Design, Synthesis and Biological Evaluation of Steroidal Glycoconjugates as Potential Antiproliferative Agents

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To systematically evaluate the impact of neoglycosylation upon the anticancer activities and selectivity of steroids, four series of neoglycosides of diosgenin, pregnenolone, dehydroepiandrosterone and estrone were designed and synthesized according to the neoglycosylation approach. The structures of all the products were elucidated by NMR analysis, and the stereochemistry of C20-MeON-pregnenolone was confirmed by crystal X-ray diffraction. The compounds' cytotoxicity on five human cancer cell lines was evaluated using a Cell Counting Kit-8 assay, and structure–activity relationships (SAR) are discussed. 2-deoxy-D-glucoside **5k** displayed the most potent antiproliferative activities against HepG2 cells with an IC_{50} value of 1.5 μM. Further pharmacological experiments on compound **5k** on HepG2 cells revealed that it could cause morphological changes and cell-cycle arrest at the G0/G1 phase and then induced the apoptosis, which might be associated with the enhanced expression of high-mobility group Box 1 (HMGB1). Taken together, these findings prove that the neoglycosylation of steroids could be a promising strategy for the discovery of potential antiproliferative agents.

Introduction

Steroids are a group of biological signaling molecules widely distributed in nature that play crucial roles in various physiological functions including the metabolism, electrolyte balance, and reproduction.[1] Because of their inherent ability to penetrate cell membranes and bind to the nuclear and membrane receptors, several types of steroids have been developed as drugs for the treatment of a large number of diseases.[2] In recent years, steroid-based anticancer agents have been extensively studied as enzyme inhibitors and cytotoxic drugs such as abiraterone[3] and galeterone,[4] so chemical modification of steroids continues to attract interest, and many synthetic strategies have been generated to extend the varieties or establish the stereochemistry of the functional groups on the steroidal skeleton.[5] Among these researches, modifications to the D-ring have exhibited some advantages in enhancing the affinity with the corresponding receptor[6] and showed improved biological effects, especially in anticancer activities.[7] Moreover, it is widely believed that the glycosylation of steroids could change their physiological properties, alter their biological activity, and even direct the molecule to the active site.[8] Thus, in this research, we attempted to combine D-ring modification with the introduction of sugar for steroidal anticancer agents screening.

Neoglycosylation, a divergent chemoselective reaction between free reducing sugars and *N*-methoxyamino-substituted acceptors, could provide a rapid strategy for differential glycosylation of a selected target scaffold and thereby avoids the need for subsequent post-glycosylation modification/deprotection to produce the desired glycoconjugates.[9] It has been reported that neoglycosylation could dramatically altered their cytotoxicity and selectivity against cancer cell lines.[10] To date, a number of natural products and small molecules, such as cardenolide,[11] colchicine,[12] lanosterol,[13] and betulinic acid,[14] have been employed for the neoglycosylation and anticancer assay. In our previous work, we found that the neoglycosides of tigogenin could significantly enhance its anticancer activity,[15] and compound **Tg29** ((3*R*)-*N*-methoxyamino-tigogenin-β-2-deoxy-D-galactoside) exhibited IC_{50} values even reaching 2.7 and 4.6 μM against HepG2 and MCF-7 cells, respectively. As a continuation of the work on the neoglycosylation of steroids, we selected four types of steroids for this research: diosgenin (a typical spirostanol sapogenin with anti-inflammatory properties);[16] pregnenolone (a steroidal precursor of cortisone, estrogen, testosterone and progesterone etc.);[17] dehydroepiandrosterone (DHEA, a steroidal hormone primarily synthesized in the adrenal gland with chondroprotective effect);[18] and estrone (a naturally occurring estrogens used for the treatment and prevention of breast cancer; Figure 1).[19] The selected steroids except diosgenin bare carbonyl groups on the D-ring which are adaptive for the desired neoglycosylation, herein we systematically explored the steroid neoglycosylation on the D-ring and evaluated their cytotoxicity *in vitro* for the first time.

At present, there are few studies on the characteristics of anticancer activity of neoglycosides. Recent studies demon-

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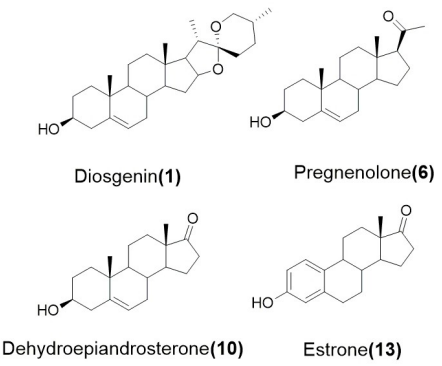


Figure 1. Structures of diosgenin, pregnenolone, dehydroepiandrosterone, estrone.

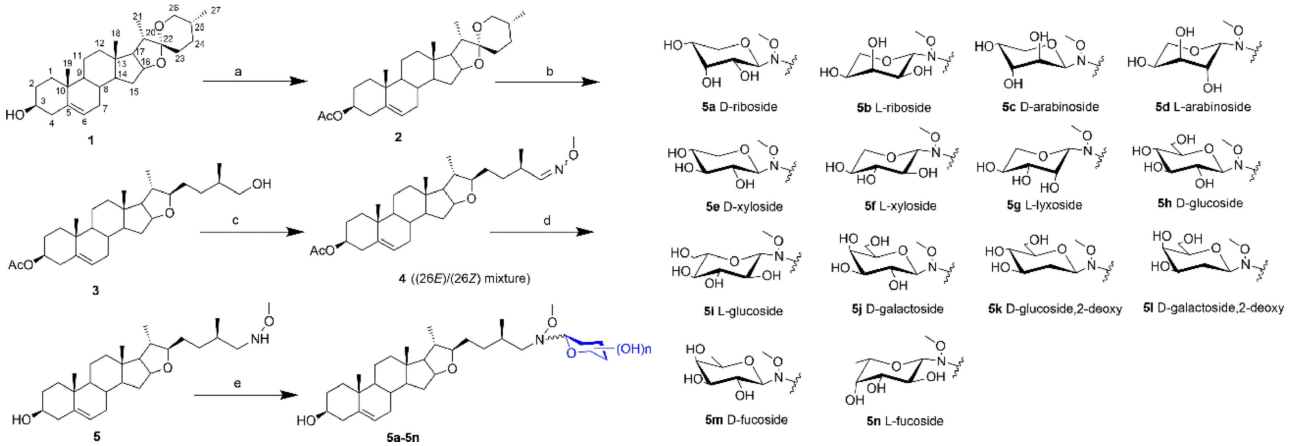
strated that HMGB1 (high mobility group box 1), a chromatin associated nuclear protein of regulated cell death and survival,[20] was found to be over-expressed in many kinds of cancers, it affects many cell behaviors, including inflammation, metastasis and invasion, by binding to the receptor for advanced glycation end products (RAGE), Toll-like receptor (TLR)-2, TLR-4 and TLR-9,[21] and then regulating downstream signaling pathways.[22] Overexpression of HMGB1 can significantly inhibit the growth of breast cancer cells *in vitro* and *in vivo*.[23] Additionally, inducing cell cycle arrest and apoptosis was considered as the predominant form of regulated cell death responsible for tumor therapies.[24] Combining all together, we established the research of neoglycosides on cell cycle arrest, apoptosis and the expression of HMGB1 which may contribute to a better understanding on the anticancer mechanisms of the steroidal neoglycosides. As a result, a 70-member steroidal MeON-neoglycosides library was constructed and their antiproliferative activities were evaluated by CCK-8 assay. The structure-activity relationships and the possible mechanisms were also discussed.

Results and Discussion

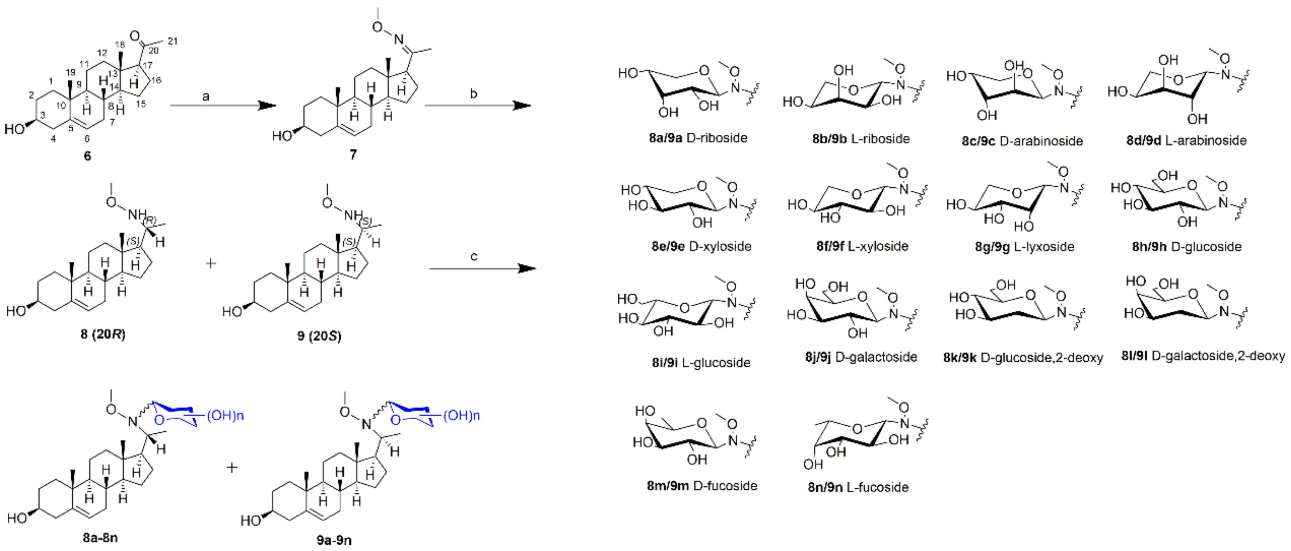
Chemistry

The synthesis of the diosgenin neoaglycon **5** was initiated by preparation of C-26 diosgenin aldehyde. Briefly, diosgenin **1** was converted into **2** with Ac_2O in DMAP at room temperature. The opening of spiroketal bond (F-ring) was carried out using $NaCNBH_3$ in the presence of acetic acid to get a C-26 primary alcohol **3** in a high yield. **3** was oxidized to C-26 diosgenin aldehyde using pyridinium chlorochromate (PCC) in CH_2Cl_2, then filtrate was reacted with methoxyamine hydrochloride salt to get **4** as a mixture of (26*E*) and (26*Z*) isomers, which was subsequently deacetylation of the acetyl groups of C-3 with KOH and followed by reduction with $NaCNBH_3$ in the presence of acetic acid to afford the requisite C-26 MeON-diosgenin **5** (Scheme 1).

Pregnenolone **6**, a typical naturally occurring pregnane-type steroid, the ketone group of D-ring can be used as reaction site directly. Therefore, C-20 ketone was linked to an alkoxyamine through reaction with methoxyamine hydrochloride salt in the presence of pyridine to get corresponding imine **7** in a polar solvent. Specifically, methoxyamine **7** was reduced by $NaCNBH_3$ to give a separable 1 : 1 mixture of MeON-pregnenolone **8/9** (*R/S*, 1 : 1; Scheme 2). The chemical shift of chiral center at C-20 for each diastereomer (**8/9**) was assigned based upon ^1H NMR,^{13}C NMR and HSQC experiment, C-20 protons (20*R*: 3.01–2.93 ppm, multiplet; 20*S*: 2.86–2.79 ppm, multiplet) and carbons (20*R*: 58.6 ppm; 20*S*: 59.7 ppm). The stereochemical structure of **8** and **9** (EtOAc) was ultimately confirmed though a single crystal X-ray diffraction experiment by using $Cu_{K\alpha}$ radiation, and the absolute configuration as 20*R* (compound **8**), 20*S* (compound **9**), respectively (Cambridge Crystallographic Data Centre 2010171, 2010608; Figure 2). The same reaction sequence was applied to get MeON-dehydroepiandrosterone **12** and MeON-estrone **15**[25] as glycosyl acceptors are illustrated in Schemes 3 and 4.



Scheme 1. Synthesis of MeON-diosgenin **5** and neoglycosides (**5a**–**5n**). a) Ac_2O, DMAP, RT, 6 h; b) $NaCNBH_3$, AcOH, RT, 4 h; c) PCC, CH_2Cl_2, RT, 3 h; $MeONH_2$·HCl, pyridine; reflux, 55 °C, 8–10 h; d) i: KOH, THF/MeOH (1 : 1), 10–12 h; ii: $NaCNBH_3$, AcOH, RT, 12 h; e) reducing sugar, MeOH/$CHCl_3$ (4 : 1), AcOH, 40 °C, 48 h.



Scheme 2. Synthesis of MeON-pregnenolones (**8** and **9**) and neoglycosides (**8a–8n** and **9a–9n**). a) $MeONH_2$·HCl, pyridine, MeOH/CH_2Cl_2 (4:1), RT, 10 h; b) $NaCNBH_3$, AcOH, RT, 10 h; c) reducing sugar, MeOH/$CHCl_3$ (4:1), AcOH, 40 °C, 48 h.

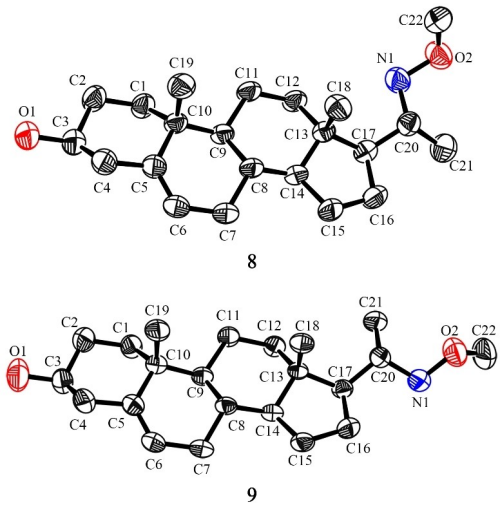


Figure 2. ORTEP diagrams at the 50% probability level of the final X-ray model of compounds **8** and **9**. (H-atoms are omitted for clarity.)

The synthesis of steroidal MeON-neoglycosides was conducted by the neoglycosylation reaction method.[9] The scope of reducing sugars selected for this study included representative pentoses (D/L-ribose; D/L-arabinose; D/L-xylose; D/L-fucose; L-lyxose), hexoses (D/L-glucose; D-galactose), 2-deoxy sugars (2-deoxy-D-glucose; 2-deoxy-D-galactose). Due to the poor solubility of the standard neoglycosylation solvent system (DMF/AcOH) for neoaglycons, we optimized the solvent system and the neoglycosylation reaction conditions, including the equivalents of reducing sugar (2–3 equiv.) and solvent ratio (MeOH/$CHCl_3$) and the amount of external proton source (acetic acid). Optimal neoglycosylation conditions of neoaglycon **15** and D-glucose could be found in entry 2 (Table 1). Finally, **15a–15n** were generated with isolated yields ranging from 28% to 69%. Using these optimal conditions (2 equiv. of sugar, 10 equiv. of AcOH in MeOH/$CHCl_3$ 4:1), compound **5a–5n**, **8a–8n** and **9a–9n**, **12a–12n** were synthesized with yields ranging from 34% to 70%, and the average yield of the product was in agreement with present study.[26] The configuration of glycosidic bond of all the MeON-neoglycosides was identified by the value of coupling constant, and the β-anomer with coupling constant $J = 5.5–9.9$ Hz was the predominant product of most MeON-neoglycosides, which is consistent with previous reports.[26] However, it is worth mentioning that in four series of steroidal MeON-neoglycosides presented highly selective for D/L-xylose, D/L-glucose, D/L-fucose to form β-anomer of MeON-neoglycosides (Table S1, Supporting Information).

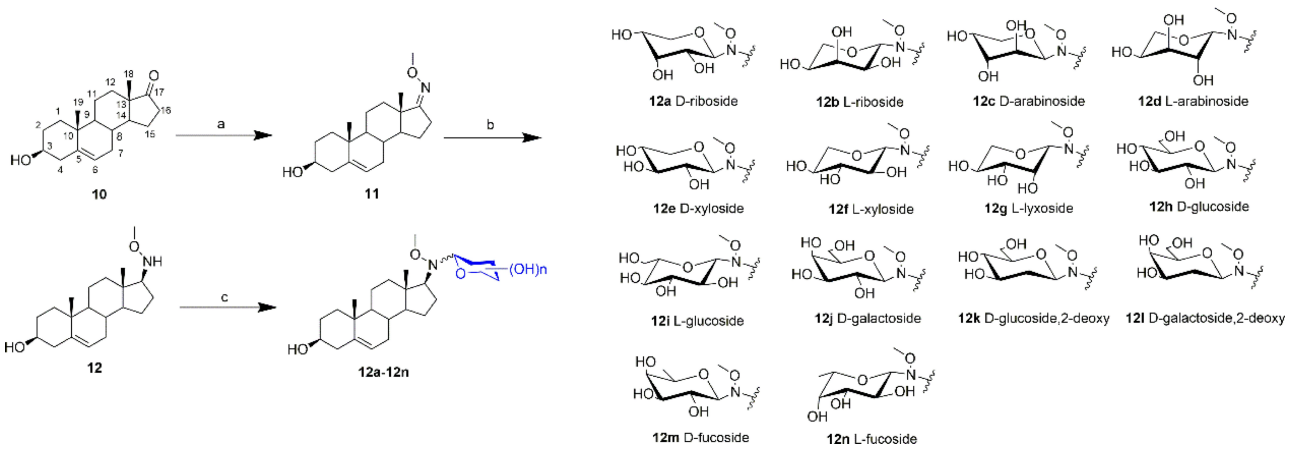
Table 1. Optimization of neoglycosylation conditions.

	D-Glucose [equiv.]	Solvent	AcOH [equiv.]	Yield [%]
1	2.0	MeOH	10	17
2	2.0	MeOH: $CHCl_3$ (4:1)	10	45
3	2.0	MeOH: $CHCl_3$ (4:1)	15	38
4	3.0	MeOH: $CHCl_3$ (4:1)	10	32

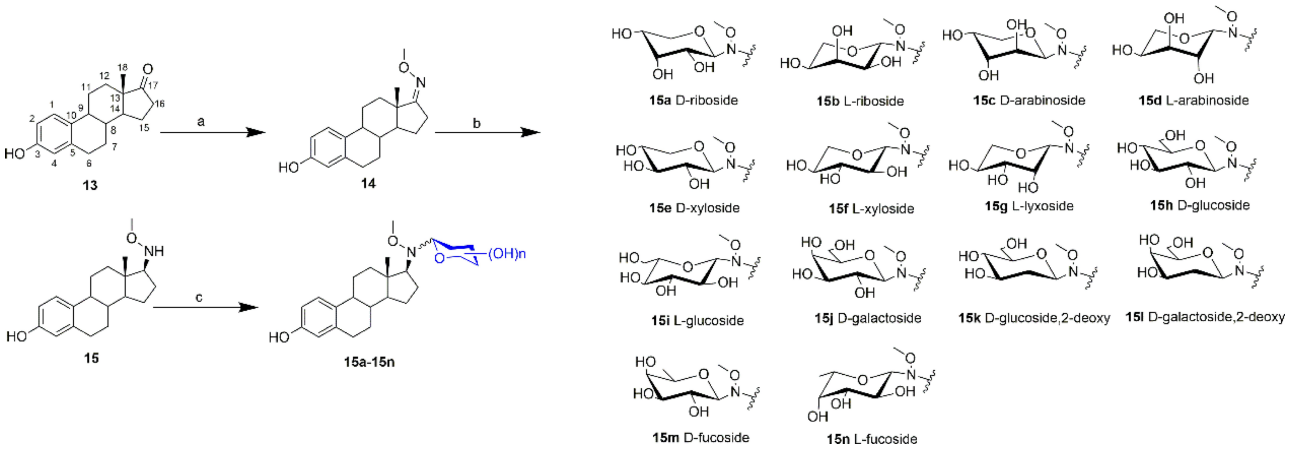
Biological evaluation

Antiproliferative activity. The *in vitro* cytotoxicity of the steroidal MeON-neoglycosides against five human cancer cell lines (A375, A549, HCT116, HepG2, MCF-7) were evaluated by CCK-8 assay.

Diosgenin, a plant steroidal saponin abundantly present in natural herbs, is used as a traditional medicine because of its anti-inflammatory activities.[27] As shown in Table 2, compared with the diosgenin, most MeON-neoglycosides of diosgenin **5a–5n** exhibited good inhibitory effects against the tested cancer cell lines, compound **5k** and **5l** with the 2-deoxysugars displayed notably antiproliferative activities and more sensitive to A549, HepG2, MCF-7 cells, especially compound **5k** showed



Scheme 3. Synthesis of MeON-dehydroepiandrosterone **12** and neoglycosides (**12a**–**12n**). a) $MeONH_2 \cdot HCl$, pyridine, $MeOH/CH_2Cl_2$ (4:1), RT, 8 h; b) $NaCNBH_3$, AcOH, RT, 10 h; c) reducing sugar, $MeOH/CHCl_3$ (4:1), AcOH, 40 °C, 48 h.



Scheme 4. Synthesis of MeON-estrone **15** and neoglycosides (**15a**–**15n**). a) $MeONH_2 \cdot HCl$, pyridine, $MeOH/CH_2Cl_2$ (1:1), RT, 8 h; b) $NaCNBH_3$, AcOH, RT, 10 h; c) reducing sugar, $MeOH/CHCl_3$ (4:1), AcOH, 40 °C, 48 h.

anticancer activities against HepG2 and A549 cells with the IC_{50} values of 1.5 and 1.7 μM, respectively. To the ribose and arabinose the sugar configuration had little influences on their antiproliferative activity, compounds with the D-ribose and L-ribose, D-arabinose and L-arabinose displayed no significantly difference in antiproliferative activity against the tested cells (Table 2) while compound **5e** with the D-xylose displayed distinct antiproliferative activity than compound **5f** with the L-xylose against HepG2 and MCF-7 cells with an IC_{50} value of 38.0 μM and 27.1 μM, respectively. Similarly, compound **5h** with the D-glucose also showed the more potent inhibitory effect than compound **5i** with the L-glucose against A375 and MCF-7 cells.

Pregnenolone, a naturally occurring steroid and it has been utilized as a template for the synthesis of steroid derivatives with improved anticancer profiles.[28] As shown in Table 2, we were surprised to find that the 20*R*-MeON-neoglycosides of pregnenolone **8a**–**8n** generally inhibited the proliferation of tested cancer cells, while almost all of the 20*S*-MeON-neoglycosides of pregnenolone **9a**–**9n** displayed no anticancer activities at the concentration of 50 μM. These similar intriguing results also appeared in our previous work,[15] almost all of the 3*S*-tigogenin neoglycosides displayed no inhibitory activity at the concentration of 100 μM, but the 3*R*-tigogenin neoglycosides showed different sensibility to the tested cells. The present results indicated that inhibitory activity appeared to be related with stereochemistry of some key positions of the skeleton.

Dehydroepiandrosterone (DHEA) is the most abundant circulating steroid hormone in the body and can be converted to either androgens or estrogens.[18] DHEA and its sulfate ester also have a variety of potential biological effects, such as anti-aging,[29] anti-inflammatory[30] and the derivates of D-ring modification of DHEA showed attractive anticancer activity.[31] Estrone, one of the three naturally occurring estrogens, is known more for its hormonal activities and the treatment and prevention of estrogen-dependent breast cancer,[32] the aromatic ring is the characteristic of estrone skeleton, it plays an important role in hormone receptor binding and activity. Similarly, the D-ring modifications of estrone have also produced various analogs which could induce autophagy and

Table 2. IC_{50} (μM)[a] values of aglycons and neoglycosides of diosgenin, pregnenolone, dehydroepiandrosterone, estrone against five human cancer lines.

Compound	Sugar	A375	A549	HCT116	HepG2	MCF-7
5	none	>50	36.9±2.6	>50	29.5±3.3	35.9±2.3
5a	D-ribose	37.6±2.1	35.7±0.7	34.0±0.5	14.9±1.6	21.5±0.8
5b	L-ribose	42.7±3.0	32.1±2.5	34.7±0.6	22.5±0.7	16.6±2.8
5c	D-arabinose	17.3±1.5	28.9±1.3	41.2±0.2	17.6±0.6	2.5±0.4
5d	L-arabinose	40.2±1.7	21.8±0.3	31.5±0.3	10.5±1.8	3.7±0.6
5e	D-xylose	>50	>50	>50	38.0±2.4	27.1±2.8
5f	L-xylose	>50	>50	>50	>50	>50
5g	L-lyxose	>50	48.4±1.2	>50	20.0±0.9	12.9±1.6
5h	D-glucose	23.0±0.7	>50	>50	>50	10.2±0.3
5i	L-glucose	>50	>50	>50	>50	>50
5j	D-galactose	18.2±1.1	>50	34.7±0.1	28.5±1.2	10.4±1.1
5k	2-deoxy-D-glucose	27.9±1.8	1.7±0.1	23.5±0.8	1.5±0.3	3.0±0.4
5l	2-deoxy-D-galactose	38.8±1.5	13.6±0.3	16.9±0.3	5.9±0.8	1.9±0.3
5m	D-fucose	>50	>50	>50	34.6±2.0	>50
5n	L-fucose	49.1±0.7	>50	>50	>50	7.9±1.0
8	none	32.7±2.1	32.9±0.5	30.5±1.6	37.7±1.2	24.2±2.1
9	none	35.0±2.8	39.7±0.7	>50	40.5±0.6	22.7±1.2
8a	D-ribose	>50	>50	>50	>50	>50
8b	L-ribose	36.6±0.9	33.0±1.0	43.2±3.1	>50	>50
8c	D-arabinose	34.9±1.5	35.7±2.4	38.7±1.1	>50	28.7±2.0
8d	L-arabinose	33.7±0.8	34.2±1.0	36.5±0.9	38.1±2.2	23.9±0.7
8e	D-xylose	25.9±2.2	27.4±1.8	30.2±0.6	39.6±1.7	22.7±1.6
8f	L-xylose	36.7±1.3	37.1±1.2	>50	47.5±1.5	38.2±0.8
8g	L-lyxose	22.3±2.6	32.4±0.6	31.8±2.2	42.3±1.9	>50
8h	D-glucose	>50	>50	>50	>50	>50
8i	L-glucose	>50	>50	>50	>50	>50
8g	D-galactose	>50	>50	>50	>50	>50
8k	2-deoxy-D-glucose	24.8±0.9	13.8±0.3	32.6±1.5	29.7±0.8	17.2±2.3
8l	2-deoxy-D-galactose	27.7±1.6	32.3±1.5	35.2±1.0	35.4±1.4	22.8±1.2
8m	D-fucose	38.2±1.8	14.5±0.7	26.8±2.8	>50	39.6±0.9
8n	L-fucose	>50	36.7±2.0	32.4±1.3	>50	32.1±1.8
12	none	>50	>50	>50	>50	>50
15	none	18.2±0.4	20.8±0.9	>50	23.3±0.6	>50
15a	D-ribose	>50	>50	>50	>50	>50
15b	L-ribose	>50	>50	>50	>50	>50
15c	D-arabinose	>50	24.2±2.6	>50	25.8±1.0	23.1±1.5
15d	L-arabinose	>50	>50	>50	>50	>50
15e	D-xylose	>50	15.9±0.4	>50	31.2±1.5	30.7±1.4
15f	L-xylose	>50	>50	>50	>50	>50
15g	L-lyxose	21.7±0.8	31.9±1.1	>50	22.6±2.8	28.2±2.3
15h	D-glucose	>50	>50	>50	>50	>50
15i	L-glucose	>50	>50	>50	>50	>50
15j	D-galactose	>50	22.4±0.7	>50	31.9±4.7	26.5±1.3
15k	2-deoxy-D-glucose	9.7±0.7	4.1±0.1	28.6±1.2	6.1±0.8	4.0±0.4
15l	2-deoxy-D-galactose	8.8±1.0	5.7±0.3	27.9±1.3	10.4±0.5	5.4±0.1
15m	D-fucose	44.2±1.6	46.6±2.4	>50	>50	>50
15n	L-fucose	41.1±3.0	33.0±0.9	>50	23.5±2.4	43.3±0.1
Sorafenib[b]		5.7±0.4	4.8±0.7	2.9±0.1	4.8±0.1	10.5±0.6

[a] Each value was determined in triplicate. The cells were continuously treated with compounds for 72h. [b] Positive control. Compounds **9a**–**9n** and **12a**–**12n** had IC_{50} > 50 μM against the tested cells (data not shown).

apoptosis in human cancer cells.[33] The structural difference between dehydroepiandrosterone (**10**) and estrone (**13**) is the unsaturation of A-ring and B-ring. In order to investigate the cytotoxic impacts of the A-ring structure of the steroidal neoglycosides, MeON-neoglycosides of dehydroepiandrosterone and estrone were examined for their antiproliferative activity. We found that all of the MeON-neoglycosides of dehydroepiandrosterone **12a**–**12n** showed no effects against the five cancer cell lines at the concentration of 50 μM. By contrast, most of MeON-neoglycosides of estrone **15a**–**15n** exhibited moderate to good inhibitory activity (Table 2).

These results indicated that the presence of the phenyl ring on the steroidal skeleton might be important for their anticancer efficacy. As shown in Table 2, compounds **15k** with the 2-deoxy-D-glucose and **15l** with the 2-deoxy-D-galactose showed significantly antiproliferative activities and more sensitive to A549, HepG2 and MCF-7 cells.

From above results, we can find that most of these MeON-neoglycosides exhibited weak to moderate activity against the tested cell lines while compounds **5k**, **5l**, **8k**, **15k** and **15l** with the 2-deoxy sugars (2-deoxy-D-glucose, 2-deoxy-D-galactose) were more sensitive to A549, HepG2 and MCF-7 cells. Among them, compound **5k** was the most potent one with the IC_{50} value of 1.5 μM against HepG2 cells. It furtherly proved that carbohydrate moieties could significantly affect their anticancer activities of neoglycosides. In addition, the configuration of

aglycon and phenyl ring on the steroidal skeleton might be critical for anticancer activities. These findings would provide us some useful information to identify more potent steroid-based anticancer agents.

*Morphological changes in HepG2 cells induced by compound **5k***. Morphological changes of cancer cells are always associated with the growth inhibition induced by cytotoxic agents.[34] After being incubated with **5k** for 48 h at different concentrations (0, 1, 5, 10 μM), the morphological changes of HepG2 cells were recorded using an inverted microscope. Compared with the control group, some of the **5k**-treated cells exhibited rounding, shrinkage, membrane blebbing, especially at high concentrations (Figure 3A). Hoechst 33342 staining was used to assess nuclear changes in HepG2 cells. We found that the chromatin is markedly shrunk after incubation with compound **5k** for 48 h (Figure 3B).

Cell-cycle distribution assay by flow cytometry. Inducing cells cycle arrest constitutes one of the most prevalent strategies used to prevent cancer development.[24a] To establish whether compound **5k** could inhibit the cell growth by interrupting the cell cycle progression, cellular DNA was analyzed by flow cytometry using propidium iodide (PI) staining. The profiles were shown in Figure 4A. Obviously, compared with the control group, the G0/G1 population of HepG2 cells was increased after treatment with **5k** from 40.67% (0 μM) to 44.38% (1 μM), 54.99% (5 μM) and 58.41% (10 μM). These results indicated that compound **5k** could induce cell cycle arrest of HepG2 cells at G0/G1 phase.

Apoptosis detection by flow cytometry. Apoptosis was generally considered as the predominant form of regulated cell death responsible for tumor therapies.[24b] In order to test whether the compound **5k** could induce apoptosis, the

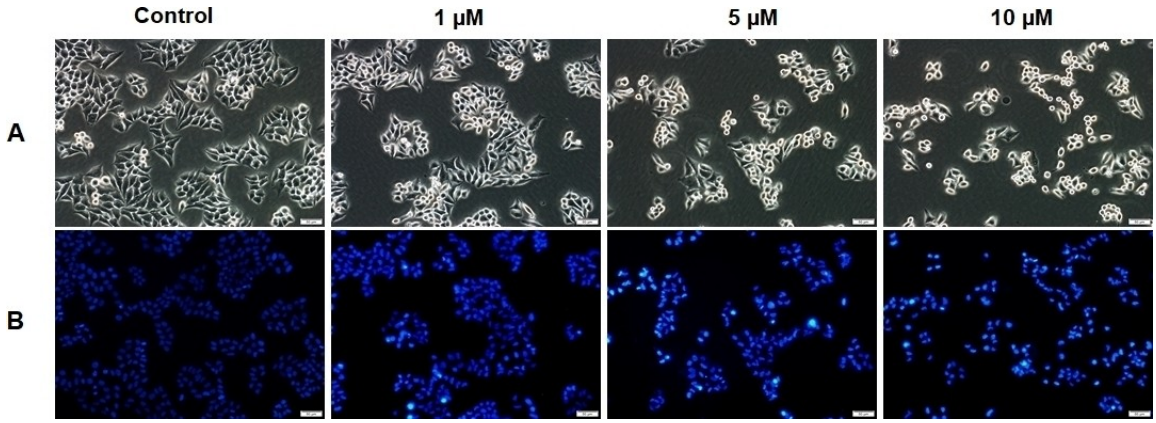


Figure 3. A) HepG2 cells' morphological changes and B) Hoechst 33342 staining after treated with compound **5k** (0, 1, 5, 10 μM) for 48 h. Scale bars: 50 μm.

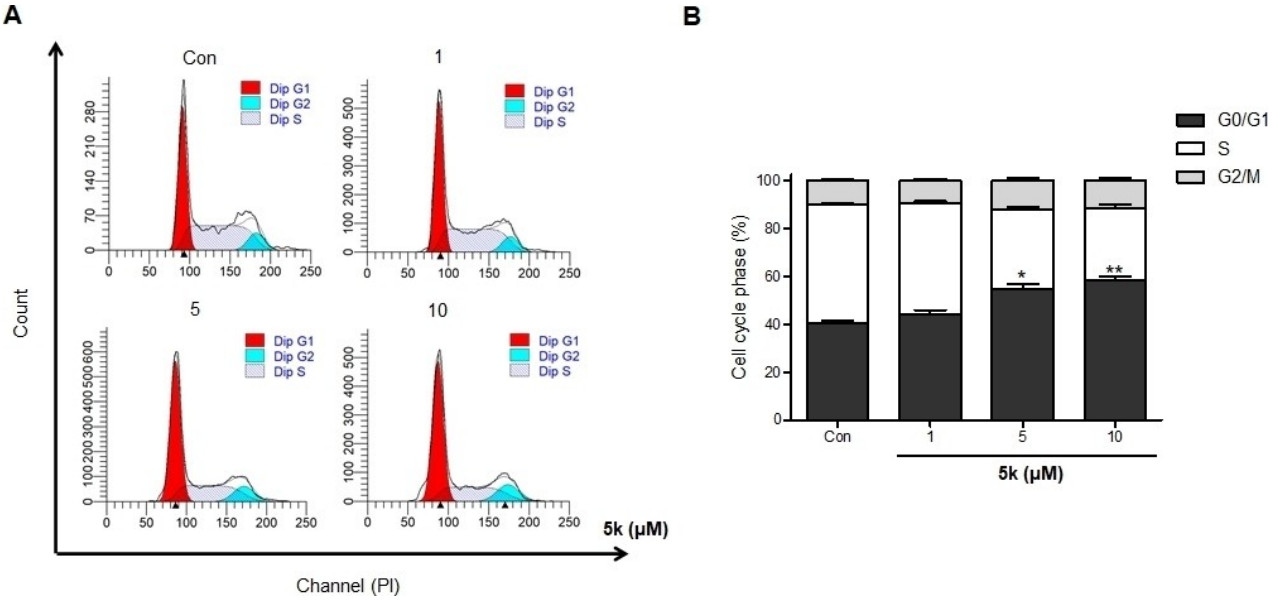


Figure 4. A) Flow cytometry analysis of the cell-cycle distribution of HepG2 cells treated with different concentrations (0, 1, 5, 10 μM) of compound **5k** for 48 h. B) Histograms displaying the percentage of cell-cycle distribution. Experiments are presented as the mean ± SD from three independent experiments. *$p < 0.05$ and **$p < 0.01$ vs. control group.

percentage of apoptotic cells was determined by flow cytometry following Annexin V-FITC and propidium iodide (PI) double staining. A dose-dependent increase in the percentage of apoptotic cells was noted after the cells were treated for 48 h with **5k** at 1, 5, and 10 μM. As shown in Figure 5A, very few (0.48%) apoptotic cells were present in the control panel, whereas the percentage of apoptotic cells significantly increased to 41.0% in neoglycoside **5k**-treated group. These results indicate that compound **5k** was a potential cancer cells apoptosis inducer.

Induction of HMGB1 expression. High-mobility group box 1 protein, a chromatin associated nuclear protein of regulated cell death and survival.[20] The expression of intracellular HMGB1 was related with the development of tumor, overexpression HMGB1 can significantly inhibit the growth of breast cancer cells *in vitro* and *in vivo*.[23] In addition, increasing the expression of HMGB1 can also lead to apoptosis and G1 phase arrest of cell cycle, promote the sensitivity of breast cancer cells to chemotherapy drugs.[35] Recent studies demonstrate that HMGB1 is an essential activator of cellular response to genotoxic stress caused by chemotherapeutic agents such as thiopurines, cytarabine and 5-fluorouracil.[36] To test whether the neoglycosides could also affect the expression of HMGB1, we first investigated the effects of the compound **5k** on HMGB1 expression, treatment of HepG2 cells with compound **5k** resulted in increased 2-fold expression of HMGB1 protein (Figure 6B) and mRNA (Figure 6C) in a concentration-dependent manner when comparing with those in control group. To verify the correlation between the cytotoxicity of the neoglycosides and the expression of HMGB1, we investigated the effects of 11 neoglycosides (against HepG2 cells with the $IC_{50} < 30$ μM) at the concentration of 30 μM on HMGB1 mRNA expression, As shown in Figure 6D, treatment with these neoglycosides upregulated HMGB1 mRNA expression by various degrees, especially for compounds **5k**, **5l**, **15k**, **15l** with significant cytotoxic activity showed the cytotoxic dependent manners.

Conclusion

In conclusion, four series of steroidal MeON-neoglycosides have been designed and synthesized by neoglycosylation, and their anticancer activities have been investigated in CCK-8 assays. Preliminary structure–activity relationship (SAR) analysis revealed that a sugar-dependent activity profile for their cytotoxicity, the configuration of aglycon and the phenyl ring on the steroidal skeleton might be critical for their anticancer activity. In particular, compound **5k** was found to be the most potent compound against HepG2 and A549 cells with the IC_{50} values of 1.5 and 1.7 μM, respectively. Moreover, compound **5k** caused morphological changes and cell-cycle arrest at the G0/G1 phase and induced apoptosis of HepG2 cells in a concentration-dependent manner. Compound **5k** could also induce HMGB1 expression in a concentration-dependent manner. Hence, compound **5k** could be a promising candidate for further exploration as a cancer chemotherapeutic agent. Collectively, our findings also suggested that neoglycosylation could provide a good strategy to explore promising lead compounds for the development of new anticancer agents.

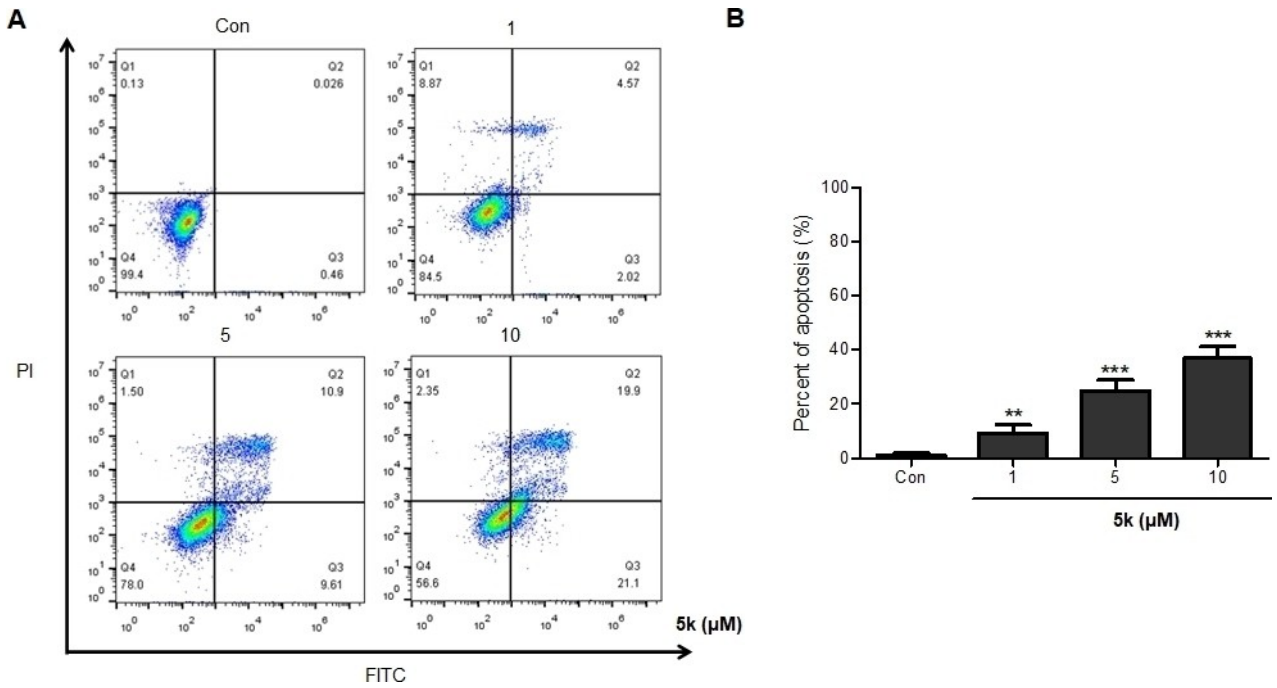


Figure 5. A) Flow cytometry analysis of the apoptosis of HepG2 cells treated with different concentrations (0, 1, 5, 10 μM) of compound **5k** for 48 h. B) Quantitative data analysis for the number of apoptotic cells [% of total] for different treatment groups. Experiments are presented as the mean ± SD from three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control group.

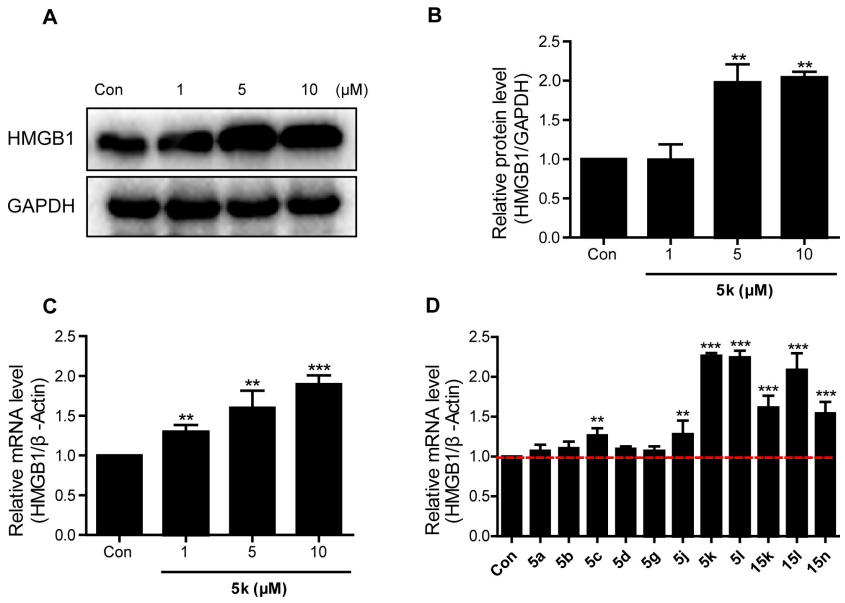


Figure 6. The expression of HMGB1 was determined by western blotting and qPCR. A), B) HepG2 cells were incubated with various concentrations (0, 1, 5, 10 μM) of compound **5k** for 48 h before cell lysis. Cell extracts were analyzed by western blotting. C) HepG2 cells were incubated with the same concentrations of compound **5k** for 48 h, and total RNA was extracted, mRNA levels were detected by using a quantitative polymerase chain reaction (qPCR). D) The expression of HMGB1 mRNA was determined by 11 neoglycosides (against HepG2 cells with $IC_{50} < 30$ μM) with treatment at a concentration of 30 μM. Experiments are presented as the mean ± SD from three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control group.

Experimental Section

Materials and methods. All starting materials and reagents were obtained from commercial suppliers as follows. All reducing sugars were purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Pregnenolone, dehydroepiandrosterone and estrone, Methoxylamine hydrochloride ($CH_3ONH_2 \cdot HCl$), Sodium cyanoborohydride ($NaCNBH_3$), 4-dimethylaminopyridine (DMAP), Pyridinium chlorochromate (PCC) Shanghai Saen Chemical Technology Co., Ltd (Shanghai, China). Diosgenin was purchased from Nanjing jingzhu Bio-Technology Co., Ltd (Nanjing, China). Reaction progress was monitored by analytical TLC was per formed on 0.50 mm Silica Gel 60 F254 plates (Qingdao Ocean Chemical Factory, Shandong, China) and were visualized by spraying with sulphuric acid in 10% EtOH. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ or C_5D_5N with Bruker AV-500 or Bruker AV-600 spectrometer (TMS as internal standard). Chemical shifts were expressed in δ values (ppm) and the coupling constants (*J*) in Hz. ^{1}H and ^{13}C NMR were assigned using 1D and 2D NMR experiments (HSQC, HMBC and NOESY). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Anomeric ratios were obtained by comparison of anomeric proton integration, where possible, are noted as α or β with the atom responsible for the shift. Mass spectrometric data were recorded on an Agilent 6530 QTOF spectrometer for electrospray ionization. Single-crystal -Xray diffraction data was collected on Bruker D8 Venture diffractometer. The figures were plotted with the aid of ORTEP program.

Synthesis of (25*R*)-3β-acetoxy-Spirost-5-en (2). Diosgenin **1** (5 g, 12 mmol) was dissolved in CH_2Cl_2 (50 mL), DMAP (250 mg, 2 mmol) and acetic anhydride (5 mL) were added. After the reaction mixture was stirred at room temperature for 6 h, water (100 mL) was added, and the mixture was extracted with CH_2Cl_2 (3×100 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution (3×50 mL), brine and dried over anhydrous Na_2SO_4, then the solids were removed by filtration. The solvent was evaporated in vacuo, and the crude product was purified by silica gel column chromatography (petroleum ether/ethyl acetate 20:1) to obtain the desired product as a white solid **2** (4.8 g, 87%). ^{1}H NMR (500 MHz, C_5D_5N): $\delta = 5.42–5.38$ (m, 1H), 4.88–4.80 (m, 1H), 4.60 (dd, $J = 14.6$, 7.7 Hz, 1H), 3.65 (dd, $J = 11.0$, 2.9 Hz, 1H), 3.56 (t, $J = 10.6$ Hz, 1H), 2.12 (s, 3H) 1.20 (t, $J = 6.3$ Hz, 3H), 1.03 (s, 3H), 0.91 (d, $J = 7.6$ Hz, 3H), 0.75 (d, $J = 5.7$ Hz, 3H).^{13}C NMR (125 MHz, C_5D_5N): $\delta = 170.6$, 140.4, 122.9, 109.6, 81.5, 74.4, 67.2, 63.2, 56.9, 50.5, 42.3, 40.8, 40.2, 38.8, 37.5, 37.3, 32.6, 32.5, 32.2, 31.9, 30.9, 29.6, 28.4, 21.6, 21.4, 19.6, 17.7, 16.7, 15.4. HRMS (ESI-MS) *m/z* calcd. for $C_{29}H_{45}O_4$: 457.3312 $[M+H]^+$; found: 457.3312.

Synthesis of (25*R*)-3β-acetoxy-furost-5-en-26-ol (3). Compound **2** (4.7 g, 10 mmol) was dissolved in AcOH (100 mL). To this solution, $NaCNBH_3$ (7 g, 111.4 mmol) was added in portions over a period of 30 min, the mixture was stirred at room temperature for 4 h. After the completion of the reaction, the mixture was poured in ice-cool water, extracted with EtOAc (3×80 mL), then the organic layer was washed with water and saturated NaCl solution (3×80 mL) successively, dried over anhydrous Na_2SO_4. The solvent was concentrated under reduced pressure, purified by silica gel column chromatography with petroleum ether/ethyl acetate (8:1) to obtain the desired product as a white solid **3** (3.7 g, 79%). ^{1}H NMR (500 MHz, C_5D_5N): $\delta = 5.96$ (t, $J = 4.9$ Hz, 1H), 5.36 (d, $J = 5.2$ Hz, 1H), 4.86–4.75 (m, 1H), 4.36 (dd, $J = 7.7$, 2.5 Hz, 1H), 3.82–3.71 (m, 2H), 3.58–3.26 (m, 1H), 2.07 (s, 3H), 1.11 (d, $J = 6.6$ Hz, 3H), 1.02 (d, $J = 6.7$ Hz, 3H), 0.98 (s, 3H), 0.89 (s, 3H).^{13}C NMR (125 MHz, C_5D_5N): $\delta =$ 170.6, 140.3, 123.0, 90.9, 83.7, 74.4, 68.0, 65.9, 57.3, 50.6, 41.2, 39.9, 38.8, 38.7, 37.5, 37.3, 37.1, 33.0, 32.6, 32.1, 32.0, 31.5, 28.4, 21.6, 21.3, 19.7, 19.5, 17.6, 16.9. HRMS (ESI-MS) *m/z* calcd. for $C_{29}H_{47}O_4$: 459.3469 $[M+H]^+$; found: 459.3469.

Synthesis of (*E*)-and (*Z*)-(25*R*)-3β-acetoxy-26-*N*-methyloxime-furost-5-en (4). Pyridinium chlorochromate (2.8 g, 13 mmol) was added to a mixture of silica gel (2.8 g, 100~200 mesh) and compound **3** (3 g, 5 mmol) in CH_2Cl_2 (100 mL) at room temperature. The reaction mixture was stirred for 3 h. The reaction mixture was

filtered and the filtrate was dissolved in MeOH (100 mL), followed by the addition of pyridine (1.8 mL) and methoxyamine hydrochloride (1.85 g). The reaction was heated to 55 °C under reflux for 8–10 h, monitored by TLC. The crude product was purified by silica gel column chromatography with petroleum ether/ethyl acetate (20:1) to obtain the desired product **4** (2.4 g, 76%) as a mixture of (26*E*) and (26*Z*) stereoisomers. ^{1}H NMR (500 MHz, C_5D_5N): $\delta = 5.36$ (d, $J = 5.2$ Hz, 1H), 4.84–4.76 (m, 1H), 4.37–4.33 (m, 1H), 3.94–3.86 (m, 3H), 3.41–3.31 (m, 1H), 1.05 (d, $J = 6.9$ Hz, 3H), 0.98 (s, 5H), 0.86 (s, 3H). ^{13}C NMR (125 MHz, C_5D_5N): $\delta = 170.6$, 155.4, 140.3, 123.0, 90.4, 83.7, 74.4, 65.8, 61.5, 57.3, 50.6, 41.2, 39.8, 38.8, 38.6, 37.6, 37.3, 35.2, 33.0, 32.6, 32.5, 32.1, 31.8, 28.4, 21.6, 21.3, 19.7, 19.4, 18.6, 16.9. HRMS (ESI-MS) *m/z* calcd. for $C_{30}H_{48}NO_4$: 486.3578 $[M+H]^+$; found: 486.3578.

Synthesis of (25*R*)-3β-hydroxy-26-*N*-methoxyamino-furost-5-en (5). Compound **4** (1.9 g, 4 mmol) was dissolved in a mixture of THF/MeOH (1:1,200 mL), KOH (3.2 g, 5.7 mmol) was added, the reaction mixture was stirred at room temperature for 10–12 h, and monitored by TLC. The solvent was concentrated under reduced pressure. The residue was purified by column chromatography (petroleum ether/ethyl acetate 15:1) to give as a white solid (1.45 g). The solid was dissolved in AcOH (100 mL) and $NaCNBH_3$ (2 g, 3 mmol) was added, then the reaction mixture was stirred at room temperature for 12 h. After quenching the reaction with saturated $NaHCO_3$ solution, and then extracted with CH_2Cl_2 (3× 100 mL). The organic layer was washed with saturated NaCl solution (3×50 mL), dried over anhydrous Na_2SO_4 and concentrated. The crude reaction mixture was purified by silica gel column chromatography with petroleum ether/ethyl acetate (10:1) to obtain white powder compound **5** (1.1 g, 76%). ^{1}H NMR (500 MHz, C_5D_5N): $\delta =$ 5.41 (d, $J = 5.1$ Hz, 1H), 4.40–4.33 (m, 1H), 3.93–3.79 (m, 1H), 3.63 (s, 3H), 3.44–3.40 (m, 1H), 3.07–2.99 (m, 1H), 2.83 (d, $J = 7.4$ Hz, 1H), 2.69–2.54 (m, 2H), 2.17–1.98 (m, 2H), 1.97–1.38 (m, 16H), 1.35–1.24 (m, 2H), 1.19–1.06 (m, 3H), 1.05 (s, 3H), 1.02 (d, $J = 4.5$ Hz, 3H), 1.01 (d, $J = 4.5$ Hz, 3H), 0.91 (s, 3H).^{13}C NMR (125 MHz, C_5D_5N): $\delta = 142.3$, 121.4, 90.7, 83.7, 71.6, 65.9, 61.8, 58.9, 57.5, 50.9, 43.9, 41.2, 40.0, 38.6, 38.2, 37.4, 33.0, 33.0, 32.9, 32.7, 32.3, 31.9, 31.7, 21.4, 20.0, 19.4, 18.7, 17.0. HRMS (ESI-MS) *m/z* calcd. for $C_{28}H_{48}NO_3$: 446.3629 $[M+H]^+$; found: 446.3635.

Synthesis of 20-*N*-methoxyiminopregnenolone (7). Compound **6** (5 g, 15.8 mmol) was dissolved in a mixture of MeOH/CH_2Cl_2 (4:1, 200 mL) followed by the addition of pyridine (5 equiv.) and methoxyamine hydrochloride (2.02 g, 24.2 mmol). The reaction mixture was stirred at room temperature for 10 h, and monitored by TLC, and then filtered to obtain an organic layer and was concentrated under reduced pressure, the crude product was purified through silica gel column chromatography with petroleum ether/ethyl acetate (20:1), obtained the desired product as a white solid **7** (4.9 g, 90%). ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 5.35$ (d, $J = 3.8$ Hz, 1H), 3.84 (s, 3H), 3.56–3.49 (m, 1H), 1.81 (s, 3H), 1.00 (s, 3H), 0.64 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 157.9$, 140.9, 121.7, 71.9, 61.4, 56.7, 56.4, 50.3, 43.8, 42.4, 38.8, 37.4, 36.7, 32.2, 31.9, 31.8, 24.4, 23.2, 21.2, 19.6, 15.7, 13.3. HRMS (ESI-MS) *m/z* calcd. for $C_{22}H_{36}NO_2$: 346.2741 $[M+H]^+$; found: 346.2726.

Synthesis of (20*R*)-*N*-methoxyaminopregnenolone (8) and (20*S*)-*N*-methoxyaminopregnenolone (9). Compound **7** (2 g, 5.8 mmol) was dissolved in AcOH (100 mL), which was subsequently reduced by $NaCNBH_3$ (10 equiv.), the reaction mixture was stirred at room temperature for 10 h. The reaction was terminated by adding saturated $NaHCO_3$ solution (100 mL), and then extracted with CH_2Cl_2 (3×100 mL). The organic layer was washed with saturated $NaHCO_3$ solution, and dried over anhydrous $MgSO_4$. Filtration and removal of the solvent, the crude product was purified by silica gel chromatography with petroleum ether/ethyl acetate (30:1) providing the two aglycons as white powder (20*R* isomer **8**: 0.94 g, 47%; 20*S* isomer **9**: 0.86 g, 43%). 20*R* isomer **8**: $[\alpha]_D^{20} = -62.0°$ ($c = 1.0$, MeOH), ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 5.34$ (s, 1H), 3.56–3.47 (m, 4H), 3.01–2.93 (m, 1H), 2.37–2.13 (m, 2H), 2.09–1.88 (m, 2H), 1.87–1.70 (m, 3H), 1.23–1.17 (m, 1H), 1.08 (s, 3H), 1.00 (s, 3H), 0.74 (s, 3H).^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 140.9$, 121.7, 71.9, 62.3, 58.6, 56.5, 53.3, 50.1, 42.4, 42.1, 39.8, 37.4, 36.6, 31.9, 31.9, 31.7, 26.7 24.4, 21.1, 19.5, 18.6, 12.3. HRMS (ESI-MS) *m/z* calcd. for $C_{22}H_{38}NO_2$: 348.2897 $[M+H]^+$; found: 348.2879. 20*S* isomer **9**: $[\alpha]_D^{20} = -30.6°$ ($c = 1.0$, MeOH), ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 5.35$–5.33 (m, 1H), 3.53 (s, 3H), 3.52–3.48 (m, 1H), 2.86–2.79 (m, 1H), 2.31–2.19 (m, 2H), 2.00–1.93 (m, 2H), 1.88–1.80 (m, 3H), 1.19 (d, $J = 6.2$ Hz, 3H), 1.00 (s, 3H), 0.69 (s, 3H).^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 140.9$, 121.7, 71.9, 62.7, 59.7, 56.5, 53.0, 50.1, 42.4, 42.2, 39.3, 37.4, 36.6, 31.9, 31.9, 31.7, 26.9, 24.4, 21.1, 19.5, 18.6, 12.3. HRMS (ESI-MS) *m/z* calcd. for $C_{22}H_{38}NO_2$: 348.2897 $[M+H]^+$; found: 348.2896.

Synthesis of 17-*N*-methoxyiminodehydroepiandrosterone (11). Compound **10** (2.8 g, 10 mmol) was dissolved in a mixture of MeOH/CH_2Cl_2 (4:1, 100 mL), followed by the addition of pyridine (5 equiv.) and methoxyamine hydrochloride (1.12 g, 13.5 mmol). The reaction mixture was stirred at room temperature for 8 h and monitored by TLC, and then filtered to obtain an organic layer and was concentrated under reduced pressure, the crude product was purified by silica gel column chromatography with petroleum ether/ethyl acetate (20:1), obtained the desired product as a white solid (2.9 g, 91%). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.37$–5.34 (m, 1H), 3.82 (s, 3H), 3.58–3.46 (m, 1H), 1.03 (s, 3H), 0.92 (s, 3H).^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 170.5$, 141.2, 121.3, 71.8, 61.4, 54.4, 50.5, 43.9, 42.4, 37.4, 36.8, 34.3, 31.8, 31.5, 31.5, 25.8, 23.5, 20.7, 19.6, 17.2. HRMS (ESI-MS) *m/z* calcd. for $C_{20}H_{32}NO_2$: 318.2428 $[M+H]^+$; found: 318.2429.

Synthesis of (17*S*)-*N*-Methoxyaminodehydroepiandrosterone (12). Compound **11** (2 g, 6.3 mmol) was dissolved in AcOH (100 mL), which was subsequently reduced by $NaCNBH_3$ (10 equiv.), the reaction mixture was stirred at room temperature for 10 h. The reaction was terminated by adding saturated $NaHCO_3$ solution (100 mL), and then extracted with CH_2Cl_2 (3×100 mL). The organic layer was washed with saturated $NaHCO_3$ solution, and dried over anhydrous $MgSO_4$. Filtration and removal of the solvent, the residue was purified by silica gel chromatography with petroleum ether/ethyl acetate (20:1), obtained the desired product as a white solid (1.8 g, 90%). ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 5.34$ (d, $J = 5.2$ Hz, 1H), 3.64–3.39 (m, 4H), 3.04 (t, $J = 8.8$ Hz, 1H), 2.36–2.16 (m, 2H), 2.05–1.74 (m, 5H), 1.69–1.16 (m, 10H), 1.11–1.04 (m, 2H), 1.01 (s, 3H), 0.99–0.91 (m, 1H), 0.75 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): $\delta = 141.0$, 121.5, 71.9, 70.2, 61.6, 53.9, 50.4, 42.7, 42.4, 38.5, 37.4, 36.7, 31.9, 31.8, 31.8, 26.1, 24.0, 21.0, 19.6, 11.8. HRMS (ESI-MS) *m/z* calcd. for $C_{20}H_{34}NO_2$: 320.2584 $[M+H]^+$; found: 320.2588.

Synthesis of 17-*N*-Methoxyiminoestrone (14). Compound **13** (2.7 g, 10 mmol) was dissolved in a mixture of MeOH/CH_2Cl_2 (1:1, 80 mL), followed by the addition of pyridine (5 equiv.) and methoxyamine hydrochloride (1.09 g, 13 mmol). The reaction mixture was stirred at room temperature for 8 h and monitored by TLC. The reaction mixture was filtered to obtain an organic layer and was concentrated under reduced pressure, the crude product was purified by silica gel column chromatography with petroleum ether/ethyl acetate (20:1), obtained the desired product as a white solid (2.8 g, 95%). ^{1}H NMR (500 MHz, C_5D_5N): $\delta = 7.28$ (d, $J = 8.4$ Hz, 1H), 7.11 (dd, $J = 8.4$, 2.6 Hz, 1H), 7.02 (d, $J = 2.5$ Hz, 1H), 3.95 (s, 3H), 0.93 (s, 3H).^{13}C NMR (125 MHz, C_5D_5N): $\delta = 170.2$, 157.2, 138.5, 131.5, 127.3, 116.7, 114.3, 61.6, 53.5, 44.8, 44.8, 39.0, 35.3, 30.3, 28.0, 27.1, 26.4, 23.5, 17.9. HRMS (ESI-MS) *m/z* calcd. for $C_{19}H_{26}NO_2$: 300.1958 $[M+H]^+$; found: 300.1938.

Synthesis of (17*S*)-*N*-methoxyaminoestrone (15). Compound **14** (1.5 g, 5 mmol) was dissolved in AcOH (100 mL), the mixture was

subsequently reduced by $NaCNBH_3$ (10 equiv.), the reaction was stirred at room temperature for 10 h. The reaction was terminated by adding saturated $NaHCO_3$ solution (100 mL), and then extracted with CH_2Cl_2 (3×100 mL). The organic layer was washed with saturated $NaHCO_3$ solution and dried over anhydrous $MgSO_4$. Filtration and removal of the solvent, the crude product was purified by silica gel chromatography with petroleum ether/ethyl acetate (20:1), obtained the desired product as a white solid (1.2 g, 80%). ^{1}H NMR (600 MHz, C_5D_5N) 7.32 (d, J=8.4 Hz, 1H), 7.11 (dd, J=8.4, 2.4 Hz, 1H), 7.03 (d, J=2.1 Hz, 1H), 3.62 (s, 3H), 3.21 (dd, J=16.4, 8.7 Hz, 1H), 2.91–2.75 (m, 2H), 2.36–2.26 (m, 1H), 2.25–2.14 (m, 2H), 1.96–1.86 (m, 1H), 1.80–1.72 (m, 1H), 1.65–0.94 (m, 10H), 0.84 (s, 3H).^{13}C NMR (125 MHz, C_5D_5N): δ=157.1, 138.5, 131.9, 127.4, 116.7, 114.3, 71.0, 61.7, 53.0, 44.7, 43.7, 39.5, 39.3, 30.4, 28.3, 27.4, 26.7, 24.1, 12.6. HRMS (ESI-MS) m/z calcd. for $C_{19}H_{28}NO_2$: 302.2115 $[M+H]^+$; found: 302.2107.

General procedure for preparation of steroidal-neoglycosides library. To a solution of aglycone (typically 0.1 mmol) and reducing sugar (2 equiv.) were dissolved in MeOH/$CHCl_3$ (4:1, 5.0 mL). External proton source AcOH (10 equiv.) was added and placed on a shaker and reaction at 40 °C for 48 h. The target neoglycoside was obtained by purified with MeOH/CH_2Cl_2 on silica gel column chromatography. The configuration of the glycosidic bond of all the steroidal neoglycosides was identified by the J value of $J_{H1'-H2'}$.

Cell culture. Five human cancer cell lines: human melanoma cell line (A375), human non-small-cell lung cancer cell line (A549), human colon cancer cell line (HCT116), human liver carcinoma cell line (HepG2), human breast adenocarcinoma cell line (MCF-7) were cultured in an atmosphere with 5% CO_2 at 37 °C, using RPMI-1640 or DMEM with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 0.1 mg/mL streptomycin as culture medium. Neoglycosides were dissolved in DMSO with a stock concentration of 100 mM. The final concentration of DMSO was 0.05%, which was nontoxic to the cells.

Cell viability assay. The CCK-8 assay was performed to examine the effect of steroidal MeON-neoglycosides on cell viability. Briefly, five human cancer cells were seeded in 96-well culture plates at a density of 6000 cells/well. After 24 h of incubation, the cells were treated with neoglycosides for 72 hours. Sorafenib was used in the subsequent experiments as a positive control. Following neoglycosides treatment, 10 μL of CCK-8 was added to each well and incubated for a further 3 h. The absorbance was measured at the wavelengths of 450 nm, and recorded the absorbance value (OD). Cell viability was calculated using the following formula: Relative cell viability=(OD value for the test group−blank OD)/(control OD value−blank OD value) ×100%. The half-maximal inhibitory concentration (IC_{50}) values were determined using GraphPad Prism 5 software.

Cell-cycle distribution analysis. Flow cytometry was employed to determine the effect of compound **5k** on the cell cycle of HepG2 cells. We used PI to stain the DNA and RNase A to hydrolyze the phosphodiester bond between the nucleotides. Briefly, HepG2 cells were seeded into six-well plates for attaching overnight. The cells were then incubated with **5k** at concentrations of 1, 5 and 10 μM for 48 h. Cells were collected and washed twice with PBS. Cells were fixed with cold 70% ethanol at 4 °C overnight. Fixed cells were washed with PBS, and then stained with 50 μg/mL propidium iodide (PI) solution containing 25 μg/mL RNase A for 30 min in the dark at room temperature. Fluorescence intensity was analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The percentages of the cells distributed in different phases of the cell cycle were analyzed using ModFit LT 3.1.

Flow cytometry analysis of apoptosis. Cell apoptosis was analyzed by using the Annexin V-FITC/PI Apoptosis kit (BD Biosciences, Franklin Lakes) according to the manufacturer's protocols. Briefly, HepG2 cells were seeded into six-well plates for attaching overnight. The cells were then incubated with **5k** at concentrations of 1, 5 and 10 μM for 48 h. Cells were collected and then washed twice with cold PBS, and then stained using the annexin V-fluorescein isothiocyanate (FITC) and PI according to the manufacturer's instructions. The stained cells were incubated for 15 min in the dark at room temperature, and the fluorescent intensity was measured using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes).

Hoechst 33342 staining. HepG2 cells were plated 6-well tissue culture plates and incubated for 24 h before the treatment. Cells were treated with **5k** for 48 h before incubation with Hoechst 33342. Removed the culture medium containing compounds and fixed the cells in 4% paraformaldehyde for 30 min at room temperature. The cells were stained with 1 mL of Hoechst 33342 for 10 min and then washed twice with PBS. The stained nuclei were observed by fluorescence microscope (Olympus, Tokyo, Japan).

Western blot analysis. The adherent and floating cells were collected and suspended in cold RIPA buffer. samples were centrifuged at 12,000 rpm at 4 °C for 10 min. The protein concentration was measured using a BCA protein assay kit. Equal amounts of protein were separated using 7.5–12.5% SDS polyacrylamide gels and transferred to PVDF membrane (Merck Millipore). The membrane was blocked with 5% skim milk in PBST (PBS with 0.05% Tween-20) for 1 h at room temperature, and then probed with primary antibody dilution overnight at 4 °C. After washing three times, the polyvinylidene fluoride (PVDF) membrane was covered with secondary antibody for 2 h at room temperature. After washing three times (5 min per wash), protein band intensity was quantifed by BandScan software (Bio-Rad), GAPDH levels were analyzed as controls for protein loading.

Quantitative real-time PCR. Total RNA from HepG2 cells was extracted using Trizol (Invitrogen). The cDNA was synthesized using an oligo(dT) primer (Generay, Shanghai, China) in a total volume of 20 μL according to the manufacturer's instructions. PCR was performed using an ABI 7900 Sequence Detection System and ChamQ SYBR Color qPCR Master Mix (Vazyme, Nanjing, China). The primer pairs used were as follows: HMGB1: 5′-GATGGGAAAGGA-GATCCTA-3′ and 5′-CTTGGTCTCCCTTTGGGG-3′. β-actin: 5′-AAGAGA-GGCATCCTCACCCT-3′ and 5′-TACATGGCTGGGGTGTTGAA-3′. The mRNA levels were normalized to β-actin. The foldchange for each gene was calculated by comparing the cycle threshold value of the gene with the Ct value of the control.

Statistical analysis. Data are presented as the mean±standard deviation (SD). All the experiments were carried out in triplicate. Comparisons of different groups was evaluated by one-way analysis of variance (ANOVA). Statistical analyses were performed using GraphPad Prism 5 software, Values of $p < 0.05$ were considered statistically significant.

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Conflict of Interest

The authors declare no conflict of interest.

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[1] M. Wehling, *Annu. Rev. Physiol.* **1997**, *59*, 365–393.
[2] a) M. Sanford, *Drugs* **2013**, *73*, 187–193; b) M. Schumacher, C. Mattern, A. Ghoumari, J. P. Oudinet, P. Liere, F. Labombarda, R. Sitruk-Ware, A. F. De Nicola, R. Guennoun, *Prog. Neurobiol.* **2014**, *113*.
[3] a) J. S. de Bono, C. J. Logothetis, A. Molina, K. Fizazi, S. North, L. Chu, K. N. Chi, R. J. Jones, O. B. Goodman, F. Saad, J. N. Staffurth, P. Mainwaring, S. Harland, T. W. Flaig, T. E. Hutson, T. Cheng, H. Patterson, J. D. Hainsworth, C. J. Ryan, C. N. Sternberg, S. L. Ellard, A. Fléchon, M. Saleh, M. Scholz, E. Efstathiou, A. Zivi, D. Bianchini, Y. Loriot, N. Chieffo, T. Kheoh, C. M. Haqq, H. I. Scher, *N. Engl. J. Med.* **2011**, *364*, 1995–2005; b) G. Attard, M. Borre, H. Gurney, Y. Loriot, C. Andresen-Daniil, R. Kalleda, T. Pham, M.-E. Taplin, *J. Clin. Oncol.* **2018**, *36*, 2639–2646.
[4] a) V. Njar, A. Brodie, *J. Med. Chem.* **2015**, *58*, 2077–2087; b) Z. Yu, C. Cai, S. Gao, N. I. Simon, H. C. Shen, S. P. Balk, *Clin. Cancer Res.* **2014**, *20*, 4075–4085.
[5] a) E. Kaasalainen, J. Tois, L. Russo, K. Rissanen, J. Helaja, *Tetrahedron Lett.* **2006**, *47*, 5669–5672; b) C. Varela, E. J. Tavares da Silva, C. Amaral, G. Correia da Silva, T. Baptista, S. Alcaro, G. Costa, R. A. Carvalho, N. A. A. Teixeira, F. M. F. Roleira, *J. Med. Chem.* **2012**, *55*, 3992–4002; c) K. Konno, T. Fujishima, S. Maki, Z. Liu, D. Miura, M. Chokki, S. Ishizuka, K. Yamaguchi, Y. Kan, M. Kurihara, N. Miyata, C. Smith, H. F. DeLuca, H. Takayama, *J. Med. Chem.* **2000**, *43*, 4247–4265.
[6] J. P. Burkhart, C. A. Gates, M. E. Laughlin, R. J. Resvick, N. P. Peet, *Bioorg. Med. Chem.* **1996**, *4*, 1411–1420.
[7] a) B. Yu, X.-J. Shi, J.-l. Ren, X.-N. Sun, P.-P. Qi, Y. Fang, X.-W. Ye, M.-M. Wang, J.-W. Wang, E. Zhang, D.-Q. Yu, H.-M. Liu, *Eur. J. Med. Chem.* **2013**, *66*, 171–179; b) M. P. Leese, B. Leblond, S. P. Newman, A. Purohit, M. J. Reed, B. V. L. Potter, *J. Steroid Biochem. Mol. Biol.* **2005**, *94*, 239–251.
[8] a) J. P. Munafo, Jr., T. J. Gianfagna, *Nat. Prod. Rep.* **2015**, *32*, 454–477; b) D. B. Salunke, B. G. Hazra, V. S. Pore, *Curr. Med. Chem.* **2006**, *13*, 813–847; c) H. Pellissier, *Tetrahedron* **2004**, *60*, 5123–5162.
[9] J. M. P. Langenhan, N. R. Guzei, I. A. Hoffmann, F. M. Thorson, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 12305–12310.
[10] a) J. M. Langenhan, J. M. Engle, L. K. Slevin, L. R. Fay, R. W. Lucker, K. R. Smith, M. M. Endo, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 670–673; b) P. Peltier-Pain, S. C. Timmons, A. Grandemange, E. Benoit, J. S. Thorson, *ChemMedChem* **2011**, *6*, 1347–1350.
[11] X. S. Li, Y. C. Ren, Y. Z. Bao, J. Liu, X. K. Zhang, Y. W. Zhang, X. L. Sun, X. S. Yao, J. S. Tang, *Eur. J. Med. Chem.* **2018**, *145*, 252–262.
[12] N. L. R. P. Aqeel Ahmed, *J A C S* **2006**, *128*, 14224–14225.
[13] M. Ukiya, T. Hayakawa, K. Okazaki, M. Hikawa, H. Akazawa, W. Li, K. Koike, M. Fukatsu, *Chem. Biodiversity* **2018**, *15*, e1800113.
[14] J. S. Thorson, *Org. Lett.* **2009**, *11*, 461–464.
[15] G. L. Li, H. J. Xu, S. H. Xu, W. W. Wang, B. Y. Yu, J. Zhang, *Fitoterapia* **2018**, *125*, 33–40.
[16] S. Hirai, T. Uemura, N. Mizoguchi, J.-Y. Lee, K. Taketani, Y. Nakano, S. Hoshino, N. Tsuge, T. Narukami, R. Yu, N. Takahashi, T. Kawada, *Mol. Nutr. Food Res.* **2010**, *54*, 797–804.
[17] W. L. Miller, R. J. Auchus, *Endocr. Rev.* **2011**, *32*.
[18] Q. Mo, S.-F. Lu, N. G. Simon, *J. Steroid Biochem. Mol. Biol.* **2006**, *99*, 50–58.
[19] H. Samavat, M. S. Kurzer, *Cancer Lett.* **2015**, *356*, 231–243.
[20] M. T. Lotze, K. J. Tracey, *Nat. Rev. Immunol.* **2005**, *5*, 331–342.
[21] a) J. E. Ellerman, C. K. Brown, M. de Vera, H. J. Zeh, T. Billiar, A. Rubartelli, M. T. Lotze, *Clin. Cancer Res.* **2007**, *13*, 2836–2848; b) G. P. Sims, D. C. Rowe, S. T. Rietdijk, R. Herbst, A. J. Coyle, *Annu. Rev. Immunol.* **2010**, *28*, 367–388; c) D. Tang, R. Kang, C. W. Cheh, K. M. Livesey, X. Liang, N. E. Schapiro, R. Benschop, L. J. Sparvero, A. A. Amoscato, K. J. Tracey, H. J. Zeh, M. T. Lotze, *Oncogene* **2010**, *29*, 5299–5310.
[22] J. R. van Beijnum, W. A. Buurman, A. W. Griffioen, *Angiogenesis* **2008**, *11*, 91–99.
[23] Y. Jiao, H. C. Wang, S. J. Fan, *Acta Pharmacol. Sin.* **2007**, *28*, 1957–1967.
[24] a) K. W. Kohn, J. Jackman, P. M. O'Connor, *J. Cell. Biochem.* **1994**, *54*, 440–452; b) G. Makin, C. Dive, *Trends Cell Biol.* **2001**, *11*, S22–S26.
[25] P. Kaspar, H. Witzel, *Steroids* **1983**, *42*, 1–9.
[26] N. S. Nandurkar, J. Zhang, Q. Ye, L. V. Ponomareva, Q. B. She, J. S. Thorson, *J. Med. Chem.* **2014**, *57*, 7478–7484.
[27] F.-C. Wu, J.-G. Jiang, *Food Funct.* **2019**, *10*, 7022–7036.
[28] a) G. Szaloki, A. Pantzou, K. C. Prousis, O. Mavrofrydi, P. Papazafiri, T. Calogeropoulou, *Bioorg. Med. Chem.* **2014**, *22*, 6980–6988; b) M. A. Elhinnawi, R. M. Mohareb, H. M. Rady, W. K. B. Khalil, M. M. Abd Elhalim, G. A. Elmegeed, *J. Steroid Biochem. Mol. Biol.* **2018**, *183*, 125–136.
[29] H. Nawata, T. Yanase, K. Goto, T. Okabe, K. Ashida, *Mech. Ageing Dev.* **2002**, *123*, 1101–1106.
[30] K. Rutkowski, P. Sowa, J. Rutkowska-Talipska, A. Kuryliszyn-Moskal, R. Rutkowski, *Drugs* **2014**, *74*, 1195–1207.
[31] a) E. Frank, Z. Mucsi, I. Zupko, B. Rethy, G. Falkay, G. Schneider, J. Wolfling, *J. Am. Chem. Soc.* **2009**, *131*, 3894–3904; b) B. L. Zhang, E. Zhang, L. P. Pang, L. X. Song, Y. F. Li, B. Yu, H. M. Liu, *Steroids* **2013**, *78*, 1200–1208; c) B. L. Zhang, L. X. Song, Y. F. Li, Y. L. Li, Y. Z. Guo, E. Zhang, H. M. Liu, *Steroids* **2014**, *80*, 92–101.
[32] L. MacCarthy-Morrogh, P. A. Townsend, A. Purohit, H. A. M. Hejaz, B. V. L. Potter, M. J. Reed, G. Packham, *Cancer Res.* **2000**, *60*, 5441–5450.
[33] a) J. A. Mobley, J. O. L'Esperance, M. Wu, C. J. Friel, R. H. Hanson, S.-M. Ho, *Mol. Cancer Ther.* **2004**, *3*, 587–595; b) S. Sinha, S. Roy, B. S. Reddy, K. Pal, G. Sudhakar, S. Iyer, S. Dutta, E. Wang, P. K. Vohra, K. R. Roy, P. Reddanna, D. Mukhopadhyay, R. Banerjee, *Mol. Cancer Res.* **2011**, *9*, 364–374.
[34] E. Ginsburg, D. Salomon, T. Sreevalsan, E. Freese, *Proc. Natl. Acad. Sci. USA* **1973**, *70*, 2457–2461.
[35] K. R. Barnes, A. Kutikov, S. J. Lippard, *Chem. Biol.* **2004**, *11*, 557–564.
[36] N. Krynetskaia, H. Xie, S. Vucetic, Z. Obradovic, E. Krynetskiy, *Mol. Pharmacol.* **2008**, *73*, 260–269.

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