Design, Synthesis, and Anti-bacterial Activity of Novel Deoxycholic Acid-Amino Alcohol Conjugates

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ABSTRACT

Novel deoxycholic acid-amino alcohol conjugates were synthesized, from conjugation of deoxycholic acid-NHS ester with amino alcohols. Various amino alcohols moieties were appended to the C24 position to yield deoxycholic acid-amino alcohol conjugates. All the synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR and mass-spectroscopy. The synthesized compounds were screened for antibacterial activity against gram positive and gram negative bacteria. The outcome illustrated that some of the novel deoxycholic acid-amino alcohol conjugates exhibited improved anti-bacterial activities. Amongst them, deoxycholic acid-amino alcohol conjugate containing (R)-2-aminocyclohexanol (**1**) demonstrated promising efficacy against both strains *S. aureus* ATCC 25923 (MIC 15 µg/mL) and *E. coli* ATCC 25922 (MIC 45 µg/mL) and was identified as a lead molecule. The potency was awfully depending on the structures of deoxycholic acid-amino alcohol conjugates.

Keywords: *Deoxycholic acid, Aminoalcohol, Antibacterial Agent, S. Aureus, E. coli*

INTRODUCTION

Bile acids and their derivatives have turned into more and more imperative in various fields such as coordination and supramolecular chemistry, materials chemistry medicine, pharmacology, nanotechnology.[1] Normally, major bile acids are conjugated with the amino acids glycine/taurine and produced by living organisms.[2] The appearances of bacterial resistance to antibiotics, a set of antimicrobial agents that has received considerable attention is membrane active cationic peptide antibiotics[3].

Key functions of bile acid comprise the regulation of lipid, glucose, cholesterol homeostasis solubilization, transport of lipids/fat and as well as enterohepatic circulation of bile acids.[4-7] Recently, literature report illustrated pharmacological applications of bile acid derivatives viz. antibacterial activity[8–12], antifungal[13-14], antitumor[15] or as drug carriers[16]. A common feature of bile acid-derived antimicrobials is their potential to exhibit a facially amphiphilic nature due to both a hydrophobic face and a hydrophilic face. Bile acids have been believed extremely helpful in the preparation of novel pharmaceutical drugs because of their intrinsic chemical and biological properties.[17-18] Structural indiscretion, the reactivity of bile acids and their assorted native biological activities make these scaffolds a fascinating and promising starting material for the synthesis of new compounds with precious biological activities.[19]

Many cholic acid-derived facial amphiphiles have been reported to get better the permeability of membranes including bacterial cell wall.[20] Bile acid–peptide conjugates are generally linked *via* amide bonds using classical methods of peptide chemistry.[21] There are multiple reports of the antibacterial activities of amides derived from cholic acid.[10, 22-24] However there is one report that bile acid amides derived from chiral amino alcohols are antimicrobials agents[25].

A number of novel antibacterial drugs have been produced in the last few years, because resistance to bacteria has become a global concern. In view of these, modern researchers are increasingly showing interest towards the synthesis of bile acid based drugs by addition of various modifications. To the best of our knowledge, there are limited reports on amino alcohol-deoxycholic acid conjugates as antibacterial agents. In the present communication, we have designed and synthesized number of deoxycholic acid-chiral amino alcohol conjugates to investigate role substituents present in deoxycholic acid-amino alcohol conjugates. Their antimicrobial activities in vitro were evaluated against Gram-positive (*Staphylococcus aureus* ATCC 25923) as well as Gram-negative (*Escherichia coli* ATCC 25922).

RESULTS AND DISCUSSION

Chemistry

We prepared deoxycholic acid-amino alcohol conjugates via the synthetic route depicted in **Scheme 1**. Activation of the carboxylic acid moiety of deoxycholic acid has been carried out with N-hydroxysuccinimide, EDCI in DMF to afford the N-succinimidyl esters **2** in excellent yield. Formation of the N-succinimidyl esters of deoxycholic was confirmed by appearance of singlet for 4H at 2.83 in ^{1}H NMR and two carbonyl peak in ^{13}C NMR at 169.1, 169.0. Subsequently the N-Hydroxysuccinimide ester of deoxycholic acid (NHS-DCA) was coupled with amino alcohol groups to yield deoxycholic acid-amino alcohol conjugates. Complete synthetic procedures and structural characterization are given in the experimental segment. Proton signals were observed between 3.80-0.70 attributes to steroidal moiety in synthesized compounds. In IR spectrum of compounds **1-7,** characteristic absorption at 2930–2920 cm^{-1} region were assigned to the ν (-CO-NH) and at 3380–3280 cm−1 region (**1-7**) assigned to the

characteristic ν(OH). In ^{13}C NMR of compounds **1–7** characteristic signals in range of 172-176 ppm attributed to amide (-CO-NH).

Biological evaluations

Deoxycholic acid-amino alcohol conjugates (**1**-**7**) were tested for antibacterial activity against Gram-positive (*Staphylococcus aureus* ATCC 25923) and Gram negative (*Escherichia coli* ATCC 25922) by employing turbidity measure and minimal zone of inhibition measure. The minimum inhibitory concentration (MIC) values of deoxycholic acid-amino alcohols conjugates are displayed in **Table 1.**

From the biological data (**Table 1**), it was observed that compound **1** (N-(3α-Hydroxy-5β-cholan-24-oyl)-(R)-2-aminocyclohexanol) showed comparatively better result against *S. aureus* with a MIC value of 15 µg/mL and against *E. coli* with a MIC value of 45µg/mL. Amusingly, amongst all evaluated compounds in this study, the derivative **3** (N-(3α-Hydroxy-5β-cholan-24-oyl)-(S)-(+)-1-Amino-2-propanol) was inactive against both bacterial strains evaluated with MIC ≥120 µg/mL.

Compound **7** (N-(3α-Hydroxy-5β-cholan-24-oyl)-(*R*)-(−)-2-Amino-1-phenylethanol), having a (*R*)-(−)-2-Amino-1-phenylethanol at C-24, showed more activity than compounds **2-6** against *S. aureus*. Whereas in case of *E. coli*, it was observed that compound **7** showed an adverse effect. The compounds having (R)-2-aminocyclohexanol and (1R, 2R)-(−)-trans-1-Amino-2-indanol moiety at C-24 (**1** and **4**) were the most active compounds against the Gram-negative bacteria. The compounds **2** and **7** were significantly less active against the Gram-negative strain nevertheless keeps the potential to inhibit the growth of the Gram-positive strain. This variation was, may be, due to the impotency of compounds **2** and **7** to pass through the

surface membrane of the Gram-negative bacteria. A little change in linker size and position of alcohol in amino alcohol-deoxycholic acid conjugates influence the activities of amino alcohol-DCA conjugates against both *S. aureus* and *E. coli* (**Table1**). Compounds **3**, **5** and **6** have lesser potency against both *S. aureus* and *E. coli*. The antibacterial activity was more manifest against Gram- positive bacteria than Gram-negative bacteria.

Minimal inhibitory concentration of synthesized compounds was also performed by diameter measurement of size of zone of inhibition in mm. The zone of inhibition results shown in **Table 2** illustrate that the majority of the tested compounds demonstrated variable inhibitory effects on the growth of the tested Gram-positive and Gram-negative bacterial strains. The antimicrobial screening of these compounds reveals that compound (**1**) exhibited a promising activity against *S. aureus*. The SAR analysis of these compounds reveals that type of chiral amino alcohol group in deoxycholic acid-amino alcohol conjugates influence, zone of inhibition to the greater extend. Compound **1** showed the maximum activity against *S. aureus* (zone of inhibition is about 16 mm) and *E. coli* (zone of inhibition is about 14 mm).

All the synthesized deoxycholic acid-amino alcohol conjugates were executed for MBC values against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. Minimum bacterial concentrations (MBCs) can be compared to concentrations of compounds required for complete inhibition of bacterial growth. The MBC values of compounds **1-7** were measured as described procedure in experimental section and are included in **Table 1**. Compound **1** and **4** exhibit low MBCs against both strains *S. aureus* and *E. coli*. However, compounds 2-3 and **5-7** appear to exhibit complete bacterial inhibition at higher concentrations. On the basis of these observations, many more deoxycholic acid-amino alcohol conjugates can be designed and synthesized from deoxycholic acid.

The structure–activity relationship (SAR) results demonstrate that preserving the hydroxyl groups and changing the side chain of the steroid nucleus, deoxycholic acid-amino alcohol based antimicrobial agents can be helpful in developing more antimicrobial agents. The high potencies of few derivatives described here together with the possibility of synthesizing a number of derivatives around 'deoxycholic acid-amino alcohol conjugates' scaffolds open up new opportunities for anti-bacterial agents.

CONCLUSIONS:

In conclusion, a number of novel deoxycholic acid-amino alcohol conjugates were designed and synthesized using *N*-succinimidyl esters of deoxycholic acid and amino alcohols in excellent yields and their antimicrobial activities were evaluated against Gram-positive (*S. aureus)* and Gram-negative bacteria (*E. coli*). Compounds **1** and **4** were demonstrated potent antimicrobial activity against both *E. coli* and *S. aureus*. The compound **1** showed very good antibacterial activity with MIC value of 45µg/mL against *E. coli* and 15µg/mL against *S. aureus*. The structure-activity relationship result established that antibacterial activities of the deoxycholic acid-amino alcohol conjugates were importantly reliant upon the substituents of amino alcohols and these associations point towards the further design and developments of new deoxycholic acid-amino alcohol conjugates with superior anti-bacterial activities.

CHEMICAL SYNTHESIS

GENERAL

All the reagents and chemicals were obtained from Sigma-Aldrich, Alfa Acer, and were used without further purification. Solvents were obtained from commercial sources, and were used without further purification unless otherwise noted. Reaction was monitored on Merck silica gel 60 F_{254} by TLC plate. Entire synthesized compounds were purified over column chromatography using silica gel 230–400 mesh of particle size. All melting points are uncorrected. IR (ATR) spectra were recorded on a PerkinElmer Spectrum Version 10.4.2 spectrophotometer. ^{1}H NMR spectra of compounds were recorded on 300 MHz Bruker NMR spectrometers using TMS as internal standard. The chemical shifts are expressed in ppm (δ) units and the coupling constants (*J*) are in Hz.

EXPERIMENTAL

Synthesis of N-Hydroxysuccinimide ester of Deoxycholic acid (NHS-DCA): A mixture of deoxycholic acid (1.0 g; 2.54 mmol), N -hydroxysuccinimide (307 mg; 2.67 mmol), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 725 mg, 3.78 mmol, 1.5 eq) in *N*,*N*-dimethylformamide (5 ml) was stirred at room temperature under N_2 conditions for 18h. The reaction mixture was diluted with 20 mL ethyl acetate and washed with water (10 mL) and with saturated brine solution (2×10 mL) and dried over anhydrous $MgSO_4$. Organic phase was concentrated under reduced pressure. Crude cholic acid *N*-succinimidyl ester (solid mass) was purified by column chromatography on silica gel, (eluent: 4:6 hexane/ethyl acetate) to provide pure cholic acid *N*-succinimidyl ester. mp190–193 ˚C, ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*: 4.24 (br s, 1H), 3.98 (m, 1H), 3.57-3.64 (m, 1H), 3.46 (br s, 1H), 2.83 (s, 4H), 2.52-2.70 (m, 2H), 1.14-1.94 (m, 24H), 1.00-1.10 (d, 3H, 21- H, J=4.2 Hz), 0.91 (s, 3H, 19-H), 0.68 (s, 3H, 18-H), ^{13}C

NMR ($CDCl_3$, 100 MHz) *δ*: 169.1, 169.0, 156.9, 73.1, 71.7, 49.1, 48.2, 47.0, 46.5, 42.0, 36.3, 36.0, 34.8, 34.0, 33.8, 33.6, 30.8, 30.4, 28.6, 27.9, 27.4, 26.0, 25.5, 24.8, 23.6, 23.1, 17.2, 12.6.

General procedure for the synthesis of bile acid-amino alcohol conjugates: N -Hydroxysuccinimide ester of Deoxycholic acid (1 mM) was dissolved in 5 mL dry DMF with stirring; the solution of amino alcohol (1.0 mmol, dissolved in 2 mL dry DMF) was, then, added dropwise. Progress of this reaction was monitored by thin layer chromatography. After being stirred at room temperature overnight, the reaction mixture was concentrated under reduced pressure, the remaining residue was, then, purified by chromatography on silica gel (eluent: Hexane/Ethyl acetate 4:6) to afford desired compounds.

N-(3α-Hydroxy-5β-cholan-24-oyl)-(R)-2-aminocyclohexanol (1): Yield (80%); m.p.128˚C; ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*: 4.01-1.0(m, 1H), 3.73-3.64 (m, 2H), 2.85-2.74 (m, 1H), 1.89-0.98 (m, 31H), 0.93(s, 3H), 0.71(s, 3H), 0.70(s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 172.2, 73.4, 71.9, 49.5, 48.2, 47.3, 47.2, 46.5, 36.2, 36.0, 35.2, 34.1, 33.7, 33.5, 31.1, 30.9, 30.7, 30.2, 30.1, 29.7, 28.9, 27.5, 27.4, 27.1, 26.1, 25.5, 25.4, 23.7, 23.0, 17.2, 12.7. FT-IR (ATR): 3328, 2925, 1710, 1451, 1372, 1210, 1043, 648 cm^{-1} ESI-MS $[M+Na]^+$ calcd for $C_{30}H_{51}NO_4Na$, 512.371; found, 512.525.

N-(3α-Hydroxy-5β-cholan-24-oyl)- (*R*)-(−)-2-Amino-1-butanol (2): Yield (70%); m.p.168˚C; ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*: 4.0(m, 1H), 3.73-3.71(m, 1H), 3.68-3.58(m, 3H), 2.36-0.70 (m, 41H) ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 176.1, 140.7, 139.0,128.6, 127.2, 125.3, 122.7, 81.8, 73.2, 71.8, 64.2, 48.3, 46.9, 46.5, 42.0, 38.5, 36.6, 36.0, 35.2, 35.1, 34.1, 33.6, 33.0, 31.6, 30.7, 30.4,

29.6, 28.6, 27.5, 27.0, 26.1, 23.6, 23.1, 17.4, 12.7. FT-IR (ATR): 3281, 2926, 1612, 1366, 1036, 656 cm^{-1}; ESI-MS $[M+H]^+$ calcd for $C_{28}H_{50}NO_4$, 464.366; found, 464.578; $[M+Na]^+$ calcd for $C_{28}H_{49}NO_4Na$ 486.355, found, 486.564.

N-(3α-Hydroxy-5β-cholan-24-oyl)-(*S*)-(+)-1-Amino-2-propanol (3): Yield (85%); m.p.110˚C; ^1H NMR ($CDCl_3$, 400 MHz) *δ*: 5.99 (d, 1H, J = 8.0 Hz), 4.59 (q, 1H), 4.0 (t, 1H, J = 4.0 Hz), 3.75 (s, 2H), 3.65-3.60 (m, 1H), 2.37-2.27 (m, 1H), 2.21-2.19 (m, 2H), 2.17-0.85(m, 23H), 0.96(s, 3H), 0.87(s, 3H); 0.69(s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*:174.3, 73.1, 71.8, 47.0, 42.0, 36.4, 36.0, 35.1, 34.1, 33.3, 31.9, 31.5,31.4, 30.5, 29.6, 28.6, 27.4, 27.41, 26.1, 23.6, 23.1, 22.6, 20.9, 17.4, 12.5. FT-IR (ATR): 3389, 2924, 1633, 1451, 1367, 1045, 598 cm-1. ESI-MS $[M+H]^+$ calcd for $C_{27}H_{47}NO_4$, 450.358; found, $[M+Na]^+$ calcd for $C_{27}H_{47}NO_4Na$, 472.340; found 450.554.

N-(3α-Hydroxy-5β-cholan-24-oyl)-(1*R*,2*R*)-(−)-*trans*-1-Amino-2-indanol (4): Yield (55%); m.p.185˚C; ^1H NMR ($CDCl_3$, 400 MHz) *δ*:7.30 (m, 5H), 6.01 (d, 1H, J = 4.0 Hz), 5.14 (q, 1H), 4.44-4.39 (m, 1H), 4.01 (d, 1H, J = 4.0 Hz), 3.75 (s, 2H), 3.67-3.51 (m, 1H),3.34 (q, 1H), 2.41-0.85 (m, 24H), 2 0.91(s, 3H), 0.71(s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 176.1, 140.7, 139.0,128.6, 127.2, 125.3, 122.7, 81.8, 73.2, 71.8, 64.2, 48.3, 46.9, 46.5, 42.0, 38.5, 36.6, 36.0, 35.2, 35.1, 34.1, 33.6, 33.0, 31.6, 30.7, 30.4, 29.6, 28.6, 27.5, 27.0, 26.1, 23.6, 23.1, 17.4, 12.7. ESI-MS $[M+H]^+$ calcd for $C_{33}H_{50}NO_4$, 524.373; found, 524.618; $[M+Na]^+$ calcd for $C_{33}H_{49}NO_4Na$, 546.355; found 546.623.

N-(3α-Hydroxy-5β-cholan-24-oyl)- *(S)*-(+)-2-Phenylglycinol (5): Yield (80%); m.p.196°C; ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*: 7.41-7.28 (m, 5H), 6.19 (d, 1H), 5.10 (d, 1H), 3.99 (s, 1H), 3.91 (d, 2H), 3.66-3.61 (m, 2H), 2.38-2.20 (m, 3H), 1.89-0.90 (m, 23H), 0.93(s, 3H), 0.86(s, 3H), 0.69(s, 3H); ^{13}C NMR ($CDCl_3$100 MHz) *δ*:173.9, 128.9, 127.9, 126.7, 73.1, 71.8, 66.8, 55.9, 48.2, 46.9, 46.5, 42.0, 36.4, 36.0, 35.2, 35.1, 34.1, 33.6, 33.3, 31.4, 30.4, 28.6, 27.4, 27.1, 26.1, 23.6, 23.1, 17.4, 12.7. FT-IR (ATR): 3292, 2928, 1371, 1041, 697 cm^{-1}. ESI-MS $[M+H]^+$ calcd for $C_{32}H_{50}NO_4$, 512.373; found, 512.617; $[M+Na]^+$ calcd for $C_{32}H_{49}NO_4Na$ 534.355 found 534.606.

N-(3α-Hydroxy-5β-cholan-24-oyl)-(*S*)-(−)-2-Amino-3-phenyl-1-propanol (6): Yield (75%); m.p.198°C; ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*: 7.36-7.23 (m, 5H), 5.90 (t, 1H, J= 8.0 Hz), 4.15 (s, 1H), 3.84-3.38 (m, 5H), 2.29-0.87 (m, 32H), 0.99(s, 3H), 0.86(s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 174.2, 128.5, 127.8, 125.8, 73.8, 73.1, 71.8, 48.3, 47.5, 47.0, 46.4, 42.0, 36.4, 36.0, 35.1, 34.1, 33.6, 35.1, 34.1, 33.6, 33.2, 31.5, 30.4, 29.6, 28.6, 27.4, 27.1, 26.1, 23.6, 23.1, 17.4, 12.7. FT-IR (ATR): 3292, 2928, 1371, 1041, 697 cm^{-1}. ESI-MS $[M+H]^+$ calcd for $C_{33}H_{52}NO_4$, 526.389; found, 526.618; $C_{33}H_{51}NO_4Na$, 548.371; found, 548.621.

N-(3α-Hydroxy-5β-cholan-24-oyl)- (*R*)-(−)-2-Amino-1-phenylethanol (7): Yield (80%); m.p.216°C; ^{1}H NMR ($CDCl_3$, 400 MHz) *δ*:7.30-7.19 (m, 5H), 5.70 (d, 1H), 4.18-4.15 (m. 1H), 3.97-3.68 (m, 4H), 2.88-2.84 (m, 2H), 2.16-2.0 (m, 4H), 1.88-1.10 (m, 21 H), 0.98 (d, 3H, J = 4.0 Hz), 0.90 (s, 3H), 0.66 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 174.2, 129.1, 128.6, 126.7, 73.1, 71.8, 64.5, 52.8, 48.2, 49.8, 42.0, 37.0, 36.4, 36.0, 35.2, 35.0, 33.6, 33.4, 31.5, 30.4, 28.6,

27.4, 27.1, 26.1, 23.6, 23.1, 17.4, 12.7, ESI-MS $[M+H]^+$ calcd for $C_{32}H_{50}NO_4$, 512.373; found, 512.615 $[M+Na]^+$ calcd for $C_{32}H_{50}NO_4Na$, 534.355 found, 534.609.

Determination of minimum inhibitory concentration (MIC):

The original solutions of the deoxycholic acid-amino alcohol conjugates were prepared using dimethyl sulfoxide (DMSO). From this concentration, a number of dilutions of each compound were made in DMSO to establish minimum inhibitory concentration (MIC). Mueller–Hinton broth (M391) was prepared and 9.9 mL of it was taken in each test tube and was sterilized after plugging. After cooling, 0.1 mL of each dilution was added to the test tubes and the final volume was made up to 10.0 mL. To each of test a tube 0.1 mL of bacterial culture broth (must contain 106 organism/ml) was added. The test tubes were shaken to homogeneously mix the inoculum with the broth. The tubes were incubated at 37˚C for 24 h. Appearance of any turbidity shows that the compound is not able to inhibit the growth of the bacteria, while no turbidity indicates the inhibition of microorganism by the sample.

Minimal Bactericidal Concentration (MBC):

MBC was recorded as the highest dilution showing of deoxycholic acid-amino alcohol conjugates and antibiotics that inhibit 99% of the bacterial inocula after 24 h incubation at 37°C. Each experiment was repeated at least three times. MBC values were determined by taking 100 µL of bacterial suspension from culture which showed no visible growth on MHA plates. Plates were, then, incubated at 37°C for period of 24 h.

To determine zone of inhibition by Kirby–Bauer's method: The antibacterial susceptibility test was done by determining zone of inhibition by Kirby–Bauer's method. Different concentration of the deoxycholic acid-amino alcohol conjugates were adsorbed on sterile filter discs. Mueller–Hinton agar was prepared and allowed to solidify. One of these discs was kept free from antibiotic and served as growth control. One mL of each bacterial culture broth were added in the Mueller-Hinton plates and spread with Help of sterile spreader. The filter paper discs adsorbed with inhibitors will be placed over the inoculated plates using sterile forceps. The plate will be incubated at 37 ˚C for 18 h, in upright position. The zone of inhibition was measure using scale.

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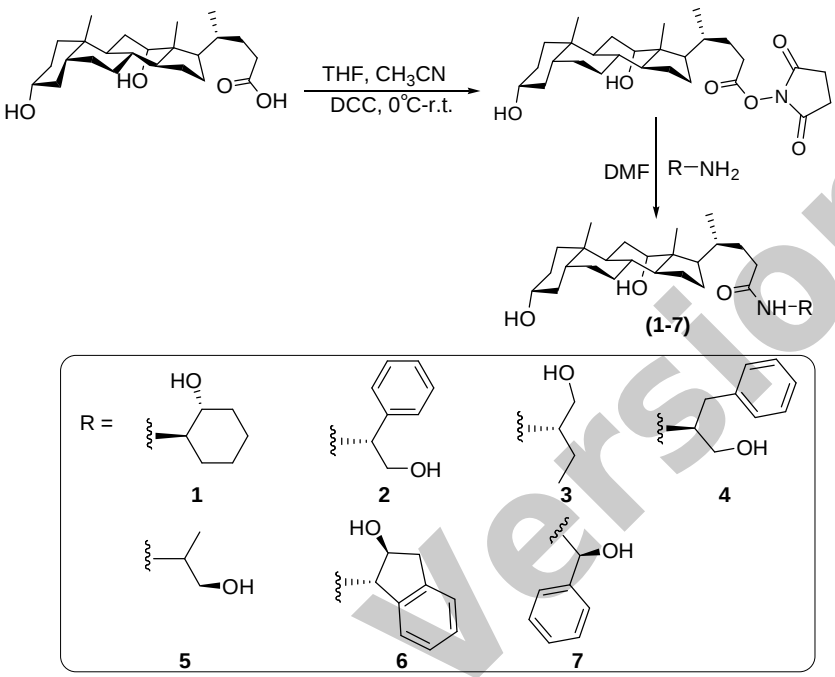


Scheme 1: Synthesis of Deoxycholic acid (DAC)-amino alcohol conjugates

Table 1: Minimum inhibitory concentration (MIC) of deoxycholic acid-amino alcohol conjugates against Gram positive (*Staphylococcus aureus* ATCC 25923) and Gram negative bacterial (*Escherichia coli* ATCC 25922)

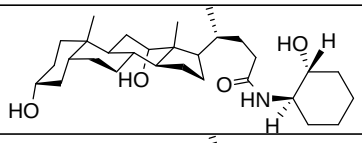
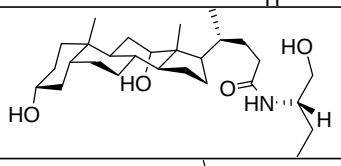
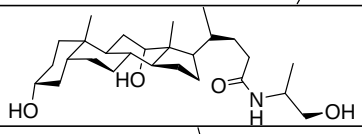
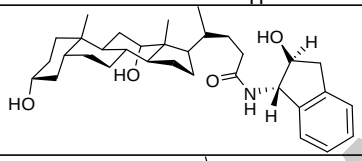
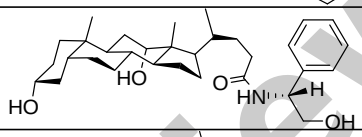
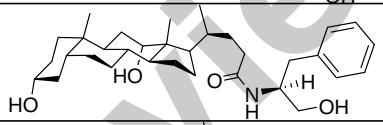
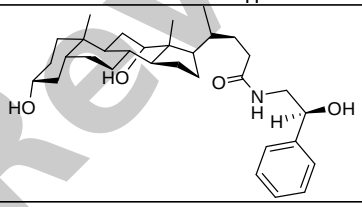
Entry	Structure	MIC (µg/mL)		MBC (µg/mL)	
		S. aureus	*E. Coli*	*S. aureus*	*E. Coli*
1		15	45	60	90
2		60	120	180	>200
3		>120	120	>200	>200
4		30	60	90	120
5		120	>120	>200	>200
6		>180	60	>200	>200
7		45	120	200	>200
8	Ampicilin	15	4	-	-

Table 2: Antibacterial activity of deoxycholic acid-amino alcohols conjugates (digit show size of zone of inhibition in mm) of different dilutions of conjugates (120, 60, 45, 30, 15 µg/mL) against *S. aureus* and *E. coli*

Entry	Zone of inhibition (mm)	
	S. aureus	*E. Coli*
1	16, 14, 13, 12, 10	14, 13, 11, -, -
2	14, 13, -, -, -	12-, -, -, -
3	-	10-, -, -, -
4	15, 14, 12, 11, -	13, 12, -, -, -
5	12, -, -, -, -	-
6	-	13, 12, -, -, -
7	14, 13, 12, -, -	10 -, -, -, -

(-) resistant