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Steroids from the rhizome of *Anemarrhena asphodeloides* and their cytotoxic activities

Yu Sun [a,b,c], Jie Wu [a,b], Xue Sun [a,b], Xiaoxiao Huang [a,b], Lingzhi Li [a,b], Qingbo Liu [a,b], ShaoJiang Song [a,b,*]

[a] School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, Liaoning, PR China
[b] Key Laboratory of Structure-Based, Drug Design & Discovery, (Ministry of Education), Shenyang 110016, Liaoning, PR China
[c] Qiqihar Medical University, Qiqihar 161006, Heilongjiang, PR China

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ABSTRACT

Cancer remains a major killer worldwide. To search for novel naturally occurring compounds that are cytotoxic to cancer cells to be used as lead structures for drug development, five new steroids (**1**–**5**) along with seven known ones (**6**–**12**) were isolated from the rhizome of *Anemarrhena asphodeloides* Bge. Their structures were established by detailed spectral studies, including 1D-NMR, 2D-NMR, HR-ESI-MS and by comparison with literature data. These compounds exhibited different levels of growth inhibition against A549, HepG2, Hep3B, Bcap37 and MCF7 cell lines in vitro. Compounds **9**, **10** and **11** showed potent inhibitory against all the tested cell lines with IC_{50} values ranging from 0.35 ± 0.15 to 25.53 ± 0.31 μM. The three compounds displayed stronger inhibitory activities against A549, HepG2 and Hep3B cell lines compared with the positive control 5-fluorouracil. The experimental data obtained permit us to identify the roles of the sugar moieties, hydroxyl group, double bond and F-ring with regard to their cytotoxic activities.

Natural products have traditionally be the main source of cytotoxic anticancer agents since the beginning of chemotherapy.[1] Around half the drugs currently in clinical use as anticancer agents come from natural products, and it has been estimated that about 60% of new chemical entities introduced in this field were originally natural products or were derived from a natural product lead compound.[2] *Anemarrhena asphodeloides* is used in China, Japan and Korea as an ingredient in herbal medicines.[3] Its rhizomes exhibit valuable bioactive effects, such as anti-oxidation, anti-osteoporosis, anti-inflammation, anti-microbial, anti-depression and anti-platelet aggregation activities.[4–9] Phytochemical and pharmacological studies have identified the presence of several classes of biologically active components such as steroidal saponins, flavonoids, phenylpropanoids, alkaloids, organic acids, anthraquinones, and xanthones.[10–17] Steroids are the main active components of the *A. asphodeloides* and they have extremely diverse structures with a broad spectrum of biological and pharmacological activities. Particular attention has been given to their potential for cancer therapy.

As part of our ongoing focus on the discovery of new anti-cancer agents from natural products,[18–21] we are interested in the anticancer activities of compounds isolated from *A. asphodeloides* and we have identified five new steroids along with seven known compounds. These compounds were screened using an in vitro activity assay.

A 70% EtOH extract prepared from the roots of *A. asphodeloides*[22] was subjected repeatedly to macroporous adsorption resin D101, column chromatography on silica gel, sephadex LH-20, RP-18 and preparative HPLC to obtain five new compounds (**1**–**5**), together with seven known ones (**6**–**12**). The structures of these compounds (**1**–**12**) are shown in Figure 1.

Compound **1**[23] was obtained as a white amorphous powder. Its molecular formula was determined as $C_{33}H_{56}O_9$ by positive ion HR-ESI-MS with a quasi-molecular ion peak at *m*/*z* 619.3833 $[M+Na]^+$ (calcd for $C_{33}H_{56}O_9Na$ 619.3817). The ^1H NMR spectrum of **1** exhibited four methyl signals at δ_H 1.15 (3H, d, *J* = 7.0 Hz), 1.03 (3H, d *J* = 6.7 Hz), 0.81 (3H, s), 0.78 (3H, s) and an anomeric signal at δ_H 4.83 (1H, d, *J* = 7.8 Hz). The β-anomeric configuration for the glucose was determined from the $^3J_{1,2}$ coupling constant of δ_H 4.83 (1H, d, *J* = 7.8 Hz).[24] The ^{13}C NMR spectrum of **1** revealed characteristic signals for four methyl groups at 17.2, 16.6, 16.2, 12.3 and an anomeric sugar carbon of sugar at δ_C 104.9. The NMR data of **1** were very similar to those of (25*S*)-26-*O*-β-D-glucopyranosyl-5β-furostan-3β,22α,26-triol[25] indicating the same partial structure for rings C, D and E. Significant differences were identified in the signals from rings A and B, where 5-H was supposed to have an α configuration. The 5-H configuration of **1** was further confirmed

* Corresponding author. Tel.: +86 24 23986088; fax: +86 24 23986510.
E-mail address: songsj99@163.com (S. Song).

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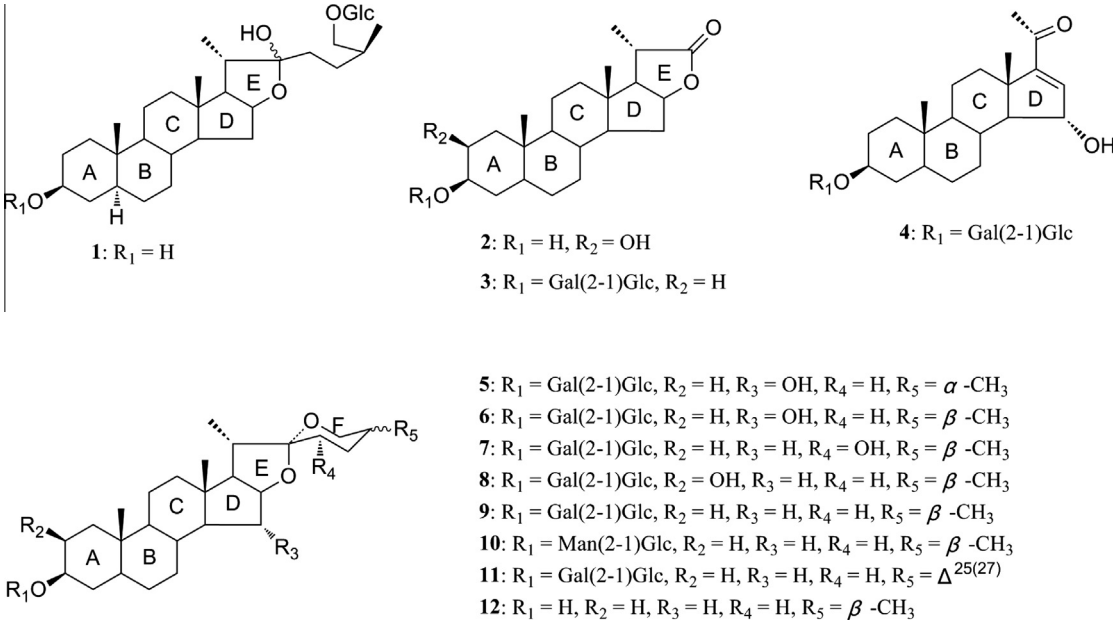

Figure 1. Compounds **1**–**12** isolated from *Anemarrhena asphodeloides*.

on the basis of the carbon chemical shift of 19-CH_3. The carbon chemical shift of 19-CH_3 is about at δ_C 12.3 for 5α compounds and is approximately at δ_C 23.9 for 5β compounds.[26] The structure was ascertained by the HMBC spectrum which showed the correlations from H-19 (δ_H 0.78) to C-10 (δ_C 35.7), C-1 (δ_C 37.2), C-5 (δ_C 44.9), C-9 (δ_C 54.3); from H-18 (δ_H 0.81) to C-12 (δ_C 40.1), C-20 (δ_C 40.5), C-13 (δ_C 40.9), C-14 (δ_C 56.2), C-17 (δ_C 63.7); from H-27 (δ_H 1.03) to C-24 (δ_C 28.1), C-25 (δ_C 34.2), C-26 (δ_C 75.0); from H-21 (δ_H 1.15) to C-20 (δ_C 40.5), C-17 (δ_C 63.7). The sugar linkage to C-26 of the aglycone was confirmed by HMBC correlation between the anomeric proton signal H-1‴ (δ_H 4.83) and the carbon signal C-26 (δ_C 75.0). Acid hydrolysis of **1** afforded D-glucose identified by thin-layer chromatography (TLC) and gas chromatography (GC) analyses.[27,28] Based on above information, the structure of compound **1** was determined and named anemarrhena S1.

Compound **2**[29] was obtained as a white amorphous powder. Its molecular formula was determined as $C_{22}H_{34}O_4$ by positive ion HR-ESI-MS with a quasi-molecular ion peak at *m*/*z* 385.2334 $[M+Na]^+$ (calcd for $C_{22}H_{34}O_4Na$ 385.2349). The ^1H NMR spectrum of **2** exhibited three methyl signals at δ_H 1.33 (3H, d, *J* = 7.6 Hz), 1.02 (3H, s) and 0.76 (3H, s). The ^{13}C NMR spectrum of **2** revealed characteristic signals for three methyl groups at δ_C 20.6, 18.0 and 13.9; one carbonyl group at δ_C 181.3. The above evidence indicated that **2** was a steroid sapogenin. The NMR data of **2** were very similar to that of 3α-hydroxy-23,24-dinor-5β-cholano-22,16-lactone[30] indicating the same partial structure for rings B, C, D and E. Comparison of the NMR spectral data with that of markogenin[31] revealed that it has the same partial structure for rings A, B, C and D. The structure was confirmed by HMBC spectrum which showed correlations from H-21 (δ_H 1.33) to C-20 (δ_C 36.1), C-17 (δ_C 59.1) and C-22 (δ_C 181.3); from H-18 (δ_H 0.76) to C-12 (δ_C 38.4), C-13 (δ_C 41.8), C-14 (δ_C 54.6) and C-17 (δ_C 59.1); from H-19 (δ_H 1.02) to C-1 (δ_C 38.4), C-2 (δ_C 67.6), C-5 (δ_C 35.3), C-9 (δ_C 41.4) and C-10 (δ_C 36.9) (Fig. 2). Based on above information, the structure of compound **2** was determined and it was named anemarrhena S2.

Compound **3**[32] was obtained as a white amorphous powder with the molecular formula $C_{34}H_{54}O_{13}$, in agreement with the positive ion HR-ESI-MS (positive) *m*/*z*: 693.3470, $[M+Na]^+$ calcd for $C_{34}H_{54}O_{13}Na$ 693.3457. The ^1H NMR spectrum of **3** showed three methyl signals at δ_H 1.28 (3H, d, *J* = 7.6 Hz), 0.94 (3H, s), 0.71 (3H, s) and two anomeric signals at δ_H 5.29 (1H, d, *J* = 7.7 Hz) and 4.91 (1H, d, *J* = 7.6 Hz). The ^{13}C NMR spectrum of **3** revealed characteristic signals for three methyl groups at δ_C 23.7, 17.7 and 13.5; one carbonyl group at δ_C 180.9; two sugar anomeric carbons of at δ_C 105.9 and 102.3. The above evidences indicated that **3** was a steroid saponin with two sugar units. The NMR data of **3** was very similar to that of timosaponin AIII[33] indicating the same partial structure for rings A, B, C and D. Comparison of the NMR spectral data with that of **2** revealed that it has the same partial structure with rings B, C, D and E. The structure was confirmed by the HMBC spectrum which showed correlations from H-21 (δ_H 1.28) to C-20 (δ_C 36.0), C-17 (δ_C 58.8) and C-22 (δ_C 180.9); from H-18 (δ_H 0.71) to C-12 (δ_C 38.1), C-13 (δ_C 41.6), C-14 (δ_C 54.2) and C-17 (δ_C 58.8). Acid hydrolysis of **3** afforded D-glucose and D-galactose in a ratio of 1:1 by thin layer chromatography (TLC) and gas chromatography (GC) analyses. The β-anomeric configuration for the glucose was determined from the $^3J_{1,2}$ coupling constant of δ_H 5.29 (1H, d, *J* = 7.7 Hz), while the β-anomeric configuration for the galactose was determined from the $^3J_{1,2}$ coupling constant of δ_H 4.91 (1H, d, *J* = 7.6 Hz). The sugar sequences and its linkage to C-3 of the aglycone were determined by HMBC correlations between the anomeric proton signal H-1′ (δ_H 4.91) and the carbon signal C-3 (δ_C 74.9), between H-1″ (δ_H 5.29) and C-2′ (δ_C 81.6). Consequently, the structure of **3** was confirmed and it was named anemarrhena S3.

Compound **4**[34] was obtained as a white amorphous powder with the molecular formula $C_{33}H_{52}O_{13}$ on the basis of a quasi-molecular ion at *m*/*z* 679.3307 $[M+Na]^+$ (calcd for $C_{33}H_{52}O_{13}$, 679.3300) in the HR-ESI-MS. In the ^1H NMR spectrum, it was found that there were three methyl signals at δ_H 1.04, 1.06 and 2.26 (each 3H); and two anomeric signals at δ_H 5.29 (1H, d, *J* = 7.7 Hz), 4.89 (1H, d, *J* = 7.5 Hz). The ^1H and ^{13}C NMR data of **4** were almost superimposable with rings A, B and C of timopregnane A.[35] The appearance of a hydroxyl group at C-15 was shown by the HMBC correlations from H-16 (δ_H 6.87) to C-15 (δ_C 76.3). The structure was further confirmed by the HMBC spectrum which showed correlations from H-16 (δ_H 6.87) to C-13 (δ_C 46.1), C-14 (δ_C 63.8), C-17 (δ_C 152.3) and C-20 (δ_C 196.6); from H-18 (δ_H 1.06) to C-13 (δ_C 46.1), C-14 (δ_C 63.8) and C-17 (δ_C 152.3); from H-21 (δ_H 2.26) to C-17 (δ_C 152.3) and C-20 (δ_C 196.6). The sugar part of **4** was deter-