New Steroidal Dimers with Antifungal and Antiproliferative Activity

Deepak B. Salunke,[†] Braja G. Hazra,*[,†] Vandana S. Pore,[†] Manoj Kumar Bhat,[‡] Pallavi B. Nahar,[§] and Mukund V. Deshpande[§]

Organic Chemistry Synthesis Division, National Chemical Laboratory, Pune 411 008, India, National Centre for Cell Science, Pune University Campus, Pune 411 007, India, and Biochemical Sciences Division, National Chemical Laboratory, Pune 411 008, India

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Bile acid-derived novel amphiphilic topology was designed and synthesized in the form of steroidal dimers. These dimers were tested for antifungal and antiproliferative activity in vitro. N^1,N^3-Diethylenetriaminebis[cholic acid amide] was found to be active against *C. albicans*, *Y. lipolytica*, and *B. poitrassi* at nanomolar concentration and did not show any effect on cell proliferation. N^1,N^2-Ethylenediaminebis[deoxycholic acid amide] totally inhibited the growth of human oral cancer (HEp-2) and human breast cancer (MCF-7) cells.

Introduction

During the last four decades, there has been tremendous increase in the frequency of fungal infections.[1] *Candida albicans* is the most dreadful human pathogen for which current drugs include amphotericin B (Amp B) and a variety of azoles. Amp B is reported to be toxic to humans and clinical resistance to azoles is increasing.[2] Therefore, there is need to screen for new antifungal therapeutics, which have high efficacy and low toxicity. The new class of membrane-disrupting drugs is ideal as antimicrobial agent because microbes are unlikely to develop resistance to them.[3]

Design of Bile Acid-Derived Facial Antimicrobial. A common feature of bile acid-derived antimicrobials is the potential to exhibit facially amphiphilic conformations containing polar and hydrophobic surfaces[4] (Figure 1). This type of amphiphilicity can be achieved by polyene macrolide antibiotics[5] such as Amp-B **1** (Figure 2) and also by peptide antimicrobial agents[6−8] having cationic residues and hydrophobic amino acids such as polymyxin-B **3**. Similar to Amp B, squalamine in the cyclic form **2** functions as an ionophore,[5,9] and squalamine mimic **4** also possesses unusual ionophoric properties.[10] Although the squalamine and polymyxin B mimics are morphologically dissimilar, they display similar activities.[11]

On the basis of these observations, we thought of an amphiphile topology that has not previously received much attention in synthetic systems: a partially rigid structure with three discrete faces, one polar face sandwiched within two nonpolar faces (Figure 3). In this case, the steroid skeleton provides a hydrophobic surface and the hydroxyl groups function as the polar headgroup. Different linkers can be used so that additional hydrophobicity can be achieved. To support our hypothesis, novel steroidal dimers **5**−**8** (Figure 4) were designed and synthesized and their antifungal activity was evaluated.

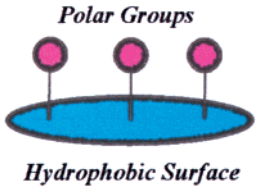

Figure 1. Schematic representation of facial amphiphile.

Chemistry. Dimeric and oligomeric steroids have tremendous applications in different areas.[12,13] The cholic acid steroidal skeleton plays a pivotal role in formation of dimeric and oligomeric forms. In the dimeric form, cholic acid exists in a rigid conformation with the steroid hydroxyl groups intramolecularly hydrogen bonded.[14] In this paper we wish to report a highly efficient synthesis, characterization, and bioevaluation of four new dimeric steroids derived from cholic acid **9** and deoxycholic acid **10**. Compounds **9** and **10** were activated as *N*-succinimidyl esters **11** and **12**, respectively (Scheme 1).[15] These esters were reacted with ethylenediamine and diethylenetriamine to afford the dimers **5** to **8** in very high yields (95−98%). ^{13}C NMR, mass spectroscopy, and C, H, N analysis confirmed the formation of dimeric compounds.

Biochemistry: Antifungal Activity. Compounds **5**−**8** were examined for antifungal activity. The MIC for compound **7** is 11.32 nM for all five *C. albicans* isolates, *B. poitrassi*, and *Y. lipolytica*. The MIC values for compound **8** ranged between 10 and 25 nM while compounds **5** and **6** did not show any zone of inhibition even up to 500 nM concentration. However, in the case of cycloheximide, the MIC value is much higher and ranged between 250 and 500 nM for isolates of *C. albicans*. In a microtiter dilution broth assay, the MIC of cycloheximide against a clinical isolate of *C. albicans* was reported to be greater than 900 nM.[16] The MIC of cycloheximide was 71 nM in *B. poitrasii* and 106 nM in *Y. lipolytica*. The difference in the toxicity of cycloheximide toward *C. albicans*, a pathogenic fungus, and *B. poitrasii* and *Y. lipolytica*, nonpathogenic fungi, can be attributed to the differences in their cell wall composition that affects the passage of the compound through the fungal cell wall. DMSO did not show any zone of

* Corresponding author. Phone: +91-20-5893153. Fax: +91-20-5893153 and +91-20-5893355. E-mail: hazra@ems.ncl.res.in.
† Organic Chemistry Synthesis Division, National Chemical Laboratory.
‡ National Centre for Cell Science, Pune University Campus.
§ Biochemical Sciences Division, National Chemical Laboratory.

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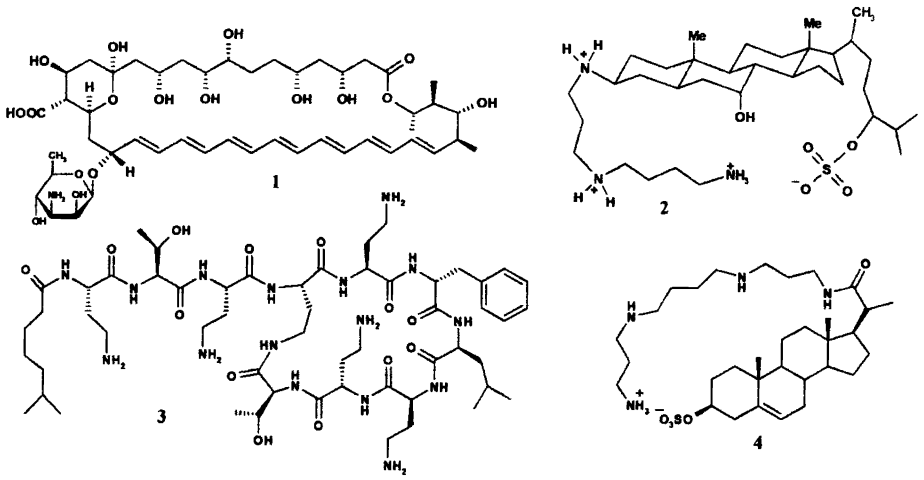


Figure 2. Amphotericin B (**1**), squalamine in salt bridged cyclic form (**2**), polymyxin B (**3**), and squalamine mimic (**4**).

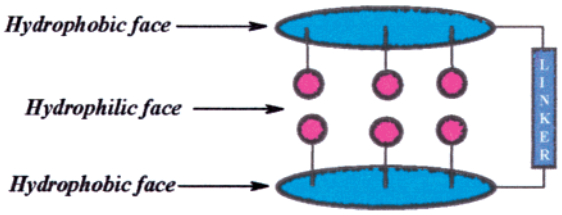
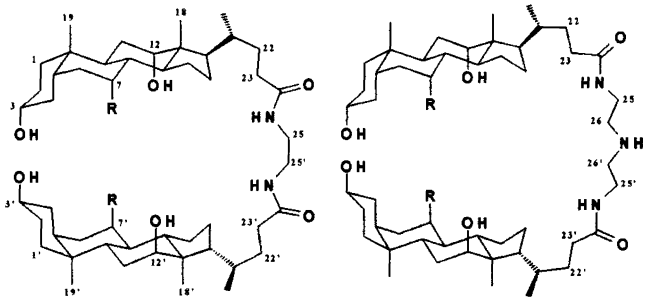


Figure 3. Schematic representation of novel facial amphiphile.

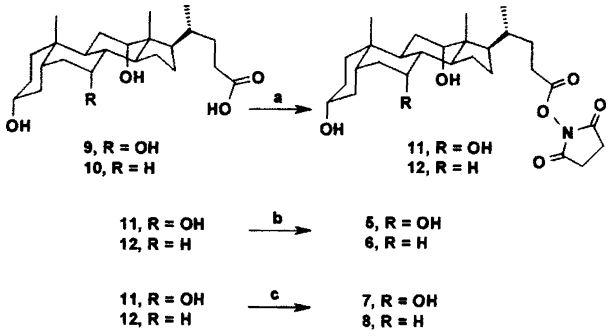


5, R = OH
6, R = H

7, R = OH
8, R = H

Figure 4. Proposed targets made from cholic acid and deoxycholic acid using different linkers such as ethylenediamine and diethylenetriamine.

Scheme 1[a]



[a] Reagents and conditions: (a) *N*-hydroxysuccinimide, DCC, THF−CH_3CN, 25 °C, 18 h, 97% yield; (b) ethylenediamine, DMF, 25 °C, 1.5 h, 96% and 97% yield; (c) diethylenetriamine, DMF, 25 °C, 1.5 h, 95% and 98%.

inhibition. The results suggest that the compounds **7** and **8** have potential as antifungal agents when compared to the standard inhibitor cycloheximide, when

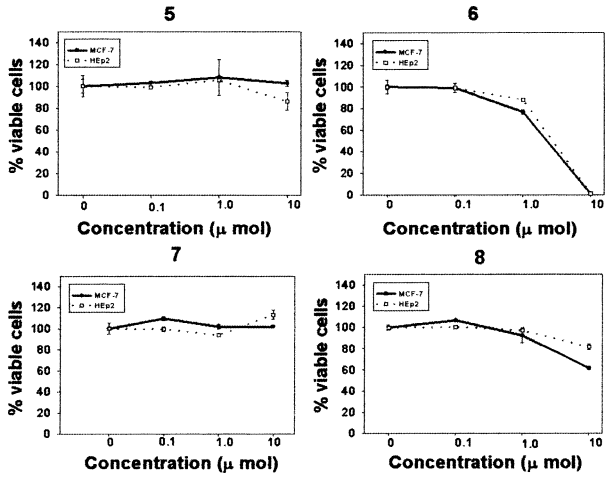


Figure 5. Graphical representation for antiproliferative activities of compounds **5**−**8**.

tested against both the pathogenic and nonpathogenic fungi.

Antiproliferative Activity. The antiproliferative activity of all the four compounds was tested against human cancer cells HEp-2 and MCF-7, which were grown in vitro (Figure 5). Compounds **5** and **7** up to a concentration of 10 μM had no effect on the survival of HEp-2 and MCF-7 cells. Compounds **6** and **8** elicited a significant reduction in proliferation of both the cell lines; however, compound **6** at 10 μM concentration totally inhibited their growth. IC_{50} values for compound **6** were between 2 and 3 μM, whereas IC_{50} values for compound **8** appear to be greater than 10 μM. The compound **8** at 10 μM concentration inhibited the growth of HEp-2 by 20% and MCF-7 by 40%.

Discussion. Compound **7** is active against *C. albicans* (Isolate 1−5), *B. poitrassi*, and *Y. lipolytica*. Compound **8** also at little higher concentration showed similar antifungal activity. Compound **5** and **6** were found to be inactive up to 500 nM concentration. Compounds **5** and **7** up to a concentration of 10 μM had no effect on the survival of HEp-2 and MCF-7 cells. Compound **8** at 10 μM concentration inhibited the growth of HEp-2 cells by 20% and MCF-7 by 40%. Compound **6** at 10 μM concentration totally inhibited the growth of both the cell lines (Table 1).

The results suggest that compound **7** has a potential as antifungal agent when tested against both pathogenic

Table 1. Comparison of Antifungal Activity and Cytotoxicity of Compounds **6** and **7**

compd	antifungal activity MIC, nM	antiproliferative activity IC-50, μM
6	>500	2.2
7	11.32	not active

and nonpathogenic fungi. Thus compound **7** may lead to a very good antifungal agent as it is selectively toxic to fungal cells at nanomolar concentration and does not have any profound effect on cell proliferation. On the other hand, compound **6**, which is not at all active against fungal cells, exhibited good antiproliferative activity.

Conclusion. Two series of steroidal dimers with different linkers were synthesized and characterized, and their biological activities were evaluated. The antifungal activity of compound **7** is very good in comparison with cycloheximide, so considering its low toxicity on cancer cell lines tested, it represents a good lead for further research in this area. Tested compounds **6** and **8** exhibited antiproliferative activity at micromolar concentrations. (IC_{50} = 2−3 μM). Compound **6** exhibited profound effect where as compound **8** inhibited cell growth partially. The growth suppressive effect of compound **6** and **8** appears to be influenced by the length of side chain present. Since compound **5** and **7** had no effect on cell survival, the absence of hydroxyl group at C-7 position plays a critical role in toxicity of these compounds toward human cells. Thus, an extensive SAR studies should be carried out so that one can reach to the appropriate explanation for the presence or absence of particular functionality in the dimers.

Experimental Section

General Methods. ^{1}H and ^{13}C NMR spectra were recorded at 300.13 or 500.13 and 75.47 or 125.78 MHz, respectively. For mass spectra, samples were introduced by the infusion method using electrospray ionization technique. Column chromatography was performed on neutral deactivated aluminum oxide. Thin-layer chromatography was performed on precoated silica gel F-254 plates.

Typical Procedure for the Synthesis of Dimers 5−8 from *N*-Succinimidyl Ester 11 of Cholic Acid 9, *N*-Succinimidyl Ester 12 of Deoxycholic Acid 10, and Polyamines Such as Ethylenediamine and Diethylenetriamine. *N*-Succinimidyl ester **11** (1.01 g, 2 mmol) was dissolved in 1.5 mL of DMF. To it was added ethylenediamine (0.073 mL, 1.1 mmol), and reaction mixture was stirred for 1.5 h at 25 °C. Reaction was quenched by the addition of crushed ice. Solid crude product was filtered, washed with cold water (2 × 5 mL), and dried under vacuum. Column chromatographic purification of the crude product (neutral deactivated alumina, eluent: $CH_3OH/CHCl_3$ (1:19)] afforded N^1,N^2-ethylenediaminebis[cholic acid amide] **5** (0.81 g, 96%). It was further recrystallized from $CH_3OH/CHCl_3$. Mp = 205 °C. In the similar way compound **12** (0.98 g, 2 mmol) and ethylenediamine (0.073 mL, 1.1 mmol) afforded N^1,N^2-ethylenediaminebis[deoxycholic acid amide] **6** (0.79 g, 97%), Mp = 170 °C ($CH_3OH/CHCl_3$). Compound **11** (1.01 g, 2 mmol) and diethylenetriamine (0.12 mL, 1.1 mmol) furnished N^1,N^3-diethylenetriaminebis[cholic acid amide] **7** (0.84 g, 95%), Mp = 168 °C ($CH_3OH/CHCl_3$). Compound **12** (0.98 g, 2 mmol) and diethylenetriamine (0.12 mL, 1.1 mmol) gave N^1,N^3-diethylenetriaminebis[deoxycholic acid amide] **8** (0.84 g, 98%), Mp = 130 °C ($CH_3OH/CHCl_3$).

Antifungal Activity. Materials and Methods. *Candida albicans* (five isolates), *Benjaminiella poitrasii*, and *Yarrowia lipolytica* were maintained on Difco yeast extract peptone glucose agar slants at 28 °C for 7 days. *C. albicans* and *Y. lipolytica* were inoculated in YPG broth at 28 °C and *B. poitrasii* at 37 °C for 24 h. Compounds **5**−**8** were solubilized in DMSO, and stock solutions of 1 mg/mL was prepared. Cycloheximide, a standard inhibitor, was also dissolved in DMSO, and varying concentrations were added onto the filter paper disks.

Antibiotic Inhibition Assay Using Disk Method. One hundred microliters of the yeast cell suspensions for all five *C. albicans* isolates, *B. poitrasii*, and *Y. lipolytica* was spread-plated on sterile YPG agar plates separately. Five sterile Whatman filter paper disks were placed on each plate. To the central disk was added DMSO as the control, and to other four disks were added different concentrations of the compounds. The plates were then incubated at 28 °C for 24 h and were observed for the zone of inhibition around the disk. The minimum concentration that gave the zone of inhibition was determined as the minimum inhibitory concentration (MIC) of the compound for that fungal culture. All the experiments were done in triplicate. Similar procedure was followed to determine the MIC of cycloheximide.

Antiproliferative Activity. Materials and Methods. Human oral squamous carcinoma HEp-2 and human mammary adenocarcinoma (MCF-7) cell lines were obtained from National Centre for Cell Science, Pune, India. Cells were maintained as a monolayer in nutrient media MEM supplemented with heat-inactivated fetal bovine serum (HyClone, Utah) (10%), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Invitrogen Life Technologies, MD). The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere. Stock solutions of all the compounds were prepared in DMSO at a concentration of 10−11.5 mM and afterward diluted to the required concentration. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved (1 mg/mL) in MEM (without phenol red) and filtered through a Millipore filter, 0.22 μm, before use.

MTT Cell Proliferation Assay. HEp-2 and MCF-7 cells were plated at a density of 15 000 cells per well in 96-well tissue culture plates. Cells were allowed to adhere for 24 h at 37 °C and then treated with various concentrations (0, 0.1, 1.0, and 10 μM) of compounds dissolved in DMSO for additional 72 h, in triplicate. In the control wells, nutrient medium, with a corresponding concentration of DMSO, only was added to the cells. Thereafter, cell proliferation was assessed by replacing treatment medium with 50 μL of media containing 1 mg/mL MTT and incubated for 4 h at 37 °C. Medium was then aspirated off, and formazan crystals were solubilized in 50 μL of 2-propanol. The optical density was read on a microplate reader at 570 nm using 630 nm as a reference filter against a blank prepared from cell free wells. Absorbance given by cells treated with the carrier DMSO alone was taken as 100% cell growth. All assays were performed in triplicates.

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Supporting Information Available: Synthetic procedures and characterization of all compounds and tabular in vitro antifungal activity data. This material is available free of charge via the Internet at http://pubs.acs.org.

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