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The Design, Synthesis, and Characterizations of Spore Germination Inhibitors Effective against an Epidemic Strain of *Clostridium difficile*

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Abstract

Clostridium difficile infections (CDI), particularly those caused by the BI/NAP1/027 epidemic strains, are challenging to treat. One method to address this disease is to prevent the development of CDI by inhibiting the germination of *C. difficile* spores. Previous studies have identified cholic amide m-sulfonic acid, CamSA, as an inhibitor of spore germination. However, CamSA is inactive against the hypervirulent strain R20291. To circumvent this problem, a series of cholic acid amides were synthesized and tested against R20291. The best compound in the series was the simple phenyl amide analogue which possessed an IC₅₀ value of 1.8 μ M, more than 225 times as potent as the natural germination inhibitor, chenodeoxycholate. This is the most potent inhibitor of *C. difficile* spore germination described to date. QSAR and molecular modeling analysis demonstrated that increases in hydrophobicity and decreases in partial charge or polar surface area were correlated with increases in potency.

Graphical Abstract

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Author Contributions

S.K.S and C.Y. contributed equally to this manuscript

Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

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Molecular formula strings (CSV)

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INTRODUCTION

Clostridium difficile infection (CDI) is primarily a nosocomial disease correlated mainly with antibiotic-associated diarrhea. These infections are caused by *Clostridium difficile*, an obligate anaerobic, Gram-positive, spore forming bacterium that is found in the gastrointestinal tract and can be transmitted between humans via the fecal–oral route.¹ In the United States alone, there are roughly 500000 cases of CDI annually with associated costs estimated to be approximately \$3 billion.^{2–4}

CDI begins with the ingestion of the *C. difficile* spores.¹ These spores are highly resistant to harsh environmental factors such as stomach acid, extreme temperatures, and pharmaceutically relevant antibiotics.⁵ As the spores travel through the gastrointestinal tract, various endogenous bile salts stimulate the spores to germinate into vegetative, toxin^{1,5–10}, producing *C. difficile* cells. In most cases, the vegetative *C. difficile* cells are able to produce two exotoxins, TcdA and TcdB, that damage the intestinal walls, leading to pseudomembranous colitis and, in severe cases, death.^{11–13}

CDIs are particularly challenging to treat. Approximately 25% of patients treated for CDI will relapse within 30 days, and the majority of these patients (45–65%) will have additional recurrences.^{14,15} Patients experiencing CDI relapse have a 30% higher mortality rate than those successfully treated.¹⁶ In addition, there has been an increase in outbreaks of severe disease which have been attributed to so-called hypervirulent strains, specifically those of the BI/NAP1/027 designation.¹⁷ Epidemiological studies have shown that infections by these strains are associated with higher mortality and now account for approximately 20–75% of all CDI.^{17,18} While many theories have been postulated, the exact reason for the increased virulence in these strains is still not fully understood.¹⁹

Treatments for CDI usually require a rigorous regimen, which normally combines decontamination of the local environment along with antibiotic therapy.⁴ However, due to *C. difficile*'s ability to form spores, complete decontamination is often difficult.^{1,5–10} CDI is treated predominately by three antimicrobial agents: metronidazole, oral vancomycin, and fidaxomicin (Figure 1).⁴ Metronidazole is considered the first line therapy for mild to moderate CDI; however, several clinically relevant *C. difficile* strains have begun to show resistance to metronidazole.²⁰ Several studies have suggested that vancomycin may be superior to the azole given its pharmacokinetic parameters and absence of any reported resistance.^{4,20} Fidaxomicin was approved in 2011 as a newer anti-CDI treatment, but its usage has been limited by drug costs.²¹ Finally, fecal transplantation for CDI relapse has been shown to be an effective, nonantibiotic therapy, although its use is limited in the US.^{22,23}

While there are a number of risk factors associated with CDI, current or recent antibiotic use is strongly correlated with the disease.¹⁶ Under normal circumstances, bacteria found naturally in the gastrointestinal tract provide a barrier against *C. difficile* colonization.^{1,5–10} Upon exposure to antibiotics, the normal gut microbiome becomes disrupted. This perturbation leads to elevated levels of taurocholate (Figure 2), a bile salt produced naturally in the gastrointestinal tract.^{6–8,24,25} In conjunction with free amino acids, taurocholate triggers the germination of *C. difficile* spores and ultimately the colonization of the human gastrointestinal tract by *C. difficile*.^{7,25} Chenodeoxycholate (CDCA) (Figure 2), another bile salt found naturally in the gastrointestinal tract, has been shown to inhibit the germination of *C. difficile* spores.^{7–9} It is thought that the balance between chenodeoxycholate and taurocholate is responsible for the control of spore germination^{1,5–10,24,25}.

Current treatments for CDI, as well as those being clinically investigated, are antibiotics that target the vegetative form of *C. difficile*. However, one potential approach to combat CDI would be to prophylactically treat high-risk patients to prevent the onset of disease.²⁶

CDI prophylaxis may be achieved by potentially inhibiting *C. difficile* spore germination. We envisioned that inhibiting the binding of taurocholate with its putative receptor should prevent the germination of *C. difficile* spores. In a previous study, one of our laboratories explored more than a dozen bile salt analogues with modifications in the side chain and/or in the cholate scaffold.²⁶ From this, cholic amide meta-sulfonic acid (CamSA), a taurocholate analogue (Figure 2), was identified which inhibited spore germination at concentrations almost four times lower than the natural inhibitor, chenodeoxycholate, in vitro.^{8,27} CamSA was subsequently shown to prevent CDI in mice by inhibiting spore germination in vivo.^{28,29} The use of modified bile salts as inhibitors of spore germination has been utilized by others, and in at least one case, it was shown that a bile salt could also be used to treat *C. difficile* ileal pouchitis in a human.^{30,86}

Although CamSA has many attractive properties, it has an IC₅₀ value in the micromolar range against *C. difficile* strain 630 spore germination. Interestingly, CamSA was unable to inhibit spore germination of R20291, a BI/NAP1/027 epidemic strain of *C. difficile* (see Table 1). Thus, we wanted to explore new compounds with activity against the epidemic *C. difficile* spores. Our approach examines modifications of the amide region of CamSA by exploring a variety of aromatic and aliphatic replacements. Here, we report the synthesis of CamSA analogues, the analysis of these agents as inhibitors of *C. difficile* spore germination and QSAR studies on these agents to identify important parameters needed for activity. This study has identified analogues that are up to 225 times more potent than chenodeoxycholate, the natural germination inhibitor, against R20291. Several analogues reported in this study account for some of the most potent antigerminants described to date so far against *C. difficile*.

RESULTS

Compound Design.

Previous studies have postulated that CspC is the receptor responsible for triggering spore germination, and thus we hypothesized that CspC is the likely site of action for CamSA.^{31,32}

Given this, we examined the sequences of CspC, from R20291, an epidemic strain that is not inhibited by CamSA and 630, a nonepidemic strain that is inhibited by CamSA with an IC₅₀ of 58 μ M. To our surprise, we found that the sequences were 99.3% conserved between both strains. An examination of the sequence differences revealed that none occurred at residues previously identified as being critical for germination activity.³¹ In addition, CspC has not been crystallized, and thus, structure-guided drug design is not currently possible. Given this, we decided to embark on an empiric investigation of a series of cholic acid amides to find agents with activity against R20291. We focused our study on amides comprised of aromatic, aliphatic, and benzylic groups.

Synthesis of the Bile Salt Analogues.

The synthesis is outlined in Scheme 1. Cholic acid (**7**) was preactivated with HBTU/NMM in DMF at room temperature and was subsequently treated in situ with a variety of substituted or unsubstituted aniline analogues (**8**) that produced Narylcholan-24-amides (**12a–n**) in 74–94% yields.^{33–35} The isolation and purification of the products (**12a–j**) were carried out by stirring each concentrated postreaction mixture with ice-cold 2–5% aqueous HCl. For compounds which contained acid labile functionalities, such as the ester or Boc group (**12k–n**), crude mixtures were precipitated with cold water and the products were further purified, if necessary, by column chromatography. The reaction between cholic acid and 4aminophenol proceeded smoothly and produced *N*-(4hydroxyphenyl) cholan-24-amide **12f**, while the same compound was not obtained via demethylation of methoxy groups (**12d**) using BBr₃/CH₂Cl₂. The generation of amine substituted products were done via deprotection of tertbutoxycarbonyl (Boc) groups of **12m** and **12n** with 1 M HCl in ethyl acetate at room temperature to produce the corresponding chloride salts (**12o** and **12p**).^{36,37} The directly linked heterocycles, pyridine and thiazole substituted cholan-24-amides (**12q** and **13**) were prepared from the activated ester of cholic acid **7** with 4-aminopyridine **8** and 2-amino-4-methylthiazole **9** in 70% and 76% yields, respectively.

The synthesis of benzyl and aliphatic analogues is also shown in Scheme 1. The *N*-benzyl and *N*-pyridinomethyl analogues of cholan-24-amides (**14a–g** and **14j–k**) were synthesized in 64–90% yields by treating the HBTU/NMM activated ester of cholic acid (**7**) with the corresponding benzylamines or pyridinomethylamines (**10**). The nitro group of compounds **14f** and **14g** was selectively reduced to give the desired amino functionality (**14h** and **14i**) in 85% and 80% yield, respectively, by high pressure hydrogenation in the presence of 10% Pd/C in MeOH.^{38,39} Aliphatic substituted cholan-24-amides (**15a–e**) and mixed arylalkyl-substituted cholan-24-amides (**15f–h**) were synthesized by the same coupling method and generated the desired products in 61–92% yields. The terminal functional groups (alkyne, cyano-, hydroxyl-, ester-, and boc-amino-) containing alkyl substituted cholan-24-amides (**15i–n**, **15p**, and **15r–s**) were synthesized similarly in good yields. The methyl ester group in compound **15n** was selectively cleaved with an aqueous THF LiOH solution to give the free acid **15o** in 76% yield.^{40–42} The benzyl groups of **15p** were selectively removed by Pd/C catalyzed hydrogenation in MeOH to generate the diacid **15q** in 74% yield.^{43,44} The Boc groups in compound **15r** and **15s** were selectively cleaved by 1 M HCl in ethyl acetate to yield the chloride salts of cholan-24-amides **15t** and **15u** in 86% and 89% yields, respectively.^{36,37} The terminally substituted heterocyclic (pyrrolidine, thiophene,

dioxoisindolin, and dioxindolin) alkyl cholan-24-amides (**15v–15z**) were prepared from the activated ester of cholic acid **7** with the appropriate amine in 65–84% yields.

Biological Activity.

All compounds were analyzed as inhibitors of spore germination using a standard optical density assay which measured germination as a decrease in absorbance at 580 nm.^{26–29} A two-step process was taken for the analysis of the biological activity of the compounds. Compounds were analyzed for their ability to inhibit spore germination of *C. difficile* R20291 at a single concentration of 125 μ M. Compounds were assessed for solubility at this concentration by visual inspection and those which were not soluble were discarded. Compounds that were able to slow spore germination >40% compared to untreated samples were then reanalyzed at different concentrations to determine their IC₅₀ values. CamSA was shown to be inactive against strain R20291 under these conditions (Table 1).

The biological activities of the compounds are shown in Tables 1–3. Not surprisingly, because CamSA is aromatic, the majority of active compounds were obtained with aromatic cholan-24-amides. The most potent compound was 12a, which is simply the unsubstituted aniline derivative (Table 1, Figure 3). Compound 12a possessed an IC₅₀ of 1.8 μ M in the spore germination assay conducted in sodium phosphate media containing 6 mM taurocholate and 12 mM glycine (Figure 3A,B). Examination of the inhibition of spore germination by 12a in the complex BHIS media containing 6 mM taurocholate gave an IC₅₀ value (1.8 μ M) identical to that in sodium phosphate buffer (Figure 3C,D). Given this, all spore germination assays were conducted in sodium phosphate buffer.

We examined the effects of electron donating or electron withdrawing substituents on the aromatic ring on the inhibitory activity of *C. difficile* spore germination via compounds 12b–p (Scheme 1). None of the substituents that we examined improved potency beyond 12a, although electron donating substituents generally gave more potent compounds than electron withdrawing substituents 12b (IC₅₀ of 9.8 μ M) compared to 12g (IC₅₀ of 16 μ M). Substitution at the ortho position yielded the most potent substituted aromatic compound (12b) with substitution on the para position being the weakest (12d). Addition of an electron donating group with H-bonding potential (e.g., hydroxyl) on the para position regained some activity (12f, IC₅₀ of 36 μ M), although the presence of an amino group at this position was inactive (12p). The data suggests that sterically larger groups on the meta- and para-positions of the aromatic ring are not tolerated for activity (12d vs 12f vs 12p).

We examined a limited number of disubstituted derivatives (12e, 12h, and 12i). Unlike 12b, the dimethoxy compound 12e was inactive, possibly due to the large group on the paraposition. Compounds 12h and 12i have both electron donating and electron withdrawing groups. These compounds are more potent (12h IC₅₀ of 24 μ M, and 12i IC₅₀ of 14 μ M) than 12e but essentially as potent as the meta-substituted fluorine compound 12g (Table 1). This suggests that the fluorine group plays a dominate role in the activity of these agents.

We examined two aromatic heterocycles (12q and 13) and found that only 12q, which has a pyridine substituent with the aromatic nitrogen at position 4 in the aromatic ring, was active. This compound is able to inhibit *C. difficile* spore germination with IC₅₀ at 30.1 μ M, which

is 14-fold less active than 12a (Table 1). The aromatic nitrogen in compound 12q causes the aromatic group to be electron deficient, and 12q displays an IC_{50} in a similar range as many of the electron deficient aromatic substitutions (i.e., 12g, 12h, etc.). Like 12f and 12p, the presence of a hydrogen bond acceptor at this location does not enhance activity.

We also examined aliphatic and benzylic substitutions on the amide nitrogen (Tables 2 and 3). All benzylic and pyridinomethyl analogues 14a–k were inactive, although we obtained an IC_{50} value for 14f to be used in our QSAR studies discussed below. All cyclic aliphatic compounds 15a–c displayed moderate activity although not as good as for 12a. The cyclohexyl analogue 15a inhibited spore germination with an IC_{50} of 95.4 μ M (53-fold higher than 12a). Decreasing the size of the ring increased activity as seen in compounds 15b (cyclopentyl), and 15c (cyclopropyl), which displayed IC_{50} values of 34.1 and 25 μ M, respectively. Placement of a methylene group between the amide bond and cyclopropyl ring (15d) did not alter the inhibition; however, branching the aliphatic group decreased potency (15e IC_{50} at 154 μ M). This suggested that there is a size constraint for the binding site of the amide nitrogen substituent. An examination of straight or branched chain containing alkyl amides with a variety of terminal functional groups such as aryl-, alkyne-, cyano-, hydroxyl-, esters-, acids-, amino-, and heterocyclic moieties (15f–z) generated inactive compounds with the exception of 15i (IC_{50} at 137 μ M), which was only modestly active.

QSAR and Molecular Modeling.

The analysis of the activity of the compounds failed to generate a clear SAR. There are a number of reasons for this including the fact that a number of compounds were insoluble and the assay is a phenotypic assay that included many factors besides strictly binding to the target receptor. Thus, we decided to examine the data by structural alignment of active versus inactive compounds followed by quantitative structure–activity relationship (QSAR) studies to determine whether activity could be predicted based upon a set of molecular descriptors.

Compounds with IC_{50} values 36 μ M or below (12a, 12b, 12f, 12g, 12h, 12i, 15b, 15c, 15d) were aligned along with a selected number of inactive or weakly active compounds (12c, 12d, 12e, 12j, 12k, 12l, 14a, 14f, 15a, 15f) using the flexible alignment protocol (using the MMF94 $\times$ molecular force field and atomic partial charges with Born solvation⁴⁵) executed in the Molecular Operating Environment (MOE, version 2016.0802, Chemical Computing Group) program. The molecular surface for each class was also calculated. The alignments are shown in Figure 4. All compounds, both active and inactive, aligned completely with the cholic acid scaffold. The only differences, as expected, were noted in the amide region of the molecule.

A comparison of the active versus inactive molecules reveals that the active agents occupy a smaller volume than the inactive species. All active agents fit within the molecular volume for the phenyl ring. Substituents on the ortho-position are tolerated, but not in the meta- or para-positions. The molecular surface of the inactive agents is larger, suggesting that the binding site for the agents is confined. An examination of the lipophilicity of the active versus inactive agents reveals that the active agents are more lipophilic (darker green color) than the inactive agents. Taken together, this analysis indicates that the size as well as

lipophilicity of the amide substituent plays a role in the activity of the agents as spore germination inhibitors.

To quantitate the effect of properties on activity, we conducted a QSAR study on the 16 compounds with which we have IC₅₀ values. Unfortunately, this low number of compounds precluded the use of a training and test set of data and thus validation was done using a leave-one-out calculation. The 343 2D and 3D molecular descriptors were calculated for each compound using MOE.⁴⁶ The IC₅₀ values were converted to negative-log10 values and the molecular descriptor values were normalized (mean-centered and divided by the descriptor's standard deviation). A set of models containing 2–7 descriptors were generated by application of a genetic function algorithm⁴⁷ via the Opdagelse Predictive Modeling Toolkit (version 1.1.0)⁴⁸ as executed in R.⁴⁹ Models for each number of descriptors were ranked by Q² values, and the top 20 models in each set were selected. Each model was analyzed by a series of statistical methods to generate R²_{adj}, Q², F-test, mean absolute error (MAE), p-value as well as variance inflation factors (VIF), and p-values for each variable in the equations.

We focused on models with four descriptors because these models are close to the 5–7 compounds per descriptor rule of thumb frequently cited for QSAR studies and have substantially better R²_{adj} (average 0.706 vs 0.865) and Q² (average 0.648 vs 0.824) values than the three-descriptor models.⁵⁰ Furthermore, there is no evidence of overfitting of these models based on VIF and p-values of the terms.

Five distinct, four-descriptor models along with their statistics are given in Table 4. These models were used to generate a consensus model in which the predicted pIC₅₀ values are the mean predicted value of each QSAR model. The consensus model gave an R² of 0.98, R²_{adj} of 0.973, and Q² of 0.927 (Figure 5). The MAE and RMSE of the consensus model was 0.081 and 0.1, respectively. Taken together, the consensus model accurately predicts the potency of the molecules against the R20291 strain.

Analysis of the molecular descriptors in the models indicates, broadly, that activity is dependent upon a mixture of charge (or partial charge) and hydrophobicity. Increases in hydrophobicity (SlogP, GCUT_SLOP1_1) are positively correlated with increases in potency, and an analysis of the percentage of importance of these descriptors indicates that between 29% and 38% of the variance captured by the model can be explained by these terms. ASA_H, which measures the water assessable surface area of hydrophobic atoms, is an indirect measure of this as well. The presence of Gasteiger partial atomic charge or polar van der Waals surface area (PEOE_VSA_FPNEG, RPCneg, PEOE_VSA_FNEG, PEOE_VSA_FNEG)⁵¹ is negatively correlated with potency, and these terms generally account for the second largest source of variance in the data. The presence of positive charge (PEOE_VSA+0, PEOE_VSA_POS) is also negatively correlated with potency. The remaining terms in the models represent molecular flexibility (KierFlex, b-single). Increasing the number of single bonds decreases activity as does an increase in flexibility as measured by the method of Kier and Hall.^{52,53}

DISCUSSION

Previously, we disclosed a cholic acid analogue, CamSA, which was a potent inhibitor of *C. difficile* spore germination and also prevented the development of CDI in a murine model of the disease.^{26,29} In these studies, CamSA was tested against *C. difficile* strains 630 and VPI 10463. To our surprise, CamSA showed no inhibitory activity against an epidemic strain, R20291. The inability of CamSA to work on this strain, coupled with an increased prevalence of these strains in clinical settings, necessitated additional analogues of CamSA to be identified. Unfortunately, the target of action for CamSA is unknown and there is little structural information on any proteins believed to be involved in spore germination in *C. difficile*, thus, an empirical approach was necessary for identifying new agents.

An analysis of a variety of cholan-24-amide analogues as spore germination inhibitors in strain R20291 revealed that the best inhibitor in our series was 12a, which is the simple phenyl derivative in which the sulfate group of CamSA was removed.⁵⁴

In general, the loss of functional groups with the potential for significant biomolecular interactions (i.e., the sulfate group) tends to result in the loss of activity. An alignment of active versus inactive compounds indicates that active agents are smaller and more hydrophobic than inactive agents. Our QSAR analyses also indicate that increasing partial charge or polar van der Waals surface area decreases activity.

A key question is why does CamSA work against strain 630 and not R20291? One possibility could be changes to the target of these agents. The CspA, CspB, and CspC proteins have been implicated in spore germination in *C. difficile*, and mutational analyses have suggested CspC as the likely taurocholate germination receptor.^{6,10,31} However, sequence analyses of CspA, CspB, and CspC reveal that these proteins are greater than 98.7% identical between the 630 and R20291 strains. Thus, the inactivity of CamSA against the R20291 strain is likely not due to alterations to a binding site in any of these proteins.

The spore germination assays are spore (cell)-based assays. Thus, differences in activity could be due to changes in transport of the molecule to the site of action. Problems with transport, either due to the lack of active uptake, the presence of efflux pumps, or poor physicochemical properties necessary for passive transport, are common in cell-based assays.^{55–57} For example, vancomycin is inactive against Gram-negative bacteria due to its inability to cross the outer membrane.⁵⁸ Recent studies on drug transport in Gram-negative bacteria have shown that changes in drug structure can convert an agent that is selective for Gram-positive into one that works on Gram-negative agents by facilitating transport.⁵⁹

Unfortunately, there is little information on transport of molecules into spores and even less for *C. difficile* spores. It has been postulated that the CspA, B, and C proteins are found in the cortex layer of the bacterial spore.⁶⁰ Thus, small molecules that bind to CspC must pass through the exosporium, coat, and outer membrane layer to reach the protein. One possible solution is that there exist proteins that enable the transport of small molecules into the cortex to facilitate germination. In *Bacillus subtilis*, GerP has been shown to enhance permeability of nutrients into the spore.⁶¹ No such protein has yet been identified in *C. difficile*; however, an AAA+ ATPase has been recently suggested to facilitate nutrient

transport.^{62,63} However, it is possible that the activity of the germination inhibitors could be the result of inhibiting such transporters.

Regardless of whether there is a change in passive or active transport in different strains of *C. difficile*, it is clear that small molecule inhibitors must be tailored to initially reach the site of action and then to inhibit germination. Our data indicates that the overall size and shape of the substitution on the amine side of the amide is fairly specific and our active compounds are more hydrophobic than CamSA.

CONCLUSIONS

We have synthesized and examined a series of cholic acid amides as inhibitors of *C. difficile* spore germination in the epidemic strain R20291. We have found that 12a is the most potent known inhibitor of spore germination described to date with an IC_{50} of 1.8 μ M. We have conducted modeling and QSAR studies which indicate that an increase in hydrophobicity and a decrease in size of the molecule enhances activity in this strain. This work also suggests that there may be strain differences in response to spore germination inhibitors and that differences in transport of molecules into the spore could be the reason for this difference. Future work will examine the *in vivo* activity of 12a in animal models of spore germination as well as the use of our QSAR model to identify potent analogues for future testing. Ultimately, we hope that these agents will find utility in the prevention of *C. difficile* infections in patients.

EXPERIMENTAL SECTION

General Comments.

Cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β cholan-24-oic acid) was purchased from MP Biomedical and silica gel for column chromatography from Sorbent Technologies, Inc. All reagents and dry solvents (DMF and EtOAc) were purchased from either Sigma-Aldrich, Acros Organics, TCI Chemicals, or ChemImpex International and were used without further purification. Thin layer chromatography (TLC) were performed on precoated (0.25 mm) silica gel plate (Sorbtech, 60 F-254), and visualization was done either by UV (254 nm), iodine staining, or ninhydrin staining. Column chromatographic purifications of compounds were performed on silica-gel (Sorbtech, 60–230 mesh, 0.063–0.20 mm). 1H and ^{13}C NMR spectra were recorded on a Varian VNMRs 600 MHz spectrometer by dissolving the compounds in deuterated solvents as chloroform-d ($CDCl_3$), methanol-d₄ (CD_3OD), or dimethyl sulfoxide-d₆ ($DMSO-d_6$), and all peaks were referenced with TMS as an internal standard. Some of the compound's spectra were recorded in multiple solvents for clarity of the aliphatic region. Chemical shifts are expressed in ppm (δ), whereas coupling constants (J) are listed in hertz (Hz) and the multiplicities are recorded by following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad signal). High-resolution mass spectra (HRMS) were recorded on a Kratos MS 80 RFA (ESI and CI) spectrometer. The purities of all testing compounds were determined to be >95% by high performance liquid chromatography (HPLC) on Beckman Coulter X-Bridge REH130 C18 column (size 4.6 mm $\times$ 150 mm) with a flow rate of 0.80 mL/min using mobile phase consisted of methanol/acetonitrile and water and monitoring with UV lamp at wavelengths

(223/234/254 nm). Melting points were determined using Mel-temp II apparatus by Laboratory Device in open capillaries and are uncorrected.

Coupling of Cholic acid with Aniline (Method A): N- (Phenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (12a).

To a 100 mL round-bottom flask, cholic acid (7, 2.085 g, 5.10 mmol) and HBTU (1.987 g, 5.23 mmol) were dissolved in anhydrous DMF (12 mL) at room temperature. NMM (0.60 mL, 5.45 mmol) was added to the solution, and the reaction content was allowed to stir for 30 min in order to generate an activated ester. During this time, the solution became light yellow. Aniline (8, 612 mg, 6.57 mmol) and an additional aliquot of NMM (0.60 mL, 5.45 mmol) were added to the above reaction mixture, and the reaction was stirred for 48 h at room temperature. The reaction mixture was concentrated using a high vacuum rotary evaporator to produce a viscous light-yellow material. Ice-cold aqueous 5% HCl solution (200 mL) was added to the residue, and the mixture was subjected to sonication for 5–15 min. A resulting white precipitate was formed, and the top aqueous layer was carefully decanted and discarded. The treatment with 5% HCl and sonication was repeated 2–3 times. The white precipitate was collected by filtration through a sintered glass funnel, washed with cold water, and dried under vacuum to produce 12a (2.241 g, 91% yield); mp 255–256 °C. TLC R_f 0.44 (AcOEt:CH₃OH, 95:5). ¹H NMR (CD₃OD, 600 MHz): δ 7.51 (d, 2H, J = 7.8 Hz), 7.27 (t, 2H, J = 7.8 Hz), 7.05 (t, 1H, J = 7.2 Hz), 3.94 (s, 1H), 3.78 (s, 1H), 3.36 (m, 1H), 2.44–2.40 (m, 1H), 2.30–2.22 (m, 3H), 2.01–1.85 (m, 5H), 1.80–1.72 (m, 2H), 1.63 (d, 1H, J = 13.2 Hz), 1.60–1.50 (m, 5H), 1.48–1.27 (m, 5H), 1.13–1.09 (m, 1H), 1.05 (d, 3H, J = 6.0 Hz), 0.97 (dt, 1H, J = 10.8 Hz), 0.90 (s, 3H), 0.70 (s, 3H). ¹H NMR (DMSO-d₆, 600 MHz): δ 9.82 (s, 1H), 7.55 (d, 2H, J = 7.8 Hz), 7.23 (t, 2H, J = 7.2 Hz), 6.96 (t, 1H, J = 7.2 Hz), 3.91 (br, 3H), 3.76 (s, 1H), 3.58 (s, 1H), 3.15 (s, 1H), 2.30 (m, 1H), 2.19–2.12 (m, 3H), 1.96 (q, 1H, J = 11.4 Hz), 1.82–1.72 (m, 4H), 1.62 (m, 2H), 1.42–1.38 (m, 3H), 1.32–1.14 (m, 8H), 0.94 (d, 4H, J = 6.0 Hz), 0.82–0.77 (m, 3H), 0.55 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 173.8, 138.6, 128.3, 123.6, 119.8, 72.6, 71.4, 67.6, 46.2, 46.1, 41.8, 41.6, 39.6, 39.0, 35.6, 35.1, 34.5, 34.5, 33.6, 31.7, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 16.4, 11.6. ¹³C NMR (DMSO-d₆, 150 MHz): δ 172.2, 139.9, 129.0, 123.3, 119.4, 71.4, 70.9, 66.7, 46.5, 46.2, 42.0, 41.8, 35.7, 35.6, 35.3, 34.8, 33.9, 31.9, 30.8, 29.0, 27.7, 26.6, 23.2, 23.1, 17.6, 12.8. HRMS (ESI, m/z): calcd for C₃₀H₄₅NO₄Na [M + Na⁺] 506.3246, found 506.3239.

N-(2'-Methoxyphenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (12b).

This compound was prepared from cholic acid 7 and 2-methoxyaniline by following method A in 93% yield; mp 207–209 °C. TLC R_f 0.61 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ¹H NMR (CD₃OD, 600 MHz): δ 7.86 (d, 1H, J = 7.2 Hz), 7.08 (t, 1H, J = 7.2 Hz), 6.97 (d, 1H, J = 7.8 Hz), 6.88 (t, 1H, J = 7.2 Hz), 3.95 (s, 1H), 3.85 (s, 3H), 3.78 (s, 1H), 3.35 (m, 1H), 2.52–2.44 (m, 1H), 2.37–2.33 (m, 1H), 2.30–2.24 (m, 2H), 2.01–1.87 (m, 5H), 1.79 (d, 1H, J = 14.4 Hz), 1.76–1.72 (m, 1H), 1.63 (d, 1H, J = 13.2 Hz), 1.57–1.49 (m, 5H), 1.46–1.31 (m, 5H), 1.14–1.05 (m, 4H), 0.99 (t, 1H, J = 13.2 Hz), 0.90 (s, 3H), 0.70 (s, 3H). ¹H NMR (DMSO-d₆, 600 MHz): δ 8.98 (s, 1H), 7.84 (d, 1H, J = 7.8 Hz), 7.01–6.95 (m, 2H), 6.83 (t, 1H, J = 7.8 Hz), 4.44 (d, 1H, J = 4.2 Hz), 4.12 (d, 1H, J = 3.6 Hz), 4.02 (d, 1H, J = 3.0 Hz), 3.78 (s, 5H), 3.17–3.12 (m, 1H), 2.38–2.34 (m, 1H), 2.26–2.07 (m, 3H), 1.92 (q, 1H, J = 12.0 Hz), 1.78–1.70 (m, 4H), 1.62–1.57 (m, 2H), 1.43–1.38 (m, 3H), 1.34–1.12 (m, 8H),

0.92 (d, 4H, $J = 6.0$ Hz), 0.85–0.75 (m, 4H), 0.54 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 173.8, 150.2, 126.8, 124.7, 122.2, 120.0, 110.4, 72.6, 71.4, 67.4, 54.8, 46.7, 46.1, 41.7, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 33.5, 31.7, 29.7, 28.1, 27.3, 26.4, 22.8, 21.8, 16.4, 11.6. ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz): δ 172.5, 150.1, 127.7, 124.7, 122.5, 120.6, 111.4, 71.6, 70.9, 66.7, 56.0, 46.6, 46.2, 41.9, 41.8, 40.1, 35.6, 35.2, 34.8, 33.7, 32.0, 30.7, 28.9, 27.7, 26.6, 23.3, 23.0, 17.5, 12.8.

***N*-(3'-Methoxyphenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (12c).**

This compound was prepared from cholic acid 7 and 3methoxyaniline by following method A in 87% yield; mp 260–262 °C. TLC R_f 0.59 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR (CD_3OD , 600 MHz): δ 7.26 (t, 1H, $J = 2.4$ Hz), 7.16 (t, 1H, $J = 7.8$ Hz), 7.04 (d, 1H, $J = 7.8$ Hz), 6.62 (dd, 1H, $J = 8.4$ and 2.4 Hz), 3.94 (s, 1H), 3.77 (d, 1H, $J = 2.4$ Hz), 3.75 (s, 3H), 3.38–3.29 (m, 1H), 2.42–2.39 (m, 1H), 2.30–2.22 (m, 3H), 2.01–1.84 (m, 5H), 1.77–1.71 (m, 2H), 1.63 (d, 1H, $J = 14.4$ Hz), 1.58–1.49 (m, 5H), 1.45–1.28 (m, 5H), 1.13–1.07 (m, 1H), 1.05 (d, 3H, $J = 6.0$ Hz), 0.96 (dt, 1H, $J = 14.4$ Hz), 0.89 (s, 3H), 0.70 (s, 3H). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 9.81 (s, 1H), 7.26 (s, 1H), 7.13 (t, 1H, $J = 8.4$ Hz), 7.07 (d, 1H, $J = 7.8$ Hz), 6.56 (dd, 1H, $J = 8.4$ and 1.8 Hz), 4.29 (d, 1H, $J = 4.2$ Hz), 4.08 (d, 1H, $J = 3.0$ Hz), 3.98 (d, 1H, $J = 3.0$ Hz), 3.75 (s, 1H), 3.67 (s, 3H), 3.57 (s, 1H), 3.14 (m, 1H), 2.31–2.26 (m, 1H), 2.21–2.09 (m, 3H), 1.98–1.93 (m, 1H), 1.82–1.69 (m, 4H), 1.60 (d, 1H, $J = 13.2$ Hz), 1.43–1.38 (m, 3H), 1.34–1.12 (m, 8H), 0.96–0.87 (m, 4H), 0.83–0.77 (m, 4H), 0.55 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 173.8, 160.1, 139.7, 129.0, 111.9, 109.1, 105.5, 72.6, 71.4, 67.4, 54.2, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 34.4, 33.6, 31.7, 29.8, 28.2, 27.3, 26.4, 22.8, 21.7, 16.4, 11.6.

***N*-(4'-Methoxyphenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (12d).**

This compound was prepared from cholic acid 7 and 4-methoxyaniline by following method A in 94% yield; mp 213–214 °C. TLC R_f 0.51 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR (CD_3OD , 600 MHz): δ 7.40 (d, 2H, $J = 9.0$ Hz), 6.84 (d, 2H, $J = 9.0$ Hz), 3.94 (s, 1H), 3.78 (m, 1H), 3.74 (s, 3H), 3.34 (m, 1H), 2.40–2.37 (m, 1H), 2.30–2.21 (m, 3H), 2.01–1.83 (m, 5H), 1.80–1.71 (m, 2H), 1.64–1.49 (m, 6H), 1.45–1.29 (m, 5H), 1.12–1.07 (m, 1H), 1.05 (d, 3H, $J = 6.6$ Hz), 0.96 (dt, 1H, $J = 13.8$ and 3.6 Hz), 0.89 (s, 3H), 0.70 (s, 3H). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 9.67 (s, 1H), 7.45 (d, 2H, $J = 8.4$ Hz), 6.81 (d, 2H, $J = 8.4$ Hz), 4.09 (br s, 3H), 3.76 (s, 1H), 3.67 (s, 3H), 3.58 (s, 1H), 3.15 (s, 1H), 2.30–2.08 (m, 4H), 1.98–1.93 (m, 1H), 1.80–1.68 (m, 4H), 1.64–1.58 (m, 2H), 1.43–1.38 (m, 3H), 1.34–1.10 (m, 8H), 0.94 (d, 4H, $J = 5.4$ Hz), 0.82–0.77 (m, 4H), 0.55 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 173.6, 156.4, 131.5, 121.7, 113.5, 72.6, 71.4, 67.6, 54.4, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.6, 35.1, 34.5, 34.4, 33.4, 31.8, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 16.4, 11.6.

***N*-(2',4'-Dimethoxyphenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (12e).**

This compound was prepared from cholic acid 7 and 2,4-dimethoxyaniline by following method A in 91% yield; mp 203–204 °C. TLC R_f 0.69 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR (CD_3OD , 600 MHz): δ 7.67 (s, 1H), 6.87 (d, 1H, $J = 9.0$ Hz), 6.61 (d, 1H, $J = 8.4$ Hz), 3.94 (s, 1H), 3.80 (s, 3H), 3.77 (s, 1H), 3.71 (s, 3H), 3.67 (m, 1H), 2.52–2.44 (m, 1H), 2.36–2.31 (m, 1H), 2.29–2.20 (m, 2H), 2.01–1.84 (m, 5H), 1.79–1.72 (m, 2H), 1.63 (d, 1H,

$J = 12.6$ Hz), 1.58–1.49 (m, 5H), 1.46–1.30 (m, 5H), 1.10 (m, 1H), 1.05 (d, 3H, $J = 6.0$ Hz), 0.97 (t, 1H, $J = 13.8$ Hz), 0.89 (s, 3H), 0.69 (s, 3H). ^1H NMR (DMSO- d_6 , 600 MHz): δ 8.96 (s, 1H), 7.66 (s, 1H), 6.88 (d, 1H, $J = 8.4$ Hz), 6.55 (d, 1H, $J = 8.4$ Hz), 4.08 (br s, 3H), 3.76 (s, 1H), 3.73 (s, 3H), 3.63 (s, 3H), 3.58 (s, 1H), 3.15 (s, 1H), 2.39 (s, 1H), 2.27–2.10 (m, 3H), 1.96 (q, 1H, $J = 10.8$ Hz), 1.77–1.60 (m, 6H), 1.41–1.15 (m, 11H), 0.92 (d, 4H, $J = 5.4$ Hz), 0.82–0.77 (m, 4H), 0.56 (s, 3H). ^{13}C NMR (CD $_3$ OD, 150 MHz): δ 173.8, 153.5, 144.0, 127.6, 111.0, 108.5, 108.3, 72.6, 71.4, 67.6, 55.3, 54.6, 46.7, 46.1, 41.8, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 34.4, 33.5, 31.7, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 16.4, 11.6.

***N*-(4'-Hydroxyphenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (12f).**

This compound was prepared from cholic acid 7 and 4aminophenol by following method A in 80% yield; mp 140–142 °C. TLC R_f 0.59 (AcOEt:CH $_3$ OH, 90:10). ^1H NMR (CD $_3$ OD, 600 MHz): δ 7.30 (d, 2H, $J = 7.2$ Hz), 6.71 (d, 2H, $J = 7.2$ Hz), 3.94 (s, 1H), 3.77 (s, 1H), 3.34 (s, 1H), 2.37 (s, 1H), 2.28–2.23 (m, 3H), 1.98–1.82 (m, 5H), 1.80–1.70 (m, 2H), 1.63 (d, 1H, $J = 11.4$ Hz), 1.56–1.49 (m, 5H), 1.42–1.29 (m, 5H), 1.16–1.11 (m, 4H), 0.95 (t, 1H, $J = 13.8$ Hz), 0.89 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (CD $_3$ OD, 150 MHz): δ 173.5, 153.9, 130.4, 122.0, 114.8, 72.7, 71.5, 67.6, 46.6, 46.1, 41.7, 41.6, 39.6, 39.0, 35.6, 35.1, 34.5, 34.5, 33.4, 31.9, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 16.4, 11.6.

***N*-(3'-Fluorophenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (12g).**

This compound was prepared from cholic acid 7 and 3fluoroaniline by following method A, and after regular work up procedure, the product was purified by column chromatography eluted from AcOEt:CH $_3$ OH (95:5, 90:10; 80:20 and 75:25) to afford a white powder (83% yield); mp 231–233 °C. TLC R_f 0.39 (AcOEt:CH $_3$ OH, 95:5). ^1H NMR (CD $_3$ OD, 600 MHz): δ 7.51 (d, 1H, $J = 14.4$ Hz), 7.27–7.21 (m, 2H), 6.77 (t, 1H, $J = 8.4$ Hz), 3.93 (s, 1H), 3.77 (s, 1H), 3.35 (m, 1H), 2.45–2.40 (m, 1H), 2.30–2.22 (m, 3H), 2.00–1.83 (m, 5H), 1.79–1.72 (m, 2H), 1.63 (d, 1H, $J = 12.0$ Hz), 1.56–1.27 (m, 10H), 1.11–1.08 (m, 1H), 1.04 (d, 3H, $J = 5.4$ Hz), 0.96 (t, 1H, $J = 14.4$ Hz), 0.89 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (CD $_3$ OD, 150 MHz): δ 173.9, 140.5, 129.7, 129.7, 114.9, 109.9, 109.7, 106.7, 106.5, 72.6, 71.5, 67.6, 46.6, 46.1, 41.7, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 33.6, 31.6, 29.8, 27.3, 26.4, 22.8, 21.8, 16.4, 11.6. HRMS (ESI, m/z): calcd for C $_{30}$ H $_{44}$ FNO $_4$ Na [$M + \text{Na}^+$] 524.3152, found 524.3130.

***N*-(4'-Fluoro-2'-methylphenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (12h).**

This compound was prepared from cholic acid 7 and 4-fluoro-2-methylaniline by following method A to afford a light-pink colored powder (91% yield); mp 134–135 °C. TLC R_f 0.64 (CH $_2$ Cl $_2$:CH $_3$ OH:NH $_4$ OH, 89:10:1). ^1H NMR (DMSO- d_6 , 600 MHz): δ 9.24 (s, 1H, 1H), 7.27 (t, 1H, $J = 6.6$ Hz), 7.01 (d, 1H, $J = 9.6$ Hz), 6.94 (t, 1H, $J = 7.8$ Hz), 4.32 (s, 1H), 4.10 (s, 1H), 3.99 (s, 1H), 3.77 (s, 1H), 3.58 (s, 1H), 3.13 (m, 1H), 2.34–2.28 (m, 1H), 2.22–2.10 (m, 6H), 1.97 (q, 1H, $J = 11.4$ Hz), 1.82–1.70 (m, 4H), 1.61 (d, 2H, $J = 10.8$ Hz), 1.44–1.36 (m, 3H), 1.35–1.13 (m, 8H), 0.96–0.92 (m, 4H), 0.83–0.77 (m, 4H), 0.56 (s, 3H). ^{13}C NMR (DMSO- d_6 , 150 MHz): δ 172.2, 160.5, 158.9, 135.3, 135.3, 133.2, 127.7, 127.6, 116.9, 116.8, 112.8, 112.7, 71.5, 70.1, 66.7, 46.6, 46.2, 41.9, 41.8, 35.7, 35.6, 35.3, 34.8, 33.2, 32.1, 30.8, 29.0, 27.8, 26.6, 23.2, 23.0, 18.3, 17.5, 12.8. ^{13}C NMR (CD $_3$ OD, 150 MHz): δ 174.5, 161.6, 160.0, 136.1, 136.1, 131.6, 127.8, 127.7, 116.4, 116.3, 112.4, 112.3, 71.6,

71.4, 67.6, 46.7, 46.1, 41.8, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 34.5, 32.8, 31.9, 29.8, 28.2, 27.3, 26.5, 22.8, 21.8, 16.9, 16.4, 11.6.

***N*-(5'-Fluoro-2'-methylphenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (12i).**

This compound was prepared from cholic acid 7 and 5-fluoro-2-methylaniline by following method A in 77% yield; mp 206–207 °C. TLC R_f 0.65 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR (CD_3OD , 600 MHz): δ 7.18 (m, 2H), 6.83 (t, 1H, $J = 7.2$ Hz), 3.95 (s, 1H), 3.78 (s, 1H), 3.60 (t, 1H, $J = 10.8$ Hz), 2.50–2.44 (m, 1H), 2.37–2.32 (m, 1H), 2.30–2.23 (m, 3H), 2.19 (s, 3H), 2.02–1.87 (m, 5H), 1.80–1.73 (m, 3H), 1.64 (d, 1H, $J = 12.6$ Hz), 1.57–1.30 (m, 9H), 1.11–1.06 (m, 4H), 0.98–0.90 (m, 4H), 0.71 (s, 3H). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 9.22 (s, 1H, 1H), 7.34 (d, 1H, $J = 7.8$ Hz), 7.15 (s, 1H), 6.82 (s, 1H), 4.11 (br s, 3H), 3.76 (s, 1H), 3.57 (s, 1H), 3.15 (s, 1H), 2.35 (s, 1H), 2.28–2.13 (s, 6H), 1.96 (s, 1H), 1.82–1.68 (m, 4H), 1.61 (s, 2H), 1.52–1.10 (m, 11H), 1.02–0.77 (m, 8H), 0.56 (s, 3H). ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz): δ 172.4, 161.3, 159.7, 138.3, 138.2, 131.7, 131.6, 126.7, 111.3, 111.1, 71.5, 70.9, 66.7, 46.6, 46.2, 42.0, 41.8, 35.7, 35.6, 35.3, 34.8, 33.3, 32.0, 30.8, 29.0, 27.8, 26.6, 23.2, 23.0, 17.6, 17.6, 12.8.

***N*-(3'-Aminocarbonylphenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (12j).**

This compound was prepared from cholic acid 7 and 3-aminobenzamide by following method A in 74% yield; mp 130–132 °C. TLC R_f 0.55 ($\text{AcOEt}:\text{CH}_3\text{OH}$, 85:15). ^1H NMR (CD_3OD , 600 MHz): δ 8.05 (s, 1H), 7.72 (d, 1H, $J = 7.8$ Hz), 7.55 (d, 1H, $J = 7.8$ Hz), 7.39 (t, 1H, $J = 7.8$ Hz), 3.94 (s, 1H), 3.77 (d, 1H, $J = 1.8$ Hz), 3.36 (m, 1H), 2.46–2.42 (m, 1H), 2.33–2.21 (m, 3H), 2.00–1.85 (m, 5H), 1.79–1.72 (m, 2H), 1.63 (d, 1H, $J = 12.6$ Hz), 1.61–1.49 (m, 5H), 1.44–1.29 (m, 5H), 1.10–1.07 (m, 1H), 1.04 (d, 3H, $J = 6.0$ Hz), 0.96 (t, 1H, $J = 13.8$ Hz), 0.88 (s, 3H), 0.69 (s, 3H). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 10.05 (s, 1H), 8.02 (s, 1H), 7.73 (d, 1H, $J = 5.4$ Hz), 7.47 (d, 1H, $J = 5.4$ Hz), 7.29 (s, 1H), 3.75 (s, 1H), 3.56 (s, 1H), 3.14 (s, 1H), 2.31 (s, 1H), 2.24–2.06 (m, 3H), 1.94 (m, 1H), 1.80–1.66 (m, 4H), 1.60 (s, 2H), 1.44–1.10 (m, 11H), 0.92 (s, 4H), 0.82–0.74 (m, 4H), 0.53 (s, 3H). ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz): δ 172.4, 168.5, 139.8, 135.3, 128.8, 122.2, 122.1, 119.1, 72.5, 70.9, 66.7, 46.6, 46.2, 41.9, 41.8, 35.7, 35.0, 35.3, 34.8, 33.9, 31.9, 30.8, 28.1, 27.7, 26.6, 23.2, 23.0, 17.6, 12.8. HRMS (ESI, m/z): calcd for $\text{C}_{31}\text{H}_{46}\text{N}_2\text{O}_5\text{Na}$ [$M + \text{Na}^+$] 549.3304, found 549.3270.

Coupling of Cholic Acid with Ethyl 3-Aminobenzoate (Method B): *N*-(3'-Ethoxycarbonylphenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (12k).

A mixture of cholic acid (7, 826 mg, 2.02 mmol) and HBTU (957 mg, 2.53 mmol) was dissolved in anhydrous DMF (6 mL) followed by the addition of NMM (0.25 mL, 2.27 mmol). The reaction was stirred at room temperature for 50 min to generate the activated ester. Ethyl 3-aminobenzoate (418 mg, 2.53 mmol) and NMM (0.20 mL) were added to the reaction, and the reaction was stirred for 72 h at room temperature. After completion, the reaction was concentrated using a high vacuum rotary evaporator to give a viscous material residue. Ice-cold water (120 mL) was added to the residue, and the mixture was subjected to sonication for 5–10 min to produce a white precipitate. The aqueous layer was carefully decanted, and the water wash and sonication was repeated. The resulting white precipitate was collected by filtration, washed with cold water, and dried under vacuum to produce 12k

(976 mg, 87% yield); mp 106–108 °C. TLC R_f 0.60 (AcOEt:CH₃OH, 90:10). ¹H NMR (CD₃OD, 600 MHz): δ 8.22 (s, 1H), 7.80 (d, 1H, J = 7.8 Hz), 7.72 (d, 1H, J = 7.8 Hz), 7.39 (t, 1H, J = 7.8 Hz), 4.34 (q, 2H, J = 7.2 Hz), 3.94 (s, 1H), 3.77 (d, 1H, J = 2.4 Hz), 3.34 (m, 1H), 2.45–2.42 (m, 1H), 2.32–2.23 (m, 3H), 2.01–1.85 (m, 5H), 1.80–1.72 (m, 2H), 1.63 (d, 1H, J = 13.2 Hz), 1.58–1.49 (m, 6H), 1.48–1–1.27 (m, 7H), 1.13–1.04 (m, 4H), 0.96 (t, 1H, J = 14.4 Hz), 0.89 (s, 3H), 0.70 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 174.0, 166.3, 139.0, 130.8, 128.5, 124.4, 124.0, 120.5, 72.6, 71.4, 67.6, 60.8, 46.6, 46.0, 41.8, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 34.4, 33.5, 31.6, 29.8, 28.2, 27.3, 26.5, 22.8, 21.7, 16.4, 13.2, 11.6. HRMS (ESI, m/z): calcd for C₃₃H₄₉NO₆Na [M + Na⁺] 578.3458, found 578.3458.

***N*-(4'-Ethoxycarbonylphenyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (12l).**

This compound was prepared from cholic acid 7 and ethyl 4-aminobenzoate by following method B in 83% yield; mp 106–108 °C. TLC R_f 0.50 (AcOEt:CH₃OH, 95:5). ¹H NMR (CD₃OD, 600 MHz): δ 7.93 (d, 2H, J = 7.8 Hz), 7.67 (d, 2H, J = 7.8 Hz), 4.31 (b, 2H), 3.93 (s, 1H), 3.77 (s, 1H), 3.35 (s, 1H), 2.48–2.42 (m, 1H), 2.33–2.23 (m, 3H), 1.99–1.87 (m, 5H), 1.79–1.72 (m, 2H), 1.63 (d, 1H, J = 12.6 Hz), 1.60–1.49 (m, 5H), 1.44–1.26 (m, 8H), 1.10 (m, 1H), 1.05 (d, 3H, J = 4.8 Hz), 0.98–89 (s, 4H), 0.69 (s, 3H). ¹H NMR (DMSO-d₆, 600 MHz): δ 10.19 (s, 1H), 7.86 (d, 2H, J = 9.0 Hz), 7.69 (d, 2H, J = 8.4 Hz), 4.30 (s, 1H), 4.25 (q, 2H, J = 6.6 Hz), 4.10 (d, 1H, J = 3.0 Hz), 4.00 (d, 1H, J = 2.4 Hz), 3.76 (s, 1H), 3.58 (s, 1H), 3.15 (s, 1H), 2.37–2.30 (m, 1H), 2.25–2.09 (m, 3H), 1.97 (m, 1H), 1.82–1.72 (m, 3H), 1.62 (m, 2H), 1.43–1.39 (m, 3H), 1.35–1.12 (m, 12H), 0.95–88 (s, 4H), 0.82–0.78 (m, 4H), 0.56 (s, 3H). ¹³C NMR (DMSO-d₆, 150 MHz): δ 174.0, 166.3, 143.2, 131.0, 130.1, 125.0, 118.7, 112.9, 72.6, 71.4, 66.6, 60.6, 46.6, 46.1, 41.7, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 33.2, 31.5, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 16.4, 13.3, 11.7.

***N*-(3'-tert-Butyloxycarbonylaminophenyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (12m).**

This compound was prepared from cholic acid 7 and 3-(tert-butyloxycarbonylamino)aniline by following method B in 83% yield; mp 134–136 °C. TLC R_f 0.37 (AcOEt:CH₃OH, 95:5). ¹H NMR (CD₃OD, 600 MHz): δ 7.67 (s, 1H), 7.18–7.14 (m, 2H), 7.08 (d, 1H, J = 7.2 Hz), 3.94 (s, 1H), 3.77 (s, 1H), 3.35 (t, 1H, J = 10.8 Hz), 2.42–2.38 (m, 1H), 2.29–2.21 (m, 3H), 2.00–1.85 (m, 5H), 1.79–1.72 (m, 2H), 1.63–1.51 (m, 6H), 1.49 (s, 9H), 1.44–1.29 (m, 5H), 1.11–1.08 (m, 1H), 1.04 (d, 3H, J = 6.0 Hz), 0.96 (t, 1H, J = 12.6 Hz), 0.89 (s, 3H), 0.69 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 173.8, 153.7, 139.6, 139.0, 128.5, 114.3, 114.2, 110.5, 79.4, 72.7, 71.5, 67.7, 46.6, 46.1, 41.7, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 33.6, 31.8, 29.8, 28.2, 27.4, 27.3, 26.4, 22.9, 21.9, 16.8, 11.7.

***N*-(4'-tert-Butyloxycarbonylaminophenyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (12n).**

This compound was prepared from cholic acid 7 and 4-(tert-butyloxycarbonylamino)aniline by following method B in 84% yield; mp 152–153 °C. TLC R_f 0.38 (AcOEt:CH₃OH, 95:5). ¹H NMR (CD₃OD, 600 MHz): δ 7.41 (d, 2H, J = 9.0 Hz), 7.30 (d, 2H, J = 8.4 Hz), 4.61 (s, 1H), 3.94 (s, 1H), 3.78 (s, 1H), 3.35 (m, 1H), 2.44–2.38 (m, 1H), 2.29–2.23 (m, 3H), 2.00–1.85 (m, 5H), 1.80–1.72 (m, 2H), 1.64 (d, 1H, J = 12.0 Hz), 1.56–1.52 (m, 3H), 1.49 (s, 9H), 1.45–1.28 (m, 6H), 1.11–1.08 (m, 1H), 1.05 (d, 3H, J = 6.6 Hz), 0.96 (t, 1H, J = 14.4 and 2.4 Hz), 0.89 (s, 3H), 0.70 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 173.6, 154.0,

135.3, 133.5, 120.5, 118.7, 79.4, 72.7, 71.5, 67.7, 46.6, 46.1, 41.7, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 34.4, 33.5, 31.8, 29.7, 28.1, 27.3, 27.3, 26.4, 22.8, 21.8, 15.4, 11.6.

Cleavage of Boc Group (Method C): N-(3'-Aminophenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide Hydrochloride (12o).

Into a reaction mixture of 12m (230 mg, 0.386 mmol) in anhydrous AcOEt (20 mL) under argon, 1 M HCl in AcOEt (0.5 mL) was added dropwise. The reaction mixture became clear after a few minutes, followed by the formation of a white solid. The reaction was stirred at room temperature, and upon completion (as determined by TLC), the solid was collected by filtration. The solid was washed with AcOEt and dried under high vacuum to give 12o (163 mg, 80% yield). TLC R_f 0.56 (CH₂Cl₂:CH₃OH:NH₄OH, 85:14:1). ¹H NMR (CD₃OD, 600 MHz): δ 8.00 (s, 1H), 7.48 (d, 1H, J = 7.8 Hz), 7.44 (t, 1H, J = 7.8 Hz), 7.10 (d, 1H, J = 7.2 Hz), 3.94 (s, 1H), 3.78 (s, 1H), 3.36 (m, 1H), 2.48–2.44 (m, 1H), 2.34–2.23 (m, 3H), 1.97–1.70 (m, 8H), 1.62–1.28 (m, 10H), 1.11–1.04 (m, 4H), 0.96 (t, 1H, J = 12.6 Hz), 0.89 (s, 3H), 0.69 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 174.2, 140.5, 130.8, 130.1, 119.5, 117.6, 114.0, 72.6, 71.5, 67.7, 46.6, 46.1, 41.7, 41.6, 39.6, 39.0, 35.6, 35.1, 34.5, 33.6, 31.6, 29.7, 28.2, 27.3, 26.5, 22.8, 21.8, 16.4, 11.62. NMR data for free base: ¹H NMR (CD₃OD, 600 MHz): δ 7.04 (s, 1H), 7.00 (t, 1H, J = 7.8 Hz), 6.80 (d, 1H, J = 7.8 Hz), 6.45 (d, 1H, J = 7.8 Hz), 3.94 (s, 1H), 3.77 (s, 1H), 3.35 (m, 1H), 2.38 (m, 1H), 2.29–2.23 (m, 2H), 2.00–1.84 (m, 5H), 1.79–1.73 (m, 2H), 1.63 (d, 1H, J = 12.6 Hz), 1.58–1.49 (m, 5H), 1.46–1.27 (m, 6H), 1.09 (m, 1H), 1.04 (d, 3H, J = 5.4 Hz), 0.95 (t, 1H, J = 13.2 Hz), 0.89 (s, 3H), 0.69 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 173.7, 147.5, 139.2, 128.9, 111.3, 110.1, 107.3, 72.7, 71.5, 67.6, 48.5, 46.6, 46.1, 41.7, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 34.5, 33.6, 31.8, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 16.4, 11.6.

N-(4'-Aminophenyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide Hydrochloride (12p).

It was prepared from 12n in a manner similar to that of the compound 12o by following method C in 84% yield. TLC R_f 0.51 (CH₂Cl₂:CH₃OH:NH₄OH, 85:14:1). ¹H NMR (CD₃OD, 600 MHz): δ 7.73 (d, 2H, J = 9.0 Hz), 7.32 (d, 2H, J = 8.4 Hz), 3.94 (s, 1H), 3.78 (s, 1H), 3.36 (m, 1H), 2.48–2.43 (m, 1H), 2.33–2.23 (m, 3H), 1.96–1.84 (m, 5H), 1.80–1.74 (m, 2H), 1.63 (d, 1H, J = 13.2 Hz), 1.57–1.28 (m, 10H), 1.11–1.09 (m, 1H), 1.05 (d, 3H, J = 6.0 Hz), 0.97 (t, 1H, J = 15.0 Hz), 0.90 (s, 3H), 0.70 (s, 3H). ¹³C NMR (DMSO-*d*₆, 150 MHz): δ 172.5, 139.7, 126.3, 124.1, 120.2, 71.4, 70.9, 66.7, 46.5, 46.2, 41.9, 41.8, 40.5, 35.7, 35.6, 35.3, 34.8, 33.9, 31.8, 30.8, 29.0, 27.7, 26.6, 23.2, 23.0, 17.6, 12.8.

N-(Pyridine-4'-yl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (12q).

This compound was prepared from cholic acid 7 and 4-aminopyridine by following method B in 70% yield; mp 230–232 °C. TLC R_f 0.28 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ¹H NMR (CD₃OD, 600 MHz): δ 8.37 (s, 2H), 7.68 (s, 2H), 3.92 (s, 1H), 3.76 (s, 1H), 3.34 (s, 1H), 2.46–2.25 (m, 4H), 2.02–1.22 (m, 18H), 1.03–0.88 (m, 8H), 0.68 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 174.6, 148.5, 147.5, 113.7, 72.6, 71.4, 67.6, 46.6, 46.1, 41.8, 41.6, 39.6, 38.0, 35.5, 35.1, 34.5, 33.7, 31.2, 29.8, 28.2, 27.3, 26.5, 22.8, 21.8, 16.4, 11.6.

***N*-(4'-Methylthiozol-2'-yl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (13).**

This compound was prepared from cholic acid 7 and 2-amino-4-methylthiazole 9 by following method B in 76% yield; mp 237–238 °C. TLC R_f 0.39 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR (CD_3OD , 600 MHz): δ 6.60 (s, 1H), 3.93 (s, 1H), 3.77 (s, 1H), 3.34 (m, 1H), 2.52–2.49 (m, 1H), 2.40–2.36 (m, 1H), 2.27–2.21 (m, 5H), 2.00–1.86 (m, 5H), 1.79–1.72 (m, 2H), 1.63 (d, 1H, J = 12.6 Hz), 1.58–1.49 (m, 5H), 1.46–1.38 (m, 3H), 1.35 (m, 1H), 1.28 (m, 1H), 1.10–1.07 (m, 1H), 1.04 (d, 3H, J = 5.4 Hz), 0.95 (t, 1H, J = 14.4 Hz), 0.88 (s, 3H), 0.68 (s, 3H). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 11.90 (s, 1H), 6.63 (s, 1H), 4.40 (s, 1H), 4.11 (s, 1H), 4.00 (s, 1H), 3.73 (s, 1H), 3.14 (s, 1H), 2.36 (m, 1H), 2.25–2.04 (m, 6H), 1.92 (m, 1H), 1.73–1.52 (m, 5H), 1.45–1.04 (m, 12H), 0.89 (s, 4H), 0.82–0.74 (m, 4H), 0.52 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 172.8, 158.1, 146.8, 107.5, 72.6, 71.4, 67.6, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.4, 35.1, 34.5, 32.2, 31.2, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 16.3, 16.6, 11.6. ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz): δ 172.2, 157.8, 146.9, 107.8, 71.5, 70.9, 66.7, 46.5, 46.2, 41.9, 41.8, 35.7, 35.6, 35.2, 34.8, 32.5, 31.6, 30.7, 28.9, 27.7, 26.6, 23.2, 23.0, 17.4, 17.3, 12.7.

***N*-(Benzyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (14a).**

This compound was prepared from cholic acid 7 and benzylamine 10 by following method A in 90% yield; mp 236–238 °C. TLC R_f 0.58 ($\text{AcOEt}:\text{CH}_3\text{OH}$, 90:10). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 8.27 (t, 1H, J = 6.0 Hz), 7.27 (t, 2H, J = 7.8 Hz), 7.20 (t, 3H, J = 7.2 Hz), 4.30 (s, 1H), 4.24–4.20 (m, 2H), 4.08 (s, 1H), 3.99 (s, 1H), 3.75 (s, 1H), 3.58 (s, 1H), 3.15 (br, 1H), 2.21–2.09 (m, 3H), 2.04–1.99 (m, 1H), 1.97–1.92 (m, 1H), 1.77–1.60 (m, 6H), 1.43–1.38 (m, 3H), 1.35–1.30 (m, 3H), 1.28–1.05 (m, 5H), 0.95–0.91 (m, 4H), 0.83–0.73 (m, 4H), 0.55 (s, 3H). ^1H NMR (CD_3OD , 600 MHz): δ 7.30–7.25 (m, 4H), 7.21 (t, 1H, J = 6.6 Hz), 4.33 (dd, 2H, J = 15.0 Hz), 3.92 (s, 1H), 3.78 (s, 1H), 3.35 (m, 1H), 2.30–2.22 (m, 3H), 2.17–2.13 (m, 1H), 2.00–1.91 (m, 2H), 1.88–1.77 (m, 4H), 1.74–1.71 (m, 1H), 1.64 (d, 1H, J = 13.2 Hz), 1.59–1.50 (m, 5H), 1.44–1.29 (m, 4H), 1.27–1.22 (m, 1H), 1.10–1.04 (m, 1H), 1.01 (d, 3H, J = 6.6 Hz), 0.96 (t, 1H, J = 14.4 Hz), 0.90 (s, 3H), 0.67 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 175.2, 138.8, 128.1, 127.2, 126.7, 72.6, 71.4, 67.6, 46.7, 46.1, 42.6, 41.8, 41.6, 39.6, 39.0, 35.4, 35.1, 34.5, 32.7, 32.0, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 16.3, 11.6. HRMS (ESI, m/z): calcd for $\text{C}_{31}\text{H}_{47}\text{NO}_4\text{Na}$ [$M + \text{Na}^+$] 520.3403, found 520.3392.

***N*-(2',4'-Dimethoxybenzyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (14b).**

This compound was prepared from cholic acid 7 and 2,4-dimethoxybenzylamine by following method A. The product was further purified by stirring with anhydrous diethyl ether, and insoluble material was collected and vacuum-dried to furnish a white powder in 85% yield; mp 222–223 °C. TLC R_f 0.54 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR (CD_3OD , 600 MHz): δ 7.08 (s, 1H), 6.48 (s, 1H), 6.42 (s, 1H), 4.23 (s, 2H), 3.90 (s, 1H), 3.79 (s, 3H), 3.75 (s, 4H), 3.34 (s, 1H), 2.23 (br s, 3H), 2.11 (s, 1H), 1.94–1.66 (m, 7H), 1.64–1.50 (m, 6H), 1.44–1.14 (m, 6H), 1.10–0.82 (m, 8H), 0.61 (s, 3H). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 7.97 (s, 1H), 6.99 (d, 1H, J = 8.4 Hz), 6.48 (s, 1H), 6.41 (d, 1H, J = 8.4 Hz), 4.08 (br s, 2H), 3.73 (s, 4H), 3.69 (s, 3H), 3.57 (s, 1H), 3.14 (br m, 1H), 2.10–2.05 (m, 2H), 2.02–1.95 (m, 2H), 1.90–1.56 (m, 6H), 1.43–1.02 (m, 12H), 0.90 (d, 4H, J = 3.6 Hz),

0.82–0.77 (m, 4H), 0.53 (s, 3H). ^{13}C NMR (DMSO- d_6 , 150 MHz): δ 173.0, 160.0, 158.0, 128.9, 119.7, 104.6, 98.5, 71.5, 70.9, 66.7, 55.8, 55.6, 46.6, 46.2, 42.0, 41.8, 37.1, 35.7, 35.6, 35.3, 34.8, 32.9, 32.3, 30.8, 29.0, 27.7, 26.6, 23.3, 23.1, 17.5, 12.8.

***N*-(3',5'-Dimethoxybenzyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (14c).**

This compound was prepared from cholic acid 7 and 3,5-dimethoxybenzylamine by following method A in 79% yield; mp 88–91 (softening) and 110–112 °C. TLC R_f 0.57 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ^1H NMR (CD₃OD, 600 MHz): δ 6.41 (s, 2H), 6.32 (s, 1H), 4.25 (s, 2H), 3.90 (s, 1H), 3.71 (s, 6H), 3.34 (s, 1H), 2.93 (s, 1H), 2.40–2.20 (br, 4H), 1.92–1.23 (m, 18H), 1.00–0.88 (2 peaks, 8H), 0.64 (s, 3H). ^1H NMR (DMSO- d_6 , 600 MHz): δ 8.25 (s, 1H), 6.35 (s, 2H), 6.31 (s, 2H), 4.14 (s, 2H), 3.76 (s, 3H), 3.70 (s, 1H), 3.67 (s, 6H), 3.57 (s, 1H), 3.15 (s, 1H), 2.22–2.08 (m, 3H), 2.04–1.90 (m, 2H), 1.76–1.59 (m, 6H), 1.44–1.18 (m, 10H), 1.13–1.06 (m, 1H), 0.90 (d, 4H, J = 4.8 Hz), 0.84–0.77 (m, 4H), 0.53 (s, 3H). ^{13}C NMR (CD₃OD, 150 MHz): δ 175.4, 161.0, 140.9, 105.1, 98.6, 72.6, 71.5, 67.7, 54.4, 46.7, 46.1, 42.8, 41.7, 41.5, 39.6, 39.0, 35.4, 35.1, 34.5, 32.7, 32.1, 29.8, 28.2, 27.3, 26.4, 22.9, 21.9, 16.4, 11.7.

***N*-(3',4'-Dichlorobenzyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (14d).**

This compound was prepared from cholic acid 7 and 3,4-dichlorobenzylamine by following method A. The product was further purified by stirring with anhydrous diethyl ether, and insoluble material was collected and vacuum-dried to furnish a white powder in 76% yield; mp 244–245 °C. TLC R_f 0.69 (AcOEt:CH₃OH, 95:5). ^1H NMR (CD₃OD, 600 MHz): δ 8.49 (t, 1H, J = 6.0 Hz), 7.43 (m, 2H), 7.18 (d, 1H, J = 8.4 Hz), 4.34–4.26 (m, 2H), 3.91 (s, 1H), 3.77 (s, 1H), 3.34 (m, 1H), 2.31–2.21 (m, 3H), 2.19–2.15 (m, 1H), 1.99–1.93 (m, 2H), 1.89–1.77 (m, 4H), 1.74–1.69 (m, 1H), 1.64 (d, 1H, J = 13.2 Hz), 1.58–1.49 (m, 5H), 1.43–1.28 (m, 4H), 1.24–1.19 (m, 1H), 1.09–1.03 (m, 1H), 1.01 (d, 3H, J = 6.0 Hz), 0.96 (t, 1H, J = 13.8 Hz), 0.89 (s, 3H), 0.65 (s, 3H). ^1H NMR (DMSO- d_6 , 600 MHz): δ 8.34 (s, 1H), 7.51 (s, 1H), 7.42 (s, 1H), 7.18 (s, 1H), 4.30–3.98 (m, 5H), 3.74 (s, 1H), 3.56 (s, 1H), 3.14 (s, 1H), 2.20–2.06 (m, 3H), 2.02 (s, 1H), 1.93 (s, 1H), 1.75–1.59 (m, 6H), 1.44–1.04 (m, 11H), 0.89 (s, 4H), 0.81–0.76 (m, 4H), 0.52 (s, 3H). ^{13}C NMR (DMSO- d_6 , 150 MHz): δ 173.3, 141.6, 131.3, 130.8, 129.6, 129.5, 127.9, 71.5, 70.9, 66.7, 46.6, 46.1, 42.0, 41.8, 41.4, 35.7, 35.5, 35.3, 34.8, 32.9, 32.2, 30.8, 29.0, 27.8, 26.6, 23.2, 23.0, 17.5, 12.7.

***N*-(4'-Aminosulfonylbzyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (14e).**

This compound was prepared from cholic acid 7 and 4-homosulfanilamide hydrochloride by following method A in 64% yield; mp 145–146 °C. TLC R_f 0.23 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ^1H NMR (DMSO- d_6 , 600 MHz): δ 8.37 (s, 1H), 7.71 (d, 2H, J = 7.2 Hz), 7.35 (d, 2H, J = 7.2 Hz), 7.28 (s, 2H), 4.27 (s, 2H), 4.11 (br s, 3H), 3.75 (s, 1H), 3.58 (s, 1H), 3.15 (s, 1H), 2.21–2.10 (m, 3H), 2.04 (m, 1H), 1.94 (m, 1H), 1.77–1.60 (m, 6H), 1.44–1.08 (m, 11H), 0.91 (d, 4H, J = 4.8 Hz), 0.83–0.78 (m, 4H), 0.55 (s, 3H). ^1H NMR (CD₃OD, 600 MHz): δ 7.82 (d, 2H, J = 7.2 Hz), 7.42 (d, 2H, J = 6.6 Hz), 4.41 (dd, 2H, J = 15.0 Hz), 3.93 (s, 1H), 3.78 (s, 1H), 3.35 (s, 1H), 2.35–2.15 (m, 4H), 1.96 (m, 2H), 1.85–1.78 (m, 4H), 1.72 (m, 1H), 1.62 (d, 1H, J = 12.6 Hz), 1.60–1.50 (m, 5H), 1.44–1.32 (m, 4H), 1.25 (m, 1H), 1.15–0.90 (m, 8H), 0.67 (s, 3H). ^{13}C NMR (CD₃OD, 150 MHz): δ 175.5, 143.5,

142.3, 127.5, 126.0, 72.6, 71.5, 67.6, 46.6, 46.1, 42.2, 41.8, 41.6, 39.6, 39.0, 35.4, 35.1, 34.5, 34.5, 32.6, 31.9, 29.8, 28.2, 27.3, 26.5, 22.8, 21.8, 16.3, 11.7.

***N*-(2'-Nitrobenzyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (14f).**

This compound was prepared from cholic acid 7 and 2nitrobenzylamine hydrochloride by following method A in 85% yield; mp 188–190 °C. TLC R_f 0.65 (AcOEt:CH₃OH, 95:5). ¹H NMR (CD₃OD, 600 MHz): δ 8.45 (s, 1H), 8.01 (d, 1H, J = 7.8 Hz), 7.65 (t, 1H, J = 6.6 Hz), 7.55 (d, 1H, J = 7.2 Hz), 7.48 (t, 1H, J = 7.2 Hz), 4.65 (s, 2H), 3.93 (s, 1H), 3.77 (s, 1H), 3.35 (s, 1H), 2.32–2.18 (m, 5H), 1.99–1.92 (m, 2H), 1.88–1.78 (m, 5H), 1.74–1.68 (m, 1H), 1.62 (d, 1H, J = 12.6 Hz), 1.60–1.50 (m, 5H), 1.44–1.35 (m, 4H), 1.26–1.19 (m, 1H), 1.10–1.06 (m, 1H), 1.00 (d, 3H, J = 5.4 Hz), 0.95 (t, 1H, J = 14.4 Hz), 0.90 (s, 3H), 0.66 (s, 3H). ¹H NMR (DMSO-d₆, 600 MHz): δ 8.37 (s, 1H), 7.97 (s, 1H), 7.68 (s, 1H), 7.48 (s, 2H), 4.48 (s, 2H), 4.30 (s, 1H), 4.08 (s, 1H), 3.98 (s, 1H), 3.75 (s, 1H), 3.57 (s, 1H), 3.37 (s, 1H), 3.15 (s, 1H), 2.22–2.06 (m, 4H), 1.94 (br, 1H), 1.78–1.55 (m, 6H), 1.44–1.04 (m, 11H), 0.91 (s, 4H), 0.78 (s, 4H), 0.54 (s, 3H). ¹³C NMR (DMSO-d₆, 150 MHz): δ 173.6, 148.4, 134.9, 134.1, 129.8, 128.6, 124.9, 71.4, 70.9, 66.7, 46.6, 46.2, 42.0, 41.8, 40.3, 35.7, 35.6, 35.3, 34.8, 32.7, 32.1, 30.8, 29.0, 27.7, 26.6, 23.2, 23.1, 17.8, 12.8. HRMS (ESI, m/z): calcd for C₃₁H₄₆N₂O₆Na [M + Na⁺] 565.3254, found 565.3259.

***N*-(4'-Nitrobenzyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (14g).**

This compound was prepared from cholic acid 7 and 4nitrobenzylamine hydrochloride by following method A in 82% yield; mp 227–228 °C. TLC R_f 0.65 (AcOEt:CH₃OH, 95:5). ¹H NMR (DMSO-d₆, 600 MHz): δ 8.44 (s, 1H), 8.13 (s, 2H), 7.45 (s, 2H), 4.33 (s, 2H), 4.07 (br, 3H), 3.74 (s, 1H), 3.56 (s, 1H), 3.14 (s, 1H), 2.22–2.02 (m, 4H), 1.94 (m, 1H), 1.78–1.54 (m, 6H), 1.44–1.14 (m, 10H), 1.08 (m, 1H), 0.90 (s, 4H), 0.82–0.72 (m, 4H), 0.52 (s, 3H). ¹H NMR (CD₃OD, 600 MHz): δ 8.16 (d, 2H, J = 8.4 Hz), 7.49 (d, 2H, J = 8.4 Hz), 4.45 (dd, 2H, J = 15.6 Hz), 3.91 (s, 1H), 3.76 (s, 1H), 3.35–3.29 (m, 1H), 2.33–2.16 (m, 4H), 2.00–1.92 (m, 2H), 1.88–1.77 (m, 4H), 1.72 (m, 1H), 1.63 (d, 1H, J = 12.6 Hz), 1.58–1.49 (m, 5H), 1.43–1.32 (m, 4H), 1.26–1.18 (m, 1H), 1.09–1.03 (m, 1H), 1.01 (d, 3H, J = 6.0 Hz), 0.95 (t, 1H, J = 12.0 Hz), 0.89 (s, 3H), 0.65 (s, 3H). ¹³C NMR (DMSO-d₆, 150 MHz): δ 173.4, 148.4, 146.7, 128.5, 123.8, 71.5, 70.9, 66.7, 46.5, 46.1, 42.0, 41.8, 40.1, 35.7, 35.5, 35.3, 34.8, 32.9, 32.1, 30.8, 28.9, 27.7, 26.6, 23.2, 23.0, 17.5, 12.7.

Reduction of Nitro Group into Amine (Method D): *N*-(2'-Aminobenzyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (14h).

Into a 500 mL high pressure Parr hydrogenating bottle, 10% Pd/C (110 mg) was placed and anhydrous CH₃OH (10 mL) was carefully added with continuous flow of argon on top of the bottleneck. A solution of 14f (506 mg, 0.93 mmol) in MeOH (140 mL) was added into the bottle, and it was subjected to hydrogenation at 65 psi for 3.5 h. After completion of the reaction (as revealed by TLC), the Pd/C was removed by filtration through a pad of Celite in a sintered filtration flask and washed with an additional 75 mL of fresh CH₃OH. The combined filtrate was concentrated in vacuo to furnish a viscous oil which formed a white solid, 14h (405 mg, 85% yield), upon drying in a high vacuum; mp 222–24 °C. TLC R_f 0.35 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ¹H NMR (CD₃OD, 600 MHz): δ 7.04 (d, 2H, J = 7.2 Hz), 7.00 (t, 1H, J = 7.8 Hz), 6.69 (d, 1H, J = 7.8 Hz), 6.62 (t, 1H, J = 7.8 Hz), 4.25 (s, 2H),

3.91 (s, 1H), 3.77 (s, 1H), 3.34 (m, 1H), 2.29–2.20 (m, 3H), 2.14–2.09 (m, 1H), 1.98–1.90 (m, 3H), 1.87–1.77 (m, 4H), 1.72–1.68 (m, 1H), 1.63 (d, 1H, $J = 12.0$ Hz), 1.58–1.49 (m, 4H), 1.43–1.27 (m, 4H), 1.24–1.19 (m, 1H), 1.16–1.06 (m, 1H), 0.98 (d, 3H, $J = 6.0$ Hz), 0.96 (m, 1H), 0.89 (s, 3H), 0.65 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 175.4, 145.4, 129.6, 128.2, 122.8, 117.5, 115.7, 72.6, 71.4, 67.6, 46.6, 46.1, 41.8, 41.6, 39.6, 39.6, 39.0, 35.4, 35.1, 34.5, 34.5, 32.7, 31.9, 29.8, 28.1, 27.3, 26.4, 22.8, 21.8, 16.3, 11.6. HRMS (ESI, m/z): calcd for $\text{C}_{31}\text{H}_{49}\text{N}_2\text{O}_4$ [$\text{M} + \text{H}^+$] 513.3693, found 513.3696.

***N*-(4'-Aminobenzyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (14i).**

This compound was prepared from 14g by following method D in 80% yield; mp 236–238 °C. TLC R_f 0.36 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ : 8.05 (s, 1H), 6.84 (s, 2H), 6.45 (d, 2H, $J = 4.8$ Hz), 4.03 (br, 5H), 3.75 (s, 1H), 3.58 (s, 1H), 3.14 (s, 1H), 2.24–12.06 (m, 3H), 1.98 (br, 2H), 1.80–1.56 (m, 6H), 1.47–1.08 (m, 11H), 0.89 (s, 4H), 0.77 (s, 4H), 0.55 (s, 3H). ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz): δ 172.8, 147.8, 128.6, 127.0, 114.1, 71.5, 70.9, 66.7, 46.6, 46.2, 42.2, 42.0, 41.8, 40.0, 35.8, 35.6, 35.3, 34.8, 33.0, 32.3, 30.8, 29.0, 27.8, 26.6, 23.4, 23.1, 17.5, 12.8.

***N*-([Pyridin-2'-yl]methyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (14j).**

This compound was prepared from cholic acid 7 and 2-(aminomethyl)pyridine by following method B in 81% yield; mp 239–241 °C. TLC R_f 0.36 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR (CD_3OD , 600 MHz): δ 8.45 (d, 1H, $J = 4.2$ Hz), 7.79 (dt, 1H, $J = 7.8$ and 1.8 Hz), 7.35 (d, 1H, $J = 7.8$ Hz), 7.29 (t, 1H, $J = 5.4$ Hz), 4.46 (s, 2H), 3.93 (s, 1H), 3.78 (s, 1H), 3.35 (m, 1H), 2.38–2.19 (m, 4H), 2.01–1.78 (m, 7H), 1.76–1.70 (m, 1H), 1.64 (d, 1H, $J = 13.2$ Hz), 1.58–1.50 (m, 4H), 1.44–1.35 (m, 4H), 1.30–1.23 (m, 1H), 1.11–1.06 (m, 1H), 1.02 (d, 3H, $J = 6.6$ Hz), 0.96 (t, 1H, $J = 13.8$ Hz), 0.90 (s, 3H), 0.68 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 175.5, 158.0, 148.2, 137.5, 122.4, 121.5, 72.6, 71.5, 67.6, 47.2, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 32.6, 31.8, 29.8, 28.2, 27.3, 26.5, 22.8, 21.8, 16.3, 11.6.

***N*-([Pyridin-3'-yl]methyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (14k).**

This compound was prepared from cholic acid 7 and 3-(aminomethyl)pyridine by following method B in 77% yield; mp 254–255 °C. TLC R_f 0.26 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 8.42 (s, 1H), 8.40 (s, 1H), 8.34 (s, 1H), 7.58 (d, 1H, $J = 6.0$ Hz), 7.30 (s, 1H), 4.30 (s, 1H), 4.23 (s, 2H), 4.07 (s, 1H), 3.99 (s, 1H), 3.76 (s, 1H), 3.58 (s, 1H), 3.15 (s, 1H), 2.22–2.08 (m, 3H), 2.05–1.92 (m, 2H), 1.80–1.55 (m, 6H), 1.44–1.14 (m, 10H), 1.08 (m, 1H), 0.89 (d, 4H, $J = 4.2$ Hz), 0.83–0.77 (m, 4H), 0.53 (s, 3H). ^{13}C NMR ($\text{DMSO}-d_6$, 150 MHz): δ 173.3, 149.1, 148.4, 135.7, 135.4, 123.8, 71.4, 70.9, 66.7, 46.6, 46.2, 42.00, 41.8, 35.7, 35.5, 35.3, 34.8, 32.9, 32.2, 30.8, 29.0, 27.8, 26.6, 23.2, 23.1, 17.5, 12.8.

***N*-(Cyclohexyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15a).**

This compound was prepared from cholic acid 7 and cyclohexylamine 11 by following method A in 92% yield; mp 140–142 °C. TLC R_f 0.58 ($\text{CH}_2\text{Cl}_2:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}$, 89:10:1). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 7.58 (s, 1H), 4.55 (br s, 3H), 3.74 (s, 1H), 3.57 (s, 1H),

3.44 (s, 1H), 3.14 (s, 1H), 2.23–1.86 (m, 5H), 1.78–1.54 (m, 10H), 1.49 (s, 1H), 1.44–1.02 (m, 16H), 0.88 (s, 4H), 0.82–0.77 (m, 4H), 0.53 (s, 3H). ¹H NMR (CD₃OD, 600 MHz): δ 3.93 (s, 1H), 3.77 (s, 1H), 3.62 (s, 1H), 3.35 (s, 1H), 2.28–2.24 (m, 2H), 2.14–2.10 (m, 1H), 2.00–1.74 (m, 11H), 1.63–1.50 (m, 7H), 1.44–1.20 (m, 10H), 1.15–0.94 (m, 5H), 0.90 (s, 3H), 0.69 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 174.9, 72.6, 71.5, 67.6, 48.7, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 32.5, 32.2, 32.2, 32.1, 29.8, 28.2, 27.3, 26.5, 25.2, 24.7, 22.8, 21.8, 16.3, 11.6.

***N*-(Cyclopentyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15b).**

This compound was prepared from cholic acid 7 and cyclopentylamine by following method A in 61% yield; mp 122–123 °C. TLC R_f 0.40 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ¹H NMR (DMSO-d₆, 600 MHz): δ 7.66 (d, 1H, J = 5.4 Hz), 4.12 (br s, 3H), 3.91 (s, 1H), 3.74 (s, 1H), 3.57 (s, 1H), 3.14 (s, 1H), 2.18–2.10 (m, 2H), 2.04–1.86 (m, 3H), 1.72 (s, 5H), 1.58 (s, 5H), 1.50–1.16 (m, 13H), 1.10 (s, 2H), 0.88 (s, 4H), 0.85–0.76 (m, 4H), 0.53 (s, 3H). ¹H NMR (CD₃OD, 600 MHz): δ 4.06 (s, 1H), 3.93 (s, 1H), 3.77 (s, 1H), 3.35 (s, 1H), 2.29–2.20 (m, 3H), 2.14–2.04 (m, 1H), 2.09–1.83 (m, 6H), 1.79–1.69 (m, 5H), 1.69–1.50 (m, 8H), 1.42–1.24 (m, 7H), 1.10–0.93 (m, 5H), 0.89 (s, 3H), 0.68 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 174.8, 72.6, 71.4, 67.6, 51.0, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 32.6, 32.1, 32.0, 29.8, 28.2, 27.3, 26.4, 23.4, 22.8, 21.8, 16.3, 11.6.

***N*-(Cyclopropyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15c).**

This compound was prepared from cholic acid 7 and cyclopropylamine by following method A in 72% yield; mp 104–106 °C. TLC R_f 0.52 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ¹H NMR (CD₃OD, 600 MHz): δ 3.92 (s, 1H), 3.77 (s, 1H), 3.35 (s, 1H), 2.61 (s, 1H), 2.27–2.17 (m, 3H), 2.05–1.73 (m, 8H), 1.63 (d, 1H, J = 12.6 Hz), 1.56–1.49 (m, 5H), 1.44–1.32 (m, 5H), 1.32–1.22 (m, 2H), 1.09 (m, 1H), 0.99–0.94 (m, 4H), 0.90 (s, 3H), 0.68 (s, 5H), 0.44 (s, 2H). ¹H NMR (DMSO-d₆, 600 MHz): δ 7.78 (s, 1H), 3.73 (s, 4H), 3.57 (s, 1H), 3.14 (s, 1H), 2.53 (s, 1H), 2.18–2.10 (m, 2H), 1.99–1.85 (m, 3H), 1.73–1.58 (m, 6H), 1.39–1.10 (m, 11H), 0.87–0.76 (m, 8H), 0.53 (s, 5H), 0.30 (s, 2H). ¹³C NMR (DMSO-d₆, 150 MHz): δ 174.2, 71.4, 70.9, 67.7, 46.5, 46.1, 42.0, 41.8, 40.0, 35.7, 35.6, 35.3, 34.8, 32.7, 32.1, 30.8, 29.0, 27.7, 26.6, 23.2, 23.0, 22.6, 17.5, 12.8, 6.1.

***N*-(Cyclopropanemethyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15d).**

This compound was prepared from cholic acid 7 and aminomethylcyclopropane by following method A; after regular work up procedure, the product was purified by column chromatography eluted from AcOEt:CH₃OH (95:5, 90:10; 85:15 and 70:30) to afford a white powder (80% yield); mp 132–134 °C. TLC R_f 0.56 (AcOEt:CH₃OH, 90:10). ¹H NMR (CD₃OD, 600 MHz): δ 3.93 (s, 1H), 3.77 (s, 1H), 3.35 (m, 1H), 3.00 (s, 2H), 2.24 (s, 3H), 2.10 (s, 1H), 1.94–1.77 (m, 7H), 1.62–1.52 (m, 6H), 1.42–1.20 (m, 6H), 1.08–0.89 (m, 8H), 0.69 (s, 3H), 0.46 (s, 2H), 0.17 (s, 2H). ¹H NMR (DMSO-d₆, 600 MHz): δ 7.79 (s, 1H), 3.74 (br s, 4H), 3.57 (s, 1H), 3.14 (s, 1H), 2.87 (s, 2H), 2.18–2.05 (m, 3H), 1.93 (s, 2H), 1.74 (s, 3H), 1.59 (s, 3H), 1.40–1.12 (m, 12H), 0.89 (s, 4H), 0.77 (s, 4H), 0.54 (s, 3H), 0.33 (s, 2H), 0.08 (s, 2H). ¹³C NMR (DMSO-d₆, 150 MHz): δ 172.9, 71.4, 70.9, 67.7, 46.6, 46.2, 43.0, 42.0, 41.8, 35.7, 35.6, 35.3, 34.8, 33.0, 32.2, 30.8, 29.0, 28.0, 27.8, 26.6, 23.2, 23.1, 17.5, 11.3, 3.5.

***N*-(Isobutyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15e).**

This compound was prepared from cholic acid 7 and isobutylamine by following method A in 72% yield; mp 115–117 °C. TLC R_f 0.51 (AcOEt:CH₃OH, 90:10). ¹H NMR (CD₃OD, 600 MHz): δ 3.92 (s, 1H), 3.77 (s, 1H), 3.34 (s, 1H), 2.96 (s, 2H), 2.24 (s, 3H), 2.11 (s, 1H), 2.05–1.20 (m, 19H), 1.15–0.70 (m, 14H), 0.68 (s, 3H). ¹H NMR (DMSO-*d*₆, 600 MHz): δ 7.71 (s, 1H), 3.97 (br s, 3H), 3.74 (s, 1H), 3.57 (s, 1H), 3.14 (s, 1H), 2.80 (s, 2H), 2.17–2.04 (m, 3H), 1.94 (s, 2H), 1.74–1.60 (m, 7H), 1.38–1.11 (m, 11H), 0.89 (s, 3H), 0.77 (s, 11H), 0.53 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 175.5, 72.6, 71.4, 67.6, 46.7, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 32.7, 32.1, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 19.2, 16.3, 11.6.

***N*-(2'-Phenylethyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (15f).**

This compound was prepared from cholic acid 7 and 2phenylethylamine by following method A in 75% yield; mp 108–110 °C. TLC R_f 0.68 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ¹H NMR (DMSO-*d*₆, 600 MHz): δ 7.83 (s, 1H), 7.22 (d, 2H, *J* = 5.4 Hz), 7.14 (s, 3H), 4.49 (br s, 3H), 3.74 (s, 1H), 3.57 (s, 1H), 3.20 (s, 2H), 3.15 (s, 1H), 2.65 (s, 2H), 2.23–1.86 (m, 5H), 1.79–1.54 (m, 6H), 1.45–1.05 (m, 11H), 0.95–0.73 (m, 8H), 0.54 (s, 3H). ¹H NMR (CD₃OD, 600 MHz): δ 7.25 (t, 2H, *J* = 7.8 Hz), 7.19–7.15 (m, 3H), 3.92 (s, 1H), 3.77 (s, 1H), 3.38–3.28 (m, 3H), 2.77 (t, 2H, *J* = 7.2 Hz), 2.29–2.20 (m, 3H), 2.09–2.04 (m, 1H), 1.99–1.92 (m, 2H), 1.90–1.72 (m, 5H), 1.63 (d, 1H, *J* = 13.2 Hz), 1.58–1.49 (m, 4H), 1.43–1.22 (m, 6H), 1.11–1.05 (m, 1H), 0.99–0.93 (m, 4H), 0.89 (s, 3H), 0.68 (s, 3H). ¹³C NMR (DMSO-*d*₆, 150 MHz): δ 173.0, 140.0, 129.0, 128.7, 126.4, 71.5, 70.9, 66.7, 46.5, 46.2, 42.0, 41.8, 40.6, 35.8, 34.8, 32.9, 32.2, 30.8, 29.0, 27.7, 26.6, 23.2, 23.0, 17.5, 12.8.

***N*-(2'-[2'',4''-Dimethoxyphenyl]ethyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15g).**

This compound was prepared from cholic acid 7 and 2,4-dimethoxyphenethylamine by following method A in 70% yield; mp 192–193 °C. TLC R_f 0.57 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ¹H NMR (CD₃OD, 600 MHz): δ 6.81 (s, 2H), 6.72 (s, 1H), 3.90 (s, 1H), 3.76 (s, 7H), 3.35 (s, 3H), 2.70 (s, 2H), 2.21 (s, 3H), 2.04–1.24 (m, 19H), 1.06 (s, 1H), 0.96 (s, 4H), 0.88 (s, 4H), 0.65 (s, 3H). ¹³C NMR (CDCl₃ + DMSO-*d*₆, 150 MHz): δ 174.1, 148.2, 147.3, 131.6, 120.6, 111.8, 111.2, 72.8, 71.6, 68.1, 55.8, 46.5, 46.2, 41.4, 40.7, 39.9, 39.8, 39.5, 39.3, 35.3, 35.1, 34.7, 33.0, 31.7, 30.3, 28.0, 27.4, 26.1, 23.1, 22.3, 17.3, 12.4.

***N*-(2',2'-Diphenylethyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15h).**

This compound was prepared from cholic acid 7 and 2,2-diphenylethylamine by following method A, and after work up procedure, the product was purified by column chromatography eluted from AcOEt:CH₃OH (90:10, 80:20 and 70:30) to afford a white powder (67% yield); mp 205–207 °C. TLC R_f 0.43 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ¹H NMR (DMSO-*d*₆, 600 MHz): δ 7.79 (s, 1H), 7.35–7.14 (m, 10H), 4.14 (s, 1H), 3.78–3.58 (m, 7H), 3.15 (s, 1H), 2.24–2.06 (m, 2H), 1.93 (s, 2H), 1.88–1.56 (m, 6H), 1.54–1.12 (m, 11H), 1.04 (m, 2H), 0.91 (m, 1H), 0.80 (s, 3H), 0.77 (s, 3H), 0.52 (s, 3H). ¹H NMR (CD₃OD, 600 MHz): δ 7.32–7.16 (m, 10H), 4.24 (t, 1H, *J* = 7.8 Hz), 3.89 (s, 1H), 3.79–3.73 (m, 3H), 3.35 (m, 1H), 2.29–2.20 (m, 2H), 2.13–2.08 (m, 1H), 1.98–1.92 (m, 3H), 1.79–1.70 (m, 4H), 1.64–1.49 (m, 6H), 1.43–1.34 (m, 3H), 1.27 (m, 1H), 1.19–1.13

(m, 2H), 1.07–1.02 (m, 1H), 0.98–0.89 (m, 7H), 0.64 (s, 3H). ^{13}C NMR (DMSO- d_6 , 150 MHz): δ 173.2, 143.4, 128.8, 128.3, 126.7, 71.4, 70.9, 66.7, 50.5, 46.6, 46.1, 43.5, 42.0, 41.8, 35.7, 35.5, 35.3, 34.8, 32.9, 32.2, 30.8, 29.0, 27.7, 26.6, 23.2, 23.1, 17.4, 12.8.

N-(Propargyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15i).

This compound was prepared from cholic acid 7 and propargylamine by following method A, and after regular work up procedure, the product was purified by column chromatography eluted from AcOEt:CH₃OH (90:10, 80:20 and 70:30) to afford a white powder (71% yield); mp 182–184 °C. TLC R_f 0.58 (AcOEt:CH₃OH, 90:10). ^1H NMR (CD₃OD, 600 MHz): δ 3.93 (s, 1H), 3.91 (s, 2H), 3.77 (s, 1H), 3.35 (t, 1H, J = 10.8 Hz), 2.55 (s, 1H), 2.30–2.22 (m, 3H), 2.13–2.08 (m, 1H), 2.00–1.70 (m, 7H), 1.63 (d, 1H, J = 13.2 Hz), 1.56–1.49 (m, 5H), 1.44–1.25 (m, 5H), 1.10 (m, 1H), 1.01–0.94 (m, 4H), 0.90 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (CD₃OD, 150 MHz): δ 175.0, 79.4, 72.6, 71.5, 70.7, 67.6, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.4, 35.1, 34.5, 32.4, 31.7, 29.8, 28.2, 28.0, 27.3, 26.4, 22.9, 21.8, 16.4, 11.7.

N-(2'-Cyanoethyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (15j).

This compound was prepared from cholic acid 7 and 3aminopropionitrile by following method A, and after regular work up procedure, the product was purified by column chromatography eluted from AcOEt:CH₃OH (95:5, 90:10; 85:15 and 70:30) to afford a white powder (67% yield); mp 206–208 °C. TLC R_f 0.45 (AcOEt:CH₃OH, 85:15). ^1H NMR (CD₃OD, 600 MHz): δ 3.93 (s, 1H), 3.77 (s, 1H), 3.38 (s, 3H), 2.64 (s, 2H), 2.25 (s, 3H), 2.13 (s, 1H), 1.95–1.73 (m, 7H), 1.62–1.52 (m, 6H), 1.37–1.29 (m, 5H), 1.09–0.96 (m, 5H), 0.89 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (CD₃OD, 150 MHz): δ 175.8, 118.1, 72.6, 71.4, 67.6, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.5, 35.2, 35.1, 34.5, 32.6, 32.5, 31.8, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 17.1, 16.3, 11.6.

N-(3'-Hydroxypropyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide (15k).

This compound was prepared from cholic acid 7 and 3amino-1-propanol by following method A in 78% yield; mp 219–221 °C. TLC R_f 0.37 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ^1H NMR (CD₃OD, 600 MHz): δ 3.93 (s, 1H), 3.77 (s, 1H), 3.56 (s, 2H), 3.35 (s, 1H), 3.23 (s, 2H), 2.30–2.23 (m, 3H), 2.10 (m, 1H), 1.98–1.62 (m, 10H), 1.56–1.50 (m, 5H), 1.42–1.26 (m, 5H), 1.08 (m, 1H), 1.01–0.94 (m, 4H), 0.90 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (CD₃OD, 150 MHz): δ 175.6, 72.6, 71.4, 67.6, 60.0, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 35.9, 35.5, 35.1, 34.5, 32.7, 32.0, 31.9, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 16.3, 11.6.

N-(4'-Ethoxy-4'-oxobutyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15l).

This compound was prepared from cholic acid 7 and ethyl 4-aminobutyrate hydrochloride by following method B and after regular work up procedure and purification to afford a white powder (53% yield); mp 74–76 °C. TLC R_f 0.71 (AcOEt:CH₃OH, 90:10). ^1H NMR (CDCl₃, 600 MHz): δ 5.89 (s, 1H), 4.11 (q, 2H, J = 7.2 Hz), 3.96 (s, 1H), 3.83 (s, 1H), 3.45 (m, 1H), 3.27 (q, 2H, J = 6.0 Hz), 2.34 (t, 2H, J = 7.4 Hz), 2.25–2.14 (m, 4H), 2.09–2.04 (m, 1H), 1.95–1.64 (m, 10H), 1.60–1.49 (m, 4H), 1.43–1.31 (m, 4H), 1.29 (m, 1H), 1.24 (t, 3H, J = 7.2 Hz), 1.15–1.07 (m, 1H), 1.00–0.95 (m, 4H), 0.88 (s, 3H), 0.67 (s, 3H). ^{13}C NMR (CDCl₃, 150 MHz): δ 174.4, 173.6, 73.0, 71.8, 68.4, 60.5, 46.4, 46.3, 41.5, 39.5, 39.4, 38.8,

35.4, 35.3, 34.7, 33.1, 31.8, 30.4, 28.1, 27.6, 26.2, 24.7, 23.2, 22.4, 17.4, 14.2, 12.4. ¹³C NMR (CD₃OD, 150 MHz): δ 175.6, 173.5, 72.6, 71.4, 67.6, 60.1, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 38.3, 35.5, 35.1, 34.4, 32.8, 31.9, 30.9, 29.8, 28.2, 27.3, 26.4, 24.4, 22.8, 21.7, 16.3, 13.1, 11.6.

N-([1'-Isopropyl-1'-methoxycarbonyl]methyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (15m).

This compound was prepared from cholic acid 7 and L-valine methyl ester by following method B, and after regular work up procedure, the product was purified by column chromatography eluted from AcOEt:CH₃OH (95:5, 90:10 and 85:15) to afford a white powder (67% yield); mp 82–84 °C. TLC R_f 0.55 (AcOEt:CH₃OH, 90:10). ¹H NMR (CD₃OD, 600 MHz): δ 4.29–4.27 (m, 1H), 3.94 (s, 1H), 3.78 (s, 1H), 3.69 (s, 3H), 3.35 (m, 1H), 2.35–2.16 (m, 4H), 2.14–2.08 (m, 1H), 2.01–1.70 (m, 7H), 1.63 (d, 1H, J = 13.2 Hz), 1.58–1.50 (m, 4H), 1.44–1.26 (m, 5H), 1.12–0.92 (m, 11H), 0.90 (s, 3H), 0.69 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 175.7, 172.4, 72.6, 71.5, 67.6, 57.8, 51.0, 47.2, 46.7, 46.1, 41.8, 41.6, 39.6, 39.0, 35.5, 35.1, 34.5, 34.4, 32.3, 31.9, 30.3, 29.8, 28.2, 27.3, 26.4, 22.8, 21.8, 18.1, 17.2, 16.3.

N-([1'-Benzyl-1'-methoxycarbonyl]methyl)-3α,7α,12α-trihydroxy-5β-cholon-24-amide (15n).

This compound was prepared from cholic acid 7 and L-phenylalanine methyl ester by following method B as a white powder in 75% yield; mp 116–118 °C. TLC R_f 0.55 (CH₂Cl₂:CH₃OH; 90:10). ¹H NMR (CD₃OD, 600 MHz): δ 8.27 (d, 1H, J = 10.2 Hz), 7.25 (m, 2H), 7.19 (m, 3H), 4.63 (dd, 1H, J = 6.0 Hz), 3.92 (s, 1H), 3.78 (s, 1H), 3.67 (s, 3H), 3.35 (m, 1H), 3.14 (dd, 1H, J = 14.4 and 4.8 Hz), 2.91 (dd, 1H, J = 13.8 and 9.6 Hz), 2.25–2.17 (m, 3H), 2.09–2.06 (m, 1H), 1.98–1.93 (m, 2H), 1.83–1.48 (m, 11H), 1.44–1.32 (m, 3H), 1.24–1.17 (m, 2H), 1.10–1.04 (m, 1H), 1.05–0.91 (m, 4H), 0.90 (s, 3H), 0.67 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 175.4, 172.2, 136.9, 128.8, 128.0, 126.4, 72.6, 71.4, 67.6, 53.8, 53.7, 51.2, 46.6, 46.0, 41.8, 41.6, 39.6, 39.0, 37.0, 35.4, 35.1, 34.5, 32.3, 31.8, 29.7, 28.1, 27.2, 26.4, 22.8, 21.7, 16.2, 11.6.

Base-Catalyzed Hydrolysis of Ester Group into Acid(Method E): N-([1'-Benzyl-1'-carboxyl]methyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (15o).

Compound 15n (275 mg, 0.482 mmol) was dissolved in THF (20 mL), and a solution of LiOH (150 mg, 6.25 mmol in 3.0 mL of H₂O) was added. The resulting solution was allowed to stir at room temperature for 48 h, and progress of the reaction was monitored by TLC (CH₂Cl₂:MeOH, 90:10). After completion of the reaction, THF was removed using rotary evaporation and ice-cold H₂O was added to dissolve the reaction material. The reaction mixture was then neutralized with dropwise addition of dilute HCl until the precipitation of product was complete. The white precipitate was collected by vacuum filtration using sintered flask, washed it with cold water, and dried under high vacuum to give 15o in 204 mg (76% yield); mp 130–32 °C. ¹H NMR (DMSO-d₆, 600 MHz): δ 12.60 (br, 1H), 8.06 (s, 1H), 7.22–7.19 (m, 5H), 4.34 (s, 1H), 3.99 (br, 3H), 3.73 (s, 1H), 3.57 (s, 1H), 3.14 (s, 1H), 3.00 (d, 1H, J = 12.0 Hz), 2.79 (t, 1H, J = 11.4 Hz), 2.22–2.00 (m, 3H), 1.92 (s, 2H), 1.77–1.55 (m, 5H), 1.49 (s, 1H), 1.43–1.16 (m, 9H), 1.05 (m, 2H), 0.96–0.77 (m, 8H), 0.52 (s, 3H). ¹H NMR (CD₃OD, 600 MHz): δ 7.24–7.20 (m, 5H), 4.63 (s, 1H),

3.91 (s, 1H), 3.77 (s, 1H), 3.34 (br s, 1H), 3.18 (d, 1H, $J = 11.4$ Hz), 2.91 (s, 1H), 2.30–2.15 (m, 3H), 2.00 (s, 1H), 1.94 (s, 2H), 1.79–1.47 (m, 10H), 1.47–1.14 (m, 4H), 1.18 (s, 2H), 1.07 (s, 1H), 0.95 (s, 4H), 0.89 (s, 3H), 0.66 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 175.3, 173.4, 137.1, 128.8, 128.0, 126.3, 72.6, 71.4, 67.0, 53.5, 46.1, 46.0, 41.8, 41.5, 39.6, 39.0, 37.0, 35.4, 35.1, 34.5, 34.4, 32.4, 31.8, 29.7, 28.1, 27.2, 26.4, 22.8, 21.8, 16.3, 11.6. HRMS (ESI, m/z): calcd for $\text{C}_{33}\text{H}_{49}\text{NO}_6\text{Na}$ [$M + \text{Na}^+$] 578.3458, found 578.3477.

***N*-(1'-Benzyloxycarboxyl-1'-[2''-benzyloxycarboxylethyl)methyl]-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15p).**

This compound was prepared from cholic acid 7 and L-glutamic acid dibenzyl ester 4-toluenesulfonate by following method B, and after regular work up procedure, the product was purified by column chromatography eluted from CH_2Cl_2 : CH_3OH (95:5, 90:10 and 80:20) to afford a white powder (61% yield); mp 68–69 °C. TLC R_f 0.58 (AcOEt : CH_3OH , 95:5). ^1H NMR (CDCl_3 , 600 MHz): δ 7.30 (s, 10H), 6.57 (s, 1H), 5.12 (s, 2H), 5.06 (s, 2H), 4.63 (s, 1H), 3.91 (s, 1H), 3.78 (s, 1H), 3.41 (s, 1H), 3.39 (s, 1H), 3.20 (br, 1H), 2.97 (s, 1H), 2.42 (m, 1H), 2.33 (m, 1H), 2.18–2.11 (m, 5H), 1.99 (d, 1H, $J = 6.0$ Hz), 1.86–1.61 (m, 9H), 1.54–1.48 (m, 4H), 1.38 (s, 4H), 1.22 (d, 1H, $J = 8.4$ Hz), 1.04 (s, 1H), 0.94 (s, 4H), 0.84 (s, 3H), 0.62 (s, 3H). ^{13}C NMR (CDCl_3 , 150 MHz): δ 174.0, 172.7, 172.0, 135.7, 135.2, 128.6, 128.6, 128.4, 128.3, 128.2, 73.1, 71.8, 68.4, 67.3, 66.5, 51.6, 46.6, 46.4, 41.6, 41.4, 39.4, 35.3, 34.8, 34.7, 32.9, 31.4, 30.4, 30.3, 28.1, 27.5, 27.2, 26.3, 23.2, 22.4, 17.5, 12.5.

Cleavage of the Benzyl Groups (Method F): *N*-(1'-Carboxyl-1'-[2''-carboxylethyl)-methyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15q).

Into a Parr hydrogenating bottle, 10% Pd/C (100 mg) was placed and anhydrous CH_3OH (10 mL) was carefully added with continuous flow of argon on top of the bottleneck. A solution of 15p (475 mg, 0.66 mmol) in CH_3OH (100 mL) was added into the bottle, and it was subjected to hydrogenation at 60 psi for 3 h and then at 75 psi for additional 3 h. After completion of the reaction (as revealed by TLC), the Pd/C was removed by filtration through a pad of Celite in sintered filtration flask and washed with additional 50 mL of CH_3OH . The combined filtrate was concentrated by rotary evaporator to furnish a viscous oil which on subjecting on high vacuum produced a white solid 15q (74% yield). TLC R_f 0.29 (AcOEt : CH_3OH , 1:1). ^1H NMR (CD_3OD , 600 MHz): δ 4.39 (m, 1H), 3.94 (s, 1H), 3.78 (s, 1H), 3.37 (m, 1H), 2.37 (t, 2H, $J = 7.8$ Hz), 2.33–2.21 (m, 3H), 2.19–2.14 (m, 2H), 2.00–1.73 (m, 7H), 1.65–1.50 (m, 6H), 1.12–1.06 (m, 1H), 1.03 (d, 3H, $J = 6.6$ Hz), 0.97 (dt, 1H, $J = 13.2$ and 3.6 Hz), 0.90 (s, 3H), 0.70 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 175.6, 174.9, 173.6, 72.7, 71.5, 67.7, 51.5, 48.5, 46.7, 46.1, 41.7, 41.6, 39.6, 39.0, 35.4, 35.1, 34.5, 34.4, 32.1, 31.8, 29.8, 29.7, 28.1, 27.3, 26.4, 26.4, 22.8, 21.7, 16.3, 11.6.

***N*-(3'-tert-Butyloxycarbonylaminopropyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15r).**

This compound was prepared from cholic acid 7 and *N*-(tert-butyloxycarbonyl)-1,3-diaminopropane by following method B. The product was purified by column chromatography using AcOEt : MeOH (95:5; 90:10; 85:15) to produce a white powder in 76% yield; mp 182–183 °C. TLC R_f 0.52 (CH_2Cl_2 : CH_3OH , 90:10). ^1H NMR (CD_3OD , 600 MHz): δ 3.93 (s, 1H), 3.77 (s, 1H), 3.35 (s, 1H), 3.17 (s, 2H), 3.04 (s, 2H), 2.30–2.23 (m, 3H), 2.10 (s, 1H), 1.98–1.70 (m, 7H), 1.61–1.50 (m, 9H), 1.41–1.22 (m, 13H), 1.10–0.93

(m, 5H), 0.89 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 175.5, 157.6, 78.5, 72.6, 71.5, 67.6, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 37.3, 36.3, 35.5, 35.1, 34.5, 32.8, 31.9, 29.8, 29.4, 28.2, 27.5, 27.3, 26.4, 22.9, 21.9, 16.4, 11.7.

***N*-(4'-tert-Butyloxycarbonylaminobutyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15s).**

This compound was prepared from cholic acid 7 and *N*-(tert-butyloxycarbonyl)-1,4-diaminobutane by following method B, and after regular work up procedure, the product was purified by column chromatography eluted from AcOEt:CH₃OH (90:10, 85:15; 80:20 and 70:30) to afford a white powder (68% yield); mp 102–103 °C. TLC R_f 0.50 (CH_2Cl_2 :CH₃OH, 90:10). ^1H NMR (CD_3OD , 600 MHz): δ 3.92 (s, 1H), 3.77 (s, 1H), 3.34 (s, 1H), 3.14 (s, 2H), 3.01 (s, 2H), 2.23 (s, 4H), 2.08–1.10 (m, 28H), 1.08–0.89 (m, 8H), 0.68 (s, 3H). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 7.69 (s, 1H), 6.74 (s, 1H), 4.20 (s, 1H), 4.06 (s, 1H), 3.98 (s, 1H), 3.74 (s, 1H), 3.57 (s, 1H), 3.15 (s, 1H), 2.95 (s, 2H), 2.85 (s, 2H), 2.20–1.92 (m, 6H), 1.80–1.00 (m, 25H), 0.89 (s, 4H), 0.77 (s, 4H), 0.54 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 175.4, 157.1, 78.4, 72.6, 71.4, 67.6, 46.6, 46.1, 41.8, 41.6, 39.6, 39.0, 38.8, 38.6, 35.5, 35.1, 34.5, 32.8, 32.0, 29.8, 27.4, 27.3, 27.0, 26.4, 26.3, 22.8, 21.8, 16.4, 11.7.

***N*-(3'-Aminopropyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide Hydrochloride (15t).**

This compound was prepared from 15r by following method C in 86% yield. TLC R_f 0.61 (CH_3OH : NH_4OH , 90:10). ^1H NMR (CD_3OD , 600 MHz): δ 3.94 (s, 1H), 3.78 (s, 1H), 3.37 (m, 1H), 3.29 (t, 2H, $J = 7.2$ Hz), 2.93 (t, 2H, $J = 6.6$ Hz), 2.32–2.15 (m, 4H), 1.97–1.72 (m, 9H), 1.64 (d, 1H, $J = 13.2$ Hz), 1.58–1.50 (m, 6H), 1.44–1.36 (m, 4H), 1.28 (m, 1H), 1.03 (d, 3H, $J = 6.0$ Hz), 0.97 (t, 1H, $J = 13.2$ Hz), 0.90 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 176.7, 72.6, 71.5, 67.6, 46.5, 46.0, 41.7, 41.7, 39.5, 39.0, 36.9, 35.8, 35.6, 35.0, 34.5, 32.3, 31.9, 29.7, 28.2, 27.3, 27.2, 26.5, 22.8, 21.7, 16.3, 11.5.

***N*-(4'-Aminobutyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24amide Hydrochloride (15u).**

This compound was prepared from 15s by following method C in 89% yield. TLC R_f 0.63 (CH_3OH : NH_4OH , 90:10). ^1H NMR (CD_3OD , 600 MHz): δ 3.93 (s, 1H), 3.78 (s, 1H), 3.36 (m, 1H), 3.21 (m, 2H), 2.93 (t, 2H, $J = 7.2$ Hz), 2.32–2.21 (m, 3H), 2.16–2.09 (m, 1H), 2.00–1.70 (m, 7H), 1.68–1.50 (m, 9H), 1.43–1.33 (m, 5H), 1.29–1.24 (m, 1H), 1.11–1.08 (m, 1H), 1.02 (d, 3H, $J = 6.6$ Hz), 0.97 (m, 1H), 0.90 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (CD_3OD , 150 MHz): δ 176.3, 72.6, 71.4, 67.6, 46.4, 46.1, 41.7, 41.6, 39.5, 39.0, 38.9, 38.6, 35.6, 35.0, 34.5, 32.2, 31.9, 29.7, 28.2, 27.3, 26.5, 25.8, 24.5, 22.8, 21.7, 16.3, 11.5.

***N*-(2'-[Pyrrolidin-1"-yl]ethyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15v).**

This compound was prepared from cholic acid 7 and 1-(2-aminoethyl)pyrrolidine by following method B in 65% yield; mp 118–120 °C. TLC R_f 0.46 (CH_2Cl_2 :CH₃OH: NH_4OH , 78:20:2). ^1H NMR (CD_3OD , 600 MHz): δ 3.93 (s, 1H), 3.78 (s, 1H), 3.54–3.47 (m, 3H), 3.36 (m, 2H), 3.29 (t, 4H $J = 6.0$ Hz), 2.32–3.25 (m, 3H), 2.23–2.07 (m, 4H), 2.00–1.72 (m, 8H), 1.63 (d, 1H, $J = 11.4$ Hz), 1.57–1.49 (m, 5H), 1.43–1.34 (m, 4H), 1.27 (m, 1H), 1.13–1.07 (m, 1H), 1.02 (d, 3H, $J = 6.0$ Hz), 0.97 (dt, 1H, $J = 14.4$ and 3.0 Hz), 0.90 (s, 3H), 0.70 (s, 3H). ^1H NMR ($\text{DMSO}-d_6$, 600 MHz): δ 7.99 (s, 1H), 4.31 (s, 1H), 4.08 (s, 1H),

3.98 (s, 1H), 3.75 (s, 1H), 3.57 (s, 1H), 3.31 (br s, 5H), 3.13 (m, 4H), 2.20–2.09 (m, 3H), 1.96–1.89 (m, 5H), 1.77–1.70 (m, 3H), 1.59 (s, 3H), 1.40–1.20 (m, 11H), 1.12 (m, 1H), 0.93–0.89 (m, 4H), 0.83–0.77 (m, 4H), 0.55 (s, 3H). ¹³C NMR (DMSO-d₆, 150 MHz): δ 174.0, 71.4, 70.8, 67.7, 54.0, 53.8, 46.4, 46.1, 41.9, 41.8, 35.7, 35.3, 34.8, 32.7, 31.8, 30.8, 29.0, 27.7, 26.7, 23.2, 23.0, 22.9, 17.5, 12.8.

***N*-(2'-[Thiophen-2"-yl]ethyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (15w).**

This compound was prepared from cholic acid 7 and 2-(2-aminoethyl)thiophene by following method A in 81% yield; mp 180–182 °C. TLC R_f 0.44 (CH₂Cl₂:CH₃OH:NH₄OH, 89:10:1). ¹H NMR (CD₃OD, 600 MHz): δ 8.04 (s, 1H), 7.16 (s, 1H), 6.89 (s, 1H), 6.83 (s, 1H), 3.91 (s, 1H), 3.76 (s, 1H), 3.37 (s, 3H), 2.97 (s, 2H), 2.22 (s, 3H), 2.06 (s, 1H), 1.93–1.28 (m, 18H), 1.15–0.81 (m, 8H), 0.67 (s, 3H). ¹H NMR (DMSO-d₆, 600 MHz): δ 7.89 (s, 1H), 7.28 (s, 1H), 6.90 (s, 1H), 6.82 (s, 1H), 4.29 (s, 1H), 4.06 (s, 1H), 3.98 (s, 1H), 3.74 (s, 1H), 3.57 (s, 1H), 3.21 (s, 2H), 3.15 (s, 1H), 2.86 (s, 2H), 2.17–1.93 (m, 5H), 1.74–1.60 (m, 6H), 1.38–1.13 (m, 11H), 0.88 (s, 4H), 0.76 (s, 4H), 0.54 (s, 3H). ¹³C NMR (DMSO-d₆, 150 MHz): δ 175.5, 141.2, 126.4, 124.8, 123.2, 72.6, 71.4, 67.6, 46.6, 46.1, 41.8, 41.6, 40.8, 40.7, 39.6, 39.0, 35.5, 35.1, 34.5, 32.7, 31.9, 29.8, 29.1, 28.2, 27.3, 26.4, 22.8, 21.8, 16.3, 11.6.

***N*-(2'-[1'',3''-Dioxoisindolin-2''-yl]ethyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (15x).**

This compound was prepared from cholic acid 7 and *N*-(2-aminoethyl)phthalimide hydrochloride by following method A in 84% yield; mp 112–114 °C. TLC R_f 0.31 (AcOEt:CH₃OH, 90:10). ¹H NMR (CD₃OD, 600 MHz): δ 7.83 (m, 2H), 7.78 (m, 2H), 3.90 (s, 1H), 3.77 (s, 3H), 3.44 (s, 2H), 3.34 (m, 1H), 2.29–2.20 (m, 2H), 2.16–2.11 (m, 1H), 1.99–1.92 (m, 3H), 1.83–1.77 (m, 2H), 1.72–1.49 (m, 8H), 1.44–1.33 (m, 4H), 1.25–1.18 (m, 2H), 1.10–1.02 (m, 1H), 0.98–0.89 (m, 7H), 0.65 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 175.9, 168.4, 133.9, 132.0, 122.7, 72.6, 71.4, 67.6, 46.6, 46.0, 41.8, 41.6, 39.6, 39.0, 37.3, 37.2, 35.5, 35.1, 34.5, 34.4, 32.6, 31.7, 29.8, 28.2, 27.2, 26.4, 22.8, 21.8, 16.2, 11.6. HRMS (ESI, m/z): calcd for C₃₄H₄₈N₂O₆Na [M + Na⁺] 603.3410, found 603.3306.

***N*-(3'-[1'',3''-Dioxoisindolin-2''-yl]propyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (15y).**

This compound was prepared from cholic acid 7 and *N*-(3-aminopropyl)phthalimide by following method A in 81% yield; mp 110–112 °C. TLC R_f 0.71 (AcOEt:CH₃OH, 95:5). ¹H NMR (CD₃OD, 600 MHz): δ 7.81 (s, 2H), 7.77 (s, 2H), 3.92 (s, 1H), 3.76 (s, 1H), 3.68 (t, 2H, J = 6.0 Hz), 3.34 (s, 1H), 3.19 (s, 2H), 2.29–2.20 (m, 3H), 2.11–2.08 (m, 1H), 1.99–1.70 (m, 10H), 1.63 (d, 1H, J = 12.6 Hz), 1.56–1.48 (m, 4H), 1.43–1.25 (m, 5H), 1.08 (m, 1H), 1.01 (d, 3H, J = 6.0 Hz), 0.95 (t, 1H, J = 13.8 Hz), 0.88 (s, 3H), 0.67 (s, 3H). ¹H NMR (DMSO-d₆, 600 MHz): δ 7.78 (s, 4H), 4.04 (s, 4H), 3.72 (s, 1H), 3.52 (s, 2H), 3.13 (s, 1H), 3.00 (s, 2H), 2.16–2.02 (m, 3H), 1.91 (s, 2H), 1.72–1.57 (m, 8H), 1.11 (m, 11H), 0.87 (s, 4H), 0.74 (s, 4H), 0.51 (s, 3H). ¹³C NMR (CD₃OD + DMSO-d₆, 150 MHz): δ 175.4, 168.3, 134.1, 132.0, 122.8, 72.4, 71.4, 67.5, 46.6, 46.1, 41.8, 41.6, 39.7, 39.2, 36.7, 35.5, 35.3, 35.2, 34.6, 34.5, 32.7, 32.0, 29.9, 28.3, 28.0, 27.4, 26.5, 22.1, 21.9, 16.5, 11.8.

***N*-(3'-[2'',3''-Dioxoindolin-1''-yl]propyl)-3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-amide (15z).**

This compound was prepared from cholic acid 7 and *N*-(3'-aminopropyl)isatin by following method B in 73% yield; mp 118–120 °C. TLC R_f 0.49 (CH₂Cl₂:CH₃OH, 90:10). ¹H NMR (CD₃OD, 600 MHz): δ 7.99 (t, 1H, J = 5.4 Hz), 7.63 (t, 1H, J = 7.8 Hz), 7.57 (d, 1H, J = 7.2 Hz), 7.14 (t, 1H, J = 7.8 Hz), 7.10 (d, 1H, J = 7.8 Hz), 3.92 (s, 1H), 3.73 (m, 3H), 3.34 (m, 1H), 3.23 (m, 2H), 2.30–2.20 (m, 3H), 2.11–2.06 (m, 1H), 1.98–1.82 (m, 5H), 1.81–1.70 (m, 3H), 1.63 (d, 1H, J = 13.2 Hz), 1.57–1.49 (m, 5H), 1.43–1.25 (m, 6H), 1.10–1.06 (m, 1H), 1.01 (d, 3H, J = 6.6 Hz), 0.96 (dt, 1H, J = 14.4 and 3.0 Hz), 0.89 (s, 3H), 0.67 (s, 3H). ¹³C NMR (CD₃OD, 150 MHz): δ 183.2, 175.5, 158.8, 150.7, 138.1, 130.3, 124.5, 123.7, 123.5, 122.7, 117.8, 110.4, 72.6, 71.5, 67.6, 46.6, 46.1, 41.6, 39.6, 39.1, 37.5, 36.7, 36.6, 35.5, 35.1, 34.5, 32.8, 31.9, 29.8, 28.2, 27.3, 26.8, 26.5, 22.9, 21.9, 16.4, 11.7. HRMS (ESI, m/z): calcd for C₃₅H₅₀N₂O₆Na [M + Na⁺] 617.3566, found 617.3553.

Bacterial Strains and Spore Preparation.

C. difficile R20291 was the kind gift of Dr. Nigel Minton (University of Nottingham). *C. difficile* cells were streaked onto BHIS (brain heart infusion supplemented with 20 mg/mL yeast extract, 0.1% L-cysteine, and 0.05% sodium taurocholate) agar to yield single colonies. Single *C. difficile* colonies were grown in BHIS (brain heart infusion supplemented with 5 mg/mL yeast extract) broth overnight and spread onto BHIS agar to obtain bacterial lawns. The plates were incubated for 7 days at 37 °C in an anaerobic environment (10% CO₂, 10% H₂, and 80% N₂). The resulting bacterial lawns were collected by flooding the plates with ice-cold deionized water. The spores were pelleted and washed three times by centrifugation at 8800g for 5 min. To remove any contaminating vegetative cells, the spores were purified through a 20–50% HistoDenz gradient at 18200g for 30 min. The resulting spore pellet was washed five times with water, resuspended in a 0.05% sodium thioglycolate solution, and stored at 4 °C.

C. *difficile* Spore Germination Assays.

Purified *C. difficile* spores were pelleted and washed with deionized water three times by centrifugation at 9400g to remove the storage buffer. The spores were heat activated at 68 °C for 30 min, then washed an additional three times to remove any spores that autogerminated. The spores were diluted to an optical density at 580 nm (OD₅₈₀) of 1.0 with a 100 mM sodium phosphate buffer, pH 6.0, containing 5 mg/mL sodium bicarbonate. To test for antagonists of spore germination, a 96-well plate was prepared by adding compounds to separate wells in triplicate along with 6 mM taurocholate and 12 mM glycine. Upon the addition of spores, the OD₅₈₀ was measured once every minute for 2 h and normalized using the OD₅₈₀ obtained at time zero [relative OD₅₈₀ = OD₅₈₀(t)/OD₅₈₀(t₀)]. The percent germination versus the log concentration of drug was plotted, and the IC₅₀ values were calculated using eq 1.

$$y = \min + \frac{(\max - \min)}{1 + \left(\frac{x}{IC_{50}}\right)^n} \quad (1)$$

Molecular Descriptors.

Semiempirical (AM1,⁶⁴ MNDO,⁶⁵ and PM3^{66–69}), traditional 2D, 2^{1/2}D,^{70,71} and VolSurf-like molecular descriptors^{72,73} were calculated for the descriptor pool. The 2D molecular descriptors, occasionally referred to as traditional descriptors, are the numerical properties evaluated from the connection tables representing a molecule and include physical properties, subdivided surface areas,^{70,74,75} atom counts, bond counts, Kier and Hall connectivity and kappa shape indices,^{51,52} adjacency and distance matrix descriptors containing BCUT⁷⁶ and GCUT descriptors, pharmacophore feature descriptors, and partial charge descriptors. The partial charge descriptors are based on the assigned atomic partial charges or the partial equalization of orbital electronegativity atomic partial charges by Gasteiger and Marsili⁵¹ that are commonly referred to as PEOE or Gasteiger atomic partial charges. A 2^{1/2}D molecular descriptor is defined herein as a 3D molecular property represented as an individual (singular) numerical value. In this case, the 2^{1/2}D molecular descriptors include measures of the conformational potential energy and its components, molecular surfaces, volumes and shapes, and conformation dependent charge descriptors. All of the 2^{1/2}D descriptors are dependent on the conformation of the molecule. A description of MOE molecular descriptors is available on the Chemical Computing Group, Inc. web site.⁴⁶

The VolSurf-like molecular descriptors^{72,73} are aligned independently and not strongly dependent on molecular conformation, and like the 2^{1/2}D molecular descriptors represent 3D molecular properties as a single numerical value. The compound is placed in a grid (with the exception of four VolSurf descriptors), a hydrophobic (dry) and hydrophilic (wet) probe visits each grid point, and the interaction energy between the probe and the compound is calculated. The grid points within an interaction energy range are considered an isocontour (isosurface), and the volume is calculated. The calculated volumes and combinations of interaction energies and volumes are used as molecular descriptors. The four nongrid VolSurf descriptors measure the molecular volume, surface area, globularity, and rugosity.

The trial descriptor pool initially contained 343 molecular descriptors divided into three main types: semiempirical, physicochemical features, and molecular interaction fields. The semiempirical molecular descriptors contain seven unique descriptors calculated by each of the AM1,⁶⁴ MNDO,⁶⁵ and PM3^{66–69} semiempirical methods. The physicochemical features (2D and 2^{1/2}D molecular descriptors) contained 243 molecular descriptors and VolSurf-like molecular interaction fields provided 76 molecular descriptors. This collection of molecular descriptors were calculated within the Molecular Operating Environment (MOE) 2016.0802⁷⁷ using the OPLS-AA molecular force field⁷⁸ and Born solvation.⁴⁵ Descriptors with no variance (the same value), near-zero variance (95% of the values are the same), and high correlation to other molecular descriptors (absolute pairwise correlations values equal to or greater than 0.90; descriptors with the largest mean absolute correlation were removed) were removed and resulted in a descriptor pool with 224 independent variables. The molecular descriptors were normalized (mean-centered and scaled) so that each descriptor had a mean of zero and a standard deviation of one. The IC₅₀ values were converted to negative-log₁₀ values.

Descriptor Selection and Model Evaluation.

Ensembles of QSAR models were constructed using a genetic algorithm^{79,80} (also known as a GA) and the previously described trial descriptor pool. A genetic function approximation,⁴⁷ a version of a genetic algorithm, was used in this study for molecular descriptor selection with an initial population of 500 models. For each evolutionary step, the top 100 models were subjected to a combination of mutagenesis (60% of the models are mutated) and crossover functions. The top models were (i) mutated, (ii) crossed-over, and (iii) crossed-over and mutated, resulting in approximately 350 “new” models, after removing duplicate models, for each evolutionary cycle. To overcome descriptor selection becoming trapped in a “local minima,” if the top 100 models remained constant for 10 generations, a new set of 300 models were generated and added to the 100 established models to inject diversity to the population. The evolution of the training set was considered complete (stable) after 1000 generations with no change in the top 100 models. Models with an explicit number of descriptor, two to seven, and a set of models where the evolutionary process dictated the number of molecular descriptors (between two and eight molecular descriptors) were selected. The constructed multiple linear regression models were evaluated and ranked based on their Q^2 values⁸¹ (leave-one-out crossvalidation) throughout as well as the end of the evolutionary process. The top 100 models were analyzed using evaluation measures such as R^2 , R^2_{adj} , Q^2 , F, mean absolute error (MAE), root-mean-square error (RMSE), p-value, relative descriptor importance/contribution to the captured variance,⁸² and variance inflation factor^{83–85}(VIF). The models were constructed and analyzed using the Opdagelse Predictive ModelingToolkit⁴⁸ (version 1.1.0) in R⁴⁹ (version 3.4.3).

Consensus Models.

Models were selected based on their R^2_{adj} and Q^2 values, molecular descriptors with variance inflation factor^{83–85} (VIF), and p-values, indicating they significantly contribute to the model, lack of cross-correlation between models’ residual values, and diversity of the models’ molecular descriptor composition. Preference was given to models with descriptor measuring physicochemical properties over those indicating the compound complies or violates a collection of measures. The consensus predicted values are the mean predicted values for the selected QSAR models.

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ABBREVIATIONS USED

CDI	Clostridium difficile infection
CamSA	cholic amide msulfonic acid
CDAD	Clostridium difficile associated disease

HBTU	(O-[benzotriazol-1-yl]-N,N,N',N'-tetramethyl-uronium hexafluorophosphate)
QSAR	quantitative structure– activity relationship
MOE	Molecular Operating Environment
NMM	N-methyl morpholine
DMF	dimethylformamide
BHIS	brain heart infusion supplemented
TLC	thin layer chromatography
NAP1	North American pulsed-field type 1
HRMS	high resolution mass spectroscopy
OD	optical density
TcdA	Clostridium difficile toxin A
TcdB	Clostridium difficile toxin B
MAE	mean absolute error
RMSE	root-meansquare error

REFERENCES

- (1). Abt MC; McKenney PT; Pamer EG Clostridium Difficile Colitis: Pathogenesis and Host Defence. Nat. Rev. Microbiol 2016, 14 (10), 609–620. [PubMed: 27573580]
- (2). McFarland LV Update on the Changing Epidemiology of Clostridium Difficile-Associated Disease. Nat. Clin. Pract. Gastroenterol. Hepatol 2008, 5 (1), 40–48. [PubMed: 18174906]
- (3). Cookson B Hypervirulent Strains of Clostridium Difficile. Postgrad. Med. J 2007, 83 (979), 291–295. [PubMed: 17488855]
- (4). McDonald LC; Gerding DN; Johnson S; Bakken JS; Carroll KC; Coffin SE; Dubberke ER; Garey KW; Gould CV; Kelly C; Loo V; Shaklee Sammons J; Sandora TJ; Wilcox MH Clinical Practice Guidelines for Clostridium Difficile Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin. Infect. Dis 2018, 66 (7), 987–994. [PubMed: 29562266]
- (5). Durre P, Physiology and Sporulation in Clostridium. In The Bacterial Spore: From Molecules to Systems; Eichenberger P, Drik A, Eds.; ASM Press: Washington, DC, 2016; pp 315–329.
- (6). Paredes-Sabja D; Shen A; Sorg JA Clostridium Difficile Spore Biology: Sporulation, Germination, and Spore Structural Proteins. Trends Microbiol. 2014, 22 (7), 406–416. [PubMed: 24814671]
- (7). Sorg JA; Sonenshein AL Bile Salts and Glycine as Cogermnants for Clostridium Difficile Spores. J. Bacteriol 2008, 190 (7), 2505–2512. [PubMed: 18245298]
- (8). Sorg JA; Sonenshein AL Chenodeoxycholate Is an Inhibitor of Clostridium Difficile Spore Germination. J. Bacteriol 2009, 191 (3), 1115–1117. [PubMed: 19060152]
- (9). Sorg JA; Sonenshein AL Inhibiting the Initiation of Clostridium Difficile Spore Germination Using Analogs of Chenodeoxycholic Acid, a Bile Acid. J. Bacteriol 2010, 192 (19), 4983–4990. [PubMed: 20675492]
- (10). Setlow P; Wang SW; Li YQ Germination of Spores of the Orders Bacillales and Clostridiales. Annu. Rev. Microbiol 2017, 71, 459–477. [PubMed: 28697670]

- (11). Leffler DA; Lamont JT Clostridium Difficile Infection. *N. Engl. J. Med* 2015, 372 (16), 1539–1548. [PubMed: 25875259]
- (12). Moir A; Cooper G, Spore Germination In The Bacterial Spore: From Molecules to Systems; Drik A, Eichenberger P, Eds.; ASM Press: Washington, DC, 2016; pp 217–236.
- (13). Chandrasekaran R; Lacy DB The Role of Toxins in Clostridium Difficile Infection. *FEMS Microbiol. Rev* 2017, 41 (6), 723–750. [PubMed: 29048477]
- (14). Hopkins RJ; Wilson RB Treatment of Recurrent Clostridium Difficile Colitis: A Narrative Review. *Gastroenterol Rep. (Oxf)* 2018, 6 (1), 21–28. [PubMed: 29479439]
- (15). Kelly CP; LaMont JT Clostridium Difficile—More Difficult Than Ever. *N. Engl. J. Med* 2008, 359 (18), 1932–1940. [PubMed: 18971494]
- (16). Desai K; Gupta SB; Dubberke ER; Prabhu VS; Browne C; Mast TC Epidemiological and Economic Burden of Clostridium Difficile in the United States: Estimates from a Modeling Approach. *BMC Infect. Dis* 2016, 16, 303. [PubMed: 27316794]
- (17). Walker AS; Eyre DW; Wyllie DH; Dingle KE; Griffiths D; Shine B; Oakley S; O'Connor L; Finney J; Vaughan A; Crook DW; Wilcox MH; Peto TE Relationship between Bacterial Strain Type, Host Biomarkers, and Mortality in Clostridium Difficile Infection. *Clin. Infect. Dis* 2013, 56 (11), 1589–1600. [PubMed: 23463640]
- (18). Stabler RA; He M; Dawson L; Martin M; Valiente E; Corton C; Lawley TD; Sebaihia M; Quail MA; Rose G; Gerding DN; Gibert M; Popoff MR; Parkhill J; Dougan G; Wren BW Comparative Genome and Phenotypic Analysis of Clostridium Difficile 027 Strains Provides Insight into the Evolution of a Hypervirulent Bacterium. *Genome Biol.* 2009, 10 (9) R102.
- (19). Smits WK Hype or Hypervirulence a Reflection on Problematic C. Difficile Strains. *Virulence* 2013, 4 (7), 592–596. [PubMed: 24060961]
- (20). Nguyen CT; Li J; Anders S; Garcia-Diaz J; Staffeld-Coit C; Hand J Comparison of Outcomes with Vancomycin or Metronidazole for Mild-to-Moderate Clostridium Difficile Associated Diarrhea among Solid Organ Transplant Recipients: A Retrospective Cohort Study. *Transpl. Infect. Dis* 2018, 20, e12867. [PubMed: 29512244]
- (21). Zhanel GG; Walkty AJ; Karlowsky JA Fidaxomicin: A Novel Agent for the Treatment of Clostridium Difficile Infection. *Can. J. Infect. Dis. Med. Microbiol* 2015, 26 (6), 305–312.
- (22). Kelly BJ; Tebas P Clinical Practice and Infrastructure Review of Fecal Microbiota Transplantation for Clostridium Difficile Infection. *Chest* 2018, 153 (1), 266–277. [PubMed: 28923757]
- (23). Cammarota G; Ianiro G; Tilg H; Rajilic-Stojanovic M; Kump P; Satokari R; Sokol H; Arkkila P; Pintus C; Hart A; Segal J; Aloï M; Masucci L; Molinaro A; Scaldaferrì F; Gasbarrini G; Lopez-Sanroman A; Link A; de Groot P; de Vos WM; Hogenauer C; Malfertheiner P; Mattila E; Milosavljevic T; Nieuwdorp M; Sanguinetti M; Simren M; Gasbarrini A European Consensus Conference on Faecal Microbiota Transplantation in Clinical Practice. *Gut* 2017, 66 (4), 569–580. [PubMed: 28087657]
- (24). Giel JL; Sorg JA; Sonenshein AL; Zhu J Metabolism of Bile Salts in Mice Influences Spore Germination in Clostridium Difficile. *PLoS One* 2010, 5 (1), e8740. [PubMed: 20090901]
- (25). Koenigsnecht MJ; Theriot CM; Bergin IL; Schumacher CA; Schloss PD; Young VB Dynamics and Establishment of Clostridium Difficile Infection in the Murine Gastrointestinal Tract. *Infect. Immun* 2015, 83 (3), 934–941. [PubMed: 25534943]
- (26). Howerton A; Ramirez N; Abel-Santos E Mapping Interactions between Germinants and Clostridium Difficile Spores. *J. Bacteriol* 2011, 193 (1), 274–282. [PubMed: 20971909]
- (27). Ramirez N; Liggins M; Abel-Santos E Kinetic Evidence for the Presence of Putative Germination Receptors in Clostridium Difficile Spores. *J. Bacteriol* 2010, 192 (16), 4215–4222. [PubMed: 20562307]
- (28). Howerton A; Patra M; Abel-Santos E A New Strategy for the Prevention of Clostridium Difficile Infection. *J. Infect. Dis* 2013, 207 (10), 1498–1504. [PubMed: 23420906]
- (29). Howerton A; Patra M; Abel-Santos E Fate of Ingested Clostridium Difficile Spores in Mice. *PLoS One* 2013, 8 (8), e72620. [PubMed: 24023628]
- (30). Weingarden AR; Chen C; Zhang NN; Graiziger CT; Dosa PI; Steer CJ; Shaughnessy MK; Johnson JR; Sadowsky MJ; Khoruts A Ursodeoxycholic Acid Inhibits Clostridium Difficile

Spore Germination and Vegetative Growth, and Prevents the Recurrence of Ileal Pouchitis Associated with the Infection. *J. Clin. Gastroenterol* 2016, 50 (8), 624–630. [PubMed: 26485102]

- (31). Francis MB; Allen CA; Shrestha R; Sorg JA Bile Acid Recognition by the Clostridium Difficile Germinant Receptor, CspC, Is Important for Establishing Infection. *PLoS Pathog.* 2013, 9 (5), e1003356. [PubMed: 23675301]
- (32). Bhattacharjee D; Francis MB; Ding X; McAllister KN; Shrestha R; Sorg JA Reexamining the Germination Phenotypes of Several Clostridium Difficile Strains Suggests Another Role for the CspC Germinant Receptor. *J. Bacteriol* 2016, 198 (5), 777–786.
- (33). Carpino LA; Imazumi H; El-Faham A; Ferrer FJ; Zhang CW; Lee YS; Foxman BM; Henklein P; Hanay C; Mugge C; Wenschuh H; Klose K; Beyersmann M; Bienert M The Uronium/Guanidinium Peptide Coupling Reagents: Finally the True Uronium Salts. *Angew. Chem., Int. Ed* 2002, 41, 441–445.
- (34). Han S-Y; Kim Y-A Recent Development of Peptide Coupling Reagents in Organic Synthesis. *Tetrahedron* 2004, 60, 2447–2467.
- (35). Albericio F; Bofill JM; El-Faham A; Kates SA Use of Onium Salt-Based Coupling Reagents in Peptide Synthesis. *J. Org. Chem* 1998, 63 (26), 9678–9683.
- (36). Gibson FS; Bergmeier SC; Rapoport H Selective Removal of an N-Boc Protecting Group in the Presence of a Tert-Butyl Ester and Other Acid-Sensitive Groups. *J. Org. Chem* 1994, 59 (11), 3216–3218.
- (37). Stahl GL; Walter R; Smith CW General Procedure for the Synthesis of Mono-N-Acylated 1,6-Diaminohexanes. *J. Org. Chem* 1978, 43 (11), 2285–2286.
- (38). Fishleigh RV; Fox KR; Khalaf AI; Pitt AR; Scobie M; Suckling CJ; Urwin J; Waigh RD; Young SC DNA Binding, Solubility, and Partitioning Characteristics of Extended Lexitropsins. *J. Med. Chem* 2000, 43 (17), 3257–3266. [PubMed: 10966744]
- (39). Aeberli P; Houlihan WJ Selective Reduction of a Nitro and Pyridyl Group. *J. Org. Chem* 1967, 32, 3211–3214.
- (40). Dayal B; Salen G; Toome B; Tint GS; Shefer S; Padia J Lithium Hydroxide/Aqueous Methanol: Mild Reagent for the Hydrolysis of Bile Acid Methyl Esters. *Steroids* 1990, 55 (5), 233–237. [PubMed: 2163126]
- (41). Corey EJ; Narasaka K; Shibasaki M A Direct, Stereocontrolled Total Synthesis of 9,11-Azo Analog of Prostaglandin Endoperoxide, PGH₂. *J. Am. Chem. Soc* 1976, 98 (20), 6417–6418. [PubMed: 965655]
- (42). Evans DA; Weber AE Synthesis of the Cyclic Hexapeptide Echinocandin-D. New Approaches to the Asymmetric Synthesis of Beta-Hydroxy Alpha-Amino Acids. *J. Am. Chem. Soc* 1987, 109 (23), 7151–7157.
- (43). Evans DA; Lundy KM Synthesis of Diphthamide: The Target of Diphtheria-Toxin Catalyzed ADP-Ribosylation in Protein Synthesis Elongation Factor-II. *J. Am. Chem. Soc* 1992, 114 (4), 1495–1496.
- (44). De Bernardo S; Tengi JP; Sasso GJ; Weigle M Clavulanate (Ro 22–5417), a New Clavam Antibiotic from Streptomyces-Clavuligerus 0.4. A Stereoselective Synthesis. *J. Org. Chem* 1985, 50 (19), 3457–3462.
- (45). Wojciechowski M; Lesyng B Generalized Born Model: Analysis, Refinement, and Applications to Proteins. *J. Phys. Chem. B* 2004, 108 (47), 18368–18376.
- (46). Lin A QuaSAR—Descriptor; Chemical Computing Group: Montreal, 2018; <http://www.chemcomp.com/journal/descr.htm> (accessed February 2018).
- (47). Rogers D; Hopfinger AJ Application of Genetic Function Approximation to Quantitative Structure-Activity-Relationships and Quantitative Structure-Property Relationships. *J. Chem. Inf. Model* 1994, 34 (4), 854–866.
- (48). Opdagel: Predictive Modeling Toolkit, 1.1.0; exeResearch LLC: East Lansing, Michigan 48823, 2018.
- (49). R Development Core Team. R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing: Vienna, Austria, 2018; <http://www.R-project.org>.
- (50). Cherkasov A; Muratov EN; Fourches D; Varnek A; Baskin II; Cronin M; Dearden J; Gramatica P; Martin YC; Todeschini R; Consonni V; Kuz'min VE; Cramer R; Benigni R; Yang CH;

- Rathman J; Terfloth L; Gasteiger J; Richard A; Tropsha A QSAR Modeling: Where Have You Been? Where Are You Going To? *J. Med. Chem* 2014, 57 (12), 4977–5010. [PubMed: 24351051]
- (51). Gasteiger J; Marsili M Iterative Partial Equalization of Orbital Electronegativity - a Rapid Access to Atomic Charges. *Tetrahedron* 1980, 36 (22), 3219–3228.
- (52). Hall LH; Kier LB In *The Molecular Connectivity Chi Indexes and Kappa Shape Indexes in Structure-Property Modeling; Reviews in Computational Chemistry*; Lipkowitz KB, Boyd DB, Eds.; VCH Publishers, Inc.: New York, 1991; Vol. 2, pp 367–422.
- (53). Hall LH; Mohney B; Kier LB The Electrotopological State: An Atom Index for QSAR. *Quant. Struct.-Act. Relat* 1991, 10 (1), 43–51.
- (54). Howerton A Anti-Germinants as a New Strategy to Prevent *Clostridium Difficile* Infections; University of Nevada Las Vegas: Las Vegas, NV, 2012.
- (55). Tucker CL High-Throughput Cell-Based Assays in Yeast. *Drug Discovery Today* 2002, 7 (18), S125–S130. [PubMed: 12546878]
- (56). An WF; Tolliday N Cell-Based Assays for High-Throughput Screening. *Mol. Biotechnol* 2010, 45 (2), 180–186. [PubMed: 20151227]
- (57). Michelini E; Cevenini L; Mezzanotte L; Coppa A; Roda A Cell-Based Assays: Fuelling Drug Discovery. *Anal. Bioanal. Chem* 2010, 398 (1), 227–238. [PubMed: 20623273]
- (58). Fernandes MM; Ivanova K; Hoyo J; Perez-Rafael S; Francesko A; Tzanov T Nanotransformation of Vancomycin Overcomes the Intrinsic Resistance of Gram-Negative Bacteria. *ACS Appl. Mater. Interfaces* 2017, 9 (17), 15022–15030.
- (59). Richter MF; Drown BS; Riley AP; Garcia A; Shirai T; Svec RL; Hergenrother PJ Predictive Compound Accumulation Rules Yield a Broad-Spectrum Antibiotic. *Nature* 2017, 545 (7654), 299–304. [PubMed: 28489819]
- (60). Donnelly ML; Li W; Li YQ; Hinkel L; Setlow P; Shen A A *Clostridium Difficile*-Specific, Gel-Forming Protein Required for Optimal Spore Germination. *mBio* 2017, 8, e02085. [PubMed: 28096487]
- (61). Butzin XY; Troiano AJ; Coleman WH; Griffiths KK; Doona CJ; Feeherry FE; Wang G; Li YQ; Setlow P Analysis of the Effects of a GERP Mutation on the Germination of Spores of *Bacillus Subtilis*. *J. Bacteriol* 2012, 194 (21), 5749–5758. [PubMed: 22904285]
- (62). Wang S; Shen A; Setlow P; Li YQ Characterization of the Dynamic Germination of Individual *Clostridium Difficile* Spores Using Raman Spectroscopy and Differential Interference Contrast Microscopy. *J. Bacteriol* 2015, 197 (14), 2361–2373. [PubMed: 25939833]
- (63). Kochan TJ; Somers MJ; Kaiser AM; Shoshiev MS; Hagan AK; Hastie JL; Giordano NP; Smith AD; Schubert AM; Carlson PE, Jr.; Hanna PC Intestinal Calcium and Bile Salts Facilitate Germination of *Clostridium Difficile* Spores. *PLoS Pathog.* 2017, 13 (7), e1006443. [PubMed: 28704538]
- (64). Dewar MJS; Zebisch EG; Healy EF; Stewart JJP Development and Use of Quantum Mechanical Molecular Models. 76. AM1: A New General Purpose Quantum Mechanical Molecular Model. *J. Am. Chem. Soc* 1985, 107 (13), 3902–3909.
- (65). Dewar MJS; Thiel W Ground States of Molecules. 38. The MNDO Method. Approximations and Parameters. *J. Am. Chem. Soc* 1977, 99 (15), 4899–4907.
- (66). Stewart JJP Optimization of Parameters for Semiempirical Methods I. Method. *J. Comput. Chem* 1989, 10 (2), 209–220.
- (67). Stewart JJP Optimization of Parameters for Semiempirical Methods II. Applications. *J. Comput. Chem* 1989, 10 (2), 221–264.
- (68). Stewart JJP Optimization of Parameters for Semiempirical Methods. III Extension of Pm3 to Be, Mg, Zn, Ga, Ge, As, Se, Cd, In, Sn, Sb, Te, Hg, Tl, Pb, and Bi. *J. Comput. Chem* 1991, 12 (3), 320–341.
- (69). Stewart JJP Optimization of Parameters for Semiempirical Methods IV: Extension of Mndo, Am1, and Pm3 to More Main Group Elements. *J. Mol. Model* 2004, 10 (2), 155–164. [PubMed: 14997367]
- (70). Labute P A Widely Applicable Set of Descriptors. *J. Mol. Graphics Modell* 2000, 18 (4–5), 464–477.

- (71). Labute P, Derivation and Applications of Molecular Descriptors Based on Approximate Surface Area. In Chemoinformatics; Bajorath J, Ed.; Humana Press: Totawa, NJ, 2004; Vol. 275, pp 261–278.
- (72). Cruciani G; Pastor M; Guba W Volsurf: A New Tool for the Pharmacokinetic Optimization of Lead Compounds. *Eur. J. Pharm. Sci* 2000, 11, S29–S39. [PubMed: 11033425]
- (73). Cruciani G; Pastor M; Mannhold R Suitability of Molecular Descriptors for Database Mining. A Comparative Analysis. *J. Med. Chem* 2002, 45 (13), 2685–2694. [PubMed: 12061872]
- (74). Stanton DT; Jurs PC Development and Use of Charged Partial Surface Area Structural Descriptors in Computer-Assisted Quantitative Structure-Property Relationship Studies. *Anal. Chem* 1990, 62 (21), 2323–2329.
- (75). Stouch TR; Jurs PC A Simple Method for the Representation, Quantification, and Comparison of the Volumes and Shapes of Chemical Compounds. *J. Chem. Inf. Model* 1986, 26 (1), 4–12.
- (76). Pearlman RS; Smith KM Novel Software Tools for Chemical Diversity. *Perspect. Drug Discovery Des* 1998, 9, 339–353.
- (77). Molecular Operating Environment, 2017.08; Chemical Computing Group Inc: Montreal, Quebec, Canada, 2017.
- (78). Jorgensen WL; Maxwell DS; Tirado-Rives J Development and Testing of the OPLS All-Atom Force Field on Conformational Energetics and Properties of Organic Liquids. *J. Am. Chem. Soc* 1996, 118 (45), 11225–11236.
- (79). Devillers J Genetic Algorithms in Molecular Modeling; Academic Press: London, 1996.
- (80). Holland JH Adaptation in Natural and Artificial Systems: An Introductory Analysis with Applications to Biology, Control, and Artificial Intelligence. Ph.D. Thesis University of Michigan, Ann Arbor, MI, 1975.
- (81). Shao J Linear Model Selection by Cross-Validation. *J. Am. Stat. Assoc* 1993, 88 (422), 486–494.
- (82). Lindeman RH; Merenda PF; Gold RZ Introduction to Bivariate and Multivariate Analysis; Scott Foresman & Co: Glenview, IL, 1980.
- (83). Belsley DA; Kuh E; Welsch RE Regression Diagnostics: Identifying Influential Data and Sources of Collinearity; John Wiley & Sons: New York, 1980.
- (84). Fox J; Monette G Generalized Collinearity Diagnostics. *J. Am. Stat. Assoc* 1992, 87 (417), 178–183.
- (85). Rousseeuw PJ; Leroy AM Robust Regression and Outlier Detection; John Wiley & Sons: New York, 1987.
- (86). Stoltz KL; Erickson R; Staley C; Weingarden AR; Romens E; Steer CJ; Khoruts A; Sadowsky MJ; Dosa PI Synthesis and biological evaluation of bile acid analogues inhibitory to *Clostridium difficile* spore germination. *J. Med. Chem* 2017, 60, 3451–3471. [PubMed: 28402634]

Figure 1.
Structures of antibiotics metronidazole (1), vancomycin (2), and fidaxomicin (3) currently used to treat *C. difficile* infections.

Figure 2.

Structures of taurocholate (4), chenodeoxycholate (5), and cholic amide meta-sulfonic acid (6).

Figure 3.

Inhibition of spore germination by 12a. (A) Inhibition of spore germination by various concentrations of 12a in sodium phosphate pH 6 buffer containing 6 mM taurocholate and 12 mM glycine. DMSO control contains no germinate, whereas taurocholate and glycine control contain no inhibitor. Some data used to generate the plot in 3B is not shown for clarity. (B) IC₅₀ plot for 12a in sodium phosphate buffer. Percent germination was calculated relative to the taurocholate and glycine control. (C) Inhibition of spore germination by various concentrations of 12a in BHIS media containing 6 mM taurocholate. DMSO control contains no germinate, whereas taurocholate control contains no inhibitor. Some data used to generate the plot in 3D are not shown for clarity. (D) IC₅₀ plot for 12a in BHIS media with taurocholate. Percent germination was calculated relative to the taurocholate only control.

Figure 4.

Molecular alignment of active (12a, 12b, 12f, 12g, 12h, 12i, 15b, 15c, 15d) and inactive or weakly active compounds (12c, 12d, 12e, 12j, 12k, 12l, 14a, 14f, 15a, 15f). Compounds were aligned using the flexible alignment protocol executed in MOE. (A) Active molecules. The molecular surface is shown faded so that the stick representation of the atoms can be seen. (B) Molecular surface area of active molecules colored for hydrophobicity. Lipophilic regions are colored green while hydrophilic groups are colored purple. (C) Inactive molecules. The molecular surface is shown faded so that the stick representation of the atoms can be seen. (D) Molecular surface area of inactive molecules colored for hydrophobicity. Lipophilic regions are colored green while hydrophilic groups are colored purple. The scale is the same as that used in B. (E) Alignment of active (yellow) and inactive (red) molecules. The molecular surfaces of each are also shown in their respective colors.

Figure 5.

Predicted versus observed pIC_{50} values for inhibition of spore germination based upon the consensus model. The error bars shown for the predicted values are the RMSE (0.1) from the consensus model. The error bars for the experimental values are those taken from Tables 1–3 after conversion into pIC_{50} .

^aReagents and reaction conditions: (A) HBTU, NMM, DMF, rt, 48–72 h, worked up with 2–5% aq HCl; (B) HBTU, NMM, DMF, rt, 48–72 h, worked up with ice-cold H₂O; (C) 1 M HCl in AcOEt, AcOEt, rt, 48–72 h; (D) 10% Pd/C, H₂, MeOH, 65 psi, 3.5 h; (E) aq LiOH, THF, rt, 48 h; (F) 10% Pd/C, H₂, MeOH, 60–75 psi, 6 h.

Scheme 1.

Synthesis of N-Aromatic Cholan-24-amides (12a–q and 13), N-Benzyl Cholan-24-amides (14a–k), and N-Aliphatic Cholan-24-amides (15a–z)^a

^a Reagents and reaction conditions: (A) HBTU, NMM, DMF, rt, 48–72 h, worked up with 2–5% aq HCl; (B) HBTU, NMM, DMF, rt, 48–72 h, worked up with ice-cold H₂O; (C) 1 M HCl in AcOEt, AcOEt, rt, 48–72 h; (D) 10% Pd/C, H₂, MeOH, 65 psi, 3.5 h; (E) aq LiOH, THF, rt, 48 h; (F) 10% Pd/C, H₂, MeOH, 60–75 psi, 6 h.

Table 1.

Spore Germination Activity of N-Aromatic (Phenyl and Heterocyclic) Cholan-24-amides (12a–b and 13)

compd ^a	R ₁	R ₂	R ₃	R ₄	X	% germination ^b	IC ₅₀ (μM) ^c
CamSA (6)	H	SO ₃ H	H	H	C	102 ± 2.2	NA
12a	H	H	H	H	C	3 ± 0.7	1.8 ± 0.11
12b	OCH ₃	H	H	H	C	8 ± 0.7	9.8 ± 3.4
12c	H	OCH ₃	H	H	C	55 ± 1	78 ± 24.4
12d	H	H	OCH ₃	H	C	79 ± 0.9	N/A ^d
12e	OCH ₃	H	OCH ₃	H	C	93 ± 2	N/A
12f	H	H	OH	H	C	20 ± 2	36 ± 3.7
12g	H	F	H	H	C	8 ± 3	16 ± 1.8
12h	CH ₃	H	F	H	C	37 ± 0.9	24 ± 6
12i	CH ₃	H	H	F	C	9 ± 1	14 ± 1.7
12j	H	CONH ₂	H	H	C	85 ± 6	N/A
12k	H	CO ₂ Et	H	H	C	109 ± 8	N/A
12l	H	H	CO ₂ Et	H	C	105 ± 11	N/A
12m	H	NH-Boc	H	H	C	89 ± 4	N/A
12n	H	H	NH-Boc	H	C	100 ± 7	N/A
12o	H	NH ₃ ⁺ Cl ⁻	H	H	C	59 ± 1	32 ± 8.1
12p	H	H	NH ₃ ⁺ Cl ⁻	H	C	82 ± 4	N/A
12q	H	H	H	H	N	17 ± 1	30.1 ± 2.8
13						84 ± 5	N/A

^aNumber corresponding to Scheme 1.^bPercent (%) germination of each compound was reported with standard deviations and tested at a final concentration of 125 μM. A 96-well plate was prepared by adding individual analogues to separate wells in triplicate along with 6 mM taurocholate and 12 mM glycine. Upon the addition of spores, the OD₅₈₀ was measured once every minute for 2 h and normalized using the OD₅₈₀ obtained at time zero [relative OD₅₈₀ = OD_{580(t)}/OD_{580(t0)}].

C. difficile spores were incubated with various concentrations of analogues along with 6 mM taurocholate and 12 mM glycine. The IC₅₀ was calculated by plotting the extent of germination versus the logarithm of the concentration of the analogue and using eq 1.

$$dN/dt = N/A$$

N/A: not applicable.

Table 2.
Spore Germination Activity of N-Benzyl and N-Pyridinemethyl Cholan-24-amides (14a–k)

compd ^a	R ₁	R ₂	R ₃	R ₄	X	Y	% germination ^b	IC ₅₀ (μM) ^c
14a	H	H	H	H	C	C	70 ± 4	N/A ^d
14b	OCH ₃	H	OCH ₃	H	C	C	85 ± 11	N/A
14c	H	OCH ₃	H	OCH ₃	C	C	99 ± 0.4	N/A
14d	H	Cl	Cl	H	C	C	111 ± 5	N/A
14e	H	H	SO ₂ NH ₂	H	C	C	109 ± 3	N/A
14f	NO ₂	H	H	H	C	C	113 ± 15	225 ± 120
14g	H	H	NO ₂	H	C	C	88 ± 3	N/A
14h	NH ₂	H	H	H	C	C	95 ± 3	N/A
14i	H	H	NH ₂	H	C	C	104 ± 2	N/A
14j	H	H	H	H	N	C	93 ± 0.5	N/A
14k	H	H	H	H	C	N	94 ± 1	N/A

^aNumber corresponding to Scheme 1.

^bPercent (%) germination of each compound was reported with standard deviations and tested at a final concentration of 125 μM. A 96-well plate was prepared by adding individual analogues to separate wells in triplicate along with 6 mM taurocholate and 12 mM glycine. Upon the addition of spores, the OD₅₈₀ was measured once every minute for 2 h and normalized using the OD₅₈₀ obtained at time zero [relative OD₅₈₀ = OD_{580(t)}/OD_{580(t0)}].

^cC, difficile spores were incubated with various concentrations of analogues along with 6 mM taurocholate and 12 mM glycine. The IC₅₀ was calculated by plotting the extent of germination versus the logarithm of the concentration of the analogue and using eq 1.

^dN/A: not applicable.

Table 3.

Spore Germination Activity of N-Alkyl Cholan-24-amides (15a–z)

compd ^a	R	% germination ^b	IC ₅₀ (μM) ^c
15a	cyclohexyl	47 ± 5	95.4 ± 21.8
15b	cyclopentyl	6 ± 0.1	34.1 ± 4.8
15c	cyclopropyl	6 ± 0.9	25.0 ± 1.7
15d	CH ₂ (cyclopropyl)	15 ± 2	24.3 ± 2.6
15e	CH ₂ CH(CH ₃) ₂	62 ± 3	154 ± 13.1
15f	CH ₂ CH ₂ Ph	104 ± 4	N/A ^d
15g	CH ₂ CH ₂ Ph(2'',4''-OCH ₃)	101 ± 0.6	N/A
15h	CH ₂ CHPh ₂	101 ± 2	N/A
15i	CH ₂ C ₂ H	59 ± 0.6	137 ± 12.3
15j	CH ₂ CH ₂ CN	112 ± 6	N/A
15k	CH ₂ CH ₂ CH ₂ OH	92 ± 2	N/A
15l	CH ₂ CH ₂ CH ₂ CO ₂ CH ₂ CH ₃	95 ± 1	N/A
15m	CH[CH(CH ₃) ₂][CO ₂ CH ₃	100 ± 1	N/A
15n	CH(CH ₂ Ph)CO ₂ CH ₃	84 ± 0.3	N/A
15o	CH(CH ₂ Ph)CO ₂ H	108 ± 7	N/A
15q	CH(CH ₂ CH ₂ CO ₂ H)CO ₂ H	114 ± 4	N/A
15s	CH ₂ CH ₂ CH ₂ CH ₂ NHBoc	117 ± 0.8	N/A
15t	CH ₂ CH ₂ CH ₂ NH ₃ +Cl ⁻	124 ± 6	N/A
15u	CH ₂ CH ₂ CH ₂ CH ₂ NH ₃ +Cl ⁻	104 ± 3	N/A
15v	CH ₂ CH ₂ (pyrrolidine-1''-yl)	101 ± 3	N/A
15w	CH ₂ CH ₂ (thiophen-2''-yl)	93 ± 5	N/A
15x	CH ₂ CH ₂ (1'',3''-dioxoisoinindolin-2''-yl)	78 ± 6	N/A

compd ^a	R	% germination ^b	IC ₅₀ (μM) ^c
15y	CH ₂ CH ₂ CH ₂ (1''-3''-dioxoisindolin-2''-yl)	82 ± 2	N/A
15z	CH ₂ CH ₂ CH ₂ (2''-3''-dioxoisindolin-1''-yl)	95 ± 5	N/A

^aNumber corresponding to Scheme 1.

^bPercent (%) germination of each compound was reported with standard deviations and tested at final aconcentration of 125 μM. A 96-well plate was prepared by adding individual CamSA analogues to separate wells in triplicate along with 6 mM taurocholate and 12 mM glycine. Upon the addition of spores, the OD₅₈₀ was measured once every minute for 2 h and normalized using the OD₅₈₀ obtained at time zero [relative OD₅₈₀ = OD₅₈₀(t)/OD₅₈₀(t₀)].

^cC. difficile spores were incubated with various concentrations of analogues along with 6 mM taurocholate and 12 mM glycine. The IC₅₀ was calculated by plotting the extent of germination versus the logarithm of the concentration of the analogue and using eq 1.

^dN/A: not applicable.

Table 4. QSAR Models for the Inhibition of Spore Germination by Cholic Acid Analogues Against Strain R20291

model	equation	R^2	R^2_{adj}	Q^2	F	p -value	VIF
1	$-0.91*b_{single} + 1.33*SlogP + -1.49*v_{dw_area} + 0.85*v_{surf_D5}$	0.97	0.95	0.93	75	6.4×10^{-8}	b_{single} , 1.8; $SlogP$, 2.6; v_{dw_area} , 3.3; v_{surf_D5} , 2.3
2	$0.88*GCUT_SLOGP_1 + -0.46*PEOE_VSA+0 + -0.98*PEOE_VSA_FPNEG + -0.82*RPCneg$	0.96	0.95	0.91	65	1.4×10^{-7}	$GCUT_SLOGP_1$, 1.3; $PEOE_VSA+0$, 1.2; $PEOE_VSA_FPNEG$, 1.9; $RPCneg$, 2.1
3	$0.61*ASA_H + 0.72*E_nb + -1.12*KierFlex + -0.62*MNDO_dipole$	0.93	0.91	0.85	37	2.5×10^{-6}	ASA_H , 2.4; E_nb , 2.5; $KierFlex$, 1.7; $MNDO_dipole$, 1.7
4	$0.72*GCUT_SLOGP_1 + -0.97*PEOE_VSA_FPNEG + -0.33*PEOE_VSA_POS + -1.07*RPCneg$	0.88	0.84	0.81	21	4.6×10^{-5}	$GCUT_SLOGP_1$, 1.3; $PEOE_VSA_FPNEG$, 1.9; $PEOE_VSA_POS$, 1.2; $RPCneg$, 2.0
5	$0.75*GCUT_SLOGP_1 + 0.35*PEOE_VSA_FNEG + -1.07*PEOE_VSA_FPNEG + -0.93*RPCneg$	0.89	0.85	0.81	22	3.4×10^{-5}	$GCUT_SLOGP_1$, 1.3; $PEOE_VSA_FNEG$, 1.2; $PEOE_VSA_FPNEG$, 1.9; $RPCneg$, 1.9