Journal Pre-proof

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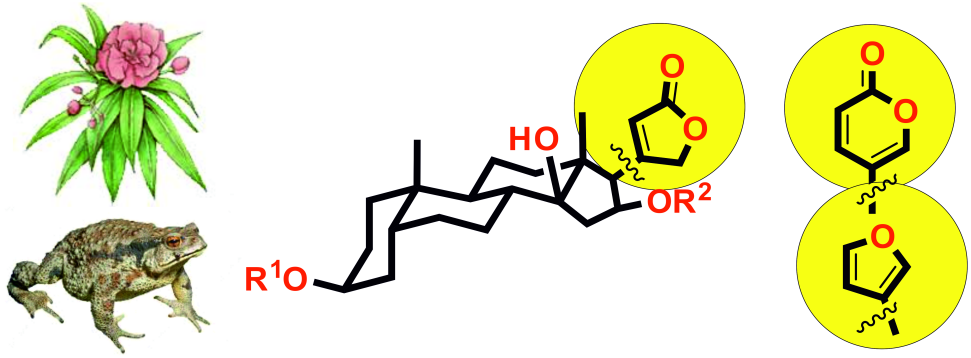


Synthesis and Evaluation of Na^+/K^+-ATP-ase Inhibiting, and Cytotoxic *in vitro* Activities of Oleandrigenin and Selected 17β-(Butenolidyl)- and 17β-(3-Furyl)- Analogues of Cardenolides and Bufadienolides

Karol Michalak,[#a] Lucie Rárová, [#b,c] Martin Kubala,[d] Tereza Štenclová, [b] Miroslav Strnad,*[b,c] and Jerzy Wicha*[a]

[a]*Institute of Organic Chemistry, Polish Academy of Sciences, ul. Marcina Kasprzaka 44/52, 01-224 Warsaw, Poland*

[b]*Laboratory of Growth Regulators, Institute of Experimental Botany of the Czech Academy of Sciences, and Faculty of Science, Palacký University, Šlechtitelů 27, CZ-78371 Olomouc, Czech Republic*

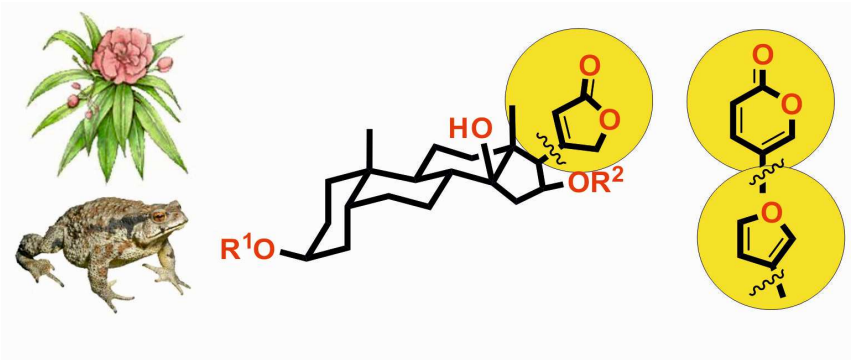
[c]*Department of Neurology, University Hospital in Olomouc, I. P. Pavlova 6, CZ-775 20 Olomouc, Czech Republic*

[d]*Department of Experimental Physics, Faculty of Science, Palacký University, Šlechtitelů 27, 783 41 Olomouc, Czech Republic*

*corresponding authors: MS (miroslav.strnad@upol.cz); JW (jerzy.wicha@icho.edu.pl)

[#] these authors contributed equally

Graphical Abstract



Abstract

Natural cardiac-active principles built upon the 14,16β-dihydroxy-5β,14β-androstane core and bearing a heterocyclic substituent at 17β, in particular, a cardenolide - oleandrin and a bufadienolide - bufotalin, are receiving a great deal of attention as potential anticancer drug. The densely substituted and sterically shielded ring D is the particular structural feature of these compounds. The first synthesis of oleandrigenin from easily available steroid starting material is reported here. Furthermore, selected 17β-(4-butenolidyl)- and 17β-(3-furyl)-14,16β-dihydroxy-androstane derivatives were *en route* synthesized and examined for their Na^+/K^+-ATP-ase inhibitory properties as well as cytotoxic activities in normal and cancer cell lines. It was found that the furyl-analogue of oleandrigenin/bufatalin (**7**) and some related 17-(3-furyl)- derivatives (**19**, **21**) show remarkably high Na^+/K^+-ATP-ase inhibitory activity as well as significant cytotoxicity *in vitro*. In addition, oleandrigenin **2** compared to derivatives **21** and **25** induced strong apoptosis in human cervical carcinoma HeLa cells after 24 h of treatment.

Introduction

Steroid cardiac-active principles[1] embracing cardenolides and bufadienolides are currently considered a promising source of anti-cancer drugs.[2-8] Both these groups of compounds are built on the 14β-androstane skeleton bearing an oxygen functional group at position 14 and a heterocyclic substituent at position 17β: 3-butenolidyl for cardenolides and 5-(α-pyranoyl) for bufadienolides. Cardenolides are known primarily as plant-derived compounds where they occur as 3-*O*-glycosides in combination with specific sugars whereas bufadienolides occur in plants as glycosides as well as in certain animals mainly amphibians as free 3-hydroxy derivatives, hemisubarates or conjugates with amino acids.

Recent researchers' attention has focused on oleandrin (**1**, Fig. 1), a glycoside of oleandrigenin (**2**) and oleandrose, a cardenolide isolated from decorative shrub *Nerium oleander* L.,[9-10] preparations which have been used in therapy since ancient times. Two herbal preparations of *Nerium oleander*, Anvirzel[11] (a hot water extract) and Breastin[12] (a cold water extract) are developed for cancer treatment.

The distinctive structural feature of the steroid core of **1** is the presence of the acetoxy group at position 16β that makes ring C densely substituted and sterically shielded. Closely related to **1** is gitoxin **3** isolated[13] from *Digitalis purpurea* L. along with its aglycone, gitoxygenin **4**, and its respective 16-*O*-formyl derivatives. Gitoxin and its 16-*O*-formate are considered[14-15] pharmacologically the most important components of *Digitalis* medications. The cytotoxic activity of **3** and **4** against various malignant cell cultures has recently been determined.[16]

Much attention is also paid[6, 17] to bufadienolides in the Chinese traditional medicine drug Ch'an Su which is obtained from the secretion of the auricular and skin glands of the toad, *Bufo bufo gargarizans*. Ch'an Su is a complex mixture of compounds of which derivatives of 16β-acetoxy- and 16β-hydroxy-androstane, bufotalin, **5**, and desacetylbufotalin **6**, are prominent ingredients.[18-20]

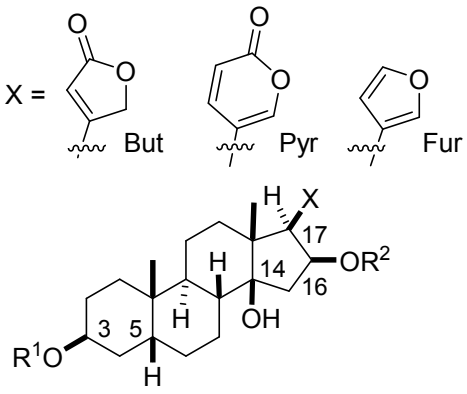
The structural differences between cardenolide **2** and bufadienolide **5** are reduced to the nature of the heterocyclic unit. Although synthetic interconversion of butenolide and α-pyrone moieties appears rather ambiguous, there are convincing indications in the literature that both these heterocycles may be related through suitable 3-furyl precursors.[21-22] The presumed common synthetic precursor, (3-furyl)-androstane derivative (**7**) is interesting in its own rights. Some 17-furyl cardenolide/butadienolide analogues are reported to exhibit cardiac activity.[23-25] Their cytotoxic activity has not been explored.

It has been shown that cardenolides bind to the regulatory site on the extracellular surface of the Na^+/K^+-ATP-ase and trigger processes responsible, e. g. for heart muscle and blood

pressure regulation, motility, cell proliferation, apoptosis and others.[26-27] It has been suggested that the α3 isoform of Na^+/K^+-ATP-ase may serve as a target for the cardiac glycoside treatment of cancer and that synthetic analogues of oleandrin are likely to provide more specific anti-cancer agents.[28]

The cytotoxic activities of oleandrin (**1**) and oleandrigenin (**2**) have been extensively studied and reviewed.[2, 12, 28-32] Bufotalin (**5**) has been recently reported to inhibit U2OS osteoblastoma cell growth *in vivo*,[33] inducing TNF-α- and TRAIL-induced apoptosis in HeLa cells.[34] It also inhibits the growth of multidrug resistant Hep G2 cells through G_2/M cell cycle arrest and induces apoptosis.[35] As one of the most effective suppressants of human cancer cells, it is considered a novel drug candidate.[36]

Insight into the biological activity of 16β-functionalized cardenolides and bufadienolides is based entirely upon studies of isolated natural compound and their simple derivatives.[37-40] Although cardenolide synthesis is receiving considerable attention[41-47] no synthetic approach to these compounds has been reported to date. The purpose of this study was to develop a synthetic route to oleandrigenin **2** from a common steroid starting material through its 3-furyl counterpart **7** and to investigate the cytotoxic and Na^+/K^+-ATP-ase inhibiting activity of selected 16β-functionalized 17β-(4-butenolidyl)- and 17β-(3-furyl)-androstane derivatives. Our model work aiming at 3-*O*-methoxy-5α-oleandrigenin was recently reported.[32, 48]



1, R^1 = Oleandrosyl, R^2 = Ac, X = But, Oleandrin
2, R^1 = H, R^2 = Ac, X = But, Oleandrigenin
3, R^1 = Tridigitoxose, R^2 = H, X = But, Gitoxin
4, R^1 = R^2 = H, X = But, Gitoxigenin
5, R^1 = H, R^2 = Ac, X = Pyr, Bufotalin
6, R^1 = R^2 = H, X = Pyr, Desacetylbufotalin
7, R^1= H, R^2 = Ac, X = Fur

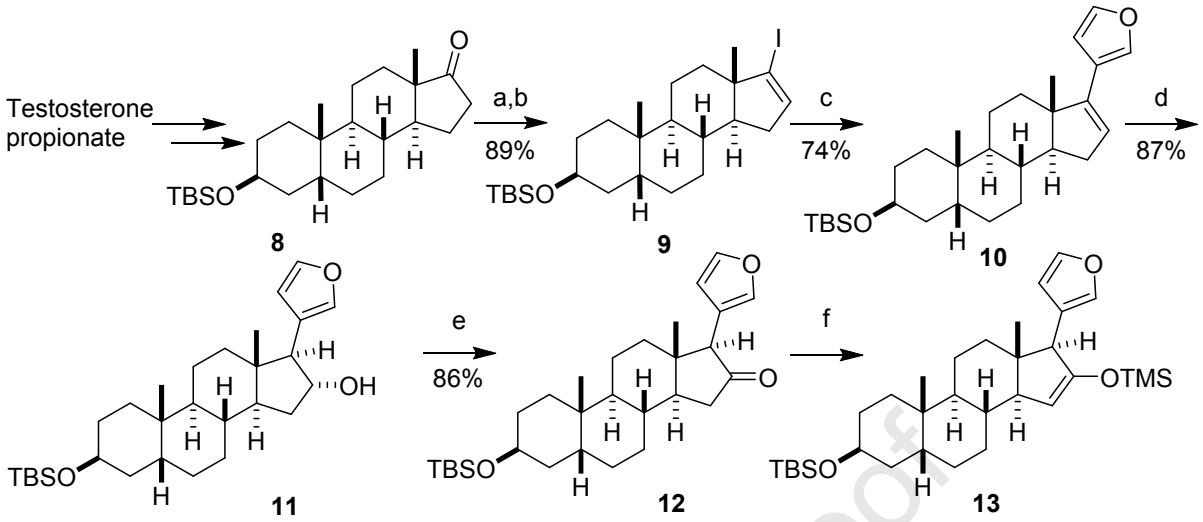
Fig. 1. Structures of selected cardenolides, bufadienolides and their 3-furyl analogues.

Results and discussion

Chemistry

The synthesis of oleandrigenin (**2**) commenced from 3-*O*-*tert*-butyldimethylsilyl (TBS) 5β-androstan-3β-ol-17-one (**8**, Scheme 1), which was prepared from testosterone propionate in five steps, essentially following the reported procedures (see, Supporting Information). Ketone **8** was converted to vinyl iodide **9** according to Barton's protocol.[49] The Suzuki-Miyaura cross-coupling reaction[50] of **9** and (3-furyl)boronic acid applying *tetrakis*-(triphenylphosphine)palladium (0) (10 mol%) and aq $NaHCO_3$ afforded **10** in a 74 % yield. Hydroboration of **10** using borane-THF complex in THF following by oxidation[51-52] of the immediate product with alkaline hydrogen peroxide afforded 16α-hydroxy derivative **11** which was subsequently oxidized with the Dess-Martin periodinane[53] to provide **12**. Ketone **12** was treated with LDA in THF at -105 °C to form enolate which was then trapped with TMSCl to provide silyl enol ether **13** as a crystalline solid.

Scheme 1. The synthesis of trimethylsilyl enol ether **13**.



Scheme 1. Reagents and conditions: a. $NH_2NH_2 \cdot H_2O$, EtOH, Et_3N, reflux; b. I_2, TMG, THF-Et_2O; c. (3-furyl)boronic acid, $(PPh_3)_4Pd(0)$, toluene-MeOH, aq $NaHCO_3$, d. $BH_3 \cdot THF$, THF, -30 °C to rt and then H_2O_2, NaOH, aq MeOH; e. Dess-Martin periodinane; f. LDA, THF, -105 °C and then TMSCl.

With silyl enol ether **13** in hand a sequence of steps employing organoselenium chemistry[54] was carried out in order to introduce a double bond at the 14,15-position (Scheme 2). First, the reaction of **13** with phenylselenyl chloride in THF at -105 °C to 0 °C gave phenylselenyl derivatives **14** as a 3:1 mixture of 15α- and 15β-epimers, respectively, as determined by ^1H NMR (95% yield). A solution of **14** in DCM was then treated with *m*-CPBA at -78 °C and the mixture was allowed to warm to rt. Under these conditions formation and thermal fragmentation of the respective phenylselenoxides occurred furnishing α,β-unsaturated ketone **15**, which was isolated in a 55% yield from ketone **12**.

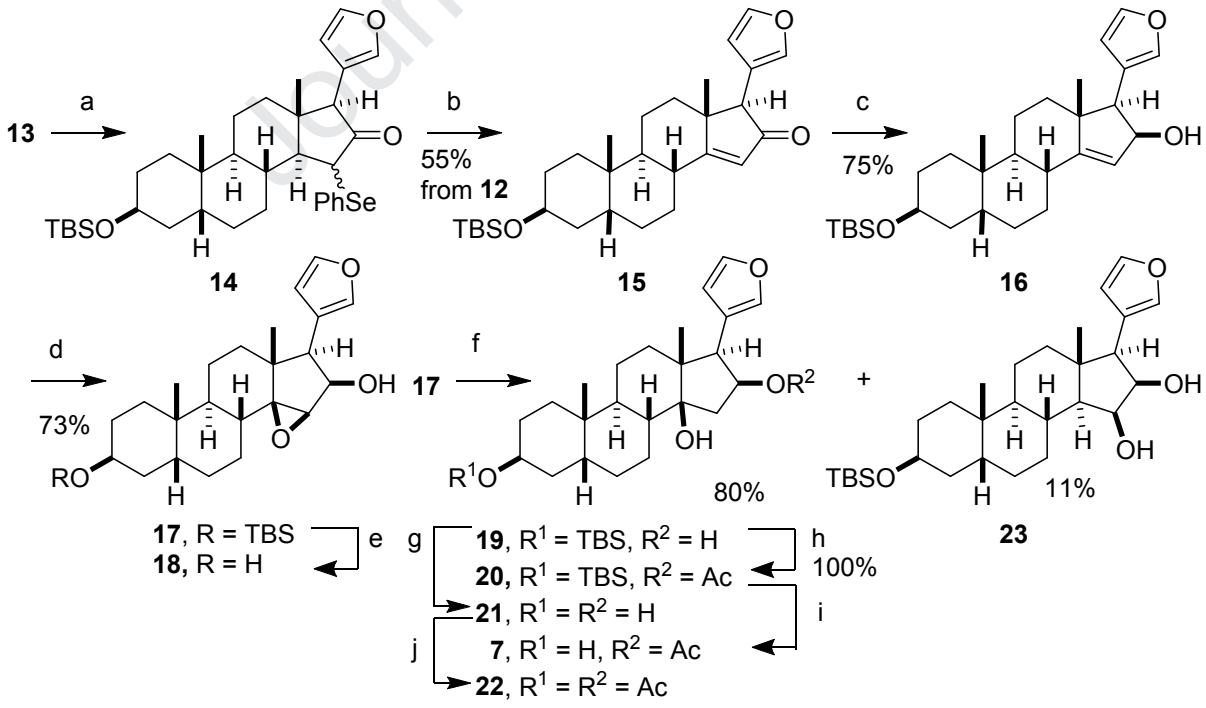
Reduction of carbonyl group in **15** was carried out using K-Selectride in Et_2O – THF at -50 °C to provide **16** in a 75% yield. Sharpless epoxidation[55-57] of **16** using titanium(IV) isopropoxide and *tert*-butylhydroperoxide in DCM in the presence of 4Å molecular sieves at -

50 °C followed by column chromatography gave epoxide **17** in 73% yield along with some unchanged **16**.

The protective TBS- group in **17** was removed using tetrabutylammonium fluoride in the usual way, to give the respective 3-hydroxy derivative **18** needed for biological activity testing. Reduction of **17** with Red-Al in THF at 60 °C afforded a mixture of two products that were separated by chromatography and identified as the required diol **19** (80% yield) and a side product, **23** (11% yield).

The secondary hydroxyl group in the diol **19** was selectively acetylated using acetic anhydride and DMAP in DCM at 0 ^{o}C to furnish **20**. The TBS protective group in **20** was removed using poly(pyridinium fluoride) [Py•$(HF)_n$] to afford the (3-furyl)-analogue of oleandrigenin **7**. On the other hand, desilylation of **19** with TBAF smoothly afforded triol **21** which was further transformed into hydroxyl-diacetate **22**.

Scheme 2. The synthesis (3-furyl)-analogue of oleandrigenin **7** and related furyl-androstane derivatives.



Scheme 2. Reagents and conditions: a. PhSeCl, THF, -105 °C; b. *m*-CPBA, DCM, -78 °C and then rt, ca. 1.5 h; c. K-Selectride, THF, -50 °C to rt; d. Ti(O*i*-Pr)$_4$, *t*-BuO$_2$H, MS 4Å, -50 °C; e. TBAF, THF, 60 °C, 2-3 days; f. Red-Al, toluene, 60 °C, chrom.; g. as e.; h. Ac$_2$O, DMAP, DCM; i. Py•(HF)$_n$, THF; j. as h.

Photochemical oxidation of the furyl derivative **20** in the presence of diethyl(isopropyl)amine,[58] which was followed by $NaBH_4$ reduction of the 5-hydroxybutenolide intermediate,[24,59] acidification and chromatography, afforded **24** (Scheme 3) in 66% yield. Removal of the TBS protective group in **24** using Py•(HF)$_n$ in THF provided oleandrigenin **2** (91% yield). Diactetate **25** was then obtained from **2** in the usual way. ^{1}H- and ^{13}C-NMR spectra, and elemental analyses of **2** and **24** confirmed their structures. M. p. and specific rotation for **2** were also in an agreement with those reported in the literature.[9, 15, 60]

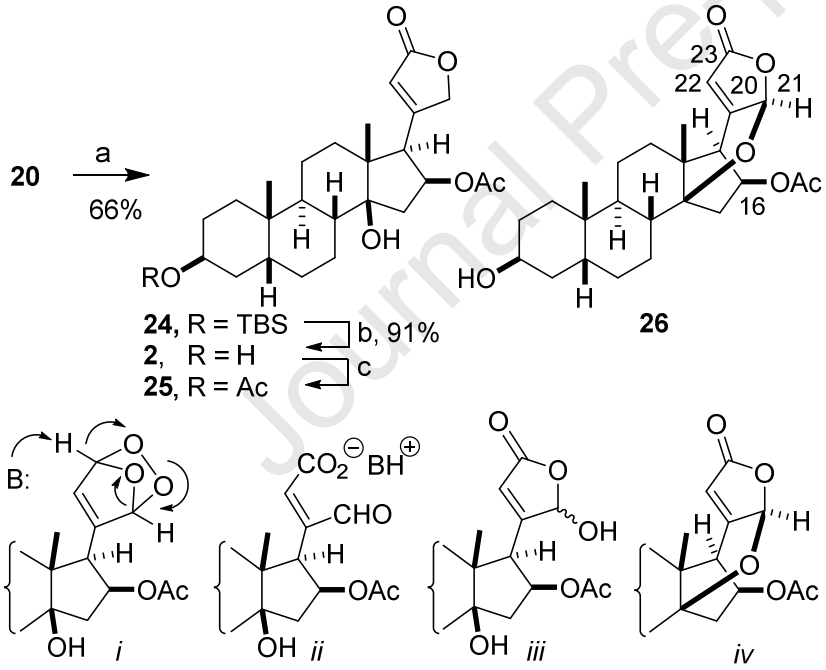
In complimentary experiments, isolation of TBS-ether **24** was skipped carrying desilylation of the crude product of the furan ring transformation. Thus, **20** was subjected consecutively to photochemical oxidation, reduction with $NaBH_4$ and acidification, and then treatment with Py•(HF)$_n$. Column chromatography of the resulting mixture gave oleandrigenin **2** along with 14,21-ether **26** in 59% and 27% yields, respectively.

^{13}C NMR spectra of **26** showed the presence of the following high field signals (δ, ppm) which were supplemented with assignments made on the grounds of the calculated spectra: δ (ppm) 170.6 (C23), 170.1 (C20), 166.8[MeC(O)O], 116.5 (C22), 98.4(C21). In the ^{1}H NMR spectrum of **26,** the following high field signals were recorded: δ (ppm) 5.95 (s, 1H), 5.84 (s, 1H), 5.52 (ddd, *J* = 9.7, 5.5, 3.9 Hz, 1H). The one bond proton-carbon correlation spectrum (ghsqc) indicated that ^{1}H signal at 5.95 ppm corresponds to ^{13}C at 98.50 ppm (C21) and that at ^{1}H 5.86 ppm corresponds to ^{13}C at 117.04 ppm (C22).

The structure of **26** was confirmed by its UV and IR spectra, and elemental analysis.

Mechanistically (Scheme 3) photooxidation of the furan ring in **20** leads to intermediate peroxide of the partial structure *i* that under the action of the bulky base (B) undergoes rearrangement to acid-aldehyde salt *ii* occurring, presumably, in the cyclic form *iii*. $NaBH_4$ reduction of *ii*/*iii* followed by chromatographic purification affords the 3-*O*-TBS butenolide **24** as the only isolable product. However, in the reaction sequence that includes Py•$(HF)_n$ treatment, unreduced *ii*/*iii* undergoes intramolecular *trans*-acetalization to provide *iv*. As a result of this process and, simultaneously, liberation of the C3 hydroxyl group, the final chromatography affords **2** along with acetal **26**.

Scheme 3. Concluding steps of the synthesis of oleandrigenin (**2**).



Scheme 3. Reagents and conditions: a. (1) O_2, hυ, DIPEA, rose bengal, (2) $NaBH_4$, MeOH then H_2SO_4, MeOH and then chromatography; b. Py•$(HF)_n$, THF; c. Ac_2O, DMAP, DCM.

Biological activities

Cytotoxicity in human cancer and noncancerous cells and ATP-ase inhibiting activities of oleandrin (**1**), oleandrigenin (**2**) and the synthesized compounds bearing a heterocyclic substituent at the 17β position and oxygen substituents at positions 14β- and 16β were determined. Cytotoxic activity was tested in three cancer cell lines: human chronic lymphoblastic leukemia (CEM), human breast adenocarcinoma (MCF7), human cervical adenocarcinoma (HeLa) and on human normal fibroblasts (BJ) (Table 1). Oleandrigenin 3-*O*-acetate (**25**) was the most active derivative of all tested compounds. **25** showed similar activity to oleandrigenin (**2**). The most sensitive adherent cancer cell line HeLa derived from cervical carcinoma was chosen for subsequent experiments to detect apoptosis using different methods (flow cytometry, caspase activity, western blotting) compared to the activity of oleandrigenin (**2**) as a positive control.

Table 1. Cytotoxic (IC_{50}; μM; 72 h) and relative ATP-ase inhibiting activities at 50 μM concentration of the synthesized compounds bearing oxygen function at C14.

	IC_{50} (μM)				ATP-ase
Compound	CEM	MCF7	HeLa	BJ	Rel. Act.(%)[1]
1	0.019 ± 0.002	0.033 ± 0.008	0.062 ± 0.002	0.026 ± 0.006	59.2 ± 3.9
2	0.2 ± 0.0	0.8 ± 0.3	0.2 ± 0.0	0.1 ± 0.0	64.8 ± 5.5
7	9.4 ± 1.7	16.1 ± 0.4	14.2 ± 0.4	4.0 ± 0.7	42.4 ± 4.7
17	34.7 ± 11.5	14.0 ± 0.3	32.3 ± 4.2	16.3 ± 0.5	74.4 ± 10.1
18	>50	>50	>50	>50	75.5 ± 3.7
19	9.9 ± 4.0	19.4 ± 1.6	26.7 ± 3.8	5.8 ± 2.8	15.1 ± 2.1
20	>50	>50	>50	>50	69.1 ± 7.3
21	3.6 ± 0.1	10.6 ± 3.6	14.5 ± 1.2	1.9 ± 0.9	7.9 ± 1.1
22	14.2 ± 0.0	20.2 ± 0.2	43.1 ± 4.1	6.2 ± 0.5	79.9 ± 5.7
24	17.3 ± 1.7	20.3 ± 3.5	34.1 ± 3.3	10.6 ± 1.8	79.3 ± 3.3
25	0.4 ± 0.0	1.7 ± 0.7	1.1 ± 0.1	0.2 ± 0.1	100.6 ± 2.7
26	>50	>50	>50	>50	83.9 ± 3.0

[1]control 100%, S.D. 3.16

Interaction with isolated Na^+/K^+-ATPase revealed that all tested species except for **25** are able to inhibit enzyme ATPase activity. Furyl derivatives **7**, **19** and **21** turned out to be even more potent inhibitors than oleandrin **1** and oleandrigenin **2** (Fig. 2), the most efficient **21** exhibiting IC_{50} = 7.9 ± 1.1 μM, and inhibition of Na^+/K^+-ATPase activity may explain the mechanism of cytotoxicity of these groups of derivatives (Table 1). On the other hand, our experiments cannot explain the submicromolar cytotoxicity of butenolides **1**, **2** and **25**, since some other molecular interactions related to NKA signaling role[61] are probably responsible for these cytotoxic effects.

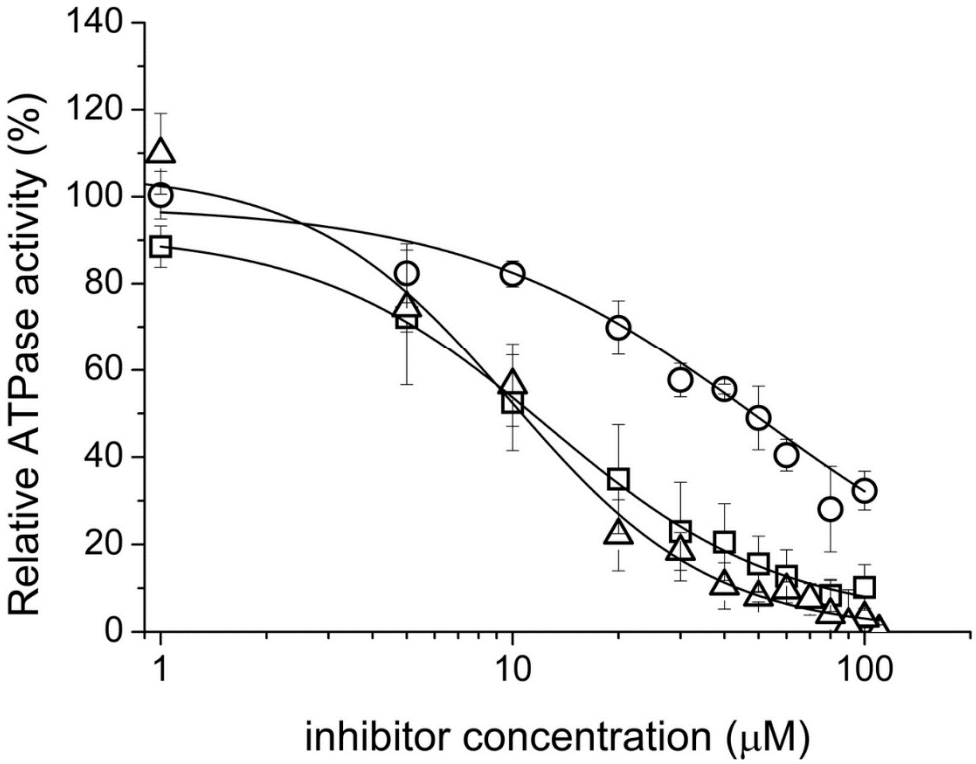
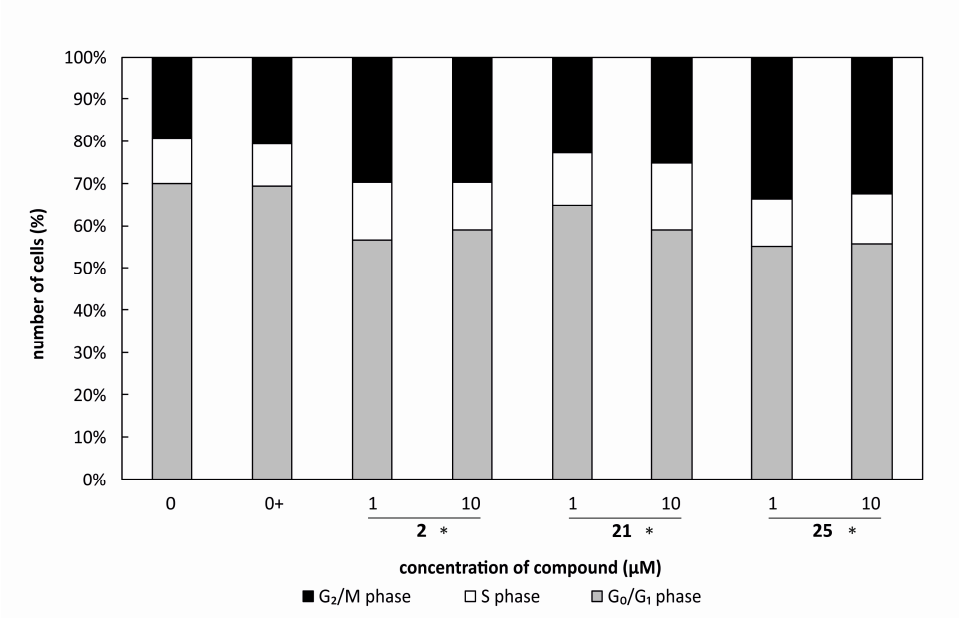


Fig. 2. Relative activity of Na^+/K^+ ATP-ase after treatment with **7** (circles), **19** (squares) or **21** (triangles) normalized to the activity of untreated enzyme.

We also examined whether our analogues of cardenolides and bufadienolides could induce apoptosis and influence the cell cycle in human cancer cells compared to the positive control, oleandrigenin (**2**). Cervical carcinoma HeLa cells were treated for 24 h, harvested, fixed and stained with propidium iodide which visualizes the DNA. The cell cycle and apoptosis of HeLa cells were then measured using BD FACS Verse. Cells in the corresponding phases of the cell cycle were compared with untreated cells (0) and cells with DMSO only (0+DMSO). The number of cells in G_0/G_1 phase decreased after treatment with all tested compound concentrations by 4.4 – 14.4% compared to untreated controls. $SubG_1$, S phase cells and G_2/M cells increased after the treatment with both cardenolides and bufadienolides. $SubG_1$ cells increased to 2-times compared to DMSO control. The largest increase (by 13.1% or 11.9%, respectively) in G_2/M cells was detected after the 24 h of treatment with 1 and 10 µM of **25** (Fig. 3).

A



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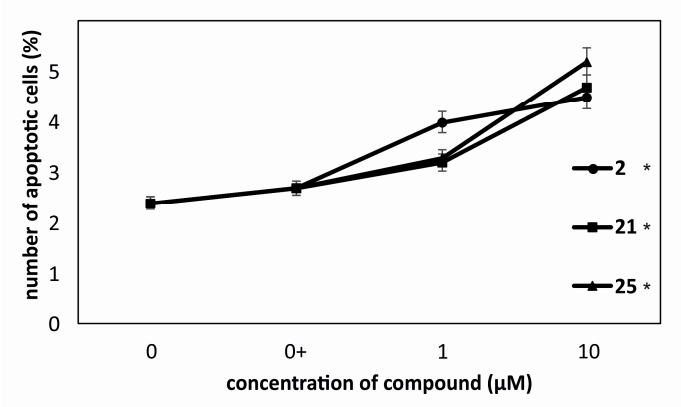


Fig. 3. Flow cytometric analysis of the cell cycle and induction of apoptosis in HeLa cells after treatments with 1 or 10 μM of different cardenolides (**21** and **25**) and oleandrigenin as a positive control (**2**) for 24 h. (A) Columns show the distribution of cells in G_0/G_1, S, G_2/M phase of the cell cycle. Error bars are omitted for clarity. (B) Graphs represent percentage of subG_1 (apoptotic) cell population. 0 refers to control cells, 0+ refers to control cells treated with DMSO only. (A, B) Analysis of variance (one-way ANOVA) between control and treated cells were significant ($p < 0.05$) marked by *.

In contrast, 5α-butenolides (3-*O*-methyl 5α-oleandrigenin, 16β-hydroxy derivative, 14α,15α-epoxide, and 14,15β-epoxy-derivative) at 15 μM concentration induced significant accumulation of apoptotic CEM cells (subG_1) after 24 h (up to 37% for 14,15β-epoxy-derivative).[32] Oleandrigenin **2** and derivatives **21** and **25** also induced twice as high caspase-3/7 activity compared to control untreated cells (Fig. 4). In our previous work, 14α,15α-epoxide increased the activity of caspase-3/7 up to 19 times.[32] Thus, the pro-apoptotic activity was weaker than the activity of butenolides published earlier.

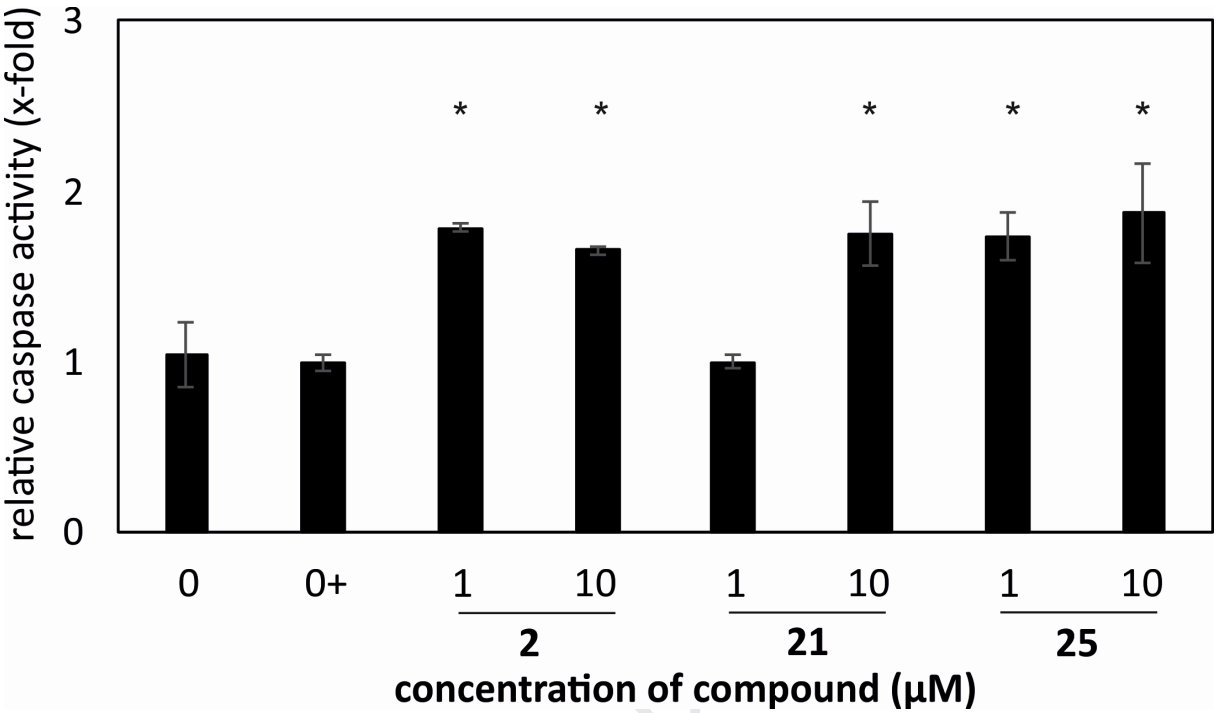


Fig. 4. Activity of caspase-3/7 in HeLa cells treated with selected cardenolides (**21** and **25**) or oleandrigenin (**2**) as a positive control for 24 h. 0+ refers to control with added DMSO only. The experiment was repeated three times in triplicate. * Differences between control and treated cells were significant ($p < 0.05$).

In addition, Western blotting with immunodetection on the membrane was used for the detection of apoptotic markers in HeLa cells after the treatment for 24 h. The initiation of apoptosis was performed by caspases-3, 6, 7 and 9 that cleave poly-ADP-Ribose-Polymerase-1 (PARP-1) into fragments.[62] In HeLa cells, the cleavage of PARP-1 was detected after the treatment with 10 µM of **2, 21** and **25** after 24 h (Fig. 5). Fragmentation of executioner caspase-7 was also demonstrated in the treated cells with the same compounds (**2, 21** and **25**). Interestingly, oleandrigenin **2** induced more pronounced apoptosis of HeLa cells and at a lower 1 µM concentration compared to 10 µM. This effect may be due to the high cytotoxicity of 10 µM **2,** which renders apoptosis initiators virtually dysfunctional. The Bcl-2 family of proteins regulates the induction or inhibition of apoptosis. Anti-apoptotic Bcl-2 was

downregulated after 24 h treatment with 1 and 10 µM of **21** and 1 µM of **25.** Furthermore, treatment with the tested compounds decreased the level of anti-apoptotic protein Mcl-1 which acts as an apical molecule in apoptosis control, promoting cell survival by interfering at an early stage in the cascade of events leading to release of cytochrome c from mitochondria.[63] p53 is a tumor suppressor gene (like Rb) which inhibits tumor development. Retinoblastoma protein (Rb) is thought to be inactivated by CDK- and cyclin-mediated phosphorylation during the late G_1 phase by preventing entry into the S phase of the cell cycle.[64] In HeLa cells, there was reduction in the expression of phosphoRb (pRb) observed after the application of all three tested compounds (**2**, **21** and **25**) except for 1µM of **21** (Fig. 5). The total Rb also decreased in these treatments. It seems that these concentrations attained cytotoxicity towards human cervical carcinoma cells. In HeLa cervical carcinoma cells, tumor suppressor protein p53 is inactivated by the viral oncoprotein E6 that plays an important role in human papilloma viruses induced tumorigenesis.[65] In our experiment, treated HeLa cells by **2**, **21** and **25** for 24 h were analyzed for level of p53. We showed that all tested compounds in both concentrations (1 and 10 µM) downregulated the expression of p53 that can lead to loss of the tumorigenic activity of this suppressor. The family of protein tyrosine kinases includes Src kinase, which plays an important role in the regulation of growth and differentiation of eukaryotic cells. The phosphorylation at Tyr416 upregulates the enzyme activity of Src kinase.[66] After the application of 1 µM **21** and **25** for 24 h, the activity of Src kinase was downregulated, which indicates inhibition of cell proliferation. In comparison, the level of total Src kinase was unchanged after the same treatment. In summary, oleandrigenin **2** and butenolides **21** and **25** showed interesting pro-apoptotic properties; compounds **21** and **25** also reduced cell proliferation.

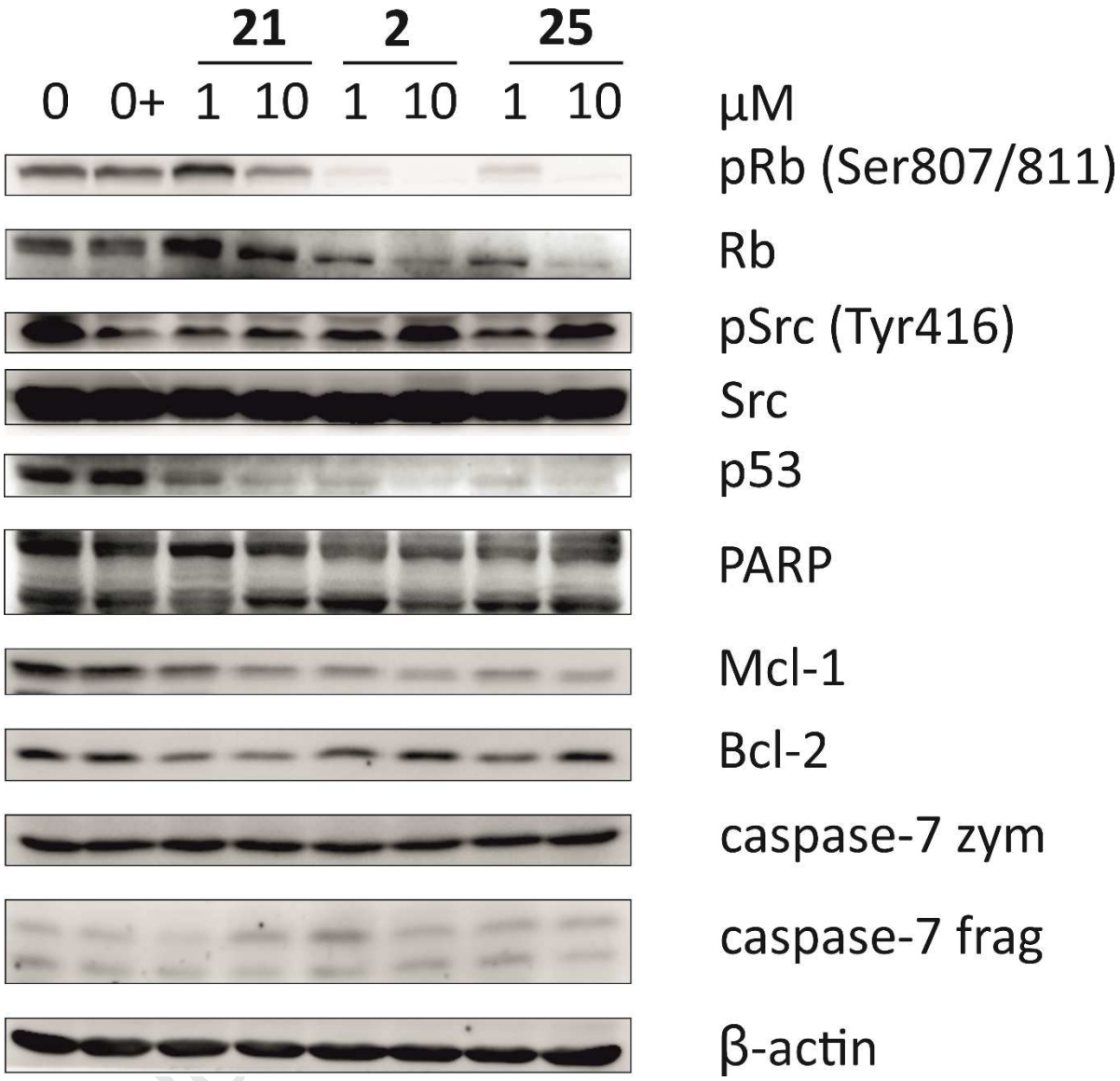


Fig. 5. Western blot analysis of apoptotic markers (PARP-1, zymogen and fragment of caspase-7, Mcl-1, Bcl-2, p53, pRb, Rb, pSrc, Src) in HeLa cells after the treatment with 1 and 10 μM cardenolides (**21** and **25**) compared to positive control oleandrigenin **2** for 24 h. 0+ refers to control with added DMSO. β-Actin was used as protein loading control. The experiment was repeated three times.

Conclusions

A synthetic approach to oleandrigenin and, potentially, to bufalin from testosterone propionate has been developed. Several analogues of oleandrigenin/bufalin differing from

natural products in the heterocyclic ring, C3-*O*-susbstituent or the mode of the ring C-functionalization were prepared and examined for cytotoxic and ATP-ase - inhibiting activities. It is shown that oleandrigenin **2**, and cardenolide **25** as well as the furyl derivative **21** induced strong apoptosis in human cervical carcinoma HeLa cells after 24 h of treatment. Furyl analogues (**7**, **19**, **21**) exhibit remarkably strong $Na^{+/}K^{+}$-ATP-ase inhibitory activity as well as significant cytotoxicity in the examined cancer cell lines. In addition, some derivatives bearing bulky and hydrophobic *tert*-butyldimethylsilyloxy-group at the C3-β position, as **19**, are shown to exhibit strong $Na^{+/}K^{+}$-ATP-ase inhibitory and cytotoxic activities.

Acknowledgements

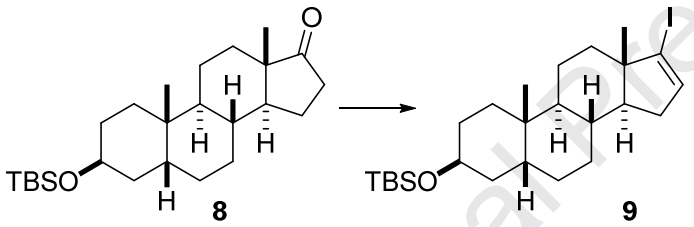
We thank Dr Jacek Wójcik of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, for taking NMR spectra of **26**, and the relevant calculations. Biological testing was supported by the European Regional Development Fund - Project ENOCH (No. CZ.02.1.01/0.0/0.0/16_019/0000868) and by grant of Czech Science Foundation No. 20-25308S and 19-01383S. We would like to thank Eva Hrdličková for excellent technical assistance.

Experimental Part

General comments

Melting points were determined on a hot-stage apparatus and are uncorrected. NMR spectra were recorded in $CDCl_3$ solutions (if not otherwise indicated): ^{1}H at 400 MHz and ^{13}C at 100 MHz on Varian Mercury 400 instrument. Chemical shifts are quoted on the δ scale taking the solvent signal as the internal standard ($CHCl_3$, ^{1}H NMR 7.26 ppm; $CDCl_3$, ^{13}C NMR 77.00

ppm). IR spectra were taken on Jasco FTIR-6200 and CD on Jasco J-715 spectrometers. HRMS (EI) were recorded using AutoSpecPremier (Waters) unit. Column chromatography was performed on Merck silica gel 60, 230-400 mesh and TLC - on aluminium sheets, Merck 60F 254. Anhydrous solvents were obtained by distillation from benzophenone ketyl (THF), $LiAlH_4$ (Et_2O) or calcium hydride (DCM). Air-sensitive reactions were performed in flame-dried glassware under argon using anhydrous solvents. Organic extracts were dried over anhydrous Na_2SO_4 and solvents were evaporated using a rotary evaporator. Poly(pyridinium fluride) [Py•$(HF)_n$] (Py ca. 30%, HF, ca. 70%) was purchased from Aldrich. The reagents and common solvents were used as they were purchased.

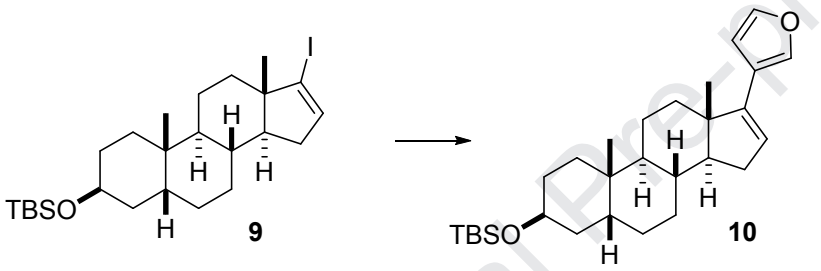


17-Iodo-3β-hydroxy-5β-androst-16-ene 3-*O*-*tert*-butyldimethylsilyl (9).

A mixture of **8** (see, SI, 5.126 g, 12.7 mmol), hydrazine hydrate (80%, 20.8 mL), Et_3N (10.4 mL) and EtOH (48 mL) was heated under reflux for 3 h. After heating was discontinued, to the hot stirred solution, water (400 mL) was added and stirring was continued for 1 h. The crystalline solid was then filtered off, washed with water (400 mL) in several portions, and air dried. The crude hydrazone was obtained (5.36 g)(colorless crystals). To a solution of that intermediate (5.36 g) in THF (50 mL) was added dropwise, within 1 h, to a solution of I_2 (6.47 g) in Et_2O (90 mL) containing TMG (9.5 mL, 76.6 mmol) stirred at 0 °C. After the addition was completed, the cooling bath was removed, stirring was continued for 1 h and then the solid material was filtered off, washed with Et_2O (3 × 20 mL) and discharged. The combined filtrates were evaporated and the oily residue was heated at 80 °C (oil bath) for 4 h, cooled and dissolved in a mixture hexane – EtOAc (3:2, 150 mL) and then partitioned with

5%HCl (100 mL). The organic layer was separated and washed consecutively with aq Na_2SO_3 and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica gel (20 g, hexane) to give **9** (5.820 g, 89%) as colorless crystals: m. p. 138 ^oC (acetone); ^1H NMR, 6.11 (dd, *J* = 3.2, 1.7 Hz, 1H), 4.04 (br.s, 1H), 2.12 (ddd, *J* = 14.9, 6.3, 3.2 Hz, 1H), 1.96 – 1.76 (m, 4H), 1.67 – 1.00 (m, 15H), 0.97 (s, 3H), 0.89 (s, 9H), 0.72 (s, 3H), 0.02 (s, 6H); ^{13}C NMR, 137.5, 113.0, 67.3, 55.1, 50.2, 40.5, 36.6, 36.5, 35.3, 34.9, 34.5, 33.7, 29.9, 28.6, 26.8, 26.2, 25.9, 23.9, 21.0, 18.1, 15.3, -4.8, -4.9.

Anal. Calcd for $C_{25}H_{43}IOSi$: C, 58.35; H, 8.42. Found: C, 58.22; H, 8.61.

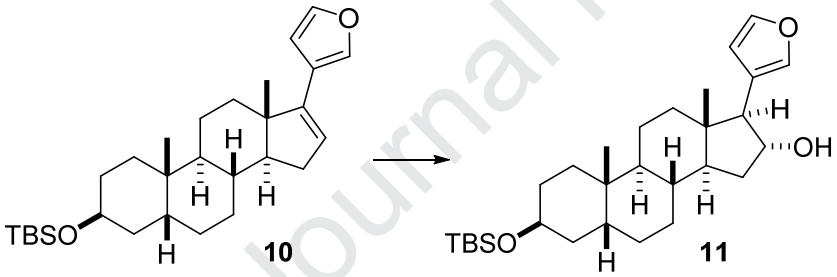


17-(3-Furyl)-3β-hydroxy-5β-androst-16-ene 3-*O*-*tert*-butyldimethylsilyl (10).

A mixture, of **9** (2.235 g, 4.32 mmol), $Pd(PPh_3)_4$ (0.495 g, 0.43 mmol), 3-furylboronic acid (0.626 g, 5.59 mmol), aq $NaHCO_3$ (2M, 6.45 ml), toluene (40 mL) and MeOH (8 mL), prepared in a Schlenk flask under an argon atmosphere, was heated on an oil bath at 80 ^oC for 28 h. It was then (after cooling) poured into water and extracted with a mixture of hexane – EtOAc, 3:2, 60 mL). The organic layer was separated, washed with brine and dried. The solvent was evaporated and the residue was taken in a small volume of DCM, diluted with hexane and transferred to a silica gel column (12 g). The column was eluted with hexane (120 mL). The eluate was evaporated to a residue (1.72g) which was dissolved in hexane and transferred to a subsequent silica gel column (80 g). The column was eluted with hexane to give a crude **10** (1.467 g, 74%) as colorless crystals. The ^1H NMR, spectrum of this product showed signals in the range 7.6 – 6.7 ppm indicating the presence of contamination. An

analytical sample was prepared as follows: a crude product (145 mg) was dissolved in EtOH (5 mL). A cloudy suspension was removed by filtration. The filtrate was concentrated to the residual volume of ca. 2 mL of and set aside for crystallization. The crystalline material was collected and air dried to give **10** (63 mg): m. p. 94 ^{o}C; ^{1}H NMR, 7.46 (s, 1H), 7.35 (t, *J* = 1.6 Hz, 1H), 6.47 (dd, *J* = 1.8, 0.8 Hz, 1H), 5.80 (dd, *J* = 3.1, 1.8 Hz, 1H), 4.04 (br.s, 1H), 2.18 (ddd, *J* = 15.5, 6.4, 3.3 Hz, 1H), 2.04 – 1.77 (m, 5H), 1.71 – 1.33 (m, 11H), 1.26 – 1.07 (m, 3H), 0.99 (s, 3H), 0.92 (s, 3H), 0.89 (s, 9H), 0.02 (s, 6H); ^{13}C NMR, 146.2, 142.5, 137.5, 125.2, 121.4, 109.4, 67.4, 57.5, 47.2, 40.5, 36.7, 35.9, 35.3, 34.5, 34.3, 31.4, 29.9, 28.62 26.90 26.5, 25.9, 23.9, 21.2, 18.1, 16.3, -4.8, -4.9.

Anal. Calcd for $C_{29}H_{46}O_2Si$: C, 76.25; H, 10.59. Found: C, 76.29 H, 10.39.

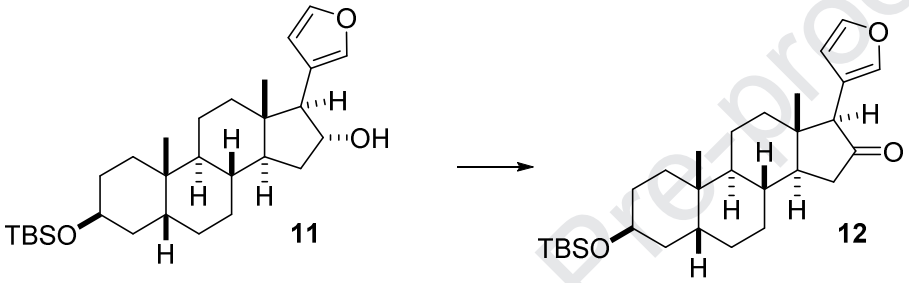


17-(3-Furyl)-5β-androstan-3β,16*α*-diol 3-*O*-*tert*-butyldimethylsilyl (11).

BH_3•THF (1M, 8.0 mL, 8 mmol) was added to a solution of crude **10** (1.467 g, ca 3.2 mmol) in THF (30 mL) stirred at -30 ^{o}C. Stirring was continued at -30 ^{o}C for 15 min., at 0 ^{o}C for 6 h and then at rt for 18 h. After that time, the mixture was cooled to -30 ^{o}C again and added consecutively was: EtOH (10.5 mL), aq NaOH (3M, 10.5 mL), and H_2O_2 (30%, 11 mL). The cooling bath was removed and the mixture was vigorously stirred for 2 h. It was then diluted with a mixture of hexane – EtOAc (3:2, 200 mL) and partitioned with water (100 mL). The organic layer was separated and washed with water (2 × 100 mL) and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica gel (50 g, hexane –

EtOAc, 92:8, ca. 1 L) to give **11** (1.322 g, 87%) as colorless crystals: m. p. 117 ^{o}C (MeOH - H_2O); ^{1}H NMR, 7.38 (t, *J* = 1.5 Hz, 1H), 7.29 (br.s, 1H), 6.30 (d, *J* = 0.8 Hz, 1H), 4.52 – 4.44 (m, 1H), 4.05 (br. s, 1H), 2.44 (d, *J* = 7.5 Hz, 1H), 1.94 – 1.75 (m, 4H), 1.73 – 1.04 (m, 17H), 0.93 (s, 3H), 0.89 (s, 9H), 0.56 (s, 3H), 0.02 (s, 6H); ^{13}C NMR, 142.7, 139.5, 123.0, 111.0, 76.8, 67.4, 58.4, 53.7, 44.9, 40.3, 38.0, 36.6, 35.7, 35.5, 35.2, 34.5, 29.9, 28.6, 26.8, 26.5, 25.8, 24.0, 20.4, 18.1, 14.4, -4.8, -4.9.

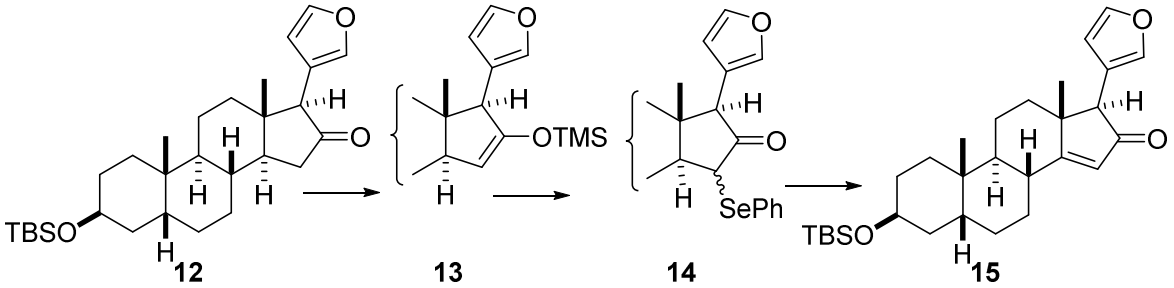
Anal. Calcd for $C_{29}H_{48}O_3Si$: C, 73.67; H, 10.23. Found: C, 73.41 H, 10.06.



17-(3-Furyl)-3β-hydroxy-5β-androstan-16-one 3-*O*-*tert*-butyldimethylsilyl (12).

Dess-Martina periodinane (1.78 g, 4.20 mmol) was added to a solution of **11** (1.003 g, 2.11 mmol) in DCM (50 mL) stirred at 0 ^{o}C. The mixture was stirred at 0 ^{o}C for 15 min and then the cooling bath was removed and stirring was continued for 2 h. After that time, EtOAc (60 mL) was added and the mixture was partitioned with aq Na_2SO_3 (40 mL). The organic layer was separated, washed with water and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica gel (12 g, hexane – EtOAc, 97:3) to give **12** (863 mg, 86%) as colorless crystals: m. p. 194 ^{o}C (MeOH); IR(film from DCM), 1740 cm^{-1}; ^{1}H NMR, 7.39 – 7.36 (m, 2H), 6.24 (dd, *J* = 1.5, 0.8 Hz, 1H), 4.06 (br.s, 1H), 3.08 (s, 1H), 2.36 (dd, *J* = 18.4, 6.9 Hz, 1H), 2.00 – 1.78 (m, 5H), 1.69 – 1.11 (m, 14H), 0.97 (s, 3H), 0.89 (s, 9H), 0.62 (s, 3H), 0.03 (s, 6H); ^{13}C NMR, 216.1, 142.4, 141.0, 117.9, 111.0, 67.3, 61.4, 50.6, 43.5, 40.3, 38.6, 37.9, 36.4, 35.3, 35.2, 34.4, 29.7, 28.6, 26.7, 26.7, 25.8, 23.9, 20.5, 18.1, 14.2, -4.9, -4.9.

Anal. Calcd for $C_{29}H_{46}O_3Si$: C, 73.99; H, 9.85. Found: C, 74.09 H, 9.87.



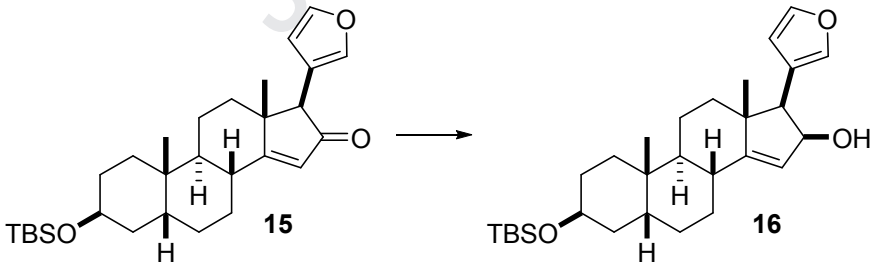
17-(3-Furyl)-3β-hydroxy-5β-androst-15-en-16-one 3-*O*-*tert*-butyldimethylsilyl (15).

A solution of LDA (4.5 mL, 1.1 eq), prepared from *i*-Pr_2NH (0.616 mL) and BuLi (2.35 M, 1.56 mL) in THF (6 mL), was added to a solution of **12** (863 mg, 1.83 mmol) in THF (10 mL) stirred at -105 ^oC (acetone-dry ice-liq N_2 bath). The mixture was stirred at -105 ^oC for 1 h and TMSCl (0.254 mL, 2.01 mmol) was added. After 15 min, the cooling bath was removed, the mixture was allowed to warm to rt and then cooled to 0 ^oC. Et_3N (1 mL) and aq $NaHCO_3$ (3 mL) were consecutively added. The mixture was vigorously stirred for 5 min, poured into water and extracted with hexane (50 mL). The extract was washed with water and brine and dried. The solvent was evaporated to give crude silyl enol ether **13** [17-(3-furyl)-5β-androst-15-en-3β,16-diol 3-*O*-*tert*-butyldimethylsilyl 16-*O*-trimethylsilyl ether] as a crystalline mass (1.22 g); m. p. 117 ^oC (MeOH - H_2O) Anal. Calcd for $C_{29}H_{48}O_3Si$: C, 73.67; H, 10.23. Found: C, 73.41 H, 10.06.

A solution of PhSeCl (350 mg, 1.82 mmol) in THF (5 mL) was added dropwise to a solution of crude silyl enol ether **13** (1.22 g) in THF (15 mL) stirred at -105 ^oC. Stirring at -105 ^oC was continued for 1h, the cooling bath was removed and the mixture was allowed to warm to 0 ^oC. The solvent was then evaporated on a rotary evaporator and the residue was chromatographed on silica gel column (18 g, hexane – EtOAc, 98:2) to give **14,** containing a small amount of **12** as an oil, (1.82 g, 95% from **12**); the ratio of epimers 15α/15β was determined as 3:1 by ^1H NMR.

A solution of *m*-CPBA (77%, 387 mg, 1.73 mmol) in DCM (5 mL) was added to a solution of **14** (1081 mg, ca 1.73 mmol) DCM (15 mL) in stirred at -78 ^{o}C. Stirring at -78 ^{o}C was continued for 1 h, the cooling bath was removed and the mixture was allowed to warm to rt. After 1.5 h, it was poured into aq Na_2SO_3 and extracted with a mixture of hexane – EtOAc, 6:4, 100 mL. The organic layer was washed with water and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica gel (37 g, hexane – EtOAc, 97.5:2.5) to give **15** as a crystalline solid containing (by TLC) a minor less polar contamination (500 mg, 58% from **14**): m. p. 180 ^{o}C (hexane); UV, λ_{max} (MeCN): 233.0 nm; IR(film from DCM), 1704, 1610 cm^{-1}; ^{1}H NMR, 7.39 (t, *J* = 1.6 Hz, 1H), 7.37 – 7.35 (m, 1H), 6.19 (dd, *J* = 1.5, 0.5 Hz, 1H), 5.86 (d, *J* = 1.6 Hz, 1H), 4.05 (br.s, 1H), 3.34 (s, 1H), 2.48 (td, *J* = 11.6, 2.3 Hz, 1H), 2.07 (dt, *J* = 12.5, 2.8 Hz, 1H), 1.99 (tt, *J* = 13.8, 4.3 Hz, 1H), 1.91 – 1.20 (m, 15H), 1.02 (s, 3H), 0.89 (s, 9H), 0.88 (s, 3H), 0.02 (s, 6H); ^{13}C NMR, 206.6, 190.6, 142.7, 141.2, 123.5, 119.2, 111.3, 67.0, 58.2, 47.6, 41.1, 40.3, 37.1, 36.0, 35.8, 34.1, 29.7, 28.6, 26.1, 25.8, 24.0, 23.6, 22.9, 21.1, 18.1, -4.9, -4.9.

Anal. Calcd for $C_{29}H_{44}O_3Si$: C, 74.31; H, 9.46. Found: C, 74.21; H, 9.29.

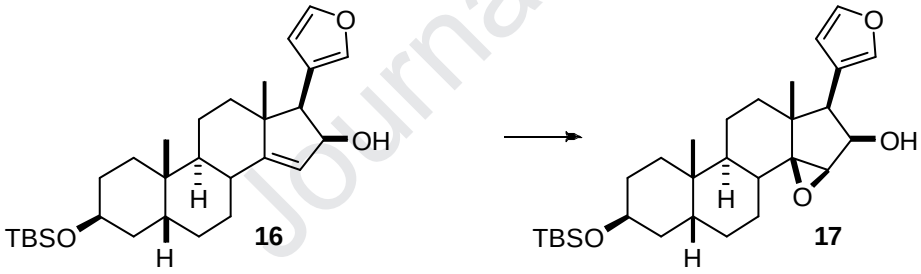


17-(3-Furyl)-5β-androst-14-en-3β,16β-diol 3-*O*-*tert*-butyldimethylsilyl (16).

K-Selectride (1M in THF, 4.3 mL) was added to a solution of **15** (406 mg, 0.86 mmol) in Et_2O (40 mL) stirred at -50 ^{o}C. Stirring was continued at -50 ^{o}C for 6 h and then at rt for 16 h. The mixture was then cooled to -30 ^{o}C and MeOH (5 mL), aq NaOH (3M, 5 mL) and H_2O_2

(30%, 5.5 mL) were consecutively added. The cooling bath was removed and the mixture was vigorously stirred for 1 h. It was then poured into water and extracted with a mixture of hexane – EtOAc (6:4, 30 mL). The organic extract was washed with water and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica gel (16 g, hexane – EtOAc, 97:3, 450 mL) to give **16** as colorless crystals (306 mg, 75%): m. p. 131 (MeOH); ^{o}C ^{1}H NMR, 7.49 – 7.47 (m, 1H), 7.40 (t, *J* = 1.6 Hz, 1H), 6.44 (d, *J* = 1.1 Hz, 1H), 4.62 – 4.57 (m, 1H), 4.02 (s, 1H), 2.79 (d, *J* = 5.7 Hz, 1H), 2.23 (tdd, *J* = 11.7, 3.0, 2.0 Hz, 1H), 1.96 (tt, *J* = 13.8, 3.9 Hz, 2H), 1.90 – 1.77 (m, 3H), 1.73 (ddd, *J* = 13.2, 6.7, 3.9 Hz, 1H), 1.65 – 1.15 (m, 15H), 0.99 (s, 3H), 0.98 (s, 3H), 0.89 (s, 9H), 0.02 (s, 6H); ^{13}C NMR, 162.2, 142.0, 140.78, 121.3, 119.8, 112.8, 77.0, 67.3, 54.7, 48.0, 40.9, 40.5, 36.3, 35.7, 35.4, 34.2, 29.8, 28.7, 26.5, 25.8, 24.2, 23.7, 23.7, 21.3, 18.1, -4.8, -4.9.

Anal. Calcd for $C_{29}H_{46}O_3Si$: C, 73.99; H, 9.85. Found: C, 73.97 H, 9.70.

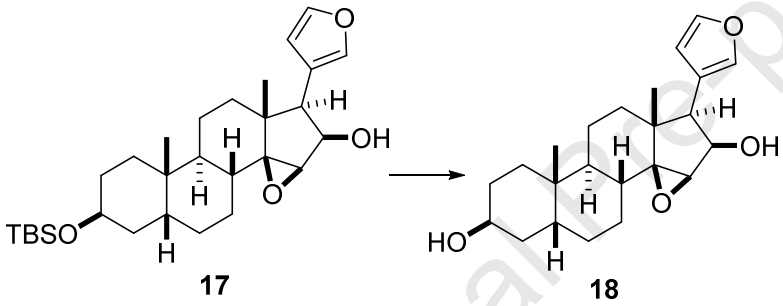


17-(3-Furyl)-14β,15β-epoxy-5β-androstan-3β,16β-diol 3-*O*-*tert*-butyldimethylsilyl (17).

Dried and powdered molecular sieves 4Å (200 mg) were added to a solution of **16** (293 mg, 0.62 mmol) in DCM (6.5 mL) and the stirred suspension was cooled to -50 ^{o}C. Ti(O*i*-Pr)$_4$ (0.276 mL, 0.93 mmol) and *t*-BuO$_2$H (4.0 M in toluene, 0.233 mL) were consecutively added and stirring -50 ^{o}C was continued for 21 h. Me$_2$S (0.45 mL) was then added followed, after 1 h, by brine (15 mL) containing NaOH (0.6 g). The cooling bath was removed and the mixture was allowed to warm to rt, and poured into water. The mixture was extracted with Et$_2$O-hexane (4:1, 3 × 30 mL). The organic extract was dried and the solvent was evaporated. The

residue was was chromatographed on silica gel (24 g, hexane – EtOAc, 93:7, 450 mL) to give consecutively unchanged **16** (25 mg, 9%) and **17** as an oil (220 mg, 73%): ^{1}H NMR, 7.38-7.36 (m, 1H), 7.34 (s, 1H), 6.55 (d, *J* = 0.9 Hz, 1H), 4.54 (br.t, *J* = 9.6 Hz, 1H), 4.06 (s, 1H), 3.50 (s, 1H), 2.93 (d, *J* = 8.9 Hz, 1H), 2.03 (td, *J* = 11.9, 3.8 Hz, 1H), 1.90-1.10 (m, 16H), 0.96 (s, 3H), 0.88 (s, 9H) overlapping 0.95-0.82 (m, 1H), 0.81 (s, 3H), 0.02 (s, 6H); ^{13}C NMR, 143.0, 142.9, 120.7, 113.8, 72.7, 72.6, 67.1, 61.6, 48.0, 44.4, 39.6, 39.2, 36.0, 35.4, 34.2, 33.0, 29.6, 28.7, 26.0, 25.8, 23.9, 21.0, 20.8, 18.1, 17.3, -4.9, -4.9.

HRMS calcd for $C_{29}H_{46}O_4Si$ (M^+) 486.3165. Found: 486.3158.

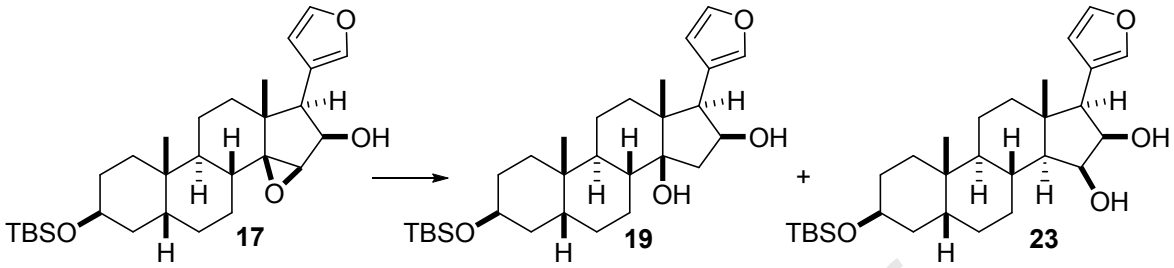


17 **18**

17-(3-Furyl)-14β,15β-epoxy-5β-androstan-3β,16β-diol (18).

In a 10 mL round bottomed flask was placed **17** (15 mg), THF (2 mL) and TBAF (60 mg). The flask was closed with a stopcock and immersed in an oil bath heated at 60 ^{o}C. The progress of reaction was monitored by TLC. After the substrate was consumed (2-3 days) the solution was allowed to cool to rt and then it was diluted with EtOAc (20 mL), and partitioned with water (20 mL). The organic layer was separated and washed consecutively with water and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica gel (1 g, hexane- EtOAc, 6:4) to give **18** as an oil:^{1}H NMR, 7.38 (t, *J* = 1.6 Hz, 1H), 7.34 (br.s, 1H), 6.55 (d, *J* = 1.0 Hz, 1H), 4.54 (t, *J* = 8.7 Hz, 1H), 4.15 (t, *J* = 2.5 Hz, 1H), 3.51 (s, 1H), 2.94 (d, *J* = 8.9 Hz, 1H), 2.05 (td, *J* = 11.9, 3.9 Hz, 1H), 1.97 – 1.81 (m, 2H), 1.81 – 1.18 (m, 14H), 0.99 (s, 3H), 0.92 (td, *J* = 11.9, 4.3 Hz, 1H), 0.82 (s, 3H); ^{13}C NMR,

143.0, 142.9, 120.7, 113.8, 72.6, 72.6, 66.8, 61.6, 48.0, 44.4, 39.4, 39.1, 36.0, 35.5, 33.3, 32.9, 29.6, 27.9, 25.7, 23.8, 21.0, 20.7, 17.3.



17-(3-Furyl)-5β,14β-androstan-3β,14,16β-triol 3-*O*-*tert*-butyldimethylsilyl (19) and 17-(3-furyl)-5β-androstan-3β,15β,16β-triol 3-*O*-*tert*-butyldimethylsilyl (23).

Red-Al (50% in toluene, 0.9 mL) was added to a solution of **17** (220 mg, 0.45 mmol) in THF (9 mL) and the mixture was heated at 60 ^{o}C for 21 h. It was then cooled and diluted with Et_2O (20 mL). The reagent excess was destroyed with aq Na_2SO_4 and the precipitate was filtered off. The filtrate was evaporated and the residue was chromatographed on silica gel (32 g, hexane – EtOAc, 92:8, 1.2 L) to give consecutively **19** as colorless crystals (177 mg, 80%) and **23** as colorless crystals (23 mg, 11%).

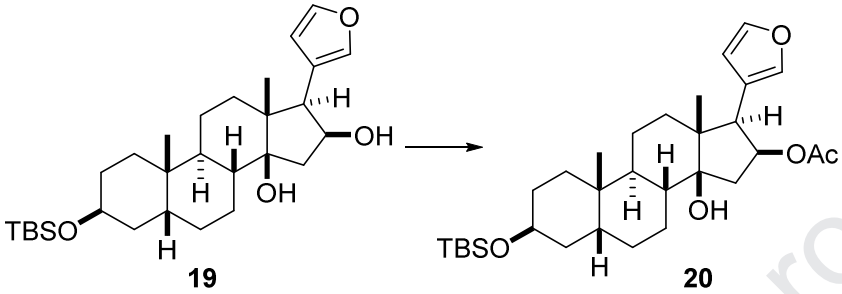
19: m. p. (hexane) 170 ^{o}C; ^{1}H NMR, 7.38 (t, *J* = 1.5 Hz, 1H), 7.30 (br.s, 1H), 6.41 (br.d, *J* = 1.0 Hz, 1H), 4.36 (dd, *J* = 11.7, 5.8 Hz, 1H), 4.05 (br.s, 1H), 3.02 (d, *J* = 6.9 Hz, 1H), 2.70 (s, 1H, OH), 2.37 (d, *J* = 5.8 Hz, 1H, OH) overlapping 2.33 (dd, *J* = 14.4, 5.9 Hz, 1H), 1.96 – 1.11 (m, 18H), 0.92 (s, 3H), 0.88 (s, 9H), 0.81 (s, 3H), 0.02 (s, 6H); ^{13}C NMR, 142.5, 141.4, 121.4, 113.8, 85.8, 73.6, 67.2, 54.8, 48.3, 41.9, 41.4, 40.8, 36.1, 35.7, 35.4, 34.3, 29.7, 28.7, 26.9, 25.8, 23.9, 21.9, 21.3, 18.1, 17.1, -4.9, -4.9.

Anal. Calcd for $C_{29}H_{48}O_3Si$: C, 71.26; H, 9.90. Found: C, 74.27; H, 9.81.

23: m. p. 141 (hexane); ^{o}C ^{1}H NMR, 7.44 (br.s, 1H), 7.39 (t, *J* = 1.6 Hz, 1H), 6.40 (d, *J* = 1.1 Hz, 1H), 4.38 – 4.31 (m, 2H), 4.05 (br.s, 1H), 2.67 (d, *J* = 4.1 Hz, 1H, OH), 2.50 (d, *J* = 2.9 Hz, 1H, OH), 2.43 (br.d, *J* = 6.5 Hz, 1H), 2.06 – 1.78 (m, 5H), 1.63 – 0.97 (m, 13H

overlapping 0.99 (s, 3H), 0.92 (s, 3H), 0.89 (s, 9H), 0.02 (s, 6H); ^{13}C NMR, 141.9, 140.9, 120.0, 113.0, 73.5, 70.9, 67.4, 59.1, 54.4, 43.4, 40.8, 39.8, 36.5, 35.3, 34.5, 31.9, 30.1, 28.6, 26.8, 26.1, 25.8, 24.0, 20.4, 18.1, 17.4, -4.8, -4.9.

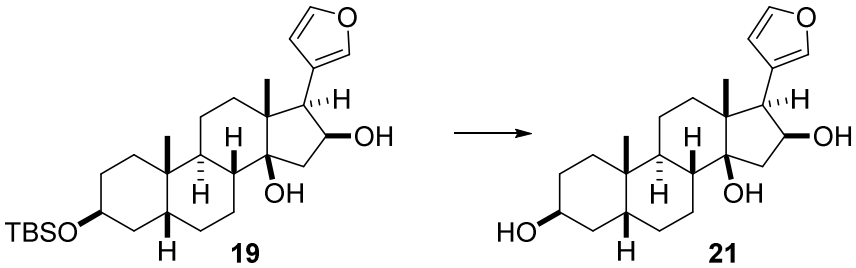
Anal. Calcd for $C_{29}H_{48}O_3Si$: C, 71.26; H, 9.90. Found: C, 71.16; H, 9.87.



17-(3-Furyl)-5β,14β-androstan-3β,14,16β-triol 16-*O*-acetyl 3-*O*-*tert*-butyldimethylsilyl (20).

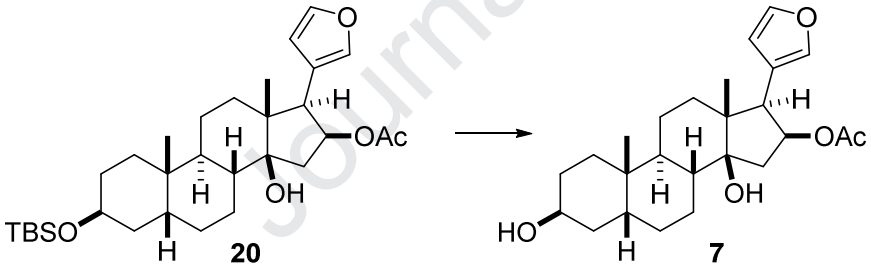
Ac_2O (0.8 mL) and DMAP (25 mg) were added to a solution of **19** (177 mg, 0.36 mmol) in DCM (8 mL) stirred at 0 ^{o}C. The mixture was stirred at 0 ^{o}C for 15 min and then set aside at 5 °C (refrigerator) for 20 h. It was then cooled to 0 ^{o}C again, MeOH (2 mL) was added and (after 15 min) the cooling bath was removed. The mixture was allowed to warm to rt, poured into water and extracted with the mixture of hexane – EtOAc (6:4, 60 mL). The organic extract was washed consecutively with water, aq $NaHCO_3$ and brine, and dried. The solvent was evaporated to give **20** as a crystalline mass (192 mg, 100%): m. p. 157 ^{o}C (hexane); IR(film from DCM), 1734 cm^{-1}; ^{1}H NMR, 7.27 (br.t, *J* = 1.5 Hz, 1H), 7.19 (br.s, 1H), 7.19 (br.s, 1H), 5.53 (td, *J* = 8.7, 1.7 Hz, 1H), 4.05 (br.s, 1H), 3.22 (d, *J* = 8.5 Hz, 1H), 2.61 (dd, *J* = 15.4, 8.9 Hz, 1H), 1.91 – 1.75 (m, 4H) overlapping 1.80 (s, 3H), 1.61 – 1.10 (m, 15H), 0.92 (s, 3H), 0.88 (s, 9H), 0.76 (s, 3H), 0.02 (s, 6H); ^{13}C NMR, 170.2, 142.0, 141.4, 121.7, 114.3, 84.5, 74.1, 67.2, 52.9, 49.0, 42.0, 40.2, 40.2, 36.0, 35.7, 35.2, 34.3, 29.7, 28.7, 26.7, 25.8, 23.8, 21.3, 21.1, 21.0, 18.1, 16.6, -4.9, -4.9.

Anal. Calcd for $C_{31}H_{50}O_5Si$: C, 70.14; H, 9.49. Found: C, 70.23; H, 9.41.



17β-(3-Furyl)-5β,14β-androstan-3β,14,16β-triol (21).

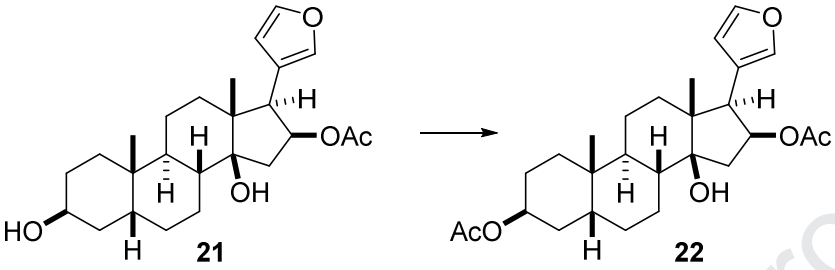
The described above procedure (preparation of **18**) was applied, eluent hex-EtOAc, 6:4 to give **21** as crystalline film: ^{1}H NMR, 7.40 (t, *J* = 1.5 Hz, 1H), 7.31 (s, 1H), 6.41 (d, *J* = 1.0 Hz, 1H), 4.38 (t, *J* = 6.3 Hz, 1H), 4.14 (br.t, *J* = 2.5 Hz, 1H), 3.04 (d, *J* = 6.9 Hz, 1H), 2.75 (s, 1H), 2.34 (dd, *J* = 14.4, 5.9 Hz, 1H), 2.00 – 1.85 (m, 4H), 1.75 (br.d, *J* = 13.6 Hz, 1H), 1.66 – 1.12 (m, 13H), 0.95 (s, 3H), 0.82 (s, 3H); ^{13}C NMR, 142.6, 141.5, 121.3, 113.8, 85.7, 73.6, 66.9, 54.8, 48.4, 41.8, 41.3, 40.8, 36.1, 35.5, 33.4, 29.7, 27.9, 26.6, 23.8, 21.8, 21.3, 17.1.



17-(3-Furyl)-5β,14β-androstan-3β,14,16β-triol 16-O-acetyl (7).

In a 10 mL round bottomed flask was placed **20** (10-30 mg), THF (1 mL) and HF•Py (0.1 mL). The flask was closed with a stopcock and left aside at rt. The progress of reaction was monitored by TLC. After the substrate was consumed (2-3 days) the mixture was diluted with EtOAc (20 mL) and partitioned with water (20 mL). The organic layer was separated and washed consecutively with aq $CuSO_4$, aq $NaHCO_3$ and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica gel (1 g, hexane –EtOAc, 6:4) to give **7** as a crystalline film: ^{1}H NMR, 7.27-7.25 (m, 1H), 7.18 (br.s, 1H), 6.49 (br.d, *J* = 1.1

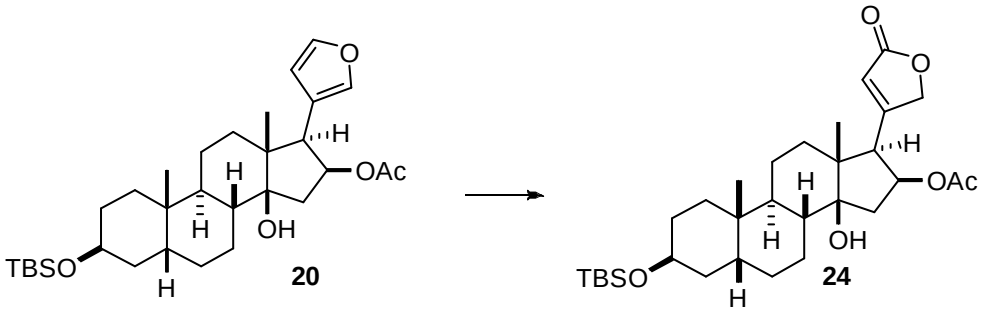
Hz, 1H), 5.51 (td, *J* = 8.7, 1.8 Hz, 1H), 4,14-4.08 (m, 1H), 3.21 (d, *J* = 8.5 Hz, 1H), 2.60 (dd, *J* = 15.4, 8.9 Hz, 1H), 1.78 (s, 3H) overlapping 1.95 – 1.70 (m, 4H), 1.69 – 1.12 (m, 15H), 0.93 (s, 3H), 0.75 (s, 3H); ^{13}C NMR, 170.1, 141.9, 141.4, 121.6, 114.3, 84.4, 74.0, 66.7, 52.8, 48.9, 41.8, 40.2, 40.0, 36.0, 35.5, 35.3, 33.3, 29.6, 27.9, 26.4, 23.7, 21.1, 21.0, 20.9, 16.6.



17-(3-Furyl)-5β,14β-androstan-3β,14,16β-triol 3β,16-di-*O*-acetyl (22).

Ac_2O (0.1 mL) was added to a solution of **21** (19 mg) and DMAP (2.5 mg) in DCM (1 mL), stirred at 0 C°. The mixture was stirred at 5 ^{o}C for 18 h and then MeOH (0.2 mL) was added and the cooling bath was removed. After 1 h the mixture was partitioned with EtOAc (20 mL) and water (20 mL). The organic layer was separated, washed consecutively with 3% HCl, aq $NaHCO_3$ and brine, and dried. The solvent was evaporated to give diacetate **22** (21 mg) as an oil: ^{1}H NMR, 7.28 (t, *J* = 1.5 Hz, 1H), 7.19 (s, 1H), 6.50 (d, *J* = 1.1 Hz, 1H), 5.54 (td, *J* = 8.7, 1.6 Hz, 1H), 5.09 (br.s, 1H), 3.23 (d, *J* = 8.4 Hz, 1H), 2.61 (dd, *J* = 15.4, 8.8 Hz, 1H), 2.04 (s, 3H), 1.94 – 1.81 (m, 4H), 1.80 (s, 3H), 1.71 – 1.14 (m, 19H), 0.95 (s, 3H), 0.77 (s, 3H).

^{13}C NMR, 170.6, 170.1, 142.0, 141.4, 121.6, 114.3, 84.4, 74.0, 70.4, 52.8, 48.9, 41.8, 40.2, 40.0, 36.8, 35.7, 35.1, 30.5, 30.4, 26.3, 25.1, 23.7, 21.4, 21.1, 21.1, 20.9, 16.6.

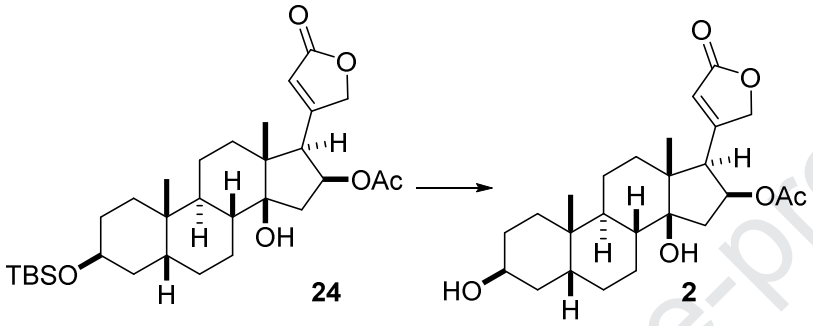


3β,14,16β-Trihydroxy-5β,14β-card-20(22)-enolide 16-*O*-acetyl 3-*O*-*tert*-butyldimethylsilyl (24).

A Schlenk flask (50 mL) was equipped an efficient stirring bar and a tungsten incandescent lamp (20 W, 140 lm, 2800 K, L4) and connected to a vacuum line and an oxygen cylinder. In the flask there were placed DIPEA (0.4 mL) and rose bengal (10.1 mg), and a solution of **20** (98.0 mg, 0.19 mmol) in DCM (16 mL). The mixture was cooled to -78 ^{o}C and vigorously stirred in an atmosphere of oxygen while irradiated until the starting material was consumed (by TLC, hexane – EtOAc, 4:1, ca. 4 h). The irradiation was then halted and the cooling bath was removed. The mixture was allowed to warm to rt and it was then flushed with argon and set aside for 16 h. The solvent was evaporated on a rotary evaporator. The residue was briefly dried on an oil pump vacuum and dissolved in MeOH (10 mL). The solution was cooled to 0 ^{o}C and $NaBH_4$ (515 mg) was added in small portions, within 1 h, while the mixture was vigorously stirred. The cooling bath was removed and the mixture was allowed to warm to rt and stirred for 1 h. After that time, it was cooled to 0 ^{o}C again and 20% H_2SO_4 was added dropwise until the mixture has decolorized. The solution was diluted with EtOAc (100 mL) and partitioned with water (100 mL). The organic layer was separated, washed consecutively with aq $NaHCO_3$ and brine, and dried. The solvent was evaporated to an oily residue. TLC of this material indicated the presence of the main product (**24**) and poorly developing contaminations. Its chromatographed on silica gel (6 g, hexane – EtOAc, 3:1) gave **24** as an oil (66.3 mg, 66%): ^{1}H NMR, 5.96 (t, *J* = 1.6 Hz, 1H), 5.47 (td, *J* = 9.4, 2.6 Hz, 1H), 4.99 (dd, *J* = 18.2, 1.7 Hz, 1H), 4.85 (dd, *J* = 18.2, 1.7 Hz, 1H), 4.04 (br.s, 1H), 3.18 (d, *J* = 8.7 Hz,

1H), 2.72 (dd, *J* = 15.6, 9.7 Hz, 1H), 1.96 (s, 3H), 1.91 – 1.10 (m, 19H), 0.92 (s, 3H), 0.91 (s, 3H), 0.87 (s, 9H), 0.01 (s, 6H); ^{13}C NMR, 174.1, 170.36, 167.86, 121.31, 84.24, 75.64, 73.94, 67.02, 56.16, 49.97, 41.84, 41.17, 39.28, 35.88, 35.59, 35.15, 34.18, 29.62, 28.62, 26.53, 25.79, 23.75, 21.10, 20.99, 20.81, 18.05, 15.92, -4.88, -4.90.

HRMS calcd for $C_{31}H_{50}O_6SiNa$ (M^++Na) 569.3274. Found: 569.3272.

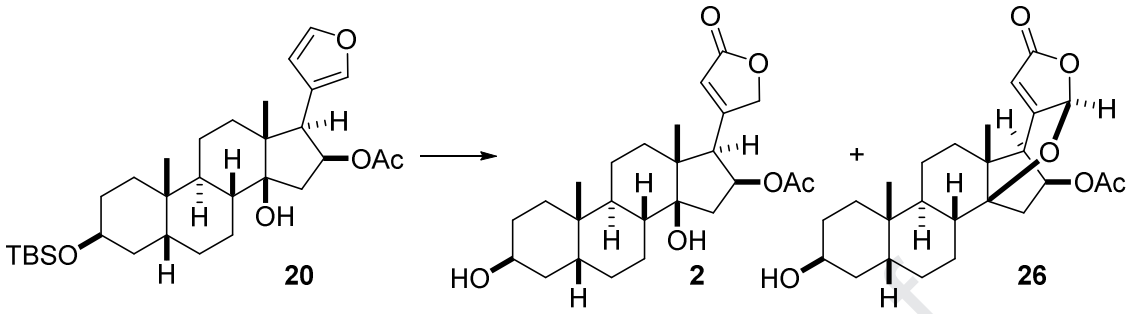


3β,14,16β-Trihydroxy-5β,14β-card-20(22)-enolide 16-*O*-acetyl (oleandrigenin) (2).

(1) Poly(pyridinium fluride) [Py•$(HF)_n$, 0.5 mL] was added to a solution of **24** (66.3 mg) in THF (5 mL) stirred at rt. The mixture was set aside for 4 days and then poured into water (30 mL) and extracted with EtOAc (50 mL). The organic extract was washed consecutively with 5% HCl, aq $NaHCO_3$ and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica gel (3 g, hexane – EtOAc, 1:1) to give **2** as colorless crystals (47.5, 91%): m. p. 232 ^{o}C (EtOAc – hexane); $[\alpha]_D^{19}$ -10.1 (c 0.68, MeOH);UV λ_{max} (MeCN): 214.7 nm; IR (KBr), 3525,(br.s), 3379 (br.s), 1789(m), 1754(s), 172shows the presence of 5(s), 1613(w) cm^{-1}; ^{1}H NMR, 5.96 (br.s, 1H), 5.47 (td, *J* = 9.4, 2.2 Hz, 1H), 4.99 (d, *J* = 18.1 Hz, 1H), 4.85 (d, *J* = 18.1 Hz, 1H), 4.13 (br.s, 1H), 3.19 (d, *J* = 8.7 Hz, 1H), 2.73 (dd, *J* = 15.6, 9.7 Hz, 1H), 1.96 (s, 3H), 1.96 – 1.09 (m, 20H), 0.95 (s, 3H), 0.93 (s, 3H); ^{13}C NMR, 174.0, 170.4, 167.8, 121.4, 84.2, 75.6, 73.9, 66.7, 56.1, 50.0, 41.8, 41.2, 39.2, 35.9, 35.4, 35.3, 33.3, 29.5, 27.9, 26.3, 23.7, 21.0, 21.0, 20.8, 15.9.

Anal. Calcd for $C_{25}H_{36}O_6$: C, 69.42; H, 8.39. Found: C, 69.46; H, 8.27

Reported (i. a.): m. p. 225-230 °C, $[\alpha]_D^{24}$ -6.9±3 (c 0.89 MeOH),[60] m. p. 223 °C, $[\alpha]_D^{18}$ -8.5 (c 1.65 MeOH)[9]



3β,14,16β-Trihydroxy-5β,14β-card-20(22)-enolide 16-*O*-acetyl (2, oleandrigenin) and 3β,16β-dihydroxy-14,21-epoxy-5β,14β,21(*R*)-card-(20,22)-enolide 16-*O*-acetyl (26).

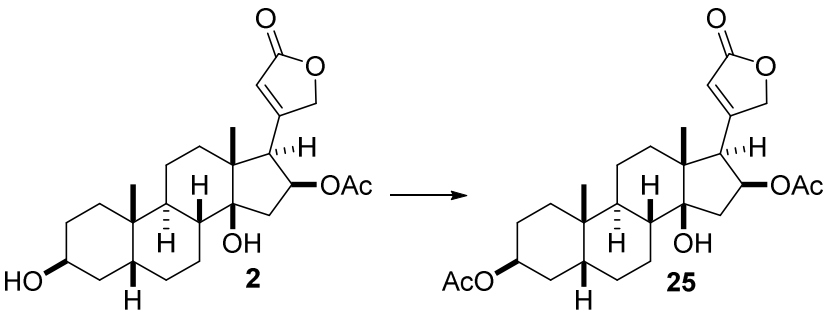
A Schlenk flask (50 mL) was equipped an efficient stirring bar and a tungsten incandescent lamp (20 W, 140 lm, 2800 K, L4) and connected to a vacuum line and an oxygen cylinder. In the flask there were placed DIPEA (0.4 mL) and rose bengal (10.5 mg), and a solution of **20** (94.1 mg, 0.18 mmol) in DCM (16 mL). The mixture was cooled to -78 °C and vigorously stirred in an atmosphere of oxygen while irradiated until the starting material was consumed (by TLC, hexane – EtOAc, 4:1, ca. 4 h). The irradiation was then interrupted, and the cooling bath was removed. The mixture was allowed to warm to rt and it was then flushed with argon. After 15 min, the solvent was evaporated on a rotary evaporator. The residue was briefly dried on an oil pump and dissolved in MeOH (10 mL). The solution was cooled to 0 °C and $NaBH_4$ (520 mg) was added in small portions, within 1 h, while the mixture was vigorously stirred. The cooling bath was removed and the mixture was allowed to warm to rt and stirred for 1 h. After that time it was cooled to 0 °C again and 20% H_2SO_4 was added dropwise until the mixture decolorized. The solution was diluted with EtOAc (50 mL) and partitioned with water (100 mL). The organic layer was separated, washed consecutively with aq $NaHCO_3$ and brine, dried and the solvent was evaporated.

The residue was dissolved in THF (5 mL) poly(pyridinium fluride) [Py•$(HF)_n$, 0.5 mL] was added. The mixture was stirred at rt for 4 days and then poured into water (30 mL) and extracted with EtOAc (50 mL). The organic extract was washed consecutively with 5% HCl, aq $NaHCO_3$ and brine, and dried. The solvent was evaporated and the residue was chromatographed on silica gel (12 g, hexane – EtOAc, 1:1, 500 mL) to give **26** as colorless crystals (after triturating with hexane) (20.9 mg, 27%) and then **2** (45.1 mg, 59%) identical to the previously obtained sample.

26: m. p. 239 ^{o}C (EtOAc – hexane); UV, λ_{max} (MeCN), 213.3 nm; IR (film from DCM), 1784, 1738, 1658 cm^{-1}; ^{1}H NMR, 5.95 (s, 1H), 5.84 (s, 1H), 5.52 (ddd, *J* = 9.7, 5.5, 3.9 Hz, 1H), 4.13 (s, 1H), 3.34 (d, *J* = 5.6 Hz, 1H), 2.90 (dd, *J* = 16.5, 10.3 Hz, 1H), 2.02 (s, 3H) overlapping 2.14 – 0.98 (m, 19H), 0.95 (s, 4H), 0.85 (s, 3H); ^{13}C NMR, 170.6, 170.1, 166.8, 116.5, 98.4, 88.8, 72.7, 66.6, 53.4, 48.9, 37.8, 36.0, 35.6, 35.4, 34.6, 33.4, 33.0, 29.7, 27.8, 26.0, 23.5, 21.0, 20.8, 20.0, 16.2.

Comparison of calculated ^{13}C NMR spectrum of the heterocyclic moiety with the experimental indicates for the assignment for ^{13}C signals: 170.6 (C-23), 170.1 (C-20), 166.8 [Me<u>C</u>(O)O], 116.5 (C-22), 98.4 (C-21); one bond proton-carbon correlation spectrum (ghsqc) shows that the ^{1}H signal at δ 5.954 ppm corresponds to ^{13}C signal δ 98.50 ppm (C-21).

Anal. Calcd for $C_{25}H_{34}O_6$: C, 69.74; H, 7.96. Found: C, 69.57; H, 8.01



3β,14,16β-Trihydroxy-5β,14β-card-20(22)-enolide 3β,16-di-O-acetyl (oleandrigenin 3-acetate) (25). [15, 67]

Ac_2O (0.1 mL) was added to a solution of **2** (5 mg) and DMAP (2.5 mg) in DCM (1 mL) stirred at 0 C°. The mixture was stirred at 5 °C for 18 h and then MeOH (0.2 mL) was added and the cooling bath was removed. After 1 h the mixture was partitioned with EtOAc (20 mL) and water (20 mL). The organic layer was separated, washed consecutively with 3% HCl, aq $NaHCO_3$ and brine, and dried. The solvent was evaporated to give acetate **25** (5 mg) as an oil film: ^{1}H NMR, 5.97 (t, *J* = 1.4 Hz, 1H), 5.48 (td, *J* = 9.4, 2.5 Hz, 1H), 5.08 (br.s, 1H), 4.98 (dd, *J* = 18.1, 1.7 Hz, 1H), 4.84 (dd, *J* = 18.1, 1.7 Hz, 1H), 3.19 (d, *J* = 8.7 Hz, 1H), 2.73 (dd, *J* = 15.7, 9.6 Hz, 1H), 2.05 (s, 3H), 1.97 (s, 3H), 1.92 – 1.06 (m, 20H), 0.96 (s, 3H), 0.94 (s, 3H).

Biological activities

Cytotoxicity assay

T-lymphoblastic leukemia CEM, cervix epithelial carcinoma HeLa, breast carcinoma MCF7 cell lines and human foreskin fibroblasts (BJ) were used to determine the cytotoxicity of tested compounds after 72 h of treatment stained by resazurin as described earlier for Calcein AM.[68] The data shown in Table 1 are means ± standard deviation (SD) obtained from at least three independent experiments performed in triplicate.

Flow cytometry

The cell cycle and apoptosis in HeLa cells treated with cardenolides and bufadienolides for 24 h was measured using flow cytometry.[68] The distribution of cells in the subG_1 (apoptotic cells), G_0/G_1, S and G_2/M peaks was analyzed. The data shown are the means of three

independent experiments performed in triplicate. The differences between control and treated cells were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel 2010. Signification was marked with asterisk ($p < 0.05$).

Caspase 3/7 activity assay

Activity of caspase 3/7 was determined in HeLa cells after 24 h of treatment with cardenolides and bufadienolides as described previously.[68] The experiments were repeated three times as biological replicates. The differences between control and treated cells were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel 2010. Signification was marked with asterisk ($p < 0.05$).

SDS–polyacrylamide gel electrophoresis and immunoblotting

HeLa cells were treated with cardenolides and bufadienolides for 24 h. Gel electrophoresis and immunoblotting were performed as described earlier with minor modifications specified below.[68] Detection and visualization of immunoblots was performed using the West Pico Supersignal chemiluminescent detection reagent (Thermo Fisher Scientific, Rockford, USA) and by CCD camera (Fujifilm, Tokio, Japan). The protein expression in treated cells was compared to that observed in untreated controls. Protein loading control was the level of β-actin. The experiments were repeated three times.

Measurement of Na^+/K^+-ATPase activity

Na^+/K^+-ATPase from porcine kidney was isolated as described previously.[69] The ATPase activity measurement of solubilized enzyme in microwell plates using pipetting station Freedom Evo (Tecan, Switzerland) and microplate reader Tecan has been described in

detail.[70] All data are presented as the specific activity of Na^+/K^+-ATPase that is standardly estimated using treatment by 500 μM ouabain, which serves as a highly specific inhibitor of Na^+/K^+-ATPase. The residual activity in the presence of ouabain is subtracted from the total ATPase activity in the absence of ouabain, yielding the specific activity of Na^+/K^+-ATPase. The values represent the means ± S.D. of four independent replicates.

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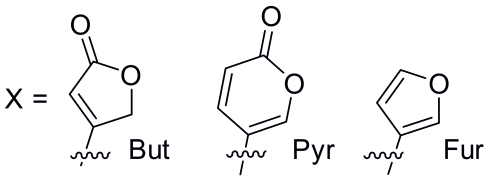
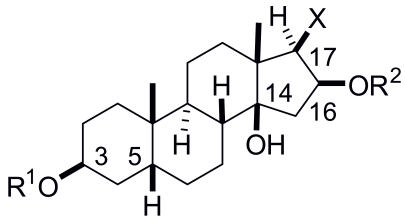
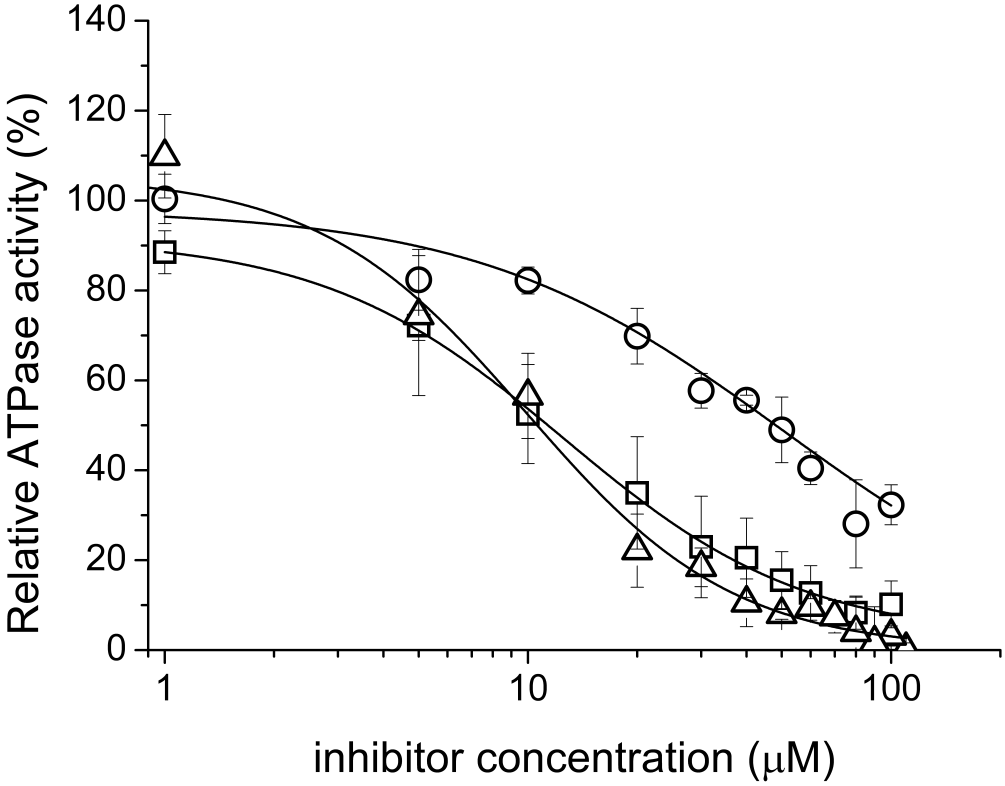




1, R^1 = Oleandrosyl, R^2 = Ac, X = But, Oleandrin
2, R^1 = H, R^2 = Ac, X = But, Oleandrigenin
3, R^1 = Tridigitoxose, R^2 = H, X = But, Gitoxin
4, R^1 = R^2 = H, X = But, Gitoxigenin
5, R^1 = H, R^2 = Ac, X = Pyr, Bufotalin
6, R^1 = R^2 = H, X = Pyr, Desacetylbufotalin
7, R^1= H, R^2 = Ac, X = Fur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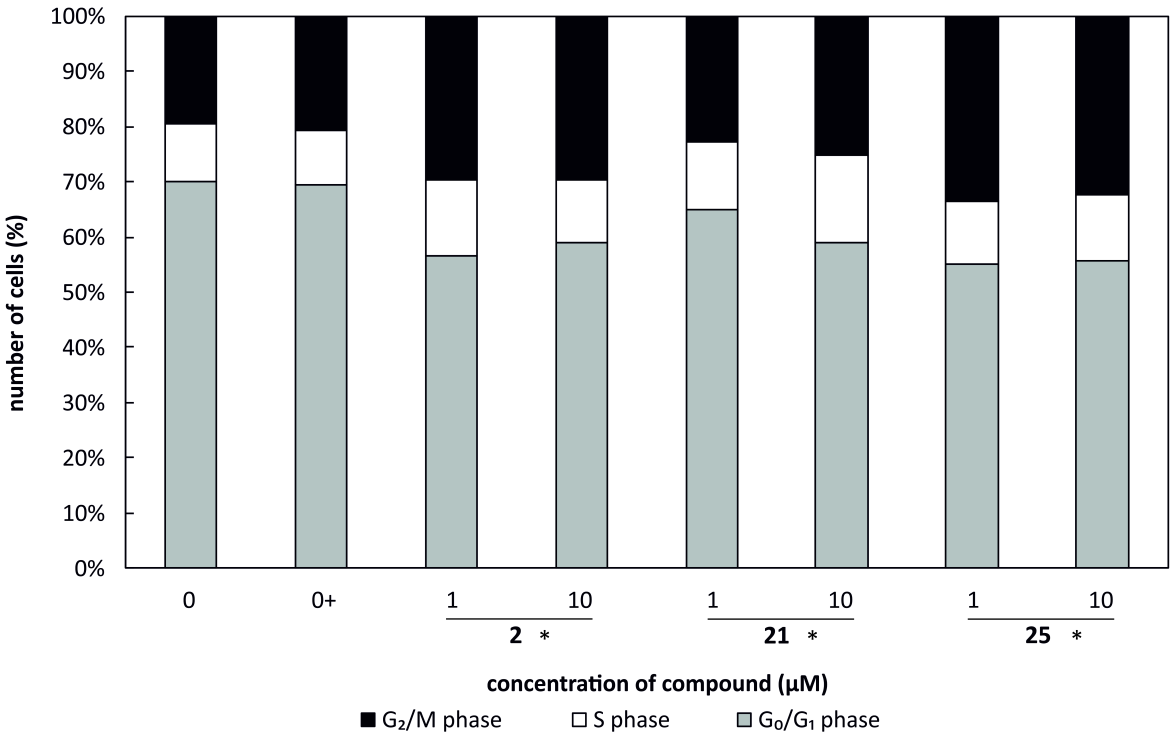
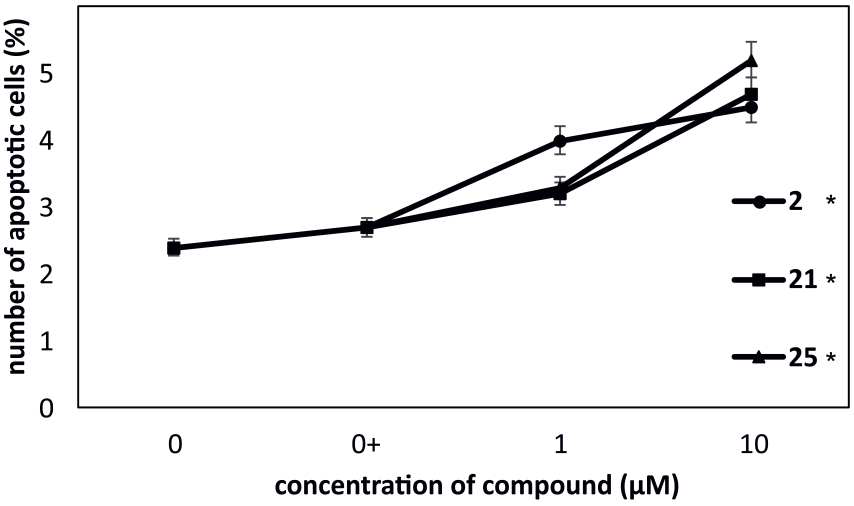
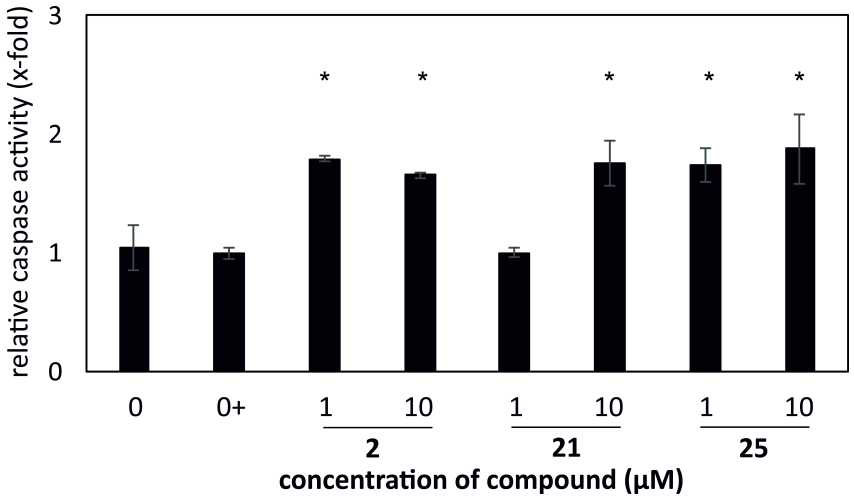
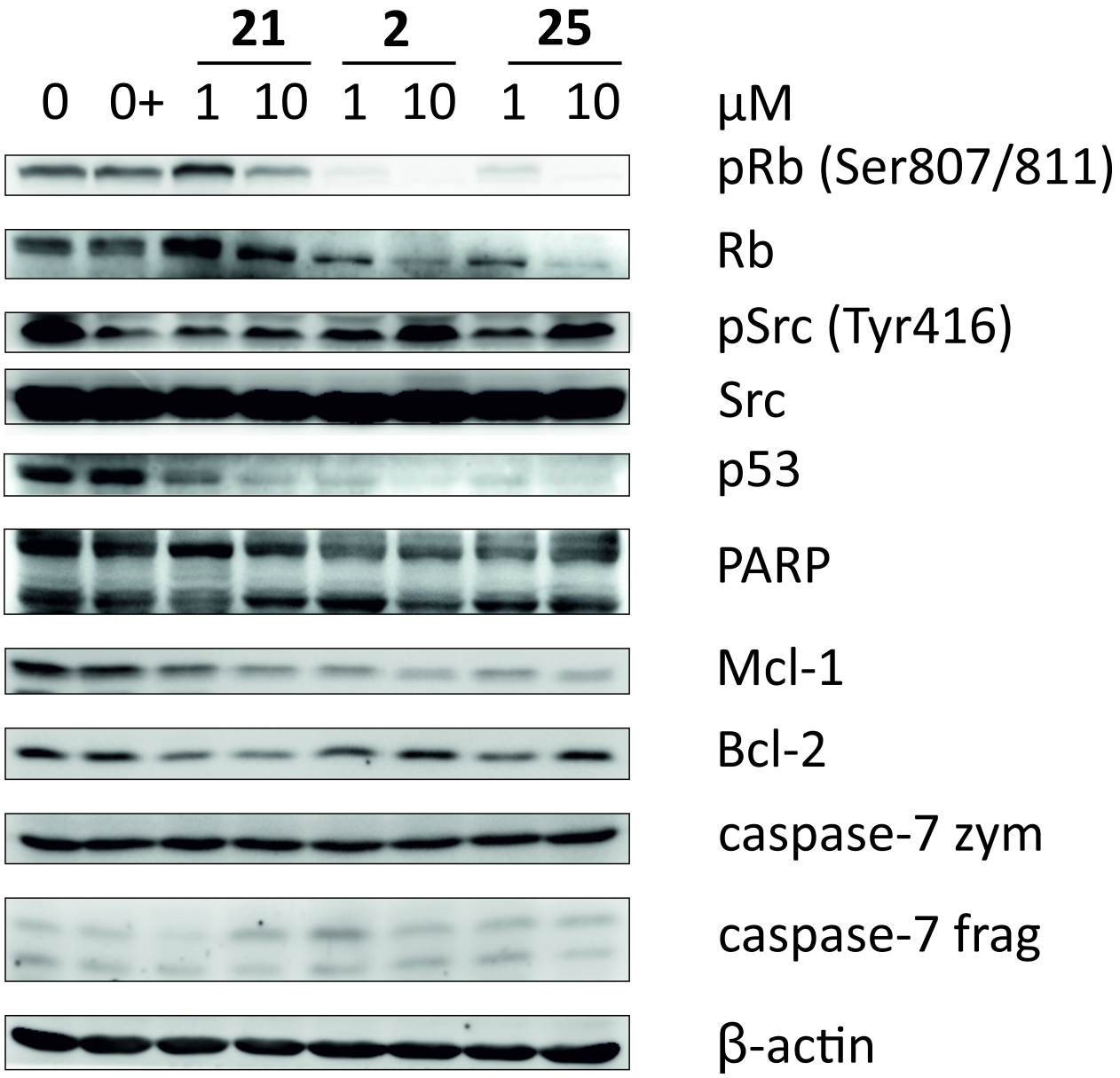
inhibitor concentration (μM)





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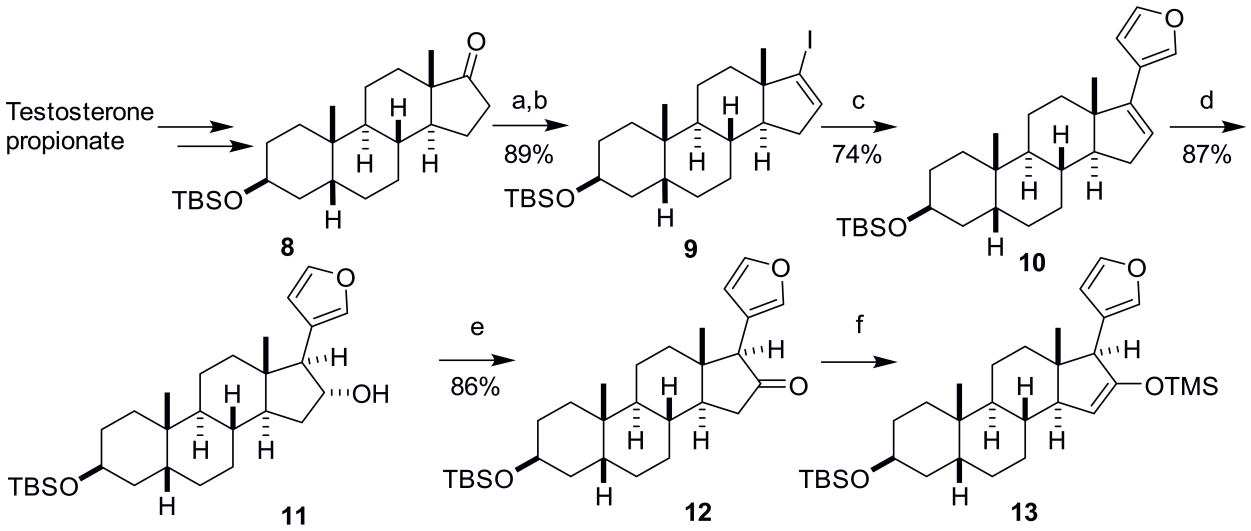

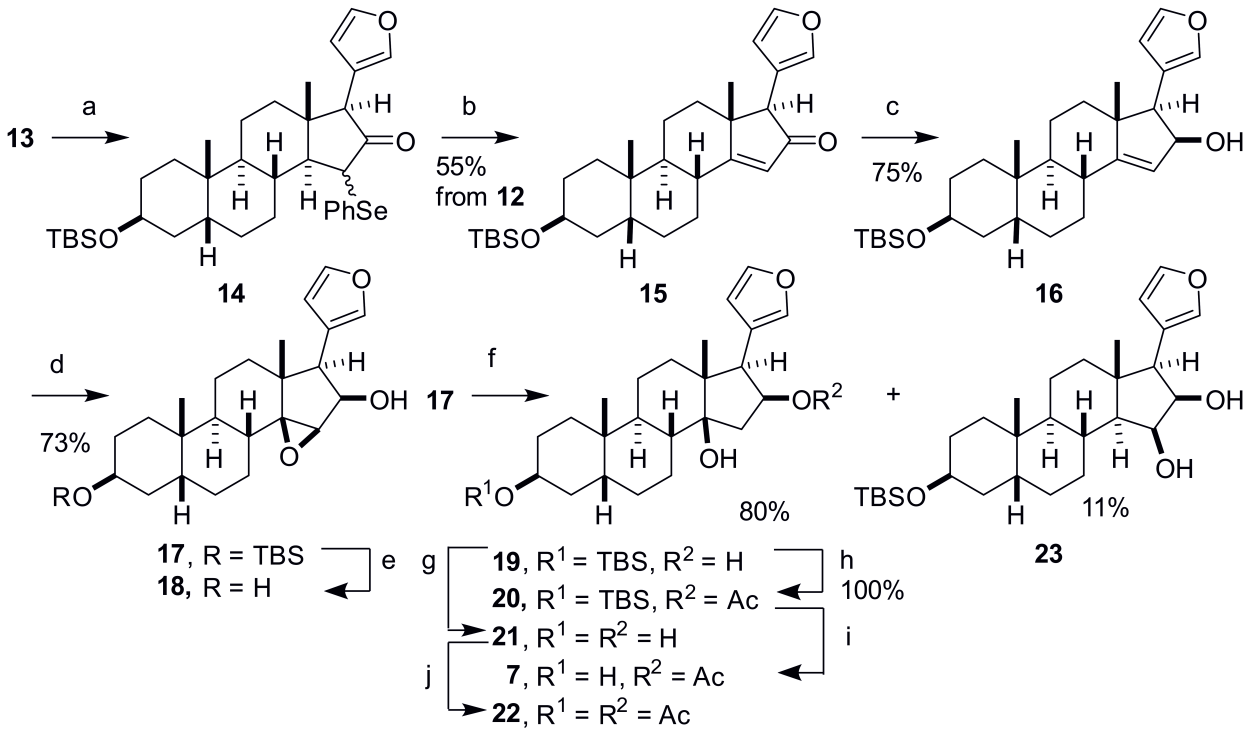

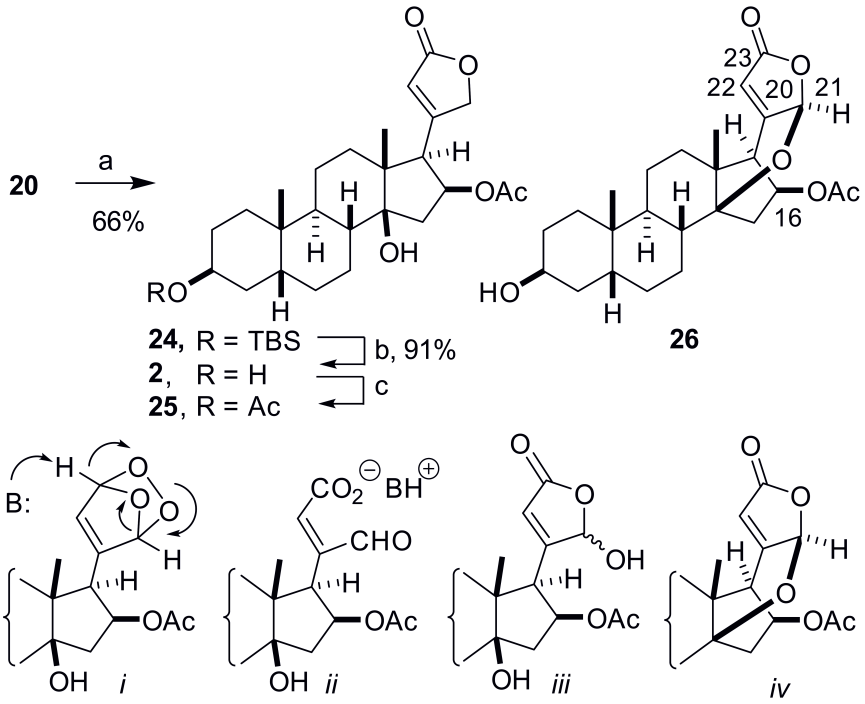

Highlights

- The first synthesis of oleandrigenin from easily available steroid developed.
- 17β-(4-butenolidyl)-, 17β-(3-furyl)-14,16β-dihydroxy-androstanes synthesized.
- Na^+/K^+-ATP-ase inhibitory and cytotoxic activities *in vitro* of new compounds.
- Derivatives **21** and **25** induced strong apoptosis in HeLa cells after 24 h.

Keywords: cardenolides; partial synthesis; furan transformation; cytotoxicity; apoptosis; ATP-ase inhibition

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: