The Design, Synthesis, and Inhibition of *Clostridioides difficile* Spore Germination by Acyclic and Bicyclic Tertiary Amide Analogs of Cholate

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Abstract: *Clostridioides difficile* infection (CDI) is a major identifiable cause of antibiotic-associated diarrhea. In our previous study (*J. Med Chem*, **2018**, *61*, 6759-6778), we have identified N-phenyl-cholan-24-amide as a potent inhibitor of spore germination. The most potent compounds in our previous work are N-arylamides. We were interested in the role that the conformation of the amide plays in activity. Previous research has shown that secondary N-arylamides exist exclusively in the coplanar *trans* conformation while tertiary N-methyl-N-arylamides exist in a non-planar, *cis* conformation. The N-methy-N-phenyl-cholan-24-amide was 17-fold less active compared to the parent compounds suggesting the importance of the orientation of the phenyl ring. To lock the phenyl ring into a *trans* conformation, cyclic tertiary amides were prepared. Indoline and quinoline cholan-24-amides were both inhibitors of spore germination; however, the indoline analogs were most potent. Isoindoline and isoquinoline amides were inactive. We found that the simple indoline derivative gave an IC$_{50}$ value of 1 μM, while the 5'-fluoro-substituted compound (**5d**) possessed an IC$_{50}$ of 400 nM. To our knowledge, **5d** is the most potent known spore germination inhibitor described to date. Taken together, our results indicate that the *trans*, coplanar conformation of the phenyl ring is required for potent inhibition.

Introduction

Clostridioides difficile is an anaerobic bacterium that can colonize the gastrointestinal tract and cause severe and potentially life-threatening colitis[1]. Patients are treated with either oral vancomycin or fidaxomicin; however, about 1 in 6 patients will experience reoccurring *C. difficile* infections even after treatment[2]. In 2019, the CDC classified *C. difficile* as one of five urgent threats to public health with about 225,000 patients hospitalized annually, resulting in almost 13,000 deaths each year[3].

C. difficile is spread from person to person via ingestion of hard to irradicate spores that are present in the environment[1]. Most individuals with healthy gut microflora do not develop *C. difficile* even if they ingest spores; however, individuals with a compromised gut microbiome are at an elevated risk for developing the infection[4-5]. This finding, along with the success of fecal transplants in the treatment of *C. difficile*, have highlighted the role of the gut microbiome in the establishment of *C. difficile* infections (CDI)[4].

CDIs begin by the germination of the ingested spore into a vegetive cell which can then replicate in the GI tract. The newly germinated cell excretes toxins that are responsible for much of the damage associated with severe infections. Details regarding spore germination in *C. difficile* is limited, but it is known that bile salts, amino acids, and ions regulate germination[6-10].

Previous research has shown that the primary conjugated bile salt, taurocholate (**1,** Figure 1), activates germination while the unconjugated bile salt, chenodeoxycholate (CDCA, **2a**) is a weak inhibitor[10-14]. The regulation of the concentration of taurocholate and CDCA in the GI tract are dictated by the gut microbiome[5]. Taurocholate is metabolized by the gut microflora into cholic acid (**2b**) which is then further metabolized into deoxycholate. Thus, under normal conditions in which the gut microbiome is robust, CDCA levels are high and taurocholate levels

are low, generating conditions which hinder *C. difficile* spore germination. However, when the gut microbiome is compromised, usually as the result of antibiotic usage or pathology, taurocholate levels are elevated and CDCA levels are low, hence promoting germination[4-5].

Given the connection between spore germination and bile salts, we and others have shown that bile salt analogs can inhibit germination and prevent the development of *C. difficile* colitis[12, 15-22]. Several natural bile salts inhibit germination *in vitro*; however, their use to treat patients has not been successful[19]. Synthetic bile salt derivatives have also been explored[15-16, 18, 21-22].

Our groups have generated numerous analogs which show potent anti-germination activity *in vitro* and have shown protective effects in rodent models of CDI[11, 15, 17-18, 20-21, 23]. One of the first agents developed is CamSA (**3a**, Figure 1) which inhibited spore germination with a K_i of 50 µM and prevented infection in both the hamster and mouse models of CDI[15, 17-18, 20-21, 24]. CamSA, however, displayed poor activity against hypervirulent strains. Additional investigation revealed that the simple phenyl substitution, CaPA (**3b**, Figure 1) gave potent inhibition (IC$_{50}$ = 1.8 µM) against a range of *C. difficile* strains and could prevent *C. difficile* infections in mouse and hamster models[15, 21]. We have further shown that the cholic acid nucleus gives the most potent inhibition of germination; however, the CDCA moiety also inhibits but with less potency[22]. Finally, other groups have explored ursodeoxycholate analogs as antigerminants[16, 25-26].

2a. R_1 = H (CDCA)
2b. R_1 = OH (CA)

3a. R = SO$_3$H (CamSA)
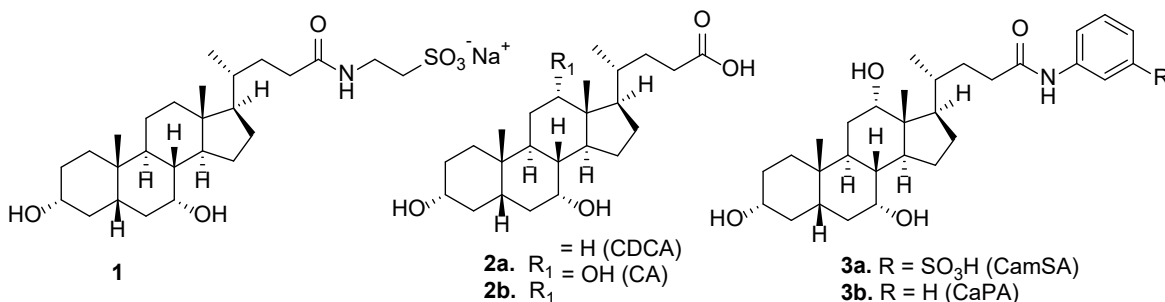**3b.** R = H (CaPA)

Figure 1. Chemical structures of sodium taurocholate (**1**), chendeoxycholic acid (CDCA, **2a**), cholic acid (CA, **2b**), CamSA (**3a**) and CaPA (**3b**).

Our previous studies have focused on secondary amide substitutions of cholic acid as spore germination inhibitors[15]. In this publication, we present our work on acyclic and cyclic tertiary amide analogs of cholic acid. We have found that the tertiary amide generated from indoline yields potent compounds with the 4-fluoro substituted indoline being the most potent spore germination inhibitor (IC_{50} = 400 nM) described to date.

Results and Discussion

Compound Design. In our previous studies, we have shown that the N-aryl containing cholan-24-amides, like CaPA, were potent antigerminants whereas N-alkyl (either open chain or cyclic) or N-arylalkyl (benzyl) analogs displayed no or modest activity[15]. We also observed that no substitution on the aromatic ring gave the most potent compounds, followed by 2'-substitution. Substitutions at the 3'- and 4'- positions resulted in inactive or weakly active compounds.

Unfortunately, the target of these antigerminants is unknown and thus the binding site must be inferred from an analysis of the structure-activity relationships of inhibitors[6]. Our work has suggested that the binding site is relatively constrained around the aromatic ring[15].

One unanswered question in our previous study was the conformation of the N-arylamide on CaPA. Examination of the structure of CaPA indicates that there are two dihedral angles, ω1 and ω2 which play a critical role in the conformation of the arylamide (Figure 2A).

Angle ω1 (R-C2-N3-H4) determines the relative orientation of the aromatic group on the nitrogen and the cholate backbone (R). Given the fact that the amide bond displays pseudo double bond character, two conformers, *cis* and *trans*, predominate. The second angle, ω2 (H4-N3-C5-C6) determines the relative orientation of the aromatic group to the plane of the amide bond.
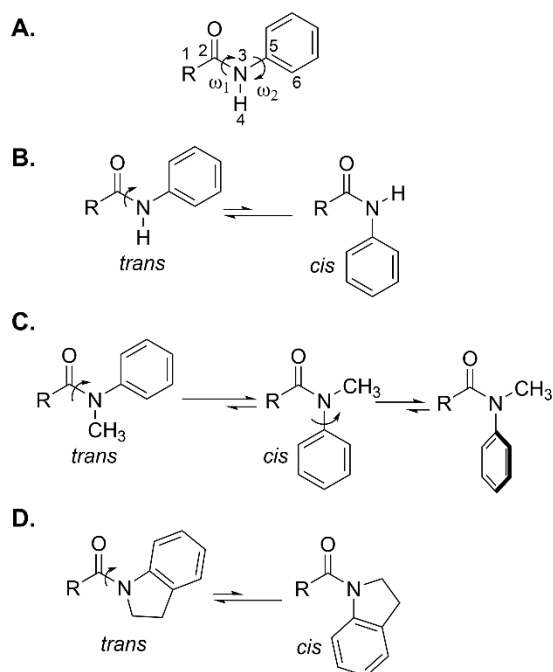
Figure 2. Conformations of secondary and tertiary aryl amides. A. Dihedral angles that control the conformation of the aryl amide. **B.** Conformation of secondary aryl amides. **C.** Conformation of N-methyl-arylamides. **D.** Conformation of indoline amides.

There have been extensive studies on the conformation of secondary N-arylamides[27-28]. These studies indicate that secondary N-arylamides exist exclusively in the trans configuration ($\omega 1$ = 0°) with the aromatic ring coplanar to the amide bond ($\omega 2$ = 0°). The calculated energy difference between the *cis* and *trans* conformers is 2-4 kcal/mol whereas the rotation at $\omega 2$ generally has a similar energy penalty. This suggests that the lowest energy solution phase conformation of CaPA would be the *trans*, coplanar conformation (Figure 2B).

Interestingly, previous studies on tertiary N-methyl-N-arylamides has shown a distinct conformational difference compared to secondary N-arylamides[27-28]. The addition of a methyl group to the amide results in a switch at $\omega 1$ from a *trans* conformation to a *cis* conformation ($\omega 1$ = 180°) and a rotation of the aromatic group such that it is perpendicular to the plane of the amide ($\omega 2$ = 45-90°, Figure 2C). The inclusion of a methyl group to generate the tert-amide may also be advantageous since previous studies have shown that tert-amides are more resistant to hydrolysis[29-]

[30]. Thus, we elected to examine N-methyl-arylamides (Figure 3A) to determine whether they possessed potent activity against spore germination.

While the presence of a N-methyl constrains the conformation of the amide, the exact energy barrier is unknown and it does not prevent the target from altering the conformation of the amide. Therefore, we chose to examine conformationally locked analogs via cyclization of the nitrogen to the phenyl ring (Figure 3B,C). Cyclization could be accomplished to generate 5,6- (indoline) or 6,6-fused ring systems (quinolone) which would lock the aromatic ring into a coplanar orientation relative to the amide. Previous studies have shown that N-acetyl indoline exists in a trans conformation with the aromatic ring coplanar to the amide (Figure 2D). We hypothesize a similar orientation for the quinolone amide[27].

To explore additional orientations of the aromatic ring relative to the amide, we also chose to prepare isoindoline or isoquinoline amides (Figure 3C). Finally, to verify if the aromatic group was still critical in the locked analogs, we removed the aromatic ring to give the pyrrolidine and piperidine amides (Figure 3D).
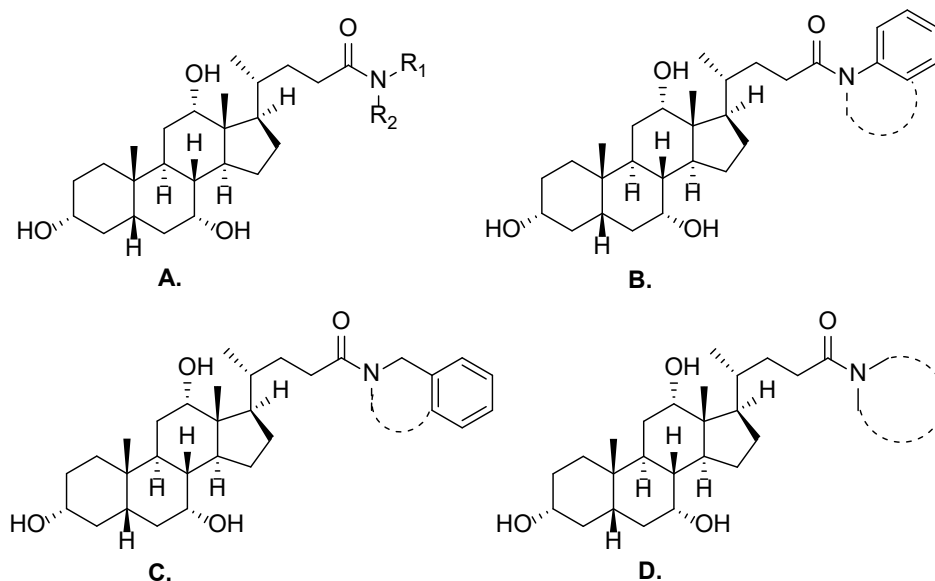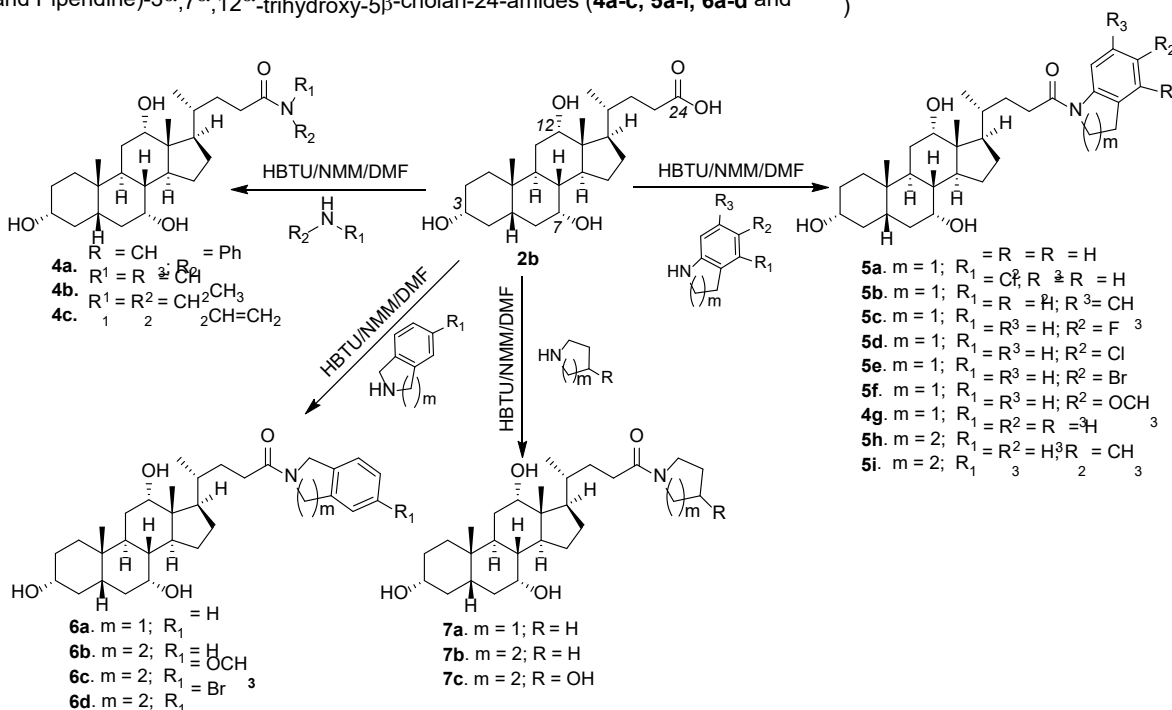
Figure 3. Acyclic and cyclic tertiary 24-amide analogs of cholic acid. **A.** Acyclic tertiary amides (R_1=alkyl, R_2=alkyl or aryl). **B.** 5,6- or 6,6-fused rings as conformationally constrained analogs. **C.** Isoindoline and isoquinoline analogs. **D.** Cyclic amide analogs.

Synthesis. The chemical synthesis of acyclic tertiary amides (**4a-c**), heterobicyclic tertiary amides (**5a-i**, and **6a-d**), and hetero monocyclic tertiary amides (**7a-c**) analogs of cholic acid are shown in scheme 1. Cholic acid (**2b**) was pre-activated with HBTU and NMM in DMF at room temperature and subsequently reacted *in situ* with the corresponding acyclic secondary amine (N-methylaniline, diethylamine or diallylamine) as previously described to produce the corresponding cholan-24-amides (**4a-c**) in 74-86% yields[15]. Likewise, the pre-activated ester of cholic acid was treated with secondary amines as indoline, 1,2,3,4-tetrahydroquinoline, isoindoline, and 1,2,3,4-tetrahydroisoquinoline to produce corresponding hetero bicyclic tertiary amides **5a-i** (62-97% yields) and **6a-d** in (79-92% yields). In a similar fashion, the cholan-24-amides (**7a-c**) were prepared in 76-89% yields from the pre-activated ester of cholic acid with cyclic aliphatic secondary amines (pyrrolidine and piperazine).

Scheme 1. Chemical Synthesis of Tertiary (Alkyl/Aryl, Alkylated, Indoline, Isoindoline, Quinoline, Isoquinoline, Pyrrolidine, and Piperidine)-3α,7α,12α-trihydroxy-5β-cholan-24-amides (**4a-c, 5a-i, 6a-d** and **7a-c**)

4a. R$_1$ = CH$_3$; R$_2$ = Ph
4b. R$_1$ = R$_2$ = CH$_3$
4c. R$_1$ = R$_2$ = CH$_2$CH=CH$_2$

5a. m = 1; R$_1$ = R$_2$ = R$_3$ = H
5b. m = 1; R$_1$ = CH$_3$; R$_2$ = R$_3$ = H
5c. m = 1; R$_1$ = R$_3$ = H; R$_2$ = F
5d. m = 1; R$_1$ = R$_3$ = H; R$_2$ = Cl
5e. m = 1; R$_1$ = R$_3$ = H; R$_2$ = Br
5f. m = 1; R$_1$ = R$_3$ = H; R$_2$ = OCH$_3$
4g. m = 1; R$_1$ = R$_2$ = R$_3$ = H
5h. m = 2; R$_1$ = R$_2$ = H; R$_3$ = CH$_3$
5i. m = 2; R$_1$ = R$_3$ = H; R$_2$ = CH$_3$

6a. m = 1; R$_1$ = H
6b. m = 2; R$_1$ = H
6c. m = 2; R$_1$ = OCH$_3$
6d. m = 2; R$_1$ = Br

7a. m = 1; R = H
7b. m = 2; R = H
7c. m = 2; R = OH

HBTU/NMM/DMF

Biological Results and Discussion. All compounds were analyzed as inhibitors of spore germination using a standard assay which measured germination as a decrease in absorbance at 580 nm[15]. A two-step process was taken for the analysis of the biological activity of the compounds. Compounds that were not soluble in buffer containing 2.5% DMSO at 125 µM were eliminated from further consideration. Soluble compounds were analyzed for their ability to inhibit spore germination of *C. difficile* hypervirulent strain R20291 at a single concentration of 125 µM. Compounds that were able to slow spore germination >60% compared to untreated samples were then reanalyzed at different concentrations to determine their IC$_{50}$ values.

Addition of a methyl group to the amide to generate N-methyl CaPA (**4a**) resulted in a 17-fold reduction in germination activity compared to unmodified CaPA (IC$_{50}$ = 32 µM vs 1.8 µM). This suggested that the amide group was necessary for optimal activity of the anti-germinants and that the *trans*, coplanar was preferred over the *cis*, non-planar conformation of the amide.

However, we could not rule out the possibility that the loss of the NH group of the amide also played a role in poor activity.

Since we have not examined any other tert-amides, we prepared symmetrical tertiary cholan-24-amide containing either two ethyl- (**4b**) or two allyl- (**4c**) groups. These agents inhibit the spore germination with IC_{50} = 29 µM and 8 µM, respectively, indicating that alkyl groups do generate active agents (Table 1). However, these agents are not as potent as compounds containing an aromatic ring but suggest that a more extensive study of acyclic tertiary amides may be warranted in the future.

We next looked at locked conformational analogs of CaPA in which the tert-amide is generated from indoline, isoindoline, quinoline, and isoquinoline (Table 2). Compound **5a** which is the locked, indoline analog of CaPA, showed strong inhibition of spore germination with an IC_{50} = 1 µM. This demonstrates that a secondary amide was not necessary for potent antigerminant activity, and that the NH was not required. Unfortunately, **5a** was not active in preventing CDI in a mouse model of infection[21]. Increasing the ring size to the quinoline (**5h**) resulted in a 22-fold loss of activity (IC_{50} = 22 µM), indicating that either the compound was too large for the binding site or the decrease in coplanarity of the aromatic ring, due to the larger ring, was detrimental to binding. Isoindoline (**6a**) and isoquinoline (**6b**) analogs displayed no activity or were insoluble in the assay conditions. We conclude that locking the ring into a coplanar, *trans* conformation results in the most potent compounds.

We next explored a series of electron-donating and withdrawing substituents on the aromatic ring of the indoline analogs. We found that the addition of 5'-fluorine (**5d**) greatly enhanced inhibition with an IC_{50} = 400 nM. This is the most potent spore germination inhibitor discovered to date. Other halides at this position (**5b** (4'-chloro), **5f** (5'-bromo)) gave inactive or weakly

active compounds, perhaps because of size. No activity was seen with a limited set of electron-donating substituents (**5c** (5'-methyl), and **5g** (6'-methoxy)).

Our previous research has indicated that the aromatic ring is a critical binding feature for potent inhibition; however, we have observed activity with amides containing small, cyclic, and acyclic groups on the nitrogen[15]. We synthesized and evaluated activity for a small series of cyclic alkyl tertiary cholan-24-amides (Table 3). The pyrrolidine containing cholan-24-amide **7a**, was a moderate inhibitor of spore germination with an IC_{50} of 38 µM. Increasing the size of the group on the amide nitrogen to piperidine analogs **7b** resulted in comparable inhibition (IC_{50} = 37 µM). These results agree with our previous work on small cyclic alkyl secondary amides. This also confirm the data from table 1 showing that alkyl-substituted cholates can be inhibitors of *C. difficile* spore germination, but are less effective than the corresponding aromatic compounds.

Conclusion

We have found that indoline cholan-24-amide and its 5'-fluoro substituted derivatives are potent antigerminants. The 5'-fluoro compound, **5d**, displays an IC_{50} value of 400 nM, which is the most potent anti-germinant described to date. The indoline analogs ae conformational constrained analogs of CaPA which lock the N-aryl amide into a productive *trans*, coplanar orientation. These results indicate that this orientation should be preserved in future compound designs to enhance potency of antigerminants. Unfortunately, the unsubstituted indoline compound **5a** did not prevent CDI in a mouse model[21]. The exact reason for this is unknown, but we speculate that **5a** is rapidly metabolized in the gut via amidases or dehydroxylation to render the compound inactive. We are currently exploring amide bioisosteres to eliminate this potential site of metabolism. We will report on these studies in due course.

Experimental Methods

General Comments. Cholic acid (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) was purchased from MP Biomedical. All other reactants, reagents and solvents were purchased from Sigma-Aldrich, Acros Organics, TCI Chemicals or Chem-Impex International and were used without further purification. Thin layer chromatography (TLC) was performed on pre-coated (0.25 mm) silica gel plate (Sorbtech, 60 F-254), and visualization was done either by UV (254 nm) or iodine staining. Column chromatographic purifications of compounds were performed on silica-gel (Sorbtech, 60-230 mesh, 0.063-0.20mm). ^{1}H and ^{13}C NMR spectra were recorded on a Varian VNMRS 600 MHz or Bruker 400 MHz spectrometer by dissolving the compounds in deuterated solvents as chloroform-*d* (CDCl$_3$), methanol-*d$_4$* (CD$_3$OD) or dimethyl sulfoxide-*d$_6$* (DMSO-*d$_6$*) and all peaks were referenced with TMS as an internal standard or to the residual solvents. 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 Thermo Orbitrap Exploris 120 using electrospray ionization (ESI) mass spectrometry

(in positive mode) at the Lumigen Instrument Center of Wayne State University and the data are presented as a mass-to-charge (m/z) ratio.

General Procedure. Cholic acid **2b** (1 equiv., 0.50-2.50 mmol scale) and HBTU (1.2-1.6 equiv.) were dissolved in anhydrous DMF at room temperature. NMM (1.1-1.8 equiv.) was added, and the reaction was stirred for 15-30 min to generate the activated ester. Secondary acyclic or cyclic amines (1.0-1.8 equiv.) either alone or dissolved in a minimal volume of DMF and NMM (0.9-2.7 equiv.) were added to the activated ester and the reaction was stirred at room temperature for 48-72 h. The solvent was removed under high vacuum rotary evaporation at 70-80°C to produce a viscous light-yellow to dark brown material. Cold, dilute HCl (2-5%, 50 ml – 200 mL) was added to the residue and sonicated which produced a white precipitate that was filtered out. In some cases, a sticky material was obtained, for which the soluble part was decanted. This process was repeated 2-3 additional times. The final product was washed with cold water, collected, and dried under vacuum to produce the pure, desired products in 62-97% yield. In cases where washing with dilute acid failed to give pure compound, silica gel column chromatography was preformed using the stated conditions.

N-(Methyl)-N-(Phenyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (4a). Compound **4a** was prepared using the general method described above. Cholic acid (1.022 g, 2.50 mmol), HBTU (3.0 mmol), N-methylaniline (2.96 mmol), and NMM (5.46 mmol) were reacted to produce the desired molecule. The product was further purified by column chromatography using CH_2Cl_2-MeOH (95:5; 90:10; 80:20; 70:30 and 50:50) to give **4a** in 927 mg (74% yield). Mp. 206-207°C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.42 (t, 2H, J = 7.6 Hz), 7.33 (t, 1H, J = 7.2 Hz), 7.18 (d, 2H, J = 7.6 Hz), 3.90 (s, 1H), 3.83 (s, 1H), 3.46-3.41 (m, 1H), 3.25 (s, 3H), 2.23-2.14 (m, 3H), 2.02-1.91 (m, 7H), 1.85-1.74 (m, 5H), 1.64-1.60 (m, 3H), 1.51-1.46 (m, 2H), 1.39 and 1.36 (2 peaks, 2H),

1.26 (br, 3H), 1.13-1.05 (m, 1H), 1.00-0.93 (m, 1H), 0.87 (s, 3H), 0.77 (s, 3H), 0.63 (s, 3H); ^{1}H NMR (CD$_3$OD, 400 MHz): δ 7.47 (t, 2H, J = 7.2 Hz), 7.39 (t, 1H, J = 7.2 Hz), 7.28 (d, 2H, J = 7.6 Hz), 3.85 (s, H), 3.77 (d, 1H, J = 2.4 Hz), 3.39-3.33 (m, 1H), 3.23 (s, 3H), 2.31-2.14 (m, 3H), 2.04-1.91 (m, 3H), 1.80-1.62 (m, 6H), 1.59-1.51 (m, 5H), 1.45-1.35 (m, 2H), 1.28-1.16 (m, 3H), 1.09-1.05 (m, 1H), 1.00-0.89 (m, 4H), 0.76 (d, 3H, J = 5.2 Hz), 0.64 (s, 3H); ^{13}C NMR (CDCl$_3$, 150 MHz): δ 173.92, 144.20, 129.71, 127.73, 127.28, 72.99, 71.87, 68.41, 47.16, 46.39, 41.55, 41.48, 39.45, 39.40, 37.38, 35.62, 35.27, 34.76, 34.67, 31.73, 31.50, 30.47, 28.12, 27.47, 26.29, 23.24, 22.46, 17.30, 12.47; ^{13}C NMR (CD$_3$OD, 100 MHz): δ 176.27, 145.35, 131.02, 129.25, 128.46, 73.93, 72.89, 69.05, 47.84, 47.43, 42.21, 42.93, 41.01, 40.48, 37.84, 36.83, 36.51, 35.90, 35.88, 33.22, 32.04, 31.19, 29.57, 28.55, 27.86, 24.19, 23.17, 17.64, 12.95. HRMS (ESI, m/z): calculated for C$_{31}$H$_{48}$NO$_4$ [M+H]$^+$: 498.3578; found, 498.3568.

N,N-(Di-ethyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (4b). Compound **4b** was prepared using the general method described above. Cholic acid (820 mg, 2.00 mmol), HBTU (2.55 mmol), diethyl amine (2.65 mmol), and NMM (4.10 mmol) were reacted to give the desired molecule **4b** in 78% yield (732 mg). mp. 202-204°C; ^{1}H NMR (CD$_3$OD, 400 MHz): δ 3.95 (s, 1H), 3.79 (d, 1H, J = 2.4 Hz), 3.42-3.33 (m, 5H), 2.41-2.37 (m, 1H), 2.33-2.23 (m, 3H), 2.04-1.87 (m, 4H), 1.85-1.74 (m, 3H), 1.67-1.51 (m, 6H), 1.47-1.30 (m, 5H), 1.20 (t, 3H, J = 7.2 Hz), 1.14-1.08 (m, 4H), 1.05-0.94 (m, 4H), 0.92 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (DMSO-d_6, 150 MHz): δ 171.45, 71.04, 70.46, 66.27, 46.15, 46.78, 41.55, 39.58, 39.26, 35.33, 35.28, 34.89, 34.40, 31.42, 30.42, 29.30, 28.55, 27.36, 26.22, 22.86, 22.63, 17.20, 14.44, 13.12, 12.35. HRMS (ESI, m/z): calculated for C$_{28}$H$_{50}$NO$_4$ [M+H]$^+$: 464.3734; found, 464.3730.

N,N'-(Di-allyl)-3α,7α,12α-trihydroxy-5β-cholan-24-amide (4c). Compound **4c** was prepared using the general method described above. Cholic acid (819 mg, 2.0 mmol) HBTU (2.50

mmol), diallyl amine (2.40 mmol), and NMM (5.46 mmol) were reacted to give the desired molecule **4c** in 86% yield (843 mg). mp. 171-173°C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 5.81-5.71 (m, 2H), 5.22-5.09 (m, 4H), 4.05-3.85 (m, 6H), 3.48-3.43 (m, 1H), 2.41-2.34 (m, 1H), 2.28-2.16 (m, 3H), 1.98-1.58 (m, 13H), 1.51-1.30 (m, 8H), 1.19-1.11 (m, 1H), 1.02-0.95 (m, 4H), 0.89 (s, 3H), 0.69 (s, 3H); ^{1}H NMR (CD$_3$OD, 400 MHz): δ 5.87-5.72 (m, 2H), 5.24-5.11 (m, 4H), 3.97 and 3.94 (2 peaks, 5H), 3.79 (d, 1H, J = 2.4 Hz), 3.39-3.35 (m, 1H), 2.46-2.39 (m, 1H), 2.33-2.22 (m, 3H), 2.03-1.73 (m, 7H), 1.66-1.51 (m, 6H), 1.47-1.29 (m, 5H), 1.17-1.06 (m, 1H), 1.03-0.91 (m, 7H), 0.71 (s, 3H); ^{13}C NMR (CDCl$_3$, 150 MHz): δ 173.83, 133.36, 132.97, 117.08, 116.62, 73.04, 71.86, 68.42, 49.22, 47.78, 47.06, 46.40, 41.52, 41.50, 39.44, 39.27, 35.71, 35.29, 34.80, 34.73, 31.46, 30.46, 30.24, 27.59, 26.23, 23.28, 22.43, 17.55, 12.49; ^{13}C NMR (CD$_3$OD, 150 MHz): δ 176.27, 134.45, 134.21, 117.51, 116.97, 73.95, 72.83, 68.97, 50.78, 49.18, 47.45, 43.16, 42.94, 40.99, 40.41, 36.87, 36.49, 35.87, 35.84, 32.85, 31.15, 30.78, 29.56, 28.70, 27.82, 24.22, 23.20, 17.84, 13.01. HRMS (ESI, m/z): calculated for C$_{30}$H$_{50}$NO$_4$ [M+H]$^+$: 488.3734; found, 488.3721.

3α,7α,12α-Trihydroxy-5β-cholan-24-(indolin-1'-yl)-amide (5a). Compound **5a** was prepared using the general method described above. Cholic acid (821 mg, 2.01 mmol), HBTU (2.50 mmol), indoline (3.56 mmol), and NMM (5.46 mmol) were reacted to give the desired molecule in 88% yield (903 mg). mp. 280-283°C; ^{1}H NMR (DMSO-d_6, 600 MHz): δ 8.07 (d, 1H, J = 6.0 Hz), 7.21 (s, 1H), 7.12 (s, 1H), 6.96 (s, 1H), 4.34 (s, 1H), 4.14-4.03 (m, 4H), 3.81 (s, 1H), 3.62 (s, 1H), 3.19 and 3.13 (2 s, 3H), 2.45 (br s, 1H), 2.32 (br s, 1H), 2.25-2.16 (m, 2H), 2.01 and 2.00 (2 peaks, 1H), 1.86-1.66 (m, 6H), 1.45-1.23 (m, 11H), 0.98 (br s, 4H), 0.86-0.81 (m, 4H), 0.61 (s, 3H); ^{13}C NMR (DMSO-d_6, 150 MHz): δ 171.34, 143.11, 131.57, 126.84, 124.66, 122.88, 115.90, 71.08, 70.48, 66.28, 47.39, 46.21, 45.79, 41.55, 41.38, 35.33, 35.19, 34.88, 34.37, 32.16,

30.43, 30.33, 28.54, 27.41, 27.34, 26.21, 22.85, 22.59, 17.22, 12.35. HRMS (ESI, m/z): calculated for $C_{32}H_{48}NO_4$ $[M+H]^+$: 510.3578; found, 510.3570.

3α,7α,12α-Trihydroxy-5β-cholan-24-(4'-chloroindolin-1'-yl)-amide (5b). Compound **5b** was prepared using the general method described above. Cholic acid (205 mg, 0.50 mmol), HBTU (0.60 mmol), and 4-chloroindoline (0.50 mmol), and NMM (2.27 mmol) were reacted to give the desired molecule. The product was purified by column chromatography using CH_2Cl_2-MeOH-NH_4OH (89:10:1) to furnish **5b** in 62% yield (170 mg). mp. 224-227°C; ^1H NMR (CD_3OD, 400 MHz): δ 8.04 (d, 1H, $J = 8.0$ Hz), 7.15 (t, 1H, $J = 7.6$ Hz), 7.019 (d, 1H, $J = 7.6$ Hz), 4.20 (t, 2H, $J = 8.4$ Hz), 3.97 (s, 1H), 3.80 (s, 1H), 3.35 (m, peak under solvent), 3.22 (t, 3H, $J = 8.4$ Hz), 2.60-2.54 (m, 1H), 2.42-2.39 (m, 1H), 2.33-2.24 (m, 2H), 2.05-1.75 (m, 9H), 1.67-1.29 (m, 15H), 1.17-1.07 (m, 4H), 1.02-0.92 (m, 5H), 0.74 (s, 3H); ^1H NMR (DMSO-d_6, 400 MHz): δ 8.02 (d, 1H, $J = 8.0$ Hz), 7.19 (t, 1H, $J = 8.0$ Hz), 7.04 (d, 1H, $J = 8.0$ Hz), 4.33 (s, 1H), 4.18-4.11 (m, 3H), 4.03 (s, 1H), 3.81 (s, 1H), 3.62 (s, 1H), 3.18-3.12 (m, peak under solvent), 2.47-2.44 (m, 1H), 2.36-2.14 (m, 3H), 2.04-1.97 (m, 1H), 1.88-1.78 (m, 4H), 1.67-1.64 (m, 2H), 1.43-1.24 (m, 11H), 0.99-0.93 (m, 4H), 0.88-0.82 (m, 4H), 0.61 (s, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ 174.95, 145.73, 131.74, 131.58, 130.70, 124.70, 116.43, 74.20, 73.03, 69.19, 48.11, 47.65, 43.34, 43.16, 40.60, 36.99, 36.63, 36.05, 36.00, 33.77, 31.98, 31.32, 29.72, 28.87, 28.46, 28.03, 24.40, 23.30, 18.03, 13.14. HRMS (ESI, m/z): calculated for $C_{32}H_{47}NO_4Cl$ $[M+H]^+$: 544.3188; found, 544.3180.

3α,7α,12α-Trihydroxy-5β-cholan-24-(5'-methylindolin-1'-yl)-amide (5c). Compound **5c** was prepared using the general method described above. Cholic acid (205 mg, 0.50 mmol), HBTU (0.60 mmol), and 5-methyl-2,3-dihydro-1H-indole (0.50 mmol), and NMM (2.27 mmol) were reacted to give the desired molecule. The product was purified by column chromatography using CH_2Cl_2-MeOH-NH_4OH (89:10:1) to furnish **5c** in 90% yield (239 mg). mp. 215-218°C; ^1H NMR

(CD$_3$OD, 400 MHz): δ 7.98 (d, 1H, J = 7.6 Hz), 7.03 (s, 1H), 6.96 (d, 1H, J = 7.6 Hz), 4.11 (t, 2H, J = 7.6 Hz), 3.97 (s, 1H), 3.80 (s, 1H), 3.16 (t, 2H, J = 7.6 Hz), 2.54-2.51 (m, 1H), 2.39-2.29 (m, 6H), 2.03-1.80 (m, 7H), 1.68-1.30 (m, 13H), 1.14-1.08 (m, 4H), 1.02-0.92 (m, 4H), 0.73 (s, 3H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ 174.26, 141.89, 134.87, 133.54, 128.68, 126.49, 117.86, 74.17, 73.00, 69.17, 49.98, 48.09, 47.63, 43.32, 43.13, 41.15, 40.59, 37.04, 36.62, 36.03, 35.99, 33.73, 32.18, 31.31, 29.71, 28.91, 28.87, 28.00, 24.39, 23.30, 21.18, 18.03, 13.15. HRMS (ESI, m/z): calculated for C$_{33}$H$_{50}$NO$_4$ [M+H]$^+$: 524.3734; found, 524.3725.

3α,7α,12α-Trihydroxy-5β-cholan-24-(5'-fluoroindolin-1'-yl)-amide (5d). Compound **5d** was prepared using the general method described above. Cholic acid (205 mg, 0.50 mmol), HBTU (0.60 mmol), and 5-fluoroindoline (0.50 mmol), and NMM (2.27 mmol) were reacted to give the desired molecule. The product was purified by column chromatography using EtOAc-MeOH (90:10) to furnish **5d** in 93% yield (246 mg). mp. >250°C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 8.06 (dd, 1H, J = 8.8 and 5.2 Hz), 7.08 (d, 1H, J = 8.4 Hz), 6.94 (td, 1H, J = 9.2 and 2.4 Hz), 4.32 (d, 1H, J = 4.0 Hz), 4.15-4.10 (m, 3H), 4.00 (d, 1H, J = 3.2 Hz), 3.81 (d, 1H, J = 2.4 Hz), 3.63 (s, 1H), 3.20-3.12 (m, 3H), 2.51-2.45 (m, 1H), 2.36-2.13 (m, 3H), 2.05-1.97 (m, 1H), 1.89-1.64 (m, 7H), 1.43-1.24 (m, 12H), 0.99-0.98 (m, 4H), 0.88-0.82 (m, 4H), 0.61 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 171.22, 139.60, 134.27, 116.54, 113.02, 112.80, 112.14, 111.90, 71.02, 70.43, 66.24, 47.73, 46.20, 45.78, 41.51, 41.38, 35.31, 35.15, 34.87, 34.39, 31.92, 30.41, 30.32, 28.55, 27.42, 27.30, 26.20, 22.85, 22.63, 17.22, 12.38. HRMS (ESI, m/z): calculated for C$_{32}$H$_{47}$NO$_4$F [M+H]$^+$: 528.3484; found, 544.3476.

3α,7α,12α-Trihydroxy-5β-cholan-24-(5'-chloroindolin-1'-yl)-amide (5e). Compound **5e** was prepared using the general method described above. Cholic acid (208 mg, 0.50 mmol), HBTU (0.60 mmol), and 5-chloro-2,3-dihydro-(1H)indole (0.50 mmol), and NMM (2.27 mmol) were

reacted to give the desired molecule. The product was purified by column chromatography using CH$_2$Cl$_2$-MeOH-NH$_4$OH (89:10:1) to furnish **5e** in 92% yield (255 mg). mp. 231-234°C; ^{1}H NMR (CD$_3$OD, 400 MHz): δ 8.07 (d, 1H, J = 8.8 Hz), 7.21 (s, 1H), 7.13 (d, 1H, J = 8.4), 4.17 (t, 2H, J = 8.4 Hz), 3.97 (s, 1H), 3.80 (s, 1H), 3.20 (t, 2H, J = 8.0 Hz), 2.58-2.53 (m, 1H), 2.45-2.38 (m, 1H), 2.33-2.24 (m, 2H), 2.05-1.75 (m, 7H), 1.67-1.29 (m, 12H), 1.17-1.06 (m, 4H), 1.02-0.92 (m, 4H), 0.73 (s, 3H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ 174.74, 143.14, 135.82, 129.79, 128.12, 126.05, 118.96, 74.20, 73.02, 69.19, 48.09, 47.64, 43.33, 43.15, 41.16, 40.60, 37.00 36.62, 36.04, 35.99, 33.66, 32.04, 31.31, 29.71, 28.86, 28.77, 28.02, 24.39, 23.30, 18.02, 13.13. HRMS (ESI, m/z): calculated for C$_{32}$H$_{47}$NO$_4$Cl [M+H]$^+$: 544.3188; found, 544.3180.

3α,7α,12α-Trihydroxy-5β-cholan-24-(5'-bromoindolin-1'-yl)-amide (5f). Compound **5f** was prepared using the general method described above. Cholic acid (206 mg, 0.50 mmol), HBTU (0.60 mmol), and 5-bromoindoline (0.50 mmol), and NMM (2.27 mmol) were reacted to give the desired molecule. The product was purified by column chromatography using CH$_2$Cl$_2$-MeOH-NH$_4$OH (89:10:1) to furnish **5f** in 91% yield (271 mg). mp. 230-233°C; ^{1}H NMR (CD$_3$OD, 400 MHz): δ 8.02 (d, 1H, J = 8.4 Hz), 7.36 (s, 1H), 7.27 (d, 1H, J = 8.4 Hz), 4.16 (t, 2H, J = 8.0 Hz), 3.96 (s, 1H), 3.80 (s, 1 H), 3.20 (t, 2H, J = 8.4 Hz), 2.59-2.52 (m, 1H), 2.44-2.34 (m, 1H), 2.30-2.23 (m, 2H), 2.05-1.83 (m, 7H), 1.80-1. 29 (m, 11H), 1.17-1.06 (m, 5H), 1.01-0.92 (m, 3H), 0.73 (s, 3H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ 174.79, 143.62, 136.19, 131.13, 129.01, 119.41, 117.18, 74.20, 73.03, 69.18, 48.10, 47.65, 43.35, 43.16, 41.17, 40.61, 37.01, 36.64, 36.05, 36.01, 33.70, 32.03, 31.33, 29.73, 28.88, 28.73, 28.03, 24.40, 23.31, 18.02, 13.14. HRMS (ESI, m/z): calculated for C$_{32}$H$_{47}$NO$_4$Br [M+H]$^+$: 588.2683; found, 544.2674.

3α,7α,12α-Trihydroxy-5β-cholan-24-(6'-methoxyindolin-1'-yl)-amide (5g). Compound **5g** was prepared using the general method described above. Cholic acid (206 mg, 0.50 mmol),

HBTU (0.60 mmol), and 6-methoxyindoline (0.50 mmol), and NMM (2.27 mmol) were reacted to give the desired molecule. The product was purified by column chromatography using CH_2Cl_2-MeOH-NH$_4$OH (89:10:1) to furnish **5g** in 97% yield (264 mg). mp. 234-236°C; ^{1}H NMR (CD$_3$OD, 400 MHz): δ 7.69 (s, 1H), 6.95 (d, 1H, J = 6.4 Hz), 6.47 (d, 1H, J = 7.2 Hz), 3.98 (br s, 2H), 3.85 (s, 1H), 3.68 (s, peak under solvent), 3.25 and 3.21 (2 peaks, 2H), 2.98 (s, 1H), 2.41 (m, 1H), 2.25-2.17 (m, 3H), 1.91-1.68 (m, 7H), 1.56-1.18 (m, 12H), 0.97-0.96 (m, 4H), 0.90-0.80 (m, 4H), 0.61 (s, 3H); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.74 (s, 1H), 7.10 (d, 1H, J = 8.4 Hz), 6.55 (d, 1H, J = 8 Hz), 4.34 (d, 1H, J = 3.6 Hz), 4.14-4.09 (m, 3H), 4.04 (s, 1H), 3.18 (d, 1H, J = 4.8 Hz), 3.05 (t, 2H, J = 8 Hz), 2.46-2.44 (m, 1H), 2.36-2.14 (m, 4H), 2.04-1.97 (m, 1H), 1.86-1.64 (m, 8H), 1.43-1.24 (m, 14 H), 0.98 (d, 4H, J = 6 Hz), 0.82 (m, 3H), 0.61 (s, 3H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ 174.56, 160.72, 145.23, 125.96, 125.14, 110.42, 104.72, 74.12, 72.97, 69.13, 56.01, 50.25, 48.08, 47.61, 43.27, 43.09, 41.12, 40.55, 36.97, 36.61, 36.00, 33.83, 32.03, 31.28, 29.69, 28.87, 28.15, 27.96, 24.38, 23.31, 18.05, 13.17. HRMS (ESI, m/z): calculated for $C_{33}H_{50}NO_5$ [M+H]$^+$: 540.3684; found, 540.3670.

3α,7α,12α-Trihydroxy-5β-cholan-24-(3',4'-dihydroquinolin-1'-(2H)yl)-amide (5h). Compound **5h** was prepared using the general method described above. Cholic acid (822 mg, 2.01 mmol), HBTU (2.50 mmol), 1,2,3,4-tetrahydroquinoline (2.55 mmol), and NMM (5.46 mmol) were reacted to give the desired molecule in 86% yield (912 mg). mp. 104-106°C; ^{1}H NMR (CDCl$_3$, 600 MHz): δ 7.19-7.12 (m, 4H), 3.94 (s, 1H), 3.84 (s, 2H), 3.75-3.71 (m, 1H), 3.45 (s, 1H), 2.71 (s, 2H), 2.57-2.54 (m, 1H), 2.43 and 2.42 (2 peaks, 1H), 2.25-2.16 (m, 2H), 1.95-1.57 (m, 17H), 1.52 (s, 2H), 1.40-1.37 (m, 3H), 1.27 and 1.26 (2 peaks, 1H), 1.11 (br s 1H), 0.99-0.88 (m, 7H), 0.66 (s, 3H); ^{1}H NMR (CD$_3$OD, 400 MHz): δ 7.19 and 7.16 (2 peaks, 4H), 3.89 (s, 1H), 3.78 (s, 2H), 3.71 and 3.69 (2 peaks, 1H), 3.36 (br, 1H), 2.72 (br s, 2H), 2.58 and 2.56 (2 peaks,

1H), 2.50 (br s, 1H), 2.28-2.23 (m, 2H), 1.96 and 1.95 (2 peaks, 4H), 1.77-1.50 (m, 11H), 1.45-1.19 (m, 5H), 1.09-0.90 (m, 8H), 0.67 (s, 3H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ 176.08, 140.28, 129.59, 127.24, 126.92, 126.01, 73.98, 72.90, 69.05, 48.00, 47.50, 43.22, 42.97, 41.03, 40.49, 36.92, 36.52, 35.91, 35.89, 33.33, 32.38, 31.21, 29.59, 28.62, 27.88, 27.56, 25.29, 24.22, 23.18, 17.70, 12.99. HRMS (ESI, m/z): calculated for C$_{33}$H$_{50}$NO$_4$ [M+H]$^+$: 524.3734; found, 524.3727.

3α,7α,12α-Trihydroxy-5β-cholan-24-(6'-methyl-3',4'-dihydroquinolin-1'-(2H)yl)-amide (5i). Compound **5i** was prepared using the general method described above. Cholic acid (207 mg, 0.50 mmol), HBTU (0.60 mmol), and 6-methyl-1,2,3,4-tetrahydroquinoline (0.50 mmol), and NMM (2.27 mmol) were reacted to give the desired molecule. The product was purified by column chromatography using CH$_2$Cl$_2$-MeOH-NH$_4$OH (89:10:1) to furnish **5i** in 95% yield (260 mg). mp. 163-167°C; ^{1}H NMR (CD$_3$OD, 400 MHz): δ 7.05 (s, 3H), 3.92 (s, 1H), 3.81 (m, 2H), 3.68-3.65 (m, 1H), 3.39-3.36 (m, 2H), 3.33 (s, 1H), 2.67 (br s, 2H), 2.60-2.55 (m, 1H), 2.50-2.45 (m, 1H), 2.28-2.20 (m, 2H), 1.95-1.93 (m, 4H), 1.81-1.46 (m, 12H), 1.43-0.97 (m, 9H), 0.90-0.87 (m, 4H), 0.66 (s, 3H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ 176.17, 137.77, 130.15, 127.96, 125.83, 74.06, 72.97, 69.14, 48.04, 47.56, 43.28, 43.05, 41.08, 40.55, 37.02, 36.60, 35.99, 35.96, 31.27, 29.65, 28.71, 27.95, 27.61, 25.36, 24.32, 23.28, 21.13, 17.79, 13.09. HRMS (ESI, m/z): calculated for C$_{34}$H$_{52}$NO$_4$ [M+H]$^+$: 538.3891; found, 538.3879.

3α,7α,12α-Trihydroxy-5β-cholan-24-(isoindolin-2'-yl)-amide (6a). Compound **6a** was prepared using the general method described above. Cholic acid (205 mg, 0.50 mmol), HBTU (0.60 mmol), and isoindoline (0.50 mmol), and NMM (2.27 mmol) were reacted to give the desired molecule. The product was purified by column chromatography using CH$_2$Cl$_2$-MeOH-NH$_4$OH (89:10:1) to furnish **6a** in 79% yield (201 mg). mp. >250°C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.35 (s, 2H), 7.31 (s, 2H), 4.84 (s, 2H), 4.62 (s, 2H), 4.34 (d, 1H, J = 4.0 Hz), 4.13 (d, 1H, J = 2.8

Hz), 4.04 (d, 1H, J = 2.8 Hz), 3.81 (s, 1H), 3.63 (s, 1H), 3.20-3.17 (m, 1H), 2.43-2.35 (m, 1H), 2.29-2.13 (m, 3H), 2.05-1.97 (m, 1H), 1.89-1.64 (m, 7H), 1.43-1.18 (m, 12H), 0.99-0.94 (m, 4H), 0.88-0.82 (m, 4H), 0.61 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 171.43, 136.97, 136.33, 127.36, 127.27, 122.99, 122.86, 71.03, 70.43, 66.24, 51.82, 51.58, 46.15, 45.76, 41.51, 41.38, 35.31, 35.20, 34.87, 34.39, 30.48, 28.55, 27.29, 26.20, 22.85, 22.63, 17.23, 12.38. HRMS (ESI, m/z): calculated for $C_{32}H_{48}NO_4$ [M+H]$^+$: 510.3578; found, 510.3571.

3α,7α,12α-Trihydroxy-5β-cholan-24-(3',4'-dihydroisoquinolin-2'-(1H)yl)-amide (6b). Compound **6b** was prepared using the general method described above. Cholic acid (824 mg, 2.01 mmol), HBTU (2.50 mmol), 1,2,3,4-tetrahydroisoquinoline (2.50 mmol), and NMM (5.46 mmol) were reacted to give the desired molecule **6b** in 91% yield (961 mg). mp. 225-228°C; ^{1}H NMR (CDCl$_3$, 600 MHz): δ 7.20-7.12 (m, 4H), 4.72 (s, 1H), 4.63 (s, 1H), 3.98 (a, 1H), 3.84 and 3.82 (2 peaks, 2H), 3.68 (m, 1H), 3.45 (s, 1H), 2.91 (s, 1H), 2.84 (s, 1H), 2.48 and 2.46 (2 peaks, 1H), 2.36-2.30 (m, 1H), 2.26-2.00 (m, 4H), 1.95-1.59 (m, 11H), 1.53-1.25 (m, 8H), 1.11 (br, 1H), 1.03-0.95 (m, 4H), 0.88 (s, 3H), 0.69 and 0.67 (2 peaks, 3H); ^{1}H NMR (CD$_3$OD, 400 MHz): δ 7.16 (s, 4H), 4.70 and 4.66 (2 s, 2H), 3.94 (d, 1H, J = 6.0 Hz), 3.77 (br s, 3H), 3.36 (s, 1H), 2.92 (s, 1H), 2.83 (s, 1H), 2.54 and 2.51 (2 peaks, 1H), 2.41 and 2.40 (2 peaks, 1H), 2.29 and 2.26 (2 peaks, 2H), 1.97-1.78 (m, 7H), 1.66-1.30 (m, 11H), 1.06-0.91 (m, 8H), 0.71 and 0.66 (2 peaks, 3H); ^{13}C NMR (CDCl$_3$, 150 MHz): δ 172.90, 172.72, 135.13, 134.05, 133.59, 132.70, 128.94, 128.29, 126.88, 126.55, 126.47, 126.32, 126.05, 73.05, 71.88, 68.44, 47.49, 46.91, 46.45, 44.25, 43.34, 41.63, 41.48, 39.72, 39.46, 39.40, 35.72, 35.61, 35.26, 34.78, 34.71, 31.33, 31.18, 30.84, 30.71, 30.53, 29.60, 28.50, 28.20, 27.63, 26.60, 26.32, 23.30, 22.46, 17.64, 17.60, 12.51; ^{13}C NMR (CD$_3$OD, 100 MHz): δ 175.34, 175.21, 136.15, 135.66, 134.49, 134.29, 129.79, 129.45, 128.05, 127.76, 127.62, 127.52, 127.22, 74.05, 72.91, 69.05, 47.94, 47.54, 47.52, 45.41, 44.84, 43.23,

43.03, 41.46, 41.06, 40.50, 37.04, 36.89, 36.53, 35.93, 35.90, 32.78, 31.61, 31.38, 31.22, 30.46, 29.61, 29.36, 28.79, 28.74, 27.91, 24.28, 23.21, 17.88, 13.03, 12.98. HRMS (ESI, m/z): calculated for $C_{33}H_{50}NO_4$ [M+H]$^+$: 524.3734; found, 524.3726.

3α,7α,12α-Trihydroxy-5β-cholan-24-(6'-methoxy-3',4'-dihydroisoquinolin-2'-(1H)yl)-amide (6c). Compound **6c** was prepared using the general method described above. Cholic acid (208 mg, 0.50 mmol), HBTU (0.60 mmol), and 6-methoxy-1,2,3,4-tetrahydroisoquinoline (0.50 mmol), and NMM (2.27 mmol) were reacted to give the desired molecule. The product was purified by column chromatography using CH_2Cl_2-MeOH-NH_4OH (89:10:1) to furnish **6c** in 88% yield (249 mg). mp. 164-166°C; ^1H NMR (CD$_3$OD, 400 MHz): δ 6.95 (t, 1H, J = 9.6 Hz), 6.65 (d, 1H, J = 8.4 Hz), 6.62 (s, 1H), 4.49 (d, 2H, J = 10.8 Hz), 3.83 (d, 1H, J = 8.0 Hz), 3.67-3.61 (m, peak under solvent), 3.25-3.21 (m, 2H), 2.78 (br s, 1H), 2.69 (br s, 1H), 2.44-2.39 (m, 1H), 2.30-2.13 (m, 3H), 1.94-1.63 (m, 8H), 1.56-1.18 (m, 12H), 1.01-0.95 (m, 4H), 0.90-0.80 (m, 4H), 0.60 and 0.55 (2 peaks, 3H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ 175.43, 175.34, 160.26, 160.03, 137.55, 137.01, 128.64, 128.33, 126.64, 126.48, 114.56, 114.31, 113.93, 113.86, 74.17, 73.02, 69.19, 55.83, 48.05, 47.65, 45.04, 44.88, 43.34, 43.14, 41.54, 41.16, 40.61, 37.14, 36.99, 36.63, 36.04, 35.99, 32.90, 31.73, 31.51, 31.32, 30.81, 29.71, 28.89, 28.83, 28.02, 24.36, 23.29, 17.98, 13.12, 13.08. HRMS (ESI, m/z): calculated for $C_{34}H_{52}NO_5$ [M+H]$^+$: 554.3840; found, 554.3833.

3α,7α,12α-Trihydroxy-5β-cholan-24-(6'-bromo-3,4-dihydroisoquinolin-2'-(1H)yl)-amide (6d). Compound **6d** was prepared using the general method described above. Cholic acid (206 mg, 0.50 mmol), HBTU (0.60 mmol), and 6-bromo-1,2,3,4-tetrahydroisoquinoline (0.50 mmol), and NMM (2.27 mmol) were reacted to give the desired molecule. The product was purified by column chromatography using CH_2Cl_2-MeOH-NH_4OH (89:10:1) to furnish **6d** in 92% yield (281 mg). mp. 162-164°C; ^1H NMR (CD$_3$OD, 400 MHz): δ 7.24-7.21 (m, 2H), 6.98 (t, 1H,

$J = 8.4$ Hz), 4.56 (s, 1H), 4.52 (s, 1H), 3.84 (d, 1H, $J = 8.8$ Hz), 3.68-3.62 (m, 3H), 3.21 (s, 1H), 2.81 (m, 1H), 2.72 (t, 1H, $J = 4.8$ Hz), 2.46-2.40 (m, 1H), 2.34-2.23 (m, 1H), 2.19-2.13 (m, 2H), 1.94-1.63 (m, 7H), 1.56-1.18 (m, 11H), 1.01-0.93 (m, 4H), 0.90-0.80 (m, 4H), 0.60 and 0.55 (2 peaks, 3H); ^{13}C NMR (CD$_3$OD, 100 MHz): δ 175.34, 175.21, 138.83, 138.31, 133.90, 133.70, 132.73, 132.40, 130.75, 130.63, 129.58, 129.27, 121.58, 121.26, 74.10, 72.96, 69.11, 49.98, 47.98, 47.59, 47.57, 45.03, 44.42, 43.28, 43.08, 43.07, 41.10, 41.02, 40.54, 37.10, 36.94, 36.61, 36.00, 32.79, 31.63, 31.39, 31.28, 30.30, 29.67, 29.23, 28.89, 28.83, 27.95, 24.38, 24.35, 23.32, 23.19, 17.97, 13.15, 13.09. HRMS (ESI, m/z): calculated for C$_{33}$H$_{49}$NO$_4$Br [M+H]$^+$: 602.2839; found, 602.2829.

3α,7α,12α-Trihydroxy-5β-cholan-24-(23yrrolidine-1'-yl)-amide (7a). Compound **7a** was prepared using the general method described above. Cholic acid (824 mg, 2.0 mmol), HBTU (2.50 mmol), pyrrolidine (2.61 mmol), and NMM (5.46 mmol) were reacted to give the desired molecule **7a** in 82% yield (761 mg). mp. 255-258°C; ^{1}H NMR (DMSO-d_6, 600 MHz): δ 4.33 (d, 1H, $J = 3.0$ Hz), 4.11 (s, 1H), 4.02 (s, 1H), 3.79 (s, 1H), 3.61 (s, 1H), 3.38 (t, 2H, $J = 6.0$ Hz), 3.25 (t, 2H, $J = 6.0$ Hz), 3.18 (br s, 1H), 2.25-2.18 (m, 2H), 2.16-2.07 (m, 2H), 1.98 (q, 1H, $J = 11.4$ Hz), 1.87-1.84 (m, 2H), 1.81-1.74 (m, 5H), 1.63 (br, 3H), 1.45-1.41 (m, 3H), 1.38-1.33 (m, 4H), 1.28-1.17 (m, 4H), 0.99-0.93 (m, 4H), 0.86-0.81 (m, 4H), 0.59 (s, 3H); ^{13}C NMR (DMSO-d_6, 150 MHz): δ 170.82, 71.03, 70.46, 66.26, 46.14, 45.92, 45.76, 45.25, 41.54, 41.38, 39.58, 35.33, 35.25, 34.89, 34.40, 30.98, 30.71, 30.42, 28.56, 27.33, 26.21, 25.70, 23.98, 22.86, 22.64, 17.21, 12.37. HRMS (ESI, m/z): calculated for C$_{28}$H$_{48}$NO$_4$ [M+H]$^+$: 462.3578; found, 462.3567.

3α,7α,12α-Trihydroxy-5β-cholan-24-(piperidin-1'-yl)-amide (7b). Compound **7b** was prepared using the general method described above. Cholic acid (819 mg, 2.00 mmol), HBTU (2.50 mmol), piperidine (2.57 mmol), and NMM (5.46 mmol) were reacted to give the desired

molecule **7b** in 89% yield (851 mg). mp. 246-249°C; ^{1}H NMR (DMSO-d_6, 600 MHz): δ 4.33 (d, 1H, J = 3.6 Hz), 4.11 (s, 1H), 4.02 (s, 1H), 3.79 (s, 1H), 3.61 (s, 1H), 3.39-3.37 (m, 4H), 3.18 (br, 1H), 2.30-2.14 (m, 4H), 1.98 (q, 1H, J = 11.4 Hz), 1.81-1.77 (m, 2H), 1.72 (br, 1H), 1.69-1.56 (m, 5H), 1.48-1.31 (m, 11H), 1.27-1.16 (m, 4H), 1.00-0.94 (m, 4H), 0.86-0.81 (m, 4H), 0.59 (s, 3H); ^{13}C NMR (DMSO-d_6, 150 MHz): δ 170.70, 71.05, 70.46, 66.26, 46.09, 46.01, 45.78, 41.80, 41.54, 41.38, 39.58, 35.33, 34.89, 34.39, 31.33, 30.42, 29.67, 28.53, 27.36, 26.21, 25.36, 24.13, 22.86, 22.63, 17.17, 12.35. HRMS (ESI, m/z): calculated for $C_{29}H_{50}NO_4$ [M+H]$^+$: 476.3734; found, 476.3724.

3α,7α,12α-Trihydroxy-5β-cholan-24-(4'-hydroxypiperdin-1'-yl)-amide (7c). Compound **7c** was prepared using the general method described above. Cholic acid (819 mg, 2.00 mmol), HBTU (2.50 mmol), 4-hydroxypiperidine (3.0 mmol), and NMM (5.46 mmol) were reacted to give desired molecule **7c** in 76% yield (749 mg). mp. 244-247°C; ^{1}H NMR (DMSO-d_6, 600 MHz): δ 4.74 (s, 1H), 4.34 (s, 1H), 4.11 (s, 1H), 4.02 (s, 1H), 3.90 (s, 1H), 3.79 (s, 1H), 3.66 and 3.61 (2 peaks, 3H), 3.18 and 3.13 (2 peaks, 2H), 2.94 (s, 1H), 2.29-2.17 (m, 4H), 1.98 (s, 1H), 1.79-1.65 (m, 8H), 1.44-1.19 (m, 13H), 0.94 (s, 4H), 0.81 (s, 4H), 0.59 (s, 3H); ^{1}H NMR (CD$_3$OD, 600 MHz): δ 4.06 (d, 1H, J = 7.8 Hz), 3.95 (s, 1H), 3.83 and 3.79 (2 peaks, 3H), 3.36 (s, 1H), 3.27 and 3.26 (2 peaks, 1H), 3.10 (s, 1H), 2.45 (s, 1H), 2.30-2.25 (m, 3H), 2.01-1.79 (m, 7H), 1.74 (br, 2H), 1.65 (d, 1H, J = 12.0 Hz), 1.58-1.51 (m, 5H), 1.46-1.29 (m, 7H), 1.12 and 1.11 (2 peaks, 1H), 1.04 (d, 3H, J = 5.4 Hz), 0.98 (t, 1H, J = 13.8 Hz), 0.91 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (CD$_3$OD, 150 MHz): δ 174.56, 73.95, 72.83, 68.97, 67.73, 47.83, 47.47, 44.52, 43.17, 42.96, 40.99, 40.42, 40.38, 37.03, 37.01, 36.49, 35.88, 35.85, 35.64, 34.81, 32.93, 31.15, 29.56, 28.78, 27.83, 24.24, 23.20, 17.79, 13.01. HRMS (ESI, m/z): calculated for $C_{29}H_{50}NO_5$ [M+H]$^+$: 492.3684; found, 492.3674.

Bacterial Strains and Spore Preparation. *C. difficile* R20291 was the kind gift of Prof. Nigel Minton (University of Nottingham). Spores were prepared according to previously published protocols[15].

***C. difficile* Spore Germination Assays.** Purified *C. difficile* spores were prepared according to previously published protocols[15]. For the germination assay, spores were diluted to an optical density (580 nm) of 1.0 with a 100 mM sodium phosphate buffer, pH 6.0, containing 5 mg/ml sodium bicarbonate. For the initial test of activity, the compound to be tested was added, in triplicate, to a 96-well plate at a final concentration of 125 μM. To each well, 6mM taurocholate and 12mM glycine was added, followed by the spores. The OD_{580} was measured once every minute for 2 hours and normalized using the OD_{580} obtained at time zero [relative OD_{580} = $OD_{580}(t)/OD_{580}(t_0)$]. Compounds displaying greater than 60% reduction in spore germination were further tested at various concentrations to determine the IC_{50} value. This was calculated from a plot of percent germination versus the log concentration of drug according to equation 1.

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

Table 1. *C. difficile* Spore germination activities of Acyclic tertiary amides **4a-c**.

4
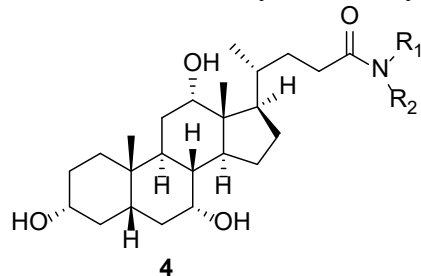
Compounds[a]	R$_1$	R$_2$	% Germination (125 μM)	IC$_{50}$ (μM)[b]
4a	CH$_3$	Ph	31 ± 2	32 ± 5
4b	CH$_2$CH$_3$	CH$_2$CH$_3$	0.05 ± 0.6	29 ± 4
4c	CH$_2$CH=CH$_2$	CH$_2$CH=CH$_2$	0 ± 0.9	8 ± 3

a. Number corresponding to scheme 1.
b. Calculated according to eq. 1.

Table 2. *C. difficile* Spore germination activities of hetero bicyclic tertiary amide analogs (**5a-i**, and **6a-d**.
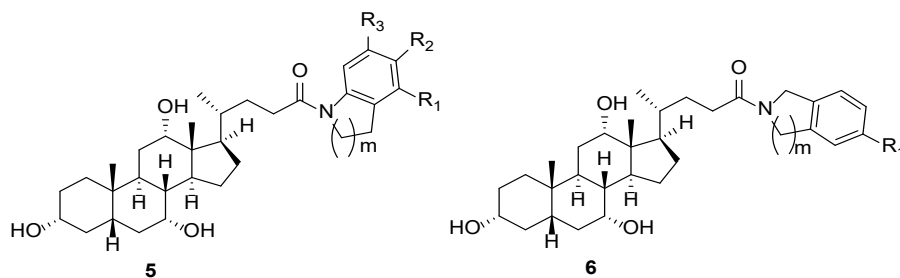
| Compounds[a]	R$_1$	R$_2$	R$_3$	m	% Germination (125 μM)	IC$_{50}$ (μM)[d]
5a	H	H	H	1	4 ± 1	1 ± 0.3
5b	Cl	H	H	1	95 ± 8	ND[e]
5c	H	CH$_3$	H	1	100[b]	ND[e]
5d	H	F	H	1	5 ± 3	0.4 ± 0.04
5e	H	Cl	H	1	70 ± 3	ND[e]
5f	H	Br	H	1	90 ± 3	ND[e]
5g	H	H	OCH$_3$	1	100[b]	ND[e]
5h	H	H	H	2	17 ± 3	22 ± 3
5i	H	CH$_3$	H	2	100b	ND[e]
6a	H	--	--	1	not tested[c]	ND[e]
6b	H	--	--	2	78 ± 2	ND[e]
6c	OCH$_3$	--	--	2	100[b]	ND[e]
6d	Br	--	--	2	100[b]	ND[e]

a. Number corresponding to scheme 1.
b. No inhibition was detected at 125 μM.
c. Compound was not tested because of solubility problems.
d. Calculated according to eq. 1.
e. Not determined

Table 3. *C. difficile* Spore germination activities of cyclic tertiary amides **7a-c**.

7
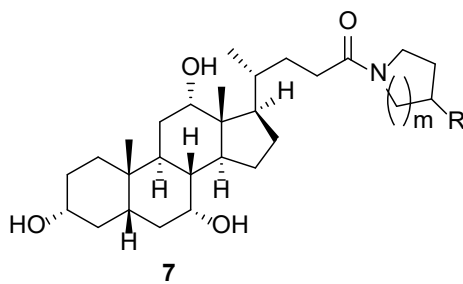
Compounds[a]	R	m	% Germination (125 µM)	IC$_{50}$ (µM)[b]
7a	H	1	29 ± 2	38 ± 4
7b	H	2	15 ± 2	37 ± 4
7c	OH	2	88 ± 1	ND[c]

a. Number corresponding to scheme 1.
b. Calculated according to equation 1.
c. Not determined.

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