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Oridonin Ring A-Based Diverse Constructions of Enone Functionality: Identification of Novel Dienone Analogues Effective for Highly Aggressive Breast Cancer by Inducing Apoptosis

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Abstract

Oridonin (**1**) has attracted considerable attention in recent years due to its unique and safe anticancer pharmacological profile. Nevertheless, it exhibits moderate to poor effects against highly aggressive cancers including triple-negative and drug-resistant breast cancer cells. Herein, we report the rational design and synthesis of novel dienone derivatives with an additional α,β -unsaturated ketone system diversely installed in the A-ring based on this class of natural scaffold that features dense functionalities and stereochemistry-rich frameworks. Efficient and regioselective enone construction strategies have been established. Meanwhile, a unique 3,7-rearrangement reaction was identified to furnish an unprecedented dienone scaffold. Intriguingly, these new analogues have been demonstrated to significantly induce apoptosis and inhibit colony formation with superior antitumor effects against aggressive and drug-resistant breast cancer cells *in vitro* and *in vivo*, while also exhibiting comparable or lower toxicity to normal human mammary epithelial cells in comparison with **1**.

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Notes

The authors declare no competing financial interest.

Supporting Information

¹H and ¹³C NMR spectra for the compounds described in this paper, and X-ray CIF files for compounds **13** and **18**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

oridonin dienone analogs; breast cancer; antiproliferation; apoptosis; drug-resistance

INTRODUCTION

Despite significant advances in the treatment of breast cancer, there are still limited therapeutic interventions available for aggressive and metastatic tumors.¹ Particularly, triple-negative breast cancer (TNBC), characterized by lacking the receptors for estrogen and progesterone, and the Her2/neu receptor, remains an unmet medical need and represents an important clinical challenge because these tumors do not respond to endocrine therapy or other available targeted agents.² In addition, resistance to chemotherapy is another major cause of the ultimate failure of breast cancer treatment.³ Therefore, effective anticancer agents with novel scaffolds or new mechanisms of action are urgently needed for the highly aggressive and drug-resistant breast cancer.

Natural products regularly serve synthetic chemists as a source of inspiration in their search for new molecular entities with unique pharmacological activity.⁴ Oridonin (**1**), isolated from the herb *Isodon rubescens* that is commonly used in Chinese traditional medicine and available over the counter in China, has attracted considerable attention in recent years due to its antitumor, antibacterial, antiviral, and anti-inflammatory activities.⁵ It has a unique and safe anticancer pharmacological profile. In China, oridonin injection was used alone or in combination with other drugs for the treatment of liver cancer⁶ and carcinoma of gastric cardia.⁷ Increasing studies have also demonstrated that **1** exerts extensive anti-neoplastic activities against various cultured human cancer cell lines through a versatile antiproliferative mechanism including regulating the cell cycle, apoptosis, and autophagy.⁸ While the antitumor activity of **1** was validated in estrogen receptor (ER)-positive breast cancer MCF-7 cells, it failed to reduce the growth of MDA-MB-231, a TNBC cell line, at the same dose range effective for MCF-7 cells,^{9a} suggesting that **1** is ineffective against the growth of highly aggressive breast cancer cells. As part of our ongoing drug discovery program based on natural products, the anticancer profile of **1** intrigued us to take advantage of its unique scaffold as a basic template to synthesize novel natural product-like oridonin derivatives to develop safe and effective anticancer agents. Recently, efficient synthetic methods based on the oridonin scaffold were successfully established by our group to obtain a series of A-ring thiazole-fused or triazole-substituted derivatives with enhanced anticancer activity and improved solubility,¹⁰ indicating that A-ring modifications appear to be tolerable for yielding biologically interesting molecules.

Structurally, oridonin is a highly oxygenated 7,20-epoxy-*ent*-kaurane-type diterpenoid that features a densely functionalized and stereochemistry-rich framework including an *exo*-methylene cyclopentanone moiety in the D-ring and a 6-hydroxyl-7-hemiacetal group in the B-ring (Figure 1). It is well known that the major structural determinant for anticancer activity of **1** is the presence of the α,β -unsaturated ketone (enone) system in the D-ring, and destruction of this enone system could counteract its anticancer activity.^{5a-b,11} Indeed, the enone system is a common and structurally important functionality which is widespread in various bioactive naturally occurring products such as ericalyxin B^{12a,b} and plakilactone C^{12c} (Figure 1). Enones have also proven useful as a key pharmacophore existing in synthetic anticancer agents as exemplified by the oleanane triterpenoids CDDO-Me (Phase I/II human clinical trials, Figure 1)¹³ and brostallicin (Phase II human clinical trials, Figure 1).¹⁴ From a biochemical point of view, the α,β -unsaturated carbonyl group, as a Michael acceptor, is an electrophilic center susceptible to nucleophilic attack (Michael addition) by a sulfhydryl group of reduced glutathione or cysteine residues in proteins, leading to the

adducts at the β -position.^{15a} Thus, alkylation of crucial cysteine residues can result in a loss of function,^{15b} or activation¹⁶ of the target proteins. For instance, eriocalyxin B, a naturally existing enone analogue of **1** isolated from *Isodon eriocalyx*, has demonstrated significant anticancer effects against various cancer cells likely through this mechanism.^{12b} In addition, a number of α,β -unsaturated ketones have exhibited preferential reactivity toward thiols rather than amino or hydroxyl groups.¹⁷ Since thiols are absent in nucleic acids, this enone system may be free of mutagenicity and carcinogenicity caused by some alkylating agents used in cancer chemotherapy.¹⁸ Meanwhile, accumulating evidence also demonstrates that dienone compounds with double α,β -unsaturated ketone functionalities, such as curcumin¹⁹ (Figure 1), have a capability to undergo two successive alkylations at the β -positions by cellular thiols which interfere with biological cascades at multiple points. This is highly deleterious for malignant cells^{17a–b,20} and may also permit selective or greater toxicity to malignant cells versus the corresponding normal cells,²¹ consequently leading to an excellent tolerability in mammal models. Inspired by these advantages, we embarked on constructions of an additional enone functionality in the A-ring of oridonin, and envisioned that the resulting dienone derivatives with α,β -unsaturated ketone substructures present in both the A- and D-rings might display enhanced anticancer activity against drug-resistant ER-positive and triple-negative breast cancer cells relative to **1**, while exhibiting less toxicity towards human normal mammary epithelial cells. In our previous work,¹⁰ the design of thiazole-fused derivatives was guided by the idea of incorporating nitrogen-containing heterocyclic ring into the A-ring to expand the core scaffold of **1**. Different from the previous strategies, the present approach focuses on the diverse construction of the enone functionality at the A-ring within the core template of oridonin. Herein, we disclose our efficient synthetic approaches to generating new oridonin dienone analogues with the enone functionality diversely installed in the A-ring and their marked anti-breast cancer activity.

RESULTS AND DISCUSSION

Chemistry

Our synthetic effort was initiated from **1** due to its natural abundance and commercial availability. To date, there is little evidence in pursuit of chemical transformations based on the A-ring of oridonin, probably due to its structural complexity with multiple chemically reactive functionalities. Thus, the goal to diversely assemble an α,β -unsaturated ketone moiety into the tetracyclic ring system of **1** while keeping key functionalities intact posed a formidable synthetic challenge. In developing efficient synthetic strategies, we attempted to employ a protecting protocol to allow regioselective reactions among several functional groups with similar reactivity and avoid the usage of nucleophilic reagents, strong bases and acids, which are chemically reactive with the key functionalities of **1**.

Our approach to synthesize oridonin analogues **6** and **7** with a 1-ketone- Δ^2 (1-ketone-2-ene) moiety in the A-ring is outlined in Scheme 1. Oxidation of **1** with Jones reagent selectively afforded the 1-oxo-oridonin derivative **2**,²² followed by treatment with 2,2-dimethoxypropane solely leading to the acetonide derivative **3** as a key building block. Although a few methods to introduce unsaturation adjacent to a carbonyl functionality have been developed over the years, the synthesis of α,β -unsaturated carbonyl compounds is often a tedious and sometimes challenging transformation.^{23a} Initially, attempts to achieve a one-step synthesis of **6** from **3** based on reported methods using several oxidizing reagents such as IBX (*o*-iodoxybenzoic acid),^{23b} 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)/*p*-toluenesulfonic acid (*p*-TsOH),^{23c} and activated manganese dioxide (MnO₂)^{23d} proved unsuccessful. In addition, although a two-step method for the synthesis of **6** utilizing PhSeCl/LDA at -78 °C followed by selenoxide elimination has been reported,²² this reaction was very complex with several side products and unreacted **3** when the same procedure was

carefully tested in our laboratory, and **6** was obtained in only 5% isolated yield. Therefore, a more reliable and efficient synthetic approach for **6** was deemed necessary, and has been achieved herein. Bromination of **2** with PyHBr_3 in dry THF at 0 °C,^{10a} followed by treatment with 2,2-dimethoxypropane, afforded 2-bromo oridonin derivative **5** as a mixture of α/β isomers in 63% yield over two steps, which further underwent a DBU-mediated elimination reaction to readily access **6** in 72% yield. It was noteworthy that the protection of the 7,14-dihydroxyl group as an acetonide was critical in this step; otherwise, **6** failed to be generated. Finally, the removal of the acetonide group in **6** with 5% HCl (aq.) successfully provided the dienone compound **7**, which could also be viewed as an ericalyxin B analogue with 14-hydroxyl functionality.

Since the electrophilic β -carbon of α,β -unsaturated ketone moiety might dictate the biological effects via nucleophilic addition, it is likely that chemically altering the reactivity of this carbon toward nucleophiles would have a profound effect on activity.²⁴ Based on this hypothesis, it was expected that introduction of an electron-withdrawing substituent such as a formyl group at the α -position of the enone system in the A-ring would increase the electrophilicity of the β -carbon, henceforth enhancing the bioactivity, in a similar fashion to that of oleanane triterpenoids (CDDO)¹³ and punaglandins.²⁵ Scheme 2 illustrates the synthesis of the dienone analogues **10** and **12** with an α -formyl enone moiety in the A-ring. It was initially intended to prepare **10** directly from **6** through a Baylis-Hillman reaction followed by oxidation of the resulting 2-hydroxymethyl group. Unfortunately, all attempts to obtain the 2-hydroxymethylated compound under several standard conditions²⁶ failed to produce the desired products.²⁷ Therefore, we pursued an alternative route to the α -formyl enone moiety through α -formylation of **3** followed by successive selenenylation and selenoxide elimination. Typically, installation of a formyl group at the α -position of the ketone can be realized by reaction with ethyl formate in the presence of strong base,^{13a,28a} but **3** immediately decomposed upon addition of a strong base such as NaOMe and *t*-BuOK. Therefore, a circuitous strategy was employed to introduce α -formyl group. Treatment of **3** with *N,N*-dimethylformamide dimethyl acetal in refluxing toluene readily afforded the enamine derivative **8** in 60% yield, which was hydrolyzed with 5% HCl aqueous solution for 15 min leading to the 2-formyl derivative **9** in 83% yield.^{28b} It should be noted that longer hydrolysis reaction time resulted in removal of the acetonide group to give **11**. Selenenylation of **9** with PhSeCl in the presence of pyridine at RT followed by 30% H_2O_2 -mediated oxidation and elimination successfully provided the desired analogue **10** with an α -formyl enone in the A-ring in 70% yield for two steps. Unexpectedly, the removal of the acetonide group in **10** with 5% HCl (aq.) in MeOH/ CH_2Cl_2 failed to give the desired product **12**. Instead, a 3,20-epoxy-*ent*-kaurane diterpenoid **13** with 2-*exo-E*-methoxymethylene moiety in the A-ring was obtained, the structure of which was unambiguously confirmed by the single crystal X-ray crystallographic analysis.²⁹ Interestingly, when THF was used as the solvent, a similar 3,20-epoxy product **14** with 2-*exo-Z*-hydroxymethylene moiety was also found, and further treatment of the isolated **14** with 5% HCl aqueous solution in methanol afforded **13**. These results suggested **14** was resulted from 3,7-rearrangement of **12**, and subsequent enol etherification of **14** led to **13**. Initially, we assumed that the rearrangement reaction was triggered by acid to result in the hydrolysis of 7-hemiacetal group to form a free 20-methylol group, which further underwent an intramolecular 1,4-conjugated Michael addition towards the unsaturated ketone moiety of the A-ring from the α -face followed by enolization or enol etherification leading to the 3,20-epoxy products **13** and **14**. Based on this assumption, to avoid the presence of an acid, we attempted to use the 7,14-diol derivative **11** without a protecting group as the substrate to synthesize **12** through the sequential selenenylation and selenoxide elimination reactions in the same fashion. To our surprise, the 3,20-epoxy product **14** instead of **12** was obtained again under these conditions, although no acid was involved in this reaction. These results

strongly indicated that **12** endowed with both α -formyl enone moiety and 7-hemiacetal group was unstable, and could automatically undergo 3,7-rearrangement reaction without the aid of acid, presumably owing to the increased electrophilicity of the β -carbon in the α -formyl enone system.

Transposition of a functional group from one carbon to another often offers a wide degree of diversity and flexibility in natural product synthesis and related drug design.^{30a} We initially considered the 1,3-enone transposition strategy in the A-ring via direct Wharton carbonyl transposition^{30b} of **6** to generate Δ^1 -3-ketone (1-ene-3-ketone) analogues **19** and **20**. Nevertheless, this approach was not feasible due to the harsh reaction conditions and the lack of regioselectivity in the enone formation. We thus developed an alternative and efficient synthetic approach in a controlled regioselective manner (Scheme 3). The synthesis of analogues **19** and **20** started with the protection of the 7,14-dihydroxyl group of **1** as an acetonide. The 1-hydroxyl group of the acetonide was then selectively activated as a mesylate **16**, which further underwent an elimination reaction³¹ in the presence of Li_2CO_3 at 110 °C to provide the 1-ene analogue **17** in 84% yield.^{10b} To introduce a hydroxyl group to the 3-position of the A-ring, we initiated a key allylic oxidation by the treatment of **17** with selenium dioxide³² in refluxing 1,4-dioxane to stereoselectively produce the 1-ene-3 β -hydroxyl analogue **18** in an excellent yield;^{10b} however, prolonged reaction time failed to give the enone product **19**. Having completed the synthesis of **18**, our attention was focused on the oxidation of the allylic alcohol. To our disappointment, neither activated MnO_2 nor Dess-Martin reagent promoted this transformation. Finally, the goal was realized by using pyridinium dichromate (PDC) to furnish the 1-ene-3-ketone analog **19** in 80% yield, followed by the removal of the protecting group to provide the desired analogue **20** bearing a 1-ene-3-ketone moiety in the A-ring.

***In Vitro* Antiproliferative Activity**

With seven novel dienone analogues including **6**, **7**, **10**, **13**, **14**, **19** and **20** in hand, their antiproliferative activities were evaluated against two breast cancer cell lines, MCF-7 (ER-positive) and MDA-MB-231 (triple-negative), with the data summarized in Table 1. **1** was also tested for comparison. The results showed that five 7,20-epoxy dienone analogues (**6**, **7**, **10**, **19** and **20**) not only exhibited significantly improved antiproliferative activity relative to **1** against ER-positive breast cancer MCF-7 cells with IC_{50} values varying from low micromolar to submicromolar range ($0.56 \pm 0.31 \mu\text{M} \sim 3.48 \pm 0.19 \mu\text{M}$), but also displayed good growth inhibitory effects on triple-negative MDA-MB-231 cells with low micromolar IC_{50} , for which **1** had only modest activity with an IC_{50} value of $28.0 \pm 1.40 \mu\text{M}$. For two 3,20-epoxy dienone compounds **13** and **14**, no obvious antiproliferative activities were observed, indicating the biological importance of the oridonin core ring system.

***In Vitro* Growth Inhibitory Activity against Drug-Resistant Breast Cancer Cells**

Resistance to chemotherapy is a major cause of the ultimate failure of breast cancer treatment. To investigate whether these dienone analogues are still effective on drug-resistant breast cancer cells, compounds **6**, **7**, **10** and **19** with potent antiproliferative effects against both MCF-7 and MDA-MB-231 cells were selected for further evaluation of growth inhibitory effects on ADR (adriamycin, a.k.a. doxorubicin)-resistant breast cancer cell MCF-7 clone (Figure 1S in Supporting Information). As shown in Figure 2, **1** displayed no growth inhibitory activity at concentrations from 1 μM to 10 μM with an IC_{50} value higher than 30 μM , while new compounds **6**, **7**, **10** and **19** were found to dose-dependently suppress the growth of MCF-7/ADR cells with IC_{50} values of $5.03 \pm 1.91 \mu\text{M}$, $5.82 \pm 2.12 \mu\text{M}$, $6.55 \pm 0.96 \mu\text{M}$, and $6.02 \pm 1.28 \mu\text{M}$, respectively (Table 2).

In Vitro Growth Inhibitory Activity on Human Normal Mammary Epithelial Cells (HMEC)

Selective toxicity for cancer, but not normal cells, is essential in the development of targeted cancer experimental therapeutics. To investigate whether the improved antiproliferative effects of analogs **6**, **7**, **10**, **19** and **20** against breast cancer cells were attributed to the undesired cell toxicities, we further examined their inhibitory effects on the growth of HMEC, and **1** was also tested for comparison. As shown in Figure 3, all of these dienone analogues exhibited comparable or lower growth inhibitory activity against HMEC cells at all tested concentrations, albeit displaying markedly enhanced anticancer activities against drug-resistant ER-positive MCF-7 and triple-negative MDA-MB-231 cancer cells when compared with **1**. Particularly, analogue **19** displayed lower toxicity at 10 μM than oridonin ($*p < 0.05$), and the IC_{50} values of analogues **19** and **20** are much higher than that of oridonin (Table 3), indicating their lower toxicities to HMEC cells.

Compounds 10 and 19 Inhibited Colony Formation of Breast Cancer Cells

Considering their potent antiproliferative activities against MDA-MB-231 cells, two structurally representative dienone analogues **10** (CYD0692) and **19** (CYD0686) were selected for colony formation assay. Both of these two compounds have demonstrated to inhibit the colony formation of highly invasive triple-negative breast cancer cells MDA-MB-231 as shown in Figure 4, and the results are consistent with their antiproliferative activity. Especially, the most promising compound **19** significantly blocked the colony formation of MDA-MB-231 cells at a submicromolar concentration.

Compounds 10 and 19 Induced Apoptosis of Breast Cancer Cells

On the basis of their promising anti-proliferative effects and their potent activities in the colony formation assay, compounds **10** and **19** were selected for further mechanistic studies to determine whether the growth inhibition induced by them in human breast cancer cells was due to apoptosis. MDA-MB-231 cells were treated with vehicle alone as control and also with **10** or **19** at different concentrations (1.0 μM , 5.0 μM or 10 μM) for 24 h and stained with FITC-Annexin V and propidium iodide (PI). The percentages of apoptotic MDA-MB-231 cells were determined by flow cytometry. As shown in Figure 5, both compounds **10** and **19** displayed significant effects to induce apoptosis of MDA-MB-231 cells in a dose-dependent manner. The findings support that the apoptosis of MDA-MB-231 cells mediated by these two compounds contributes to their antiproliferative effects.

Compounds 10 and 19 Regulated Apoptotic Related Proteins

Previous studies have demonstrated that **1** induces apoptosis of cancer cells by modulating a series of transcription factors, protein kinases as well as pro- and/or anti-apoptotic proteins such as NF- κB ,⁹ MAPK,^{33a} Bax and Bcl-2.^{33b} To elucidate the potential mechanisms contributing to apoptosis induction by the new derivatives **10** and **19**, several proteins related to apoptosis were determined by Western blot assay. As shown in Figure 6, treatment of MDA-MB-231 cells with compounds **10** and **19**, respectively, at low concentrations (2.5 μM -10 μM) led to the down-regulation of antiapoptotic protein Bcl-2 levels and the up-regulation of the pro-apoptotic protein Bax. In addition, they also induced a significant decrease of NF- κB (p65) protein expression, suggesting that NF- κB inhibition might contribute to the reduction of Bcl-2/Bax ratio. Meanwhile, compounds **10** and **19** also triggered PARP cleavage from its full-length form (116 kDa) to the cleaved form (25 kDa) as indicated by appearance of PARP fragments and activated caspase-3 in a dose-dependent manner, which might be either partially or totally responsible for the proteolytic cleavage of PARP. For comparison, exposure to high doses of **1** (10 μM -30 μM) also led to down-regulation of NF- κB (p65), Bcl-2, and PARP (116 kDa), and up-regulation of Bax and cleaved PARP (25 kDa); nevertheless, it did not activate caspase-3 cleavage from the

concentrations of 10 μ M to 30 μ M. These preliminary results indicated that the dienone derivatives **10** and **19** induced the apoptosis in MDA-MB-231 cells likely by regulating unique apoptotic pathways. Other than apoptosis, oridonin has also been found to suppress tumor cell proliferation and induce cancer cell death through cell cycle arrest,^{9a,33b} autophagy,^{9b} and necrosis.^{33a} Therefore, more extensive mechanism studies on the new dienone analogues are ongoing.

Compound 19 Suppressed Growth of Xenograft Tumors in Mice

In our *in vivo* studies, dienone analogue **19** was further evaluated for its antitumor activity in suppression of tumor growth in the triple-negative MDA-MB-231 xenograft model due to its potent antiproliferative and colony formation inhibitory effects in MDA-MB-231 cells as well as lower toxicity in HMEC cells. Meanwhile, compound **19** was selected for further *in vivo* efficacy studies because of its good *in vitro* dose-response relationship. As shown in Figure 7A, compound **19** at 5.0 mg/kg was much more efficacious in suppressing xenograft tumor growth as compared to oridonin at the same dosage ($p < 0.0001$). Meanwhile, compound **19** was also found to be well tolerated during experiments and showed no significant loss of body weight (Figure 7B). These results suggest that compound **19** is a promising anticancer drug candidate with potent antitumor activity and good tolerability for further clinical development.

CONCLUSIONS

In summary, our efficient synthetic methodologies to access several kinds of oridonin analogues with diverse enone functionality presented in the A-ring have been achieved in moderate to good yields through regioselective enone construction strategies starting from oridonin. A key α -bromination/HBr elimination sequence was applied to introduce a double bond to the carbonyl functionality to achieve analogues **6** and **7**. The α -formyl enone analogue **10** was prepared through the hydrolysis of enamine **8** followed by sequential selenenylation and selenoxide elimination, while analogue **12** with both an α -formyl enone system and a 7-hemiacetal group proven to be unstable, and spontaneously underwent a novel 3,7-rearrangement reaction to give unprecedented 3,20-epoxy products **13** and **14**. Different from the conventional protocols, the goal to generate the 1-ene-3-ketone analogues **19** and **20** was realized through 1-ene functionality formation with subsequent successive oxidations of allylic methylene. Intriguingly, dienone analogues **6**, **7**, **10** and **19** have demonstrated enhanced antiproliferative effects against ER-positive MCF-7 and TNBC MDA-MB-231 cells as well as drug-resistant MCF-7/ADR clones, while exhibiting comparable or lower toxicity to normal cells relative to **1**. In our preliminary mechanism studies, dienone analogues **10** and **19** were found to significantly inhibit colony formation and induce apoptosis of MDA-MB-231 cells in a dose-dependent manner through regulating a series of apoptotic related proteins. Meanwhile, analogue **19** has demonstrated more efficacious antitumor activity than oridonin and excellent tolerability in MDA-MB-231 xenograft-bearing nude mice, indicating the potential of these new dienone analogues for the treatment of highly aggressive triple negative and drug-resistant breast cancers.

EXPERIMENTAL SECTION

General

All commercially available starting materials and solvents were reagent grade, and used without further purification. Oridonin was purchased from Shanxi Huike, China. Reactions were performed under a nitrogen atmosphere in dry glassware with magnetic stirring. Preparative column chromatography was performed using silica gel 60, particle size 0.063–0.200 mm (70–230 mesh, flash). Analytical TLC was carried out employing silica gel 60

F254 plates (Merck, Darmstadt). Visualization of the developed chromatograms was performed with detection by UV (254 nm). NMR spectra were recorded on a Bruker-600 (^1H , 600 MHz; ^{13}C , 150 MHz) spectrometer or Bruker-300 (^1H , 300 MHz; ^{13}C , 75 MHz). ^1H and ^{13}C NMR spectra were recorded with TMS as an internal reference. Chemical shifts were expressed in ppm, and J values were given in Hz. High-resolution mass spectra (HRMS) were obtained from Thermo Fisher LTQ Orbitrap Elite mass spectrometer. Parameters include the following: Nano ESI spray voltage was 1.8 kV; Capillary temperature was 275 °C and the resolution was 60,000; Ionization was achieved by positive mode. Melting points were measured on a Thermo Scientific Electrothermal Digital Melting Point Apparatus and uncorrected. Purity of final compounds was determined by analytical HPLC, which was carried out on a Shimadzu HPLC system (model: CBM-20A LC-20AD SPD-20A UV/VIS). HPLC analysis conditions: Waters μ Bondapak C18 (300 \times 3.9 mm); flow rate 0.5 mL/min; UV detection at 270 and 254 nm; linear gradient from 30% acetonitrile in water (0.1% TFA) to 100% acetonitrile (0.1% TFA) in 20 min followed by 30 min of the last-named solvent. All biologically evaluated compounds are > 96% pure.

Synthesis of (3*S*,3*aR*,3*a*¹*R*,6*aR*,7*S*,7*aR*,11*aS*,11*bS*)-7-hydroxy-5,5,8,8-tetramethyl-15-methylene-3,3*a*,7,7*a*,8,11*b*-hexahydro-1*H*-6*a*,11*a*-(epoxymethano)-3,3*a*¹-ethanophenanthro[1,10-*de*][1,3]dioxine-11,14(2*H*)-dione (6)

To a solution of **4** (80 mg, 0.18 mmol) in acetone (4 mL) was added *p*-TsOH (5 mg) and 2,2-dimethoxypropane (0.32 mL) at rt. The resulting mixture was stirred at rt for 2 h. The reaction mixture was then diluted with water and extracted with dichloromethane. The extract was washed with saturated NaHCO_3 solution and brine, dried over anhydrous Na_2SO_4 , filtered, and evaporated to afford compound **5** (83 mg, 95%) as a colorless gel. To a solution of **5** (50 mg, 0.10 mmol) in toluene (5 mL) was added DBU (20 mg, 0.13 mmol) at rt. The resulting mixture was stirred at 110 °C for 4 h, and diluted with water and extracted with EtOAc. The organic extract was washed with 3 N HCl aqueous solution and brine, dried over anhydrous Na_2SO_4 , filtered, and evaporated to give an oily residue, which was purified using preparative TLC developed by 30% EtOAc in hexane to afford the desired product **6** as a colorless amorphous gel (30 mg, 72%). $[\alpha]_D^{25} -54$ (c 0.10, CH_2Cl_2); HPLC purity 98.7% ($t_R = 19.78$ min); ^1H NMR (600 MHz, CDCl_3) δ 6.80 (d, 1H, $J = 9.6$ Hz), 6.17 (s, 1H), 5.84 (d, 1H, $J = 10.2$ Hz), 5.59 (s, 1H), 5.41 (d, 1H, $J = 12.0$ Hz), 4.88 (s, 1H), 4.24 (dd, 1H, $J = 1.2$ Hz, 10.2 Hz), 4.08 (m, 2H), 3.08 (d, 1H, $J = 9.0$ Hz), 2.53 (m, 1H), 2.00 (m, 3H), 1.67 (s, 3H), 1.62 (m, 3H), 1.42 (s, 3H), 1.36 (s, 3H), 1.27 (s, 3H); ^{13}C NMR (150 MHz, CDCl_3) δ 204.7, 196.5, 162.1, 150.4, 126.6, 120.8, 101.3, 95.7, 71.7, 69.9, 65.1, 56.5, 55.9, 47.4, 45.8, 40.1, 35.9, 30.4, 30.2, 30.1, 25.4, 25.0, 19.3. HRMS Calcd for $\text{C}_{23}\text{H}_{29}\text{O}_6$: $[\text{M} + \text{H}]^+$ 401.1959; found 401.1957.

Synthesis of (4*aR*,5*S*,6*S*,6*aR*,9*S*,11*aS*,11*bS*,14*R*)-5,6,14-trihydroxy-4,4-dimethyl-8-methylene-4,4*a*,5,6,9,10,11,11*a*-octahydro-1*H*-6,11*b*-(epoxymethano)-6*a*,9-methanocyclohepta[*a*]naphthalene-1,7(8*H*)-dione (7)

To a solution of **6** (8.0 mg, 0.02 mmol) in a mixture of MeOH (2 mL) and CH_2Cl_2 (0.5 mL) was added 5% HCl aqueous solution (0.5 mL) at rt. The resulting mixture was stirred at rt for 4 h. The reaction mixture was then diluted with water and extracted with dichloromethane. The extract was washed with saturated NaHCO_3 (aq.) solution and brine, dried over anhydrous Na_2SO_4 , filtered, and evaporated to give an oily residue. The residue was purified using preparative TLC developed by 50% EtOAc in hexane to afford the desired product **7** as a colorless amorphous gel (6.5 mg, 89%). $[\alpha]_D^{25} -56$ (c 0.10, CH_2Cl_2); HPLC purity 99.0% ($t_R = 16.02$ min); ^1H NMR (600 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD} = 5:1$) δ 6.88 (d, 1H, $J = 9.6$ Hz), 6.21 (s, 1H), 5.87 (d, 1H, $J = 10.2$ Hz), 5.63 (s, 1H), 4.97 (s, 1H), 4.27 (m, 2H), 4.06 (dd, 1H, $J = 1.2$ Hz, 10.2 Hz), 3.96 (d, 1H, $J = 8.4$ Hz), 3.04 (d, 1H, $J = 9.6$ Hz), 2.52 (m, 1H), 2.10 (m, 2H), 2.03 (d, 1H, $J = 8.4$ Hz), 1.62 (m, 1H), 1.48 (m, 1H), 1.39 (s,

3H), 1.27 (s, 3H); ^{13}C NMR (150 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD} = 5:1$) δ 206.7, 197.3, 161.8, 150.8, 126.8, 121.2, 97.9, 72.3, 72.2, 65.2, 61.4, 56.8, 50.0, 45.9, 42.7, 35.7, 29.8, 29.4, 23.9, 18.9; HRMS Calcd for $\text{C}_{20}\text{H}_{25}\text{O}_6$: $[\text{M} + \text{H}]^+$ 361.1646; found 361.1544.

Synthesis of (3*S*,3*aR*,3*a*¹*R*,6*aR*,7*S*,7*aR*,11*aS*,11*bS*,*Z*)-10-((dimethylamino)methylene)-7-hydroxy-5,5,8,8-tetramethyl-15-methyleneoctahydro-1*H*-6*a*,11*a*-(epoxymethano)-3,3*a*¹-ethanophenanthro[1,10-*de*][1,3]dioxine-11,14(2*H*)-dione (8)

To a solution of **2** (250 mg, 0.68 mmol) in acetone (10 mL) was added *p*-TsOH (20 mg) and 2,2-dimethoxypropane (1.0 mL) at rt. The resulting mixture was stirred at rt for 2 h. The reaction mixture was then diluted with water and extracted with dichloromethane. The extract was washed with saturated NaHCO_3 (aq.) solution and brine, dried over anhydrous Na_2SO_4 , filtered, and evaporated to afford compound **3** as a colorless gel (263 mg, 95%). To a solution of **3** (230 mg, 0.57 mmol) in DMF (4 mL) was added DMF-DMA (136 mg, 1.14 mmol) at rt. The resulting mixture was refluxed at 110 °C for 36 h. The solvent was then removed under vacuum to give a brown oily residue, which was further purified using preparative TLC developed by 66% EtOAc in hexane to afford the desired product **8** as a brown gel (156 mg, 60%). ^1H NMR (600 MHz, CDCl_3) δ 7.42 (s, 1H), 6.14 (s, 1H), 5.55 (s, 1H), 5.20 (d, 1H, $J = 12.0$ Hz), 4.87 (s, 1H), 4.31 (d, 1H, $J = 10.2$ Hz), 4.04 (d, 1H, $J = 10.2$ Hz), 3.87 (m, 1H), 3.07 (s, 6H), 3.04 (d, 1H, $J = 9.6$ Hz), 2.47 (m, 3H), 1.97 (m, 2H), 1.66 (s, 3H), 1.62 (m, 1H), 1.56 (m, 2H), 1.34 (s, 3H), 1.23 (s, 3H), 1.00 (s, 3H); HRMS Calcd for $\text{C}_{26}\text{H}_{36}\text{NO}_6$: $[\text{M} + \text{H}]^+$ 458.2537; found 458.2541.

Synthesis of (3*S*,3*aR*,3*a*¹*R*,6*aR*,7*S*,7*aR*,11*aS*,11*bS*,*Z*)-7-hydroxy-10-(hydroxymethylene)-5,5,8,8-tetramethyl-15-methyleneoctahydro-1*H*-6*a*,11*a*-(epoxymethano)-3,3*a*¹-ethanophenanthro[1,10-*de*][1,3]dioxine-11,14(2*H*)-dione (9)

To a solution of **8** (200 mg, 0.43 mmol) in THF (5 mL) was added 5% HCl aqueous solution (0.5 mL) at rt. The resulting mixture was stirred at rt for 15 min. The reaction mixture was then diluted with water and extracted with dichloromethane. The extract was washed with saturated NaHCO_3 (aq.) solution and brine, dried over anhydrous Na_2SO_4 , filtered, and evaporated to give an oily residue. The residue was further purified using preparative TLC developed by 60% EtOAc in hexane to afford the desired product **9** (100 mg, 83%) as a pale pink gel. ^1H NMR (300 MHz, CDCl_3) δ 14.72 (d, 1H, $J = 3.3$ Hz), 8.39 (s, 1H), 6.19 (s, 1H), 5.60 (s, 1H), 5.29 (d, 1H, $J = 12.0$ Hz), 4.90 (s, 1H), 4.30 (dd, 1H, $J = 1.2$ Hz, 9.9 Hz), 4.09 (dd, 1H, $J = 0.9$ Hz, 9.9 Hz), 3.92 (m, 1H), 3.09 (d, 1H, $J = 9.6$ Hz), 2.55 (m, 1H), 2.29 (d, 1H, $J = 15.0$ Hz), 2.05 (m, 3H), 1.84 (m, 1H), 1.67 (s, 3H), 1.60 (m, 2H), 1.37 (s, 3H), 1.29 (s, 3H), 1.04 (s, 3H); ^{13}C NMR (75 MHz, CDCl_3) δ 204.6, 185.4, 184.8, 150.4, 120.7, 109.2, 101.2, 95.7, 71.6, 70.0, 64.4, 58.1, 56.0, 48.3, 43.7, 40.1, 39.9, 33.2, 30.5, 30.3, 30.0, 25.3, 20.6, 19.8; HRMS Calcd for $\text{C}_{24}\text{H}_{31}\text{O}_7$: $[\text{M} + \text{H}]^+$ 431.2064; found 431.2063.

Synthesis of (3*S*,3*aR*,3*a*¹*R*,6*aR*,7*S*,7*aR*,11*aS*,11*bS*)-7-hydroxy-5,5,8,8-tetramethyl-15-methylene-11,14-dioxo-2,3,3*a*,7,7*a*,8,11,11*b*-octahydro-1*H*-6*a*,11*a*-(epoxymethano)-3,3*a*¹-ethanophenanthro[1,10-*de*][1,3]dioxine-10-carbaldehyde (10)

To a stirring solution of phenylselenenyl chloride (33.6 mg, 0.175 mmol) in CH_2Cl_2 (3 mL) at 0 °C was added pyridine (0.017 mL, 0.208 mmol). The solution was stirred for 45 min, and then a solution of α -keto aldehyde **9** (60 mg, 0.139 mmol) in CH_2Cl_2 (2 mL) was added. The mixture was stirred at 0 °C for 15 min and at rt for 45 min. It was then extracted twice with 1 N HCl (aq.). The organic phase was dried over MgSO_4 , filtered, and concentrated under reduced pressure. The crude product was further purified using the preparative TLC developed by hexane/EtOAc (1:1) to afford the selenide as a yellow gel (60.0 mg, 74%). To a stirring solution of the above selenide (60.0 mg, 0.102 mmol) in CH_2Cl_2 (5.8 mL) was added 35% H_2O_2 (aq.) solution (0.10 mL, 1.2 mmol). The mixture was vigorously stirred for

5 min, followed by the addition of another portion of 35% H₂O₂ (aq.) solution (0.10 mL, 1.2 mmol) with vigorous stirring for another 5 min. The reaction mixture was then extracted twice with water. The extract was dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude product was further purified using the preparative TLC developed by hexane/EtOAc (1:1) to afford **10** (43 mg, 97%) as a pale yellow gel. [α]_D²⁵ -102 (c 0.10, CH₂Cl₂); HPLC purity 98.1% (*t*_R = 18.33 min); ¹H NMR (600 MHz, CDCl₃) δ 9.83 (s, 1H), 7.59 (s, 1H), 6.18 (s, 1H), 5.61 (s, 1H), 5.42 (d, 1H, *J* = 12.6 Hz), 4.89 (s, 1H), 4.33 (dd, 1H, *J* = 1.2 Hz, 10.2 Hz), 4.09 (m, 2H), 3.10 (d, 1H, *J* = 9.0 Hz), 2.56 (m, 1H), 2.06 (m, 2H), 2.00 (d, 1H, *J* = 8.4 Hz), 1.67 (s, 3H), 1.56 (m, 3H), 1.52 (s, 3H), 1.36 (s, 3H), 1.32 (s, 3H); ¹³C NMR (150 MHz, CDCl₃) δ 204.5, 195.2, 188.0, 168.4, 150.0, 133.0, 121.3, 101.5, 95.8, 71.3, 69.8, 64.9, 56.1, 55.7, 47.0, 46.5, 40.0, 36.2, 30.0, 29.7, 25.3, 24.2, 19.0; HRMS Calcd for C₂₄H₂₉O₇: [M + H]⁺ 429.1908; found 429.1897.

Synthesis of (3*S*,4*aR*,5*S*,6*aR*,9*S*,11*aS*,11*bS*,14*R*,*E*)-5,14-dihydroxy-2-(methoxymethylene)-4,4-dimethyl-8-methyleneoctahydro-1*H*-3,11*b*-(epoxymethano)-6*a*,9-methanocyclohepta[*a*]naphthalene-1,6,7(2*H*,8*H*)-trione (13)

To a solution of **10** (5 mg, 0.011 mmol) in a mixture of MeOH (2 mL) and CH₂Cl₂ (0.5 mL) was added 5% HCl aqueous solution (0.2 mL) at rt. The resulting mixture was stirred at rt for 2 h. The reaction mixture was then diluted with water and extracted with dichloromethane. The extract was washed with saturated NaHCO₃ (aq.) solution and brine, dried over anhydrous Na₂SO₄, filtered, and evaporated to give an oily residue. The residue was purified using preparative TLC developed by EtOAc to afford the desired product **13** as a colorless amorphous gel (3.5 mg, 78%). [α]_D²⁵ -110 (c 0.10, CH₂Cl₂); HPLC purity 98.3% (*t*_R = 14.58 min); ¹H NMR (300 MHz, CDCl₃) δ 7.59 (s, 1H), 6.18 (s, 1H), 5.47 (s, 1H), 4.67 (m, 2H), 4.43 (d, 1H, *J* = 0.9 Hz), 4.33 (s, 1H), 4.22 (m, 1H), 3.94 (s, 3H), 3.91 (m, 1H), 3.09 (m, 1H), 2.92 (m, 1H), 1.62 (m, 3H), 1.57 (m, 1H), 1.52 (s, 3H), 0.99 (s, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 205.5, 201.4, 196.7, 156.3, 146.0, 118.6, 115.4, 75.1, 74.2, 71.7, 66.3, 62.1, 61.5, 51.7, 51.0, 45.3, 42.0, 38.2, 30.9, 28.5, 21.8, 20.1; HRMS Calcd for C₂₂H₂₇O₇: [M + H]⁺ 403.1751; found 403.1768.

Synthesis of (3*S*,4*aR*,5*S*,6*aR*,9*S*,11*aS*,11*bS*,14*R*,*Z*)-5,14-dihydroxy-2-(hydroxymethylene)-4,4-dimethyl-8-methyleneoctahydro-1*H*-3,11*b*-(epoxymethano)-6*a*,9-methanocyclohepta[*a*]naphthalene-1,6,7(2*H*,8*H*)-trione (14)

To a solution of **10** (15 mg, 0.035 mmol) in THF (2 mL) was added 5% HCl (aq.) solution (0.3 mL) at rt. The resulting mixture was stirred at rt for 4 h. The reaction mixture was then diluted with water and extracted with dichloromethane. The extract was washed with saturated NaHCO₃ (aq.) solution and brine, dried over anhydrous Na₂SO₄, filtered, and evaporated to give an oily residue. The residue was purified using preparative TLC developed by 5% methanol in dichloromethane to afford the desired product **14** as a pale pink amorphous gel (11 mg, 80%). [α]_D²⁵ -104 (c 0.1, CH₂Cl₂); HPLC purity 96.6% (*t*_R = 4.47 min); ¹H NMR (300 MHz, CDCl₃) δ 7.99 (br s, 1H), 7.13 (s, 1H), 6.26 (s, 1H), 5.49 (s, 1H), 4.61 (d, 1H, *J* = 6.0 Hz), 4.54 (d, 1H, *J* = 4.5 Hz), 4.47 (s, 1H), 3.83 (m, 4H), 3.11 (d, 1H, *J* = 1.2 Hz), 3.05 (d, 1H, *J* = 3.9 Hz), 1.89 (m, 1H), 1.75 (m, 2H), 1.57 (m, 2H), 1.48 (s, 3H), 0.96 (s, 3H); ¹³C NMR (75 MHz, CDCl₃) δ 205.1, 200.4, 199.4, 160.1, 144.8, 120.3, 113.2, 71.6, 65.8, 60.9, 51.4, 50.6, 45.6, 41.2, 38.7, 31.1, 29.6 (2C), 22.5, 20.0. HRMS Calcd for C₂₁H₂₅O₇: [M + H]⁺ 389.1595; found 389.1591.

Synthesis of (3S,3aR,3a¹R,6aR,7S,7aR,11S,11aS,11bS)-7-hydroxy-5,5,8,8-tetramethyl-15-methylene-14-oxodecahydro-1H-6a,11a-(epoxymethano)-3,3a¹-ethanophenanthro[1,10-de][1,3]dioxin-11-yl methanesulfonate (16)

To a solution of oridonin (500 mg, 1.36 mmol) in acetone (20 mL) was added *p*-TsOH (20 mg) and 2,2-dimethoxypropane (3.0 mL) at rt. The resulting mixture was stirred at rt for 2 h. The reaction mixture was then diluted with water and extracted with dichloromethane. The extract was washed with saturated NaHCO₃ (aq.) solution and brine, dried over anhydrous Na₂SO₄, filtered, and evaporated to afford compound **15** as a colorless gel (520 mg, 93%); To a solution of **15** (277 mg, 0.68 mmol) in dichloromethane was added Et₃N (138 mg, 1.37 mmol) and MsCl (94 mg, 0.82 mmol) dropwise at 0 °C. The mixture was stirred at rt overnight, diluted with water, and extracted with dichloromethane. The organic extract was washed with saturated NaHCO₃ (aq.) solution and brine, dried over anhydrous Na₂SO₄, filtered, and evaporated to give an oily residue. The crude residue was further purified by silica gel column; elution with 50% EtOAc in hexane afforded the desired product **16** as a colorless amorphous gel (264 mg, 80%). ¹H NMR (600 MHz, CDCl₃) δ 6.16 (s, 1H), 5.82 (d, 1H, *J* = 12.0 Hz), 5.58 (s, 1H), 4.76 (dd, 1H, *J* = 6.0 Hz, 12.0 Hz), 4.14 (d, 1H, *J* = 4.2 Hz), 3.92 (m, 1H), 3.07 (d, 1H, *J* = 9.0 Hz), 2.99 (s, 3H), 2.51 (m, 1H), 2.05 (m, 1H), 1.89 (m, 2H), 1.77 (m, 3H), 1.63 (s, 3H), 1.52 (m, 1H), 1.38 (d, 1H, *J* = 7.2 Hz), 1.33 (s, 3H), 1.19 (s, 3H), 1.18 (s, 3H), 1.16 (d, 1H, *J* = 7.2 Hz); ¹³C NMR (150 MHz, CDCl₃) δ 205.1, 150.4, 120.5, 101.0, 94.6, 84.7, 72.9, 69.9, 62.3, 59.5, 55.9, 50.0, 40.6, 40.1, 38.2, 33.2, 33.0, 31.5, 30.1 (2C), 26.4, 25.4, 22.4, 18.8; HRMS Calcd for C₂₄H₃₆O₈S: [M+H]⁺ 483.2047; found 483.2052.

Synthesis of (3S,3aR,3a¹R,6aR,7S,7aR,11aR,11bS)-7-hydroxy-5,5,8,8-tetramethyl-15-methylene-2,3,3a,7,7a,8,9,11b-octahydro-1H-6a,11a-(epoxymethano)-3,3a¹-ethanophenanthro[1,10-de][1,3]dioxin-14-one (17)

To a solution of **16** (34 mg, 0.07 mmol) in DMF (5 mL) was added LiBr (18 mg, 0.21 mmol) and Li₂CO₃ (15 mg, 0.21 mmol) at rt. The resulting mixture was stirred at 115 °C for 2 h. The reaction mixture was then diluted with water and extracted with EtOAc. The organic extract was washed with saturated NaHCO₃ (aq.) solution and brine, dried over anhydrous Na₂SO₄, filtered, and evaporated to give an oily residue. The crude residue was further purified by silica gel column; elution with 25% EtOAc in hexane afforded the desired product **17** as a colorless powder (25 mg, 84%). HPLC purity 99.8% (*t*_R = 18.30 min). ¹H NMR (600 MHz, CDCl₃) δ 6.16 (s, 1H), 5.77 (m, 1H), 5.56 (s, 1H), 5.41 (d, 1H, *J* = 12.0 Hz), 5.19 (dd, 1H, *J* = 3.0 Hz, 10.2 Hz), 4.82 (s, 1H), 3.99 (d, 1H, *J* = 10.2 Hz), 3.90 (dd, 1H, *J* = 8.4 Hz, 12.0 Hz), 3.81 (d, 1H, *J* = 9.6 Hz), 3.06 (d, 1H, *J* = 9.0 Hz), 2.53 (m, 1H), 1.95 (d, 1H, *J* = 17.4 Hz), 1.76 (m, 4H), 1.65 (s, 3H), 1.56 (m, 1H), 1.50 (d, 1H, *J* = 8.4 Hz), 1.35 (s, 3H), 1.18 (s, 3H), 1.05 (s, 3H); ¹³C NMR (150 MHz, CDCl₃) δ 204.5, 150.5, 130.3, 124.1, 120.4, 101.2, 95.4, 72.0, 70.1, 64.8, 57.9, 56.3, 49.1, 41.1, 40.3, 38.1, 32.2, 31.1, 30.3, 30.1, 25.5, 22.1, 17.3; HRMS Calcd for C₂₃H₃₁O₅: [M + H]⁺ 387.2166; found 387.2169.

Synthesis of (3S,3aR,3a¹R,6aR,7S,7aS,9S,11aR,11bS)-7,9-dihydroxy-5,5,8,8-tetramethyl-15-methylene-2,3,3a,7,7a,8,9,11b-octahydro-1H-6a,11a-(epoxymethano)-3,3a¹-ethanophenanthro[1,10-de][1,3]dioxin-14-one (18)

A mixture of **17** (20 mg, 0.05 mmol) and SeO₂ (16 mg, 0.15 mmol) in 1,4-dioxane (4 mL) was stirred at 100 °C for 16 h. The reaction mixture was then filtered, and the filtrate was diluted with water and extracted with dichloromethane. The extract was washed with brine, dried over anhydrous Na₂SO₄, filtered, and evaporated to give an oily residue. The residue was purified using preparative TLC developed by 50% EtOAc in hexane to afford the desired product **18** as a colorless amorphous gel (16 mg, 76%). HPLC purity 99.7% (*t*_R =

17.31 min); ^1H NMR (600 MHz, CDCl_3) δ 6.16 (s, 1H), 6.00 (dd, 1H, $J = 6.0$ Hz, 10.2 Hz), 5.56 (s, 1H), 5.42 (m, 2H), 4.82 (s, 1H), 3.94 (m, 2H), 3.84 (d, 1H, $J = 9.6$ Hz), 3.06 (d, 1H, $J = 9.6$ Hz), 2.53 (m, 1H), 1.87 (d, 1H, $J = 9.0$ Hz), 1.82 (m, 2H), 1.71 (m, 2H), 1.65 (s, 3H), 1.57 (m, 1H), 1.35 (s, 1H), 1.24 (s, 3H), 1.01 (s, 3H); ^{13}C NMR (150 MHz, CDCl_3) δ 204.1, 150.3, 130.9, 128.6, 120.6, 101.2, 95.5, 72.6, 71.5, 70.0, 64.5, 56.1, 51.0, 48.8, 40.3, 38.2, 36.7, 30.2, 30.1, 25.9, 25.4, 21.9, 17.3; HRMS Calcd for $\text{C}_{23}\text{H}_{31}\text{O}_6$: $[\text{M} + \text{H}]^+$ 403.2115; found 403.2118.

Synthesis of (3S,3aR,3a¹R,6aR,7S,7aS,11aR,11bS)-7-hydroxy-5,5,8,8-tetramethyl-15-methylene-3,3a,7,7a,8,11b-hexahydro-1H-6a,11a-(epoxymethano)-3,3a¹-ethanophenanthro[1,10-de][1,3]dioxine-9,14(2H)-dione (19)

To a solution of **18** (10 mg, 0.025 mmol) in dichloromethane (2 mL) was added PDC (11.2 mg, 0.03 mmol) at rt. The resulting mixture was stirred at rt for 4 h. The reaction mixture was then filtered, and the filtrate was diluted with water and extracted with dichloromethane. The extract was washed with saturated NaHCO_3 (aq.) solution and brine, dried over anhydrous Na_2SO_4 , filtered, and evaporated to give an oily residue. The residue was purified using preparative TLC developed by 50% EtOAc in hexane to afford the desired product **19** as a colorless amorphous gel (8.0 mg, 80%). $[\alpha]_D^{25} -100$ (c 0.10, CH_2Cl_2); HPLC purity 97.5% ($t_R = 18.62$ min); ^1H NMR (600 MHz, CDCl_3) δ 6.29 (d, 1H, $J = 10.2$ Hz), 6.19 (s, 1H), 6.00 (d, 1H, $J = 10.2$ Hz), 5.60 (s, 1H), 5.54 (d, 1H, $J = 12.0$ Hz), 4.84 (s, 1H), 4.16 (d, 1H, $J = 10.2$ Hz), 4.07 (m, 1H), 4.01 (d, 1H, $J = 10.2$ Hz), 3.10 (d, 1H, $J = 8.4$ Hz), 2.58 (m, 1H), 1.93 (d, 1H, $J = 7.8$ Hz), 1.88 (m, 2H), 1.76 (m, 1H), 1.66 (s, 3H), 1.60 (m, 2H), 1.37 (s, 3H), 1.36 (s, 3H), 1.27 (s, 3H); ^{13}C NMR (150 MHz, CDCl_3) δ 204.1, 203.2, 149.9, 142.6, 129.8, 121.2, 101.5, 95.4, 70.9, 69.8, 64.1, 56.2, 55.7, 48.3, 44.6, 40.1, 38.8, 30.1, 29.9, 25.4, 23.9, 22.4, 17.1; HRMS Calcd for $\text{C}_{23}\text{H}_{29}\text{O}_6$: $[\text{M} + \text{H}]^+$ 401.1959; found 361.1962.

Synthesis of (4aS,5S,6S,6aR,9S,11aS,11bR,14R)-5,6,14-trihydroxy-4,4-dimethyl-8-methylene-4,4a,5,6,9,10,11,11a-octahydro-3H-6,11b-(epoxymethano)-6a,9-methanocyclohepta[a]naphthalene-3,7(8H)-dione (20)

To a solution of **19** (15 mg, 0.037 mmol) in a mixture of MeOH (2 mL) and CH_2Cl_2 (0.5 mL) was added 5% HCl aqueous solution (0.5 mL) at rt. The resulting mixture was stirred at rt for 4 h. The reaction mixture was then diluted with water and extracted with dichloromethane. The extract was washed with saturated NaHCO_3 (aq.) solution and brine, dried over anhydrous Na_2SO_4 , filtered, and evaporated to give an oily residue. The residue was purified using preparative TLC developed by 66% EtOAc in hexane to afford the desired product **20** as a colorless amorphous gel (11.5 mg, 85%). $[\alpha]_D^{25} -128$ (c 0.10, CH_2Cl_2); HPLC purity 98.2% ($t_R = 14.87$ min); ^1H NMR (600 MHz, CDCl_3) δ 6.31 (d, 1H, $J = 10.2$ Hz), 6.22 (s, 1H), 6.13 (d, 1H, $J = 11.4$ Hz), 6.02 (d, 1H, $J = 10.8$ Hz), 5.63 (s, 1H), 4.92 (s, 1H), 4.17 (d, 1H, $J = 10.2$ Hz), 4.06 (dd, 1H, $J = 1.8$ Hz, 10.2 Hz), 3.98 (m, 1H), 3.10 (d, 1H, $J = 9.0$ Hz), 2.58 (m, 1H), 1.95 (d, 1H, $J = 9.0$ Hz), 1.91 (m, 2H), 1.65 (m, 3H), 1.34 (s, 3H), 1.26 (s, 3H); ^{13}C NMR (150 MHz, CDCl_3) δ 206.0, 202.8, 150.5, 142.7, 130.0, 122.2, 97.7, 72.4, 72.1, 64.8, 61.7, 55.6, 51.4, 44.4, 42.5, 39.2, 29.4, 23.6, 22.0, 17.5; HRMS Calcd for $\text{C}_{20}\text{H}_{25}\text{O}_6$: $[\text{M} + \text{H}]^+$ 361.1646; found 361.1651.

In Vitro Determination of Effects of Synthesized Compounds on Cancer Cell Proliferation

Breast cancer cell lines MCF-7 and MDA-MB-231 were seeded in 96-well plates at a density of 1×10^4 cells/well and treated with DMSO, 0.01 μM , 0.1 μM , 1 μM , 5 μM , 10 μM , and 100 μM of individual compound for 48 h, and then 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL in PBS) was added to each well and further incubated for another 4 h. Then, MTT solution was removed

and 150 μ L of DMSO was added to each well. Absorbance of all wells was determined by measuring OD at 550 nm after 10 min incubation on a 96-well GlowMaxate Absorbance Reader (Promega, Madison, WI). Each individual compound was tested in quadruplicate wells for each concentration.

Colony Formation Assay

Breast cancer MDA-MB-231 cells were seeded in 6-well tissue culture plates with a density of 800 cells/per well and maintained in regular culture media. After 24 h, the cells were treated with compounds **19** and **10** at different concentrations (0.625 μ M, 1.25 μ M, 2.5 μ M, 5 μ M, and 10 μ M, respectively) or DMSO as the vehicle. The culture media with the compounds were changed every 72 h. At the end of two weeks, the wells were washed twice with PBS buffer and 2 mL of 0.01% crystal violet staining buffer was added to each well and incubated for 10 min. The wells were then washed with PBS for 5 min for three times, and allowed to dry. Photographs were then taken and the density of the entire culture well area was digitally measured using the GelCountTM instrument (Oxford Optronix, UK). Experiments were performed in triplicate and the density data were analyzed with one-way ANOVA using GraphPad Prizm 5 software package. Error bars represent standard deviation.

Cell Apoptosis Assay

Breast cancer MDA-MB-231 cells were incubated in 6-well plates (2.5×10^5 cells/well). Cells were then treated with DMSO, oridonin or new compounds at different concentrations for 24 h, and then both adherent and floating cells were collected, washed once with PBS. Resuspended cells were incubated with 100 μ L PBS containing 1% BSA and 100 μ L Annexin V and dead cell detection reagent at room temperature for 20 min. Apoptosis was measured immediately using the Muse Cell Analyzer with the MuseTM Apoptosis Kit (Catalog No. MCH100105).

Western Blot Analysis

Breast cancer MDA-MB-231 cells were treated with DMSO, oridonin or compounds **10** and **19**, respectively. After 48 h of treatment, cells were harvested and lysed. Protein concentrations were quantified by the method of Bradford with bovine serum albumin as the standard. Equal amounts of total cellular protein extract (30 μ g) was separated by electrophoresis on SDS-polyacrylamide gels and transferred to PVDF membranes. After blocking with 5% non-fat milk, the membrane was incubated with the desired primary antibody overnight at the following dilution: anti-Bcl-2 (1:200), anti-Bax (1:1000), anti-PARP (1:10000), anti-NF- κ B (1:2000), anti-caspase-3 (1:1000) and β -actin (1:20000). Subsequently, the membrane was incubated with appropriate secondary antibody. The immunoreactive bands were visualized by enhanced chemiluminescence as recommended by the manufacturer.

In Vivo Antitumor Efficacy Determination

All drugs were dissolved in 50% DMSO with 50% polyethylene glycol for *in vivo* administration. Body weights and tumors volume were measured daily and tumor volume was calculated according to the formula $V = 0.5 \times L \times W^2$, where L = length (mm) and W = width (mm). All procedures including mice and *in vivo* experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of UT M. D. Anderson Cancer Center (MDACC). 25 female nude mice were obtained from MDACC and were used for orthotopic tumor studies at 4 to 6 weeks of age. The mice were maintained in a barrier unit with 12 h light-dark switch. Freshly harvested MDA-MB-231 cells (2.5×10^6 cells per mouse, resuspended in 100 μ L PBS) were injected into the fat pad of the 3rd mammary gland of mice, and then randomized into 3 groups. The mice were treated 5 days/week for 10

days with 5 mg/kg of compound **19, 1** or vehicle through intraperitoneal injection, when the tumor volume reached 200 mm³.

Statistical Analysis

Statistical significance was determined using Student's t-test in drug-resistant breast cancer cell and HMEC viability assay or one way ANOVA in *in vivo* experiments. * represents a *p* value less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS USED

TNBC	triple-negative breast cancer
SAR	Structure-Activity Relationships
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
IC₅₀	half maximal inhibitory concentration
PI	propidium iodide
HRMS	High-resolution mass spectrometry
HPLC	high performance liquid chromatography
TFA	trifluoroacetic acid
DMSO	dimethyl sulfoxide
TLC	thin layer chromatography
NMR	nuclear magnetic resonance
TMS	tetramethylsilane
THF	tetrahydrofuran
EtOAc	ethyl acetate
DMF	<i>N,N</i> -dimethylformamide
<i>p</i>-Ts	4-toluenesulfonyl
Py	pyridine
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
LDA	lithium diisopropylamide
DMF-DMA	<i>N,N</i> -dimethylformamide dimethyl acetal
Ms	methanesulfonyl

PDC	pyridinium dichromate
PBS	phosphate-buffered saline
BCA	bicinchoninic acid
BSA	bovine serum albumin
SDS	sodium dodecyl sulfate
PVDF	polyvinylidene difluoride
HMEC	human normal mammary epithelial cell

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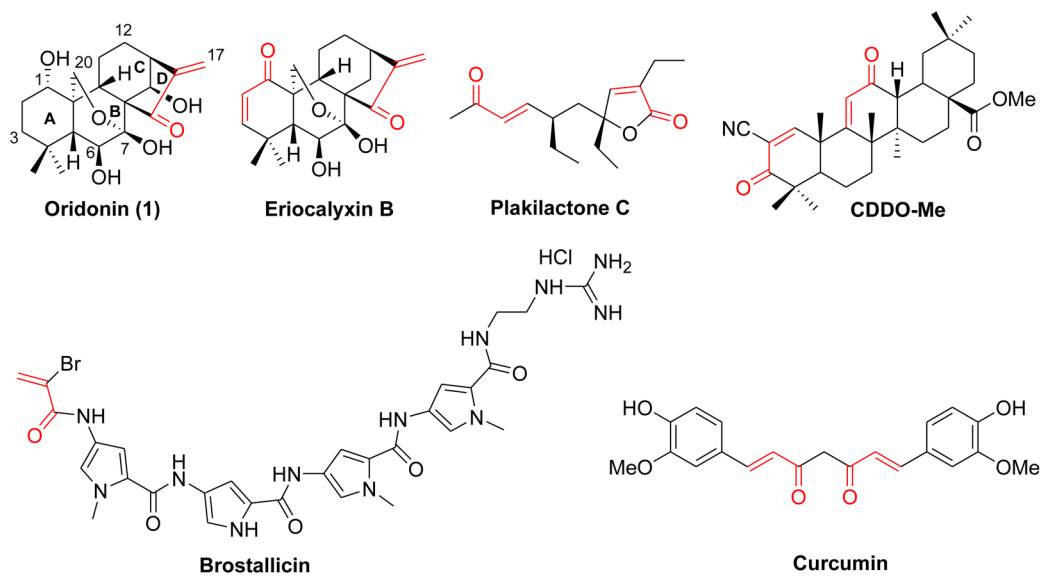


Figure 1.
Structures of oridonin and representative bioactive enone or dienone compounds

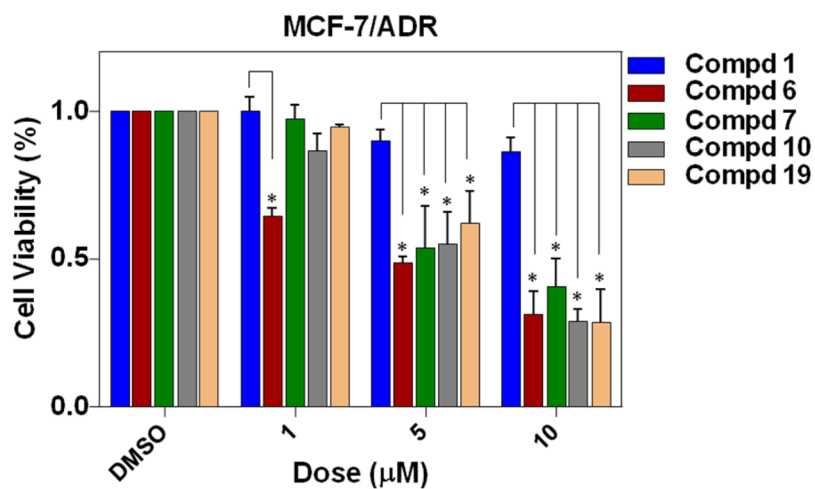


Figure 2. Growth inhibitory effects of compounds **1**, **6**, **7**, **10** and **19** on drug-resistant breast cancer cells. MCF-7/ADR cells were treated with varying concentrations of drugs for 48 h. Values are mean \pm SE of three independent experiments. Statistical significance was determined using Student's t-test. * $p < 0.05$, in comparison with corresponding value of oridonin treatment at the same concentration.

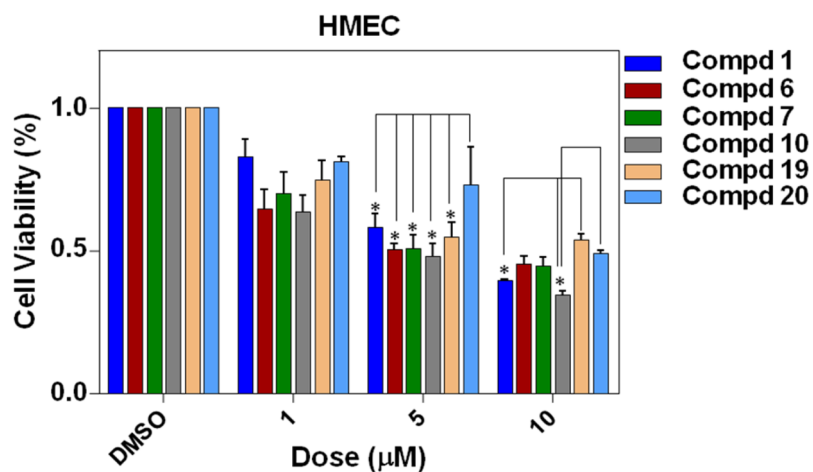


Figure 3. Effects of compounds **1**, **6**, **7**, **10**, **19** and **20** on normal human mammary epithelial cell (HMEC) proliferation. Human mammary epithelial cells (HMEC) were treated with varying concentrations of drugs for 24 h. Values are mean \pm SE of three independent experiments. Statistical significance was determined using Student's t-test. * $p < 0.05$, in comparison with corresponding value of oridonin treatment at the same concentration.

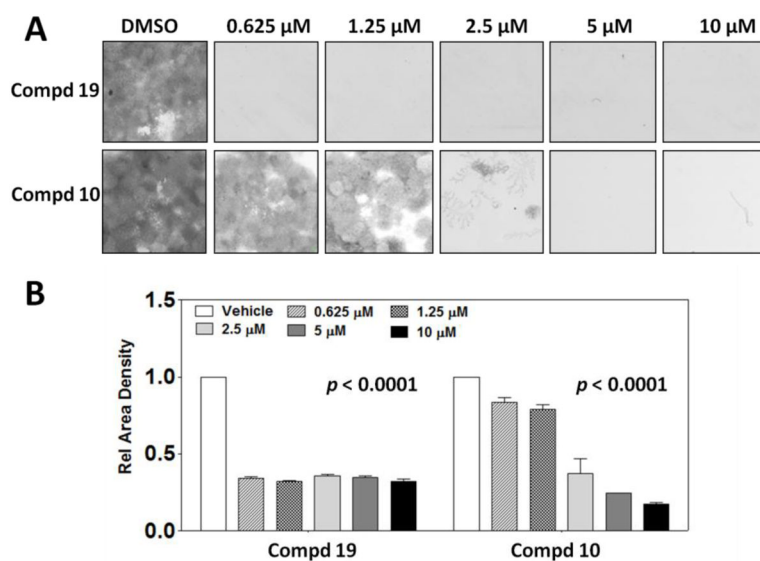


Figure 4. Inhibitory effects of compounds **10** and **19** on colony formation of breast cancer cells. Colony formation assay was performed to measure the capacity of MDA-MB-231 cells to form colonies. (A) **10** and **19** were used to treat the cells at various concentrations as indicated. Culture areas were scanned and digitally quantified with GelCount™ instrument. (B) Density data from scanning the stained culture areas were graphed as shown. Error bars represent standard deviations ($p < 0.0001$). Experiments were performed in triplicate, and the statistical significance was obtained with one-way ANOVA.

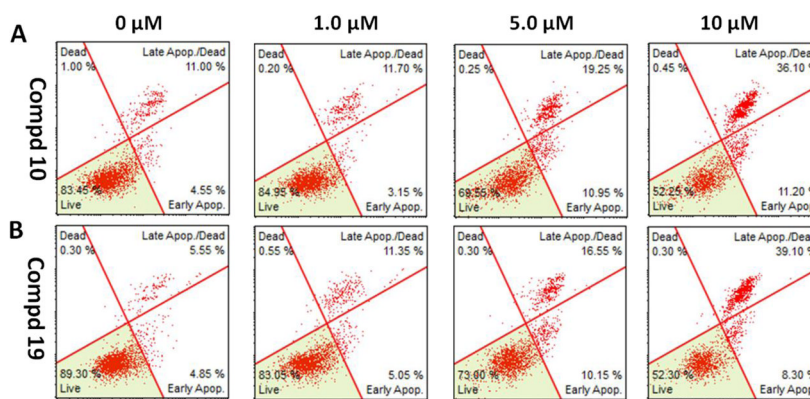


Figure 5. Induction of apoptosis on MDA-MB-231 cells by compounds **10** and **19**. (A) Flow cytometry analysis of apoptotic MDA-MB-231 cells induced by **10** at different concentrations. (B) Flow cytometry analysis of apoptotic MDA-MB-231 cells induced by **19** at different concentrations. Cells were treated with vehicle, **10** or **19** at 1.0 μM, 5.0 μM, and 10 μM concentrations, respectively, for 24 h. The values are means ±SE of at least three independent experiments.

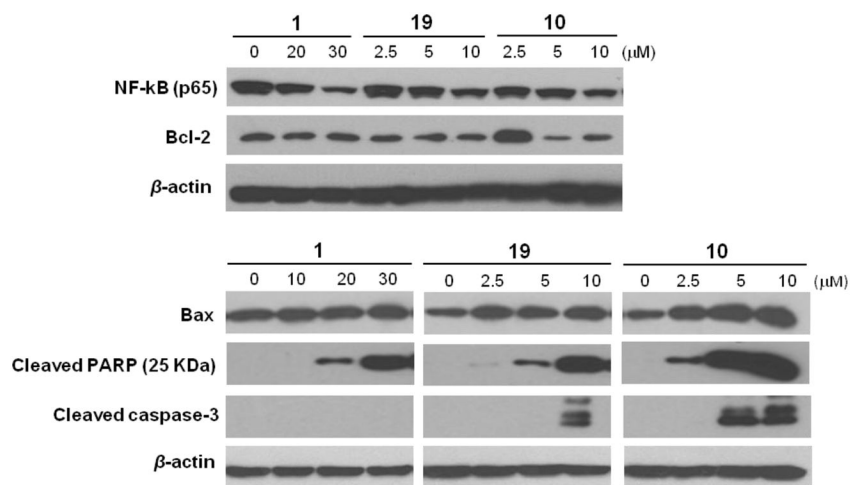


Figure 6. Western blot analysis of biological markers for apoptosis induction by compounds **10** and **19** and **1** in the MDA-MB-231 cells at different concentrations (48 h).

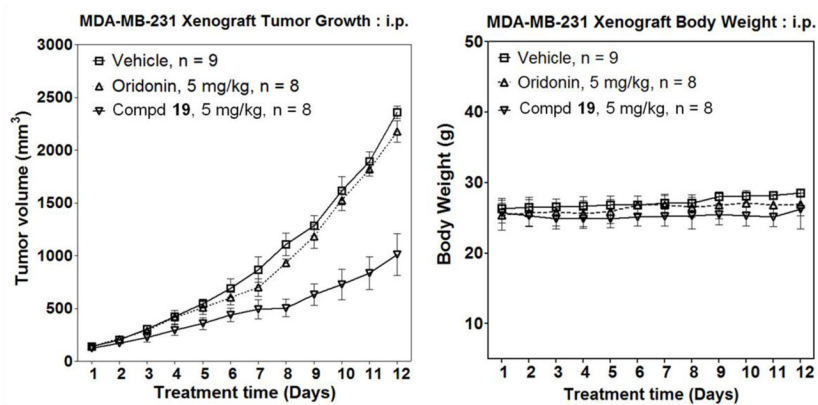
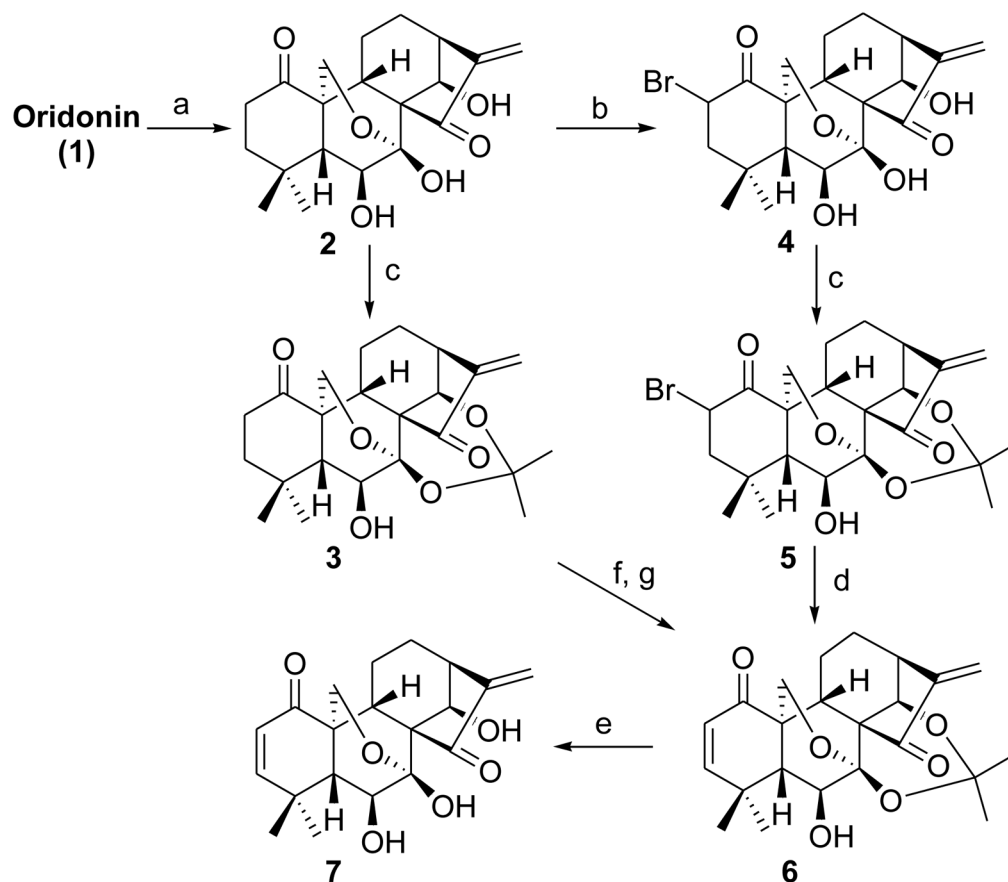
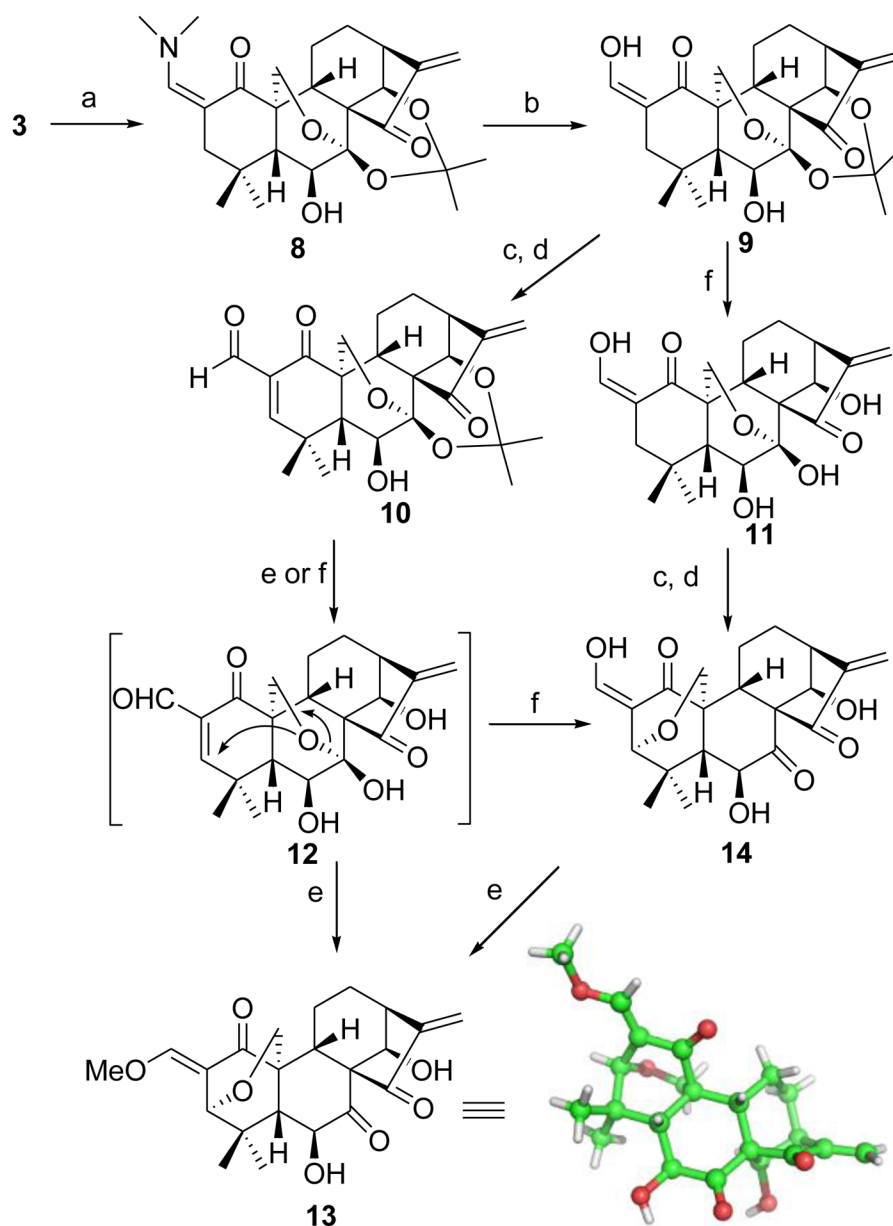


Figure 7. *In vivo* therapeutic efficacy of compound **19** compared to **1** in inhibiting growth of xenograft tumors (triple-negative breast cancer cell line MDA-MB-231) in mice (i.p.) at 5 mg/kg: (A) average tumor size changes; (B) average body weight changes. Values are mean \pm SE of three independent experiments. Statistical significance was determined using one-way ANOVA ($p < 0.0001$).



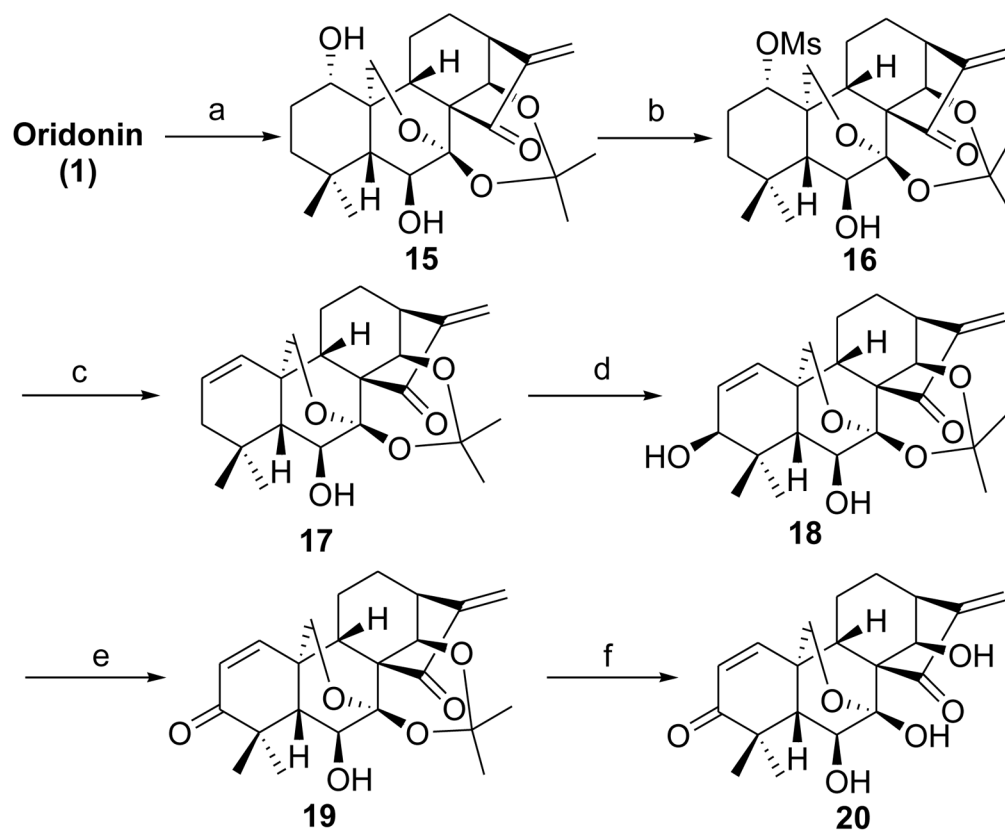
Scheme 1. Synthesis of the dienone analogues 6 and 7^a

^a Reagents and conditions: a) Jones reagent, acetone, 2 h, rt, 82%; b) PyHBr₃, THF, 0 °C, 3 h, 66%; (c) 2,2-dimethoxypropane, cat. *p*-TsOH, acetone, 1 h, rt, 95%; (d) DBU, toluene, reflux, 4 h, 72%; (e) 5% HCl (aq), MeOH/CH₂Cl₂, 0.5 h, 89%; (f) PhSeCl, LDA, THF, -78 °C; (g) 10% H₂O₂ (aq), pyridine, CH₂Cl₂, rt, 5% (2 steps).



Scheme 2. Synthesis of the dienone analogues 10, 13 and 14^a

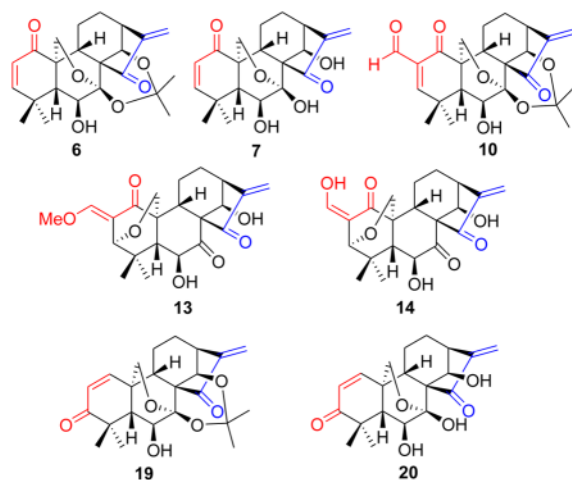
^aReagents and conditions: a) DMF-DMA, toluene, reflux, 24 h, 60%; b) 5% HCl (aq), THF, rt, 10 min, 83%; c) PhSeCl, pyridine, CH₂Cl₂, 0 °C, 1 h; d) 30% H₂O₂ (aq.), CH₂Cl₂, rt, 0.5 h, 70% (two steps) for **10** from **9**, 60% (two steps) for **14** from **11**; e) 5% HCl (aq), MeOH/CH₂Cl₂, rt, 1 h, 80–82%; f) 5% HCl (aq), THF, rt, 2 h, 80% for **14**, 73% for **11**.

**Scheme 3. Synthesis of the dienone analogues 19 and 20^a**

^aReagents and conditions: a) MeCH(OMe)₂, cat. *p*-TsOH, acetone, rt, 2 h, 93%; b) MsCl, Et₃N, CH₂Cl₂, rt, overnight, 80%; c) LiBr, Li₂CO₃, DMF, 110 °C, 2 h, 84%; d) SeO₂, 1,4-dioxane, 110 °C, 16 h, 76%; e) PDC, CH₂Cl₂, rt, 4 h, 80%; f) 5% HCl (aq), MeOH, CH₂Cl₂, rt, 1 h, 85%.

Table 1

Antiproliferative effects of oridonin and the dienone analogues against human breast cancer cell lines.



Compounds	IC ₅₀ (μM) ^a	
	MCF-7	MDA-MB-231
1	4.36 ± 1.41	28.0 ± 1.40
6	0.56 ± 0.31	3.49 ± 1.21
7	1.31 ± 0.25	2.23 ± 0.68
10	1.28 ± 0.47	3.46 ± 1.33
13	10.2 ± 3.07	> 30 ^b
14	> 30	> 30
19	0.98 ± 0.19	5.6 ± 1.06
20	3.48 ± 2.16	9.39 ± 0.48

^aBreast cancer cell lines: MCF-7 and MDA-MB-231. Software: MasterPlex ReaderFit 2010, MiraiBio, Inc. Values are mean ± SE of three independent experiments.

^bIf a specific compound is given a value > 30, it indicates that a specific IC₅₀ cannot be calculated from the data points collected, meaning 'no effect'.

Table 2

Growth inhibitory effects of oridonin and the selected dienone analogues against drug-resistant breast cancer MCF-7/ADR cells.

Compounds	IC ₅₀ (μM) ^a
1	>30
6	5.03 ± 1.91
7	5.82 ± 2.12
10	6.55 ± 0.96
19	6.02 ± 1.28

^aBreast cancer cell line: MCF-7/ADR. Software: MasterPlex ReaderFit 2010, MiraiBio, Inc. Values are mean ± SE of three independent experiments.

^bIf a specific compound is given a value > 30, it indicates that a specific IC₅₀ cannot be calculated from the data points collected, meaning 'no effect'.

Table 3

Effects of oridonin and the selected dienone analogues on normal human mammary epithelial cell (HMEC) proliferation.

Compounds	IC ₅₀ (μM) ^a
1	5.60 ± 0.38
6	5.11 ± 0.18
7	4.95 ± 0.63
10	4.51 ± 0.54
19	10.2 ± 0.24
20	10.1 ± 0.68

^a Cell line: HMEC. Software: MasterPlex ReaderFit 2010, MiraiBio, Inc. Values are mean ± SE of three independent experiments.