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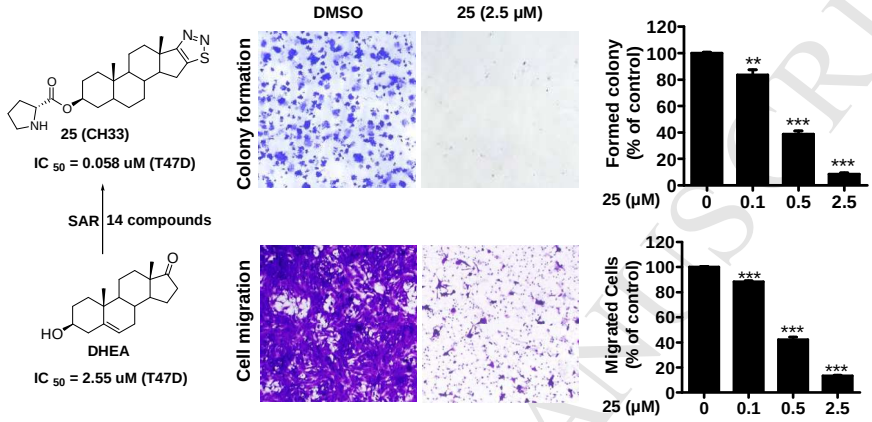

A series of novel D-ring fused 1,2,3-thiadiazole dehydroepiandrosterone (DHEA) derivatives were synthesized and investigated for their antitumor activity *in vitro* and *in vivo*.

Synthesis and biological evaluation of D-ring fused 1,2,3-thiadiazole dehydroepiandrosterone derivatives as antitumor agents

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ABSTRACT

A series of D-ring fused 1,2,3-thiadiazole DHEA derivatives were synthesized and investigated for their activity against the growth of various tumor cell lines using the sulforhodamine B (SRB) assay. It is amazing that for these compounds, T47D cell line was much more sensitive than other tumor cell lines. The most potent saturated N-heterocyclic derivatives showed similar antitumor effect with the positive control compound ADM (adriamycin) on T47D cells, that was 44-60 folds more potent than the lead compound DHEA. Most compounds with potent antitumor activity displayed low toxicity on normal human fibroblasts (HAF). Especially compound **25** (**CH33**) showed an IC_{50} of 0.058 μM on T47D cells and its selectivity index (SI) between HAF and T47D was 364, which was 214 folds better than ADM (SI = 1.7). The apoptosis, colony formation and

Abbreviations: IC_{50}, half maximal inhibitory concentration; SAR, structure-activity relationship; SI, selectivity index; SRB, sulforhodamine; RTKs, receptor tyrosine kinases; HE, hematoxylin and eosin; HAF, human fibroblasts; ADM, adriamycin; DHEA, dehydroepiandrosterone.

transwell migration assays of **25** were performed on T47D cell line. The primary mechanism study showed that **25** caused a dose-dependent induction of apoptosis, and induced phosphorylation of EphA2 and EphB3 in T47D cells. The *in vivo* antitumor effect of **25** was also observed in T47D tumor-bearing mice without obvious toxicity.

1. Introduction

Steroids are widely distributed in natural world and display a variety of biological activities, especially antitumor activity [1,2]. Heterocycles are considered as privileged scaffolds in drug discovery and widely used in medicinal chemistry [3,4]. The introduction of A- and D-ring fused heterocycles into the steroid derivatives often result in amelioration of biological properties [5,6], for example, enhancing the antitumor effect [7–10].

Dehydroepiandrosterone (DHEA) also known as androstenolone or prasterone, is the most abundant circulating steroid hormone in humans [11]. DHEA and its sulfate ester also have a variety of potential biological effects, such as anti-aging [12], anti-inflammatory [13], immunomodulatory [14], antiviral [15,16], antidepressant [17] and anticancer effects [18,19]. As heterocycles were fused with the D-ring of DHEA, the antitumor activity was greatly improved. For instance (Fig. 1), compound **A**, that was obtained by introducing p-methoxyphenylpyrazoline fragment displayed more cytotoxic activity than cisplatin on malignant human cell lines [20]; a series of D-ring fused thiazole, thiazole imines, imidazo thiazoles and pyridine were synthesized by Liu et al. showed good cytotoxicity against three tumor cell lines (EC109, EC9706 and MGC 803), especially compound **B** possessed the most potent antitumor activity [21,22]; compound **C** was synthesized by fusing pyrazole-scaffold with D-ring exhibited the best antitumor activity with IC_{50} values of 20-1.4 nM against four cell lines, and 1.03 μM against a tamoxifen resistant breast cancer cell line, and a preliminary structure-activity relationship (SAR) showed that with a linker in 3-position was favorable to increase the bioactivity [23].

Thiadiazoles occur widely in nature in four isomeric forms, that is 1,2,3-thiadiazole, 1,2,5-thiadiazole, 1,2,4-thiadiazole and 1,3,4-thiadiazole. Many drugs containing thiadiazole nucleus are available in the market for instance acetazolamide, methazolamide, cefazoline, sulfamethazole, etc. In recent decades, thiadiazole and their derivatives have attracted much

attentation because of their wide range of biological activities [24]. Especially the 1,3,4-thiadiazole derivatives have attracted widespread attention due to their diverse biological activity such as antimicrobial, antifungal, antiviral, antitubercular, anti-inflammatory, analgesic, antiepileptic, and antitumor activity, and been described in recent reviews [25–27]. However, there are only a few researches performed on the 1,2,3-thiadiazole derivatives and their biological activity [28–32]. In our laboratory, researches are focused on exploring the 1,2,3-thiadiazole DHEA derivatives and their antitumor activity.

In order to obtain novel potent antitumor agents, a series of compounds with D-ring fused 1,2,3-thiadiazole of DHEA were synthesized. Their inhibitory activity against the growth of T47D cells and other 8 different tumor cell lines was evaluated using a SRB assay. DHEA and its derivative were screened by flow cytometry to determine their apoptotic behaviors, and their inhibitory effects on tumor cell migration and colony formation ability were also tested. The primary anticancer mechanism was studied and the *in vivo* antitumor evaluation in T47D tumor-bearing mice was also carried out.

Fig. 1. Chemical structures of D-ring fused heterocycles DHEA antitumor agents

2. Chemistry

A series of D-ring fused 1,2,3-thiadiazole DHEA derivatives were synthesized according to the pathways described in Scheme 1–2.

Compound **1** was prepared by protection of the 3-hydroxyl group of DHEA with acetyl group under Ac_2O and DMAP in CH_2Cl_2. The intermediate **2** was afforded by reaction of **1** with aminourea hydrochloride and sodium acetate in EtOH. Compound **4** was obtained by condensation of **2** under thionyl chloride, then hydrolysis with K_2CO_3 in MeOH. Reduction of the double bond at B-ring of **1** under H_2 and palladium in carbon in autoclave gave compound **5**. Compound **8** (**CH21**) was prepared in a manner similar to that of compound **4**.

Compounds **9**–**13** were obtained by reaction of compound **8** with corresponding acyl chlorides under DMAP in CH_2Cl_2. Compounds **20**－**25** were obtained by condensation of **8** with corresponding N-Boc-amino-carboxylic acids under 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI), 1-hydroxybenzotriazole (HOBt) and DMAP in CH_2Cl_2, then deprotection of

the Boc group under boron trifluoride etherate in Et_2O.

Scheme 1. Synthesis of derivatives **4** and **8**. Reagents and conditions: (a) Ac_2O, DMAP, CH_2Cl_2, rt, 5 h, 98%; (b) aminourea hydrochloride, CH_3COONa, EtOH, rt, 6 h, 92% for **2** and 95% for **6**; (c) $SOCl_2$, CH_2Cl_2, rt, 5 h, 82% for **3** and 78% for **7**; (d) K_2CO_3, MeOH, rt, 4h, 93% for **4** and 95% for **8**; (e) H_2 (4.0 Mp), 5% Pd/C, MeOH, 40 ^oC, 16 h, 87%.

Scheme 2. Synthesis of derivatives **9-13** and **20-25**. Reagents and conditions: (a) acyl chlorides, DMAP, CH_2Cl_2, rt, 4 h, 83−90% for **9**−**13**; (b) N-Boc-amino-carboxylic acids, EDCI, HOBt, DMAP, CH_2Cl_2, rt, 8 h, 86−98% for **14**−**19**; (c) boron trifluoride etherate, Et_2O, rt, 2 h, 72−82% for **20**−**25**.

3. Result and discussion

3.1. Antiproliferative activity

The first round synthetic DHEA derivatives **4** and **8** were evaluated for their *in vitro* antitumor activity in SRB assay against human breast cancer cell lines (T47D, MDA-MB-231 and MCF-7), human prostate cancer cell lines (DU-145 and LNCaP), human colon carcinoma cell lines (HCT-116 and HT-29), human promyelocytic leukemia cell line (HL-60) and human T lymphocyte cell line (Jurkat). It is amazing that for these tested compounds (DHEA、EPI、**4** and **8**), T47D was the most sensitive tumor cell line. However for other various tumor cell lines, these compounds just showed weak or even no inhibition activity (Table 1). All these tested compounds displayed potent antiproliferative activity against T47D cells. Especially compound **8**, which was about 2 and 4 folds higher activity than EPI and DHEA respectively. Thus, we considered the D-ring fused 1,2,3-thiadiazole as an effective modification and the subsequent evaluation should be focused on the most sensitive T47D cell line.

Table 1

IC_{50} values of DHEA and its derivatives **4** and **8** against the growth of T47D cells and other various tumor cell lines

From SRB assay after 96 h of treatment; [a]IC_{50} data are an average of at least 3 independent experiments, variation ± 10%; [b] Epiandrosterone (EPI) is a reduzate of DHEA.

The second round synthetic analogues were obtained by modification of the C3-hydroxyl group of compound **8** and screened using the SRB assay on T47D cell line (Table 2). The results showed that the alkyl esters (**7**, **9**, **10**, **20** and **21**) and aromatic N-heterocyclic esters (**11**, **12** and **13**) in position C-3 were not favorable substituted groups for improving antitumor activity, which resulted in a lower inhibition of cell growth compared with **8**. Derivatives (**22**, **23**, **24** and **25**) bearing a saturated N-heterocycle in position C-3, possessed more potent antiproliferative activity than **8**. It is interesting that for the five-membered saturated N-heterocycle, the unnatural D-proline derivative **25** (IC_{50} = 0.058 μM) possessed much higher antiproliferative activity than its natural L-proline derivative **24** (IC_{50} = 0.24 μM). The most potent saturated N-heterocyclic derivatives **22**, **23** and **25** showed similar antitumor effect with the positive control compound ADM (adriamycin) on T47D cells, that was 44-60 folds more potent than DHEA (IC_{50} = 2.55 μM).

In order to further investigate the selectivity between T47D tumor cell line and other tumor cells, we tested the most potent compounds **22** and **25** and its L-proline isomer **24** against the growth of these tumor cell lines (Table 3). The results revealed that selectivity was in accordance with the former (Table 1), T47D was the most sensitive tumor cells in the all tested tumor cell lines. Compound **25** (**CH33**) possessed the best selectivity, which was 43- to 793-fold for T47D over other tumor cells.

Table 2

IC_{50} values of DHEA and its derivatives (**7**, **8** and **26**-**43**) against the growth of T-47D tumor cell line and normal human fibroblast (HAF) cells.

From SRB assay after 96 h of treatment; [a]IC_{50} data are an average of at least 3 independent experiments; [b]the

selectivity indexes (SI) were calculated by IC_{50} values in HAF cells divided by IC_{50} values in T47D cancer cell line; [c]data from Table 1; [d]ND = not determined; [e]ADM is Adriamycin.

Table 3

IC_{50} values of compounds **22**, **24** and **25** against the growth of T47D cells and other various tumor cell lines.

From SRB assay after 96 h of treatment; [a]IC_{50} data are an average of at least 3 independent experiments, variation ± 10%; [b]data from Table 2; [c]Selectivity is the IC_{50} values of T47D cells divide IC_{50} values of other tumor cell lines.

3.2. Selectivity

One of the major hindrances for druggability of compounds with effective antitumor activity is their toxicity to normal cells. Thus, it is important to measure cytotoxicity on normal cells in anticancer drug discovery.

Compound **8** and its derivatives were chosen for selectivity test on a normal human fibroblast (HAF) cell line using the SRB assay. The selectivity indexes (SI) were calculated by IC_{50} values in HAF cells divided by IC_{50} values in T47D cell line. The results (Table 2) revealed that most of the tested compounds were less toxic on HAF in comparison with the tumor cells. To our delight, the SI of compounds **22**, **24** and **25** were satisfactory. Especially compound **25** possessed the highest selectivity (SI = 364), which was 214 folds better than the positive control compound ADM (SI = 1.7), although the two compounds possessed almost equivalent antiproliferative activity against T47D cells.

3.3. Colony formation

Colony formation assay is the gold standard for measuring the effect of cytotoxic agents on tumor cells *in vitro* [33]. It is closer to physiology and growth of tumor *in vivo* to mimic individual tumor cell development into macroscopic cell clones [34]. So the colony formation assay on T47D cell line was performed to confirm the antiproliferative activity of compound **25**. The colony formation ability of T47D cells was significantly inhibited by **25** (Fig. 2, IC_{50} = 0.35 μM) in a dose-dependent manner, which was much more effective than DHEA.

Fig. 2. Compound **25** and DHEA inhibited the colony formation of T47D cell line. After treatment by various doses of **25** and DHEA in 6-well plates for 7 days, cells were fixed and stained with crystal violet and the numbers of cell colonies were counted. **P < 0.01, ***P < 0.001, versus the control group (0 μM).

3.4. Migration

Migration is an important step during the metastasis of cancer. The antimetastatic activity of compound **25** was measured by *in vitro* transwell migration assay. The cell migration ability of T47D cells was significantly inhibited by **25** (Fig. 3, IC_{50} = 0.42 μM) in a dose-dependent manner, which was much more effective than DHEA.

Fig. 3. Compound **25** and DHEA inhibited the cell migration of T47D cell line. After 12 h incubation with various doses of **25** and DHEA, non-migrated cells were removed and migrated cells were fixed with cold 4% paraformaldehyde and stained with 0.2% crystal violet. Images were taken and cells from three random areas per filter were counted. **P < 0.01, ***P < 0.001, versus the control group (0 μM).

3.5. Apoptosis and cell cycle

Previous results of cellular cytotoxicity and colony formation assay indicated that compound **25** inhibited cancer cell proliferation. Many factors cause cell proliferation inhibition, including cell cycle arrest and induction of apoptosis [35–37]. So we evaluated whether the antiproliferative activity of the compound was resulted by cell apoptosis or cell cycle arrest using flow cytometry. Results indicated that **25** did not induce cell-cycle arrest (Fig. 4A), but caused a dose-dependent induction of apoptosis on T47D cells (Fig. 4B). The apoptotic cells rose from 5.74% to 30.14% with doses of **25** increased from 0 to 12.5 μM after 24 h treatment. These results suggested that this compound inhibited cell proliferation through inducing apoptosis in the cell line mainly.

Fig. 4. (A) Compound **25** didn't induce cell cycle arrest in T47D cell line. Cells were stained with PI and analyzed using flow cytometry after treatment with **25** for 24 h. (B) Compound **25** mediated T47D cells apoptosis in dose-dependent manner. Cells were treated with different dose of **25** for 24 h and then the percentages of apoptosis

cells were analyzed using flow cytometry by staining with PI and Annexin V-FITC.

3.6. Phospho-receptor tyrosine kinase array analysis

Receptor tyrosine kinases (RTKs) are a group of cell surface receptors that regulate a diverse array of cellular functions including cellular proliferation, survival, differentiation, migration, and metabolism [38]. We analyzed the activation of RTKs comprehensively of T47D cells using a phospho-RTK array after compound **25** treated for 24 h. A remarkable increase in the phosphorylation of EphA2 and EphB3 was detected (Fig. 5). According to previous studies, there is accumulating evidence to suggest that EPH expression and function in the absence of kinase activity is tumor promoting, whereas EPH forward signaling (phosphorylated Eph) is tumor suppressive [39-42]. So, the phosphorylation of EphA2 and EphB3 may play pivotal role in the proliferation suppression, migration inhibition and apoptosis-inducing of **25** in T47D cells and further in-depth mechanism research is also required.

Fig. 5. Compound **25** increased the phosphorylation of EphA2 and EphB3 in T47D cells. After treated with 2.5 μM **25** for 24 h, T47D cells were lysed and analyzed for the activation of RTKs comprehensively using a phospho-RTK array.

3.7. In vivo antitumor activity

In order to determine the efficacy of compound **25** *in vivo*, T47D subcutaneous xenograft growth model and orthotopic xenograft growth and metastasis model were evaluated. Results of subcutaneous xenograft growth model suggested that the tumor growth of T47D was significantly inhibited by **25** (20mg/kg/day) compared with the control group (Fig. 6A, left), and there was almost no obvious effect on body weight in the compound treated mice (Fig. 6A, right). The tumor volume and body weight of **25** treated mice in orthotopic xenograft model displayed the same results with subcutaneous xenograft model (Fig. 6B), moreover, the compound displayed remarkable antimetastatic ability, the number of metastasized T47D nodules in the lungs of the compound (20 mg/kg/day) treated mice reduced to about 40% compared with the control group (Fig. 6C). HE staining results of liver and kidney showed that **25** presented no obvious influence

to the anatomical morphologies of mice (Fig. 6D).

Fig. 6. (A) T47D cells subcutaneous xenograft mice were intraperitoneally treated with compound **25** (20 mg/kg/day) or DMSO (served as control) for 19 days. Tumor volume and mice body weight were measured every other day. N = 8; ns, no significant; ***P < 0.001. (B) T47D cells orthotopic xenograft mice intraperitoneally treated with **25** (20 mg/kg/day) or DMSO (served as control) for 16 days. Tumor volume and mice body weight were measured every three days. N = 5; ns, no significant; *P < 0.05. (C) Lungs were fixed, photographed, and amount of metastasis nodules were manually counted using a dissecting microscope. *P < 0.05. (D) Liver and kidney hematoxylin and eosin (HE) staining of control and **25** treatment groups.

4. Conclusion

In this study we synthesized a novel class of D-ring fused 1,2,3-thiadiazole DHEA derivatives and screened them for antiproliferative activity using the SRB assay on various cancer cell lines. It is amazing that for these compounds, T47D cell line was much more sensitive than other tumor cell lines. Compound **25** was an unnatural D-proline modified derivative, which possessed the most potent antiproliferative activity on T47D cells and the best selectivity between the tumor cell line and HAF cells. The colony formation and metastatic ability of T47D cells was significantly inhibited by **25** in a dose-dependent manner, which was much more effective than lead compound DHEA. The primary mechanism study showed that **25** did not induce cell-cycle arrest, but caused a dose-dependent induction of apoptosis, and induced phosphorylation of EphA2 and EphB3 in T47D cells. *In vivo* antitumor activity evaluation displayed that the tumor volume was significantly inhibited and there was no obvious change in body weight of **25** treated mice in subcutaneous xenograft model and orthotopic xenograft model. Moreover, the compound also displayed remarkable antimetastatic ability, the number of metastasized T47D nodules in the lungs reduced to about 40% compared with the control group, and the HE staining results showed that the compound presented no obvious influence to the anatomical morphologies of mice.

In conclusion, we report D-ring fused 1,2,3-thiadiazole DHEA derivatives as a series of new chemical entities for the first time. Especially **25**, which exhibited potent antitumor and

antimetastatic activity *in vitro* and *in vivo*, and could be used as a promising lead for the development of a new class of antitumor and antimetastatic agents.

5. Experimental section

5.1. General

All reagents and chemicals were purchased from commercial suppliers and used without further purification unless otherwise stated. When needed, the reactions were carried out in oven-dried glassware under a positive pressure of dry N_2. Column chromatography was performed on silica gel (QinDao, 200-300 mesh) using the indicated eluents. Thin-layer chromatography was carried out on silica gel plates (QinDao) with a layer thickness of 0.25mm. Melting points were determined using the MEL-TEMP 3.0 apparatus and uncorrected. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on Bruker AM-400 spectrometer with $CDCl_3$ or DMSO-d_6 as solvent and tetramethylsilane (TMS) as the internal standard. All chemical shift values were reported in units of δ (ppm). The following abbreviations were used to indicate the peak multiplicity: *s* = singlet; *d* = doublet; *t* = triplet; *m* = multiplet; *br* = broad. High-resolution mass data were obtained on a BrukermicroOTOF-Q II spectrometer.

*5.2. 3β-Acetoxy-5-androstene-17-one (**1**)*

To a solution of DHEA (5 g, 17.2 mmol) in dry CH_2Cl_2 (40 mL), Ac_2O (15.6 g, 103.3 mmol) and DMAP (315 mg, 2.58 mmol) were added at room temperature. The reaction mixture was stirred for 5 h under nitrogen atmosphere at room temperature and concentrated. The residue was poured into H_2O (30 mL) and extracted with AcOEt (20 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4 and concentrated to give compound **1** (5.61 g, 98%) as a white solid without purified. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (d, *J* = 4.7 Hz, 1H), 4.63–4.56 (m, 1H), 2.45 (dd, *J* = 19.2, 8.9 Hz, 1H), 2.33 (dd, *J* = 13.1, 2.7 Hz, 2H), 2.15–2.05 (m, 2H), 2.03 (s, 3H), 1.96 (dd, *J* = 12.1, 5.8 Hz, 1H), 1.86 (s, 3H), 1.65 (d, *J* = 8.3 Hz, 3H), 1.33–1.24 (m, 2H), 1.18–1.12(m, 1H), 1.05 (s, 3H), 0.88 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 220.88, 170.41, 139.87, 121.84, 73.65, 51.64, 50.10, 47.46, 38.04, 36.90, 36.68, 35.79, 31.342, 37.37, 30.73, 27.66, 21.85, 21.38 , 20.28, 19.31, 13.51.

*5.3. 3β-Acetoxy-17-(2-carbamoylhydrazono)-5-androstene (**2**)*

To a solution of compound **1**（400 mg, 1.2 mmol）in absolute EtOH (20 mL), aminourea hydrochloride (675 mg, 6 mmol) and anhydrous CH_3COONa (492 mg, 6 mmol) were added at room temperature. The reaction mixture was stirred for 6 h at room temperature and concentrated. The residue was poured into H_2O (30 mL) and extracted with AcOEt (20 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 20/1, v/v) to give compound **2** (431 mg, 92%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H), 5.43 (d, *J* = 5.1 Hz, 1H),4.73–4.63 (m, 1H), 2.38 (dd, *J* = 13.1, 5.2 Hz, 2H), 2.27–2.14 (m, 1H), 2.02 (s, 3H), 0.86 (s, 3H), 0.84 (s, 3H).

*5.4. 3β-Acetoxy-androsta-5,16-dieno[17,16-d][1,2,3]thiadiazole (**3**)*

To a solution of compound **2** (435 mg, 1.12 mmol) in dry CH_2Cl_2 (10 mL), thionyl chloride (2 mL) was added at 0 ^{o}C. The reaction mixture was stirred for 5 h at room temperature and then poured into H_2O (30mL) and extracted with CH_2Cl_2 (10 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 40/1, v/v) to give compound **3** (335 mg, 82%) as a white solid. IR (ATR) cm^{-1}: 2930, 2840, 1719, 728. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.42 (d, *J* = 5.1 Hz, 1H), 4.68–4.56 (m, 1H), 3.05 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.63 (dd, *J* = 15.5, 11.9 Hz, 1H), 2.48 (dd, *J* = 10.2, 2.4 Hz, 1H), 2.42–2.29 (m, 2H), 2.28–2.19 (m, 1H), 2.15–2.06 (m, 1H), 2.04 (s, 3H), 1.13 (s, 3H), 1.12 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.86, 170.48, 153.88, 140.30, 121.49, 73.65, 62.91, 50.41, 41.54, 38.09, 36.89, 36.82, 34.24, 30.96, 30.62, 27.67, 27.45, 21.41, 20.34, 19.29, 17.71.

*5.5. 3β-Hydroxy-androsta-5,16-dieno[17,16-d][1,2,3]thiadiazole (**4**)*

To a solution of **3** (230 mg, 0.62 mmol) in MeOH (10 mL), K_2CO_3 (428 mg, 3.1 mmol) was added at room temperature. The reaction mixture was stirred for 4 h at room temperature and concentrated. The residue was poured into H_2O (30mL) and acidified to pH 5-6 with hydrochloric acid (1M), then extracted with AcOEt (20 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (CH_2Cl_2/MeOH, 30/1, v/v) to give compound **4** (190mg, 93%) as a white solid; mp: 198–200 ^{o}C. IR (ATR) cm^{-1}: 3420, 2920, 2853, 720. ^{1}H NMR (400 MHz, $CDCl_3$) δ

5.42–5.35 (m, 1H), 3.61–3.49 (m, 1H), 3.07–3.01 (m, 1H), 2.67–2.59 (m, 1H), 2.50–2.47 (m, 1H), 2.36–2.19 (m, 3H), 2.12–2.06 (m, 1H), 1.13 (s, 3H), 1.10 (s, 3H). ^{13}C NMR (100MHz, $CDCl_3$) δ 180.06, 154.06, 141.57, 120.61, 77.48, 77.16, 76.84, 71.66, 63.15, 50.63, 42.31, 41.68, 37.20, 36.93, 34.39, 31.66, 31.10, 30.80, 27.57, 20.51, 19.48, 17.82. HRMS (ESI): calcd for $C_{19}H_{27}N_2OS$ $[M + H]^+$; 331.1839; found 331.1830.

*5.6. 3β-Acetoxy-5α-androstan-17-one (**5**)*

To a solution of **1** (200 mg, 0.6 mmol) in MeOH (15 mL) in an autoclave, Pd/C (100 mg, 5%) and the H_2 were added and maintained at 4.0 Mp. The reaction mixture was stirred at 40 °C for 16 h, then fitered with diatomite, and the filter cake was washed with CH_2Cl_2 (15 mL × 3). The filtrate was concentrated and the residue was purified by silica gel column chromatography (petroleum ether/AcOEt, 5/1, v/v) to give compound **5** (172 mg, 87%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.68 (s, 1H), 2.42 (dd, *J* = 19.2, 8.9 Hz, 1H), 2.11–2.03 (m, 1H), 2.01 (d, *J* = 2.3 Hz, 3H), 0.84 (d, *J* = 2.7 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 221.14, 170.60, 73.45, 54.27, 51.31, 47.73, 44.61, 36.67, 35.81, 35.61, 34.99, 33.91, 31.49, 30.77, 28.24, 27.37, 21.74, 21.42, 20.43, 13.79, 12.18.

*5.7. 3β-Acetoxy-17-(2-carbamoylhydrazono)-5α-androstan (**6**)*

By a similar procedure described for compound **2**, compound **6** was obtained as a white solid; yield: 95%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (s, 1H), 4.73–4.63 (m, 1H), 2.38 (dd, *J* = 13.1, 5.2 Hz, 2H), 2.27–2.14 (m, 1H), 2.02 (s, 3H), 0.86 (s, 3H), 0.84 (s, 3H).

*5.8. 3β-Acetoxy-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**7**)*

By a similar procedure described for compound **3**, compound **7** was obtained as a white solid; yield: 78%; mp: 184–186 °C. IR (ATR) cm^{-1}: 2917, 2853, 1716, 1245, 728. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.75–4.65 (m, 1H), 3.02 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.59 (dd, *J* = 15.5, 11.9 Hz, 1H), 2.43 (dd, *J* = 12.0, 3.1 Hz, 1H), 2.25–2.18 (m, 1H), 2.02 (s, 3H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.09, 170.76, 153.99, 73.54, 62.96, 54.83, 44.86, 41.91, 36.60, 35.92, 34.41, 34.33, 34.04, 31.32, 28.31, 27.50, 27.50, 21.54, 20.81, 18.06, 12.33. HRMS (ESI): calcd for $C_{21}H_{30}N_2NaO_2S$ $[M + Na]^+$; 397.1920; found 397.1922.

*5.9. 3β-Hydroxy-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**8**)*

By a similar procedure described for compound **4**, compound **8** was obtained as a white solid; yield: 95%; mp: 208–210 °C. IR (ATR) cm^{-1}: 3410, 2923, 2847, 1720, 735. ^1H NMR (400 MHz, $CDCl_3$) δ 3.66–3.56 (m, 1H), 3.02 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.59 (dd, *J* = 15.5, 11.9 Hz, 1H), 2.48–2.38 (m, 1H), 2.25–2.17 (m, 1H), 1.10 (s, 3H), 0.90 (s, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ 180.15, 154.06, 71.17, 63.06, 54.96, 45.06, 41.92, 38.15, 36.84, 35.93, 34.44, 34.36, 31.53, 31.41, 28.44, 27.51, 20.86, 18.07, 12.43. HRMS (ESI): calcd for $C_{19}H_{28}N_2NaOS$ $[M + Na]^+$; 355.1815; found 355.1818.

*5.10. General procedure for the preparation of compounds **9–13***

To a solution of compound **8**（150 mg, 0.45 mmol）in dry CH_2Cl_2 (20 mL), acyl chloride (9.0 mmol) and DMAP (55 mg, 0.45 mmol) were added at room temperature. The reaction mixture was stirred for 4 h at room temperature under nitrogen atmosphere, then poured into H_2O (30 mL) and extracted with CH_2Cl_2 (20 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (petroleum ether/AcOEt, 3/1, v/v) to give the desired product.

*5.10.1. 3β-Propionyloxy-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**9**)*

White solid; yield: 88%; mp: 178–180 °C. IR (ATR) cm^{-1}: 2920, 2847, 1727, 1196. ^1H NMR (400 MHz, $CDCl_3$) δ 4.75–4.67 (m, 1H), 3.02 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.59 (dd, *J* = 15.5, 12.0 Hz, 1H), 2.43 (dd, *J* = 11.9, 3.2 Hz, 1H), 2.29 (d, *J* = 7.6 Hz, 2H), 2.24–2.17 (m, 1H), 1.13 (t, *J* = 7.6 Hz, 3H), 1.09 (s, 3H), 0.92 (s, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ 180.11, 174.18, 154.01, 73.31, 62.97, 54.84, 44.87, 41.91, 36.62, 35.94, 34.42, 34.34, 34.08, 31.33, 28.33, 28.06, 27.53, 27.50, 20.82, 18.07, 12.35, 9.31. HRMS (ESI): calcd for $C_{22}H_{32}N_2NaO_2S$ $[M + Na]^+$; 411.2077; found 411.2109.

*5.10.2. 3β-Methylsulfonyloxy-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**10**)*

White solid; yield: 90%; mp: 166–167 °C. IR (ATR) cm^{-1}: 2919, 2855, 1728, 728. ^1H NMR (400 MHz, $CDCl_3$) δ 4.69–4.58 (m, 1H), 3.04 (d, *J* = 6.3 Hz, 1H), 3.00 (s, 3H), 2.60 (dd, *J* = 15.5, 11.9 Hz, 1H), 2.44 (dd, *J* = 11.4, 3.2 Hz, 1H), 2.21 (d, *J* = 29.5 Hz, 1H), 2.02 (d, *J* = 14.0 Hz, 1H), 1.09 (s, 3H), 0.92 (s, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ 179.98, 153.95, 81.78, 62.85, 54.65, 44.98, 41.87, 38.94, 36.62, 35.68, 35.19, 34.34, 34.24, 31.21, 28.69, 28.18, 27.47, 20.81, 18.05, 12.28. HRMS (ESI): calcd for $C_{20}H_{30}N_2NaO_3S_2$ $[M + Na]^+$; 433.1590; found 433.1612.

*5.10.3. 3β-Nicotinoyloxy-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**11**)*

White solid; yield: 83%; mp: 223–225 °C. IR (ATR) cm^{-1}: 2923, 2850, 1724, 1280, 730. ^1H NMR (400 MHz, $CDCl_3$) δ 9.24 (s, 1H), 8.79 (d, *J* = 4.3 Hz, 1H), 8.41 (d, *J* = 7.7 Hz, 1H), 7.54–7.45 (m, 1H), 5.06–4.95 (m, 1H), 3.03 (dd, *J* = 15.5, 6.4 Hz, 1H), 2.61 (dd, *J* = 15.5, 11.9 Hz, 1H), 2.46 (dd, *J* = 11.7, 3.1 Hz, 1H), 2.28–2.20 (m, 1H), 2.00 (d, *J* = 12.5 Hz, 1H), 1.11 (s, 3H), 0.98 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.07, 164.88, 153.98, 153.35, 150.99, 137.11, 126.73, 123.32, 74.81, 62.94, 54.82, 44.90, 41.91, 36.60, 35.97, 34.41, 34.33, 34.07, 31.32, 28.32, 27.56, 27.50, 20.84, 18.07, 12.41. HRMS (ESI): calcd for $C_{25}H_{32}N_3O_2S$ $[M + H]^+$; 438.2210; found 438.2232.

*5.10.4. 3β-Isonicotinoyloxy-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**12**)*

White solid; yield: 89%; mp: 235–237 °C. IR (ATR) cm^{-1}: 2914, 2851, 1721, 1280, 727. ^1H NMR (400 MHz, $CDCl_3$) δ 8.78 (d, *J* = 5.2 Hz, 2H), 7.90 (d, *J* = 5.5 Hz, 2H), 5.04–4.94 (m, 1H), 3.03 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.61 (dd, *J* = 15.5, 11.9 Hz, 1H), 2.45 (dd, *J* = 11.7, 3.2 Hz, 1H), 2.27–2.20 (m, 1H), 2.04–1.98 (m, 1H), 1.11 (s, 3H), 0.97 (s, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ 180.07, 164.71, 153.98, 150.62, 150.62,138.13, 122.97, 122.97, 75.25, 62.95, 54.82, 44.90, 41.92, 36.59, 35.98, 34.41, 34.35, 34.00, 31.32, 28.33, 27.51, 27.50, 20.86, 18.08, 12.41. HRMS (ESI): calcd for $C_{25}H_{32}N_3O_2S$ $[M + H]^+$; 438.2210; found 438.2250.

*5.10.5. 3β-[(Pyrazine-2-carbonyl)oxy]-5α-androst-16-eno [17,16-d][1,2,3]thiadiazole (**13**)*

White solid; yield: 89%; mp: 222–224 °C. IR (ATR) cm^{-1}: 2916, 2853, 1712, 733. ^1H NMR (400 MHz, $CDCl_3$) δ 9.30 (s, 1H), 8.74 (d, *J* = 5.6 Hz, 2H), 5.15–5.04 (m, 1H), 3.03 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.60 (dd, *J* = 15.5, 12.0 Hz, 1H), 2.45 (dd, *J* = 11.8, 3.2 Hz, 1H), 2.28–2.20 (m, 1H), 2.06–2.01 (m, 1H), 1.10 (s, 3H), 0.97 (s, 3H).^{13}C NMR (100 MHz, $CDCl_3$) δ 180.04, 163.57, 153.95, 147.58, 146.38, 144.50, 143.97, 75.83, 62.92, 54.78, 44.94, 41.89, 36.61, 35.97, 34.39, 34.31, 33.93, 31.29, 28.28, 27.49, 27.43, 20.82, 18.05,12.40. HRMS (ESI): calcd for $C_{24}H_{30}N_4NaO_2S$ $[M + Na]^+$; 461.1982; found 461.1986.

*5.11. General procedure for the preparation of compounds **14–19***

To a solution of compound **8** (250 mg, 0.75 mmol) in dry CH_2Cl_2 (20 mL), N-BOC-amino-carboxylic acid (3.0 mmol), HOBT (405 mg, 3.0 mmol), EDCI (575 mg, 3.0 mmol) and DMAP（366 mg, 3.0 mmol）were added at room temperature. The reaction mixture was stirred

for 8 h at room temperature under nitrogen atmosphere, then poured into water (30 mL) and extracted with CH_2Cl_2 (20 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel column chromatography (petroleum ether/AcOEt, 3/1, v/v) to give the desired product.

*5.11.1. 3β-{2-[(tert-butoxycarbonyl)amino]acetoxy}-5α-androst-16-eno[17,16-d][1,2,3] thiadiazole (**14**)*

White solid; yield: 93%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.01 (s, 1H), 4.83–4.72 (m, 1H), 3.89 (d, *J* = 4.7 Hz, 2H), 3.04 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.62 (dd, *J* = 15.4, 12.0 Hz, 1H), 2.46 (dd, *J* = 11.6, 3.4 Hz, 1H), 2.27–2.16 (m, 1H), 1.48 (s, 9H), 1.12 (s, 3H), 0.94 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.97, 169.87, 155.71, 153.91, 79.91, 74.64, 62.82, 54.66, 44.70, 42.67, 41.79, 36.41, 35.78, 34.27, 34.19, 33.82, 31.18, 28.33, 28.16, 27.40, 27.31, 20.70, 17.97, 12.22.

*5.11.2. 3β-{[4-((tert-butoxycarbonyl)amino)butanoyl]oxy}-5α-androst-16-eno[17,16-d][1,2,3] thiadiazole (**15**)*

White solid; yield: 96%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.76–4.67 (m, 1H), 4.65 (d, *J* = 11.9 Hz, 1H), 3.15 (d, *J* = 6.3 Hz, 2H), 3.01 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.59 (dd, *J* = 15.5, 12.0 Hz, 1H), 2.43 (dd, *J* = 11.8, 3.3 Hz, 1H), 2.31 (t, *J* = 7.3 Hz, 2H), 2.26–2.16 (m, 1H), 1.43 (s, 9H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.98, 172.82, 155.94, 153.92, 79.17, 73.51, 62.83, 54.67, 44.73, 41.78, 39.96, 36.46, 35.80, 34.27, 34.19, 33.92, 32.00, 31.20, 28.41, 28.19, 27.39, 25.33, 20.69, 17.97, 14.14, 12.23.

*6.11.3. 3β-{[1-(tert-butoxycarbonyl)piperidine-3-carbonyl]oxy}-5α-androst-16-eno [17,16-d][1,2,3]thiadiazole (**16**)*

White solid; yield: 98%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.78–4.65 (m, 1H), 3.99 (d, *J* = 13.4 Hz, 2H), 3.01 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.92–2.77 (m, 2H), 2.59 (dd, *J* = 15.4, 12.0 Hz, 1H), 2.49–2.35 (m, 2H), 2.26–2.16 (m, 1H), 1.45 (s, 9H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.00, 173.01, 154.71, 153.91, 79.66, 73.46, 62.84, 54.67, 44.71, 41.79, 41.59,41.57, 36.44, 35.81, 34.28, 34.20, 33.90, 33.87, 31.21, 28.43, 28.19, 27.37, 27.35, 27.33, 24.33, 20.70, 17.97, 12.24.

*5.11.4. 3β-{[1-(tert-butoxycarbonyl)piperidine-4-carbonyl]oxy}-5α-androst-16-eno [17,16-d][1,2,3]thiadiazole (**17**)*

White solid; yield: 96%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.78–4.64 (m, 1H), 4.12 (d, *J* = 7.1 Hz, 1H), 3.94–3.83 (m, 1H), 3.01 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.86–2.76 (m, 1H), 2.59 (dd, *J* = 15.4, 12.0 Hz, 1H), 2.46–2.34 (m, 2H), 2.27–2.15 (m, 1H), 2.02 (d, *J* = 15.8 Hz, 1H), 1.46 (s, 9H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 179.98, 174.15, 154.72, 153.91, 79.55, 73.42, 62.83, 54.67, 44.71, 41.79, 41.29, 36.45, 35.81, 34.27, 34.20, 33.91, 31.20, 28.44, 28.19, 28.01, 27.98, 27.40, 27.37, 20.71, 17.97, 12.25.

5.11.5. 3β-[N-tert-butoxycarbonyl-(L-prolyl)oxy]-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole ***(18)***

White solid; yield: 98%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.80–4.68 (m, 1H), 4.31–4.16 (m, 1H), 3.59–3.34 (m, 2H), 3.02 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.59 (dd, *J* = 15.1, 12.2 Hz, 1H), 2.44 (d, *J* = 9.1 Hz, 1H), 2.28–2.11 (m, 2H), 1.46 (s, 3H), 1.42(s, 6H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 179.81, 172.60, 155.17, 153.34, 79.13, 73.75, 62.50, 59.11, 54.25, 46.61, 44.44, 41.79, 36.25, 35.80, 34.49, 34.07, 31.07, 30.82, 29.88, 28.52, 28.38, 27.66, 24.28, 23.50, 20.77, 18.32, 12.30.

5.11.6. 3β-[N-tert-butoxycarbonyl-(D-prolyl)oxy]-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole ***(19)***

White solid; yield: 86%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.80–4.67 (m, 1H), 4.33–4.14 (m, 1H), 3.61–3.35 (m, 2H), 3.02 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.65–2.53 (m, 1H), 2.43 (d, *J* = 11.4 Hz, 1H), 2.30–2.11 (m, 2H), 1.46 (s, 3H), 1.42 (s, 6H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.96, 172.71, 154.36, 153.88, 79.77, 73.85, 62.82, 59.30, 54.68, 46.33, 44.72, 41.79, 36.43, 35.81, 34.28, 34.21, 34.01, 31.19, 31.00, 28.45, 28.39, 28.21, 27.39, 23.53, 20.71, 17.96, 12.25.

5.12. General procedure for the preparation of compounds ***20–25***

One of compounds **14**–**19** (0.4 mmol) was dissolved in anhydrous diethyl ether (20 mL) and boron trifluoride etherate (5 mL) was added. The reaction mixture was stirred for 2 h at room temperature and then quenched with saturated aqueous $NaHCO_3$ (20 mL). The mixture was extracted with AcOEt (20 mL × 3). The combined organic extract was washed with brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography (CH_2Cl_2/MeOH, 10/1, v/v) to give the desired product.

*5.12.1. 3β-(2-Aminoacetoxy)-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**20**)*

White solid; yield: 77%; mp: 163–165 °C. IR (ATR) cm^{-1}: 3378, 2918, 2853, 1718, 1280, 728. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.82–4.71 (m, 1H), 3.45 (s, 2H), 3.01 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.59 (dd, *J* = 15.5, 11.9 Hz, 1H), 2.43 (dd, *J* = 11.7, 3.2 Hz, 1H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.04, 173.69, 153.97, 74.19, 62.92, 54.79, 44.82, 44.19, 41.87, 36.54, 35.88, 34.37, 34.29, 34.01, 31.28, 28.27, 27.47, 27.47, 20.79, 18.04, 12.30. HRMS (ESI): calcd for $C_{21}H_{32}N_3O_2S$ $[M + H]^+$; 390.2210; found 390.2211.

*5.12.2. 3β-[(4-Aminobutanoyl)oxy]-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**21**)*

White solid; yield: 72%; mp: 170–172 °C. IR (ATR) cm^{-1}: 3342, 2918, 2855, 1719, 1685, 728. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.77–4.65 (m, 1H), 3.02 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.73 (t, *J* = 7.0 Hz, 1H), 2.59 (dd, *J* = 15.5, 11.9 Hz, 1H), 2.43 (dd, *J* = 12.0, 3.0 Hz, 1H), 2.39–2.26 (m, 2H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.06, 173.01, 154.01, 73.60, 71.07, 62.90, 54.75, 44.81, 41.86, 41.10, 38.13, 36.54, 35.89, 34.35, 34.26, 34.01, 32.09, 31.28, 28.27, 27.48, 20.78, 18.06, 12.32. HRMS (ESI): calcd for $C_{23}H_{36}N_3O_2S$ $[M + H]^+$; 418.2523; found 418.2528.

*5.12.3. 3β-[(Piperidine-3-carbonyl)oxy]-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**22**)*

White solid; yield: 75%; mp: 179–181 °C. IR (ATR) cm^{-1}: 3527, 2923, 2853, 1722, 728. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 (s, 1H), 4.75 (s, 1H), 3.63–3.10 (m, 4H), 3.01 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.90 (s, 1H), 2.59 (dd, *J* = 15.4, 12.0 Hz, 1H), 2.43 (dd, *J* = 11.9, 2.9 Hz, 1H), 1.09 (s, 3H), 0.92 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.02, 171.99, 154.06, 74.95, 62.96, 54.81, 45.42, 44.99, 44.82, 41.90, 38.08, 36.50, 35.90, 34.41, 34.29, 33.78, 31.31, 28.27, 27.51, 27.30, 25.30, 20.95, 20.84, 18.07, 12.34. HRMS (ESI): calcd for $C_{25}H_{38}N_3O_2S$ $[M + H]^+$; 444.2679; found 444.2679.

*5.12.4. 3β-[(Piperidine-4-carbonyl)oxy]-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**23**)*

White solid; yield: 82%; mp: 175–177 °C. IR (ATR) cm^{-1}: 3340, 2917, 2852, 1717, 728. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.79–4.61 (m, 1H), 3.14–3.05 (m, 2H), 3.02 (dd, *J* = 15.5, 6.4 Hz, 1H), 2.68–2.60 (m, 2H) , 2.48–2.33 (m, 2H), 2.25–2.18 (m, 1H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (100MHz, $CDCl_3$) δ 180.07, 174.73, 153.98, 73.26, 62.93, 54.79, 45.87, 44.83, 41.88, 41.81, 36.56, 35.91, 34.38, 34.30, 34.02, 31.30, 29.32, 29.29, 28.29, 27.47, 20.79, 18.04, 12.33. HRMS

(ESI): calcd for $C_{25}H_{38}N_3O_2S$ $[M + H]^+$; 444.2679; found 444.2686.

*5.12.5. 3β-[(L-prolyl)oxy]-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**24**)*

White solid; yield: 74%; mp: 82–84 °C. IR (ATR) cm^{-1}: 3391, 2917, 2853, 1719, 728. ^1H NMR (400 MHz, $CDCl_3$) δ 4.83–4.66 (m, 1H), 3.79–3.65 (m, 1H), 3.13–3.04 (m, 1H), 3.01 (dd, *J* = 15.5, 6.3 Hz, 1H), 2.95–2.84 (m, 1H), 2.58 (dd, *J* = 15.3, 12.1 Hz, 1H), 2.45–2.40 (m, 1H), 2.25–2.09 (m, 2H), 1.08 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.10, 175.05, 153.99, 74.12, 62.96, 59.99, 54.82, 47.15, 44.85, 41.92, 36.58, 35.94, 34.41, 34.34, 33.99, 31.33, 30.54, 28.31, 27.51, 27.47, 25.58, 20.83, 18.07, 12.36. HRMS (ESI): calcd for $C_{24}H_{36}N_3O_2S$ $[M + H]^+$; 430.2523; found 430.2546.

*5.12.6. 3β-[(D-prolyl)oxy]-5α-androst-16-eno[17,16-d][1,2,3]thiadiazole (**25**)*

White solid; yield: 81%; mp: 148–150 °C. IR (ATR) cm^{-1}: 3389, 2931, 2853, 1719, 728. ^1H NMR (400 MHz, $CDCl_3$) δ 4.80–4.68 (m, 1H), 3.71–3.68 (m, 1H), 3.10–3.06 (m, 1H), 3.04–2.99 (m, 1H), 2.91–2.85 (m, 1H), 2.59 (dd, *J* = 15.5, 11.9 Hz, 1H), 2.43 (dd, *J* = 11.8, 3.2 Hz, 1H), 2.25–2.18 (m, 1H), 2.16–2.07 (m, 1H), 1.09 (s, 3H), 0.91 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.07, 175.17, 153.97, 74.00, 62.94, 60.08, 54.80, 47.21, 44.85, 41.89, 36.55, 35.91, 34.39, 34.32, 33.98, 31.30, 30.53, 28.29, 27.49, 27.45, 25.61, 20.82, 18.06, 12.35. HRMS (ESI): calcd for $C_{24}H_{36}N_3O_2S$ $[M + H]^+$; 430.2523; found 430.2543.

5.13. Biological assay

5.13.1. Cell lines and culture conditions

Tumor cell lines used in this study were obtained from the American Type Culture Collection (ATCC). LNCaP, DU145, HCT116, HT29, T47D, MCF7, HL60 and Jurkat cell lines were cultured in RPMI 1640 medium, MDA-MB-231 cell line was cultured in MEM medium, and HAF cell line was cultured in DMEM medium with an additional final concentration of 2 mM L-glutamine. Medium was supplemented with 10% FBS, and all tumor cells were incubated at 37 °C and 5% CO_2 incubator.

5.13.2. Cell Viability assay

Adherent cell viability was determined by SRB assay which was described previously [43]. In brief, cells (including LNCaP, DU-145, HCT-116, HT-29, T47D, MDA-MB-231, MCF7 and HAF)

were seeded into 96-well plates. After 24 h, the cells were treated with various doses of the compounds for 96 h. Then fixed and washed after dyeing with SRB. After dried, 10 mM Tris-based solution was added and absorbance was measured at 515 nm. Suspension cell viability was determined by MTS assay which was described previously [44]. In brief, cells (including HL60 and Jurkat) were seeded into 96-well plates. After 24 h, the cells were treated with various doses of the compounds. MTS was added and incubated after 96 h treatment and absorbance was measured at 490 nm. The IC_{50} was calculated using GraphPad software.

5.13.3. In vitro transwell migration assay

Transwell migration assay was performed as previously reported [45]. The inhibition of tumor cell migration was assessed by the Boyden chamber (Corning Falcon) migration assay with 8.0 μm pore. Briefly, T47D cells were collected, centrifuged, and resuspended with serum-free medium. The top chambers were seeded with 5×10^4 cells in serum-free 1640 medium containing different dose of compounds. The bottom chambers were filled with complete medium supplemented with different dose of compounds. After 12 h incubation, non-migrated cells were removed, and migrated cells were fixed and stained. Images were taken and cells from three random areas per filter were counted.

5.13.4. Cell cycle analysis

Cell cycle analysis was conducted by PI staining as described previously [43]. T47D cells were plated in 6 cm dishes and were treated with different dose of compound for 24 h. After ethanol fixation, cells were washed in PBS once and suspended in PBS with RNAase and propidium iodide (PI) in dark for 30 min. Then cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences).

5.13.5. Cell apotosis analysis

Apoptotic cells were monitored with Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) as described previously [44]. T47D cells were plated in 6 cm dishes and were treated with different dose of compound for 24 h. Cells were washed with cold PBS, harvested and re-suspended in 1×binding buffer, and incubated with Annexin V fluorescein isothiocyanate and propidium iodide for 15 min in dark at room temperature. Then 1 × binding buffer was added and analyzed immediately with flow cytometry (FACS Calibur, BD Biosciences).

5.13.6. Colony formation assay

T47D cells were trypsinized and seeded 2000 per well in 6-well dishes. Cells were allowed to attach overnight and then exposed to different dose of compound for a week. After fixed for 20 min, cells were stained. The number of cell colonies was calculated and analyzed as the ratio of the number of treated samples to untreated samples. Triplicate wells were set up for each concentration.

5.13.7. Subcutaneous and orthotopic xenograft animal model of T47D

These assays were performed as described previously [46,47]. In subcutaneous xenograft animal model, 2×10^6 T47D cells were implanted subcutaneously on 8-week-old female nude mice. On day 3, mice were divided into two groups (n=8) randomly. Compound (20 mg/kg) or DMSO was injected intraperitoneally every day. The tumor length, width and body weight were evaluated every other day and xenograft tumor growth rate was measured as the following equation, volume = length × width^2 × 0.52. Mice were continually observed until they were sacrificed at the twenty-first day. The liver and kidney were fixed and prepared for H&E staining. In orthotopic xenograft animal model, 1×10^5 T47D cells were injected subcutaneously into the 4th abdominal mammary fat pad on 8-week-old female nude mice. On day 3, mice were divided into two groups (n = 5) randomly. Compound (20 mg/kg/day) or DMSO was injected intraperitoneally every day. The tumor length, width and body weight were evaluated every three days and xenograft tumor growth rate was measured as the following equation, volume = length × width^2 × 0.52. Mice were continually observed until they were sacrificed at the eighteenth day. Lung metastases were manually counted using a dissecting microscope.

5.13.8. Phospho-receptor tyrosine kinase array analysis

A Human Phospho-RTK Array Kit (R&D Systems) was used to measure the relative level of tyrosine phosphorylation of 49 different receptor tyrosine kinases (RTKs). T47D cells were plated in 10 cm dishes and treated with 2.5 μM compound or DMSO for 24 h. Cells were lysed with Lysis Buffer 17 and diluted to 1000ug/mL with Array Buffer 1. The arrays were blocked with Array Buffer 1 for 1 h at room temperature on a rocking platform shaker and then incubated with 1 mL lysate overnight at 4°C on a rocking platform shaker. The arrays were washed, incubated with Anti-Phospho-Tyrosine-HRP Detection Antibody, treated with Chemi Reagent Mix, and

exposed to film.

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Appendix A. Supplementary data

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Table 1

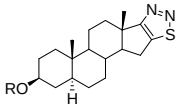
IC_{50} values of DHEA and its derivatives **4** and **8** against the growth of T47D cells and other various tumor cell lines.

Compound	IC_{50} (μM)[a]								
	T47D	MDA-MB-231	MCF-7	DU145	LNCaP	HCT116	HT29	HL-60	Jurkat
DHEA	2.55	> 50	> 50	> 50	> 50	> 50	> 50	> 50	46.5
EPI[b]	1.23	> 50	> 50	> 50	> 50	> 50	> 50	> 50	28.8
4 (**CH12**)	1.36	>50	24.1	> 50	> 50	> 50	> 50	> 50	> 50
8 (**CH21**)	0.67	20.1	> 50	35.6	48.1	> 50	> 50	> 50	22.7

From SRB assay after 96 h of treatment; [a]IC_{50} data are an average of at least 3 independent experiments, variation ± 10%; [b] Epiandrosterone (EPI) is a reduzate of DHEA.

Table 2

IC_{50} values of DHEA and its derivatives (**7**, **8** and **26**-**43**) against the growth of T-47D tumor cell line and normal human fibroblast (HAF) cells.



Compound	R	IC_{50} (μM)[a]		SI[b]	Compound	R	IC_{50} (μM)[a]		SI[b]
		T47D	HAF				T47D	HAF	
8 (**CH21**)	H	0.67 ± 0.11[c]	39.05 ± 4.15	32	**21**		1.15 ± 0.10	18.0 ± 0.22	16
7		2.17 ± 0.09	> 50	ND[d]	**22**		0.042 ± 0.028	2.95 ± 0.28	70
9		2.66 ± 0.85	> 50	ND	**23**		0.051 ± 0.010	1.82 ± 0.03	36
10		2.83 ± 0.49	> 50	ND	**24**		0.24 ± 0.06	36.0 ± 2.05	150
11		2.25 ± 0.30	> 50	ND	**25** (**CH33**)		0.058 ± 0.016	21.1 ± 5.06	364
12		3.28 ± 0.51	> 50	ND	**DHEA**		2.55 ± 0.01	> 50	ND
13		3.04 ± 0.99	> 50	ND	**ADM**[e]		0.040 ± 0.018	0.068 ± 0.064	1.7
20		0.92 ± 0.05	26.1 ± 0.77	28					

From SRB assay after 96 h of treatment; [a]IC_{50} data are an average of at least 3 independent experiments; [b]the selectivity indexes (SI) were calculated by IC_{50} values in HAF cells divided by IC_{50} values in T47D cancer cell line; [c]data from Table 1; [d]ND = not determined; [e]ADM is Adriamycin.

Table 3

IC_{50} values of compounds **22**, **24** and **25** against the growth of T47D cells and other various tumor cell lines.

Compound	IC_{50} (μM)[a]									
	T47D[b]	MDA-MB-231	MCF-7	DU145	LNCaP	HCT116	HT29	HL-60	Jurkat	Selectivity[c]
22	0.042	1.24	1.41	0.92	4.28	2.01	2.12	5.33	2.57	30-126
24	0.24	7.69	15.0	24.3	36.2	8.52	13.6	31.5	1.41	6-150
25 (**CH33**)	0.058	9.66	27.1	17.7	31.6	11.7	16.5	46.0	2.49	43-793

From SRB assay after 96 h of treatment; [a]IC_{50} data are an average of at least 3 independent experiments, variation ± 10%; [b]data from Table 2; [c]Selectivity is the IC_{50} values of T47D cells divide IC_{50} values of other tumor cell lines.

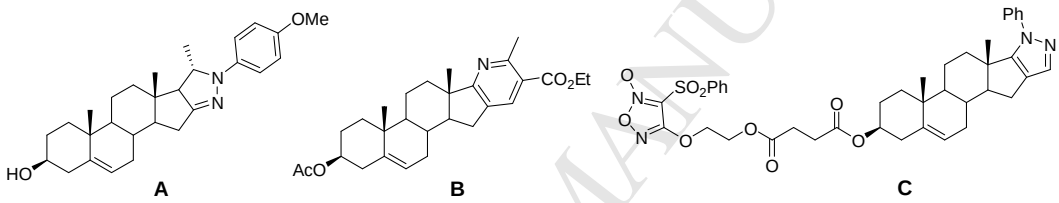


Fig. 1. Chemical structures of D-ring fused heterocycles DHEA antitumor agents

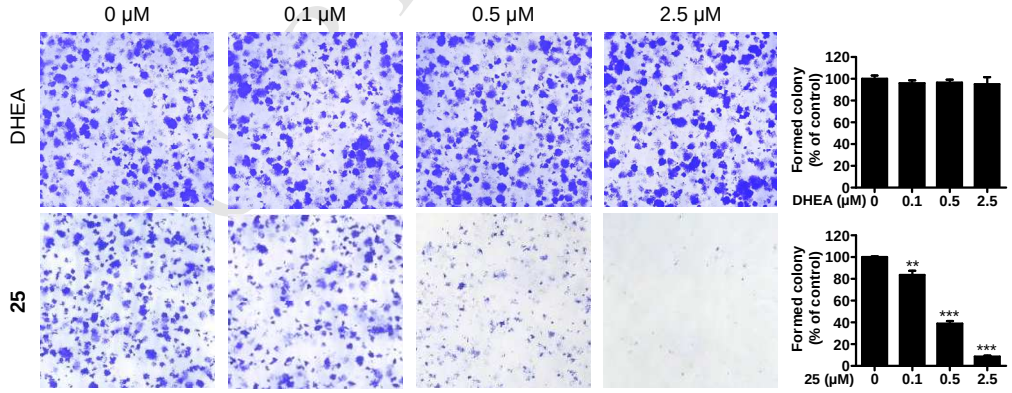


Fig. 2. Compound **25** and DHEA inhibited the colony formation of T47D cell line. After treatment by various doses of **25** and DHEA in 6-well plates for 7 days, cells were fixed and stained with crystal violet and the numbers of cell colonies were counted. **P < 0.01, ***P < 0.001, versus the control group (0 μM).

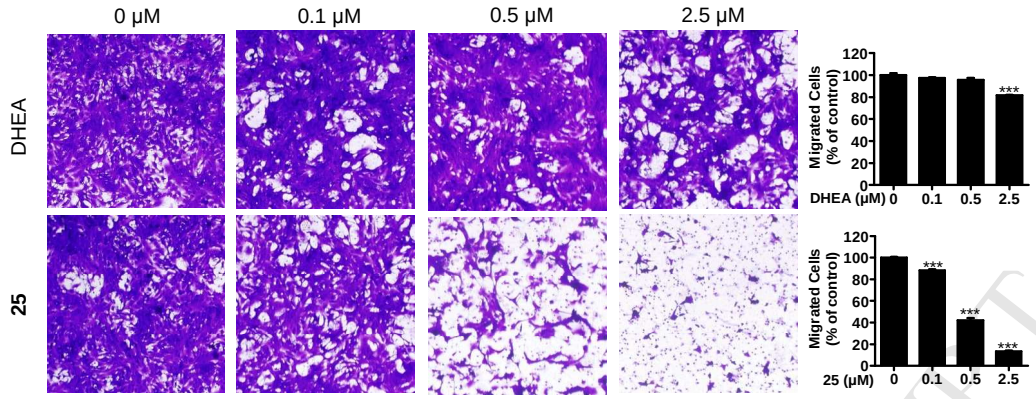


Fig. 3. Compound **25** and DHEA inhibited the cell migration of T47D cell line. After 12 h incubation with various doses of **25** and DHEA, non-migrated cells were removed and migrated cells were fixed with cold 4% paraformaldehyde and stained with 0.2% crystal violet. Images were taken and cells from three random areas per filter were counted. **P < 0.01, ***P < 0.001, versus the control group (0 μM).

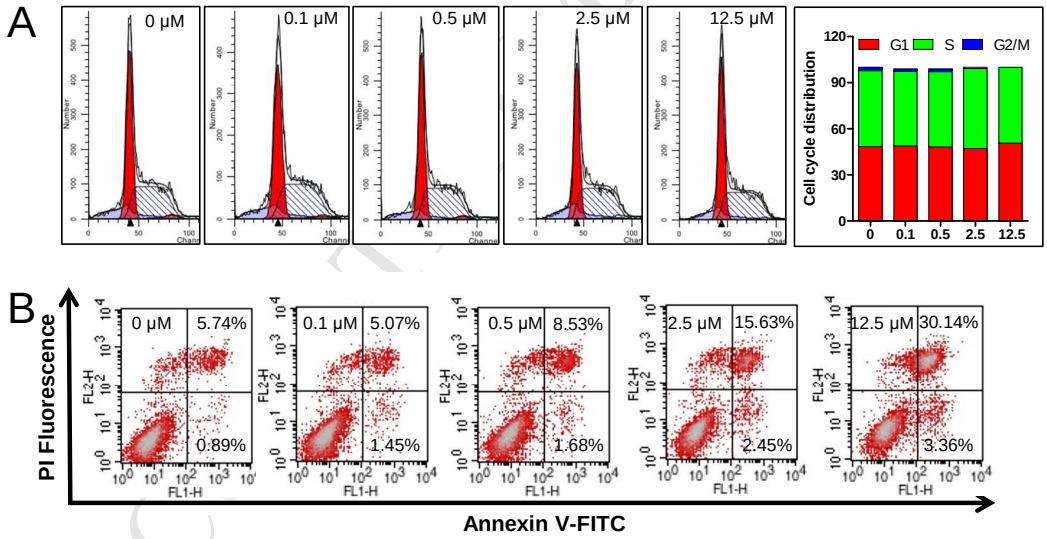


Fig. 4. (A) Compound **25** didn't induce cell cycle arrest in T47D cell line. Cells were stained with PI and analyzed using flow cytometry after treatment with **25** for 24 h. (B) Compound **25** mediated T47D cells apoptosis in dose-dependent manner. Cells were treated with different dose of **25** for 24 h and then the percentages of apoptosis cells were analyzed using flow cytometry by staining with PI and Annexin V-FITC.

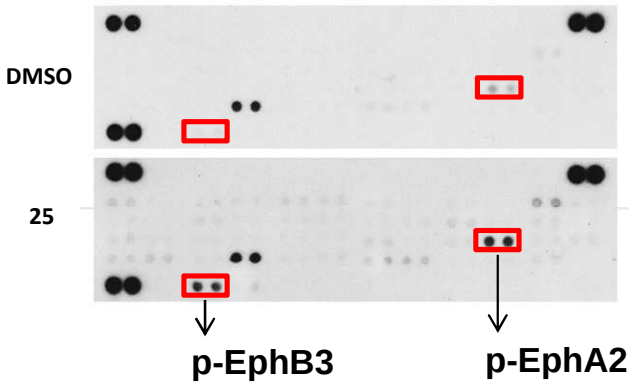


Fig. 5. Compound **25** increased the phosphorylation of EphA2 and EphB3 in T47D cells. After treated with 2.5 μM **25** for 24 h, T47D cells were lysed and analyzed for the activation of RTKs comprehensively using a phospho-RTK array.

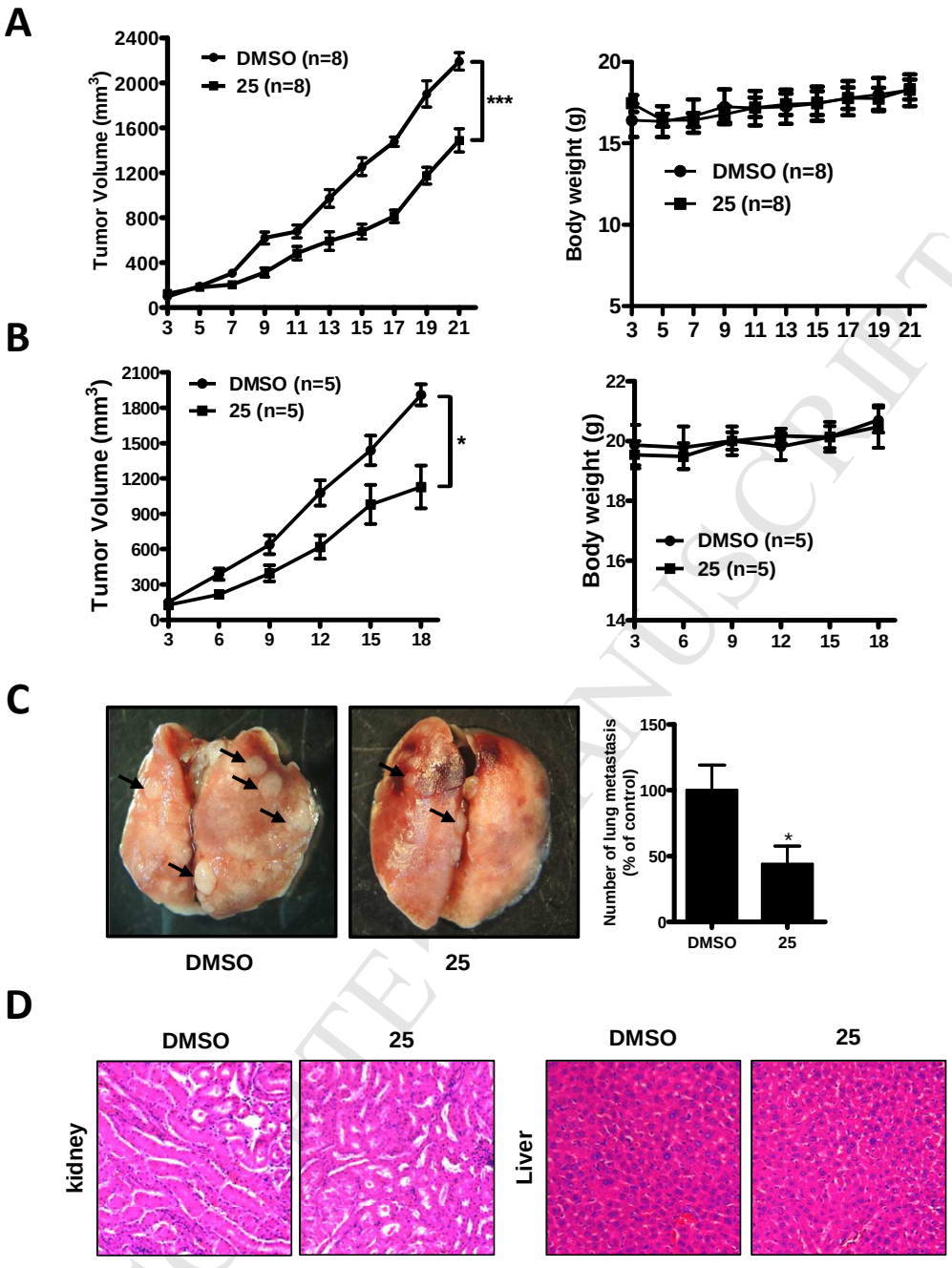
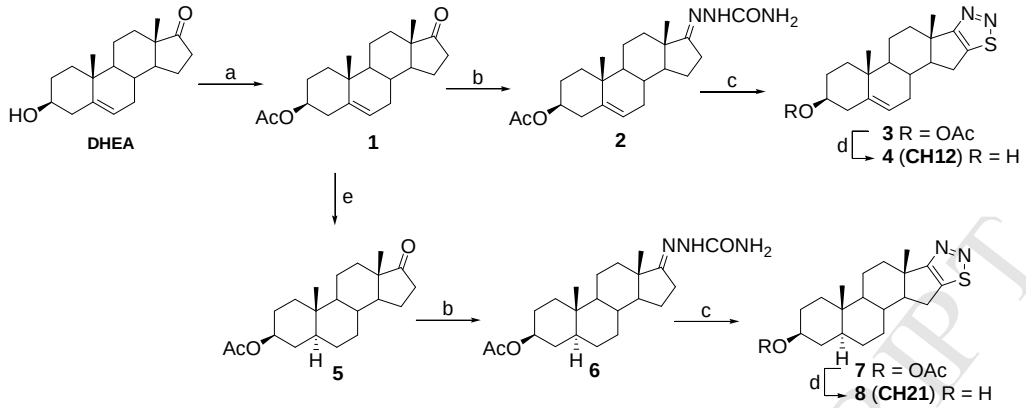
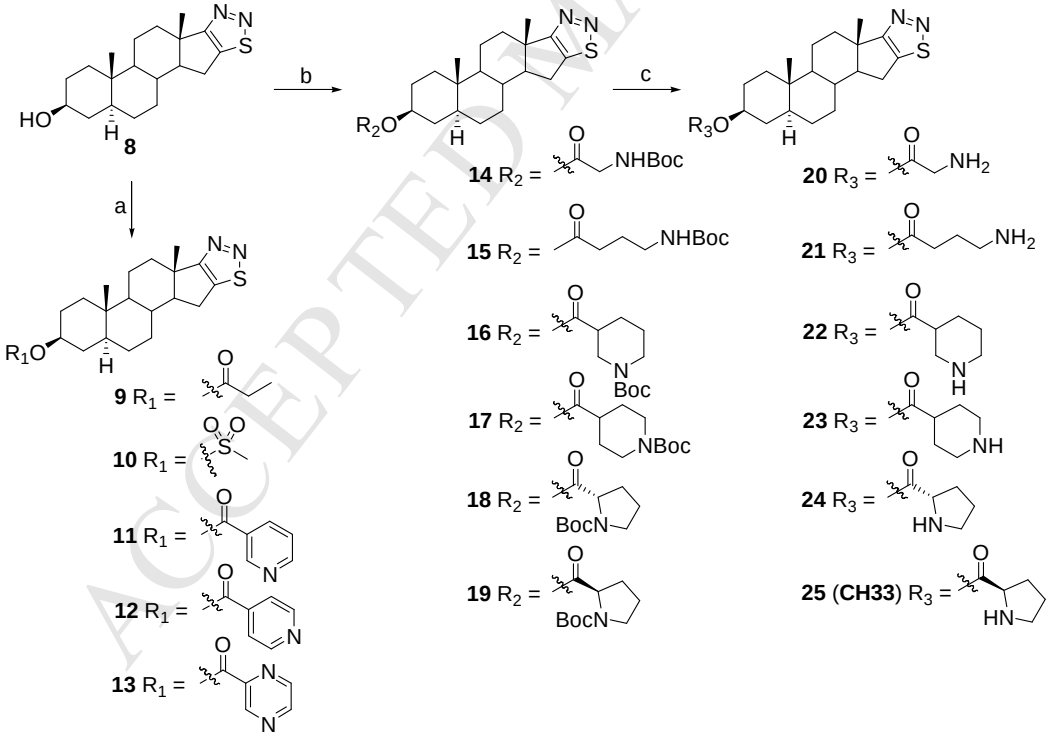


Fig. 6. (A) T47D cells subcutaneous xenograft mice were intraperitoneally treated with compound **25** (20 mg/kg/day) or DMSO (served as control) for 19 days. Tumor volume and mice body weight were measured every other day. N = 8; ns, no significant; ***P < 0.001. (B) T47D cells orthotopic xenograft mice intraperitoneally treated with **25** (20 mg/kg/day) or DMSO (served as control) for 16 days. Tumor volume and mice body weight were measured every three days. N = 5; ns, no significant; *P < 0.05. (C) Lungs were fixed, photographed, and amount of metastasis nodules were manually counted using a dissecting microscope. *P < 0.05. (D) Liver and kidney hematoxylin and eosin (HE) staining of control and **25** treatment groups.



Scheme 1. Synthesis of derivatives **4** and **8**. Reagents and conditions: (a) Ac_2O, DMAP, CH_2Cl_2, rt, 5 h, 98%; (b) aminourea hydrochloride, CH_3COONa, EtOH, rt, 6 h, 92% for **2** and 95% for **6**; (c) $SOCl_2$, CH_2Cl_2, rt, 5 h, 82% for **3** and 78% for **7**; (d) K_2CO_3, MeOH, rt, 4h, 93% for **4** and 95% for **8**; (e) H_2 (4.0 Mp), 5% Pd/C, MeOH, 40 ^oC, 16 h, 87%.



Scheme 2. Synthesis of derivatives **9**-**13** and **20**-**25**. Reagents and conditions: (a) acyl chlorides, DMAP, CH_2Cl_2, rt, 4 h, 83−90% for **9**−**13**; (b) N-Boc-amino-carboxylic acids, EDCI, HOBt, DMAP, CH_2Cl_2, rt, 8 h, 86−98% for **14**−**19**; (c) boron trifluoride etherate, Et_2O, rt, 2 h, 72−82% for **20**−**25**.

A series of novel dehydroepiandrosterone derivatives were synthesized.

The antitumor activity was investigated against T47D tumor cell line.

Compound **25** showed the most potent antitumor activity.

The apoptosis, colony formation and migration assays were performed.

In vivo antitumor effect was observed in T47D tumor-bearing mice.