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ABSTRACT

Here, we report the structural optimization of a hit natural compound, 2-ME₂ (2-methoxyestradiol), which exhibited inhibitory activity but low potency on tubulin polymerization, anti- angiogenesis, MCF-7 proliferation and metastasis in vitro and in vivo. A novel series of 3,17-modified and 17-modified analogs of 2-ME₂ were synthesized and investigated for their antiproliferative activity against MCF-7 and another five different human cancer cell lines leading to the discovery of **9i**. **9i** bind to tubulin colchicine site tightly, inhibited tubulin polymerization and disrupted cellular microtubule networks. Cellular mechanism studies revealed that **9i** could induce G2/M phase arrest by down-regulated expression of p-Cdc2, P21 and cell apoptosis by regulating apoptosis-related proteins (Parp, Caspase families) in a dose-dependent manner. Importantly, **9i** significantly inhibited HUVEC tube formation, proliferation, migration and invasion. The inhibitory effect against angiogenesis in vivo was confirmed by zebrafish xenograft. Furthermore, **9i** could effectively inhibit the proliferation and metastasis of MCF-7 cells in vitro and in zebrafish xenograft. The satisfactory physicochemical property and metabolic stability of **9i** further indicated that it can act as a promising and potent anti-angiogenesis, inhibiting proliferation and metastasis of breast cancer agent via targeting tubulin colchicine binding site.

1. Introduction

Breast cancer, one of the most popular cancers around the world, has been the leading cause of tumor-related death for females [1]. Although some novel therapy strategy has prolonged the survival time of breast cancer patients, the treatment of breast cancer remains extremely unfavorable and suppressing breast cancer progression, especial metastasis, is still very imperious. According to the American Cancer Society in 2018, breast cancer metastasis was regarded as the main cause of therapeutic failure and responsible for over 80% of patient mortality [2]. Breast cancer metastasis is a complicated process in which breast cancer cells disseminate from the primary tumor to distant secondary organs through the lumina or blood vessels. Recent progression has focused on the understanding of the molecular signaling pathway that regulates breast cancer metastasis, which displayed that angiogenesis may be a potential therapeutic target for breast cancer metastasis prevention and inhibition [3,4]. It is well known that angiogenesis is critical in the development, progression, and metastasis of breast cancer, and aids in supplying nutrients to promote cancer cells growth and metastasis. Angiogenesis for breast cancer is thought as a trustworthy predictor of poor prognosis and a key risk factor for breast cancer metastasis [5,6]. Thus, there is an urgent need to explore novel and strong antiangiogenic molecules with the activity of significantly inhibiting breast cancer growth and metastasis.

Tubulin, the necessary protein for the formation of the mitotic spindle and mitotic division of cell, has been an important target for the treatment of breast cancer in the past decades [7]. Tubulin inhibitors, including microtubule stabilizers such as taxanes, and microtubule destabilizers like vinca alkaloids and colchicine, disrupt microtubule dynamic and arrest cells in G2/M phase, eventually cancer cells or human umbilical vein endothelial cells (HUVEC) will undergo apoptosis, which can lead to tumor growth suppression and angiogenesis inhibition [8–12]. Also, different research suggested that tubulin could regulate the expression of angiogenesis related growth factors, such as vascular

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endothelial growth factor (VEGF) and Hypoxia-inducible factor 1 (HIF-1) [13–15]. Considering the vital role of tubulin for breast cancer progression and metastasis, a number of tubulin inhibitors have been approved to treat breast cancer in clinic including paclitaxel, epothilone, vinfulnine and eribulin (Fig. 1) [16]. In general, these inhibitors bind to the taxane or vinca alkaloid binding sites in tubulin with a large molecular weight, complicated chemical structures, relative hydrophobicity, poor water solubility and strong neurotoxicity, which has limited their further use in clinic [17]. Tubulin inhibitors that bind to the colchicine binding site can largely overcome the above drawbacks and have therapeutic advantages over taxanes and vinca alkaloids. Unfortunately, there is no FDA approved drugs that specifically target the colchicine binding site of tubulin for the cancer treatment till date. And more, these reported tubulin colchicine binding site inhibitors, such as LG-25 and G-1 with potent anti-breast cancer growth in vitro and in vivo, almost never involved in angiogenesis and metastasis [18-20]. Therefore, there is still an urgent need to discover potent tubulin colchicine binding site inhibitors with novel chemical diversity, strong anti-angiogenesis, suppressing proliferation and metastasis of breast cancer.

Nature resources such as micro-organisms and plants provide a vast abundance of metabolites with numerous activities, which make natural products an ideal starting point for drug screening and the identification of novel chemotherapeutics [21]. Considering the advantages of natural products in discovering novel leading compounds and our continuous interest in tubulin [7,22–25], an in-house natural products library with about 1000 compounds have been screened to identify hits of antitubulin polymerization, anti-angiogenesis, suppressing proliferation and metastasis of breast cancer for further optimization. From the screening, 2-ME₂ was identified as a preliminary hit containing the moderate inhibitory effect on tubulin polymerization and MCF-7 proliferation with the IC_{50} value of 36.824 and 3.901 $\mu M,$ respectively. Preliminary biological evaluation showed that this hit could, to some extent, inhibit angiogenesis and breast cancer migration. As shown in Fig. 2, these results has encouraged us to further optimize hit (2-ME₂) structure for improving the potency of inhibiting tubulin polymerization, anti-angiogenesis, intervention of breast cancer growth and metastasis, which has led to the discovery of lead compound (9i).

2. Results and discussion

2.1. Chemistry

To explore the preliminary structure-activity relationships of various sulfone substituents on the 17β -oxygen atom, we prepared a series of 3-O-sulfamate- 17β -sulfonic acid ester **9a-9h** endowed with electrondonating and withdrawing groups. 2-Methoxyestradiol was reacted with benzyl chloride under the presence of sodium hydroxide to afford 2-methoxy-3-O-benzyl estradiol **6** (Scheme 1). Sulfonylation of **6** by reaction with corresponding sulfonyl chloride under dry pyridine proceeded smoothly to give **7** in excellent yield. This was followed by deprotection with Pd/C under a hydrogen atmosphere to generate compound **8**. Finally, sulfamoylation of **8** was achieved by reaction with a solution of sulfamoyl chloride in DMA following the method of Okada and co-workers [26] to give sulfamate **9a-9h**.

We also wished to evaluate the impact of replacement of the 17-*O*sulfamate with different functional groups, such as methyl, acetyl, nitrogen-containing heterocycles and *N*, *N*-dimethylformamide, on antiproliferative activity. The synthesis of this comprehensive series of 3-*O*-sulfamate estradiol derivatives are thus illustrated in Scheme 2.

After some investigation, it was determined that compound 7i, 7j, 7o, 7n could be achieved by a nucleophilic substitution reaction of 6 with corresponding halide, in the presence of NaH or pyridine. Subsequently, 9i, 9j, 9o and 9n was obtained using the aforementioned deprotection and sulfamoylation conditions. Attempts to introduce an unreactive pyrimidine or pyridine bromides using the same approach and Ullmann coupling reaction [27], however, proved unsuccessful. Gratifyingly, the expected aryl ether 7k was accessed in 85% yield by Buchwald-Hartwig cross-coupling reaction conditions [28]. Likewise, 7k and 7m was converted into the corresponding phenols under standard catalytic hydrogenolytic conditions, and the nitro group of 7l was simultaneously reduced into amino, which all in turn were sulfamoylated to give 9k, 9l, 9m respectively.

With above 3-O-sulfamte compounds in hand, we want to better understand the role played by sulfamate on the key interactions with the binding site. Thus, carbamate was used as a potential bioisostere to replace sulfamate and a small series 3-O-carbamate analogs were synthesized from the appropriate aryl ether **8**, as shown in Scheme 3. Compound **8** was reacted with trichloroacetyl isocyanate to afford *N*trichloroacetyl carbamates **10**, which were then hydrolyzed with K_2CO_3 in MeOH/THF/H₂O to yield **11a**, **11i**, **11n**, **11o** respectively.



Fig. 1. Representive tubulin inhibitors against breast cancer.



Fig. 2. Identification of hit compound (2-ME₂) from our natural compound library and further optimizations to discover lead compound (9i).



Scheme 1. Reagents and conditions: a. RSO₂Cl, dry pyridine, rt; b. Pd/C, H₂, MeOH, THF, rt; c. NH₂SO₂Cl, DMA, 0 °C to rt.

2.2. Biology

2.2.1. Antiproliferative activity and structure activity relationships

All sulfamates and carbamates synthesized were evaluated, together with $2-ME_2(1)$ and 4, for their cytotoxic effects on MCF-7 (human breast adenocarcinoma) and another five individual cancer cell lines including U937 (histiocytic lymphoma cancer), A549 (human non-small-cell lung

carcinoma), MGC-803 (human gastric cancer), HepG2 (human liver cancer) and Hela (human cervix carcinoma) cells using the SRB and CCK-8 assay. The results obtained from this in vitro screening are presented in Table 1. In the sub-groups of 3-O-sulfamate-17-O-sulfonate derivatives (**9a-9h**), **9a** displayed the most promising antiproliferative activity with IC₅₀ of from 0.133 μ M to 0.633 μ M. Introduction of an ethyl- or CF₃-substituent (**9b-9c**) on the sulfonyl caused a slight



Scheme 2. Reagents and conditions: a. CH₃I, NaH, DMF, rt, overnight; b. 2-bromoquinoline, NaH, TBAI, dry DMF, 100 °C, 6 h; c. Pd (OAc)₂, CsCO₃, BINAP, toluene, 100 °C; d. NMe₂COCl, NaH, MeCN,100 °C, 10 h; e.CH₃COCl, dry pyridine, rt, 30 min; f. Pd/C, H₂, MeOH, THF, rt; g. NH₂SO₂Cl, DMA, 0 °C to rt.



Scheme 3. Reagents and conditions: a. Cl₃CC(O)NCO, dry THF, rt; b. K₂CO₃, H₂O/MeOH/ THF, rt.

reduction in activity. In contrast, the phenyl sulfonate derivatives (9d-9h), without any exception, was 10- to 40- fold less active than 9a, suggesting that the increased steric bulk around the hydrogen bond acceptor sulfonyl reduced its accessibility. Based on the evidence of above results, the steric hindrance of 17-position appears to be critical for antiproliferative activity. This could also be explained by another

example of *N*, *N*-dimethyl formate **9n**, which led to a substantial loss of activity relative to corresponding carbamate derivative **9p**. The most striking compound is sulfamate **9i**, in which a small group methyl ether was directly attached at the 17-position, affording >50% growth inhibition at concentrations below 150 nM in all cell lines, especially for MCF-7 with about 56 folds increasing in contrast with 2-ME₂. This

Table 1

Biological evaluation of synthesized com	pounds and	$12ME_2$.
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Compound	$IC_{50} \pm SD (\mu M)^{a}$					
	U937	A549	MGC-803	MCF7	HepG2	Hela
2ME ₂	1.261	3.216	4.123	3.901	0.307	0.616
9a	0.159	0.138	0.133	0.165	0.633	0.124
9b	0.409	0.663	0.111	0.920	1.217	0.169
9c	0.542	1.087	0.216	0.579	0.957	0.511
9d	3.552	> 5	> 5	> 5	> 5	> 5
9e	> 5	> 5	> 5	> 5	> 5	> 5
9f	> 5	> 5	> 5	> 5	> 5	> 5
9g	> 5	> 5	> 5	> 5	> 5	> 5
9h	> 5	> 5	> 5	> 5	> 5	> 5
9i	0.139	0.101	0.141	0.069	0.075	0.063
9j	> 5	> 5	> 5	> 5	> 5	> 5
9k	> 5	> 5	> 5	> 5	> 5	> 5
91	> 5	> 5	> 5	> 5	> 5	> 5
9m	> 5	> 5	> 5	> 5	> 5	> 5
9n	> 5	> 5	> 5	> 5	> 5	> 5
90	2.33	4.212	1.263	4.383	8.099	1.961
9p	0.778	2.265	0.382	1.256	6.207	0.580
8a	> 5	> 5	> 5	> 5	> 5	> 5
8b	2.473	5.926	2.654	2.284	4.392	2.837
8f	> 5	> 5	> 5	> 5	> 5	> 5
8i	> 5	> 5	> 5	> 5	> 5	> 5
81	> 5	> 5	> 5	> 5	> 5	> 5
8n	> 5	> 5	> 5	> 5	> 5	> 5
80	> 5	> 5	> 5	> 5	> 5	> 5
11a	> 5	> 5	> 5	> 5	> 5	> 5
11i	> 5	> 5	> 5	> 5	> 5	> 5
111	> 5	> 5	> 5	> 5	> 5	> 5
11n	> 5	> 5	> 5	> 5	> 5	> 5
110	> 5	> 5	> 5	> 5	> 5	> 5
4	0.333	0.686	0.113	0.228	0.614	0.419

 $^{\rm a}\,$ Each compound was tested in triplicate; the data are presented as the mean \pm SD.

unexpected result might be due to methyl ether which is functioned as a sterically unhindered bond acceptor group, once again confirming steric size of the 17-subtituent is important. Unsurprisingly, the corresponding non-sulfamoylated analogue 11i, was dramatically less potent than 9i in all cell lines, indicating that 3-O-sulfamate substitution is highly favored. Actually, most of the phenol series compounds proved to possess modest or no activity (IC₅₀ > 5 μ M), with the exception of the 17ethyl sulfonate 8b, displaying fairly good activity with a IC₅₀ of 2.473 µM against the U937 cell line. These findings in the 3-O-carbamate series were similar to those obtained in the phenol series, wherein all derivatives show uniformly IC_{50} of $>5\ \mu\text{M}.$ Based on this finding, it revealed that replacement of the 3-O-sulfamate with a carbamate group was deleterious to retain good antiproliferative activity, while 17-Ocarbamate compounds have been reported to deliver maintained or improved activity in some cases compared to 17-O-sulfamate analogues. In an attempt to improve water solubility of 2ME₂ analogues, several pyridinyl group was introduced at 17-position, derived from its potential character to form H-bond with water or binding site. Unfortunately, all compounds bearing aromatic heterocyclic at 17-position exhibited weak activity with IC₅₀ values of $>5 \,\mu$ M against tested cell lines. Collectively, the structural optimization and SAR studies led to the discovery of 9i that exhibited high potency on MCF-7. Further in-depth in vitro and in vivo activity assays were subsequently performed with compound 9i.

2.2.2. 9i bind to the colchicine site of tubulin and inhibit the microtubule polymerization

It has been reported that $2ME_2$ occupies exactly the same binding site of β -tubulin that is occupied by colchicine [19]. In this paper, molecular docking was performed to elucidate the binding mode of compound **9i** with tubulin-microtubule system by docking **9i** into the active site of tubulin (PDB code: 1SA0). The docking pose in Fig. 3 (panel A-C) show that compound **9i** could be well-anchored into the hydrophobic pocket of β -tubulin subunit. While Lys 352 residues are in close interaction with

3-sulfamate group of **9i** to form a hydrogen bond (β-tubulin, O···H-N: 3.08 Å). Its interaction is further stabilized by hydrogen bond formed between 17-methoxy group and the amine group of the side chain Lys 254 (β-tubulin, O…H—N: 3.41 Å), indicating the important role of methoxy group for inhibitory activity. As shown in Fig. 3 (panel D), 9i efficiently bind to the [³H]colchicine binding domain of tubulin compared with the positive control CA-4, which demonstrated that 9i directly interacted with tubulin by the pocket of colchicine site. The in vitro tubulin polymerisation inhibition activity of 9i was evaluated subsequently, with colchicine (4 µM) as a positive control group. Purified and unpolymerized tubulin was incubated with 9i at different concentrations (2 μ M, 4 μ M, 8 μ M). The fluorescence intensity becoming stronger with time indicated the occurrence of tubulin polymerization. It was found that the increased tendency of the fluorescence intensity was obviously slowed down with higher concentrations of 9i (Fig. 2, panel E). The IC₅₀ value of compound **9i** was 4.875 μ M against tubulin that was more potent than colchicine with IC_{50} of 6.6 μ M [29]. It revealed that **9i** was a novel tubulin polymerization inhibitor. As tubulin polymerization and microtubule assembly plays a vital role in the maintenance of cellular morphology and basic cellular functions, an immunofluorescence assay was carried out to reveal whether compound 9i could disrupt the microtubule dynamics in living cells. As shown in Fig. 3 (panel F), the microtubule network of MCF-7 cells in the control group was arranged and organized normally, with filamentous microtubule surrounding the cell nucleus. Compound 9i produced moderate disrupt of the spindle formation at a concentration of 50 nM. When the concentration increased (100 and 200 nM), the microtubule spindles around the cell center were significantly reduced, cell shape change from spindle to round, irregular or multiangular, and some spot-like disordered structures were observed clearly, similar to CA-4. These morphological changes of microtubules indicated that 9i dramatically interfered with mitosis of MCF-7 and inhibited tubulin polymerization, which possibly eventually lead to tumor cell death.

2.2.3. 9i induces G2/M phase arrest and regulated the expression of G2/M-related proteins in MCF cells.

It has been reported that most tubulin polymerization inhibitors disrupt the cell cycle at G2/M phase [30]. Thus, the effect of compound **9i** on cell cycle progression was investigated in MCF-7 cells by flow cytometry analysis, using propidiumiodide (PI) staining. MCF cells were treated with **9i** at different concentrations (0, 50, 100 and 200 nM) for 12 h and 100 nM **9i** at different times (0, 6 h, 12 h, 24 h) respectively. As shown in Fig. 4 (panel A-D), the number of cells arrested at G2/M phase was increased, depending on concentration, from 16.3% (control) to 38.1% (50 nM), 54.0% (100 nM), and 75.1% (200 nM) accordingly. Similarly, the accumulation of MCF-7 cells at G2/M phase was markedly increased with a longer exposure to compound **9i**. These results suggested that **9i** could induce cell cycle arrest at the G2/M phase in a dose and time-dependent manner.

To acquire the underlying mechanisms of G2/M phase arrest induced by compound **9i**, we detected the protein level of Cyclin B1, Cdc2 and P21, which effectively control cell cycle progression through increasing the G2/M fraction [31]. As depicted in Fig. 4 (panel E-F), the results demonstrated that levels of p-Cdc2 and P21 were down-regulated in a dose-dependent manner. Meanwhile, compound **9i** obviously increased Cyclin B1 expression with increasing concentrations (0, 50, 100, 200 nM).

2.2.4. 9i induced caspase-dependent apoptosis

Mitotic arrest of cancer cells by tubulin inhibitor is generally associated with cellular apoptosis [32]. The effects of compound **9i** on MCF-7 cell apoptosis were evaluated in an Annexin V-APC/PI double staining assay and analyzed by a flow cytometry. The results were illustrated in Fig. 5 (panel A-B). Compared with the control group (6.65%, early and late apoptosis), the apoptosis rates increased gradually from 20.90% to 21.31% and 39.58% after being treated with 9i at 50, 100, and 200 nM



Fig. 3. Compound **9i** bind to the colchicine-binding site of tubulin and inhibited microtubule polymerization. (A) Molecular docking results of compound **9i** binding mode within the $\alpha\beta$ -tubulin heterodimer. The α - and β - tubulin subunits are represented as green ribbons. (B) 2D binding models and hydrogen-bond interactions. (C) 3D binging models of **9i** in different configuration. (D) Effect of compound **9i** on tubulin binding of [³H] colchicine. (E) Tubulin polymerization inhibitory activity of **9i**. (F) The effects of **9i** and CA-4 on the organization of celluar microtubule network of MCF-7 cells observed under a confocal microscope. The photomicrographs shown are representative of at least three independent experiments performed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for 48 h, respectively. These results confirmed that 9i clearly induced cell apoptosis in MCF-7 cells in a dose-dependent manner.

Caspases, a family of cysteine-aspartic proteases, are the central executioners of apoptosis. As a critical step in the process of apoptotic cellular death, caspase activation is mediated by various inducers [33]. caspase-3 is responsible for chromatin condensation and DNA fragmentation, which represents the hallmark of apoptosis. The caspase cascade usually begins with the activation of casase-8 followed by caspase-3, which eventually trigger nuclear fragmentation. The cleavage of poly (ADP-ribose) polymerase (Parp) is an important indicator of apoptosis and generally considered to be an indicator of caspase-3 activation [34]. In order to explore the intrinsic mechanism of the apoptosis effects produced by 9i, western blotting assay was conducted on MCF-7 cells threated with 9i at different concentrations for 48 h. β -Actin was used as a loading control. We observed that **9i** induced activation of the initiator caspase-9 in a dose-dependent manner (Fig. 5, panel C-D). Meanwhile, the activation of executioner caspase-3 and cleavage of its substrate Parp during the apoptotic process were also investigated. The effect of 9i on caspase-3 and Parp cleavage was consistent with concentration at which it causes G2/M arrest in MCF-7 cells.

2.2.5. 9i inhibited migration, invasion and tube formation of vascular endothelial cells

Recently, several clinical trials are ongoing to evaluate the potential of vascular disrupting agents or antiangiogenic drugs in combination with conventional therapies to treat breast cancer metastasis [35]. Many tubulin binding agents, especially CA-4, show vascular-disrupting and antiangiogenic effects against tumor endothelium [36], and for that reason we examined effects of **9i** on endothelial cells in vitro. Specially, human umbilical vein endothelial cells (HUVECs) seeded on matrigel was used as a model to study the antivascular activity of **9i**. Endothelial cell migration to the tumor site is essential for the process of angiogenesis. Microtubules-targeting agents have also been reported to prevent the formation of new blood vessels or interfere with cell migration at low doses.

The antivascular activity of **9i** was evaluated by a HUVEC tube formation assay. As shown in Fig. 6 (panel A-B), relative to the control, **9i** effectively reduced the width and length of "tubule-like" networks formed by HUVEC after 6 h incubation at the concentrations of 1 μ M and 10 μ M. We then perform image analysis to obtain a quantitative determination of the segment length, the area and the number of meshes, the percent of area covered by HUVECs, and the number of branching points after 6 h treatment [35].



Fig. 4. Compound **9i** induced G2/M arrest in MCF-7 cells. (A) Cells were treated with indicated concentrations of **9i** for 24 h and profiles were obtained by flow cytometry. (B) The percentage of cell cycle from (A) were illustrated in plots. (C) Cells were treated with 100 nM **9i** for 0, 6, 12, 24 h respectively and analyzed by flow cytometry. (D) The percentage of cell cycle from (C) were illustrated in plots. (E) MCF-7 cells were treated with **9i**, then cells were harvested and the protein expressions of Cyclin B1, p-Cdc2 and P21 were detected by western blotting assay. (F) Quantitative analysis of the expression of Cyclin B1, p-Cdc2 and P21 from experiments as in (E). Data are presented as mean \pm SD of at least three independent experiments.

A wound healing assay was then performed to evaluate the capacity of **9i** to prevent cell motility. As shown in Fig. 6 (panel C-D), after exposed to compound **9i** at 0.1, 1 and 10µM for 48 h, **9i** obviously decrease the closure of would scratching in a confluent monolayer of HUVEC cells, with a dose-response relationship observed. Then, HUVECs cells were treated with 0.1, 1 and 10 µM of **9i**, and the migration ability was further detected by transwell assay. It was found that, with the increase in the concentration of **9i**, the number of migrated endothelial cells gradually decrease (Fig. 6, E-F), and the invasion ability of cells gradually weakened. These results collectively indicated that **9i** has significant effect on prohibiting HUVECs tubular structure formation and migration.

2.2.6. 9i inhibited migration and invasion of MCF-7 cells

Metastasis is the leading cause of morbidity and mortality for breast cancer patients. In this work, we evaluated whether **9i** could suppress migration and invasion of MCF-7 cells by wound healing and transwell assay respectively. As shown in Fig. 7 (panel A-B), after treatment for 48 h at indicated doses, **9i** apparently inhibit the wound healing of MCF-7 cells compared with 0 h. Transwell assays show cell quantity from upper side of transwell membrane to bottom side were obviously decreased with the increase in the compound's concentrations, confirming that **9i** could hinder the MCF-7 cells invasion (Fig. 7, panel C-D).

2.2.7. Antiangiogenic effects in zebrafish embryos

The zebrafish model for antiangiogenesis is a well-studied and adopted tumor model system used for drug screening purposes. The advantages of zebrafish xenografts for antiangiogenesis screening includes low cost, transparent embryos, rapid propagation and the ability to perform large number of transplants. Moreover, major molecular pathways regulating angiogenesis in mammalian system are conserved in zebrafish. Except for angiogenic potential, it also allows evaluation of metastatic potential of cancer [37]. The effect of compound 9i on embryonic angiogenesis in zebrafish was determined, in which the vascular endothelial cells were labeled with green fluorescent protein (GFP). Tg (flk: EGFP) Zebrafish embryos (12hpf) were treated with different doses of compound 9i (0.5, 1, 1.5µM) and 30hpf zebrafish was imaged by fluorescent microscopy to observe the affection of applied agents on intersegmental vessels (ISVs) development. As shown in Fig. 8, the normally developing embryos in the control group had 22-24 ISVs, whereas, for zebrafish embryos treated with 0.5 and 1 µM of 9i, the number and length of ISVs considerably reduced with concentration increasing, while preserving fluorescence in the doral aorta and major cranial vessels. At the doses \geq 1.5 µM, ISVs formations were seriously impaired and developmental defects were observed, indicating a dosedependent inhibition pattern. Our results suggest that 9i is a powerful vasoactive reagent and could greatly blocked the growth of angiogenesis at low doses.



Fig. 5. 9i induces MCF-7 cells apoptosis. (A) Apoptosis ratio detection by Annexin/ PI double staining assay through flow cytometry analysis treated with indicated concentrations of compound 9i for 48 h. The diverse cell stages were given as live (Q4), early apoptotic (Q3), late apoptotic (Q2) and necrotic cell (Q1). (B) The quantitative analysis of apoptotic rate at early and advanced stages of MCF-7 cells. (C) Western blot analysis of the apoptosis related proteins. (D) The quantitative analysis of the protein levels. The data was presented as the mean \pm SD of three independent tests.

2.2.8. In vivo anti-tumor and -metastasis effects of compound 9i in a zebrafish xenograft

To evaluate in vivo anti-tumor and -metastasis activity, we investigated the proliferation and metastasis of MCF-7 Cells induced by 9i using the zebrafish xenograft model, a well-studied and adopted in vivo platform for drug screening and translational research in human carcinomas. Accordingly, Cell-Tracker Red CMTPX labeled MCF-7 cells were microinjected and developed into the volk of Tg(flk: EGFP) embryos, and different concentrations of 9i were added. 72 h after xenotransplantation, zebrafish were processed by fluorescence microscopy to evaluate the effects of 9i on the tumor mass development and cancer cells metastasis. 2ME₂ was used as a positive control. As shown in Fig. 9, when tumor cells were microinjected into zebrafish embryos, after 72 h the MCF-7 cells in the control group disseminated and widely migrated away from the primary area, and some tumor cells even migrated in the blood vessel of the zebrafish tail. However, treatments with 9i could markedly blocked MCF-7 cells migration and invasion in zebrafish xenografts. In addition to the anti-metastasis effect, comparison of the fluorescence intensities and size of MCF-7 cells to untreated xenografts at 72 hpf indicated that 9i could suppress the growth of tumor in a dosedependent manner. The tumor almost completely disappeared upon 1.5 μ M dosages of **9i**, which was further evidenced by the result of reduced fluorescence intensities and decreased tumor area compared with the control group and 2-ME₂ group. All these research showed the potential of 9i to be the candidate to inhibit the metastasis and proliferation of breast cancer.

2.2.9. Physicochemical property and metabolic stability of compound 9i

To evaluate the drug-likeness of compound **9i**, various physicochemical and metabolic parameters had been tested. As shown in Table 2, **9i** has a polar surface area (PSA) of 87.65 placed between 80 and 90 Å, well within the range considered appropriate for passive absorption. Distribution coefficients displayed an excellent drug-like cLogD value which was 3.5. Aqueous solubility ranged from a moderate 45–90 µg/mL at pH 2.0 for **9i** to a more acceptable value of up to 210 µg/mL at pH 6.5. On the whole, the lead compound **9i** possessed favorable solubility and permeability properties. Then metabolic liabilities of compound **9i** were also assessed with human (HLM) liver microsomes. The in vitro degradation half-life in HLM was 86 min for **9i**, and there was promising value for intrinsic clearance (Clint) which was 105 µL/min/mg protein.

3. Conclusions

In the current investigation, a total of 27 novel 3,17-modified and 17modified analogs of $2-ME_2$ were designed, synthesized and evaluated. Among them, the 3-O-sulfamate-17-methoxy derivative **9i** exhibited a potent inhibitory activity against the proliferation, migration, and invasion of MCF-7 breast cancer cells. Molecular mechanisms illustrated that compound **9i** could disrupt microtubule networks, induce cells MCF-7 apoptosis and arrest MCF-7 cells at G2/M phase. Scratch and transwell assays showed that **9i** dose-dependently suppress the proliferation, migration and invasion of HUVECs. Tube formation assays



Fig. 6. Compound **9i** displayed antivascular activity in vitro. (A) Typical images depicting the vascular structure formation of HUVEC cells by treatments with **9i** for 6 h. (B) Quantitative evaluation of indicated concentration **9i** on standard parameters of HUVEC tubule formation. Mean \pm SEM, three experiments. ** p < 0.01; *** p < 0.001. (C) Confluent HUVECs in a monolayer were wound, and images were captured at 0 h and 48 h after treatments with **9i**. (D) Inhibitory rates of **9i** on the HUVECs migration. (E) The invasion suppressing effects of **9i** against HUVECs cells by transwell assay. (F) Quantitative analysis of the migration ability. * p < 0.05, ** p < 0.01; *** p < 0.001, vs. control; n = 3.

revealed that **9i** severely impaired the tube forming capacity of HUVECs. Molecular docking, radioligand binding assay, tubulin polymerization assay in vitro and immunofluorescence studies in cells suggested **9i** could bind to tubulin colchicine binding site, cause disruption of tubulin polymerization, and interfered with the normal formation of mitotic spindles via the depolymerization of microtubules. In addition, the evaluation of in vivo activity illustrated that **9i** obviously inhibit the proliferation and metastasis of MCF-7 cells, and angiogenesis in transgenic zebrafish. Together with the promising physicochemical property and metabolic stability, **9i** may serve as a potential treatment agent as tubulin inhibitor with the activity of anti-angiogenesis, inhibiting

proliferation and metastasis of breast cancer.

4. Experimental procedures

4.1. Biological section

MCF-7, MGC-803, Hela and HepG-2 cell lines were cultured in a base medium (90% DMEM and 10% FBS), A549 and U937 were cultured in a base medium (90% PRMI-1640 and 10% FBS) at 37 $^{\circ}$ C, 5% CO₂ and a saturated humidity atmosphere. All the mediums were supplemented with 10% FBS and 1% ampicillin/streptomycin (all cells were purchased



Fig. 7. Effects of **9i** on MCF-7 cells migration and invasion in vitro. (A) Images of MCF-7 cells migration inhibited by **9i** determined in wound-healing assay. All tested samples were treated with serum-free DMEM. (B) Inhibitory rates of **9i** on MCF-7 cells migration. (C) Inhibition of cellular invasion by **9i** in transwell assay. (D) Quantitative analysis of the migration ability of MCF-7 cells after the treatment of **9i**. The experiments were repeated three times and results were indicated as means \pm SD. * p < 0.05, ** p < 0.01; *** p < 0.001 vs untreated control.



Fig. 8. Anti-angiogenic effects of **9i** in zebrafish embryos assay. (A) Inhibitory effects of compound **9i** on the angiogenesis of transgenic zebrafish. Representative images from three independent experiments were shown. The left angiogenic vessels were magnified and shown in the right section. (B) Histogram showed the numbers of zebrafish ISVs per field under confocal microscopy. Results represented the means \pm SD. ** p < 0.01; *** p < 0.001 compared to untreated control.



Fig. 9. (A) Inhibitory effects of **9i** on the proliferation and metastasis of MCF-7 cells in zebrafish xenograft models. CMTPX labeled MCF-7 cells (red) were microinjected into zebrafish embryos, and indicated concentration of **9i** were added. 2ME₂ was used as a positive control. (B) Quantification of the fluorescent area of the tumor xenografts, representing total MCF-7 cells in zebrafish. (C) Fluorescence intensity of the tumor xenografts in trunk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table	2
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Physicochemical parameters and metabolic stability of compound 9i.

MW ^a	PSA ^a	cLogD ^a	Solubilit mL) pH 2.0	д ^ь (µg∕ рН 6.5	Degradation half-life (min) in human	In Vitro Clint (μL/ min/mg protein) in human
395.18	87.65	3.5	45–90	105–210	86	105

^a Calculated using ChemAxon JChem software;

^b Kinetic solubility determined by nephelometry (SolpH).

from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China).

4.1.1. Cell lines growth inhibition. SRB procedure

A549, MGC-803, MCF-7, HepG2 and Hela cells in exponential growth phase were seeded in 96-well plates with appropriate cell line density (3×10^3 – 4×10^3 cells/well). Then cells were incubated in a 5% CO₂ incubator at 37 °C for 24 h. When the cells adhered, different concentrations of compounds as shown in the Figures and Tables were added to every well. After incubation for another 72 h, cell proliferation was quantified using SRB (Sulforhodamine B) to determine the number of viable cells. Briefly, cells were fixed by addition of cold trichloroacetic acid (100 µL, 0.4%) to the wells (10% (w/v) for 30 min at 4 °C. Plates were washed with water and dried at 65 °C. Sulforhodamine B solution (100 µL) at 0.4% (w/v) in 1% acetic acid was added to each well, and the resulting mixtures were further incubated for 20 min at room temperature. Unbound dye was removed by washing five times with 1% acetic

acid. Bonded dye was solubilized in 10 mM unbuffered trisbase, and the absorbance was read at a wavelength 560 nm according to color intensity using quant universal microplate spectrophotometer (Biotek, Winooski, VT). Cell viability (%) was calculated using the following equation: cell viability (%) = (a treatment/a control) \times 100%. The data represented the mean of three independent experiments in triplicate and were expressed as means \pm SD. The IC₅₀ was calculated by the Logit method.

4.1.2. CCK-8 assay

U937 Cells were seeded in 96-well plates at a density of 8×10^3 per well and exposed to different concentrations of compounds for 72 h. Subsequently, 10 μL CCK-8 solution was added to each well, and co-incubated for another 3–4 h at 37 °C. The absorbance was determined at 450 nm. Data are calculated with GraphPad Prism5 software.

4.1.3. Immunofluorescent staining

MCF-7 cells (3 × 10⁴ cells/well) were plated onto square coverslip (1 cm side), after 12 h of incubation, cells were treated with different concentration of **9i**, CA-4 and the vehicle control 0.1% DMSO for 48 h, respectively. The cells were rinsed twice with PBS and fixed by 4% paraformaldehyde for 20 min, permeabilized using 0.5% Triton X-100 for another 5 min and blocked with 5% BSA for 40 min. Subsequently, microtubule detection was performed using a fluorescent antibody against α -tubulin in 5% BSA and incubated for 5–6 h at room temperature or overnight at 4 °C. To stain cell nucleus, crystals were incubated with Hoechst for 10 min. After washing with PBST to remove redundant stain, the samples were visualized under an Olympus laser scanning confocal microscope.

4.1.4. Cell cycle distribution assay

MCF-7 cells (15 × 10⁴ cells/well) were seeded in 6-well culture plate. After 24 h incubation at 37 °C, exponentially cells were treated with indicated concentration of compound **9i** for 12 h and 24 h respectively. The cells were harvested and washed with cold PBS, then fixed with 75% ethanol in PBS at -20 °C for overnight. Then the fixed cells were washed twice with PBS buffer and subsequently stained with 500 µL staining solution containing fluorescein isothiocyanate propidium iodide (PI, 5 mg) for 15 min in darkness. Samples were immediately tested for DNA content with flow cytometry instrument (Becton, Dickinson and Company, USA). The percentage of cell cycle phases was analyzed by the software supplied in the instrument.

4.1.5. Cell apoptosis assay

MCF-7 cells at 8 ×10⁴ cells/well were seeded in 12-well cell culture plates overnight, and then the cultured cells were incubated with a dose range of compounds **9i** for 48 h. The treated or untreated cells were washed twice with PBS and stained by Annexin V-PE and PI for 15 min without light exposure according to the manufacturer's protocol. Apoptosis was quantified using a flow cytometer (Becton, Dickinson and Company, USA).

4.1.6. Western blot assay

Exponentially growing MCF-7 cells were seeded in 10 mm dishes at a density of 6×10^5 /dish and incubated overnight. The cultured cells were treated with indicated concentrations of **9i** for 48 h. After the cells were washed with cold PBS, the supernatant was removed. Cells were scraped off the tissue culture dish, and then lysed by 100 µL ice-cold RIPA lysis buffer for 20 min, with occasional agitation. The supernatant was collected by centrifuging at 12,000g for 10 min at 4 °C. The protein concentration in the supernatant was determined by using a BCA protein assay kit. After BCA analysis to quantify proteins, samples were prepared in SDS-PAGE loading buffer, then boiled for 10 min at 100 °C. Western blot analyses were conducted after separation by SDS-PAGE electrophoresis and transfer to nitrocellulose filter (NC) membranes. Immunoblotting was performed according to the antibody manufacturers' recommendations.

4.1.7. Tube formation assay

Matrigel matrix (Basement Membrane Matrix, BD Biosciences) was thawed at 4 °C overnight. At this point, 50 μ L of the matrix solution was added to each well of a 96-well plate. After gelling at 37 °C for 30 min, the matrix was overlaid with 200 μ L of medium containing 1 \times 10⁵ HUVECs per well, which was incubated for 6 h to allow capillary tubes to form. Different concentrations of **9i** were added in the cultures and incubated for 6 h to monitor the morphological changes of cells and tubes. The disappearance of existing vasculature was photographed at 10 \times magnification. Values were expressed as percent change form control cultures grown with complete medium. Standard dimensional parameters (percent area covered by HUVECs and total length of the HUVEC network per field) were noted, and standard topological parameters (number of meshes and branching points per field) were estimated.

4.1.8. Would healing migration assays

MCF-7 cells were seeded in 6-well plates and cultivated for 24 h. Scratches were made in confluent monolayer using 200 μ L pipette tips and washed with PBS to remove the detached cells. The media containing different concentrations of **9i** were added to the scratched monolayers. The migrated cells were photographed under a light microscope at indicated time points from the scratch. The migration distance of cells migrated to the wound area was measured manually and compared with the initial distance.

4.1.9. Transwell cell invasion experiment

The upper surface of transwell membrane was precoated with 40 μL

Matrigel gel and incubated for 45 min at 37 °C for gelling. Then, cells were trypsinized and seeded at 6×10^4 per upper chamber, and cultured in 1% fetal bovine serum medium containing different concentrations of **9i**. The chamber was then placed in a 24-well plate to which 10% FBS had been added. After 48 h incubation at 37 °C, both chambers were washed with PBS three times, and then fixed with 4% paraformaldehyde for 15 min, followed by staining with crystal violet. The non-invasive cells on the upper surface membrane were wiped with cotton swabs. The image of the cells on the bottom face was recorded and quantified under an inverted microscope. The results were the means calculated from three replicates of each experiment.

4.1.10. Zebrafish angiogenesis assay

The transgenic flk: enhanced GFP zebrafish embryos were generated by pair-wise mating, collected and sorted into the 6-well plate (n = 30 per well) with 1 mL of aquaculture water (0.2 g/L of Instant Ocean Salt in distilled water) and raised at 28 °C. Then the embryos staged at 12 h post fertilization (hpf) were treated with the indicated concentrations of **9i**, which were added into embryo water. About 30 embryos were screened for each concentration gradient. At 30hpf, the treated embryos were anesthetized with 0.02% tricaine in embryo water and photographed. The numbers of ISVs were recorded under confocal microscopy. Results were obtained from three independent determinations and presented as mean \pm SD.

4.1.11. Zebrafish xenografts injection and treatment

MCF-7 cells were labeled with Red CMTPX according to the manufacturer's protocol. Before the microinjections, *Tg*(flk:EGFP) embryos were kept at 28 °C and manually dechorionated few hours. At 48 hpf, 4 nL of cells suspension containing 400 labelled cells was microinjected into the yolk of anesthetized embryos by a pneumatic pico pump (PV820, World Precision Instruments, USA). After injection, embryos were incubated to recover for at least 1 h at 28 °C, the embryos were distributed to 6-well plates with 20 embryos placed in each well. The injected xenografts were exposed to the indicated doses of **9i** and maintained at 35 °C. DMSO (0.3%) was used as a vehicle control and 2ME₂ as a positive control. The survival and development of the xenografted embryos was recorded every day until the end of experiment. At one day post injection (dpi), fluorescent microscopy was used to examine MCF-7 cells size, death and migration in caudal region of anesthetized zebrafish.

4.2. General chemistry (the detailed information is in Supplementary Information)

All chemicals were purchased from commercial sources and used without further purification unless otherwise noted. Solvents and reagents were dried and purified according to the methods described in the literature. Column chromatography (CC) was carried out on silica gel (200–300 mesh, Qingdao Ocean Chemical Company, China). Thinlayer chromatography (TLC) analyses were carried out on silica gel GF254 (Qingdao Ocean Chemical Company, China) glass plates.

¹HNMR and ¹³CNMR were respectively recorded on a Bruker AV-400 (400 MHz) nuclear magnetic resonance spectrometer at 400 and 100 MHz as deuterated chloroform (CDCl₃) or deuterated dimethyl sulfoxide (DMSO- d_6) solutions. Chemical shifts were reported in parts per million (ppm) relative to tetramethyl silanes as internal standard. High-resolution mass spectra (HRMS) were recorded with a Q-TOF micro mass spectrometer.

4.2.1. General procedure for synthesis of compound 7a-7h.

A solution of compound **6** (200 mg, 0.51 mmol) in pyridine (5 mL) was stirred and corresponding substituted sulfonyl chloride (3.06 mmol) was added portionwise. The mixture was stirred for 30 min at room temperature. Ethyl acetate (3×15 mL) and water 15 mL was added and the organic layer was separated, washed with water then brine, dried

(MgSO₄) and evaporated. The residue was purified by flash chromatography using mixtures of petroleum ether and ethyl acetate (typically between 30:1 and 5:1 by volume) to give **7a-7h**.

4.2.2. General procedure for preparation of compound 8

Pd/C (80 mg, 10%) was added to a solution of 7 (0.426 mmol) in MeOH (15 mL) and THF (15 mL). The mixture was stirred under a H_2 atmosphere for 1 h at room temperature and the catalyst was filtered off. The solvents were removed under reduced pressure to give the crude product, which was used in next step without further purification.

4.2.3. General procedure for preparation of sulfamate 9a-9h from 8

An ice-cold solution of sulfamoyl chloride (200 mg, 1.732 mmol) in DMA (1.5 mL) was added to **8** (crude product). After 3 h of stirring at room temperature, ethyl acetate (3×10 mL) and water (10 mL) was added, the organic layer was separated and washed with water and brine, dried (MgSO₄), and concentrated. The residue was purified by silica gel chromatography using mixtures of petroleum ether and ethyl acetate (typically between 2:1 and 3:1 by volume) to afford **9a-9h** as white solids with a yield between 85% and 92%.

2-Methoxy-17β-O-methanesulfonylestra-1,3,5(10)-trien-3-ol (**8a**): m.p. 167.5–168.9 °C; ¹H NMR (400 MHz, CDCl₃) δ 6.80 (s, 1H), 6.67 (s, 1H), 5.48 (s, 1H), 4.60 (dd, J = 9.0, 7.9 Hz, 1H), 3.89 (s, 3H), 3.04 (s, 3H), 2.80 (dd, J = 9.7, 6.7 Hz, 2H), 2.35–2.18 (m, 3H), 2.11–2.03 (m, 1H), 1.93–1.74 (m, 3H), 1.56–1.28 (m, 6H), 0.90 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 144.65, 143.59, 131.23, 129.34, 114.62, 108.06, 89.46, 56.09, 49.08, 43.99, 43.33, 38.53, 38.28, 36.40, 28.87, 27.97, 27.15, 26.31, 23.05, 11.78. Purity: 97.54%.

2-Methoxy-17β-O-ethanesulfonylestra-1,3,5(10)-trien-3-ol (**8b**): m. p. 154.5–156.2 °C; ¹H NMR (400 MHz, CDCl₃) δ 6.80 (s, 1H), 6.67 (s, 1H), 5.47 (s, 1H), 4.60 (dd, J = 9.0, 8.0 Hz, 1H), 3.89 (s, 3H), 3.16 (q, J = 7.4 Hz, 2H), 2.84–2.75 (m, 2H), 2.34–2.18 (m, 3H), 2.08 (t, J = 6.0 Hz, 1H), 1.92–1.76 (m, 3H), 1.53–1.31 (m, 9H), 0.90 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 144.65, 143.59, 131.28, 129.36, 114.62, 108.07, 88.97, 56.09, 49.13, 45.59, 44.01, 43.37, 38.56, 36.42, 28.89, 28.03, 27.16, 26.34, 23.06, 11.81, 8.35. Purity: 98.27%.

2-Methoxy-17β-O-(4-methoxybenzene sulfonyl) estra-1,3,5(10)-trien-3ol (**8f**): m.p. 84.7–86.5 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.88 (d, J = 8.9 Hz, 2H), 7.03 (d, J = 8.9 Hz, 2H), 6.77 (s, 1H), 6.65 (s, 1H), 4.36 (t, J = 8.4 Hz, 1H), 3.92 (s, 3H), 3.87 (s, 3H), 2.87–2.66 (m, 2H), 2.27–2.10 (m, 2H), 2.04–1.92 (m, 1H), 1.88–1.78 (m, 2H), 1.76–1.65 (m, 2H), 1.49–1.40 (m, 2H), 1.28 (dq, J = 7.1, 4.7 Hz, 2H), 1.20–1.10 (m, 2H), 0.86 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 163.52, 144.63, 143.55, 131.27, 129.98, 129.30, 128.80, 114.60, 114.25, 108.04, 89.68, 56.06, 55.69, 49.04, 43.96, 43.27, 38.44, 36.16, 28.84, 27.72, 27.13, 26.26, 23.00, 11.77. Purity: 98.14%.

2-Methoxy-3-sulfamoyloxy-17β-O-methanesulfonylestra-1,3,5(10)triene (**9a**): This compound was prepared according to abovementioned general procedures and the resultant crude solid was purified by flash chromatography (petroleum ether/ethyl acetate 2:1) to give a white powder (yield 90%); m.p. 192.2–193.0 °C ¹H NMR (400 MHz, DMSO) δ 7.83 (s, 2H), 6.99 (s, 2H), 4.54 (t, J = 8.2 Hz, 1H), 3.77 (s, 3H), 3.17 (s, 3H), 2.74 (d, J = 5.0 Hz, 2H), 2.39 (d, J = 9.3 Hz, 1H), 2.19 (d, J = 8.6Hz, 2H), 1.89 (d, J = 9.1 Hz, 1H), 1.85–1.66 (m, 3H), 1.46–1.26 (m, 6H), 0.80 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 149.47, 138.52, 136.78, 128.21, 122.94, 110.50, 88.63, 55.87, 48.16, 43.72, 42.70, 37.73, 37.44, 35.52, 28.10, 27.43, 26.48, 25.52, 22.54, 11.55. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₀H₂₉NO₇S₂, 482.1385, found 482.1383. Purity: 98.28%.

2-Methoxy-3-sulfamoyloxy-17 β -O-ethanesulfonylestra-1,3,5(10)-triene (**9b**): m. p. 202.8–205.1 °C; ¹H NMR (400 MHz, DMSO) δ 7.78 (s, 2H), 7.00 (d, J = 2.9 Hz, 2H), 4.53 (t, J = 8.2 Hz, 1H), 3.78 (s, 3H), 3.29 (s, 2H), 2.75 (d, J = 4.5 Hz, 2H), 2.41 (s, 1H), 2.21 (d, J = 6.2 Hz, 2H), 1.93–1.68 (m, 4H), 1.45–1.26 (m, 9H), 0.82 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 149.47, 138.51, 128.23, 122.92, 110.54, 88.44, 55.90, 48.17, 44.19, 43.70, 42.76, 37.76, 35.65, 28.10, 27.50, 26.46, 25.54, 22.54,

11.57, 8.06. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₁H₃₁NO₇S₂, 496.1542, found 496.1546. Purity: 97.62%.

2-Methoxy-3-sulfamoyloxy-17β-O-trifluoromethanesulfonylestra-1,3,5 (10)-triene(9c): Compound 6 (267 mg, 0.68 mmol) was reacted with trifluoromethyl sulfonyl chloride (0.2 mL, 1.7 mmol) in the presence of DMAP (498 mg, 4.08 mL) in anhydrous DCM (12 mL) to give 7c as a white solid, which continue the hydrogenation and sulfamoyl reaction according to general procedures. The resultant crude solid was purified by flash chromatography (petroleum ether/ethyl acetate 5:1) to give a white powder (250 mg, 85%); m. p. 156.0–158.1 °C; ¹H NMR (400 MHz, DMSO) δ 7.83 (s, 2H), 7.00 (s, 2H), 4.27 (d, *J* = 6.3 Hz, 1H), 3.78 (s, 3H), 2.80–2.68 (m, 2H), 2.39–2.15 (m, 2H), 1.97–1.79 (m, 4H), 1.75–1.62 (m, 2H), 1.54–1.15 (m, 5H), 0.83 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 149.47, 138.65, 136.79, 128.27, 122.92, 110.58, 71.44, 55.88, 47.25, 45.98, 43.56, 38.35, 33.86, 33.43, 28.25, 27.41, 25.92, 23.68, 17.49, 13.42. HRMS (ESI-TOF) *m*/*z* [M + Na]⁺ calcd for C₂₀H₂₆F₃NO₇S₂, 536.1103, found 536.1106. Purity: 97.03%.

2-Methoxy-3-sulfamoyloxy-17 β -O-benzenesulfonylestra-1,3,5(10)-

triene (**9d**): m.p. 103.7–104.8 °C; ¹H NMR (400 MHz, DMSO) δ 7.94 (d, J = 7.8 Hz, 2H), 7.80 (d, J = 12.1 Hz, 3H), 7.69 (t, J = 7.6 Hz, 2H), 6.95 (d, J = 10.6 Hz, 2H), 4.37 (t, J = 8.0 Hz, 1H), 3.75 (s, 3H), 2.70 (d, J = 4.8 Hz, 2H), 2.25 (d, J = 12.2 Hz, 1H), 2.11 (t, J = 10.8 Hz, 1H), 1.89 (d, J = 6.2 Hz, 1H), 1.76 (d, J = 11.3 Hz, 1H), 1.62 (s, 2H), 1.48 (d, J = 12.2 Hz, 1H), 1.02 (t, J = 11.5 Hz, 1H), 0.76 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 149.45, 138.41, 136.73, 136.23, 134.16, 129.61, 128.11, 127.57, 122.89, 110.47, 89.63, 55.84, 47.91, 43.56, 42.66, 37.61, 35.25, 28.05, 27.35, 26.39, 25.40, 22.46, 11.51. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₅H₃₁NO₇S₂, 544.1542, found 544.1546. Purity: 98.29%.

2-Methoxy-3-sulfamoyloxy-17β-O-(4-methylbenzene)sulfonylestra-1,3,5(10)-triene (**9e**): m. p. 108.2–110.4 °C ¹H NMR (400 MHz, DMSO) δ 7.81 (s, 4H), 7.48 (d, J = 7.9 Hz, 2H), 6.95 (d, J = 9.1 Hz, 2H), 4.34 (t, J = 8.1 Hz, 1H), 3.75 (s, 3H), 2.70 (d, J = 4.9 Hz, 2H), 2.43 (s, 3H), 2.25 (t, J = 15.5 Hz, 1H), 2.12 (t, J = 11.3 Hz, 1H), 1.88 (s, 1H), 1.76 (d, J = 11.4 Hz, 1H), 1.58 (dd, J = 20.7, 9.9 Hz, 3H), 1.37–1.09 (m, 6H), 0.76 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 149.45, 144.68, 138.44, 136.75, 133.40, 130.01, 128.13, 127.58, 122.89, 110.48, 89.39, 55.85, 47.93, 43.56, 42.66, 37.61, 35.33, 28.05, 27.35, 26.40, 25.41, 22.46, 21.10, 11.52. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₆H₃₃NO₇S₂, 558.1698, found 558.1699. Purity: 97.33%.

2-Methoxy-3-sulfamoyloxy-17β-O-(4-methoxybenzene)sulfonylestra-1,3,5(10)-triene (9f): m. p. 100.8–102.1 °C; ¹H NMR (400 MHz, DMSO) δ 7.86 (d, J = 8.7 Hz, 2H), 7.82 (s, 2H), 7.18 (d, J = 8.7 Hz, 2H), 6.96 (d, J = 8.4 Hz, 2H), 4.30 (t, J = 8.1 Hz, 1H), 3.88 (s, 3H), 3.75 (s, 3H), 2.70 (d, J = 4.8 Hz, 2H), 2.27 (d, J = 14.1 Hz, 1H), 2.12 (t, J = 11.0 Hz, 1H), 1.88 (s, 1H), 1.76 (d, J = 11.7 Hz, 1H), 1.58 (dd, J = 21.3, 9.8 Hz, 3H), 1.25 (ddd, J = 22.3, 16.8, 8.6 Hz, 5H), 1.05 (t, J = 11.5 Hz, 1H), 0.75 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 163.34, 149.45, 138.45, 136.74, 129.89, 128.13, 127.66, 122.90, 114.71, 110.48, 89.13, 55.84, 55.82, 47.98, 43.58, 42.62, 37.62, 35.36, 28.06, 27.37, 26.40, 25.42, 22.47, 11.51. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₆H₃₃NO₈S₂, 574.1648, found 574.1646. Purity: 98.05.

2-Methoxy-3-sulfamoyloxy-17 β -O-(4-aminobenzene)sulfonylestra-1,3,5(10)-triene(**9** g): Compound **7** g was prepared using the general procedure described above by the addition of compound **6**(200 mg, 0.51 mmol) and 4-nitrobenzene sulfonyl chloride (678 mg, 3.06 mmol) in dried pyridine (5 mL). In the hydrogenation step, the nitro group was simultaneously reduced to amino group according to general procedure. The crude product from the sulfamoyl rection was purified by column chromatography (petroleum ether/ethyl acetate 5:1) to give a white powder (yield 88%); m. p. 127.8–129.4 °C; ¹H NMR (400 MHz, DMSO) δ 10.37 (s, 1H), 8.59 (s, 1H), 7.82 (d, *J* = 8.6 Hz, 2H), 7.48 (s, 2H), 7.33 (d, *J* = 8.6 Hz, 2H), 6.72 (s, 1H), 6.42 (s, 1H), 4.30 (t, *J* = 8.1 Hz, 1H), 3.70 (s, 3H), 2.60 (d, *J* = 4.3 Hz, 2H), 2.19 (d, *J* = 11.9 Hz, 1H), 2.02 (d, *J* = 10.0 Hz, 1H), 1.86 (s, 1H), 1.72 (d, *J* = 9.4 Hz, 1H), 1.56 (d, *J* = 11.9 Hz, 3H), 1.23 (ddd, *J* = 32.3, 15.7, 7.0 Hz, 6H), 0.75 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 145.55, 144.56, 144.35, 129.87, 129.03, 128.15, 127.98, 116.56, 115.52, 109.73, 89.20, 59.72, 55.76, 47.97, 43.33, 42.71, 38.07, 35.44, 28.24, 27.35, 26.72, 25.68, 22.49, 20.73, 14.05, 11.57. HRMS (ESI-TOF) $m/z \ [M + Na]^+$ calcd for $C_{25}H_{32}N_2O_7S_2$, 559.1651, found 559.1649. Purity: 98.72%.

2-Methoxy-3-sulfamoyloxy-17β-O-naphthalenesulfonylestra-1,3,5(10)triene (**9h**): m.p. 113.1–114.6 °C; ¹H NMR (400 MHz, DMSO) δ 8.67 (s, 1H), 8.24 (dd, J = 18.6, 8.4 Hz, 2H), 8.12 (d, J = 8.0 Hz, 1H), 7.90 (d, J = 8.7 Hz, 1H), 7.83–7.68 (m, 4H), 6.95 (s, 1H), 6.90 (s, 1H), 4.42 (t, J = 8.2 Hz, 1H), 3.71 (s, 3H), 2.68 (d, J = 5.4 Hz, 2H), 2.20 (d, J = 11.9 Hz, 1H), 2.08 (t, J = 10.7 Hz, 1H), 1.90 (s, 1H), 1.77–1.51 (m, 4H), 1.31 (dd, J = 21.9, 11.2 Hz, 3H), 1.17 (d, J = 7.2 Hz, 2H), 0.99 (t, J = 11.5 Hz, 1H), 0.79 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 149.43, 138.40, 136.71, 134.75, 133.14, 131.55, 129.77, 129.55, 129.52, 129.29, 128.09, 127.95, 127.91, 122.87, 122.39, 110.47, 89.72, 55.83, 55.45, 47.86, 43.51, 42.70, 37.58, 35.28, 28.04, 27.40, 26.37, 25.36, 22.47, 11.55. HRMS (ESI-TOF) *m*/*z* [M + Na]⁺ calcd for C₂₉H₃₃NO₇S₂, 594.1698, found 594.1701. Purity: 97.12%.

2-Methoxy-17β-O-methylestra-1,3,5(10)-trien-3-ol (**8i**): NaH (113 mg, 4.71 mmol) was added to the solution of compound 6 (308 mg, 0.785 mmol) in DMF (3 mL) and stirring was continued overnight under the atmosphere of argon. Then water (15 mL) was added carefully and the suspension was extracted with ethyl acetate (3 \times 15 mL). The combined organic layers were washed with brine, dried (MgSO₄) and concentrated. The residue was purified by flash chromatography (petroleum ether/ethyl acetate 35:1) to give 7i as a white powder (303 mg, 95%). The hydrogenation step was conducted according to general procedure to afford compound 8i. m. p. 229.8-231.1 °C; ¹H NMR (400 MHz, DMSO) & 8.62 (s, 1H), 6.76 (s, 1H), 6.44 (s, 1H), 3.71 (s, 3H), 3.32-3.19 (m, 4H), 2.78–2.53 (m, 2H), 2.28 (d, J = 5.1 Hz, 1H), 2.09 (d, J = 8.6 Hz, 1H), 2.02–1.86 (m, 2H), 1.77 (d, J = 9.2 Hz, 1H), 1.62 (d, J = 7.2 Hz, 1H), 1.46–1.10 (m, 7H), 0.72 (s, 3H). $^{13}\mathrm{C}$ NMR (101 MHz, DMSO) δ 145.54, 144.31, 130.21, 128.23, 115.55, 109.69, 89.80, 57.05, 55.75, 49.55, 43.68, 42.76, 38.28, 37.45, 28.35, 27.23, 26.93, 26.21, 22.61, 11.56. Purity: 99.17%.

2,17β-dimethoxy-3-sulfamoyloxyestra-1,3,5(10)-triene (9i): This compound was prepared using general sulfamoyl procedure described above and the resultant crude solid was purified by flash chromatography (petroleum ether/EtOAc 3:1) to give a white powder (yield 86%). m. p. 181.3–182.8 °C; ¹H NMR (400 MHz, DMSO) δ 7.81 (s, 2H), 6.98 (s, 2H), 3.76 (s, 3H), 3.31–3.23 (m, 4H), 2.72 (d, *J* = 5.0 Hz, 2H), 2.34 (s, 1H), 2.18 (s, 1H), 2.05–1.89 (m, 2H), 1.81 (d, *J* = 11.9 Hz, 1H), 1.63 (d, *J* = 7.4 Hz, 1H), 1.44–1.17 (m, 7H), 0.72 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 149.46, 138.80, 136.72, 128.22, 122.93, 110.41, 89.73, 57.05, 55.83, 49.55, 43.94, 42.69, 37.84, 37.36, 28.17, 27.21, 26.62, 25.96, 22.60, 11.51. HRMS (ESI-TOF) *m*/*z* [M + Na]⁺ calcd for C₂₀H₂₉NO₅S, 418.1766, found 418.1769. Purity: 98.94%.

2-Methoxy-3-sulfamoyloxy-17β-O-(isoquinolin-3-yl)estra-1,3,5(10)triene (**9***j*): m.p.118.1–120.0 °C ¹H NMR (400 MHz, CDCl₃) δ 7.99 (d, J = 8.8 Hz, 1H), 7.83 (d, J = 8.2 Hz, 1H), 7.73 (d, J = 8.0 Hz, 1H), 7.66–7.58 (m, 1H), 7.42–7.35 (m, 1H), 7.08 (s, 1H), 6.96 (s, 1H), 6.92 (d, J = 8.8 Hz, 1H), 5.26 (t, J = 8.4 Hz, 1H), 5.04 (s, 2H), 3.89 (s, 3H), 2.85 (dd, J = 8.7, 3.9 Hz, 2H), 2.65–2.47 (m, 1H), 2.31 (t, J = 8.3 Hz, 2H), 2.05–1.92 (m, 2H), 1.91–1.79 (m, 1H), 1.65–1.34 (m, 7H), 1.00 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 162.33, 148.93, 146.68, 140.58, 138.41, 136.79, 130.29, 129.33, 127.36, 127.21, 124.99, 124.10, 123.76, 113.56, 110.58, 83.51, 56.37, 49.96, 44.52, 43.10, 38.18, 37.28, 28.67, 28.09, 27.08, 26.46, 23.42, 12.27. HRMS (ESI-TOF) *m*/z [M + Na]⁺ calcd for C₂₈H₃₂N₂O₅S, 531.2032, found 531.2035. Purity: 98.46%.

2-Methoxy-3-sulfamoyloxy-17 β -O-(5-aminopyridin-2-yl)estra-1,3,5 (10)-triene (9 l): m.p. 121.2–123.4 °C; ¹H NMR (400 MHz, DMSO) δ 9.10 (s, 1H), 8.57 (s, 1H), 7.96 (d, J = 2.7 Hz, 1H), 7.51 (dd, J = 8.8, 2.8 Hz, 1H), 6.98 (s, 2H), 6.79–6.73 (m, 2H), 6.45 (s, 1H), 4.85 (t, J = 8.1 Hz, 1H), 3.70 (s, 3H), 2.72–2.58 (m, 2H), 2.34–2.23 (m, 2H), 2.15 (t, J = 8.2 Hz, 1H), 1.84–1.76 (m, 2H), 1.71 (s, 1H), 1.37 (dd, J = 17.0, 9.0 Hz,

7H), 0.88 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 160.03, 145.56, 144.34, 139.13, 132.85, 130.17, 129.57, 128.28, 115.59, 110.65, 109.76, 83.22, 55.78, 49.14, 43.69, 42.63, 38.31, 36.84, 28.38, 27.67, 26.96, 26.09, 22.87, 12.10. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₄H₃₁N₃O₅S, 496.1984, found 496.1983. Purity: 99.42%.

2-Methoxy-3-sulfamoyloxy-17β-O-(pyridin-2-yl)estra-1,3,5(10)-triene (9 k): Method as for 7 l using compound 6 (240 mg, 0.61 mmol), Cs₂CO₃ (299 mg, 0.92 mmol), BINAP (76 mg, 0.12 mmol), 2,4-dichloropyridine (132 µL, 1.22 mmol) and Pd (OAc)₂ (14 mg, 0.06 mmol) in toluene (10 mL) at 100 °C for 6 h. Flash column chromatography (petroleum ether/ ethyl acetate 15:1) afforded compound 7 k (262 mg, 85%). Further hydrogenation and sulfamoyl reaction according to general procedure give 9 k as a white solid. m. p. 186.2–189.0 °C; ¹H NMR (400 MHz, DMSO) δ 8.13 (s, 1H), 7.81 (s, 2H), 7.67 (t, J = 7.2 Hz, 1H), 6.99 (s, 2H), 6.93 (s, 1H), 6.78 (d, J = 7.7 Hz, 1H), 4.94 (s, 1H), 3.76 (s, 3H), 2.75 (s, 2H), 2.40–2.15 (m, 4H), 1.81 (dd, J = 32.6, 22.0 Hz, 4H), 1.48–1.25 (m, 7H), 0.91 (s, 3H). $^{13}{\rm C}$ NMR (101 MHz, DMSO) δ 163.96, 149.96, 147.34, 139.47, 139.24, 137.22, 128.74, 123.44, 117.22, 111.48, 110.96, 83.39, 56.34, 49.61, 44.44, 43.05, 38.36, 37.23, 28.70, 28.12, 27.15, 26.34, 23.36, 12.58. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₄H₃₀N₂O₅S, 481.1875, found 481.1878. Purity: 98.27%.

2-Methoxy-3-sulfamoyloxy-17β-O-(6-chloropyrimidin-4-yl)estra-1,3,5 (10)-triene (**9** m): white solid. m. p. 219.2–221.8 °C; ¹H NMR (400 MHz, DMSO) δ 8.65 (s, 1H), 7.81 (s, 2H), 7.16 (s, 1H), 6.99 (s, 2H), 5.01 (t, J = 8.0 Hz, 1H), 3.76 (s, 3H), 2.75 (d, J = 5.0 Hz, 2H), 2.37 (d, J = 12.3 Hz, 2H), 2.23 (d, J = 10.0 Hz, 1H), 1.89–1.79 (m, 2H), 1.74 (s, 1H), 1.62–1.36 (m, 7H), 0.90 (s, 3H). HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₃H₂₈ClN₃O₅S, 516.1438, found 516.1438. Purity: 97.14%.

2-Methoxy-17 β -dimethyl formyl estra-1,3,5(10)-trien-3-ol (8n): To a solution of compound 6 (396 mmol, 1.0 mmol) in acetonitrile (12 mL), was added N, N-dimethylcarbamic chloride (158 µL, 1.72 mmol) and NaH (242 mg, 10.09 mmol). The mixture was stirred at 100 $^\circ$ C for 10 h before being evaporated to remove acetonitrile. The residue was extracted with ethyl acetate (3 \times 20 mL) and the combined organic layers were washed with brine, dried (MgSO₄) and concentrated. Flash chromatography on silica (petroleum ether/ethyl acetate 3:1) afforded compound **7n** (421 mg,90%) as a white powder. Then general hydrogenation procedure gives compound 8n. ¹H NMR (400 MHz, CDCl₃) δ 6.78 (s, 1H), 6.64 (s, 1H), 5.62 (s, 1H), 4.62 (t, J = 8.4 Hz, 1H), 3.85 (s, 3H), 2.92 (s, 6H), 2.81–2.72 (m, 2H), 2.23 (dd, J = 15.1, 6.7 Hz, 3H), 1.94–1.83 (m, 2H), 1.72 (d, J = 8.9 Hz, 1H), 1.60–1.24 (m, 7H), 0.85 (s, 3H). ¹³C NMR (101 MHz, CDCl₂) δ 156.77, 147.64, 146.40, 137.50, 132.97, 128.89, 128.48, 127.71, 127.32, 114.75, 109.96, 83.49, 71.16, 56.39, 49.69, 44.16, 42.92, 38.59, 37.01, 36.35, 35.85, 29.16, 28.15, 27.32, 26.44, 23.27, 12.29. Purity: 97.41%.

2-Methoxy-3-sulfamoyloxy-17β-dimethylcarbamoylestra-1,3,5(10)triene(**9**n): white solid. m. p. 200.1–202.6 °C; ¹H NMR (400 MHz, DMSO) δ7.81 (s, 2H), 6.98 (s, 2H), 4.49 (t, J = 8.1 Hz, 1H), 3.76 (s, 3H), 2.83 (d, J = 16.2 Hz, 6H), 2.74 (d, J = 4.9 Hz, 2H), 2.36 (d, J = 10.3 Hz, 1H), 2.21 (s, 1H), 2.11 (d, J = 8.2 Hz, 1H), 1.81 (t, J = 8.7 Hz, 2H), 1.67 (s, 1H), 1.40–1.22 (m, 7H), 0.81 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 155.61, 149.45, 138.71, 136.74, 128.24, 122.93, 110.48, 82.55, 55.85, 48.95, 43.84, 42.30, 37.84, 36.40, 35.85, 35.37, 28.18, 27.75, 26.59, 25.74, 22.75, 12.02. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₂H₃₂N₂O₆S, 475.1981, found 475.1983. Purity: 98.64%.

2-Methoxy-17β-acetoxyestra-1,3,5(10)-trien-3-ol (80): m. p. 190.6–192.1 °C; ¹H NMR (400 MHz, CDCl₃) δ 6.81 (s, 1H), 6.67 (s, 1H), 5.49 (s, 1H), 4.72 (dd, J = 9.0, 7.9 Hz, 1H), 3.88 (s, 3H), 2.80 (dd, J =9.7, 6.7 Hz, 2H), 2.33–2.17 (m, 3H), 2.09 (s, 3H), 1.89 (ddd, J = 7.9, 5.6, 3.0 Hz, 2H), 1.81–1.70 (m, 1H), 1.60–1.27 (m, 7H), 0.86 (s, 3H). ¹³C NMR (101 MHz, CDCl₃) δ 171.25, 144.61, 143.50, 131.61, 129.48, 114.62, 108.11, 82.73, 56.08, 49.81, 44.11, 42.92, 38.56, 36.95, 28.97, 27.60, 27.30, 26.52, 23.24, 21.20, 12.10. Purity: 98.38%.

2-Methoxy-3-sulfamoyloxy-17 β -acetoxyestra-1,3,5(10)-triene (**90**): m. p. 214.3–215.1 °C; ¹H NMR (400 MHz, DMSO) δ 7.82 (s, 2H), 6.99 (d, J = 4.2 Hz, 2H), 4.61 (t, J = 8.3 Hz, 1H), 3.77 (s, 3H), 2.74 (d, J = 4.8 Hz,

2H), 2.36 (d, J = 8.8 Hz, 1H), 2.15 (ddd, J = 14.9, 13.6, 7.1 Hz, 2H), 2.02 (s, 3H), 1.80 (dd, J = 20.3, 12.1 Hz, 2H), 1.73–1.62 (m, 1H), 1.47–1.24 (m, 7H), 0.80 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 170.79, 149.96, 139.15, 137.25, 128.71, 123.43, 110.95, 82.34, 56.34, 49.54, 44.29, 42.87, 38.28, 36.89, 28.66, 27.71, 27.10, 26.22, 23.24, 21.34, 12.39. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₁H₂₉NO₆S, 446.1716, found 446.1715. Purity: 99.04%.

2-*Methoxy-3-sulfamoyloxy-17β-carbamoylestra-1,3,5(10)-triene* (**9***p*): This compound was prepared using the method of Potter et al [17]. m. p. 184.9–189.2°C; ¹H NMR (400 MHz, DMSO) δ 7.82 (s, 2H), 6.98 (s, 2H), 6.41 (d, *J* = 21.4 Hz, 2H), 4.47 (t, *J* = 8.3 Hz, 1H), 3.76 (s, 3H), 2.73 (s, 2H), 2.36 (d, *J* = 8.6 Hz, 1H), 2.20 (s, 1H), 2.07 (d, *J* = 7.9 Hz, 1H), 1.81 (t, *J* = 9.4 Hz, 2H), 1.65 (s, 1H), 1.52–1.20 (m, 7H), 0.78 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 156.78, 149.45, 138.75, 136.71, 128.22, 122.92, 110.45, 81.23, 55.84, 49.14, 43.86, 42.24, 37.84, 36.50, 28.17, 27.44, 26.59, 25.77, 22.65, 11.89. HRMS (ESI-TOF) *m/z* [M + Na]⁺ calcd for C₂₀H₂₈N₂O₆S, 447.1668, found 447.1667. Purity: 98.25%.

4.2.4. General procedure for preparation of carbamates (compounds 11).

Trichloroacetyl isocyanate (0.21 mL, 1.7 mmol) was added to a solution of corresponding compound **8** (0.87 mmol) in anhydrous THF. Stirring was continued for 10 h at room temperature prior to water was added. The product was extracted with ethyl acetate (3×15 mL) and the combined organic layers were washed with brine, dried (MgSO₄) and concentrated to give the desired crude product **10**, which was used for the next step directly.

A solution of K_2CO_3 (516 mg, 3.73 mmol) in water (12 mL) was added to a solution of above crude product in THF (12 mL) and MeOH (12 mL). The mixture was stirred for 3 h at room temperature and at this time a large amount of white solid is precipitated. Filtered and obtained the target product **11** with the yield greater than 85% over two steps.

2-Methoxy-3-carbamoyl-17β-O-methanesulfonylestra-1,3,5(10)-triene (**11a**): m.p.206.2–208.1 °C; ¹H NMR (400 MHz, DMSO) δ 6.89 (s, 1H), 6.66 (s, 1H), 4.55 (t, J = 8.3 Hz, 1H), 3.72 (s, 3H), 3.18 (s, 3H), 2.78–2.65 (m, 2H), 2.39 (s, 1H), 2.27–2.13 (m, 2H), 1.95–1.87 (m, 1H), 1.82–1.70 (m, 3H), 1.43 (p, J = 11.6 Hz, 4H), 1.29 (dd, J = 15.1, 7.5 Hz, 2H), 0.82 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 146.09, 145.04, 130.31, 128.71, 116.09, 110.35, 89.25, 79.66, 56.32, 48.65, 43.97, 43.25, 38.68, 37.92, 36.09, 28.80, 27.94, 27.30, 26.26, 23.05, 12.09. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₁H₂₉NO₆S, 446.1716, found 446.1719. Purity: 97.69%.

2-Methoxy-3-carbamoyl-17 β -O-(4-methylbenzene)sulfonylestra-1,3,5 (10)-triene (**110**): ¹H NMR (400 MHz, DMSO) δ 6.88 (s, 1H), 6.65 (s, 1H), 4.62 (t, J = 8.4 Hz, 1H), 3.71 (s, 3H), 2.75–2.66 (m, 2H), 2.35 (d, J = 9.5 Hz, 1H), 2.20 (dd, J = 14.8, 6.4 Hz, 1H), 2.16–2.08 (m, 1H), 2.03 (s, 3H), 1.85–1.77 (m, 2H), 1.73–1.65 (m, 1H), 1.48–1.28 (m, 7H), 0.81 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 170.31, 165.97, 160.54, 149.51, 139.07, 136.15, 127.79, 123.44, 109.63, 81.89, 55.51, 49.08, 43.81, 42.42, 37.97, 36.45, 28.19, 27.22, 26.78, 25.83, 22.77, 20.87, 11.92. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₂H₂₉NO₅, 410.2046, found 410.2015. Purity: 97.81%.

2,17β-Dimethoxy-3-carbamoylestra-1,3,5(10)-triene (11i): ¹H NMR (400 MHz, DMSO) δ 6.75 (s, 1H), 6.44 (s, 1H), 3.70 (s, 3H), 3.29–3.24 (m, 4H), 2.70–2.57 (m, 2H), 2.27 (d, J = 5.0 Hz, 1H), 2.10 (s, 1H), 2.03–1.88 (m, 2H), 1.80–1.72 (m, 1H), 1.67–1.55 (m, 1H), 1.39–1.17 (m, 7H), 0.71 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 145.58, 144.49, 130.08, 128.24, 115.59, 109.73, 89.81, 57.05, 55.76, 49.56, 43.69, 42.77, 38.29, 37.47, 28.36, 27.23, 26.94, 26.22, 22.61, 11.56. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₁H₂₉NO₄, 382.2097, found 382.2094. Purity: 98.34%.

2-Methoxy-3-carbamoyl-17 β -O-(5-aminopyridin-2-yl)estra-1,3,5(10)triene (111): m.p. 152.8–154.2 °C; ¹H NMR (400 MHz, DMSO) δ 8.61 (s, 1H), 8.42 (s, 1H), 8.07 (d, J = 2.6 Hz, 1H), 7.76 (dd, J = 8.8, 2.7 Hz, 1H), 6.76 (s, 1H), 6.69 (d, J = 8.8 Hz, 1H), 6.46 (s, 1H), 5.87 (s, 2H), 4.83 (t, J= 8.1 Hz, 1H), 3.71 (s, 3H), 2.74–2.58 (m, 2H), 2.27 (d, J = 8.8 Hz, 2H), 2.14 (s, 1H), 1.83–1.65 (m, 3H), 1.49–1.21 (m, 7H), 0.88 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 159.05, 156.69, 146.04, 144.80, 136.90, 131.52, 131.08, 130.68, 128.75, 116.06, 110.74, 110.20, 83.52, 56.23, 49.64, 44.19, 43.10, 38.80, 37.35, 28.88, 28.18, 27.47, 26.59, 23.36, 12.59. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₅H₃₁N₃O₄, 460.2315, found 460.2317. Purity: 98.64%.

2-Methoxy-3-carbamoyl-17β-dimethylcarbamoylestra-1,3,5(10)-triene (**11n**): m.p.237.6–238.8 °C; ¹H NMR (400 MHz, DMSO) δ 6.87 (s, 1H), 6.64 (s, 1H), 4.49 (t, J = 8.3 Hz, 1H), 3.70 (s, 3H), 2.84 (d, J = 18.3 Hz, 6H), 2.76–2.65 (m, 2H), 2.40–2.28 (m, 1H), 2.21 (t, J = 8.6 Hz, 1H), 2.16–2.04 (m, 1H), 1.81 (d, J = 11.0 Hz, 2H), 1.71–1.60 (m, 1H), 1.51–1.24 (m, 7H), 0.82 (s, 3H). ¹³C NMR (101 MHz, DMSO) δ 156.12, 146.05, 144.94, 130.51, 128.73, 116.08, 110.28, 99.99, 83.10, 79.69, 56.28, 49.46, 44.09, 42.86, 38.77, 36.97, 36.36, 28.87, 28.24, 27.41, 26.47, 23.25, 12.57. HRMS (ESI-TOF) m/z [M + Na]⁺ calcd for C₂₃H₃₂N₂O₅, 439.2311, found 439.2310. Purity: 97.39%.

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.

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Appendix A. Supplementary data

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