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Structural Modifications that Increase Gut Restriction of Bile Acid Derivatives

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Abstract

Bile acid derivatives have been investigated as possible therapeutics for a wide array of conditions, including several for which gut-restricted analogs would likely be preferred. These include the prevention of *Clostridioides difficile* infection (CDI) and the treatment of inflammatory bowel disease (IBD). The design of gut-restricted bile acid analogs, however, is complicated by the highly efficient enterohepatic circulation system that typically reabsorbs these compounds from the digestive tract for subsequent return to the liver. Herein, we report that incorporation of a sulfate group at the 7-position of the bile acid scaffold reduces oral bioavailability and increases fecal recovery in two pairs of compounds designed to inhibit the germination of *C. difficile* spores. A different approach was necessary for designing gut-restricted bile acid-based TGR5 agonists for the treatment of IBD, as the incorporation of a 7-sulfate group reduces activity at this receptor. Instead, building on our previous discovery that incorporation of a 7-methoxy group into chenodeoxycholic acid derivatives greatly increases their TGR5 receptor potency, we determined that an *N*-methyl-D-glucamine group could be conjugated to the scaffold to obtain a compound with an excellent mix of potency at the TGR5 receptor, low oral exposure, and good fecal recovery.

1. Introduction

Though their primary function is to help absorb lipids and fat-soluble vitamins from the gastrointestinal tract, bile acids also play an important role in carrying signals from the gut to the rest of the body as well as between the gut microbiota and the host.[1] Bile acids are natural ligands for an array of receptors including the TGR5 (also known GPBAR-1 or M-BAR), farnesoid X (FXR), pregnane X (PXR), constitutive androstane (CAR), sphingosine-1-phosphate 2 (S1PR2), and vitamin D3 receptors (VDR).[2, 3] Through activation of these receptors, bile acids help regulate glucose, lipid, and energy metabolism.[4] They can also have significant anti-apoptotic effects.[5-7] As a result, endogenous bile acids and their synthetic derivatives have been investigated as possible therapeutics for a wide range of diseases including nonalcoholic steatohepatitis (NASH),[8, 9] primary biliary cholangitis,[10, 11] obesity,[12] diabetes,[13] acute kidney injury,[14] myocardial infarction,[15] sepsis,[16] and amyotrophic lateral sclerosis.[17]

We have been working to design bile acid derivatives to serve as therapeutics for two additional indications: prevention of *Clostridioides difficile* infection (CDI)[18-20] and TGR5 agonists as treatments for inflammatory bowel disease (IBD).[21] As both of these diseases are focused primarily in the gastrointestinal tract, especially the colon, it is likely that gut-restricted therapeutics would be preferred, allowing higher drug concentrations to reach the colon, while minimizing undesired effects that could potentially be caused by systemic exposure. Unfortunately, designing gut-restricted analogs of bile acids is particularly difficult due to natural active and passive transport mechanisms that efficiently recycle these compounds from the gut into the liver.

Each day, approximately 12 g of bile acids are secreted from the liver into the gut as part of the digestion process.[22] Most of the bile acids (approximately 95%) are then reabsorbed from the gut,

with only a small amount lost in the feces.[23] The absorption of bile acids from the intestine occurs primarily in the ileum via an active transport mechanism mediated by the apical sodium-dependent bile acid transporter (ASBT), but passive absorption also contributes to the high efficiency of this process.[23, 24] Attempts to design a gut-restricted bile acid-derived therapeutic need to incorporate structural features designed to block both of these transport mechanisms. In addition, since any structural modifications need to maintain therapeutic efficacy, this required us to take differing approaches to gut-restricting our potential therapeutics for CDI and IBD. Our design approach was also heavily influenced by current understanding about bile acids and their absorption from the gastrointestinal tract.

Chemical structure of bile acids. The primary human bile acids chenodeoxycholic acid (CDCA) and cholic acid are synthesized in the liver from cholesterol. Both are comprised of four rings with α-hydroxy groups at the 3- and 7-positions, but cholic acid has an additional α-hydroxy at the 12-position (Figure 1). They each also contain a side chain bearing a carboxylic acid at the 24-position. Bile acids are subsequently conjugated to either glycine or taurine to produce analogs such as taurochenodeoxycholic acid (Figure 1). This conjugation process changes the physiochemical properties of the compounds, converting relatively weak acids (pK_a about 5) to much stronger acids ($pK_a < 2$ for taurine conjugates).[25] Conjugation also increases the solubility of the bile acids and their active transport by ASBT, while generally reducing their susceptibility to passive absorption.[26] From the liver, the conjugated bile acids are secreted into bile and stored in the gallbladder. After a meal, the bile acids are released into the small intestine to aid in digestion. Conjugated bile acids are then either reabsorbed from the gut or deconjugated by bacterial enzymes. Bacteria can also further metabolize primary bile acids to form secondary bile acids such as lithocholic acid (LCA) or ursodeoxycholic acid (UDCA).[25] These bile acid modifications can

lead to compounds with substantially different pharmacological properties than their parent compounds, for instance LCA is over 20 times more potent at the TGR5 receptor than is CDCA.[27]

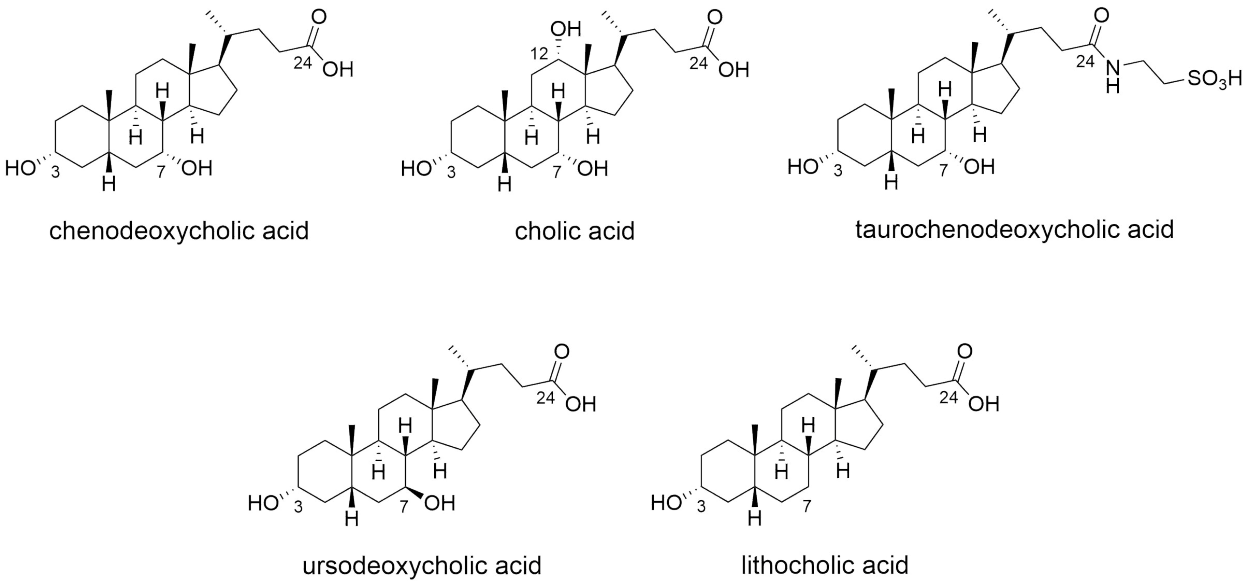


Figure 1. Structures of representative of bile acids.

Blocking active transport by ASBT. ASBT, also known as the ileal bile acid transporter (IBAT), is a 348 amino acid protein that is highly expressed in the terminal ileum, biliary epithelium, and renal proximal tubules.[28] Studies by Polli and coworkers into how modifications to the bile acid scaffold affected transport by ASBT found that structural changes to the 3-position are better tolerated than alterations at the 7-position.[29] For example, 3-nicotinoyl glycochenodeoxycholate (**1**) is an ASBT substrate while 7-nicotinoyl glycochenodeoxycholate (**2**) is not (Figure 2). The chemical substitution at the 24-position also plays a role in determining the susceptibility of a bile acid to transport by ASBT. In general, conjugated bile acids are better substrates than their unconjugated analogs.[26] However, in certain circumstances, significant modifications to this position can be made that still allow for transport by ASBT. For instance, the acyclovir prodrug

acyclovir valylchenodeoxycholate (**3**, Figure 2) is an ASBT substrate with 2-fold greater oral bioavailability than acyclovir itself.[30]

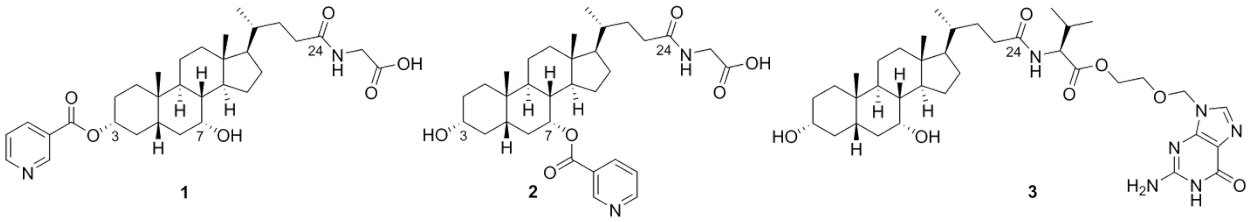


Figure 2. Compounds **1** and **3** are ASBT substrates, while **2** is not.

Blocking passive transport from the gut. While most medicinal chemists are familiar with Lipinski's[31] and Veber's[32] rules for designing druglike, orally bioavailable compounds, the rational design of druglike, non-absorbable compounds presents its own set of challenges. Successful case studies can result in compounds that look very different from each other. For instance, it can be possible to reduce oral bioavailability by making compounds either highly hydrophilic or highly lipophilic. Other techniques that have been successful include increasing the molecular weight, polar surface area, charges, or the number of rotatable bonds in a compound.[33, 34] One method that has met with success, the kinetophore approach, involves identifying portions of the scaffold of interest which can be modified without significantly reducing potency at the target receptor. Once such a site is identified, additional moieties (kinetophores) that can reduce absorption can be appended to that location.[33]

Herein, we describe two different approaches that we have found to significantly increase the gut restriction of bile acid analogs. We have previously hypothesized that the addition of a 7-sulfate group to a set of bile acid derivatives designed to inhibit the germination of *C. difficile* spores in the colon would decrease their oral bioavailability. This would likely work by both blocking active transport by ASBT and by reducing passive absorption by increasing the molecular weight and

polar surface area while also introducing a negative charge to the compounds.[18] This hypothesis is supported by research showing that both 7-sulfated UDCA and 7-sulfated cholic acid are poorly absorbed from the intestines.[35, 36] We have also shown that this modification can be incorporated into our derivatives without significant reduction in their ability to inhibit spore germination. In this study, we examined the pharmacokinetic properties of two sets of these derivatives and confirmed that 7-sulfation does indeed lower oral exposure and increase the fecal recovery of our compounds.

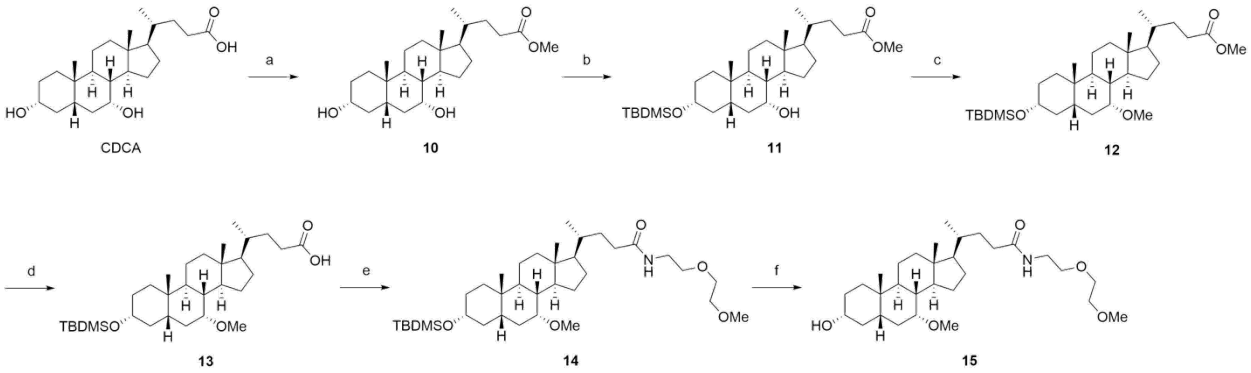
However, 7-sulfation was unsuitable for designing TGR5 agonists with reduced oral bioavailability, as this modification also greatly lowers their potency at the receptor. Our previous SAR studies have shown that incorporating a methoxy group at this position instead increases TGR5 agonist potency 40-150 fold compared to a free hydroxyl moiety.[21] In this study, we show that this modification also decreases oral exposure and increases fecal recovery, albeit only modestly. To further reduce oral bioavailability, we turned to a kinetophore-based approach. After first determining that several potential kinetophores could be conjugated to the 24-position of the bile acid scaffold with only a moderate detrimental effect on TGR5 potency, we next examined the pharmacokinetic properties of two of the resulting derivatives to determine the effect of the kinetophores on oral exposure and fecal recovery.

2. Chemistry

The pharmacokinetic studies discussed in this manuscript were conducted using a mix of new and previously reported bile acid derivatives. The *C. difficile* spore germination inhibitors **4**-**7** and TGR5 agonists **8**-**9** were synthesized as previously described (Figure 3).[18, 21] To prepare TGR5 agonist **15**, compound **13** was first synthesized in four steps from CDCA using our reported

procedure (Scheme 1).[21] Next, compound **13** was coupled to 2-(2-methoxyethoxy)ethanamine with HATU to furnish amide **14**. Finally, removal of the TBDMS protecting group with TBAF afforded the desired amide **15**.

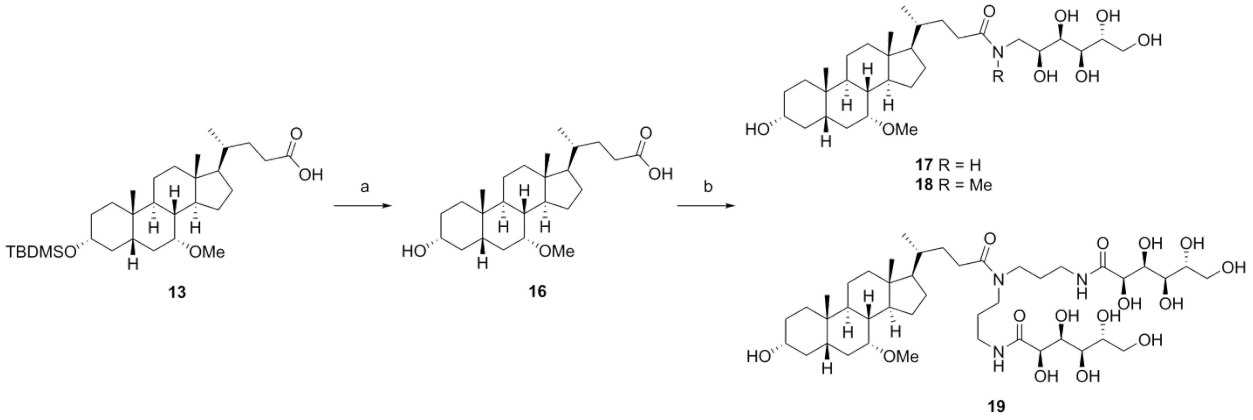
Scheme 1: Synthesis of amide 15[a]



[a]Reagents and conditions: (a) PTSA, methanol, rt; (b) TBDMSCl, imidazole, DMF, rt; (c) methyl trifluoromethanesulfonate, 2,6-di-*tert*-butylpyridine, DCM; (d) aq. LiOH, THF, MeOH, rt; (e) 2-(2-methoxyethoxy)ethanamine, HATU, DIEA, DCM, rt; (f) TBAF, THF.

The synthetic route to TGR5 agonists **17-19** began instead with the removal of the TBDMS protecting group of **13** with TBAF to afford carboxylic acid **16** (Scheme 2). This acid was then coupled to either D-glucamine, *N*-methyl-D-glucamine, or N,N-[Iminobis(trimethylene)]bis-D-gluconamide) using HATU to obtain amides **17-19**.

Scheme 2: Synthesis of amides 17-19[a]



[a]Reagents and conditions: (a) TBAF, THF. b) amine (D-glucamine, *N*-methyl-D-glucamine, or N,N-[Iminobis(trimethylene)]bis-D-gluconamide), HATU, DIEA, DMA or DMF, rt.

3. Pharmacokinetic Studies

All animal studies were approved by the Institutional Animal Care and Use Committees at the University of Minnesota (IACUC). All animals are housed in the RAR facility, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. The IACUC membership follows the Animal Welfare Act, which is enforced by the US Department of Agriculture. In addition, our IACUC policies are compliant with the Public Health Service (PHS) Policy on the Humane Care and Use of Laboratory Animals. Finally, our animal protocols were also approved by the US Army Medical Research and Development Command Animal Care and Use Review Office (ACURO).

Animal dosing and sample collection. Four male C57Bl/6 mice (8-12 weeks, Jackson Laboratories, Bar Harbor, ME) were placed in metabolic cages (Harvard Apparatus, Holliston, MA) and allowed to acclimate for at least 72 h. Animals were dosed with 5 mg/kg PO of test compound and at specified time points following dosing (5, 15, 60 and 120 min), 30 µl of blood was sampled from the lateral tail vein of each mouse. Blood from the 4 animals were pooled (120 µl total volume) in

order to obtain sufficient sample volume for analysis. Animals were replaced into the metabolic cage and housed for an additional 24 h for collection of fecal pellets. Pooled blood samples were centrifuged at 16,000 x g for 2 min. and resulting plasma (>50 µl) was stored frozen at ≤ -20° C, along with the corresponding fecal pellets, until processing and analysis by LC-MS/MS.

Sample Processing and LC/MS/MS Quantitation. Fecal pellets were weighed and then homogenized in 10 ml ice-cold acetonitrile (ACN) using a mortar and pestle. The suspension was rocked at room temperature for ≥30 min, and centrifuged at 1,000 x g for 5 min. This cycle was repeated at least 6 times until the recovered supernatant was colorless. The final supernatant was removed, the volume recorded, and stored at 4° C.

LC-MS/MS analysis was performed using a Quattro Ultima triple quadrupole mass spectrometer (Waters, Milford, MA) coupled with a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system by operating in electrospray in the positive or negative ion mode (ES+/-). Mass-to-charge ratio (m/z) transitions for the analyte and internal standard were determined. For liquid chromatographic separation, either isocratic or gradient elution using 0.1% formic acid in water and 0.1% formic acid in ACN as solvents was performed using a Synergi Polar-RP column (75 × 2 mm, 4 µm; Phenomenex, Torrance, CA).

Working solutions of analytical standards were prepared by serial dilution of stock solutions (made from 1 mg/ml master stock in DMSO) with 100% acetonitrile to obtain working concentrations at 0.1, 0.3, 0.9, 2.7, 8.0, 24.1, 72.2, 216.7 and 650 µg/µl. Calibration standards were prepared by spiking 50 µL of freshly prepared working solutions into the blanks to achieve standards with concentrations of 1.0, 3.0, 9.0, 27.0, 80.0, 241.0, 722.0, 2167.0 and 6500 ng/ml. Quality control (QC) samples were prepared by spiking 50 µl of freshly prepared working solutions into blanks to

obtain 0.1, 0.9, 24.1 and 650 ng/µl and these dilutions were used to obtain the limit of quantitation (LoQ), low quality control (LQC), medium quality control (MQC) and high quality control (HQC).

Plasma and fecal extract samples (50 µl) were placed in 1.5- ml Eppendorf tubes, 100 µl 100% ice cold ACN was added with a spiked amount of internal standard (final concentration 500 ng/ml), and the mixture was vortexed. Samples were left to stand at 4 °C for at least 30 min and then centrifuged at 16,000 x g for 5 min and the supernatant was recovered. Supernatants (100 µl) were transferred to a clean tube and evaporated to dryness under a nitrogen stream at 25 °C for approximately 10-15 min. Samples were reconstituted with 100 µl of HPLC elution starting buffer. Processed samples were then transferred to 12 x 32 mm glass vials with 400 µl inserts and loaded into 96-vial auto-sampler plates from where 7.5 µl was injected into the LC/MS/MS system. Sample analysis was performed using MassLynx Software (v4.1, Waters, Milford, MA). Data was plotted and AUC calculations were performed using Prism 5.0 (GraphPad, San Diego, CA).

4. Results and Discussion

4.1 Pharmacokinetic evaluation of C. difficile spore germination inhibitors

Clostridioides difficile (formerly *Clostridium difficile*) is an anaerobic bacterium that can cause a potentially lethal infection of the colon. This condition, *Clostridioides difficile* infection (CDI), usually occurs when spores of *C. difficile* germinate in the intestine of a patient whose native gut microbiota has been disrupted after the administration of antibiotics.[37] Spores of *C. difficile* use environmental signals to help determine if they are in the right environment to germinate. One of the most important of these signals is the absolute and relative concentrations of different bile acids. In particular, the presence of cholic acid derivatives such as taurocholic acid (TCA)

promotes germination, while CDCA derivatives are typically inhibitory to germination.[38] However, naturally occurring inhibitors such as CDCA are not very potent (~500 μM)[18] and their pharmacokinetic properties prevent them from effectively inhibiting spore germination in the colon, as they are efficiently absorbed from the digestive tract by active and passive mechanisms. We have been developing bile acid derivatives that not only inhibit the germination of *C. difficile* spores more potently than natural inhibitors, but are also designed to be gut-restricted so that significantly higher concentrations of these compounds can be reached in the colon. To do this, we have incorporated a 7-sulfate group into our compounds. As discussed in the introduction section, we hypothesized that this modification would significantly reduce the oral bioavailability of our compounds by blocking their absorption from the intestine by both active and passive transport mechanisms.

To test our hypothesis, we evaluated the pharmacokinetic properties of two pairs of compounds, UDCA derivatives **4**-**5**, and CDCA derivatives **6**-**7** (Figure 3). All four compounds inhibited the germination of *C. difficile* spores promoted by 2000 μM of TCA at concentrations of 50 μM or less. *In vitro* data from a Caco-2 model of intestinal permeability showed that compounds **5** and **7** were 60- and 25-fold less permeable, respectively, than their corresponding nonsulfated analogs **4** and **6**, consistent with compounds that are likely to have poor oral bioavailability (Figure 3).[18] In addition, while both compounds **4** and **6** had transport ratios ($P_{app(A-B)}/P_{app(B-A)}$) > 5, consistent with the behavior of actively transported bile acids in a Caco-2 assay,[39] the transport ratios for compounds **5** and **7** were ≤ 1, suggesting that these compounds may no longer substrates of ASBT.

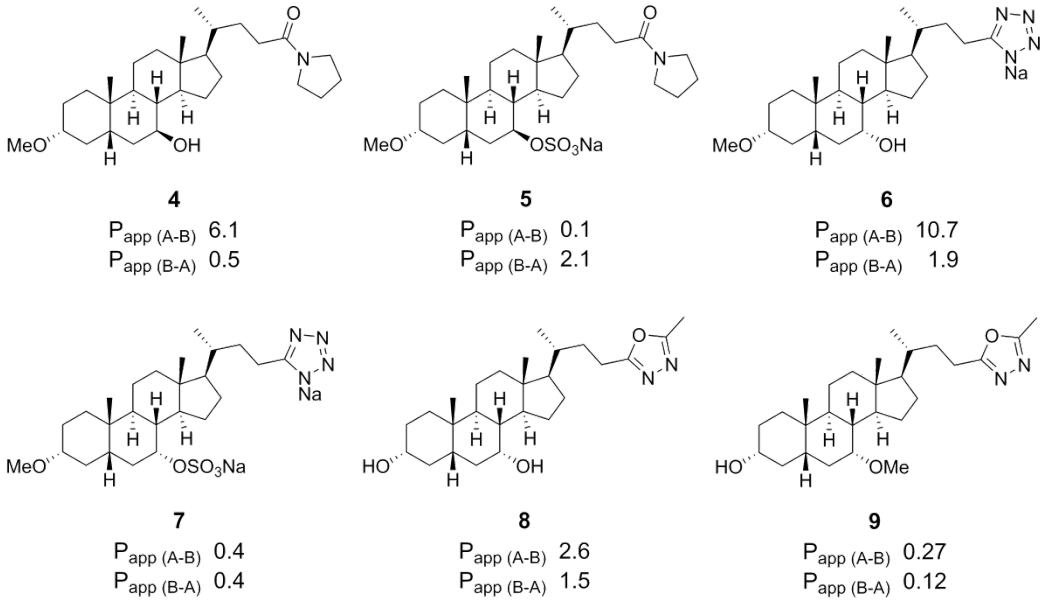


Figure 3. Structures and Caco-2 permeability (10^{-6} cm/s) of *C. difficile* spore germination inhibitors **4**-**7** and TGR5 agonists **8**-**9**. Caco-2 data for compounds **4**-**7** previously published in reference 18.

We designed our pharmacokinetic studies to determine whether there was significant oral absorption of test compounds while also minimizing the number of animals used in the experiments. In keeping with this goal, there was no IV arm to the studies. Similarly, blood was only drawn at 4 time points to avoid having to use additional animals because of IACUC restrictions on the amount of blood that could be drawn from a single animal. Male C57Bl/6 mice (n=4) were dosed (5 mg/kg PO) with test compound and placed in metabolic cages. At 15, 30, 60 and 120 min following the oral dose, blood was collected (30 µl) from the lateral tail vein of each mouse and pooled. In order to determine the amount of compound that passed through the gastrointestinal tract intact, animals were left in the metabolic cages for 24 hours after dosing and fecal pellets recovered. The resulting plasma and extractions from the collected fecal pellets were then analyzed for compound levels by LC-MS/MS (Figure 4).

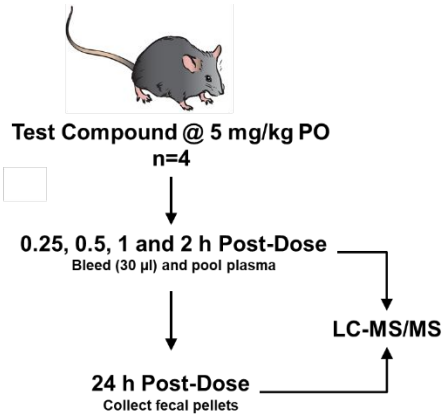


Figure 4. Pharmacokinetic testing scheme.

Plasma concentrations observed after administering compounds **4**-**5** and **6**-**7** are shown in Figure 5a and 5b, respectively. Plasma exposure levels (AUC) and fecal recovery amounts are listed in Table 1. With both sets of compounds, the incorporation of a 7-sulfate group led to a reduction in oral exposure, but the extent of this reduction varied significantly between the two scaffolds, with an approximately 2-fold reduction in AUC seen between amides **4** and **5**, and a nearly 8-fold reduction seen between tetrazoles **6** and **7**. In both cases, a roughly 10-fold improvement in fecal recovery was observed after the incorporation of a 7-sulfate group. In particular, tetrazole **7** had very high fecal recovery (89%), suggesting that its relatively low oral exposure was due to gut restriction rather than rapid metabolism either in the gut or after absorption. As there was significant fecal recovery of both amide **5** and tetrazole **7**, which can only be achieved by the compounds passing through the colon, we are currently investigating the ability of these compounds to prevent CDI in animal models.

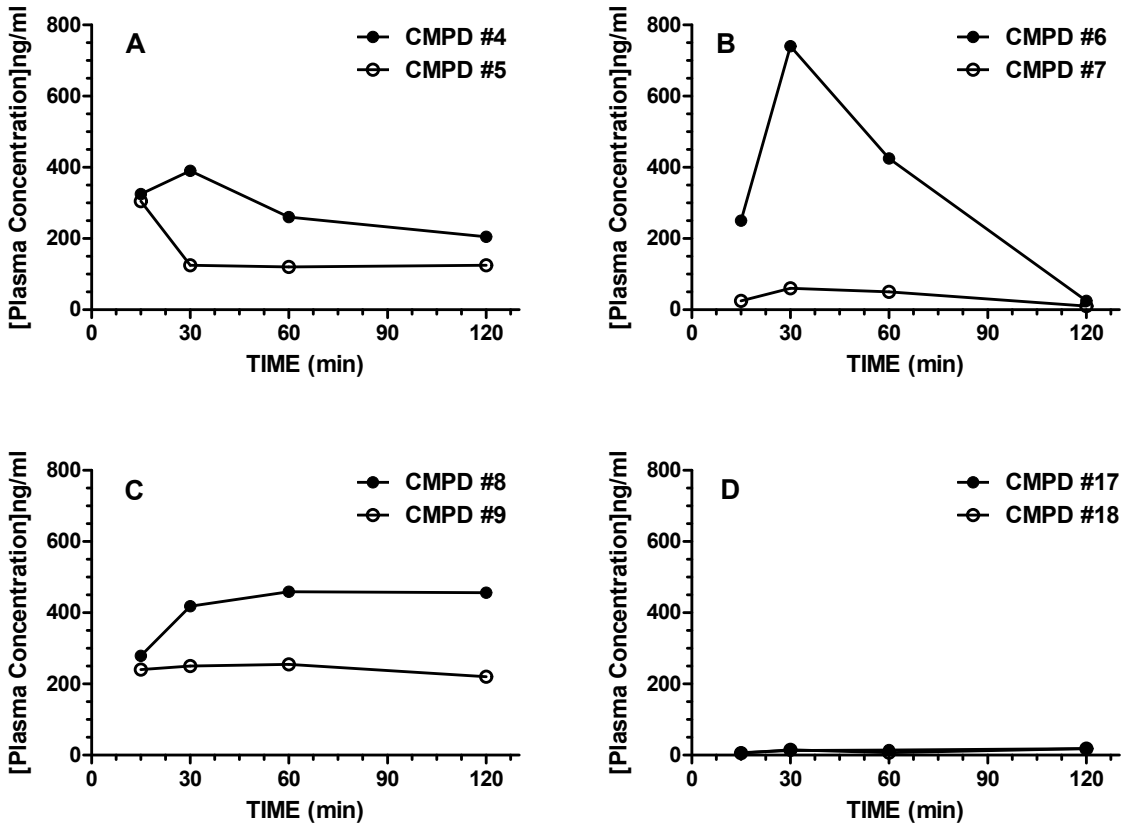


Figure 5. Pooled plasma samples collected from mice dosed with test compounds were analyzed by LC-MS/MS to determine compound levels. Each data point represents plasma from 4 individual mice orally dosed with 5 mg/kg.

Compound #	AUC ([ng/ml]h)	Fecal Recovery (%)
4	29,625	3.0
5	14,291	29.1
6	37,728	7.7
7	4,842	89.1
8	45,833	0.5
9	25,664	7.1
17	1,515	11.7
18	1,248	39.7

Table 1. Plasma exposure levels (AUC) of test compounds and recovery from feces.

4.2 TGR5 agonists with reduced oral exposure

The TGR5 receptor is an important regulator of both metabolism and the human immune system.[40, 41] Modulators of this receptor have been explored as therapeutics for diseases including IBD, diabetes, obesity, and NASH.[42, 43] This receptor is one of the key mechanisms by which the human microbiota interact with their host, as bacterial metabolism coverts the primary bile acids CDCA and cholic acid, relatively weak TGR5 agonists, into secondary bile acids like LCA and deoxycholic acid, which are much stronger agonists.[41] We have recently discovered that methylation of the 7-hydroxy of CDCA derivatives substantially increases their potency at the TGR5 receptor. This methylation, along with additional modifications at the C24-position of the bile acid scaffold, has allowed us to identify agonists that are over 10-fold more potent at the TGR5 receptor than other previously disclosed bile acid derivatives.[21] However, systemic exposure of TGR5 agonists has been associated with significant side effects, including excess gallbladder filling and blood pressure reduction.[44, 45] As a result, it is likely that gut-restricted compounds would be preferred for most clinical indications.[40]

We began our search for TGR5 agonists with reduced systemic exposure by first determining the effect of 7-methylation on the oral bioavailability of a pair of bile acid derivatives, 1,3,4-oxadiazole derivative **8** and its 7-methylated analog **9** (Figure 3). Both compounds are selective TGR5 agonists, with no activity at either the FXR or VDR receptors, and demonstrate no cytotoxicity against HEK293 cells at 10 μM. As previously reported, data from this pair of compounds clearly shows the significant effect that 7-methylation has on TGR5 activity, as a 40-fold increase in agonist potency is observed with this modification (Table 2), making compound **9** the most potent bile acid-derived TGR5 agonist reported to date.[21] We expected that 7-methylation

could also help reduce systemic exposure, as it could block or reduce ASBT-mediated transport, though its effect on passive permeability was predicted to be more modest. To help test this hypothesis, we evaluated both compounds **8** and **9** in a Caco-2 assay (Figure 3). Interestingly, compound **8** was somewhat less permeable in the apical to basolateral direction than either compounds **4** or **6**, with a reduced transport ratio of 1.7, suggesting that the introduction of the 1,3,4-oxadiazole moiety may reduce ASBT transport by itself. Methylation of the 7-hydroxy moiety (compound **9**) lowered permeability in both the apical to basolateral and basolateral to apical directions by approximately 10-fold.

To further evaluate the effect of 7-methylation on the pharmacokinetic properties of our compounds, we examined both compounds **8** and **9** in a mouse model. Using the same pharmacokinetic testing scheme described above, 1,3,4-oxadiazole derivative **8** had high oral exposure (Figure 5c, Table 1), with the largest AUC of any of the eight compounds we examined, and had negligible fecal recovery (<1%). In contrast, oral exposure with 7-methylated derivative **9** was more limited, with an observed AUC of approximately half that of compound **8** and with greater fecal recovery (7%). While this result suggests that blocking the 7-position with a methyl does help reduce oral absorption, additional modifications are clearly necessary to identify truly gut-restricted compounds. Unfortunately, it also suggests that Caco-2 permeability data is of limited utility in predicting the oral bioavailability of bile acids in a mouse model as, for instance, compounds **4** and **9** have similar oral exposures (Table 1) despite a difference in apical to basolateral permeability of over 20-fold.

To achieve our goal of reducing oral exposure, we attempted to identify a location on the bile acid scaffold to which a kinetophore could be appended. This task was aided by the observation that 7-

methylation significantly reduced the relative importance of substitutions at the C-24 position in determining potency. For instance, there is a 7-fold difference in potency between 1,3,4-oxadiazole **8** and pyrrolidine amide **20**, which both have free hydroxy groups at the 7-position, but this drops to a 2-fold difference in potency with 7-methylated derivatives **9** and **21** (Table 2). To further confirm this finding, 7-methylated CDCA (compound **16** in Scheme 2) was synthesized and evaluated in a TGR5 agonist assay. With an EC_{50} of 76.8 nM, this compound is an astonishing 200-fold more potent than CDCA. Thus, the 70-fold difference in potency between 1,3,4-oxadiazole **8** and CDCA is reduced to only a 15-fold difference in potency between the methylated analogs of these compounds, **9** and **16**. These combined results suggest that the C-24 position might be a good location to attach a kinetophore, as long as the scaffold also contains a 7-methoxy group. To explore this possibility, we synthesized the 2-(2-methoxyethoxy)ethanamino derivative **15** (Scheme 1) and determined that it was a TGR5 agonist with an EC_{50} of 49.5 nM (Table 2). While compound **15** is less potent than **9** or **21**, it is still one of the most active bile acid-derived TGR5 agonists ever reported,[21] suggesting that it may be a good starting point for further exploration.

After determining that the C24-position of the bile acid side chain might be an acceptable location to attach a kinetophore, we next searched for a suitable moiety. As a starting point, we chose D-glucamine, which has been previously used to help gut-restrict compounds including with the clinical candidate canosimibe[33] as well as with a recently published family of non-steroidal TGR5 agonists.[46] Incorporation of this group into a scaffold significantly increases the molecular weight and polar surface area of the resulting molecule, as well as the number of rotatable bonds, hydrogen bond donors, and hydrogen bond acceptors, all of which should generally lower oral absorption. To test this idea, the D-glucamine conjugate **17** was synthesized from acid **16** (Scheme 2) and

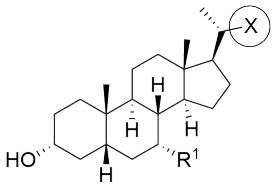
evaluated in a TGR5 agonist assay. Gratifyingly, this compound proved to be a more potent agonist than **15**, with an EC_{50} of 33.3 nM (Table 2), suggesting that a large, highly polar conjugate could be tolerated at the C24-position.

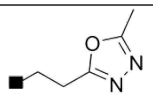
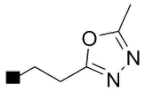
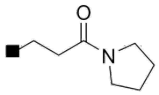
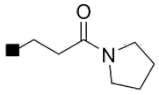
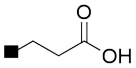
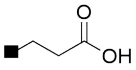
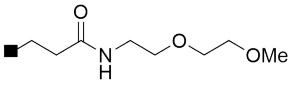
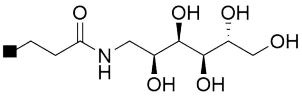
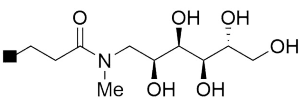
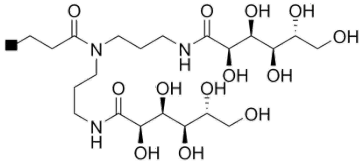
We next evaluated the pharmacokinetic properties of the D-glucamine amide **17** in a mouse study. As shown in Figure 5d and Table 1, this compound had very little oral bioavailability, with a much lower AUC than the other bile acids derivatives that we had evaluated to that point. However, the fecal recovery of this compound was disappointing at 11.7%. The lack of large amounts of observed compound in either the blood or feces strongly suggests that the compound was being metabolized, either in the gut or after absorption. Because D-glucamine amides were used to successfully gut restrict other compounds,[33, 46] there is no evidence that this moiety is inherently metabolically unstable, suggesting that the metabolism may be specific to the bile acid scaffold. Bacteria naturally deconjugate taurine and glycine from bile acids and it is possible that D-glucamine may also be deconjugated, as in all three cases the substrate would be a secondary amide.

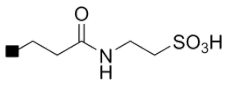
To further differentiate our conjugates from natural substrates of bacterial metabolism, we next synthesized a pair of tertiary amides, the *N*-methyl-D-glucamine conjugate **18** and the *N*,*N*-[iminobis(trimethylene)]bis-D-gluconamide conjugate **19** (Scheme 2). While the highly bulky derivative **19** exhibited greatly diminished activity at the TGR5 receptor (EC_{50} of 567 nM, Table 2), the *N*-methyl-D-glucamine analog **18** was only modestly less potent than **17**, with an EC_{50} of 62.3 nM. Promisingly, in a mouse pharmacokinetic study compound **18** had low exposure, with less than 1/30th the AUC of 1,3,4-oxadiazole derivative **8** (Figure 5d, Table 1), and much better fecal recovery (39.7%) than **17.** As a result, we next intend to evaluate compound **18** in animal

models of IBD. Interestingly, the structural modifications that were made to our TGR5 agonists to lower their oral exposure also completely eliminated their ability to block the germination of spores of *C. difficile*, as both compounds **17** and **18** failed to inhibit spore germination at concentrations as high as 200 μM (see Figure S1 in the Supporting Information).

Table 2: TGR5 Agonist Activity of CDCA Analogs



Compound	R^1	X	EC_{50} (nM)[a]
8	-OH		223[b]
9	-OMe		5.6 ± 0.7[b]
20	-OH		1600[b]
21	-OMe		10.5 ± 1.7[b]
CDCA	-OH		15600[b]
16	-OMe		76.8
15	-OMe		49.5
17	-OMe		33.3 ± 6.3
18	-OMe		62.3
19	-OMe		567

TLCA	-H		1005.4 ± 80.0

[a]Data represent mean of at least two experiments. ± SEM is given when N ≥ 3. [b]Data previously reported in reference [21]. All compounds were full agonists, with an E_{max} similar to positive control (TLCA). Data from individual experiments are listed in Table S1.

5. Conclusions

We have been working to identify gut-restricted bile acid derivatives that can be used as therapeutics for the prevention of CDI and the treatment of IBD. In this study, we identified two different modifications that can be used to reduce the oral bioavailability of bile acid derivatives: sulfation of the C7-hydroxy group and conjugation of a kinetophore to the side chain of the bile acid. Sulfation led to lowered oral bioavailability of two different analogs designed to inhibit the germination of *C. difficile* spores in the intestine, though by differing amounts. It also increased fecal recovery of the compounds approximately 10-fold, demonstrating that it can be used to increase delivery of bile acid derivatives to the colon. However, while the incorporation of a 7-sulfate group did not adversely affect the ability of the bile acid derivatives to inhibit the germination of *C. difficile* spores, this modification greatly reduced activity at the TGR5 receptor. To identify bile acid-derived TGR5 agonists with reduced oral exposure that might be suitable treatments for IBD, we began by determining that it was possible to conjugate a 2-(2-methoxyethoxy)ethanamine to the bile acid with only a modest effect on potency as long as the scaffold also incorporated a 7-methoxy moiety. We then determined that more polar amines could also be employed as well, with an *N*-methyl-D-glucamine conjugate providing a satisfactory mix of potency at the TGR5 receptor, low oral exposure, and good fecal recovery.

6. Experimental Procedures

6.1 TGR5 agonist assay

Dose response curves for each compound were determined by assaying cAMP production in cells expressing human TGR5 receptor as previously reported.[21] The EC_{50} is the average of at least two experiments.

6.2 Compound synthesis and characterization

NMR spectra were recorded using a Bruker 400 spectrometer. ^{1}H NMR data are reported as follows: chemical shift in parts per million downfield of tetramethylsilane (TMS), multiplicity (s = singlet, bs = broad singlet, d = doublet, t = triplet, q = quartet, quint = quintet and m = multiplet), coupling constant (Hz), and integrated value. All materials, reagents, and solvents were obtained from commercial suppliers and were used without further purification unless otherwise noted. The purity of all new compounds was determined to be ≥95% by LC-MS/ELSD analysis.

(*R*)-4-((3*R*,5*R*,7*R*,8*R*,9*S*,10*S*,13*R*,14*S*,17*R*)-3-((*tert*-butyldimethylsilyl)oxy)-7-methoxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-(2-(2-methoxyethoxy)ethyl)pentanamide (14). To a solution of compound **13**[21] (114.5 mg, 0.220 mmol) in DCM (4.0 mL) was added DIEA (0.075 mL, 0.431 mmol) and HATU (87.5 mg, 0.230 mmol), followed 10 min. later by 2-(2-methoxyethoxy)ethanamine (0.030 mL, 0.241 mmol). After stirring at rt overnight, the reaction mixture was purified by flash column chromatography on silica gel (35-100% EtOAc in hexanes as eluent) to obtain **14** as a clear, colorless oil (131.7 mg, 96% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.06-5.96 (m, 1H), 3.67-3.59 (m, 2H), 3.59-3.51 (m, 4H), 3.50-3.43 (m, 2H), 3.43-3.36 (m, 1H), 3.41 (s, 3H), 3.24 (s, 3H), 3.20-3.14 (m, 1H), 2.28-

2.15 (m, 2H), 2.12-2.00 (m, 1H), 1.95-1.73 (m, 6H), 1.66-0.97 (m, 15H), 0.96-0.86 (m, 1H), 0.92 (d, *J* = 6.4 Hz, 3H), 0.88 (s, 3H), 0.875 (s, 9H), 0.63 (s, 3H), 0.040 (s, 3H), 0.036 (s, 3H).

(*R*)-4-((3*R*,5*S*,7*R*,8*R*,9*S*,10*S*,13*R*,14*S*,17*R*)-3-hydroxy-7-methoxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-(2-(2-methoxyethoxy)ethyl)pentanamide (15). Compound **14** (124.1 mg, 0.200 mmol) was dissolved in 1 M TBAF in THF (2.5 mL, 2.5 mmol) and stirring continued for 2 days. The reaction mixture was diluted with water (60 mL) and extracted with EtOAc (60 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by silica gel column chromatography (0−7 % MeOH in EtOAc as eluent) to obtain **15** (60.9 mg, 60% yield) as a clear oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.10-6.00 (m, 1H), 3.65-3.59 (m, 2H), 3.59-3.52 (m, 4H), 3.50-3.37 (m, 3H), 3.40 (s, 3H), 3.25 (s, 3H), 3.21-3.16 (m, 1H), 2.30-0.82 (m, 26H), 0.92 (d, *J* = 6.4 Hz, 3H), 0.90 (s, 3H), 0.64 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 173.7, 77.5, 72.1, 71.9, 70.2, 70.0, 59.1, 55.9, 55.8, 50.3, 42.4, 41.9, 39.6, 39.4, 39.1, 38.5, 35.5, 35.3, 35.0, 33.7, 33.5, 31.7, 30.9, 28.2, 27.8, 23.7, 22.9, 20.8, 18.4, 11.7. LC/MS (ESI): m/z calcd. $C_{30}H_{54}NO_5$ ($M+H^+$) 508.4, found 508.4.

(*R*)-4-((3*R*,5*S*,7*R*,8*R*,9*S*,10*S*,13*R*,14*S*,17*R*)-3-hydroxy-7-methoxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)pentanoic acid (16). Compound **13** (581.1 mg, 1.12 mmol) was dissolved in 1 M TBAF in THF (6.0 mL, 6.0 mmol) and stirring continued for 5 days. The reaction mixture was diluted with 1 M HCl water (100 mL) and extracted with DCM (2 x 100 mL). The combined organic layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude material was purified by silica gel

column chromatography (80-100% EtOAc in hexanes as eluent) to obtain **16** (372.7 mg, 82% yield) as white solid.

^{1}H NMR (400 MHz, DMSO-D_6) δ: 11.92 (bs, 1H), 4.34 (bs, 1H), 3.25-3.16 (m, 1H), 3.18 (s, 3H), 3.16-3.12 (s, 1H), 2.29-2.18 (m, 1H), 2.16-2.04 (m, 1H), 2.03-0.99 (m, 23H), 0.94-0.90 (m, 1H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.86 (s, 3H), 0.61 (s, 3H). ^{13}C NMR (100 MHz, DMSO-D_6) δ: 174.9, 76.8, 70.1, 55.5, 55.4, 49.9, 41.9, 41.3, 39.3, 38.9, 38.1, 35.1, 34.9, 34.6, 33.3, 30.7, 30.6, 30.5, 27.6, 27.3, 23.2, 22.7, 20.4, 18.1, 11.5. LC/MS (ESI): m/z calcd. $C_{25}H_{41}O_4$ (M-H) 405.6, found 405.3.

(*R*)-4-((3*R*,5*S*,7*R*,8*R*,9*S*,10*S*,13*R*,14*S*,17*R*)-3-hydroxy-7-methoxy-10,13-dimethylhexadecahydro-1*H*-cyclop)enta[*a*]phenanthren-17-yl)-N-((2*S*,3*R*,4*R*,5*R*)-2,3,4,5,6-pentahydroxyhexyl)pentanamide (17). To a solution of **16** (41.9 mg, 0.103 mmol) in DMA (1.0 mL) was added DIEA (0.040 mL, 0.23 mmol) and HATU (42.4 mg, 0.112 mmol), followed 5 min. later by D-glucamine (21.2 mg, 0.117 mmol). After stirring at rt overnight, the reaction mixture was purified by reverse-phase chromatography (10-100% ACN in water as eluent, C_{18} column) to obtain **17** (43.5 mg, 74% yield) as a white solid after lyophilization.

^{1}H NMR (400 MHz, DMSO-D_6) δ: 7.70 (t, *J* = 5.5 Hz, 1H), 4.73 (d, *J* = 4.5 Hz, 1H), 4.44 (d, *J* = 5.6 Hz, 1H), 4.37 (d, *J* = 6.0 Hz, 1H), 4.35 (d, *J* = 4.8 Hz, 1H), 4.32 (t, *J* = 5.6 Hz, 1H), 4.25 (d, *J* = 6.3 Hz, 1H), 3.62-3.51 (m, 3H), 3.51-3.33 (m, 3H), 3.28-3.10 (m, 3H), 3.18 (s, 3H), 3.06-2.94 (m, 1H), 2.17-0.97 (m, 25H), 0.97-0.86 (m, 1H), 0.88 (d, *J* = 6.4 Hz, 3H), 0.86 (s, 3H), 0.60 (s, 3H). ^{13}C NMR (100 MHz, DMSO-D_6) δ: 172.9, 76.8, 72.0, 71.8, 71.4, 70.0, 69.4, 63.2, 55.4, 55.3, 49.8, 41.8, 41.2, 39.7, 39.2, 38.8, 38.0, 35.0, 34.9, 34.5, 33.2, 32.1, 31.5, 30.5, 27.5, 27.3, 23.2, 22.6, 20.3, 18.2, 11.4. LC/MS (ESI): m/z calcd. $C_{31}H_{56}NO_8$ ($M+H^+$) 570.4, found 570.4.

(*R*)-4-((3*R*,5*S*,7*R*,8*R*,9*S*,10*S*,13*R*,14*S*,17*R*)-3-hydroxy-7-methoxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl)-*N*-methyl-*N*-((2*S*,3*R*,4*R*,5*R*)-2,3,4,5,6-pentahydroxyhexyl)pentanamide (18). To a solution of **16** (58.6 mg, 0.144 mmol) in DMF (1.5 mL) was added DIEA (0.050 mL, 0.29 mmol) and HATU (55.2 mg, 0.145 mmol), followed 5 min. later by *N*-methyl-D-glucamine (31.1 mg, 0.159 mmol). After stirring at rt overnight, the reaction mixture was purified by reverse-phase chromatography (10-100% ACN in water as eluent, C_{18} column) to obtain **18** (70.3 mg, 29% yield) as a white solid after lyophilization. ^{1}H NMR (400 MHz, DMSO-D_6) δ: 4.88 (major rotamer, d, J = 5.1 Hz, 0.6H), 4.71 (minor rotamer, d, J = 5.0 Hz, 0.4H), δ: 4.50 (major rotamer, d, J = 5.4 Hz, 0.6H), 4.46 (minor rotamer, d, J = 5.4 Hz, 0.4H), 4.42-4.24 (m, 4H), 3.79-3.70 (m, 1H), 3.62-3.09 (m, 9H), 3.17 (s, 3H), 3.00 (minor rotamer, 1.2H), 2.79 (major rotamer, s, 1.8H); 2.37-0.97 (m, 25H), 0.94-0.81 (m, 4H), 0.86 (s, 3H), 0.60 (s, 3H). LC/MS (ESI): m/z calcd. $C_{32}H_{58}NO_8$ ($M+H^+$) 584.4, found 584.5.

(2*R*,3*S*,4*R*,5*R*)-2,3,4,5,6-pentahydroxy-*N*-(3-((*R*)-4-((3*R*,5*S*,7*R*,8*R*,9*S*,10*S*,13*R*,14*S*,17*R*)-3-hydroxy-7-methoxy-10,13-dimethylhexadecahydro-1*H*-cyclopenta[a]phenanthren-17-yl)-*N*-(3-((2*R*,3*S*,4*R*,5*R*)-2,3,4,5,6-pentahydroxyhexanamido)propyl)pentanamido)propyl)hexanamide (19). To a solution of **16** (43.0 mg, 0.106 mmol) in DMA (1.0 mL) was added DIEA (0.040 mL) and HATU (43.7 mg, 0.115 mmol), followed 5 min. later by N,N-[iminobis(trimethylene)]bis-D-gluconamide (60.0 mg, 0.123 mmol). After stirring at rt overnight, the reaction mixture was purified by reverse-phase chromatography (10-100% ACN in water as eluent, C_{18} column) to obtain **19** (43.7 mg, 47% yield) as a white solid after lyophilization.

^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.86-7.68 (m, 2H), 5.43-5.35 (m, 2H), 4.60-4.52 (m, 2H), 4.52-4.44 (m, 2H), 4.44 (d, J = 7.1 Hz, 2H), 4.37-4.29 (m, 3H), 4.03-3.95 (m, 2H), 3.95-3.87 (m, 2H), 3.62-3.54 (m, 2H), 3.53-3.44 (m, 4H), 3.42-3.34 (m, 2H), 3.29-3.07 (m, 8H), 3.18 (s, 3H), 3.07-2.97 (m, 2H), 2.35-2.10 (m, 2H), 2.07-0.97 (m, 27H), 0.98-0.82 (m, 1H), 0.90 (d, J = 6.5 Hz, 3H), 0.86 (s, 3H), 0.61 (s, 3H). ^{13}C NMR (100 MHz, DMSO-D_6) δ: 172.4, 172.3, 172.2, 76.8, 73.6, 73.5, 72.30, 72.26, 71.39, 71.38, 70.0 (2C), 63.2 (2C), 55.6, 55.3, 49.8, 44.9, 42.4, 41.9, 41.3, 39.2, 38.8, 38.0, 35.8, 35.7, 35.04, 35.03, 34.5, 33.2, 31.2, 30.5, 29.2, 28.7, 27.6, 27.4, 27.2, 23.2, 22.6, 20.3, 18.3, 11.4. LC/MS (ESI): m/z calcd. $C_{43}H_{78}N_3O_{15}$ ($M+H^+$) 876.5, found 876.6.

Supporting Information

Supplementary data to this article can be found online at

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Conflict of Interest

There is no conflict of interest to declare.

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