). The antiproliferative activity of these derivatives against MCF-7 cells was moderate. HCC1428 cells were strongly inhibited by compounds **10** and **11**, with IC_{50} values of 3.1 and 1.5 μM, respectively, compared with adriamycin (IC_{50} = 1.1 μM). And HEPG-2 cells were highly susceptible to compounds **9**, **11** and **21**, with IC_{50} values of 2.1, 0.90 and 2.6 μM, respectively, compared with adriamycin (IC_{50} = 1.6 μM).

The SARs of these derivatives were analyzed and summarized. Compound **9** containing an α, β-unsaturated ketone group in A-ring



Scheme 3. Introduction of a Mannich base or five-membered unsaturated lactone in both the A- and D-rings simultaneously. *Reagents and conditions:* (a) O_3, dimethyl sulfide, DCM-MeOH (1:1), 82%; (b) LiHMDS, eschenmoser's salt, dry THF, 27%; (c) 1) LiHMDS, CS_2, dry THF; 2) CH_3I, 78%; (d) 1) $(CH_3)_3SI$, n-BuLi, dry THF; 2) 0.5 M H_2SO_4, MeOH, 75%.

was slightly more active than compound **17** with a five-membered unsaturated lactone against most of the tested cancer cells, except for K562 cells. The antiproliferative activity of **17** ($IC_{50} = 10$ μM) was slightly better than **9** ($IC_{50} = 4.7$ μM) against K562 cells. Introduction of a Mannich base group in D-ring could obviously increase antiproliferative activity against most of the tested cancer cell lines (**9**, **19** be compared with **10** and **20**, respectively). This might be due to the influence of base group on their relative lysosome localization and also the Mannich base group could be eliminated slowly to form another functional group of α, β-unsaturated ketone. There were exceptions: the activity of **10** was slightly decreased compared with **9** against HEPG-2 cells and the activity of **20** was slightly decreased compared with **19** against MCF-7 cells. Compound **18** with a Mannich side chain on the five-membered unsaturated lactone group obviously diminished antiproliferative activity compared with **17**. By elimination of this dimethylamine group to C—C double bond (**19**), the activity was increased compared with **18**. Compounds **11** and **21** with an *exo*-methylene cyclopentanone in D-ring significantly increased antiproliferative activity compared with **9** and **19**, respectively. Compound **11** with two α, β-unsaturated ketone groups exhibited the best antiproliferative activity ($IC_{50} = 0.4$ μM against K562), but poor selectivity to normal cells.

2.5. Structural optimization of compound **9**

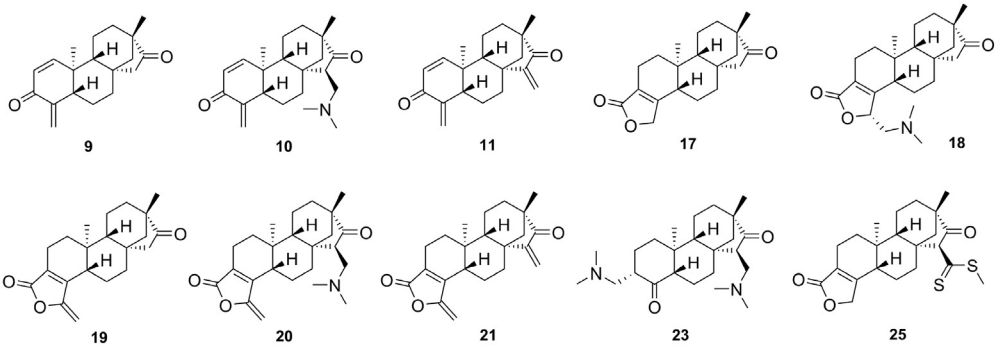
Although compound **11** had the most potent activity, the poor selectivity impeded its development. Compound **9** displayed a moderate antiproliferative activity, but its structure was easy to be modified. So, we chose compound **9** as a basic skeleton for further modifications to decrease toxicity and enhance activity. As shown in Scheme 4, Firstly, two Mannich side chains including pyrrolidinyl and piperidinyl were introduced into the D-ring to produce Mannich bases **26** and **27**, respectively. Then, diverse heterocycles were tried to prepare to increase structural diversity. Lactone compound **28** was synthesized by oxidation of compound **9** with *m*-CPBA. This reaction was highly site selective, presumably because quaternary carbon migrates more easily than secondary carbon. Next, oxime **29** was gotten by treatment of compound **9** with hydroxylamine. The oxime **29** was further etherified with different alkyl bromides (RBr) to produce oxime ethers **30**—**32**. The oxime **29** was converted to mesylate **33** by MsCl, followed by treatment with acid in methanol, exclusively leading to lactam **34**. This procedure appeared to undergo a Beckmann rearrangement, and the proposed mechanism was shown in Fig. S5. To study the effect of substitutions on the amide, the lactam **34** was subsequently alkylated with methyl iodide, and benzyl bromide, to furnish compounds **35** and **36**, respectively.

2.6. Further antiproliferative activity evaluation and SAR analysis

The antiproliferative activity of each of these derivatives was then evaluated (Table 2). Except for compounds **28** and **34**, the antiproliferative activity of the other derivatives was improved significantly against all the tested cancer cell lines compared to their parent compound **9**. Compounds **30** and **31** showed the best antiproliferative activity among all these derivatives, with IC_{50} value ranges of 0.29—0.92 and 0.47—1.3 μM, respectively, which were clearly superior to those of the positive control adriamycin. In addition to **30** and **31**, compounds **35** and **36** also exhibited potently antiproliferative activity against K562 cells, with IC_{50} values of 0.72 and 0.90 μM, respectively. And compound **29** exhibited significantly antiproliferative activity against HEPG-2 cells, with IC_{50} value of 0.61 μM.

The SARs of these novel derivatives were analyzed and summarized. Compounds **26** and **27** with different Mannich side chains

Table 1
Cytotoxicities of representative compounds against various human cancer cell lines[a].



Compds	IC_{50} (μM)					
	K562	A549	MCF-7	HCC1428	HEPG-2	HUVEC
9	10 ± 1.3	3.2 ± 0.28	11 ± 0.91	5.7 ± 0.37	2.1 ± 0.11	15 ± 1.2
10	0.80 ± 0.065	2.0 ± 0.17	4.0 ± 0.26	3.1 ± 0.29	4.6 ± 0.39	5.9 ± 0.55
11	0.40 ± 0.037	1.4 ± 0.11	5.6 ± 0.43	1.5 ± 0.16	0.90 ± 0.10	1.5 ± 0.17
17	4.7 ± 0.38	5.6 ± 0.47	12 ± 1.4	17 ± 2.1	> 20	> 20
18	14 ± 1.5	> 20	15 ± 1.9	> 20	11 ± 1.3	> 20
19	7.7 ± 0.87	13 ± 1.5	7.0 ± 0.81	16 ± 1.8	10 ± 1.2	18 ± 2.1
20	2.1 ± 0.27	2.8 ± 0.25	11 ± 1.2	5.7 ± 0.63	9.5 ± 1.3	16 ± 1.3
21	1.2 ± 0.13	2.0 ± 0.19	4.4 ± 0.48	6.2 ± 0.54	2.6 ± 0.29	7.1 ± 0.73
23	7.9 ± 0.81	16 ± 1.8	15 ± 1.3	> 20	15 ± 1.6	> 20
25	17 ± 2.0	8.1 ± 0.92	> 20	> 20	11 ± 1.4	> 20
ADR[b]	0.74 ± 0.053	1.7 ± 0.085	1.1 ± 0.088	1.1 ± 0.18	1.6 ± 0.10	4.3 ± 0.25

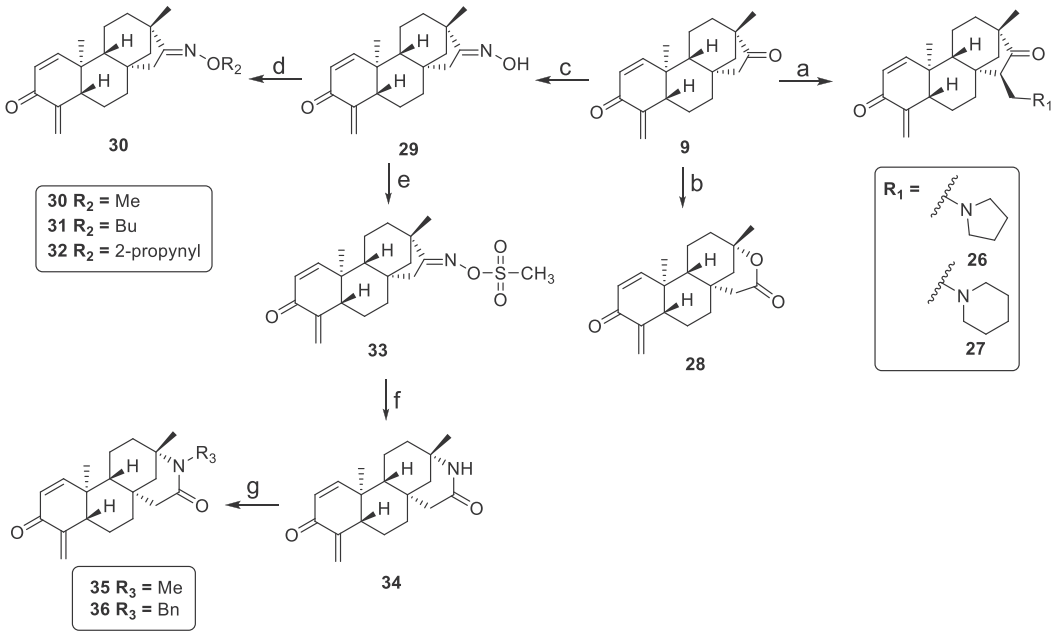
[a]Results are expressed as the mean IC_{50} ± standard deviation (SD) in μM. [b]Positive control, ADR represent adriamycin. The experiments were performed three times.

showed enhanced antiproliferative activity compared with the parent compound **9**. And there was no significant difference in activity among compounds with different Mannich side chains. Introduction of a lactone or lactam group in D-ring couldn't improve the antiproliferative activity. However, the antiproliferative activity of the methyl or benzyl substituted derivatives of lactam (**35** and **36**) was improved significantly, suggesting that introduction of N-methyl or N-benzyl protected lactam was beneficial to the improvement of activity. Oxime derivatives **29**–**32**, especially oxime methyl ether **30** and oxime butyl ether **31**, showed the most potent antiproliferative activity. Compound **30** exhibited the best activity and remarkable antiproliferation of all the tested cancer cell lines. In particular, the A549 cell lines had the lowest IC_{50} value of 0.29 μM (Fig. 4A). The toxicities of compound **30** to normal human mammary epithelial cells (HUVEC) and normal human lung cells (Beas-2b) were much low, and the IC_{50} values were 2.9 μM and 14 μM, respectively (Tables 2 and S1). It showed a good selectivity.

2.7. Covalent bond formation reactivity of candidate *30*

Covalent inhibitors have the potential to cross-react with proteins. Such 'off-target' activity could lead to unanticipated toxicities in drug development. Previous studies suggested that the suitable reactivity window of covalent drugs, in terms of $t_{1/2}$ with GSH, is from 25 to 400 min [5]. Once this selectivity range is breached, potential off-target effects would be observed. Thus, the reaction rate of compound **30** with GSH was detected to evaluate the selectivity and druggability.

Compound **30** was incubated with 1.5–2 equivalence of GSH at 37 °C in 0.5 mL of DMSO-d_6 and 0.05 mL of sodium phosphate deuterium oxide buffer (pH 7.4). The sample was analyzed by ^{1}H NMR at 10 time points for quantitatively describing the reactivity of the covalent warhead with GSH (Fig. 3). Based on the disappearance of ^{1}H NMR peaks **a**, **b** and **c** and generation of new peaks **d** and **e** during the reaction, the half-life value of compound **30** with GSH was identified from 30 to 60 min, in line with the reactivity window (25~400 min) suggested for covalent drugs. The results indicated that the covalent warhead of compound **30** is mild and it showed a suitable drug-like property. Additionally, the adduct product was also validated by HRMS analysis of the reaction at 6 h, indicating the dominant component of the sample was the adduct **37**. ESI-HRMS (*m*/*z*): calcd for $C_{22}H_{29}O_3S_2$ $[M + H]^+$, 405.1553; found, 405.1552.



Scheme 4. Diverse modifications on the D-ring. *Reagents and conditions:* (a) LiHMDS, eschenmoser's salt, dry THF, 32% for **26**, 37% for **27**; (b) *m*-CPBA, K_2CO_3, DCM, 89%; (c) $NH_2OH^{\cdot}HCl$, $NaHCO_3$, DCM-MeOH, 82%; (d) NaH, alkyl bromides, dry DMF, 72% for **30**, 85% for **31**, 75% for **32**; (e) TEA, MsCl, DCM; (f) concentrated HCl, MeOH, 65% for two steps; (g) NaH, alkyl bromides, dry DMF, 93% for **35**, 80% for **36**.

2.8. The effects of candidate **30** on lysosomes

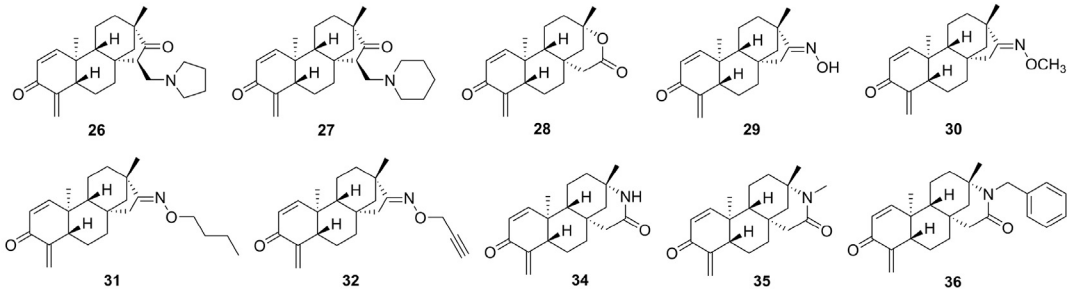
Thus, compound **30** was chosen as a promising candidate to investigate potential antiproliferative mechanisms in A549 cell lines. The cells were stained with Lysotracker Red to examine the effect of compound **30** on the acidic lysosomes. As shown in Fig. 4B and videos, the Lyso-Red staining became much weaker in response to compound **30**, demonstrating that abnormal changes occurred in the lysosomes. Similar changes in lysotracker fluorescent intensity were noted in cells exposed to compound **30** by quantitative flow cytometry analysis (Fig. 4C). In addition, lysosome associated membrane protein 1 (LAMP1), a type I transmembrane lysosomal glycoprotein, showed weaker fluorescence intensity and became dispersive in cells after compound **30** treatment compared with the integral lysosomes in the control (Fig. 4D). The result was consistent with the observation of the decreased expression level of LAMP1 measured by western blotting (Fig. 4E and S6). These results indicated that compound **30** targeted and damaged lysosomes. Any damage to the lysosomes would impair autophagic degradation, the final step of autophagy, and increase the numbers of autophagosomes [25]. As shown in Fig. 4E and S6, compound **30** hardly had any effects on Beclin-1 and LC3B—I, which indicated that compound **30** did not induce autophagy. However, compound **30** increased the accumulation of LC3BII and p62/SQSTM1, substrates of autophagy that are degraded in lysosomes, demonstrating that lysosome dysfunction was caused by compound **30**. To further determine whether compound **30** interrupted the fusion of autophagosomes with lysosomes, a pH-sensitive tandem mRFP-GFP-LC3 adenovirus construct was transfected into A549 cells before exposure to compound **30**. Hydroxychloroquine, a weak base that could increase lysosomal pH and inhibit the activity of lysosomes [26,27], was selected as a positive reference. Rapamycin, an inducer of autophagy, markedly activated autophagy flux, which could be observed by the presence of red puncture dots. As shown in Fig. 4F and S7, compound **30**-treated cells exhibited the green/yellow fluorescence of LC3 puncta clearly, which was similar to hydroxychloroquine-treated cells. These data indicated that compound **30** impaired autophagosome—lysosome fusion, through damaging to the lysosomes.

Lysosome membrane permeabilization (LMP), caused by dysfunction of lysosomes, is thought to trigger cancer cell death through apoptosis and apoptosis-like pathways that involve the release of certain cathepsins from lysosomes into cytoplasm [28]. Subcellular fractionation of the cells was carried out to directly measure the expression levels of cathepsins in cytoplasm and lysosomes. The cathepsin B (CTSB) activity in the cytosolic and lysosomal fractions was tested using Z-RR-AMC (a CTSB substrate). As shown in Fig. 5A, the levels of Z-RR-AMC were higher in the cytosolic fraction treated with compound **30** than that without treatment with compound **30** by 85.3%. The result indicated that compound **30** damaged lysosomes and induced lysosome membrane permeabilization and certain cathepsins were released from lysosomes to cytoplasm. In addition, we evaluated whether inhibition of cathepsins attenuated cell death to investigate the function of these cathepsins in compound **30**-induced A549 cytotoxicity. For these experiments, cells were pre-incubated with a cysteine (thiol) proteinase inhibitor (E64d), a CTSB inhibitor (CA074Me), a CTSL inhibitor (CLI), a CTSD inhibitor (pepstatin A), and a pan-caspase inhibitor (Z-VAD-fmk), prior to exposure to compound **30**. From Fig. 5B, we could see CA074Me and Z-VAD-fmk partly rescued cell death induced by compound **30**, indicated that inhibition of cathepsins could attenuate compound **30**-induced cell death. These results further proved that the cell death induced by compound **30** was caused by lysosomal disruption.

2.9. The in vivo efficacy of candidate **30**

Xenotransplantation of human tumor cells in zebrafish has been an important model utilized by cancer biologists for many years [29]. Because of the extensive advantages of high reproductive ability, superior imaging qualities, and little immunorejection [30], we selected the zebrafish human tumor xenograft model to assess

Table 2
Cytotoxicities of representative compounds against various human cancer cell lines[a].



Compds	IC_{50} (μM)					
	K562	A549	MCF-7	HCC1428	HEPG-2	HUVEC
26	1.7 ± 0.12	2.5 ± 0.32	7.4 ± 0.83	5.6 ± 0.48	1.1 ± 0.10	13 ± 1.1
27	1.9 ± 0.17	1.3 ± 0.10	8.5 ± 0.79	3.7 ± 0.28	2.2 ± 0.21	14 ± 1.3
28	9.4 ± 0.63	9.0 ± 0.47	16 ± 1.5	8.9 ± 0.75	7.0 ± 0.65	17 ± 1.9
29	1.2 ± 0.11	2.4 ± 0.12	3.5 ± 0.21	4.8 ± 0.27	0.61 ± 0.046	4.9 ± 0.41
30	0.41 ± 0.029	0.29 ± 0.017	0.92 ± 0.067	0.51 ± 0.056	0.39 ± 0.042	2.9 ± 0.27
31	0.55 ± 0.051	0.47 ± 0.031	0.89 ± 0.076	1.3 ± 0.11	0.91 ± 0.083	2.7 ± 0.21
32	4.2 ± 0.11	3.0 ± 0.097	2.3 ± 0.19	5.1 ± 0.42	3.6 ± 0.27	7.6 ± 0.58
34	4.9 ± 0.52	18 ± 1.7	> 20	14 ± 1.2	6.9 ± 0.58	> 20
35	0.72 ± 0.049	4.2 ± 0.36	9.8 ± 1.1	4.5 ± 0.47	2.1 ± 0.15	6.7 ± 0.59
36	0.90 ± 0.078	2.6 ± 0.23	3.6 ± 0.31	2.3 ± 0.20	1.3 ± 0.11	6.8 ± 0.54
ADR[b]	0.74 ± 0.053	1.7 ± 0.085	1.1 ± 0.088	1.1 ± 0.18	1.6 ± 0.10	4.3 ± 0.25

[a]Results are expressed as the mean IC_{50} ± standard deviation (SD) in μM. [b]Positive control, ADR represent adriamycin. The experiments were performed three times.

the efficacy and toxicity of compound **30** in vivo. Specifically, A549 cells were stained with cell tracker CM-Dil (red fluorescence color) and then transplanted into zebrafish embryos. Following transplantation, different concentrations of compound **30** were treated. Taxol was used as a positive control. As shown in Fig. 6A and B, the A549-cell xenografts treated with Taxol (1 μg/mL) or compound **30** (0.1 μg/mL and 0.2 μg/mL) showed reduced fluorescence intensities compared with the control group, indicating that compound **30** could significantly inhibit the proliferation of A549 cells in zebrafish xenografts. It's worth noting that compound **30** at a low concentration of 0.2 μg/mL could effectively inhibit the proliferation, similar to the positive control Taxol at 1 μg/mL.

Potential toxicity of compound **30** in vivo was then assessed by treatment of healthy zebrafish embryos with different concentrations of compound **30**. As shown in Fig. 6C, compound **30** exhibited no apparent toxicity towards zebrafish embryos at a dose of 2 μg/mL, but resulted in substantial toxicity at a dose of 4 μg/mL or higher, which was about 10–20 times higher than the effective dosage (0.2 μg/mL). These data suggested a potential application of compound **30** in oncotherapy. The relevant ethical protocols used for the in vivo study for zebrafish were followed by the institutional guidelines of Animal Care and Use Committee at Shandong University.

3. Conclusions

In summary, with the help of the beyerane skeleton of isosteviol, efficient and concise synthetic approaches have been developed for the introduction of α, β-unsaturated ketone and five-membered unsaturated lactone groups in the A-ring; these groups have been previously reported as active antitumor groups in natural and synthetic products. These new synthetic templates would also enrich the natural scaffold-based compound library. In addition, modifications of the D-ring with diverse nitrogen-containing groups generated a series of novel molecules. As expected, these new molecules demonstrated potent antiproliferative effects against various human cancer cell lines, in particular, compound **30** has an IC_{50} value of 0.29 μM against A549 cell lines and has a lower toxicity to normal human mammary epithelial cells (HUVEC) and normal human lung cells (Beas-2b) with IC_{50} values of 2.9 μM and 14 μM, respectively. And compound **30** also has a suitable drug-like property due to the mild reactivity with GSH ($t_{1/2}$: 30–60 min), which is important to minimize toxicities. Further research into the mechanism of action of compound **30** indicated that it targeted and damaged lysosomes and induced LMP to cause cell death. And it also exhibited significantly antiproliferative effect in xenograft zebrafish, thus confirming the in vivo activity of compound **30**. These studies suggest that compound **30** has the potential to be

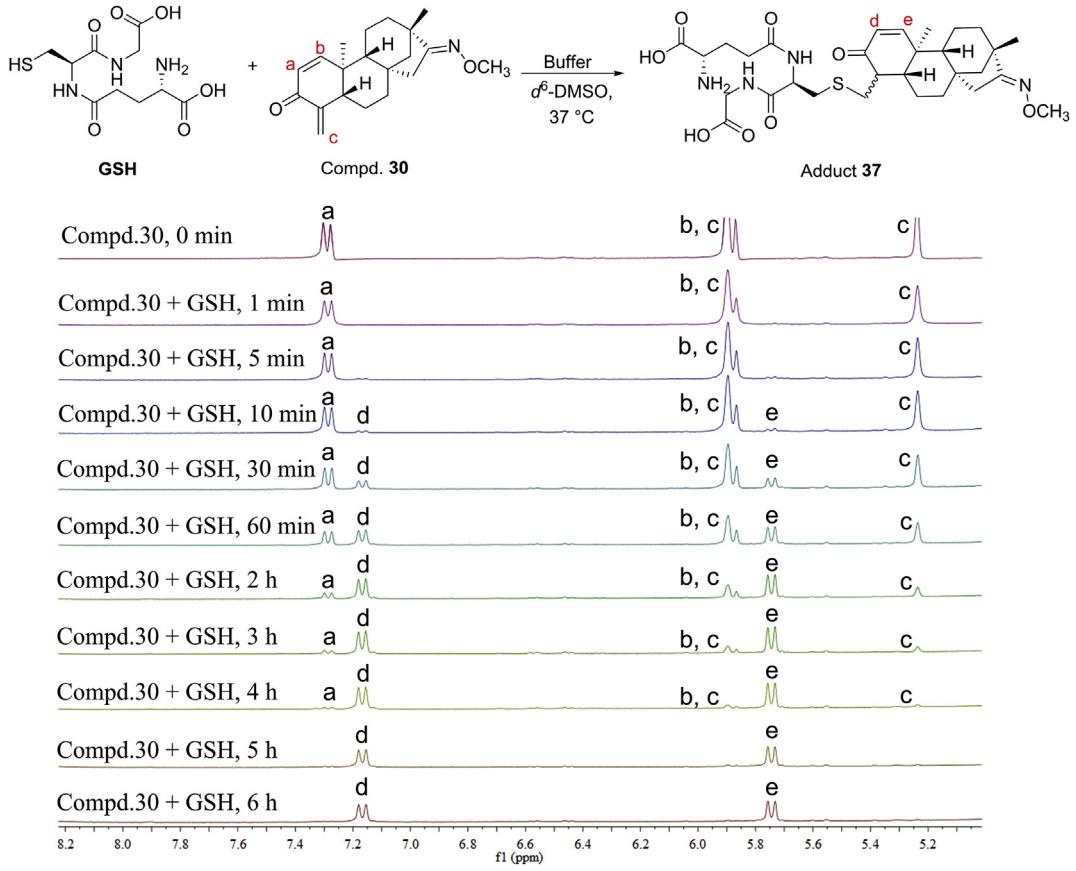


Fig. 3. Reactivity of compound **30** with GSH determined by ^{1}H NMR.

developed as a novel anticancer agent.

4. Experimental section

4.1. General material and methods for chemistry

All commercially available reagents were used without further purification. Anhydrous solvents were dried through routine protocols. All reactions were carried out under a nitrogen atmosphere in dry glassware with magnetic stirring. Column chromatography was carried out on 200–300 mesh silica gel (Qingdao Haiyang Chemical, China). Analytical TLC was carried out employing 0.25 mm silicagel plates (GF254) and visualization under UV light. The NMR spectra were recorded on a Bruker 400 (^{1}H, 400; ^{13}C, 101 MHz) or Bruker 600 (^{1}H, 600 MHz; ^{13}C, 150 MHz) spectrometer. Chemical shifts were expressed in ppm, and *J* values were given in Hz. High-resolution mass spectra were measured using a Thermo Fisher Finnigan LTQ Orbitrap Elite mass spectrometer. Ionization was achieved using the positive mode. The purity of the final compounds was verified using an HPLC system (Agilent Technologies 1200) equipped with a G1311A isopump, a G1322A degasser, and a G1315D DAD detector using an Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm). All compounds evaluated for biological effects were >95% pure.

4.1.1. (4R,4aS,6aR,9S,11aR,11bS)-4,9,11b-Trimethyl-8-oxotetradecahydro-6a,9-methanocyclohepta[a]naphthalene-4-carboxylic acid (**2**)

Isosteviol **2** was obtained by hydrolysis of stevioside **1** (4.0 g) with 30 mL of 10% sulfuric acid at 95 °C for 5 h. After cooling to room temperature, the reaction mixture was filtered and washed with saturated $NaHCO_3$ solution and H_2O. The crude product was recrystallized by ethanol as colorless crystals, yield 90%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.63 (dd, J = 18.6, 2.4 Hz, 1H), 2.15 (d, J = 13.3 Hz, 1H), 1.90–1.53 (m, 9H), 1.49 (dd, J = 13.6, 2.9 Hz, 1H), 1.45–1.32 (m, 3H), 1.24 (s, 3H), 1.22–1.11 (m, 3H), 1.07–1.00 (m, 1H), 0.97 (s, 3H), 0.95–0.85 (m, 1H), 0.77 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 183.8, 57.1, 54.8, 54.4, 48.9, 48.6, 43.8, 41.6, 39.9, 39.6, 38.3, 37.8, 37.4, 29.1, 21.7, 20.5, 20.0, 19.0, 13.4. ESI-MS *m/z* 317 [M − H]$^{-}$.

4.1.2. (4aR,6aR,9S,11aR,11bR)-9,11b-Dimethyl-4-methylenedodecahydro-6a,9-methanocyclohepta[a]naphthalen-8(7H)-one (**5**)

To a solution of compound **2** (200 mg, 0.62 mmol) in anhydrous dichloromethane (2 mL) was added $(COCl)_2$ (60 μL, 0.68 mmol) and DMF (1 μL) at 0 °C under N_2 protection. The reaction mixture was allowed to stir at room temperature for 3 h. Then, the solution was removed under reduced pressure to give a yellow solid **3**, which was dissolved in 3 mL of anhydrous toluene. Sodium omadine (111 mg, 0.74 mmol) and DMAP (15 mg, 0.12 mmol) were added, and the mixture was stirred under reflux. After 4 h, the mixture was filtered and concentrated under reduced pressure to give a yellow oil **4**, which was then dissolved in 1.5 mL of anhydrous CH_2Cl_2. To the mixture, *m*-CPBA (107 mg, 0.62 mmol) in 2 mL of CH_2Cl_2 was dropped slowly at −78 °C under N_2 protection. Two hours later, the mixture was removed to room temperature and 4 mL of anhydrous toluene was added. Then the reaction mixture was further stirred under reflux overnight. When finished, 20 mL of dichloromethane was added and the mixture was washed with saturated $NaHCO_3$, water and brine, dried over $MgSO_4$, and concentrated under

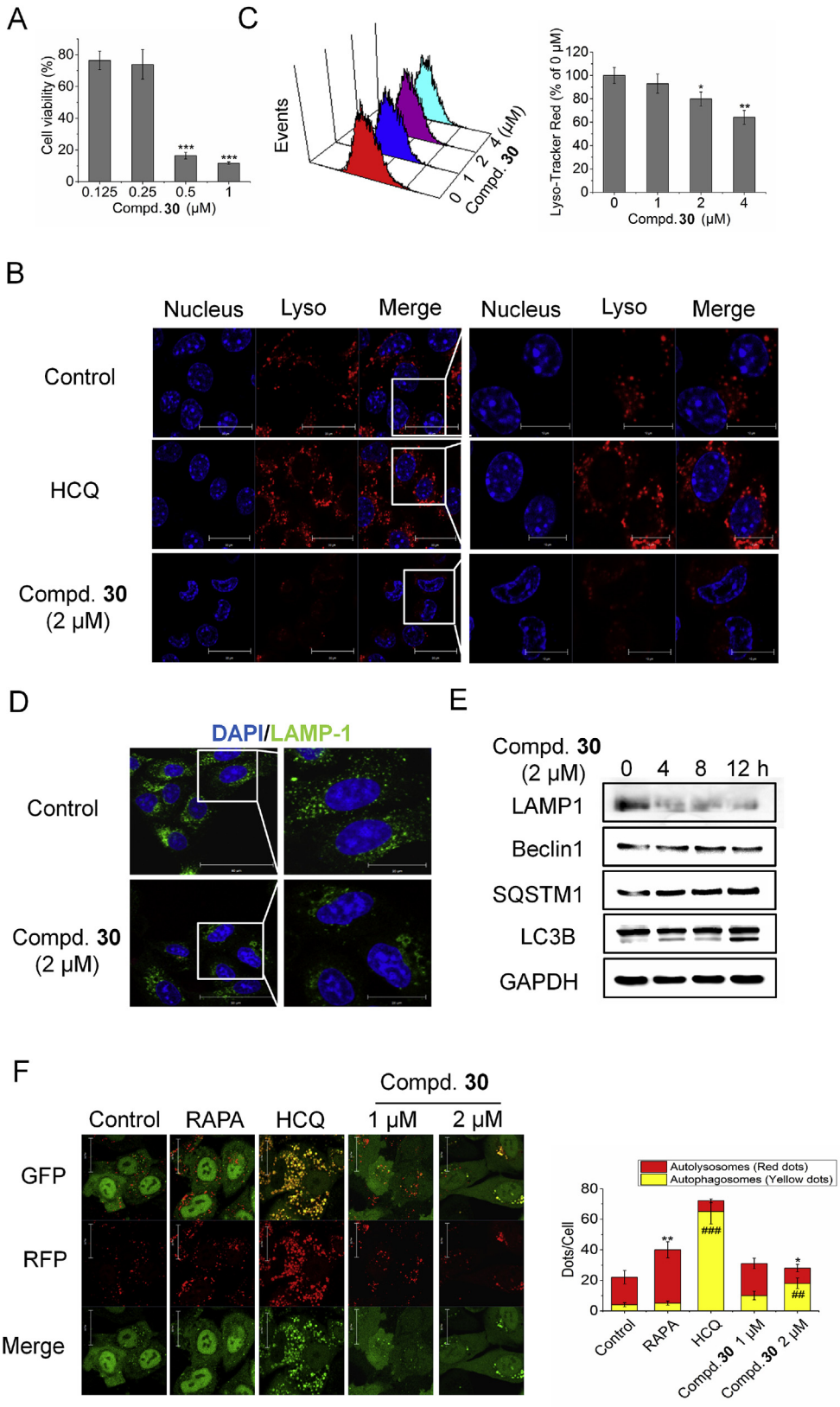


Fig. 4. Compd. **30** targeted lysosomes in A549 cells. (A) Cytotoxicity of Compd. **30** in A549 cells. Cells were treated with 0.125−1 μM Compd. **30** for 48 h. The cell viability rate, which is denoted as a percentage of untreated control (Compd. **30**, 0 μM) at the concurrent time point was estimated by the MTT assay. (B, C) Lysosomal rupture assayed using the Lyso-Tracker Red-uptake method. Cells were captured by confocal microscopy. The intensity of lysosomal fluorescence from 10,000 cells per sample was measured by flow cytometry. Cells with decreased red fluorescence ("pale" cells) were gated, and their percentages are indicated. (D) Immunofluorescence staining of LAMP-1 (green) and DAPI (blue) in A549 cells after treatment with compounds. Bars = 50 and 20 μm. (E) Western blotting analysis of proteins in A549 cells. (F) Cells were transfected with a pcDNA3.1-mRFP-GFP-LC3 plasmid, then treated with the indicated compounds. The GFP/mRFP-LC3 signals were visualized using a confocal microscopy. Representative patterns (Bar = 20 μm) are displayed. All values were mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. control.

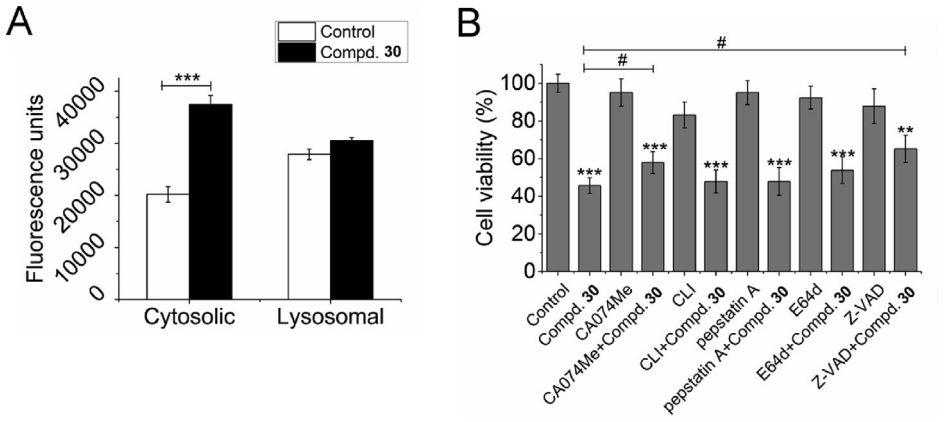


Fig. 5. Compd. **30** induced lysosome-mediated cell death in A549 cells. The concentration of compd. **30** used in this experiment was 2 μM. (A) Cell fractionation was performed to separate lysosomal and cytosolic fractions. The increase in cytosolic CTSB activity was measured after subcellular fractionation of samples in A549 cells. (B) CA074Me, E64d, and Z-VAD-fmk attenuated A549 cell death induced by Compd. **30**, as assessed by MTT assays. Data are expressed as the mean ± S.D. (n = 3). $^{**}P < 0.01$ and $^{***}P < 0.001$, vs. control and $\#P < 0.05$ vs. Compd. **30** group.

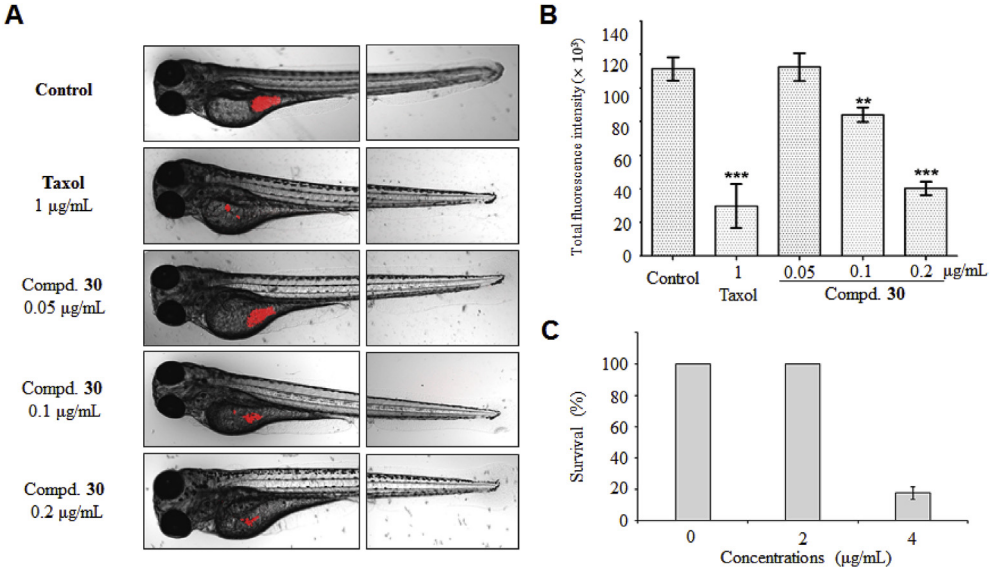


Fig. 6. Inhibitory effect of compound 30 on the proliferation of A549 cells in zebrafish xenograft model. CM-Dil-labeled A549 cells (red) were microinjected into zebrafish embryos, and different concentrations of compound 30 were added. Taxol was used as a positive control. After 48 h of incubation, zebrafish were imaged under a confocal microscopy. (A) Representative fluorescent images of A549 cells xenografted zebrafish with treatment of Taxol, compound 30 or negative control. (B) Quantification of fluorescence intensity of the tumor xenografts, representing the number of A549 cells. The results are presented as the mean ± S.D. (n = 15). $^{*}P < 0.05$ and $^{**}P < 0.01$, vs. control. (C) The toxicity of compound 30 to healthy zebrafish embryos incubated with serial concentrations of compound 30.

reduced pressure to give the crude product, which was then purified by flash chromatography to give compound **5** (96 mg, 57%) as a white solid, mp 93–95 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.72 (s, 1H), 4.45 (s, 1H), 2.70 (dd, J = 18.6, 3.8 Hz, 1H), 2.32–2.23 (m, 1H), 1.98 (td, J = 13.0, 5.9 Hz, 1H), 1.83 (d, J = 18.7 Hz, 1H), 1.79–1.71 (m, 3H), 1.62–1.54 (m, 5H), 1.50–1.31 (m, 5H), 1.29–1.17 (m, 2H), 1.06 (td, J = 12.9, 4.9 Hz, 1H), 0.99 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 150.7, 105.9, 54.5, 53.3, 52.3, 49.0, 48.9, 40.0, 39.6, 39.6, 39.3, 37.5, 36.6, 23.0, 22.6, 21.1, 20.0, 13.1. ESI-HRMS (*m/z*): calcd for $C_{19}H_{29}O$ $[M + H]^+$, 273.2213; found, 273.2215.

*4.1.3. (3S,4aS,6aR,9S,11aR,11bS)-3-Hydroxy-9,11b-dimethyl-4-methylenedodecahydro-6a,9-methanocyclohepta[a]naphthalen-8(7H)-one (**7**)*

To a stirred solution of compound **5** (136 mg, 0.50 mmol) in dichloromethane (5 mL) was added *t*-BuOOH (70 wt % in water, 212 μL, 1.50 mmol) and SeO_2 (28 mg, 0.25 mmol) at room temperature, and the mixture was stirred at room temperature. After 5 h, the reaction mixture was quenched with water and extracted with dichloromethane (15 mL × 2). The combined extracts were washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure to give the crude product. The crude product was purified by flash chromatography to give pure compound **7** (130 mg, 90%) as a white solid, mp 135–137 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.94 (d, J = 1.1 Hz, 1H), 4.60 (d, J = 1.3 Hz, 1H), 4.26 (s, 1H), 2.68 (dd, J = 18.6, 3.7 Hz, 1H), 2.34 (d, J = 11.9 Hz, 1H), 1.83 (d, J = 18.6 Hz, 1H), 1.76–1.71 (m, 2H), 1.67–1.35 (m, 12H), 1.22 (qd, J = 13.2, 5.4 Hz, 1H), 0.98 (s, 3H), 0.66 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 151.7, 109.4, 73.1, 54.5, 53.0, 48.9, 48.9, 45.7, 39.9, 39.6, 39.4, 37.4, 33.4, 29.4, 22.1, 21.0, 20.0, 12.3. ESI-HRMS (*m/z*): calcd for $C_{19}H_{29}O_2$ $[M + H]^+$, 289.2162; found, 289.2165.

*4.1.4. (4aS,6aR,9S,11aR,11bS)-9,11b-Dimethyl-4-methylenedecahydro-6a,9-methanocyclohepta[a]naphthalene-3,8(2H,7H)-dione (**8**)*

To a stirred solution of compound **7** (50 mg, 0.17 mmol) in anhydrous dichloromethane (4 mL) was added PCC (73 mg, 0.34 mmol). The mixture was stirred at room temperature for 3 h and then diluted with saturated $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with warter and brine, dried over $MgSO_4$, and concentrated under reduced pressure to give the crude product. The crude product was purified by flash chromatography to give the compound **8** (49 mg, 97%) as a white amorphous solid, mp 119–121 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.84 (d, J = 0.7 Hz, 1H), 5.09 (d, J = 0.8 Hz, 1H), 2.68 (dd, J = 18.6, 3.8 Hz, 1H), 2.54 (ddd, J = 18.2, 6.6, 2.1 Hz, 1H), 2.48–2.36 (m, 1H), 2.20 (dd, J = 11.4, 2.4 Hz, 1H), 2.02 (ddd, J = 13.3, 7.6, 2.0 Hz, 1H), 1.88 (d, J = 18.6 Hz, 1H), 1.84–1.72 (m, 2H), 1.71–1.58 (m, 4H), 1.54–1.29 (m, 6H), 1.01 (s, 3H), 0.83 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.3, 199.7, 148.6, 118.2, 54.2, 52.5, 50.1, 48.9, 48.5, 39.3, 39.3, 37.3, 37.3, 36.9, 36.1, 22.2, 21.2, 19.9, 13.0. ESI-HRMS (*m/z*): calcd for $C_{19}H_{27}O_2$ $[M + H]^+$, 287.2006; found, 287.2009.

*4.1.5. (4aS,6aR,9S,11aR,11bS)-9,11b-Dimethyl-4-methylene-4a,5,6,9,10,11,11a,11b-octahydro-6a,9-methanocyclohepta[a]naphthalene-3,8(4H,7H)-dione (**9**)*

To a stirred solution of compound **8** (300 mg, 1.05 mmol) in anhydrous dioxane (15 mL) was added DDQ (362 mg, 1.57 mmol) at room temperature. The mixture was stirred under reflux overnight. After filtration and concentrated under reduced pressure, the crude product was obtained. The crude product was then purified by flash chromatography to give compound **9** (176 mg, 59%) as a white amorphous solid, mp 150–152 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 (d, J = 10.1 Hz, 1H), 6.17–6.04 (s, 1H), 5.96 (d, J = 10.1 Hz, 1H), 5.22 (s, 1H), 2.65 (dd, J = 18.6, 3.8 Hz, 1H), 2.57 (dd, J = 11.5, 2.3 Hz, 1H), 2.01–1.87 (m, 2H), 1.82–1.73 (m, 1H), 1.72–1.65 (m, 3H), 1.64–1.51 (m, 4H), 1.45 (dd, J = 12.1, 4.6 Hz, 1H), 1.42–1.33 (m, 1H), 1.02 (s, 3H), 0.93 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 199.7, 189.1, 159.0, 145.8, 127.0, 118.7, 54.0, 51.1, 49.0, 48.9, 48.1, 41.1, 40.0, 39.4, 37.0, 21.7, 21.0, 19.8, 16.2. ESI-HRMS (*m/z*): calcd for $C_{19}H_{25}O_2$ $[M + H]^+$, 285.1849; found, 285.1851.

*4.1.6. (4aS,6aS,7R,9S,11aR,11bS)-7-((Dimethylamino)methyl)-9,11b-dimethyl-4-methylene-4a,5,6,9,10,11,11a,11b-octahydro-6a,9-methanocyclohepta[a]naphthalene-3,8(4H,7H)-dione (**10**)*

To a magnetically stirred solution of ketone **9** (200 mg, 0.70 mmol) in dry THF (15 mL) maintained at −78 °C under an argon atmosphere was added dropwise with LiHMDS (1.0 mL of a 1 M THF solution, 1.05 mmol) and the resulting mixture left to stir at −78 °C for 1 h. After this time, Eschenmoser's salt (390 mg, 2.10 mmol) was added in one portion. The reaction mixture was then allowed to warm to room temperature and stirred over 16 h before being quenched with HCl (10 mL of a 3 M aqueous solution) and then washed with diethyl ether (10 mL × 2). The separated aqueous phase was basified to pH 14, then extracted with dichloromethane (10 mL × 2). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford the product **10** (204 mg, 85%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16 (d, J = 10.2 Hz, 1H), 6.10 (s, 1H), 5.97 (d, J = 10.1 Hz, 1H), 5.24 (s, 1H), 2.65–2.45 (m, 3H), 2.25 (s, 6H), 2.07 (dt, J = 14.0, 3.1 Hz, 1H), 1.97 (d, J = 11.3 Hz, 1H), 1.86 (dd, J = 12.8, 2.5 Hz, 1H), 1.78 (dd, J = 12.1, 2.3 Hz, 1H), 1.70 (dd, J = 15.0, 4.8 Hz, 2H), 1.61 (dd, J = 12.7, 3.7 Hz, 1H), 1.46–1.37 (m, 3H), 1.33 (dd, J = 13.8, 3.7 Hz, 2H), 0.99 (s, 3H), 0.95 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.4, 159.3, 146.0, 127.2, 118.7, 58.8, 52.6, 51.9, 51.3, 50.3, 48.3, 45.9, 42.3, 41.4, 37.0, 34.0, 29.8, 22.1, 20.2, 20.2, 16.2. ESI-HRMS (*m/z*): calcd for $C_{22}H_{31}NO_2$ $[M + H]^+$, 342.2428; found, 342.2432.

*4.1.7. (4aS,6aS,9S,11aS,11bS)-9,11b-Dimethyl-4,7-dimethylene-4a,5,6,9,10,11,11a,11b-octahydro-6a,9-methanocyclohepta[a]naphthalene-3,8(4H,7H)-dione (**11**)*

To a magnetically stirred solution of tertiary amine **10** (50 mg, 0.15 mmol) in diethyl ether-dichloromethane (4 mL of a 3:1 v/v mixture) was added iodomethane (234 μL, 3.60 mmol) under a nitrogen atmosphere at room temperature and the resulting mixture was stirred at room temperature for 16 h. Then, the mixture was concentrated under reduced pressure to give a yellow oil. This oil was dissolved in CH_2Cl_2 (3 mL) and basic alumina (300 mg) was added to the mixture. The resulting suspension was stirred at room temperature for 0.5 h and then concentrated under reduced pressure. The ensuing solid was loaded on the top of a column of TLC-grade alumina. Elution with CH_2Cl_2 afforded **11** (26 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.06 (d, J = 10.2 Hz, 1H), 6.26–6.08 (m, 2H), 5.97 (d, J = 10.1 Hz, 1H), 5.51 (s, 1H), 5.27 (s, 1H), 2.65 (ddd, J = 6.0, 4.5, 2.4 Hz, 1H), 2.09–2.02 (m, 1H), 2.01–1.94 (m, 1H), 1.93–1.89 (m, 1H), 1.88–1.81 (m, 1H), 1.76–1.65 (m, 2H), 1.63–1.55 (m, 3H), 1.51 (dd, J = 11.5, 2.5 Hz, 1H), 1.36 (dd, J = 13.2, 5.3 Hz, 1H), 1.07 (s, 3H), 0.85 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 209.7, 189.1, 158.3, 154.6, 145.8, 127.0, 119.0, 116.8, 53.2, 50.4, 50.3, 47.1, 44.1, 41.7, 37.6, 36.1, 22.1, 21.7, 20.1, 13.8. ESI-HRMS (*m/z*): calcd for $C_{20}H_{25}O_2$ $[M + H]^+$, 297.1849; found, 297.1849.

*4.1.8. (4a′R,6a′R,9′S,11a′R,11b′R)-9′,11b′-Dimethyl-4′-methylenedodecahydro-7′H-spiro[[1,3]dioxolane-2,8'- [6a,9] methanocyclohepta[a]naphthalene] (**12**)*

In a 25 mL flask with a Dean-Stark distiller and bubble condenser provided with a calcium chloride valve, the compound **5** (136 mg, 0.50 mmol) was dissolved in cyclohexane (10 mL) together with 1,2-ethanediol (0.5 mL) and camphorsulfonic acid (2 mg). The reaction mixture was vigorous stirred under reflux until the material disappear. After cooled to room temperature, the reaction mixture was quenched with 10 mL of saturated $NaHCO_3$ and extracted with dichloromethane (15 mL × 2). The combined extracts were washed with H_2O and brine, dried over $MgSO_4$ and concentrated under reduced pressure to give the crude product. The crude product was purified by flash chromatography to give the product **12** (152 mg, 96%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.69 (s, 1H), 4.42 (s, 1H), 3.92–3.79 (m, 4H), 2.33–2.21 (m, 2H), 2.02–1.91 (m, 1H), 1.78–1.71 (m, 2H), 1.62–1.53 (m, 7H), 1.48–1.19 (m, 5H), 1.13 (dd, J = 12.5, 4.5 Hz, 1H), 1.07–1.01 (m, 2H), 0.85 (s, 3H), 0.71 (s, 3H). ESI-MS *m/z* 317 $[M + H]^+$.

*4.1.9. (4a′S,6a′R,9′S,11a′R,11b′S)-9′,11b′-Dimethyldecahydro-7′H-spiro[[1,3]dioxolane-2,8'- [6a,9]methanocyclohepta[a]naphthalen]-4′(1′H)-one (**13**)*

The compound **12** (550 mg, 1.74 mmol) in a solution of CH_2Cl_2–MeOH (15 mL:15 mL) was cooled to −78 °C, and ozone was passed into the mixture until it turned a light blue color (10 min). Nitrogen was bubbled through the solution to remove excess ozone, and then dimethyl sulfide (2.5 mL) was added slowly. After 30 min, the reaction mixture was allowed to warm to room temperature and stirred for 2 h. When finished, the solvent was removed under reduced pressure to give the crude product, which was then purified by flash chromatography to give the compound **13** (398 mg, 72%) as a colorless oil.^{1}H NMR (400 MHz, $CDCl_3$) δ 3.97–3.72 (m, 4H), 2.33–2.25 (m, 2H), 2.22 (dd, J = 14.4, 2.8 Hz, 1H), 2.10 (d, J = 8.7 Hz, 1H), 1.98–1.80 (m, 3H), 1.78–1.71 (m, 1H), 1.70–1.45 (m, 6H), 1.41–1.15 (m, 5H), 1.03 (dd, J = 11.4, 2.7 Hz, 1H), 0.84 (s, 3H), 0.75 (s, 3H). ESI-MS *m/z* 319 $[M + H]^+$.

*4.1.10. (4a′S,6a′R,9′S,11a′R,11b′S)-3′-(Bis(methylthio)methylene)-9′,11b′-dimethyldecahydro-7′H-spiro[[1,3]dioxolane-2,8′- [6a,9] methanocyclohepta[a]naphthalen]-4′(1′H)-one (**14**)*

To a stirred solution of compound **13** (50 mg, 0.16 mmol) in anhydrous THF (2 mL) cooled at −78 °C under nitrogen atmosphere and kept for 15 min, was added dropwise LiHMDS (241 μL of a 1 M THF solution, 0.23 mmol) and the resulting mixture was left to stir at −78 °C for 1 h. After this time, redistilled carbon disulfide (48 μL, 0.78 mmol) was added. The reaction mixture was then allowed to stir at room temperature. After 1 h, methyl iodide (34 μL, 0.55 mmol) was added and the stirring was continued for a further 5 h. Then the reaction was quenched with saturated NH_4Cl, extracted with EtOAc, washed with water and brine. The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure to give a crude product, which was purified by flash chromatography to give the compound **14** (58 mg, 88%) as a yellow solid, mp 139–141 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.99–3.78 (m, 4H), 3.24 (ddd, *J* = 16.4, 5.8, 2.3 Hz, 1H), 2.53 (ddd, *J* = 16.6, 12.6, 6.2 Hz, 1H), 2.33 (s, 6H), 2.22 (dd, *J* = 14.4, 2.6 Hz, 1H), 2.17–2.10 (m, 1H), 1.97 (ddd, *J* = 13.1, 6.1, 2.1 Hz, 1H), 1.85–1.72 (m, 2H), 1.67–1.52 (m, 6H), 1.48–1.39 (m, 1H), 1.34 (d, *J* = 10.4 Hz, 1H), 1.27–1.19 (m, 2H), 1.03 (dd, *J* = 11.4, 2.5 Hz, 1H), 0.86 (s, 3H), 0.82 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.6, 140.9, 118.4, 65.3, 64.2, 60.5, 54.9, 53.8, 48.3, 45.2, 40.9, 40.5, 39.1, 38.2, 35.6, 29.5, 21.1, 20.1, 19.2, 18.2, 17.8, 14.0. ESI-HRMS (*m/z*): calcd for $C_{23}H_{35}O_3S_2$ $[M + H]^+$, 423.2022; found, 423.2031.

*4.1.11. (3b′S,5a′R,8′S,10a′R,10b′S)-8′,10b′-Dimethyl-1′-(methylthio)-3b′,4′,5′,8′,9′,10′,10a′,10b′,11′,12′-decahydro-6′H-spiro[[1,3] dioxolane-2,7′- [5a,8]methanocyclohepta [5,6]naphtho [1,2-c]furan] (**16**)*

To a stirred solution of trimethylsulfonium iodide (249 mg, 1.20 mmol) in dry THF (5 mL), under nitrogen atmosphere at −20 °C, was added *n*-BuLi (515 μL, 1.20 mmol, 2.0 M in hexane) and the mixture was stirred at −20 °C for 2 h. Then cooled to −78 °C, compound **14** (170 mg, 0.40 mmol) in THF (2 mL) was added dropwise, and the mixture was stirred for an additional 1.5 h. Then the mixture was warmed to room temperature and quenched by adding water slowly. The resulting mixture was extracted with EtOAc, washed with water and brine, dried over $MgSO_4$ and concentrated under reduced pressure to give compound **15**, which was dissolved in the 0.5 M H_2SO_4/MeOH (25 mL) without purification and the stirring was continued for a further 5 h at room temperature. Then EtOAc was added and the mixture was washed with saturated $NaHCO_3$, water and brine, dried over $MgSO_4$ and concentrated under reduced pressure to give the crude product, which was purified by flash chromatography to give the compound **16** (203 mg, 89%) as a yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.13 (d, *J* = 1.8 Hz, 1H), 3.92–3.80 (m, 4H), 2.58 (dd, *J* = 17.5, 6.6 Hz, 1H), 2.45 (ddd, *J* = 17.5, 12.3, 6.2 Hz, 1H), 2.35 (dd, *J* = 14.4, 2.8 Hz, 1H), 2.30 (s, 3H), 2.20–2.14 (m, 1H), 1.94 (dd, *J* = 13.1, 6.8 Hz, 1H), 1.80–1.75 (m, 1H), 1.74–1.69 (m, 2H), 1.64–1.60 (m, 3H), 1.56–1.51 (m, 2H), 1.39–1.33 (m, 1H), 1.24–1.13 (m, 3H), 1.09 (dd, *J* = 11.4, 2.7 Hz, 1H), 0.86 (s, 3H), 0.70 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 140.7, 138.6, 128.1, 125.1, 118.5, 65.2, 64.0, 55.1, 52.1, 48.9, 45.1, 43.6, 40.7, 40.0, 36.3, 35.5, 35.0, 22.7, 21.2, 20.0, 18.5, 17.2, 11.7. ESI-HRMS (*m/z*): calcd for $C_{23}H_{33}O_3S$ $[M + H]^+$, 389.2145; found, 389.2142.

*4.1.12. (3bS,5aR,8S,10aR,10bS)-8,10b-Dimethyl-3b,4,5,8,9,10,10a,10b,11,12-decahydro-5a,8-methanocyclohepta [5,6] naphtho [1,2-c]furan-1,7(3H,6H)-dione (**17**)*

Compound **16** was dissolved in 0.5 M H_2SO_4/MeOH and the mixture was stirred for 4 h at 60 °C to quantitative give the compound **17** as a white solid, mp 221–223 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.82–4.56 (m, 2H), 2.77 (dd, *J* = 18.6, 3.8 Hz, 1H), 2.40–2.29 (m, 2H), 2.26–2.13 (m, 1H), 1.96 (dd, *J* = 13.3, 6.5 Hz, 1H), 1.89 (d, *J* = 18.7 Hz, 1H), 1.82 (ddd, *J* = 13.3, 6.8, 2.7 Hz, 1H), 1.73–1.58 (m, 6H), 1.56–1.20 (m, 6H), 1.01 (s, 3H), 0.76 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 174.3, 163.0, 124.3, 70.5, 54.3, 51.9, 49.4, 48.9, 46.5, 40.1, 39.5, 37.0, 36.7, 34.1, 21.4, 21.1, 19.9, 17.7, 12.3. ESI-HRMS (*m/z*): calcd for $C_{20}H_{27}O_3$ $[M + H]^+$, 315.1955; found, 315.1956.

*4.1.13. (3R,3bS,5aR,8S,10aR,10bS)-3-((Dimethylamino)methyl)-8,10b-dimethyl-3b,4,5,8,9,10,10a,10b,11,12-decahydro-5a,8-methanocyclohepta [5,6]naphtho [1,2-c]furan-1,7(3H,6H)-dione (**18**)*

To a stirred solution of compound **17** (50 mg, 0.16 mmol) in anhydrous THF, maintained at −78 °C under nitrogen atmosphere, was added LiHMDS (240 μL of a 1 M THF solution, 0.24 mmol) and the resulting mixture left to stir at −78 °C for 10 min. After this time, Eschenmoser's salt (90 mg, 0.48 mmol) was added, in one portion, to the reaction mixture and the stirring was continued for a further 1 h at −78 °C. Then the reaction was quenched with saturated NH_4Cl and extracted with dichloromethane. The combined extracts were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure to give the crude product, which was purified by flash chromatography to give the compound **18** (38 mg, 65%) as a white solid, mp 151–153 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.95 (d, *J* = 7.1 Hz, 1H), 2.84 (dd, *J* = 13.9, 1.1 Hz, 1H), 2.78 (dd, *J* = 18.7, 3.6 Hz, 1H), 2.37 (s, 6H), 2.34–2.26 (m, 2H), 2.24–2.17 (m, 2H), 1.97–1.79 (m, 3H), 1.78–1.61 (m, 5H), 1.56–1.47 (m, 2H), 1.45–1.36 (m, 2H), 1.36–1.28 (m, 2H), 1.01 (s, 3H), 0.75 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 173.2, 163.6, 125.5, 81.0, 62.2, 54.3, 52.0, 49.5, 48.9, 46.4, 46.0, 40.3, 39.5, 36.9, 33.7, 21.5, 21.1, 19.8, 17.8, 12.4. ESI-HRMS (*m/z*): calcd for $C_{23}H_{34}NO_3$ $[M + H]^+$, 372.2533; found, 372.2539.

*4.1.14. (3bS,5aR,8S,10aR,10bS)-8,10b-Dimethyl-3-methylene-3b,4,5,8,9,10,10a,10b,11,12-decahydro-5a,8-methanocyclohepta [5,6] naphtho [1,2-c]furan-1,7(3H,6H)-dione (**19**)*

To a stirred solution of compound **18** (30 mg, 0.08 mmol) in diethyl ether-dichloromethane (4 mL of a 3:1 v/v mixture) was added iodomethane (100 μL, 1.60 mmol) under a nitrogen atmosphere at room temperature, and the resulting mixture was stirred for 16 h. Silica gel TLC analysis indicated the consumption of the starting material, and the mixture was concentrated under reduced pressure to give a crude intermediate. Then the intermediate was dissolved in dichloromethane (3 mL) and basic alumina (300 mg) was added to the mixture. The resulting suspension was stirred at room temperature for 0.5 h. Then, the reaction mixture was diluted with dichloromethane (20 mL) and filtered. The solvent was concentrated under reduced pressure and the residue was purified by flash chromatography to give the pure compound **19** (15 mg, 55%) as a white solid, mp 211–213 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.15 (d, *J* = 2.5 Hz, 1H), 5.03 (d, *J* = 2.6 Hz, 1H), 2.77 (dd, *J* = 18.6, 3.8 Hz, 1H), 2.51–2.37 (m, 2H), 2.35–2.23 (m, 1H), 2.18 (dd, *J* = 12.2, 2.9 Hz, 1H), 1.99 (dd, *J* = 13.4, 6.6 Hz, 1H), 1.90 (d, *J* = 18.7 Hz, 1H), 1.87–1.81 (m, 1H), 1.78–1.61 (m, 5H), 1.51 (dd, *J* = 11.7, 3.8 Hz, 1H), 1.47–1.38 (m, 2H), 1.34 (dd, *J* = 12.7, 4.6 Hz, 1H), 1.30–1.26 (m, 1H), 1.01 (s, 3H), 0.78 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.9, 154.5, 150.8, 129.5, 96.6, 54.3, 51.8, 49.2, 48.9, 46.3, 40.1, 39.0, 37.1, 37.0, 33.7, 22.0, 21.2, 19.8, 17.9, 12.5. ESI-HRMS (*m/z*): calcd for $C_{21}H_{27}O_3$ $[M + H]^+$, 327.1955; found, 327.1957.

*4.1.15. (3bS,5aS,6R,8S,10aS,10bS)-6-((Dimethylamino)methyl)-8,10b-dimethyl-3-methylene-3b,4,5,8,9,10,10a,10b,11,12-decahydro-5a,8-methanocyclohepta [5,6]naphtho [1,2-c]furan-1,7(3H,6H)-dione (**20**)*

To a stirred solution of compound **19** (30 mg, 0.09 mmol) in dry

THF (3 mL) maintained at −78 °C under a nitrogen atmosphere was added dropwise LiHMDS (100 μL of a 1 M THF solution, 0.10 mmol) and the resulting mixture was stirred at −78 °C for 1 h. Then, N,N-dimethylmethyleneammonium iodide (50 mg, 0.27 mmol) was added in one portion. The mixture was allowed to stir at room temperature for 16 h. The reaction was quenched with water and extrated with two 20-mL portions of dichloromethane. The combined extract was washed with brine, dried over MgSO4, filtered and concentrated under reduced pressure to afford the crude product. The crude product was purified by flash chromatography to give the pure compound 20 (15 mg, 43%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.15 (s, 1H), 5.05 (s, 1H), 2.64−2.46 (m, 3H), 2.40 (dd, J = 19.4, 6.8 Hz, 1H), 2.23 (s, 6H), 2.08 (d, J = 11.7 Hz, 2H), 2.00−1.90 (m, 2H), 1.86−1.72 (m, 2H), 1.62 (d, J = 9.6 Hz, 1H), 1.45−1.32 (m, 5H), 1.28−1.18 (m, 3H), 0.96 (s, 3H), 0.79 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.9, 153.4, 150.1, 128.1, 95.5, 57.8, 52.9, 51.9, 51.0, 46.9, 45.4, 44.7, 40.1, 36.2, 35.8, 33.7, 32.2, 21.4, 19.3, 19.0, 16.7, 11.0. ESI-HRMS (*m/z*): calcd for $C_{24}H_{34}NO_3$ $[M + H]^+$, 384.2533; found, 384.2532.

4.1.16. (3bS,5aS,8S,10aS,10bS)-8,10b-Dimethyl-3,6-dimethylene-3b,4,5,8,9,10,10a,10b,11,12-decahydro-5a,8-methanocyclohepta [5,6] naphtho [1,2-c]furan-1,7(3H,6H)-dione (**21**)

To a stirred solution of compound **20** (20 mg, 0.05 mmol) in diethyl ether-dichloromethane (4 mL of a 3:1 v/v mixture) was added iodomethane (62 μL, 1.00 mmol) under a nitrogen atmosphere at room temperature, and the mixture was stirred for 16 h. Silica gel TLC analysis indicated the consumption of the starting material and the reaction mixture was concentrated under reduced pressure to give a crude intermediate. The intermediate was dissolved in dichloromethane (3 mL) and basic alumina (100 mg) was added to the mixture. The resulting suspension was stirred at room temperature for 0.5 h. Then the mixture was diluted with dichloromethane, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography to give the pure compound **21** (6.5 mg, 37%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.15 (s, 1H), 5.43 (s, 1H), 5.18 (d, J = 2.7 Hz, 1H), 5.05 (d, J = 2.7 Hz, 1H), 2.54 (dd, J = 12.6, 3.5 Hz, 1H), 2.41 (dd, J = 19.5, 6.5 Hz, 1H), 2.33−2.19 (M, 2H), 2.10−2.03 (m, 1H), 2.02−1.91 (m, 2H), 1.89−1.78 (m, 2H), 1.74 (dd, J = 13.8, 4.6 Hz, 1H), 1.59−1.56 (m, 1H), 1.52−1.47 (m, 2H), 1.34−1.29 (m, 3H), 1.06 (s, 3H), 0.75 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 153.8, 153.4, 128.8, 115.6, 95.2, 52.5, 52.1, 45.8, 45.1, 36.7, 36.5, 35.9, 33.0, 28.7, 21.3, 20.8, 19.0, 16.7, 10.4. ESI-HRMS (*m/z*): calcd for $C_{22}H_{27}O_3$ $[M + H]^+$, 339.1955; found, 339.1957.

4.1.17. (4aS,6aR,9S,11aR,11bS)-9,11b-Dimethyldecahydro-6a,9-methanocyclohepta[a]naphthalene-4,8(1H,7H)-dione (**22**)

The compound **5** (100 mg, 0.37 mmol) was dissolved in dichloromethane-methanol (10 mL:10 mL). The mixture was cooled to −78 °C and kept for 15 min. Then ozone was passed into the mixture until it turned blue. After 5 min, nitrogen was bubbled through the solution to remove excess ozone, and then dimethyl sulfide (1 mL) was added slowly. After 30 min, the reaction mixture was allowed to warm to room temperature and stirred for 10 min. Then the solvent was removed under reduced pressure to give the crude product, which was purified by flash chromatography to afford the pure compound **22** (82 mg, 82%) as a white solid, mp 135−137 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.64 (dd, J = 18.7, 3.8 Hz, 1H), 2.35−2.26 (m, 2H), 2.24−2.16 (m, 1H), 2.01−1.91 (m, 1H), 1.90−1.80 (m, 3H), 1.79−1.72 (m, 1H), 1.71−1.54 (m, 4H), 1.53−1.34 (m, 6H), 1.22 (ddd, J = 26.8, 13.3, 5.4 Hz, 1H), 0.98 (s, 3H), 0.74 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 212.6, 59.6, 54.3, 53.1, 48.9, 48.7, 43.0, 40.9, 39.2, 38.9, 38.0, 37.2, 22.1, 21.1, 19.9, 18.8, 13.8. ESI-HRMS (*m/z*): calcd for $C_{18}H_{27}O_2$ $[M + H]^+$, 275.2006; found, 275.2008.

4.1.18. (3S,4aS,6aS,7R,9S,11aS,11bS)-3,7-Bis((dimethylamino) methyl)-9,11b-dimethyldecahydro-6a,9-methanocyclohepta[a] naphthalene-4,8(1H,7H)-dione (**23**)

To a stirred solution of compound **22** (100 mg, 0.36 mmol) in dry THF (10 mL) cooled under nitrogen at −78 °C, was added dropwise LiHMDS (760 μL of a 1 M THF solution, 0.76 mmol), and the resulting mixture was stirred for 1 h at −78 °C. Then, N,N-dimethylmethyleneammonium iodide (333 mg, 1.80 mmol) was added in one portion. The mixture was then allowed to stir at room temperature for 16 h. When finished, the reaction was quenched with water, and extrated with dichloromethane. The extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography to afford the pure product **23** (38 mg, 27%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.70 (dd, J = 11.7, 9.5 Hz, 1H), 2.57 (dd, J = 14.9, 7.1 Hz, 1H), 2.48−2.41 (m, 2H), 2.38−2.23 (m, 3H), 2.20 (s, 12H), 1.99 (dt, J = 13.8, 3.0 Hz, 1H), 1.95−1.87 (m, 1H), 1.79−1.64 (m, 5H), 1.63−1.46 (m, 4H), 1.39 (dd, J = 13.0, 5.4 Hz, 1H), 1.33 (dd, J = 12.2, 1.9 Hz, 1H), 1.27−1.20 (m, 1H), 1.16 (dd, J = 13.8, 4.0 Hz, 1H), 0.96 (s, 3H), 0.76 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 214.7, 61.8, 58.7, 55.8, 55.8, 52.9, 51.6, 48.1, 47.7, 46.0, 45.7, 43.4, 41.4, 37.3, 33.8, 33.4, 24.5, 20.3, 20.3, 19.1, 13.6. ESI-HRMS (*m/z*): calcd for $C_{24}H_{41}N_2O_2$ $[M + H]^+$, 389.3163; found, 389.3169.

4.1.19. Methyl(4aS,6aS,7S,9S,11aS,11bS)-3-(bis(methylthio) methylene)-9,11b-dimethyl-4,8-dioxotetradecahydro-6a,9-methanocyclohepta[a]naphthalene-7-carbodithioate (**24**)

To a stirred solution of compound **22** (100 mg, 0.36 mmol) in dry THF (5 mL) cooled at −78 °C under a nitrogen atmosphere was added dropwise LiHMDS (910 μL of a 1 M THF solution, 0.76 mmol), and the mixture was stirred for 1 h at −78 °C. Then, redistilled carbon disulfide (108 μL, 1.80 mmol) was added. The reaction was allowed to stir at room temperature. After 1 h, iodomethane (135 μL, 2.16 mmol) was added and the stirring was continued for a further 5 h. Then the reaction was quenched with saturated NH_4Cl, extracted with dichloromethane. The extracts were washed with water, brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography to give the pure compound **24** (133 mg, 78%) as a yellow solid, mp 155−157 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.29 (s, 1H), 3.33−3.19 (m, 1H), 2.68 (d, J = 12.1 Hz, 1H), 2.66−2.56 (m, 4H), 2.36 (s, 3H), 2.34 (s, 3H), 2.25 (dd, J = 11.4, 2.8 Hz, 1H), 1.99 (d, J = 14.5 Hz, 2H), 1.87−1.76 (m, 2H), 1.76−1.65 (m, 2H), 1.56−1.44 (m, 3H), 1.33 (d, J = 11.4 Hz, 1H), 1.25 (t, J = 7.1 Hz, 1H), 1.16 (td, J = 14.0, 4.3 Hz, 1H), 1.06 (s, 3H), 1.01 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 218.4, 201.3, 143.4, 139.5, 73.6, 59.9, 56.0, 52.4, 48.9, 44.9, 40.8, 37.7, 37.6, 32.3, 29.1, 20.4, 20.3, 19.6, 19.5, 18.3, 17.9, 14.1. ESI-HRMS (*m/z*): calcd for $C_{23}H_{32}O_2S_4$ $[M + H]^+$, 469.1358; found, 469.1361.

4.1.20. Methyl(3bS,5aS,6S,8S,10aS,10bS)-8,10b-dimethyl-1,7-dioxo-1,3,3b,4,5,6,7,8,9,10,10a,10b,11,12-tetradecahydro-5a,8-methanocyclohepta [5,6]naphtho [1,2-c]furan-6-carbodithioate (**25**)

To a stirred solution of trimethylsulfonium iodide (129 mg, 0.63 mmol) in dry THF (5 mL), under a nitrogen atmosphere at −20 °C, was added dropwise *n*-BuLi (320 μL, 0.63 mmol, 2.0 M in hexane), and the mixture was stirred at −20 °C for 2 h. Then the mixture was cooled to −78 °C and compound **24** (100 mg, 0.21 mmol) in THF (1 mL) was added dropwise. The resulting mixture was stirred for an additional 1.5 h. Then the mixture was warmed to room temperature and quenched by adding water slowly. The mixture was extracted with dichloromethane. The extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was dissolved in the 0.5 M H_2SO_4/MeOH (5 mL) without purification and the

mixture was stirred for a further 5 h at 60 °C. Then the mixture was diluted with dichloromethane and washed with $NaHCO_3$, water and brine, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give the crude product, which was purified by flash chromatography to afford the pure compound **25** (65 mg, 75%) as a yellow solid, mp 234–236 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.73 (d, J = 16.9 Hz, 1H), 4.65 (d, J = 17.2 Hz, 1H), 4.41 (s, 1H), 2.64 (s, 3H), 2.36 (d, J = 10.5 Hz, 2H), 2.28–2.17 (m, 1H), 2.07–1.95 (m, 2H), 1.89 (d, J = 13.6 Hz, 1H), 1.77–1.68 (m, 2H), 1.57–1.46 (m, 3H), 1.40 (d, J = 12.2 Hz, 1H), 1.35–1.23 (m, 4H), 1.07 (s, 3H), 0.98 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 218.1, 174.2, 162.8, 124.2, 74.0, 70.5, 54.8, 52.6, 48.8, 46.5, 45.1, 37.4, 36.9, 33.8, 33.4, 21.8, 20.4, 20.4, 19.5, 17.6, 12.3. ESI-HRMS (*m/z*): calcd for $C_{22}H_{29}O_3S_2$ $[M + H]^+$, 405.1553; found, 405.1552.

4.1.21. (4aS,6aS,7R,9S,11aR,11bS)-9,11b-Dimethyl-4-methylene-7-(pyrrolidin-1-ylmethyl)-4a,5,6,9,10,11,11a,11b-octahydro-6a,9-methanocyclohepta[a]naphthalene-3,8(4H,7H)-dione (**26**)

To a stirred solution of compound **9** (50 mg, 0.18 mmol) in dry THF (3 mL) cooled at −78 °C under a nitrogen atmosphere, was added dropwise LiHMDS (220 μL of a 1 M THF solution, 0.22 mmol) and the mixture was stirred at −78 °C for 1 h. Then, 1-methylenepyrrolidin-1-ium iodide (114 mg, 0.54 mmol) was added in one portion. The reaction mixture was then allowed to stir for 16 h at room temperature. Silica gel TLC analysis indicated the consumption of the starting material. The reaction was quenched with water, and extracted with dichloromethane. The extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography to afford the pure compound **26** (21 mg, 32%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.15 (d, J = 10.2 Hz, 1H), 6.13–6.06 (m, 1H), 5.97 (d, J = 10.1 Hz, 1H), 5.20 (s, 1H), 2.92–2.76 (m, 1H), 2.68–2.46 (m, 6H), 2.18 (d, J = 14.2 Hz, 1H), 1.96 (d, J = 12.9 Hz, 1H), 1.85 (dd, J = 12.0, 2.5 Hz, 1H), 1.81–1.65 (m, 7H), 1.60 (dd, J = 12.3, 4.0 Hz, 1H), 1.46–1.36 (m, 3H), 1.32 (dd, J = 13.7, 3.8 Hz, 1H), 1.28–1.24 (m, 1H), 1.01 (s, 3H), 0.94 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.4, 159.3, 146.1, 127.2, 118.5, 54.7, 52.6, 51.3, 50.4, 48.3, 42.3, 41.5, 37.1, 33.7, 24.0, 22.1, 20.3, 16.2. ESI-HRMS (*m/z*): calcd for $C_{24}H_{34}NO_2$ $[M + H]^+$, 368.2584; found, 368.2589.

4.1.22. (4aS,6aS,7R,9S,11aR,11bS)-9,11b-Dimethyl-4-methylene-7-(piperidin-1-ylmethyl)-4a,5,6,9,10,11,11a,11b-octahydro-6a,9-methanocyclohepta[a]naphthalene-3,8(4H,7H)-dione (**27**)

To a stirred solution of compound **9** (50 mg, 0.18 mmol) in dry THF (3 mL) maintained at −78 °C under nitrogen protection was added dropwise LiHMDS (220 μL of a 1 M THF solution, 0.22 mmol) and the mixture was stirred at −78 °C for 1 h. Then 1-methylenepiperidin-1-ium iodide (122 mg, 0.54 mmol) was added in one portion. The reaction mixture was allowed to stir at room temperature for 16 h. Then the reaction was quenched with water, and extracted with dichloromethane. The extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography to afford the pure compound **27** (25 mg, 37%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.14 (d, J = 10.2 Hz, 1H), 6.12 (s, 1H), 5.98 (d, J = 10.1 Hz, 1H), 5.25 (s, 1H), 3.35–2.67 (m, 5H), 2.62 (d, J = 13.8 Hz, 1H), 2.06–1.67 (m, 10H), 1.64 (dd, J = 12.6, 3.8 Hz, 1H), 1.54–1.36 (m, 5H), 1.34–1.24 (m, 3H), 1.09 (s, 3H), 0.98 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.1, 158.7, 145.5, 127.4, 119.0, 54.5, 51.8, 51.7, 51.2, 50.2, 48.8, 42.4, 41.4, 37.0, 33.5, 29.4, 24.5, 24.5, 21.9, 20.2, 20.2, 16.5. ESI-HRMS (*m/z*): calcd for $C_{25}H_{36}NO_2$ $[M + H]^+$, 382.2741; found, 382.2746.

4.1.23. (3S,6aR,8aS,12aS,12bR)-3,12a-Dimethyl-9-methylene-2,3,7,8,8a,9,12a,12b-octahydro-3,6a-methanonaphtho [2,1-d] oxocine-5,10(1H,6H)-dione (**28**)

To a stirred solution of **9** (50 mg, 0.18 mmol) in dry dichloromethane (2 mL) under ice bath was added potassium carbonate (30 mg, 0.22 mmol) and 3-chloroperoxybenzoic acid (50 mg, 0.22 mmol). Then the reaction mixture was allowed to stir at room temperature overnight. Then 20 mL of dichloromethane was poured into the resulting mixture, which was washed with water and brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to give a crude product. The crude product was purified by flash chromatography to give the compound **28** (47 mg, 89%) as a colorless oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.18 (d, J = 10.1 Hz, 1H), 6.13 (dd, J = 2.0, 1.2 Hz, 1H), 5.98 (d, J = 10.1 Hz, 1H), 5.27–5.20 (m, 1H), 3.07 (dd, J = 18.7, 2.7 Hz, 1H), 2.56 (ddd, J = 11.7, 4.5, 2.2 Hz, 1H), 2.15 (d, J = 18.7 Hz, 1H), 2.13–2.09 (m, 1H), 1.93 (ddd, J = 13.3, 4.4, 2.1 Hz, 1H), 1.77–1.73 (m, 1H), 1.69 (dd, J = 13.7, 2.7 Hz, 1H), 1.67–1.63 (m, 1H), 1.63–1.59 (m, 1H), 1.57–1.54 (m, 1H), 1.52 (dd, J = 13.7, 2.7 Hz, 1H), 1.49 (dd, J = 13.5, 3.6 Hz, 1H), 1.46–1.43 (m, 1H), 1.40 (s, 3H), 1.39–1.37 (m, 1H), 1.01 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 188.9, 171.6, 158.9, 145.2, 126.9, 118.7, 80.3, 51.4, 49.1, 47.5, 41.3, 40.8, 38.8, 38.5, 35.6, 28.2, 19.6, 19.2, 16.9. ESI-HRMS (*m/z*): calcd for $C_{19}H_{25}O_3$ $[M + H]^+$, 301.1798; found, 301.1801.

4.1.24. (4aS,6aR,9S,11aR,11bS,E)-8-(Hydroxyimino)-9,11b-dimethyl-4-methylene-4a,5,6,7,8,9,10,11,11a,11b-decahydro-6a,9-methanocyclohepta[a]naphthalen-3(4H)-one (**29**)

To a 10 mL round-bottomed flask was added **9** (50 mg, 0.18 mmol), MeOH (1.5 mL), CH_2Cl_2 (1.5 mL), $NH_2OH^{\cdot}HCl$ (15 mg, 0.22 mmol) and $NaHCO_3$ (30 mg, 0.36 mmol) at room temperature. A reflux condenser was fitted to the flask and the mixture was heated to 40 °C for 12 h after which time TLC analysis indicated the starting material was consumed. Then 20 mL of dichloromethane was added to the mixture, which was washed with brine, dried over $MgSO_4$ and filtered. The solvent was then removed under reduced pressure and the residue was purified by flash chromatography to give the product **29** (43 mg, 82%) as a white solid, mp 118–120 °C. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.13 (d, J = 10.1 Hz, 1H), 6.10 (s, 1H), 5.96 (d, J = 10.0 Hz, 1H), 5.22 (s, 1H), 3.00 (d, J = 18.1 Hz, 1H), 2.54 (d, J = 11.2 Hz, 1H), 2.11 (d, J = 18.7 Hz, 1H), 1.94–1.88 (m, 1H), 1.77 (d, J = 13.5 Hz, 1H), 1.73–1.68 (m, 1H), 1.66–1.59 (m, 2H), 1.57 (d, J = 12.0 Hz, 1H), 1.54–1.49 (m, 2H), 1.46–1.38 (m, 2H), 1.37–1.33 (m, 1H), 1.16 (s, 3H), 0.99 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.2, 169.9, 159.4, 145.9, 126.7, 118.5, 56.0, 51.1, 48.0, 44.1, 41.1, 41.0, 39.2, 38.6, 37.4, 21.9, 21.5, 21.0, 16.0. ESI-HRMS (*m/z*): calcd for $C_{19}H_{26}NO_2$ $[M + H]^+$, 300.1958; found, 300.1961.

4.1.25. (4aS,6aR,9S,11aR,11bS,E)-8-(Methoxyimino)-9,11b-dimethyl-4-methylene-4a,5,6,7,8,9,10,11,11a,11b-decahydro-6a,9-methanocyclohepta[a]naphthalen-3(4H)-one (**30**)

Oxime **29** (40 mg, 0.13 mmol) was dissolved in anhydrous DMF (2 mL). Sodium hydride (60% in mineral oil, 16 mg, 0.39 mmol) was then added under nitrogen atmosphere at 0 °C and the reaction mixture was stirred under room temperature for 1 h until hydrogen evolution ceased. The flask with the oxime sodium salt was cooled to 0 °C and iodomethane (40 μL, 0.65 mmol) was added dropwise. The reaction mixture was stirred at room temperature for 2 h before being quenched with saturated NH_4Cl, and then extracted with dichloromethane. The combined organic extracts was washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure to give the crude product, which was purified by flash chromatography to give the compound **30** (30 mg, 72%) as a white solid, mp 140–142 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.12 (d, J = 10.1 Hz, 1H), 6.09 (s, 1H), 5.95 (d, J = 10.1 Hz, 1H), 5.20 (s, 1H),

3.84 (s, 3H), 2.90 (dd, J = 18.6, 2.5 Hz, 1H), 2.52 (d, J = 10.5 Hz, 1H), 2.01 (d, J = 18.7 Hz, 1H), 1.94–1.85 (m, 1H), 1.78–1.67 (m, 2H), 1.65–1.46 (m, 6H), 1.45–1.36 (m, 1H), 1.31 (dd, J = 11.5, 2.9 Hz, 1H), 1.14 (s, 3H), 0.97 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 188.4, 167.3, 158.7, 145.1, 125.8, 117.5, 60.6, 55.2, 50.2, 47.2, 43.0, 40.2, 40.1, 38.4, 37.8, 37.0, 21.1, 20.6, 20.1, 15.2. ESI-HRMS (m/z): calcd for $C_{20}H_{28}NO_2$ $[M + H]^+$, 314.2115; found, 314.2120.

4.1.26. (4aS,6aR,9S,11aR,11bS,E)-8-(Butoxyimino)-9,11b-dimethyl-4-methylene-4a,5,6,7,8,9,10,11,11a,11b-decahydro-6a,9-methanocyclohepta[a]naphthalen-3(4H)-one (**31**)

Oxime **29** (10 mg, 0.033 mmol) was dissolved in anhydrous DMF (1 mL). Sodium hydride (60% in mineral oil, 2.6 mg, 0.06 mmol) was then added under nitrogen atmosphere at 0 °C and the reaction mixture was stirred at room temperature for 30 min. After this time, 1-bromobutane (5.4 μL, 0.05 mmol) was added to the reaction mixture and the stirring was continued for a further 2 h at room temperature. Then the mixture was quenched with water and extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$ and concentrated under reduced pressure to give the crude product, which was purified by flash chromatography to give the compound **31** (10 mg, 85%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.13 (d, J = 10.1 Hz, 1H), 6.09 (dd, J = 1.9, 1.4 Hz, 1H), 5.95 (d, J = 10.1 Hz, 1H), 5.21 (s, 1H), 4.09–3.98 (m, 2H), 2.92 (dd, J = 18.6, 3.3 Hz, 1H), 2.52 (dd, J = 11.1, 2.3 Hz, 1H), 2.01 (d, J = 18.6 Hz, 1H), 1.93–1.85 (m, 1H), 1.77–1.73 (m, 1H), 1.72–1.68 (m, 1H), 1.65–1.56 (m, 5H), 1.53 (dd, J = 11.4, 2.8 Hz, 1H), 1.50–1.46 (m, 2H), 1.45–1.40 (m, 1H), 1.40–1.35 (m, 2H), 1.31 (dd, J = 11.4, 3.3 Hz, 1H), 1.14 (s, 3H), 0.98 (s, 3H), 0.93 (t, J = 7.4 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 189.3, 167.8, 159.6, 146.0, 126.7, 118.3, 73.4, 56.1, 51.1, 48.1, 43.9, 41.1, 41.0, 39.3, 38.7, 38.0, 31.3, 22.1, 21.6, 21.0, 19.3, 16.0, 14.0. ESI-HRMS (m/z): calcd for $C_{23}H_{34}NO_2$ $[M + H]^+$, 356.2584; found, 356.2580.

4.1.27. (4aS,6aR,9S,11aR,11bS,E)-9,11b-Dimethyl-4-methylene-8-((prop-2-yn-1-yloxy)imino)-4a,5,6,7,8,9,10,11,11a,11b-decahydro-6a,9-methanocyclohepta[a]naphthalen-3(4H)-one (**32**)

To a stirred solution of compound **29** (50 mg, 0.17 mmol) in dry DMF (2 mL) was added sodium hydride (60% in mineral oil, 13.6 mg, 0.34 mmol) at ice bath, and the mixture was stirred for 30 min at room temperature. Then, 3-bromopropyne (22 μL, 0.25 mmol) was added to the reaction mixture and the stirring was continued for a further 2 h at room temperature. Silica gel TLC analysis indicated the consumption of the starting material. The reaction was quenched with water, and extracted with dichloromethane. The extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography to afford the pure compound **32** (42 mg, 75%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.13 (d, J = 10.1 Hz, 1H), 6.10 (s, 1H), 5.96 (d, J = 10.1 Hz, 1H), 5.21 (s, 1H), 4.64 (d, J = 2.3 Hz, 2H), 2.95 (dd, J = 18.7, 3.3 Hz, 1H), 2.53 (dd, J = 11.3, 2.2 Hz, 1H), 2.44 (t, J = 2.3 Hz, 1H), 2.07 (d, J = 18.7 Hz, 1H), 1.93–1.86 (m, 1H), 1.76 (dd, J = 12.6, 3.0 Hz, 1H), 1.74–1.69 (m, 1H), 1.68–1.64 (m, 1H), 1.62 (dd, J = 12.8, 3.5 Hz, 1H), 1.55 (dd, J = 11.6, 2.6 Hz, 2H), 1.52–1.46 (m, 2H), 1.44–1.39 (m, 1H), 1.32 (dd, J = 11.5, 3.3 Hz, 1H), 1.15 (s, 3H), 0.98 (s, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 189.3, 169.9, 159.5, 145.9, 126.7, 118.4, 80.1, 74.0, 61.1, 56.0, 51.1, 48.1, 44.2, 41.1, 41.1, 39.2, 38.6, 38.1, 22.0, 21.5, 21.0, 16.1. ESI-HRMS (m/z): calcd for $C_{22}H_{28}NO_2$ $[M + H]^+$, 338.2115; found, 338.2116.

4.1.28. (3S,6aR,8aS,12aS,12bR)-3,12a-Dimethyl-9-methylene-1,2,3,4,7,8,8a,9,12a,12b-decahydro-10H-3,6a-methanonaphtho [2,1-d]azocine-5,10(6H)-dione (**34**)

To a flame dried 10 mL round bottomed flask was added oxime **29** (50 mg, 0.17 mmol) and anhydrous dichloromethane (2 mL). The flask was flushed with nitrogen and cooled to −78 °C. Triethylamine (118 μL, 0.85 mmol) and MsCl (27 μL, 0.34 mmol) were added. After 30 min, TLC analysis indicated that the starting material was consumed and then the mixture was quenched with saturated $NaHCO_3$, extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to give the mesylate ester **33**, which was then carried on without further purification. Then to another 10 mL round-bottomed flask containing mesylate ester **33** in MeOH (2.5 mL) and toluene (0.5 mL) was added concentrated HCl (56 μL, 0.68 mmol). The mixture was then heated to 60 °C and stirred for 90 min. Then the mixture was cooled to room temperature and 20 mL of dichloromethane was added to the mixture, which was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to give the crude product. The crude product was purified by flash chromatography to give the product **34** (32.5 mg, 65%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (d, J = 10.1 Hz, 1H), 6.10 (dd, J = 1.8, 1.2 Hz, 1H), 6.07 (s, 1H), 5.95 (d, J = 10.1 Hz, 1H), 5.22 (s, 1H), 2.88 (dd, J = 18.5, 2.1 Hz, 1H), 2.55 (dd, J = 11.4, 2.3 Hz, 1H), 2.03 (d, J = 18.5 Hz, 1H), 1.89–1.84 (m, 1H), 1.77–1.67 (m, 2H), 1.66–1.60 (m, 2H), 1.59–1.54 (m, 1H), 1.53–1.49 (m, 1H), 1.48–1.44 (m, 1H), 1.43–1.40 (m, 1H), 1.32–1.27 (m, 2H), 1.23 (s, 3H), 1.02 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.2, 173.0, 159.7, 145.6, 126.8, 118.6, 52.1, 51.8, 50.1, 49.2, 42.1, 41.0, 40.5, 39.9, 36.1, 28.9, 20.0, 19.6, 17.3. ESI-HRMS (m/z): calcd for $C_{19}H_{26}NO_2$ $[M + H]^+$, 300.1958; found, 300.1961.

4.1.29. (3S,6aR,8aS,12aS,12bR)-3,4,12a-Trimethyl-9-methylene-1,2,3,4,7,8,8a,9,12a,12b-decahydro-10H-3,6a-methanonaphtho [2,1-d]azocine-5,10(6H)-dione (**35**)

To a 10 mL flame-dried round-bottomed flask of lactam **34** (50 mg, 0.17 mmol) in anhydrous DMF (3 mL) under nitrogen protection was added sodium hydride (60% in mineral oil, 34 mg, 0.85 mmol) and the mixture was stirred for 30 min at room temperature. Then iodomethane (105 μL, 1.70 mmol) was added dropwise and the mixture was raised to 90 °C for 5 h. After this time, TLC analysis indicated the consumption of starting material and the reaction was carefully quenched with water. The resulting mixture was extracted with dichloromethane and the extracts were washed with brine, dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to give the crude compound, which was purified by flash chromatography to give the product **35** (49 mg, 93%) as a white solid, mp 128–130 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (d, J = 10.1 Hz, 1H), 6.10 (s, 1H), 5.95 (d, J = 10.1 Hz, 1H), 5.22 (s, 1H), 2.96 (dd, J = 18.4, 2.7 Hz, 1H), 2.88 (s, 3H), 2.54 (dd, J = 11.6, 2.3 Hz, 1H), 2.15 (d, J = 18.4 Hz, 1H), 2.05–1.96 (m, 1H), 1.85–1.79 (m, 1H), 1.73 (dd, J = 13.1, 2.7 Hz, 1H), 1.70–1.64 (m, 1H), 1.63–1.56 (m, 2H), 1.43 (dd, J = 13.1, 2.9 Hz, 1H), 1.40–1.34 (m, 2H), 1.30 (s, 3H), 1.26–1.23 (m, 2H), 1.00 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.3, 171.3, 159.8, 145.6, 126.8, 118.6, 55.7, 51.8, 50.8, 50.2, 42.2, 41.2, 41.1, 35.9, 34.8, 27.5, 27.1, 19.9, 19.5, 17.3. ESI-HRMS (m/z): calcd for $C_{20}H_{28}NO_2$ $[M + H]^+$, 314.2115; found, 314.2120.

4.1.30. (3S,6aR,8aS,12aS,12bR)-4-Benzyl-3,12a-dimethyl-9-methylene-1,2,3,4,7,8,8a,9,12a,12b-decahydro-10H-3,6a-methanonaphtho [2,1-d]azocine-5,10(6H)-dione (**36**)

To a 10 mL flame-dried round-bottomed flask of lactam **34** (20 mg, 0.07 mmol) in anhydrous DMF (2 mL) under nitrogen atmosphere was added sodium hydride (60% in mineral oil, 14 mg, 0.35 mmol) and the mixture was stirred for 30 min at room temperature. Then benzyl bromide (84 μL, 0.70 mmol) and tetrabutylammonium iodide (5.2 mg, 0.014 mmol) were added to the reaction mixture and the reaction mixture was heated to 90 °C for 5 h. After TLC analysis had indicated the consumption of starting

material, the reaction was carefully quenched with water. Then dichloromethane (20 mL) was poured into the mixture which was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography to give the product **36** (21 mg, 80%) as a white solid, mp 186–188 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.37–7.28 (m, 5H), 6.18 (s, 1H), 6.02 (d, J = 10.1 Hz, 1H), 5.31 (s, 1H), 5.06 (d, J = 15.7 Hz, 1H), 4.34 (d, J = 15.7 Hz, 1H), 3.14 (dd, J = 18.6, 2.2 Hz, 1H), 2.61 (d, J = 11.2 Hz, 1H), 2.37 (d, J = 18.5 Hz, 1H), 1.98 (d, J = 9.6 Hz, 1H), 1.93–1.84 (m, 2H), 1.82–1.76 (m, 1H), 1.71 (d, J = 11.2 Hz, 2H), 1.53–1.37 (m, 6H), 1.25 (s, 3H), 1.11 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 189.3, 171.8, 159.8, 145.6, 139.7, 128.6, 127.1, 126.9, 126.8, 118.6, 56.9, 51.8, 51.5, 50.2, 44.9, 42.1, 41.4, 41.1, 37.3, 35.0, 28.1, 20.0, 19.4, 17.4. ESI-HRMS (*m/z*): calcd for $C_{26}H_{32}NO_2$ $[M + H]^+$, 390.2428; found, 390.2430.

4.2. General material and methods for biology

The final compounds were more than 95% pure and dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM, and was stored at −20 °C. LC3B antibodies, MTT, Hoechst33342 and Z-VAD-fmk were purchased from Sigma Co, (USA). Primary antibodies for Beclin-1 were obtained from Cell Signaling Technology, (Boston, MA). LAMP1 was obtained from Abcam (Cambridge, UK). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), SQSTM1 antibody and secondary biotin-conjugated goat anti-mouse IgG or anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lyso-Tracker Red and DAPI were purchased from Beyotime Institute of Biotechnology. Z-RR-AMC, E64d, CA074Me, CLI and pepstatin A were purchased from Enzo Biochem Inc.

4.2.1. Cell culture and treatments

Human breast adenocarcinoma cell lines MCF-7 and HCC1428, the human non-small cell lung cancer A549 cell line, and human hepatocellular carcinoma cell lines HepG-2 and SMMC7721 were purchased from Shanghai Institute for Biological Sciences (SIBS), China Academy of Sciences (Shanghai, China). The human myelogenous leukemia K562 cell line was purchased from the Department of Pharmacology, the Institute of Hematology of Chinese Academy of Medical Sciences (Tianjin, China). These cells were cultured in RPMI-1640 (HyClone) medium containing 10% FBS, 100 units/mL of penicillin G, and 100 μg/mL of streptomycin in a stable environment at 37 °C and 5% CO_2. After cell confluence reached 50%–80%, the cells were treated with compounds as indicated. Control cells were subjected to DMSO treatment.

4.2.2. MTT assay

Cells were seeded into 96-well plates at 3–5 × 10^3/well and incubated with compounds for the indicated times. After incubation, cells were supplied with MTT (5.0 mg/mL) solution, and the plates were incubated for an additional 4 h at 37 °C. Cell growth response to the chemicals was determined at 570 nm on a microplate reader (Bio-Rad 680). All experiments were performed in triplicate in three independent experiments. The cell viability inhibitory ratio was calculated by comparison with the vehicle control using the formula: Cell viability ratio (%) = × 100%.

4.2.3. Lysosomal integrality assay

Lyso-Tracker Red (100 nM) for 30 min was used for lysosomal integrity assays according to the manufacturer's instructions. Flow cytometry was performed on a FACScan cytometry (FACSCalibur, Becton Dickinson, USA). Data were analyzed using CELLQUEST software (Verity Software House, Topsham, Maine, USA). For fluorescence staining, cells were seeded onto 24-well plates. After the indicated treatment, cells were incubated with Lyso-Tracker Red and Hoechst33342 (10 μg/mL) and captured under an LSM 700 confocal microscope using a Plan-Apochromat 63 × /1.40 Oil lens (Carl Zeiss). For dynamic imaging, cells were seeded onto 24-well plates, then incubated with Lyso-Tracker Red. Cells were added to Compd. **30** and immediately captured under a Cytation 5 imaging reader (Biotek).

4.2.4. Immunofluorescence staining

Cells were seeded onto 12-mm round, glass cover slips in 24-well plates. After the indicated treatment, cells were fixed with cold methanol: acetone (1:1) for 5 min and washed twice with cold PBS. Then, cells were incubated with 3% goat serum (in 0.1% Triton X-100) for 20 min to prevent nonspecific antibody binding. After removing the liquid, cover slips were incubated with the antibody overnight, washed in PBS three times, and incubated with FITC-conjugated goat anti-mouse secondary antibodies for 1 h. Then, cells were washed in PBS three times and counter-stained with DAPI for 15 min. Fluorescence images were captured under an LSM 700 confocal microscope using a Plan-Apochromat 63 × /1.40 Oil lens and ZEN 2009 software (Carl Zeiss).

4.2.5. Autophagy assay

To determine the formation of autophagosomes and autolysosomes, a mRFP-GFP-LC3 reporter plasmid (1 μL/mL) was transfected into A549 cells. After 24 h, cells were washed with PBS and incubated in complete medium for 12 h. Cells were then treated with compounds for the indicated times. Images were acquired using confocal microscopy.

4.2.6. Western blotting assay

A western blotting assay was used to analyze the expressions of proteins. The cells were dispersed in RIPA lysis buffer containing fresh protease inhibitor mixture (50 μg/mL aprotinin, 0.5 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM glycerolphosphate) and centrifuged. The supernatants were collected and the total proteins were quantified by BCA assay. Equal amounts of protein extracts were separated on 9%–12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in TBS buffer for 1 h prior to incubation with specific antibodies at 4 °C. Membranes were then washed with TBST and incubated with peroxidase-conjugated appropriate secondary antibodies. Immunoblot proteins were visualized by an enhanced chemiluminescence detection system.

4.2.7. Enzyme activities

Cells were homogenized with a tight-fitting handheld homogenizer in 0.6 mL of subcellular fractionation buffer (20 mM HEPES-KOH, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 8 mM dithiothreitol, and 1 mM PMSF, pH 7.5). Debris and nuclei were pelleted at 750 g and 4 °C, for 12 min. The supernatant was centrifuged at 10,000×g for 35 min to pellet lysosomes and other organelles. The pellet was rinsed and washed once to obtain the lysosomal fraction. The supernatant was cleared of microsomes by an extra spin at 100,000×g for 1 h and collected as the cytosolic fraction. The CTSB activity of the lysed fraction was measured. Equal amounts of protein (10 μg) were added to 200 μL of CTSB reaction buffer with the fluorescent substrate Z-RR-AMC (50 μM; ex. = 365 nm, em. = 449 nm). CTSB activity was measured after incubation (15 min, 37 °C), as described previously.

4.2.8. Zebrafish assay

Zebrafish embryos were used to determine the antitumor effects of the tested compounds. All experiments were carried out in compliance with institutional guidelines. Wild-type zebrafish were

obtained from the Biology Institute of Shandong Academy of Science, maintained at 28 °C with 10 h dark/14 h light cycles. Zebrafish embryos were generated by natural pairwise mating and raised at 28 °C in embryonic medium (5.0 mM NaCl, 0.17 mM KCl, 0.4 mM $CaCl_2$, 0.16 mM $MgSO_4$). A549 cells labeled red fluorescence by CM-Dil at a density of ~10^7 cells/mL were then microinjected into zebrafish larvae (at 48 h post fertilization) to establish zebrafish embryo xenograft model. After 4 h of incubation, zebrafish embryos with the same injection spot were selected and incubated in a six-well plate with 15–20 embryos per well. Then different concentrations of compound 30 (5 nL) were added, and Taxol (Sigma) was used as a positive control. After 48 h postfertilization, zebrafish were anesthetized with 0.01% tricaine and imaged under a confocal microscopy (Olympus, Japan).

To determine the toxicity of compound 30 to zebrafish, healthy zebrafish embryos collected at 24 hpf, were incubated with serial concentration of compound 30 for 48 h, and the survival state was monitored by microscopic observation.

4.2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD) for triplicate experiments and analyzed by one-way ANOVA with Dunnett's multiple comparison test or an unpaired Student's t-test. *P*-value < 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS 14.0 statistical software program (SPSS, Inc, Chicago, IL).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work was financially supported by the National Natural Science Foundation of China (81874293 and 81630093); Focus on research and development plan in Shandong Province (2017CXGC1403); Natural Science Foundation of Shandong Province (ZR2019BH073).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112896.

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