Bioorganic & Medicinal Chemistry Letters 24 (2014) 1600–1604

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis and cytotoxic effect of pseudodiosgenyl saponins with thio-ring F

Xin Zan [a,1], Jian Gao [a,1], Guofeng Gu [b], Shanshan Liu [c], Bin Sun [a], Lei Liu [a], Hong-Xiang Lou [a,*]

[a] Department of Natural Product Chemistry, Key Lab of Chemical Biology of MOE (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, 44 West Wenhua Road, Jinan 250012, PR China
[b] National Glycoengineering Research Center, Shandong University, Jinan 250100, PR China
[c] School of Oceanology, Shandong University, Weihai 264209, PR China

ARTICLE INFO

Article history:
Received 7 October 2013
Revised 3 January 2014
Accepted 18 January 2014
Available online 28 January 2014

Keywords:
Steroid saponins
Pseudodiosgenyl conjugates
Cytotoxicity
Apoptosis

ABSTRACT

Both the sugar moieties and aglycons of steroid saponins play important roles for their bioactivities. In order to test the biological contribution of the glycosyl residue and search new saponins with notable anticancer activity, mono- and di-saccharide pseudodiosgenyl saponins **22**–**28** together with two pseudodiosgenyl conjugates **29** and **30** were conveniently synthesized, all of which were based on the aglycon **7** bearing the thio-ring F. The cytotoxicity on human cancer cells (MCF-7, HepG-2, A549) for all of the synthesized compounds **7** and **22**–**30** was evaluated by MTT method. The thio-aglycon **7** when conjugated with sugars exhibited potent cytotoxicity, and the introduction of D-glucosamine into aglycon **7** led to the most potent compound **28**. Furthermore, DAPI staining, AV/PI staining, AO-relocation, AO-uptake and LysoTracker Red-uptake assays demonstrated that the cell death caused by neosaponin **28** was at least partially through apoptosis involving lysosomal membrane permeabilization.

Steroid saponins, widespread in Chinese medicinal herbs, possess a wide range of structural diversities and biological activities, such as cAMP phosphodiesterase inhibition, antifungal, antiviral as well as antitumor activities.[1–3] Both the sugar moieties and aglycons play important roles for their bioactivities.[4,5] For example, solamargine, solasonine and khasianine, which have the identical aglycon but variant sugar moieties showed different antiproliferative effects.[6] Conversely, indioside E and some of its trisaccharide analogs also exhibited different anticancer activities.[7]

Solamargine (SM), the most powerful integrant in clinically antitumor drug BEC (a mixture of glycoalkaloids from the Devil's Apple plants),[8] comprises a very labile nitrogen ring-F contained in aglycon, namely salasodine. Consequently, there was surprisingly few reports about the synthesis of SM and its analogs.[9] In addition, the bioisosteres of SM, for example, dioscin and 26-thiodioscin (Fig. 1), also exhibited good anticancer activity.[9–12] So far as we know, there is no saponin except 26-thiodioscin containing the aglycon **7** with the ring F in which the oxygen was substituted with sulfur has been reported.[12] In view of these precedents, we herein exploited the synthesis of the pseudodiosgenyl conjugates **22**–**30** containing the aglycon **7** to look for new saponins with pronounced cytotoxic activity and to value the biological contribution of sugar moieties for saponins. The simplified residues installed to the aglycon **7** may facilitate industrial-scale preparation of the biological saponins. Furthermore, the in vitro anticancer activity of all the synthesized compounds **7** and **22**–**30** was assayed and the preliminary apoptosis mechanism of the most potent saponin **28** was also revealed in the manuscript.

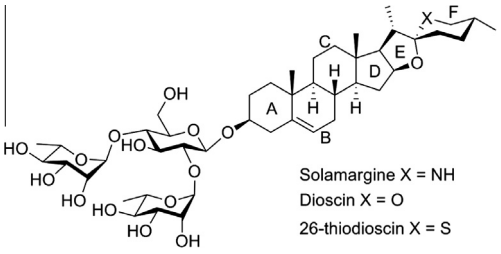
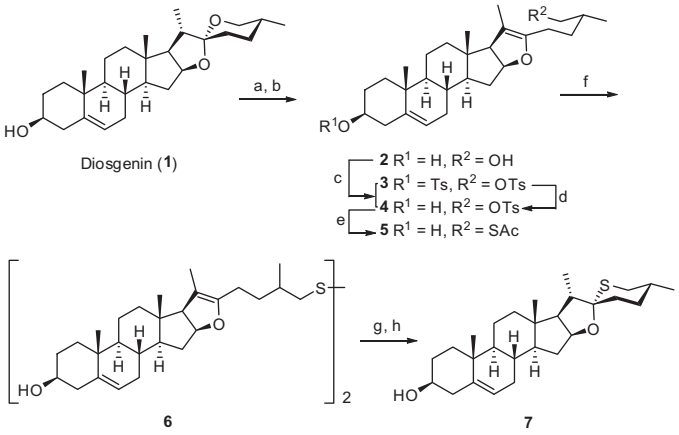


Figure 1. Structures of solamargine, dioscin and 26-thiodioscin.

As outlined in Scheme 1, synthesis of aglycon **7** was conducted according to the reported methods.[13–16] Ring F opening of diosgenin **1** with Ac_2O/AcCl/Pyr. in refluxing *p*-xylene, followed by hydrolysis of the acetyl group gave diol **2** in 79% yield over two steps.[15] The yield of this ring opening reaction was safeguarded by using a small quantity of Pyr. as a scavenger for the excess acetic and hydrochloric acid generated from the reaction. Thereafter,

* Corresponding author. Tel.: +86 531 88382012; fax: +86 531 88382019.
E-mail address: louhongxiang@sdu.edu.cn (H.-X. Lou).
[1] These authors contributed equally to this work.

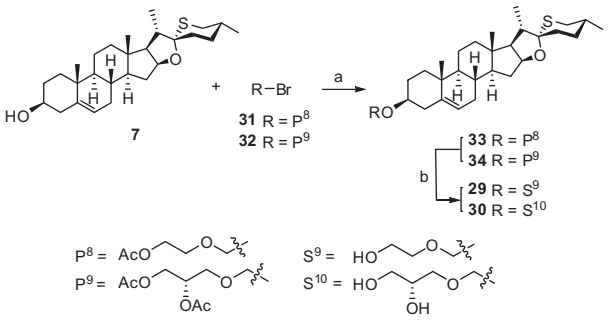
http://dx.doi.org/10.1016/j.bmcl.2014.01.055
0960-894X/



Scheme 1. Reagents and conditions: (a) Ac_2O, AcCl, Pyr., *p*-xylene, reflux 10 h; (b) KOH, CH_3OH/H_2O, 79% over two steps; (c) TsCl, Pyr., 0 °C, 12 h, 33% for **4**, 55% for **3**; (d) H_2O/acetone 3:7, reflux 1.5 h, 84%; (e) CH_3COSK, DMF, rt, 24 h, 85%; (f) KOH, H_2O/CH_3OH, rt, 12 h, 92%; (g) Zn, AcOH, reflux; (h) KOH, acetone, 90% over two steps.

treatment of **2** with 2.5 equiv *p*-tosyl chloride (*p*-TsCl) at 0 °C afforded the desired 26-*O*-*p*-tosylpseudodiosgenin **4** (33% yield) together with 3β,26-di-*O*-*p*-toluenesulfonate derivate **3** (55% yield), and the undesired **3** was selectively hydrolyzed in refluxing aqueous acetone for 2 h to give **4** with the 84% overall yield from **2**.[16] Replacing the 26-*O*-*p*-tosyl group in **4** for thioacetyl using potassium thioacetate in DMF (→**5**), followed by alkaline hydrolysis yielded the disulphide dimer **6**.[13] Finally, the thio-ring F pseudodiosgenin **7** was achieved from **6** through the reaction with zinc powder in acetic acid and subsequent hydrolysis in 44% overall yield over eight steps starting from diosgenin **1**.

With the key thio-aglycon **7** in hand, we commenced to prepare the new saponins **22**–**28** (Scheme 2). In the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf), **7** was glycosylated with mono- and di-saccharide trichloroacetimidates **8**–**13** at 0 °C to afford the protected intermediates **15**–**20**, which was in situ transformed to target neosaponins **22**–**27** through saponification with CH_3ONa. En route to compound **28**, the commercial D-glucosamine hydrochloride was readily converted to imidate **14** according to reported method.[17] **14** was then coupled with aglycon **7** catalyzed by TMSOTf to give **21**. Thereafter, deacetylation with CH_3ONa and hydrolysis of the phthalic anhydride with CH_3NH_2 provided the compound **28** in 79% yield over three steps. All of the above

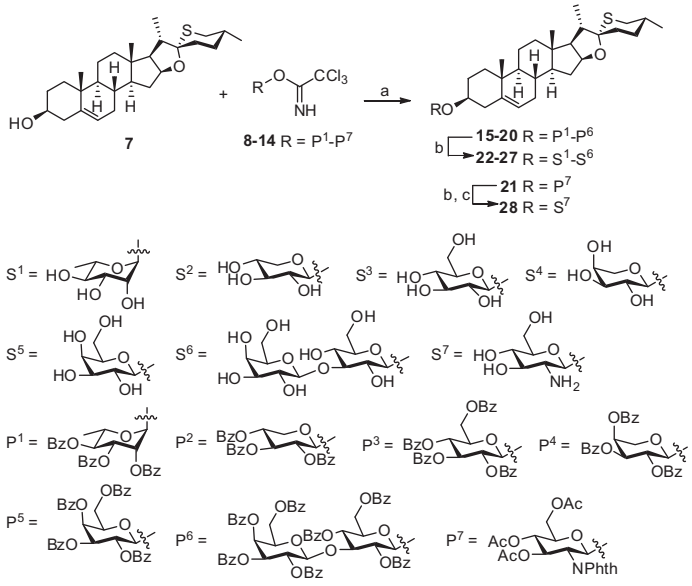


Scheme 3. Reagents and conditions: (a) DIEA, CH_2Cl_2, rt, 12 h, 85% for **33**, 80% for **34**; (b) 0.5 N NaOMe, CH_2Cl_2/CH_3OH 2:1, rt, 3 h 90% for **29**, and 80% for **30**.

Table 1
The cytotoxicity of compounds **7**, **22**–**30** on three tumor cell lines and one normal cell line[a]

Compound	IC_{50} (μM)[b]			
	Cancer cell			Normal cell
	MCF-7	HepG-2	A549	HL7702
7	34.90	37.67	30.48	47.22
22	14.46	15.26	13.57	36.53
23	15.23	11.40	12.66	34.63
24	26.10	24.42	21.24	23.52
25	20.92	38.21	31.37	51.55
26	15.47	15.24	10.25	47.30
27	31.33	36.07	32.25	67.26
28	6.08	11.43	6.03	31.57
29	>70	>70	>70	>70
30	16.37	16.82	10.75	20.71
SM	8.12	2.67	8.18	13.23

[a] Values are means of three independent experiments.
[b] IC_{50} values were determined at 24 h.



Scheme 2. Reagents and conditions: (a) TMSOTf, CH_2Cl_2, 0 °C, MS 4 Å, 30 min, 84–88% for **15–21**; (b) 0.5 N NaOMe, CH_2Cl_2/CH_3OH 2:1, 3 h, 90–93% for **22–27**; (c) aqueous 40% CH_3NH_2, CH_3OH, 4 h, 79% from **21**.

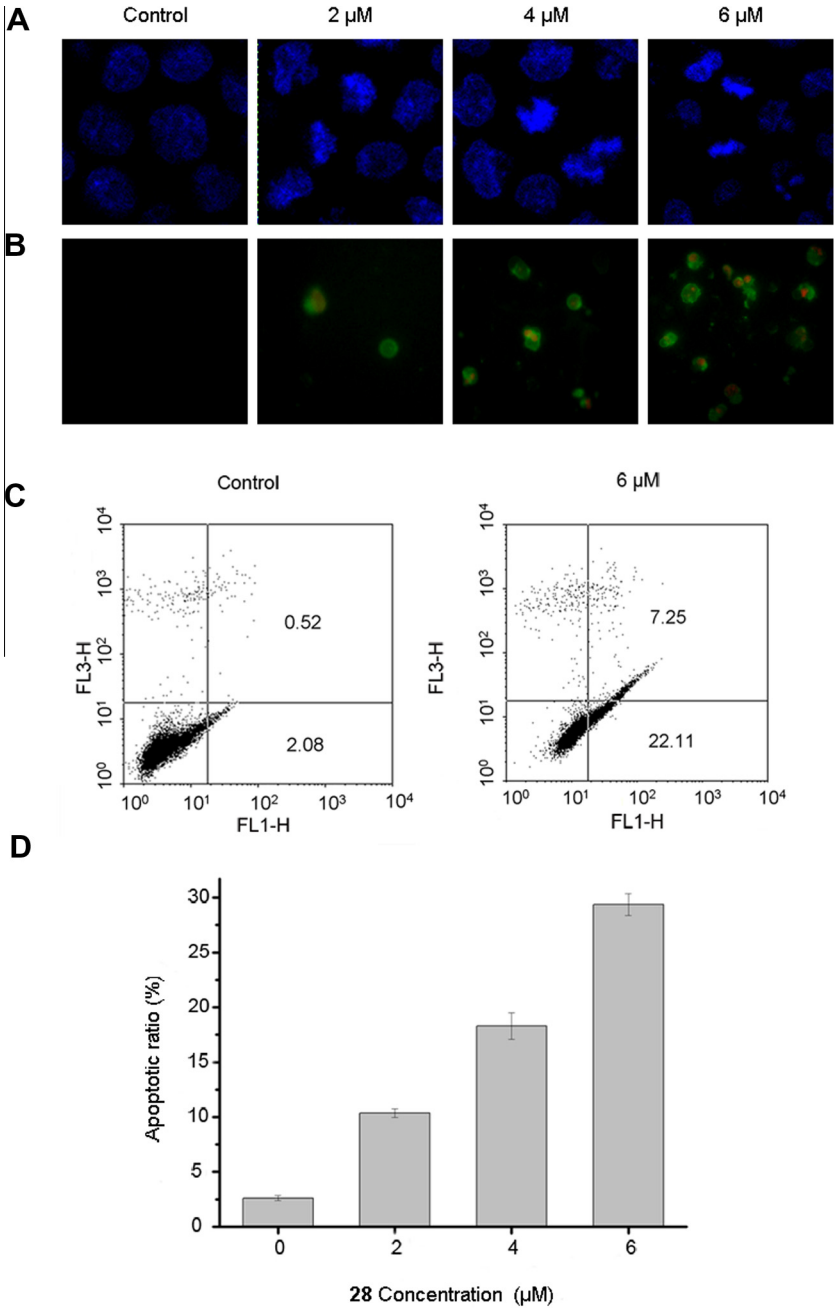


Figure 2. Induction of apoptosis in A549 cells by saponin **28**. (A) DAPI staining of A549 cells after 24 h treatment with serial concentrations of **28**. (B) Annexin-V/PI uptake of A549 cells after **28** treatment. (C, D) Quantitation of **28**-induced apoptosis in A549 cells by flow cytometric analysis. The values are means ± standard deviations (indicated by the bars) of three independent experiments. FL1-H stands for green fluorescence signal received by the photomultiplier tube (PMT), means the amount of apoptotic cells stained with Annexin V; FL3-H stands for red fluorescence signal received by the photomultiplier tube, means the amount of necrotic cells stained by PI.

resultant glycosidic linkages were stereo-controlled owing to the neighboring group participation, which were confirmed by ^{1}H NMR spectra[18] (α glycosidic linkage for **22**, $J_{1,2}$ = 1.2 Hz; **25**, $J_{1,2}$ = 6.6 Hz. β glycosidic linkages for **23**, **24** and **26**–**28**, $J_{1,2}$ ranging from 6.2 to 7.1 Hz). The anomeric C-H coupling constants of **22**–**28** proved conformation of their glycosyl residues as follows: **22**, $^1C_4$, $^1J_{C,H}$ = 171 Hz; **23**–**28**, $^4C_1$, $^1J_{C,H}$ ranging from 161 to 163 Hz.

With hope to simplify the structure of sugar residue and to expurgate the unnecessary glycosidic linkage, two pseudodiosgenyl conjugates containing a chain with free hydroxyl groups were designed and assembled (Scheme 3). Coupling of **7** with **31** and **32**, prepared from 1,3-dioxolane and 1,3-dioxolan-4-yl-methanol,[19,20] followed by deprotection provided the compounds **29** and **30**, respectively.

Cytotoxic activity of the synthesized aglycon **7** and novel thiopseudodiosgenyl conjugates **22**–**30** against three cancer cell lines, including human breast adenocarcinoma cell (MCF-7), human hepatocellular liver carcinoma cell (HepG-2), and human lung adenocarcinoma cell (A549), together with the normal cell line human hepatocyte (HL7702) was assayed by MTT method.[21] As shown in Table 1, Saponin **28** bearing D-glucosamine as sugar residue displayed the strongest antiproliferative activity against MCF-7 and A549 cell lines with IC_{50} of 6.03 and 6.08 μM, respectively, whereas **28** was less cytotoxic to the normal HL7702 (IC_{50} = 31.57 μM). Compounds **22**, **23** and **26**, containing L-rhamnose, D-xylose, and D-galactose, respectively, exhibited moderate cytotoxicity on three cancer cell lines with IC_{50} ranging from 10.24 to 15.47 μM and weak antiproliferative effect to

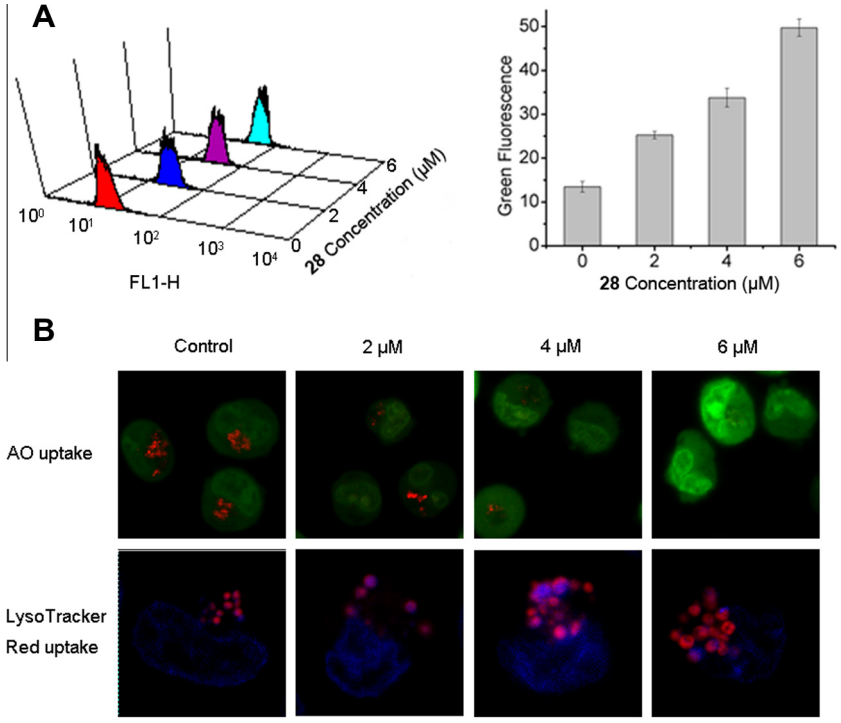


Figure 3. The effect of saponin **28** on lysosomal membrane permeabilization was evaluated with AO relocation analysis, AO-uptake and LysoTracker Red uptake methods. (A) Lysosomal rupture measured by AO relocation analysis. Increase in green-AO fluorescence, indicating AO was released from ruptured lysosomes to the cytosol, and was detected by flow cytometry. The values are means ± standard deviations (indicated by the bars) of three independent experiments. (B) Lysosomal rupture was measured by AO-uptake and LysoTracker Red uptake and visualized by fluorescence microscopy. FL1-H stands for Green Fluorescence signal received by the photomultiplier tube (PMT), means the amount of AO is present in the cytosol.

HL7702 (>30 μM). In addition, compound **30** containing a chain with two free hydroxyl groups also exhibited the comparable anticancer effect with compounds **22**, **23** and **26**. The other saponins **24**, **25**, and **27** showed a little better anticancer effect than aglycon **7**. Compound **29** did not show the antiproliferative effect against the three cancer cells at a concentration of 70 μM. These results indicated that introducing glycosyl residue to aglycon **7** could improve its cytotoxicity, and incorporating D-glucosamine to **7** leaded to the most potent neosaponin **28**. Incorporation of glycosyl residue to the aglycon **7** might be advantageous for the uptake of compounds into cell. Furthermore, the enhanced alkalinity of glycoside **28** after the introduction of D-glucosamine might facilitate its accumulation in protonated form within acidic vesicles (lysosomes), which could induce lethal lysosomal destabilization and cell death.[22]

As we know, two types of prelethal reaction of cell death have been characterized: apoptosis and oncosis.[23,24] In addition, our group has demonstrated that some biological saponins, for example, SM and indioside E, could cause cell death through both of these two patterns.[7,10,25] Encouraged by the good anticancer activity and lower toxicity to normal cell HL7702 of neosaponin **28**, we performed further test to elucidate whether its cytotoxic effect of A549 was associated with apoptosis or not. After A549 cells were incubated with 2, 4 and 6 μM concentrations of neosaponin **28** for 24 h, apoptotic characteristics were observed by DAPI staining.[26] Morphological changes, such as chromatin condensation, cell shrinkage, membrane blebbing, and apoptotic body formation, were canonical apoptotic markers (Fig. 2A and B). In addition, A549 cells double stained with Annexin V and propidium iodide (PI) were analyzed by flow cytometry to quantitate the extent of **28**-induced apoptosis.[27] Annexin V staining provides the possibility to detect the presence of phosphatidylserine on the outer leaflet of the cell membrane, a characteristic related to apoptosis. Necrotic cells can be stained by PI, and exhibited red fluorescence. As shown in Figure 2C and D, the proportion of apoptotic cells stained with Annexin V increased after the treatment of **28**. All of the above results suggested that the cytotoxicity of compound **28** against A549 cells was at least partly induced by the apoptosis. It is noteworthy that the 28-induced anticancer activity on A549 could be only partially the result of apoptosis, and the oncosis may be also involved, which is actually our ongoing research project.

To assess the effect of **28** on lysosomal membrane permeabilization, three assays including AO-relocation, AO-uptake and LysoTracker Red-uptake were applied.[25,28] AO, a metachromatic fluorophore that accumulates in normal lysosomes, exhibits a low level of green fluorescence when cloistered inside of the lysosome. When lysosomes are disrupted, AO is present in the cytosol, manifesting a much brighter green fluorescence.[29] AO relocation method demonstrated that lysosomes had been disrupted since enhanced cytosolic green fluorescence was detected by flow cytometry after AO-loaded cells were exposed to **28** for 2 h (Fig. 3A). AO-uptake method verified the results of AO relocation method by showing decreased numbers of AO-accumulating lysosomes after 2 h exposure to **28**. The loss of the punctate red spots (lysosomes with intact integrity) depended on the concentrations of **28** (Fig. 3B). LysoTracker Red-uptake methods also demonstrated lysosomal destabilization by showing lysosomes became swollen and trended rupture after 2 h exposure to **28**, while the nucleus stained with DAPI appeared apoptotic characteristics (Fig. 3B). These results show that compound **28** caused lysosomal vacuolation, disrupted lysosomal membrane integrity and initiated an early lysosomal destabilization pathway leading to apoptosis.

In summary, nine pseudodiosgenyl conjugates containing the aglycon **7** with a thio-ring F were facilely assembled. Preliminary anticancer evaluation indicated that the incorporation of glycosyl

residue to the aglycon **7** could improve its antiproliferative effect pronouncedly, and incorporation of the D-glucosamine generated the most potent neosaponin **28**. Compound **28**-induced apoptosis on A549 cell was involved lysosomal vacuolation and disruption of lysosomal membrane integrity. However, the anticancer activity of saponin **28** may be also related to the other mechanism, such as oncotic necrosis, which is the ongoing exploitation in our group.

Acknowledgment

This work was supported by grants from Natural Science Foundation of China (No. 81172956).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.01.055.

References and notes

1. Tang, Y.; Li, N.; Duan, J.; Tao, W. *Chem. Rev.* **2013**, *113*, 5480.
2. Saleem, M.; Nazir, M.; Ali, M. S.; Hussain, H.; Lee, Y. S.; Riaz, N.; Jabbar, A. *Nat. Prod. Rep.* **2010**, *27*, 238.
3. Song, G.; Yang, S.; Zhang, W.; Cao, Y.; Wang, P.; Ding, N.; Zhang, Z.; Guo, Y.; Li, Y. *J. Med. Chem.* **2009**, *52*, 7368.
4. Křen, V.; Martínková, L. *Curr. Med. Chem.* **2001**, *8*, 1303.
5. Podolak, I.; Galanty, A.; Sobolewska, D. *Phytochem. Rev.* **2010**, *9*, 425.
6. Chang, L.-C.; Tsai, T.-R.; Wang, J. J.; Lin, C.-N.; Kuo, K.-W. *Biochem. Biophys. Res. Commun.* **1998**, *242*, 21.
7. Gao, J.; Li, X.; Gu, G.; Sun, B.; Cui, M.; Ji, M.; Lou, H.-X. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 622.
8. Cham, B. E. *Int. J. Clin. Med.* **2012**, *3*, 115.
9. Wei, G.; Wang, J.; Du, Y. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2930.
10. Sun, L.; Zhao, Y.; Yuan, H.; Li, X.; Cheng, A.; Lou, H. *Cancer Chemother. Pharmacol.* **2011**, *67*, 813.
11. Wang, Y.; Zhang, Y.; Zhu, Z.; Zhu, S.; Li, Y.; Li, M.; Yu, B. *Bioorg. Med. Chem.* **2007**, *15*, 2528.
12. Chen, P.; Wang, P.; Song, N.; Li, M. *Steroids* **2013**, *78*, 959.
13. Quan, H.-J.; Koyanagi, J.; Ohmori, K.; Uesato, S.; Tsuchido, T.; Saito, S. *Eur. J. Med. Chem.* **2002**, *37*, 659.
14. Uhle, F. C. *J. Org. Chem.* **1962**, *27*, 2797.
15. Zha, X.; Sun, H.; Hao, J.; Zhang, Y. *Chem. Biodivers.* **2007**, *4*, 25.
16. Uhle, F. C. *J. Am. Chem. Soc.* **1961**, *83*, 1460.
17. Kretzschmar, G.; Stahl, W. *Tetrahedron* **1998**, *54*, 6341.
18. Deng, S.; Yu, B.; Xie, J.; Hui, Y. *J. Org. Chem.* **1999**, *64*, 7265.
19. Koizumi, Y.; Seki, S.; Tsukuda, S.; Sakamoto, S.; Tagawa, S. *J. Am. Chem. Soc.* **2006**, *128*, 9036.
20. Radi, S.; Lazrek, H. B. *Bull. Korean Chem. Soc.* **2002**, *23*, 437.
21. Carmichael, J.; DeGraff, W. G.; Gazdar, A. F.; Minna, J. D.; Mitchell, J. B. *Cancer Res.* **1987**, *47*, 936.
22. Boya, P.; Kroemer, G. *Oncogene* **2008**, *27*, 6434.
23. Trump, B. E.; Berezesky, I. K.; Chang, S. H.; Phelps, P. C. *Toxicol. Pathol.* **1997**, *25*, 82.
24. Majno, G.; Joris, I. *Am. J. Pathol.* **1995**, *146*, 3.
25. Sun, L.; Zhao, Y.; Li, X.; Yuan, H.; Cheng, A.; Lou, H. *Toxicol. In Vitro* **2010**, *24*, 1504.
26. Kapuscinski, J. *Biotech. Histochem.* **1995**, *70*, 220.
27. Bacsó, Z.; Everson, R. B.; Eliason, J. F. *Cancer Res.* **2000**, *60*, 4623.
28. Yuan, X.-M.; Li, W.; Dalen, H.; Lotem, J.; Kama, R.; Sachs, L.; Brunk, U. T. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 6286.
29. Gorria, M.; Tekpli, X.; Rissel, M.; Sergent, O.; Huc, L.; Landvik, N.; Fardel, O.; Dimanche-Boitrel, M.-T.; Holme, J. A.; Lagadic-Gossmann, D. *Toxicol. Appl. Pharmacol.* **2008**, *228*, 212.