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Synthesis of chimeric tetrapeptide-linked cholic acid derivatives: Impending synergistic agents

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ABSTRACT

Tetrapeptides derived from glycine and β-alanine were hooked at the C-3β position of the modified cholic acid to realize novel linear tetrapeptide-linked cholic acid derivatives. All the synthesized compounds were tested against a wide variety of microorganisms (Gram-negative bacteria, Gram-positive bacteria and fungi) and their cytotoxicity was evaluated against human embryonic kidney (HEK293) and human mammary adenocarcinoma (MCF-7) cell lines. While relatively inactive by themselves, these compounds interact synergistically with antibiotics such as fluconazole and erythromycin to inhibit growth of fungi and bacteria, respectively, at 1–24 μg/mL. The synergistic effect shown by our novel compounds is due to their inherent amphiphilicity. The fractional inhibitory concentrations reported are comparable to those reported for Polymyxin B derivatives.

The dramatically rising prevalence over the past few decades of multidrug-resistant microbial infections has become a serious health problem. In order to circumvent this situation, there is an urgent need to develop new antimicrobial therapeutics. The class of membrane-disrupting drugs is ideal as antimicrobial agents because microbes are unlikely to develop resistance to them.[1,2] As the outer membrane or cell wall of microbes provides a protective barrier against many types of antibiotics,[3] amphipathic molecules that can act synergistically with various hydrophobic antibiotics as outer membrane permeabilizers may represent a new class of antibiotic agents.

Design of novel amphipathic molecules: A literature survey of antimicrobial steroids reveals that several amino cholesterol derivatives exhibit profound antimicrobial activity.[4] The in vitro antibacterial properties of bile acids against certain Gram-positive microorganisms are well known.[5] Consequently, the preparation of various bile acid-based aminosterols was reported with a view to examine their activity as anti-microbial agents.[6] A recent approach to combat against pathogens is to introduce a polycationic chain onto a steroid scaffold. One such chimeric natural product namely squalamine[7] **1** has attracted considerable attention because of its potent antimicrobial activity against a broad spectrum of microorganisms[8,9] (Fig. 1). Soon after the isolation of squalamine, Regen reported[10] rapid construction of squalamine mimic **2** which displays extraordinary antimicrobial properties. Naturally occurring steroid-amino acid conjugates such as bufotoxin **3** and polymastiamide A **4**, exhibit in vitro antimicrobial activity.[11] Several peptides have been identified that increase the permeability of the outer membranes of Gram-negative bacteria and sensitize these organisms to hydrophobic antibiotics.[12] The best studied of these peptides are the polymyxin B (PMB) **5** derivatives. Savage and co-workers[13] designed a class of cationic steroid antibiotics (CSA) **6** as PMB mimics which display antibacterial activities comparable or superior to that of Squalamine **1,** bufotoxin **3** or PMB **5**.

A number of intuitive assumptions can be drawn from this wide-ranging literature survey.

- A common feature of steroid derived antimicrobials is their potential to exhibit facially amphiphilic conformations containing polar and hydrophobic surfaces.[14]
- The overall amphiphilicity of the molecule plays a key role in determining the level of antimicrobial activity.
- A generic structure with fine-tuning of the molecular amphiphilicity may lead to novel molecules capable of selectively permeabilizing the microbial membranes.

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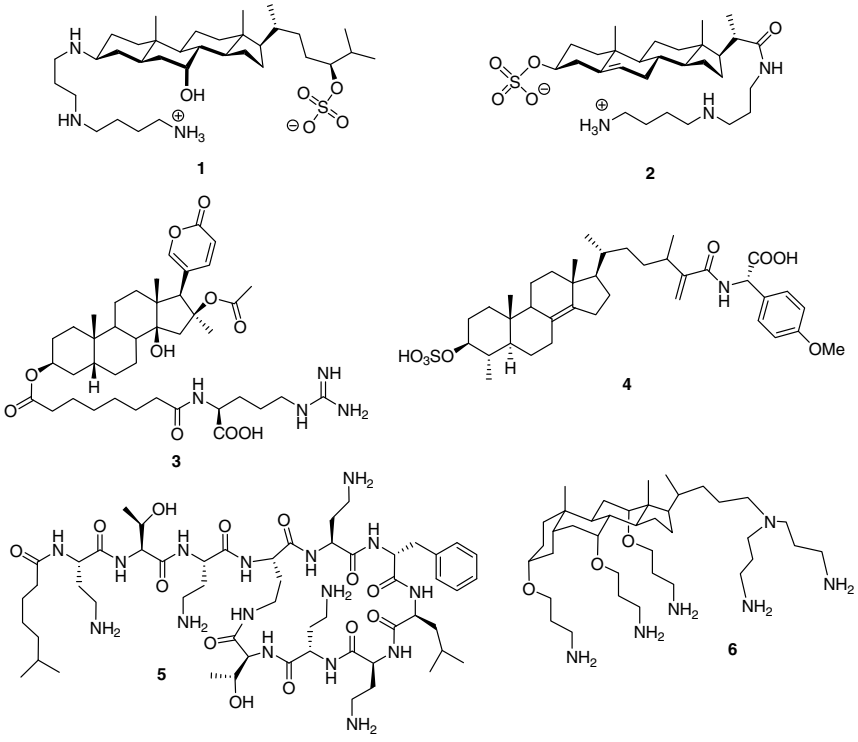


Figure 1. Squalamine **1**, steroid polyamine conjugate **2**, steroid-amino acids conjugates **3** and **4**, polymyxin B **5**, and cationic steroid antibiotics **6**.

With this in view, novel cholic acid-tetrapeptide conjugates of glycine and β-alanine were synthesized and tested against a wide variety of microorganisms. Cholic acid has been chosen because of its natural amphiphathic nature.[15] It differs from the conventional head-to-tail amphiphiles because the polar and non-polar domains are separated along the longitudinal axis of the molecule, which gives rise to distinct polar and non-polar faces.[16] A polypeptide segment has been introduced on to the cholic acid to have combination of a hydrophilic functional moiety as well as a hydrophobic carrier in the same molecule. Synthetic variations of the amino acid residues can produce a library of compounds with variable amphiphilicity.

Chemistry: We undertook the synthesis and evaluation of a new family of cholic acid-polypeptide conjugates by exploiting a convergent approach, using classical solution phase synthesis. According to the literature procedures,[17] suitably protected monomers **9**, **11** and **10**, **12** were synthesized from glycine **7** and β-alanine **8**, respectively. The stepwise elongation was performed in dichloromethane (DCM) using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) activation to furnish Boc-$(Gly)_2$-OBn **13** in 76% yield and Boc-$(\beta\text{-Ala})_2$-OBn **14** in 74% yield (Scheme 1). Removal of the *tert*-butoxycarbonyl (Boc) group from peptides **13** and **14** was performed in trifluoroacetic acid (TFA)/DCM, to afford compounds **15** and **16** in quantitative yield. On the other hand, catalytic hydrogenolysis of the benzyl (Bn) group from peptides **13** and **14**, furnished compounds **17** and **18** in excellent yields. Compounds **19** and **20** were obtained by fragment condensation of **15** with **17** and **16** with **18** in DMF using EDCI activation in the presence of 1-hydroxybenzotriazole (HOBt) as catalyst. These tetrapeptides were purified by column chromatography to furnish compounds **19** and **20** as white solids in 73% and 70% yields, respectively.

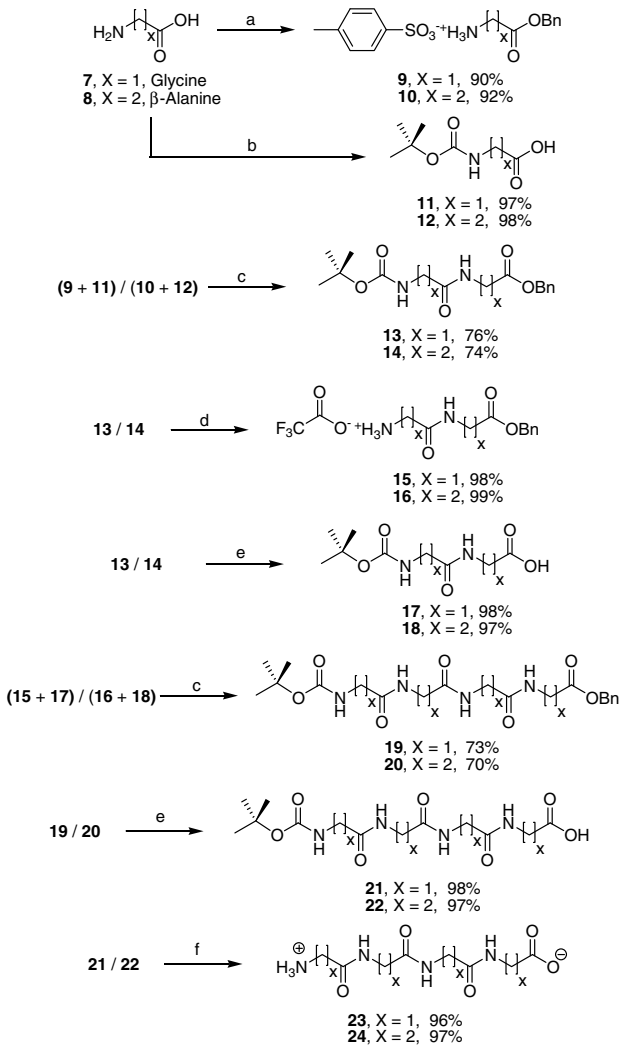
The Benzyl groups of Boc-$(Gly)_4$-OBn **19** and Boc-$(\beta\text{-Ala})_4$-OBn **20** were removed by a similar catalytic hydrogenation reaction to furnish compounds **21** and **22** in 98% and 97% yield, respectively. Finally, the Boc groups of compounds **21** and **22** were removed using 2M HCl:Et_2O to afford the desired peptides **23** and **24** in 96% and 97% yield, respectively. Following the modified procedure (using EDCI and HOBt), we have drastically reduced the reaction time (7–8 h) compared to the earlier reported method, which required 3 days for the reaction to complete.[18]

Synthesis of the cholic acid intermediates, which are the building blocks for realization of the desired conjugates, is depicted in Scheme 2. 3β-Amino methyl cholate **26** was synthesized from cholic acid **25** in four steps in a straightforward way with an overall yield of 77%.[19] The amine functionality in compound **26** was protected using Boc anhydride to afford compound **27** in excellent yield (Scheme 2).

Reduction of the methyl ester of compound **27** was carried out using LAH to afford C-24 hydroxy compound **28** in 74% yield. Acylation of the C-7 and C-24 hydroxyl groups of compound **28** was carried out using acetic anhydride and a catalytic amount of *N*,*N*-dimethylaminopyridine (DMAP) to furnish compound **29** in 87% yield. The protected C-3β amino functionality of compound **29** was unmasked using TFA to afford compound **30** in almost quantitative yield.

Coupling of 3β-amino cholic acid intermediates **26** and **30** with Boc-$(Gly)_4$-OH **21** and Boc-$(\beta\text{-Ala})_4$-OH **22** under mild condition using EDCI/HOBt and Et_3N in DMF provided compounds **31, 32** in 74% and 71% yield and compounds **37**, **38** in 75% and 74% yield, respectively (Scheme 3). Subsequent hydrolysis of the methyl ester functionality in compound **32** and C-7 and C-24 acetates in compound **38** (LiOH 2M, CH_3OH) and aqueous work-up provided the corresponding acid **35**, and hydroxy compound **40** in 89% and 84% yield, respectively. Cleavage of the Boc group in compounds **31**, **32**, **35**, **37** and **40** was accomplished with 2M HCl:Et_2O to afford free amino compounds **33**, **34**, **36**, **39**, and **41**, respectively, in yields ranging from 89–96%. It is worth mentioning here that all the compounds **31–41** (Scheme 3) are new and characterized fully by IR, ^1H NMR and ^{13}C NMR spectroscopy.

Antimicrobial activity: Tetrapeptides **19–24,** Cholic acid **25**, and cholic acid-tetrapeptide conjugates **31–41** were examined for



Scheme 1. Synthesis of polypeptide chains. Reagents and conditions: (a) benzyl alcohol, *p*TSA, toluene, reflux, 4 h; (b) Boc anhydride, 1 N NaOH, dioxane:water, 0–25 °C, 30 min; (c) EDCI, HOBt, Et_3N, DCM, 0–25 °C, 6 h; (d) TFA, DCM, 0–25 °C, 2 h; (e) H_2, Pd-C, MeOH, 25 °C, 1 h; (f) 2M HCl:Et_2O, 0–25 °C, 1.5 h.

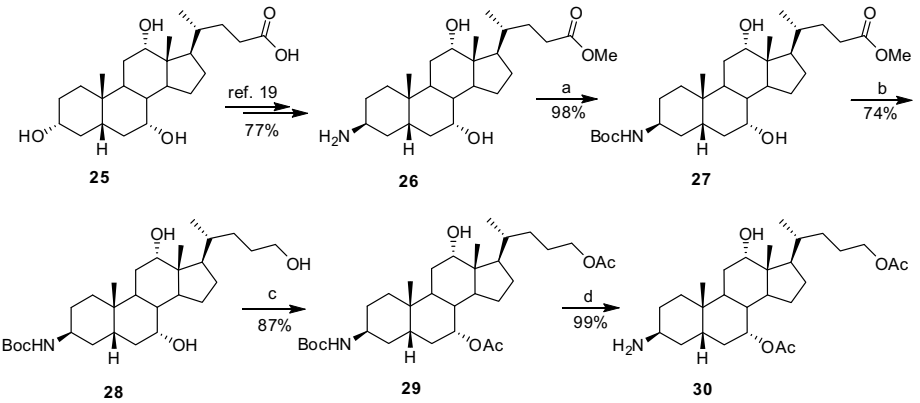
in vitro antifungal as well as antibacterial activity against the fungal strains viz., *Candida albicans*, *Cryptococcous neoformans*, *Benjaminiella poitrasii*, *Yarrowia lipolytica*, *Fusarium oxysporum* strains and bacterial strains *Escherichia coli*, and *Staphylococcus aureus*, respectively, to find out the minimum inhibitory concentration (MIC) values (Table 1). These compounds were also tested for their ability to permeabilize the outer membrane of Gram-negative bacteria such as *E. coli* causing sensitization to hydrophobic antibiotics that inefficiently cross the outer membrane. We also demonstrated such permeabilization by cholic acid derivatives with *C. albicans*, a pathogenic fungus (Table 2).

In the preliminary bioevaluation, tetrapeptides **19**–**24** as well as cholic acid **25** did not show any appreciable antifungal or antibacterial effect. In comparison the tetrapeptide-linked cholic acid derivatives **31**–**41** showed good to moderate activity against *C. albicans*, *B. poitrasii* and *F. oxysporum* (columns A, C and E) whereas these compounds were less active towards bacteria (columns F and G) than fungi. The antifungal activity of most of the compounds was found to be similar to that of fluconazole (MIC, 32 μg/mL). The difference in the toxicity of the synthesized compounds against a wide variety of microorganisms can be attributed to the differences in their cell wall/cell membrane compositions which affect the passage of these compounds through cell wall/cell membrane.[20]

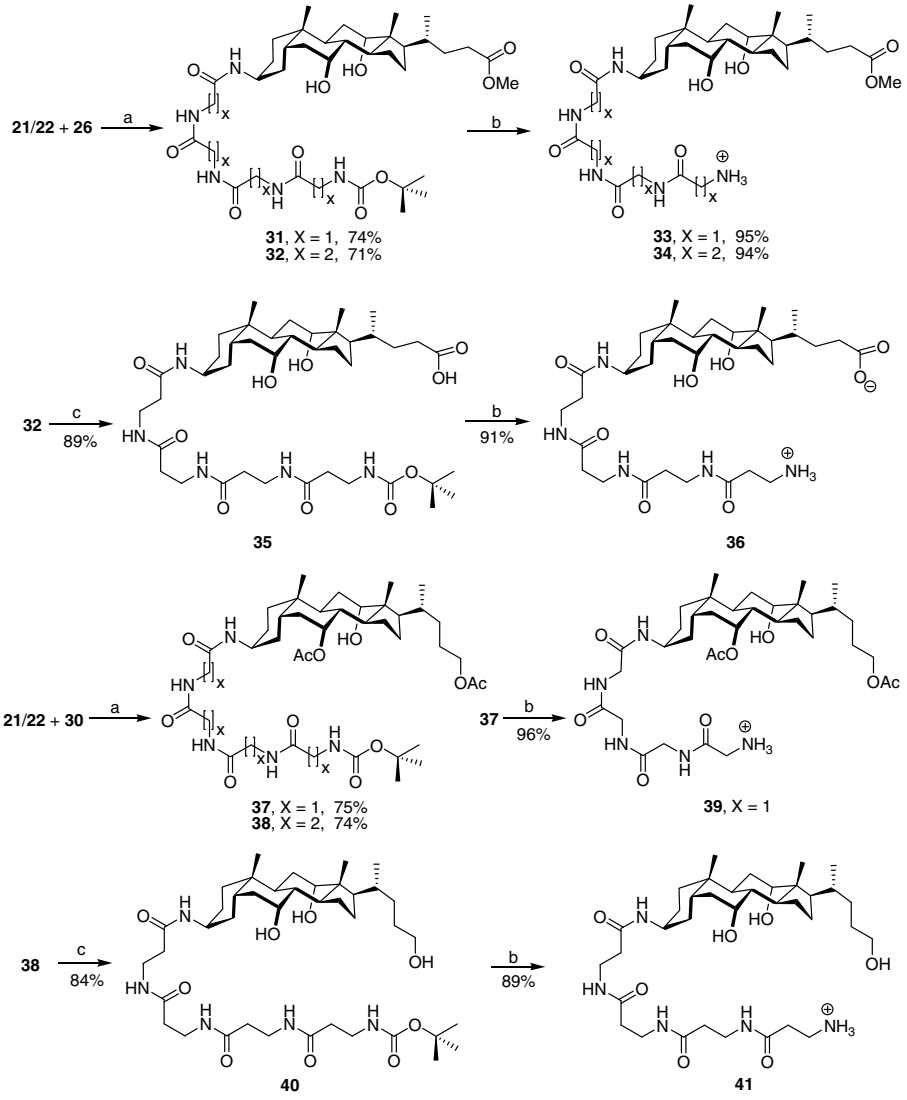
To characterize synergism of **19**–**25** and **31**–**41** with fluconazole (an antifungal agent) and erythromycin (a hydrophobic antibacterial agent), we determined the concentrations of these compounds necessary to lower the MIC values of the antibiotics to 1 μg/mL (a concentration at which many clinically useful antibiotics are active).[12b] This measurement entailed incubating a known population of yeast suspension (*C. albicans*) for 48 h in YPG and bacterial cells (*E. coli*) for 24 h in a nutrient broth with fluconazole and erythromycin, respectively, with incrementally varied concentrations of the synthesized compounds as described by Savage et al.[12b] Tetrapeptides **19**–**24** and cholic acid **25** did not showed any appreciable synergism, on the other hand, almost all the cholic acid conjugates exhibited very good synergism. This suggests that the synergism effect shown by our novel compounds is due to their inherent amphiphilicity.

To quantify the synergistic behaviour of our compounds with fluconazole and erythromycin, fractional inhibition concentration (FIC) values were also calculated.[21] Synergism between antibiotics is indicated by FIC values of less than 0.5. In the present case all the synthesised compounds **31**–**41** displayed FIC values of less than 0.5 with both fluconazole and erythromycin and many of the FICs shown in Table 2 are comparable to those reported for PMB derivatives.[12a]

Antiproliferative activity: Bile acids are known to promote proliferation and metastasis of cells of cancer origin and inhibit the proliferation of cells of non-cancer origin.[22] They are also known to be extremely toxic at high doses, presumably damaging cell mem-



Scheme 2. Synthesis of steroid backbone. Reagents and conditions: (a) Boc anhydride, Et_3N, dioxane:water, 25 °C 6 h; (b) LAH, THF, 0–25 °C, 1 h; (c) Ac_2O, DMAP, Et_3N, DCM, 25 °C, 8 h; (d) TFA, DCM, 0–25 °C, 2 h.



Scheme 3. Syntheses of cholic acid-tetrapeptide conjugates. Reagents and conditions: (a) EDCI, HOBt, Et_3N, DMF, 0–25 °C, 6 h; (b) 2 M HCl:Et_2O, 0- 25 °C, 1.5 h; (c) 2M LiOH, MeOH, 25 °C, 12 h.

Table 1
Minimum inhibitory concentration (MIC) of modified steroids

Entry	Compound	Antimicrobial activity MIC (μg/mL)						
		Fungal strains					Bacterial strains	
		A	*B*	*C*	*D*	*E*	*F*	*G*
1	**25**	>128	>128	128	>128	>128	>128	>128
2	**31**	16	>128	64	>128	64	96	32
3	**32**	32	>128	16	96	64	>128	>128
4	**33**	32	>128	64	>128	128	>128	96
5	**34**	128	>128	24	>128	32	96	64
6	**35**	32	>128	48	>128	64	>128	>128
7	**36**	32	>128	24	>128	64	32	>128
8	**37**	64	>128	32	>128	64	64	>128
9	**38**	32	>128	64	96	>128	>128	32
10	**39**	64	>128	16	>128	32	>128	64
11	**40**	128	>128	>128	>128	64	>128	>128
12	**41**	64	>128	64	>128	>128	32	>128
13	AmpB	2	16	16	16	16	NT	NT
14	Fluconazole	32	32	32	64	8	NT	NT
15	Tetracycline	NT	NT	NT	NT	NT	8	16
16	Erythromycin	NT	NT	NT	NT	NT	64	32

A, *C. albicans*; B, *C. neoformans*; C, *B. poitrasii*; D, *Y. lipolytica*; E, *F. oxysporum*; F, *E. coli*; G, *S. aureus*; NT, Not Tested.

Table 2
MIC, Permeabilization and FIC Data for **19–25**, and **31–41** with *C. albicans* and *E. coli*

Entry	Compound	a (μg/ml)	a′ (μg/ml)	a″	b (μg/ml)	b′ (μg/ml)	b″
1	**19**	>128	128	1.03	>128	128	1.03
2	**20**	>128	128	1.03	>128	64	0.53
3	**21**	>128	64	0.53	>128	128	1.03
4	**22**	>128	64	0.53	>128	>128	1.03
5	**23**	128	>128	1.03	128	64	0.53
6	**24**	>128	128	1.03	>128	>128	1.03
7	**25**	>128	64	0.53	>128	64	0.52
8	**31**	16	8	0.53	96	24	0.265
9	**32**	32	6	0.22	>128	16	0.14
10	**33**	32	6	0.22	>128	12	0.11
11	**34**	128	3	0.055	96	8	0.099
12	**35**	32	12	0.41	>128	16	0.14
13	**36**	32	1	0.062	32	4	0.14
14	**37**	64	12	0.22	64	24	0.39
15	**38**	32	8	0.28	>128	10	0.093
16	**39**	64	6	0.125	>128	8	0.078
17	**40**	128	12	0.125	>128	20	0.172
18	**41**	64	6	0.125	32	8	0.265

a: MIC of the synthesized compounds against *C. albicans*.
a′: Concentration of the synthesized compounds required to lower the MIC of fluconazole from 32 to 1 μg/mL (a concentration at which many clinically useful antibiotics are active).
a″: FIC values with fluconazole.
b: MIC of the synthesized compounds against *E. coli*.
b′: Concentration of the synthesized compounds required to lower the MIC of erythromycin from 64 to 1 μg/mL.
b″: FIC values with erythromycin.

branes and mitochondrial membranes.[23] At low doses, bile acids stimulate the cell-signalling effects involving various pathways.[24] Hence we tested the cytotoxicity of the synthesized compounds in two different cell lines, one of cancer origin (human mammary carcinoma: MCF-7) and the other of non-cancer origin (human embryonic kidney: HEK293). Concentration of compounds needed to reduce the population growth of HEK293 and MCF-7 cells by 50% (IC_{50}) in vitro was evaluated using the MTT assay[25] (Table 3). Graphical representation of antiproliferative activities of all the compounds is included in the Supporting information.

Of all the cholic acid-tetrapeptide conjugates tested, none showed any substantial cytotoxicity up to 80 μM concentration. Incorporation of peptide residues on the cholic acid derivatives drastically reduced its cytotoxicity towards HEK293 cell line. Moreover, it was found that all the compounds except **33** and **40** enhanced the proliferation of MCF-7 cells and not HEK293 cells. This confirms the observation that cholic acid and its derivatives promote the proliferation of cells from cancerous origin and not normal cells. This study also confirm that the synthesized cholic acid-peptide conjugates are not toxic to the cell lines tested at concentrations up to 80 μM. While relatively inactive by themselves, these compounds interact synergistically with antibiotics such as fluconazole and erythromycin to inhibit growth of fungi and bacteria, respectively, at 1–24 μg/mL.

Table 3
Cytotoxicity of modified steroids

Entry	Compound	IC_{50} (μM)	
		HEK293	MCF-7
1	**25**	50	>1000
2	**31**	300	600
3	**32**	100	>1000
4	**33**	500	80
5	**34**	600	700
6	**35**	>1000	>1000
7	**36**	200	>1000
8	**37**	500	200
9	**38**	150	80
10	**39**	150	800
11	**40**	>1000	800
12	**41**	>1000	800

In conclusion, a generic structure wherein fine-tuning of the molecular amphiphilicity is possible, have been designed utilizing amphiphilic nature of cholic acid. We have observed that, incorporation of linear tetrapeptide fragment on the cholic acid skeleton not only reduced its toxicity to the tested cell lines but also demonstrated very good synergism effect with the known antibiotics. The synergism of the most active compounds **36** (1 and 4 μg/mL) and **41** (6 and 8 μg/mL) greatly improves the activity of fluconazole and erythromycin against *C. albicans* and *E. coli*, respectively. To conclude, we have demonstrated that molecular amphiphilicity is one of the important factor which accounts for the easy transport of the molecules through the membranes.

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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.2008.09.013.

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