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Anti-Germinants as a New Strategy to Prevent Clostridium Difficile Infections

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ANTI-GERMINANTS AS A NEW STRATEGY TO PREVENT *CLOSTRIDIUM DIFFICILE* INFECTION

by

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ABSTRACT

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Clostridium difficile infections (CDI) have emerged as a leading cause of hospital-associated complications. CDI is the major cause of antibiotic-related cases of diarrhea and nearly all cases of pseudomembranous colitis. The infective form of *C. difficile* is the spore, a dormant and hardy structure that forms under stress. Germination of *C. difficile* spores into toxin producing bacteria in the GI tract of susceptible patients is the first step in CDI establishment. Patient susceptibility occurs with a disruption of the natural gut microbiota by broad-spectrum antibiotics. Antibiotic treatments usually resolve CDI but refractory cases are on the rise. Of great concern is the high incidence of recurrence due to persistence of spores in the gut following antibiotic treatment and/or spore re-ingestion. Besides surface decontamination there are currently no protocols for prevention of CDI.

C. difficile spores must germinate to cause disease. Therefore, a logical approach to preventing CDI is to prevent spore germination. Unlike other *Bacillus* and *Clostridia*, the genome of *C. difficile* does not encode for any known germination binding site(s). Small molecules are typically required to activate spore germination in *Bacillus* and *Clostridia*. *C. difficile* germinates in the

presence of taurocholate, a natural bile salt, and glycine, an amino acid. The natural bile salt, chenodeoxycholate, has been shown to inhibit spore germination *in vitro*. We used structure activity analysis to define the microenvironment of the putative *C. difficile* germination binding site(s). Amino acids and amino acid analogs were analyzed for activation or inhibition of *C. difficile* spore germination.

To determine which functional groups of bile salts are necessary and sufficient to activate or inhibit spore germination, we prepared bile salt analogs of taurocholate and chenodeoxycholate. This analysis elucidated specific functional groups recognized by *C. difficile* spores. Furthermore, many bile salt analogs are able to bind but are not recognized by the putative *C. difficile* germination binding site(s). During this structure analysis, we discovered that a *meta*-benzene sulfonic acid derivative of taurocholate (CamSA) was a strong inhibitor of spore germination *in vitro*. CamSA is stable and non-toxic based on pharmacokinetic *in vitro* studies. CamSA showed no acute toxicity at the highest concentrations tested. More importantly, a single dose of CamSA prevents CDI in mice. Ingested *C.* difficile spores were quantitatively recovered from feces and intestines of CamSA-protected mice. Using CamSA as a probe, we were able to establish when onset of disease occurs in mice after infection with *C. difficile* spores. The results presented in this dissertation project support a mechanism whereby the anti-germination effect of CamSA is responsible for preventing CDI signs.

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CHAPTER 1

INTRODUCTION

1.1. *Clostridium difficile* Background and Significance

Clostridium difficile is a pathogenic bacterium from the Phylum *Firmicutes* of the Kingdom Eubacteria in the Domain Bacteria (Fig. 1.1). This phylum contains two major Classes: *Bacilli* and *Clostridia* (1, 2). The Class *Clostridia* consists only of organisms that are obligate anaerobes, Gram-positive and endospore-forming bacteria (3). Microorganisms, that like *C. difficile* are obligate anaerobes, are intolerant of molecular oxygen and use other molecules such as nitrate as a final electron acceptor for cellular respiration (4, 5). For example, vegetative *C. difficile* cells die in the presence of oxygen but flourish in the anaerobic, high nutrient environment of the mammalian colon (6, 7). Like others in the Class *Clostridia, C. difficile* is Gram-positive due to a thick cell wall made up of peptidoglycan, which absorbs the primary stain, crystal violet, and appears purple by light microscopy (Fig. 1.2) (8). In contrast, Gram-negative bacteria have cell walls made up mostly of lipopolysaccharides that do not retain the primary stain but absorb the counter stain, safranin, and appear red under microscopy (Fig. 1.2) (9). The Gram-staining technique was developed by Hans Christian Gram to separate bacteria into two large groups based on physical properties of cell wall constituents (9). Also like other bacteria in its Class, *C. difficile* produces and releases dormant spores under stressful conditions as a survival mechanism (10). The spores survive in aerobic conditions where the

mother vegetative cells cannot, thereby providing a survival strategy outside of the host (10).

Further classification of *C. difficile* in the Order *Clostridiales,* Family *Clostridiaceae* is due to the usage of glucose as a source of carbon (Fig. 1.1) (11). The Genus *Clostridium* contains approximately 150 species found in diverse anaerobic environments such as soils, aquatic sediments, and intestinal tracts of mammals (12). *C. difficile* is an opportunistic pathogen of the mammalian gastrointestinal (GI) tract. *C. difficile* is the etiological agent of *C. difficile* infection (CDI), which is the leading cause of antibiotic-associated diarrhea in the healthcare setting in the United States and Canada (10, 13, 14).

C. difficile was first identified in 1935 during an examination of indigenous colonic flora of healthy newborn infants and was originally named *Bacillus*

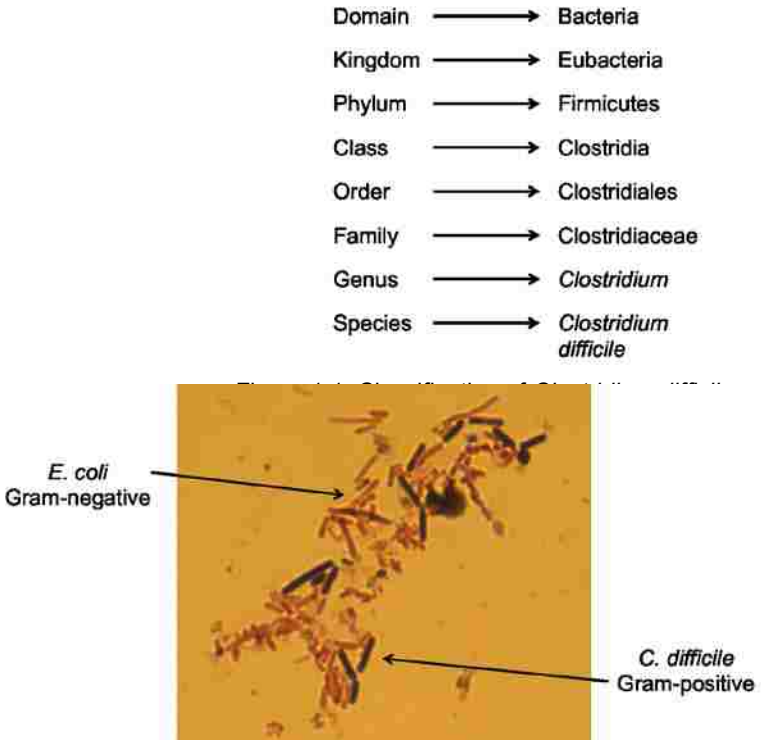


Figure 1.2. Representation of Gram staining. Comparison of *C. difficile* a Gram-positive bacterium (shown as purple) and *E. coli* a Gram-negative bacterium (shown as pink/red).

difficilis due to its difficulty to grow in culture (6). The origin of *C. difficile* in neonates is due to hospital contamination and it is poorly understood how newborns carry the bacterium asymptomatically (6, 15). Neonates are not the only symptomless carriers of *C. difficile*. One study found that nearly 20% of hospitalized patients were asymptomatic carriers of *C. difficile* (16). Although it would be reasonable to expect that *C. difficile* carriers would have a higher risk of developing disease after antibiotic therapy, studies have shown humans that carry *C. difficile* asymptomatically are actually at decreased risk of developing CDI (17-20). This phenomenon is not understood, but speculation exists that the host immune system is responsible. Studies have suggested that an increase of immunoglobulin G (IgG) antibodies or an overall antibody response to *C. difficile* is responsible for symptom-less carriers remaining symptom-less (21, 22).

C. difficile colonization and infection is also a concern in domestic and food animals. Outbreaks of CDI in piglets have been reported for more than twenty-five years and the mortality rate during outbreak is reported to be as high as 16% (23-25). *C. difficile* has been found to colonize and infect feedlot cattle, veal calves, and farm-raised deer (26-30). Since infection of humans with *C. difficile* is by the oral route, contamination of food animals and processing plants is an important concern.

C. difficile has been isolated from the feces of domesticated cats and dogs. Environmental cross infection of *C. difficile* from domestic animals to humans has been a growing concern in the last five years. Recent reports show that dogs used as pet therapy in hospitals can acquire and shed *C. difficile*

spores after contact with contaminated patients (31, 32). *C. difficile* has been isolated from cats and dogs presenting signs of diarrhea although the extent of disease in these animals is still unclear (33, 34).

CDI primarily affects the cecum and the colon of mammalian hosts. CDI presentation in patients can range from mild to severe diarrhea, pseudomembranous colitis and fulminant colitis (35). Pseudomembranous colitis, the characteristic symptom of CDI, was first described in 1893, however; it was not until 1978 that this symptom was associated with *C. difficile* (36, 37). Purportedly early CDI symptoms are persistent mild to moderate diarrhea with or without abdominal pain and can begin within the first 48 hours of *C. difficile* infection (20, 35). The progression to severe disease typically includes profuse diarrhea, abdominal distention and in some cases, occult colonic bleeding occurs. In up to 4% of severe cases, fulminant colitis leads to toxic megacolon (colonic dilation), bowel perforation, sepsis, shock and death (10, 12).

Since the late 1970s, the rate of CDI progression to severe symptoms and death has been increasing annually (38, 39). In the past decade, a highly virulent epidemic *C. difficile* strain has emerged, BI/NAP1/027 (13, 39, 40). The BI/NAP1/027 strain has been associated with increased disease severity, more deaths, higher incidences of recurrence and a wider range of antibiotic resistance than other strains of *C. difficile* (13). Indeed, approximately 500,000 patients are reported to have CDI annually in the United States and 20,000 succumb to this disease (41). A recent study reported CDI responsible for 25% more nosocomial infections than methicillin-resistant *Staphylococcus aureus* (MRSA) (42).

CDI is a significant burden on the health care system with hospital costs estimated at greater than $3.2 billion in the United States annually (35, 43, 44). The economic burden is due in part to increased hospital stay of up to two weeks per patient (44, 45) and the high incidence of recurrence in patients with CDI. Recurrence of CDI has been defined as either a relapse of infection by the original strain of *C. difficile* or re-infection of susceptible patients exposed to new strains (35). Recurrences happen at an alarming frequency of up to 55% (46, 47). Furthermore, the risk of recurrence increases with each episode and is greater than 60% with more than two CDI episodes (48-50). Extreme cases have reported more than 10 CDI recurrences per patient (51).

1.2. *Clostridium difficile* Infections

CDI is primarily a nosocomial infection with the elderly and immunocompromised patients being at the highest risk (52). High incidences of CDI are reported in cancer patients with severe neutropenia, transplant patients, and AIDS patients (53-56). Susceptibility to CDI has been observed in patients administered broad-spectrum antibiotics (57). However in the last few years, community-acquired CDI in patients previously considered at low risk have been reported and are a growing concern (58-61).

In the 1990s an estimated 30 cases of hospital-acquired CDI was reported per 100,000 patients per year and less than seven cases of community-acquired CDI per 100,000 patients per year (62). An overlapping and broadening study that ended in 2005 found that the rates for each type of acquired CDI had increased. This report found that there were more than 40 cases of hospital-

acquired CDI and approximately 15 cases of community-acquired CDI per 100,000 patients per year (61). This study did not find common risk factors in patients; such as antibiotic exposure or recent hospital admittance (61). It is important to note that standardized surveillance and reporting of CDI cases and risk factors has been a problem (63). The study found that *C. difficile* strains endemic to the hospital were responsible for hospital-acquired CDI and that different strains were common in community-acquired cases (61). These findings suggest that patients with community-acquired CDI were not infected by patients with hospital-acquired CDI spreading spores at home or in the community. The source of community-acquired CDI remains a mystery. These findings make identification of the community exposure to *C. difficile* spores an important issue. As discussed previously, food animals and domestic animals can be affected by *C. difficile* and can transmit spores (26, 29, 32). One group reported that many strains found in animals were not the same as human strains of *C. difficile* (64). However, they also found that epidemiologically important strains were becoming more common in animals, particularly the hypervirulent strain BI/NAP1/027 (64). Although classification of *C. difficile* as a zoonotic infection is still unclear, these studies suggest the potential risks.

Since *C. difficile* has been isolated from food animals, another potential reservoir for spore transmission is contaminated food products and drinking water. *C. difficile* spores have been recovered from up to 20% of retailmeat in the United States and Canada. In these cases, *C. difficile* was approximately 5% of the total isolated microorganisms (64, 65). These incidences of contaminated

food suggest that community-acquired CDI could be a food borne illness. Community-acquired CDI is still poorly understood but it is possible that multiple factors may be involved. It has been suggested that genetically-determined differences in the immune system, differences in the normal gut flora of some individuals, and administration of medications (other than antibiotics) that affect the colonization resistance of the natural flora are possible situations that could allow the development of CDI in unlikely patients (59).

The mode of transmission is the highly infective *C. difficile* spore. The environment, hospital personnel and patients are potential reservoirs for transmission of spores and infection (66-69). *C. difficile* spores are ingested from contaminated matter and will either germinate and cause disease or will be retained in asymptomatic carriers. Spores and/or cells are excreted in the feces of infected patients and transferred person to person via the fecal oral route (Fig. 1.3) (63, 70). Contamination of surfaces and transmission between patients is a cycle that can lead to outbreaks of CDI and is of serious concern to healthcare settings. Patients are intermittently exposed to *C. difficile* spores during hospital stays and the length of stay has been correlated to increased risk of CDI (16).

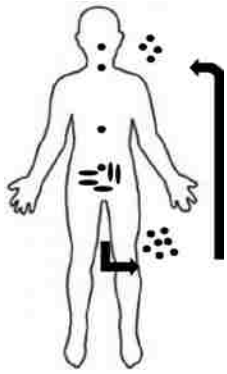


Figure 1.3. Representation of the *Clostridium difficile* infection cycle. Mammalian hosts ingest spores (black circles) that survive the GI tract to germinate in the lower intestine (black ovals) and cause disease. As a survival mechanism vegetative cells produce spores that are released in the feces. The same or different mammal ingests excreted spores thereby continuing the cycle.

*C. difficile s*pores are metabolically dormant structures that are highly resistant to chemicals, temperature, desiccation and can persist in aerobic environments (71). Spores can persist on hospital surfaces for extended periods of time and can be reservoirs for disease transmission (35, 72). To colonize and cause disease in susceptible patients, *C. difficile* spores are ingested and evade the host's immune system allowing spores to germinate into vegetative bacteria and colonize the gut (Fig. 1.3) (35). In a healthy individual, indigenous intestinal bacteria resist *C. difficile* colonization (73). Patients undergoing aggressive antibiotic treatment become susceptible to *C. difficile* due to the disruption of the normal microflora resulting in a loss of colonization resistance (74). Most broad-spectrum antibiotics can predispose a patient for *C. difficile* infection (35). The key factors that lead to outgrowth of *C. difficile* and onset of disease are ingestion of *C. difficile* spores, susceptibility due to antibiotic use, and loss of natural protection.

As *C. difficile* spores germinate and multiply, toxins are released that cause characteristic pathology of CDI. *C. difficile* cells release low levels of toxins steadily during exponential phase (up to six hours). However, during late exponential phase (eight hours) into stationary phase (24 – 48 hours) the amount of toxin quadruples in production (75-77). Most *C. difficile* strains produce toxin A (TcdA) and toxin B (TcdB) and some produce a third binary toxin, CDT. Non-toxigenic strains exist naturally and have not been shown to cause disease (20). The two large Clostridial toxins, TcdA and TcdB, are widely accepted as the main virulence factors of *C. difficile*. Specialized assays for detection of these toxins are used in the diagnosis of CDI (35).

The major toxins, TcdA and TcdB, are among the largest (>270 kDa) bacterial toxins and are related to the *Clostridium sordellii* lethal and hemorrhagic toxins and to the *Clostridium novyi* alpha toxin (78). The toxins have 49% amino acid similarity and are both highly expressed during stationary phase growth (79). Genes for both toxins are located on the pathogenicity region of the *C. difficile* chromosome (78). TcdA and TcdB are internalized into host epithelial cells after binding to an unknown surface binding site(s) (78). After internalization, toxins modify intestinal epithelial cell actin cytoskeleton using UDP glucose-dependent glycosylation of Rho family proteins (80). TcdA and TcdB disrupt the tight junctions between epithelial cells and recruit neutrophils to the site of infection resulting in diarrhea and characteristic colitis (78). The roles and necessity for both toxins during CDI has been under investigation. However, using a recently

designed *C. difficile* genetic manipulation system a recent report provides evidence for the importance of both toxins in a hamster model of CDI (81, 82).

TcdA is an enterotoxin capable of triggering host cell fluid secretion, severe inflammation, and inducing epithelial damage, which are the characteristics of pseudomembranous colitis (83). Until a *C. difficile* strain was engineered in a recent study, there have been no reports of naturally occurring TcdA+B- strains (82).

TcdB is a very potent cytotoxin capable of mucosal necrosis, reduction of epithelial integrity, and triggering apoptosis (84, 85). Although most *C. difficile* strains clinically isolated are TcdA+B+ or TcdA-B+ the role of TcdB is not well characterized (86). Furthermore, strains that do not produce TcdA still cause severe pseudomembranous colitis and death in patients (87).

The third toxin is called binary toxin CDT and it was first described in 1988 (88). Binary toxin CDT is in the same family as *Clostridium botulinum* C2 toxin, *Clostridium perfringens* iota toxin and *Clostridium spiroforme* iota-like toxin (89, 90). Binary toxin CDT is capable of modifying the cellular actin cytoskeleton by ADP-ribosyltransferase activity (91). Unlike TcdA and TcdB, the binary toxin is composed of two different proteins, a binding component and a catalytic component. Both components are necessary for toxicity (89, 92, 93). Not all *C. difficile* strains encode for binary toxin (94) and some strains that encode for it have mutated genes (95). The epidemic strain BI/NAP1/027 produces a fully active binary toxin (96). The hypervirulence of this strain may be due in part to the addition of this toxin to its virulence repertoire. Evidence of increased

virulence of strains in humans due to binary toxin production is unclear. Some reports have shown that binary toxin is found in less than 10% of clinical isolates where as other reports show that binary toxin is present in 65% of isolates of *C. difficile* (86, 88, 91, 97). The presence of the toxin in clinical isolates does not define whether binary toxin itself is responsible for increased *C. difficile* virulence or disease severity. Using the hamster model of CDI, a recent study, found that a strain that produced only binary toxin was able to colonize the gut of animals but was unable to cause diarrhea or death (98). Although the *C. difficile* binary toxin is unable to cause disease when produced alone, it is possible that a synergistic effect may occur in the presence of TcdA and/or TcdB (98). Another possibility is that binary toxin may target a specific protein or immune response that is only present in the human GI tract. More research is needed to fully understand the role of binary toxin CDT in *C. difficile* strains.

Once CDI is diagnosed, treatment options become paramount. The first step is to discontinue antibiotic therapy that the patient is undergoing (35). Vancomycin and metronidazole are the most commonly used treatments for initial episodes of CDI (35). An expensive new antibiotic was recently approved by the FDA for treatment of CDI; Fidaxomicin (Dificid) can be administered if vancomycin and metronidazole fail to treat CDI (99). Other antibiotics are under investigation as potential treatment options but the cure rates are variable (100). Currently, treatment of CDI has a failure rate of up to 38% with first line antibiotics (46). Antibiotics are only effective against metabolically active, toxin-producing bacterium and not on dormant spores (101). Therefore, current

treatment regimens may not completely eradicate the organism from the gut allowing for recurrence due to persistence of spores (101). Some researchers have proposed treatment with immunoglobulin G (IgG) antibodies due to the observation that asymptomatic patients have increased IgG titers (21, 22). Other studies have shown that deliberately colonizing animals with non-toxigenic *C. difficile* strains can partially prevent CDI (102, 103).

There is a high incidence of recurrence of CDI due to relapse and/or re-infection (35). Typical treatment of recurrent CDI is re-administration of the first line of antibiotics (35). However there is great concern about long-term usage of metronidazole and vancomycin because of metronidazole-induced neuropathy and increased drug resistance of other pathogens to vancomycin (100, 104). The newly approved antibiotic, fidaxomicin, is ineffective against recurrent CDI (105). Furthermore by using antibiotics to treat CDI and recurrent CDI, the normal gut flora is not allowed to recover and a vicious circle ensues.

Alternative treatments have been described for recurrent CDI but have variable results. In severe cases of CDI, radical procedures are sometimes necessary to combat the disease (35, 106). Fecal transplantation has been termed the 'ultimate probiotic' and has been used in patients with CDI that have exhausted all other treatment options (107). Stool samples from donors closely associated with the patient are obtained, diluted with saline, homogenized with a blender and filtered before being instilled into the recipient via enema or nasogastric tube (106-108). Colectomy is also used as a last resort to combat CDI and/or recurrent CDI. Removing part of or the entire colon can remove the

diseased tissue as well as massive amounts of the infection. Up to 8% of severe CDI patients require this type of aggressive treatment (109). However, surgery as an aggressive treatment for CDI is complicated by the compromised immune system of the patient. Patients with CDI as a complication to other illnesses, such as cancer, are rarely treated with surgery due to the high risk of not surviving the colectomy (109). Furthermore, total colectomy may not completely remove *C. difficile* from the GI tract. In rare instances, CDI returned to the small bowel of patients that underwent total colectomies, presenting comparable symptoms as seen in the colon (110-112). Small bowel CDI is not well understood however; others and we have found that although *C. difficile* is prominent in the cecum and colon, spores and cells are also found in the upper GI tract of animals (113, 114). It is likely that *C. difficile* spores can persist in the small intestine and begin outgrowth after surgery resulting in small bowel CDI.

Careful use of antibiotics, early and reliable *C. difficile* testing, isolation of CDI patients, and proper decontamination of hospital surfaces are the current preventative measures for outbreaks of *C. difficile* (35). A recent study from a hospital following CDI prevention guidelines showed the persistence of *C. difficile* spores in stools, on skin and in the environment of CDI patients up to six weeks after diarrhea was resolved (115). Glove use during contact with all patients is not enforced in health care settings and could potentially lead to the transmission of spores (35). Spores are not affected by typical disinfectants; therefore specific *C. difficile* sporicidal-labeled cleaning agents are required for cleaning contaminated surfaces (35). Unfortunately patients may not begin to show signs

of CDI until after discharge from the hospital by which time a new patient may inhabit the contaminated room (63). This persistence of spores provides potential reservoirs for transmission and recurrence in patients and hospital outbreaks (115). As described, prevention of CDI in the healthcare setting is a serious problem. In even the strictest settings, outbreaks can still occur. As the number of cases increases yearly, researchers are trying to understand *C. difficile* and find proper ways to eradicate it from hospitals.

1.3. Sporulation and Germination

Endospore-forming bacteria produce spores during stressful conditions like nutrient deprivation (10). Cells respond to signals from the external and internal environments and initiate sporulation processes. Sporulation initiator and regulator proteins are activated and subsequently activate a cascade of sporulation proteins and sigma (σ) factors (116). The mother bacterial cell produces a septum that separates the cellular material from a portion of the cell pole (Fig. 1.4). The prespore is then engulfed inside the mother cell for application of the synthesized spore cortex and spore coat (Fig. 1.4). After spore maturation, the mother cell is lysed resulting in the release of the free spore (Fig. 1.4) (116). Sporulation has been well studied in *Bacillus subtilis* but not as thoroughly for *C. difficile*. Analyses have shown that many but not all sporulation genes are conserved between *B. subtilis* and *C. difficile* (116). An interesting difference is that *B. subtilis* requires up to 3 hours to transition from initiation of sporulation to forespore formation whereas *C. difficile* can take 8-20 hours for the same transition (116). This wide and variable range of time for *C. difficile*

sporulation may explain the variation seen in the amount of spores produced by different strains *in vitro* (117, 118).

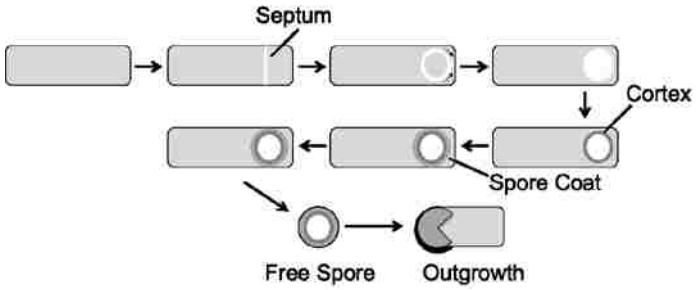


Figure 1.4. Representation of the sporulation cycle of *Clostridium difficile*. After sporulation has been triggered, vegetative bacterial cells produce a septum at one pole of the mother cell. This section separated by the septum is then engulfed creating the prespore. In stages, the cortex and then the spore coat are produced on the spore. The mature spore is released after lysis of the mother cell. When the free spore is triggered to germinate, the spore outgrows into the vegetative bacteria. Adapted from Paredes *et al* 2005 (116)

The newly formed *C. difficile* spore is covered by a loosely associated exosporium that may be important to surface attachment by thin proteinaceous structures (119, 120). Adhesion to animal cells by pathogens is an important step in colonization and infection. After the normal gut flora is disturbed by antibiotic therapy, it is likely that *C. difficile* spores make contact with and adhere to previously occupied niches in the intestinal cell lining (119). Using the exosporium proteinaceous projections, *C. difficile* spores attach to cells allowing the spore to anchor itself to the intestinal wall (119).

The exosporium covers a complex, dense polypeptide component called the spore coat (Fig. 1.5). Unlike other *Bacillus* and *Clostridia*, the genome of *C. difficile* only encodes 18 of the 70 conserved spore coat proteins (121, 122). It has been postulated that like *B. subtilis*, the spore coat in *C. difficile* has surface proteins rich in cysteine residues that form a rigid lattice around the inner spore

(121). These cysteine residues form disulfide bonds under aerobic (oxidative conditions) and this mesh is weakened in the anaerobic (reducing) environment of the host gut (121). The identity of these proteins is currently unknown but they have likely diverged from the common ancestor of *Bacillus* and *Clostridia*. The spore coat likely plays a part in the passage of small molecules (germinants) to the inner layers of the spore (123). The spore coat is also responsible for protection of the inner spore layers against harsh chemicals and lysozyme (124).

The spore cortex is separated from the coat by an outer membrane. The cortex is comprised of highly cross-linked peptidoglycan layers forming a cage-like structure around the spore's inner membrane and spore core (Fig. 1.5) (125). The spore cortex plays an important role during germination of spores. As the spore core becomes hydrated (during germination), hydrolytic enzymes are released to degrade the cortex. This step is essential for proper spore germination so that the spore core can become fully hydrated without the physical barrier of the cortex (116, 123).

The inner membrane is a very rigid lipid bilayer and is the location of germination receptors (Ger) that are found in almost all *Clostridia* and *Bacillus* species, except *C. difficile* (95). Although *C. difficile* lacks known germination proteins, it is expected that receptors, unique to *C. difficile*, are also located on the inner membrane of the spore (126, 127). The inner membrane's rigidity is suspected to be due to immobile polycrystalline structures (Fig. 1.5) (128). The inner membrane has very low permeability to protect DNA in the core. Even water is prevented from crossing the membrane in a dormant spore (129, 130).

The inner spore core is the location of dehydrated RNA, DNA, and metabolic enzymes required for degradation of the spore layers and for outgrowth (Fig. 1.5). The spore core is anhydrous and filled with a calcium-dipicolinic acid complex that confers heat resistance (131). Acid-soluble proteins (SASP) are associated with spore DNA to resist ultraviolet light radiation and SASPs make up 10-20% of the total protein in the spore core (131). Due to their unique structure and durability, *C. difficile* spores are metabolically dormant structures that are highly resistant to harsh chemicals, temperature, desiccation and can persist in aerobic environments (71).

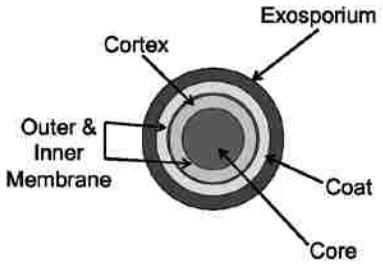


Figure 1.5. Generalized *C. difficile* spore structure.

Like other *Bacilli* and *Clostridia*, when *C. difficile* spores encounter favorable nutrient-rich environments they undergo a series of events termed germination that lead to metabolically active vegetative bacteria (125, 132). Small molecules and combinations of molecules trigger the irreversible first step in the commitment to germinate (128). In fact *Bacillus* and *Clostridia* recognize a number of different small molecules including but not limited to: amino acids, carbohydrates, nucleosides, salts, and bile acids (125, 133-136). In most *Bacillus* and *Clostridia*, it is believed that these molecules traverse the outer layers of the

spore to bind to proteinaceous germination (Ger) receptors located on the inner membrane (126). The germinant activates the receptors and water is transported to the spore core to partially rehydrate the molecules inside. Upon water entry, the spore's large store of Ca^{2+}-dipicolinic acid (DPA) complex is released. The small acid-soluble proteins associated with DNA are hydrolyzed and the amino acids released are used for outgrowth (131). Lytic enzymes disintegrate the spore coat and cortex resulting in total hydration of the spore core contents (126, 131). During the spore germination process, spore-specific characteristics such as refractility, resistance and dormancy are lost (125). The cell escapes from the spore coats, becomes metabolically active, resumes vegetative growth and multiplies (126, 131). In stressful conditions or as a survival strategy, vegetative cells can renew the sporulation process as described before.

The Ger proteins are highly conserved in all sequenced *Bacillus* and *Clostridia spp.* when using Basic Local Alignment Search Tools (BLAST). Surprisingly, *C. difficile* genes encode analogs for most spore-specific proteins except for Ger receptors and most spore coat proteins (95). *C. difficile* must germinate to cause disease, therefore the *C. difficile* germination binding site(s) may be very divergent from other species or they use a different set of proteins to detect germinants (95). The lack of known germination receptor analogs in *C. difficile* has impeded the use of genetic tools to design mutants. Recently a mariner-based transposon system has been designed for mutagenesis in *C. difficile.* However, preliminary phenotypic screening of the mutant library has yielded a single clone that is defective in sporulation and/or germination (137).

Genetic manipulation of *C. difficile* is still in the infancy of general use and research in *C. difficile* germination will take time. An alternative approach to study *C. difficile* spore germination is kinetic methods and chemical probes (127, 134).

1.4. *Clostridium difficile* Spore Germination

Since early cultivation of *C. difficile*, researchers have found that bile salts (bile acids) are required for optimal growth *in vitro* (138, 139). A recent article revealed that *C. difficile* spores recognize glycine, an amino acid, and taurocholate, a natural primary bile salt, as germinants (140). Furthermore, chenodeoxycholate another natural primary bile salt, has been shown to inhibit *C. difficile* spore germination *in vitro* (141). Deoxycholate, a secondary bile salt, has been shown to inhibit the growth and multiplication of vegetative *C. difficile* cells (134, 140). The dynamics between the chemistry of the GI tract and indigenous bacteria play a crucial role in establishment of *C. difficile.* Unfortunately, many details are not well understood (7, 142).

Primary bile salts are synthesized in the liver of mammals from cholesterol and secreted into the duodenum via bile ducts (Fig. 1.6) (143). The basic bile salt structure has three six-membered rings and one five-membered ring with hydroxyl groups and methyl groups at specific locations. Before secretion into the GI tract, bile salts are conjugated to glycine or taurine thereby increasing their solubility and decreasing passive absorption (Fig. 1.7) (143, 144). High amounts of conjugated bile salts in the intestinal lumen increases the removal of cholesterol and other lipids and aids in the digestion of fats for absorption.

Indigenous bacteria in the GI tract alter bile salts by deconjugation and dehydroxylation generating secondary bile salts (Fig. 1.6) (143, 144).

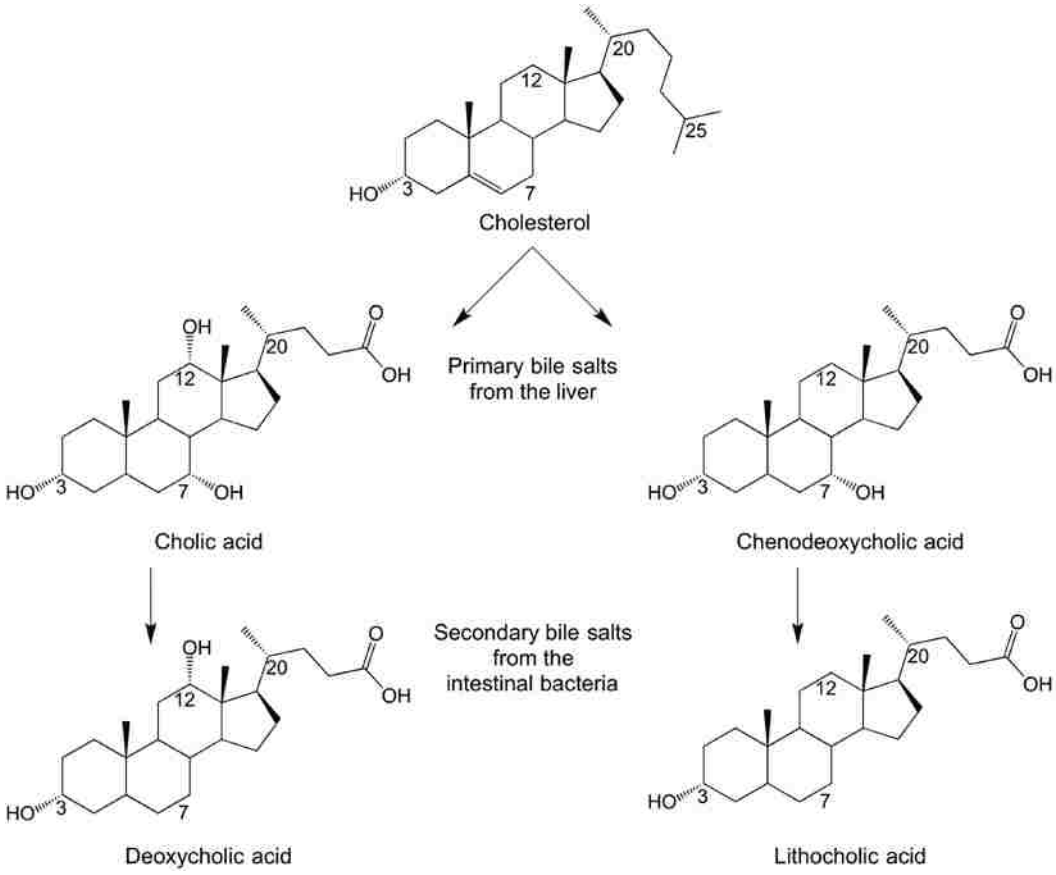


Figure 1.6. Conversion of cholesterol to bile acids.

The majority of bile acids are reabsorbed and recycled to the liver from the distal ileum however; approximately 400-800 mg of bile passes to the cecum (145, 146). The concentration of primary bile salts in the upper GI tract is estimated at 10 mM and 2 mM in the lower GI tract (147). In the lower intestine the indigenous bacterial flora transform primary bile acids into secondary bile acids. Glycocholate is also deconjugated, resulting in an increase of free glycine (144, 148). Researchers have hypothesized that in a healthy person, ingested

spores encounter high levels of primary bile salts in the upper GI tract but may be inhibited from outgrowth due to the aerobic environment and the presence of chenodeoxycholate (140, 141). In the lower intestine where normal flora transform primary bile salts into secondary bile salts, specifically deoxycholate, growth is inhibited and cells are excreted (140). Chenodeoxycholate has a reabsorption rate ten times greater than taurocholate in the anaerobic colon where *C. difficile* spores likely germinate (149). Although chenodeoxycholate is more readily absorbed, taurocholate is deconjugated by normal flora thus decreasing the concentration of taurocholate in a healthy gut (149).

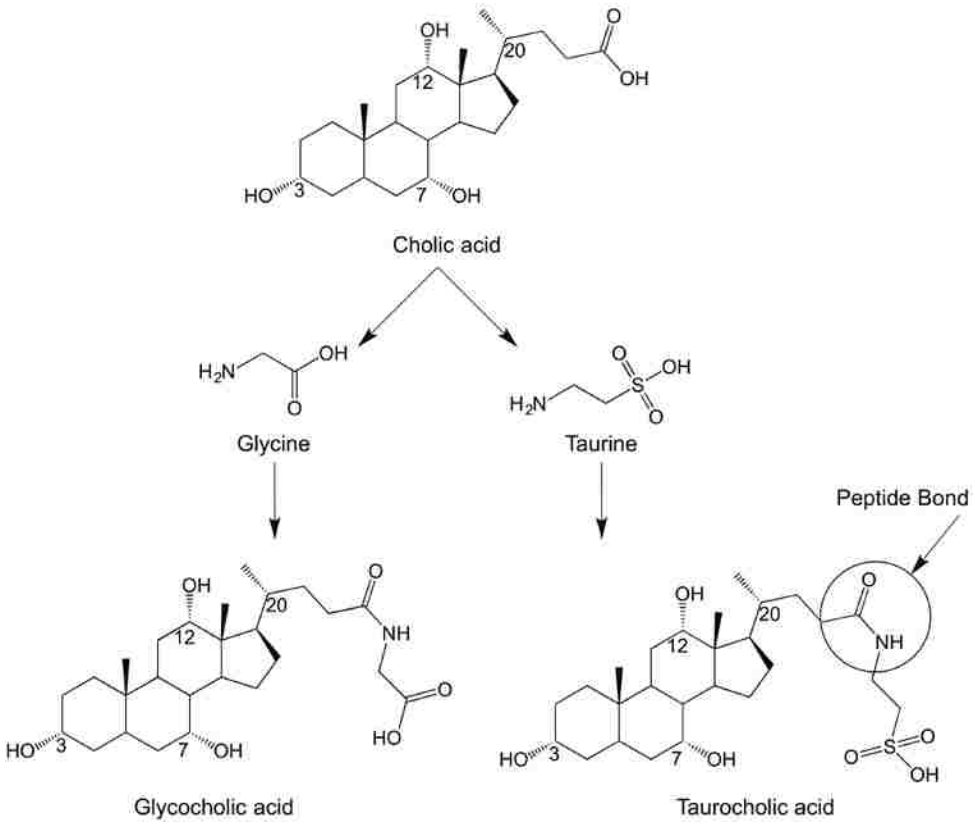


Figure 1.7. Synthesis of conjugated bile acids.

Following antibiotic treatment, the indigenous flora is disrupted. Previous reports show that after antibiotic treatment, the amount of secondary bile salts is reduced and primary bile salts increase in animals (7, 140). The authors postulate that bile salt shift could allow for outgrowth and colonization by *C. difficile* (7). Because chenodeoxycholate is more readily absorbed, the concentration of chenodeoxycholate is likely too low to prevent germination in response to the high level of taurocholate. In fact, a recent study has shown that *C. difficile* spores germinate more efficiently in GI tract extracts, high in primary bile salts, taken from antibiotic treated animals than from GI extracts from untreated animals which contained a mixture of primary and secondary bile salts (7). Although the concentrations of specific bile salts are unknown in antibiotic treated mammals, it is clear that disruption of the indigenous microflora result in susceptibility to *C. difficile*.

Neither glycine nor taurocholate has been previously described to activate spore germination in *Bacillus* and *Clostridium* species, suggesting a novel mode of germinant recognition in *C. difficile* spores. *C. difficile* spores have adapted for survival in a variety of conditions but will germinate in the presence of specific molecular signals found in the appropriate environment, the intestinal tract (126, 131). Highly conserved *Bacillus* and *Clostridium* germination receptors have not been identified in *C. difficile.* However, recent *in vitro* analyses provide evidence of receptor-like germination binding site(s) (127). Taurocholate is used by the mammalian body to emulsify fatty acids and therefore could activate *C. difficile* spore germination by non-specific disruption of spore membranes. However, our

lab has previously shown that *C. difficile* spores do not germinate in the presence of highly concentrated surfactants such as sodium dodecyl sulfate or Triton X-100 (127). Kinetic analysis of *C. difficile* spore germination in the presence of taurocholate and glycine shows that spores bind taurocholate and glycine in a complex cooperative mechanism (127). This study shows that the binding of one germinant increases the affinity for the second. Similar cooperativity, with different germinants, has been shown with *Bacillus cereus* and *Clostridium sordellii* (133, 136). The presence of germination receptors is also evidenced by the ability of germinants to saturate the binding site(s) (127). Furthermore, chenodeoxycholate, another fat emulsifier, is a competitive inhibitor of taurocholate and is recognized specifically by the same binding site(s) (127, 141). Competitive inhibitors of *C. difficile* spore germination are important for potential CDI prophylaxis treatments, especially those with a strong affinity for the binding site even when present at low concentrations. Anti-germinants as drugs would bind to *C. difficile* spores, preventing germination, and allowing the spore to be excreted harmlessly. These reports provide evidence that *C. difficile* likely encodes unknown receptor proteins to bind germinants and that other molecules compete for these binding sites, inhibiting spore germination (127).

1.5. Aims of This Study

The initial aim of this study was to perform a structure-activity analysis of *C. difficile* spores and analogs of glycine, taurocholate, and chenodeoxycholate. This type of analysis provides evidence of functional group requirements for recognition by the putative germination binding site(s) in *C. difficile* spores. Due

to the scarcity of genetic tools, many of the metabolic capabilities encoded by *C. difficile* remain poorly understood (150). This has precluded the use of molecular microbiology to identify putative germination binding site(s) encoded by *C. difficile*. As an alternative to genetic manipulation, molecular probes can be used to study the mechanism of *Bacillus* and *Clostridium* spore germination (127, 133, 151). This provides mechanistic information, even when the identity of the germination binding site(s) is unknown (136).

While mapping the interactions between *C. difficile* spores and germinants we discovered a bile salt analog, CamSA, capable of strongly inhibiting spore germination *in vitro* (Fig. 1.8). As a secondary, we hypothesized that by screening CamSA analogs we could determine the functional groups essential for CamSA's potency. Furthermore, during this second structure activity analysis we hoped to discover compounds more potent than CamSA at inhibiting *C. difficile* spore germination *in vitro.*

Based on *in vitro* structure activity analyses, CamSA was chosen as a lead compound for further study. The third aim was to characterize CamSA *in vitro* for stability, oral bioavailability and cytotoxicity. The final aim was to challenge animals with *C. difficile* spores or vegetative cells and treat these animals with CamSA. We hypothesized that CamSA could prevent signs of CDI in mice by preventing *C. difficile* spore germination into toxin-producing bacteria.

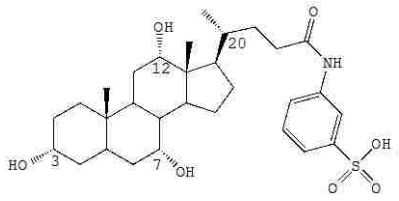


Figure 1.8. Structure of CamSA.

CHAPTER 2

MAPPING INTERACTIONS BETWEEN GERMINANTS AND *CLOSTRIDIUM DIFFICILE* SPORES

2.1. Introduction

Germination of *C. difficile* spores is the first required step in establishing CDI (152, 153). Taurocholate, a bile salt, and glycine, an amino acid, have been shown to be important germinants of *C. difficile* spores (Fig. 2.1) (140). The question that has remained unanswered is how glycine and taurocholate interact with the putative binding sites. Structure-activity relationship analysis of germinant analogs allows a better understanding of the microenvironment of the *C. difficile* germination binding site(s) (127, 133, 151). This approach provides mechanistic information, even when the identity of the germination binding site(s) is unknown by identifying essential functional groups for recognition (136). In the current work, 30 amino acid analogs and 22 taurocholate analogs were tested as activators or inhibitors of *C. difficile* spore germination. Activators of the germination pathway identify functional groups essential for binding and activation of the *C. difficile* germination binding site(s). On the other hand, inhibiting agents provide structural details about functional groups that allow only binding. Inhibition assays serve as an indirect method to map physiochemical configurations in the receptor binding site(s). Competitive inhibitors most likely bind to the same site as the cognate germinant. Strong competitive inhibitors complement the germinant binding site shape, size, hydrophobicity, and

hydrogen bonding pattern. Inactive compounds yield information on functional group changes that interfere with germinant binding (151).

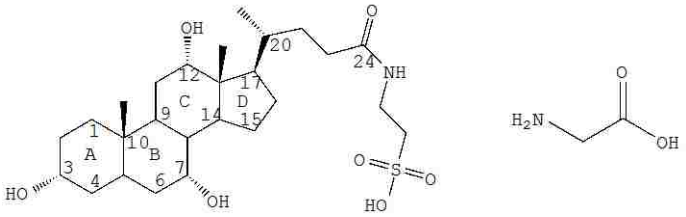


Figure 2.1. The structure of taurocholate (left) and glycine (right). The four rings of the cholate backbone are labeled A-D.

As expected, changing glycine and taurocholate functional groups affected the germination of *C. difficile* spores. Structure-activity relationship analysis allowed the determination of which taurocholate and amino acid functional groups are necessary and sufficient to bind to and/or activate *C. difficile* spores. The data suggests either the presence of multiple amino acid germination binding site(s) or that the putative glycine binding site(s) recognizes structurally diverse amino acids. Furthermore, the putative taurocholate germination binding site(s) recognizes its cognate germinant through multiple molecular interactions. In fact, one interesting molecule was discovered that is four times more active than the natural inhibitor, chenodeoxycholate, at inhibiting *C. difficile* spore germination.

2.2. Materials and Methods

2.2.1. General Comments

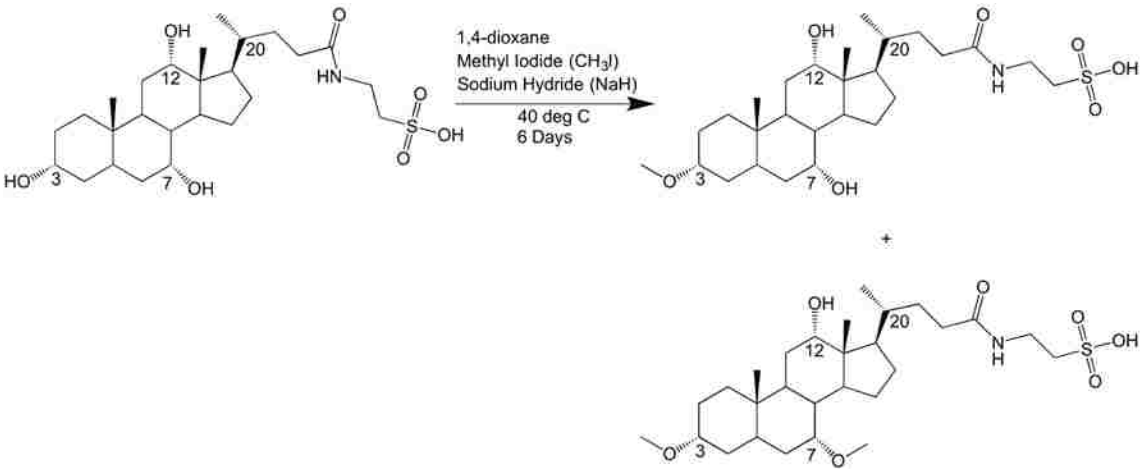
Taurocholate and amino analogs were purchased from Sigma Aldrich Corporation (St. Louis, MO), Steraloids (Newport, RI) or were synthesized in the

Abel-Santos laboratory. Reagents for synthesis were purchased from Sigma Alrdrich Corporation (St. Louis, MO) or Alfa Aesar (Ward Hill, MA). Thin layer chromatography silica gel 60 F_{254} was purchased from EMD Chemicals (Gibbstown, NJ). Silica gel for column chromatography was purchased from Fisher Scientific (Pittsburg, PA).

2.2.2. Synthesis of Methoxylated Taurocholate Analogs

Two methoxylated taurocholate analogs, 3-methoxy-7,12-dihydroxytaurocholate [T09] and 3,7-dimethoxy-12-hydroxytaurocholate [T10], were prepared following published procedures (154) (Scheme 2.1). To a solution of taurocholate (1 equivalent – 1mM) in dry 1,4-dioxane, methyl iodide (50 eq.) and sodium hydride (4 eq.) was added under nitrogen. The reaction mixture was heated to 40 °C for 48 h with stirring. After the initial 48 h, sodium hydride (4 eq.) was added daily to the reaction mixture for four additional days. The reaction mixture was then diluted with dichloromethane and washed once with 1 M HCl and twice with water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The resulting residue was purified by silica gel column chromatography eluted with a step gradient from 100% dichloromethane (DCM) to 60% dichloromethane/acetone. Two different compounds were obtained. Final yield for each compound was less than 10%. ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry showed that one compound had a single methoxy group and the second compound had two methoxy groups and were greater than 95% pure. The compounds were tentatively identified 3-methoxy-7,12-dihydroxytaurocholate

[T09] and 3,7-dimethoxy-12-hydroxytaurocholate [T10], as expected from published OH reactivity (155, 156).

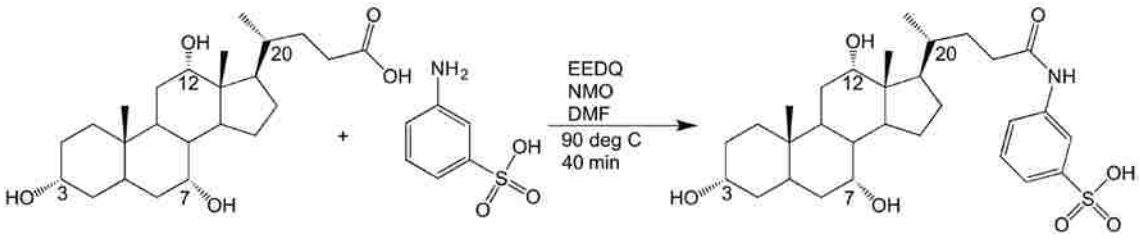


Scheme 2.1. Synthesis of methoxylated taurocholate analogs.

2.2.3. Synthesis of T11 to T16 and T18 to T21

The taurocholate analogs were prepared following published procedures (157, 158). Cholic acid (1 eq. - 1mM) was activated with 1.4 eq. of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and 1.3 eq. of *N*-methylmorpholine (NMO) in dimethylformamide (DMF) (Scheme 2.2). After the mixture was stirred for 5 min, 1.2 eq. of the appropriate amino sulfonic acid or amino acid was added. The reaction mixture was heated to 90 °C for 40 min and then cooled to room temperature. The solution was poured into 100 ml of ice-cold diethyl ether, resulting in a precipitate. The ether suspension was kept at 4 °C overnight. The ether layer was decanted and the resinous residue was dissolved in 25 ml 0.2 N NaOH-MeOH and poured into 100 ml cold diethyl ether. The ether solution was kept at 4 °C for at least 2 h and the resulting precipitate was filtered and washed with diethyl ether. If necessary, the product was recrystallized by

dissolving in hot ethanol to saturation, followed by the addition of ethyl acetate until a precipitate appeared. The solution was kept at -20 °C for 2 h to allow complete precipitation and then filtered to retrieve the product. The precipitated residue was further purified by silica gel column chromatography eluted with a step gradient from 100% DCM to 100% ethanol (EtOH). Two compounds CA2APA [T19] and CA2ABA [T21] were obtained as side products of the synthesis of CAAPA [T18] and CAABA [T20], respectively. Percent yields were typically 30 – 80%. Compound structures were verified by ^{1}H-NMR, Fourier transform infrared spectroscopy (FTIR), and mass spectrometry. Synthesized compounds were determined to be >95% pure based on HPLC-MS and NMR analysis. Representative verification data for CamSA [T15] includes ^{1}H NMR, FT-IR, and MS (Fig. 2.2 and 2.3). ^{1}H NMR (400 MHz, methanol-d_4, ppm) δ = 7.93 (s, 1H), 7.78 (d, 1H, *J* = 12.00 Hz), 7.53 (d, 1H, *J* = 8 Hz), 7.34 (t, 1H, *J* = 16.00 Hz), 3.96 (s, 1H), 3.80 (s, 1H), 3.34 (m, 3H), 2.41 (m, 1H), 2.27 (m, 3H), 1.88 (m, 5H), 1.76 (m, 2H), 1.50 (m, 12H), 1.06 (m, 4H), 0.98 (m, 1H) 0.91 (s, 3H), 0.72 (s, 3H). MS m/z 562.42 $[M-H]^-$.



Scheme 2.2. Synthesis taurocholate analogs.

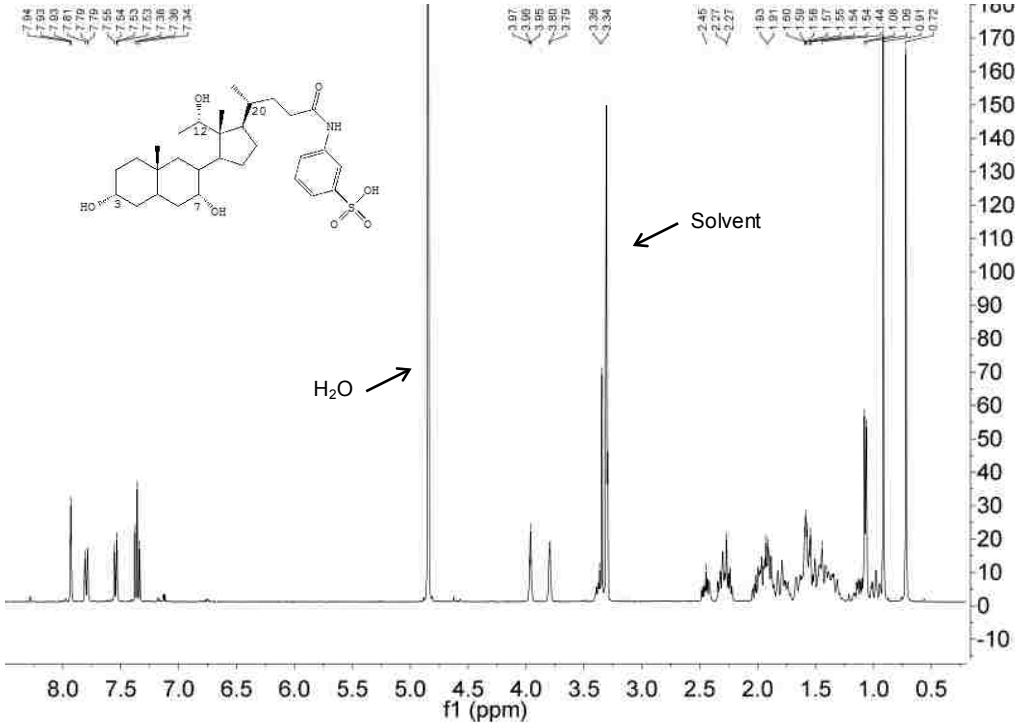


Figure 2.2. ^{1}H NMR spectrum of CamSA [T15]. Water and residual solvent peaks are from CD_3OD.

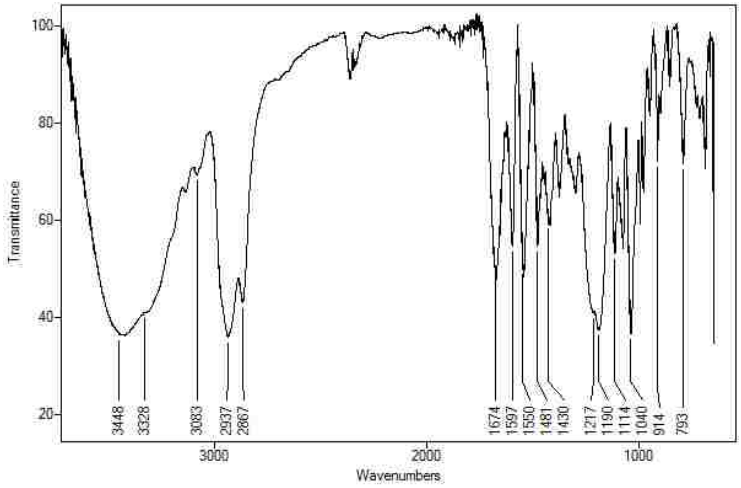
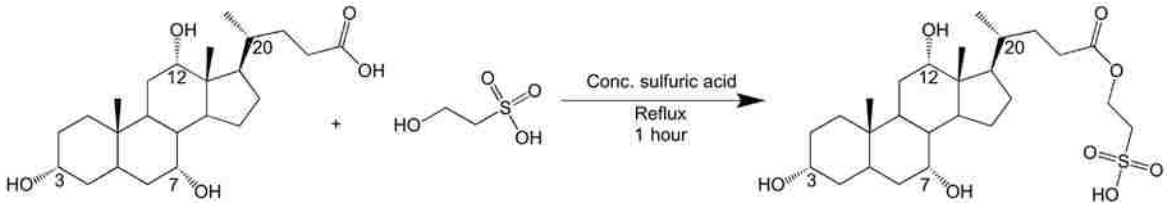


Figure 2.3. FT-IR spectra of CamSA [T15].

2.2.4. Synthesis of CaHESA [T22]

Conjugation of cholate to the sulfonic acid alkyl linker by an ester was prepared following established protocols for Fischer esterification (159-161). To a solution of cholate (1 eq. - 1mM) and hydroxyl ethane sulfonic acid (4 eq.) concentrated sulfuric acid was added dropwise and refluxed for 1 h (Scheme 2.3). The reaction mixture was poured into cold diethyl ether, and a precipitate formed immediately. The diethyl ether suspension was left overnight at 4°C. The precipitate was filtered, dissolved in 0.2 N NaOH-MeOH, precipitated a second time in diethyl ether and kept at 4°C for at least 2 h. The crude precipitate was filtered and purified by silica gel column chromatography eluted by step gradient from 100% DCM to 30% DCM/EtOH. The final yield was 20%. The compound structure was verified and determined > 95% pure by ^{1}H-NMR, FTIR, and mass spectrometry.



Scheme 2.3. Synthesis of taurocholate analog, compound T22.

2.2.5. Bacterial Strains and Spore Preparation

Clostridium difficile strain 630 was obtained from American Tissue Culture Collection (ATCC) (BAA-1382). *C. difficile* strain 630 is an epidemic strain clinically isolated from a patient with pseudomembranous colitis in Zurich, Switzerland in the early 1980s and is highly virulent and transmissible (122). *C.*

difficile cells were plated on BHIS (brain heart infusion salt) agar supplemented with 1% yeast extract, 0.1% L-cysteine-HCl and 0.05% sodium taurocholate to yield single-cell colonies. Single *C. difficile* colonies were grown in BHIS broth until exponential phase (approximately four hours) and spread plated onto agar to obtain bacterial lawns. The plates were incubated for five days at 37 °C in an anaerobic environment (5% CO_2, 10% H_2, and 80% N_2). The resulting bacterial lawns were collected by flooding the plates with ice-cold deionized water. Spores were pelleted by centrifugation at 8,800 x g for five minutes and resuspended in fresh deionized water. After two washing steps the spores were separated from vegetative and partially sporulated forms by centrifugation through a 20% to 50% HistoDenz gradient at 18,200 x g for 30 minutes with no brake. The spore pellet was washed five times with water, resuspended in sodium thioglycolate (0.5 g/L) and stored at 4 °C.

Immediately before *in vitro* germination and inhibition assays, spores were transferred to fresh tubes, centrifuged and washed with deionized water five times to remove storage buffer. Purified spores were heat activated at 68 °C for 30 minutes and washed another five times with water. Spores were diluted in germination buffer (100 mM sodium phosphate buffer at pH 6.0 containing 5 mg/ml sodium bicarbonate) to an optical density at 580 nm (OD_{580}) of 1.0. The spore strongly refracts light at 580 nm. With the loss of the spore coat and hydration of the spore core (during germination), there is concomitant loss of refractility, resulting in a decrease in optical density (162, 163).

2.2.6. Endospore Staining Method

To determine spore purity, a sample was removed and stained using the Shaeffer-Fulton staining method (122, 164). The Schaeffer-Fulton method of endospore staining consists of two stains; a primary stain (malachite green) and a counter stain (safranin). Slides smeared with spore/cell sample are heat fixed. Then slides are flooded with malachite green and heated over an open flame for five minutes. The slide is rinsed and a generous amount of safranin is added and kept at room temperature (RT) for 1 minute before rinsing. The slide is then viewed by light microscopy. The malachite green stain irreversibly binds to the spore coat resulting in visualized green spores whereas the malachite green is washed from vegetative cell walls. Safranin, a red stain, is then used to stain the vegetative cells resulting in a contrast of green spores and red vegetative cells (Fig. 2.4). Spore preparations were generally >95% pure after HistoDenz gradient.

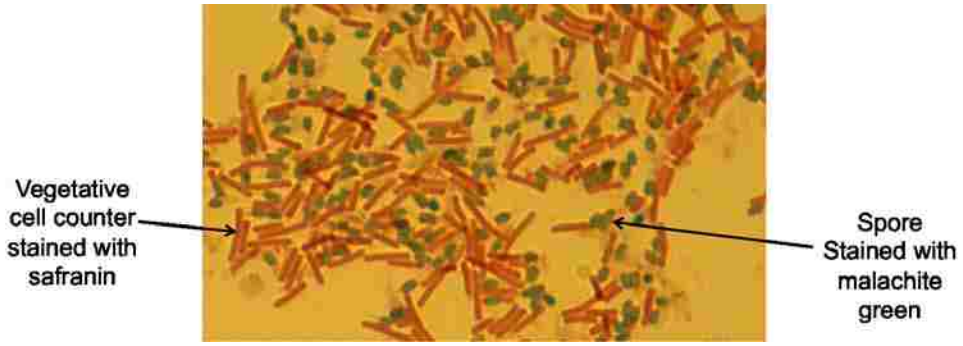


Figure 2.4.*C. difficile* stained using the Schaeffer-Fulton endospore staining method.

2.2.7. Activation of *C. difficile* Spore Germination

To test for taurocholate activators of spore germination, spore suspensions at OD_{580} of 1.0 were individually supplemented with 6 mM of a taurocholate analog and 12 mM glycine (127). To test for amino acid activators of

spore germination, spore suspensions were supplemented with 12 mM of an amino acid analog and 6 mM taurocholate. As control, spores were treated with taurocholate at 6 mM and glycine at 12 mM. Spore germination was evaluated based on the decrease in OD_{580}. The OD_{580} was measured each minute for 90 min at 30 °C and was repeated in triplicate. A 90% decrease in optical density was determined for spores treated with taurocholate and glycine (control). This was considered maximum germination and was set at 100%. The time point for maximum germination was set as a reference point to compare analogs. The percent germination at this reference time point for all analogs was calculated and compared to spores germinated with taurocholate and glycine. As expected, a decrease in optical density similar to taurocholate/glycine was observed in the presence of active germinants (Fig. 2.5A). The resulting data were fitted using the four-parameter logistic function of SigmaPlot v.9 to obtain the half maximal effective concentration (EC_{50}) (165, 166). The EC_{50} value is a relative number used to compare analogs.

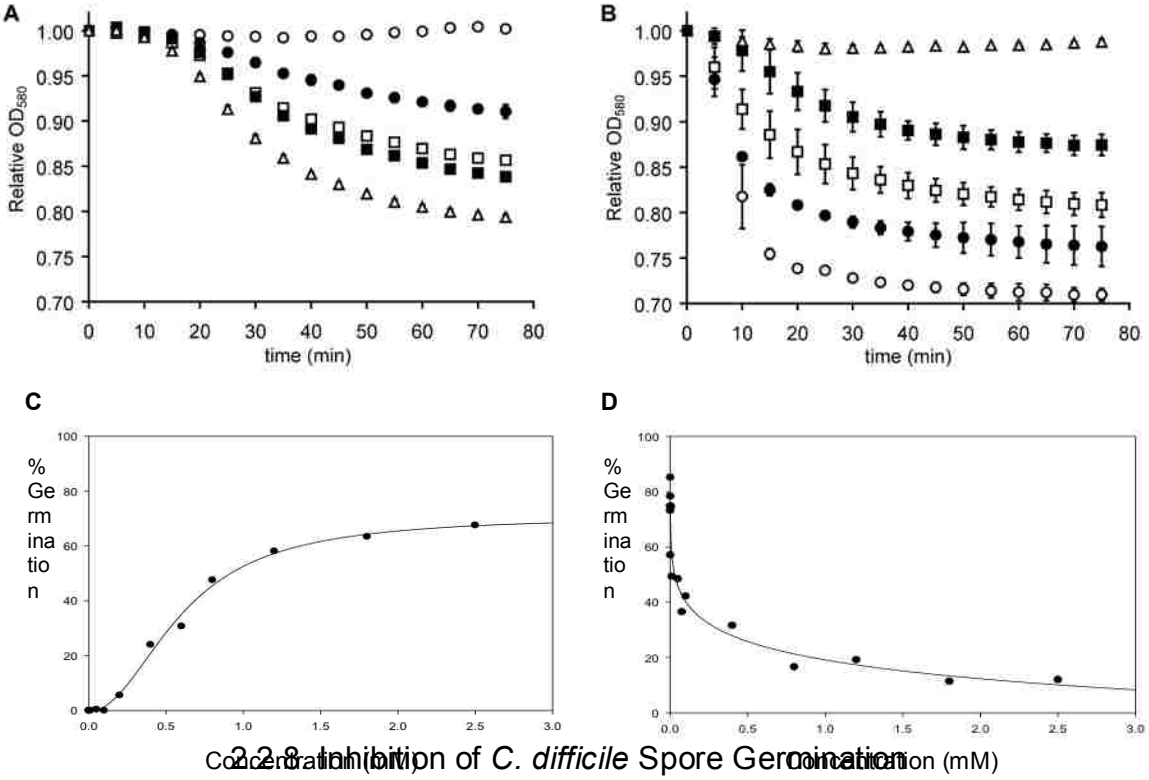


Figure 2.5 Germination of spores by taurocholate and glycine and inhibition of germination with *C. difficile* spores. A) Activation of germination. *C. difficile* spores were treated with a fixed concentration of taurocholate (6 mM) and glycine was added at 0 mM (o), 8 mM (●), 10 mM (□), 12 mM (■), and 14 mM (△) final concentrations. For clarity, the data are shown at five minute intervals and for only five glycine concentrations. B) Inhibition of germination. *C. difficile* spores were incubated with 0 mM (o), 0.0005 mM (●), 0.001 mM (□), 0.075 mM (■), and 7.5 mM (△) concentrations of CamSA [T15] and supplemented with taurocholate (6 mM) and glycine (12 mM). For clarity, the data are shown at five minute intervals and for only five CamSA [T15] concentrations. Although data were collected for 90 minutes, only 75 minutes are shown in both graphs for clarity. The error bars indicate standard deviations, n = 3. C) Calculation of EC_{50} for compound T11. D) Calculation of IC_{50} for compound T15, CamSA. For both C and D graphs the percent germination was determined as described and plotted versus concentration. The data was fitted using a four-parameter logistic function to obtain values for EC_{50} and IC_{50}.

2.2.8. Inhibition of *C. difficile* Spore Germination

To test for inhibitors of spore germination, spore aliquots of OD_{580} of 1.0 were individually supplemented with various concentrations of a taurocholate analog or an amino acid analog. Spore suspensions were incubated for 15 min at room temperature while the OD was monitored. If no germination was detected, taurocholate and glycine were added to 6 and 12 mM final concentrations, respectively. Relative OD_{580} values were obtained every minute for 90 min after germinant addition. As before, a 90% decrease in OD_{580} was determined for spores treated with taurocholate and glycine only and set as a reference point for comparisons. As expected, there was less change in OD_{580} over time in the presence of active germination inhibitors at increasing concentration (Fig. 2.5B). The resulting data were fitted using the four-parameter logistic function of SigmaPlot v.9 to obtain the half maximal inhibitory

concentration (IC_{50}) values (165, 166). The IC_{50} value is a relative number used to compare analogs.

2.2.9. *C. difficile* Spore Germination in BHIS Medium

To test for germination in complex media, spores were resuspended in BHIS alone and with combinations of taurocholate, chenodeoxycholate, glycocholate, glycine, L-arginine, and L-phenylalanine. Bile salts were added to a final concentration of 6 mM and amino acids were added at a final concentration of 12 mM. Relative OD_{580} values were obtained every minute for 90 min after germinant addition.

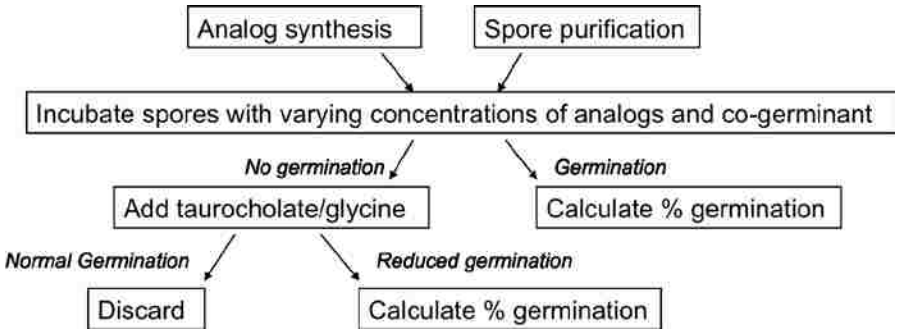


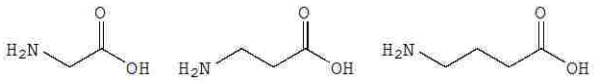
Figure 2.6. Flow chart used to identify germinants or inhibitors of *C. difficile* spore germination.

2.3. Results and Discussion

2.3.1. Effects of Amino Acids and Analogs with *C. difficile* Spores

Glycine [A01] has a methylene bridge that separates the carboxylic and amino groups and is the simplest of the 20 common amino acids (Fig. 2.7). To find determinants required for glycine recognition, taurocholate-treated spores were individually supplemented with 30 different glycine analogs. Each of the glycine analogs differs from the parent compound by a single modification in

either the length of the alkyl chain, substitutions to the amino group, changes in the carboxylate group or changes in the side chain. β-Alanine [A02] and γ-aminobutyric acid [A03] have an ethylene and a propylene bridge between the amino and carboxylate group, respectively (Fig. 2.7). These changes progressively increase the distance between the amino and carboxylate groups. β-Alanine and γ-aminobutyric acid are effective as glycine as co-germinants of *C. difficile* spores (Fig. 2.13). Thus, lengthening the chain between the amino and carboxylate functional groups does not interfere with recognition by the putative glycine germination binding site(s).



A01 A02 A03

Figure 2.7. Amino acids and alkyl chain length. Glycine (A01) and analogs A02 – A03.

Aminomethylphosphonic acid [A04] is a glycine analog in which the carboxylate has been changed to a phosphonic acid (Fig. 2.8). This substitution exchanges a carbon atom for phosphorus while retaining the negative charge. A04 significantly decreased *C. difficile* spore germination (Fig. 2.13). Furthermore, methylation of the carboxylate in glycine methyl ester [A05] (Fig. 2.8) resulted in less than 10% germination compared to glycine-triggered germination (Fig. 2.13). A05 was previously shown to be unable to trigger germination in *C. difficile* spores and this finding supports that claim (127). Any other modification of the carboxylate (glycine ethyl ester [A06], glycinamide [A07], and glycine hydroxamate [A08]) (Fig. 2.8) resulted in compounds that were

unable to activate or inhibit *C. difficile* spore germination. The sum of these data suggests that there is a specific requirement for a carboxylate functional group for recognition by the glycine germination binding site(s) to activate germination.

The distance between the amino and carboxylate of diglycine [A09] is similar to that of γ-aminobutyric acid [A03] (Fig. 2.8). However, whereas γ-aminobutyric acid is a good activator of *C. difficile* spore germination, diglycine has no effect. Thus, the addition of an internal amide must interfere with compound binding. Possibly there is a requirement for a more hydrophobic linker between the two functional-group ends. Glycine anhydride [A10] is the result of the dehydration of diglycine, forming a cyclic diamide (Fig. 2.8). Without a free amine or carboxylate, this compound is unable to activate or inhibit *C. difficile* spore germination. The rigidity and bulkiness of the analog may prevent interaction with the glycine binding site.

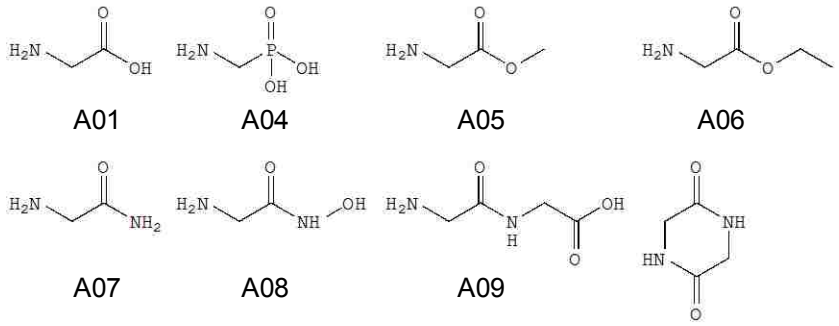


Figure 2.8. Amino acids with modifications to carboxyl group. Glycine (A01) and analogs A04 - A10.

Alkylation (sarcosine [A11], *N,N*-dimethylglycine [A12], and betaine [A13]), acetylation (*N*-acetylglycine [A14]), or other modification (nitrilotriacetic acid [A15]) to the amino group of glycine resulted in compounds that can neither activate nor inhibit *C. difficile* spore germination (Fig. 2.9). This suggests that activation of *C. difficile* spores by glycine has a requirement for a free primary amino group regardless of the presence of an unmodified carboxylate group.

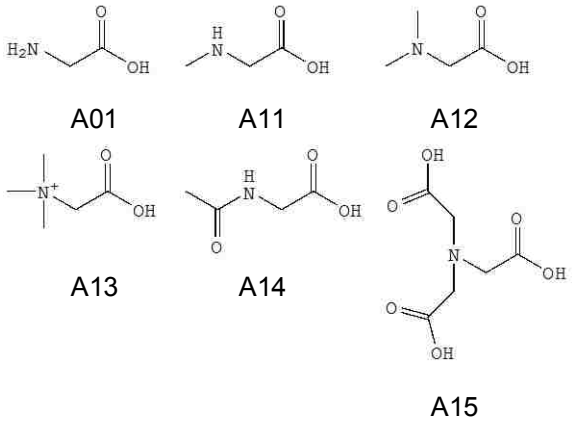


Figure 2.9. Amino acids with modifications to the amino group. Glycine (A01) and analogs A11-A15.

To test the effect of side chain substitution in amino acid recognition by the *C. difficile* germination binding site(s), we exposed taurocholate-treated *C. difficile* spores to other amino acid analogs (Fig. 2.10). L-Alanine [A16] has been shown to act as a germinant and/or co-germinant in other sporulating bacteria (133, 167). The stereoisomer, D-alanine [A17], has been shown to inhibit alanine-mediated germination in *Bacillus* (168). In *C. difficile*, L-alanine was as efficient at triggering germination as glycine [A01] (Fig. 2.13). Interestingly, D-alanine was unable to inhibit the germination of spores treated with L-alanine and taurocholate. D-Alanine was also inactive as an activator of *C. difficile* spore

germination. This implies that stereochemistry is important for recognition and binding of amino acids.

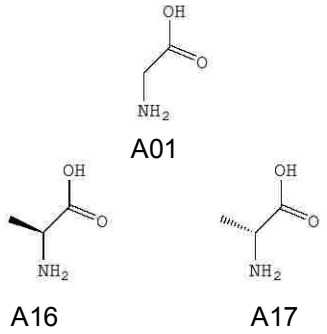


Figure 2.10. Alanine amino acids. Glycine (A01), L-alanine (A16) and D-alanine (A17).

To determine whether amino acid analogs with longer linear alkyl side chains were able to activate *C. difficile* spore germination, taurocholate-treated spores were exposed to L-2-aminobutyric acid [A18] and L-norvaline [A19] (Fig. 2.11). Both of these amino acid analogs were able to activate germination to levels similar to those with glycine [A01] (Fig. 2.13). L-valine [A20] is a branched isomer of L-norvaline and has similar chemical and physical properties (Fig. 2.11). However, this slight difference in structure reduced *C. difficile* spore germination by more than 90% compared to that of L-norvaline. Similarly, L-isoleucine [A21] and L-leucine [A22] are poor germinants of *C. difficile* spores compared to L-norvaline (Fig. 2.13). The data suggest that branched alkyl side chains are unable to be recognized by the putative amino acid germination binding site(s) in *C. difficile.*

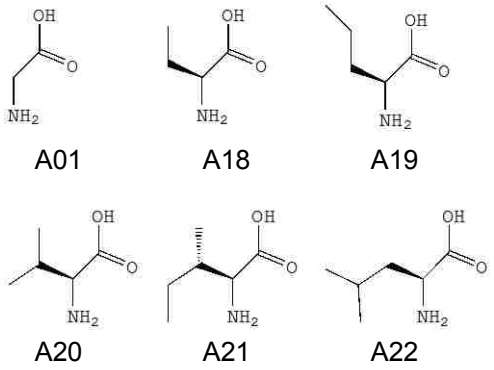


Figure 2.11. Amino acid with linear alkyl side chains. Glycine (A01) and analogs A18 - A22.

L-Cysteine [A23] is an amino acid with a methanethiol side chain (Fig. 2.12). L-Serine [A24], on the other hand, is an L-cysteine analog in which the thiol group is substituted for a hydroxyl group. Interestingly, whereas L-cysteine is a good germinant of *C. difficile* spores, the more polar L-serine is almost inactive (Fig. 2.13). The germination activity of L-cysteine is not due solely to hydrophobicity, since the more hydrophobic L-methionine [A25] is a poor germinant compared to L-cysteine (Fig. 2.13). This suggests that *C. difficile* recognize the thiol group specifically as a determinant for germination.

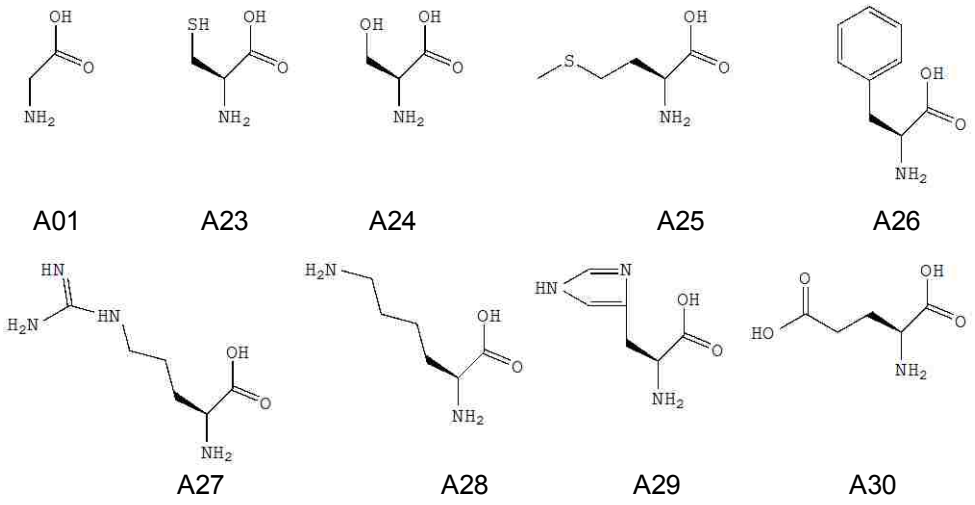


Figure 2.12. Amino acids with modified alkyl side chains. Glycine (A01) and amino acids A23 - A30.

Surprisingly, L-phenylalanine [A26], with a bulky aromatic side chain, is as effective as glycine as a germinant of *C. difficile* spores (Fig. 2.12 and 2.13). A reasonable expectation is that since branched amino acids are inactive, the bulkier side chain of L-phenylalanine would also be restricted from the binding site. The possibility that the putative glycine binding site(s) is able to accommodate phenyl but not branched alkyl side chains cannot be completely ruled out. However, that possibility is unlikely due to their sizes relative to glycine. Hence, we postulate that aromatic amino acids are recognized by a separate binding site in *C. difficile* spores. Indeed, other *Bacillus* and *Clostridium* strains are able to recognize structurally different germinants by encoding multiple binding site(s) (125, 126, 136, 151).

L-Arginine [A27] is also a strong co-germinant for *C. difficile* spores. Although L-arginine has a linear alkyl chain, it also contains a positively charged, branched guanidinium group (Fig. 2.12). Similarly, L-lysine [A28] is linear with a

positively charged amino aside chain, yet L-lysine is a weak germinant compared to L-arginine (Fig. 2.13). L-Histidine [A29] contains an aromatic side chain, like L-phenylalanine [A26], but it is positively charged (at physiological pH), like the L-arginine side chain (Fig. 2.12). However, unlike L-phenylalanine or L-arginine, L-histidine could not efficiently activate *C. difficile* spore germination. L-Aspartic acid [A30] has a short acidic side chain and was similarly unable to affect *C. difficile* spore germination. Since L-arginine has physicochemical properties that are very different from those of the other amino acids that are able to activate *C. difficile* spore germination, it suggests there is a specific recognition site for L-arginine binding.

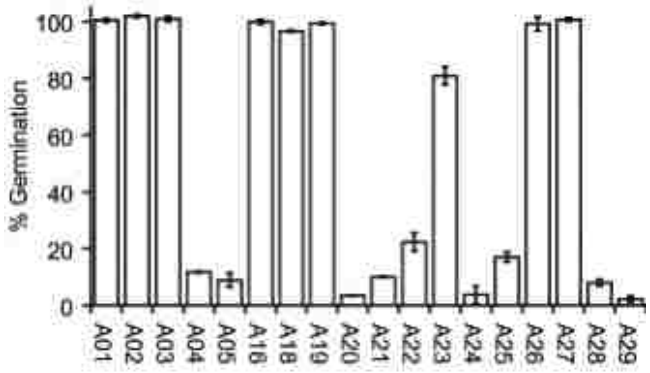


Figure 2.13. Comparison of amino acids as activators of *C. difficile* spore germination. Spores were treated with taurocholate (6 mM) and amino acid analogs at 12 mM. Germination was determined by the decrease in the OD_{580} for 90 min at 30 °C. The percent germination for each analog was calculated based on glycine/taurocholate germination set as 100%. The error bars indicate standard deviations, n = 3. Significant difference ($p < 0.01$) observed between glycine and each analog showing less than 85% germination (A04, A05, A20, A21, A23, A24, A25, A28, and A29).

All glycine analogs and amino acids were further tested for the ability to inhibit *C. difficile* spore germination. Spores were treated with each analog or amino acid in the presence of taurocholate and glycine. However, no individual amino acid analog inhibited *C. difficile* spore germination.

In conclusion, multiple amino acid analogs are able to activate germination and the data suggests that there may be separate binding sites for phenylalanine and arginine. Furthermore, no amino acids were able to inhibit *C. difficile* spore germination.

2.3.2. Effects of Amino Acid Combinations on *C. difficile* Spore Germination

Earlier studies (140) reported that only glycine was able to trigger *C. difficile* spore germination in the presence of taurocholate. This study reportedly used three combinations of defined media to narrow down active germination stimulators. Although glycine was supplemented with only seven amino acids, phenylalanine and arginine were in a mixture supplemented with 15 other amino acids. The presence of multiple weak germinants in the defined media containing phenylalanine and arginine could mask their ability to stimulate germination. Furthermore, in those experiments, L-phenylalanine and L-arginine were supplemented at concentrations (1.21 mM and 1.15 mM, respectively) lower than those used for the current experiments (12 mM) (140).

Glycine, L-arginine, and L-phenylalanine individually or in pairs do not trigger *C. difficile* spore germination in the absence of taurocholate. However, a cocktail of L-phenylalanine, L-arginine, and glycine (all at 12 mM) was able to effectively trigger *C. difficile* spore germination in the absence of taurocholate. Chenodeoxycholate (6 mM) is not able to inhibit the germination of *C. difficile* spores treated with amino acids only. Other Clostridia have been shown to use amino acids alone as germination signals (136).

In conclusion, the data indicate that *C. difficile* spores can be triggered to germinate in the presence of glycine, phenylalanine and arginine. This amino acid cocktail does not require taurocholate for activation nor is it inhibited by chenodeoxycholate.

2.3.3. Effects of BHIS Medium on *C. difficile* Spore Germination

When *C. difficile* spores are resuspended in BHIS medium, germination is very slow, even though BHIS contains a complex amino acid mixture that includes glycine, L-arginine, and L-phenylalanine (Fig. 2.14). It is possible that BHIS contains amino acids that are weak germinants and that compete with glycine, L-phenylalanine, and L-arginine for binding. Binding of these alternative substrates causes a fraction of the spore population to germinate. Similar behavior has been observed in the Abel-Santos lab in the germination of *C. sordellii* spores (136). Interestingly, supplementing BHIS with taurocholate [T01] augmented *C. difficile* spore germination (Fig. 2.14). The taurocholate-enhanced spore germination in BHIS was inhibited by chenodeoxycholate. Thus, in conclusion, amino-acid-only *C. difficile* spore germination occurs *in vitro* only when a limited number of amino acids are present and is disfavored with complex amino acid mixtures.

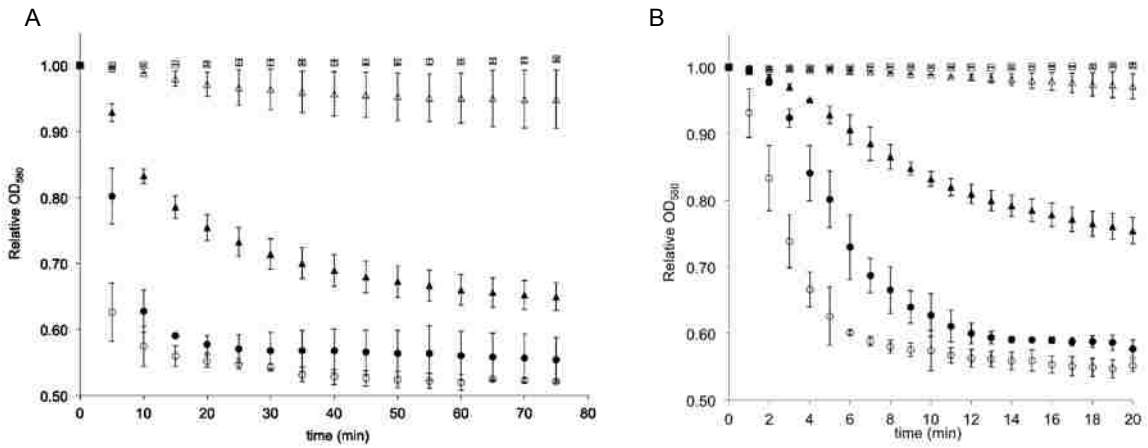


Figure 2.14. Germination kinetic graph showing behavior of *C. difficile* spores and germinants in buffer and complex media. A) *C. difficile* spores were suspended in germination buffer and treated with L-Phe, L-Arg, and Gly (each at 12 mM) (o) or L-Phe, L-Arg, Gly (each at 12 mM) and chenodeoxycholate (6 mM) (●). Purified spores were also suspended in BHIS medium (□), BHIS supplemented with 12 mM taurocholate (▲), BHIS supplemented with 12 mM taurocholate and 12 mM chenodeoxycholate (△). For clarity, data are shown at 5-min intervals and only for 75 min. B) Same as A except all time points are shown up to 20 minutes. For both graphs, the error bars indicate standard deviations, n = 3.

2.3.4. Effect of Taurocholate Hydroxyl Groups on *C. difficile* Spore Germination and Inhibition

Taurocholate [T01] is a natural bile salt that has hydroxyl groups at positions 3, 7 and 12 of the cholate backbone (Fig. 2.15). All three hydroxyl groups are in the alpha configuration. Taurocholate also has a side chain consisting of taurine attached to the cholate backbone by an amide bond. Taurocholate activates *C. difficile* spore germination with a 50% effective concentration (EC_{50}) of 15.9 mM (127). Taurocholate analogs were tested for the ability to activate germination in the presence of glycine and for the ability to inhibit germination in the presence of taurocholate and glycine.

To understand the importance of hydroxyl groups on the cholate backbone of taurocholate, analogs T02 to T08 were tested as activatorss of *C. difficile* spore germination. These analogs differ from taurocholate [T01] in the number, placement, or stereochemistry of the hydroxyl groups (Fig. 2.15). Taurodeoxycholate [T02] lacks only the hydroxyl group at the 7 position. This change was sufficient to reduce germination by more than 70% (Table 2.1).

Meanwhile, taurochenodeoxycholate [T03] is only missing the hydroxyl at position 12 and was able to induce germination only to 10% of the level of taurocholate. Tauroursodeoxycholate [T04] is an isomer of taurochenodeoxycholate in which the 7-hydroxyl is in the beta configuration. Alteration of the stereochemistry of this one hydroxyl group further decreased germination activity from 10% for taurochenodeoxycholate to 3% for tauroursodeoxycholate (Table 2.1).

As expected, taurolithocholate [T05] and taurocholanate [T06], which lack hydroxyls at positions 7 and 12 and 3,7 and 12, respectively are unable to activate germination (Fig. 2.15). Taurohyocholate [T07] and taurohyodeoxycholate [T08] are isomers of taurocholate [T01] and taurodeoxycholate [T02], respectively, in which the 12-hydroxyl groups are moved to the 6 position (Fig. 2.15). Neither of these compounds is able to significantly activate germination of *C. difficile* spores (Table 2.1). The sum of these data suggests that both the 7 and 12 α-hydroxyls of taurocholate are important determinants for binding and activation of *C. difficile* spores.

The 3-hydroxyl position of the cholate molecule is more nucleophilic than the other two hydroxyls. Similarly, the 7-hydroxyl is more reactive than the 12-hydroxyl (155, 156). Thus, methylation of taurocholate yielded two compounds that we putatively identified as 3-methoxy-7,12-dihydroxytaurocholate [T09] and 3,7-dimethoxy-12-hydroxytaurocholate [T10] (Fig. 2.15). Interestingly, 3-methoxy-7,12-dihydroxytaurocholate neither induces nor inhibits *C. difficile* spore germination (Table 2.1). As expected, 3,7-dimethoxy-12-hydroxytaurocholate

was also inactive. This suggests that the ability to donate a 3-hydroxyl hydrogen bond is essential for recognition of taurocholate as a germinant for *C. difficile* spores.

To determine if analogs differing in the number, location, or stereochemistry of the hydroxyl groups can inhibit taurocholate-mediated germination, *C. difficile* spores were treated with taurocholate [T01], glycine [A01], and compounds T02 to T10 (Fig. 2.15). Only taurochenodeoxycholate [T03], tauroursodeoxycholate [T04] and taurohyodeoxycholate [T08] showed germination-inhibitory properties (Table 2.1). All three inhibitors have the common feature of lacking the 12-hydroxyl group. Since the 12-hydroxyl group was necessary for triggering spore germination, these results suggest that this hydroxyl is necessary for activation of germination but not for binding of taurocholate to the putative *C. difficile* germination binding site(s). The inhibitory compounds also have hydroxyl groups at either the 6 or 7 position (but not both), indicating that having one (but not two) hydroxyls in the B ring is important for inhibition of taurocholate-mediated germination of *C. difficile* spores.

In conclusion, bile salt analogs with hydroxyl groups at positions 3 and 12 promoted *C. difficile* spore germination activation, whereas the hydroxyl group at position 7 is optimal for binding.

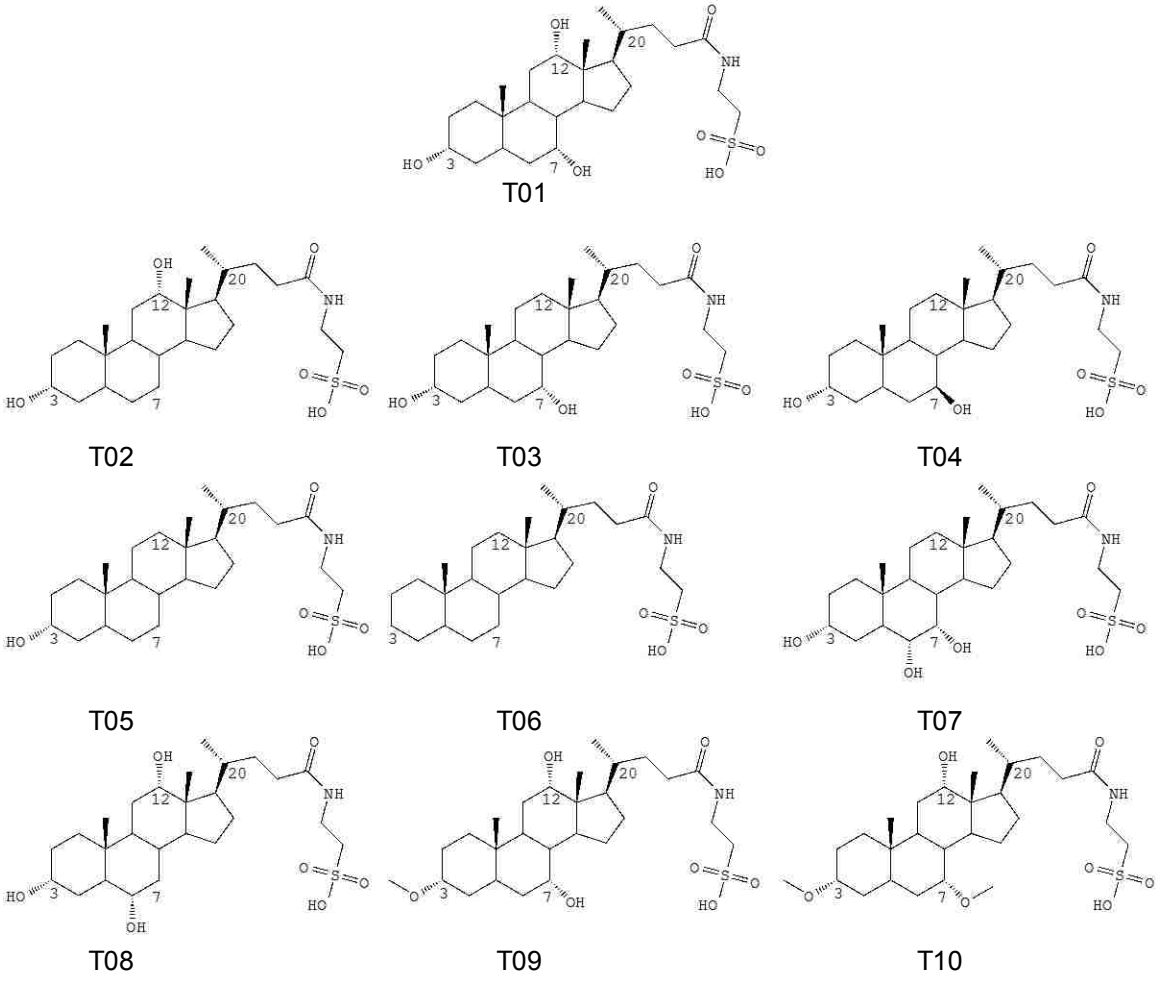
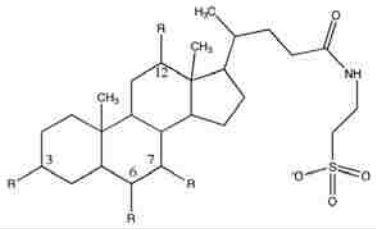


Figure 2.15. Taurocholate analogs with hydroxyl group modifications. Taurocholate (T01) and taurocholate analogs T02 - T10.

Table 2.1. Effect of taurocholate hydroxyl groups on *C. difficile* spore germination.



No.	Group at position: 3	6	7	12	% Germination[a]	IC_{50} (mM)[b]
T01	OH	H	OH	OH	97.9 (1.9)	NA
T02	OH	H	H	OH	24.3 (1.8)	NA
T03	OH	H	OH	H	9.6 (0.4)	0.51 (0.06)
T04	OH	H	β-OH	H	3.2 (1.7)	0.32 (0.05)
T05	OH	H	H	H	2.3 (0.9)	NA
T06	H	H	H	H	NA[c]	NA
T07	OH	OH	OH	H	1.3 (0.5)	NA
T08	OH	OH	H	H	2.3 (2.4)	0.41 (0.20)
T09	OCH_3	H	OH	OH	NA	NA
T10	OCH_3	H	OCH_3	OH	NA	NA

[a] *C. difficile* spores were individually treated with 6 mM glycine and 12 mM taurocholate analogs T01 to T10. The percent germination was calculated based on taurocholate/glycine germination set as 100%. Standard deviations are shown in parentheses.

[b] *C. difficile* spores were incubated with various concentrations of taurocholate analogs for 15 min prior to the addition of 6 mM taurocholate and 12 mM glycine. The IC_{50} was calculated by plotting the extent of germination versus the logarithm of analog T02 to T10 concentrations. Standard deviations are shown in parentheses.

[c] NA, no change in absorbance after 90 minutes under the conditions tested thus no statistics could be performed.

2.3.5. Effect of Taurocholate Side Chain on *C. difficile* Spore Germination and Inhibition

To determine the taurine side chain functional groups responsible for recognition by the *C. difficile* germination binding site(s), analogs T11 to T22 were tested for the ability to induce spore germination in the presence of glycine (Fig. 2.16). All of these compounds differ from taurocholate in the structure of the side chain and retain the cholate backbone with hydroxyl groups at the 3, 7, and 12 positions.

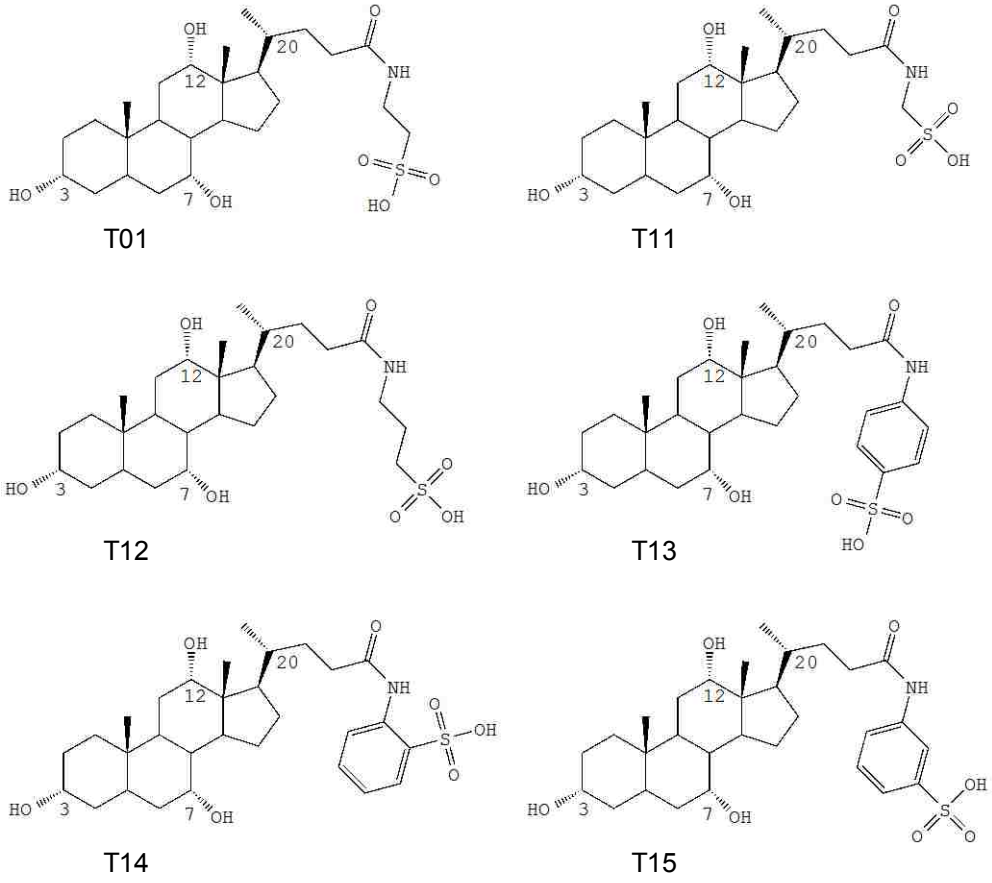


Figure 2.16. Taurocholate analogs with modified side chains. Taurocholate (T01) and analogs T11 - T15.

Compound T11 is a taurocholate analog in which the alkyl linker between the sulfonate and the amide was shortened by one methylene (Fig. 2.16). Compound T12, on the other hand, is a taurocholate analog in which the linker was lengthened by one methylene. The results show that spores treated with compound T11 germinate to levels comparable with those spores treated with taurocholate [T01] (Table 2.2). In contrast, the longer alkyl chain, compound T12, does not activate germination. To further analyze the necessity for the alkyl linker in germination, three analogs containing a benzene ring in place of the ethylene

linker of taurine were synthesized. These analogs differed in the position of the sulfonate, *para* [T13], *ortho* [T14], or *meta* (CamSA) [T15], with respect to the amino group in the benzene ring (Fig. 2.16). These three taurocholate analogs were unable to activate *C. difficile* spore germination (Table 2.2). This suggests that *C. difficile* spores are activated by taurocholate analogs with shorter, but not longer or bulkier, linkers.

Hypotaurocholate [T16] differs from taurocholate by a substitution of a sulfonate for a sulfinate (Fig. 2.17). This analog was unable to activate *C. difficile* spore germination (Table 2.2). Interestingly, glycocholate [T17] is a compound T11 analog in which a sulfonate has been substituted for a carboxylate (Fig. 2.17). In our hands, glycocholate like compound T11, is able to significantly activate *C. difficile* spore germination in buffer (Table 2.2). A previous report had determined that glycocholate is not a germinant for *C. difficile* spores in BHIS medium (140). Indeed, when we tested glycocholate in BHIS medium, the *C. difficile* spore percent germination dropped almost 10-fold. However, addition of glycine to BHIS medium partially restored glycocholate-mediated germination. Hence, compound mixtures in BHIS medium seem to intrinsically inhibit *C. difficile* spore germination.

Compound T18 is a carboxylated analog of taurocholate [T01] (Fig. 2.17). Interestingly, whereas taurocholate, compound T11, and glycocholate [T17] are able to activate *C. difficile* spore germination, compound T18 is inactive. Similarly, compound T19, compound T20, and compound T21, with longer side chains, are also inactive (Table 2.2). These results suggest that carboxylate is

able to partially substitute for sulfonate, but is not optimal for activation of *C. difficile* germination.

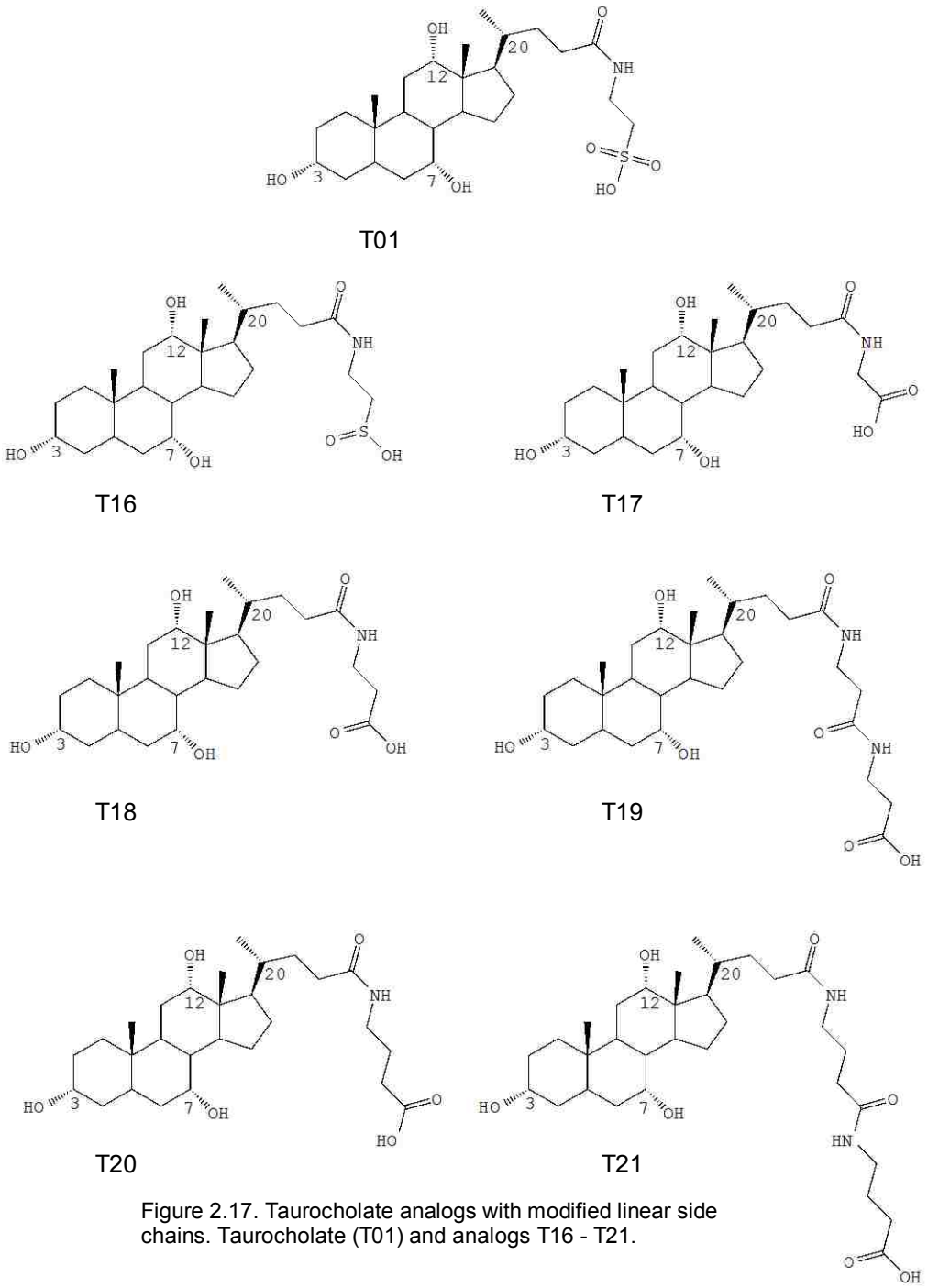


Figure 2.17. Taurocholate analogs with modified linear side chains. Taurocholate (T01) and analogs T16 - T21.

Compound T22 is a taurocholate analog in which an amide group is substituted for an ester (Fig. 2.18). Compound T22 was unable to trigger germination in *C. difficile* spores (Table 2.2). This suggests that the ability of the amide group to form hydrogen bonds is necessary for *C. difficile* spore germination.

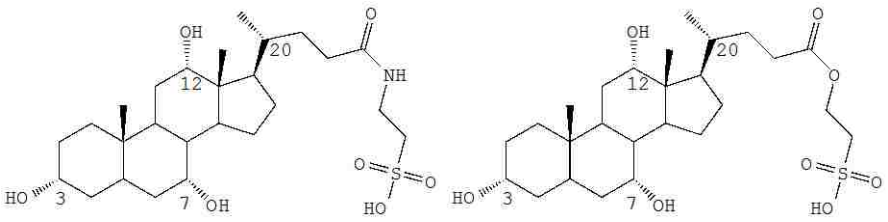


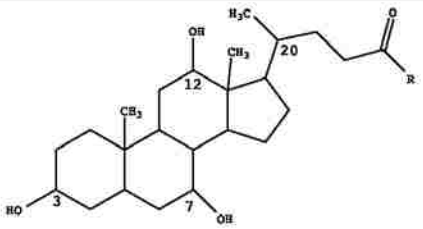
Figure 2.18. Taurocholate analog with an ester side chain linkage. Taurocholate (T01) and analog T22.

To determine whether taurocholate analogs with modified side chains [T11 to T22] can inhibit *C. difficile* spore germination, the effects of these analogs on *C. difficile* spores treated with taurocholate [T01] and glycine [A01] were analyzed. Taurocholate analogs with one less carbon [T11] or one more carbon [T12] in the taurine side chain were unable to inhibit germination. Interestingly, while the addition of a benzene ring in the linker with the sulfonate in the *para* [T13] or the *ortho* [T14] position does not inhibit spore germination. CAmSA [T15] has an IC_{50} of 58.3 μM is the strongest inhibitor of *C. difficile* spore germination reported so far (Table 2.2). The conversion of the sulfonate functional group to a sulfinate [T16] resulted in a compound with slight inhibitory activity at an IC_{50} of 640 μM (Table 2.2). Interestingly, compounds with longer alkyl linkers followed by

a carboxylate [T20 and T21] are able to inhibit *C. difficile* spore germination, whereas shorter carboxylate end groups [T17 to T19] are inactive. Compound T20 has an IC_{50} of 762 μM, whereas the longer side chain, Compound T21, containing two amide groups has an IC_{50} of approximately 3,000 μM (Table 2.2). Replacement of the amide group of taurocholate with an ester [T22] results in a compound with slight inhibitory activity (IC_{50} of 1,322 μM).

In conclusion, recognition and activation of *C. difficile* spore germination by compounds containing the taurine side chain is optimal however a shorter side chain is also capable of activating germination. Longer and bulkier side chains are not able to activate *C. difficile* spore germination, but several were able to inhibit germination *in vitro*. Furthermore, compound T15 is a potent inhibitor of *C. difficile* spore germination.

Table 2.2. Effect of taurocholate side chain (R) on *C. difficile* spore germination.



No.	R	% Germination[a]	IC_{50} (mM)[b]
T01	NH(CH2)SO3H	97.9 (1.9)	NA
T11	NHCH2SO3H	96.5 (2.1)	NA
T12	NH(CH2)3SO3H	NA[c]	NA
T13	NH(*p*-(C6H4))SO3H	NA	NA
T14	NH(*o*-(C6H4))SO3H	NA	NA
T15	NH(*m*-(C6H4))SO3H	NA	0.058 (0.035)
T16	NH(CH2)2SO2H	NA	0.64 (0.11)
T17	NHCH2CO2H	59.5 (4.0)	NA
T18	NH(CH2)2CO2H	NA	NA
T19	NH(CH2)2CONH(CH2)2CO2H	NA	NA
T20	NH(CH2)3CO2H	NA	0.76 (0.11)
T21	NH(CH2)3CONH(CH2)3CO2H	NA	2.9 (0.41)
T22	O(CH2)2SO3H	NA	1.3 (0.05)

[a] *C. difficile* spores were individually treated with 6 mM glycine and 12 mM taurocholate analogs T11 to T22. The percent germination was calculated based on taurocholate/glycine germination set as 100%. Standard deviations are shown in parentheses.

[b] *C. difficile* spores were incubated with various concentrations of taurocholate analogs for 15 min prior to the addition of 6 mM taurocholate and 12 mM glycine. The IC_{50} was calculated by plotting the extent of germination versus the logarithm of analog T11 to T22 concentrations. Standard deviations are shown in parentheses.

[c] NA, no change in absorbance after 90 minutes under the conditions tested thus no statistics could be performed.

2.4. Conclusions

In this study, taurocholate and glycine analogs were used to better understand how the *C. difficile* germination binding site(s) recognizes its germinants. Chemical probes can reveal the chemical, physical, and spatial requirements of the germination binding site(s). The present study has shown that the *C. difficile* germination binding site(s) recognizes a number of amino acid side chains and that the putative glycine binding site(s) requires both a free

carboxylate and a free amino group to recognize glycine, but the binding site is flexible enough to accommodate longer separations between the two functional groups. Linear alkyl amino acid side chains are recognized but their branched isomers are not. The Abel-Santos lab has observed and reported similar branched-chain restriction in the recognition of inosine analogs of *B. cereus* spores (169). These size and polarity restrictions also suggest the existence of separate binding sites for L-phenylalanine, L-arginine, and possibly L-cysteine.

It is reasonable to hypothesize that the binding region for L-alanine in *C. difficile* is divergent enough from the L-alanine binding site in *Bacillus* to impede the binding and inhibition by D-alanine. Because none of the amino acid analogs were able to compete with glycine to inhibit *C. difficile* spore germination, the functional groups in the amino acid moieties are needed for both binding and activation of the putative amino acid germination binding site(s).

Few bile salt analogs were able to activate *C. difficile* spore germination. Hydroxyl groups at positions 3 and 12 seem to be required for both binding and activation of *C. difficile* spore germination. In contrast, hydroxyl groups in the B ring appear to be important only for binding. Hence, the data imply that there is a requirement for hydrogen bonding with hydroxyls at specific locations and configurations in the *C. difficile* germination binding pocket.

Recognition of the taurine side chain seems to be even more restricted. Even small changes in the linker length and rigidity, amide bond, or oxidation state of the sulfonate group had a large effect on *C. difficile* spore germination. Although the sulfonate group is optimal for spore germination activation, it can be

partially substituted with a carboxylate as long as the alkyl chain is short. These data suggest that the binding site for taurocholate recognizes the taurine side chain for optimal germination.

In contrast to germinant specificity, the putative taurocholate binding site(s) was more flexible in regard to inhibitor binding. The *meta*-sulfonic benzene derivative CamSA [T15] is active at concentrations approximately 275-fold lower than taurocholate and is almost 4 times more active than the natural inhibitor chenodeoxycholate (127, 141). The benzene ring is a rigid functional group with little free rotation. We speculate that the sulfonate in the *meta* position is able to fit tightly into the sulfonate binding pocket but the overall binding site(s) does not recognize the benzene ring to trigger germination. This is further confirmed by the inactivity of the *ortho* and *para* isomers that would spatially place the sulfonate in different locations. The rigidity and positioning of the *m*-sulfonate probably provides an entropic advantage over alkyl sulfonates. It is possible that longer alkyl side chains are too flexible to allow the sulfonate moiety to bind efficiently to the putative taurocholate binding site(s). The discovery of CamSA and its strong inhibitor effect has revealed a new path to designing compounds for CDI prophylaxis.

The putative taurocholate and glycine binding site(s) in *C. difficile* recognizes multiple functional groups in their respective germinants. Hence, even subtle changes in the germinant structure can be detrimental to the binding ability of the germination binding site(s) of *C. difficile* spores. In conclusion, a structure activity relationship analysis of *C. difficile* spores and analogs of glycine and

taurocholate can reveal necessary moieties in the germinant structure. This analysis can also elucidate the physical and chemical spatial characteristics in the microenvironment of the binding sites.

CHAPTER 3

MAPPING INTERACTIONS BETWEEN INHIBITORS AND *CLOSTRIDIUM DIFFICILE* SPORES

3.1. Introduction

The microenvironments of the putative taurocholate and glycine binding sites in *C. difficile* spores limit the functional groups allowed to bind and/or activate spore germination. Besides physical requirements, chemical requirements are also essential for binding. For the *C. difficile* putative glycine binding site(s), both the amino and carboxylate group must be unmodified for interactions with the binding site(s). Interestingly, it seems there may be separate binding sites for the amino acids L-phenylalanine, L-arginine, and possibly L-cysteine. Of the 30 amino acids analyzed in structure activity analyses, none were able to inhibit *C. difficile* spore germination.

The *C. difficile* putative taurocholate germination binding site(s) can be efficiently activated by specific functional groups. However, the germination binding site(s) is able to bind to a broader range of compounds that are unable to activate germination. In fact the *meta*-benzene sulfonic acid derivative of taurocholate, CamSA [T15], is able to competitively inhibit taurocholate-mediated *C. difficile* spore germination at low concentrations (134). We suggest that the rigidity of the benzene ring in CamSA allows the compound to fit the sulfonic acid directly in its binding pocket. Because the benzene ring has different physiochemical properties than an alkyl chain, it may not be recognized to activate germination. This prediction is supported by the fact that the *C. difficile*

germination binding site(s) does not recognize the *para* and the *ortho* analogs of CamSA (134).

Based on the structure activity relationship defined for germinants and *C. difficile* spores, a new series of analogs were synthesized and assessed for ability to inhibit germination (134). Bile salt analogs that inhibited spore germination in the previous study were used as a basis for designing new analogs for testing. By manipulating bile salt structures that showed activity in the previous study, we hypothesized that a pattern would emerge that could be used to chemically define the bile salt binding region in *C. difficile* spores. Taurocholate analogs with long linear side chains containing a carboxylate moiety [T20 – T22] were found to inhibit *C. difficile* spore germination (Table 2.2). For this study, a series of linear side chains with longer alkyl groups and/or different functional groups were produced and analyzed for activation of *C. difficile* spore germination or inhibition of germination.

CamSA [T15] does not have a linear side chain but instead has a benzene ring with a sulfonic acid group in the *meta* position. CamSA is a potent inhibitor of *C. difficile* spore germination (134). This discovery was interesting since the side chain structure of CamSA is quite different from taurocholate, the germinant (Fig. 3.1A). Furthermore, the *para* and *ortho* analogs of CamSA had no effect on *C. difficile* spore germination. To better understand the interactions between CamSA and *C. difficile* spores, a series of CamSA analogs were synthesized and assessed for germinant and anti-germinant activity.

The natural bile salt, chenodeoxycholate, has been reported to act as a competitive inhibitor of taurocholate-mediated *C. difficile* spore germination (127, 141). Taurocholate is a conjugated natural bile salt that consists of cholate (cholic acid) linked to a taurine side chain by an amide bond (Fig. 3.1A). The structure of chenodeoxycholate is similar to taurocholate except that chenodeoxycholate is not linked to a taurine side chain (Fig. 3.1B). Taurocholate also has three hydroxyl groups on the cholate backbone and all are in the alpha configuration. Chenodeoxycholate has two hydroxyl groups at position 3 and position 7 also in the alpha configuration but not at position 12. To better understand how chenodeoxycholate interacts with *C. difficile* spores, cholate and chenodeoxycholate analogs were tested for activation and inhibition of spores *in vitro.* We hypothesized that the placement and configuration of the hydroxyl groups are essential for recognition. Furthermore, cholate and chenodeoxycholate are not linked to a side chain but each has a free carboxylic acid. A series of analogs with modifications to the carboxylic acid were also assessed for activity with *C. difficile* spores.

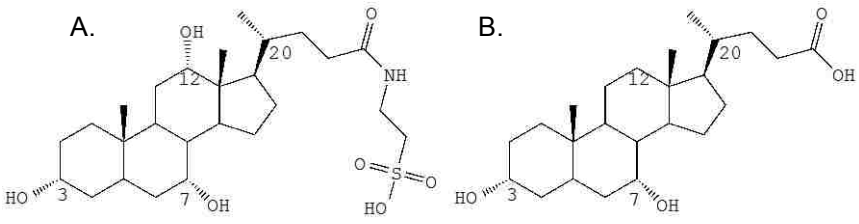


Figure 3.1. Natural bile salts. A) taurocholate, B) chenodeoxycholate

Taurochenodeoxycholate is an analog of taurocholate that is missing the hydroxyl group at position 12. As described in the previous study,

taurochenodeoxycholate [T03] is able to inhibit *C. difficile* spore germination but is not as potent as chenodeoxycholate. This data would suggest that the hydroxyl group at position 12 may not be essential for binding but may be necessary for activation of germination. Using chenodeoxycholate as a backbone, a variety of bile salt analogs with different alkyl linkers were synthesized. These taurochenodeoxycholate analogs were synthesized based on whether taurocholate analogs were found to have activity with *C. difficile in vitro*. For example the *meta*-benzene sulfonic side chain was found to have activity when linked to the cholate backbone, CamSA. So for this study, an analog of CamSA was prepared using the chenodeoxycholate backbone. These analogs were synthesized and tested to potentially discover more potent inhibitors of *C. difficile* spore germination *in vitro* than CamSA.

Cholesterol analogs are found in many different plants and vary in function. Some plant-derived cholesterol analogs are potent toxins that are fatal to those who ingest them and some are use medically (176, 177, 181). Others serve as growth hormones and are essential to plant metabolic activity making them useful agriculturally (172, 173). Epibrassinolide is in a class of steroid plant hormones called brassinosteroids originally isolated from *Brassica napus*, rapeseed (170). Epibrassinolide stimulates various physiological plant cell processes to bulk up foliage, is effective in fighting pathogens and prevents fungal infections in plants (171, 172). Ursolic acid, found in high concentrations in apple peels, has been found to inhibit muscular atrophy-associated gene expression in humans and in mice (173, 174). In mice, ursolic acid prevents

muscle weakening during fasting and also helps grow muscle in normal-diet mice (174). Since epibrassinolide has shown antimicrobial effects in plants and ursolic acid has positive effects in mammals and both are analogs of bile salts, they were analyzed for the ability to activate and/or inhibit *C. difficile* spore germination. They are not close analogs of taurocholate and chenodeoxycholate like the other bile salt analogs analyzed in this study. However, the focus of this project is to understand the microenvironment of the *C. difficile* germination binding site(s) and to discover potent inhibitors of *C. difficile* spore germination. By studying the effects of related analogs, we can determine if the compounds screened should be broadened to include other cholesterol derivatives.

Some steroids found in plants have important impacts on biological systems. Digoxigenin, ouabain, and digitoxin are three such steroids. Digitoxin is found in the foxglove plant from the genus *Digitalis* and has been used since 1785 to treat heart arrhythmia and congestive heart failure regardless of its toxic side effects (175, 176). Digoxigenin (DIG) is found in the *Digitalis* plants and is used widely in molecular biology as a probe for non-radioactive immunoassays (177). Ouabain is found in the seeds of plants from the genus *Strophanthus* and *Acokanthera* (178, 179). Ouabain is a toxic cardiac glycoside and affects cell-to-cell cooperation by targeting the sodium pump (180). These toxins are similar in structure to bile salts and contain an α,β-unsaturated lactone. This type of lactone can form an irreversible adduct by Michael addition to cysteine residues (181-184). Since the amino acid sequence of the germination receptor for *C. difficile* is unknown (95), we hypothesized that bile salt analogs containing an

α,β-unsaturated lactone can be used as chemical probes to crosslink with the amino acids that participate in binding. However, analogs containing the α,β-unsaturated lactone must first be recognized by the *C. difficile* germination binding site(s). Purification of the germination binding site(s) is out of scope of this project but finding compounds active with *C. difficile* spores is the focus of this dissertation. Therefore, the effect these three toxins have on *C. difficile* spore germination was assessed *in vitro*.

Screening bile salt analogs for *in vitro* activity with *C. difficile* spores provides data that can be used to elucidate the mechanistic activity of small molecules and the putative *C. difficile* germination binding site(s). More importantly, there is the potential use of anti-germinants in CDI prevention. Thorough characterization of the germination binding site(s) *in vitro* will help to design binding site(s) specific molecules to prevent *C. difficile* germination.

3.2. Materials and Methods

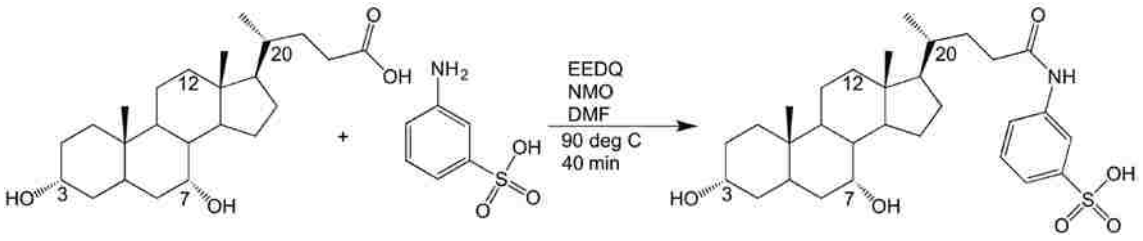
3.2.1 General Comments

Taurocholate and bile salt analogs were obtained as described in Chapter 2 as well as thin layer chromatography silica gel and silica gel for column chromatography. Bacterial growth medium was obtained from BD (Franklin Lakes, NJ).

3.2.2. General Synthesis of Side-Chain Modifications

Unless otherwise stated, all the taurocholate and taurochenodeoxycholate analogs were prepared following published procedures and resulted in up to 65% yield (157, 158). Cholic acid (1 eq. - 1mM) or chenodeoxycholic acid (1 eq. -

1mM) was activated with 1.4 eq. of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and 1.3 eq. of *N*-methylmorpholine (NMO) in dimethylformamide (DMF) (Scheme 3.1, as an example). After the mixture was stirred for 5 min, 1.2 eq. of the appropriate side chain reagent was added. The reaction mixture was heated to 90 °C for 40 min and then cooled to room temperature.



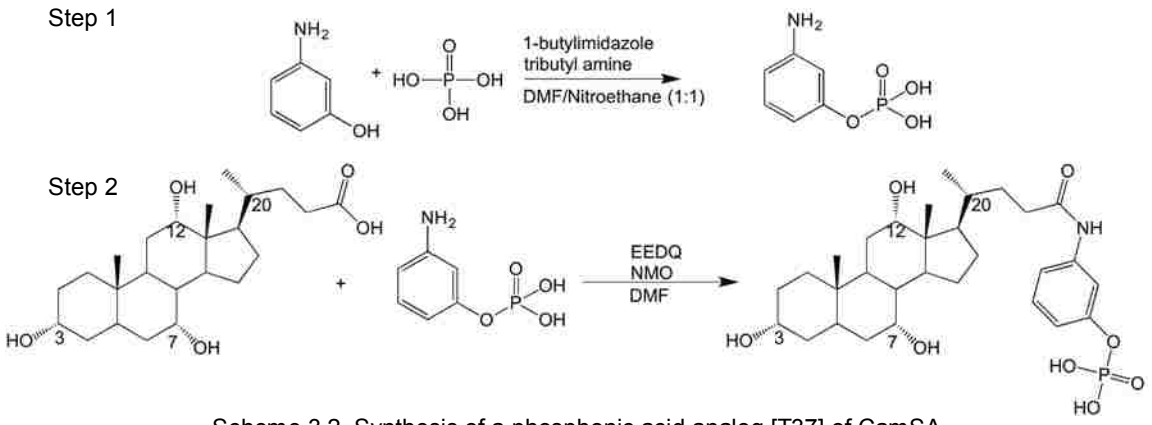
Scheme 3.1. Coupling reaction of chenodeoxycholate and *meta*-aminobenzene sulfonic acid

For polar compounds, the solution was poured into 100 ml of ice-cold diethyl ether, resulting in a precipitate. The ether suspension was kept at 4 °C overnight. The ether layer was decanted and the resinous residue was dissolved in 25 ml 0.2 N NaOH-MeOH and poured into 100 ml cold diethyl ether. The ether solution was kept at 4 °C for at least 2 h and the resulting precipitate was filtered and washed with diethyl ether. If necessary, the product was recrystallized by dissolving to saturation in hot ethanol, followed by the addition of ethyl acetate until a precipitate appeared. The solution was kept at -20 °C for 2 h to allow complete precipitation and then filtered to retrieve the product. For nonpolar compounds, the reaction mixture was diluted with ethyl acetate and washed three times with water. The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The resulting residues for both

polar and nonpolar compounds were further purified by silica gel column chromatography eluted with a step gradient from 100% ethyl acetate to 100% methanol (MeOH). Percent yields were typically 30 – 80%. Compound structures were verified and determined to be >95% pure by ^{1}H-NMR, Fourier transform infrared spectroscopy (FT-IR), and mass spectrometry.

3.2.3. Synthesis of the Phosphonic Acid Derivative of CamSA [T37]

To synthesize a phosphonic acid analog of CamSA [T37], the published procedure for dehydrating phosphoric acid with an alcohol was first followed (185). A solution of 3-aminophenol (1 eq. - 1mM), phosphoric acid (1 eq.), 1-butylimidazole (0.1 eq.), tributyl amine (1 eq.), and DMF/nitroethane (50/50 v/v) was refluxed under azeotropic conditions at 155 °C for six hours (Scheme 3.2, step 1).



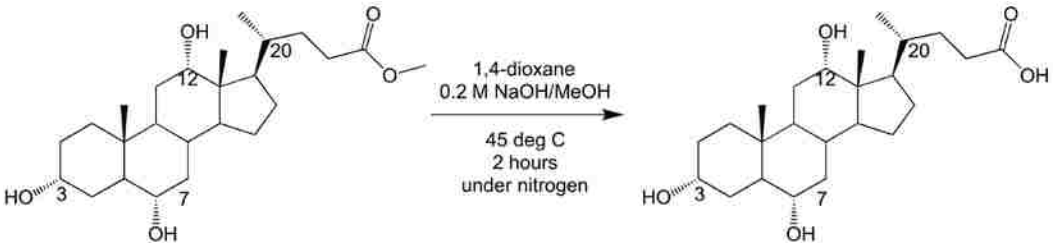
Scheme 3.2. Synthesis of a phosphonic acid analog [T37] of CamSA

Dowex 50WX2-200 cation exchange resin was directly added to the crude mixture and stirred at room temperature for one hour. The resin was removed by filtration and the solvent was not removed. The filtrate contained the crude product. The production of 3-aminophenylphosphoric acid was verified by TLC

(mobile phase 100% ethyl acetate). The 3-aminophenylphosphoric acid (filtrate in DMF) was conjugated to cholate using the coupling procedures described above (157, 158) (Scheme 3.1, step 2). The product was purified by silica gel column chromatography eluted as before. The final yield was 15%. The structure was verified and determined to be >95% pure by ^{1}H-NMR, FT-IR, and mass spectrometry.

3.2.4. Preparation of Hyodeoxycholic acid [T95]

To a solution of hyodeoxycholic acid methyl ester [T96] in 15 mls 1,4-dioxane, 5 mls of 0.2 M NaOH/MeOH was added and stirred at 45 °C for two hours under nitrogen (Scheme 3.3). The de-protected hyodeoxycholic acid [T95] was purified (85% yield) by silica gel chromatography in 90% dichloromethane/10 % MeOH. T96 was verified and found to be >95% pure by ^{1}H-NMR, FT-IR, and mass spectrometry.

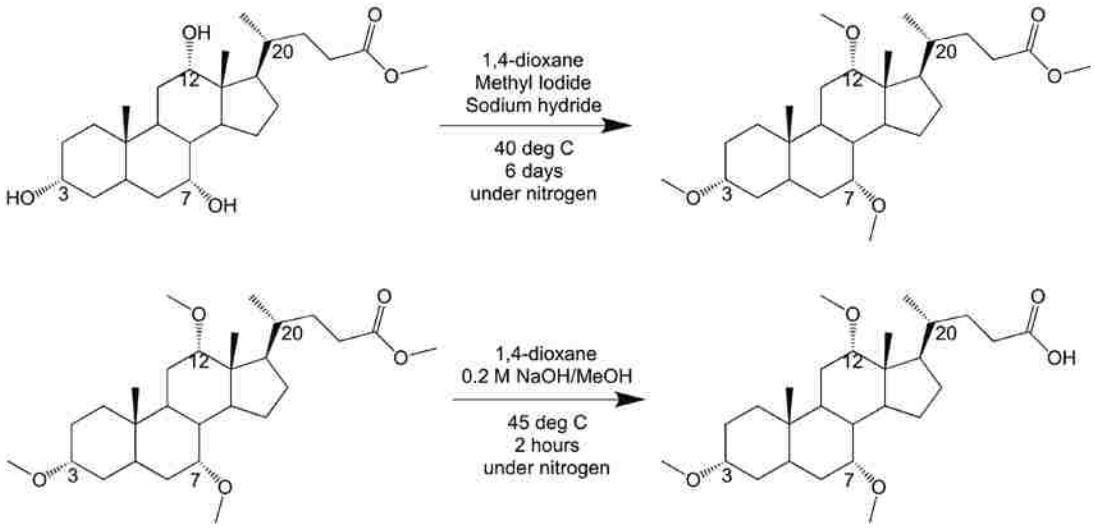


Scheme 3.3. Preparation of hyodeoxycholic acid [T96]

3.2.5. Preparation of Tri-methoxy Cholic Acid [T89]

Tri-methoxy cholic acid [T89] was prepared following published procedures (154). A similar procedure was used to prepare methoxylated taurocholate analogs [T09 and T10] in Chapter 2 (section 2.2.2). The previous procedure used taurocholate as the starting reagent whereas; this procedure

uses methyl cholate and requires a deprotection step. To a solution of methyl cholate (1 eq. - 1mM) in dry 1,4-dioxane, methyl iodide (50 eq.) and sodium hydride (4 eq.) were added under nitrogen. The reaction mixture was heated to 40 °C for 48 h with stirring (Scheme 3.4). After the initial 48 h, sodium hydride (4 eq.) was added daily to the reaction mixture for four days. The reaction mixture was diluted with dichloromethane and washed with 1 M HCl and twice with water. The organic layer was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The crude product was dissolved in 15 mls 1,4-dioxane and 5 mls of 0.2 M NaOH/MeOH was added. The mixture was heated to 45 °C under nitrogen for two hours. After heating the 1,4-dioxane was removed under reduced pressure and the sample was worked up a nonpolar compound as described before. The crude residue was purified by silica gel column chromatography eluted with 90% chloroform and 10% MeOH. The purified compound (10% yield) was verified and found to be >95% pure by ^{1}H NMR, mass spectrometry and FT-IR spectroscopy.



Scheme 3.4. Reaction scheme for the synthesis of compound T89

3.2.6. Preparation of *C. difficile* Spores

Clostridium difficile strain 630 was obtained from American Tissue Culture Collection (ATCC) (BAA-1382). *C. difficile* cells were plated on BHIS (brain heart infusion salt broth) agar supplemented with 1% yeast extract, 0.1% L-cysteine-HCl and 0.05% sodium taurocholate to yield single-cell colonies. Single *C. difficile* colonies were grown in BHIS broth until exponential phase (approximately four hours) and spread plated onto agar to obtain bacterial lawns. The plates were incubated for five days at 37 °C in an anaerobic environment (5% CO_2, 10% H_2, and 80% N_2). The resulting bacterial lawns were collected by flooding the plates with ice-cold deionized water. Spores were pelleted by centrifugation at 8,800 x g for five minutes and resuspended in fresh deionized water. After two washing steps the spores were separated from vegetative and partially sporulated forms by centrifugation through a 20% to 50% HistoDenz gradient at 18,200 x g for 30 minutes with no brake. The spore pellet was washed five times with water, resuspended in sodium thioglycolate (0.5 g/L) and stored at 4 °C. Immediately before *in vitro* germination activation or inhibition assays, spores were transferred to fresh tubes, centrifuged, and washed with deionized water five times to remove storage buffer.

The methods for endospore staining and the activation or inhibition of *C. difficile* spore germination were described in Chapter 2 (please refer to the Methods section 2.2.6 – 2.2.8).

3.3. Results and Discussion

3.3.1. Taurocholate Analogs with Linear Side Chains

Based on preliminary structure activity analyses of taurocholate analogs and their interactions with *C. difficile* spores, new analogs were designed and tested for their ability to act as activators or inhibitors of *C. difficile* spore germination. *C. difficile* strain 630 used previously (Chapter 2) was also used for these studies.

The analysis of linear side chains discussed in chapter 2 revealed that shorter [T11] but not longer [T12] alkyl side chains of taurocholate analogs could activate spore germination (Table 2.2). Some longer alkyl side chains that contain a carboxylic acid were able to inhibit *C. difficile* spore germination [T20 and T21] (Table 2.2 and Fig. 3.2). To further characterize the recognition of long alkyl side chains by the *C. difficile* germination binding site(s), taurocholate analogs T23-T29 were synthesized and tested *in vitro*. Compounds T23 and T25 differ by one carbon in the alkyl chain and both have carboxylic acids at the end of the alkyl side chain (Fig. 3.2). T23 and T25 have longer alkyl side chains than previously tested analogs [T17, T18, and T20] (Fig. 3.2). Neither T23 nor T25 were able to activate *C. difficile* spore germination. T23 did inhibit spore germination but with an IC_{50} of 5.3 mM. The analog tested previously [T20], which has one less carbon in the alkyl chain, has an IC_{50} of 0.76 mM, seven fold more potent than T23 (Fig. 3.2). Interestingly, T25 with one more carbon in the alkyl chain than T23 inhibited *C. difficile* spore germination with an IC_{50} of 2.3 mM. These data suggest that an even number of carbons in the alkyl chain [T17

= 2C, T20 = 4C, and T25 = 6C] is able to bind more efficiently than their odd numbered counterparts [T18 = 3C and T23 = 5C].

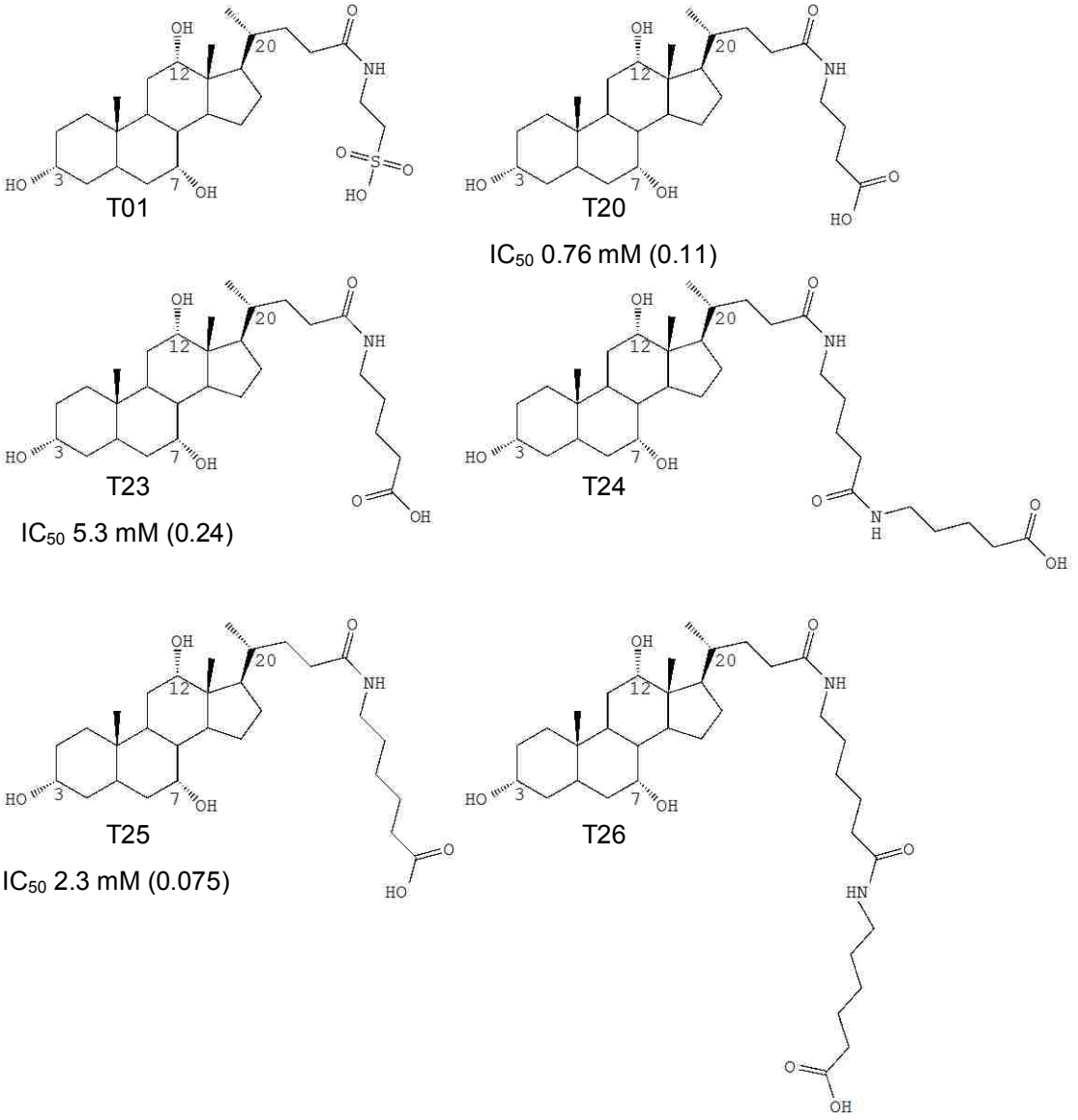


Figure 3.2. Taurocholate analogs with linear side chains. Taurocholate [T01], taurocholate analog from Chapter 2 [T20] and taurocholate analogs [T23 – T26]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

Similar to the synthesis of compounds T18 and T20, side products [T24 and T26] were produced during the synthesis of compounds T23 and T25. These

compounds were also analyzed for the ability to activate and/or inhibit *C. difficile* spore germination. Although compound T21, the side product from T20, was able to inhibit spore germination as described previously (Table 2.2), compounds T24 and T26 were unable to activate nor inhibit spore germination (Fig. 3.2). The alkyl chains in compounds T21, T24 and T26 have an total even number of carbons connected by two amide bonds. The total chain length for T21 has 8 carbons, T24 has 10 carbons and T26 has 12 carbons. The sum of these data suggests that shorter alkyl side chains containing a carboxylic acid with an even number of carbons in the chain are able to bind but not activate *C. difficile* spore germination.

Compound T27 is similar to taurocholate [T01] except the sulfonic acid is replaced with a sulfate group resulting in an organosulfate (Fig. 3.3). This rendered compound T27 unable to bind to *C. difficile* spores. Compound T27 neither activated nor inhibited spore germination, suggesting three but not four oxygen atoms in the alkyl functional group are recognized by the *C. difficile* germination binding site(s). Alternatively, the thioether linkage of compound T27 could by hydrolyzed. Compound T28 is a taurocholate analog containing a thioester bond (Fig. 3.3). Compound T28 is also an analog of the ester compound [T22] described in chapter 2. Unlike taurocholate, T28 is unable to activate *C. difficile* spore germination. Compound T28 is also unable to inhibit spore germination whereas the analog T22 inhibits spore germination. It is possible that the reason the thioester bond is not recognized is likely due to the

limited ability of the sulfur ester to participate in electron delocalization. This makes the thioester bond less stable and likely to be hydrolyzed to the free thiol.

Compound T29 is a taurocholate analog with one less carbon in the alkyl chain. It is a close analog of T11, an activator of *C. difficile* spore germination (Table 2.2). Compound T29 differs from both taurocholate and T11 in the functional group in the alkyl side chain. Instead of a sulfonic acid, T29 has a phosphonic acid moiety (Fig. 3.3). Unlike taurocholate and T11, T29 neither activated nor inhibited *C. difficile* spore germination. Phosphoric acids have a pKa of 2 whereas sulfonic acids have a pKa of -10. Therefore, phosphoric acids are weaker acids than sulfonic acids. The putative *C. difficile* taurocholate germination binding site(s) seems to prefer the stronger acid for recognition.

In conclusion, these data indicate that the taurine side chain connected by an amide bond is optimal for *C. difficile* spore germination.

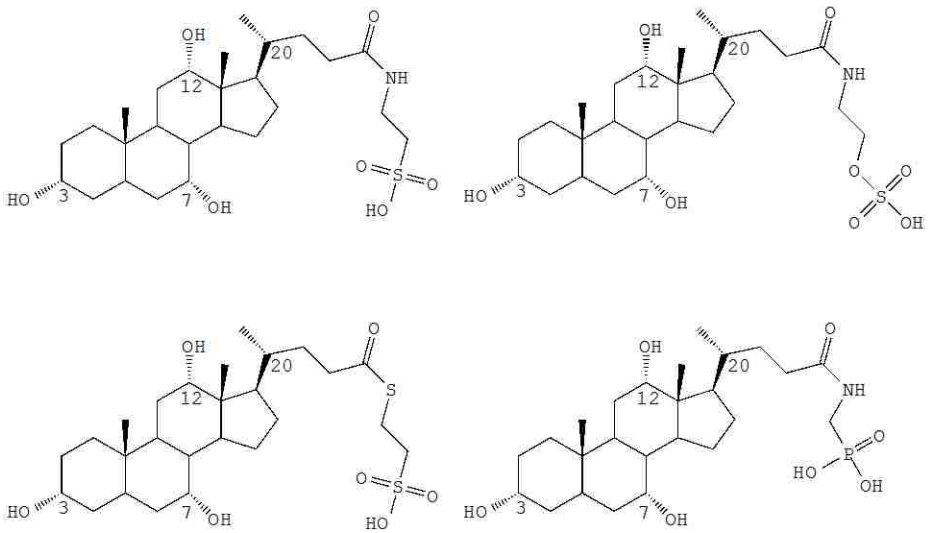
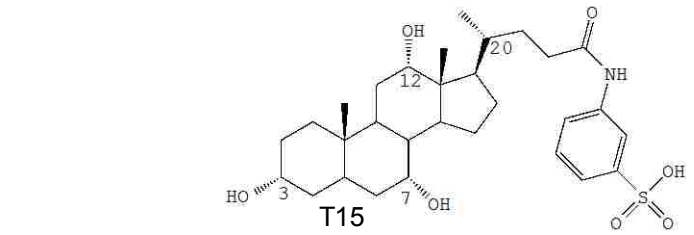


Figure 3.3. Taurocholate analogs with linear side chains and modified functional groups. Taurocholate [T01] and taurocholate analogs [T27 – T29]. No IC_{50} value is listed because these analogs had no activity under the conditions tested.

3.3.2. CamSA Analogs with Unsubstituted Aromatic Side Chains

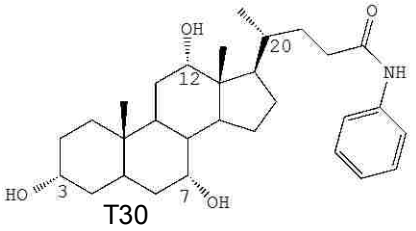
CamSA, the potent inhibitor discovered and described in chapter 2 has an aromatic ring in the side chain with a sulfonic acid in the *meta*-position. To determine if the aromaticity of CamSA is responsible for CamSA's strong effects, compounds T30 and T31 were synthesized. Compound T30 is similar to CamSA, but lacks the sulfonic acid on the benzene ring. Compound T30 inhibits *C. difficile* spore germination with an IC_{50} of 0.27 mM, five fold higher than CamSA (Fig. 3.4). Thus, it appears that the aromatic ring does have some effect on binding and inhibition of spore germination. However, this suggests that the sulfonic acid makes the inhibitor more potent.

Compound T31 has a pyridine substituent with the aromatic nitrogen at position-3 in the aromatic ring. Compound T31 is unable to activate or inhibit *C. difficile* spore germination (Fig. 3.4). The aromatic nitrogen in T31 causes the molecule to be electron deficient as opposed to the electron rich benzene. Although, the electron deficiency may explain the difference between T30 and T31, the sulfonic acid moiety of CamSA creates an electron deficient aromatic ring. CamSA and compound T31 cannot be compared directly since the sulfonic acid is a substituent on the aromatic ring and compound T31 has aromatic nitrogen. In conclusion, these data indicate that if no substituent is present on an aromatic ring bound to the cholate backbone, then the *C. difficile* binding site(s) is able to recognize to the aromatic ring but not a pyridine.



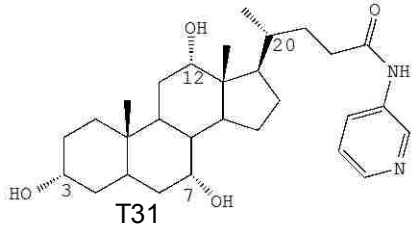
T15

IC_{50} 0.058 mM (0.035)



T30

IC_{50} 0.27 mM (0.070)



T31

Figure 3.4. CamSA analogs with unsubstituted aromatic side chains. CamSA [T15] and CamSA analogs [T30 – T31]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

3.3.3. CamSA Analogs with Mono-substituted Aromatic Side Chains

Sulfonic acids are highly polar and very strong acids. In fact, sulfonic acids are stronger acids than their carboxylic analogs. When the sulfonic acid was substituted for a carboxylic acid in the alkyl side chain analog of taurocholate [T17, T18, and T20], we found that the sulfonic acid is optimal for activation of *C. difficile* spore germination. The sulfonic acid can be substituted for a carboxylate as long as the alkyl chain is short, two carbons [T17]. Longer alkyl chains with carboxylic acids, described previously, were not able to activate *C. difficile* spore germination but were able to bind and inhibit spore germination. To determine the effect of carboxylic acid substituted aromatic rings on *C. difficile* spore germination, compounds T32-T37 were synthesized and tested for the ability to activate or inhibit *C. difficile* spore germination.

Compound T32 is an analog of CamSA with a carboxylic acid in the *meta* position on the aromatic ring (Fig. 3.5). Compound T32 is unable to activate spore germination but was did inhibit *C. difficile* spore germination with an IC_{50} of 3.1 mM (Fig. 3.5). CamSA is approximately 50 fold more potent than T32 at inhibiting spore germination. The data suggests that the sulfonic acid is optimal for binding and preventing *C. difficile* spores from germinating. This is likely due to the stronger acidity of the sulfonic acid group and their stronger electron withdrawing properties as substituents on aromatic rings. It should be noted that aromatic rings have a very modest effect on acidity.

Compound T33 is the *ortho* analog of T32 and the carboxylic analog of compound T14 (Chapter 2). As expected, compound T33 was not recognized by *C. difficile* spores and was unable to affect spore germination (Fig. 3.5). Compound T34 is the *para* analog of T32 and the carboxylic acid analog of T13. Interestingly, compound T34 is able to bind and inactivate *C. difficile* spores with an IC_{50} of 1.4 mM, which is 2-fold lower than the *meta* analog [T32] (Fig. 3.5). This finding is surprising since the *para* analog of CamSA [T13] was unable to bind to *C. difficile* spores. CamSA is more potent an inhibitor of *C. difficile* spore germination since it is more active at concentrations 25-fold lower than T34.

Compound T35 is a non-aromatic analog of compound T32. Compound 35 has a cyclohexane group side chain with a carboxylic acid substituent on the third carbon from the amide bond (equivalent to the *meta* position of an aromatic compound). Compound T35 is seven fold more potent as an inhibitor (IC_{50} 0.47 mM) of *C. difficile* spore germination than compound T32. We expected that the

aromaticity of the substituent was partially responsible for activity. However, it seems that as long as there is an electronegative group on a ring structure then the ring does not need to be aromatic. This does not fully explain why an unsubstituted aromatic ring [T30] was able to inhibit *C. difficile* spore germination. The unsubstituted aromatic ring is about 2-fold more potent than compound T35 suggesting that either the presence of an aromatic ring or the carboxylic acid is sufficient for binding (Fig. 3.5). However, in the presence of both an aromatic ring and carboxylic acid [T32 and T34] the activity decreases. In comparison to CamSA, the sulfonic acid on an aromatic ring is optimal for binding and inhibition of *C. difficile* spore germination.

Compound T36 is an analog of compound T32 except that the carboxylic acid has been converted to an ester (Fig. 3.5). Carboxylic acids are weak acids that can act as hydrogen bond acceptors and donors whereas esters are less polar and can only act as hydrogen bond acceptors. The change from a carboxylic acid to an ester for compound T36 resulted in complete loss of binding to *C. difficile* spores (Fig. 3.5). Ester groups are unstable and undergo hydrolysis. If the ester group in compound T36 is hydrolyzed, the resulting compound is a carboxylic acid [T32]. It is expected that the product of the hydrolysis would inhibit *C. difficile* spore germination (similar to T32). Since compound T36 did not inhibit spore germination, it is possible the ester group was not hydrolyzed. Therefore, compound T36 was not recognized by *C. difficile* spores. The data suggests that the presence of a carbonyl, but loss of hydrogen bond donating ability results in complete loss of *C. difficile* binding.

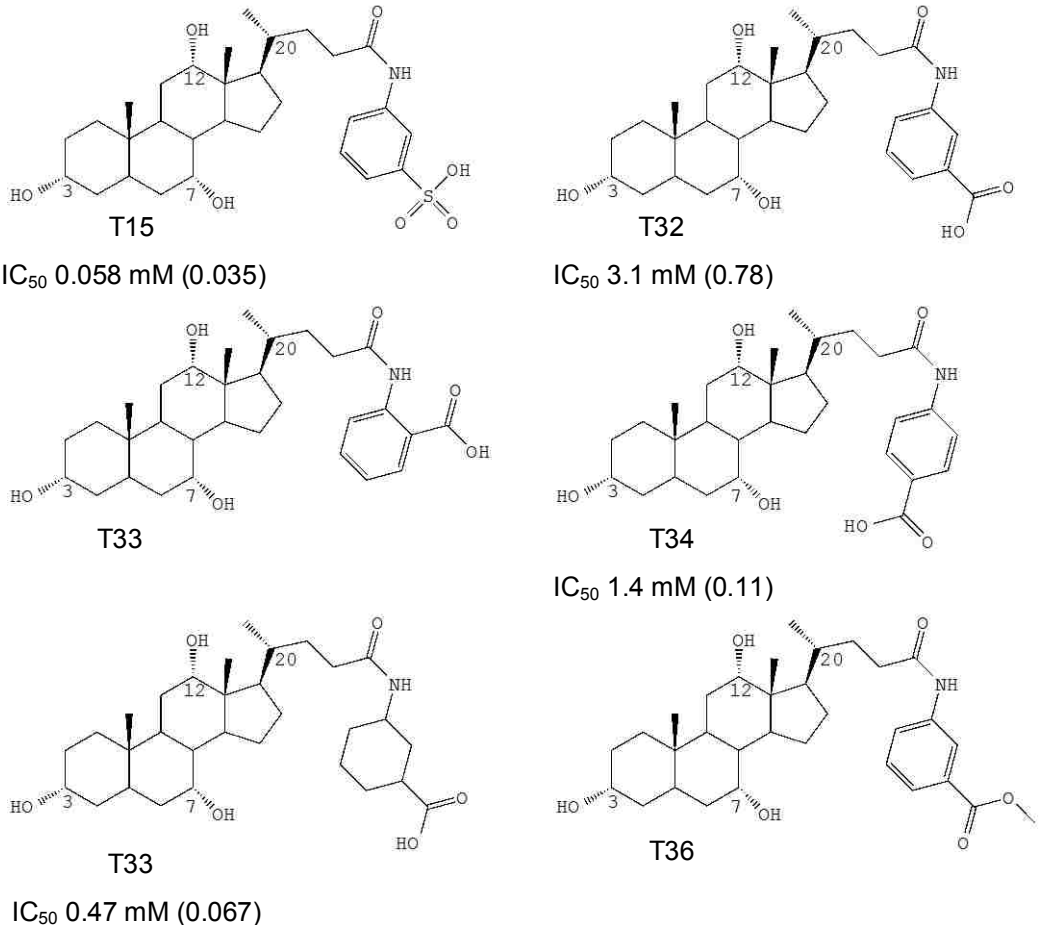


Figure 3.5. CamSA analogs with mono-substituted aromatic side chains. CamSA [T15] and CamSA analogs [T32 – T36]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

The sum of these data suggests that the aromatic ring of CamSA can be partially substituted with carboxylic acid analogs and in the presence of a carboxylic acid the ring does not have to be aromatic. The sulfonic acid substituent on an aromatic compound remains optimal for *C. difficile* binding.

To determine if the *C. difficile* germination binding site(s) recognizes substituents other than carboxylic or sulfonic acids, compounds T37 - T44 were synthesized. Phosphoric acids (pKa = ~2) like carboxylic acids (pKa = 1.5 – 4.0) are weaker acids than sulfonic acids (pKa = -10). Compound T37 contains a

phosphoric acid in the *meta* position on the benzene ring (Fig. 3.6). Whereas the carboxylic acid derivative [T32] of CamSA was able to inhibit *C. difficile* spore germination, T37 is unable to bind to *C. difficile* spores. Based on acidity, we would expect compound T37 to be comparable to the carboxylic acid analog [T32]. It is possible that compound T37 inhibits at concentrations higher than the concentration tested in these experiments. A more likely explanation is that the binding region in *C. difficile* spores for bile salts is a specialized region that simply does not recognize the phosphoric acid moiety.

Compounds T38 and T39 have hydroxyl groups in the *meta* or *ortho* position, respectively (Fig. 3.6). Neither compound was able to bind to *C. difficile* spores. Oxygen atoms in the alkyl substituents of analogs have been shown to be optimal for *C. difficile* spore recognition. However, since hydroxyl groups are unable to bind, it is possible that an sp^2 hybridized oxygen group is recognized specifically. Another explanation is the electron donating effect (through resonance) hydroxyl groups have on aromatic rings. As described previously, electron withdrawing groups are predicted to be optimal for spore recognition. The finding that compounds with hydroxyl groups do not affect *C. difficile* spore germination supports that hypothesis.

Taurocholate and CamSA have a sulfonic acid group in the alkyl chain. To determine if sulfur alone is sufficient for *C. difficile* spore recognition, compounds T40 and T41 were synthesized and assessed for activation or inhibition of spore germination. Both compounds [T40 and T41] have a sulfhydryl group in either the *meta* or *para* position, respectively. Neither T40 nor T41 was able to activate or

inhibit spore germination (Fig. 3.6). It is likely that the acidic characteristics of the sulfonic acid make it an optimal germinant for *C. difficile*. As expected, compound T42, containing a thiol ether, was also unable to be recognized by the putative *C. difficile* bile salt germination binding site(s). The sulfhydryl and thiol ether provide electron donating effects to the aromatic ring. These data support the need for an electron withdrawing group on the aromatic ring and an acidic substituent for optimal recognition by *C. difficile* spores.

The data suggests that electronegative compounds are favored by the *C. difficile* germination binding site(s). Compound T43 has an amine group at the *meta* position on the benzene ring. Unlike carboxylic and sulfonic acids, amine groups are basic and much less electronegative. Amine group substituents of an aromatic ring are less basic and increase the reactivity of the ring due to the amine's electron donating property by resonance. As expected, compound T43 did not activate *C. difficile* spore germination. Surprisingly, T43 inhibited spore germination with an IC_{50} of 0.53 mM (Fig. 3.6). It is possible that the increased reactivity of the aromatic ring [T30] outweighed the basic nature of the amine. However, this is unlikely since hydroxyl groups and sulfahydryl groups were unable to bind to *C. difficile* spores. Albeit a weak inhibitor, it is unclear at this time why compound T43 is able to affect *C. difficile* spore germination. More *in vitro* studies with amine substituted aromatic rings will need to be performed for a better understanding of this finding.

Compound T44 contains an aromatic ring with a methyl substituent in the *meta* position. The methyl substituent has an electron donating effect by

induction. As expected, compound T44 did not affect *C. difficile* spore germination (Fig. 3.6). This data supports our earlier prediction that optimal analogs for recognition by *C. difficile* spores will have electron withdrawing substituents on the aromatic ring.

In conclusion, the combined data from CamSA analogs with mono-substituted aromatic rings supports that the *meta*-benzene sulfonic acid analog produces optimal inhibition of *C. difficile* spore germination. There are limited instances when other functional groups are able to bind to the putative binding site(s). None of CamSA analogs with mono-substituted aromatic rings were able to activate *C. difficile* spore germination *in vitro*. Except for compound T43, the data suggests that an electronegative substituent with electron withdrawing properties is able to be bind but not activate *C. difficile* spore germination. Furthermore, the aromaticity of the analog may play a role in recognition and the presence of an sp^2 hybridized oxygen in the substituent is almost always required. More acidic substituents are also favored over weaker acid substituents. These data provide some insight to the chemical requirements of the putative taurocholate germination binding site(s) in *C. difficile*. However, these findings also show that binding and recognition of bile salts is quite complicated and multiple factors exist to determine if a compound is able to bind or not.

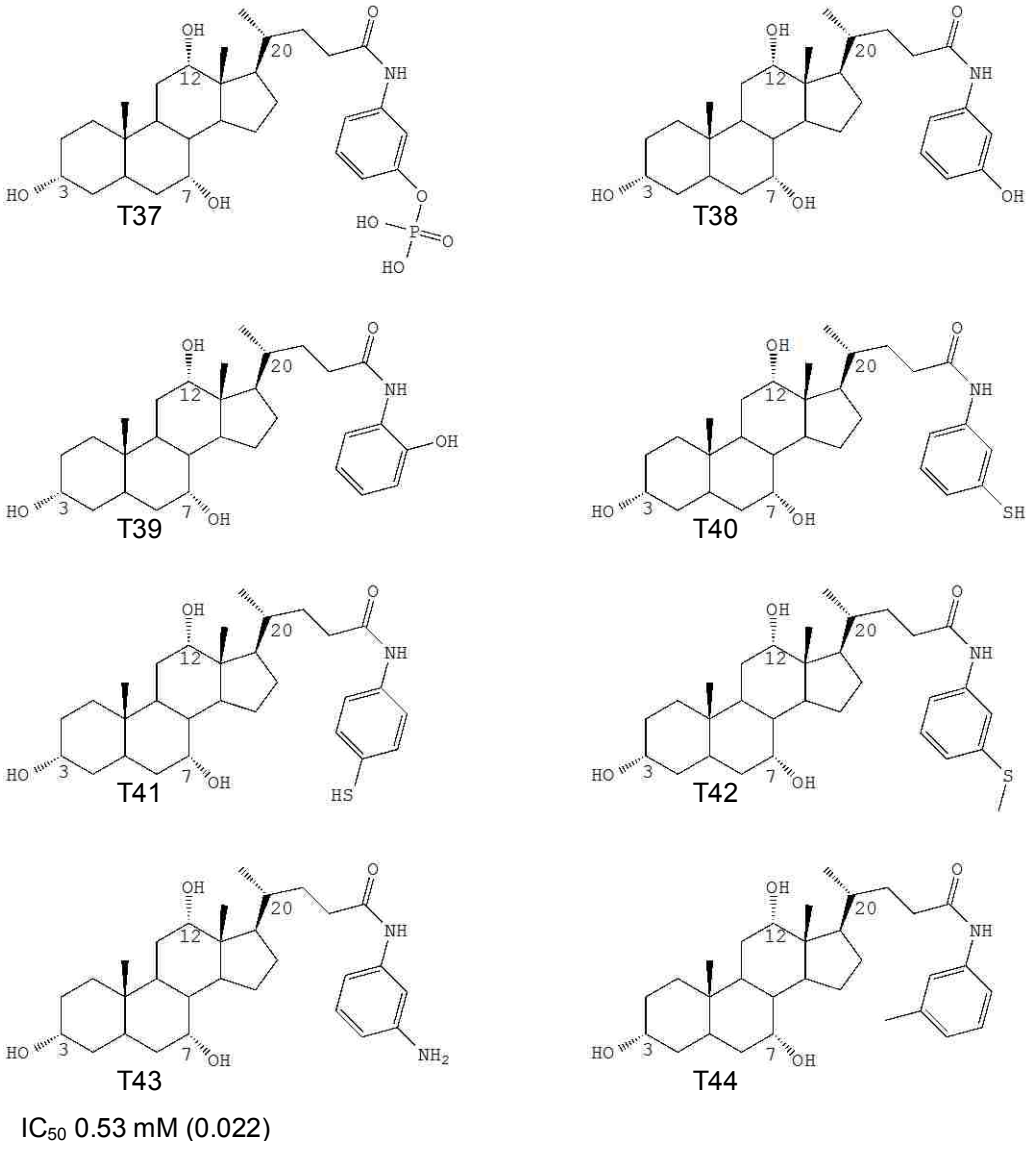


Figure 3.6. CamSA analogs [T37 – T44] with mono-substituted aromatic side chains. The corresponding IC_{50} value is listed below the compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

3.3.4. CamSA Analogs with Di-substituted Monocyclic Aromatic Rings

CamSA and the CamSA analogs discussed above have single substitutions in the aromatic ring. To determine if aromatic rings with di-substitutions are able to activate or inhibit *C. difficile* spore germination, compounds T45 to T48 were synthesized. Each of these analogs has an

aromatic ring with a *meta*-sulfonic acid or *meta*-carboxylic acid substituent and a second substituent. Compounds T45 and T46 have a sulfonic acid in the *meta* position. Compound T45 is di-substituted with a methyl group in the *ortho* position (from the amide bond) (Fig. 3.7). Unlike CamSA, compound T45 was unable to unable to activate and unable to inhibit *C. difficile* spore germination. Compound T46 is di-substituted with a methoxy group at the *ortho* position. Interestingly, compound T46 activates spore germination with an EC_{50} of 4.6 mM and does not inhibit germination (Fig. 3.7). This finding was not expected since the methoxy group in T46 would affect the aromatic ring as an electron donating group due to resonance. In previous experiments, almost all compounds that are active with *C. difficile* spores have electron withdrawing groups. Compound T46 does have the sulfonic acid moiety at the *meta* position which is electron withdrawing. The recognition is not strong as reflected by the high EC_{50} but there does seem to be some interaction that will need further defining in future experiments.

Compounds T47 and T48 have a carboxylic acid in the *meta* position on the aromatic ring. Compound T47 is the analog of T45 and has a methyl group in the *ortho* position. Compound T47 is a very weak activator of *C. difficile* spore germination with an EC_{50} of 16 mM (Fig. 3.7). Compound T45 was not tested at concentrations higher than 12 mM so it is possible that it has very weak activity like T47. Compound T48 has a methyl group in the *para* position. Compound T48 inhibited spore germination with an IC_{50} 11 mM (Fig. 3.7). Both compounds [T47 and T48] are recognized by *C. difficile* but their activity is extremely weak.

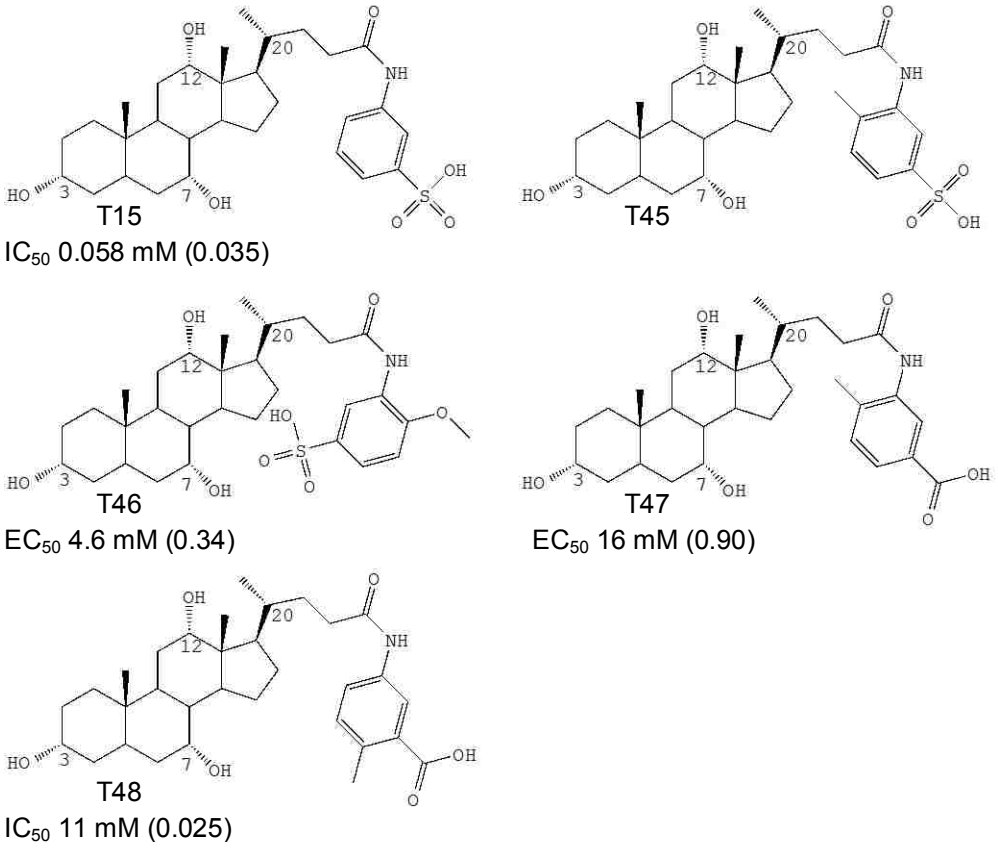


Figure 3.7. CamSA analogs with di-substituted monocyclic aromatic rings. CamSA [T15] and CamSA analogs [T45 – T48]. The corresponding EC_{50} or IC_{50} value is below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

3.3.5. CamSA Analogs with Tri-substituted Monocyclic Aromatic Rings

CamSA analogs with di-substituted aromatic rings provided evidence that the *C. difficile* germination binding site(s) could recognize more than one aromatic substitution. To determine if tri-substituted aromatic rings could affect spore germination compounds T49-T50 were synthesized. Both tri-substitute analogs have a sulfonic acid in the *meta* position from the nitrogen in the amide bond. Compound T49 has two methyl group substitutions, *ortho* and *para* to the amino group, whereas compound T50 has two methyl groups, *meta* and *para* (Fig. 3.8). Both compounds were able to bind to the putative *C. difficile*

germination binding site(s) and inhibit spore germination. Compound T49 inhibited *C. difficile* spore germination with an IC_{50} of 5.6 mM and compound T50 inhibited spore germination with an IC_{50} of 1.1 mM (Fig. 3.8). Neither compounds activated spore germination *in vitro*. In conclusion these data show that two [T49 and T50] but not one methyl group [T45] is recognized by the *C. difficile* spore germination binding site(s). Methyl groups are electron donating groups to the aromatic ring. Two methyl group substituents will increase the electron donating effect therefore adding stability to the ring. Although the data suggests some interaction does occur, the recognition by *C. difficile* spores is weak.

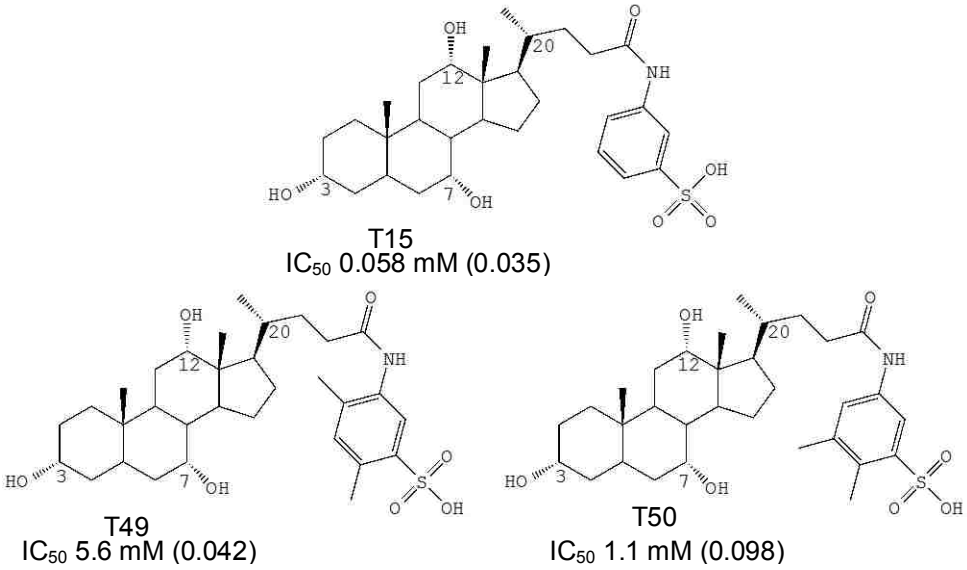
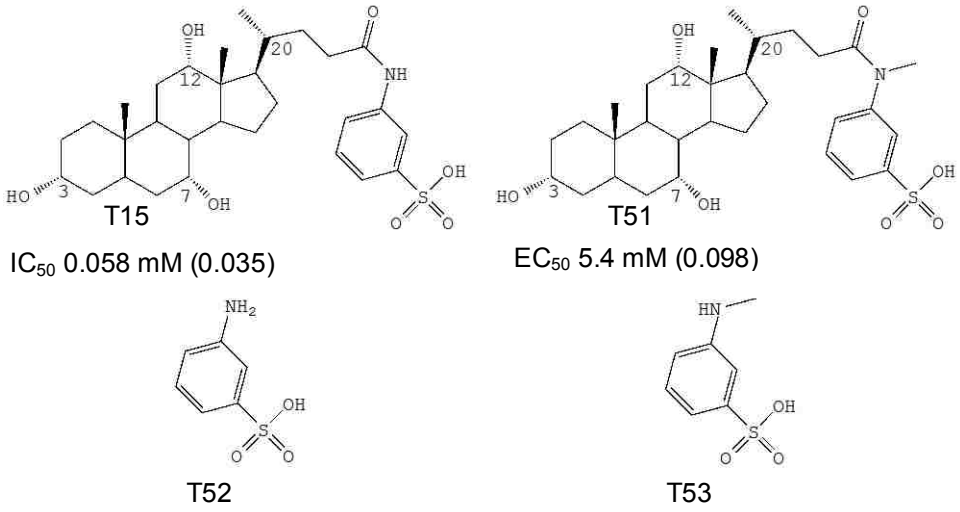


Figure 3.8. CamSA analogs with tri-substituted monocyclic aromatic rings. CamSA [T15] and CamSA analogs [T49 – T50]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses.

3.3.6. CamSA Analog with Methylated Amidate Group

Conjugated bile salts have the cholate backbone attached to taurine or glycine by an amide bond. Bile salt hydrolases produced by enteric bacteria hydrolyze the amide bond in bile salts thereby releasing free taurine or glycine (186-188). This deconjugation aids in the enterohepatic circulation of bile salts in the gut. In a previous report, the alkylation of the nitrogen in the amido group of a conjugated bile salt produced an analog resistant to deconjugation (188). In CamSA [T15], the cholate backbone is attached to a *meta*-benzene sulfonic acid by an amide bond. Since CamSA is a potent inhibitor of *C. difficile* spore germination *in vitro*, CamSA is a good candidate for *in vivo* studies. To have optimal efficiency in the mammalian gut, CamSA will have to remain a conjugated bile salt to be effective in preventing *C. difficile* spore germination. However, modifying the structure of CamSA may reduce its inhibitory activity. A CamSA analog with an alkylation of the nitrogen in the amide group [T51] was synthesized for *in vitro* testing. After modification to the amine group in CamSA, compound T51 was no longer able to inhibit *C. difficile* spore germination. Unexpectedly, compound T51 activated spore germination with an EC_{50} of 5.8 mM (Fig. 3.9). Compound T51 is not a strong germinant but is able trigger germination. In conclusion, the data indicates that compounds with a free amine are adequate for binding but not necessary for activation. Although the mechanism used by compound T51 to activate *C. difficile* spore germination is unclear, compound T51 cannot be used as a preventative of CDI even if it can resist bile salt hydrolase.

Since deconjugation of bile salts by bile salt hydrolases results in the release of the deconjugated alkyl group, compounds T52 and T53 were tested for effect on *C. difficile* spore germination (Fig. 3.9). Neither T52 nor T53 was able to bind to the putative germination binding site(s). This data suggests the cholate backbone is essential to the activity of CamSA and compound T51 in recognition by *C. difficile* spores.



IC_{50} 0.058 mM (0.035)

EC_{50} 5.4 mM (0.098)

Figure 3.9. CamSA analog with methylated amidate group. CamSA [T15], CamSA analog [T51] and starting reagents [T52 and T53]. The corresponding EC_{50} value is below each compound with standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

3.3.7. Bile Salt Analogs with Polycyclic Aromatic Hydrocarbons

To determine if polycyclic aromatic hydrocarbons in the alkyl chain with and without substituents are able to bind to *C. difficile* spores, compounds T54-T65 were synthesized and tested *in vitro*. Naphthalene is the simplest polycyclic aromatic hydrocarbon made up of two fused benzene rings. Two analogs were synthesized using 1-aminonaphthalene and 2-aminonaphthalene coupled with cholic acid by an amide bond [T54 and T55] (Fig. 3.10). Neither compound T54

nor T55 was recognized by the *C. difficile* germination binding site(s). Compound T56 is cholic acid conjugated to 2-aminoanthracene. Like compounds T54 and T55, compound T56 was unable to bind to *C. difficile* spores (Fig. 3.10). Pyrene is a fluorescent polycyclic aromatic hydrocarbon. Compound T57 is a cholate backbone conjugated by an amide bond to 1-aminopyrene. Unfortunately, compound T57 was neither able to activate nor inhibit *C. difficile* spore germination (Fig. 3.10). Polycyclic aromatic hydrocarbons are very rigid and flat structures. The fact that they are unable to bind to *C. difficile* spores is not surprising since the spatial requirements for bile salts is likely limited in size and shape. Therefore, rigid, bulky polycyclic aromatic hydrocarbons without substituents are unable to bind to the *C. difficile* bile salt germination binding site(s).

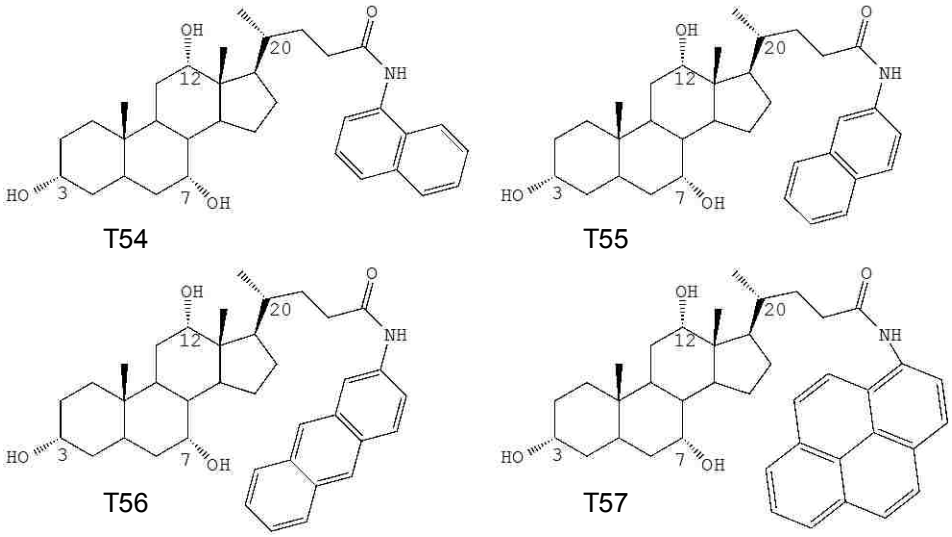


Figure 3.10. Bile salt analogs with unsubstituted polycyclic aromatic hydrocarbons. Bile salt analogs [T54 – T57]. Those without a listed value had no activity under the conditions tested.

Compounds T58-T61 contain naphthalene with one substituent on the rings. Compound T58 has a hydroxyl bonded to the carbon at position 2 from the amino group (Fig. 3.11). Compound T58 was unable to activate or inhibit *C. difficile* spore germination. This was expected since neither T54 nor T55 was able to bind to *C. difficile* spores. Furthermore, aromatic rings with hydroxyl groups in previous experiments [T38 and T39] did not affect *C. difficile* spore germination. Likely, compound T58 is unable to bind due to the rigidity of the naphthalene ring and the presence of a hydroxyl substituent. Compound T59 contains a sulfonic acid at position 1 from the amino group. Compound T59 was not recognized by the germination binding site(s) (Fig. 3.11). Although compound T59 has a sulfonic acid that has been shown to be optimal for *C. difficile* germination binding, the functional group is on the carbon next to the amide bond (equivalent to the *ortho* position in a monocyclic aromatic ring). Steric hindrance and rigidity of the side chain are likely why compound T59 is not recognized.

Compounds T60 and T61 both have a carboxylic acid substituent on the naphthalene side chain. In compound T60, the carboxylic acid is on the first carbon from the amino group and is an analog to T59. Compound T60 neither activated nor inhibited *C. difficile* spore germination similar to compound T59 (Fig. 3.11). Steric hindrance resulting from the carboxylic acid so near the amide bond and the rigidity of the polycyclic aromatic hydrocarbon are likely responsible for this inactivity. In compound T61, the naphthalene side group has a carboxylic acid on the fourth carbon from the amino group. Interestingly, compound T61 was able to bind to *C. difficile* spores and trigger germination with an EC_{50} of 1.3

mM (Fig. 3.11). Compound T61 is unable to prevent spore germination. It is unclear how this rigid structure is able to activate *C. difficile* spore germination. We predict that the polycyclic aromatic hydrocarbon is not directly responsible for the effect but may act to position the carboxylic acid in a binding region capable of recognizing it and activating germination. Glycocholate [T17] is also an analog of taurocholate that has a carboxylic acid substituent in the side chain and is able to effectively activate spore germination. Furthermore, compound T47 has a di-substituted aromatic side chain with a carboxylic acid and weakly activates *C. difficile* spore germination. Although the sulfonic acid is optimal for binding, carboxylic acids have been shown to also bind to the putative taurocholate binding site(s). Compound T61 probably interacts with the germination binding site(s) in a similar way to these compounds.

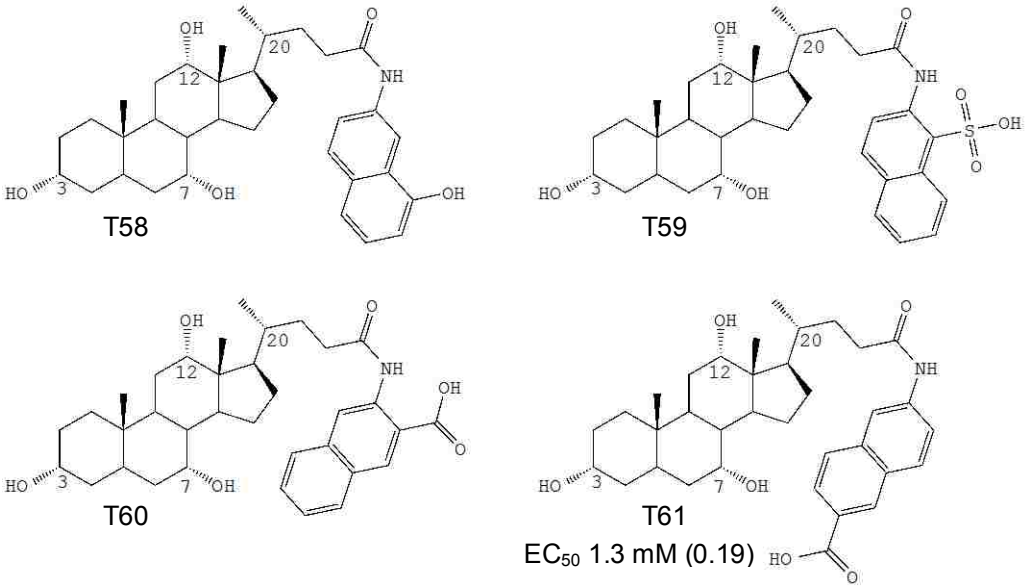


Figure 3.11. Bile salt analogs with monosubstituted polycyclic aromatic hydrocarbons. Bile salt analogs [T58 – T61]. The corresponding EC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

Compounds T62-T64 each contain naphthalene and two functional group substituents. Compound T62 has a hydroxyl on the carbon at position 2 from the amino group and a sulfonic acid at position 4. Compound T62 was neither able to active nor inhibit *C. difficile* spore germination (Fig. 3.12). Hydroxyl groups in the side chains seem to interfere with binding since none of the analogs discussed that have hydroxyl moieties are able to affect *C. difficile* spore germination. Compound T63 has two sulfonic acids, one at the first position and one at position 5 from the amino in the amide bond. Compound T63 was not recognized by *C. difficile* germination binding site(s) (Fig. 3.12). Since compound T59 and T60 discussed above were unable to affect *C. difficile* spore germination, it is not surprising that T63 was also ineffective. The steric effects caused by the functional group so near the amide bond in the alkyl chain is most likely responsible for the inability to bind to spores.

Compound T64 also has two sulfonic acids on the naphthalene side group but at position 2 and 5 from the amino. Compound T64 was unable to inhibit taurocholate-mediated spore germination. Interestingly, compound T64 triggered germination with an EC_{50} of 5.7 mM (Fig. 3.12). Similar to compound T61, compound T64 has an electronegative group at the position-5 on the second aromatic ring. Both of these compounds were able to activate spore germination *in vitro*. It is likely that the bulkiness and rigidity of these fused rings with the electronegative group at position-5 is able to come into contact with a specific region of the binding pocket that triggers germination. This effect is very interesting and will require future experiments to explain the interaction between

these polycyclic aromatic hydrocarbons and *C. difficile* spores. To rule out non-specific effects caused by the polycyclic aromatic hydrocarbon, compound T65 was also tested for germinant and inhibitor activity with *C. difficile* spores. The unconjugated di-substituted naphthalene ring was unable to bind to *C. difficile* spores. Compounds not bound to cholate [T52, 53, and T65] were unable to affect binding. Therefore, the cholate backbone is necessary for recognition by the putative taurocholate binding site(s) in *C. difficile* spores.

In conclusion, polycyclic aromatic hydrocarbons without substituents are unable to bind to the putative taurocholate binding site(s) in *C. difficile* spores. Substituted naphthalene produced variable results and the interactions of these compounds with potential binding sites are not clearly understood.

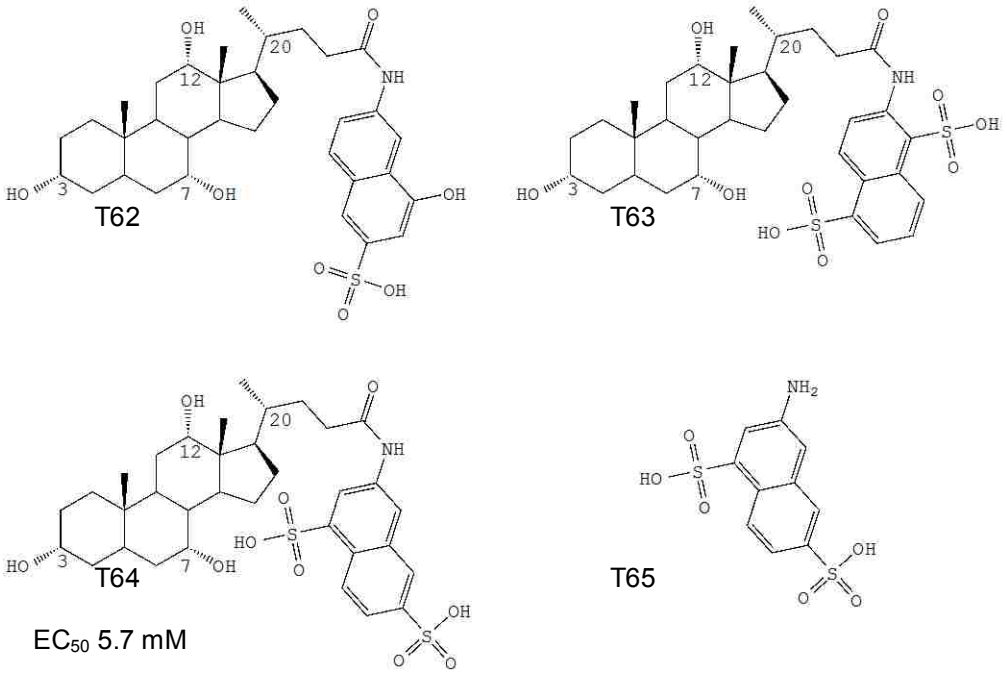


Figure 3.12. Bile salt analogs with di-substituted polycyclic aromatic hydrocarbons. Bile salt analogs [T62 – T64] with di-substituted naphthalene side chains and unconjugated starting compound [T65]. The corresponding EC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

3.3.8. Taurochenodeoxycholic Acid Derivatives

Chenodeoxycholate has been shown to inhibit *C. difficile* spore germination *in vitro* (127, 141). Chenodeoxycholate is a natural bile salt that resembles cholic acid except that chenodeoxycholate does not have a hydroxyl group at position-12 on the cholate backbone. Taurochenodeoxycholate [T03] is the conjugated derivative of chenodeoxycholate and inhibits *C. difficile* spore germination with an IC_{50} of 0.51 mM (Fig. 3.13). Using the chenodeoxycholate backbone, a series of analogs were synthesized. These compounds are also analogs of taurocholate and CamSA derivatives discussed previously. In fact, analogs using the chenodeoxycholate backbone were not synthesized unless taurocholate and CamSA analogs were active with *C. difficile* spores.

In the previous study, a taurocholate analog that had one less carbon in the alkyl chain [T11] was able to activate *C. difficile* spore germination as effectively as taurocholate. The effect of this same alkyl chain coupled to chenodeoxycholate [T66] was assessed for activation or inhibition of *C. difficile* spore germination. Compound T66 activated *C. difficile* spore germination with an EC_{50} of 0.31 mM (Fig. 3.13). Compound T66 is approximately 50 fold more active than taurocholate. Although this compound is an analog of the inhibitor, taurochenodeoxycholate [T03], compound T66 is an germinant like its cholate analog compound T11. In fact, compound T66 is active at concentrations more than two-fold lower than T11 (Fig. 3.13). No hydroxyl group at position-12 of the cholate backbone does not prevent the molecule from activating germination as long as the side chain is short. As the alkyl chain is lengthened as in

taurochenodeoxycholate, the hydroxyl is required to activate spore germination. However, the longer alkyl side chains are recognized and able to bind but their activation properties are lost.

Compound T67 is the taurochenodeoxycholate derivative of compound T16. Compound T67 has a sulfinic acid instead of a sulfonic acid as a substituent of the alkyl chain. Similar to compound T16, compound T67 did not activate spore germination but inhibited with an IC_{50} of 5.3 mM (Fig. 3.13). Compound T67 is a weak inhibitor compared to T16, which is active at concentrations eight-fold lower.

Compound T68 has two carbons in the alkyl chain like taurochenodeoxycholate but instead of a sulfonic acid, T68 has a carboxylic acid group. Like taurochenodeoxycholate, T68 inhibited *C. difficile* spore germination but was more than seven-fold less effective, with an IC_{50} of 3.9 mM (Fig. 3.13). Furthermore, the taurocholate derivative [T17] activated spore germination albeit less effective than taurocholate. This data suggests that the carboxylic acid is not optimal for binding. Furthermore, this data suggests that if the alkyl chain is longer and contains a carboxylic acid [T17] then a hydroxyl at position-12 is necessary for activation of *C. difficile* spore germination. Based on the data to this point, it seems that there is a direct correlation between acidic functional group, linear chain length and the hydroxyl at position-12 on the cholate backbone. Very simple modifications to the structure result in an analog capable of binding and activating *C. difficile* spore germination and a compound simply able to bind.

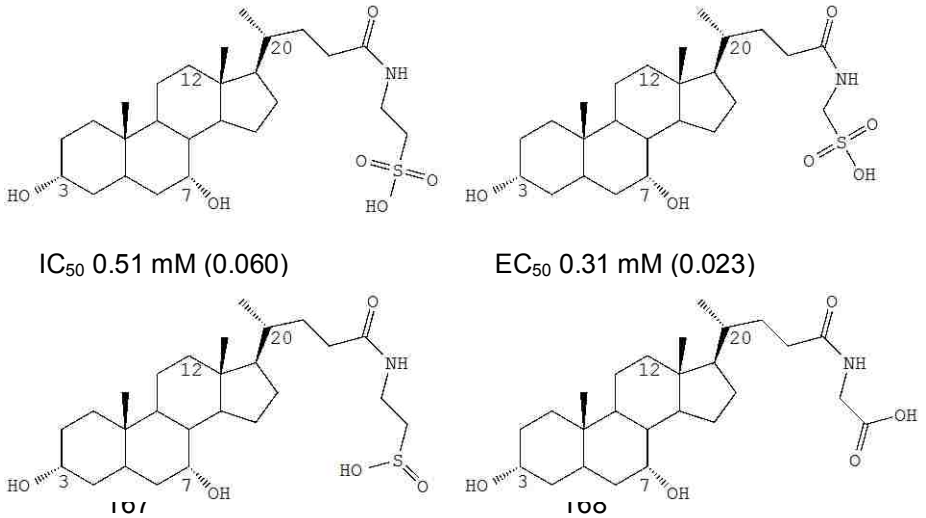


Figure 3.13. Taurochenodeoxycholic acid derivatives. Taurochenodeoxycholate [T03] and analogs [T66 – T68]. The corresponding EC_{50} and IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

Taurocholate analogs with longer alkyl side chains and a carboxylic acid functional group [T20, T21, and T23] inhibited *C. difficile* spore germination. To determine if the hydroxyl group at position-12 on the cholate backbone is essential for this inhibition, compounds T69 – T72 were synthesized. Compound T69 inhibited *C. difficile* spore germination with an IC_{50} of 0.64 mM comparable to its analog compound T20, which has an IC_{50} of 0.76 mM (Fig. 3.14). Compound T70 is the side product formed during synthesis of T69 and is an analog of compound T21. Like compound T21, compound T69 (IC_{50} 1.9 mM) is a weak inhibitor of spore germination (Fig. 3.14). Compounds T71 and T72 are analogs of compounds T23 and T24. Compound T71 inhibited spore germination with an IC_{50} of 1.1 mM and compound T72 inhibited with an IC_{50} of 8.6 mM (Fig. 3.14). Compound T71 is a more effective inhibitor of *C. difficile* spore germination than its analog T23. Compound T24 had no activity with spores but compound T72 is

a weak inhibitor of *C. difficile* spore germination. Taurochenodeoxycholate analogs with linear side chains [T69 – T72] were unable to activate spore germination. Like discussed previously, even number of carbons in the alkyl chain result in more effective binding. This finding was supported by the taurochenodeoxycholate analogs discussed here. The data also supports the earlier observation that no hydroxyl group at position 12 results in no interference with the binding of the bile salt to the putative germination binding site(s). Furthermore, the earlier hypothesis is supported that longer alkyl side chains containing a carboxylic acid are unable to activate *C. difficile* spore germination.

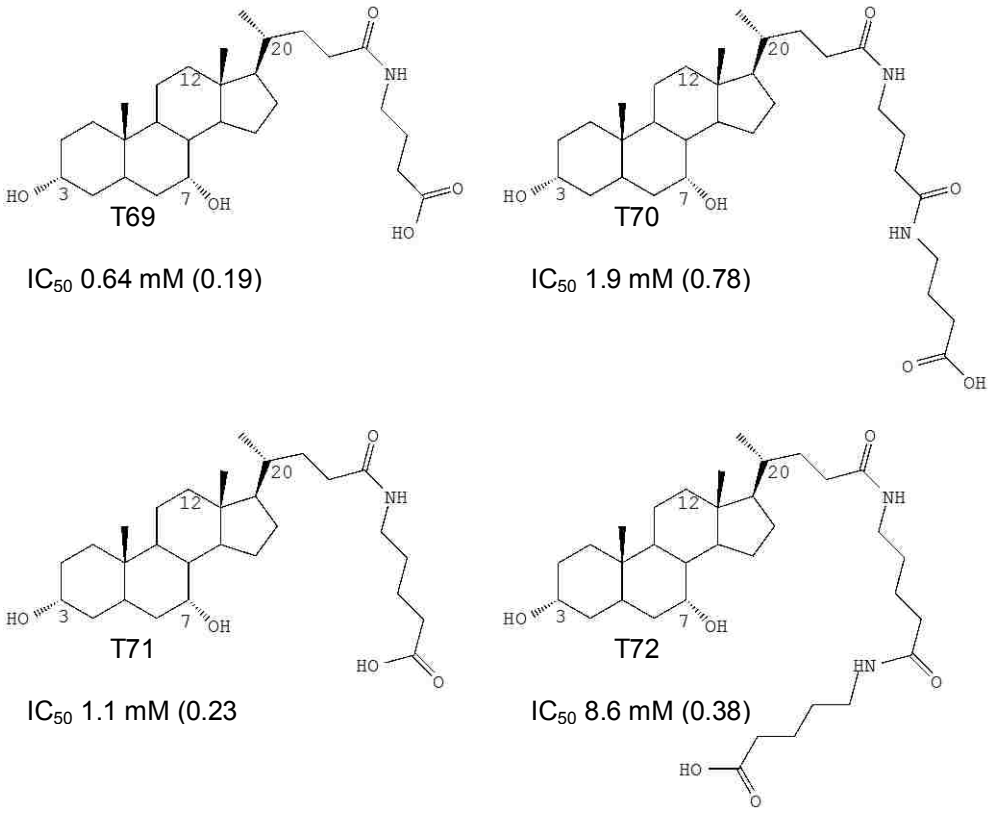


Figure 3.14. Taurochenodeoxycholic acid derivatives with long side chains. Taurochenodeoxycholate analogs [T69 – T72]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses

CamSA consists of a cholate backbone linked to *meta*-benzene sulfonic acid by amide bond. Since chenodeoxycholate and taurochenodeoxycholate inhibit *C. difficile* spore germination, we predicted that a CamSA analog with the chenodeoxycholate backbone would provide a more active inhibitor. Based on the activity of CamSA analogs described previously, CamSA analogs using chenodeoxycholate backbone were prepared and tested for activity with *C. difficile* spores *in vitro.*

Compound T73 is a CamSA analog that does not have the hydroxyl group at position-12 on the cholate backbone. Like CamSA, compound T73 did not activate spore germination. Unlike CamSA, compound T73 is a weak inhibitor of *C. difficile* spore germination with an IC_{50} of 6.5 mM (Fig. 3.15). CamSA is 100 fold more active than compound T73. No hydroxyl group at position-12 resulted in a significant decrease of potency in this CamSA analog. These data suggest that the hydroxyl group at position-12 is optimal (compared to other analogs tested) for binding when the side chain contains an aromatic ring and sulfonic acid.

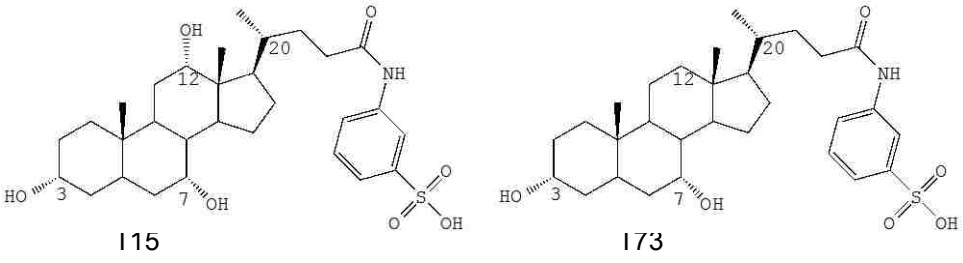


Figure 3.15. CamSA [T15] and CamSA analog T73. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses.

Compound T74 is an analog of compound T30 and contains an unsubstituted aromatic ring. As previously discussed compound T30 was able to inhibit *C. difficile* spore germination. Compound T74 was not recognized by *C. difficile* spores therefore was neither able to activate nor inhibit spore germination (Fig. 3.16). This data suggests that the hydroxyl is necessary for binding when an unsubstituted aromatic ring is part of the analog.

Compound T75 is the analog of compound T32 and has a carboxylic acid at the *meta* position on the aromatic ring. Compound T32 was a weak inhibitor of *C. difficile* spore germination (Fig. 3.16). Compound T75 is a more potent inhibitor of *C. difficile* spore germination than T32 and is active at concentrations three fold lower (IC_{50} 0.93 mM). Interestingly, the analog missing the hydroxyl is more potent than with the hydroxyl in this instance. In previous analogs the hydroxyl group seemed to be helpful for binding and analogs missing the hydroxyl group were less active. Compound T76 is the analog of compound T34 with the carboxylic acid in the *para* position on the benzene ring. Compound T76 was not recognized by *C. difficile* spores (Fig. 3.16). This data suggests that an analog without a hydroxyl at position-12 that has an aromatic ring substituted with a carboxylic acid at the *meta* position only is able to be recognized by the *C. difficile* germination binding site(s). Analogs that do not have a carboxylic acid at the *meta* position require the hydroxyl group at position-12 for optimal binding.

During analysis of CamSA analogs in previous studies, it was observed that the analog of compound T32 with a non-aromatic cyclic substituent [T35] inhibited spore germination. To determine if the loss of the hydroxyl group on the

cholate backbone would interfere with this binding, compound T77 was synthesized. Compound T77 did not inhibit *C. difficile* spore germination however it did activate spore germination with an EC_{50} of 7.4 mM (Fig. 3.16). This is a weak germinant and is active at concentrations 16 fold lower than T32 and 8 fold lower than its aromatic analog T75. This data contradicts earlier observations that the hydroxyl at position-12 seemed to be necessary for activation of *C. difficile* germination. Therefore although a hydroxyl at position-12 is optimal, it is not essential for weak germination activation. On the other hand, there may be other factors that affect the activity of compound T77 that are unclear at this time.

Compound T43 was found to inhibit *C. difficile* spore germination although it does not have an electron withdrawing group on the aromatic ring. To determine if the hydroxyl group at position-12 interferes with the ability of the compound to bind, compound T78 was prepared. Compound T78 is an analog of compound T43 that has an aromatic ring with an amine substituent at the *meta* position. Unlike compound T43, compound T78 neither activated nor inhibited spore germination (Fig. 3.16). As discussed earlier, the ability of T43 to bind is currently unclear. This data suggests that the hydroxyl group at position-12 is necessary for the analog to be recognized. As postulated earlier, the mechanism by which compound T43 binds to *C. difficile* spores may be different than other analogs. More studies with similar compounds are necessary to understand this mechanism better.

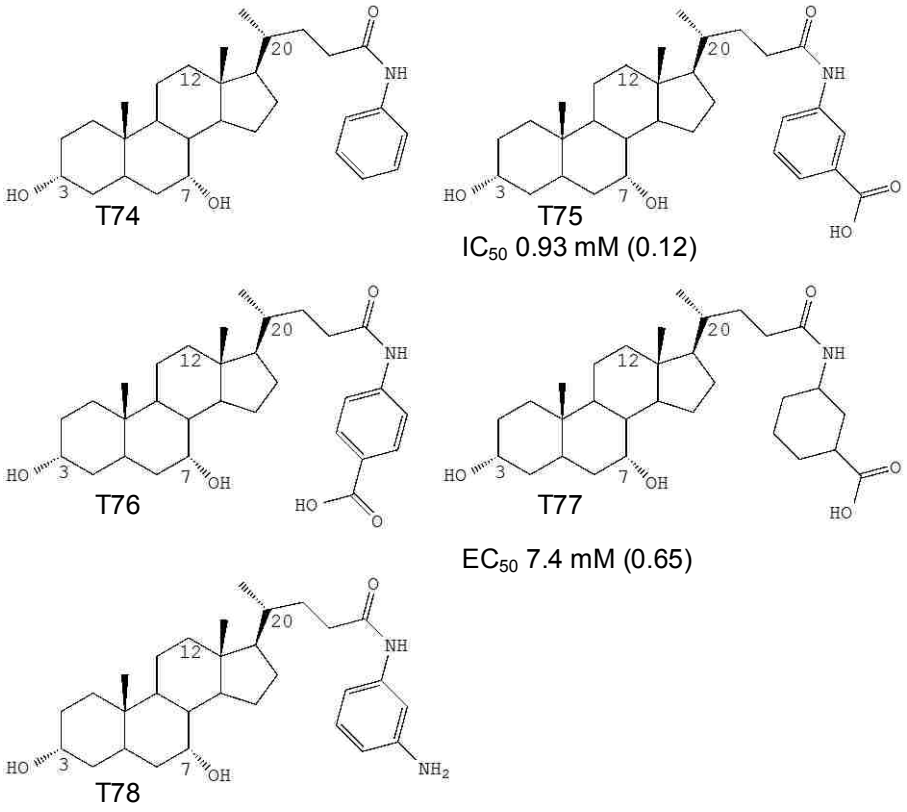


Figure 3.16. Bile salt analogs [T74-T78]. The corresponding EC_{50} and IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

Bile salt analogs with multiple substituents on the aromatic ring and polycyclic aromatic hydrocarbons with specific substituents were found previously to either activate or inhibit *C. difficile* spore germination. Analogs of these active compounds were synthesized using the chenodeoxycholate backbone and tested for activity *in vitro* with *C. difficile* spores. Compound T79 is an analog of compound T48 and has a carboxylic acid group at the *meta*-position and a methyl group at the *para*-position of the aromatic ring. Compound T79 does not activate *C. difficile* spore germination (Fig. 3.17). Like the compound T48, compound T79 prevents *C. difficile* spore germination. Interestingly,

compound T79 is a potent inhibitor with an IC_{50} of 0.092 mM (Fig. 3.17). Compound T79 is active at concentrations 120 fold lower than its analog compound T48, which differs only by the hydroxyl at position-12. Furthermore, T79 is the only inhibitor discovered thus far that can compare to CamSA activity *in vitro.* We would expect that potent inhibitors would require the stronger sulfonic acid. However, these data suggest that as long as there is not a hydroxyl at position-12, the side aromatic group can have a carboxylic acid at the *meta* position and a methyl at the *para* position and be active with *C. difficile* spores.

Compounds T80 and T81 are analogs of compounds T49 and T50 that consist of tri-substituted aromatic side chains with sulfonic acids in the *meta* position. Compound T80 has two methyl groups at the *ortho* and *para* position on the aromatic ring. This analog inhibited spore germination with an IC_{50} of 0.92 mM, six fold more potent than its analog T49 (Fig. 3.17). In contrast to the significant difference between the potency of compound T80 and T49, compound T81 and compound T50 have very similar IC_{50}s, 0.75 mM and 1.1 mM respectively (Fig. 3.17). Neither T80 nor T81 was able to activate spore germination. In some cases the hydroxyl group seems to be nonessential for binding to the *C. difficile* germination binding site(s) but in some cases as just described analogs without the hydroxyl group are bind more efficiently. The chemical properties of the side alkyl group seem to play a part in whether the hydroxyl group is necessary or not. However, with the limited number of compounds used for these studies, a direct correlation cannot be drawn at this time.

The CamSA analog containing naphthalene [T64], was shown to activate *C. difficile* spore germination. Although this compound is not a potent activator, the fact that it is able to activate spore germination is an interesting discovery. To determine the effect the hydroxyl group has on activation, compound T82 was synthesized and analyzed in *C. difficile* spore germination assays. Unlike compound T64, compound T82 was unable to activate spore germination. In contrast, T82 inhibited spore germination with an IC_{50} of 12 mM (Fig. 3.17). Similar to compound T64, T84 is weakly recognized. As previously observed, the hydroxyl at position-12 seems to be optimal for activation of germination. Furthermore, the surprising finding that compound T64 was recognized regardless of its bulkiness is supported by the recognition of compound T82 by the *C. difficile* spore germination binding site(s). In conclusion, none of the CamSA analogs were superior to CamSA as an inhibitor of *C. difficile* spore germination

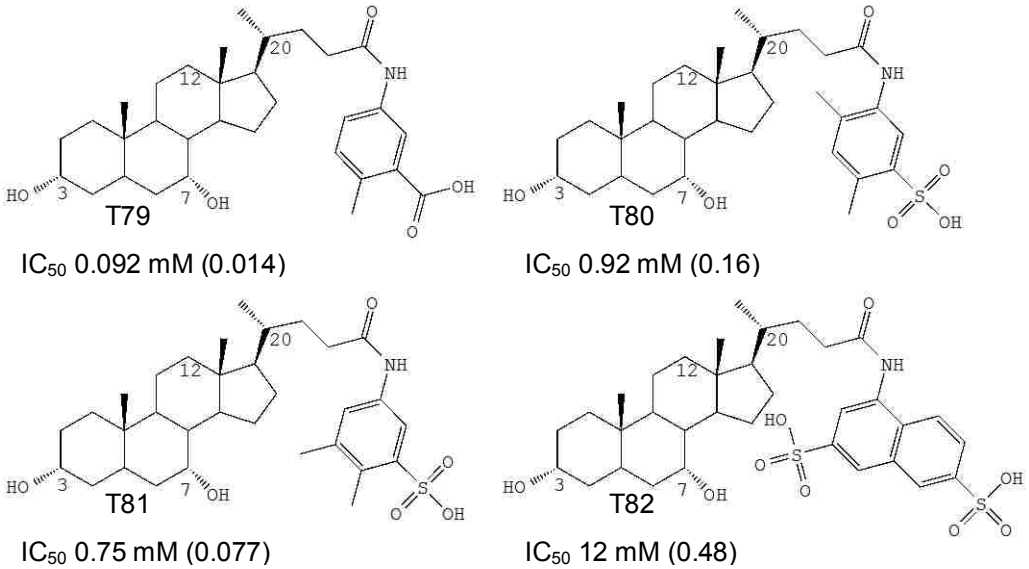


Figure 3.17. Bile salt analogs [T79-T82]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses

3.3.9. Cholic Acid Derivatives

To further characterize the differences between germination and inhibition of simple bile salts, we tested a series of cholic acid derivatives. These analogs differ in the cholic acid alkyl chain length, position of the hydroxyl groups, configuration of hydroxyl groups, methylation of the hydroxyl groups and alterations to the carboxylic acid functional group. The analogs differ from cholic acid or chenodeoxycholate (Fig. 3.18) in up to three functional group changes.

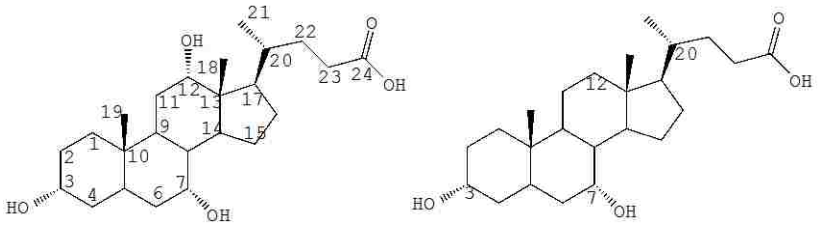
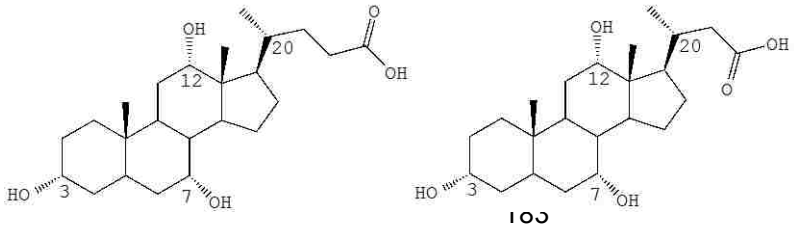


Figure 3.18. Cholic acid (left) and chenodeoxycholate (right)

Cholic acid has an alkyl side chain bonded to a carbon at position-17 of the D ring. This alkyl chain is branched and has a carboxylic acid at position-24 (Fig. 3.18). To determine if a shorter alkyl chain affects *C. difficile* spore germination, compound T83 was tested for activation and inhibition of *C. difficile* spores (Fig. 3.19). Interestingly, unlike cholic acid compound T83 did not induce *C. difficile* spore germination however, it did inhibit spore germination with an IC_{50} of 6.3 mM (Fig. 3.19). This suggests that the longer alkyl chain length is necessary for activation of germination but the shorter chain does not prevent binding by *C. difficile* spores.



T83

Cholic acid IC_{50} 6.3 mM (0.0070)

Figure 3.19. Cholic acid analog T83. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses.

The alkyl side chain in cholic acid has a carboxylic acid group. To determine if the carboxylic acid is required for binding to *C. difficile* spores, two analogs were tested for activation and inhibition of *C. difficile* spore germination. In compound T84, the carboxylic acid has been reduced to an alcohol. In compound T85, the carboxylic acid has been esterified to form the ester derivative. Neither compound T84 nor T85 was able to induce *C. difficile* spore germination but both inhibited germination (Fig. 3.20). Compound T84 was able to inhibit germination with an IC_{50} of 0.33 mM and compound T85 had an IC_{50} of 0.059 mM (Fig. 3.20). Compound T85 is a potent inhibitor of spore germination and is active at almost the same concentration as CamSA. This data suggests that the carbonyl group is necessary for activation of *C. difficile* spore germination. Either the carbonyl group or the hydroxyl group is sufficient for binding and inhibiting *C. difficile* spore germination. A free carboxylic acid seems to be necessary for activation. Carboxylic acids are able to participate in hydrogen bonding and form salt bridges, which may be essential for activation.

Since compound T85 is a very strong inhibitor, analogs of compound T85 with shorter alkyl chain lengths were assessed for inhibition or activation of *C.*

difficile spore. Compound T86 has one less carbon in the alkyl chain and T87 has two fewer carbons in the alkyl chain. Neither compound T86 nor T87 activated *C. difficile* spore germination. However, both bile salts inhibited spore germination. Compound T86 inhibited *C. difficile* spore germination with an IC_{50} of 0.154 mM and compound T87 has an IC_{50} of 0.660 mM (Fig. 3.20). Compound T85 discussed above is more potent than compounds T86 and T87 however; the ester functional group in these compounds is recognized for binding. These data support the earlier observation that the carbonyl group may be the functional group recognized by *C. difficile* spores and that the loss of hydrogen bonding prevents activation of germination. Furthermore the data suggests optimal binding to *C. difficile* spores requires a longer not shorter alkyl side chain.

To determine if a longer alkyl ether linkage interferes with binding, Compound T88 was tested. Compound T88 has an ester with an ethoxy group. Surprisingly, compound T88 is a very strong inhibitor of *C. difficile* spore germination. The IC_{50} for compound T88 is 0.0082 mM (Fig. 3.20). In fact, T88 is seven-fold more potent than CamSA. Compound T88 is the most potent inhibitor of *C. difficile* spore germination discovered in this dissertation project. Evidently the *C. difficile* germination binding site(s) recognizes the ester functional group but without the ability to hydrogen bond, the bile salt does not activate spore germination.

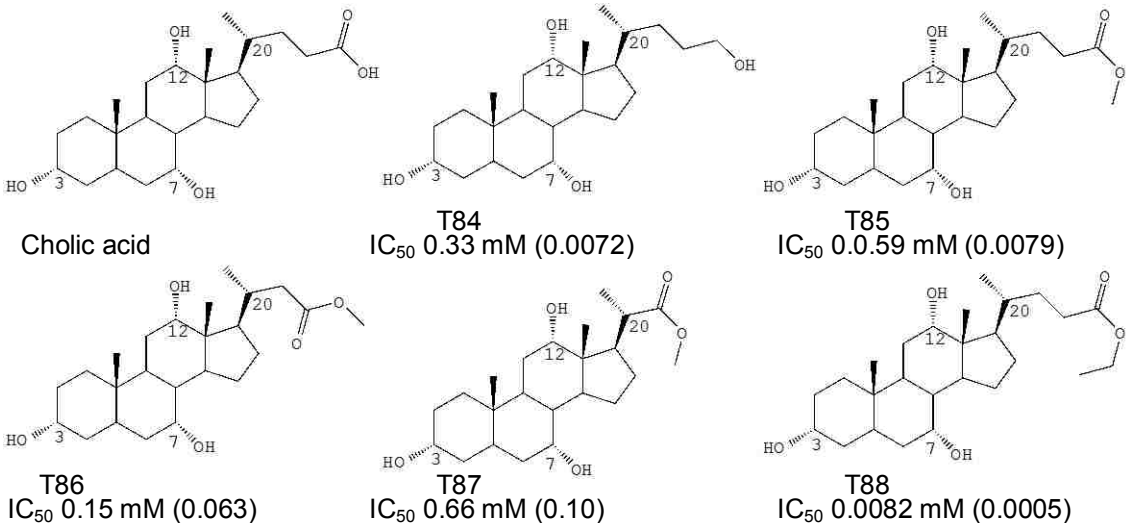


Figure 3.20. Cholic acid analogs [T84 – T88]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

Cholic acid has three hydroxyl groups at positions 3, 7 and 12. To assess whether the specific recognition of these hydroxyl is important for *C. difficile* spore germination, compound T89 was prepared. In compound T89, all three hydroxyls have been converted to methoxy groups. Compound T89 was neither able to activate nor inhibit spore germination (Fig. 3.21). Since compound T89 was unable to bind to *C. difficile* spores, the data suggests that hydrogen bonding ability of hydroxyl groups is essential for recognition. This is supported by previous data showing that the loss of hydrogen bonding ability in taurocholate analogs [T09 and T10] resulted in loss of recognition.

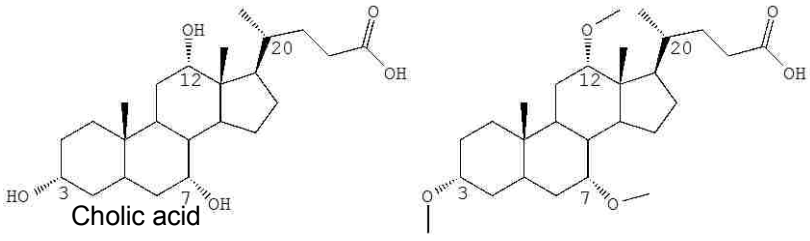


Figure 3.21. Cholic acid analog [T89].

To determine the effects of modification to multiple functional groups have on *C. difficile* recognition, a series of bile salt analogs were obtained and analyzed *in vitro* for activity. Compounds T90 – T94 have hydroxyl groups at position 3 and 12 but not at position 7. Furthermore, these compounds differ in stereochemistry of the hydroxyl groups and two compounds have methyl esters instead of carboxylic acids. Compound T90 differs from cholic acid only in the loss of a hydroxyl group at position-7. Compound T90 inhibited *C. difficile* spore germination with an IC_{50} of 0.19 mM (Fig. 3.22). The hydroxyl group at position-12 in compound T91 is in the beta configuration. Compound T91 neither activated nor inhibited *C. difficile* spore germination. The hydroxyl group at position-3 in compound T92 is in the beta configuration. Compound T92 inhibited spore germination with an IC_{50} of 0.095 mM (Fig. 3.22). Compound T92 is a potent inhibitor of *C. difficile* spore germination and CamSA is active at concentrations only 1.5-fold lower. Compounds T90 – T92 were unable to trigger spore germination. These data suggest that no hydroxyl group at position-7 results in loss of ability to activate *C. difficile* spore germination but not the ability to bind. The configuration of the hydroxyl at position-12 is important for recognition but the configuration of the hydroxyl at position-3 is not important for recognition. However, the optimal configuration for the hydroxyl at position-3 is the alpha configuration. These statements represent the data when there is no hydroxyl at position-7 in cholic acid.

Compound T93 is an analog of compound T90 that contains a methyl ester instead of a carboxylic acid. Similar to compound T90, compound T93 was

unable to activate germination but inhibited *C. difficile* spore germination with an IC_{50} of 0.097 mM (Fig. 3.22). Compound T93 is a potent inhibitor of *C. difficile* spore germination and is two fold more active than compound T90. This finding supports earlier data showing that ester derivatives are more potent inhibitors of *C. difficile* spore germination. Furthermore, the data supports that the hydroxyl group at position 7 is not important for recognition but is important for activation.

Compound T94 is an analog of compound T91 but with a methyl ester and the hydroxyl at position-12 is in the beta configuration. Compound T91 was unable to activate *C. difficile* spore germination. However, compound T94 inhibited germination with an IC_{50} of 0.095 mM (Fig. 3.22). Like compound T93, compound T94 is a potent inhibitor of *C. difficile* spore germination. These findings are interesting since the analog of compound T94 with a free carboxylic acid and a hydroxyl at position-12 in the beta configuration [T91] was unable to bind to *C. difficile* spores. Based on results discussed above, the alpha configuration of the hydroxyl at position-12 seemed to be essential for binding. These results show that as long as there is an ester group the configuration of the hydroxyl is not important for binding. Furthermore, the activity of methyl esters is supported by these findings. The strong interaction between the ester and the binding region in *C. difficile* spores overcomes suboptimal configuration of the hydroxyl group.

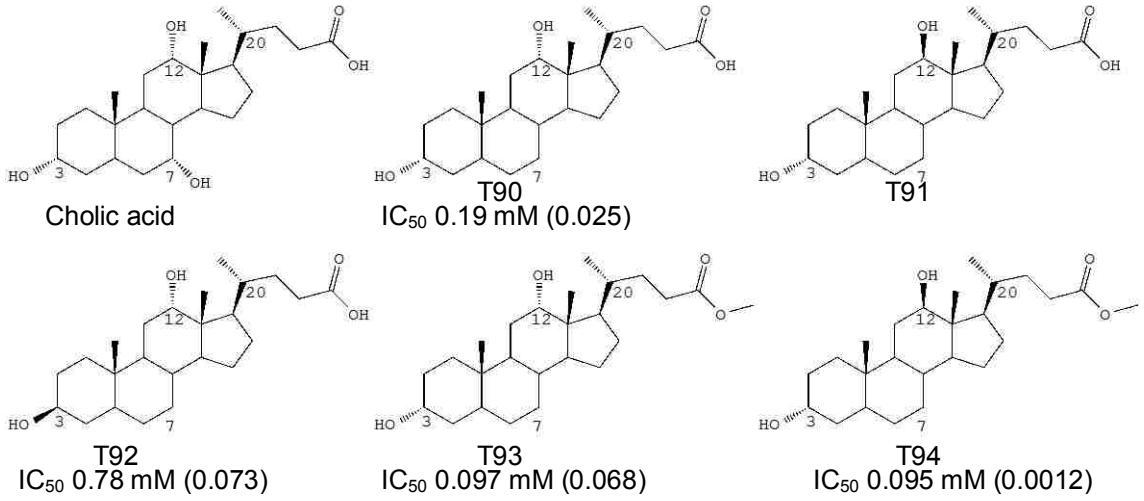
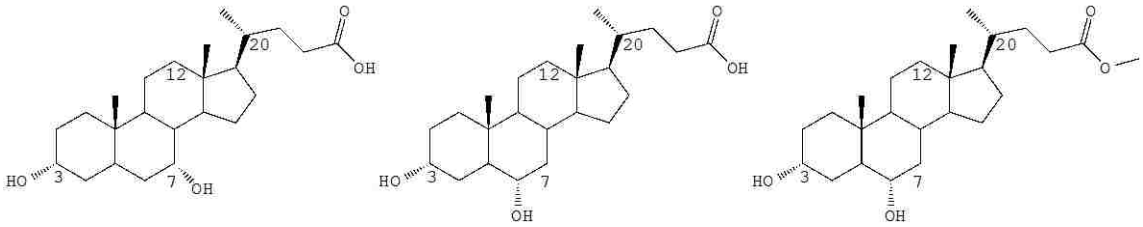


Figure 3.22. Cholic acid analogs [T90 – T94]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

Compounds T95 and T96 both lack a hydroxyl group at position 12 and position 7. However, they both have two hydroxyls in the alpha configuration at position-3 and position-6. Compound T95 inhibited *C. difficile* spore germination with an IC_{50} of 1.2 mM (Fig. 3.23). Compound T96 is the methyl ester analog of compound T95. Compound T96 inhibited spore germination with an IC_{50} 0.037 mM, more than 33 times lower concentration than T95 (Fig. 3.23). Furthermore, T96 is approximately 1.5-fold more potent than CamSA. Neither compound T95 nor T96 was able to activate spore germination. This data suggests that the hydroxyl at position 12 is not necessary for binding. The hydroxyl at position-7 is also not essential as long as there is a hydroxyl at position-6. Furthermore, a hydroxyl at position-6 does not interfere with binding to the *C. difficile* germination binding site(s). Ester functional groups increase the activity of a compound significantly as observed in this data set.



Cholic acid T95 T96

IC_{50} 1.2 mM (0.11) IC_{50} 0.037 mM (0.021)

Figure 3.23. Cholic acid analogs [T95 and T96]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses.

Compounds T97 – T99 have only one hydroxyl group and no carboxylic acid. Compound T97 has an alpha hydroxyl group at position-3 and the carboxylic acid is reduced to an alcohol. Compound T97 was neither able to activate nor inhibit *C. difficile* spore germination (Fig. 3.24). Since compound T84 was able to bind to *C. difficile* spores with an alcohol alkyl chain, it is unlikely that the alcohol is solely responsible for the loss of binding. Optimal binding in *C. difficile* spores is a multi-faceted approach. Only one hydroxyl group paired with an alcohol functional group in the alkyl chain is probably does not have enough optimal functional groups for binding. Compound T98 has a hydroxyl group in the alpha configuration at position-12 and a methyl ester. Compound T98 was unable to activate spore germination however inhibited with an IC_{50} of 0.37 mM (Fig. 3.24). As discussed previously, the methyl ester compound is strongly recognized by the *C. difficile* germination binding site(s) therefore, the presence of this functional group outweighs the less optimal conditions of few hydroxyls on the cholic acid backbone. Hydroxyl groups at positions-3 and -7 are not necessary for binding as long as there is a methyl ester present on the compound. Compound T99 has a hydroxyl at position-3 but it is in the beta

configuration. Compound T99 also has a methyl ester functional group in the alkyl chain. As expected, compound T99 did not trigger germination but was able to inhibit *C. difficile* spore germination with an IC_{50} of 1.3 mM (Fig. 3.24). This data suggests that a hydroxyl at position 3 also requires an ester group for efficient binding. Hydroxyls at positions-7 and -12 are not necessary as long as there is a methyl ester functional group. Furthermore, the IC_{50} for compound T99 is higher than other methyl esters. It is probable that the methyl ester activity is able to overcome the suboptimal binding ability resulting from less hydroxyl groups on the cholate backbone. However, since the hydroxyl at position-3 is in the beta configuration and not in the optimal alpha configuration, the recognition is not as sensitive as other analogs. In conclusion, the data shows that as long as there is an ester in the side chain of the compound, the requirements for hydroxyl groups at specific positions and configurations are not essential.

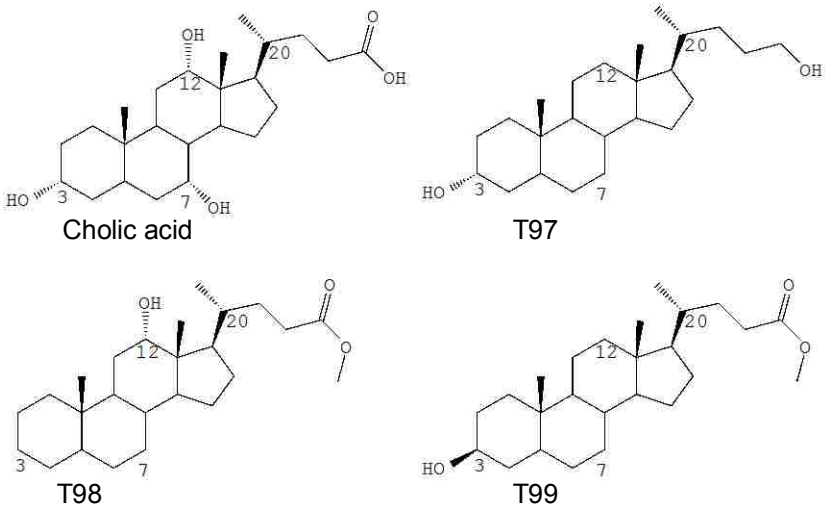


Figure 3.24. Cholic acid analogs [T97 – T99]. The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

Table 3.1. Effect of bile salt analogs on *C. difficile* spore germination.

Compound No.	Modifications to taurocholate side chain	EC_{50}[a] (stdev) mM	IC_{50}[b] (stdev) mM
T23	$NH(CH_2)_4COOH$	NA	5.3 (0.24)
T24	$NH(CH_2)_4CONH(CH_2)_4COOH$	NA	NA
T25	$NH(CH_2)_5COOH$	NA	2.3 (0.075)
T26	$NH(CH_2)_5CONH(CH_2)_5COOH$	NA	NA
T27	$NH(CH_2)_2OSO_3H$	NA	NA
T28	$S(CH_2)_2SO_3H$	NA	NA
T29	$NHCH_2PO_3H$	NA	NA
T30	NHC_6H_5	NA	0.27 (0.070)
T31	NHC_5H_4N	NA	NA
T32	NH(*m*-(C_6H_4))COOH	NA	3.1 (0.78)
T33	NH(*o*-(C_6H_4))COOH	NA	NA
T34	NH(*p*-(C_6H_4))COOH	NA	1.4 (0.11)
T35	NH(*m*-(C_6H_8))COOH	NA	0.47 (0.069)
T36	NH(*m*-(C_6H_4))$COOCH_3$	NA	NA
T37	NH(*m*-(C_6H_4))OPO_3H_2	NA	NA
T38	NH(*m*-(C_6H_4))OH	NA	NA
T39	NH(*o*-(C_6H_4))OH	NA	NA
T40	NH(*m*-(C_6H_4))SH	NA	NA
T41	NH(*p*-(C_6H_4))SH	NA	NA
T42	NH(*m*-(C_6H_4))SCH_3	NA	NA
T43	NH(*m*-(C_6H_4))NH_2	NA	0.53 (0.022)
T44	NH(*m*-(C_6H_4))CH_3	NA	NA
T45	NH(*o*-CH_3-*m*-(C_6H_4))SO_3H	NA	NA
T46	NH(*o*-OCH_3-*m*-(C_6H_4))SO_3H	4.6 (0.34)	NA
T47	NH(*o*-CH_3-*m*-(C_6H_4))COOH	16 (0.90)	NA
T48	NH(*p*-CH_3-*m*-(C_6H_4))COOH	NA	11 (0.025)
T49	NH(*o,p*-$(CH_3)_2$-*m*-(C_6H_4))SO_3H	NA	5.6 (0.042)
T50	NH(*p,m*-$(CH_3)_2$-*m*-(C_6H_4))SO_3H	NA	1.1 (0.098)
T51	NCH_3(*m*-(C_6H_4))SO_3H	5.4 (0.10)	NA
T52	NH_2(*m*-(C_6H_4))SO_3H	NA	NA
T53	$NHCH_3$(*m*-(C_6H_4))SO_3H	NA	NA
T54	NH(1-$C_{10}H_8$)	NA	NA
T55	NH(2-$C_{10}H_8$)	NA	NA
T56	NH($C_{14}H_{10}$)	NA	NA
T57	NH($C_{16}H_{10}$)	NA	NA
T58	NH($C_{10}H_8$)-3-OH	NA	NA
T59	NH($C_{10}H_8$)-1-SO_3H	NA	NA
T60	NH($C_{10}H_8$)-1-CO_2H	NA	NA

T61	$NH(C_{10}H_8)$-5-CO_2H	1.3 (0.19)	NA
T62	$NH(C_{10}H_8)$-3-OH-5-SO_3H	NA	NA
T63	$NH(C_{10}H_8)$-1,6-SO_3H	NA	NA
T64	$NH(C_{10}H_8)$-2,6-SO_3H	5.7 (0.26)	NA
T65	$NH_2(C_{10}H_8)$-1,5-SO_3H	NA	NA
Compound No.	Modifications to taurochenodeoxycholate side chain	EC50 (stdev) mM	IC50 (stdev) mM
T66	$NHCH_2SO_3H$	0.31 (0.023)	NA
T67	$NH(CH_2)_2SO_2H$	NA	5.3 (0.20)
T68	$NH(CH_2)_2COOH$	NA	3.9 (0.80)
T69	$NH(CH_2)_4COOH$	NA	0.64 (0.19)
T70	$NH(CH_2)_4CONH(CH_2)_4COOH$	NA	1.9 (0.78)
T71	$NH(CH_2)_5COOH$	NA	1.1 (0.23)
T72	$NH(CH_2)_5CONH(CH_2)_5COOH$	NA	8.6 (0.38)
T73	NH(*m*-(C_6H_4))SO_3H	NA	6.5 (0.20)
T74	NHC_6H_5	NA	NA
T75	NH(*m*-(C_6H_4))COOH	NA	0.93 (0.12)
T76	NH(*p*-(C_6H_4))COOH	NA	NA
T77	NH(*m*-(C_6H_8))COOH	7.4 (0.65)	NA
T78	NH(*m*-(C_6H_4))NH_2	NA	NA
T79	NH(*p*-CH_3-*m*-(C_6H_4)COOH	NA	0.092 (0.014)
T80	NH(*o,p*-$(CH_3)_2$-*m*-(C_6H_4))SO_3H	NA	0.92 (0.16)
T81	NH(*p,m*-$(CH_3)_2$-*m*-(C_6H_4))SO_3H	NA	0.75 (0.077)
T82	$NH(C_{10}H_8)$-2,6-SO_3H	NA	12 (0.48)
Compound No.	Modifications to cholic acid	EC50 (stdev) mM	IC50 (stdev) mM
T83	Shorter alkyl chain	NA	6.3 (0.0070)
T84	Alcohol side chain	NA	0.33 (0.0072)
T85	Methyl ester side chain	NA	0.059 (0.0079)
T86	Methyl ester side chain - shorter alkyl chain by 1 carbon	NA	0.15 (0.063)
T87	Methyl ester side chain - shorter alkyl chain by 2 carbon	NA	0.66 (0.10)
T88	Ethyl ester side chain	NA	0.0082 (0.00050)
T89	Methoxylated hydroxyl groups	NA	NA
T90	Hydroxyls at 3 (α) and 12 (α)	NA	0.19

			(0.025)
T91	Hydroxyls at 3 (α) and 12 (β)	NA	NA
T92	Hydroxyls at 3 (β) and 12 (α)	NA	0.78 (0.073)
T93	Methyl ester with hydroxyls at 3 (α) and 12(α)	NA	0.097 (0.068)
T94	Methyl ester with hydroxyls at 3 (α) and 12 (β)	NA	0.095 (0.0012)
T95	Hydroxyls at 3 (α) and 6 (α)	NA	1.24 (0.11)
T96	Methyl ester with hydroxyls at 3 (α) and 6 (α)	NA	0.037 (0.021)
T97	Alcohol with hydroxyl at 3 (α)	NA	NA
T98	Methyl ester with hydroxyl at 12 (α)	NA	0.37 (0.051)
T99	Methyl ester with hydroxyl at 3 (β)	NA	1.3 (0.54)

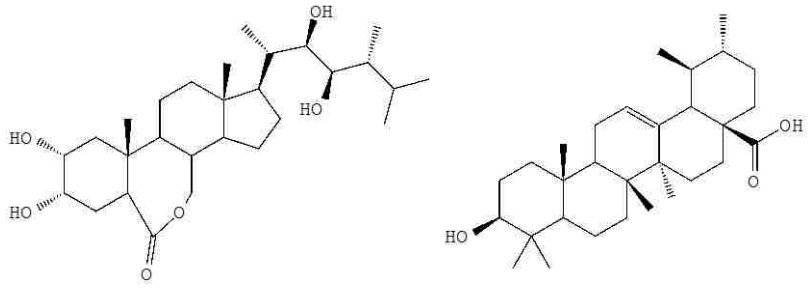
[a] *C. difficile* spores were individually treated with 6 mM glycine and bile salt analogs. Standard deviations are shown in parentheses.
[b] *C. difficile* spores were incubated with bile salt analogs for 15 min prior to the addition of 6 mM taurocholate and 12 mM glycine. Standard deviations are shown in parentheses.
[c] NA, no change in absorbance after 90 minutes under the conditions tested thus no statistics could be performed.

3.3.10. Plant-Derived Cholesterol Analogs

Bile salts found in mammals are essentially cholesterol analogs that are synthesized from cholesterol in the liver (143). Cholesterol analogs are also found in plants and vary in function. Some plant-derived cholesterol analogs are potent toxins and fatal when ingested and some are used medically. Others serve as growth hormones and are essential to plant metabolic activity making them useful agriculturally. During bile salt analog screening for *C. difficile* spore binding, several cholesterol analogs derived from plants were available for purchase. Although they differ from bile salts structurally, there are similarities in ring orientation or alkyl side chains. The focus of this project is to map the interactions between bile salt analogs and *C. difficile* spores therefore, all data is helpful to better understand the putative germination binding site(s).

Epibrassinolide is in a class of steroid plant hormones called brassinosteroids that stimulate various physiological plant cell processes and prevent infection (171, 172). When tested for activation or inhibition of *C. difficile* spore germination, epibrassinolide was unable to trigger germination. However, epibrassinolide inhibited *C. difficile* spore germination with an IC_{50} of 0.076 mM (Fig. 3.25). Epibrassinolide is a slightly less potent inhibitor of *C. difficile* germination than CamSA. It is unclear at this time how epibrassinolide is able to bind to the putative germination site(s). Although previous studies have shown that the *C. difficile* germination binding site(s) is very sensitive to methyl and ethyl esters, no cyclic esters were studied. It is possible that this sensitivity toward esters allows the germination binding site(s) to overcome the other differences in the structure of epibrasssinolide.

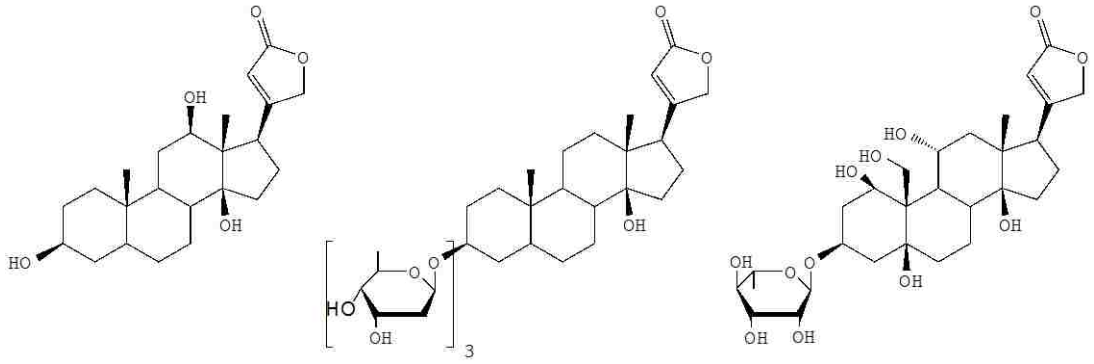
Ursolic acid, found in high concentrations in apple peels, has been found to inhibit muscular atrophy-associated gene expression in humans and in mice (173, 174). Ursolic acid was unable to bind *C. difficile* spores (Fig. 3.25). It is likely that the lack of hydroxyls and the bulkiness of the uppermost rings prevent proper *C. difficile* binding.



IC_{50} 0.076 mM (0.0015)

Figure 3.25. Plant-derived cholesterol analogs. Epibrassinolide (left) and ursolic acid (right). The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

Some steroids found in plants have important impacts on biological systems. Digoxigenin, ouabain, and digitoxin are three such steroids (175-180). These toxins contain the four cholesterol rings and an α,β–unsaturated lactone. An α-methylene-γ-lactone is an electrophilic functional group capable of participating in Michael additions with biological nucleophiles such as sulfhydryl groups of cysteine residues forming an irreversible adduct (181-184). These compounds may be used as molecular probes to irreversibly bind the binding site(s) for characterization. Unfortunately, neither digoxigenin nor digitoxin was able to bind to *C. difficile* spores. Although ouabain did not trigger *C. difficile* spore germination, it did inhibit with an IC_{50} of 7.3 mM (Fig. 3.26). Ouabain has multiple functional groups on the cholate backbone and a side chain of L-rhamnose sugar. Besides the cholate backbone the structure of ouabain is quite different than taurocholate and other bile salt analogs tested. The mechanism by which ouabain binds to *C. difficile* spores is unclear. However, ouabain could potentially be used as a probe to determine the binding site(s) residues in *C. difficile*.



IC_{50} 7.3 mM (0.050)

Figure 3.26. Plant-derived steroids. Digoxigenin (left), digitoxin (middle) and ouabain (right). The corresponding IC_{50} value is listed below each compound with the standard deviation in parentheses. Those without a listed value had no activity under the conditions tested.

3.3.11. Fluorinated Cholic Acid Derivative

The cholic acid derivative containing 17 fluorines was synthesized for a different project. However, since the *C. difficile* germination binding site(s) are unknown, all data generated from *in vitro* kinetic studies will help characterize the binding region for bile salts. The fluorinated compound was unable to activate *C. difficile* spore germination and was also unable to inhibit germination (Fig. 3.27). The large concentration of fluorine in the alkyl chain will increase the overall negativity of the compound. This strong negative charge and the bulky side group likely affected this compound from being able to bind to *C. difficile* spores.

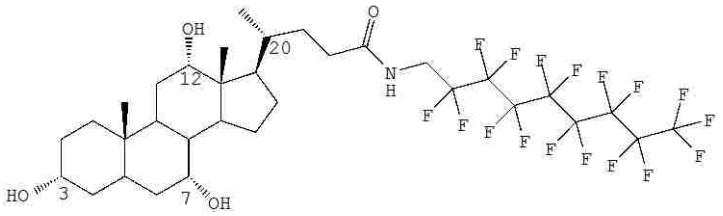


Figure 3.27. Fluorinated cholic acid derivative. This compound had no activity under the conditions tested.

3.4. Conclusions

The putative taurocholate binding site(s) in *C. difficile* spores is a very complicated structure. The microenvironment is quite complex with respect to functional groups that are recognized for binding and activating germination and binding but inhibiting germination. Unfortunately, the data and results generated from this project were not able to completely describe the requirements for *C. difficile* bile salt recognition by the putative bile salt binding site(s). Although we hoped to find obvious patterns in functional groups and spatial arrangements necessary for binding, only a few final conclusions can be drawn. Many compounds will require further screening to understand how they interact with the germination binding site(s). On the other hand, until this project very little was known about the microenvironment of the *C. difficile* taurocholate germination binding site(s). The fact that some correlations between binding and recognition were observed during this study is a stepping-stone in *C. difficile* spore germination research.

Taurocholate, the natural bile salt activator of *C. difficile* spore germination, has a linear (taurine) side chain bound to cholate by an amide bond. The number of carbons in the taurine side chains is two carbons. Our results suggest that inhibitors with an even number of carbons in the alkyl side chain are more active than their odd numbered counterparts. Furthermore, the activity diminishes with increased chain length. Although some odd numbered alkyl chain analogs were able to interact with *C. difficile* spores, their activity required much higher concentrations.

In chapter 2, the *meta*-benzene sulfonic acid analog (CamSA, [T15]) was found to be a potent inhibitor of taurocholate-mediated germination. In this study, CamSA analogs were produced and assessed for activity with *C. difficile* spores in attempt to understand why CamSA is an effective inhibitor. The aromatic ring in CamSA is partially responsible for activity since an analog with no sulfonic acid substituent was able to inhibit spore germination. However, the sulfonic acid moiety is optimal for binding. Phenolic groups negatively affected the ability of the analog to bind to the *C. difficile* germination binding site(s). In general, the putative germination binding site(s) requires electronegative functional groups that cause an electron withdrawing effect on aromatic rings. Strong acid functional groups, specifically sulfonic acids, are preferred over weak acid functional groups. Oxygen atoms in the functional group (except hydroxyl) on aromatic rings are necessary for recognition. In contrast, sulfur is not essential since carboxylic acids are recognized by the *C. difficile* germination binding site(s).

Since CamSA could be used as a preventative for CDI in animal studies, it will come into contact with bile salt hydrolases in the gut of animals. To make CamSA more stable, the amide linkage was methylated (188). However, this modified analog was no longer able to inhibit spore germination but activated germination. Although it is unclear how compound T51 activates germination, the fact that it no longer inhibits spore germination renders it impractical for animal studies or bile salt hydrolase studies.

Polycyclic aromatic hydrocarbons (PAH) consist of two or more fused benzene rings that are flat and very rigid molecules. When cholate was coupled with PAHs with two to four fused aromatic rings, the analog was weakly recognized or not at all by *C. difficile* spores. Interestingly, two bile salt analogs containing naphthalene and a carboxylic acid or a sulfonic acid at position 4 and 5 were recognized and activated *C. difficile* spore germination, albeit weakly. Acid moieties at other positions (of naphthalene) had no effect on *C. difficile* spore germination. Clearly, there is an interaction that can be made with the *C. difficile* binding pocket when the molecule has the acid in this specific placement on naphthalene. It is unclear at this time how this interaction occurs. We expect that the *C. difficile* binding region for bile salts is dynamic in structure allowing functional groups to bind that have certain size, shape, and chemical properties.

Taurochenodeoxycholate is a natural bile salt that inhibits *C. difficile* spore germination *in vitro* and differs from taurocholate in the lack of a hydroxyl group at position-12 in the cholate backbone. Taurochenodeoxycholate analogs with linear side chains have similar activity on *C. difficile* spore germination. The hydroxyl at position-12 is not necessary for binding and activating germination as long as the chain is short. Longer alkyl side chains with carboxylic acid functional groups are unable to activate spore germination but are able to bind to the *C. difficile* germination binding site(s). Similar to the linear chain taurocholate analogs, taurochenodeoxycholate linear analogs were more effective when the carbons in the alkyl chain were an even number.

Most compounds that were able to bind as the cholate (backbone) analogs were also able to bind as the chenodeoxycholate analogs. In some cases, no hydroxyl group at position-12 resulted in increased activity. A compound was discovered in this study that is only about 1.5-fold less potent than CamSA. More studies will be needed to determine if this compound is a candidate for pharmacokinetics and animal studies.

The hydroxyl group at position-12 is necessary for activation of germination. Except one analog, all other analogs that activated spore germination as the cholate analog were unable to activate germination without the hydroxyl group. Correlations between hydroxyl groups and side chains are difficult to make conclusively with the data provided. More analogs will need to be tested for activity in *C. difficile* spore germination to define patterns and explain inconsistencies.

Cholic acid is an unconjugated bile salt with a carboxylic acid moiety. The side chain length is important for activation of *C. difficile* spore germination. The carboxylic acid moiety and the length of the alkyl chain are optimal for activation of germination by cholic acid. Modifications to the side chain did not prevent recognition by *C. difficile* spores. As long as an oxygen atom was present, the bile salt was able to bind and inhibit spore germination. In fact, an ester functional group in place of the carboxylic acid resulted in potent inhibition of *C. difficile* spore germination. An ester with an ethoxy group inhibited spore germination at concentrations eight fold lower than CamSA. This is the most potent inhibitor discovered to in this project. The sum of these data suggest that

carboxylic acids on unconjugated bile salts are optimal for activation of *C. difficile* spore germination, longer not shorter alkyl chains are optimal for recognition and ester groups with ethoxy groups are potent inhibitors of *C. difficile* spore germination.

The results from these studies suggest that the hydroxyl group at position-12 is not essential for *C. difficile* spore recognition. The hydroxyl group at position-7 is essential for activation of *C. difficile* spore germination when there are hydroxyls at position-3 and -12 or at position-3 and -6 as well as a free carboxylic acid moiety on the alkyl chain. However, the hydroxyl group at position-7 is not essential for binding unless the hydroxyl at position-12 is in the beta configuration. The alpha configuration of the hydroxyl at position-12 is optimal for binding when there is a carboxylic acid and no hydroxyl at position-7. This requirement is void however if the carboxylic acid functional group is replaced with an ester group. The strong recognition of the ester overcomes the unrecognized configuration of the hydroxyl at position-12. The hydroxyl at position-3 can be in either configuration for recognition by *C. difficile* spores but the alpha configuration is preferred. As long as there is an ester in the alkyl chain there can be one hydroxyl at either position-3 or position-12 and the bile salt can be recognized. The results from these studies provide strong evidence that the *C. difficile* germination binding site(s) is quite sensitive to ester functional groups.

Although many of the cholic acid analogs were able to bind to *C. difficile* spores, none were able to activate spore germination. The requirements for activation by cholic acid are very specific. There must be three hydroxyl groups

all in the alpha configuration and located on position-3, -7, and -12. A carboxylic acid functional group must be present on the alkyl chain. Any changes to these requirements result in loss of activation but not binding, suggesting a combined role for these germinant functional groups. In conclusion, the functional groups and arrangements of functional groups are not flexible for activation of *C. difficile* spore germination. Ester containing compounds are highly active at binding to *C. difficile* spores, suggesting a specific binding region that this functional group is able to occupy.

This project analyzed many bile salt analogs in attempt to map the interactions between bile salts and *C. difficile* spores. Some correlations between functional groups and activity were discovered however many interactions are still uncharacterized. Another focus of this study was to identify compounds that can inhibit *C. difficile* spore germination *in vitro*. Many inhibitors were discovered however only a few are as good as or better than CamSA. Unfortunately, the bile salts and analogs that were superior to CamSA are also very expensive commercially and difficult to produce. Based on cost and activity, CamSA is the best current candidate for pharmacokinetic characterization and animal studies of CDI.

CHAPTER 4

IN VITRO CHARACTERIZATION OF CAMSA

4.1. Introduction

The *meta*-benzene sulfonic acid derivative of taurocholate, CamSA, is a potent inhibitor of *C. difficile* spore germination *in vitro*. In fact, CamSA is active at concentrations 250-fold lower than taurocholate, the natural germinant. Furthermore, CamSA is 4-fold lower than chenodeoxycholate, the natural inhibitor. CamSA was also chosen as the lead compound for *in vitro* characterization due to the high yields obtained and the relative ease of purification. The procedure of coupling cholic acid to *meta*-aminobenzene sulfonic acid yields >80% of pure CamSA. The sodium salt of CamSA has high solubility in water and DMSO compared to other analogs. The ethyl and methyl esters of cholate derivatives [T85, T88, and T96] have very low IC_{50}s *in vitro.* However, these compounds are highly insoluble in water and are expensive comparatively to produce. Based on these criteria CamSA is the most practical choice for characterization *in vitro* for potential CDI chemotherapeutic use.

Pharmacokinetic analysis of anti-germinants is a necessary step in determining potential novel therapeutic agents for CDI. The fate of a drug depends upon its absorption, distribution, metabolism, and excretion (ADME). *In vitro* characterization can predict the ADME parameters prior to *in vivo* testing (189). Furthermore, guidelines describe that potential drugs must be safe, delivered to the target site, manufactured at minimal cost, and stable (190, 191). The bioavailability of any drug must be determined to ensure proper distribution

and dosage to target tissues/organs and limited distribution outside of the target areas (189, 192). Oral medications must also be stable to the changing environments of the gastro-intestinal tract (193). Because, *C. difficile* spores germinate in the GI tract and the resulting infection is localized to the intestine, proper treatment should target the intestinal tract and have low absorption into the blood and other tissues (35).

Mammalian cell culture has been used extensively to predict the cytotoxicity of potential drugs on different tissues (194-196). Comparison of basal cell parameters to varying drug concentration allows researchers to predict whether organ-specific toxin events may occur. Assessing the cytotoxicity of drugs for CDI on epithelial cells and immune cells provides information about toxicity to cells in direct contact with the bacterium and the drug.

C. difficile toxins cause severe damage to the epithelial cells in the intestinal tract resulting in characteristic symptoms of CDI (78). Due to the drastic difference in *in vitro* growth conditions between cultured mammalian cells and *C. difficile*, toxins secreted during bacterial growth are often used to assess the effects on cultured cells (197, 198). This is an indirect way to measure whether *C. difficile* spores have germinated into toxin-producing cells and whether toxin production is sufficient to affect the viability of cultured mammalian cells.

Based on the criteria described above, CamSA was assessed for several pharmacokinetic parameters *in vitro* prior to animal studies. The effect of CamSA on bacterial proliferation was observed using common enteric bacteria. CamSA stability in simulated gut microenvironments was determined. The apparent

permeability of CamSA through mammalian intestinal lining was predicted. Finally using cell culture, CamSA was assessed for cytotoxicity and for the ability to prevent toxin-induced cell death. The purpose of this project was to determine if CamSA is a good candidate for animal studies.

4.2. Materials and Methods

4.2.1. General comments

Bile salts and amino analogs were purchased from Sigma Aldrich Corporation (St. Louis, MO). CamSA was synthesized in the Abel-Santos laboratory as described previously. Thin layer chromatography silica gel 60 F_{254} was purchased from EMD Chemicals (Gibbstown, NJ). Bacterial cultures, tissue cultures, and media were purchased from the ATCC (Manassas, VA). CellTiter-Glo luminescent cell viability assay was purchased from Promega (Madison, WI).

4.2.2. Effect of CamSA on Bacterial Growth

Laboratory strains of *Bacillus cereus*, *E. coli*, *Bifidobacterium longum*, *Lactobacillus gasseri*, *Clostridium difficile* strains 630, and *C. difficile* strain VPI 10463 were inoculated from freezer stock onto appropriate agar medium as directed by ATCC. Plates were incubated overnight at 37 °C either aerobically (*L. gasseri*, *B. cereus,* and *E. coli*) or anaerobically (*C. difficile* and *B. longum*). Single cell clones were carefully selected and used to inoculate 5 mL of liquid medium. Inoculated broth was shaken at 37 °C for approximately four hours until optical density at 580 nm reached 0.8 representing exponential phase of growth (199). Bacteria were sub-cultured (1:100) into fresh media supplemented with 0 or 10 mM CamSA, taurocholate, or chenodeoxycholate. Optical density was

recorded at time 0 and then at 1, 2, 3, 4, 6, 8, and 24 hours post inoculation. Growth to stationary phase was determined when optical density reached OD_{580} >1.5. Bacterial growth was monitored on a ThermoElectron Biomate 5 spectrophotometer at 580 nM. Cultures supplemented with bile salts were compared to the control cultures grown under normal conditions.

4.2.3. Stability of CamSA in Artificial Gastric and Intestinal Juice

CamSA was analyzed for stability in simulated gastric and intestinal juices as published (193). Artificial gastric juice (no pepsin) is a 0.05 M sodium chloride solution adjusted to pH 1.5 with HCl. Artificial intestinal juice (no pancreatin) is a 0.05 M sodium dihydrogen phosphate buffer at pH 6.8 (193). CamSA (100 mg) was added to 1 ml of either artificial intestinal or artificial gastric juice and incubated at 37 °C for 24 hours. Aliquots were taken at 4, 8, 12, and 24 hours. Samples (1 µl) were spotted on silica thin layer chromatography (TLC) plates and allowed to dry. Plates were developed with 75% ethyl acetate/methanol. TLC plates were visualized by spraying with 10% wt/vol phosphomolybdic acid (PMA)/ethanol solution followed by heating at 100 °C for 2 minutes. Quantification of CamSA was determined using a GE Healthcare Typhoon 9410 Variable Mode Imager and analyzed using ImageQuant TL 5.2 software.

4.2.4.Stability of CamSA with Bile Salt Hydrolase-Producing Bacteria

Bifidobacterium longum, *Lactobacillus gasseri*, and *Escherichia coli* DH5-α were obtained from ATCC. *B. longum* was streaked for single colonies on tryptic soy agar (TSA) supplemented with 5% defibrinated sheep blood and incubated overnight anaerobically at 37 °C. *L. gasseri* was streaked for single colonies on

Difco *Lactobacillus* MRS (de Man, Rogosa, Sharpe) agar. *E. coli* was streaked for single colonies on Luria-Bertani (LB) agar. Both *L. gasseri* and *E. coli* were incubated overnight aerobically at 37 °C. Individual colonies were chosen to inoculate appropriate media and adjusted to an OD_{580} of 1.0 with fresh media supplemented with 6 mM CamSA. As control, bacterial cultures were separately incubated with taurocholate, cholic acid, taurodeoxycholate, and chenodeoxycholate. Bacterial cultures/bile salt mixtures were incubated at 37 °C for 24 hours. Samples were taken at 4, 8, 12, and 24 hours for analysis. Bile salt stability was monitored by TLC and quantified as above. Percent conjugated bile salts were derived by comparing the intensity of cholic acid TLC spots obtained following incubation to the intensity of the TLC spot for cholic acid obtained at the beginning of incubation. A known concentration of cholic acid spotted on TLC was set at 100%.

4.2.5. *In vitro* Caco-2 Permeability Assays

Caco-2 permeability assays of CamSA were performed by Apredica, LLC (Watertown, MA). Briefly, CamSA was dissolved in DMSO and added to Caco-2 cell cultures to 10 μM final concentration. CamSA was analyzed for both apical to basolateral permeability and basolateral to apical permeability across a Caco-2 cell monolayer. After a 2 hour incubation, CamSA concentrations in the apical and basolateral sides of the Caco-2 monolayers was determined by HPLC-MS.

4.2.6. Cytotoxicity of CamSA

Vero, Caco-2, and J774A.1 cell lines stored in growth medium supplemented with 5% (v/v) DMSO were thawed rapidly from liquid nitrogen

storage. Cell lines were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (complete medium) at 37 °C in a humid incubator containing air at 95% and CO_2 at 5% (200). To remove cells from tissue culture flasks, 1X trypsin-EDTA (1mM EDTA) was added and incubated with cells for 5 minutes. Complete medium was added and using a cell scraper, monolayers were gently removed. Cells were recovered by 800 x g for 5 minutes at room temperature and the cell pellet was resuspended in fresh media. A sample of cell suspension was treated with trypan blue to determine basal cell viability (201). Trypan blue is a dye exclusion method that selectively stains dead or dying cells that do not have an intact (viable) cell membrane (201). Trypan blue treated cells were inserted into a hemocytometer and viewed by light microscopy for enumeration of live cells (usually >90% live cells). Culture cells were plated in 12-well or 96-well tissue culture treated plates at a density of 10^5 cells/ml and allowed to attach overnight. Spent media was removed and fresh media supplemented with CamSA in DMSO at a final concentration of 50 or 200 µM was added to the wells. As negative control, cells were supplemented with media containing DMSO. As positive control cells were treated with 10% EtOH. Plates were incubated overnight as described above.

The 12-well plates containing cell cultures were used as a qualitative method to visualize cytotoxicity by cell rounding and trypan blue. Following overnight treatment with 0, 50 or 200 µM CamSA or 10% EtOH, media was removed from wells and PBS containing 0.4% trypan blue was added. After 5 minutes, cells were visualized using a light microscope and observed for blue

stained cells. The number of blue cells in CamSA-treated wells was compared to the number of blue cells in the negative and positive control wells. Morphological change (rounding) was also determined by comparing CamSA-treated to positive and negative control cells.

The 96-well plates cultured with mammalian cells were used as a quantitative method using the CellTiter Glo Luminescent cell viability assay. This assay quantitates the concentration of ATP, which indicates metabolically active cells (202). After overnight treatment, the 96-well plates were equilibrated to room temperature for 30 minutes before addition of the CellTiter-Glo reagent. Luminescence was read with an integration time of 1 second per well using a Tecan Infinite 200 plate reader and iControl software. All experiments were done in triplicate.

4.2.7. *C. difficile* Toxin-Induced Cell Death

C. difficile spores were washed five times with nanopure water, heat activated at 68 °C for 30 minutes and washed five more times with water. Spore pellets were resuspended in 0.1 M sodium phosphate buffer at pH 6.0 supplemented with 0.5% sodium bicarbonate (germination buffer) to an OD_{580} of 1.0. Spores were diluted five-fold in BHI broth containing 6 mM taurocholate/12 mM glycine (germinants), germinants supplemented with CamSA at 50 μM (CamSA 50) or 200 μM (CamSA 200), or germinants supplemented with chenodeoxycholate at 200 μM (CDCA 200). The resulting mixtures were incubated at 37 °C anaerobically overnight. The following day, the cells/spores

were removed by centrifugation and the supernatants were filtered through 0.2 µm sterile filter (Fig. 4.1).

In parallel, cell lines were cultured in 96-well plates as described above. Fresh media containing no bacterial supernatant or bacterial supernatant at varying concentrations (percent v/v) were added to the cultured vero and Caco-2 cells (Fig. 4.1). Cells were also treated with BHI as an added control. After overnight incubation, the plates were treated as before for the CellTiter Glo viability assay and luminescence was obtained using a plate reader and software as described.

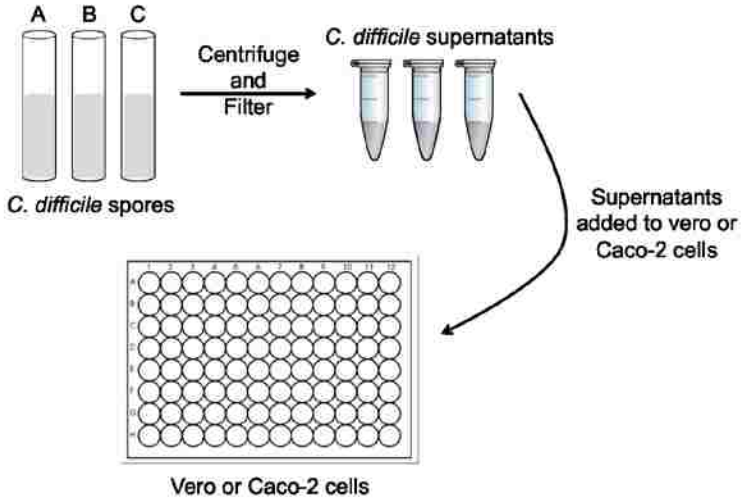


Figure 4.1. Representation of the method for treating culture cells with supernatants derived from *C. difficile* spores. *C. difficile* spores in BHI were supplemented with taurocholate and glycine (test tube A above), taurocholate, glycine and CamSA (test tube B above), and taurocholate, glycine, and chenodeoxycholate (test tube C above). After overnight incubation of *C. difficile* spores with supplements, the supernatants were recovered by centrifugation and sterile filtered. Supernatants were titrated into wells containing mammalian cells in growth medium.

4.2.8. Statistical Analysis

Standard deviations represent at least three independent measures, unless otherwise stated. Analysis of data by 1-Way ANOVA for nonparametric analysis had a statistical significance set at a p value of < 0.05. Student's paired *t*-test was

used to determine the significance of difference of means and the p value was set at < 0.05.

4.3. Results and Discussion

4.3.1. Effect of CamSA on Bacterial Growth

Three of the bacterial strains (*B. longum, L. gasseri* and *E. coli*) chosen for this study are indigenous human intestinal bacteria and two strains cause disease in humans (*B. cereus* and *C. difficile*). Indigenous bacteria resist colonization of *C. difficile* and these same bacteria are compromised during antibiotic treatment (74, 142, 203). Since the natural flora is imperative to the resistance of infection by *C. difficile*, therapies to counteract or prevent CDI should not damage this natural barrier (142, 204). *B. longum* and *L. gasseri* are both Gram-positive bacterium that harmlessly populate the human intestinal tract and aid in bile salt degradation (205, 206). *E. coli* is a Gram-negative bacterium common to the human intestine (207). Most *E. coli* strains are non-pathogenic (like the strain used in this study) although some strains are associated with infection and disease (208). *E. coli*, *B. longum*, and *L. gasseri* are exposed to bile salts regularly (144, 209). In fact, *B. longum*, and *L. gasseri* with other indigenous bacteria aid in important bile salt modifications in the gut. Modifications include epimerization, deconjugation by bile salt hydrolases, oxidation, reduction, hydroxylation and dehydroxylation (144, 210). Modified bile salts aid in digestion and the removal of cholesterol from enterohepatic circulation (144, 210). Associations between *E. coli* and bile are not well understood but *E. coli* is bile salt resistant (144). *E. coli*, *B. longum*, and *L. gasseri* were each assessed for

growth in the presence of taurocholate, chenodeoxycholate or CamSA. As expected, these three normal gut bacteria were unaffected by bile salts supplemented in the growth medium (Table 4.1). These results indicate that although CamSA is a bile salt analog, it retains essential bile salt characteristics that allow it to be tolerated by enteric bacteria tested in this study. Furthermore, CamSA was tolerated by both Gram-positive and Gram-negative bacteria.

The Gram-positive bacterium, *B. cereus*, is an important bacterium that produces toxins associated with food spoilage and subsequent food-borne illness (211, 212). It is postulated that *B. cereus* is a transitory species in the natural gut flora (213). *Bacillus cereus* is widely distributed in the environment and toxins ingested from spoiled food cause severe vomiting and diarrhea (211, 212, 214). Since *B. cereus* has been found in the feces of humans, cells and/or spores likely encounter bile salts during transit through the GI tract (211, 213, 215). Previous reports have shown that *B. cereus* is unable to grow in the presence of bile salts (216, 217). *B. cereus* vegetative cells added to control media resulted in normal growth with OD_{580} saturation at 24 hours. However, *B. cereus* did not grow in media supplemented with taurocholate, chenodeoxycholate or CamSA (Table 4.1). This suggests that CamSA is similar to taurocholate and chenodeoxycholate in preventing the growth of *B. cereus* (216, 217).

In vitro kinetic data suggests that CamSA prevents *C. difficile* spore germination but other bile salts have been shown to inhibit vegetative growth (140, 141). Recovery of *C. difficile* in growth medium is enhanced by the addition of the bile salt, taurocholate (138, 218, 219). Other bile salts such as

chenodeoxycholate prevent the vegetative growth of *C. difficile* in culture (140, 220). CamSA prevents *C. difficile* spore germination *in vitro* however the effect on vegetative growth was not studied. Since *C. difficile* is the focus of this project, effect of CamSA on vegetative growth was determined. Two strains of *C. difficile* were observed for growth in the presence of taurocholate, chenodeoxycholate or CamSA. *C. difficile* strain 630 and VPI 10463 grew to saturation in the presence of taurocholate. However, neither grew in the presence of chenodeoxycholate as expected (140, 220). CamSA supplemented in *C. difficile* growth medium did not affect either *C. difficile* strain tested and each grew normally (Table 4.1). These data support previous reports of taurocholate and chenodeoxycholate effects on *C. difficile* growth. Although CamSA inhibits spore germination *in vitro*, CamSA does not affect the vegetative growth of *C. difficile*. In conclusion, this data supports the proposed mechanism that CamSA inhibits spore germination and has no affect as an antibacterial agent.

Table 4.1. Effect of Bile Salts on Bacterial

Bacterial Strain	Growth without bile salt	Growth with CamSA	Growth with taurocholate	Growth with cheno-deoxycholate
Bacillus cereus	Yes	No	No	No
Escherichia coli	Yes	Yes	Yes	Yes
Bifidobacterium longum	Yes	Yes	Yes	Yes
Lactobacillus gasseri	Yes	Yes	Yes	Yes
Clostridium difficile strain 630	Yes	Yes	Yes	No
Clostridium difficile strain VPI 10463	Yes	Yes	Yes	No

4.3.2. Stability of CamSA in Simulated Gut Microenvironments

CamSA is a taurocholate analog with an amide bond linking cholic acid to *meta*-benzene sulfonic acid. To be effective CamSA must survive the changing environments of the GI tract. If CamSA is going to be administered orally, it must be stable to the extremely low pH of gastric juices located in the stomach. CamSA was incubated with simulated gastric juice at pH 1.5. Degradation of CamSA was not observed after 24 hours in gastric juice. CDI is localized to the intestinal tract (12), therefore effective treatment must be stable in intestinal juices. CamSA was incubated with simulated intestinal juice and no degradation of CamSA was evident even after 24 hours. Hence, CamSA is stable in simulated gastric and intestinal juices at the pH likely encountered during the oral route of drug delivery to the intestinal tract.

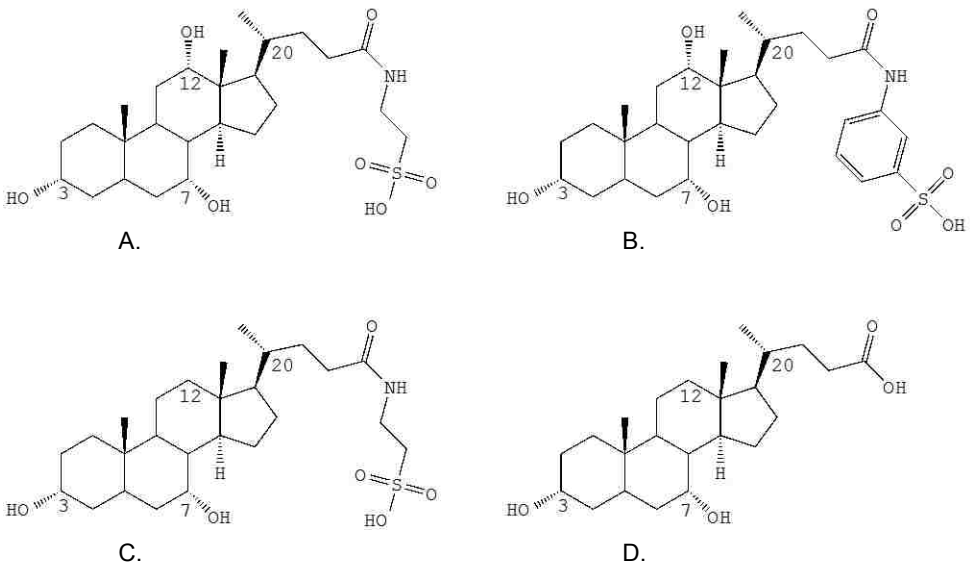
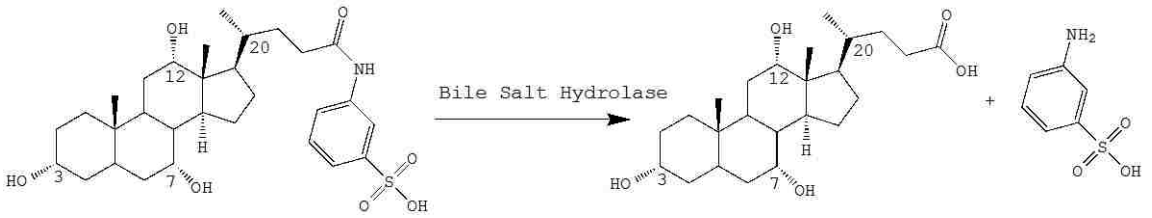


Figure 4.2. Bile salts used for bile salt hydrolase deconjugation studies. A) taurocholate, B) CamSA, C) taurochenodeoxycholate, and D) chenodeoxycholate

Bacterial bile salt hydrolases (BSHs) can hydrolyze conjugated bile salts (144, 187). *Bifidobacterium longum* and *Lactobacillus gasseri* are two intestinal bacteria commonly used as test strains for BSH production (187, 205, 221). CamSA is a conjugated bile salt similar to the natural bile salt that activates *C. difficile* spore germination, taurocholate (Fig. 4.2). Taurochenodeoxycholate is the conjugated derivative of chenodeoxycholate (Fig. 4.2). After four hours incubation with a culture of *B. longum,* approximately 25% of CamSA is hydrolyzed to cholic acid (Scheme 4.1 and Fig. 4.3A). Taurocholate is degraded quickly in the first four hours resulting in almost 60% degradation (Fig. 4.3A, [◆] $p < 0.005$). Taurochenodeoxycholate is hydrolyzed faster than CamSA but slower than taurocholate; within four hours 36% was hydrolyzed (Fig. 4.3A, [*] $p < 0.005$). After 24 hours incubation with *B. longum,* 79% of CamSA and 83% of taurocholate are hydrolyzed to cholic acid. There was no significant difference determined between CamSA and taurocholate degradation after 24 hours (Fig. 4.3A [**]). Taurchenodeoxycholate is hydrolyzed at a slower rate than either CamSA or taurocholate by *B. longum* as only 55% had been hydrolyzed after 24 hours (Fig 4.3A [★] $p < 0.01$). In the first four hours following incubation with *L. gasseri*, 1% of CamSA, 7% of taurocholate, and 14% of taurochenodeoxycholate was hydrolyzed to cholic acid (Fig. 4.3B). This degradation by *L. gasseri* in the first four hours for CamSA and taurocholate was significantly different than the degradation by *B. longum* in the first four hours (Fig. 4.3A and B [●] $p < 0.05$) CamSA and taurocholate are less sensitive to degradation in the presence of *L. gasseri* than to *B. longum* after 24 hours (Fig. 4.3A and B [+] $p < 0.03$). Less than

30% of CamSA was hydrolyzed to cholic acid after 24 hours by *L. gasseri* (Fig. 4.3B [i] $p < 0.05$). In contrast, 64% of taurochenodeoxycholate had been hydrolyzed after 24 hours (Fig. 4.3B [iii] $p < 0.05$). Taurocheneodeoxycholate is more sensitive to degradation by *L. gasseri* than it is to *B. longum* ($p < 0.05$). Chenodeoxycholate is not a conjugated bile salt and showed no degradation when incubated with either *B. longum* or *L. gasseri*. *E. coli* does not produce BSH and bile salts were stable after 24 hour incubation with *E. coli* cultures.



Scheme 4.1. Schematic representation of the hydrolysis of CamSA by bile salt hydrolase.

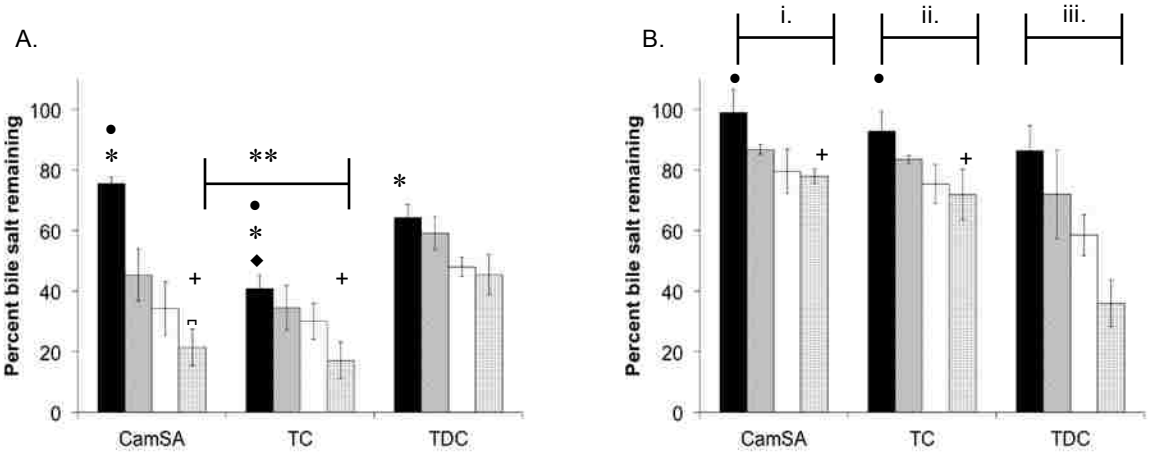


Figure 4.3 Incubation of CamSA with bile salt hydrolase-producing bacteria. A) Incubation of bile salts with *B. longum.* B) Incubation of bile salts with *L. gasseri*. Data indicates percent of conjugated bile salt remaining after incubation with bacteria. Data is represented by black bars (4 hours), patched bars (8 hours), white bars (12 hours), and dotted bars (24 hours). Statistical analysis of bile salt degradation at different time points was compared using 1-way ANOVA. Analysis of bile salts at 24 hours was compared using the student's paired *t* test. For both analyses, the p was set at < 0.05. *B. longum* comparisons: degradation of taurocholate within the four hours (⬥) $p < 0.005$, each bile salt after four hours (*) $p < 0.005$, CamSA and taurocholate after 24 hours (**), each bile salt after four hours (▯) $p < 0.01$. Degradation by *B. longum* and *L. gasseri* comparisons: CamSA after four hours and taurocholate after four hours (•) $p < 0.05$; CamSA after 24 hours and taurocholate after 24 hours (+), CamSA at four and 24 hours (i.) $p < 0.05$, taurocholate at four and 24 hours (ii.) $p < 0.05$, significant increase in taurocholate between four and 24 hours (iii.) $p < 0.05$.

Bile salt hydrolases produced by enteric bacteria will degrade CamSA when present. However, indigenous bacteria are severely compromised due to antibiotic exposure allowing the outgrowth of *C. difficile* (142). Based on these data, we predict that CamSA would only minimally be degraded following antibiotic therapy. Once therapy ceases and the normal flora replenish the

intestinal lumen, CamSA will be hydrolyzed and excreted. Further metabolic *in vivo* data will be needed to support this hypothesis.

4.3.3. CamSA Predicted Oral Bioavailability

To prevent *C. difficile* spores from germinating, CamSA needs to be retained in the intestinal lumen. Furthermore, there is less chance of toxic side effects to non-targeted organs if the anti-germinant therapeutic drug has a low oral bioavailability (192). Caco-2 permeability serves as an *in vitro* surrogate assay for intestinal permeability (222). Potential drugs are added to the apical side and the amount of permeation is determined on the basolateral side (Fig. 4.4). The permeation from apical to basolateral suggests the absorption of the drug across the gut wall and the rate of transport can be calculated. The opposite is used to determine basolateral to apical permeation. The permeability coefficient (P_{app}) is obtained by dividing the rate of permeation by the initial drug concentration and the area of the monolayer. The permeability ranking for P_{app} less than 0.5 is low permeability, moderate permeability is 0.5 to 5 and high permeability is greater than 5. The basolateral to apical permeability suggests whether the drug will undergo active efflux. The receiver side buffer is removed for analysis.

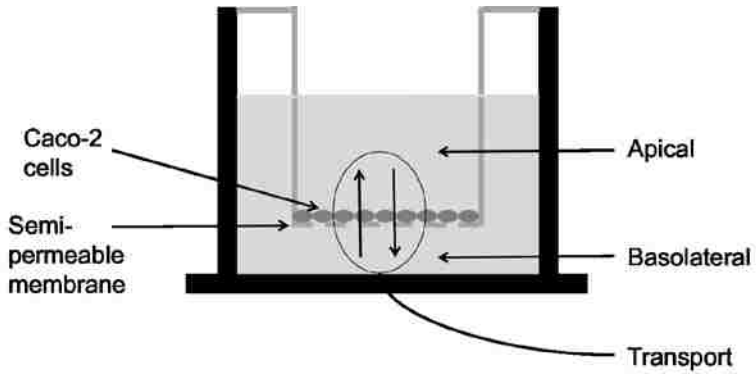


Figure 4.4. Caco-2 permeability assay

Natural bile salts are regulated in the GI tract by active and passive transport for reabsorption in enterohepatic circulation (143). Cholic acid, chenodeoxycholate, taurocholate and other bile salts have high permeability in simulated gut wall experiments (143, 223, 224). Furthermore, natural bile salts enhance the permeability of certain molecules across cell monolayers due to their ability to form micelles at high concentrations (223). The critical micelle concentration is currently not known for CamSA. In a Caco-2 permeability assay, CamSA displayed an apical to basolateral permeability coefficient (P_{app}) of 0.00 x 10^{-6} cm/s. The P_{app} of CamSA was compared to ranitidine which has a P_{app} of 0.3 x 10^{-6} cm/s and warfarin which has a P_{app} of 42.9 x 10^{-6} cm/s. Based on the permeability scale, CamSA has low to no permeability from Caco-2 cells' apical to basolateral side.

Bile salts can enhance permeability of molecules across the intestinal cell lining by forming micelles (143, 223, 224). The concentration of CamSA used for these experiments was significantly lower (10 μM) than the critical micelle concentration (CMC) of other bile salts. The CMCs for taurocholate and chenodeoxycholate are estimated to be 7 mM and 8 -12 mM, respectively (225,

226). These data show that at low concentrations, CamSA does not traverse the intestinal lining. However, at higher concentrations CamSA may form micelles like other bile salts. The CMC of CamSA will need to be determined in future studies.

CamSA displayed a basolateral to apical P_{app} of 10.9 x 10^{-6} cm/s. The basolateral to apical P_{app} was also compared to ranitidine which has a P_{app} of 1.8 x 10^{-6} cm/s and warfarin which has a P_{app} of 16.0 x 10^{-6} cm/s. Based on the permeability scale described above, CamSA has high permeability from the basolateral cell side to the apical side. It is likely that CamSA is actively transported to the apical surface of the Caco-2 monolayers. In the Caco-2 permeability assay, CamSA at 10 μM is not absorbed by the apical surface of the monolayer and is effluxed from the basolateral.

From both data sets the efflux ratio (R_E) can be calculated by dividing the basolateral P_{app} by the apical P_{app}. An R_E of greater than 2 indicates a significant efflux activity and indicates a candidate substrate for active transporters such as P-glycoprotein (227). Ranitidine has a R_E of 6, warfarin has a R_E of 0.4 and CamSA has a R_E of 11. The sum of this data suggests that CamSA has low bioavailability and will remain in the lumen of the gut.

An *in vitro* ADME-Tox test was also conducted to estimate the percent recovery of CamSA from either the apical to basolateral permeability or basolateral to apical permeability. In both assays CamSA was recovered at 100% indicating low binding to and low accumulation inside Caco-2 cells. The recovery of CamSA at 100% also indicates low metabolism by Caco-2 cells (228,

229). The ADME-Tox test further supports that CamSA will be stable and remain in the intestinal lumen.

4.3.4. Cytotoxicity of CamSA

Cell culture can be a useful tool to predict toxicity of therapeutic agents to particular tissues or to immune cells (194-196). There are many ways to determine cell viability and/or cell death using *in vitro* assays all with limitations (230). To minimize variability, we utilized visual observation of rounded cells, trypan blue exclusion, and ATP production to determine cell viability. Rounding of mammalian cells in culture upon exposure to *C. difficile* toxin is used as a visual determination of sensitivity of cells due to the destruction of the actin cytoskeleton (87, 92, 231, 232). It is a rapid method to determine the dose of toxin necessary to induce cell rounding (231). The affect CamSA has on the morphology of mammalian cells was determined. Trypan blue is a dye exclusion method that selectively stains dead or dying cells that do not have an intact (viable) cell membrane (201). ATP concentration assays provide evidence of cell viability due to its presence in all metabolically active cells. When cell are dying the amount of ATP decreases allowing for distinction between viable and non-viable cells (202).

Vero and Caco-2 epithelial cell lines were chosen for cytotoxicity studies because they are the commonly used cell lines used to test for *C. difficile* toxin production and toxin damage (92, 197, 198). Vero cells are an immortalized cell lineage from kidney epithelial cells originating from the African green monkey. Caco-2 carcinoma cells are human colon epithelial cells. Murine macrophages

(J774.1) are immune cells that are used extensively in the Abel-Santos laboratory to study spore phagocytosis (135, 233). Recent studies have analyzed macrophage responses to *C. difficile* spores and to *C. difficile* cellular proteins (234, 235). Therefore for potential future studies, the cytotoxicity of CamSA was determined for a murine macrophage cell line.

Cultures of macrophages, vero cells and Caco-2 cells were treated with DMSO as a negative control or 10% ethanol as a positive control for non-viable cell experiments. All cell lines treated with DMSO appeared healthy (non-rounded) and very few were stained blue by trypan blue exclusion (Fig. 4.5A). In contrast, cells treated with ethanol appeared rounded and stained blue by trypan blue (Fig. 4.5B). CamSA was added to cells at either 50 µM or 200 µM. CamSA treated cells were undistinguishable from DMSO-treated cells (Fig. 4.5C). In conclusion, based on the lack of cell rounding and trypan blue staining experiments, CamSA did not induce cell death at the concentrations tested.

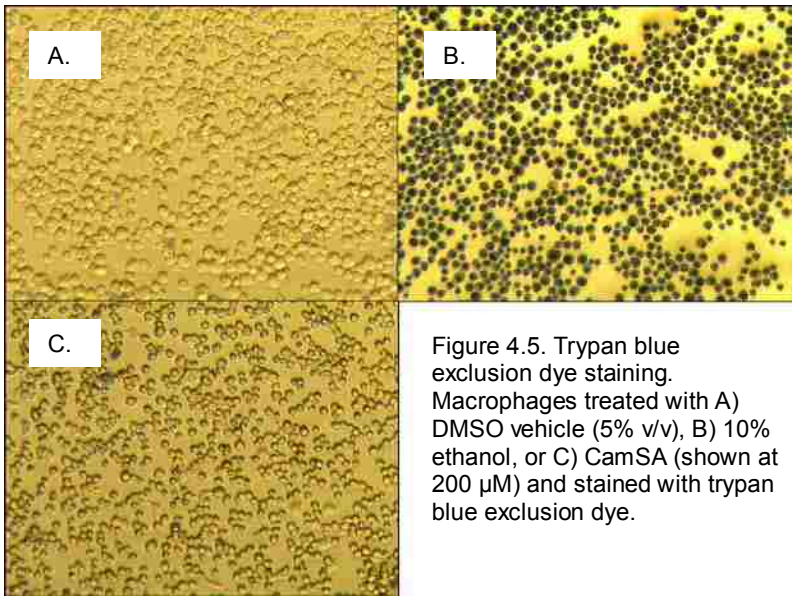


Figure 4.5. Trypan blue exclusion dye staining. Macrophages treated with A) DMSO vehicle (5% v/v), B) 10% ethanol, or C) CamSA (shown at 200 µM) and stained with trypan blue exclusion dye.

Vero and Caco-2 cells were treated as above and assayed for cell viability due to ATP concentration. Wild type cells and those treated with DMSO resulted in high basal concentrations of ATP, whereas cells treated with ethanol resulted in very low amounts of ATP (Fig. 4.6). As described above, CamSA treated cells were similar to the DMSO treated cells with high levels of ATP (Fig. 4.6). In conclusion, these combined *in vitro* experimental results indicate that CamSA at 50 µM and 200 µM is not cytotoxic to vero cells, Caco-2 cells or murine macrophages.

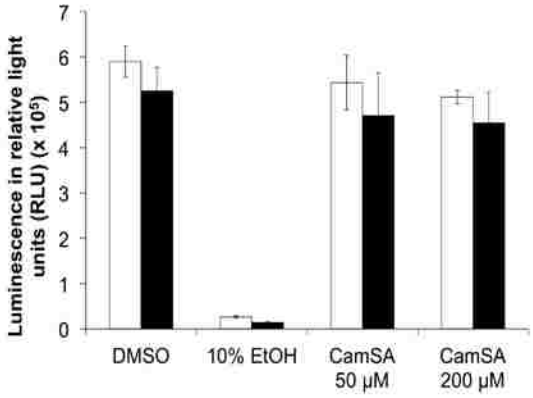


Figure 4.6. Cytotoxicity cell viability assay. Luminescence due to ATP in vero cells (white bars) and Caco-2 cells (black bars) after treatment with DMSO, 10% EtOH, or CamSA at 50 µM and 200 µM. Significant differences are observed between 10% ethanol treated cells and cells treated with DMSO or CamSA (both cell lines) $p < 0.005$. No significant difference is observed between DMSO treated and CamSA at either concentration for both cells lines.

4.3.5. CamSA protection of Caco-2 and Vero Cells

Cell culture is an indirect method to determine protection from *C. difficile* induced cell death (78). *C. difficile* does not tolerate aerobic conditions that are required for the culturing of tissue cells and tissue cells will not tolerate the anaerobic conditions necessary for *C. difficile* growth. Therefore, tissue culture experiments rely on the production of *C. difficile* toxin secreted into medium

during *C. difficile* vegetative growth (197, 198, 236). Toxin production and secretion increases when bacteria are in stationary growth phase and is controlled by an optimum temperature of 37 °C (77, 237, 238). Since toxin is only secreted by metabolically growing and dividing *C. difficile* cells, halting germination should result in less toxin production (78).

C. difficile spores were grown in medium supplemented with taurocholate and glycine (germinants) to induce spore germination. Because vegetative *C. difficile* produces toxins, spores treated with germinants serve as a positive control for toxin production. Spores were also grown in medium supplemented with germinants and 50 μM (CamSA 50) or 200 μM (CamSA 200) of CamSA or chenodeoxycholate. Spores treated with germinants grew normally whereas only slight growth was observed for CamSA treated spores. Chenodeoxycholate inhibits spore germination and also prevents *C. difficile* cell growth and indeed no growth was observed (140, 141).

Supernatants were recovered from *C. difficile* supplemented as described and titrated into vero and Caco-2 cell medium. The supernatant from *C. difficile* spores treated with germinants affected vero and Caco-2 cells in a dose-dependent manner. As the concentration of germinant supernatant increased, luminescence due to the presence of ATP decreased (Fig. 4.7A and B). The decrease in ATP corresponds to fewer viable cells (202). In both cell types, addition of 5% (v/v) *C. difficile* germinant supernatant resulted in greater than 95% decrease in viable cells (Fig. 4.7A and B). This data is supported by

previous reports indicating toxin production during vegetative *C. difficile* cell growth and toxin-induced cell death of vero and Caco-2 cells (197, 198, 236).

CamSA inhibits *C. difficile* spore germination *in vitro* with an IC_{50} of 58 µM. Therefore, toxin concentration should be less in supernatants from *C. difficile* spores treated with CamSA. CamSA 50 supernatants were titrated into mammalian cell cultures at 0.5%, 1% and 5% (v/v). As the amount of CamSA 50 supernatants increased, a decrease in cell viability was observed in both cell lines (Fig. 4.7A and B [i.a, and i.b] $p < 0.01$). A significant difference was observed between germinant supernatants and CamSA 50 supernatants at 1% and 5% (Fig. 4.7A and B [1%, *], [5%, **] $p < 0.05$). The data suggests that a fraction of *C. difficile* spores germinated when supplemented with CamSA 50 supernatants and CamSA at 50 µM was able to partially protect mammalian cells from toxin-induced cell death.

Vero cells treated with CamSA 200 supernatants were better protected from toxin-induced cell death at 1% than CamSA 50 supernatants ($p < 0.05$) (Fig. 4.7A [◆]). Caco cells treated with 1% CamSA 200 or 5% CamSA 200 supernatants were better protected than those treated with CamSA 50 (Fig. 4.7B [1%, +], [5%, ++] $p < 0.05$). Interestingly, Caco-2 cells seem to be more sensitive to toxin than Vero cells. More vero cells (60%) than Caco-2 cells (28%) remained viable after treatment with 5% CamSA 200 supernatants (Fig. 4.7A and B). Caco-2 sensitivity to *C. difficile* toxin has been previously reported (197). In conclusion, the sum of these data show that vero and Caco-2 cells treated with CamSA supernatants are better protected from toxin-induced cell death than when

treated with germinant supernatants. Vero and Caco-2 cells treated with supernatant containing chenodeoxycholate did not differ from untreated cultured cells. This is likely due to the inability of *C. difficile* to grow in the presence of chenodeoxycholate.

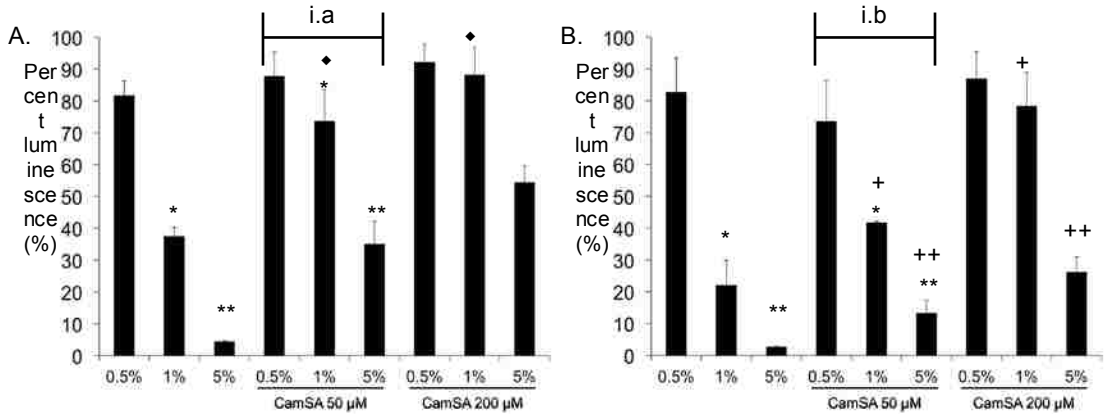


Figure 4.7. Toxin-induced cell viability assay. Luminescence due to ATP in A) vero cells and B) Caco-2 cells. Percent luminescence compared to untreated control cells. Cells were treated with titrated amounts of supernatant from *C. difficile* grown with or without CamSA. Statistical analysis of ATP reduction per treatment was compared using 1-way ANOVA. Analysis of ATP reduction due to concentration was compared using the student's paired *t* test. For both analyses, the p was set at < 0.05. Comparison of CamSA 50 supernatant percent ATP for vero cells (i.a $p < 0.01$) and Caco-2 cells (i.b: $p < 0.01$). Comparison of CamSA 50 supernatants and germinant supernatants at 1% (*) and 5% (**) for both cell lines. Comparison of vero cells treated with CamSA 50 and CamSA 200 at 1% (◆) $p < 0.05$. Comparison of Caco-2 cells treated with CamSA 50 and CamSA 200 at 1% (+) and 5% (++) $p < 0.05$.

4.4 Conclusions

Since the first step in the establishment of CDI is the germination of *C. difficile* spores in the microflora-depleted gut of hospitalized patients, anti-germination compounds could be used in combination therapies to supplement antibiotic treatments in immunocompromised patients. Once the antibiotic regime is completed, re-establishment of the normal gut flora will prevent *C. difficile* spore germination and anti-germination therapy can also be discontinued.

CamSA is stable to all tested GI tract microenvironments tested except incubation with bile salt hydrolase-producing bacteria. *Lactobacilli* and *Bifidobacterium* are two main human enteric bacteria that produce bile salt hydrolases (187, 205, 221). Antibiotics disrupt the normal microflora dynamics of the gut allowing for outgrowth of *C. difficile* (74, 142). A recent study in mice

shows that shifts occur in the bacterial population during antibiotic treatment and that different antibiotics can individually affect the resulting population shift (142). The indigenous bacteria in humans and mice differ significantly, for example *Bifidobacterium* is commonly found in humans. However, this bacterium was not found to colonize mice (142, 209). Although there is difference in the natural flora between mice and humans, the study suggests that the overall dynamics of the gut can be changed during and after antibiotic treatment. These changes may play an important role in CDI and CDI recurrence. CamSA is degraded by bile salt hydrolase-producing bacteria, which populate the normal gut. CamSA is more sensitive to *Bifidobacterium* degradation than to *Lactobacilli* degradation. In fact, a recent paper reported there are actually four related bile salt hydrolases produced by enteric bacteria (239). Each hydrolase has activity with all the bile salts tested however certain enzymes are more effective with specific substrates (239). This could explain the differences we observed with *L. gasseri* and *B. longum*. Antibiotic treatment disrupts the normal flora thus allowing CamSA to remain stable in the gut of antibiotic treated mammals. We hypothesize that after antibiotic treatment is terminated *Lactobacilli* and *Bifidobacterium* will be able to re-colonize the intestines in the presence of CamSA and degrade any remaining CamSA. This is further supported by the inability of CamSA to affect normal growth of *Lactobacilli* and *Bifidobacterium in vitro*. Future studies will address this hypothesis.

Orally-administered drugs typically need to penetrate the epithelial membrane of the GI tract to be systemically circulated. Low bioavailability usually

leads to increased time in the GI tract (189). Furthermore, oral drugs must be stable to changing GI tract microenvironments. In contrast to systemic infections, CDI is a localized intestinal infection. Thus, CDI-targeting drugs require low permeability and high GI tract stability for maximum efficacy. *In vitro* assays indicate that CamSA does not traverse cells in a permeability assay, suggesting that CamSA will be retained in the intestinal tract of mammals. The poor bioavailability of CamSA will also reduce toxic effects to other organs since CamSA is unlikely to circulate outside of the intestinal lumen. The results of the ADME-tox test indicate that CamSA (recovered at 100%) does not adhere to or accumulate in Caco-2 cells. Since CamSA does not appear to physically interact with Caco-2 cells, the concentration of CamSA in the lumen of the gut will not be affected. Furthermore, Caco-2 cells do not metabolize CamSA. The stability of CamSA to gut tissue will prevent toxicity due to metabolites and the effective concentration will not be altered. Collectively, these data show that CamSA will have low toxicity and high stability in the mammalian GI tract.

CamSA has been shown to inhibit *C. difficile* spore germination *in vitro* and these studies support that mechanism of action. CamSA has no effect on the growth of vegetative *C. difficile* cells so the mode of action of CamSA is not as an antibacterial agent. This is further supported by the cell culture studies involving *C. difficile* toxin produced when spores germinate into metabolically active cells. Increasing concentrations of CamSA resulted in less spore germination therefore led to less toxin production. Cell cultures treated with CamSA were protected from *C. difficile* toxin-induced cell death. CamSA, up to 200 μM, was not

observed to be cytotoxic. The *in vitro* data supports further characterization of CamSA in animal models of CDI.

CHAPTER 5

A NEW STRATEGY FOR THE PREVENTION OF *CLOSTRIDIUM DIFFICILE* INFECTION

5.1. Introduction

Clostridium difficile infection (CDI) is the major identifiable cause of antibiotic-associated diarrhea in hospitals (13, 14). In the US alone, CDI develops in over 500,000 patients with up to 20,000 deaths per year (41). The yearly health care burden has been estimated to be greater than $3 billion in the United States (43, 44). A recent study reported that CDI is responsible for 25% more nosocomial infections than methicillin-resistant *Staphylococcus aureus* (MRSA) (240). The rate of CDI progression to severe symptoms and death has been increasing annually since the 1970s (38, 39).

The use of animal models to study the pathophysiology of CDI is a necessary step in understanding the disease. *In vitro* data is helpful to predict certain spore-host interactions such as pharmacokinetic analysis described previously. Mammalian studies are essential to elucidate specific host-pathogen relationships in CDI (241-243). Until recently, there has been a lack of information regarding *C. difficile* environmental adaptation *in vivo.* In fact, a recent study shows that previously uncharacterized genes and cellular pathways are only identified *in vivo* (244). The host-pathogen relationship is very dynamic and cannot fully be understood by *in vitro* studies alone. Therefore, several animal models for CDI have been designed to study the *in vivo* effects of the infection and the disease.

Historically, Syrian hamsters have been the chosen animal model to study CDI *in vivo* (242, 245, 246). Hamsters are highly susceptible to infection with *C. difficile* after antibiotic exposure and also present signs of recurrence (51, 242, 245, 246). The hamster model is the classical model to study the effects that antibiotic exposure has on developing CDI signs and also the effects antibiotics have on treating the established disease (51, 242, 245, 247, 248). Unfortunately, because hamsters are extremely responsive to CDI, the disease progresses from severe to lethal very rapidly, usually within 48 hours (51, 204, 249). This intense presentation is uncharacteristic of human disease since human CDI can range from asymptomatic to severe (70). Therefore, the hamster model has limitations for studies that focus on human clinical and pathological conditions. The hamster model is the current established model to study CDI recurrence in animals (51, 204, 250). Due to their high susceptibility to *C. difficile*, hamsters that get mild CDI (or respond to treatment for CDI) and recover typically show signs of recurrence 10-15 days post infection (51, 250).

A new model for studying CDI has recently been reported utilizing gnotobiotic piglets(251). The basis for this model is the high frequency of *C. difficile* infections that occur on swine farms and affect newborn piglets (23, 24). The progression of the disease and disease presentation in pigs has been compared to human disease (24, 251). Severity of disease varies between piglets infected with *C. difficile* spores, which is similar to the symptoms described in humans (251). The authors report a flexible model that can be tailored according to specific research needs (251). Gnotobiotic piglets are

considered germ-free and lack indigenous gut bacteria. This model is adequate for studying disease characteristics however, due to the lack of natural gut flora the model is inappropriate when studying colonization resistance. In humans, the gut becomes susceptible to CDI only after antibiotic exposure and destruction of the natural gut bacteria (12, 41). The natural bacteria resist colonization by *C. difficile* (74). Therefore when studying the dynamics of natural bacteria, *C. difficile*, and prevention of CDI and CDI recurrence, the current gnotobiotic piglet model is inadequate. The authors postulate that conventional pigs could be used however, there are no current models describing conventional pigs in CDI-gut dynamics studies. Other drawbacks to using this model are the expense of acquiring gnotobiotic pigs (from cesarean section), appropriate housing (space limitations) and maintenance (rearing) (251, 252).

In recent years, mouse models have been more extensively used for animal studies of CDI due to the relative ease of handling and low expense compared to hamsters. Mice are not as sensitive to infection with *C. difficile* and although disease signs can become severe and animals reach a clinical endpoint, lethality is less common (142, 241). Unlike in hamsters, disease severity in the mouse can be varied with bacterial inoculum size (241). The flexibility of controlling disease in mice allows researchers to characterize the host-pathogen interaction, specifically the fate of the *C. difficile* spore inside the mammalian GI tract. One drawback to using the murine model in CDI research is that CDI recurrence has not been established in mice. A recent study reported an induced CDI relapse model in mice (253). The report more appropriately

describes a re-infection model since the authors re-challenge mice with *C. difficile* after they recover from the initial bout of disease (253). As described before, human recurrence has been defined as relapse of the same strain due to persistence in the gut or re-infection by the same or different strain due to susceptibility (35). To our knowledge there have been no reports of relapse in mice due to persistence of *C. difficile* spores in the gut. The lack of a recurrence model is a slight drawback when choosing the murine model of CDI however; the potential ability to study the fate of *C. difficile* spores using anti-germination therapy outweighs the caveats.

In the current study, CamSA was administered to mice to determine if the compound has acute toxicity at high doses. Following toxicity studies, animals were treated with anti-germinants and *C. difficile* spores and observed for signs of disease. Disease progression and severity was monitored by visual observation of signs of disease and the ratio of spores and cells in the GI tract and feces compared to untreated (healthy) animals.

5.2. Materials and Methods

5.2.1. Animals

The Institutional Animal Care and Use Committee at the University of Nevada, Las Vegas, reviewed and approved all animal protocols used in this study. All experiments were performed according to the National Institutes of Health guidelines in the Guide for Care and Use of Laboratory Animals. Based on previous CDI murine studies, C57BL/6N mice are susceptible to *C. difficile* (241, 253). Weaned Female C57BL/6N mice were purchased from Harlan

Laboratories (Indianapolis, IN). Animals were housed in groups of five mice per cage in the UNLV animal care facility. All water, food, bedding and cages were autoclaved prior to contact with animals. Upon arrival, mice were allowed to acclimate for one week prior to experimentation. Animal manipulations were performed in a biosafety level 2 laminar flow hood.

5.2.2. Toxicity of CamSA in Mice

CamSA and chenodeoxycholate were dissolved in DMSO to a concentration of 100 mg/ml. Groups of five mice were treated by oral gavage for thee consecutive days with 50 mg/kg body weight (b.w.) of CamSA or chenodeoxycholate. A control group was administered DMSO. Weight changes were recorded twice daily and mice were observed for adverse reactions such as vomiting, diarrhea, hair loss, weight loss, difficulty breathing, and lethargy. Other groups of mice were treated with 300 mg/kg CamSA or chenodeoxycholate and observed as above.

5.2.3. Preparation of *C. difficile* for Infection

5.2.3.1. Purified Spores by Abel-Santos Laboratory Method

C. difficile strain 630 and VPI 10463 were obtained from ATCC. Each strain was plated onto BHI agar supplemented with 1% yeast extract, 0.1% L-cysteine HCl, and 0.05% sodium taurocholate to yield single cell clones. Individual *C. difficile* colonies were grown in BHI broth until turbid and re-plated to obtain bacterial lawns. Plates were incubated for seven days at 37 °C in an anaerobic environment (5% CO_2, 10% H_2 and 85% N_2). Spores were harvested by washing and gentle scraping of the bacterial lawns from plates with ice-cold

nanopure water. The cells and spores were centrifuged at 8,800 x g for five minutes at 4 °C. Pellets were washed three times with nanopure water. To separate spores from bacterial cells, bacterial pellets were centrifuged through a 20% to 50% HistoDenz gradient at 18,200 x g for 30 minutes at 4 °C with no brake. The resulting purified spores were washed five times with nanopure water.

To determine spore purity, selected samples were stained using the Shaeffer-Fulton staining method (122, 164). Spore preparations were generally >95% pure after HistoDenz gradient. Before infection, purified spores were heat activated at 68 °C for 30 minutes and washed another five times with water. Purified *C. difficile* spores were resuspended in water to obtain an OD_{580} of 1.0. A spore aliquot was serially diluted onto BHI agar supplemented with cysteine and taurocholate to determine colony-forming units (CFU).

5.2.3.2. *C. difficile* Preparation based on Chen *et al* 2008 (241)

A sample of *C. difficile* 630 and VPI 10463 were purified by published procedure (241). Difco cooked meat medium was inoculated with frozen culture of 630 or VPI 10463 and incubated anaerobically at 37 °C for 36 hours. Mixtures of spores and cells were harvested by centrifugation at 8,800 x g for 5 min at 4 °C and washed three times with nanopure water. No further purification or heat activation was performed. CFUs were enumerated as described above. This preparation of *C. difficile* is hereto referred to as Chen *et al*.

To determine if 36 hours is an impractical time for VPI 10463 to sporulate, a separate culture was grown as above and allowed to incubate for 13 days.

After this extended period of time, a very low percentage (<10%) of spores were present by endospore staining.

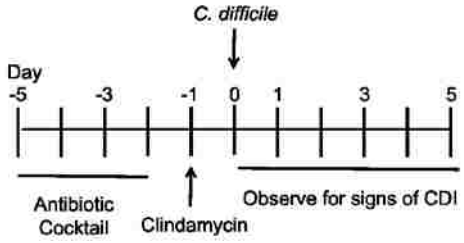
5.2.3.3. Spore Preparation based on Douce *et al* 2010 (254)

C. difficile spores were prepared based on previous procedures designed for the hamster model of CDI (254). Strain VPI 10463 was plated onto BHI agar supplemented with 1% yeast extract, 0.1% L-cysteine HCl, and 0.05% sodium taurocholate to yield single cell clones. Individual *C. difficile* colonies were grown in BHI broth until turbid and re-plated to obtain bacterial lawns. Plates were incubated for seven days at 37 °C in an anaerobic environment (5% CO_2, 10% H_2 and 85% N_2). Prior to spore harvest, plates with bacterial lawns were incubated at room temperature in a BSL-2 laminar flow hood overnight in air to maximize sporulation. Spores were harvested by the addition of 100% ethanol and gentle scraping the bacterial lawns from plates. Plates were washed once more with 1 X PBS and spores and cells were harvested by centrifugation at 8,800 x g for 5 min at 4 °C. The resulting pellet was washed three times with nanopure water. This preparation yielded approximately 25% of VPI 10463 spores. No further purification or heat activation was performed. CFUs were enumerated as above. This preparation is hereto referred to as Douce *et al*.

5.2.4. Optimization of *C. difficile* Inoculum in Mice

C. difficile 630 and VPI 10463 strains prepared above were used to infect mice as published (241). The murine CDI induction model was based on published procedures (241). 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) was prepared in autoclaved water and sterile filtered (241). For three consecutive days, mice were allowed to drink the antibiotic cocktail *ad libitum* (Scheme 5.1). The antibiotic water was refreshed daily. After three days of antibiotic water, all 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 (24 hours before *C. difficile* infection). Mice, in groups of five, were challenged by oral gavage with either 10^5 or 10^8 CFUs of water suspensions of *C. difficile* strain 630 spores or VPI 10463 spores, preparation by Chen *et al*. 2008 (241) or preparation by Douce *et al*. 2010 (254). A control group of five mice received water by oral gavage.



Scheme 5.1. Representation of induction of CDI in mice

All animals were observed daily for signs of CDI: decreased weight, hunched posture, wet tail, lethargy, anal redness, and increased soiling of cages. Disease signs were scored using a scoring rubric (Table 5.1). Animals scoring 2 or less were considered non-diseased animals scoring 3-4 were considered to have mild CDI. Animals scoring 5-6 were considered to have moderate CDI.

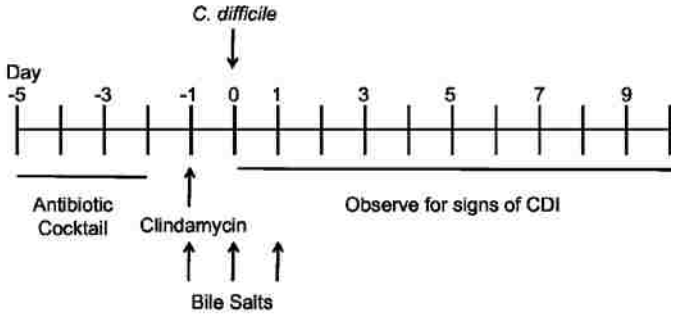
Animals scoring >6 were considered to have severe CDI and were immediately sacrificed.

Table 5.1. Scoring rubric for CDI in mice

Scoring Rubric	Score
Pink anogenital area	1
Red anogenital area	2
Lethargy	1
Diarrhea/Increase in soiled bedding	1
Wet tail	2
Hunchback posture	2
8-15% loss of body weight	1
>15% loss of body weight	2

5.2.5. Preliminary Testing of Anti-Germinants for CDI Prevention

Antibiotic-treated mice (five per group) were administered CamSA at 300 mg/kg, taurocholate 300 mg/kg, chenodeoxycholate at 50 mg/kg or compound T88 at 300 mg/kg in DMSO as vehicle one day prior to spore challenge, the day of spore challenge, and one day following spore challenge for a total of three consecutive doses (Scheme 5.2). Control animals received neat DMSO. Bile salts and DMSO were administered to mice by oral gavage. *C. difficile* strain 630 and VPI 10463 was administered at 10^8 CFUs by oral gavage. Animals were observed twice daily for signs of infection and scored appropriately. Animals that reached clinical endpoint were sacrificed. Cages and water sources were changed daily.

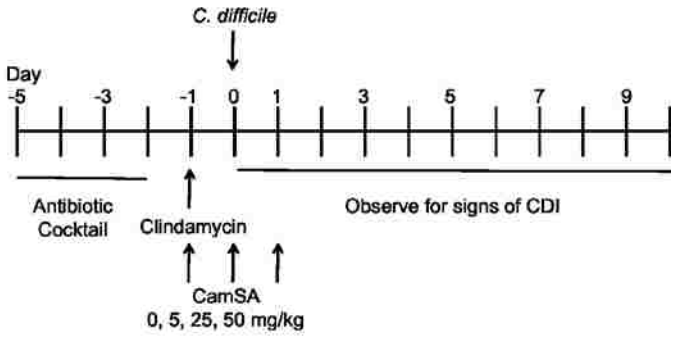


Scheme 5.2. Representation of induction of CDI in mice and preliminary test with bile salts

5.2.6. Minimum Effective Dose (MED) of CamSA

Twenty-four hours prior to spore challenge, groups of five antibiotic-treated mice received 0 mg/kg (neat DMSO), 5 mg/kg, 25 mg/kg, or 50 mg/kg CamSA by oral gavage. The day of infection, animals received 10^8 CFUs of *C. difficile* strain 630 spores by oral gavage. One hour post infection, animals received a second dose of the corresponding bile salt or DMSO (Scheme 5.3). A third dose of bile salt or DMSO was administered 24 hours post-infection. Animals were observed twice daily for signs of infection and scored appropriately. Cages were changed and feces were collected once daily throughout the experiment. The contents were stored at 4 °C. Aliquots of feces were heated to 68 °C for 30 minutes. Heated and unheated feces were diluted as necessary in water and plated on *Clostridium difficile* selective agar (CDSA). Plates were incubated anaerobically for 48 hours and colonies were counted to enumerate CFUs. CFUs obtained from unheated samples represent the sum of *C. difficile* vegetative cells and spores. CFUs obtained from heated samples represent the number of *C. difficile* spores only. The presence of *C. difficile* colonies was verified by PRO disk. Surviving animals were allowed to recover for

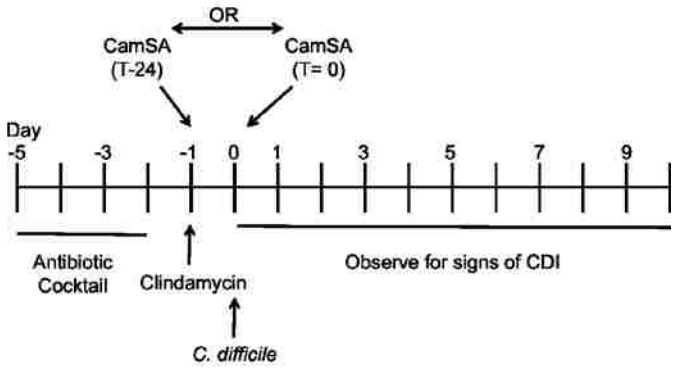
14 days followed by a second course of antibiotics. Surviving mice were injected with three consecutive doses of clindamycin. Animals were monitored for another 14 days to observe for signs of CDI relapse.



Scheme 5.3. Representation of induction of CDI in mice and MED of CamSA

5.2.7. CamSA Dosage Regime

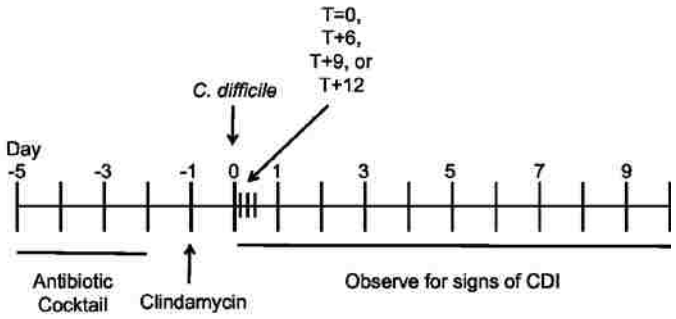
Antibiotic regimen was repeated with two groups of mice (5 per group). A single 50 mg/kg dose of CamSA was administered by oral gavage to one group of mice 24 hours (T-24) before challenge with 10^8 CFUs of *C. difficile* 630 spores. The second group received a single 50 mg/kg dose of CamSA at 10 minutes (T0) following spore challenge (Scheme 5.4). Animals were observed for CDI signs as before. Animals that reached clinical endpoint were sacrificed. Cages and water sources were changed daily to minimize re-ingestion of excreted *C. difficile*.



Scheme 5.4. Representation of the induction of CDI in mice and CamSA administered as a single dose

5.2.8. Onset of CDI Signs in Mice

Antibiotic treated mice were challenged by oral gavage with 10^8 CFUs of *C. difficile* strain 630 spores. Individual groups of five mice were treated with a 300 mg/kg dose of CamSA at the time of infection (T+0) or post-infection. Animals administered CamSA post-challenge were dosed at 6 hours (T+6), 9 hours (T+9), and 12 hours (T+12) (Scheme 5.5). A second 300 mg/kg dose of CamSA was administered 24 hours after the first dose. Mice were observed for signs of CDI twice daily and scored accordingly. Animals that reached clinical endpoint were sacrificed. Cages and water sources were changed daily to prevent re-ingestion of excreted *C. difficile*.



Scheme 5.5. Representation of the induction of CDI in mice and CamSA administered at different time points

5.2.9. Fate of *C. difficile* in the Murine GI Tract

Seven groups of mice with four mice per group, one group with three mice and one group of one mouse were treated with antibiotics as described before. All animals except group 1 were challenged by oral gavage with 10^8 CFUs of *C. difficile* strain 630 spores (Table 5.2). Group 1 was sacrificed on Day 0. Groups 2 - 5 were sacrificed 2, 4, 24, or 48 hours post-challenge with *C. difficile* spores. Groups 6 – 9 were treated with a 50 mg/kg dose of CamSA for one day prior to infection and at the time infection for at total of two doses. Animals administered CamSA were sacrificed 24, 48, 72 or 96 hours post-challenge with *C. difficile* spores. Cages were changed every two hours and feces were collected. The contents were stored at 4 °C. GI tracts of sacrificed animals were observed for signs of disease. Gastrointestinal tracts were removed in blocks and GI tract contents were flushed with autoclaved water and stored at 4 °C. Heated and unheated feces and GI tract contents were diluted as necessary in water and plated on *Clostridium difficile* selective agar (CDSA). Plates were incubated anaerobically for 48 hours and colonies were counted to enumerate CFUs (Fig. 5.1). CFUs obtained from unheated samples represent the sum of *C. difficile* vegetative cells and spores. CFUs obtained from heated samples represent the number of *C. difficile* spores only. The presence of *C. difficile* was verified by PRO disk (Fig. 5.1).

Figure 5.1. CDSA and PRO disk. Photograph of *C. difficile* growth on a CDSA plate (left) and verification of the presence of *C. difficile* by PRO disk (right upper). Right lower PRO disk is a sample negative for *C. difficile.*

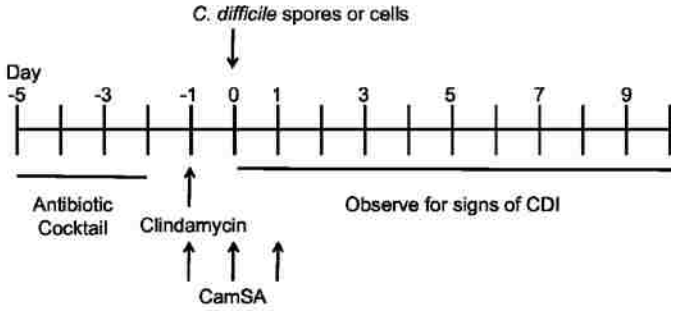
Table 5.2. Groups of animals used to determine the fate of spores in the GI tract of mice treated with CamSA.

Mouse Group	Number	*C. difficile*	CamSA	Time from spore challenge to sacrifice (hours)
Group 1	1	-	-	-
Group 2	4	Yes	No	2
Group 3	4	Yes	No	4
Group 4	4	Yes	No	24
Group 5	4	Yes	No	48
Group 6	3	Yes	Yes	24
Group 7	4	Yes	Yes	48
Group 8	4	Yes	Yes	72
Group 9	4	Yes	Yes	96

5.2.10. Effect of CamSA on Mice Challenged with Vegetative *C. difficile*

BHI broth was inoculated with a freezer stock of *C. difficile* 630 and allowed to grow overnight in an anaerobic chamber at 37 °C. The overnight culture was diluted 10-fold into fresh BHI broth and incubated for four hours at

37°C to reach exponential growth phase (75, 255). The presence of vegetative bacterial cells and absence of spores was verified by microscopic observation of Shaeffer-Fulton stained samples (122, 164). The bacterial culture was harvested by centrifugation at 13,700 x g for 1 min. The resulting pellet was resuspended in 1 ml sterile water and serially diluted onto BHI agar containing cysteine and taurocholate to determine CFUs. To prevent oxygen induced cellular death, the remaining bacterial culture was capped inside the anaerobic chamber prior to overnight storage at 4 °C. Antibiotic treated mice received three consecutive 50 mg/kg doses of CamSA and were challenged with 10^8 CFU *C. difficile* 630 vegetative cells, as above (Scheme 5.6). Animals were observed for CDI signs as previously described. Animals that reached clinical endpoint were sacrificed. Cages and water sources were changed daily to minimize re-ingestion of excreted *C. difficile*.



Scheme 5.6. Representation of induction of CDI in mice using vegetative cells or spores and administration of CamSA

5.2.11. Statistical Analysis

Mice survival (n=5) was analyzed by Kaplan-Meier survival analysis. Statistical comparison to untreated infected controls was calculated using log-

rank test. Signs of severity were analyzed as box-whisker plots. Data was expressed as mean ± standard deviation in box-whisker plots. Analysis of severity data by 1-Way ANOVA for nonparametric analysis had a statistical significance set at a p value of <0.01. Standard deviations represent at least three independent measures, unless otherwise stated. Recovered CFU and recovered spores represent mean values from a pool of five animals. Student's unpaired *t*-test was used to determine the significance of difference of means.

5.3. Results and Discussion

5.3.1. CamSA Toxicity in Mice

To determine acute toxicity of CamSA, we used the fixed dose procedure in mice (256). The fixed dose procedure allows researchers to define acute toxicity of a potential drug at pre-set doses and uses a minimal number of animals (256). Instead of dosing animals with a gradual increased concentration of compound, researchers determine a high limit and that concentration is administered to assess acute toxicity (256). If the compound results in acute toxicity at the high limit, a lower dose (usually a log lower) is assessed for toxicity and so forth. CamSA was administered to mice at the high limit of 300 mg/kg body weight (b.w.) for three consecutive days with no physical adverse effects. A higher dose of CamSA was not administered. A 300 mg/kg dose of chenodeoxycholate caused immediate death, probably due to observed precipitation of chenodeoxycholate upon interaction with mouse saliva and gastric juice. Chenodeoxycholate at 50 mg/kg did not cause any observable side effects.

5.3.2. Optimization of *C. difficile* Inoculum for Infection

C. difficile strain 630 is a highly virulent and transmissible epidemic strain clinically isolated from a patient with pseudomembraneous colitis in Zurich, Switzerland in early 1982 (122). Reference strain VPI 10463 is a toxigenic strain isolated from an abdominal wound and first described in 1982 (257, 258). These strains were chosen for the animal studies in this project. Various *C. difficile* growth, sporulation, and harvesting procedures exist for preparation of *C. difficile* for infection of animals (142, 241, 254). To determine the optimal preparatory procedure for infection of mice, three different procedures were followed to obtain *C. difficile* for infecting animals. The first procedure is the defined Abel-Santos preparation of purified *C. difficile* spores (127, 134). Both 630 and VPI 10463 strains produced spores and purified spores (>95% pure) were recovered using this procedure. When mice were challenged with 10^5 CFU of *C. difficile* strain 630 or VPI 10463 purified spores, mild CDI signs developed (score of 3-4) within 48 hours (Fig. 5.2). At 10^8 CFU, both *C. difficile* 630 and VPI 10463 purified spores caused severe CDI signs in the mouse (score > 6) and animals reached clinical endpoint within 48 hours (Fig 5.2). These data support previous reports showing increased severity of disease in mice with increased inoculation of *C. difficile* (241, 259). Both strains produced spores with this method however the yield of spores obtained from harvesting strain 630 was substantially higher than VPI 10463 (>75%). This finding is supported by previous sporulation studies that have found *C. difficile* strain VPI 10463 to produce fewer spores than 630 and

other *C. difficile* strains (117, 118, 260). In this study, both *C. difficile* strains caused disease in mice with a similar dose-dependent response.

C. difficile strain VPI 10463 was also grown as described in Chen *et al.* (241). This published procedure is the guideline for inducing CDI in mice. In our hands, this preparation yielded non-viable vegetative bacteria and did not cause CDI signs at either 10^5 or 10^8 CFU inoculums (Fig 5.2). The procedure describes that cultures were grown for only 36 hours (241). After 36 hours, medium inoculated with strain 630 contained mostly spores however, VPI 10463 inoculated broth contained almost entirely vegetative cells. Others and we have found that *C. difficile* typically requires nearly seven days for maximum sporulation (127, 134, 253, 259). To determine if the amount of time was insufficient, VPI 10463 was grown for 13 days in conditions described by Chen *et al* (241) however no spores were observed. Interestingly, *C. difficile* strain 630 was able to efficiently sporulate under the same conditions and within 36 hours. This data further supports that *C. difficile* strain 630 yields more spores than VPI 10463. Due to limited number of animals available, strain 630 spores prepared from this procedure were not used to infect mice.

The third method described in Douce *et al*. (254) was used to prepare *C. difficile* strain VPI 10463 for infection of animals. This procedure differs from the Abel-Santos procedure only slightly. *C. difficile* is grown the same but the harvest procedure is slightly different using ethanol for the removal of bacterial lawns as opposed to water. Also, this procedure does not define a purification step for separating spores from cells after harvest. *C. difficile* VPI 10463 produced spores

although not in high yield. It is interesting to note that VPI 10463 did not produce spores when grown in liquid media based on Chen *et al*. (241) however, the same strain produced spores when grown on solid media based on Douce *et al*. (254) and the Abel-Santos method (127, 134). Animals treated with either 10^5 or 10^8 CFU inoculums of *C. difficile* VPI 10463 prepared from this protocol induced similar dose response severity seen with purified spores described before (Fig 5.2). At the lower dose of *C. difficile*, animals developed mild CDI signs and recovered. At the higher dose animals developed severe symptoms and reached clinical endpoint within 48 hours (Fig 5.2). *C. difficile* strain VPI 10463 prepared from this method yielded viable and lethal cells and/or spores.

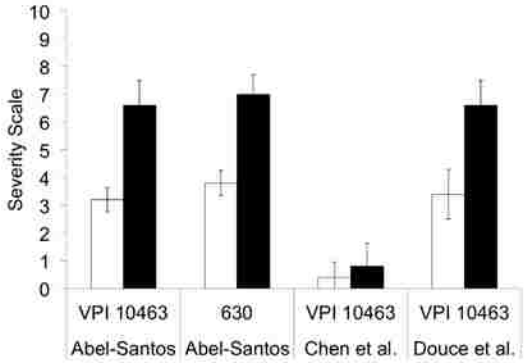


Figure 5.2. Comparison of CDI severity in mice after 48 hours by *C. difficile* preparations. White bars represent the severity in mice inoculated with *C. difficile* at 10^5 CFUs and black bars represent the severity in mice inoculated with *C. difficile* at 10^8 CFUs. VPI 10463 and 630 are *C. difficile* strains. Five mice per group. (127, 134, 241, 254)

C. difficile strain 630 consistently yielded larger amounts of infectious spores than *C. difficile* strain VPI 10463. Hence, *C. difficile* strain 630 was used in nearly all subsequent experiments. The preparation described by Douce et al. (254) does not provide a method for separating spores from cells. No histodenz

gradient was used to separate spores from cells therefore the final pellet was a mixture of spores and cells. Although it is expected that harvesting *C. difficile* with ethanol will kill vegetative cells (261) it is not a guarantee. Also ethanol may not affect toxins remaining in the spore/cell pellet. Furthermore without heat activation prior to infection, it is possible that persistent vegetative cells or toxins will induce disease in mice (142, 249). This variability is unacceptable when studying anti-germinants and their ability to prevent disease by preventing spore germination *in vivo.* Therefore, the Abel-Santos method of spore purification was used for further experiments. The large (10^8 CFUs) inoculum of spores ensures homogeneous CDI onset, fast CDI sign progression and allows testing the limits of anti-germination therapy.

5.3.3. Preliminary Testing of Bile Salts for CDI Prevention

As a preliminary test of bile salt effects on CDI progression and severity, groups of mice were administered antibiotics and three consecutive days of bile salts. All but one group of animals (control) were infected with purified *C. difficile* spores and observed for signs of disease. A second control group was challenged with spores but received only DMSO (untreated). Mice that were not challenged with *C. difficile* spores did not develop signs of CDI. All infected mice that were not treated with bile salts developed severe signs of CDI by 48 hours post challenge and were sacrificed (Fig. 5.3).

Taurocholate has been shown activate *C. difficile* spore germination *in vitro* (127, 140). To determine effects of taurocholate *in vivo*, a group of mice were administered taurocholate at 300 mg/kg. Mice that received taurocholate

developed CDI similar to control infected animals. All but one mouse reached clinical endpoint by 48 hours. That mouse developed slightly delayed signs and reached clinical endpoint at 72 hours post challenge (Fig. 5.3). Despite this one mouse in the taurocholate group, the mice were comparable to untreated mice. Disease severity was equally severe and onset of disease was similar. Therefore the data suggests that an excess of germinant in the gut of animals challenged with *C. difficile* spores does not affect disease severity or progression. Another possible explanation is that the inoculum (10^8 CFU) used in this study to induce CDI in mice was excessive. This overloading of *C. difficile* could hide any subtle differences the addition of germinants may have on disease progression and severity.

Chenodeoxycholate is a natural bile salt and a known inhibitor of *C. difficile* spore germination *in vitro* (127, 141). In toxicity experiments discussed previously, chenodeoxycholate was lethal at 300 mg/kg b.w but not at 50 mg/kg. Infected animals treated with chenodeoxycholate at 50 mg/kg showed a delay in onset of disease by 24 hours. By 72 hours post challenge, 80% of chenodeoxycholate-treated animals had reached clinical endpoint (Fig. 5.3). One animal developed moderate signs of disease and eventually recovered. Due to the insoluble nature of chenodeoxycholate described previously, it is possible that concentrated bile salt did not reach the GI tract consistently and remain soluble to interact with *C. difficile* spores. Furthermore, chenodeoxycholate has been shown to be absorbed at a high rate from the colon and recycled to the liver for enterohepatic circulation (149). It is possible that soluble chenodeoxycholate

was absorbed before interaction with *C. difficile* spores. CamSA and compound T88 are bile salt analogs that are potent inhibitors of *C. difficile* spore germination *in vitro* (134). To determine if CamSA and compound T88 can prevent CDI in the murine model, each was administered at 300 mg/kg to mice challenged with purified *C. difficile* spores. All mice treated with CamSA remained healthy and did not present signs of CDI (Fig. 5.3). Mice treated with CamSA closely compared to non-challenged mice. Since there was a 24 hour delay in CDI for mice treated with chenodeoxycholate, CamSA treated mice were observed for 10 days following spore challenge. Even after 10 days CamSA treated mice remained healthy and showed no signs of CDI. Interestingly, even though compound T88 is a strong inhibitor of *C. difficile* spore germination *in vitro*, T88 treated mice developed severe signs of CDI similar to untreated and taurocholate treated mice. Unlike chenodeoxycholate, compound T88 treated mice did not display a delayed onset of CDI but reached clinical endpoint by 48 hours post-infection (Fig. 5.3). These preliminary results show that CamSA, but not compound T88, is able to prevent CDI signs in mice challenged with spores from *C. difficile* strain 630. This experiment was repeated with *C. difficile* strain VPI 10463 and yielded similar results.

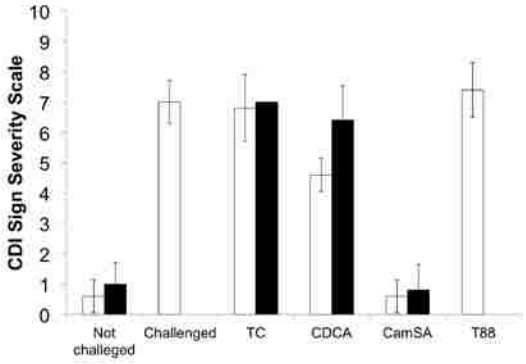


Figure 5.3. Preliminary study for the prevention of CDI in mice. Comparison of CDI severity after 48 hours (white bars) and 72 hours (black bars) of animals administered *C. difficile* and treated as indicated. Animals in the challenged and T88 groups reached clinical endpoint after 48 hours. TC = taurocholate, CDCA = chenodeoxycholate. Five mice per group. No significant difference was observed between not challenged and CamSA-treated animals. Other groups were statistically significant compared to not challenged or CamSA-treated animals, $p < 0.001$.

5.3.4. Minimum Effective Dose of CamSA

To determine the minimum effective dose (MED) of CamSA required to prevent CDI in the murine model, groups of animals were administered increasing doses of CamSA. Within 48 hours following spore challenge, all untreated mice (0 mg/kg CamSA) developed severe CDI signs and reached the clinical endpoint (Fig. 5.4). Mice treated with 5 mg/kg CamSA developed moderate to severe CDI, although signs onset was delayed by approximately 24 hours (Fig. 5.4). Animals presenting moderate CDI signs eventually recovered. Mice treated with 25 mg/kg CamSA developed mild CDI signs with delayed signs onset (Fig. 5.4). All animals recovered before reaching the clinical endpoint. In contrast, all animals treated with 50 mg/kg CamSA showed no sign of CDI and were undistinguishable from non-infected controls (Fig. 5.4). All surviving CamSA-treated animals were treated with a second course of antibiotics, but no relapse signs were observed even after 14 days.

Mice treated with 50 mg/kg chenodeoxycholate, a naturally occurring *C. difficile* spore germination inhibitor, developed moderate to severe CDI signs. In fact, animals treated with 50 mg/kg chenodeoxycholate show the same pattern of CDI signs as animals treated with 5 mg/kg CamSA. The data shows that mice treated with 50 mg/kg are completely prevented from developing CDI. In fact there is a dose dependent correlation between CamSA and disease severity.

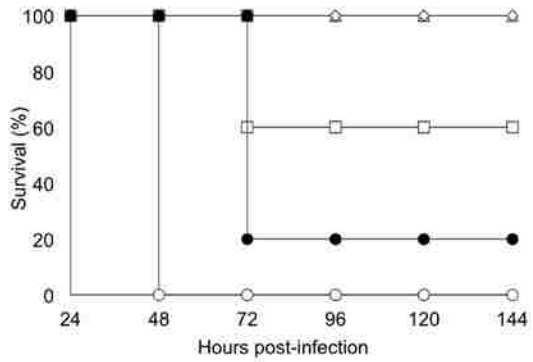


Figure 5.4. Kaplan-Meier survival plot for *C. difficile* infected mice treated with increasing doses of CamSA. Groups of five mice were treated with three doses of 0 (O), 5 (□), 25 (◊) or 50 (Δ) mg/kg CamSA. A separate group was treated with 50 mg/kg chenodeoxycholate (●). Log-rank test p=0.0027

Feces were collected from the cages of untreated and CamSA-treated animals every two hours up to 10 hours and then 24 hours post-challenge with *C. difficile* spores. Untreated animals (0 mg/kg CamSA) presenting signs of CDI started to excrete large amounts of vegetative cells at two hours post-challenge reaching a maximum between 8 and 10 hours (Fig. 5.5A). Diseased animals continued to excrete lower amounts of vegetative cells up to the clinical endpoint. Although some *C. difficile* spores were excreted in diseased animals, the amounts were negligible compared to excreted vegetative cells.

Mice treated with 5 mg/kg CamSA excreted mostly vegetative cells and developed CDI similar to untreated animals. Interestingly, the maximum amount of shed bacteria was delayed compared to untreated animals (Fig. 5.5B). This delay correlated with the delay in signs onset (Fig. 5.4). With a dose of 25 mg/kg CamSA, the proportion of excreted *C. difficile* spores increased and disease signs were milder (Fig. 5.4 and 5.5B). These mice displayed maximum shedding 72 hours following infection. The feces of animals treated with 50 mg/kg CamSA contained almost exclusively spores (Fig. 5.5A and B) and these mice did not develop CDI (Fig. 5.4). In these animals, *C. difficile* spore excretion started 2 hours following infection and continued for 96 hours, peaking 72 hours following challenge (Fig. 5.5A). In fact, 120 hours post-infection, the sum of excreted *C. difficile* spores was quantitatively identical to the number of spores given.

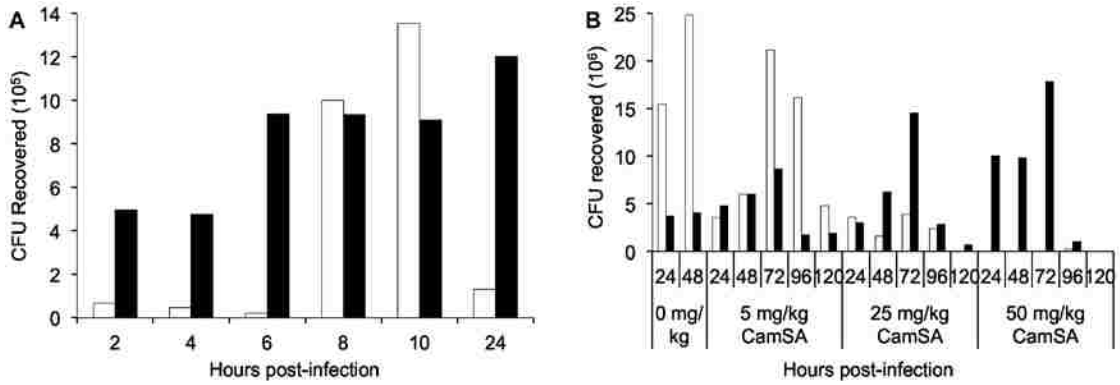


Figure 5.5. Spore excretion from mice. Feces were collected from cages housing five mice challenged with *C. difficile* spores and treated with 0 and 50 mg/kg CamSA. A) White bars represent *C. difficile* vegetative cells excreted by untreated animals. The amount of *C. difficile* spores present in untreated animals was negligible. Black bars represent *C. difficile* spores released from CamSA treated animals. The amount of *C. difficile* vegetative cells in CamSA treated animals was negligible. B) Feces were collected from cages containing groups of five mice infected with *C. difficile* spores and treated with 0, 5, 25, and 50 mg/kg doses of CamSA. White bars represent *C. difficile* vegetative cells and black bars represent *C. difficile* spores. Both graphs represent CFU per mg feces. Feces were collected per cage housing five animals. Therefore, no standard deviations could be obtained.

5.3.5. Refining CamSA Dosage Regime

Since CamSA prevented CDI in mice at 50 mg/kg when given in three consecutive doses, the next practical step was to determine if CamSA is able to prevent CDI with one dose. The fecal data from diseased mice, described above, suggests that at approximately 8 – 10 hours post infection there is an explosion of *C. difficile* growth. We hypothesize that this correlates with spore germination. Therefore, if a single dose of CamSA is to be effective it must be administered before 8 hours. Animals were administered 50 mg/kg CamSA 24 hours before *C. difficile* spore challenge (T-24) and 10 minutes after spore challenge (T=0). CamSA completely protected animals from CDI with a single 50 mg/kg dose when administered at time of infection (Fig. 5.6). These mice remained free of signs even after repeated antibiotic treatments (Fig. 5.7B). Hence, this single dose of CamSA was sufficient to prevent CDI signs without any signs of relapse.

However, when the single 50 mg/kg CamSA dose was administered 24 hours prior to challenge with *C. difficile* spores, animals developed moderate to severe CDI with delayed illness onset. Three animals reached clinical endpoint 48 to 72 hours days post-challenge (Fig. 5.6). Disease severity was more variable in mice treated with a single dose of CamSA 24 hours before infection (Fig. 5.7A). All animals remained healthy 24 hours post infection. Two animals displayed severe signs 48 hours following spore challenge whereas three presented only mild signs (Fig. 5.7A). Of these, two mice began to recover 72 hours following infection and one mouse worsened to reach the clinical endpoint (Fig. 5.7A). Animals that did not reach clinical endpoint eventually recovered.

Since CamSA was administered in only one dose 24 hours before infection, it is likely that CamSA had been degraded or excreted at the time of *C. difficile* spore challenge. Permeability data (discussed in chapter 4) suggests that CamSA will remain in the intestinal lumen and will not be absorbed. This supports the likelihood of excretion or degradation. A potential explanation for the variability in severity is that not all CamSA was excreted by 24 hours, resulting in a varied concentration in the mouse gut. This is further supported by the dose dependent response in mice. CamSA is able to prevent CDI in mice with three consecutive doses or with one single dose as long as it is administered at the time of *C. difficile* challenge.

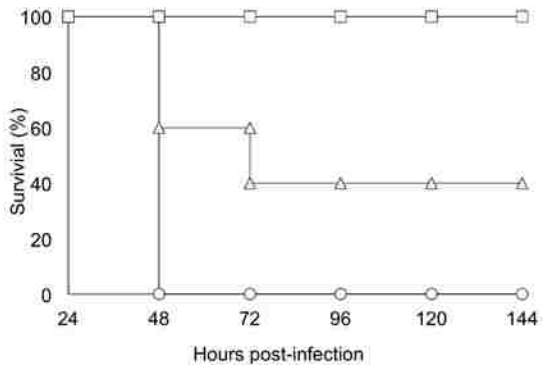


Figure 5.6. Kaplan-Meier survival plot for *C. difficile* infected mice treated with single doses of CamSA. Groups of five mice were treated with DMSO (O), a single 50 mg/kg dose of CamSA 24 hours prior to *C. difficile* spore challenge (Δ), or a single dose of CamSA at the time of infection (□). Log-rank test p=0.0495.

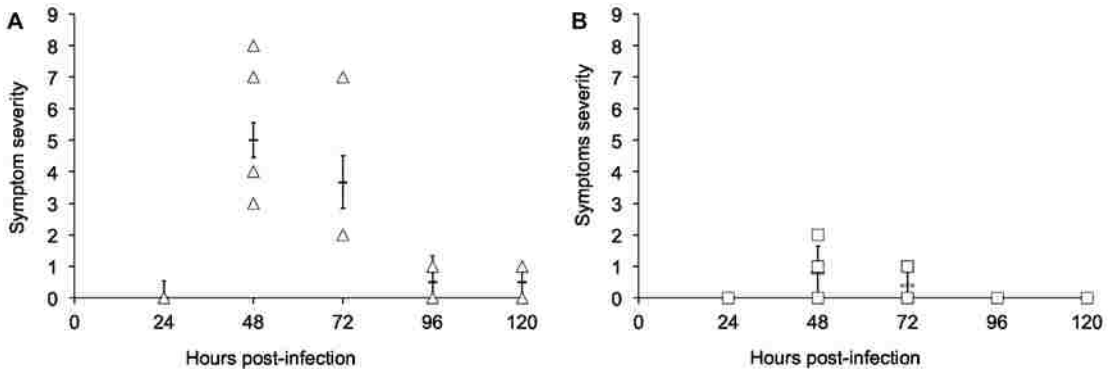


Figure 5.7. CDI severity graphs. Animals challenged with *C. difficile* spores and treated with a single 50 mg/kg dose of CamSA A) 24 hours prior to *C. difficile* spore challenge (Δ), or B) at the time of infection (□). Severity of CDI signs was scored using the Rubicon scale discussed above. Analysis of data by 1-way ANOVA analysis $p<0.01$ compared to untreated mice.

5.3.6. Timing of CDI Onset Using CamSA

To determine the onset of CDI in mice, animals were challenged with *C. difficile* spores and treated with CamSA at the time of infection (T=0), at 6 hours (T+6), 9 hours (T+9), or 12 hours (T+12) post-infection. All animals treated with CamSA at T=0 and T+6 were fully protected from CDI and presented no signs of disease (Fig. 5.8). In contrast, all animals treated with CamSA at T+9 and T+12 developed severe CDI undistinguishable from untreated mice and reached the clinical endpoint 48 hours post infection (Fig. 5.8). This data correlates with the fecal data discussed previously. In untreated mice there is a spike in *C. difficile* excreted at the 8 and 10 hour time points (Fig. 5.8). Since mice were completely protected up to six hours post challenge, it is likely that *C. difficile* spore germination or the commitment to germinate is between 6 and 9 hours in the mouse gut. Mice treated with CamSA after this time had no effect on disease progression and based on *in vitro* data CamSA does not affect vegetative growth.

These data suggest that the mechanism by which CamSA protects mice from CDI is by inhibition of *C. difficile* spore germination *in vivo*.

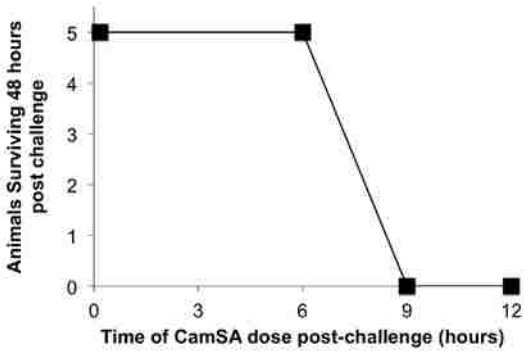


Figure 5.8. Graph showing the number of surviving animals 48 hours post challenge with *C. difficile* spores. Groups of five mice were treated with CamSA at the time of challenge (T=0) or 6 hours (T+6), 9 hours (T+9), or 12 hours (T+12) following spore challenge. Five mice per group. All animals either survived or all animals reached clinical endpoint at the times indicated therefore no error bars are shown.

5.3.7. Fate of *C. difficile* in GI Tract of Mice Treated with CamSA

The GI tracts of diseased and CamSA treated animals were removed in blocks and observed for signs of disease. The contents of the GI tracts were analyzed for the presence of *C. difficile* spores and cells. As previously reported, GI contents from animals with CDI signs contained almost exclusively (>98%) *C. difficile* vegetative cells unevenly distributed throughout the intestinal tract (262). Furthermore, the cecum and colon of these animals looked inflamed and swollen (Fig. 5.9). Intestinal content was watery and purulent. The small number of *C. difficile* spores recovered from diseased animals was localized to the cecum and colon (97%) of diseased animals. Approximately 3% of *C. difficile* spores were recovered from other gastrointestinal organs (stomach, duodenum, ileum, and jejunum).

Similar to the hamster CDI model (262), ingested *C. difficile* spores accumulated in the lumen of the cecum and the colon of CamSA-treated mice. The combined upper GI blocks contained less than 0.5% of total spores recovered in CamSA-treated mice (Fig. 5.10). Indeed, in animals treated with 50 mg/kg CamSA, the GI contents contained almost exclusively *C. difficile* spores (Fig. 5.11). These spores narrowly localized to the cecum and colon at every time point tested. The cecum and colon of these animals looked healthy and were undistinguishable from non-infected animals (Fig. 5.9 lower). *C. difficile* spores remained in the cecum and colon for 72 hours after spore challenge. By 96 hours, the amount of spores recovered from the cecum and colon of CamSA treated animals decreased considerably (Fig. 5.11).

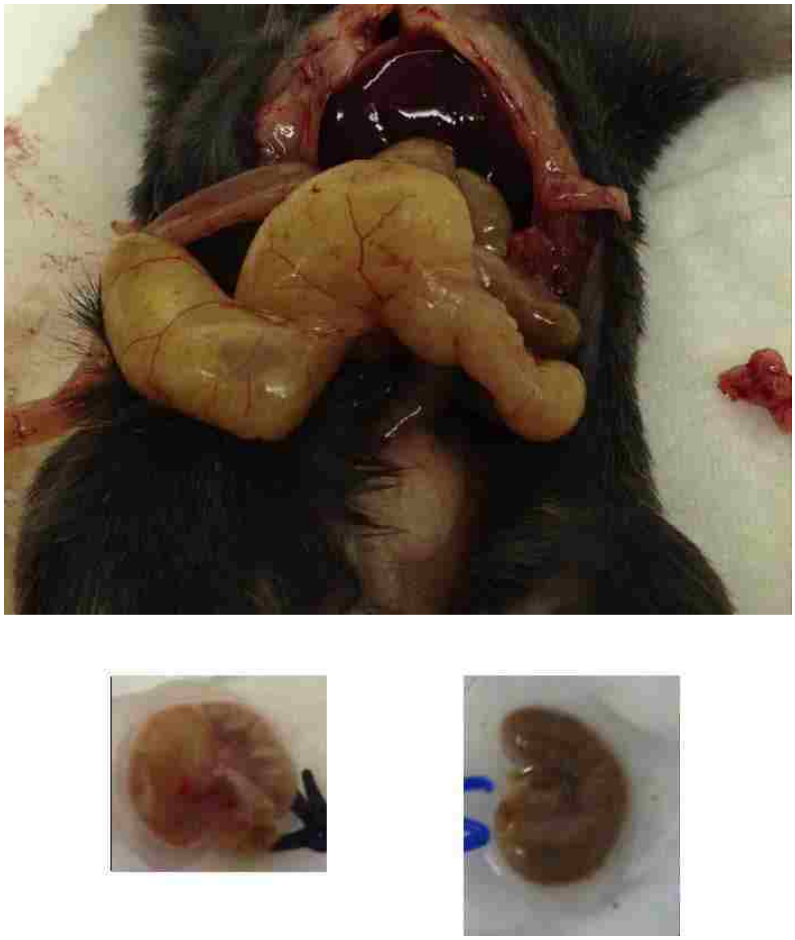


Figure 5.9. Necropsy photos. Disease mouse during necropsy (top) showing the swollen and gas filled cecum. Cecum from diseased mouse (bottom left) and from CamSA treated mouse (bottom right).

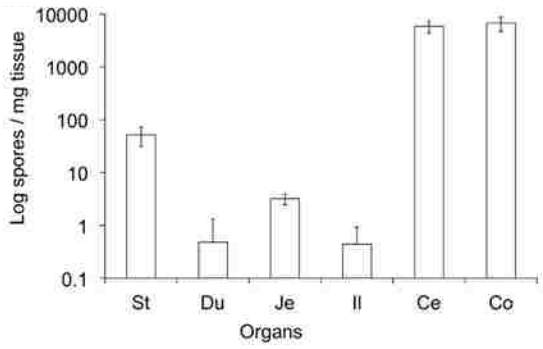


Figure 5.10. Distribution of *C. difficile* spores in the GI tract of CamSA-treated animals. Significant amount of spores were found in the cecum (Ce) and colon (Co). The stomach (St), duodenum (Du), jejunum (Je), and ileum (Il) showed negligible amounts of spores. No vegetative *C. difficile* cells were found in any of the GI tract structures.

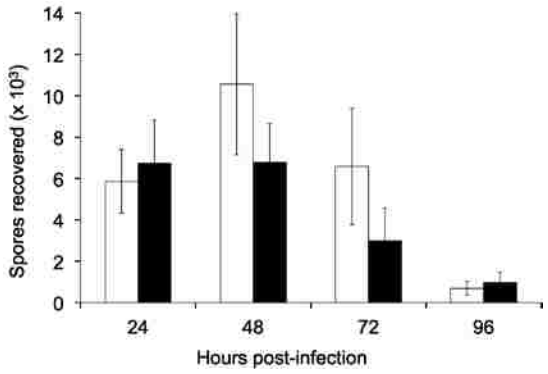


Figure 5.11. Amount of *C. difficile* spores recovered at different time points following spore challenge. White bars represent the cecum and black bars represent the colon of mice treated with 50 mg/kg CamSA.

5.3.8. CDI from Vegetative *C. difficile* Cells

Murine models can develop CDI signs when infected with vegetative *C. difficile* cells (142). Vegetative cells were prepared and used to challenge groups of five mice treated with three consecutive doses of 50 mg/kg CamSA. While CamSA prevents CDI in spore-challenged mice, the same dose was unable to prevent CDI in vegetative cell-challenged mice. Three animals became moribund between 48 and 72 hours after challenge (Fig. 5.12). Two other animals developed moderate CDI signs and eventually recovered. This data further supports the hypothesis that CamSA is able to prevent CDI in mice due to anti-germination activity.

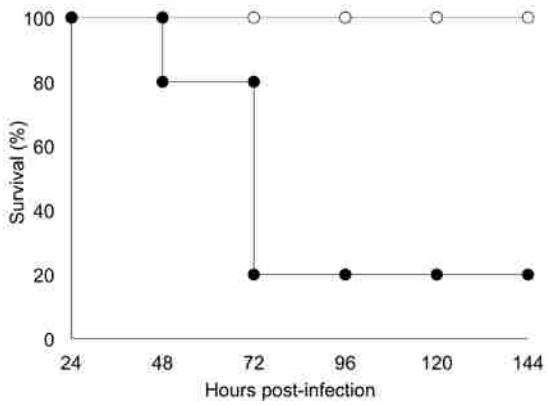


Figure 5.12. Mice treated with *C. difficile* spores or vegetative cells. Kaplan-Meier survival plot for animals treated with 50 mg/kg CamSA and challenged with (O) *C. difficile* spores or (●) or *C. difficile* vegetative cells.

5.4. Conclusions

CamSA was able to "prophylactically" prevent murine CDI caused by two different *C. difficile* strains and ameliorate CDI signs in a dose dependent manner. Furthermore, a single dose of CamSA was sufficient to prevent CDI signs without any signs of relapse. No signs of inflammation or damage to the GI tract were seen in CamSA-treated mice. It is noteworthy that CamSA gave complete protection from CDI against unnaturally massive *C. difficile* spore inoculums. A 75 kg human will have to ingest hundreds of grams of infected feces to reach the same level of inoculum. And yet, even in these extreme conditions CamSA was able to completely prevent CDI.

CamSA prevented CDI in mice at concentrations that were more than six times lower than the dose administered that showed no toxicity. Under the same conditions, chenodeoxycholate was not able to prevent CDI. In fact, animals treated with 50 mg/kg chenodeoxycholate show the same symptom patterns as animals treated with 5 mg/kg CamSA. At higher concentrations,

chenodeoxycholate showed acute toxicity, likely due to low solubility in biological fluids. Another compound, compound T88, that showed potent inhibitory activity *in vitro* was unable to prevent CDI in mice. In fact, compound T88-treated animals resembled untreated animals in regards to disease onset and severity. Taurocholate is the natural bile salt germinant for *C. difficile* spores (138, 140). Animals treated with *C. difficile* spores and taurocholate did not present signs of disease more severe than untreated animals. Furthermore, the onset of disease in taurocholate treated animals was no different than untreated mice. It is possible that disease caused by the high inoculum of *C. difficile* spores hid any minor affect taurocholate had on germination and disease progression. Future studies will need to be performed to determine if taurocholate has an effect on murine CDI with lower concentrations of *C. difficile* spores.

When CamSA was given at sub-optimal concentrations, CDI onset was delayed and signs were less severe than in untreated mice. Similar results were obtained when an optimal CamSA dose was administered 24 hours prior to infection. In both cases, late CDI onset and reduced symptom severity could be attributed to the partial inhibition of *C. difficile* spore germination by lowered intestinal CamSA concentrations. These data suggest that CDI severity is linked to the number of *C. difficile* spores able to germinate in the GI tract of mice. This argument is supported by the ratio of *C. difficile* vegetative cell to spores in intestine and feces, which correlated with CDI symptom severity. Thus, CamSA treatment can prevent CDI and ameliorate signs in a concentration dependent manner. Vegetative *C. difficile* cells are not believed to be involved in human CDI

transmission but can infect mice (67, 263-265). CamSA was able to prevent infections from dormant *C. difficile* spores, but not from actively dividing *C. difficile* cells. Although not clinically relevant, infection of mice with vegetative *C. difficile* cells bypasses spore germination requirements and suggests that CamSA prevents CDI by inhibiting *C. difficile* spore germination and not by targeting metabolic processes in the vegetative bacterium.

In our hands, *C. difficile* preparations from published procedures for mouse model of CDI (241) produced no detectable spores and did not result in infection. It is possible that in this case, most *C. difficile* cells died due to air exposure or that post stationary phase vegetative cells will not cause disease. In contrast, when we prepare *C. difficile* vegetative cells for infection, the cells are manipulated only in an anaerobic environment and were administered to animals immediately to minimize oxygen exposure.

Since CamSA blocks *C. difficile* spore germination *in vivo*, the fate of ingested *C. difficile* spores can be followed without interference from germination and/or re-sporulation. CamSA-treated mice excreted *C. difficile* vegetative cells and/or spores in a dose dependent manner. Ingested *C. difficile* spores were quantitatively recovered from feces, cecum, and colon contents of mice treated with 50 mg/kg CamSA, further supporting the role of CamSA anti-germination activity in CDI prevention. Thus, CamSA treated mice are able to shed almost all of the ingested spores before they can be activated for germination. Interestingly, ingested *C. difficile* spores rapidly transit through the stomach and small intestine, but remain in the colon and cecum for up to four days. The mechanism

of dormant *C. difficile* spore accumulation in the lower intestine is not understood, but suggests that ingested *C. difficile* spores can form a transitory reservoir that is slowly released from the lower intestine. Although the amount of unattached spores in the intestines is small, it is tempting to speculate that these spore reservoirs serve as a focal point for CDI relapse.

CamSA dosage was effective in preventing CDI when administered up to six hours following spore challenge, but ineffective when administered nine hours post-challenge. The observation of this narrow three-hour window correlates *C. difficile* spore germination with maximum *C. difficile* shedding in symptomatic mice. These data suggest that some germinated *C. difficile* cells are excreted soon after germination, while the remaining *C. difficile* vegetative cells lead to CDI onset.

CamSA has strong prophylactic properties against CDI, but can also be used as a probe to address mechanistic details on CDI initiation. The sum of this data suggests that ingested spores rapidly transit through the GI tract and accumulate in the lower intestine. Six to nine hours after ingestion *C. difficile* spores germinate and the newly germinated cells establish infection. *C. difficile* vegetative cells start shedding almost immediately after germination and continue throughout the infection. In contrast, non-germinated *C. difficile* spores are slowly shed over a four day period. The timing of *C. difficile* spore germination and persistence of ungerminated spores in the lower intestine will have profound implication in the prophylactic treatment of CDI.

It is clear that if *C. difficile* spores germinate in the gut of a patient, CamSA will be ineffective against CDI. In these experiments, CamSA only failed when we purposely pushed the limits of effectiveness to test the mechanism of *C. difficile* spore germination *in vivo*. In these cases, CamSA was not optimized as a CDI prophylactic. Instead, by giving single CamSA doses at different time points after spore challenge, we were able to determine when and where *C. difficile* spores germinated in the mouse gut. Subtly changing the experimental setup allows CamSA to play two separate roles: as a prophylactic agent against CDI and as a chemical probe for *C. difficile* spore germination.

Because CDI onset roughly coincides with antibiotic dosage, one can determine a priori when a patient will be at risk for CDI. In a clinical setting, one would not rely on a single CamSA dose. CamSA treatment will be started before the first antibiotic dose. Our data suggests that one CamSA dose every six hours will be sufficient to provide continuous protection from CDI. A six-hour interval between overlapping CamSA doses could indefinitely prevent C. difficile spore germination and thus compensate for the narrow efficacy window of CamSA. Since CamSA does not affect bacterial growth, anti-germination treatment can be continued until the intestinal microbiota has recovered. A multi-dose CamSA regime is further supported by the low toxicity exhibited by CamSA. Four therapeutic doses a day is not uncommon in hospitals and can certainly be improved with better CamSA formulations (e.g. controlled and/or delayed release capsules).

In conclusion, there are multiple lines of evidence to support that CamSA prevents CDI by inhibiting *C. difficile* spore germination *in vivo*, thus allowing ingested spores to be shed before they can establish infection. To our knowledge, CamSA is the first reported *C. difficile* spore anti-germinant that also protects mice from CDI. Furthermore, this is also the first study defining the temporal and spatial distribution of ingested *C. difficile* spores using a chemical probe. This approach represents a new paradigm that could be further developed and used in CDI management. Instead of further compromising the microbiota of CDI patients with strong antibiotics, anti-germination therapy could serve as a microbiota surrogate to curtail *C. difficile* colonization of antibiotic-treated patients.

CHAPTER 6

Conclusions and Future Directions

Spore germination mechanisms have been studied and described for *Bacillus* (131, 266, 267). The activation of spore germination typically requires the detection of small molecules called germinants (126, 131). These germinants are recognized and bind to a very sensitive proteinaceous biosensor located on the inner membrane of the spore (167, 268). These germination (Ger) binding site(s) are highly conserved in *Bacillus* and *Clostridium* and all sequenced sporulating bacteria have analogs of these Ger binding site(s) (by Basic Local Alignment Search Tool – BLAST) (95). The exception is *Clostridium difficile*. *C. difficile* encodes for all spore-specific proteins except Ger binding site(s) and spore coat proteins (95). It is possible that the germination binding site(s) in *C. difficile* have diverged from other sporulating bacteria or a different set of proteins is used as germination binding site(s). In any case, the identity of the germination binding site(s) for *Clostridium difficile* is currently unknown (95).

C. difficile spores must germinate to cause disease and a recent article identifies glycine (amino acid) and taurocholate (bile salt) as germinants (140). Furthermore, the bile salt, chenodeoxycholate, has been shown to inhibit *C. difficile* germination *in vitro* (141). Neither glycine nor taurocholate has been reported to activate germination in *Bacillus and Clostridium*, suggesting a novel mode of germination recognition in *C. difficile* spores. Kinetic analysis using taurocholate and glycine reveals that *C. difficile* spores bind germinants by a complex cooperative mechanism. The affinity of the spore for one germinant is

affected by the binding of the other germinant (127). Furthermore, chenodeoxycholate is a competitive inhibitor of taurocholate-mediated spore germination (127). Similar cooperativity of germinants is described for *Bacillus cereus* and *Clostridium sordellii* spores (133, 136). Thus, *C. difficile* likely encodes unknown germination binding site(s) proteins to bind to these germinants (127).

Due to the scarcity of genetic tools to understand *C. difficile* spore germination, an alternative approach is to use chemical probes to study the mechanism of spore germination (127, 133, 136, 151). The current dissertation project has described chemical probes to elucidate necessary epitopes of amino acids and bile salts capable of being recognized by the putative *C. difficile* germination binding site(s). To characterize the amino acid germination binding site(s), 30 amino acids and amino acid analogs were analyzed for germinant or inhibitor behavior with *C. difficile* spores. The results suggest that the binding region for amino acids has very little flexibility for the size of the molecule and functional groups allowed. There is also evidence for multiple binding site(s) that recognize specific amino acids. Besides glycine, L-phenylalanine, L-argnine and L-cysteine are able to activate *C. difficile* spore germination, probably by binding to separate binding sites (134). The limited number of functional groups on glycine, the optimal amino acid for germination, restricts the number of practical analogs that can be tested. Furthermore, none of the amino acids tested were able to inhibit *C. difficile* spore germination. As potential anti-germination therapy, amino acids are unlikely candidates.

Taurocholate is the natural bile salt that activates *C. difficile* spore germination (140). Taurocholate has multiple functional groups available for characterization. In this study, more than 100 bile salt analogs were analyzed for germinant and inhibitor behavior with *C. difficile* spores. The bile salt binding site(s) is quite complicated and is able to bind to multiple epitopes. The functional groups recognized for activation of spore germination are more specific than the functional groups that are simply able to bind (inhibition). Many functional groups and combinations are able to bind but not activate the *C. difficile* germination binding site(s). During this study, correlations were found between molecules that can activate and inhibit spore germination. Full characterization of the binding region for bile salts is far from completion. Based on this study, specific functional groups and interactions warrant further analysis.

Activity-based protein profiling using compounds to crosslink to germination binding site(s) can be used to determine the amino acid sequence of the binding region (270). This is an important technique that could be used to determine the bile salt binding region in *C. difficile* spores since the germination binding site(s) are currently unknown (95). The plant cholesterol derivative, Ouabain, contains an α-methylene-γ-lactone group which is an electrophilic functional group capable of participating in Michael additions with biological nucleophiles such as the sulfhydryl groups of cysteine residues forming an irreversible adduct (181-184). Ouabain was able to bind to *C. difficile* spores and inhibit germination. Ouabain could potentially be used as a probe to determine the binding site(s) binding residues in *C. difficile*.

In general, a number of inhibitors of *C. difficile* spore germination were discovered during this project. In the big picture, compounds able to inhibit *C. difficile* spore germination could potentially be used as anti-germinant therapy in high risk patients. The lack of prophylactic treatment for CDI is an important problem and current medications only combat the established disease (35, 269). Therefore, the best inhibitors were assessed to determine the most practical approach for further characterization. CamSA was chosen for pharmacokinetic characterization and *in vivo* studies due its potency as an inhibitor of *C. difficile* spore germination and inexpensive synthesis.

A patient becomes susceptible to *C. difficile* infection when taking antibiotic therapy and certain groups of people are at high risk for disease, such as the elderly and immunocompromised patients (41). This susceptibility is due to the disruption of indigenous bacteria that aid in the resistance to pathogen colonization (74, 142). An optimal anti-germination therapeutic should not further damage the natural flora in GI tract. CamSA does not have antibacterial effects on the enteric Gram-negative and Gram-positive bacteria tested in this study. Specifically, CamSA does not affect the vegetative growth of *C. difficile*. The proposed mechanism of CamSA is anti-germination activity with *C. difficile* spores. The results support this hypothesis since CamSA is unable to prevent proliferation of vegetative *C. difficile* cells. Future studies should include the effects CamSA has on other bacteria known to be important in colonization resistance (203, 271). For example, *Lactobacillus delbrueckii* ssp. *bulgaricus* has been shown to reduce cytotoxicity of *C. difficile* toxin *in vitro* (272). A more

detailed screen of CamSA with enteric bacteria *in vitro* will provide a better understanding of the effect CamSA will have on the indigenous gut populations.

CamSA is stable to the microenvironments of the gut that it will likely encounter during transit. Simulated gastric and intestinal fluids can be used to determine the stability of compounds to the changing pH of the GI tract (193). CamSA is stable to these simulated GI tract fluids. The experiments performed in this project did not determine the effect of common GI tract proteases (besides hydrolases describe below) on CamSA degradation. In the future, the effect proteases have on the stability of CamSA should be assessed. Bacteria in the GI tract secrete bile salt hydrolases that degrade bile salts (186, 187). CamSA is especially sensitive to degradation to the bile salt hydrolases produced by *B. longum*. Antibiotic therapy compromises and/or destroys the natural flora of the GI tract (142, 273). CamSA administered as a prophylactic alongside antibiotics will likely remain stable in the gut. After the cessation of antibiotics and CamSA, the natural bacterial flora can repopulate the gut and degrade any remaining CamSA. The direct effects of antibiotic agents on the stability of CamSA were not tested during this project. However, subsequent animal studies suggest that antibiotic medications have no affect on the activity of CamSA.

CamSA was analyzed in an *in vitro* gut permeability assay. This assay predicted that CamSA is impermeable from the gut lumen into cells. Also, the assay predicted that CamSA is actively transported into the lumen. These studies were performed with a very low concentration of CamSA. Bile salts can form micelles at high concentrations and readily traverse cellular membranes (223-

225). This critical micelle concentration (CMC) has been used to enhance the permeability of some molecules (225, 226). The critical micelle concentration of CamSA will need to be determined to be able to directly compare CamSA with previously studied bile salts. At the concentrations tested CamSA has low bioavailability, which is beneficial for treating a disease that is localized to the intestinal tract. CamSA was recovered at 100% in these permeability assays. This suggests that the human intestinal cells used in the study do not degrade CamSA and that CamSA does not adhere to these cells. As predicted earlier, this data further supports that CamSA will remain stable in the lumen of the gut.

Mammalian cell cultures were used to determine the cytotoxicity of CamSA *in vitro.* CamSA did not adversely affect the viability of two types of epithelial cells or an immune cell line. The epithelial cell lines were also used to determine toxicity due to vegetative growth of *C. difficile.* This is an indirect method to study toxin effect on cell morphology and viability. Based on previous studies, we hypothesized that *C. difficile* releases toxins after spore germination and that CamSA could reduce the toxins produced by preventing spore germination. Although assays were not performed to detect toxins secretion, epithelial cells treated with *C. difficile* germination medium caused morphology changes and cell death comparable to published results (87, 92, 198). CamSA reduced *C. difficile* toxin-induced cell death in a dose dependent manner. In future experiments, the concentration of toxin A and toxin B can be determined by enzyme-linked immunosorbent assay (ELISA) or Western blot. The direct effect CamSA has on toxin production can then be assessed. We predict that the

total concentration of toxins secreted will decrease with increasing CamSA concentration.

Taurocholate is the natural bile salt germinant of *C. difficile* spores (138, 140). We predicted that inoculating mice with *C. difficile* spores and administering taurocholate would result in increased disease severity or a faster onset of disease signs. Unfortunately, we did not see a difference between taurocholate-treated mice and untreated mice. Both presented signs of CDI within 24 hours and were moribund within 48 hours. The most probable explanation for these observations is that the *C. difficile* inoculum was too high to identify affects taurocholate had on spore germination *in vivo*. To test this hypothesis, future studies will have to be performed varying the inoculum of *C. difficile* spores administered to animals and the concentration of taurocholate administered. This will provide evidence that either exogenous taurocholate has effect or no effect on *C. difficile* spore germination *in vivo*. To our knowledge this type of *in vivo* study of taurocholate and *C. difficile* spores has not been reported.

CamSA has low bioavailability based on *in vitro* permeability assays, which reduces the risk of toxic effects on non-target organs (192). When CamSA was administered to mice in high concentrations, there were no observable toxic effects such as respiratory distress, tremors, salivation, coma, or death (256). Toxicity of CamSA to internal organs is a necessary next step to determining CamSA's safety. The major organs need to be assessed for size, morphology changes, and signs of toxicity (256). If metabolized, CamSA will likely be degraded to the cholate backbone and the *meta*-aminobenzenesulfonic acid

substituent. Cholate is a natural bile salt and the effects of increased concentration of cholate in the system will have to be determined. The substituent is not a natural substance and currently the oral, inhalational, and dermal toxicity and lethal dose has not been determined. The internal toxicity to major organs and blood vessels will need to be determined. Although CamSA is expected to remain in the intestine, the metabolites may not. The concentration of CamSA breakdown products in the blood, liver, and heart will provide a clearer picture of the fate of CamSA in the mammalian body (192).

A recent study shows that the biodiversity of the murine gut changes with the administration of different antibiotics and after infection with *C. difficile* (142). The natural flora of the gut resists colonization of *C. difficile* and certain bacterial populations are more resistant to infection (74, 142, 271). It is possible that this change in population dynamics of the natural flora after CDI provides a susceptible environment for CDI recurrence. Therefore for optimal protection, CamSA should not alter the natural flora either during or after treatment. In future murine studies, the population dynamics of the gut flora in mice treated with CamSA alone and CamSA during infection with *C. difficile* should be compared to untreated animals. We do not expect that CamSA alone will affect the indigenous bacterial populations. We do predict that the natural gut flora of untreated animals repopulates animals that have been treated with CamSA and infected with *C. difficile.* In other words, we expect to see the bacterial populations in the gut revert to the natural microbial community. The proposed study will provide a clearer understanding of the interactions between CamSA

and the microbiota of the gut. If the hypothesis is supported, it is likely that CamSA will also be able to prevent CDI recurrence.

To determine if CamSA is active *in vivo*, CamSA was administered to mice challenged with *C. difficile* spores. CamSA prevents CDI in these mice in a dose dependent manner. Furthermore, CamSA can prevent CDI in mice with a single dose when administered at the time of infection. *C. difficile* spores used to challenge mice did not germinate in the gut of mice treated with CamSA. This provides evidence of the anti-germinant ability of CamSA. Using CamSA as a chemical probe, the onset of CDI was determined to be between 6 and 9 hours post-challenge in the murine model. There are currently no prophylactic treatments for CDI making the discovery of CamSA as an *in vivo* anti-germinant an important step toward preventing the disease. Furthermore, to our knowledge no other studies have reported the timing of *C. difficile* spore germination in the murine gut before this study.

The risk of CDI recurrence increases with each episode and is greater than 60% in patients with more than two CDI episodes (48-50). Currently no prophylactic protocols exist for CDI and CDI recurrence (35). Recurrence has been defined as a relapse of CDI with the same strain as the initial episode or re-infection with a new strain (35). Recurrence of CDI is possibly due to the persistence of *C. difficile* spores in the gut following treatment and/or re-infection due to susceptible patients coming into contact with spores in the environment (101, 274-276). The mechanism of recurrence is poorly understood and represents a gap in *C. difficile* research. Mouse and Syrian hamsters have both

been successful animal models for CDI research (37, 241, 277). Relapse models in animals are more difficult to induce and to study. One study of relapse in the mouse has been reported; however, upon close inspection of the article, a model for reinfection is described (253). The authors induced CDI in mice and after they recovered, the mice were re-challenged with either the initial strain of *C. difficile* spores or a different strain (253). Admittedly, re-infection is one possible cause for recurrent CDI, due to suppressed patient immune systems and accessibility to contaminated surfaces (35). However, it is also likely that spores persist in the gut following CDI episodes and the spores begin outgrowth when therapy has ceased, causing relapse. CDI relapse has traditionally been studied in hamsters because the relapse induced in the hamster model mimics human disease more closely than the mouse (51, 250, 269, 278). A future direction from his current study will be to determine if CamSA can prevent CDI in the hamster model of disease and whether CamSA can prevent recurrence in hamsters. CamSA is able to prevent CDI in mice in a dose dependent manner and I hypothesize that CamSA will prevent CDI in hamster in a similar way. Furthermore based on the finding that CamSA-treated mice excrete *C. difficile* spores quantitatively, I hypothesize that CamSA will prevent recurrence in the hamster model. Any persistent spores remaining in the gut following antibiotic treatment will be prevented from germination and will be excreted. This project has provided evidence that anti-germination therapeutic compounds can prevent CDI in mice. The continuation of this project will lead the way for the re-defined management and prevention of *C. difficile* infection.

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Dissertation Title: Anti-germinants as a New Strategy to Prevent *Clostridium difficile*
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