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# Antifungal activity of 2α,3β-functionalized steroids stereoselectively increases with the addition of oligosaccharides

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ABSTRACT

Invasive fungal infections pose a significant problem to the immune-compromised. Moreover, increased resistance to common antifungals requires development of novel compounds that can be used to treat invasive fungal infections. Naturally occurring steroidal glycosides have been shown to possess a range of functional antimicrobial properties, but synthetic methodology for their development hinders thorough exploration of this class of molecules and the structural components required for broad spectrum antifungal activity. In this report, we outline a novel approach to the synthesis of glycoside-linked functionalized 2α,3β-cholestane and spirostane molecules and present data from in vitro screenings of the antifungal activities against human fungal pathogens and as well as mammalian cell toxicity of these derivatives.



The occurrences of life-threatening fungal infections have continued to rise over the last two decades, particularly within populations of immune compromised individuals.[1,2] Further, some clinical therapeutics used in the treatment of invasive fungal infections can be cytotoxic and a growing number of organisms are exhibiting increased drug resistance to common antifungals.[3–5] Finally, cross-continental travel, combined with both the increase in immune compromised populations and increased drug resistance, essentially ensures that opportunistic fungal pathogens will continue to present significant treatment challenges.[3,5–7] Considering these concerns, the design and synthesis of novel antifungal agents remains an area of intense significance.

The exploration of the biological properties of natural products has successfully elucidated classes of molecules that have potent antimicrobial activities.[8–11] One such class of natural products that have shown considerable promise as antimicrobials is the saponin.[2] These natural products are typically isolated from plant or marine species,[9] and are generally characterized as steroidal glycosides. Furthermore, there have been reports that indicate that saponins with functionalized 2,3-spirostane moieties linked to an oligosaccharide exhibit broad spectrum antifungal activity, through yet to be defined mechanisms.[12–14] However, the major obstacle to the systematic study of 2,3-spirostane glycosides as potential antifungals is the availability of synthetic preparations of these target compounds.

In addition to the aforementioned class of saponins, there have also been reports showing that synthetic modifications to cholesterol have also yielded a number of promising antimicrobial compounds. Among these are 6,5-fused cholestane oxazoles, oxygenated cholesterol derivatives, cholesterol-bound fatty acid derivatives and cholesterol-hydrazone derivatives.[8,15,16] However, oligosaccharide derivatives of functionalized 2,3-cholestanes have not yet been explored with respect to their potential for antifungal activity.

In keeping pace with these findings, we have begun to explore the possibility that further synthetic modifications to functionalized spirostane and cholestane molecules might bring forth insight into the development of a novel class of structurally diverse compounds that have antifungal activity. Recently, we published our approaches to the synthesis of functionalized 2,3-cholestane and spirostane molecules and the in vitro evaluation of their antifungal activities against pathogenic fungi.[17,18] Most of these molecules exhibited little or no capacity for antifungal activity. We then hypothesized that a functionalized steroid moiety may be a key contributor to antifungal activity, but interactions between the steroid and fungal cell wall or membrane may require the presence of an oligosaccharide, as seen in previously reported active saponins. In this report, we use our previously synthesized functionalized 2α,3β-steroids as building blocks to demonstrate that the addition of sugar residues in the 3β-position of functionalized steroids

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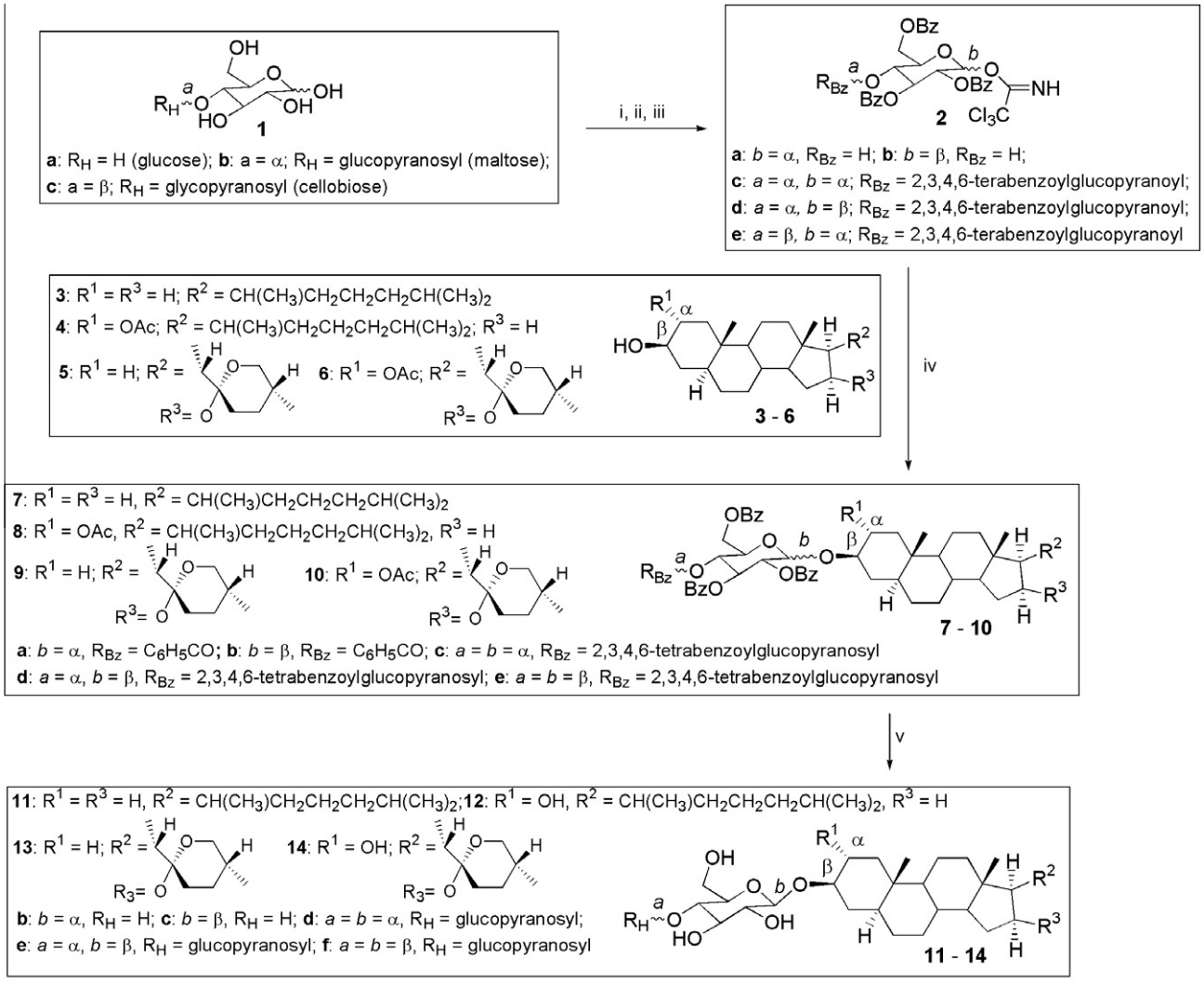



**Scheme 1.** Condensed reaction pathway for the preparation of compounds **11**–**14**.

**Table 1**
3β-substituted cholestane derivatives and the $MIC_{50}$ for three yeast species

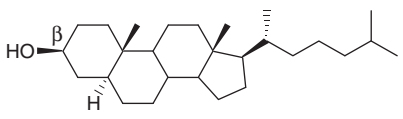
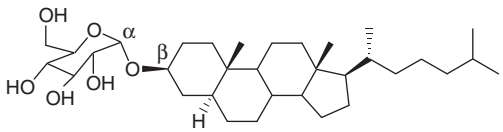
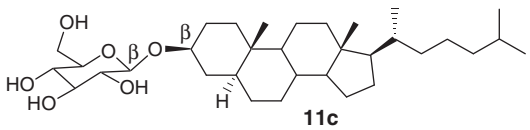
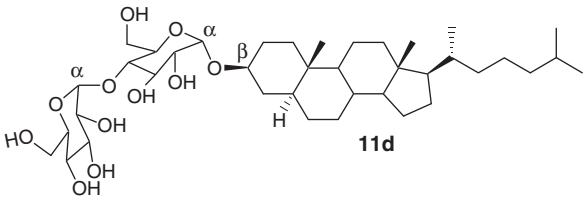
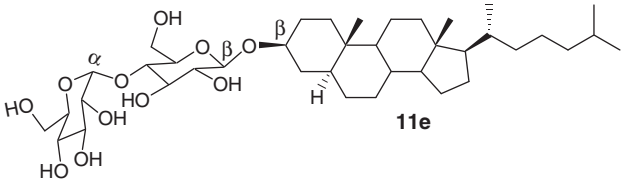
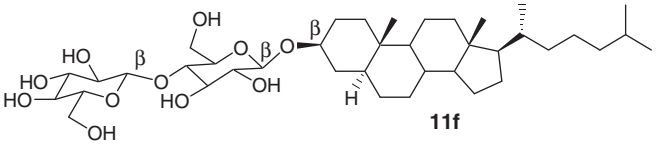
| Structure | $MIC_{50}$[a,e] (μg/mL) | | |
|---|---|---|---|
| | *Candida albicans*[b] | *Candida glabrata*[c] | *Cryptococcus neoformans*[d] |
|  **11a** | NA | NA | NA |
|  **11b** | NA | NA | NA |
|  **11c** | NA | NA | NA |

**Table 1** (*continued*)

| Structure | MIC$_{50}$[a,e] (μg/mL) | | |
|---|---|---|---|
| | *Candida albicans*[b] | *Candida glabrata*[c] | *Cryptococcus neoformans*[d] |
|  **11d** | NA | NA | NA |
|  **11e** | NA | 4 | 4 |
|  **11f** | NA | >125 | >125 |

**Previously reported in Ref. 17.

NA denotes compounds that had (1) a slight reduction in turbidity to no change and; (2) had less than a 50% reduction in growth compared to controls, as measured spectroscopically by absorption at 530 nm.

[a] MIC values are reported only for compounds displaying (1) a prominent decrease in turbidity by visual comparison to the control wells containing no antifungal and; (2) a >50% reduction in fungal growth compared to controls containing no antifungal, as measured spectroscopically by absorption at 530 nm.

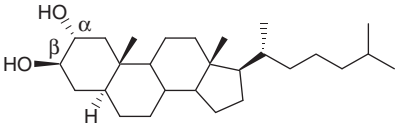
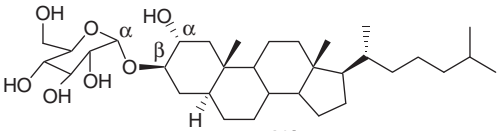
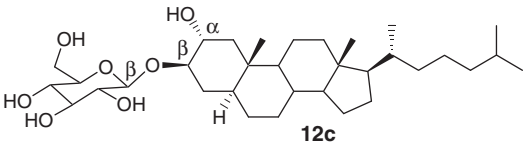
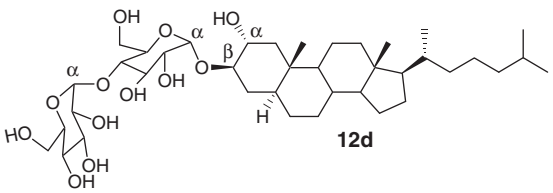
[b] ATCC no. 10231.

[c] ATCC no. 48435.

[d] ATCC no. 36556.

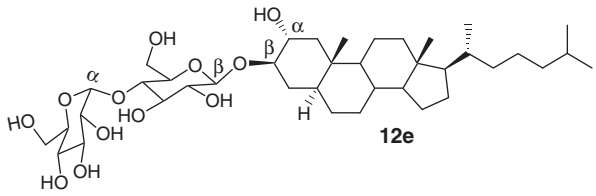
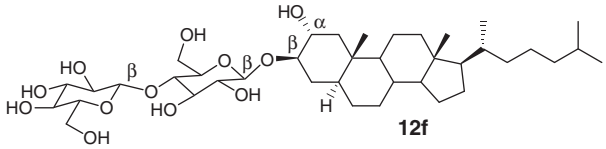
[e] All values were determined after incubation at 35 °C for 48 h.

**Table 2**
2α,3β-substituted cholestane derivatives and the MIC$_{50}$ for three yeast species

| Structure | MIC$_{50}$[a,e] (μg/mL) | | |
|---|---|---|---|
| | *Candida albicans*[b] | *Candida glabrata*[c] | *Cryptococcus neoformans*[d] |
|  **12a** | NA | NA | 32 (25% inhibition) |
|  **12b** | NA | NA | NA |
|  **12c** | NA | NA | NA |
|  **12d** | NA | NA | NA |

(*continued on next page*)

**Table 2** (*continued*)

| Structure | $MIC_{50}$[a,e] (μg/mL) | | |
|---|---|---|---|
| | *Candida albicans*[b] | *Candida glabrata*[c] | *Cryptococcus neoformans*[d] |
|  **12e** | NA | NA | 32 |
|  **12f** | NA | 8 | 4 |

**Previously reported in Ref. 17.

NA denotes compounds that had (1) a slight reduction in turbidity to no change and; (2) had less than a 50% reduction in growth compared to controls, as measured spectroscopically by absorption at 530 nm.

[a] MIC values are reported only for compounds displaying (1) a prominent decrease in turbidity by visual comparison to the control wells containing no antifungal and; (2) a >50% reduction in fungal growth compared to controls containing no antifungal, as measured spectroscopically by absorption at 530 nm.

[b] ATCC no. 10231.

[c] ATCC no. 48435.

[d] ATCC no. 36556.

[e] All values were determined after incubation at 35 °C for 48 h.

**Table 3**
3β-substituted spirostane derivatives and the $MIC_{50}$ for three yeast species

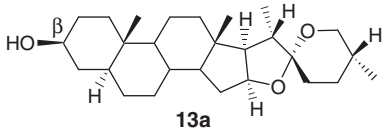
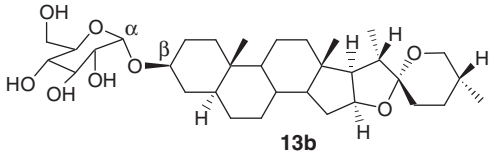
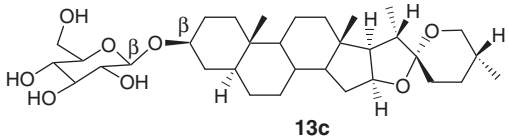
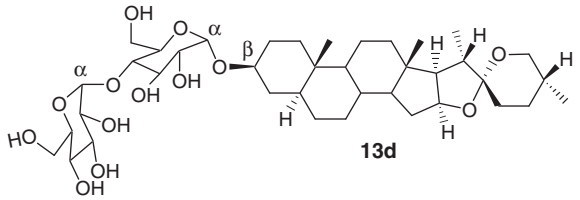
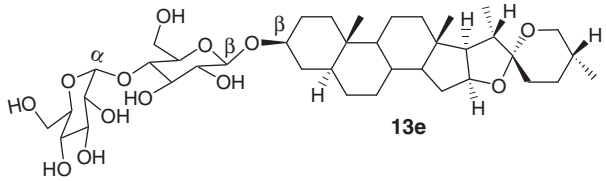
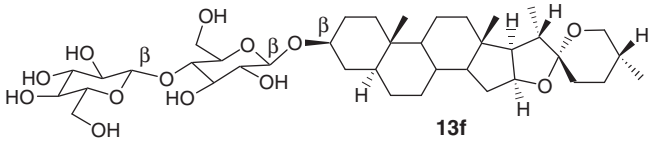
| Structure | $MIC_{50}$[a,e] (μg/mL) | | |
|---|---|---|---|
| | *Candida albicans* [b] | *Candida glabrata*[c] | *Cryptococcus neoformans*[d] |
|  **13a** | NA | >62 | NA |
|  **13b** | NA | NA | NA |
|  **13c** | 8 | 8 | 8 |
|  **13d** | NA | NA | NA |
|  **13e** | 16 (25% inhibition) | 32 | 1 |

**Table 3** (*continued*)

| Structure | MIC$_{50}$[a,e] (μg/mL) | | |
|---|---|---|---|
| | *Candida albicans* [b] | *Candida glabrata*[c] | *Cryptococcus neoformans*[d] |
|  **13f** | NA | 16 | 125 |

**Previously reported in Ref. 18

NA denotes compounds that had (1) a slight reduction in turbidity to no change and; (2) had less than a 50% reduction in growth compared to controls, as measured spectroscopically by absorption at 530 nm.

[a] MIC values are reported only for compounds displaying (1) a prominent decrease in turbidity by visual comparison to the control wells containing no antifungal and; (2) a >50% reduction in fungal growth compared to controls containing no antifungal, as measured spectroscopically by absorption at 530 nm.

[b] ATCC no. 10231.

[c] ATCC no. 48435.

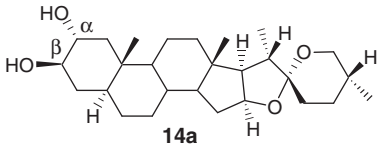
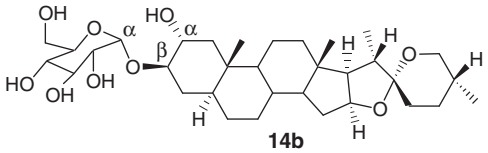
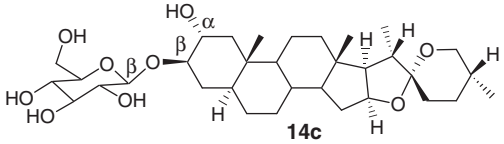
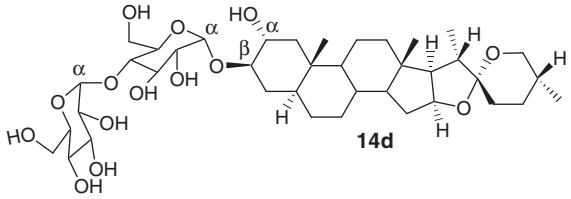
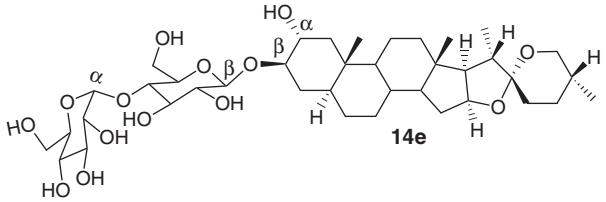
[d] ATCC no. 36556.

[e] All values were determined after incubation at 35 °C for 48 h.

contributes to increased antifungal activity in a stereoselective manner in three species of pathogenic yeasts, *Candida albicans, Crypotococcus neoformans*, and *Candida glabrata*.

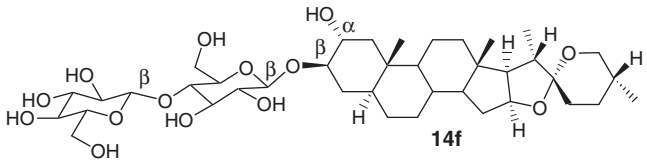
Although there are many procedures for glycosylation of steroids,[19] it was our imperative to select a simple, reliable, and above all α/β selective glycosylation method that could then be applied to the preparation of a wide variety of functionalized steroids. For these purposes, several classic glycoside donors, such as halofens, thioethers, phosphates, etc.[20] were explored on the example of α/β glycosylation of cholesterol. We obtained the best results using *O*-2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl-trichloroacetimidate (**2a**, Scheme 1) as a glycosylation reagent.[21,22] This approach utilizes the classic Schmidt's glycosylation reaction conditions.[23] Considering our ability to use this method and have control in the preparation of α/β glucose derivatives of cholesterol, we then selected this method for the preparation of other functionalized

**Table 4**
2α,3β-substituted spirostane derivatives and the MIC$_{50}$ for three yeast species

| Structure | MIC$_{50}$[a,e] (μg/mL) | | |
|---|---|---|---|
| | *Candida albicans* [b] | *Candida glabrata*[c] | *Cryptococcus neoformans*[d] |
|  **14a** | NA | NA | NA |
|  **14b** | NA | NA | NA |
|  **14c** | NA | NA | NA |
|  **14d** | NA | NA | NA |
|  **14e** | 16 | 8 | 0.5 |

(*continued on next page*)

**Table 4** (*continued*)

| Structure | $MIC_{50}$[a,e] (μg/mL) | | |
|---|---|---|---|
| | *Candida albicans* [b] | *Candida glabrata*[c] | *Cryptococcus neoformans*[d] |
|  **14f** | NT | NT | NT |

**Previously reported in Ref. 18

NA denotes compounds that had (1) a slight reduction in turbidity to no change and; (2) had less than a 50% reduction in growth compared to controls, as measured spectroscopically by absorption at 530 nm.

NT denotes not yet tested.

[a] MIC values are reported only for compounds displaying (1) a prominent decrease in turbidity by visual comparison to the control wells containing no antifungal and; (2) a >50% reduction in fungal growth compared to controls containing no antifungal, as measured spectroscopically by absorption at 530 nm.

[b] ATCC no. 10231.

[c] ATCC no. 48435.

[d] ATCC no. 36556.

[e] All values were determined after incubation at 35 °C for 48 h.

steroids presented in this report (**11**–**14**, Scheme 1). Briefly, the corresponding α or β trichloroacetamidates were prepared as glycosylation reagents (**2**, Scheme 1) from commercially available mono and disaccharides in three steps. The first step of the preparation was total benzoylation of the free saccharide hydroxy groups with benzoyl chloride in pyridine.[24] The second step involved an elegant and selective perbenzoylated saccharide C-1 ester hydrolysis with ethanolamine in dimethylsulfoxide-ethyl acetate.[25] Finally, in the third step of the procedure, the prepared C-1 deprotected perbenzoylated saccharide was used in a reaction with trichloroacetamide in dichloromethane with potassium carbonate as a base catalyst (Scheme 1). This reaction was highly efficient, and if it was conducted under kinetic conditions, only the corresponding β acetamidates were prepared. On the other hand, if the reaction was conducted under thermodynamic conditions, then the corresponding α acetamidate was the dominant product.[26] Purification of the product involved a simple and fast silica gel filtration followed with the immediate coupling to the corresponding functionalized steroid.

All of the functionalized steroids used in the preparation of our glycosylated products (**11–14**, Scheme 1) were prepared in several steps from commercially available materials using our previously developed and published methods of synthesis.[17,18] Thus, the previously prepared 2α,3β substituted steroids **3–6** (Scheme 1) were glycosylated with trichloroacetamidate glycoside donors **2** (Scheme 1) under TMSOTf catalyzed conditions (Scheme 1). It was demonstrated in many instances that sialic acid donors are good catalysts for glycosylation.[27] However, the opposite isomer of trichloroacetamidate must be used to prepare the steroid derivative with our desired stereochemistry. It is important to emphasize that these glycosylation reactions are very sensitive to moisture; therefore the reaction must be carried out in the presence of molecular sieves. The final step in the preparation of our glycosylated steroids involved the hydrolysis of all ester groups of compounds **7**–**10** (Scheme 1). This reaction must also be conducted in a manner to control for excess moisture, due to the fact that upon isolation, the resulting glycosylated steroid will swell in an excess of water, making the isolation of the targeted compounds exceptionally difficult.[28] To circumvent this problem and simplify the isolation and purification of glycosidic steroids **11**–**14**, we performed the hydroxyl deprotection in 'water-free' conditions. We developed a simple and highly efficient method of 'dry' hydrolysis. In 'dry' hydrolysis, trans-esterification followed by ion-exchange neutralization generates a clean reaction containing the dry saponin and the easily removable methyl benzoate. In this case, the dry saponin is a white powder. In contrast, under normal hydrolysis conditions, the saponin product would be a viscous, semi-solid material from which it would be difficult to remove water and other impurtities. A dichloromethane solution of peresterified steroids **7–10** were slowly titrated with freshly prepared 10% $CH_3O$-Na/$CH_3OH$ until the solution reached pH 10. The reaction mixture was neutralized with dry acidic dowex (50WX8-200), and the product was then purified by silica gel chromatography.

The antifungal activity of the resulting glycosylated steroids shown in Scheme 1 were evaluated in vitro using *C. albicans*,*C. neoformans*, and *C. glabrata*. All assays were done in accordance with NCCLS reference documents.[29] The results of these screenings are summarized in Tables 1–4. Of the analogs tested, none of the 2α,3β functionalized steroids that contained either a mono or disaccharide that was α-linked to the 3β-position of the steroid showed any antifungal activity against the three yeasts tested, while a number of the β-linked derivatives showed promise. Specifically, one of the four steroid derivatives having a β-linked glucose showed antifungal activity against all three species at 8 μg/mL (**13c**, Table 3). However, the most promising results were observed in all but one class of molecules when the addition of the disaccharide maltose (1,4α-linked disaccharide) was β-linked to cholestane, spirostane and 2,3-functionalized spirostanes ( **11e**, **13e**, and **14e** Tables 1, 3 and 4). Antifungal activities of these three derivatives ranged from 32–0.5 μg/mL, depending on the fungal species assayed. The exception to this observation was seen in the 2α,3β-functionalized cholestane group, where greater antifungal activity was observed in the disaccharide cellibiose (1,4β-linked disaccharide) (**12f** Table 2). Additionally, the presence of a hydroxyl group in the 2α-position of the spirostane derivatives increased the antifungal activity against all three yeasts (compounds **13e** vs **14e**). These findings indicate that the stereochemistry of the glycosidic bond to the steroid may be a key factor in the development of novel antifungals. Current work is underway to determine the antifungal activities of 2α,3α,2β,3β and 2β,3α-functionalized steroids and glycosidic steroids. Finally, in vitro mammalian cell toxicity studies were done using two cell lines (Vero cells (ATCC no. CRL-1651) and rabbit skin cells (ATCC no. CCL-68) on all derivatives that had antifungal activity of less than 32 μg/mL at a range

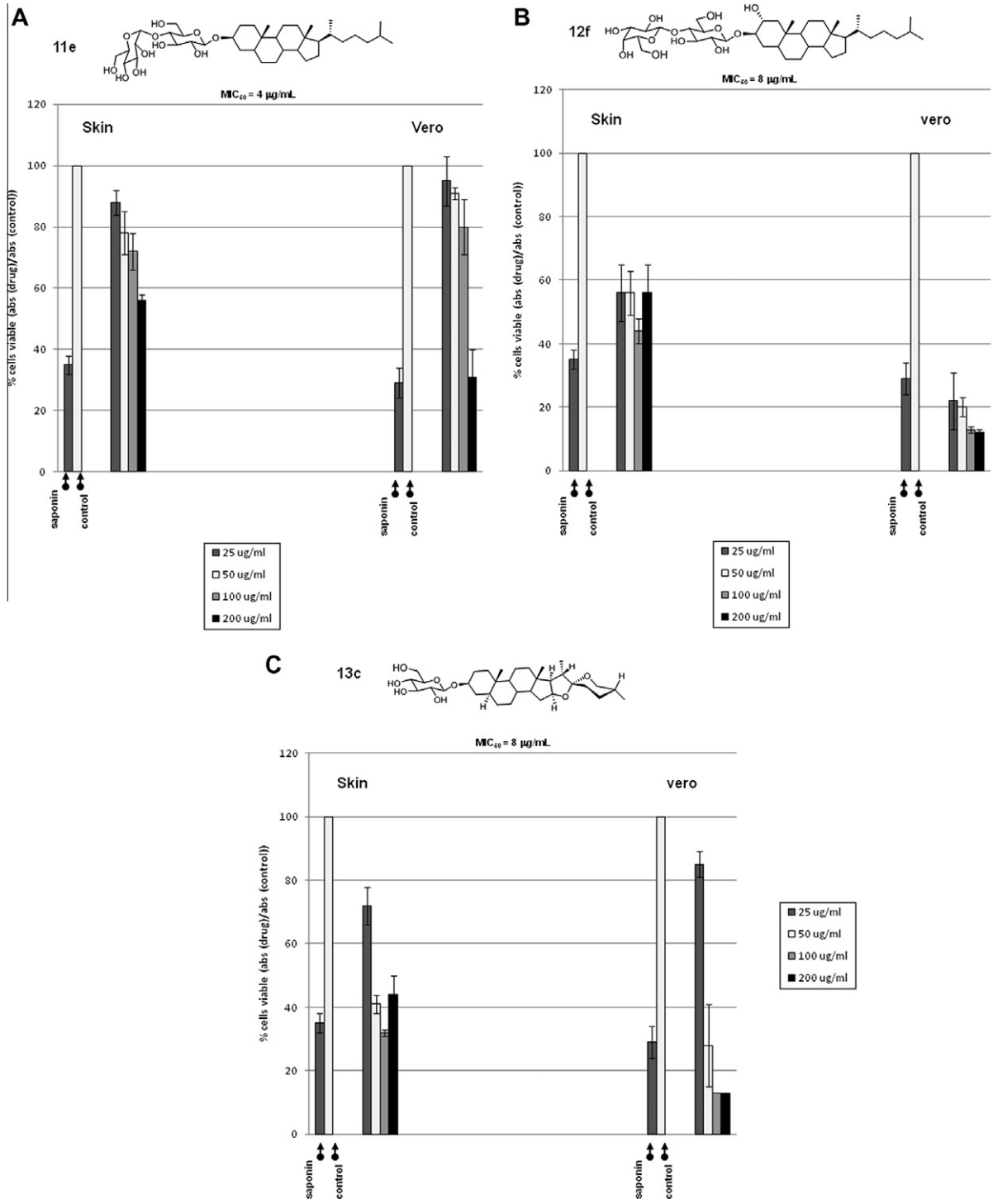



**Figure 1.** Evaluation of mammalian cell toxicity of active compounds. Cytotoxicity was determined using noncancerous vero cells (African green monkey) and rabbit skin cells in accordance with Promega CellTiter 96 Non-RadioactivCell Proliferation Assay (cat # G4000) on compounds **11e**, **12f**, **13c**, **13e** and **14f**. Compounds were diluted in media to the testing dilution amounts: 200 µg/ml, 100 µg/ml, 50 µg/ml and 25 µg/ml. All assays were done in triplicate and the average values are presented, with ±SD. Cells were incubated in the presence of the compounds for 24 h at 37 °C and 5% $CO_2$. Tetrazolium dye solution was added to each well and allowed to incubate for 1–4 h. Stabilization/Stop solution was added and allowed to sit at room temperature for 1 h. Formazan product was scored spectrophotometrically with an automatic plate reader set at 570 nm. 1× saponin was used as a control to measure cell death. Wells containing 1% DMSO and no drug were used as a positive control and the average absorbance at 570 nm were set to 100%. The percent of cells viable were calculated as a ratio between the average absorption at 570 nm of the drug containing wells/average absorption at 570 nm of the control (no drug). Ratios were multiplied by 100 to give the values as a percentage. It is important to note that the MIC value shown at the top of each panel does not reflect the MIC for each fungal species analyzed. Rather, this value is the highest MIC value of the three species tested.

of at least 5–10 times greater than the minimum inhibitory concentration in accordance with Promega CellTiter 96 Non-RadioactivCell Proliferation Assay (cat # G4000). This included compounds **11e, 12f, 13c, 13e** and **14f**. The results of those studies are summarized in Figure 1. Two of the five compounds assayed (**12f** and **13c)** showed significant toxicity in both cell lines utilized. In summary, several promising antifungal agents were identified through the course of these assays. Further work determining the

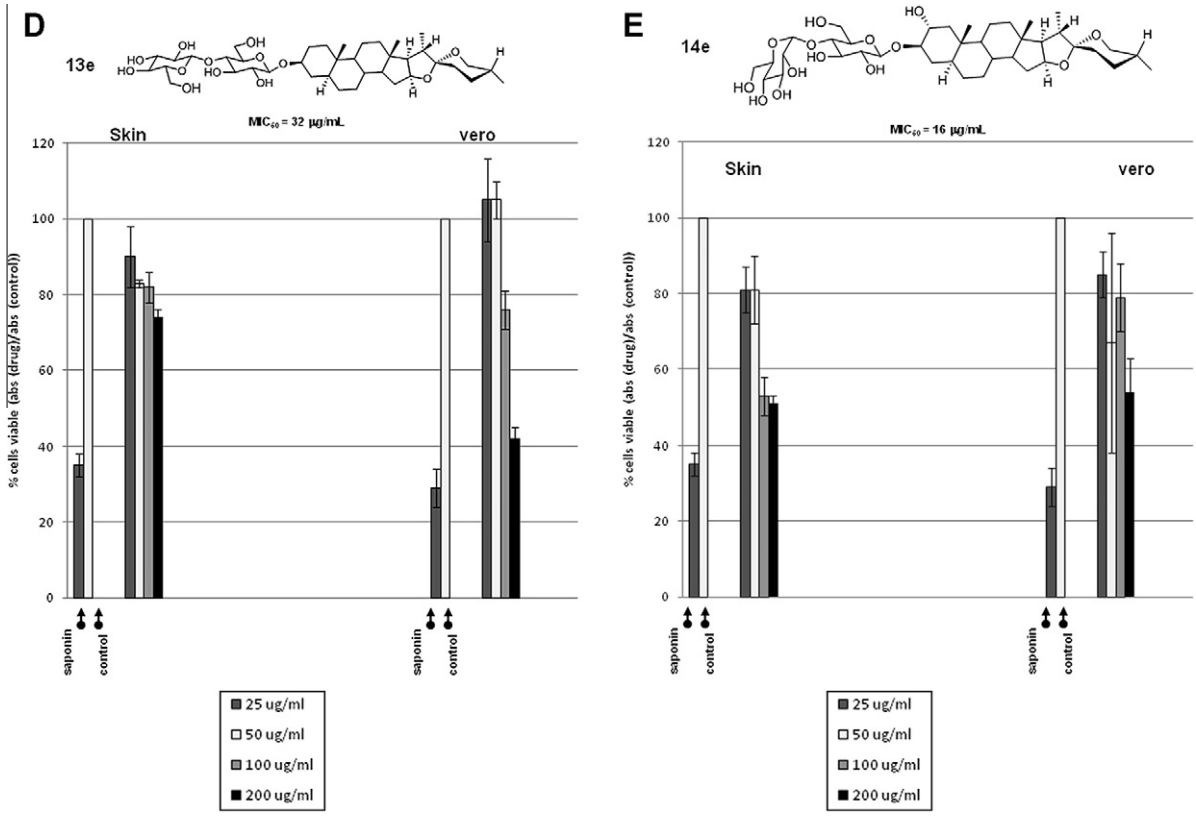



**Fig. 1** (*continued*)

role of stereochemistry of the steroid moiety with respect to antifungal activity is currently underway.

Detailed information regarding the syntheses, spectroscopic characterization and biological evaluation of the steroid-glycosides presented in this paper can be found in the Supplementary data provided.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.015.

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