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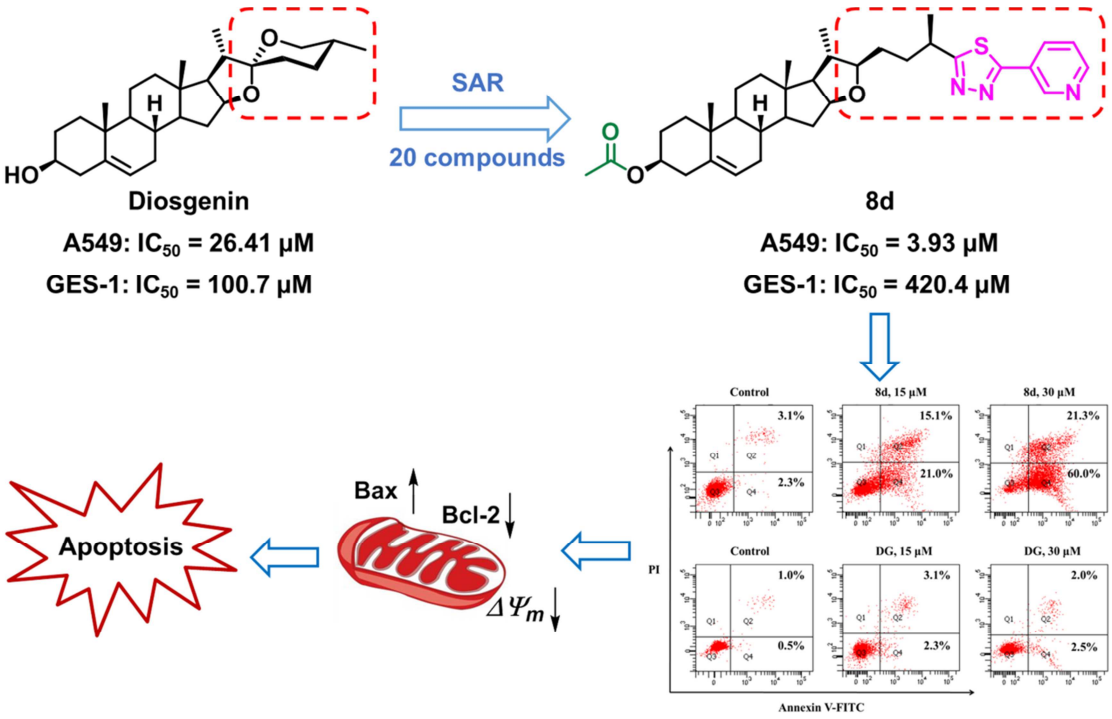
Graphical Abstract



Novel diosgenin derivatives containing 1,3,4-oxadiazole/thiadiazole moieties as potential antitumor agents: Design, synthesis and cytotoxic evaluation

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Abstract

Diosgenin, a naturally occurring steroidal saponin, has been confirmed to possess potent anticancer properties. In the current work, two series of novel diosgenin derivatives bearing 1,3,4-oxadiazole (**6a**–**6e** and **7a**–**7e**) or 1,3,4-thiadiazole (**8a**–**8e** and **9a**–**9e**) moieties were designed, synthesized and evaluated for their cytotoxicities in four human cancer cell lines (HepG2, A549, MCF-7 and HCT-116) and normal human gastric epithelial cells (GES-1) using the MTT assay *in vitro*. The results showed that compounds **8d** and **9d** exhibited significant cytotoxic activities against the HepG2 and A549 cells, being more potent than their parent compound diosgenin. Furthermore, the 1,3,4-thiadiazole series of compounds generally exhibited stronger cytotoxicity compared with the 1,3,4-oxadiazole series against HepG2 and A549 cells, and the substitution of 3-pyridyl group at the C5 position of the 1,3,4-thiadiazole ring was the preferred option for these compounds to display significant cytotoxic activities. Compound **8d** showed potent cytotoxic activity against A549 cell line (IC_{50} = 3.93 μM) and was 6.7-fold more potent than diosgenin (IC_{50} = 26.41 μM). Moreover, compound **8d** displayed low toxicity against GES-1 cells (IC_{50} = 420.4 μM), showing specificity between normal and tumor cells. Further cellular mechanism studies in A549 cells indicated that compound **8d** triggered the mitochondrial-mediated apoptosis by decreasing mitochondrial membrane potential, which was associated with up-regulation of Bax, down-regulation of Bcl-2 and activation levels of the caspase cascade. The above results indicated that compound **8d** may be used as a promising skeleton for antitumor agents with improved efficacy.

Key words: Diosgenin derivatives; 1,3,4-Oxadiazole; 1,3,4-Thiadiazole; Antitumor; Apoptosis.

1. Introduction

The incidence and mortality of cancer are rapidly growing worldwide. According to the latest Annual World Cancer Report, there were approximately 18 million new cases and 9.6 million cancer deaths worldwide in 2018 [1]. Current studies showed that chemotherapy was still one of the most effective methods for cancer treatment. However, undesirable side effects seriously impede its clinical applications [2, 3]. Therefore, new drugs killing cancer cells without obvious side effects are of great importance for cancer chemotherapy. Natural products have been a rich source of compounds for drug discovery and the majority of anticancer drugs currently used in clinic are derived from natural product scaffolds [4].

Naturally occurring steroids have been shown to possess potent antitumor activity and provide privileged structures for further modifications [5]. During the last few years, our research groups have been working on structural modifications of steroids to obtain more active compounds as potential antitumor agents, and some derivatives with highly promising cytotoxic activities against a number of cancer cell lines were found [6-8].

Diosgenin (DG, Fig. 1), a steroidal saponin abundantly present in the roots of wild yam (*Dioscorea villosa*), is used as a traditional medicine because of its activities of antitumor [9-13], antidiabetes [14], anti-inflammatory [15] and anti-Parkinson's Disease [16]. More recently, it has been reported that DG inhibited cancer cell proliferation and induced apoptosis in multiple cancer cell lines including colorectal cancer [9], hepatocellular carcinoma [10], breast cancer [11], lung cancer [12] and prostate cancer [13]. Although DG has extensive cytotoxic activity, the use of DG for cancer therapy was hampered due to its moderate potency. Therefore, it is important to carry out structural modification of DG to obtain promising anticancer compounds.

Heterocycles are considered as privileged scaffolds in drug discovery and widely used in medicinal chemistry [17, 18]. Among them, 1,3,4-oxadiazole and 1,3,4-thiadiazole are two well-known pharmacophores and have drawn much attention due to their diverse pharmacological activities. Multiple studies have demonstrated that 1,3,4-oxadiazole/thiadiazole containing Zibotentan (an anticancer drug candidate in late stage clinical trials) and compounds A-D exhibited significant cytotoxic activity against various cancer cell lines [19-23] (Fig. 2). Hence, the incorporation of these two moieties in the designed compounds may give new effective antitumor agents.

Aiming at finding promising new leads with potential antitumor activity, it was considered worthwhile to design and synthesize novel diosgenin derivatives that contain the 1,3,4-oxadiazole or 1,3,4-thiadiazole ring systems. Accordingly, twenty novel diosgenin 1,3,4-oxadiazolyl/thiadiazolyl derivatives were designed,

synthesized, characterized and evaluated for their cytotoxicity against four tumor cell lines and one normal cell line.

2. Results and discussion

2.1 Chemistry

The synthetic route for the target compounds is shown in Scheme 1. The acetylation of DG (**1**), using dry dichloromethane as a co-solvent, afforded intermediate **2** according to the published method [24] with some modifications. Next, intermediate **2** was converted into intermediate **3** by reductive opening of the cyclic spiroketal using $NaBH_3CN$ in AcOH at room temperature in 81.0% yield [6]. Intermediate **3** was oxidized using Jones reagent to give the key intermediate **4** in 76.0% yield. Subsequently, the intermediate **4** was coupled with the appropriate acylhydrazines in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium-tetrafluoroboratetetrabutyl (TBTU) as a coupling catalyst to afford intermediates **5a**–**5e** [25]. The cyclodehydration of intermediates **5a**–**5e** with $POCl_3$ at 80 ^oC furnished the corresponding compounds **6a**–**6e** in good yields according to the method described in the literature [26], and subsequent deacetylation led to the 3*β*-hydroxy derivatives **7a**–**7e**. On the other hand, the Lawesson reagent (LR) induced dehydrosulfurization of intermediates **5a**–**5e** in refluxing toluene for 1 h furnished compounds **8a**–**8e** in good yields [26]. Finally, deacetylation of compounds **8a**–**8e** afforded the 3*β*-hydroxy analogs **9a**–**9e**. All the synthesized compounds were well characterized by their ESI-HRMS, ^1H NMR, and ^{13}C NMR spectral data.

2.2 Biological evaluation

2.2.1 In vitro cytotoxic activity

The *in vitro* cytotoxicities of the synthesized diosgenin derivatives against human hepatoma (HepG2), lung carcinoma (A549), breast cancer (MCF-7), colorectal cancer (HCT-116) and normal gastric (GES-1) cell lines were evaluated by the MTT assay, and Mitomycin C was used as the positive control. The IC_{50} values are presented in Table 1. The results showed that compounds **8d** and **9d** exhibited marked cytotoxicities against the HepG2 and A549 cell lines, and were more potent than their parent compound DG and Mitomycin C.

For HepG2 cell line, the 1,3,4-oxadiazolyl series of compounds **6a**–**6e** (R_2 = Ac) and **7a**–**7d** (R_2 = H) displayed lower cytotoxic activities than DG (IC_{50} = 33.87 μM). Only compound **7e** (IC_{50} = 29.24 μM) showed similar cytotoxicity to DG. The substitutions (CH_3, phenyl, *p*-methoxyphenyl and 1-naphthyl) on the 1,3,4-thiadiazole moiety led to compounds **8a**–**8e** and **9a**–**9e** without significant improvement in cytotoxicity except for compounds **8d** and **9d**. These two compounds possessing 3-pyridyl group displayed significant cytotoxic activities with IC_{50} values of 11.73 and 8.83 μM, respectively. Specifically, the cytotoxicity of compound **8d** was about 2.9-fold more potent than those of compounds **8a** (IC_{50} = 34.25 μM) and DG. For compound **9d**, the cytotoxicity was 2.6–3.8-fold higher than those of compounds **9a** (IC_{50} = 33.16 μM), **9b** (IC_{50} = 32.14 μM), **9c** (IC_{50} = 23.05 μM) and **9e** (IC_{50} = 32.90 μM), and was about 3.8-fold more potent than that of DG. Furthermore, compounds **8d** and **9d** were less toxic to non-cancer cells GES-1 with IC_{50} values of 420.4 and 354.4 μM, respectively. These data showed that the incorporation of a 3-pyridine ring at the C5 position of the 1,3,4-thiadiazole ring led to significant improvement in cytotoxic activity when compared with methyl and aryl rings.

For A549 cell line, compounds **8d** (IC_{50} = 3.93 μM) and **9d** (IC_{50} = 7.68 μM) also possessed stronger cytotoxicity than that of DG (IC_{50} = 26.41 μM). Among these compounds, compound **8d** was the most active compound, which was 6.7-fold more potent than DG. Moreover, compound **8d** (IC_{50} = 420.4 μM) showed lower cytotoxicity against normal cells GES-1 than DG (IC_{50} = 100.7 μM). Compound **9d** was 2.0–4.8-fold more potent than compounds **9a** (IC_{50} = 36.71 μM), **9b** (IC_{50} = 15.04 μM), **9c** (IC_{50} = 16.4 μM) and **9e** (IC_{50} = 18.26 μM). The results also suggested that the substitution of 3-pyridyl group at the C5 position of the 1,3,4-thiadiazole ring was beneficial for compounds to display potent cytotoxicity against A549 cell line.

For MCF-7 cell line, the 20 synthesized derivatives exhibited lower cytotoxicity compared with DG (IC_{50} = 23.91 μM) with the exception of compound **7a** (IC_{50} = 20.37 μM). For HCT-116 cell line, compound **8d** (IC_{50} = 29.56 μM) showed higher cytotoxicity than DG (IC_{50} = 49.11 μM).

Taken together, the 1,3,4-thiadiazole series of compounds generally exhibited stronger cytotoxicity compared with the 1,3,4-oxadiazole series against the HepG2 and A549 cell lines. The above results suggested that the group with 3-pyridyl substitution at the C5 position of the 1,3,4-thiadiazole ring was the preferred substitution pattern for compounds with potent cytotoxic activities.

The partition coefficient (log *P*) between two solvents *n*-octanol and water has been used as a classical descriptor for lipophilicity. Compound **8d** exhibited potent cytotoxic activity against A549 cell line. Therefore,

the calculated log *P* (clog *P*) values of compound **8d** and DG were calculated using SwissADME web tool [27, 28]. The results showed that the clog *P* value of compound **8d** (6.35) was relatively higher than that of DG (5.03). Compound **8d** with the medium clog *P* value showed stronger cytotoxicity, which may attribute to the increase of lipophilicity.

To gain insight into the cellular mechanism of our synthesized compounds, the most potent compound **8d** was selected for further studies in A549 cells by performing DAPI, Annexin V/propidium iodide (PI) staining, mitochondrial membrane potential ($\Delta\Psi$m) and Western blot assays.

2.2.2 Apoptosis study

Previous studies have reported that DG exhibited cytotoxic activity against DU145 and K562 cells by inducing apoptosis *in vitro* [29, 30]. Compared with the control group, some of the **8d**-treated cells exhibited rounding, shrinkage, membrane blebbing and the formation of apoptotic bodies, which were hallmarks of apoptotic cells (Fig. 3A). However, those changes were not obviously observed in the DG group. Changes of the morphological characters in A549 cells were further studied by DAPI staining under fluorescence microscopy to confirm that the growth inhibitory activity of **8d** was related to the inducing of apoptosis. As shown in Fig. 3B, cells of the untreated group were stained homogeneously, while some **8d**-treated cells exhibited bright chromatin condensation and nuclear fragmentation, a hallmark of apoptosis. The above observation indicated that the treatment of **8d** induced the apoptosis of A549 cells.

To further verify whether **8d** could induce apoptosis in A549 cells, **8d**-incubated A549 cells were stained with Annexin V (AV) and propidium iodide (PI). As shown in Fig. 4, the percentage of total died cells (apoptotic and necrotic cells) increased to 36.1% and 81.3% after treatment with **8d** at the concentrations of 15 and 30 μM, respectively, in comparison with the control (5.4%). Moreover, the apoptotic ratios of **8d**-treated cells were significantly increased than those of DG-treated cells. These findings suggested that **8d** exerted its antiproliferative effects by inducing apoptosis.

Subsequently, the expression levels of cleaved caspase-9, 3 and cleaved PARP were evaluated by Western blot analysis in A549 cells. Fig. 5 showed that the expression levels of cleaved caspase-9, cleaved caspase-3 and cleaved PARP were activated after treated with **8d**. However, the expression levels of those proteins did not change after treated with DG in A549 cells.

2.2.3 Measurement of mitochondrial membrane potential (MMP)

Mitochondrion plays an important role in regulating cellular functions, and mitochondrial dysfunction has been identified as one of the characteristic events of apoptosis [8]. In order to determine whether **8d**-induced apoptosis was involved in a disruption of mitochondrial membrane integrity, we analyzed MMP changes of the A549 cells by staining with the dye JC-1 and analyzing the cells by flow cytometry. As shown in Fig. 6, **8d** induced a concentration-dependent increase in depolarized cell population from 0.4% of control to 7.5% and 26.0%, respectively. The results indicated that apoptosis in A549 cells induced by **8d** was associated with the mitochondrial (intrinsic) pathways.

*2.2.4 **8d** induces apoptosis through mitochondrial pathway*

The mitochondria-dependent apoptotic pathway, also called the intrinsic pathway, is modulated by the Bcl-2 family of proteins, including Bax (pro-apoptotic protein) and Bcl-2 (anti-apoptotic protein), which could induce cytochrome *c* (cyto-*c*) released into the cytosol, resulting in the activation of the caspase-9, -3, and PARP cleavage, finally triggering the execution of apoptosis [31]. Therefore, to reveal the molecular mechanism of **8d**, we examined the expression of Bcl-2, Bax, and cyto-*c* by Western blot analysis. As shown in Fig. 7, in comparison with untreated cells, **8d** could increase the level of Bax and decrease the level of Bcl-2 in a concentration-dependent manner. Moreover, the expression of cyto-*c* remarkably increased in a dose-related fashion (Fig. 7). These results indicated that **8d** could induce the apoptosis of A549 cells via increasing the level of Bax and decreasing the level of Bcl-2, leading to the release of cyto-*c* and activation of caspase-9, -3, and PARP cleavage, which triggered the execution of apoptosis.

The above results indicated that compound **8d** showed low cytotoxicity to normal human cells and potential anti-cancer activity against A549 cells by inducing apoptosis through mitochondrial pathway.

3. Conclusion

In the present work, twenty novel diosgenin 1,3,4-oxadiazolyl/thiadiazolyl derivatives were synthesized and tested for their cytotoxic activities *in vitro*. Derivatives **8d** and **9d** showed stronger cytotoxic effects than their parent compound diosgenin in the HepG2 and A549 cell lines. The 1,3,4-thiadiazole series of compounds generally exhibited stronger cytotoxicity compared with the 1,3,4-oxadiazole series against the HepG2 and A549 cell lines, and the substitution of 3-pyridyl group at the C5 position of the 1,3,4-thiadiazole ring was the

preferred option for these compounds to display significant cytotoxic activities. Compound **8d** displayed the most potent cytotoxicity against A549 cells (IC_{50} = 3.93 μM) and low cytotoxicity against normal GES-1 cells (IC_{50} = 420.4 μM). Further studies on the cellular mechanism of compound **8d** showed that it caused morphological changes, decreased mitochondrial membrane potential, and induced apoptosis through mitochondrial pathway in A549 cells. The results of the current study imply that compound **8d** is a lead compound for further research.

4. Experimental protocols

4.1 Chemistry

Diosgenin was purchased from Xi'an Xiaocao Plant Technology Co., Ltd. Reagents and solvents were purchased from commercial sources and used as received. Melting points (mp) were determined on a WRS-2 melting apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ with Bruker AVANCE NEO 600 spectrometer (TMS as internal standard). The values of the chemical shifts were expressed in δ values (ppm) and the coupling constants (J) in Hz. ESI-HRMS data were obtained using an AB SCIEX TripleTOF 4600 instrument. Reaction progress was monitored by analytical thin layer chromatography (TLC) on silica gel HSGF254 plates (Yantai Zifu Chemical Group Co. China). Silica gel (100–200 or 200–300 mesh, Qingdao city, China) was used for column chromatography.

*4.1.1 Synthesis of (25R)-3β-acetoxy-5-en-spirostan (**2**).*

Ac_2O (14.8 g, 144.9 mol) was added to a magnetically stirred solution of diosgenin (30 g, 72.4 mmol) in CH_2Cl_2 (210 mL) and pyridine (60 mL). After the mixture was stirred at room temperature for 6 h, water (300 mL) was added, and then extracted with CH_2Cl_2 (100 mL). The combined organic layers were washed with saturated $NaHCO_3$, brine and dried over anhydrous Na_2SO_4, then the solids were removed by filtration. The solvent was evaporated in vacuo, and the crude material was purified by reflux with anhydrous ethanol (240 mL) to provide compound **2** (32.1 g, 97.0%). White amorphous powder, mp 194.8–195.6 ^{o}C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 5.37 (d, J = 4.9 Hz, 1H, H-6), 4.60 (m, 1H, H-3), 4.41 (dd, J = 14.8, 7.2 Hz, 1H, H-16), 3.47 (dd, J = 10.1, 4.1 Hz, 1H, H-26), 3.37 (t, J = 11.0 Hz, 1H, H-26), 2.03 (s, 3H, Ac-CH_3), 1.03 (s, 3H, 19-CH_3), 0.97 (d, J = 7.0 Hz, 3H, 21-CH_3), 0.78 (s, 3H, 18-CH_3), 0.78 (d, J = 5.6 Hz, 3H, 27-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 170.7 (Ac-CO), 139.8 (C-5), 122.5 (C-6), 109.4 (C-22), 80.9 (C-16), 74.0 (C-3), 67.0 (C-26), 62.2 (C-17), 56.6

(C-14), 50.1 (C-9), 41.8 (C-20), 40.4 (C-12), 39.9 (C-13), 38.2 (C-4), 37.1 (C-1), 36.9 (C-10), 32.2 (C-7), 32.0 (C-15), 31.8 (C-8), 30.4 (C-23), 29.8 (C-25), 29.0 (C-2), 27.9 (C-24), 21.6 (Ac-CH_3), 21.0 (C-11), 19.5 (C-19), 17.3 (C-27), 16.4 (C-18), 14.7 (C-21). ESI-HRMS: *m*/*z* 457.3330 $[M+H]^+$ (Calcd for $C_{29}H_{45}O_4$, 457.3312).

*4.1.2 Synthesis of (22R,25R)-3β-acetoxy-5-en-furostan-26-ol (**3**).*

To a magnetically stirred solution of appropriate compound **2** (20 g, 43.9 mmol) in CH_2Cl_2 (80 mL) was added acetic acid (20 mL) and $NaBH_3CN$ (5.53 g, 87.8 mmol). After 8 h, the mixture was alkalified by saturated Na_2CO_3 solution. The organic layer was washed with water and brine and dried over anhydrous Na_2SO_4, then the solids were removed by filtration. The solvent was evaporated in vacuo, and the crude material was purified by column chromatography over the silica gel with petroleum ether–ethyl acetate (20:1) to get intermediate **3** (16.3 g, 81.0%). White amorphous powder, mp 107.2–108.6 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 5.36 (d, *J* = 5.0 Hz, 1H, H-6), 4.59 (m, 1H, H-3), 4.30 (m, 1H, H-16), 3.49 (dd, *J* = 10.6, 6.0 Hz, 1H, H-26), 3.43 (dd, *J* = 10.6, 6.0 Hz, 1H, H-26), 3.32 (td, *J* = 8.3, 3.7 Hz, 1H, H-22), 2.02 (s, 3H, Ac-CH_3), 1.03 (s, 3H, 19-CH_3), 1.00 (d, *J* = 6.7 Hz, 3H, 21-CH_3), 0.91 (d, *J* = 6.8 Hz, 3H, 27-CH_3), 0.80 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 170.7 (Ac-CO), 139.8 (C-5), 122.5 (C-6), 90.5 (C-22), 83.3 (C-16), 74.0 (C-3), 68.2 (C-26), 65.2 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-4), 38.1 (C-20), 37.1 (C-1), 36.8 (C-10), 35.9 (C-25), 32.4 (C-7), 32.1 (C-15), 31.7 (C-8), 30.6 (C-24), 30.3 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.8 (C-11), 19.5 (C-19), 19.1 (C-21), 16.8 (C-27), 16.6 (C-18). ESI-HRMS: *m*/*z* 459.3472 $[M+H]^+$ (Calcd for $C_{29}H_{47}O_4$, 459.3469).

*4.1.3 Synthesis of (22R,25R)-3β-acetoxy-5-en-furostan-26-oic acid (**4**).*

To a stirred solution of compound **3** (10.0 g, 21.8 mmol) in a mixture of THF (50 mL) and acetone (50 mL), Jones reagent was added dropwise at room temperature until the solution remained orange. Then the reaction mixture was stirred for 2 h at room temperature. Upon completion, the precipitate was removed by filtration and the solvent was evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (50 mL) and washed with brine (3×50 mL) and dried over anhydrous Na_2SO_4, then concentrated under reduced pressure. The residue was purified on silica gel chromatography with petroleum ether-ethyl acetate (10:1) to give intermediate **4** (7.63 g, 76.0%). White amorphous powder, mp 124.5–125.2 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 5.37 (d, *J* = 4.8 Hz, 1H, H-6), 4.60 (m, 1H, H-3), 4.33 (m, 1H, H-16), 3.35 (td, *J* = 8.6, 2.4 Hz, 1H, H-22), 2.52 (m, 1H, H-25), 2.03 (s, 3H, Ac-CH_3), 1.18 (d, *J* = 6.9 Hz, 3H, 27-CH_3), 1.03 (s, 3H, 19-CH_3), 1.00 (d, *J* = 6.7 Hz, 3H, 21-CH_3), 0.79

(s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 181.2 (C-26), 170.7 (Ac-CO), 139.8 (C-5), 122.5 (C-6), 90.4 (C-22), 83.7 (C-16), 74.0 (C-3), 65.0 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 39.5 (C-25), 38.2 (C-4), 38.2 (C-20), 37.1 (C-1), 36.8 (C-10), 32.3 (C-7), 32.1 (C-15), 31.7 (C-8), 31.6 (C-24), 31.1 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.8 (C-11), 19.5 (C-19), 19.0 (C-21), 17.4 (C-27), 16.5 (C-18). ESI-HRMS: *m*/*z* 473.3220 $[M+H]^+$ (Calcd for $C_{29}H_{45}O_5$, 473.3262).

*4.1.4 General procedure for synthesis of compounds **5a**–**5e**.*

To a solution of compound **4** (0.6 g, 1.28 mmol) and the appropriate acylhydrazines (2.56 mmol) in CH_2Cl_2 (5 mL) was added TBTU (0.82 g, 2.56 mmol) and DIPEA (0.33 g, 2.56 mmol) and stirred for 2 h at room temperature. When the substrates disappeared (as detected by TLC), the mixture was washed with water (50 mL) and brine (50 mL) and dried over anhydrous Na_2SO_4. The solvent was concentrated under vacuum. The crude product was purified by flash chromatography on silica gel using petroleum ether-ethyl acetate (2:1) as eluent to yield compounds **5a**–**5e**.

*4.1.4.1 (22R,25R)-N-Acetyl-3β-acetoxy-5-en-furostan-26-carbohydrazide (**5a**).*

White amorphous powder, yield 74.5%, mp 221.9–223.0 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 9.24 (brs, 1H, NH), 9.15 (d, *J* = 4.7 Hz, 1H, NH), 5.36 (d, *J* = 3.3 Hz, 1H, H-6), 4.59 (m, 1H, H-3), 4.30 (m, 1H, H-16), 3.28 (t, *J* = 6.4 Hz, 1H, H-22), 2.52 (m, 1H, H-25), 2.03 (s, 3H, 2′-CH_3), 2.02 (s, 3H, Ac-CH_3), 1.13 (d, *J* = 6.6 Hz, 3H, 27-CH_3), 1.02 (s, 3H, 19-CH_3), 0.96 (d, *J* = 6.1 Hz, 3H, 21-CH_3), 0.76 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 173.6 (C-26), 170.7 (Ac-CO), 167.0 (C-1′), 139.8 (C-5), 122.5 (C-6), 91.0 (C-22), 83.7 (C-16), 74.0 (C-3), 64.7 (C-17), 57.1 (C-14), 50.1 (C-9), 40.8 (C-12), 39.6 (C-13), 38.4 (C-25), 38.3 (C-20), 38.2 (C-4), 37.1 (C-1), 36.8 (C-10), 32.2 (C-7), 32.1 (C-15), 31.6 (C-8), 29.8 (C-24), 29.8 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.8 (C-2′), 20.8 (C-11), 19.5 (C-19), 18.7 (C-21), 17.9 (C-27), 16.5 (C-18). ESI-HRMS: *m*/*z* 529.3650 $[M+H]^+$ (Calcd for $C_{31}H_{49}N_2O_5$, 529.3636).

*4.1.4.2 (22R,25R)-N-Benzoyl-3β-acetoxy-5-en-furostan-26-carbohydrazide (**5b**).*

White amorphous powder, yield 63.1%, mp 106.3–107.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 9.75 (d, *J* = 3.5 Hz, 1H, NH), 9.54 (d, *J* = 3.5 Hz, 1H, NH), 7.79 (d, *J* = 7.7 Hz, 2H, H-2′ and H-6′), 7.44 (t, *J* = 7.7 Hz, 2H, H-4′), 7.33 (d, *J* = 7.7 Hz, 2H, H-3′ and H-5′), 5.34 (d, *J* = 4.3 Hz, 1H, H-6), 4.57 (m, 1H, H-3), 4.29 (m, 1H,

H-16), 3.27 (t, *J* = 7.8 Hz, 1H, H-22), 2.59 (m, 1H, H-25), 2.00 (s, 3H, Ac-CH_3), 1.12 (d, *J* = 6.6 Hz, 3H, 27-CH_3), 0.98 (s, 3H, 19-CH_3), 0.94 (d, *J* = 6.5 Hz, 3H, 21-CH_3), 0.74 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 174.2 (C-26), 170.6 (Ac-CO), 164.4 (C-28), 139.7 (C-5), 132.1 (C-4′), 131.8 (C-1′), 128.6 (2C, C-3′ and C-5′), 127.4 (2C, C-2′ and C-6′), 122.4 (C-6), 90.8 (C-22), 83.6 (C-16), 73.9 (C-3), 64.7 (C-17), 57.0 (C-14), 50.0 (C-9), 40.7 (C-12), 39.5 (C-13), 38.5 (C-25), 38.3 (C-20), 38.1 (C-4), 37.0 (C-1), 36.8 (C-10), 32.2 (C-7), 32.0 (C-15), 31.6 (C-8), 31.0 (C-24), 30.7 (C-23), 27.8 (C-2), 21.5 (Ac-CH_3), 20.7 (C-11), 19.4 (C-19), 18.7 (C-21), 17.8 (C-27), 16.4 (C-18). ESI-HRMS: *m/z* 591.3784 $[M+H]^+$ (Calcd for $C_{36}H_{51}N_2O_5$, 591.3792).

*4.1.4.3 (22R,25R)-N-(p-Methoxybenzoyl)-3β-acetoxy-5-en-furostan-26-carbohydrazide (**5c**).*

White amorphous powder, yield 48.7%, mp 108.8–111.0 °C. ^{1}H NMR (600 MHz, $CDCl_3$, *δ*): 9.26 (brs, 1H, NH), 9.20 (brs, 1H, NH), 7.77 (d, *J* = 8.6 Hz, 2H, H-2′ and H-6′), 6.86 (d, *J* = 8.6 Hz, 2H, H-3′ and H-5′), 5.35 (d, *J* = 3.3 Hz, 1H, H-6), 4.58 (m, 1H, H-3), 4.31 (m, 1H, H-16), 3.82 (s, 3H, 4′-OCH_3), 3.30 (t, *J* = 6.7 Hz, 1H, H-22), 2.58 (m, 1H, H-25), 2.02 (s, 3H, Ac-CH_3), 1.16 (d, *J* = 6.6 Hz, 3H, 27-CH_3), 1.00 (s, 3H, 19-CH_3), 0.96 (d, *J* = 6.6 Hz, 3H, 21-CH_3), 0.75 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 173.9 (C-26), 170.7 (Ac-CO), 164.0 (C-28), 162.8 (C-4′), 139.8 (C-5), 129.3 (2C, C-2′ and C-6′), 124.1 (C-1′), 122.5 (C-6), 113.9 (2C, C-3′ and C-5′), 91.0 (C-22), 83.7 (C-16), 74.0 (C-3), 64.7 (C-17), 57.1 (C-14), 55.5 (C-4′-OCH_3), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.6 (C-25), 38.4 (C-20), 38.2 (C-4), 37.1 (C-1), 36.8 (C-10), 32.3 (C-7), 32.1 (C-15), 31.6 (C-8), 31.1 (C-24), 30.7 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.8 (C-11), 19.4 (C-19), 18.8 (C-21), 17.9 (C-27), 16.5 (C-18). ESI-HRMS: *m/z* 621.3910 $[M+H]^+$ (Calcd for $C_{37}H_{53}N_2O_6$, 621.3898).

*4.1.4.4 (22R,25R)-N-(Pyridyl-3-carbonyl)-3β-acetoxy-5-en-furostan-26-carbohydrazide (**5d**).*

White amorphous powder, yield 67.8%, mp 124.9–126.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$, *δ*): 9.81 (brs, 1H, NH), 9.32 (brs, 1H, NH), 9.01 (s, 1H, H-2′), 8.69 (brs, 1H, H-6′), 8.12 (d, 1H, *J* = 7.9 Hz, H-4′), 7.33 (dd, *J* = 7.9, 4.5 Hz, 1H, H-5′), 5.34 (d, *J* = 4.0 Hz, 1H, H-6), 4.58 (m, 1H, H-3), 4.33 (m, 1H, H-16), 3.32 (m, 1H, H-22), 2.64 (m, 1H, H-25), 2.01 (s, 3H, Ac-CH_3), 1.15 (d, *J* = 6.7 Hz, 3H, 27-CH_3), 0.98 (s, 3H, 19-CH_3), 0.97 (d, *J* = 6.9 Hz, 3H, 21-CH_3), 0.75 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 174.8 (C-26), 170.7 (Ac-CO), 163.0 (C-28), 152.7 (C-2′), 148.7 (C-6′), 139.8 (C-5), 135.2 (C-4′), 127.8 (C-5′), 123.5 (C-3′), 122.4 (C-6), 91.2 (C-22), 83.8 (C-16), 74.0 (C-3), 64.5 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.5 (C-25), 38.2 (C-4), 38.2 (C-20), 37.1 (C-1), 36.8 (C-10), 32.2 (C-7), 32.1 (C-15), 31.6 (C-8), 31.0 (C-24), 30.4 (C-23),

27.8 (C-2), 21.5 (Ac-CH_3), 20.7 (C-11), 19.4 (C-19), 18.6 (C-27), 17.8 (C-21), 16.5 (C-18). ESI-HRMS: *m/z* 592.3769 $[M+H]^+$ (Calcd for $C_{35}H_{50}N_3O_5$, 592.3745).

*4.1.4.5 (22R,25S)-N-(1′-Naphthoyl)-3β-acetoxy-5-en-furostan-26-carbohydrazide (**5e**).*

White amorphous powder, yield 70.6%, mp 157.7–158.6 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 8.36 (m, 1H, Ar-H), 7.92 (d, *J* = 8.2 Hz, 1H, Ar-H), 7.84 (m, 1H, Ar-H), 7.70 (d, *J* = 8.2 Hz, 1H, Ar-H), 7.51 (m, 2H, Ar-H), 7.42 (t, *J* = 8.2 Hz, 1H, Ar-H), 5.37 (d, *J* = 4.9 Hz, 1H, H-6), 4.59 (m, 1H, H-3), 4.36 (m, 1H, H-16), 3.35 (t, *J* = 8.4 Hz, 1H, H-22), 2.68 (m, 1H, H-25), 2.02 (s, 3H, Ac-CH_3), 1.19 (d, *J* = 6.7 Hz, 3H, 27-CH_3), 1.00 (s, 3H, 19-CH_3), 0.98 (d, *J* = 6.7 Hz, 3H, 21-CH_3), 0.82 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 174.1 (C-2′), 170.7 (Ac-CO), 166.2 (C-5′), 139.8 (C-5), 133.8 (Ar-C), 131.6 (Ar-C), 131.2 (Ar-C), 130.5 (Ar-C), 128.4 (Ar-C), 127.5 (Ar-C), 126.7 (Ar-C), 126.0 (Ar-C), 125.4 (Ar-C), 124.7 (Ar-C), 122.5 (C-6), 91.4 (C-22), 84.0 (C-16), 74.0 (C-3), 64.5 (C-17), 57.1 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.7 (C-25), 38.6 (C-20), 38.2 (C-4), 37.1 (C-1), 36.8 (C-10), 32.3 (C-7), 32.1 (C-15), 31.7 (C-8), 31.0 (C-24), 30.5 (C-23), 27.9 (C-2), 21.5 (Ac-CH_3), 20.7 (C-11), 19.4 (C-19), 18.5 (C-21), 17.9 (C-27), 16.5 (C-18). ESI-HRMS: *m/z* 641.3959 $[M+H]^+$ (Calcd for $C_{40}H_{53}N_2O_5$, 641.3949).

*4.1.5 General procedure for synthesis of compounds **6a–6e**.*

N,*N*′-Disubstituted hydrazine (**5a–5e**, 0.40 mmol) was heated in $POCl_3$ (5 mL) at 80 °C for 1 h until the starting material was not observed by TLC. The reaction mixture was cooled to room temperature, carefully poured onto crushed ice and made basic with saturated $NaHCO_3$ solution. The resulting precipitate was filtered, dried and purified by silica gel chromatography to obtain compounds **6a–6e**.

*4.1.5.1 (22R,25R)-3β-Acetoxy-5-en-25-(2′-[5′-methyl]-1′,3′,4′-oxadiazolyl) furostan (**6a**).*

White amorphous powder, yield 75.0%, mp 120.4–121.7 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 5.36 (d, *J* = 4.8 Hz, 1H, H-6), 4.58 (m, 1H, H-3), 4.28 (m, 1H, H-16), 3.28 (m, 1H, H-22), 3.07 (dd, *J* = 14.9, 7.1 Hz, 1H, H-25), 2.49 (s, 3H, 5′-CH_3), 2.02 (s, 3H, Ac-CH_3), 1.34 (d, *J* = 7.1 Hz, 3H, 27-CH_3), 1.02 (s, 3H, 19-CH_3), 0.96 (d, *J* = 7.1 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 170.7 (Ac-CO), 170.5 (C-2′), 163.6 (C-5′), 139.8 (C-5), 122.5 (C-6), 89.8 (C-22), 83.4 (C-16), 74.0 (C-3), 65.2 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-20), 38.0 (C-4), 37.1 (C-1), 36.8 (C-10), 32.3 (C-7), 32.1 (C-15), 32.0 (C-25), 31.9

(C-24), 31.7 (C-8), 31.1 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.8 (C-11), 19.5 (C-19), 19.0 (C-27), 18.4 (C-21), 16.5 (C-18), 11.1 (5′-CH_3). ESI-HRMS: *m*/*z* 511.3562 $[M+H]^+$ (Calcd for $C_{31}H_{47}N_2O_4$, 511.3530).

*4.1.5.2 (22R,25R)-3β-Acetoxy-5-en-25-(2′-[5′-phenyl]-1′,3′,4′-oxadiazolyl) furostan (**6b**).*

White amorphous powder, yield 81.5%, mp 97.2–98.6 °C. ^{1}H NMR (600 MHz, $CDCl_3$, *δ*): 8.04 (dd, *J* = 7.9, 1.3 Hz, 2H, H-2″ and H-6″), 7.50 (m, 3H, H-3″, H-4″ and H-5″), 5.36 (d, *J* = 3.5 Hz, 1H, H-6), 4.59 (m, 1H, H-3), 4.28 (m, 1H, H-16), 3.30 (dd, *J* = 13.6, 7.3 Hz, 1H, H-22), 3.20 (m, 1H, H-25), 2.02 (s, 3H, Ac-CH_3), 1.43 (d, *J* = 7.0 Hz, 3H, 27-CH_3), 1.02 (s, 3H, 19-CH_3), 0.96 (d, *J* = 6.5 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 170.7 (Ac-CO), 170.3 (C-2′), 164.7 (C-5′), 139.8 (C-5), 131.6 (C-4″), 129.1 (2C, C-3″ and C-5″), 126.9 (2C, C-2″ and C-6″), 124.3 (C-1″), 122.5 (C-6), 89.9 (C-22), 83.4 (C-16), 74.0 (C-3), 65.1 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-4), 38.1 (C-20), 37.1 (C-1), 36.8 (C-10), 32.3 (C-7), 32.2 (C-15), 32.1 (C-25), 31.7 (C-8), 31.2 (C-24), 31.1 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.7 (C-11), 19.5 (C-19), 19.0 (C-27), 18.5 (C-21), 16.5 (C-18). ESI-HRMS: *m*/*z* 573.3714 $[M+H]^+$ (Calcd for $C_{36}H_{49}N_2O_4$, 573.3687).

*4.1.5.3 (22R,25R)-3β-Acetoxy-5-en-25-(2′-[5′-p-methoxyphenyl]-1′,3′,4′-oxadiazolyl) furostan (**6c**).*

White amorphous powder, yield 73.2%, mp 69.8–71.3 °C. ^{1}H NMR (600 MHz, $CDCl_3$, *δ*): 7.97 (dd, *J* = 8.7, 2.9 Hz, 2H, H-2″ and H-6″), 6.98 (dd, *J* = 8.7, 2.9 Hz, 2H, H-3″ and H-5″), 5.36 (d, *J* = 2.0 Hz, 1H, H-6), 4.59 (m, 1H, H-3), 4.28 (m, 1H, H-16), 3.86 (s, 3H, H-4″-OCH_3), 3.30 (m, 1H, H-22), 3.16 (m, 1H, H-25), 2.02 (s, 3H, Ac-CH_3), 1.41 (d, *J* = 6.8 Hz, 3H, 27-CH_3), 1.02 (s, 3H, 19-CH_3), 0.96 (d, *J* = 6.8 Hz, 3H, 21-CH_3), 0.76 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 170.7 (Ac-CO), 169.8 (C-2′), 164.6 (C-5′), 162.3 (C-4″), 139.8 (C-5), 128.7 (2C, C-3″ and C-5″), 122.5 (C-6), 116.8 (C-1″), 114.5 (2C, C-2″ and C-6″), 89.9 (C-22), 83.4 (C-16), 74.0 (C-3), 65.1 (C-17), 57.0 (C-14), 55.6 (C-4″-OCH_3), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-4), 38.1 (C-20), 37.1 (C-1), 36.8 (C-10), 32.3 (C-7), 32.2 (C-15), 32.1 (C-25), 31.7 (C-8), 31.2 (C-24), 31.0 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.7 (C-11), 19.4 (C-19), 19.0 (C-27), 18.5 (C-21), 16.5 (C-18). ESI-HRMS: *m*/*z* 603.3795 $[M+H]^+$ (Calcd for $C_{37}H_{51}N_2O_4$, 603.3972).

*4.1.5.4 (22R,25R)-3β-Acetoxy-5-en-25-(2′-[5′-3″-pyridyl]-1′,3′,4′-oxadiazolyl) furostan (**6d**).*

White amorphous powder, yield 82.4%, mp 76.6–78.0 °C. ^{1}H NMR (600 MHz, $CDCl_3$, *δ*): 9.25 (s, 1H,

H-2″), 8.76 (d, *J* = 3.5 Hz, 1H, H-6″), 8.34 (d, 1H, *J* = 7.9 Hz, H-4″), 7.46 (dd, *J* = 7.9, 3.5 Hz, 1H, H-5″), 5.36 (d, *J* = 4.8 Hz, 1H, H-6), 4.58 (m, 1H, H-3), 4.28 (m, 1H, H-16), 3.31 (dd, *J* = 12.6, 7.5 Hz, 1H, H-22), 3.22 (dd, *J* = 14.4, 7.2 Hz, 1H, H-25), 2.02 (s, 3H, Ac-CH_3), 1.44 (d, *J* = 6.9 Hz, 3H, 27-CH_3), 1.02 (s, 3H, 19-CH_3), 0.96 (d, *J* = 6.9 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 170.9 (C-2′), 170.6 (Ac-CO), 162.6 (C-5′), 152.3 (C-2″), 147.8 (C-6″), 139.8 (C-5), 132.4 (C-4″), 123.9 (C-5″), 122.4 (C-6), 120.8 (C-3″), 89.8 (C-22), 83.4 (C-16), 74.0 (C-3), 65.1 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.4 (C-13), 38.2 (C-4), 38.0 (C-20), 37.1 (C-1), 36.8 (C-10), 32.3 (C-7), 32.1 (C-15), 32.1 (C-25), 32.1 (C-24), 31.7 (C-8), 31.1 (C-23), 27.8 (C-2), 21.5 (Ac-CH_3), 20.7 (C-11), 19.4 (C-19), 18.9 (C-27), 18.4 (C-21), 16.5 (C-18). ESI-HRMS: *m*/*z* 574.3615 $[M+H]^+$ (Calcd for $C_{35}H_{48}N_3O_4$, 574.3639).

*4.1.5.5 (22R,25S)-3β-Acetoxy-5-en-25-(2′-[5′-(naphthalen-1″-yl)]-1′,3′,4′-oxadiazolyl) furostan (**6e**).*

White amorphous powder, yield 82.0%, mp 146.6–147.9 °C. ^{1}H NMR (600 MHz, $CDCl_3$, *δ*): 9.20 (d, *J* = 8.5 Hz, 1H, Ar-H), 8.15 (d, *J* = 7.3 Hz, 1H, Ar-H), 8.02 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.92 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.67 (m, 1H, Ar-H), 7.58 (m, 2H, Ar-H), 5.36 (d, *J* = 4.9 Hz, 1H, H-6), 4.59 (m, 1H, H-3), 4.30 (m, 1H, H-16), 3.34 (m, 1H, H-22), 3.27 (m, 1H, H-25), 2.03 (s, 3H, Ac-CH_3), 1.49 (d, *J* = 6.9 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.97 (d, *J* = 6.8 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 170.7 (C-2′), 169.9 (Ac-CO), 164.7 (C-5′), 139.8 (C-5), 134.0 (Ar-C), 132.5 (Ar-C), 130.2 (Ar-C), 128.8 (Ar-C), 128.4 (Ar-C), 128.2 (Ar-C), 126.8 (Ar-C), 126.4 (Ar-C), 125.0 (Ar-C), 122.5 (C-6), 120.9 (Ar-C), 89.9 (C-22), 83.5 (C-16), 74.0 (C-3), 65.2 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-4), 38.1 (C-20), 37.1 (C-1), 36.8 (C-10), 32.4 (C-25), 32.2 (C-7), 32.1 (C-15), 32.1 (C-23), 31.7 (C-8), 31.2 (C-24), 27.9 (C-2), 21.6 (Ac-CH_3), 20.8 (C-11), 19.5 (C-19), 19.0 (C-27), 18.6 (C-21), 16.6 (C-18). ESI-HRMS: *m*/*z* 623.3829 $[M+H]^+$ (Calcd for $C_{40}H_{51}N_2O_4$, 623.3843).

*4.1.6 General procedure for synthesis of compounds **7a–7e**.*

Compounds **6a–6e** (0.2 mmol) was dissolved in MeOH (6 mL), respectively, and KOH (33.6 mg, 0.6 mmol) was added. The mixture was stirred at room temperature for 1 h. Upon completion, the mixture was diluted with water and acidified with dilute HCl. The precipitate was filtered, washed with water and dried.

*4.1.6.1 (22R,25R)-3β-Hydroxy-5-en-25-(2′-[5′-methyl]-1′,3′,4′-oxadiazolyl) furostan (**7a**).*

White amorphous powder, yield 91.0%, mp 162.2–163.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 5.33 (d, J = 4.3 Hz, 1H, H-6), 4.28 (m, 1H, H-16), 3.51 (m, 1H, H-3), 3.28 (dd, J = 13.5, 7.8 Hz, 1H, H-22), 3.06 (dd, J = 14.1, 7.2 Hz, 1H, H-25), 2.49 (s, 3H, 5′-CH_3), 1.34 (d, J = 6.9 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.96 (d, J = 6.9 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 170.5 (C-2′), 163.6 (C-5′), 141.0 (C-5), 121.5 (C-6), 89.8 (C-22), 83.4 (C-16), 71.8 (C-3), 65.2 (C-17), 57.1 (C-14), 50.2 (C-9), 42.4 (C-4), 40.8 (C-12), 39.5 (C-13), 38.0 (C-20), 37.4 (C-1), 36.7 (C-10), 32.3 (C-7), 32.1 (C-15), 32.0 (C-25), 31.9 (C-24), 31.7 (C-8), 31.1 (C-23), 31.1 (C-2), 20.8 (C-11), 19.5 (C-19), 19.0 (C-27), 18.3 (C-21), 16.5 (C-18), 11.1 (5′-CH_3). ESI-HRMS: m/z 469.3427 $[M+H]^+$ (Calcd for $C_{29}H_{45}N_2O_3$, 469.3425).

*4.1.6.2 (22R,25R)-3β-Hydroxy-5-en-25-(2′-[5′-phenyl]-1′,3′,4′-oxadiazolyl) furostan (**7b**).*

White amorphous powder, yield 93.6%, mp 127.4–128.5 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 8.04 (dd, J = 8.0, 1.3 Hz, 2H, H-2″ and H-6″), 7.50 (m, 3H, H-3″, H-4″ and H-5″), 5.34 (d, J = 5.2 Hz, 1H, H-6), 4.29 (m, 1H, H-16), 3.52 (m, 1H, H-3), 3.31 (m, 1H, H-22), 3.20 (m, 1H, H-25), 1.43 (d, J = 7.1 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.96 (d, J = 6.9 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 170.3 (C-2′), 164.7 (C-5′), 141.0 (C-5), 131.6 (C-4″), 129.1 (2C, C-3″ and C-5″), 126.9 (2C, C-2″ and C-6″), 124.3 (C-1″), 121.5 (C-6), 89.9 (C-22), 83.5 (C-16), 71.9 (C-3), 65.2 (C-17), 57.1 (C-14), 50.2 (C-9), 42.4 (C-4), 40.8 (C-12), 39.5 (C-13), 38.1 (C-20), 37.4 (C-1), 36.8 (C-10), 32.4 (C-7), 32.2 (C-24), 32.1 (C-15), 32.1 (C-25), 31.8 (C-23), 31.7 (C-8), 31.2 (C-2), 20.8 (C-11), 19.6 (C-19), 19.0 (C-27), 18.5 (C-21), 16.6 (C-18). ESI-HRMS: m/z 531.3589 $[M+H]^+$ (Calcd for $C_{34}H_{47}N_2O_3$, 531.3581).

*4.1.6.3 (22R,25R)-3β-Hydroxy-5-en-25-(2′-[5′-p-methoxyphenyl]-1′,3′,4′-oxadiazolyl) furostan (**7c**).*

White amorphous powder, yield 92.5%, mp 150.3–151.6 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 7.97 (dd, J = 8.8, 1.9 Hz, 2H, H-2″ and H-6″), 6.98 (dd, J = 8.8, 1.9 Hz, 2H, H-3″ and H-5″), 5.34 (d, J = 5.0 Hz, 1H, H-6), 4.29 (m, 1H, H-16), 3.87 (s, 3H, H-4″-OCH_3), 3.52 (m, 1H, H-3), 3.31 (m, 1H, H-22), 3.18 (m, 1H, H-25), 1.42 (d, J = 7.0 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.96 (d, J = 6.7 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 169.8 (C-2′), 164.6 (C-5′), 162.3 (C-4″), 141.0 (C-5), 128.7 (2C, C-3″ and C-5″), 121.6 (C-6), 116.8 (C-1″), 114.5 (2C, C-2″ and C-6″), 89.9 (C-22), 83.5 (C-16), 71.9 (C-3), 65.2 (C-17), 57.1 (C-14), 55.6 (C-4″-OCH_3), 50.2 (C-9), 42.4 (C-4), 40.8 (C-12), 39.5 (C-13), 38.1 (C-20), 37.4 (C-1), 36.8 (C-10), 32.4 (C-7), 32.2 (C-15), 32.1 (C-25), 31.8 (C-8), 31.8 (C-24), 31.2 (C-23), 29.5 (C-2), 20.8 (C-11), 19.6

(C-19), 19.0 (C-27), 18.5 (C-21), 16.6 (C-18). ESI-HRMS: *m/z* 561.3684 $[M+H]^+$ (Calcd for $C_{35}H_{49}N_2O_4$, 561.3687).

*4.1.6.4 (22R,25R)-3β-Hydroxy-5-en-25-(2′-[5′-3″-pyridyl]-1′,3′,4′-oxadiazolyl) furostan (**7d**).*

White amorphous powder, yield 93.0%, mp 81.6–83.0 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 9.25 (s, 1H, H-2″), 8.76 (d, *J* = 2.8 Hz, 1H, H-6″), 8.36 (d, 1H, *J* = 7.9 Hz, H-4″), 7.47 (dd, *J* = 7.9, 2.8 Hz, 1H, H-5″), 5.34 (d, *J* = 4.8 Hz, 1H, H-6), 4.29 (m, 1H, H-16), 3.50 (m, 1H, H-3), 3.31 (dd, *J* = 13.5, 7.5 Hz, 1H, H-22), 3.22 (dd, *J* = 14.3, 7.1 Hz, 1H, H-25), 1.44 (d, *J* = 7.0 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.96 (d, *J* = 6.7 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 171.0 (C-2′), 162.5 (C-5′), 152.1 (C-2″), 147.7 (C-6″), 141.0 (C-5), 134.5 (C-4″), 124.0 (C-5″), 121.5 (C-6), 120.7 (C-3″), 89.8 (C-22), 83.5 (C-16), 71.8 (C-3), 65.1 (C-17), 57.1 (C-14), 50.2 (C-9), 42.4 (C-4), 40.8 (C-12), 39.5 (C-13), 38.1 (C-20), 37.4 (C-1), 36.8 (C-10), 32.4 (C-7), 32.1 (C-15), 32.1 (C-25), 32.1 (C-24), 31.8 (C-23), 31.7 (C-8), 31.1 (C-2), 20.8 (C-11), 19.6 (C-19), 19.0 (C-27), 18.5 (C-21), 16.6 (C-18). ESI-HRMS: *m/z* 532.3534 $[M+H]^+$ (Calcd for $C_{33}H_{46}N_3O_3$, 532.3534).

*4.1.6.5 (22R,25S)-3β-Hydroxy-5-en-25-(2′-[5′-(naphthalen-1″-yl)]-1′,3′,4′-oxadiazolyl) furostan (**7e**).*

White amorphous powder, yield 91.8%, mp 153.6–154.7 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 9.20 (d, *J* = 8.5 Hz, 1H, Ar-H), 8.15 (d, *J* = 7.3 Hz, 1H, Ar-H), 8.02 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.92 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.67 (m, 1H, Ar-H), 7.58 (m, 2H, Ar-H), 5.34 (d, *J* = 4.9 Hz, 1H, H-6), 4.30 (m, 1H, H-16), 3.52 (m, 1H, H-3), 3.34 (m, 1H, H-22), 3.27 (m, 1H, H-25), 1.49 (d, *J* = 6.9 Hz, 3H, 27-CH_3), 1.00 (s, 3H, 19-CH_3), 0.97 (d, *J* = 6.7 Hz, 3H, 21-CH_3), 0.78 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 169.9 (C-2′), 164.7 (C-5′), 141.0 (C-5), 134.0 (Ar-C), 132.5 (Ar-C), 130.2 (Ar-C), 128.8 (Ar-C), 128.4 (Ar-C), 128.2 (Ar-C), 126.8 (Ar-C), 126.4 (Ar-C), 125.0 (Ar-C), 121.6 (C-6), 121.0 (Ar-C), 89.9 (C-22), 83.5 (C-16), 71.9 (C-3), 65.2 (C-17), 57.1 (C-14), 50.2 (C-9), 42.4 (C-4), 40.8 (C-12), 39.5 (C-13), 38.1 (C-20), 37.4 (C-1), 36.8 (C-10), 32.4 (C-7), 32.2 (C-15), 32.1 (C-25), 32.1 (C-24), 31.8 (C-23), 31.2 (C-8), 30.5 (C-2), 20.8 (C-11), 19.6 (C-19), 19.0 (C-27), 18.6 (C-21), 16.6 (C-18). ESI-HRMS: *m/z* 581.3777 $[M+H]^+$ (Calcd for $C_{38}H_{49}N_2O_3$, 581.3738).

*4.1.7 General procedure for synthesis of compounds **8a–8e**.*

To a solution of the appropriate compounds **5a–5e** (0.23 mmol) in dry toluene (6 mL) was added Lawesson reagent (186 mg, 0.46 mmol) and the reaction mixture was refluxed for 1 h. When TLC showed the completion

of the reaction, the solvent was removed in vacuo. The crude product was purified by flash chromatography on silica gel using petroleum ether-ethyl acetate (10:1) as eluent.

*4.1.7.1 (22R,25R)-3β-Acetoxy-5-en-25-(2′-[5′-methyl]-1′,3′,4′-thiadiazolyl) furostan (**8a**).*

White amorphous powder, yield 75.6%, mp 92.2–93.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 5.35 (d, J = 3.8 Hz, 1H, H-6), 4.57 (m, 1H, H-3), 4.27 (dd, J = 13.1, 7.5 Hz, 1H, H-16), 3.32 (m, 1H, H-22), 3.26 (m, 1H, H-25), 2.72 (s, 3H, 5′-CH_3), 2.01 (s, 3H, Ac-CH_3), 1.37 (d, J = 6.9 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.94 (d, J = 7.2 Hz, 3H, 21-CH_3), 0.76 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 176.7 (C-2′), 170.7 (Ac-CO), 164.7 (C-5′), 139.8 (C-5), 122.4 (C-6), 90.0 (C-22), 83.4 (C-16), 74.0 (C-3), 65.1 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-4), 37.9 (C-20), 37.1 (C-1), 36.8 (C-10), 36.5 (C-25), 32.3 (C-7), 32.1 (C-15), 31.7 (C-8), 31.3 (C-24), 30.8 (C-23), 27.8 (C-2), 21.5 (Ac-CH_3), 20.7 (C-11), 19.4 (C-19), 18.9 (C-27), 18.5 (C-21), 16.5 (C-18), 15.8 (5′-CH_3). ESI-HRMS: m/z 527.3300 $[M+H]^+$ (Calcd for $C_{31}H_{47}N_2O_3S$, 527.3302).

*4.1.7.2 (22R,25R)-3β-Acetoxy-5-en-25-(2′-[5′-phenyl]-1′,3′,4′-thiadiazolyl) furostan (**8b**).*

White amorphous powder, yield 72.0%, mp 66.4–67.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 7.94 (dd, J = 5.1, 1.7 Hz, 2H, H-2″ and H-6″), 7.46 (m, 3H, H-3″, H-4″ and H-5″), 5.36 (d, J = 3.5 Hz, 1H, H-6), 4.60 (m, 1H, H-3), 4.30 (dd, J = 13.0, 7.1 Hz, 1H, H-16), 3.40 (m, 1H, H-22), 3.30 (m, 1H, H-25), 2.02 (s, 3H, Ac-CH_3), 1.45 (d, J = 6.6 Hz, 3H, 27-CH_3), 1.02 (s, 3H, 19-CH_3), 0.94 (d, J = 6.7 Hz, 3H, 21-CH_3), 0.78 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 176.2 (C-2′), 170.7 (Ac-CO), 168.2 (C-5′), 139.8 (C-5), 131.0 (C-4″), 130.5 (C-1″), 129.2 (2C, C-3″ and C-5″), 128.0 (2C, C-2″ and C-6″), 122.5 (C-6), 90.0 (C-22), 83.4 (C-16), 74.0 (C-3), 65.1 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-4), 38.0 (C-20), 37.1 (C-1), 36.8 (C-10), 36.6 (C-25), 32.3 (C-7), 32.1 (C-15), 31.7 (C-8), 31.3 (C-24), 30.9 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.7 (C-11), 19.5 (C-19), 19.0 (C-27), 18.6 (C-21), 16.6 (C-18). ESI-HRMS: m/z 589.3434 $[M+H]^+$ (Calcd for $C_{36}H_{49}N_2O_3S$, 589.3458).

*4.1.7.3 (22R,25R)-3β-Acetoxy-5-en-25-(2′-[5′-p-methoxyphenyl]-1′,3′,4′-thiadiazolyl) furostan (**8c**).*

White amorphous powder, yield 74.0%, mp 136.9–138.2 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 7.87 (dd, J = 8.7 Hz, 2H, H-2″ and H-6″), 6.96 (d, J = 8.7 Hz, 2H, H-3″ and H-5″), 5.36 (br.s, 1H, H-6), 4.59 (m, 1H, H-3), 4.29 (m, 1H, H-16), 3.86 (s, 3H, H-4″-OCH_3), 3.39-3.30 (m, 2H, H-22, H-25), 2.02 (s, 3H, Ac-CH_3), 1.44 (d, J

= 6.9 Hz, 3H, 27-CH_3), 1.02 (s, 3H, 19-CH_3), 0.95 (d, *J* = 6.8 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 175.4 (C-2′), 170.7 (Ac-CO), 167.9 (C-5′), 161.9 (C-4″), 139.8 (C-5), 129.5 (2C, C-3″ and C-5″), 123.2 (C-1″), 122.5 (C-6), 114.6 (2C, C-2″ and C-6″), 89.7 (C-22), 83.4 (C-16), 74.0 (C-3), 65.2 (C-17), 57.0 (C-14), 55.6 (C-4″-OCH_3), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-4), 38.0 (C-20), 37.1 (C-1), 36.8 (C-10), 32.4 (C-7), 32.1 (C-15), 31.7 (C-25), 31.7 (C-8), 31.3 (C-24), 30.9 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.8 (C-11), 19.5 (C-19), 19.5 (C-27), 19.0 (C-21), 16.6 (C-18). ESI-HRMS: *m*/*z* 619.3524 $[M+H]^+$ (Calcd for $C_{37}H_{51}N_2O_4S$, 619.3564).

*4.1.7.4 (22R,25R)-3β-Acetoxy-5-en-25-(2′-[5′-3″-pyridyl]-1′,3′,4′-thiadiazolyl) furostan (**8d**).*

White amorphous powder, yield 68.9%, mp 72.0–73.1 °C. ^{1}H NMR (600 MHz, $CDCl_3$, *δ*): 9.11 (brs, 1H, H-2″), 8.71 (brs, 1H, H-6″), 8.32 (d, *J* = 7.6 Hz, 1H, H-4″), 7.44 (dd, *J* = 7.6, 4.5 Hz, 1H, H-5″), 5.36 (brs, 1H, H-6), 4.59 (m, 1H, H-3), 4.29 (dd, *J* = 12.6, 6.9 Hz, 1H, H-16), 3.45 (m, 1H, H-22), 3.33 (m, 1H, H-25), 2.02 (s, 3H, Ac-CH_3), 1.47 (d, *J* = 6.9 Hz, 3H, 27-CH_3), 1.02 (s, 3H, 19-CH_3), 0.95 (d, *J* = 6.5 Hz, 3H, 21-CH_3), 0.78 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 176.9 (C-2′), 170.7 (Ac-CO), 164.7 (C-5′), 151.5 (C-2″), 148.7 (C-6″), 139.8 (C-5), 135.0 (C-4″), 127.0 (C-3″), 124.1 (C-5″), 122.5 (C-6), 90.0 (C-22), 83.5 (C-16), 74.0 (C-3), 65.1 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-4), 38.0 (C-20), 37.1 (C-1), 36.8 (C-10), 36.6 (C-25), 32.3 (C-7), 32.1 (C-15), 31.7 (C-8), 31.3 (C-24), 30.9 (C-23), 27.9 (C-2), 21.6 (Ac-CH_3), 20.7 (C-11), 19.5 (C-19), 19.0 (C-27), 18.4 (C-21), 16.6 (C-18). ESI-HRMS: *m*/*z* 590.3415 $[M+H]^+$ (Calcd for $C_{35}H_{48}N_3O_3S$, 590.3411).

*4.1.7.5 (22R,25S)-3β-Acetoxy-5-en-25-(2′-[5′-(naphthalen-1″-yl)]-1′,3′,4′-thiadiazolyl) furostan (**8e**).*

White amorphous powder, yield 75.5%, mp 73.5–74.2 °C. ^{1}H NMR (600 MHz, $CDCl_3$, *δ*): 8.68 (dd, *J* = 8.3, 5.0 Hz, 1H, Ar-H), 7.97 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.91 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.78 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.55 (m, 3H, Ar-H), 5.36 (d, *J* = 4.9 Hz, 1H, H-6), 4.59 (m, 1H, H-3), 4.31 (m, 1H, H-16), 3.50 (m, 1H, H-22), 3.35 (m, 1H, H-25), 2.02 (s, 3H, Ac-CH_3), 1.51 (d, *J* = 6.8 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.98 (d, *J* = 6.8 Hz, 3H, 21-CH_3), 0.78 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, *δ*): 177.0 (C-2′), 170.7 (Ac-CO), 167.2 (C-5′), 139.8 (C-5), 134.0 (Ar-C), 131.3 (Ar-C), 130.7 (Ar-C), 129.7 (Ar-C), 128.6 (Ar-C), 127.8 (Ar-C), 127.3 (Ar-C), 126.7 (Ar-C), 125.8 (Ar-C), 125.1 (Ar-C), 122.5 (C-6), 90.0 (C-22), 83.5 (C-16), 74.0 (C-3), 65.1 (C-17), 57.0 (C-14), 50.1 (C-9), 40.8 (C-12), 39.5 (C-13), 38.2 (C-4), 38.0 (C-20), 37.1 (C-1), 36.8 (C-10), 36.5 (C-25),

32.3 (C-7), 32.1 (C-15), 31.7 (C-8), 31.3 (C-24), 31.0 (C-23), 27.9 (C-2), 21.5 (Ac-CH_3), 20.7 (C-11), 19.4 (C-19), 19.0 (C-27), 18.5 (C-21), 16.5 (C-18). ESI-HRMS: *m/z* 639.3617 $[M+H]^+$ (Calcd for $C_{40}H_{51}N_2O_3S$, 639.3615).

*4.1.8 General procedure for synthesis of compounds **9a–9e**.*

Compounds **8a–8e** (0.15 mmol) were used for the synthesis as described in Section 4.1.6.

*4.1.8.1 (22R,25R)-3β-Hydroxy-5-en-25-(2′-[5′-methyl]-1′,3′,4′-thiadiazolyl) furostan (**9a**).*

White amorphous powder, yield 92.0%, mp 135.5–136.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 5.32 (d, J = 3.7 Hz, 1H, H-6), 4.27 (dd, J = 13.1, 7.4 Hz, 1H, H-16), 3.50 (m, 1H, H-3), 3.32 (m, 1H, H-22), 3.26 (m, 1H, H-25), 2.72 (s, 3H, 5′-CH_3), 1.37 (d, J = 7.0 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.94 (d, J = 7.0 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 176.8 (C-2′), 164.7 (C-5′), 141.0 (C-5), 121.5 (C-6), 90.0 (C-22), 83.4 (C-16), 71.8 (C-3), 65.2 (C-17), 57.1 (C-14), 50.2 (C-9), 42.4 (C-4), 40.8 (C-12), 39.5 (C-13), 37.9 (C-20), 37.4 (C-1), 36.7 (C-10), 36.5 (C-25), 32.3 (C-7), 32.1 (C-15), 31.7 (C-8), 31.3 (C-24), 31.0 (C-2), 30.8 (C-23), 20.8 (C-11), 19.5 (C-19), 19.0 (C-27), 18.5 (C-21), 16.5 (C-18), 15.8 (5′-CH_3). ESI-HRMS: *m/z* 485.3185 $[M+H]^+$ (Calcd for $C_{29}H_{45}N_2O_2S$, 485.3196).

*4.1.8.2 (22R,25R)-3β-Hydroxy-5-en-25-(2′-[5′-phenyl]-1′,3′,4′-thiadiazolyl) furostan (**9b**).*

White amorphous powder, yield 93.6%, mp 134.0–135.4 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 7.94 (dd, J = 5.1, 1.6 Hz, 2H, H-2″ and H-6″), 7.47 (m, 3H, H-3″, H-4″ and H-5″), 5.34 (d, J = 4.5 Hz, 1H, H-6), 4.29 (m, 1H, H-16), 3.51 (m, 1H, H-3), 3.42 (m, 1H, H-22), 3.30 (m, 1H, H-25), 1.46 (d, J = 6.9 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.95 (d, J = 6.5 Hz, 3H, 21-CH_3), 0.79 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 176.3 (C-2′), 168.2 (C-5′), 141.0 (C-5), 131.0 (C-4″), 130.5 (C-1″), 129.2 (2C, C-3″ and C-5″), 128.0 (2C, C-2″ and C-6″), 121.5 (C-6), 90.0 (C-22), 83.4 (C-16), 71.8 (C-3), 65.2 (C-17), 57.1 (C-14), 50.2 (C-9), 42.2 (C-4), 40.8 (C-12), 39.5 (C-13), 38.0 (C-20), 37.4 (C-1), 36.8 (C-10), 36.6 (C-25), 32.4 (C-7), 32.1 (C-15), 31.7 (C-8), 31.3 (C-24), 31.2 (C-2), 30.9 (C-23), 20.8 (C-11), 19.5 (C-19), 19.0 (C-27), 18.5 (C-21), 16.6 (C-18). ESI-HRMS: *m/z* 547.3324 $[M+H]^+$ (Calcd for $C_{34}H_{47}N_2O_2S$, 547.3353).

*4.1.8.3 (22R,25R)-3β-Hydroxy-5-en-25-(2′-[5′-p-methoxyphenyl]-1′,3′,4′-thiadiazolyl) furostan (**9c**).*

White amorphous powder, yield 92.5%, mp 193.6–194.2 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 7.87 (dd, J = 8.7 Hz, 2H, H-2″ and H-6″), 6.97 (d, J = 8.7 Hz, 2H, H-3″ and H-5″), 5.34 (d, J = 2.0 Hz, 1H, H-6), 4.59 (m, 1H, H-3), 4.29 (m, 1H, H-16), 3.86 (s, 3H, H-4″-OCH_3), 3.51 (m, 1H, H-3), 3.39-3.30 (m, 2H, H-22, H-25), 1.44 (d, J = 6.9 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.96 (d, J = 6.8 Hz, 3H, 21-CH_3), 0.77 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 175.3 (C-2′), 167.9 (C-5′), 161.8 (C-4″), 141.0 (C-5), 129.5 (2C, C-3″ and C-5″), 123.3 (C-1″), 121.6 (C-6), 114.6 (2C, C-2″ and C-6″), 89.7 (C-22), 83.4 (C-16), 71.9 (C-3), 65.2 (C-17), 57.1 (C-14), 50.2 (C-9), 42.4 (C-4), 40.8 (C-12), 39.6 (C-13), 38.0 (C-20), 37.4 (C-1), 36.8 (C-10), 36.6 (C-25), 32.4 (C-7), 32.1 (C-15), 31.8 (C-8), 31.7 (C-2), 31.4 (C-24), 30.9 (C-23), 20.8 (C-11), 19.6 (C-19), 19.5 (C-21), 19.0 (C-27), 16.6 (C-18). ESI-HRMS: *m*/*z* 577.3559 $[M+H]^+$ (Calcd for $C_{35}H_{48}N_3O_3S$, 577.3558).

*4.1.8.4 (22R,25R)-3β-Hydroxy-5-en-25-(2′-[5′-3″-pyridyl]-1′,3′,4′-thiadiazolyl) furostan (**9d**).*

White amorphous powder, yield 91.4%, mp 135.9–137.2 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 9.12 (brs, 1H, H-2″), 8.72 (brs, 1H, H-6″), 8.33 (d, J = 7.7 Hz, 1H, H-4″), 7.45 (m, 1H, H-5″), 5.33 (brs, 1H, H-6), 4.30 (m, 1H, H-16), 3.51 (m, 1H, H-3), 3.45 (m, 1H, H-22), 3.34 (m, 1H, H-25), 1.47 (d, J = 6.8 Hz, 3H, 27-CH_3), 1.01 (s, 3H, 19-CH_3), 0.96 (d, J = 6.7 Hz, 3H, 21-CH_3), 0.79 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 177.0 (C-2′), 164.7 (C-5′), 151.5 (C-2″), 148.7 (C-6″), 141.0 (C-5), 135.0 (C-4″), 127.1 (C-3″), 124.2 (C-5″), 121.5 (C-6), 89.9 (C-22), 83.5 (C-16), 71.8 (C-3), 65.1 (C-17), 57.1 (C-14), 50.2 (C-9), 42.4 (C-4), 40.8 (C-12), 39.5 (C-13), 38.0 (C-20), 37.4 (C-1), 36.8 (C-10), 36.6 (C-25), 32.4 (C-7), 32.1 (C-15), 31.8 (C-8), 31.7 (C-2), 31.3 (C-24), 30.9 (C-23), 20.8 (C-11), 19.6 (C-19), 19.0 (C-27), 18.5 (C-21), 16.6 (C-18). ESI-HRMS: *m*/*z* 548.3304 $[M+H]^+$ (Calcd for $C_{33}H_{46}N_3O_2S$, 584.3305).

*4.1.8.5 (22R,25S)-3β-Hydroxy-5-en-25-(2′-[5′-(naphthalen-1″-yl)]-1′,3′,4′-thiadiazolyl) furostan (**9e**).*

White amorphous powder, yield 92.5%, mp 86.5–87.8 °C. ^{1}H NMR (600 MHz, $CDCl_3$, δ): 8.68 (dd, J = 8.3, 5.4 Hz, 1H, Ar-H), 7.97 (d, J = 8.3 Hz, 1H, Ar-H), 7.91 (d, J = 8.3 Hz, 1H, Ar-H), 7.78 (d, J = 8.3 Hz, 1H, Ar-H), 7.55 (m, 3H, Ar-H), 5.32 (br.s, 1H, H-6), 4.30 (m, 1H, H-16), 3.50 (m, 1H, H-3), 3.48 (m, 1H, H-22), 3.34 (m, 1H, H-25), 1.50 (d, J = 6.9 Hz, 3H, 27-CH_3), 1.00 (s, 3H, 19-CH_3), 0.97 (d, J = 6.9 Hz, 3H, 21-CH_3), 0.78 (s, 3H, 18-CH_3). ^{13}C NMR (150 MHz, $CDCl_3$, δ): 177.0 (C-2′), 167.2 (C-5′), 141.0 (C-5), 134.0 (Ar-C), 131.3 (Ar-C), 130.7 (Ar-C), 129.7 (Ar-C), 128.6 (Ar-C), 127.8 (Ar-C), 127.3 (Ar-C), 126.7 (Ar-C), 125.8 (Ar-C), 125.1 (Ar-C), 121.5 (C-6), 90.0 (C-22), 83.5 (C-16), 71.8 (C-3), 65.2 (C-17), 57.1 (C-14), 50.2 (C-9), 42.4 (C-4), 40.8

(C-12), 39.5 (C-13), 38.0 (C-20), 37.4 (C-1), 36.7 (C-10), 36.5 (C-25), 32.4 (C-7), 32.1 (C-15), 31.7 (C-8), 31.3 (C-24), 31.0 (C-2), 31.0 (C-23), 20.8 (C-11), 19.5 (C-19), 19.0 (C-27), 18.5 (C-21), 16.6 (C-18). ESI-HRMS: *m*/*z* 597.3497 $[M+H]^+$ (Calcd for $C_{38}H_{49}N_2O_2S$, 597.3509).

4.2 Cell Culture

Human hepatoma (HepG2), lung carcinoma (A549), breast cancer (MCF-7), colorectal cancer (HCT-116) and gastric epithelial (GES-1) cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium (HyClone, Utah, USA) supplemented with 10% FBS (Gibco BRL, Grand Island, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (HyClone, Utah, USA) in a humidified atmosphere of 5% CO_2 at 37 ^oC.

4.3 In vitro cytotoxic activity

Cytotoxic activities of all compounds against HepG2, A549, MCF-7, HCT-116 and GES-1 cell lines were evaluated by the MTT method. The cells (5×10^3/well) were seeded into the 96-well plates filled with culture medium containing various concentrations of test samples. They were incubated at 37 ^oC, 5% CO_2 for 48 h. After incubation, the cells were incubated with 10 μL of MTT (Sigma Chemical Co., Ltd., USA) solution (5 mg/mL in PBS) for 4 h. Subsequently, the growth medium was replaced with 100 μL of DMSO and the absorbance values were measured at 492 nm using microplate reader (Bio-Rad iMARK, USA). The IC_{50} was defined as the concentration of the compound that inhibited cell proliferation by 50%. Three replicates were performed.

4.4 Determination of morphological changes of cells

For cell morphological analysis, A549 cells were grown in 6-well plates (2×10^5 cells/well). After stabilization for 24 h, compound **8d** (15 or 30 μM) and DG (30 μM) were cultured together with the cells for 48 h. Control cells were incubated with 0.1% DMSO. A phase contrast microscopy (Olympus, Tokyo, Japan) was used to observe the cellular morphology.

For DAPI staining experiments, A549 cells were grown in 6-well plates (2×10^5 cells/well). After 24 h of

incubation, the cells were then treated with compound **8d** (15 or 30 μM) and DG (30 μM) for 48 h. Control cells were exposed to 0.1% DMSO. The cells were fixed with 4% formaldehyde for 1 h at 4 °C. After fixation, the cells were treated with 5 μM DAPI (4',6-diamidino-2-phenylindole) at 37 °C in the dark for 10 min. Then the cells were observed using a fluorescence microscope [32].

4.5 Apoptosis analysis by flow cytometry

A549 cells were incubated with compound **8d** and DG at different concentrations (0, 15 and 30 μM). After 48 h, the cells were washed twice in PBS and resuspended in the Annexin V-FITC/PI staining solution according to the manufacturer's instruction (BD Pharmingen Co., Ltd., USA). The cells were detected to analyze apoptosis by a flow cytometry (Beckman Coulter, USA).

4.6 Analysis of mitochondrial membrane potential

A549 cells were incubated with compound **8d** for 48 h, then washed with PBS and stained with the lipophilic cationic dye JC-1 according to the manufacturer's instruction (KGA601, KeyGEN Biotech). The percentage of cells with healthy or collapsed mitochondrial membrane potentials was monitored by flow cytometry analysis.

4.7 Western Blot Analysis

After treatment with compound **8d** (0, 7.5, 15 and 30 μM) and DG (0, 15 and 30 μM), A549 cells were harvested and lysed in RIPA buffer and boiled for 10 min at 100 °C. Equal amount of protein (30 μg) were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA and probed with a 1:1000 dilution of primary antibody against Cl-caspase-3 (Cell Singaling Technology, Inc., Danvers, MA, USA), Cl-caspase-9 (Cell Singaling Technology Inc), PARP (Cell Singaling Technology, Inc), Bcl-2 (Santa Cruz Biotechnology, Inc), Cytochrome *c* (Proteintech Group, Inc), Bax (Proteintech Group, Inc), β-actin (Ding-Guo Biotech Ltd., Beijing). Then the membranes were incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody for 2 h. Positive bands were visualized on a X-ray film using an enhanced chemiluminescence system (Kodak).

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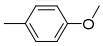
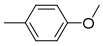
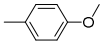
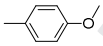
Table 1

Cytotoxic activities of compounds **6a-6e**, **7a-7e**, **8a-8e**, and **9a-9e** in human cells.

compound	R_1	R_2	IC_{50}[a] (μM)				
			HepG2	A549	MCF-7	HCT-116	GES-1
6a	CH_3	Ac	57.64±1.17	34.38±1.22	44.81±2.17	78.82±2.81	98.24±1.17
6b		Ac	63.24±1.34	84.49±2.57	>100	>100	82.34±3.56
6c		Ac	-	-	-	-	151.0±6.31
6d		Ac	82.56±2.07	33.55±1.34	>100	72.53±2.51	62.40±2.14
6e		Ac	-	-	-	-	70.08±4.37
7a	CH_3	H	34.23±0.87	30.36±1.09	20.37±1.03	51.63±2.01	72.77±4.16
7b		H	39.51±1.01	64.16±2.77	52.97±2.47	>100	119.7±5.43
7c		H	-	-	-	>100	-
7d		H	>100	98.13±3.07	>100	>100	198.9±6.43
7e		H	29.24±1.09	31.26±1.11	69.66±2.44	>100	298.07±7.34
8a	CH_3	Ac	34.25±1.12	40.27±1.36	47.72±1.39	>100	75.78±4.35
8b		Ac	-	-	-	-	-
8c		Ac	-	-	-	-	-
8d		Ac	11.73±0.67	3.93±0.37	>100	29.56±1.02	420.4±5.37
8e		Ac	-	>100	-	>100	-
9a	CH_3	H	33.16±1.14	36.71±1.35	33.75±1.25	48.89±1.38	107.4±2.36
9b		H	32.14±1.03	15.04±0.87	27.37±1.05	48.91±1.43	527.0±5.57
9c		H	23.05±1.02	16.40±0.97	>100	>100	208.7±5.38
9d		H	8.83±0.67	7.677±0.57	>100	51.65±2.87	354.4±7.17
9e		H	32.90±1.47	18.26±0.95	63.77±2.43	46.61±1.58	200.7±5.62
Diosgenin	–	–	33.87±1.37	26.41±1.42	23.91±1.34	49.11±2.11	100.7±4.53
Mitomycin C	–	–	32.63±1.22	11.03±1.04	16.71±1.02	11.03±1.01	21.12±1.01

[a] IC_{50}: concentration of the tested compound that inhibits 50% of cell growth. All data are presented as means ± SD of three independent experiments.

"-" not active.

1. Legends for Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig. 5 and Scheme 1

Fig. 1. Structure of diosgenin.

Fig. 2. Examples of 1,3,4-oxadiazole/thiadiazole-based antitumor compounds and designed diosgenin 1,3,4-oxadiazolyl/thiadiazolyl derivatives.

Fig. 3. Compound **8d** and DG induced apoptosis in A549 cells. (A) Morphological changes of A549 cells were observed with a phase contrast microscope (Scale bar: 50 μm). (B) The nuclear morphology changes of A549 cells were detected by DAPI staining (Scale bar: 50 μm).

Fig. 4. A549 cells were treated with compound **8d** and DG (0, 15, and 30 μM) for 48 h, and apoptosis was measured by flow cytometry after staining with Annexin V/PI, ***P < .001 versus control.

Fig. 5. Effect of compound **8d** and DG on the expression of apoptosis-related proteins in A549 cells was assayed by Western blot analysis. β-actin was employed as loading control, ***P< .001 versus control. A representative result from three separate experiments is shown.

Fig. 6. Compound **8d** induced mitochondrial depolarization in A549 cells, **P< .01; ***P< .001 versus control.

Fig. 7. The expression levels of apoptosis-related proteins, including Bax, Bcl-2, and cyto-*c* were assayed by Western blot analysis. *β*-actin was employed as loading control, **P< .01; ***P< .001 versus control.

Scheme 1. Synthesis of diosgenin 1,3,4-oxadiazolyl/thiadiazolyl derivatives. Reagents and conditions: (a) Ac_2O, dry pyridine, dry CH_2Cl_2, rt, 6 h; (b) $NaBH_3CN$, AcOH, CH_2Cl_2, 8 h; (c) Jones reagent, THF/acetone (1/1), rt; (d) appropriate acylhydrazines, TBTU, DIPEA, CH_2Cl_2, rt, 2 h; (e) $POCl_3$, 80 ^oC, 1 h; (f) KOH, MeOH, rt, 1 h; (g) LR, toluene, 110 ^oC, 1 h; (h) KOH, MeOH, rt, 1 h.

2. Graphics for Fig. 1, Fig. 2, Fig. 3, Fig. 4, Fig. 5 and Scheme 1

Fig. 1

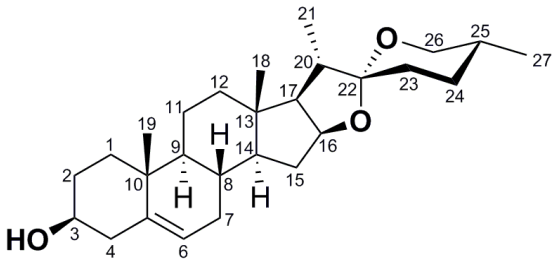


Fig. 2

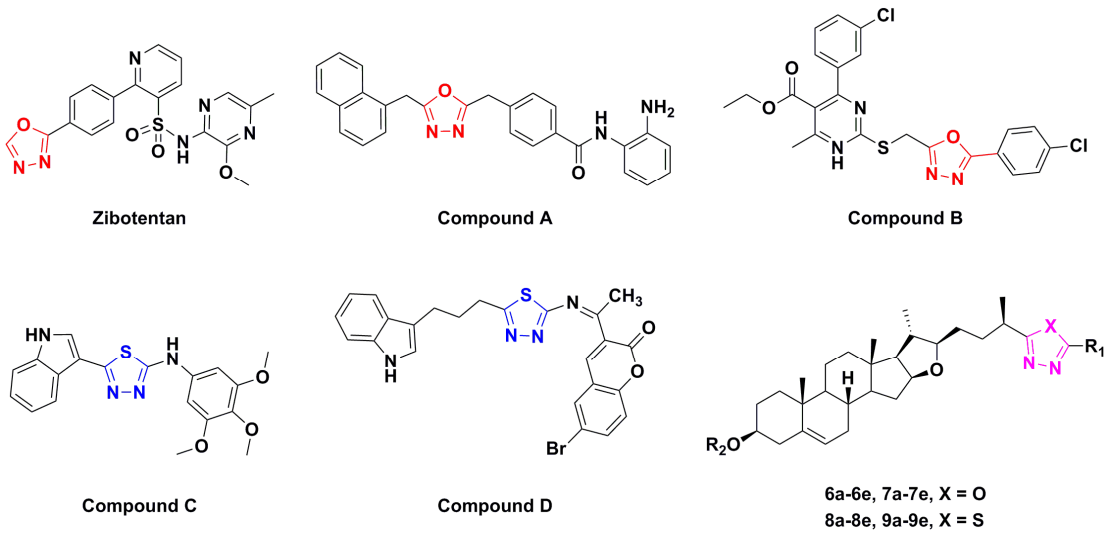


Fig. 3

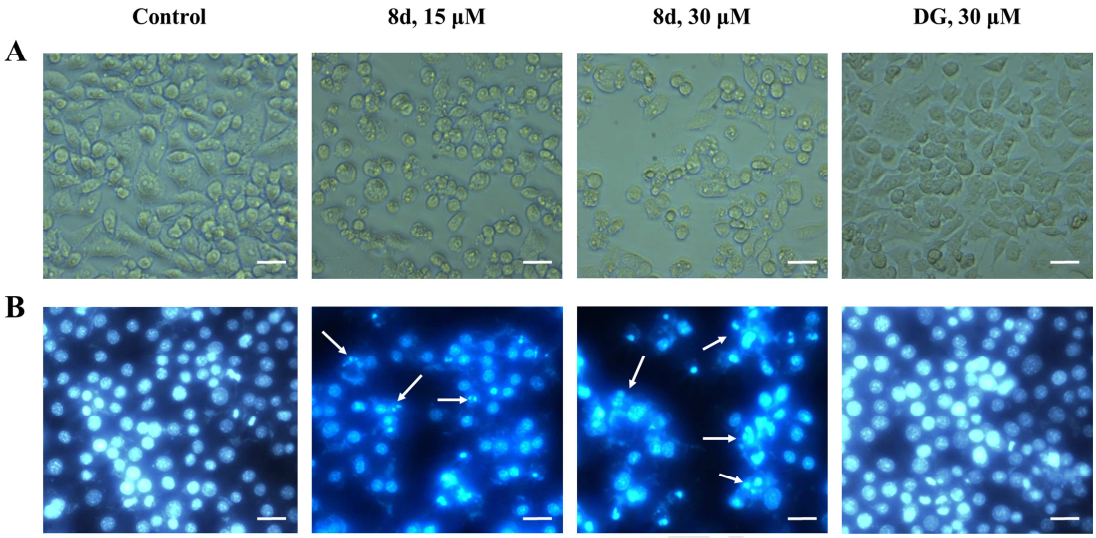


Fig. 4

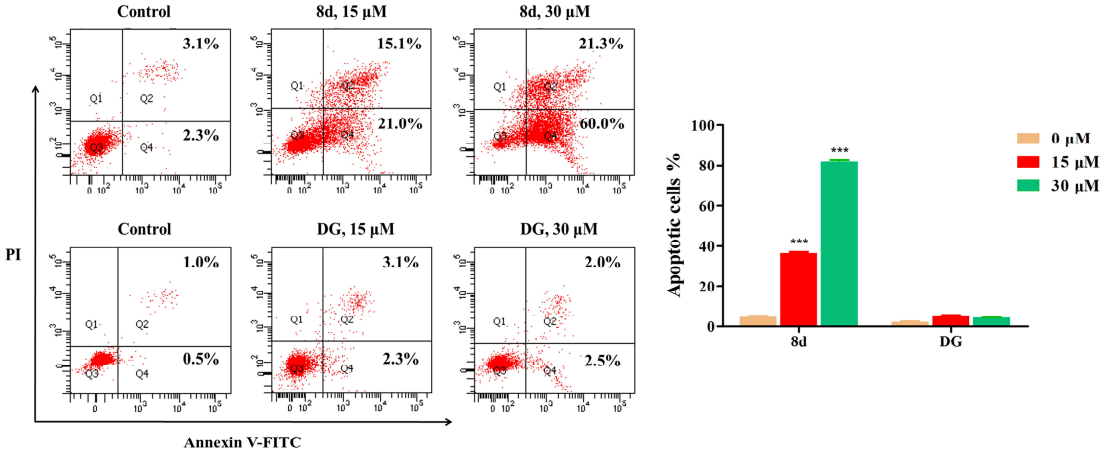


Fig. 5

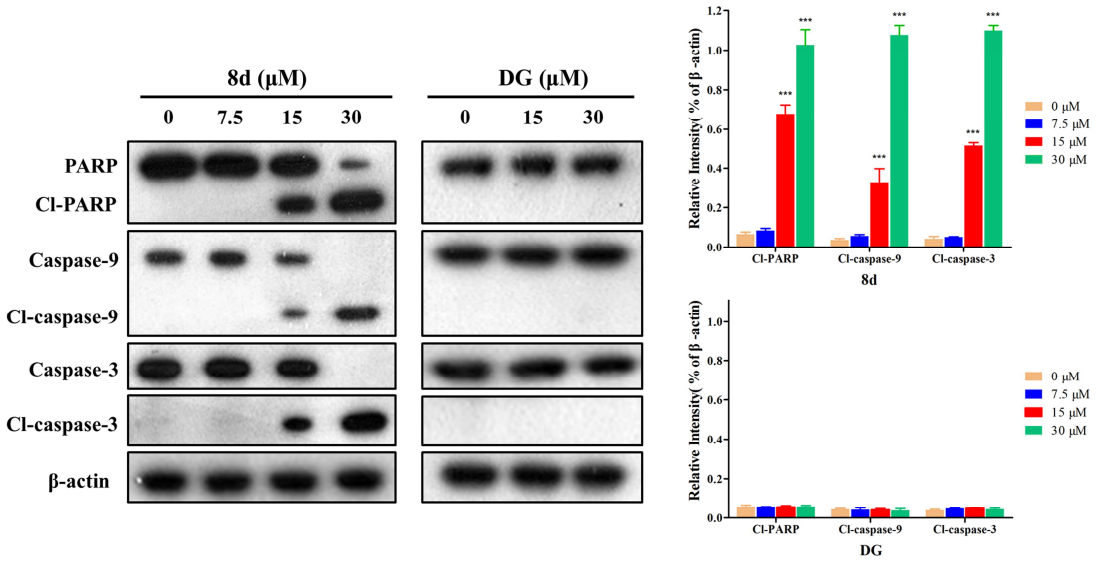


Fig. 6

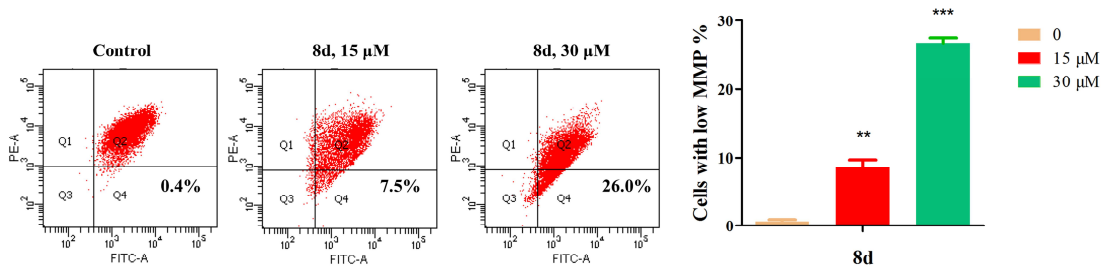
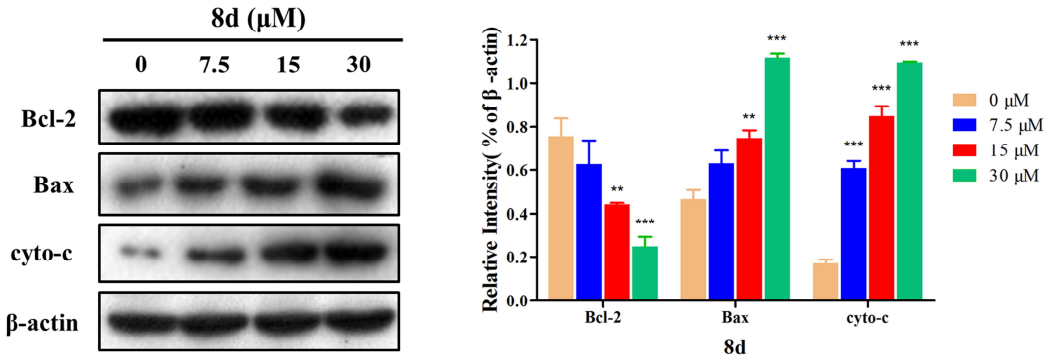
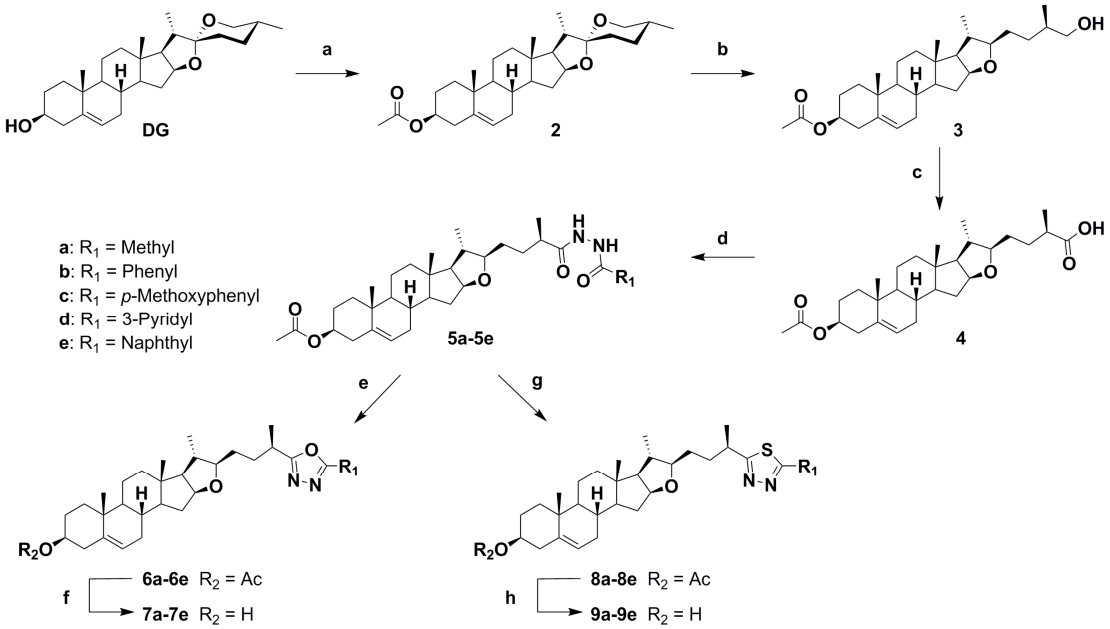


Fig. 7



Scheme 1



Highlights

- 20 novel diosgenin 1,3,4-oxadiazolyl/thiadiazolyl derivatives were designed and synthesized.
- The compounds were evaluated for their cytotoxic activity.
- Compound **8d** exhibited potent cytotoxic activity against A549 cell line.
- Compound **8d** induced A549 cells apoptosis via the mitochondria-related pathway.

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: