5,6-Fused Heterocycle Cholate Derivatives as Spore Germination Inhibitors of *Clostridioides difficile*

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Abstract: *Clostridioides difficile* infection (CDI) is an anaerobic bacterium that is responsible for most antibiotic-associated cases of diarrhea. CDI begins with the ingestion of an environmentally stable spore that germinates within the GI tract under specific conditions. We have shown that the bile salt analog N-phenyl-cholan-24-amide (**1**) can inhibit spore germination and prevent CDI in animal models of infection. Unfortunately, **1**, was stable in the gut of antibiotic-treated animals but was rapidly degraded by gastrointestinal micoflora in the normal gut preventing its use to prevent CDI. We hypothesized that the source of the instability was the amide group. To explore this hypothesis, we removed the amide by either converting it to an amine or to 5,6-fused heterocycles. We found that reduction of the amide to an amine resulted in a significant loss of activity, but conversion to benzimidazole (**6a**), benzothiazole (**6b**), or benzoxazole (**6c**) gave compounds with good antigermination activity. Exploration of other sterane groups such as chenodeoxycholate and deoxycholate gave active compounds only for the benzimidazole series. Examination of **6b** showed that it was stable for 24 hours in the presence of feces taken from healthy mice validating the hypothesis that an amide bioisostere would increase the stability of the compound. Mice treated with **6a** and **6b** also showed no signs of toxicity up to 300 mg/kg daily for 7 days. Each compound was tested for its ability to prevent CDI in a murine model. Compound **6b** but not **6a** was able to prevent CDI when given at a dose of 50 mg/kg.

Introduction

Clostridioides difficle (*C. difficile*) is a spore-forming, anaerobic bacterium infecting the gastrointestinal track leading to significant morbidity and mortality. *C. difficile* infection (CDI) is a growing public health problem where recent data has shown that it afflicts almost 224,000 patients in the United States[1]. CDI is usually treated by antibiotics such as vancomycin or fidaxomicin; however, approximately 20% of patients develop relapsing CDI necessitating continued antibiotic treatment [2]. Unfortunately, antibiotic use has been shown to reduce resistance to *C. difficile* colonization increasing the likelihood of future reinfection. Given this, alternative methods for the treatment and prevention of CDI are critically needed.

C. difficile is spread from patient to patient via environmentally stable spores[3]. Patients possessing a healthy gut microbiome are generally protected from developing CDI even if spores are ingested. However, if the gut microbiome is altered, usually due to antibiotics, resistance to *C. difficile* colonization is compromised[4-5]. The colonization of *C. difficile* begins by the germination of the spore into a vegetative cell. Spore germination is regulated by the composition of bile salts, amino acids, and ions in the gut[6-9]. The composition and concentration of bile salts is dependent on the gut metabolome, and thus, alterations in the gut microbiome result in changes to bile salts in the gastrointestinal tract.

Previous studies have shown that cholate (CA) and taurocholate are inducers of spore germination[10-12]. Taurocholate is deconjugated by species in the gut microbiome to yield CA which is then metabolized to yield deoxycholate. Thus, in a healthy gut microbiome, taurocholate and CA levels are low resulting in an environment which does not favor germination. When the gut microbiome is disrupted by antibiotics, metabolism of taurocholate is diminished leading to elevated levels of taurocholate resulting in an environment which is conducive to spore germination.

Given the connection between bile salts and spore germination, we and others have examined bile salt analogs as potential inhibitors of spore germination[13-18]. Our research has focused on examining cholon-24-amides, although we have shown that chenodeoxy- and deoxycholate derivatives also give active agents[15]. We have extensively explored CaPa (**1**) and CamSA (**2**) and have recently found that CaIn (**3**) possesses an IC_{50} of 38-740 nM depending on the strain tested (Figure 1)[13]. CaIn is the most potent spore germination inhibitor described to date. CamSA and CaPA prevent CDI in mouse models of disease, but unfortunately CaIn displays no activity *in vivo* perhaps due to rapid metabolism[13-14, 16]. We have shown that CamSA and CaPA are stable in feces obtained from antibiotic-treated mice; however, they are rapidly metabolized in feces from healthy animals[14]. As noted above, all the agents that we have produced contain a metabolically labile amide group. In addition to the fact that amide hydrolysis decreases the concentration of the active agent, hydrolysis generates CA which is an inducer of germination, and aniline analogs that are potentially toxic. Since we envision these agents will be used prophylactically to prevent CDI or its recurrence, it is critical that our compounds be stable in a healthy gut microbiome. This requires the elimination of the amide. In this paper, we report on derivatives of CaPA lacking the amide group. We find that 5,6-fused heterocyclic analogs are potent germination inhibitors and display activity in a mouse model of CDI.

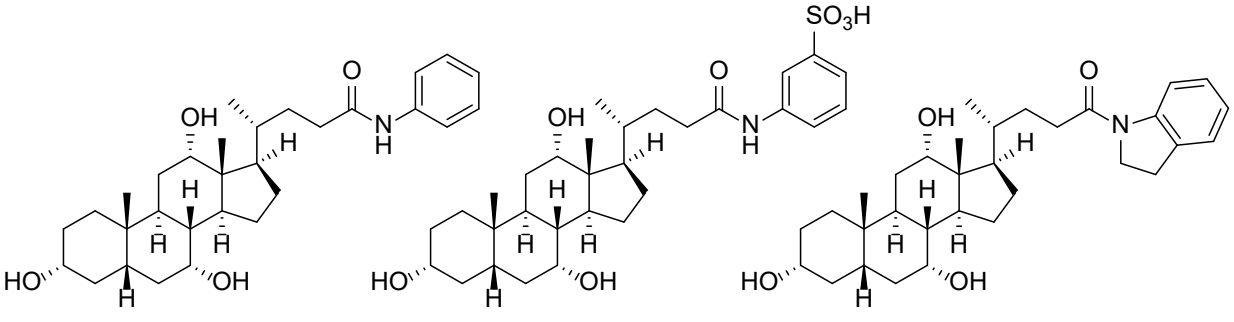


Figure 1. Structure of spore germination inhibitors CaPA, CamSA, and CaIn.

Results and Discussion

Compound Design. Despite their prevalence in many clinically used drugs, amides are enzymatically label which can result in rapid metabolism of therapeutic agents[19]. To improve metabolic stability of compounds, a variety of amide bioisosteres or derivatives have been examined[19]. We initially examined CaPA analogs with *N*-methyl and reverse amide bioisosteres; however, these modifications greatly reduced the anti-germination activity (data not shown). Hence, we have focused on two approaches (Figure 2). The first is reduction of the carbonyl group of the amide to yield the corresponding amine. The second approach is to use heterocyclic bioisosteres such as imidazole, thiazole, or oxazole. Since our previous studies have demonstrated a strong preference for an aromatic ring in potent anti-germinants, we have elected to examine 5,6-fused heterocycles such as benzimidazole, benzothiazole, and benzoxazole derivatives[17]. Lastly, while our most potent compounds are derived from cholate, we have also shown that spore germination inhibitors can be prepared from chenodeoxy- and deoxycholate[15]. Thus, we will also examine heterocyclic derivatives of these bile salts as well.

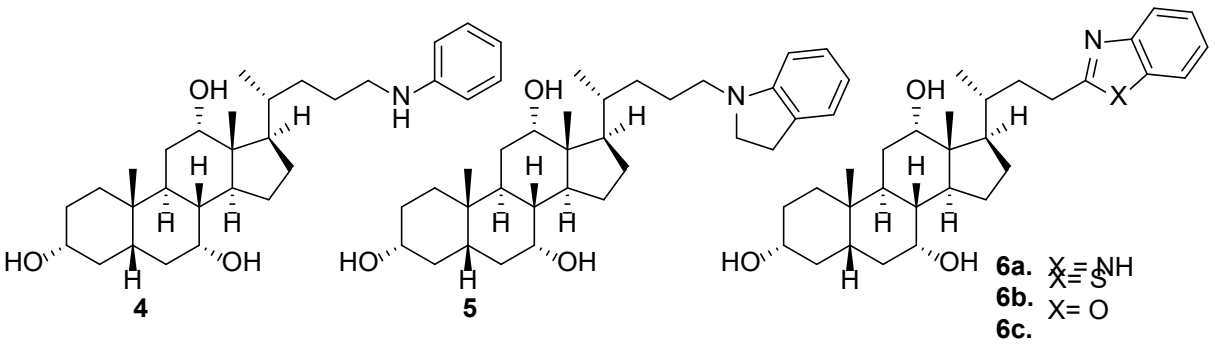
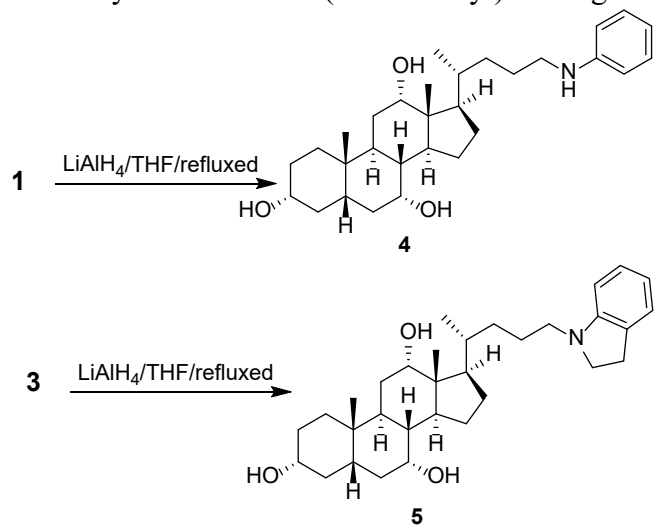


Figure 2. Amide bioisosteres of spore germination inhibitors.

Chemical Synthesis. The synthesis of the reduced amides was accomplished according to scheme 1. CaPA (**1**) and CaIn (**3**) were selectively reduced using refluxing $LiAlH_4$ in THF to give the corresponding secondary and tertiary amines, **4** and **5**, respectively in low yields.

Scheme 1. Synthesis of N-Phenylamino- and N-(Indolin-1'-yl) Analogs of Cholic acid (**4** and **5**)

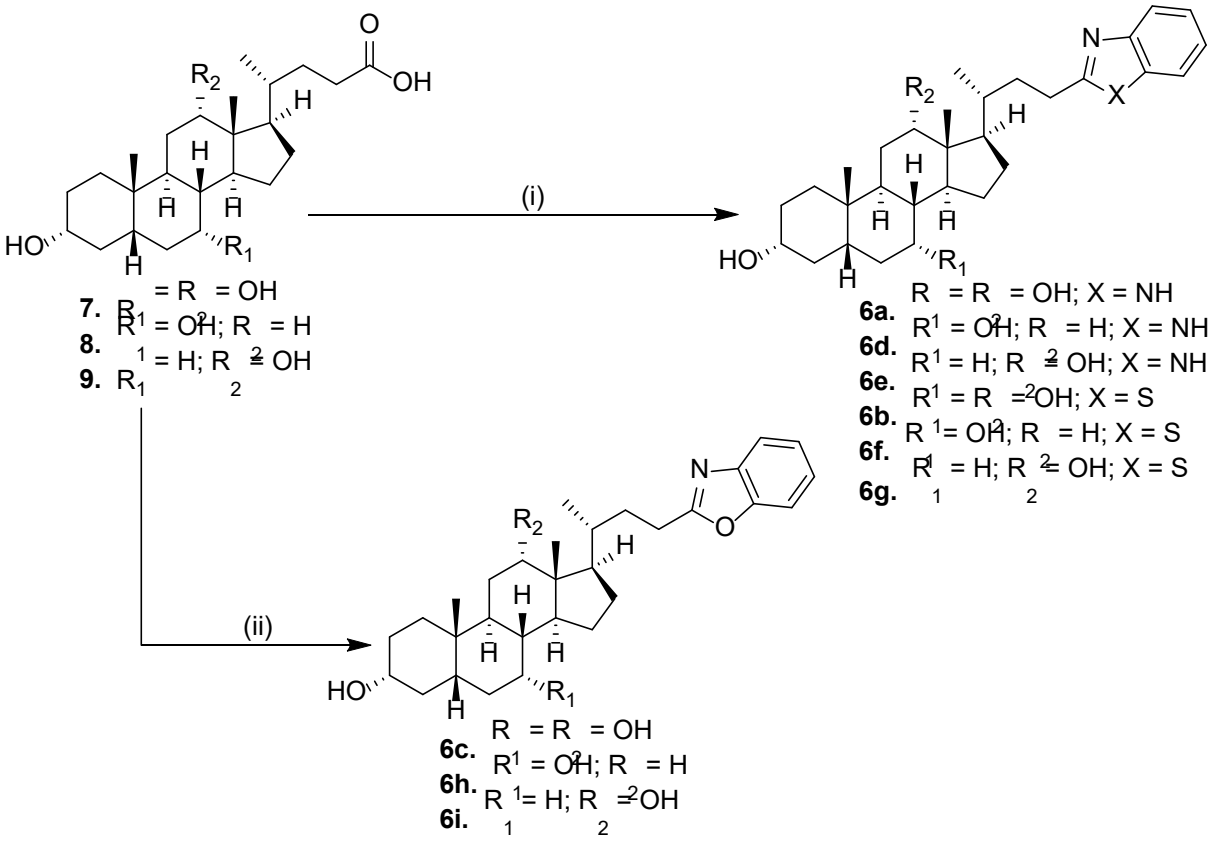


The synthesis of heterocyclic derivatives (**6a-i**) is outlined in Scheme 2. Cholic acid (**7**), chenodeoxycholic acid (**8**) and deoxycholic acid (**9**) were converted into their activated esters using of HBTU/NMM in anhydrous DMF and were subsequently reacted with either 1,2-phenylenediamine or 2-aminothiophenol under microwave irradiation conditions at 140°C. This generated the corresponding amide which was cyclized *in situ* to yield the desired benzimidizaoles and benzothiazoles in 53-73% yields. A similar approach for the synthesis of benzoxazoles was unsuccessful. However, using the method of Nguyen et al, we were able to prepare the desired benzoxazoles of cholic acid, chenodeoxycholic acid, and deoxycholic acid by direct heating, in the absence of solvents, with 2-aminophenol at 230-250ºC to give the desired products in 24-38% yields[20].

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, 17]. A two-step process was taken for the analysis of the biological activity of the compounds. All compounds tested were soluble in buffer containing 2.5% DMSO at 125 μM. Compounds were tested for their ability to inhibit spore germination of *C. difficile* R20291 at a single concentration of

125 μM. Compounds that were able to inhibit spore germination by at least 60% were reanalyzed at different concentrations to determine their IC_{50} values.

Scheme 2. Syntheses of 23-(Benzimidazol-2'-yl- **6a**, **6d**, **6e**), 23-(Benzothiazol-2'-yl- **6b**, **6f** ,**6g**), and 23-(Benzoxazol-2'-yl- **6c**, **6h**, **6i**) 3α,7α,12α-trihydroxy-5β-norcholanes.



Reagents and reaction conditions: (i). HBTU, NMM, DMF, 1,2-phenylenediamine or 2-aminothiophenol, microwave reaction at 140°C for 30 min (4 times), 53-73% yields; (ii). 2-hydroxyaniline, heated without the solvent at 230-250°C for 1h, 24-38% yield;

We first examined the reduced amide derivative (**4**, **5**) for their ability to inhibit spore germination (Table 1). The reduce amide of CaPA (**4**) was a weak inhibitor, while that of CaIn (**5**) was more potent with an IC_{50} of 33 μM. Despite this activity, **5** was approximately 100-fold less potent than the parent compound (**3**). These results indicate that the amide was critical for activity either because of the presence of hydrogen bond donors or acceptors, or because of the conformational rigidity the functional group provides to spore germination inhibitors.

Previous researchers have utilized heterocycles as amide bioisosteres because they are stable, can mimicking hydrogen bonding patterns, and possess the conformations observed in the amide[19].

The inhibition of 5,6-fused heterocycles are shown in table 2. The 23-(benzimidazol-2'-yl) norcholanes (**6a, 6d,** and **6e**) derived from the cholic, chenodeoxycholic, and deoxycholic acids were the best inhibitors with IC_{50} values of 4, 12, and 6 μM respectively. The 23-(benzothiazol-2'-yl) norcholane (**6b**) obtained from cholic acid was a potent inhibitor of spore germination (IC_{50} = 6 μM), but the other benzothiazoles derived from chenodeoxycholic acid and deoxycholic acid were not. Finally, 23-(benzoxazol-2'-yl) norcholane **6c** inhibited spore germination with an IC_{50} value of 6 μM, but like the benzothiazole derivatives, other bile salts gave only weakly active inhibitors.

The 5,6-fused heterocycles were prepared to counteract bile salt hydrolase deconjugation activity produced by members of the gut microbiome. As expected, CamSA and CaPA were hydrolyzed by undisturbed murine microbiota[13-14]. In contrast, a model benzazole derivative, compound **6b**, exhibited multiday stability to hydrolysis and deconjugation.

These results are both similar and different from our previous studies. In our earlier work, we found that inhibitors derived from cholic acid are superior inhibitors when compared to those created from chenodeoxy- or deoxycholic acid[15]. This trend is clearly observed for the benzothiazole and benzoxazole series, but not in the benzimidazoles. All bile salts used for the synthesis of benzimidazoles displayed good inhibition.

We calculated the logP and logD of all the compounds in Table 2 using the Chemaxon property calculator and found a linear inverse correlation between percent germination and these properties (R^2=0.74 and 0.67 respectively). Our data indicates that the more water-soluble analogs are more potent. Benzimidazoles increase the polarity because of the presence of hydrogen bond donors and acceptors but they also have a pKa of approximately 6. Since the germination assay is run at pH 6, about 50% of the molecules are charged greatly increasing their water solubility. Most *C. difficile*

spores germinate in the ileum where the pH varies from 6 to 7, which is also the pH range of maximum spore germination.

It is interesting that the cholic acid heterocycles all have essentially the same activity. We expected to see variances given the differences in the properties of the heterocycles. Benzimidazoles contain a H-bond donor and are protonated near physiological pH, while the benzothiazole is larger than the other two. Unfortunately, the target for these spore germination inhibitors is unknown and thus a rationale for this similarity cannot be explained at this time. Our previous studies have shown that the conformation and distance of the aromatic ring from the steroid is critical for activity[17]. Thus, we hypothesize that the 5,6-fused heterocycles lock the aromatic group into a productive binding conformation, while also maximizing the interactions of the target with the hydroxyl groups of the cholate.

Given the potency of these agents, we selected compounds **6a** and **6b** for testing in mice. Both compounds show no toxicity even when administered at 300 mg/kg for seven consecutive days. Post-mortem analysis showed no damage to intestine, liver, heart, or kidneys (data not shown).

Since the compounds were not toxic, we examined whether they could prevent CDI. Mice were orally given hypervirulent *C. difficile* R20291 spore followed by either the compounds at 50 mg/kg or DMSO as a negative control. Treatment with compounds or DMSO were given at days 0, 1, and 2. Mice were scored for disease severity each day. Under these conditions, untreated animals (Fig. 3, white bars) showed fast infection onset, progression, followed by remission. Untreated animals showed moderate to severe CDI signs that peaked two days post-challenge. Surviving animals started to recover by day 3 and were asymptomatic by day 4.

When used as a CDI prophylactic, **6a** failed to prevent murine CDI onset (light grey bars). Furthermore, animals treated with **6a** displayed very heterogeneous CDI sign severities. By day 2 post-challenge, some treated animals became very sick and needed to be culled. In contrast, other animals in the same cohort remained asymptomatic. By day 3, surviving animals showed much less sign variability and clinical signs of *C. difficile* infection were generally resolved by days 4 and 5.

In contrast, compound **6b** (dark grey columns) was able to both delay CDI onset and significantly reduced sign severity compared to untreated animals (p=2*10^{-3}). CDI signs for animals treated with compound **6b** showed maximum symptomatology at day 3 post-challenge, but even then, treated animals only developed mild CDI. Indeed, comparing the symptomatology of untreated animals at day 2 with **6b**-treated animals at day 3 revealed that treated animals showed statistically milder CDI than untreated animals. Even though animals treated with compound **6b** seem to show signs of CDI relapse between days 4 and 5, the change in sign severities were statistically insignificant (*ns*).

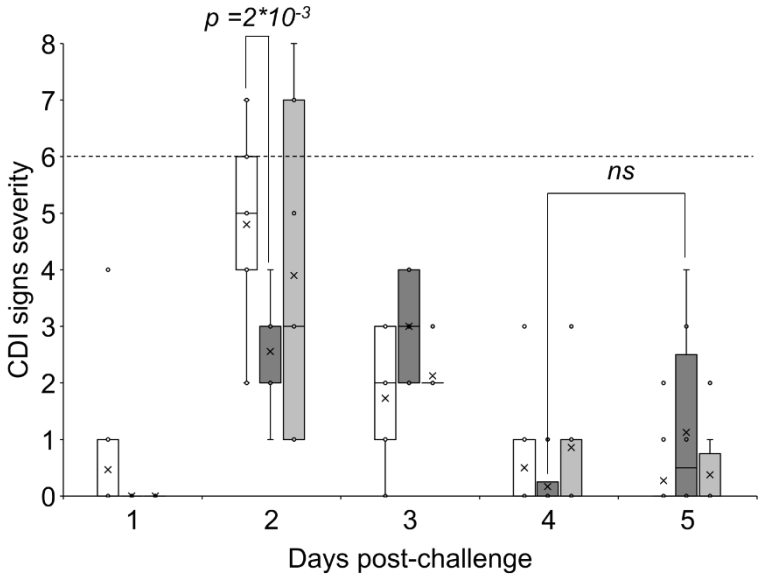


Figure 3. Ability of 6a and 6b to protected mice from CDI. Starting on the day of *C. difficile* spore challenge and following once daily for three days, infected animals were treated with DMSO (white columns), 50 mg/kg of **6a** (light grey columns) or **6b** (dark grey columns). Daily multigroup comparison between control, **6a**-treated and **6b**-treated animals was determined by one-way ANOVA followed by Holm analyses. Only animals treated with **6b** showed statistical difference to control (p=2*10^{-3}). Intra-day pairwise comparisons between animals treated with

compound **6b** was determined by student t-test. No difference in sign severity (*ns*) was observed between days 4 and 5 for animals treated with **6b**. Dashed line represent the clinical endpoint for murine CDI[13].

Conclusions

Our results indicate that non-hydrolysable heterocyclic bile salt analogs display anti-germinant and CDI prophylaxis activity, validating our hypothesis that heterocycles could be used to replace the amide group. By substituting cholate and deoxycholate with the same heterocyclic moieties, we will be able to better understand the role of sterane hydroxyl groups in targeting *C. difficile* spores.

It is intriguing that even though compounds **6a** and **6b** have similar strong anti-germination activity, only compound **6b** protects animals from CDI. A possibility for these differences in CDI prophylactic activity could be due to selective degradation of compound **6a** by the gut microbiota. We have shown that **6a** is not hydrolyzed and dehydroxylation would convert it into compound **6d** which is still active. It is possible that other bile salt modifications, such as sulfation or gluconidation, could be involved in the selective inactivation of **6a** but not **6b**[21].

Another possibility for the differential activity of compounds **6a** and **6b**, could be their PK characteristics. We have previously shown that CamSA undergoes enterohepatic circulation (EHC), thus allowing multiday CDI protection with a single CamSA dose[14]. If compound **6a** is not uptaken by the EHC, its excretion timeframe could be accelerated compared to compound **6b**. As a result, the concentration of compound **6a** in the intestine would decrease quickly allowing *C. difficile* spores to germinate and establish infection. Alternatively, since approximately 50% of **6a** would be charged in the ileum, it is possible that **6a** binds tightly to negatively charged ions in the intestines preventing its interaction with the spore. We are currently exploring these scenarios to improve our pipeline of anti-germinants to CDI prophylaxis. We will report on these studies in due course.

Experimental Section

General Comments. Cholic acid, chenodeoxycholic acid, and deoxycholic acid were purchased from MP Biomedical and Chem-impex Internationals. 1,2-Phenylenediamine, 2-aminothiophenol, and 2-aminophenol were purchased from Sigma-Aldrich or Matrix Scientific. Silica gel for column chromatography was purchased from Sorbent technologies, Inc. All other reagents and solvents were purchased from Sigma-Aldrich, Acros Organics, TCI Chemicals or Chem-Impex International and were used without further purification. Microwave vials were obtained from Biotage. Microwave reactions were conducted using a Biotage Initiator with settings dependent on the solvent or as listed. 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 Bruker 400 MHz spectrometer by dissolving the compounds in deuterated solvents as chloroform-*d* ($CDCl_3$), methanol-d_4 (CD_3OD) or dimethyl sulfoxide (DMSO-d_6) and all peaks were referenced with TMS as an internal standard or to the residual solvents. 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). Some of the compound's spectra were recorded in multiple solvents for clarity of the aliphatic region. The purities of all compounds were checked by ^{1}H-NMR. Melting points were determined using Mel-temp II apparatus by Laboratory Device, in open capillaries and are uncorrected.

(3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-10,13-Dimethyl-17-((R)-5-(phenyl-amino)-pentan-2-yl)hexadecahydro-1H-cyclopenta[a]phenanthrene-3,7,12-triol (4). Compound **1** (543 mg, 1.12 mmol) was dissolved in anhydrous THF (30 mL), and a solution 2.4M $LiAlH_4$ in THF (2.5 mL) was added dropwise with stirring under argon environment. To the thick solution, an additional

amount of 10 mL of THF was added to decrease viscosity. The reaction mixture was stirred for 30 min at room temperature, then refluxed for 6h, and stored at room temperature overnight. The reaction was quenched by the slow, dropwise addition of methanol (10 mL) followed by water (5 mL). A white, turbid solution with some precipitate was formed after the quench and this mixture was concentrated to dryness by rotary evaporation. The resulting white solid was dried under high vacuum for several hours. The solid was stirred with $CHCl_3$ (100 mL), the organic layer decanted, and the residue stirred with EtOAc (100 mL). The mixture was filtered to remove the solid and the ethyl acetate layer was combined with the chloroform layer. These organic extracts were concentrated to produce a white solid. The product was purified by repeated column chromatography using a gradient of CH_2Cl_2-MeOH-NH_4OH (97.5:2.0:0.5; 94.5:5.0:0.5; 89:10:1; and 70:28:2) followed by CH_2Cl_2-MeOH (50:50) to give **4** in 41% yield (217 mg). mp. 175-177°C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.16 (t, 2H, J = 7.6 Hz), 6.68 (t, 1H, J = 7.2 Hz), 6.59 (d, 2H, J = 8.4 Hz), 3.99 (s, 1H), 3.84 (s, 1H), 3.46-3.41 (m, 1H), 3.11-3.01 (m, 2H), 2.28-2.16 (m, 2H), 1.98-1.86 (m, 5H), 1.80-1.66 (m, 8H), 1.60-1.57 (m, 1H), 1.51-1.46 (m, 3H), 1.41-1.22 (m, 5H), 1.18-1.10 (m, 2H), 1.01-0.94 (m, 4H), 0.89 (s, 3H), 0.69 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 148.54, 129.22, 117.10, 112.72, 73.04, 71.97, 68.44, 47.40, 46.50, 44.51, 41.91, 41.49, 39.73, 39.61, 35.47, 35.24, 34.72, 34.62, 33.29, 30.57, 28.30, 27.62, 26.63, 26.28, 23.21, 22.54, 17.74, 12.56. HRMS (ESI, m/z): calcd for $C_{30}H_{48}NO_3$ $[M+H]^+$ 470.3634, found 470.3632.

(3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-17-((R)-5-(Indolin-1-yl)pentan-2-yl)-10,13-dimethyl-hexadecahydro-1H-cyclopenta[a]phenanthrene-3,7,12-triol (5). Compound **5** was prepared from **3** (511 mg, 1.0 mmol) and 2.4M $LiAlH_4$ in THF (1.4 mL) by following above method for **4**. The crude product was subjected to repeated column chromatography using a gradient of CH_2Cl_2-MeOH-NH_4OH (94.5:5.0:0.5; 89:10:1; 78:20:2) followed by CH_2Cl_2-MeOH; 50:50 to furnish a white solid **5** in 26% yield (131 mg). mp. 87-88°C; ^{1}H NMR ($CDCl_3$, 600 MHz): δ 7.05 and

7.04 (2 peaks, 2H), 6.62 (s, 1H), 6.44 (d, 1H, J = 7.2 Hz), 3.97 (s, 1H), 3.82 (s, 1H), 3.45 (m, 1H), 3.34-3.30 (m, 2H), 2.99 (s, 2H), 2.95 and 2.94 (2 peaks, 2H), 2.20 and 2.18 (2 peaks, 2H), 1.88-1.68 (m, 11H), 1.60-1.38 (m, 9H), 1.26 and 1.25 (2 peaks, 1H), 1.15-0.92 (m, 7H), 0.87 (s, 3H), 0.67 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 152.75, 130.00, 127.24, 124.33, 117.25, 106.89, 73.18, 71.85, 68.47, 53.04, 49.82, 47.31, 46.37, 41.58, 41.47, 39.47, 39.44, 35.62, 35.34, 34.77, 34.64, 33.41, 30.28, 28.58, 28.08, 27.69, 26.30, 23.96, 23.25, 22.46, 17.77, 12.49.

(3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-17-((R)-4-(1H-benzo[d]imidazol-2-yl)butan-2-yl)-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,7,12-triol (**6a).** Cholic acid (818 mg, 2.0 mmol) and HBTU (793 mg, 2.1 mmol) were dissolved in anhydrous, room temperature DMF (5.0 mL). NMM (0.30 mL) was added, and the reaction was stirred for 30 min at room temperature to generate an activated ester. Meanwhile in a 30 mL microwave vial, 1,2-phenylenediamine (217 mg, 2.0 mmol) was dissolved in DMF (8.0 mL) containing NMM (0.30 mL) at room temperature. The generated activated ester was added to the microwave vial containing the amine along with an additional amount of DMF (3.0 mL) and NMM (0.30 mL). The vial was sealed and subjected to microwave heating at very high absorbance to a temperature of 140ºC for 30 min. The progress of the reaction was monitored by TLC. Microwave heating was conducted a total of four times and then the reaction was at room temperature overnight. The reaction was transferred to a 250 mL round bottom flask and solvent was removed on a high vacuum rotary evaporator to produce a viscous liquid. Ice cold water (150 mL) was added to the flask and subjected to sonication for 15 min to produce a white precipitate. The water layer was decanted, and the precipitate was treated with ice cold water and sonication 3 additional times. The product was purified by repeated column chromatography over silica gel to elute from CH_2Cl_2-MeOH-NH_4OH (89:10:1; 78:20:2) to yield a white solid. The resulting solid was sonicated with 100 mL of $CHCl_3$ and the insoluble product was filtered and vacuum dried to give **6a** in 54% yield (528 mg). mp. 287-290ºC; ^{1}H NMR (DMSO-d_6,

400 MHz): δ 12.22 (br, 1H), 7.45 (dd, 2H, *J* = 5.2 and 3.2 Hz), 7.10 (dd, 2H, *J* = 5.6 and 3.2 Hz), 4.31 (s, H), 4.14 (d, 1H, *J* = 2.4 Hz), 4.00 (d, 1H, *J* = 2.0 Hz), 3.82 (s, 1H), 3.62 (s, 1H), 3.20 (br s, 1H), 2.88-2.80 (m, 1H), 2.74-2.67 (m, 1H), 2.22-2.14 (m, 2H), 2.05-1.98 (m, 1H), 1.91-1.79 (m, 4H), 1.67 and 1.64 (2 peaks, 2H), 1.44-1.14 (m, 11H), 1.05-0.96 (m, 4H), 0.88-0.82 (m, 4H), 0.60 (s, 3H); ^{1}H NMR (CD_3OD, 400 MHz): δ 7.50 (dd, 2H, *J* = 6.0 and 3.2 Hz), 7.20 (dd, 2H, *J* = 6.0 and 3.2 Hz), 3.99 (s, 1H), 3.81 (s, 1H), 3.41-3.36 (m, 1H), 3.03-2.95 (m, 1H), 2.87-2.79 (m, 1H), 2.35-2.24 (m, 2H), 2.07-1.96 (m, 5H), 1.84-1.75 (m, 2H), 1.69-1.37 (m, 10H), 1.32-1.29 (m, 1H), 1.16-1.11 (m, 4H), 1.03-0.93 (m, 4H), 0.72 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 155.64, 138.64, 121.03, 114.26, 70.99, 70.41, 66.22, 46.04, 45.75, 41.50, 41.38, 35.28, 34.85, 34.36, 34.19, 30.39, 28.55, 27.33, 26.21, 25.49, 22.78, 22.60, 17.12, 12.33.

(3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-17-((R)-4-(benzo[d]thiazol-2-yl)butan-2-yl)-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,7,12-triol (6b). This compound was prepared using the method described above for **6a**. Cholic acid (819 mg, 2.0 mmol), HBTU (793 mg, 2.1 mmol), 2-aminothiophenol (253 mg, 2.0 mmol), and NMM (0.90 mL) were used in the reaction and the crude product was subjected to column chromatography using a gradient of CH_2Cl_2-MeOH (90:10; 80:20; 70:30) to give **6b** in 72% yield (716 mg). mp. 87-90ºC (softening), 115-117ºC; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.95 (d, 1H, *J* = 8.0 Hz), 7.83 (d, 1H, *J* = 8.0 Hz), 7.44 (t, 1H, *J* = 7.2 Hz), 7.33 (t, 1H, *J* = 7.6 Hz), 3.99 (s, 1H), 3.85 (s, 1H), 3.48-3.43 (m, 1H), 3.22-3.16 (m, 1H), 3.15-3.07 (m, 1H), 2.93-1.85 (m, 11H), 1.80-1.57 (m, 7H), 1.52-1.49 (m, 2H), 1.45-1.27 (m, 3H), 1.16-1.09 (m, 4H), 1.01-0.94 (m, 1H), 0.89 (s, 3H), 0.68 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 173.03, 155.21, 135.16, 125.85, 124.60, 122.47, 121.49, 73.01, 71.96, 68.40, 47.01, 46.56, 41.88, 41.51, 39.71, 39.59, 35.92, 35.47, 35.27, 34.74, 34.69, 31.24, 30.58, 28.33, 27.60, 26.57, 23.24, 22.52, 17.60, 12.55.

(3R,5S,7R,8R,9S,10S,12S,13R,14S,17R)-17-((R)-4-(benzo[d]oxazol-2-yl)butan-2-yl)-10, 13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,7,12-triol (6c). Cholic acid (818 mg, 2.0 mmol) and 2-aminophenol (262 mg, 2.4 mmol) were placed in a microwave vial without solvent and mixed well. The microwave vial was sealed and heated conventionally with stirring under argon at 230-250ºC for 1h. The vial was cooled to room temperature, opened and 20 mL of cold water was added. A glass-like material developed at the bottom of the vial and this material was crushed into small pieces with the help of spatula and sonication. All the material from the vial was transferred into 250-mL round bottom flask and 150 mL additional cold water was added. The mixture was stirred and further sonicated for 15 minutes. The water layer was decanted, and the remaining material was treated with water and sonication three additional times. The product was collected by filtration, dried *in vacuo*, and purified by column chromatography using a gradient of CH_2Cl_2-MeOH (95:5; 90:10; 80:20; 70:30) to give **6c** in 24% yield (231 mg). mp. 110-112ºC; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.67-7.65 (m, 1H), 7.49-7.46 (m, 1H), 7.30-7.28 (m, 2H), 4.00 (s, 1H), 3.86 (d, 1H, J = 1.6 Hz), 3.49-3.43 (m, 1H), 3.04-2.96 (m, 1H), 2.90-2.85 (m, 1H), 2.29-2.18 (m, 2H), 2.08-1.86 (m, 5H), 1.84-1.59 (m, 11H), 1.52-1.50 (m, 2H), 1.43-1.29 (m, 3H), 1.20-1.07 (m, 4H), 1.03-0.96 (m, 1H), 0.90 (s, 3H), 0.70 (s, 3H); ^{1}H NMR (CD_3OD, 400 MHz): δ 7.62-7.59 (m, 1H), 7.57-7.54 (m, 1H), 7.36-7.31 (m, 2H), 3.96 (s, 1H), 3.79 (d, 1H, J = 2.4 Hz), 3.48-3.36 (m, 1H), 3.06-2.99 (m, 1H), 2.93-2.87 (m, 1H), 2.33-2.23 (m, 2H), 2.07-1.89 (m, 5H), 1.82-1.73 (m, 2H), 1.67-1.47 (m, 8H), 1.45-1.35 (m, 2H), 1.31-1.28 (m, 1H), 1.17-1.06 (m, 4H), 1.00-0.91 (s, 4H), 0.70 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.95, 150.77, 141.30, 124.39, 124.05, 119.44, 110.26, 73.05, 71.92, 68.40, 46.79, 46.53, 41.73, 41.54, 39.60, 39.54, 35.51, 35.32, 34.79, 34.75, 32.88, 30.54, 28.25, 27.60, 26.43, 25a54, 23.28, 22.48, 17.48, 12.51.

(3R,5S,7R,8R,9S,10S,13R,14S,17R)-17-((R)-4-(1H-benzo[d]imidazol-2-yl)butan-2-yl)-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,7-diol (6d). This compound

was prepared according to the method described for **6a** with the following modifications and reagents. Chenodeoxycholic acid (786 mg, 2.0 mmol), HBTU (795 mg, 2.1 mmol), 1,2-phenylenediamine (219 mg, 2.0 mmol), and NMM (0.90 mL) were reacted according to the method above. After sonication, the isolated product was dissolved in CH_2Cl_2 (75 mL) and MeOH (10 mL) and treated with activated charcoal and stirred for 30 min at room temperature to remove colored impurities. The product was then subjected to silica column chromatography using CH_2Cl_2-MeOH-NH_4OH (89:10:1; and 78:20:2) followed by CH_2Cl_2-MeOH (50:50) to give a white powder. This product was stirred with $CHCl_3$ (50 mL), insoluble solid was collected and vacuum dried to furnish **6d** in 53% yield (491 mg). mp. 160-163 (softening), 166-168°C; ^{1}H NMR (CD_3OD, 400 MHz): δ 7.48-7.46 (m, 2H), 7.18-7.16 (m, 2H), 3.78 (s, 1H), 3.39-3.34 (m, 1H), 2.98-2.91 (m, 1H), 2.82-2.75 (m, 1H), 2.27 (q, 1H, J = 12.4 Hz), 2.03-1.66 (m, 6H), 1.74 (m, 1H), 1.66-1.56 (m, 2H), 1.53-1.50 (m, 5H), 1.38-1.22 (m, 6H), 1.12-1.06 (m, 4H), 1.02-0.92 (m, 4H), 0.68 (s, 3H); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 12.15 (br, 1H), 7.46-7.44 (m, 2H), 7.10 (dd, 2H, J = 5.6 and 2.8 Hz), 4.32 (br s, 1H), 4.11 (s, 1H), 3.64 (s, 1H), 3.20 (br s, 1H), 2.88-2.83 (m, 1H), 2.72-2.66 (m, 1H), 2.21 (q, 1H, J = 12.4 Hz), 1.95-1.69 (m, 7H), 1.46-1.33 (m, 8H), 1.26-1.14 (m, 6H), 1.01 and 0.99 (2 peaks, 4H), 0.91-0.84 (m, 4H), 0.61 (s, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ 157.36, 139.66 (br), 123.04, 115.24 (br), 72.83, 68.99, 57.24, 51.50, 43.68, 43.14, 41.03, 40.74, 40.46, 36.99, 36.54, 36.19, 35.89, 35.80, 34.02, 31.36, 29.30, 26.75, 24.61, 23.39, 21.77, 18.98, 12.17; ^{13}C NMR (DMSO-d_6, 100 MHz): δ 155.52, 120.94, 70.29, 66.13, 55.50, 49.98, 41.92, 41.39, 35.28, 35.16, 34.78, 34.69, 34.10, 32.26, 30.53, 27.83, 25.44, 23.12, 22.67, 20.23, 18.31, 11.63.

(3R,5R,8R,9S,10S,12S,13R,14S,17R)-17-((R)-4-(1H-benzo[d]imidazol-2-yl)butan-2-yl)-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,12-diol (**6e).** This compound was prepared using the method described above for **6d**. Deoxycholic acid (786 mg, 2.0 mmol), HBTU (795 mg, 2.1 mmol), 1,2-phenylenediamine (219 mg, 2.0 mmol), and NMM (0.90 mL) were used in

the reaction and the product isolated from sonication was decolorized with activated charcoal. The product was subjected to column chromatography using CH_2Cl_2-MeOH-NH_4OH (89:10:1; 78:20:2) followed by CH_2Cl_2-MeOH (50:50) to give a white powder. The material was stirred with $CHCl_3$ (50 mL), the insoluble white solid was collected and vacuum dried to furnish **6e** in 59% yield (547 mg). mp. 298-301°C; ^{1}H NMR (CD_3OD, 400 MHz): δ 7.47 (br s, 2H), 7.16 (dd, 2H, *J* = 6.0 and 3.2 Hz), 3.97 (s, 1H), 3.55-3.48 (m, 1H), 2.99-2.92 (m, 1H), 2.83-2.76 (m, 1H), 1.98-1.85 (m, 5H), 1.83-1.75 (m, 2H), 1.65-1.59 (m, 3H), 1.53-1.37 (m, 8H), 1.28-1.06 (m, 7H), 1.01-0.92 (m, 4H), 0.69 (s, 3H); ^{1}H NMR (DMSO-d_6, 400 MHz): δ 12.16 (br, 1H), 7.45 (dd, 2H, *J* = 5.2 and 3.2 Hz), 7.10 (dd, 2H, *J* = 5.6 and 3.2 Hz), 4.45 (s, 1H), 4.23 (d, 1H, *J* = 3.2 Hz), 3.82 (s, 1H), 3.37 and 3.18 (2 broad peaks, 1H), 2.88-2.81 (m, 1H), 2.73-2.66 (m, 1H), 1.89-1.82 (m, 5H), 1.66-1.60 (m, 3H), 1.56-1.46 (m, 3H), 1.39-1.30 (m, 8H), 1.23-1.17 (m, 2H), 1.11-0.98 (m, 5H), 0.91-0.85 (m, 4H), 0.60 (s, 3H); ^{13}C NMR (CD_3OD, 100 MHz): δ 157.46, 123.04, 74.03, 72.54, 49.28, 48.08, 47.59, 43.62, 37.45, 37.21, 36.93, 36.43, 35.81, 35.30, 34.83, 31.08, 29.91, 28.70, 28.39, 27.45, 26.75, 24.86, 23.69, 17.72, 13.17; ^{13}C NMR (DMSO-d_6, 100 MHz): δ 155.59, 120.96, 70.98, 69.90, 47.46, 46.12, 45.98, 41.57, 36.27, 35.62, 35.16, 34.13, 33.78, 32.91, 30.21, 28.59, 27.21, 26.94, 26.06, 25.49, 23.47, 23.06, 17.08, 12.43.

(3R,5S,7R,8R,9S,10S,13R,14S,17R)-17-((R)-4-(benzo[d]thiazol-2-yl)butan-2-yl)-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,7-diol (**6f**)**.** This compound was prepared using the method described above for **6a**. Chenodeoxycholic acid (786 mg, 2.0 mmol), HBTU (795 mg, 2.1 mmol), 2-aminothiophenol (252 mg, 2.0 mmol), and NMM (0.90 mL) were used in the reaction and the crude product was subjected to column chromatography using a gradient of CH_2Cl_2-MeOH (95:5; 90:10; 80:20; 70:30) to give **6f** in 71% yield (688 mg). mp. 93-95°C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.96 (d, 1H, *J* = 8.0 Hz), 7.84 (d, 1H, *J* = 8.0 Hz), 7.44 (t, 1H, *J* = 7.6 Hz), 7.34 (t, 1H, *J* = 7.6 Hz), 3.85 (s, 1H), 3.46 (br s, 1H), 3.21-3.14 (m, 1H), 3.07-2.99 (m, 1H), 2.21 (q, 1H,

J = 12.8 Hz), 2.05-1.91 (m, 4H), 1.85-1.82 (m, 2H), 1.73-1.61 (m, 4H), 1.53-1.08 (m, 14H), 1.06 (d, 3H, *J* = 6.0 Hz), 0.99 (m, 1H), 0.91 (s, 3H), 0.70 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.91, 153.26, 135.16, 125.84, 124.59, 122.49, 121.48, 73.01, 68.51, 55.83, 50.49, 42.77, 41.51, 39.93, 39.67, 39.46, 36.03, 35.62, 35.35, 35.06, 34.65, 32.87, 31.28, 30.71, 28.26, 23.73, 22.79, 20.60, 18.50, 11.82.

(3R,5R,8R,9S,10S,12S,13R,14S,17R)-17-((R)-4-(benzo[d]thiazol-2-yl)butan-2-yl)-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,12-diol (**6g).** This compound was prepared using the method described above for **6a**. Deoxycholic acid (786 mg, 2.0 mmol), HBTU (795 mg, 2.1 mmol), 2-aminothiophenol (253 mg, 2.0 mmol), and NMM (0.90 mL) were used in the reaction and the crude product was subjected to column chromatography using a gradient of CH_2Cl_2-MeOH (95:5; 90:10; 80:20; 70:30) to give **6g** in 73% yield (701 mg). mp. 84-86 ºC (softening), 93-95ºC; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.96 (d, 1H, *J* = 8.0 Hz), 7.84 (d, 1H, *J* = 8.0 Hz), 7.44 (t, 1H, *J* = 7.6 Hz), 7.34 (t, 1H, *J* = 7.6 Hz), 4.00 (s, 1H), 3.61 (t, 1H, *J* = 10.8 Hz), 3.23-3.15 (m, 1H), 3.08-3.00 (m, 1H), 2.06-1.99 (m, 1H), 1.95-1.73 (m, 6H), 1.69-1.55 (m, 6H), 1.51 (br, 2H), 1.43-1.25 (m, 8H), 1.19-1.04 (m, 5H), 0.99 (td, 1H, *J* = 13.6 and 2.8 Hz), 0.91 (s, 3H), 0.68 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 172.89, 153.23, 135.15, 125.85, 124.60, 122.49, 121.48, 73.12, 71.78, 48.28, 47.31, 46.56, 42.10, 36.47, 36.06, 35.92, 35.37, 35.24, 34.13, 33.68, 31.34, 30.52, 28.73, 27.57, 27.14, 26.14, 23.67, 23.16, 17.55, 12.77.

(3R,5S,7R,8R,9S,10S,13R,14S,17R)-17-((R)-4-(benzo[d]oxazol-2-yl)butan-2-yl)-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,7-diol (**6h).** This compound was prepared using the method described for **6c**. Chenodeoxycholic acid (786 mg, 2.0 mmol) and 2-aminophenol (655 mg, 6.0 mmol) were used in the reaction. The product was purified by column chromatography using a gradient of EtOAc-MeOH (100:0; 95:5; 90:10; 80:20 and 70:30) to give **6h**

in 36% yield (336 mg). mp. 78-80°C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.68-7.66 (m, 1H), 7.48-7.46 (m, 1H), 7.30-7.28 (m, 2H), 3.86 (s, 1H), 3.49-3.44 (m, 1H), 3.02-2.95 (m, 1H), 2.87-2.81 (m, 1H), 2.25-1.92 (m, 7H), 1.85-1.82 (m, 2H), 1.73-1.58 (m, 5H), 1.50-1.47 (m, 2H), 1.44-1.12 (m, 9H), 1.05-0.94 (m, 4H), 0.91 (s, 3H), 0.67 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.83, 150.78, 141.33, 124.39, 124.05, 119.49, 110.24, 72.04, 68.55, 55.74, 50.47, 42.76, 41.50, 39.87, 39.65, 39.45, 35.58, 35.33, 35.06, 34.63, 32.98, 32.86, 30.67, 28.21, 25.58, 23.72, 22.78, 20.60, 18.39, 11.81.

(3R,5R,8R,9S,10S,12S,13R,14S,17R)-17-((R)-4-(benzofuran-2-yl)butan-2-yl)-10,13-dimethylhexadecahydro-1H-cyclopenta[a]phenanthrene-3,12-diol (6i). This compound was prepared using the method described for **6c**. Deoxycholic acid (786 mg, 2.0 mmol) and 2-aminophenol (655 mg, 6.0 mmol) were used in the reaction. The product was purified by column chromatography using a gradient of EtOAC-MeOH (100:0; 95:5; 90:10; and 80:20) to give **6i** in 38% yield (352 mg). mp. 72-74°C; ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.67-7.65 (m, 1H), 7.48-7.46 (m, 1H), 7.30-7.28 (m, 2H), 4.00 (s, 1H), 3.65-3.58 (m, 1H), 3.04-2.96 (m, 1H), 2.89-2.81 (m, 1H), 2.13-2.03 (m, 2H), 1.95-1.73 (m, 6H), 1.69-1.58 (m, 5H), 1.52-1.50 (m, 3H), 1.44-1.26 (m, 7H), 1.16-1.07 (m, 5H), 1.02-0.95 (m, 1H), 0.91 (s, 3H), 0.69 (s, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 167.78, 150.80, 141.37, 124.40, 124.05, 119.51, 110.25, 73.17, 71.84, 48.29, 47.23, 46.56, 42.10, 36.45, 36.06, 35.29, 35.23, 34.13, 33.71, 32.89, 30.51, 28.73, 27.51, 27.13, 26.13, 25.62, 23.66, 23.16, 17.44, 12.78.

Bacterial Strains and Spore Preparation. *C. difficile* R20291 was the kind gift of Prof. 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% CO_2, 10% H_2, and 80% N_2). 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 8,800 x g for five minutes. To remove any contaminating vegetative cells, the spores were purified through a 20% to 50% HistoDenz gradient at 18,200 x g for 30 minutes. 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 9,400 x g to remove the storage buffer. The spores were heat activated at 68°C for 30 minutes, then washed an additional three times to remove any spores that autogerminated. The spores were diluted to an optical density at 580 nm (OD_{580}) 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 a final concentration of 125 μM into separate wells in triplicate along with 6mM taurocholate and 12mM glycine. Upon the addition of 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} = $OD_{580}(t)/OD_{580}(t_0)$]. Selected compounds were further tested for germination inhibition at increasing concentrations to determine the concentration that reduces spore germination by 50% (IC_{50}). The IC_{50} value was determined from a plot of percent germination versus the log concentration of the drug according to equation 1.

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

Animals: All procedures involving animals in this study were performed in accordance with the Guide for Care and Use of Laboratory Animals outlined by the National Institutes of Health. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada, Las Vegas (Permit Number: R0914-297). Weaned female

mice (strain C57BL/6) were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in groups of five per cage at the University of Nevada, Las Vegas animal care facility. Upon arrival at the facility, animals were allowed to acclimate for at least one week prior to the start of experimentation. All bedding, cages, food, and water were autoclaved prior to contact with animals. All post-challenge manipulations were performed within a biosafety level 2 laminar flow hood.

Metabolism of compound 6b by Fecal Microbiota. To determine the effect of antibiotics on microbiota-mediated compound **6b** degradation, we first provided mice (n=10) with autoclaved and sterile-filtered water containing kanamycin (0.4 mg/ml), gentamycin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml), and vancomycin (0.045 mg/ml). Animals were allowed to drink the antibiotic cocktail *ad libitum* for three consecutive days with the antibiotic water refreshed daily to ensure biome disruption. Mice received autoclaved water for the remainder of the experiment. A single dose of clindamycin (10 mg/kg) was administered by intraperitoneal (IP) injection on the fourth day of the experiment. Feces were collected from animals prior to treating with antibiotics, one day after the clindamycin shot, and one week after the clindamycin shot. Approximately 100 mg of feces was suspended in artificial gastric juice (5 ml) with or without 10 mM compound **6b** to produce a fecal slurry. Immediately after mixing, 100 µl of each slurry was removed, centrifuged, and 0.5 µl samples of the supernatant were spotted on silica thin layer chromatography (TLC) plates in triplicate and allowed to air dry (t = 0). Fecal slurries were maintained at 37 °C in an anaerobic chamber. Samples were collected and spotted on TLC plates after incubation for 0.5, 1, 2, 3, 4, 6, 8, 24, 48, 72 and 96 hours. Pure 10 mM **6b** and cholate solutions were spotted on each TLC plate as controls. Compound **6b** spots were preliminarily identified by exposing to UV-light. TLC plates were then developed with 75% ethyl acetate/methanol. Compound **6b** and cholate were visualized by spraying with 10% w/v

phosphomolybdic acid (PMA)/ethanol solution followed by heating at 100 °C for 2 minutes. The relative amount of compound **6b** was determined by using a GE Healthcare Typhoon 9410 Variable Mode Imager and analyzed using ImageQuant TL 5.2 software. Percent compound **6b** was derived by dividing the intensity of TLC spots obtained at different times by the intensity of the TLC spot before incubation.

Seven-day compound toxicity regimen in mice: To test for acute and sub-chronic toxicity, animals were given saturating 300 mg/kg of bile salt analog (dissolved in DMSO) once per day for 7 days. Bile salt analogs were administered via oral gavage with a total volume of 50 µL per dose. Neat DMSO was used as a control in one cage of mice. Weight changes were recorded daily. Body weight loss in mice was calculated as percent change from the weight on day 0. Mice were observed for signs of distress daily. At the end of the 7 days, animals were sacrificed and necropsied to investigate for potential anatomical abnormalities.

Murine CDI Prevention Model: The murine CDI model used in this study was adapted from Chen *et al*[22]. For 3 consecutive days, mice were given an antibiotic cocktail containing kanamycin (0.4 mg/ml), gentamycin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml), and vancomycin (0.045 mg/ml) *ad libitum* (14. Mice were then given autoclaved DI water for the remainder of the experiment. On the day prior to *C. difficile* challenge (day -1), mice were given an intraperitoneal (IP) injection of 10 mg/kg clindamycin. None of the animals develop clinical symptoms during antibiotic treatment. On the day of infection (day 0), experimental groups were challenged with 10^8 *C. difficile* spores via oral gavage and given 50 mg/kg daily gavage doses of either **6a** or **6b** at 0-, 24-, and 48-hours post-challenge. One group of five infected mice was given neat DMSO and served as a positive infection control for CDI.

Mice were observed for signs of CDI twice daily and disease severity was scored according to a CDI sign rubric adapted from previously published work[23-25]. According to the rubric, disease

signs were scored as followed: pink anogenital area (score of 1), red anogenital area (score of 2), lethargy (score of 1), diarrhea/increase in soiled bedding (score of 1), wet tail (score of 2), hunchback posture (score of 2), 8-15% loss of body weight (score of 1), > 15% loss of body weight (score of 2). Animals scoring 2 or less were undistinguishable from non-infected controls and were considered non-diseased. Animals scoring 3-4 were considered to have mild CDI with signs consisting of pink anogenital area, lethargy, an increase of soiled bedding and minor weight loss. Animals scoring 5-6 were considered to have moderate CDI with signs consisting of mild CDI signs plus red anogenital area and hunchback posture. Animals scoring > 6 were considered to have severe CDI and were immediately euthanized. These animals displayed signs described above plus wet tail and severe weight loss.

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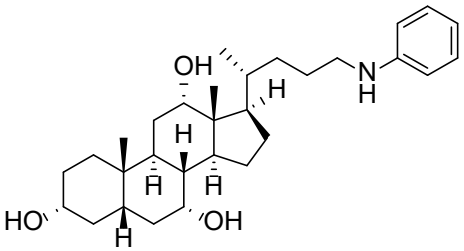
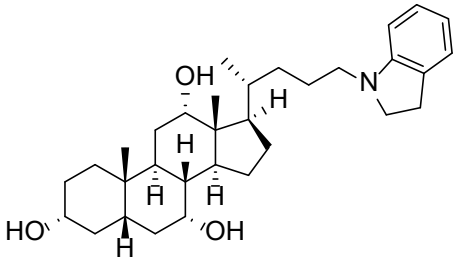
Table 1. *C. difficile* spore germination activities of reduced amides **4** and **5**



4

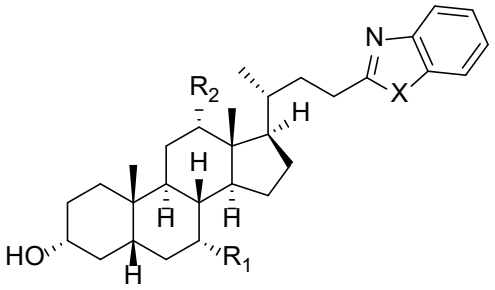


5

Compounds[a]	**% Germination (125 µM)**[b]	**IC_{50} (µM)**[c]
4	62 ± 6	ND[d]
5	26 ± 3	33 ± 49

a. Number corresponding to scheme 1.
b. Each experiment was conducted in triplicate.
c. IC_{50} was calculated according to equation 1.
d. ND. Not determined

Table 2. Spore germination activities of 5,6-fused heterocycle derivatives of cholate, chendeoxycholate, and deoxycholate.



Compounds[a]	R_1	R_2	X	% Germination (125 µM)[b]	IC_{50} (µM)[c]
6a	OH	OH	NH	0 ± 3	4 ± 0.3
6d	OH	H	NH	18 ± 2	12 ± 7
6e	H	OH	NH	23 ± 5	6 ± 1
6b	OH	OH	S	0 ± 6	6 ± 4
6f	OH	H	S	88 ± 5	ND[d]
6g	H	OH	S	81 ± 2	ND
6c	OH	OH	O	0 ± 4	6 ± 3
6h	OH	H	O	80 ± 4	ND
6i	H	OH	O	83 ± 8	ND

a. Number corresponding to scheme 2.
b. Each experiment was conducted in triplicate.
c. IC_{50} was calculated according to equation 1.
d. ND. Not determined.