Synthesis of a molecularly tethered dual function germinant and antimicrobial agent.

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Clostridium difficile (*C. difficile)* bacteria are the leading cause of nosocomial diarrhoea in the UK. The problem with the removal of *C. difficile* from hospitals is that it can sporulate and therefore be difficult to remove/kill using conventional methods. The spores enter the body *via* the faecal-oral route and in the presence of germinants (taurocholate), germinate into vegetative cells in the intestine, cause infection and produce symptoms *via* the release of two main toxins.

The project's aim was to produce polymeric steroid-based antimicrobial materials which will be able to germinate spores and then destroy the resulting vegetative cells. Deoxycholic acid, lithocholic acid and cholic acid were chemically manipulated to do this.

Various methods were tried to attach di-amines with varying tertiary amine-based groups to the parent bile acids, with success found using ethyl chloroformate to activate the carboxylic acid group *via* an anhydride group, with yields up to 90 %.

Once synthesised, the bile amides were screened for germinatory activity. The variables included the chain length and the nature of the groups on the tertiary amine. Once germination had been achieved the tertiary amine group was quaternized using various alkyl halides to introduce potential antimicrobial functionality.

From the manipulation of the tertiary amine, several compounds were found to be germinants. Several quaternized materials also displayed antimicrobial activity. Work was undertaken to attach acryloyl groups to the 3-OH group of chemically modified lithocholic acid, with success of then polymerising the monomer.

Co-germinants are amino acids, such as glycine and alanine which assist taurocholate in the germination of *C. difficile* spores. It was therefore attempted to produce a polymerizable glycine analogue which could be incorporated into the steroidal polymer to produce a germinatory surface. Boc-Lys-OH was converted to its acrylamide derivative with a view to the incorporation of a tethered glycine equivalent into a steroid polymer.

Key words; *Clostrdium difficile*, polymer, co-germinants, bile acids, nosocomial.

Acknowledgements

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On a separate note, I am also grateful to all my friends who work at Aston University; you have made this a very special experience.

This thesis is dedicated to my parents.

"Research is what I'm doing when I don't know what I'm doing".

Wernher von Braun.

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Abbreviations:

AIBN - Azo-isobutyronitrile.

Boc- *N*-(tert-butyloxycarbonyl).

CDAD – *Clostridium difficile* associated disease.

CDI - *Clostridium difficile* infection.

C. diff – Clostridium difficile.

Ca^{2+} DPA – Calcium diplionic acid.

$CDCl_3$ – Deuterated chloroform.

DCC – Dicyclohexyl carbodiimide.

DCM – Dichloromethane.

DMSO – Dimethylsulfoxide.

DMT-MM - 4-(4, 6-dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium.

MeOD – Deuterated methanol.

MIC – Minimum inhibitory concentration.

PDMAEMA - Poly (N, N-dimethylaminoethyl methacrylate).

THF – Tetrahydrofuran.

TLC – Thin layer chromatography

Synthesis of a molecularly tethered dual function germinant and antimicrobial agent

Aim: To produce a polymer surface which is capable of germinating *Clostridium difficile (C. difficile)* spores and killing the resultant vegetative bacterium.

1.0 Introduction:

1.1.1 *Clostridium difficile*:

Infections arising from pathogenic microorganisms are a significant concern in the world today, especially in a hospital setting, with medical devices and surfaces being major reservoirs of infections (Munoz-Bonilla and Fernandez-Garcia, 2012). These pathogens end up killing more people in the world than any other single cause. *C. difficile* is a Gram positive, spore forming, obligate anaerobic, rod shaped bacteria found asymptomatically in 3-15% of most healthy adult populations (Liggins et al., 2011, Heeg et al., 2012) but more importantly is a nosocomial infection affecting both humans and animals (Sorg and Sonenshein, 2010).

C. difficile was first discovered in 1935 by (Hall IC, 1935), while they were trying to understand the development of natural gut flora in neonates. It wasn't until 1978, however, that it was discovered that the bacterium was responsible for pseudomembranous colitis (Badger et al., 2012). In humans, *C. difficile* is found in the intestinal tract and it is also found in soil. In recent years, there has been a sharp rise in cases of *C. difficile* infection, with numbers of fatal cases and incidences due to antibiotic resistance increasing steadily, and the emergence of a hyper-virulent strain of *C. difficile* (Cecil, 2012).

Infections associated with the healthcare environment are widely regarded as the most frequent adverse events of hospital life (Hook et al., 2012). *C. difficile* is one of the most important healthcare associated infections in the UK, U.S.A and Canada (Koo et al., 2010). *C. difficile* associated disease (CDAD) is the most usual cause of watery diarrhoea (Pyrek, 2013), colitis and pseudomembranous colitis, due to the toxins produced by the bacterium. It is also the leading cause of diarrhoea in HIV infected patients (Surawicz, 2000, Sorg and Sonenshein, 2008b, Howerton et al., 2010). There were

over 14,000 reported cases of *C. difficile* in the financial 2012/2013 year in the United Kingdom and in America where in 2000, they spent more than $1.1 billion on the treatment of the disease (Badger et al., 2012). The incidence of the disease is rising and with 30% of sufferers not surviving the infection (2010, Koo et al., 2010), this is a large morbidity count. It is only the vegetative form of *C. difficile* which is able to produce the toxins which cause the symptoms (Howerton et al., 2010). Only the metabolically active, vegetative bacteria can be killed by alcohol gels, not the spores which are able to survive and resist many environmental stresses and are therefore, difficult to remove (Pyrek, 2013, Leggett et al., 2012). As *C. difficile* is an obligate anaerobe, the vegetative cells are extremely sensitive to oxygen, and if left in an aerobic environment will very quickly start to sporulate (Sorg and Sonenshein, 2008a). Due to the spore's resistance to normal cleaning measures, including alcohol stations and the fact that it is they which are spread, they are known as the infectious stage of *C. difficile* (Heeg et al., 2012). It is not known exactly how long the spores are able to survive for but there has been the suggestion in the literature that they can survive for a very long time, some literature suggests this can be many years (Kennedy et al., 1994).

One of the key problems with the diagnosis of the disease is that there is not one single identifying assay that is sensitive, specific and rapid, thus delays, or false positive results are seen on a regular basis (Hookman, 2009, McFarland, 2008).

C. difficile is predominantly found in hospitals, however, community infections are now also being reported (Wilcox et al., 2008). Spread through the faecal-oral route, generally by healthcare professionals not being as hygienic as they could be, the spores of *C. difficile* are ingested, and by their nature they are able to resist the stomach acid. A major predisposing factor for CDAD (*C. difficile* associated diarrhoea) is the overuse of broad range antibiotics (to which *C. difficile* is becoming increasingly resistant, but natural gut flora is not) and as a result the natural normal gut bacterial flora of the patient is significantly diminished. The natural gut flora would normally suppress the *C. difficile* growth, but as it has been depleted, the vegetative cells are able to colonise the gut, grow unchecked and produce toxins which can have severe symptoms. It has been shown that 25% of hospital cases of

antibiotic associated diarrhoea is caused by *C. difficile* vegetative cells and that per gram of faeces, approx. 100 spores are released (Wheeldon et al., 2008a).

1.1.2 Sporulation of *Clostridium difficile*.

When stressed (presence of air, lack of nutrients), *C. difficile* is able to form a spore which is very hard to kill and difficult to remove from environmental surfaces. Harsh disinfectants including peracetic acid or chlorine based disinfectants (such as chlorhexidine) (Fordtran, 2006) are the only materials able to do this, but it would be impractical to wash a hospital in bleach or acid. It has been shown that chlorine based disinfectants have sporicidal activity (Pyrek, 2013), but they are unpleasant to use and have associated health and safety issues. In addition, bleach is corrosive and the use of these disinfectants is therefore not ideal. Unfortunately, less harmful disinfectants such as 70% alcohol has no effect on the spores of *C. difficile*, but it will kill vegetative cells.

There are several different, clinically relevant microorganisms which are able to sporulate. When sporulation takes place it is usually categorised into seven unique stages illustrated in fig 1. At the start of germination, the exosporium produces small filaments which are able to attach the whatever surface the spore is on, thus preventing it being washed away (Sorg and Sonenshein, 2008a).

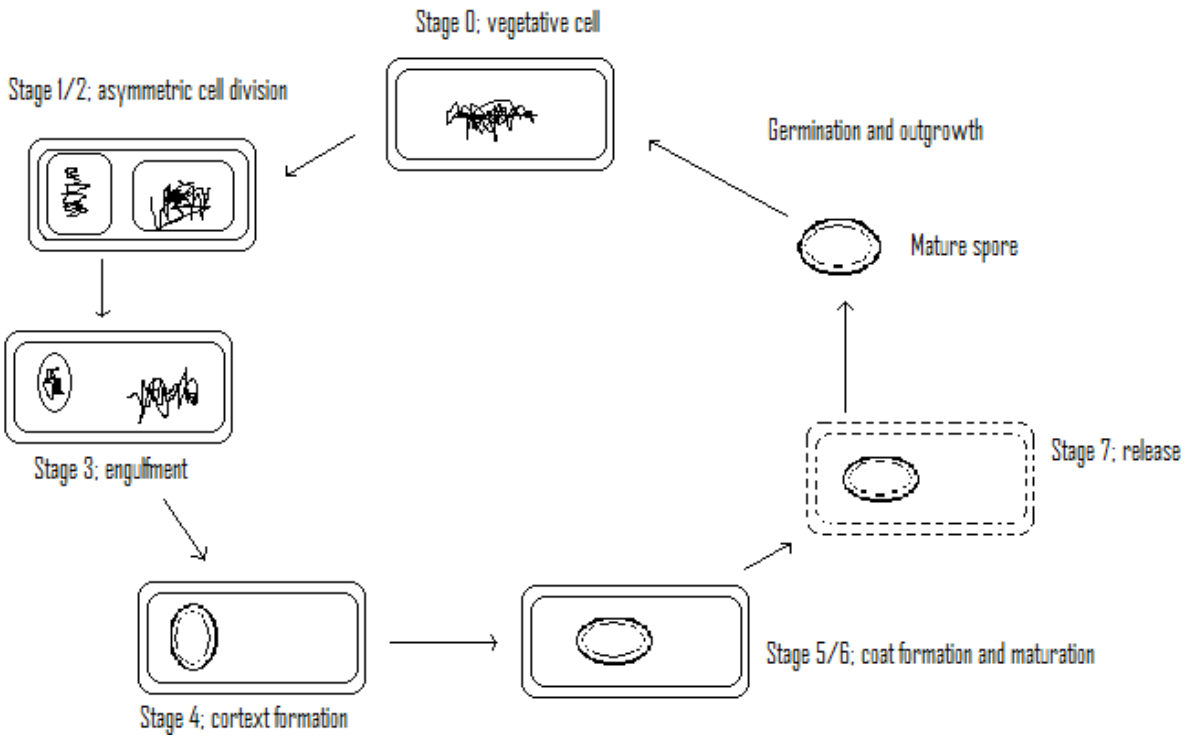

Fig 1; Key steps in the sporulation of *Clostridium difficile*, modified from (Leggett et al., 2012), squiggly line represents cell DNA.

1.1.3 Germination of *Clostridium difficile* cells.

When most bacterial spores (excluding *C. difficile*) germinate it is usually done through the Ger receptor family of proteins. What distinguishes *C. difficile* spores from other bacteria is the lack of these proteins. It is still assumed that the receptor sites in *C. difficile* spores are proteinaceous (Sorg and Sonenshein, 2010), but no one is sure of how many different receptor sites there may be. At the time of writing there is no known route of germination for these bacteria, only suggested hypothesis. Germination is defined as, "the irreversible loss of spore-specific characteristics and ultimately leads to vegetative cell growth"(Heeg et al., 2012). Germination can be observed in *C. difficile* spores spectrophotometrically. When germination starts to occur there is a sudden decrease in the absorbance at 600 nm. This indicates that the spore is going from phase-bright to phase-dark (when viewed using a phase contrast microscope). Without spore germination, no colonisation of the gut occurs. If no colonisation occurs, there is no release of toxins and therefore no infection. If the key factors of

germination can be established, then it may be easier to stop germination occurring in the first place. The presence of specific bile acids and co-germinants are the compounds which make the *C. difficile* spores start to germinate, specifically (sodium) taurocholate (a secondary bile salt) and the amino acid glycine. The mechanism of binding of each of the co-germinants is *via* a cooperative mechanism, whereby the particular affinity of one co-germinant directly affects the binding of another (Howerton et al., 2010).

The germination process has been fully studied in other spore forming bacteria such as *Bacillus subtilis*. In these bacteria, germination is initiated by a proteinaceous germination receptor which is encoded by a tricistronic operon. *C. difficile* does not have this receptor and therefore the exact mechanism of germination is not completely understood, and with a scarcity of genetic tools available, it will be this way for the foreseeable future (Howerton et al., 2010, Paredes-Sabja et al., 2008).

Although it is not known how it recognises germinants, the process that occurs after recognition is well researched. Initially Ca^{2+}-dipicolinic acid is released, as well as H^+, K^+ and Na^+ (Heeg et al., 2012, Sorg and Sonenshein, 2010) which causes an uptake of water. This redistribution of ions causes the lytic enzymes already present to become activated; these then degrade the spore cortex (a thick peptidoglycan layer). The uptake of water ultimately degrades the cortex and therefore the outgrowth of the bacterium can occur (Sorg and Sonenshein, 2010).

1.1.4 NAP1/BI/027, the hyper-virulent form of *C. difficile.*

Some literature suggests there has recently been an increase in number of infections due to the emergence of the hyper- virulent strain of the bacterium known as NAP1/BI/027 (Costello et al., 2008) (>35 % of all infections are caused by this strain). This strain has a higher antibacterial resistance (specifically against fluoroquinones (Cecil, 2012)) and is associated with increased number of CDI (*C. difficile* infection) which with it, has an increased mortality rate of 1-2.5 % (Liggins et al., 2011). Some papers suggest the reason for this increased mortality rate is the ability of this strain to produce toxins A and B quicker and in larger quantities (hyperproduction). The bacterium is able to do this due

to a mutation in its *tcdC* gene (Badger et al., 2012), which also allows it to germinate at a higher rate compared to other strains of the bacteria. Due to this mutation a rise in antibacterial resistance (fluoroquinones, levofloxacin, and moxifloxacin) has also been found. The fact that *C. difficile* is already very adept at going from a spore to a vegetative state (hypersporulation) means the spread of this bacteria can be very quick indeed. (Heeg et al., 2012), strongly argue against this idea of a new strain, however, and with no definitive review there is conflict on this particular strain in the literature.

1.1.5 Toxins and symptoms produced from *Clostridium difficile/CDI.*

Once the bacteria have colonised the small intestine (naturally occurs in 1-15% of healthy adults, 80 % in neonates) (Badger et al., 2012)), they start to produce two known toxins, TcdA (enterotoxin, 308 kDa) and TcdB (cytotoxin, 270 kDa, more important in pathogenesis in animals(Sorg and Sonenshein, 2010)), with a possibility of a third toxin TcdC, about which little is known, other than perhaps a synergistic relationship with the other two toxins and it being a binary toxin (Young and Hanna, 2014). TcdA causes cytotoxic loss of epithelial barrier function and specifically targets Rho GTPase by being absorbed into the intestinal epithelial cells. This simply means that the junctions between the epithelial cells are loosened and therefore destructive leukotrienes and cytokines are released and cause damage (Hookman, 2009). Within minutes of exposure to TcdA there is considerable damage to the mitochondria of the cells, the levels of ATP are greatly reduced and there is an abundance of oxygen radicals which are likely to cause even more damage (Hookman, 2009). TcdB merely has a synergistic relationship with toxin A and helps it to function. Thus, toxin A is the major virulence factor. The other problem encountered with the toxins is that they are extremely unstable and are degraded within 2 hours of being outside of the host's body. Therefore, if a person is suspected of *C. difficile* infection, it is imperative that they have their stool sample checked as soon as possible to see if there is any toxin present in the stool sample. Failure to do so can cause false negatives to be found, thus delaying of what could be critical treatment (Pyrek, 2013).

The most common effect of these toxins is colitis, but more severe symptoms such as toxic megacolon, ileal perforation, fulminant colitis, brain empyema, colonic perforation, sepsis and

colectomy have also been reported (Koo et al., 2010, Pyrek, 2013, Guo et al., 2012). The toxins bind to the specific receptor on the colonocyte and this causes endocytosis of toxin-receptor complex into the cell. Once the complex is inside the cell, it causes a drop in pH and a refolding of the toxin means it is inserted into the trans membrane domain of the endosomal membrane. This is followed by a translocation and release of catalytic domain into the cytosol *via* a mediated pore. Finally this causes uridine diphosphatase-glucose dependent monoglycosylation of Rho-GTPase targets (Fordtran, 2006, Hookman, 2009).

1.1.6 Current treatments for *Clostridium difficile* infection.

There are currently two main drug treatments for *C. difficile* infections, vancomycin (fig 2) which at high doses has been shown to give patients more severe *C. difficile* diarrhoea (Surawicz, 2000) and metronidazole (fig 2). Both of these drugs are becoming less effective, with recurrence rates being as high as 20-25% (Mahony et al., 1999, Koo et al., 2010), with 50 % of those recurring sufferers going on to relapse more than once (Badger et al., 2012). Although vancomycin has been shown to be the better treatment for severe CDI, even with these drugs, there is up to a 20% relapse rate, which requires a second round of treatment with antibiotics (Mahony et al., 1999). There has been a suggestion that vancomycin is the better treatment but there have still been deaths even when it was used as a treatment.

1

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Fig 2: Two current treatments for *C. difficile*, vancomycin (compound **1**) and metronidazole (compound **2**) (Tsutsumi et al., 2014)

Metronidazole is a nitro aromatic prodrug which, when reduced on the 5 nitrogen becomes active against the bacteria. It is a very low molecular weight drug and therefore it is easily transferred across the cell membrane (Tsutsumi et al., 2014). Due to its relative low cost, compared to vancomycin, it is usually the first prescribed antibiotic for the treatment of *C. difficile* associated disease.

Vancomycin is a hydrophilic (and therefore poorly absorbed in the GI tract), high molecular weight, rigid glycopeptide antibiotic, consisting of a glycosylated hexapeptide chain and cross linked aromatic rings by aryl ether bonds. Vancomycin works by tightly binding to the Ala-Ala sub-unit of the precursor DP-*N*-acetylmuramylpentapeptide of peptidoglycan, which produces a complex through the formation of hydrogen bonding. The resulting complex inhibits the biosynthesis of peptidoglycan, a key component of the bacterial cell wall (Tsutsumi et al., 2014).

Other than the widespread overuse of antibiotics (all antibiotics have been implicated in this, other than aminoglycosides, which do not cause CDI) (Williams and Spencer, 2009), there are several other risk factors when it comes to the prevalence of *C. difficile* (specifically colitis) in hospitals. These

factors include; advanced age (>65), gender (male) (Drekonja et al., 2011), impaired immune system such as HIV/AIDS, malnutrition and post pyloric tube feeding (Fordtran, 2006, Badger et al., 2012).

There are some variants of *C. difficile* which don't have the usual symptoms of diarrhoea, which can make it particularly difficult to diagnose and therefore successfully treat. It has also been shown that there are, in fact, specific antibiotics which can make the infection worse: penicillin, cephalosporin and clindamycin being the main culprits, as well as other treatments such as glucocorticoids (Liggins et al., 2011). Clindamycin was the first drug to be reported to produce severe diarrhoea when being used to treat anaerobic infections (Tedesco, 1984).

There are several new antibiotics which are currently being tested as a treatment to *C. difficile*, such as fidaxomicin (shown below, fig 3), the first drug to be approved for the treatment of *C. difficile* by the F.D.A in over 20 years (Stranges et al., 2013). It is a poorly absorbed macrocycle which is highly specific against the whole family of *clostridiaceae*. It works by inhibiting DNA transcription. The main advantage this drug has over other drugs is that it has a cure rate which is similar to vancomycin, but with reduced relapse rates and is still as effective at concentrations 4 times lower than both vancomycin and metronidazole (Zhang et al., 2012, Lancaster and Matthews, 2012). Other drugs currently being tested are nitazoxanide (fig 3), rifaximin, ramoplanin and tigecycline (Koo et al., 2010). The problem with fidaxomicin is the fact that it is expensive compared with other treatments ($2500 per course) (Lancaster and Matthews, 2012) and its recurrence rates with the hyper-virulent strain of *C. difficile* are the same as vancomycin and metronidazole (Chen et al., 2014).

Another school of thought is the use of probiotics, thereby replacing the natural gut flora which is decreased by broad range antibiotics (Koo et al., 2010). However, a systematic review in 2008 by Pillai and Nelson found there to be no significant evidence for the use of probiotics in treating of *C. difficile* infection (Pillai A, 2008, Hookman, 2009), but in 2013 Schoster et al found there were at least 5 probiotic strains which would be complementary to the treatment of both *Clostridium difficile* and *Clostridium perfringens* (Schoster et al., 2013), two of which are recommended to be taken forward for further testing. In this area at least, there would appear to a lot of disagreement in the literature

with no definitive answer. One of the newest class of antibiotics, specifically acting against *C. difficile* are the diarylacylhydrazones (fig 4) which have a protonophoric (prevention of movement of protons across cell membranes) mechanism (Chen et al., 2014).

Further study is being done to develop a vaccine for the disease, by inducing an immune response to the toxins, but this is still in early stages (Sougioultzis et al., 2005). There are also phase two trials going on to see if, by introducing non-toxigenic *C. difficile* into the body, they will take up the same niche as the toxigenic versions, therefore bringing the numbers of toxigenic bacteria down to a more manageable level (Cecil, 2012). The only current successful treatment of *C. difficile* infection is the use of faecal transplantation, which has a recorded success rate of 92 % (Drekonja et al., 2011), in recurring infections.

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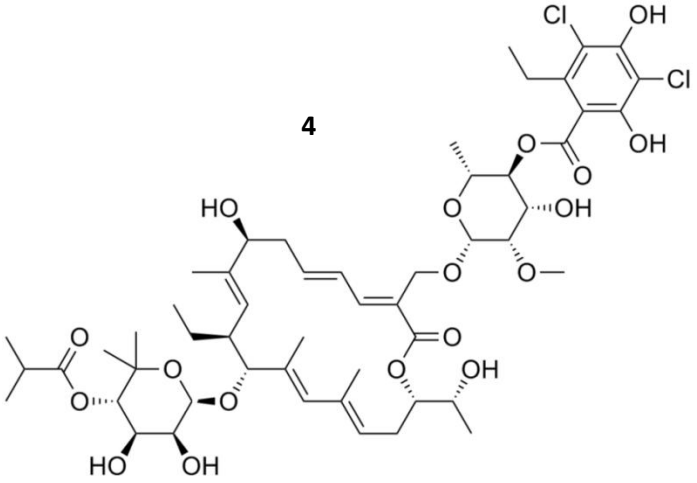

Fig 3: Structure of nitazoxanide (compound **3**) and fidaxomicin (compound **5**), new and emerging antibacterial drugs used in the treatment of *Clostridium difficile* (Zhang et al., 2012).

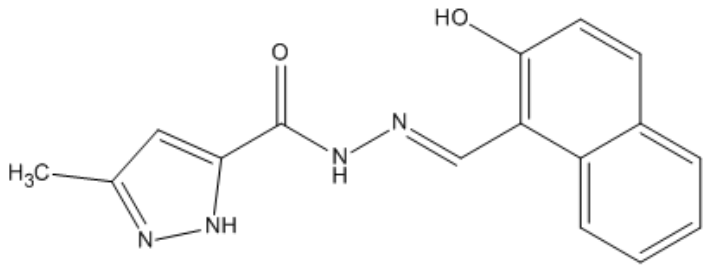

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Fig 4: Diarylacylhydrazones (compound **3**), a recently approved antibiotic in the treatment of *C. difficile.*

1.2.1 Bile acids introduction:

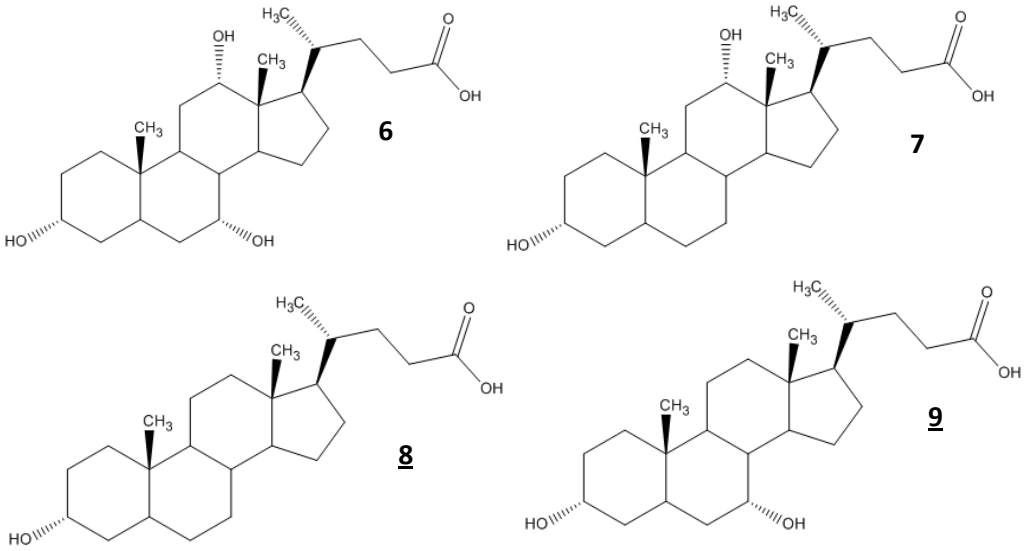

Fig 5: Key bile acids, cholic acid (compound **6**), deoxycholic acid (compound **7**), lithocholic acid (compound **8**), chenodeoxycholic acid (compound **9**).

Bile acids belong to the coprostane family (Zhu and Nichifor, 2002) and are based on steroidal structures with several chiral centres. In nature they are biosynthesised from cholesterol (El Kihel et al., 2008) in eukaryotic cells and are absent from the prokaryotes (Liggins et al., 2011). The reason that there are so many closely related steroid structures is because there are so many different biochemical pathways set up for the conversion of cholesterol into highly water soluble membranolytic compounds (Hofmann and Hagey, 2008). They are used in a number of

pharmaceutical products, such as carriers for drugs which are specific to the liver and the dissolving of gallstones (Kagedahl et al., 1997), due to their non-toxicity (Koivukorpi et al., 2007, Hu et al., 2005). Various analogues of bile acids have exhibited antibacterial, antifungal (Tamminen and Kolehmainen, 2001) and antiviral activity as well as a metal complexing ability (Joachimiak et al., 2008). There are 4 saturated rings which are lettered in the steroidal backbone (fig 6) (Hofmann and Hagey, 2008), from left to right, a-d (fig 6);

Work has established that the nature of the groups chemically attached to C-24 greatly dictates the kind of properties that the compound has. If there is a hydrophobic component to it then they have a tendency to be more active against gram negative bacteria (Guan et al., 2000). If there is a particularly short chain, they are able to make the outer cell membrane permeable to other materials, and in fact kill gram positive bacteria.

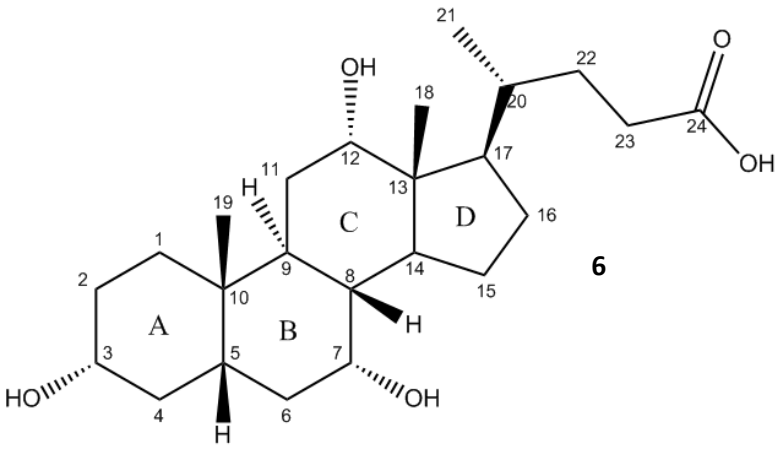

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Fig 6: Naming and numbering system of the steroidal rings (compound **6**).

The inherent skeletal structure of the bile acid with its four rings, covering an area of ~ 10 x 6 Å (Davis and Joos, 2003) seems to be very important as there have not been any structures which germinate the bacteria without this steroidal backbone. There would also appear to be a direct correlation with increasing amounts of hydroxyl groups in the 3, 7 and 12 positions and germinating ability i.e. cholic acid is a better germinator than lithocholic acid (Liggins et al., 2011).

Bile acids are an unusual class of molecules, due to their large, rigid but ultimately curved structure (Gao and Dias, 1999, Zhu and Nichifor, 2002). Each of the hydroxyl groups in cholic acid is chemically different in reactivity. Bile acids have a unique amphilicity and because of this can form micelles very easily (Idziak et al., 1999, Hu et al., 2005, Zhu and Nichifor, 2002) and are known emulsifiers (Zhang et al.). This is due to the positions of the methyl groups on the β face pointing "up" due to being in the axial position while the hydroxyl groups on the α face all point "down", due to the equatorial position of 3-hydroxyl and the axial positions of 7 and 12 (in cholic acid, one of the most common bile acids found) as shown in Fig 7. As there is a *cis* AB ring junction, this produces the curvature of the structure. There are several structural variants with regard to the carbon chain length attached to C20, with an addition of an extra four carbons being the most common in mammals (Fig 7). These are known as cholanes, where the four rings are all completely saturated. In the body these structures are usually conjugated to the amino acids glycine or taurine (Hofmann and Hagey, 2008, Zuluaga et al., 1999). Over the last couple of years there have been many modified bile acid drugs produced, which have both anticancer and antibacterial activity (Huang et al., 2009). It has also been shown that the introduction of steroid based drugs with antibiotics in the delivery system can lower the MIC (minimum inhibitory concentration) of said antibiotic (Fig 8). The steroid based compounds are able to do this by being inhibitors of the enzymes which are key in digestive processes in the liver and thus increase the bioavailability of the potential drug molecules (Ahonen et al., 2010).

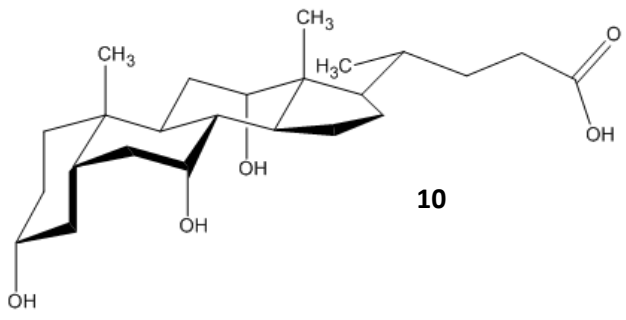

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Fig 7: 3D structure of cholic acid, showing the curvature of the steroid structure (compound **10**).

Fig 8: A steroid based drug which helps to lower the MIC of other antibiotics (Savage and Li, 2004) (compound **11**).

Lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid) (**8**) and deoxycholic acid (3α, 12α-dihydroxy-5β-cholan-24-oic acid) (**7**) are both secondary bile acids which are metabolised from primary bile acids naturally *via* enteric bacteria modification of either chenodeoxycholic acid (**9**) or cholic acid (**6**) in the liver, specifically a 7-α-dehydroxylation (by dehydroxylase enzymes) to form a double bond intermediate at the 7-position, which is then hydrogenated to produce the deoxycholic acid (Scheme 1) (Stellwag and Hylemon, 1979).

Scheme 1: Proposed pathway for 7-dehydroxylation of cholic acid by liver biosynthetic enzymes (Stellwag and Hylemon, 1979).

1.2.2 Germinants and inhibitors of *Clostridium difficile* spores.

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Fig 9: Structure of progesterone (compound **12**), an inhibitor of *C. difficile* germination (Liggins et al., 2011).

Progesterone (fig 9) has been shown to be an inhibitor of *C. difficile* spore germination, which means it stops the spore from germinating into a vegetative cell. This shows that there is clearly some interaction of the steroidal body with the bacteria, as there are no hydroxyls present in this compound, indicating that the presence of hydroxyls in lithocholic, deoxycholic and cholic acid may have some important function in promotion of germination. This has also been shown in other steroid based materials such as corticosterone, which has a hydroxyl group in position 11 and has decreased inhibitory effect compared to similar structures without the hydroxyl group present (Liggins et al., 2011). Movement of the double bond (as in pregnenolone (**13)**) or exchanging the ketone group for a sulfonic acid group (fig 10, compound **14**) does not appear to make a difference; these materials are still inhibitors of *Clostridium difficile* germination.

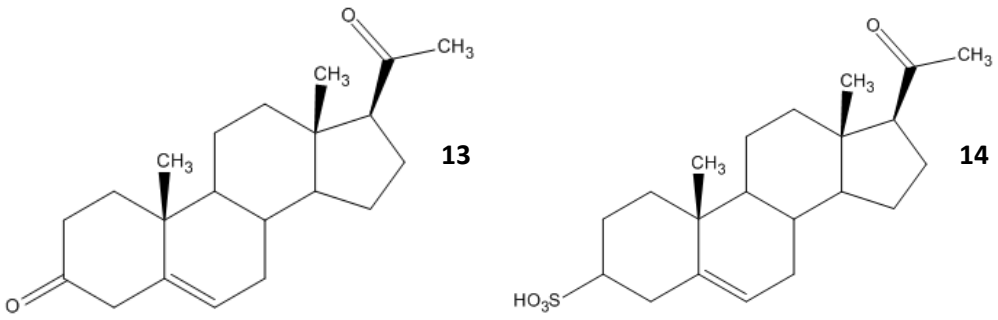


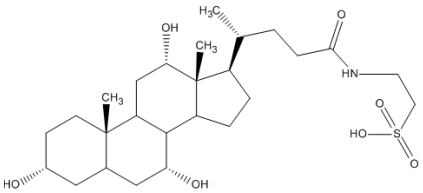
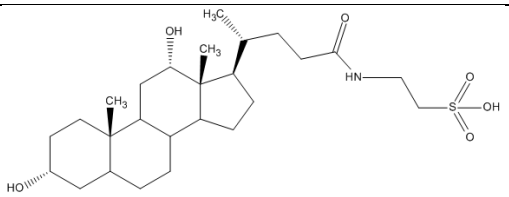
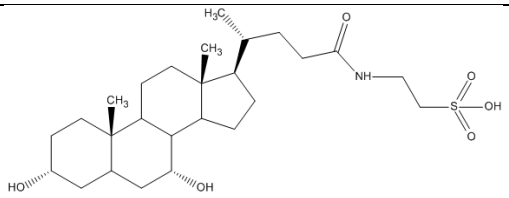
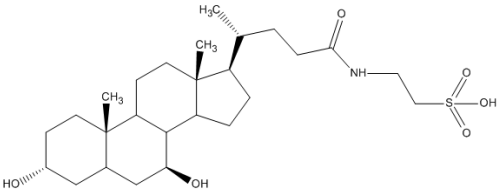
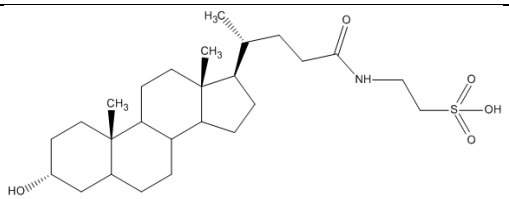
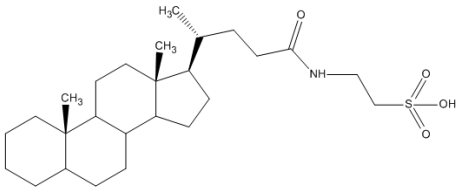
Fig 10: Structure of pregnenolone (compound **13**) and dehydroepiandrosterone (compound **14**), two inhibitors of *C. difficile* spore germination.

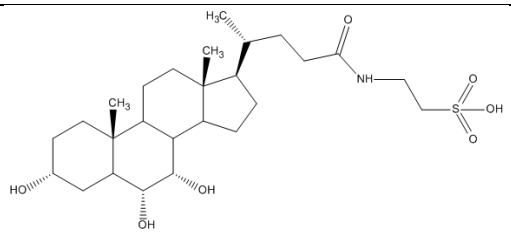
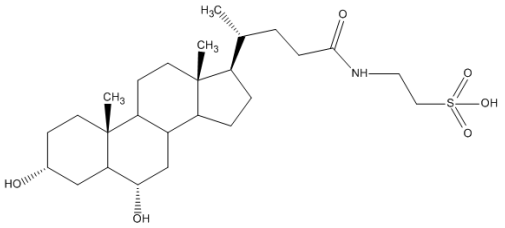
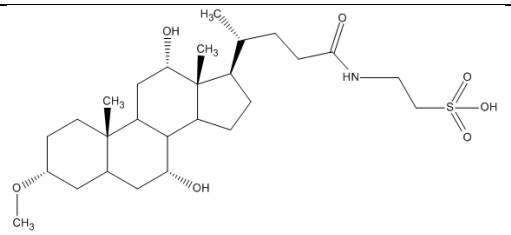
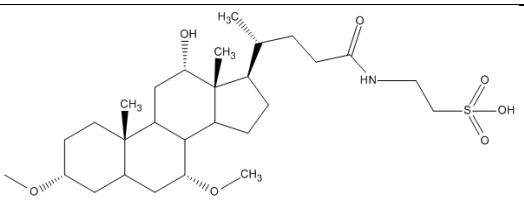
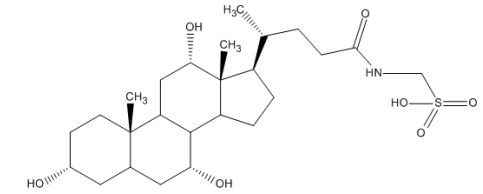
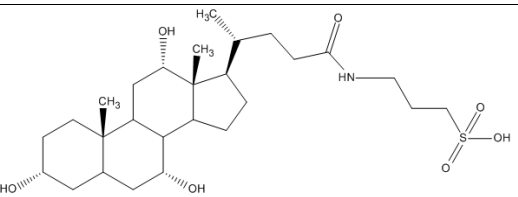
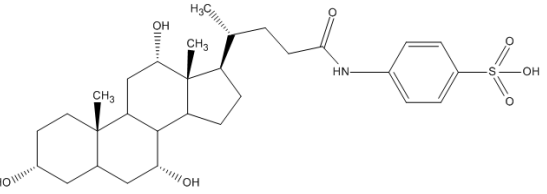
Chenodeoxycholate (**9)** has been shown to be a germination inhibitor of certain strains of *C. difficile* (Heeg et al., 2012). Unlike other materials which are germinants, it does not have a hydroxyl group at position 12, suggesting that although the 12 hydroxyl is not important for the binding of a material to *C. difficile* spores, it is in fact very important if the compound is to cause *C. difficile* spores to germinate.

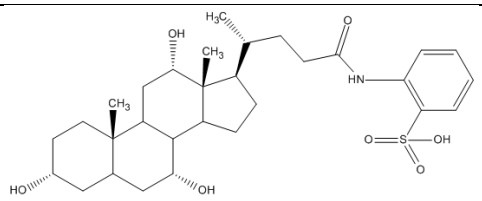
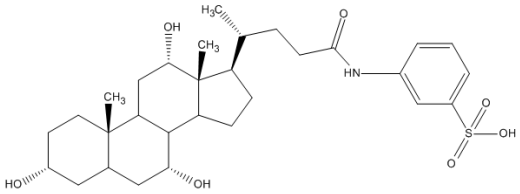
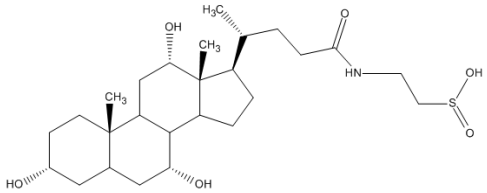
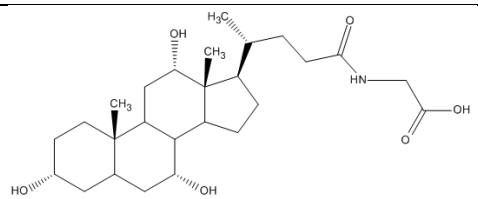
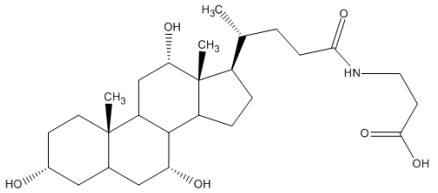
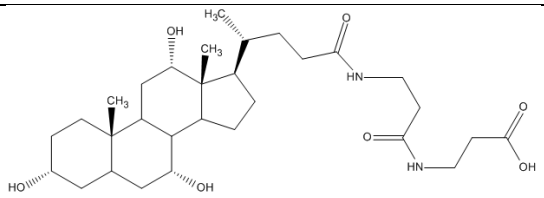
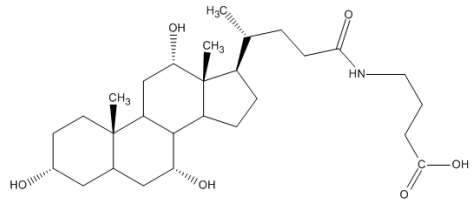
Chenodeoxycholate is a competitive inhibitor and therefore binds to *C. difficile* spore and prevents other known germinating compounds such as sodium taurocholate from binding at the active site. This suggests that the inhibitors most likely bind to the same place as the germinants do (Sorg and Sonenshein, 2010, Heeg et al., 2012). However, there may be other allosteric receptor sites where it may bind, thus changing the shape of the primary binding site, preventing germinants from attaching to the binding site. Chenodeoxycholate is absorbed through the colonic epithelium where it is usually metabolised to lithocholic acid, a poorly soluble bile acid with no germinating ability. When the gut flora are diminished due to the overuse of antibiotics, the dehydroxylation does not occur very quickly and chenodeoxycholate is absorbed 10 x quicker by the colonic epithelium than cholate (Sorg and Sonenshein, 2010).

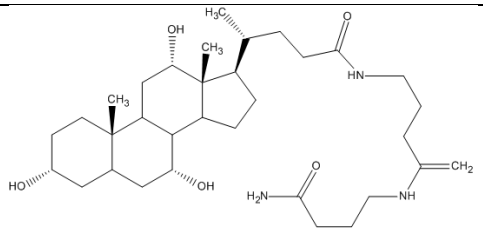
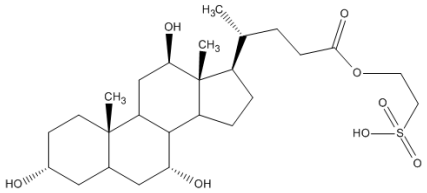
Howerton et al synthesised several different isomers of taurocholate to try and establish some structure activity relationship. Below in table 1, is a summary of the compounds which were tested.

Table 1: Structures tested by Howerton et al for their germinating ability of *C. difficile* (Howerton et al., 2010).

Compound number	Structure	% Germination of *C. difficile*
15		97.9 %
16		Reduction of 70 %
17		10 % of initial germination ability
18		3 % of initial germination ability
19		No germination
20		No germination

21		No germination
22		No germination
23		No germination
24		No germination
25		96.5 %
26		No germination
27		No germination

28		No germination
29		No germination
30		No germination
31		59.5 %
32		No germination
33		No germination
34		No germination

35		No germination
36		No germination.

Compound **15** is the structure for taurocholate. This has three hydroxyl groups in the 3, 7 and 12 positions. Taurocholate is a natural bile salt which is a very good germinant of *C. difficile* spores with a 50 % effective concentration at 15.9 nM. In the body this compound is usually present as the sodium salt and is the gold standard for germinating ability. The other compounds tested in Howerton *et al* are all synthetic analogues of this compound. Previous work in this area had shown that sodium taurocholate had significant solubility issues and thus was not appropriate for this project.

Compound **16** is lacking the hydroxyl at position 7. This removal has lowered its germinating ability by 70 % (relative to taurocholate), thus showing that the 7-OH is very important in germination of *C. difficile* spores. Compound **17** is similar to compound **16** but is missing the 12 hydroxyl instead of 7. This hydroxyl is clearly more important as a germinatory factor as without it, as germination is only 10 % of taurocholate. Compound **18** is a further analogue of compound **17** but the 7 hydroxyl has been changed from the alpha configuration to the beta configuration. By inverting the stereochemistry of this substituent the germinating ability of the compound falls from 10 % to 3%, showing how important the stereochemistry is.

Taurolithocholate (compound **19**) lacks hydroxyls at both position 7 and 12, while taurocholanate (compound **20**), has no hydroxyl groups present at all. Unsurprisingly both of these compounds are unable to induce any germinatory effects, thus showing the importance of these hydroxyl groups.

In compound **21** and compound **22** the 12 hydroxyl group has been moved to the 6 position on the steroid backbone. By moving the hydroxyl group, this severely inhibited any germination of *C. difficile* spores.

The Howerton group discovered that if taurocholate is O-methylated, two different compounds are produced, 3-methoxy-7, 12-dihydroxytaurocholate (compound **23**) and 3, 7-dimethoxy-12-hydroxytaurocholate (compound **24**). Neither of these compounds were able to induce or inhibit germination of *C. difficile* spores.

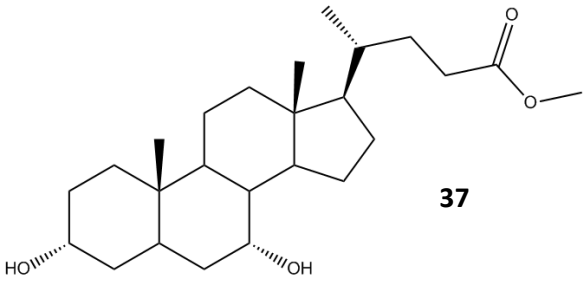
Compounds **25- 36** were all variations on the taurine side chain to see if manipulation of this side chain would have any effect on the germinatory ability of the compounds to germinate *C. difficile* spores.

Compound **25** has one less carbon in its taurine side chain and this results in a marginal germination decrease from 97.9 % for taurocholate to 96.5 %. Whilst removal of this carbon doesn't hinder germination, it doesn't show any real inhibitory value either. However, addition of an extra carbon in the chain, compound **26**, negates any germinatory ability. This would suggest that the putative germination receptor is relatively small so any expansion in size of the chain, or indeed, insertion of extra groups, such as benzene rings or extra amide groups (compounds **27-30, 32-35**), will leave the compound completely unable to germinate *C. difficile* spores.

Compound **31** is similar to compound **25** but the change is conversion from a sulphonic acid group to a carboxylic acid group. This conversion reduced germinating ability to 59.5 %, while conversion of the amide of taurocholate to an ester removes any germinatory activity whatsoever.

Lithocholic acid has been shown to be an inhibitor of germination in some literature, even in the presence of taurocholate. The reason it is not used as a medicine is because of its poor solubility and the implication of its role in colorectal carcinogenesis (Sorg and Sonenshein, 2010). The 7 hydroxyl could be very important when it comes to germinating, but not binding, much like deoxycholic acid. Lithocholic acid does not have the 7-OH and is not a germinant. Deoxycholic acid is an inhibitor of

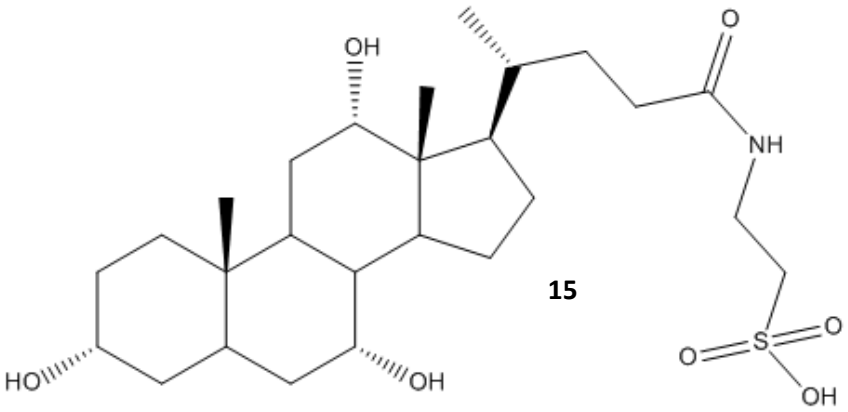
vegetative growth, where no growth was recorded at all (Paredes-Sabja et al., 2008, Wilson, 1983). Cholic acid, which has all three hydroxyls present, has been shown to be a very good germinator of *C. difficile* spores, although not as good as (sodium) taurocholate. The potential analogues of this compound could prove to be very good at causing germination of the spores, converting them into vegetative cells.



37

Fig 11: Methyl chenodeoxycholate (compound **37**).

Taurocholate (fig 12) has been shown to be one of the most effective bile acid germinant of *C. difficile* spores in a clinical setting, with or without the presence of co-germinants. It is so far the best germinating material known, but extensive exposure to the material is required for germination to occur (Wheeldon et al., 2008a).



15

Fig 12: Taurocholate (compound **15**), a compound usually conjugated with sodium in the body.

(Sodium) taurocholate is a naturally occurring bile salt which has hydroxyl groups on the 3, 7 and 12 positions, respectively. If the hydroxyl group at position 7 is missing, the material is known as taurodeoxycholate (**16)**; this has been shown to germinate *C. difficile* spores as well, but at a rate 70% less than standard (sodium) taurocholate. It would therefore appear that the key groups for germination are the 7 and the 12 hydroxyls in the alpha position. However the hydrogen bonding ability of the 3-OH is also important for binding and therefore germinating ability.

Removing the amide bond and replacing it with an ester in taurocholate negates any germinating ability of the compound as well, thus showing that the amide group, and presumably therefore, the group's ability to form hydrogen bonds is very important to producing a germinatory response (Howerton et al., 2010, Aher et al., 2009, Tiller et al., 2001).

Although there is a lot of data on the materials which will and will not cause germination of spores, there are other factors which may be important to germination but which have not been fully investigated at this point. These factors include but are not limited to pH, temperature and pressure. Although inside the body all three of these are likely to be pretty stable, outside of the body, which is the area this project is focussed, these factors can be highly variable (Wheeldon et al., 2008a).

1.2.3 Amides overview:

Amides are ubiquitous in nature and are found to exist in lots of complex synthetic and natural compounds, such as proteins, used to join individual amino acids together, known as peptide bonds (Allen et al., 2012, Allen et al., 2009). These bonds can be produced both *via* chemical and biological means (Allen et al., 2010). However, there are difficulties with the synthesis of this bond, such as low yield, racemisation, degradation and difficult purification (Montalbetti and Falque, 2005).

Amide bonds are strong because they are able to form different resonance forms of the bond. In nature this bond can be formed using enzymes and therefore has minimal amount of waste material associated with it, unlike modern day methods which often involve the use of inorganic catalysts or ineficient reagents. This in turn makes the whole process environmentally unfriendly and costly. There

are many ways of synthesising an amide bond, with different reagents and conditions and yields. Often what the amide is attached to will dictate the preferred method of amide coupling.

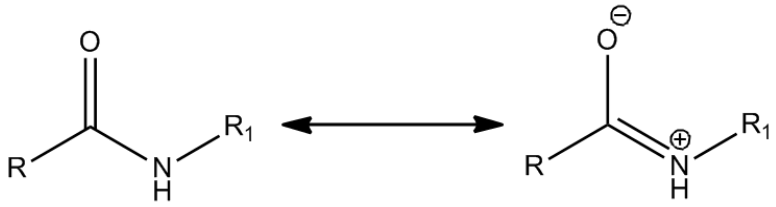


Fig 13: Resonance forms of an amide bond.

1.2.4 Amide bond formation

While the use of acid chlorides and *p*-nitrophenyl-esters have been proven to be the highest yielding reagents, the execution of the synthesis can be difficult, whereas production of an anhydride, although generally lower yielding, is a much simpler methodology (Bellini et al., 1990, Cravotto et al., 2005). Anhydrides are species which will readily react with many different nucleophilic species, especially alcohols to form esters and amines to form amides. The advantage ethyl chloroformate (an anhydride forming reagent) has over DCC (dicyclohexyl carbodiimide); (the more traditional reactive species) is that dicyclohexylurea is formed as a by-product, which can be difficult to remove. With anhydrides, this is not the case and therefore, this has the advantage, especially if using an acid which is more valuable (Montalbetti and Falque, 2005).

Scheme 2: Carbodiimide induced amide formation mechanism (McMurry, 2004).

There are several different types of carbodiimides, depending on the reaction type. DCC (dicyclohexylcarbodiimide) as shown in scheme 2 produces a urea which is very poorly soluble and is generally removed by filtration. This coupling method is therefore used mostly in solution phase chemistry. If the requirement is to use solid phase chemistry, then DIC (diisopropylcarbodiimide) is used as the resulting urea can be removed by DCM washes (Montalbetti and Falque, 2005).

Acid chlorides are very reactive species and will readily react with amines to produce amide bonds. They are often introduced into the reaction with *N*, *N*-dimethylaminopyridine (DMAP) which behaves as a catalyst. Pyridine can also be used as the solvent for many acid chloride reactions and produces and intermediate acylpyridinium salt. Acid chlorides do have limitations however, they are easily hydrolysable, can produce racemic mixtures of compounds and can potentially unwanted side reactions. This potential for racemisation and side reactions to occur can sometimes be limited by the

use of acyl fluorides or acyl bromides, as they are less moisture sensitive and are more reactive towards amines.

1.2.5 Aminolysis reaction

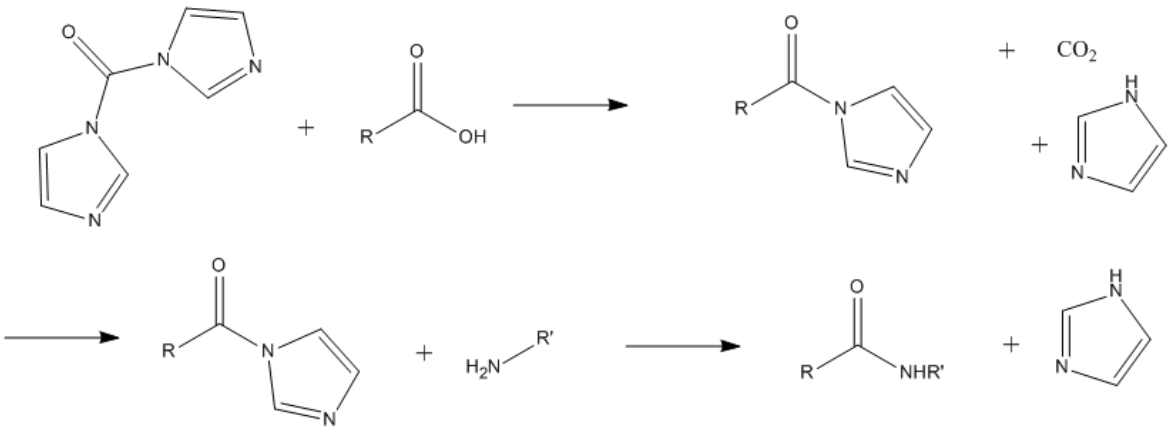
Aminolysis (scheme 3) is simply the heating up of a methyl ester of a bile acid with an amine, at a temperature over 180 ^{o}C, producing methanol as a by-product. It has been shown that this reaction has a rate limiting step which is the collapse of the tetrahedral intermediate. Unsurprisingly, the rate of disappearance of the ester was equal to the rate of formation of the alcohol, in this case methanol (Talvik et al., 1999).

Scheme 3: Aminolysis reaction (Talvik et al., 1999)

1.2.6 N, N'-Carbonyldiimidazole

There are many reagents which are useful in the coupling of a carboxylic acids and amines (not specifically bile acid), as this is a key step in peptide chemistry. Literature suggests that the reagent which was most likely to work on bile acids would be *N, N'*-carbonyldiimidazole (scheme 4) (Larrivée-Aboussafy et al., 2009, Woodman et al., 2008). Carbonyldiimidazoles are generally preferred to the use of acid chlorides because the intermediate acyl imidazole compounds are a lot more stable (Larrivée-Aboussafy et al., 2009). The advantages of carbonyldiimidazole are the fact that it is inexpensive. The only by-products produced are carbon dioxide and imidazole, which in relative terms are reasonably benign, and therefore, if the reaction needed to be scaled up at some point, would not be a problem (Woodman et al., 2008). The same paper also suggests that the use of imidazole· HCl

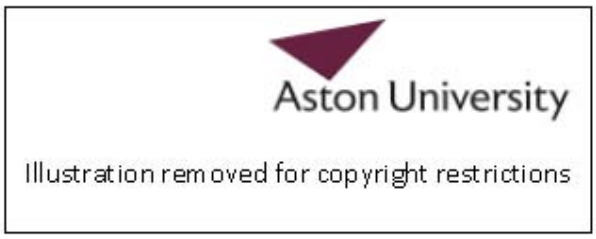
would improve the rate of reaction because of the presence of a proton donating compound, protonating the imidazole, which would therefore make it more reactive.



Scheme 4: Formation of amide bond using N, N'-carbonyldiimidazole.

1.2.7 DMT-MM

Care must be taken when choosing the solvent system for the reaction. Alcohol-based solvents for example, are likely to form the corresponding esters and therefore not appropriate. One paper has reported a way around this by using 4-(4, 6-dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) as a reagent (scheme 5). This enables the use of alcohols and water as solvents. They are good solvents because they are very inexpensive and are much greener than the solvents usually used in this kind of chemistry.

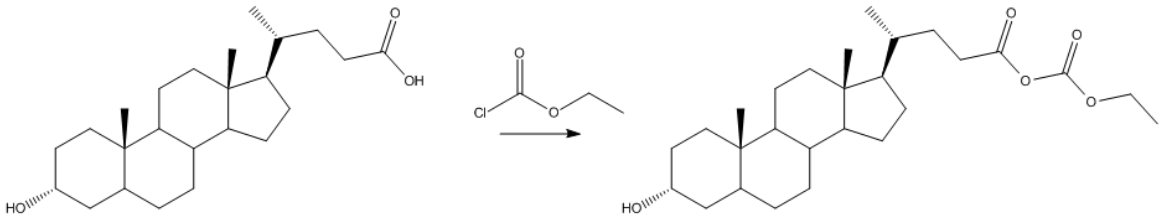


(Kunishima et al., 2001)

Scheme 5: Amide bond formation using DMT-MM

The same paper suggests that this method has higher selectivity for primary compared to secondary amines and greater yields in comparison to simply using carbonyldiimidazole.

1.2.8 Ethyl chloroformate



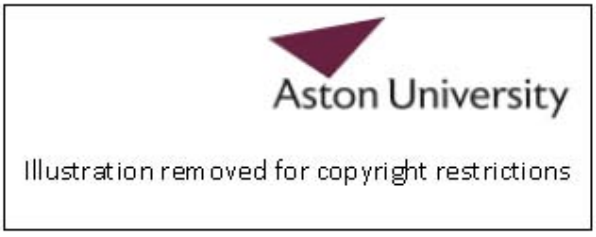
Scheme 6: Production of mixed anhydride using ethyl chloroformate.

Ethyl chloroformate can be used in acid coupling reactions (scheme 6), which has the advantage of not having to use the methyl ester, as opposed to the aminolysis reaction which does. Use of ethyl chloroformate gives rise to the production of a mixed acid anhydride, which is a more reactive group than the acid (McMurry, 2004). Heating is not required for the amide bond to be formed, thus preventing the problems associated with aminolysis, such as degradation of materials, thus making it easier to purify.

1.3.0 Co-germinants of *Clostridium difficile* spores:

As well as bile acids being germinants of *C. difficile* spores, there is a group of compounds, which comprise of amino acids or analogues thereof, which are known as co-germinants. While it is generally accepted that a suitable bile amide will germinate the *C. difficile* spore on its own, if a co-germinant is added then this can help increase the germination rate of the spore; it is a synergistic relationship between the two compounds. As a general rule, single co-germinants are not able to induce germination without the presence of a bile acid analogue.

Table 2: List of amino acids and small molecular weight compounds which act as co-germinants or inhibitors of *C. difficile* spore germination.

Glycine-compound **38**	
β–alanine-compound **39**	
γ –butyric acid-compound **40**	
Aminomethylphosphonic acid-compound **41** An inhibitor of germination	
L-alanine-compound **42**	
D-alanine-compound **43**	
L-cysteine-compound **44**	

L-phenylalanine-compound **45**	
L-serine-compound **46**	
Dodecylamine-compound **47**	

Glycine is probably the most well-known co-germinant of *C. difficile* but it would appear that there can be a considerable amount of manipulation of the structure of the amino acid and it will still act as a co-germinant. An important factor is simply how hydrophobic/hydrophilic the compound is. Examples include β-alanine and γ-butyric acid (Table 2). One paper seemed to indicate that it is not the chain length between the amino and carboxyl groups in the co-germinant which is important but the fact that there is a carboxyl and a free primary amino group inherent in the amino acid (Howerton et al., 2010). Another key point is that although it does not seem to matter how long the chain between the amino group and the carboxylic acid is, if there is any branching in that chain then this dramatically reduced the germination properties of the co-germinant. There are exceptions to this rule however, with L-phenylalanine and L-arginine also being shown to be good co-germinants (Howerton et al., 2011).

One analogue of glycine, aminomethylphosphonic acid (Table 2), has been shown to actually inhibit the germination of *C. difficile*. While it retains the overall negative charge, the replacement of the carboxylic acid carbon with phosphorus is clearly the reason for this removal of activity. The same literature suggest that if any modification is made to the free amino group of glycine, then this abolishes any co-germinating ability, showing that the free amino group is as important, if not more so, than having the free carboxylic acid moiety. Furthermore, if the glycine's carboxylic acid is

methylated, this decreases germination ability even more (10 % of the original value), suggesting that the free acid group is key to its co-germinating ability (Howerton et al., 2010).

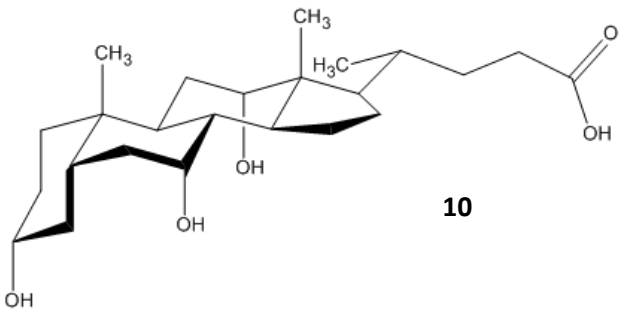
L-Alanine (Table 2) is an amino acid which has been shown to be as good a co-germinant as glycine. What is interesting is that, although the L form of alanine was a co-germinant, the D version was not a co-germinant for *C. difficile,* therefore implying that the stereochemistry is very important in determining whether the material is a co-germinant (Howerton et al., 2010). Therefore, it is probable that stereochemistry is also important in germinating materials, much like the conversion of hydroxyls from alpha to beta, as discussed earlier, negating any germinating ability.

L-Cysteine and L-phenylalanine are both as good at co-germinating *C. difficile* as glycine, yet L-serine is inactive against the bacterium. The fact that L-phenylalanine is such a hydrophobic amino acid implies that perhaps it will be attaching to a different receptor site to glycine, as it is difficult to imagine both amino acids fitting in the same site. In other spore forming bacteria, where more is known about the receptor sites, there are numerous different sites for differing reagents, so it is plausible that *C. difficile* also has these different receptor sites (Howerton et al., 2010). All these factors together seem to imply that there are many different factors for germination, and that only a few of them need to be fulfilled before germination will take place.

While isolated amino acids will not affect germination on their own, if multiple groups of amino acids are introduced into the bacterium's environment (L-phenylalanine, L-arginine and glycine) then this will promote germination of the bacteria. It has also been shown that the bacterium will germinate in the presence of Ca-DPA (Calcium diplionic acid), a key chemical in controlling germination), potassium ions and in the presence of the surfactant dodecylamine (table 2), with no bile acid analogues present at all (Paredes-Sabja et al., 2008, Wilson, 1983).

1.4.1 Protecting groups for bile acids:

Due to the inherent nature and shape of bile acids, the C-3 hydroxyl group is much more open to attack due to the steric hindrances which "protect" C-7 and C-12 to some extent.



10

Fig 14: Positions of the hydroxyls in compound **10**.

There are quite a few reactive functional groups in the bile acid structure where reactions can take place, so there is often the need for use of protecting groups, either the carboxylic acid groups or the hydroxyl groups. For the hydroxyl groups the simplest way to protect them is through the use of acetate groups, which are easy to prepare, highly stable and easily removed *via* hydrolysis when they are no longer required (Kuhajda et al., 1996, Gao and Dias, 1999). As there is a CH_2 (C4) unit in a 1,3 diaxial position, then this means that the 7-OH is more sterically hindered than the 12- OH, which is itself more sterically hindered than the 3- OH (Davis and Joos, 2003). By varying the specific reaction conditions, different groups can be protected/deprotected. Acetylation of the 3-OH, 7-OH and 12-OH can be achieved by using Ac_2O at room temperature. This protects all three of the OH groups. It is relatively easy, however, to selectively remove the acetate group from the C-3 hydroxyl group. This can be done through the use of K_2CO_3 in methanol at room temperature, with a yield of 99 % (Gao and Dias, 1999). Therefore, if specific manipulation at 3-OH is required, there is no chance of production of diprotected or triprotected compounds being formed as the other hydroxyl groups will still be protected. Another method for acetylation is $HCOOH/HClO_4/55^{o}/Ac_2O$ (Malik et al., 1986). If protection of both C-3 and C-7 is required, whilst leaving C-12 open for manipulation, this is relatively easy to do as well.

Other possibilities are to convert the hydroxyl groups into ketones, which can be done chemically using potassium chromate and sodium acetate. To convert specific hydroxyl groups to ketones using bacteria, this can be achieved using *Acinetobacter calcoaceticus* (Giovannini et al., 2008).

To manipulate the hydroxyl groups, the carboxylic acid group will need to be protected to prevent any unwanted side reaction from occurring. The easiest way to do this is to convert the acid into the methyl ester. The conversion to the methyl ester can be done by several different methods; however, the method of choice seems to be HCl/MeOH. Use of $TMSCHN_2$ in methanol has been reported, with the advantage of this method leaving already protected acetate hydroxyl groups unaffected (Gao and Dias, 1999), as opposed to the HCl/MeOH which can cause cleavage of acid sensitive protecting groups.

Another method for producing ketones is to use Jones' reagent (CrO_3) (Fieser and Rajagopalan, 1950) and acetone. Once the ketone has been produced, it is possible to further protect it as a cyclic ketal by the use of ethylene glycol. To produce the ketone specifically at C-7, the conditions needed are room temperature and the use of AcCl and CH_3OH. Conversion of alcohol to ketone at only the C-7 position can also be achieved by use of NBA(N-bromoacetamide), acetone, H_2O at room temperature for 3 hours (Gao and Dias, 1999).

There are also specific chemical protecting group reagents; these include tert-butyloxycarbonyl (Boc) and 9-fluorenylmethyloxycarbonyl (Fmoc). One way of attaching a Boc group is to use di-tert-butyl dicarbonate which is attacked by the nucleophilic amine to produce the protected amide.

Trifluoroacetates are another method of protecting hydroxyl groups in bile acids. It has been shown however, that trifluoroacetates are much more prone to hydrolysis than acetate groups. The advantage being however, that the terminal carboxylic acid does not need to be converted to the methyl ester first before manipulation. Again, like the acetate protection groups, the solvent used dictates which hydroxyl group is specifically protected (Gao and Dias, 1999). Reaction conditions often involve TFAA, THF, then $NaHCO_3$, MeOH and THF (Gao and Dias, 1999).

During protection with acetates, it was found that pure products could not be isolated without the use of column chromatography, so a new methodology was required to get around this problem (Gao and

Dias, 1999). Use of formate as a protecting group meant that the product was in a crystalline state, which was able to have characterised and well defined melting points.

1.5.1 Stereochemistry of bile acids.

Inversion of stereochemistry (fig 15) is very important in designing drugs and other molecules. One form of a drug may be life-saving, whereas another isomer may have no effect/unwanted effect. Conversion of hydroxyl groups from α to β can be done, specifically for the hydroxyl group at C-7 (Compound 48).

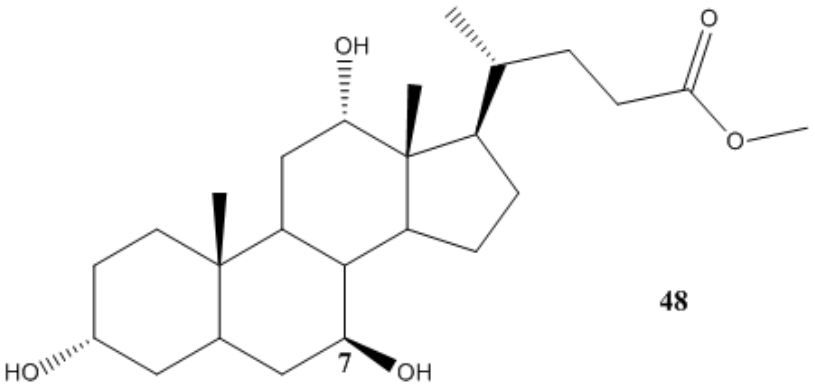


Fig 15: Inversion of stereochemistry at the 7 position in methyl cholate, compound **48**.

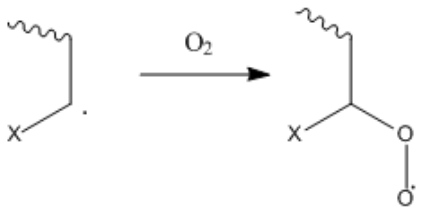
This inversion of stereochemistry is achieved by first oxidising the alcohol to the ketone using potassium dichromate and then performing a stereospecific reduction using potassium tert amyl alcohol.

1.6.1 Polymer overview:

Synthetic polymers are prepared by the polymerisation of monomeric units. The easiest way to do this is by a process called free radical polymerisation, which is the industry standard for about 40-45 % of industrial polymers (Nesvadba, 2012). The advantages of free radical polymerisation compared with any other form of polymerisation are that it is able to be performed in the majority of solvents and the

presence of trace oxygen (Nesvadba, 2012). There are many initiators of free radical polymerisation but the most well-known (and used in this project) is AIBN (azo-isobutyronitrile) (Scheme 7). The most important features of this azo class of initiators are the fact that they are symmetrical dialkyldiazenes which have tertiary alkyl groups to stabilize the radical which is formed. By having these features on the free radical initiator, the decomposition temperature of the compound can be fine-tuned to the needs of the project. They also have a greater tendency (compared to other photo initiators) to undergo chain transfer reactions. The propagating organic radicals are as a general rule, planar. While it was stated earlier that free radical polymerisations are tolerant of trace levels of oxygen, fully saturated oxygen environments do stop the polymerisation entirely, due to formation of peroxy radicals being formed (Scheme 8).

Scheme 7: Mechanism of AIBN breakdown to produce a reactive radical species (Clayden, 2009).

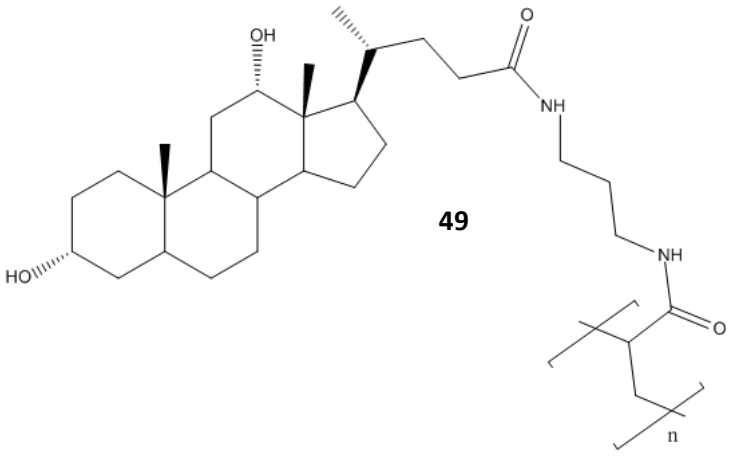


Scheme 8; Peroxy radical formation by allowing free radical polymerisation to occur in an oxygen environment.

1.6.2 Bile acid derived polymers.

The first literature relating to polymerisation of bile acids was in 1988 (not free radical) (Zuluaga et al., 1999). This was carried out in toluene at 90-100 ^{o}C using p-toluene sulfonic acid as the catalyst to induce ester formation. The initial bile acid was cholic acid and it was observed that some crosslinking would occur at both positions 7 and 12; the amount of this crosslinking was directly related to the temperature at which the material was heated. A lot of work has gone into the addition of polymerizable groups onto either hydroxyl groups or the C24 carboxylic acid groups, and therefore producing polymerizable bile acids. As stated earlier, these bile acid structures have a unique amphilicity which means that they can potentially bind to the polymer matrix through both the carboxylic acid side chain and the hydroxyl groups (Idziak et al., 1999).

Other research groups (Avoce et al., 2003) have worked on producing polymeric materials by attaching amines with polymerizable units to the carboxylic acid tail (fig 16).



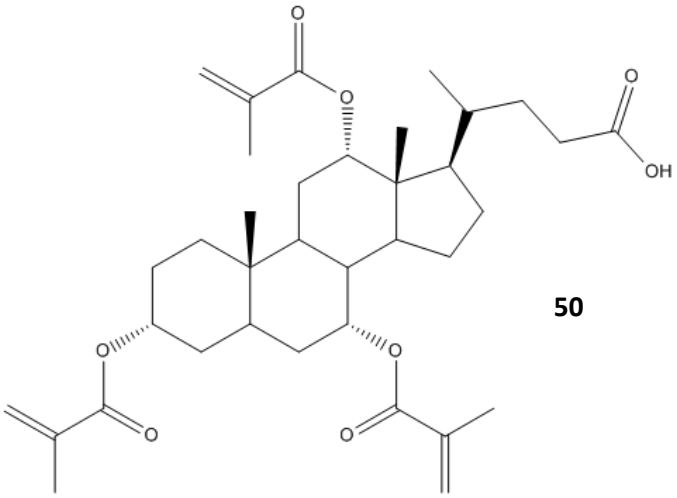
49

Fig 16: A polymerized bile acid (compound **49**).

One possible way of producing a polymeric bile amide species is to attach an acryloyl group to the hydroxyls located on the bile amide structure. The 3-OH is the most reactive of the three hydroxyl groups and therefore would be preferentially attacked. If needed though, all three hydroxyl groups can have acryloyl groups attached but the conditions required to do so are much harsher.

It is also possible to react elsewhere on the steroid compound. If ethylene glycol has been attached to the carboxylic acid group to produce an ester it is then possible to react on the primary hydroxyl at the end of the ethylene glycol chain (Zhu and Nichifor, 2002). If the carboxylic acid is not the required attachment site, then this can be converted to the methyl ester and as well as protecting the acid group from attack it will also improve the solubility of the material.

Polymethacrylates (fig 17) are widely used in biomedical technology; however, there is a problem with their toxicity. When attached to bile acids and polymerised they produce super branched macromolecules which are self-organising and possess good thermal and mechanical properties (Hao et al., 2006). The main advantage of attaching a natural product to this methacrylate group is that the biocompatibility of the overall compound is improved (Avoce et al., 2003).



50

Fig 17: Polymethacrylated cholic acid (compound **50**)

1.6.3 Antimicrobial polymers

It is a well reported fact that the majority of bacterial cell walls are negatively charged. Therefore, if the polymer is to be antimicrobial, the way to do this is to make the polymer itself positively charged; thus the wide usage of polymers with quaternary ammonium ions incorporated into the structure (Munoz-Bonilla and Fernandez-Garcia, 2012). There is a collection of polymers, known as protonated

polyethyleneimines which have a track record of killing bacteria because of their unique structures (Dhende et al., 2011) fig 18.

51

Fig 18; Polyethyleneimines, an antimicrobial cationic polymer (compound **51**).

It was found that when polymers have a charge to chain length ratio which is roughly equal (carbons between charged nitrogen's), this improved its antimicrobial function. However, it also increased its toxicity towards mammalian cells (fig 18).

Polymers are ideal as antimicrobial agents due to their low toxicity, plus the fact they are reusable as no materials are released and it is inherently antimicrobial. Due to the mode of action of these materials (due to quaternized nitrogen), the risk of bacterial resistance is minimal because the cell is completely destroyed, thus minimal chance of survival. In an age when the treatments are becoming less and less effective, prevention is better than cure. The use of a antimicrobial surface would reduce the microbial load on said surface and would be integral to a hygiene routine which would ultimately break the nosocomial infection loop (Page et al., 2009). However, eventually it could be surmised that resistance to quaternized material would eventually occur and this could present another problem in the future. This could potentially be minimised by use of several different alkylating agents for quaternization so resistance is slowed down considerably.

PDMAEMA (poly N, N-dimethylaminoethyl methacrylate) (fig 19) has been shown to prevent any effect of the cytotoxins of *C. difficile* once they have been released (Munoz-Bonilla and Fernandez-Garcia, 2012). Although this is an interesting finding, the fact that this cannot be taken orally means that it is not the optimum solution to the problem currently faced.

52

Fig 19: PDMAEMA (poly N, N-dimethylaminoethyl methacrylate, compound **52**), an antimicrobial polymer (Lu et al., 2007).

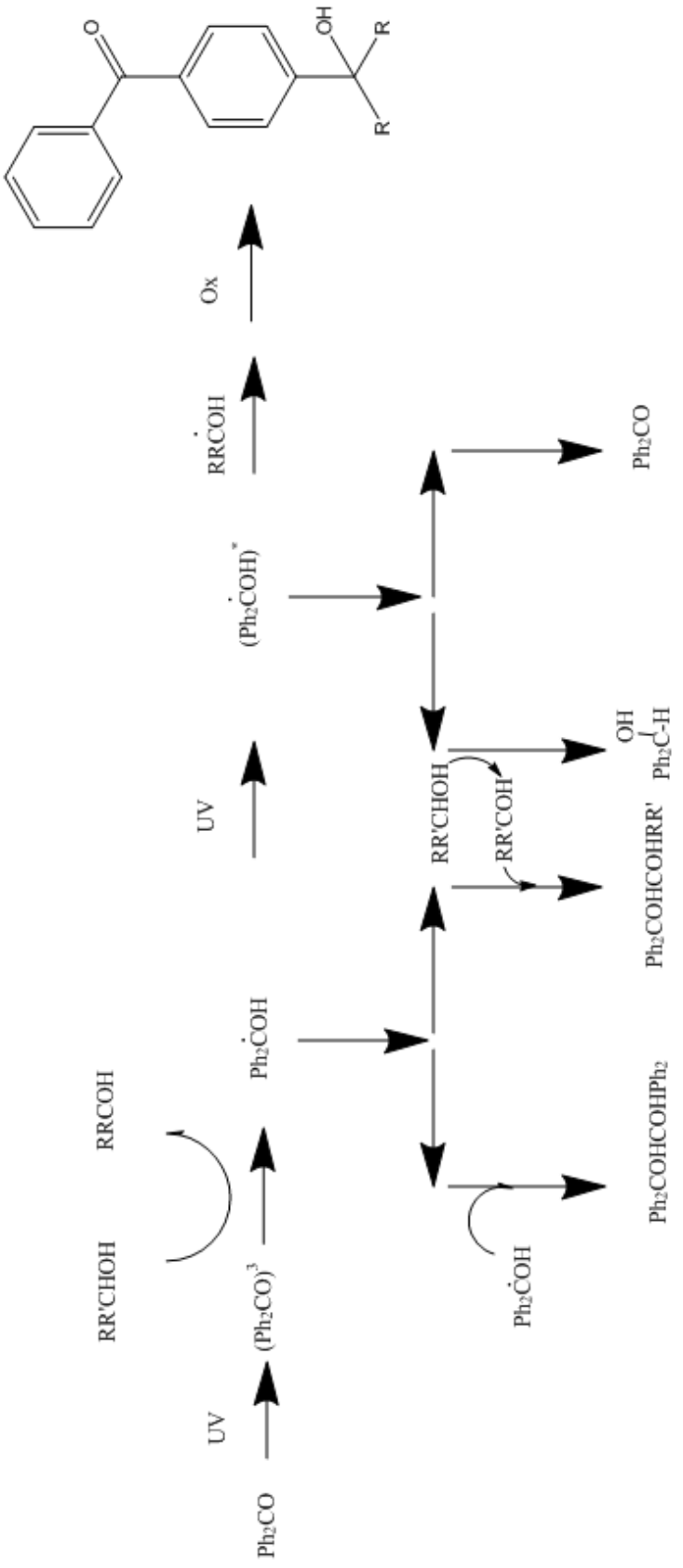
1.7.1 Benzophenone introduction:

Ultraviolet light has been the primary methodology for the curing of coatings since the early 1980s. Benzophenone has been widely used as the type 2 initiator (hydrogen abstraction-type) of such curing reactions (Cheng and Shi, 2011).

An alternative way of producing polymeric materials is through the use of UV radiation to produce hydrogen abstraction off a triplet benzophenone, thus producing free radicals which can come together to form a polymer (Lin et al., 1988). The photochemistry of benzophenone has been heavily investigated because of its ability to be both an electron acceptor, hydrogen abstracting agent or to work as an energy transfer donor (Muldoon et al., 2001).

One of the advantages of this hydrogen abstraction is the fact that although oxygen does have a minor inhibitory effect on the reaction, it is not completely inhibitory like in most other polymerisation techniques (excluding AIBN methodology discussed earlier). The choice of solvents is key in this reaction as it defines the molecular interactions which will occur (Castro et al., 2000).

Benzophenones have an interesting chemistry. Both the phenyl groups interact with the carbonyl group through both σ and π bonds, creating a molecular orbital which incorporates the entire structure. This produces a carbonyl group which loses its individual character because of the delocalization, thus allowing hydrogen abstraction to occur more easily (Castro et al., 2000). One of the most developed reactions involves the use of isopropanol as the solvent, the reaction scheme for the ground state and electronically excited radical are shown in scheme 9.



Scheme 9; Ground and excited state of benzophenone, with all possible outcomes upon exposure to UV light. R = Phenol.

Benzophenone absorbs wavelengths which are approximately 350 nm. This promotes one electron from a non-bonding sp^2 orbital on the oxygen to and anti-bonding π^* orbital on the carbonyl group. This causes the oxygen to become electron deficient and to become electrophilic, interacting with C-H σ bonds, thus abstracting the hydrogen. If any group comes near the carbonyl in this excited state, electron transfer can occur and the ketyl radicals formed can recombine to produce a new C-C bond (Dorman and Prestwich, 1994).

If benzophenone is left in direct sunlight in solution (ethanol), this is enough for a reaction to occur. The major products of this sort of reaction are benzopinacol and isobenzopinacol (fig 20) (Pitts et al., 1959). However, once again, the choice of solvents is highly important with cyclohexene completely retarding the benzopinacol formation. It was further found that cross coupling reactions would only occur if hydrogen abstraction was easily achieved, most noticeably this occurs with alcohols, toluene and cumene (Weiner, 1971). It has also been shown (Qu, 2002), that it is easier for the benzophenone to abstract from tertiary bonded hydrogen, than a secondary bonded hydrogen, which in turn is easier to be abstracted than a primary (Dorman and Prestwich, 1994).

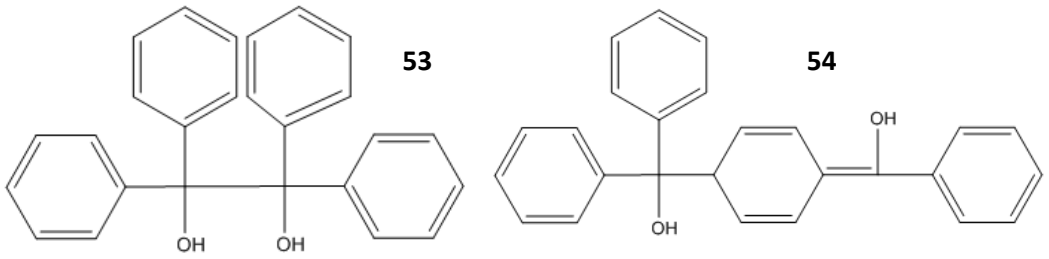


Fig 20: Structure of benzopinacol (compound **53**) and isobenzopinacol (compound **54**), two products from photo initiation of benzophenone.

To produce a material with germinating ability and be photochemically active, amino-benzophenone can be chemically attached to a bile acid. Thus a potential germinant material would be attached to a material (benzophenone) which could be attached to any organic surface by subjecting the material to ultraviolet light. This would be another way of producing a polymeric potential germinant/antimicrobial surface which could be applied almost anywhere required.

As an added advantage to the already proven properties of benzophenone as a photo initiator, literature suggests that poly (benzophenone) can be potentially antimicrobial against *S. aureus* and *E. coli*, which could mean that any potential germinant polymer may also end up killing other unwanted bacteria as well (Hong and Sun, 2009). Also in that same literature it was suggested that incorporation of the benzophenone moiety into the polymer structure can cause mechanical weakness in the polymer's properties.

1.8.1 Quaternizable materials/antimicrobials:

A lot of work has been done on the introduction of a polycationic chain into a steroid structure scaffold with the hope of it being antimicrobial (Aher et al., 2009, Tiller et al., 2001). Active compounds are able to disrupt and/or make the cell membranes of prokaryotes much more permeable. There has been a lot of research going into making dry state polycations. If dried, polycations are rendered useless, as they cannot penetrate the cell membrane and disrupt it. If there was a long spacer group however, the polycation would be able to reach the membrane and therefore cause the damage, with biological activity only being present if there is also a polar head group (Bouloussa et al., 2008, Aher et al., 2009). It has been reported that the biological activity of quaternary ammonium compounds is very much dependent on the organic species which is attached the nitrogen, the number of nitrogens present within the molecule and the counter ion to the positively charged nitrogen (Munoz-Bonilla and Fernandez-Garcia, 2012).

The mechanism of action of cationic materials has been studied and the process seems to go thus: adsorption onto cell surface, diffusion through the cell wall, binding to cytoplasmic membrane, release of K^+ ions and other cytoplasmic constituents which leads to precipitation of cells contents due to loss of ionic integrity (Dhende et al., 2011) and thus death (Ikeda et al., 1984). This absorption will occur quicker with polymers as opposed to monomer units because of the higher density of cationic charges in a single area.

It has been shown that polymers which contain quaternary ammonium moieties can potentially be antimicrobial. This is a potentially important area (Perichaud et al., 2001). However, research also shows that if this polymer is cross-linked or is insoluble, then any antimicrobial activity is lost as the material has not got the range of movement to find the bacterial cell wall and disrupt it (Tiller et al., 2001). This method of making a surface antibacterial has the distinct advantage that there is no chance of the bacteria developing a resistance to it as to do so would require alterations to the bulk membrane structure which is a very difficult task for a cell to undertake (Guan et al., 2000). Therefore, in a polymer surface, this would be ideal as any microorganisms that landed on the surface would be immediately eradicated. It has the other advantage of not being selective, so as well as being active against *C. difficile* it would kill other unwanted harmful microorganisms. In fact, there have been polymers produced (fig 21) which are aimed at *S. aureus* which have been alkylated and have been successful at killing the bacteria.

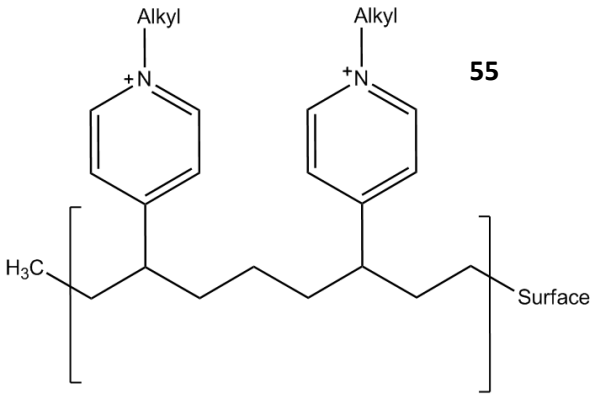


Fig 21: An antimicrobial polymer, with quaternary nitrogen ions, used in the prevention of *S. aureus*. Modified from (Tiller et al., 2001) (compound **55**).

Another material which has been used as an antimicrobial agent is copper. Although clearly not a quaternized material it has been shown (Wheeldon et al., 2008b) that copper surfaces are antimicrobial within a certain time frame (24-48 hours). What is interesting is that although usually the antimicrobials were aimed at killing the vegetative cells after germination, copper has been shown to also be sporicidal.

The problem with current literature is that, although work has been done on antimicrobial agents and other scientists have done work on germinatory effects of bile acids on *C. difficile,* none that could be found that specifically worked on both germination and then killing of *C. difficile* cells/spores. The antimicrobials which have been made (not bile acid based) are always tested on other gram positive bacteria, so there is clearly a gap in the literature which this project hopes to fill.

1.8.2 Antimicrobial bile amides.

The advantages of polyamines (in particular) are found to be generally of low toxicity and have a defined structure for pharmaceutical characterization (Aher et al., 2009). An example of an amino sterol with antimicrobial properties is shown below in the form of squalamine, a natural bile acid originally found in the stomach tissue of the dogfish shark (Randazzo et al., 2009) (fig 22). There are several mimics of squalamine which have been reported (Kikuchi et al., 1997), all based on steroidal structures, including cholic, deoxycholic and lithocholic acid. Although the exact mechanism of antibacterial activity in squalamine is unknown, it is postulated that it works by perforating the membranes of the bacteria.

Hydrophobicity of the bile acid compound is important in antimicrobial action, with there being an optimum logP value. Basicity of the bile acid analogues proved to be important for antimicrobial activity and, as with hydrophobicity, there is an optimum value. As well as activity against gram positive bacteria, there has been a large amount of work done on the antimicrobial activity of bile acid analogues against gram negative bacteria. This same research (Cravotto et al., 2005), has shown that some dimeric bile acids are antifungal and anti-proliferative. Gram-positive bacteria differ from gram-negative bacteria in the structure of their cell walls. The cell walls of gram-positive bacteria are made up of twenty times as much murein or peptidoglycan than gram-negative bacteria. These complex polymers of sugars and amino acids cross-link and layer the cell wall. The outer cell membrane of a prokaryote has an overall negative charge (Guan et al., 2000).

56

Fig 22: Squalamine (compound **56)**, a steroid based antibiotic, effective against gram negative rods and gram positive cocci (Aher et al., 2009).

Another material which has shown antimicrobial activity, specifically against gram positive bacteria is lithocholyl-N-2-(aminoethyl) amide Fig 23.

57

Fig 23: Lithocholyl-N-2-(2-aminoethyl) amide (compound **57**), a bile amide which has shown *in vitro* activity against gram positive bacteria (Ahonen et al., 2010).

The aim of this project is, at the very least, to better understand the important structural characteristics which are essential for germination of *C. difficile.* By building up a structural database using both lithocholic, deoxycholic and to a lesser extent, cholic acid, the important factors should emerge. If a compound which is a good germinant can be found, then incorporating this compound into a

polymeric surface (while still retaining germinating ability), this would be key. There is also a need to be able to kill the vegetative cells, so incorporation of an antimicrobial entity into this polymer would be required.

2.0 Experimental:

2.1.1 Instrumentation

Proton NMR were obtained using a Bruker AC250 instrument (250 MHz) as solutions and referenced from δ TMS = 0.00 pm. Carbon NMR (CPD and DEPT) were obtained using a Bruker AC250 instrument (62.9 MHz) as solutions and referenced from δ TMS = 0.00 pm. All analysis of carbon NMR incorporates both CPD and DEPT data and used data from (Waterhous et al., 1985) for assignments of the carbon NMR. Infrared spectra were recorded as KBr discs on a Mattson 3000 FTIR spectrometer or Thermo Scientific Nicolet 1s5 with 1D5 ATR attachment as a solid sample. Atmospheric pressure chemical ionisation mass spectrometry (APCI) was obtained using a Hewlett-Packard 5989B quadropole instrument connected to an electrospray 59987A unit with an APCI accessory and automatic injection using a Hewlett-Packard 1100 series auto sampler. ESI spectra were obtained using a Thermofisher LTQ orbitrap XL at the EPSRC facility at Swansea University. Melting points were obtained using a Reichert-Jung Thermo Galen hot stage microscope and are corrected. The work done on the anthranilamide based materials was done using a Leica Microsystems GmbH TCS SP5 II system with an upright DMI6000B microscope. The UV source for the benzophenone attachment work was a Proxima Direct 36 Watt UV, 220-240 V/50 Hz Nail curer. All work was carried out at Aston University, either in the medicinal chemistry laboratory or the NMR suite. All experiments were COSHH assessed, using the standard practices in the medicinal chemistry laboratory. All materials were sourced from Sigma-Aldrich or Fisher with no further purification. For the ethyl chloroformate amide formation reactions, all materials analysed by TLC (thin layer chromatography) had an R_f of 0.2-0.3 and were all single spots in an eluent of 8:2 EtOAc/MeOH. For quaternization reaction materials analysed by TLC 100 % MeOH, single spots with R_f of 0.1-0.2. When removing solvents under vacuum on the rotary evaporator, ethyl acetate was taken off at 55 ^{o}C. All TLCs were visualised using vanillin as a stain. All synthetic chemistry work was done by Matthew Justin Hird. All biological testing work was done between Christian Lowden, Kristian Poole and Amber Lavender under the supervision of Dr Tony Worthington.

When referring to carbon numbers in the experimental, the numbers are associated with the labelled bile acid below (fig 24)

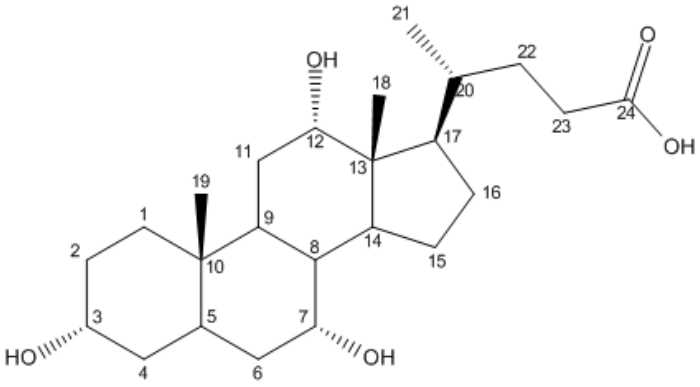
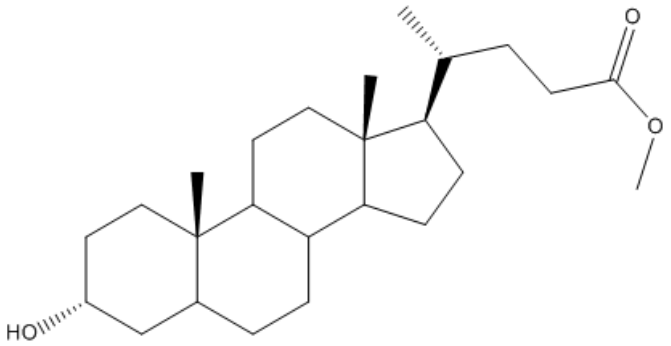


Fig 24: The carbon numbering system for a steroid based structure (in this case cholic acid). This numbering system will be used in the bile acid derived compounds reported in this thesis.

Due to the nature of the structures associated with this project, full assignment can be very difficult. In particular the majority of the steroid based hydrogens will not be explicitly stated, only the key peaks which define compound structure. There are a large amount of overlapping peaks starting from ~ 0.95 ppm and finishing at ~ 2.50 ppm which are associated with protons attached to carbons 1, 2, 4-6, 8-11, 14-17, and 20-23. For ease, as these peaks are not affected by structural changes they will not be explicitly dealt with and a range will be provided.

2.2.1 Ester preparation

Preparation of methyl lithocholate (Comp 58)



Lithocholic acid (5.0 g, 0.01329 mol) was added to methanol (90 mL) to produce a suspension. Acetyl chloride (0.5 mL, 0.006 mol) was then added. The solution was heated and stirred at 80 ^{o}C for 40 minutes as a homogeneous solution, then allowed to cool overnight in an ice bath. This was added to water (150 mL) and the resulting precipitate was collected by filtration, washed with water (3 x 20 mL) and dried under vacuum. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 5 g, 0.01328 mol, 96.5 %.

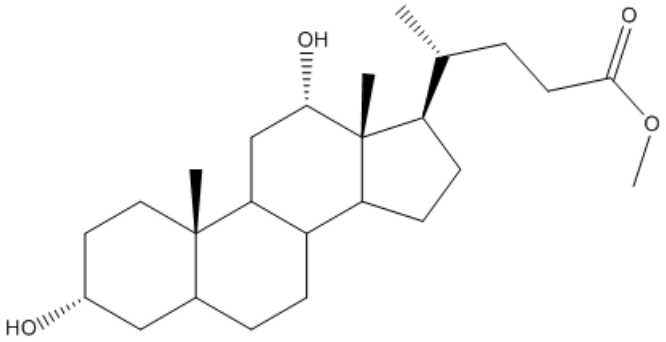
Melting point: 75-76 ^{o}C. Lit 71-73 ^{o}C, (Huong et al., 2009).

^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.66 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 0.95-2.39 (m, 33H, steroidal backbone CH/CH_2), 3.65 (m, 1H, 3-CH), 3.69 (s, 3H, O-CH_3) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.6 (CH_3, C21), 20.8 (CH_2, C11), 23.7 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 30.9 (CH_2, C22), 31.0 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.4 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 40.1 (CH_2, C12), 40.4 (CH, C9), 42.0 (CH, C5), 42.7 (C, C13), 51.5 (O-CH_3), 55.9 (CH, C17), 56.5 (CH, C14), 71.9 (CH, C3), 174.8 (CO, C24) ppm.

IR; 3347 (OH stretch), 2937 (C-H), 2859 (O-CH_3), 1733 (C=O), 1640, 1436, 1206, 1043 (R_2CH-OH) cm^{-1}.

Preparation of methyl deoxycholate (comp 59)



Deoxycholic acid (5.0 g 0.01 mol) was dissolved in methanol (30 mL) and treated with acetyl chloride (0.5 mL, 0.006 mol). The solution was heated and stirred at 80 ^oC for 40 minutes then allowed to cool overnight in an ice bath. The resultant crystals were collected by vacuum filtration to produce a white crystalline powder. This was washed with water (2 x 20 mL) and dried under vacuum. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 1.81 g, 0.004 mol, 35 %.

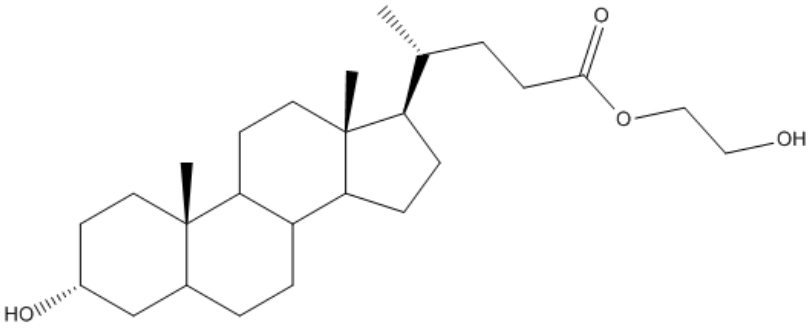
Melting point: 70-72 ^oC. Lit 67-70 ^oC (Anelli et al., 2009).

^1H NMR ($CDCl_3$) (250 MHz) δ= 0.68 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.60 (m, 1H, 3-CH), 3.67 (s, 3H, O-CH_3), 3.99 (s, 1H, 12-CH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.8 (CH_3, C18), 17.3 (CH_3, C21), 23.2 (CH_3, C19), 26.6 (CH_2, C15), 26.1 (CH_2, C7), 27.1 (CH_2, C6), 27.4 (CH_2, C16), 28.7 (CH_2, C11), 30.5 (CH_2, C2), 30.9 (CH_2, C23), 31.1 (CH_2, C22), 33.7 (CH, C9), 34.1 (C, C10), 35.1 (CH_2, C1), 35.2 (CH, C20), 36.0 (CH_2, C4), 36.4 (CH, C8), 42.0 (CH, C5), 46.5 (C, C13), 47.3 (CH, C17), 48.3 (CH, C14), 51.5 (O-CH_3), 71.8 (CH, C3), 73.1 (CH_2, C12), 174.7 (CO, C24) ppm.

IR; 3474 (OH stretch) 2985 (C-H), 2852 (O-CH_3), 1743 (C=O), 1450, 1380, 1040 (R_2CH-OH) cm^{-1}.

Preparation of 2-hydroxyethyl (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoate (compound 60)



Lithocholic acid (0.5 g, 0.001 mol) was dissolved in ethylene glycol (15 mL 0.24 mol) and acetyl chloride (0.1 mL 0.001 mol) was added. The reaction was sealed under argon and heated to 100 ^oC overnight. Water (50 mL) was added and the solution was allowed to cool for 1 hour in an ice bath, which produced precipitate which was washed with water (3 x 20 mL) and dried under vacuum. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.43 g, 0.001 mol, 78 %.

Melting point: 75-77 ^oC.

^1H NMR ($CDCl_3$) (250 MHz) δ= 0.63 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 0.95-2.47 (m, 33H, steroidal backbone CH/CH_2), 3.56 (m, 1H, 3-CH), 3.83 (t, 2H, CH_2, J= 5.0), 4.21 (t, 2H, CH_2, J= 5.0) ppm.

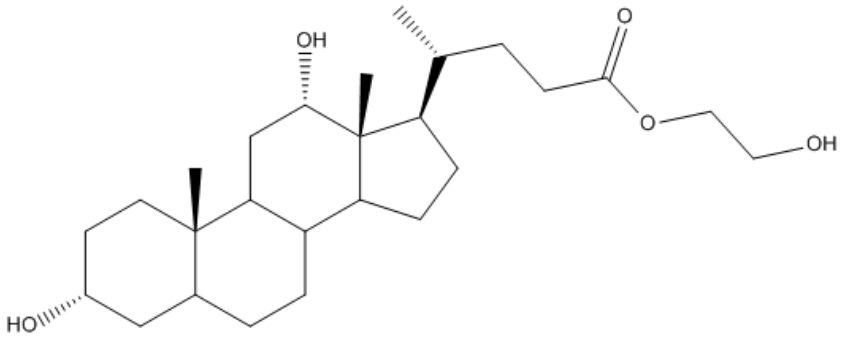
^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.2 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 30.9 (CH_2, C22), 31.1 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.8 (CH_2, C1), 36.4 (CH, C8), 40.1 (CH_2, C4), 40.4 (CH_2, C12), 41.0 (CH, C9), 42.0 (CH, C5), 42.7 (C, C13), 55.9 (CH, C17), 56.5 (CH, C14),

61.3 (CH_2, C(=O)O<u>C</u>H_2 or <u>C</u>H_2OH), 65.97 (CH_2, C(=O)O<u>C</u>H_2 or <u>C</u>H_2OH), 71.9 (CH, C3), 174.7 (CO, C24) ppm.

IR= 3334 (OH), 2857 (alkyl), 1727.11 (C=O), 1441, 1168 (C-O ester stretch), 1000 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 438.3579 $(M+NH_4)^+$; calculated for $C_{26}H_{48}NO_5$ 438.3578; 0.3 ppm.

<u>Preparation of 2-hydroxyethyl (4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoate (compound 61).</u>



Deoxycholic acid (0.5 g, 0.001 mol) was dissolved in ethylene glycol (5 mL 0.06 mol) and acetyl chloride (0.1 mL 0.001 mol) was added. The reaction was sealed under argon and heated to 100 ^oC overnight. Water (50 mL) was added and the solution was allowed to cool for 1 hour in an ice bath, which produced a precipitate which was washed with water (3 x 20 mL) and dried under vacuum. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.21 g, 0.0004 mol, 38 %.

Melting point: 128-131 ^oC.

^{1}H NMR ($CDCl_3$) (250 MHz) $CDCl_3$ δ= 0.69 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 0.98 (d, 3H, 21-CH_3, J = 5.0), 1.00-2.43 (m, 33H, steroidal backbone CH/CH_2), 3.39 (broad s, 1H, 3-CH), 3.65 (m, 1H, 12-CH), 3.85 (t, 2H, CH_2, J= 5.0), 4.20 (t, 2H, CH_2, J= 5.0) ppm.

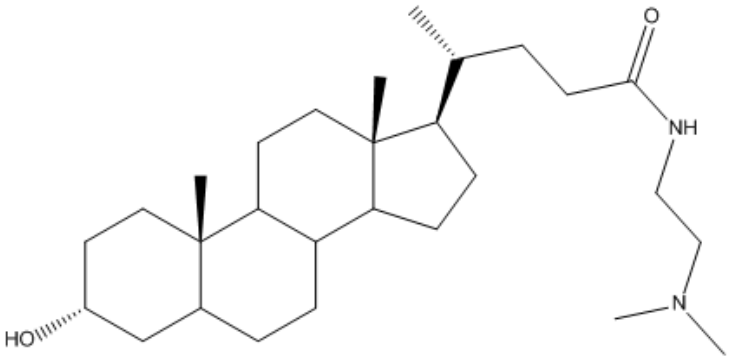
^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.2 (CH_3, C21), 20.8 (CH_2), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.1 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C11), 30.9 (CH_2, C2), 31.1 (CH_2, C23), 34.5 (CH, C9), 35.3 (C, C10), 35.8 (CH_2, C1), 36.4 (CH, C20), 40.1 (CH), 40.4 (C, C13), 42.0 (CH_2 C5), 42.7 (CH), 55.9 (CH_2, C(=O)O<u>C</u>H_2 or <u>C</u>H_2OH), 56.5 (CH_2, C(=O)O<u>C</u>H_2 or <u>C</u>H_2OH), 61.3 (CH_2), 65.9 (CH, C3), 71.8 (CH_2, C12), 174.7 (CO, C24) ppm.

IR= 3509 (OH), 3309 (OH), 2925 (alkyl), 2857 (alkyl), 1718 (C=O), 1449, 1356, 1292, 1189 (C=O ester stretch), 1027 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 454.3527 $(M+H)^+$; calculated for $C_{26}H_{48}NO_5$ 454.3527; 0.0 ppm.

2.3.1 Amide formation

Preparation of (4R)-N-(2-dimethylaminoethyl)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 62)



A mixture of lithocholic acid (1.0 g 0.005 mol) and N, N-dimethylethylenediamine (0.16 mL 0.001 mol) was dissolved in toluene (20 mL). The solution was heated at reflux for 24 hours. Analysis by TLC indicated that the reaction had not gone to completion so a further (0.32 mL 0.003 mol) of N, N-dimethylethylenediamine was added. Water (100 mL) was added to the solution, causing material to precipitate out. The precipitate was collected by vacuum filtration and the crude product was recrystallized from ethyl acetate to produce a white powder.

Yield 0.85 g, 0.001 mol, 69%.

Melting point: Not recorded.

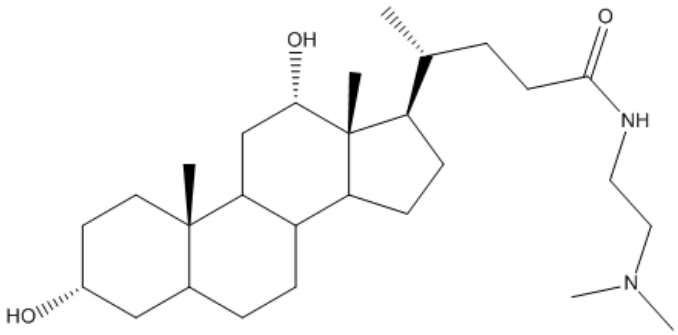
^{1}H NMR (250 MHz) ($CDCl_3$) δ= 0.64 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 0.94-2.30 (m, 33H, steroidal backbone CH/CH_2), 2.21 (s, 6H, 2 x CH_3), 2.42 (t, 2H CH_2 J=5.0), 3.34 (q, 2H, CH_2, J= 7.5), 3.63 (m, 1H, 3-CH), 6.20 (broad s, 1H, NH) ppm.

^{13}C NMR (62.9 MHz) ($CDCl_3$) δ= 12.0 (CH_3, C18), 18.3 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 31.7 (CH_2, C22), 33.5 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.5 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 36.6 (CH_2, NH<u>C</u>H_2 or <u>C</u>H_2N(CH_3)$_2$), 40.2 (CH_2, C12), 40.4 (CH, C9), 42.1 (CH, C5), 42.7 (C, C13), 45.1 (CH_3, N<u>C</u>H_3), 56.0 (CH, C17), 56.5 (CH, C14), 57.9 (CH_2, NH<u>C</u>H_2 or <u>C</u>H_2N(CH_3)$_2$), 71.8 (CH, C3), 173.7 (CO, C24) ppm.

IR; 3377 (OH), 3293 (NH) 2929 (C-H), 2870 (C-H), 1646 (C=O), 1543, 1445 cm^{-1}.

MS (+ESI) m/z= Found 447.3943 $(M+H)^+$; calculated for $C_{28}H_{51}N_2O_2$ 447.3945; 0.5 ppm.

Preparation of (4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-(2-dimethylaminoethyl)pentanamide (compound 63)



A mixture of deoxycholic acid (1.0 g 0.002 mol) and N, N-dimethylethylenediamine (0.16 mL 0.001 mol) was dissolved in toluene (20 mL). The solution was heated at reflux for 24 hours. Analysis by TLC (EtOAc: MeOH 4:1) indicated that the reaction had not gone to completion so a further (0.16 mL 0.001 mol) of N, N-dimethylethylenediamine was added and left for a further 24 hours. Solvent was then evaporated under reduced pressure, then triturated with hot water (~10 mL) and washed with water (3 x 20 mL), then dried under vacuum. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield 0.310 g, 0.0006 mol, 24%.

Melting point: Not recorded.

^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.68 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 1.01 (d, 3H, 21-CH_3, J= 6.5), 0.97-2.43 (m, 33H, steroidal backbone CH/CH_2), 2.26 (s, 6H, 2 x CH_3), 2.46 (t, 2H, CH_2, J= 5.0), 3.34 (m, 2H, CH_2, J=5.0), 3.61 (m, 1H, 3-CH), 3.97 (broad singlet, 1H, 12-CH), 6.44 (broad singlet, 1H, NH) ppm.

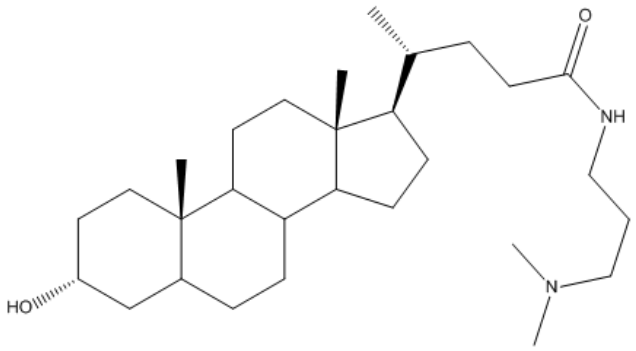
^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.7 (CH_3, C18), 17.5 (CH_3, C21), 23.1 (CH_3, C19), 23.6 (CH_2, C15), 26.1 (CH_2, C7), 27.1 (CH_2, C6), 27.8 (CH_2, C16), 28.5 (CH_2, C11), 30.5 (CH_2, C2), 31.6 (CH_2, C23), 33.3 (CH_2, C22), 33.6 (CH, C9), 34.1 (C, C10), 35.2 (CH_2, C1), 36.0 (CH, C20), 36.4 (CH_2,

C4), 42.0 (CH, C5), 44.9 (C, C13), 46.5 (CH_3, N-$\underline{C}H_3$), 47.3 (CH, C17), 48.2 (CH, C14), 57.9 (CH_2 $NH\underline{C}H_2$ or $\underline{C}H_2N(CH_3)_2$), 71.7 (CH, C3), 73.0 ($CH_2$, C12), 172.7 (CO, C24) ppm.

IR= 3305, (OH), 2929 (alkyl), 2861 (alkyl), 1720 (C=O), 1044 (R_2CH-OH) cm^{-1}

MS (+ESI) m/z= Found 463.3888 $(M+H)^+$; calculated for $C_{28}H_{51}N_2O_3$ 463.3894; 1.3 ppm.

Preparation of (4R)-N-[3-(dimethylamino)propyl]-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 64)



Following the method of (Liu et al., 2001). A mixture of methyl lithocholate (0.5 g 0.001 mol) and 3-dimethylamino-propylamine (3 mL, 0.02 mol) was heated and stirred at 140 °C for 24 hours in an argon environment. Analysis by TLC (thin layer chromatography, 8:2 ethyl acetate/methanol) indicated that the starting material had been consumed. Ice water (3 mL) was added to the material and left to stir for two hours at room temperature. The resulting solid was then collected by filtration, washed with water (3 x 20 mL) and left to dry overnight under vacuum to produce off-brown crystals. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield, 0.41 g, 0.0008 mol, 69 %.

Melting point: 177.0-178.3°C.

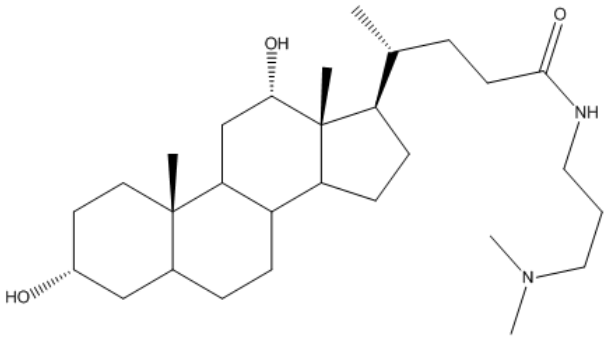
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.64 (s, 3H, 18-CH_3), 0.92 (d, 3H, 19-CH_3), 0.95-2.20 (m, 33H, steroidal backbone CH/CH_2), 2.25 (s, 6H, 2 x CH_3), 2.39 (t, 2H, CH_2, J=6.3), 3.33 (q, 2H, CH_2, J=5.6), 3.63 (m, 1H, 3-CH), 6.95 (s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.3 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 24.3 (CH_2, C7), 26.4 (CH_2, side chain $CH_2\underline{C}H_2CH_2$), 27.1 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 31.7 (CH_2, C22), 34.5 (C, C10), 35.3 (CH, C20), 35.5 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 40.1 (CH_2, C12), 40.3 (CH, C9), 42.0 (CH, C5), 42.7 (C, C13), 43.0 (CH_3 N-$\underline{C}H_3$), 55.3 (CH, C17), 55.9 (CH, C14), 56.4 (CH_2 NH$\underline{C}H_2$ or $\underline{C}H_2N(CH_3)_2$), 71.9 (CH, C3), 174.9 (CO, C24) ppm.

IR= 3310-3318 (OH-NH), 2730 (alkyl), 2859 (alkyl), 2946 (alkyl), 1648 (C=O), 1047 (CH-OH) cm^{-1}.

MS (ES +APCI) m/z= Found 461.4105 $(M+H)^+$; calculated for $C_{29}H_{53}N_2O_2$ 461.4107; 0.4 ppm.

Preparation of (4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-[3-(dimethylamino)propyl]pentanamide (compound 65)



A mixture of methyl deoxycholate (0.5 g 0.002 mol) and 3-dimethylamino-1-propylamine (3.63 mL 0.03 mol) was heated at 100 ^{o}C for 5 days then allowed to cool to ambient temperature. Ice water (40 mL) was then added and left to stir for 2 hours and the precipitate collected by vacuum filtration,

washed with water (3 x 20 mL) and dried under vacuum. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield, 0.41 g, 0.0008 mol, 73 %.

Melting point: 123-127 ^{o}C.

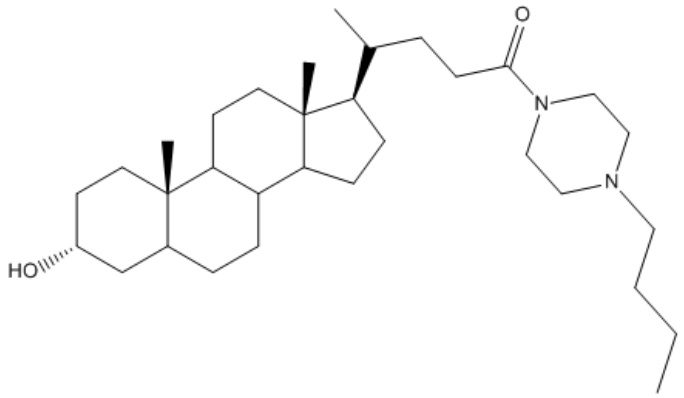
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.64 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 1.00-2.23 (m, 33H, steroidal backbone CH/CH_2), 2.23 (s, 6H, 2 x CH_3), 2.42 (t, 2H, CH_2, J= 5.0), 3.325 (q, 2H, CH_2, J=7.5), 3.63 (m, 1H, 3-CH), 6.21 (broad singlet, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.3 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 31.7 (CH_2, C22), 33.5 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.5 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 36.6 (CH_2), 40.2 (CH_2, C12), 40.4 (CH, C9), 42.1 (CH, C5), 42.7 (C, C13), 45.1 (CH_2, NH<u>C</u>H_2 or <u>C</u>$H_2N(CH_3)_2$), 56.0 (CH, C17), 56.5 (CH_2, NH<u>C</u>H_2 or <u>C</u>$H_2N(CH_3)_2$), 57.9 (CH, C14), 71.8 (CH, C3), 173.7 (CO, C24) ppm.

IR= 3376 (NH and OH), 2938 (alkyl), 2861 (alkyl), 1643 (C=O), 1544, 1444, 1378, 1045 (R_2CH-OH) cm^{-1}.

MS (+ES APCI) m/z= Found 477.4064 $(M+H)^{+}$; calculated for $C_{29}H_{53}N_2O_3$ 477.4056; 1.7 ppm.

Preparation of 1-(4-butylpiperazin-1-yl)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentan-1-one (compound 66)



Following a modified method (Fini et al., 1992), lithocholic acid (0.5 g 0.001 mol) was dissolved in tetrahydrofuran (20 mL) with triethylamine (0.4 mL 0.002 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.2 mL 0.001 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours, solvent removed under reduced pressure then re-dissolved in dichloromethane and washed with water. 1-Butylpiperazine (0.25 mL 0.001 mol) was added and the solution was stirred for 24 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. Solvent was evaporated under reduced pressure. TLC EtOAc: MeOH 4:1 R_f 0.2 (single spot).

Yield; 0.16 g, 0.0003 mol, 23.5 %.

Melting point: 172.7-173.1 ^{o}C.

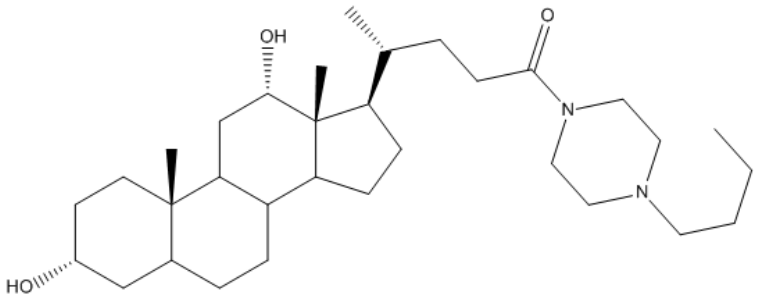
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.61 (s, 3H, 18-CH_3), 0.88 (s, 3H, 19-CH_3), 1.00-2.25 (m, 33H, steroidal backbone CH/CH_2), 1.34 (m, 4H, CH_2), 2.35 (m, 6H, CH_2), 3.45 (broad singlet, 2H, CH_2), 3.60 (broad singlet, 2H, CH_2) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 14.0 (CH_3, $CH_2\underline{C}H_3$), 18.5 (CH_3, C21), 20.6 (CH_2, C11), 20.8 (CH_2), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 28.8 (CH_2), 30.2 (CH_2, C2), 30.5 (CH_2, C22), 31.4 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.6 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 40.1 (CH_2, C12), 40.4 (CH, C9), 41.4 (CH, C5), 42.1 (C, C13), 42.8 (CH_2), 45.6 (CH_2), 52.9 (CH_2), 53.4 (CH_2), 53.4 (CH_2), 56.0 (CH, C17), 56.5 (CH, C14), 58.3 (CH_2), 71.7 (CH, C3), 172.1 (CO, C24) ppm.

IR= 3381, (OH), 2916 (alkyl), 2852 (alkyl), 1616 (C=O), 1437, 1253, 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 501.4410 $(M+H)^+$; calculated for $C_{32}H_{57}N_2O_2$ 501.4415; 0.9 ppm.

Preparation of (4R)-1-(4-butylpiperazin-1-yl)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentan-1-one (compound 67)



Deoxycholic acid (0.5 g 0.001 mol) was dissolved in tetrahydrofuran (20 mL) with triethylamine (0.4 mL 0.001 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.2 mL 0.001 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours, solvent removed under reduced pressure then re-dissolved in dichloromethane and washed with water then 1-butylpiperazine (0.25 mL 0.001 mol) was added and the solution was stirred for 24 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over

magnesium sulfate. Solvent was evaporated under reduced pressure. TLC EtOAc: MeOH 4:1 R_f 0.2 (single spot).

Yield; 0.04 g, 0.0004 mol, 6 %.

Melting point: 92.3 – 93.5 ^{o}C.

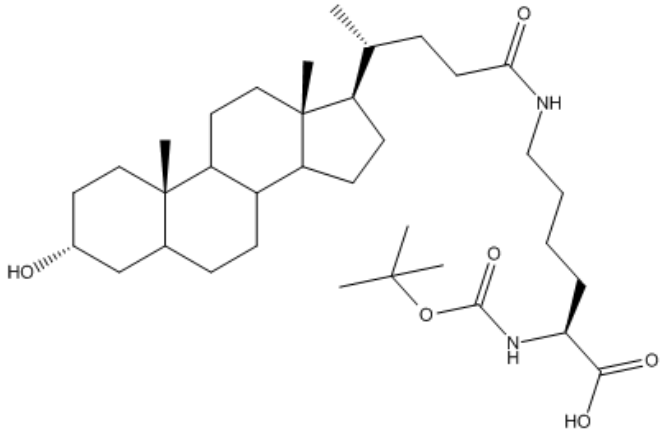
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.68 (s, 3H, 18-CH_3), 0.89 (s, 3H, 19-CH_3), 0.98 (d, 3H, 21-CH_3 J=7.5), 1.00-2.35 (m, 33H, steroidal backbone CH/CH_2), 2.38 (m, 6H, CH_2), 3.48 (broad singlet, 2H, CH_2), 3.62 (broad singlet, 2H, CH_2), 3.98 (broad singlet, 1H, 12-CH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.7 (CH_3, C18), 14.0 (CH_3 $CH_2\underline{C}H_3$), 17.5 (CH_3, C21), 20.6 (CH_2), 23.1 (CH_3, C19), 23.6 (CH_2, C15), 26.1 (CH_2), 27.5 (CH_2, C7), 28.6 (CH_2, C6), 28.8 (CH_2, C16), 30.1 (CH_2, C11), 30.5 (CH_2, C2), 31.3 (CH_2, C23), 33.6 (CH_2, C22), 34.1 (CH, C9), 35.2 (C, C10), 35.2 (CH_2, C1), 36.0 (CH, C20), 36.4 (CH_2, C4), 41.4 (CH_2), 42.0 (CH, C5), 45.6 (CH_2), 46.5 (CH_2), 47.2 (CH, C17), 48.3 (CH, C14), 52.8 (CH_2), 53.4 (CH_2), 71.8 (CH, C3), 73.1 (CH_2, C12), 171.9 (CO, C24) ppm.

IR= 3300 (OH), 2930 (alkyl), 2861 (alkyl), 1594 (C=O), 1443, 1282, 1162 (C=O ester stretch), 1011 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 517.4359 $(M+H)^{+}$; calculated for $C_{32}H_{57}N_2O_3$ 517.4364; 0.9 ppm.

Preparation of (2S)-2-(tert-butoxycarbonylamino)-6-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]hexanoic acid (compound 68)



Lithocholic acid (0.5 g 0.001 mol) was dissolved in tetrahydrofuran (20 mL) with triethylamine (0.4 mL 0.002 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.2 mL 0.001 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours, solvent removed under reduced pressure then re-dissolved in dichloromethane and washed with water then Boc-Lys-OH (0.32 g 0.001 mol) was added and the solution was stirred for 24 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The crude material was purified by column chromatography (9:1 chloroform/methanol). The organic layers were combined and dried over magnesium sulfate. The solvent was removed under reduced pressure to produce viscous oil. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.19 g, 0.0003 mol, 24 %.

Melting point: Oil.

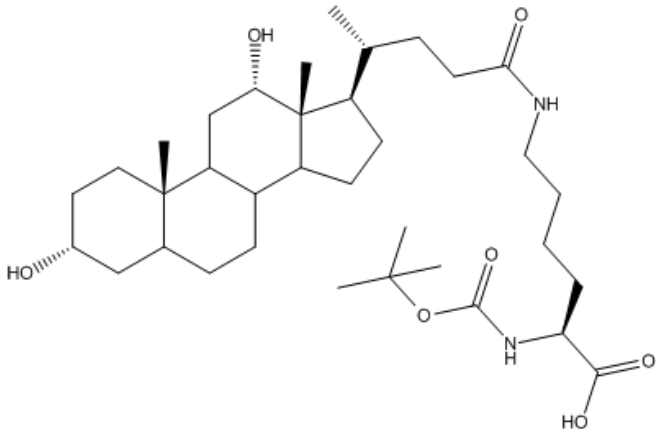
^{1}H NMR ($CDCl_3$ + D_2O shake) (250 MHz) δ= 0.63 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 1.00-2.25 (m, 33H, steroidal backbone CH/CH_2), 1.45 (s, 9H, BOC), 3.22 (broad s, 2H, CH_2), 3.63 (m, 1H, 3-CH), 4.25 (broad singlet, 1H, 12-CH) ppm.

^{13}C NMR ($CDCl_3$ + D_2O shake) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 20.8 (CH_2, C11), 22.5 (CH_2), 23.4 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 28.3 (CH_3 Boc), 28.9 (CH_2), 29.3 (CH_2), 30.2 (CH_2, C2), 31.8 (CH_2, C22), 32.3 (CH_2, C23), 33.3 (C, C10), 34.5 (CH, C20), 35.3 (CH_2, C1), 35.5 (CH, C8), 35.8 (CH_2, C4), 36.1 (CH_3), 39.2 (CH_2, C12), 40.2 (CH, C9), 40.4 (CH, C5), 42.0 (C, C13), 42.7 (CH_2), 53.4 (CH NHCHCOOH), 55.9 (CH_2, C17), 56.5 (CH, C14), 71.8 (CH, C3), 155.0 (CH_2), 174.7 (CO), 175.6 (CO, C24) ppm.

IR= 3343 (OH), 2921 (alkyl), 2857 (alkyl), 1705 (C=O), 1616, 1364, 1010 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 603.4376 $(M+H)^+$; calculated for $C_{35}H_{59}N_2O_6$ 603.4379; 0.4 ppm

Preparation of (2S)-2-(tert-butoxycarbonylamino)-6-[[(4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]hexanoic acid (compound 69)



Deoxycholic acid (0.5 g 0.001 mol) was dissolved in tetrahydrofuran (15 mL) with triethylamine (0.20 mL 0.001 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.2 mL 0.001 mol) was then added dropwise over a ten minute period. Once added, the cold water was

removed and the solution was stirred for 2 hours. After 2 hours Boc-Lys-OH (0.3 g 0.001 mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The crude material was purified by column chromatography (9:1 chloroform/methanol). The organic layers were combined and dried over magnesium sulfate. The solvent was removed under reduced pressure to produce a white solid. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.09 g, 0.0001 mol, 11 %.

Melting point: 97-100 ^{o}C.

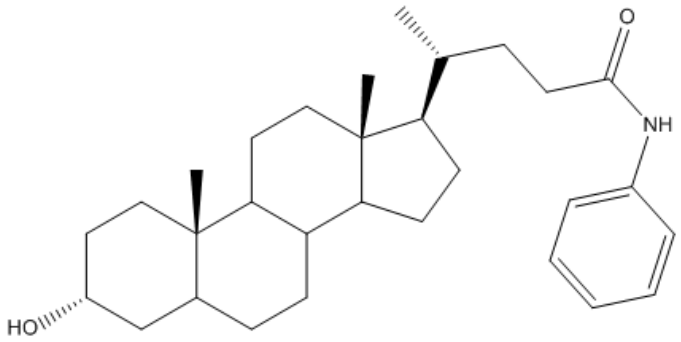
^{1}H NMR ($CDCl_3$ + D_2O shake) (250 MHz) δ= 0.67 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 0.99 (d, 3H, 21-CH_3, J= 7.5), 1.00-2.17 (m, 33H, steroidal backbone CH/CH_2), 1.45 (s, 9H, BOC), 3.24 (broad singlet, 2H, CH_2), 3.64 (m, 1H, 3-CH), 4.01 (s, 1H, 12-CH), 4.28 (broad singlet, 1H, CH) ppm.

^{13}C NMR ($CDCl_3$ + D_2O shake) (62.9 MHz) δ= 12.6 (CH_3, C18), 17.5 (CH_3, C21), 22.3 (CH_3, Boc), 23.0 (CH_2), 23.7 (CH_3, C19), 26.2 (CH_2, C15), 27.1 (CH_2, C7), 27.5 (CH_2), 28.3 (CH_2, C6), 28.7 (CH_2, C16), 30.1 (CH_2, C11), 31.5 (CH_2, C2), 32.8 (CH_2, C23), 33.5 (CH_2, C22), 34.1 (CH, C9), 35.2 (C, C10), 35.3 (CH_2, C1), 35.9 (CH, C20), 39.0 (CH_2), 42.0 (CH, C5), 46.2 (C, C13), 46.4 (CH, C17), 48.2 (CH, C14), 53.4 (CH_3), 71.8 (CH, C3), 73.4 (CH_2, C12), 155.8 (COOH), 174.8 (CONH, C24) ppm.

IR= 3334 (OH), 2933 (alkyl), 2865 (alkyl), 1697 (C=O), 1646 (C=O), 1530, 1360, 1159 (C=O ester stretch), 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 619.4322 $(M+H)^+$; calculated for $C_{35}H_{59}N_2O_7$ 619.4328; 0.9 ppm.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-phenyl-pentanamide (compound 70)



Based on the literature (Joachimiak et al., 2008) Lithocholic acid (2.0 g 0.005 mol) was dissolved in tetrahydrofuran (60 mL) with triethylamine (0.64 mL, 0.006 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.54 mL, 0.005 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours aniline (0.54 mL, 0.005 mol) and DMAP (20 mg) were added and the solution was stirred for a further 24 hours. Water (50 mL) was then added and the solution was extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. The solvent was evaporated under reduced pressure and the product was recrystallized from ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.2 (single spot).

Yield; 1.22 g, 0.002 mol, 51 %.

Melting point: 212.6-214.1 ^{o}C.

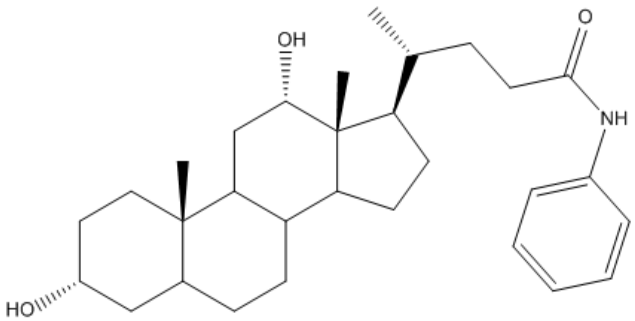
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.65 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 1.01-2.50 (m, 33H, steroidal backbone CH/CH_2), 3.63 (m, 1H, 3-CH), 7.09 (t, 1H, Ar-CH, J= 5.0), 7.32 (t, 2H, Ar-CH, J = 7.5), 7.505 (d, 2H, Ar-CH, J= 7.5) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 31.6 (CH_2, C22), 34.5 (C, C10), 35.3 (CH, C20), 35.4 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 40.2 (CH_2, C12), 40.4 (CH, C9), 42.1 (CH, C5), 42.7 (C, C13), 55.0 (CH, C17), 56.5 (CH, C14), 71.8 (CH, C3), 119.7 (Ar-CH), 129.0 (Ar-CH), 171.6 (CO, C24) ppm.

IR= 3292 (OH), 2929 (alkyl), 2861 (alkyl), 1663 (C=O), 1539, 1437, 1309, 753 (aromatic) cm^{-1}.

MS (+ESI) m/z= Found 452.3519 $(M+H)^+$; calculated for $C_{30}H_{46}N_2O_2$ 452.3523; 0.9 ppm.

Preparation of (4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-phenyl-pentanamide (compound 71)



Deoxycholic acid (2.0 g 0.005 mol) was dissolved in tetrahydrofuran (60 mL) with N-methylmorpholine (1.2 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.40 mL, 0.003 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours aniline (0.52 mL, 0.005 mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the resultant precipitate was collected by vacuum filtration. The product was recrystallized from ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 1.02 g, 0.002 mol, 42.8 %.

Melting point: 219.1 - 221.9 ^{o}C.

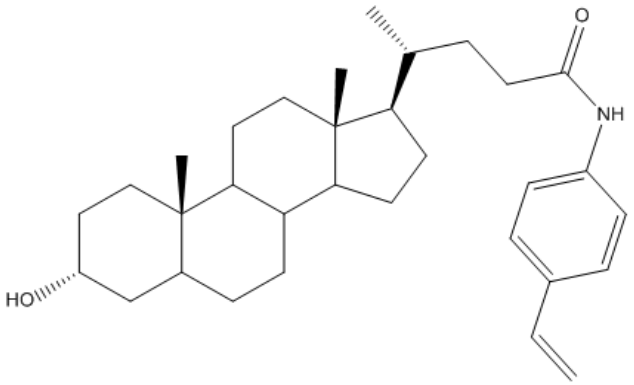
^{1}H NMR (DMSO) (250 MHz) δ= 0.60 (s, 3H, 18-CH_3), 0.85 (s, 3H, 19-CH_3), 0.98-2.43 (m, 33H, steroidal backbone CH/CH_2), 3.81 (broad s, 1H, 12-CH), 4.19 (d, 1H, 3-OH, J= 2.5), 4.45 (d, 1H, 12-OH, J= 5.0), 7.00 (t, 1H, Ar-CH, J= 7.5), 7.27 (t, 2H, Ar-CH, J= 7.5), 7.57 (d, 2H, Ar-CH, J= 7.5), 9.82 (s, 1H, NH) ppm.

^{13}C NMR (DMSO) (62.9 MHz) δ= 12.4 (CH_3, C18), 17.1 (CH_3, C21), 23.0 (CH_3, C19), 23.4 (CH_2, C15), 26.0 (CH_2, C7), 26.9 (CH_2, C6), 27.1 (CH_2, C16), 30.2 (CH_2, C11), 31.4 (CH_2, C2), 32.9 (CH_2, C23), 33.4 (CH_2, C22), 33.8 (CH, C9), 35.0 (C, C10), 35.6 (CH_2, C1), 36.2 (CH, C20), 45.9 (CH, C5), 46.1 (C, C13), 47.4 (CH, C17), 69.9 (CH), 70.9 (CH, C3), 75.1 (CH_2, C12), 118.9 (Ar-CH), 122.8 (Ar-CH), 128.5 (Ar-CH), 139.3 (Ar-C), 171.6 (CO, C24) ppm.

IR= 3411 (OH), 2921 (alkyl), 2861 (alkyl), 1663 (C=O), 1590, 1548, 1441, 1249, 1040 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 468.3467 $(M+H)^{+}$; calculated for $C_{30}H_{46}NO_3$ 468.3472; 1.1 ppm.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-(4-vinylphenyl)pentanamide (compound 72)



Lithocholic acid (1.0 g 0.005 mol) was dissolved in tetrahydrofuran (30 mL) with triethylamine (0.64 mL 0.006 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.19 mL, 0.0017 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 4-vinylaniline (0.37 mL 0.003 mol) and 10 mg of 4-(dimethylamino)pyridine was added and the solution was stirred for a further 24 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with hydrochloric acid (3 x 100 mL 3 mol). The organic layer was dried over magnesium sulfate. Solvent was evaporated under reduced pressure to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.27 g, 0.0005 mol, 20.61%

Melting point: 188.8 – 193.9 ^{o}C.

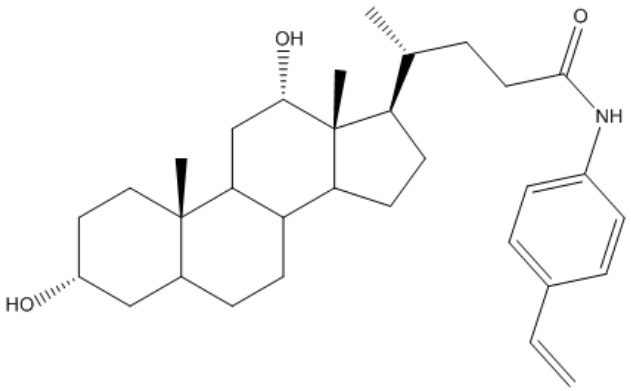
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.65 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 1.03-2.50 (m, 33H, steroidal backbone CH/CH_2), 3.63 (m, 1H 3-CH), 5.19 (d, 1H =CH- J= 10.0), 5.67 (d, 1H, CH, J= 17.5), 6.67 (dd, 1H, CH, J= 12.5 and 22.5), 7.11 (Broad s, 1H, NH), 7.365 (d, 2H, Ar-CH, J= 7.5), 7.485 (d, 2H, Ar-CH, J= 7.5) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 34.5 (C, C10), 34.6 (CH, C20), 35.3 (CH_2, C1), 35.4 (CH, C8), 35.8 (CH_2, C4), 40.2 (CH_2, C12), 40.4 (CH, C9), 42.0 (CH, C5), 42.7 (C, C13), 56.0 (CH, C17), 56.5 (CH, C14), 71.8 (CH, C3), 119.5 (Ar-CH), 120.6 (Ar-CH), 136.0 (Ar-CH), 144.0 (Ar-C) ppm.

IR= 3449 (OH), 2925 (alkyl), 2857 (alkyl), 1667 (C=O), 1513, 1313, 1245, 1027 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 478.3671 $(M+H)^+$; calculated for $C_{32}H_{48}NO_2$ 478.3680; 1.8 ppm.

Preparation of (4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-(4-vinylphenyl)pentanamide (compound 73)



Deoxycholic acid (2.0 g 0.005 mol) was dissolved in 1, 4 dioxane (60 mL) with triethylamine (1.32 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.4 mL, 0.003 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 1 hour, 4-vinylaniline (0.31 g, 0.002 mol) and DMAP (10 mg) were added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the resultant precipitate was collected by vacuum filtration. This product was re-dissolved in ethyl acetate and washed with 3 M HCl (3 x 100 mL). The organic layer was dried over

magnesium sulfate. The solvent was evaporated under reduced pressure to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield not recorded.

Melting point: 98.7-101.5 ^{o}C.

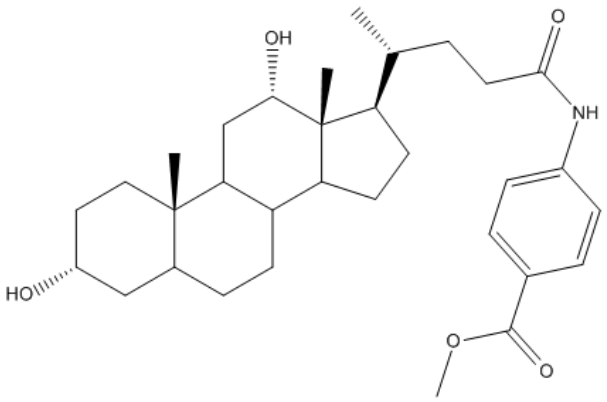
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.68 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.62 (m, 1H, 3-CH), 3.99 (broad singlet, 1H, 12-CH), 5.185 (d, 2H, =CH-, J= 12.5), 5.675 (d, 2H, CH, J= 17.5), 6.675 (dd, 1H, CH, J values = 10 and 17.5), 7.355 (d, 2H, Ar-CH, J= 7.5), 7.505 (d, 2H, Ar-CH, J= 7.5) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.7 (CH_3, C18), 17.5 (CH_3, C21), 23.1 (CH_2), 23.7 (CH_3, C19), 26.1 (CH_2, C15), 27.1 (CH_2, C7), 27.5 (CH_2, C6), 28.5 (CH_2, C16), 30.5 (CH_2, C2), 31.3 (CH_2, C23), 33.6 (CH_2, C22), 34.1 (CH, C9), 35.1 (C, C10), 36.0 (CH_2, C1), 36.4, 42.0 (CH, C5), 46.5 (C, C13), 46.9 (CH, C17), 48.2 (CH, C14), 71.8 (CH, C3), 112.8 (Ar-CH), 119.66 (Ar or vinyl CH), 126.8 (Ar or vinyl CH), 133.5 (Ar-C), 136.1 (Ar or vinyl-CH), 137.7 (Ar-C), 171.9 (CO, C24) ppm.

IR= 3300 (OH), 2925 (alkyl), 2861 (alkyl), 1663 (C=O), 1599, 1245, 1040 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 494.3623 $(M+H)^{+}$; calculated for $C_{32}H_{48}N_1O_3$ 494.3629; 1.2 ppm.

Preparation of methyl 4-[[(4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]benzoate (compound 74)



Deoxycholic acid (2.0 g 0.005 mol) was dissolved in 1, 4-dioxane (60 mL) with triethylamine (1.32 mL 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.40 mL 0.003 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours methyl 4-aminobenzoate (0.84 g 0.005 mol) was added and the solution was stirred for a further 24 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. Solvent was evaporated under reduced pressure to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.57 g, 0.001 mol, 21 %.

Melting point: 124.3-127.6 ^{o}C.

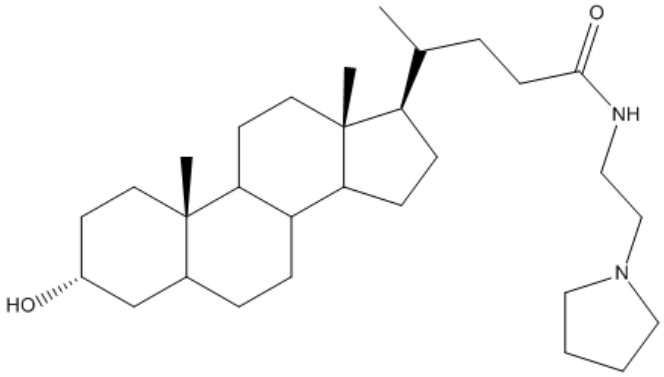
^{1}H NMR (250 MHz) (DMSO) δ= 0.60 (s, 3H, 18-CH_3), 0.85 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.81 (s, 3H, O-CH_3), 4.20 (d, 1H, 3-OH, J= 5.0), 4.45 (d, 1H, 12-OH, J= 5.0), 7.72 (d, 2H, Ar-CH, J= 10.0), 7.895 (d, 2H, Ar-CH, J= 7.5), 10.20 (broad singlet, 1H, NH) ppm.

^{13}C NMR (62.9 MHz) (MeOD) δ= 13.2 (CH_3, C18), 17.7 (CH_3, C21), 23.7 (CH_3, C19), 24.9 (CH_2, C15), 27.5 (CH_2, C7), 28.4 (CH_2, C6), 28.7 (CH_2, C16), 29.9 (CH_2, C11), 33.0 (CH_2, C22), 34.8 (CH, C9), 35.1 (C, C10), 35.3 (CH_2, C1), 36.4 (CH, C20), 36.9 (CH_2, C4), 37.2 (CH, C8), 37.5 (CH_3 O-CH_3), 43.6 (CH, C5), 52.4 (CH_2), 72.5 (CH, C3), 74.0 (CH_2, C12), 120.1 (Ar-CH), 126.2 (Ar-CH), 131.5 (Ar-CH), 144.7 (Ar-C), 168.2 (COOCH) 175.5 (CONH, C24) ppm.

IR= 3478 (OH), 2935 (alkyl), 2865 (alkyl), 1691, 1669 (C=O), 1593, 1536, 1430, 1275, 1175 (C-O ester stretch), 1038 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 526.3522 $(M+H)^+$; calculated for $C_{32}H_{48}N_1O_5$ 526.3527; 1 ppm.

4-[(3R,10S,13R,17R)-3-Hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-(2-pyrrolidin-1-ylethyl)pentanamide (compound 75)



Lithocholic acid (0.5 g 0.001 mol) was dissolved in tetrahydrofuran (20 mL) with triethylamine (0.20 mL, 0.001 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.20 mL, 0.001 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours at room temperature. After 2 hours 1-(2-aminoethyl)pyrollidine (0.25 mL, 0.002 mol) was added and the solution was stirred for 3 hours. Water (50 mL) was then added and the solution was extracted with ethyl acetate (3 x 50 mL). The

organic layers were combined and dried over magnesium sulfate. The solvent was evaporated under reduced pressure to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.56 g, 0.001 mol, 91.8 %.

Melting point: 144.1- 145.1 ^{o}C.

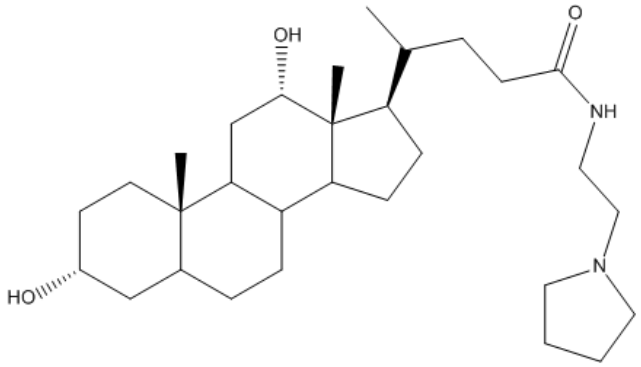
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.64 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 0.94 (d, 3H, 21-CH_3, J= 7.5), 1.00-2.26 (m, 33H, steroidal backbone CH/CH_2), 1.80 (m, 4H, CH_2), 2.54 (broad singlet, 4H, CH_2), 2.60 (t, 2H, CH_2, J=5.0), 3.365 (q, 2H, CH_2, J= 7.5), 3.63 (m, 1H, 3-CH), 6.13 (broad s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 20.8 (CH_2, C11), 23.4 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (C H_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 31.7 (CH_2, C22), 33.5 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.5 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 37.7 (CH_2), 40.2 (CH_2, C12), 40.4 (CH, C9), 42.1 (CH, C5), 42.7 (C, C13), 53.8 (CH_2, NH-$\underline{C}H_2$ or $CH_2N(CH_3)_2$), 54.9 (CH_2, NH-$\underline{C}H_2$ or $CH_2N(CH_3)_2$), 56.0 (CH, C17), 56.5 (CH, C14), 71.8 (CH, C3), 173.9 (CO, C24) ppm.

IR= 3415 (NH), 3310 (OH), 2933 (alkyl), 2865 (alkyl), 2872 (alkyl), 1648 (C=O), 1548, 1444, 1378, 1265 (C-O ester stretch), 1064 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 473.4096 $(M+H)^+$; calculated for $C_{30}H_{53}N_2O_2$ 473.4102; 1.2 ppm.

Preparation of 4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-(2-pyrrolidin-1-ylethyl)pentanamide (compound 76)



Deoxycholic acid (2.0 g 0.005 mol) was dissolved in 1, 4-dioxane (60 mL) with triethylamine (1.28 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.37 mL, 0.003 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 1-(2-aminoethyl) pyrrolidine (0.77 mL, 0.006 mol) was added and the solution was stirred for 3 hours at room temperature. Water (50 mL) was then added and the solution was extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. The solvent was evaporated under reduced pressure to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.2 (single spot).

Yield; 1.2 g, 0.0025 mol, 49 %.

Melting point: 155.3-158.7 ^{o}C.

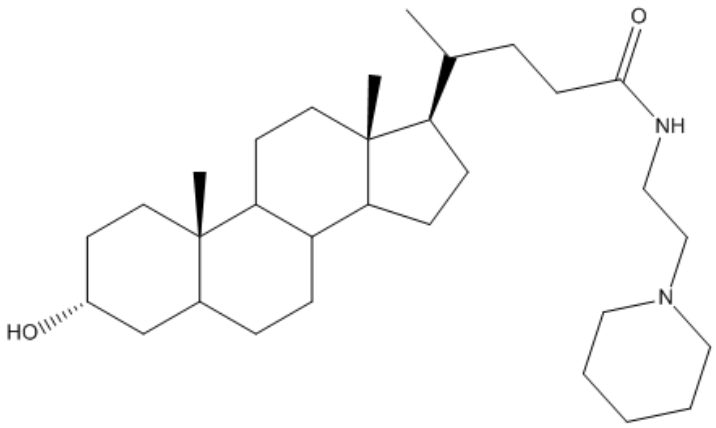
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.66 (s, 3H, 18-CH_3), 0.89 (s, 3H, 19-CH_3), 0.975 (d, 3H, 21-CH_3, J= 7.5), 1.00-2.30 (m, 33H, steroidal backbone CH/CH_2), 1.78 (m, 4H, CH_2), 2.42-2.58 (m, 6H, CH_2), 3.35 (m, 2H, CH_2), 3.58 (m, 1H, 3-CH), 3.95 (broad s, 1H, 12-CH), 6.49 (broad s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.7 (CH_3, C18), 17.5 (CH_3, C21), 23.3 (CH_3, C19), 23.7 (CH_2, C15), 26.1 (CH_2, C7), 27.1 (CH_2, C6), 28.6 (CH_2, C16), 30.4 (CH_2, C11), 30.5 (CH_2, C2), 33.3 (CH_2, C23), 33.6 (CH_2, C22), 34.1 (CH, C9), 35.2 (C, C10), 35.3 (CH_2, C1), 36.0 (CH, C20), 36.5 (CH_2, C4), 37.9 (CH, C8), 42.1 (CH_2), 46.5 (C, C13), 46.8 (CH_2, C17), 48.2 (CH_2, C14), 53.9 (CH_2), 55.0 (CH_2), 71.5 (CH, C3), 73.0 (CH_2, C12), 173.9 (CO, C24) ppm.

IR= 3287 (OH), 2916 (alkyl), 2865 (alkyl), 1641 (C=O), 1539, 1441, 1040 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 489.4046 $(M+H)^+$; calculated for $C_{30}H_{53}N_2O_3$ 489.4051; 1 ppm.

Preparation of 4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-[2-(1-piperidyl)ethyl]pentanamide (compound 77)



From a modification of a method.(Liu et al., 2001) Methyl lithocholate 0.5g (0.001mol) 1,2 amino-ethyl piperidine 0.36 mL (0.002mol) were heated at and stirred at 150°C for 48 hours in a argon environment. TLC (Ethyl acetate: Methanol 4:1) indicated partial consumption of starting material so another 1 mL (0.007mol) of 1,2 amino-ethyl piperidine was added and the reaction was put on with the same conditions overnight. TLC indicated that starting material had been consumed but that the mixture was not pure. Purified by column chromatography (1:1 EtOAc/MeOH).

Yield; 0.01 g, 0.00001 mol, 1.6%.

Melting point: 85.0-89.9 °C.

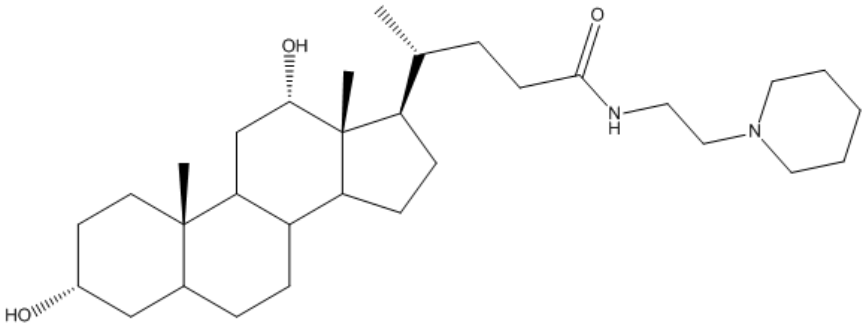
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.59 (s, 3H, 18-CH_3), 0.87-0.89 (s, 3H, 19-CH_3 J=5.5), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 1.72 (m, 4H, CH_2), 2.68 (t, 2H, CH_2, J=5.6), 3.42-3.44 (q, 2H, CH_2, J=5.6), 3.58 (m, 1H, 3-CH), 7.18 (s/t, 1H, NH) ppm.

^{13}C NMR (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 20.8 (CH_2, C11), 23.4 (CH_3, C19), 24.2 (CH_2, C15), 24.3 (CH_2), 25.9 (CH_2), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 31.8 (CH_2, C22), 33.6 (CH_2, C23), 34.5 (C, C10), 35.34 (CH, C20), 35.5 (CH_2, C1), 35.8 (CH, C8), 35.9 (CH_2, C4), 36.45 (Unknown C), 40.1 (CH_2, C12), 40.4 (CH, C9), 42.0 (CH, C5), 42.7 (C, C13), 54.2 (CH, C17), 56.0 (CH, C14), 56.4 (CH_2), 57.2 (CH_2), 71.8 (CH, C3), 173.6 (CO, C24) ppm.

IR= 3418 (NH), 3465 (OH) 2935 (alkyl), 2865 (alkyl), 1641 (C=O) cm^{-1}.

MS (ES) m/z= Found 487.4347 $(M+H)^+$; calculated for $C_{31}H_{54}N_2O_3$ 487.4264; 0.8 ppm.

Preparation of (4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-[2-(1-piperidyl)ethyl]pentanamide (compound 78)



Methyl deoxycholate (1.0 g 0.002 mol) was dissolved in methanol (10 mL) with 1-(2-aminoethyl)piperidine (3.15 g, 0.024 mol) was added and the solution was stirred for 5 days at reflux. The solvent was evaporated under reduced pressure and the product was recrystallized from ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.56 g, 0.0011 mol, 45 %.

Melting point: 158.5-161.1 ^{o}C.

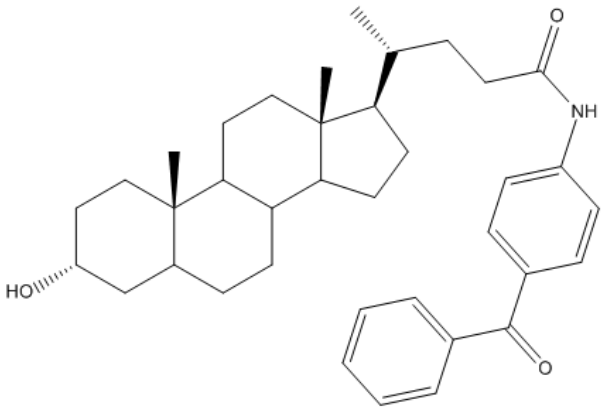
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.66 (s, 3H, 18-CH_3), 0.90 (s, 3H, 19-CH_3), 0.99 (d, 3H, 21-CH_3, J= 5.0), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 2.52 (m, 2H, CH_2), 3.385 (q, 2H, CH_2, J= 5.0), 3.60 (m, 1H, 3-CH), 3.97 (s, 1H, 12-CH), 6.72 (broad s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.7 (CH_3, C18), 17.5 (CH_3, C21), 23.1 (CH_3, C19), 23.7 (CH_2), 23.8 (CH_2), 25.2 (CH_2, C15), 26.1 (CH_2) 27.1 (CH_2, C7), 27.5 (CH_2, C6), 28.6 (CH_2, C16), 30.5 (CH_2, C2), 31.6 (CH_2, C23), 33.3 (CH_2, C22), 33.6 (CH, C9), 34.1 (C, C10), 35.2 (CH_2, C1), 35.2 (CH, C20), 35.4 (CH_2, C4), 36.0 (CH, C8), 36.5 (CH, C5), 42.0 (C, C13), 46.5 (CH, C17), 47.0 (CH_2), 48.3 (CH, C14), 54.2 (CH_2), 57.4 (CH_2), 71.6 (CH, C3), 73.0 (CH_2, C12), 173.9 (CO, C24) ppm.

IR= 3300 (OH), 2925 (alkyl), 2861 (alkyl), 1642 (C=O), 1543, 1437, 1313, 1040 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 503.4202 $(M+H)^{+}$; calculated for $C_{31}H_{55}N_2O_3$ 503.4207; 1 ppm.

Preparation of (4R)-N-(4-benzoylphenyl)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 79)



Lithocholic acid (2.0 g 0.005 mol) was dissolved in tetrahydrofuran (60 mL) with N-methylmorpholine (1.07 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl

chloroformate (0.54 mL, 0.005 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours amino-benzophenone (1.5 g, 0.007 mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the solution was extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL) and 2 M hydrochloric acid solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. The solvent was evaporated under reduced pressure and the product was triturated with ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield 0.57g, 0.001 mol, 19 %.

Melting point: 228.6-230.8 ^{o}C.

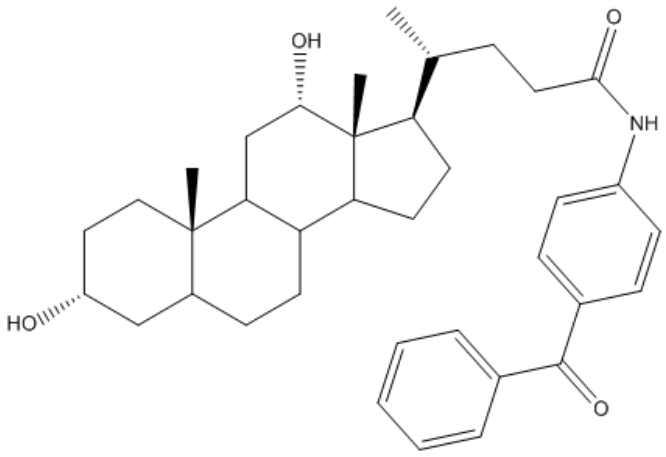
^{1}H NMR (d6 DMSO) (250 MHz) δ= 0.62 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 0.92 (d, 2H, CH_2 J= 7.5), 1.00-2.50 (m, 33H, steroidal backbone CH/CH_2), 4.44 (d, 1H, 3-OH, J= 5.0), 7.52-7.805 (m, 9H, Ar-CH), 10.20 (s, 1H, NH) ppm.

^{13}C NMR (d6 DMSO) (62.9 MHz) δ= 11.8 (CH_3, C18), 18.3 (CH_3, C21), 20.3 (CH_2, C11), 23.2 (CH_3, C19), 23.8 (CH_2, C15), 26.1 (CH_2, C7), 26.8 (CH_2, C6), 27.7 (CH_2, C16), 30.3 (CH_2, C2), 31.1 (CH_2, C22), 33.4 (CH_2, C23), 34.1 (C, C10), 34.9 (CH, C20), 35.1 (CH_2, C1), 35.3 (CH, C8), 41.4 (CH, C5), 42.2 (C, C13), 55.5 (CH, C17), 56.0 (CH, C14), 69.8 (CH, C3), 118.1 (Ar-CH), 128.4 (Ar-CH), 129.3 (Ar-CH), 131.0 (Ar-CH), 131.1 (Ar-CH), 132.1 (Ar-CH), 137.5 (Ar-C), 143.5 (Ar-C), 172.3 (CO, C24), 194.4 (Ar-CO) ppm.

IR= 3488 (NH), 3249 (OH), 2925 (alkyl), 2857 (alkyl), 1675 (C=O), 1586, 1296, 1245, 1168, 1031 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 556.3781 $(M+H)^+$; calculated for $C_{37}H_{50}NO_3$ 556.3785; 0.8 ppm.

Preparation of (4R)-N-(4-benzoylphenyl)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 80)



Deoxycholic acid (2.0 g 0.005 mol) was dissolved in 1, 4-dioxane (60 mL) with N-methylmorpholine (1.07 mL 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.69 mL 0.006 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours amino-benzophenone (1.5 g 0.007 mol) was added and the solution was stirred for a further 24 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL) and 2 M hydrochloric acid (3 x 50 mL). The organic layer was dried over magnesium sulfate. Solvent was evaporated under reduced pressure and triturated with ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.24 g, 0.0004 mol, 8 %.

Melting point: 220-227.6 ^{o}C.

^{1}H NMR (d6 DMSO) (250 MHz) δ= 0.60 (s, 3H, 18-CH_3), 0.85 (s, 3H, 19-CH_3), 0.97 (d, 3H, 21-CH_3, J= 5.0), 1.00-2.50 (m, 33H, steroidal backbone CH/CH_2), 3.80 (s, 1H, 3-CH), 4.03 (s, 1H, 12-CH),

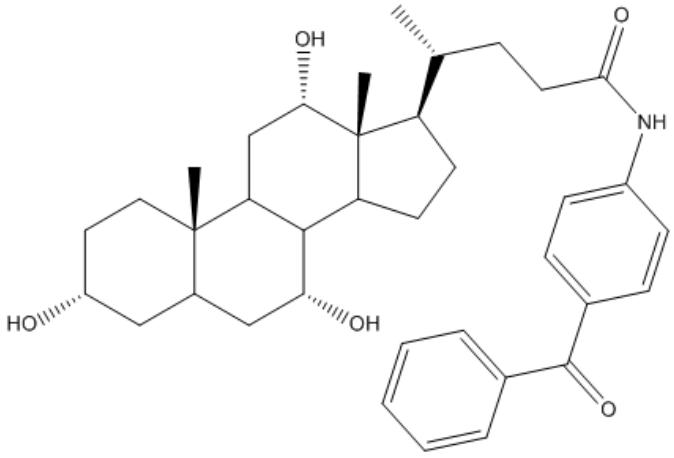
4.21 (d, 1H, 3-OH, J= 2.5), 4.46 (d, 1H, 12-OH, J= 5.0), 7.52-7.79 (multiple overlapping multiplets, 9H, Ar-CH), 10.26 (s, 1H, NH) ppm.

^{13}C NMR (d6 DMSO) (62.9 MHz) δ= 12.4 (CH_3, C18), 17.0 (CH_3, C21), 23.0 (CH_3, C19), 26.9 (CH_2, C7), 27.2, (CH_2, C2), 32.9 (CH_2, C22), 33.5 (CH, C9), 33.7 (C, C10), 35.0 (CH_2, C1), 35.6 (CH, C20), 45.9 (C, C13), 46.1 (CH, C17), 47.2 (CH, C14), 70.9 (CH, C3), 118.1 (Ar-CH), 128.4 (Ar-CH), 129.3 (Ar-CH), 131.1 (Ar-CH), 132.1 (Ar-CH), 137.5 (Ar-C), 143.5 (Ar-C), 172.4 (CONH, C24), 194.5 (Ar-CO) ppm.

IR= 3462 (OH), 2921 (alkyl), 2861 (alkyl), 1675 (C=O), 1586 (C=O), 1441, 1279, 1168.78 (C-O ester stretch), 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 572.3730 $(M+H)^+$; calculated for $C_{37}H_{50}NO_4$ 572.3734; 0.8 ppm.

Preparation of (4R)-N-(4-benzoylphenyl)-4-[(3R,7R,10S,12S,13R,17R)-3,7,12-trihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 81)



Cholic acid (2.0 g 0.004 mol) was dissolved in 1, 4-dioxane (60 mL) with N-methylmorpholine (1.07 mL 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.66 mL 0.006 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours amino-benzophenone (1.44 g 0.007

mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL) and 2 M hydrochloric acid (3 x 50 mL). The organic layer was dried over magnesium sulfate. Solvent was evaporated under reduced pressure and triturated with ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.77 g, 0.001 mol, 31 %.

Melting point: 250.5-254.8 ^{o}C.

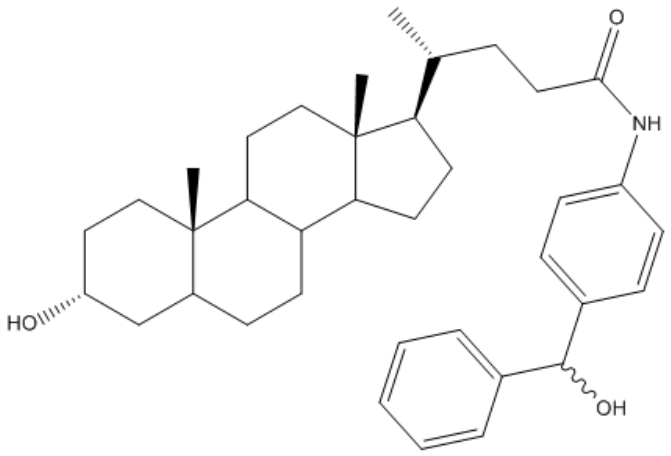
^{1}H NMR (d6 DMSO) (250 MHz) δ= 0.61 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3), 0.995 (d, 2H, CH_2 J= 7.5), 1.31-2.50 (m, 33H, steroidal backbone CH/CH_2), 3.2, 3.63, 3.81 (broad s, 3H, 7, 3, 12-C<u>H</u>), 4.02, 4.13, 4.34 (d, 3H, 7, 3, 12-O<u>H</u>, J= 5.0), 7.56-7.77 (m, 9H, Ar-CH), 10.27 (s, 1H, NH) ppm.

^{13}C NMR (d6 DMSO) (62.9 MHz) δ= 12.3 (CH_3, C18), 17.1 (CH_3, C21), 22.6 (CH_3, C19), 22.7 (CH_2, C15), 26.1 (CH, C9), 27.2 (CH_2, C16), 28.5 (CH_2, C11), 30.3 (CH_2, C3), 31.2 (CH_2, C23), 33.5 (CH_2, C22), 34.3 (CH_2, C6), 34.8 (C, C10), 35.1 (CH_2, C1), 35.2 (CH, C20), 41.3 (CH, C8), 41.4 (CH, C14), 45.7 (CH, C5), 46.0 (C, C13), 66.2 (CH, C7), 66.3 (CH, C3), 70.4 (CH), 70.9 (CH, C12), 118.1 (Ar-CH), 128.4 (Ar-CH), 129.3 (Ar-CH), 130.9 (Ar-CH), 131.1 (Ar-CH), 137.5 (Ar-C), 143.5 (Ar-C), 172.4 (C, CONH), 194.4 (Ar-CO) ppm.

IR= 3462 (OH), 2933 (alkyl), 2870 (alkyl), 1671 (C=O), 1594, 1164, 1279, 1091 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 588.3680 $(M+H)^+$; calculated for $C_{37}H_{50}NO_5$ 588.3684; 0.6 ppm.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-[4-[hydroxy(phenyl)methyl]phenyl]pentanamide (compound 82)



Compound **22** (0.25 g, 0.0006 mol) was dissolved in tetrahydrofuran (10 mL) with sodium borohydride (0.34 g, 0.008 mol). The solution was stirred for 2 days at room temperature. Water (10 mL) was then added and the precipitate collected by vacuum filtration, washed with water (3 x 20 mL) and dried under vacuum. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield 0.18 g, 0.003 mol. 72 %.

Melting point: 211.2 – 217.6 ^{o}C.

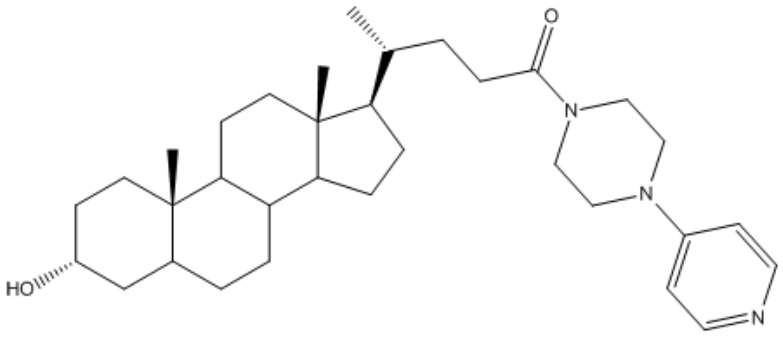
^{1}H NMR (d6 DMSO) (250 MHz) δ= 0.61 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 0.92 (d, 3H, 21-CH_3 J= 7.5), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 4.43 (s, 1H, 3-OH), 5.62 (s, 1H, CH-O), 5.80 (broad s, 1H, OH), 7.27-7.47 (m, 9H, Ar-CH), 9.79 (s, 1H, CH) ppm.

^{13}C NMR (d6 DMSO) (62.9 MHz) δ= 11.8 (CH_3, C18), 18.3 (CH_3, C21), 20.3 (CH_2, C11), 23.2 (CH_3, C19), 23.8 (CH_2, C15), 26.1 (CH_2, C7), 27.7 (CH_2, C6), 30.3 (CH_2, C2), 31.3 (CH_2, C22), 34.1 (C, C10), 34.9 (CH, C20), 35.3 (CH_2, C1), 41.4 (CH, C5), 42.2 (C, C13), 55.5 (CH, C17), 56.0 (CH, C14), 69.8 (CH, C3), 73.8 (CH_2), 79.1 (CH_2), 118.7 (Ar-CH), 126.1 (Ar-CH), 126.4 (Ar-CH), 127.9 (Ar-CH), 137.9 (Ar-C), 140.2 (Ar-CH), 145.7 (Ar-C), 171.4 (CO, C24) ppm.

IR= 3424 (NH), 2925 (alkyl), 2861 (alkyl), 1663 (C=O), 1599, 1535, 1407, 1300, 1031 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 575.4207 (M + NH_4); calculated for $C_{37}H_{55}N_2O_3$ 575.4207; 0.6 ppm.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-1-[4-(4-pyridyl)piperazin-1-yl]pentan-1-one (compound 83).



From a modification (El Kihel et al., 2008), lithocholic acid (0.5 g, 0.001 mol) was dissolved in tetrahydrofuran (15 mL) with triethylamine (0.20 mL, 0.001 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.2 mL, 0.001 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 1-(4-pyridyl)piperazine (0.21 mL, 0.001 mol) was added and the solution was stirred for 24 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). Due to impurities, re-dissolved in a methanol/water solution (20 mL 50/50) and extracted with chloroform (4 x 20 mL). The organic layer was dried over magnesium sulfate. Solvent was evaporated under reduced pressure to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.03 g, 0.00005 mol, 4 %.

Melting point: 150-157 ^oC.

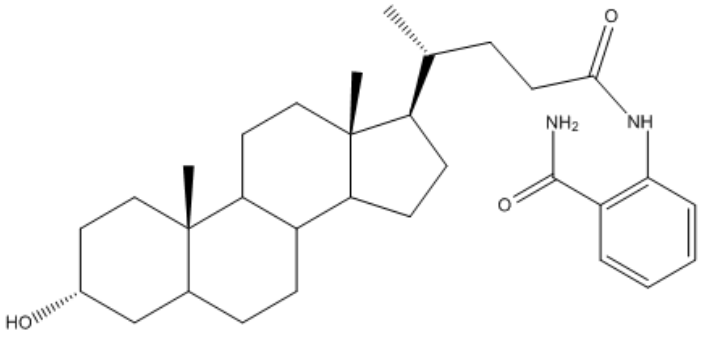
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.65 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 1.00-2.40 (m, 33H, steroidal backbone CH/CH_2), 3.35 (m, 4H, CH_2), 3.64 (m, 2H, CH_2), 6.68 (d, 2H, Ar-CH, J = 5.0), 8.31 (s, 2H, Ar-CH) ppm.

^{13}C NMR (62.9 MHz) δ= 12.0 (CH_3, C18), 18.6 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.3 (CH_2, C15), 26.3 (CH_2, C7), 27.1 (CH_2, C6), 28.3 (CH_2, C16), 30.2 (CH_2, C2), 31.3 (CH_2, C22), 34.5 (C, C10), 35.4 (CH, C20), 35.8 (CH_2, C1), 36.4 (CH_2, C4), 40.1 (CH_2, C12), 40.4 (CH, C9), 40.7 (CH, C5), 42.1 (C, C13), 42.7 (CH_2 N-$\underline{C}H_2$), 44.7 (CH_2, N-$\underline{C}H_2$), 45.8 (CH_2, N-$\underline{C}H_2$), 45.9 (CH_2, N-$\underline{C}H_2$), 55.9 (CH, C17), 56.5 (CH, C14), 71.8 (CH, C3), 108.5 (Ar-CH), 129.7 (Ar-CH), 149.9 (Ar-C), 150.0 (Ar-CH), 154.6 (Ar-CH), 172.7 (CO, C24) ppm.

IR= 3387 (OH), 2925 (alkyl), 2852 (alkyl), 1641 (C=O), 1445, 1236, 1044, 989 (R_2CH-OH) cm^{-1}.

MS (ES) m/z= Found 522.4048 $(M+H)^+$; calculated for $C_{31}H_{57}N_2O_4$ 521.4313; 1.5 ppm.

Preparation of 2-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]benzamide (compound 84)



Lithocholic acid (2.0 g 0.005 mol) was dissolved in 1, 4 dioxane (40 mL) with triethylamine (1.40 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.54 mL, 0.005 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 2-aminobenzamide (0.7 g, 0.005 mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the solution was

extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. The solvent was evaporated under reduced pressure and the product was recrystallized from ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 1.41 g, 0.002 mol, 53 %.

Melting point: 112.1-113.0 ^{o}C.

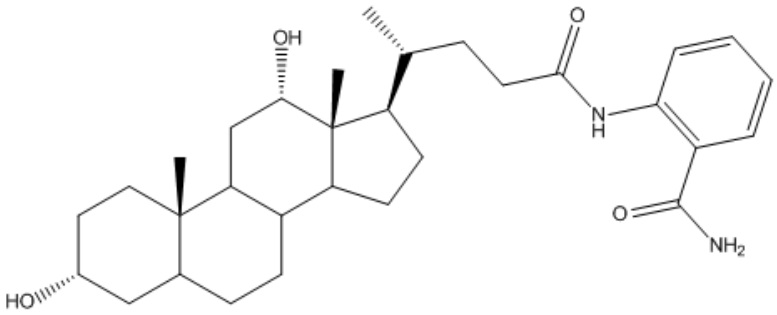
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.63 (s, 3H, 18-CH_3), 0.90 (s, 3H, 19-CH_3), 1.0-2.50 (m, 33H, steroidal backbone CH/CH_2), 3.63, (m, 1H, 3-CH), 5.92 (broad singlet, 1H, split NH_2), 6.39 (broad singlet, 1H, split NH_2), 7.05 (t, 1H, Ar-CH), 7.51 (t, 1H, Ar-CH), 8.625 (d, 1H, Ar-CH, J= 7.5), 11.15 (s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 20.8 (CH_2, C11), 23.4 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.4 (CH_2, C1), 35.4 (CH, C8), 35.8 (CH_2, C4), 36.4 (CH_2), 40.1 (CH_2, C12), 40.4 (CH, C9), 42.1 (CH, C5), 42.7 (C, C13), 55.9 (CH, C17), 56.4 (CH, C14), 71.8 (CH, C3), 118.4 (Ar-C), 121.5 (Ar-CH), 122.4 (Ar-CH), 127.3 (Ar-CH), 133.3 (Ar-CH), 140.3 (Ar-C), 171.5 (<u>C</u>ONH_2 Or <u>C</u>ONH), 172.8 (<u>C</u>ONH_2 Or <u>C</u>ONH) ppm.

IR= 3432 (NH), 3351 (OH), 1663 (C=O), 1509, 1381, 1283, 1023 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 495.3577 $(M+H)^+$; calculated for $C_{31}H_{47}N_2O_3$ 495.3581; 0.8 ppm.

Preparation of 2-[[(4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]benzamide (compound 85)



Deoxycholic acid (2.0 g 0.005 mol) was dissolved in 1, 4-dioxane (40 mL) with triethylamine (1.40 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.54 mL, 0.005 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 2-aminobenzamide (0.7 g, 0.005 mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the solution was extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. The solvent was evaporated under reduced pressure and the product was triturated with diethyl ether and then vacuum filtered to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 1.78 g, 0.003 mol, 68 %.

Melting point: 119.8 – 125.7 ^{o}C.

^{1}H NMR (d6 DMSO) (250 MHz) δ= 0.60 (s, 3H, 18-CH_3), 0.85 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.80 (s, 1H, 12-CH), 4.22 (d, 1H, 3-OH, J= 5.0), 4.47 (d, 1H, 12-OH, J=

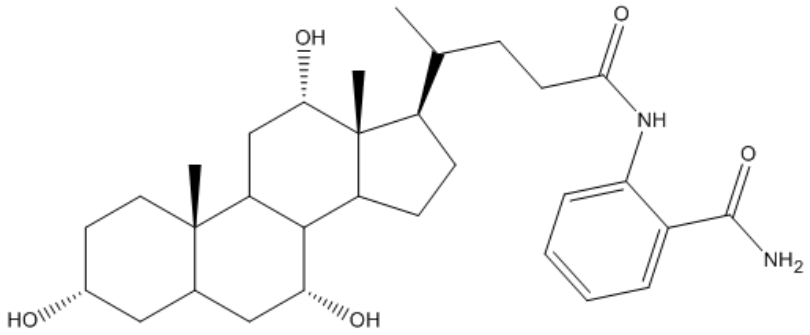
5.0), 7.10 (t, 1H, Ar-CH, J= 10.0), 7.48 (t, 1H, Ar-CH, J= 10.0), 7.72 (s, 1H, split NH_2), 7.79 (d, 1H, Ar-CH, J= 7.5), 8.25 (s, 1H, NH_2), 8.47 (d, 1H, CH, J= 7.5), 11.71 (s, 1H, NH) ppm.

^{13}C NMR (d6 DMSO) (62.9 MHz) δ= 12.4 (CH_3, C18), 15.1 (CH_2), 16.9 (CH_3, C21), 23.0 (CH_3, C19), 24.4 (CH_2, C15), 26.0 (CH_2, C7), 26.9 (CH_2, C6), 27.1 (CH_2, C16), 28.6 (CH_2, C11), 30.2 (CH_2, C2), 31.3 (CH_2, C23), 32.9 (CH_2, C22), 33.7 (CH, C9), 34.7 (C, C10), 34.9 (CH_2, C1), 35.1 (CH, C20), 36.2 (CH_2, C4), 38.4 (CH, C8), 45.9 (CH, C5), 46.1 (C, C13), 47.4 (CH, C17), 64.8 (CH), 69.9 (CH, C3), 70.9 (CH_2, C12), 119.2 (Ar-CH), 119.9 (Ar-CH), 122.0 (Ar-CH), 128.4 (Ar-C), 132.1 (Ar-C), 139.7 (Ar-CH), 170.7 ($\underline{C}ONH_2$ Or $\underline{C}ONH$), 171.4 ($\underline{C}ONH_2$ Or $\underline{C}ONH$) ppm.

IR= 3343 (OH), 2925 (alkyl), 2861 (alkyl), 1663 (C=O), 1189.78 (C=O ester stretch), 1518, 1441, 1381, 1270, 1023 (RCHOH), 750 cm^{-1}.

MS (ES) m/z= Found 511.3525 $(M+H)^+$; calculated for $C_{31}H_{47}N_2O_4$ 511.3530; 1.0 ppm.

Preparation of 2-[4-[(3R,7R,10S,12S,13R,17R)-3,7,12-trihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoylamino]benzamide (compound 86)



Cholic acid (2.0 g 0.005 mol) was dissolved in 1, 4-dioxane (40 mL) with triethylamine (1.40 mL 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.54 mL 0.005 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 2-aminobenzamide (0.7 g 0.005 mol) was added and the

solution was stirred for a further 48 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. Solvent was evaporated under reduced pressure and recrystallized from ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield: 0.692 g, 0.001 mol, 26.8 %.

Melting point: 122.8 – 129.9 ^{o}C.

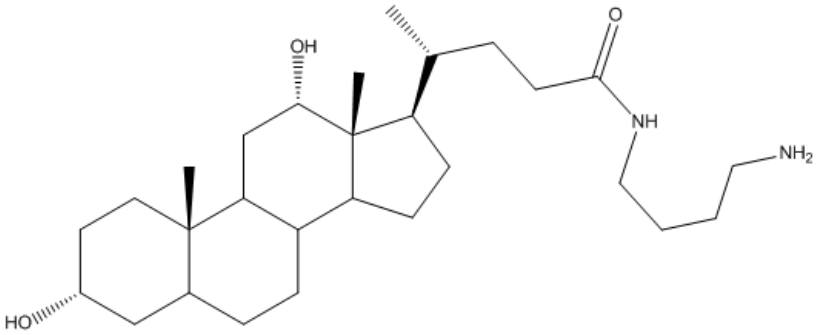
^{1}H NMR (d6 DMSO) (250 MHz) δ= 0.60 (s, 3H, 18-CH_3), 0.82 (s, 3H, 19-CH_3), 0.99 (d, 3H, 21-CH_3, J= 7.5), 1.35-2.50 (m, 33H, steroidal backbone CH/CH_2), 3.63 (s, 1H, 3-CH), 3.80 (s, 1H, 12-CH), 4.00 (d, 1H, 7-OH), 4.14 (s, 1H, 3-OH), 4.34 (s, 1H, 12-OH), 7.10 (t, 1H, Ar-CH, J= 7.50), 7.51 (t, 1H, Ar-CH, J= 15), 7.79 (d, 1H, Ar-CH), 8.25 (s, 1H, NH), 8.49 (d, 1H, Ar-CH), 11.70 (s, 1H, NH) ppm.

^{13}C NMR (d6 DMSO) (62.9 MHz) δ= 12.3 (CH_3, C18), 17.0 (CH_3, C21), 22.6 (CH_3, C19), 22.7 (CH_2, C15), 26.1 (CH, C9), 27.2 (CH_2, C16), 28.5 (CH_2, C11), 30.3 (CH_2, C3), 31.3 (CH_2, C23), 34.3 (CH_2, C22), 34.8 (C, C10), 35.0 (CH_2, C1), 35.2 (CH, C20), 41.3 (CH_2, C4), 41.4 (CH, C8), 45.7 (CH), 46.0 (CH), 66.2 (CH, C7), 70.4 (CH, C3), 70.9 (CH, C12), 119.3 (Ar-C), 119.98 (Ar-CH), 122.10 (Ar-CH), 128.5 (Ar-CH), 132.1 (Ar-CH), 139.7 (Ar-C), 170.8 (<u>C</u>ONH_2 Or <u>C</u>ONH), 171.5 (<u>C</u>ONH_2 Or <u>C</u>ONH) ppm.

IR= 3462 (OH), 2933, 2870 (alkyl), 1671 (C=O), 1189, 1091 (R_2CH-OH) cm^{-1}.

MS (ES) m/z= Found 527.3472 $(M+H)^{+}$; calculated for $C_{31}H_{47}N_2O_5$ 527.3479; 1.4 ppm.

Preparation of (4R)-N-(4-aminobutyl)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 87)



Methyl deoxycholate (1.0 g 0.009 mol) was dissolved in methanol (10 mL) with 1,4-diaminobutane (2.47 mL 0.02 mol) was added and the solution was stirred for a further 72 hours. Water (50 mL) was then added and the resultant precipitate was collected by vacuum filtration, washed with water (3 x 20 mL) and dried under vacuum to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.29 g, 0.0006 mol, 25 %.

Melting point: 137.4-141 ^{o}C.

^{1}H NMR (d6 DMSO) (250 MHz) δ= 0.58 (s, 3H, 18-CH_3), 0.84 (s, 3H, 19-CH_3), 0.91 (d, 3H, 21-CH_3, J= 5.0), 1.00-2.23 (m, 33H, steroidal backbone CH/CH_2), 2.99 (q, 2H, CH_2, J=7.5), 3.6 (m, 1H, 3-CH), 3.78 (s, 1H, 12-CH), 7.73 (broad s, 1H, NH) ppm.

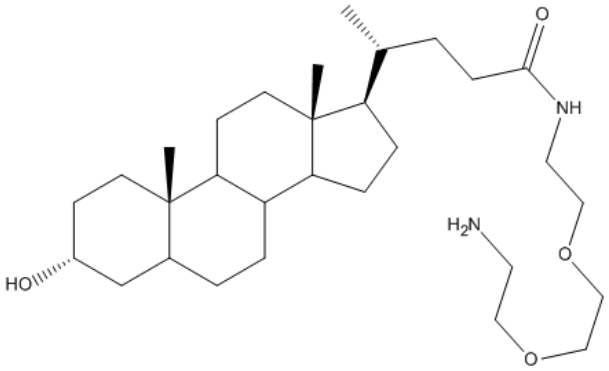
^{13}C NMR (d6 DMSO) (62.9 MHz) δ= 12.4 (CH_3, C18), 17.0 (CH_3, C21), 23.0 (CH_3, C19), 23.4 (CH_2, C15), 26.0, (CH_2, NH-$\underline{C}H_2$ or $\underline{C}H_2N(CH_3)_2$ or $CH_2\underline{C}H_2CH_2$), 26.6 (CH_2, CH_2, NH-$\underline{C}H_2$ or $\underline{C}H_2N(CH_3)_2$ or $CH_2\underline{C}H_2CH_2$), 26.9 (CH_2, CH_2, NH-$\underline{C}H_2$ or $\underline{C}H_2N(CH_3)_2$ or $CH_2\underline{C}H_2CH_2$), 27.1 (CH_2, C7), 28.5 (CH_2, C16), 30.2 (CH_2, C11), 30.5 (CH_2, C2), 31.7 (CH_2, C23), 32.5 (CH_2, C22), 32.8 (CH, C9), 33.7 (C, C10), 35.0 (CH_2, C1), 35.1 (CH, C20), 35.6 (CH_2, C4), 36.2 (CH, C8), 41.3 (CH, C5), 41.5 (CH),

45.9 (C, C13), 46.1 (CH, C17), 47.4 (CH, C14), 69.8 (CH, C3), 69.8 (CH_2, C12), 172.2 (CO, C24) ppm.

IR= 3322 (OH), 2929 (alkyl), 2857 (alkyl), 1624 (C=O), 1539, 1437, 1360, 1040 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 463.3886 $(M+H)^+$; calculated for $C_{28}H_{51}N_2O_3$ 463.3894; 1.8 ppm.

Preparation of (4R)-N-[2-[2-(2-aminoethoxy)ethoxy]ethyl]-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 88)



Lithocholic acid (2.0 g, 0.005 mol) was dissolved in Toluene (40 mL). 2, 2'-(Ethylenedioxy)bis(ethylamine) (7.8 mL, 0.052 mol) was added and the solution was heated at reflux for 5 days. Water (50 mL) was then added and resultant precipitate collected by vacuum filtration, with further washes of water (3 x 20 mL). Dried under vacuum to produce an off-white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield, 1.62 g, 0.003 mol, 60.22 %

Melting point: 81.1-85.5 ^oC.

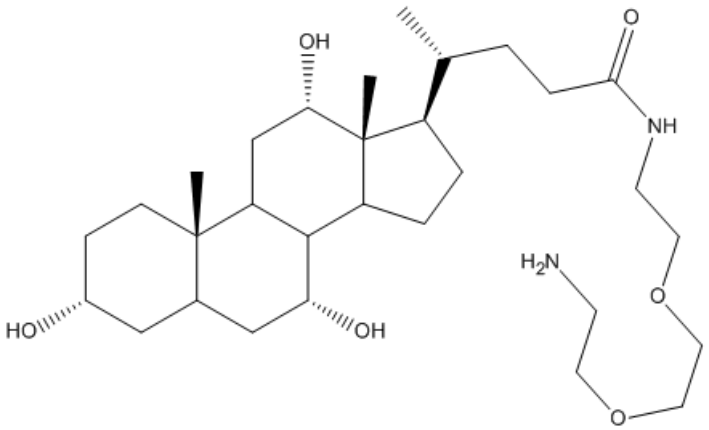
^1H NMR ($CDCl_3$) (250 MHz) δ= 0.64 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 1.00-2.29 (m, 33H, steroidal backbone CH/CH_2), 2.91 (broad s, 2H, CH_2), 3.46-3.63 (multiple overlapping multiplets, 10H, CH_2), 6.24 (broad s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 19.9 (CH_2), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 31.7 (CH_2, C22), 33.4 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.5 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 39.0 (CH_2, C12), 39.1 (CH, C9), 40.2 (CH_2, NH-CH_2 or OCH_2), 40.4 (CH_2, CH_2, NH-CH_2 or OCH_2), 42.1 (CH, C5), 42.7 (C, C13), 56.0 (CH, C17), 56.5 (CH, C14), 70.1 (CH_2), 70.5 (CH, C3), 71.7 (CH_2), 173.7 (CO, C24) ppm.

IR= 3356 (OH), 2921 (alkyl), 2857 (alkyl), 1641 (C=O), 1552, 1441, 1300, 1104 (C=O ester stretch), 1053 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 507.4149 $(M+H)^+$; calculated for $C_{30}H_{55}N_2O_4$ 507.4156; 1.4 ppm.

Preparation of (4R)-N-[2-[2-(2-aminoethoxy)ethoxy]ethyl]-4-[(3R,7R,10S,12S,13R,17R)-3,7,12-trihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 89)



Cholic acid (2.0 g 0.005 mol) was dissolved in tetrahydrofuran (40 mL) with triethylamine (1.78 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.52 mL, 0.005 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours this material was added to a flask containing 2, 2' (ethylenedioxy)bis(ethylamine) (7.25 mL 0.04 mol) drop by drop and the solution was stirred for 24 hours. Water (50 mL) was then added and the solution was extracted with ethyl acetate (3 x 50 mL).

The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. The solvent was evaporated under reduced pressure to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.05 g, 0.00009 mol, 1.9 %.

Melting point: 134.0-136.0 ^{o}C.

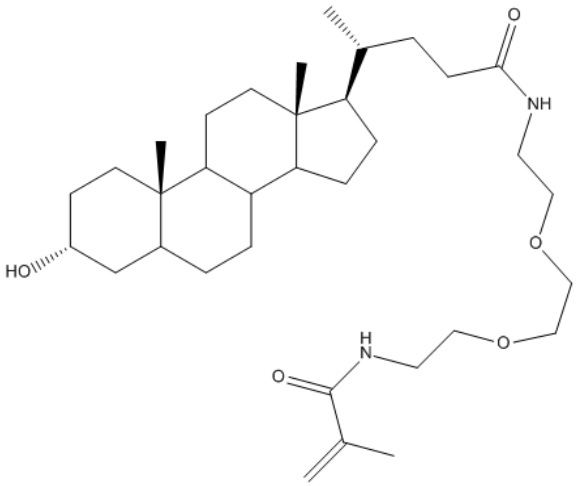
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.67 (s, 3H, 18-CH_3), 0.88 (s, 3H, 19-CH_3), 1.28-1.92 (m, 33H, steroidal backbone CH/CH_2), 2.63 (broad s, 1H, split NH_2), 3.04 (broad s, 1H, split NH_2), 3.44-3.62 (multiple overlapping multiplets, 12H, CH_2), 3.83 (s, 1H, 3-CH), 3.96 (s, 1H, 12-CH), 6.73 (broad s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.4 (CH_3, C18), 14.2 (CH_2), 17.5 (CH_3, C21), 21.0 (CH_2), 22.4 (CH_3, C19), 23.3 (CH_2, C15), 26.3 (CH, C9), 27.6 (CH_2, C16), 28.1 (CH_2), 30.5 (CH_2, C11), 31.8 (CH_2, C3), 32.9 (CH_2, C23), 34.8 (CH_2, C6), 35.4 (C, C10), 39.1 (CH_2, C1), 39.5 (CH, C20), 39.6 (CH_2), 41.5 (CH_2, C4), 46.3 (CH, C5), 46.4 (C, C13), 60.4 (CH_2), 68.4 (CH, C7), 69.9 (CH_2), 70.1 (CH_2), 71.8 (CH, C3), 73.1 (CH, C12), 174.4 (C, CO) ppm.

IR= 3334 (OH), 2925 (alkyl), 2861 (alkyl), 1646 (C=O), 1535, 1449, 1368, 1066, 1040 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= No corresponding peak found.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-[2-[2-[2-(2-methylprop-2-enoylamino)ethoxy]ethoxy]ethyl]pentanamide (compound 90)



Using a modification of a method from (Hu et al., 2005) product from compound **88** (0.5 g 0.0009 mol) was dissolved in anhydrous tetrahydrofuran (10 mL) with triethylamine (0.20 mL, 0.001 mol). Methacrylic anhydride (0.30 mL, 0.0018) was then added. The solution was stirred for 48 hours, protected from sunlight by tin foil at ambient temperature. Water (30 mL) was then added and the resulting precipitate was collected by vacuum filtration, washed with water (3 x 20 mL) and dried under vacuum. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.07 g, 0.0001 mol, 12.5 %.

Melting point: 57.3 – 59.0 ^{o}C.

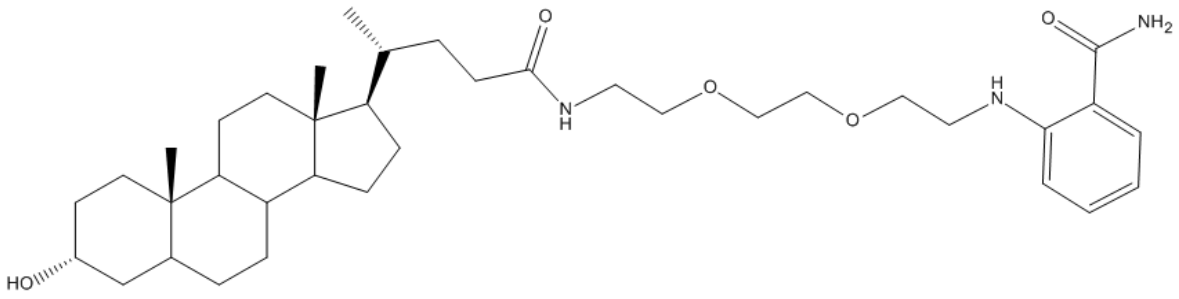
^{1}H NMR (d6 DMSO) (250 MHz) δ= 0.60 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 1.84 (s, 3H, acryloyl-CH_3), 3.154-3.50 (m, 13H, CH/CH_2), 4.42 (d, 1H, 3-OH, J= 5.0), 5.31 (s, 1H, =CH), 5.64 (s, 1H, =CH), 7.78 (broad s, 1H, NH), 7.91 (broad s, 1H, NH) ppm.

^{13}C NMR (d6 DMSO) (62.9 MHz) δ= 11.8 (CH_3, C18), 18.2 (CH_3, C21), 18.6 (CH), 20.3 (CH_2, C11), 23.2 (CH_3, C19), 23.8 (CH_2, C15), 26.1 (CH_2, C7), 26.8 (CH_2, C6), 27.7 (CH_2, C16), 30.3 (CH_2, C2), 31.5 (CH_2, C22), 32.2 (CH_2, C23), 34.1 (C, C10), 34.9 (CH, C20), 35.1 (CH_2, C1), 35.3 (CH, C8), 41.4 (CH, C5), 42.2 (C, C13), 55.2 (CH, C17), 56.0 (CH, C14), 68.8 (CH_2, CH_2, NH-CH_2 or OCH_2), 69.1 (CH_2, CH_2, NH-CH_2 or OCH_2), 69.5 (CH_2, CH_2, NH-CH_2 or OCH_2), 69.8 (CH, C3), 118.9 (=CH_2), 139.8 (=C), 167.4 (CO), 172.5 (CO, C24) ppm.

IR= 3415 (NH), 3292 (OH), 2933 (alkyl), 2861 (alkyl), 1658 (C=O), 1590 (C=O), 1394, 1249 (C-O ester stretch), 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 575.4412 $(M+H)^+$; calculated for $C_{34}H_{59}N_2O_5$ 575.4418; 1.1 ppm.

Preparation of 2-[2-[2-[2-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]ethoxy]ethoxy]ethylamino]benzamide (compound 91)



Compound **88** (0.5 g 0.0009 mol) was dissolved in ethanol (15 mL) and isatoic anhydride (0.17 g, 0.001 mol) was added. The solution was heated at reflux for 24 hours then allowed to cool. Water (50 mL) was then added and the resulting precipitate was collected by vacuum filtration and washed with hot water (3 x 20 mL) to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.15 g, 0.0002 mol, 24.5 %.

Melting point: 72.5-74.7 ^oC.

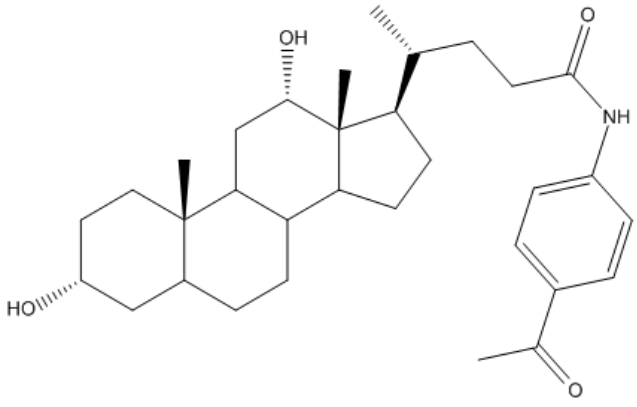
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.63 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.43 (t, 2H, CH_2, J= 7.5), 3.52 (t, 2H, CH_2, J= 5.0), 3.63 (m, 8H, CH_2), 5.96 (broad s, 1H, NH), 6.66 (overlapping multiplets, 2H, Ar-CH), 7.20, (t, 2H, Ar-CH, J= 5.0), 7.36 (d, 1H, Ar-CH J = 12.5 and 15) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.3 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 31.7 (CH_2, C22), 33.5 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.4 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 39.1 (CH_2, C12), 39.3 (CH, C9), 40.1 (CH_2, CH_2, NH-CH_2 or OCH_2), 40.4 (CH_2, CH_2, NH-CH_2 or OCH_2) 42.1 (CH, C5), 42.7 (C, C13), 55.9 (CH, C17), 56.4 (CH, C14), 69.8 (CH_2), 70.0 (CH_2), 70.1 (CH_2), 70.2 (CH_2), 71.8 (CH, C3), 116.0 (Ar-CH), 116.5 (Ar-CH), 117.3 (Ar-CH), 127.3 (Ar-CH), 132.2 (Ar-CH), 148.7 (Ar-CH), 169.3 (CO), 173.7 (CO, C24) ppm.

IR= 3330 (OH), 2929 (alkyl), 2861 (alkyl), 1624 (C=O), 1535, 1432, 1258, 1087 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 626.4523 $(M+H)^+$; calculated for $C_{37}H_{60}N_3O_5$ 626.4527; 0.7 ppm.

Preparation of (4R)-N-(4-acetylphenyl)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 92)



Deoxycholic acid (2.0 g 0.005 mol) was dissolved in 1, 4 dioxane (60 mL) with triethylamine (1.32 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.40 mL,

0.003 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 4'-aminoacetophenone (0.75 g, 0.005 mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the resulting precipitate was collected by vacuum filtration. The crude product was triturated with ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 0.65 g, 0.001 mol, 25 %.

Melting point: Gradual softening from 194.1-206.9 ^{o}C.

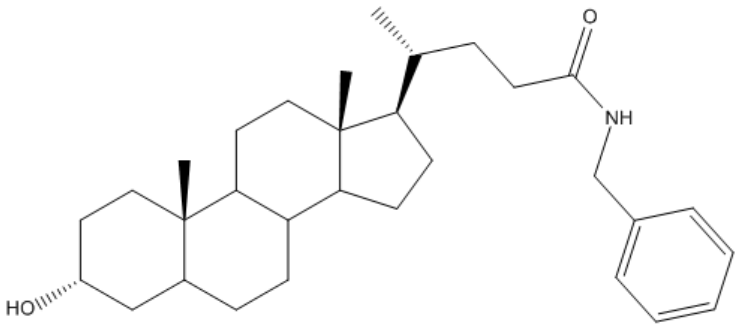
^{1}H NMR (d6 DMSO) (250 MHz) δ= 0.62 (s, 3H, 18-CH_3), 0.86 (s, 3H, 19-CH_3), 1.00-2.50 (m, 33H, steroidal backbone CH/CH_2), 2.52 (s, 3H, ketone-CH_3), 3.81 (broad s, 1H, 12-CH), 4.21 (d, 1H, 3-OH, J =2.5), 4.46 (d, 1H, 12-OH, J= 2.5), 7.73 (d, 2H, Ar-CH, J= 10.0), 7.92 (d, 2H, Ar-CH, J= 7.5), 10.21 (s, 1H, NH) ppm.

^{13}C NMR (62.9 MHz) δ= Due to solubility issues, there was not a strong enough concentration of material for a successful carbon NMR to be completed.

IR= 3475 (OH), 2921 (alkyl), 2861 (alkyl), 1684 (C=O), 1646 (C=O), 1594, 1539, 1407, 1044 cm^{-1}.

MS (+ESI) m/z= Found 510.3570 $(M+H)^{+}$; calculated for $C_{32}H_{48}NO_4$ 510.3578; 1.3 ppm.

Preparation of (4R)-N-benzyl-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 93)



Lithocholic acid (2.0 g 0.005 mol) was dissolved in tetrahydrofuran (60 mL) with N-methylmorpholine (1.07 mL 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.51 mL 0.005 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 4-benzylamine (1.7 mL 0.01 mol) was added and the solution was stirred for a further 24 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The crude product was recrystallized from dichloromethane to produce a white powder.

Yield; 1.27 g, 0.002 mol, 51 %.

Melting point: 189.9-191.6 ^{o}C.

^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.64 (s, 3H, 18-CH_3), 0.91 (d, 3H, 19-CH_3, J= 2.5), 0.92 (s, 3H, 21-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.62 (m, 1H, 3-CH), 4.445 (d, 2H, CH_2, J= 7.5), 7.32-7.34 (multiple overlapping aromatic multiplets, 5H, CH) ppm.

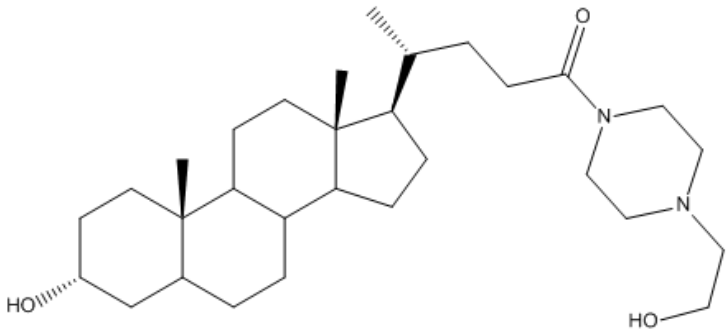
^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.5 (CH_2, C2), 31.8 (CH_2, C22), 33.6 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.4 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2,

C4), 40.2 (CH_2, C12), 40.4 (CH, C9), 42.1 (CH, C5), 42.7 (C, C13), 43.6 (CH_2, CH_2-Ar), 56.0 (CH, C17), 56.5 (CH, C14), 71.8 (CH, C3), 127.5 (Ar-CH), 127.8 (Ar-CH), 128.7 (Ar-CH), 138.4 (Ar-C), 173.3 (CO, C24) ppm.

IR= 3398 NH), 3322 (OH), 2929 (alkyl), 2857 (alkyl), 1629 (C=O), 1552, 1445, 1364, 1228, 1044 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 466.3672 $(M+H)^+$; calculated for $C_{31}H_{48}NO_2$ 466.3680; 1.6 ppm.

Preparation of ethane; (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-1-[4-(2-hydroxyethyl)piperazin-1-yl]pentan-1-one (compound 94)



Lithocholic acid (4.0 g 0.01 mol) was dissolved in tetrahydrofuran (120 mL) with triethylamine (1.30 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (1.02 mL, 0.009 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 1-(2-hydroxyethylpiperazine) (1.5 mL, 0.01 mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the solution was extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. The solvent was evaporated under reduced pressure and the product was recrystallized from ethyl acetate to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.25 (single spot).

Yield; 2.27 g, 0.004 mol, 43 %.

Melting point: 153.0-154.8 ^{o}C.

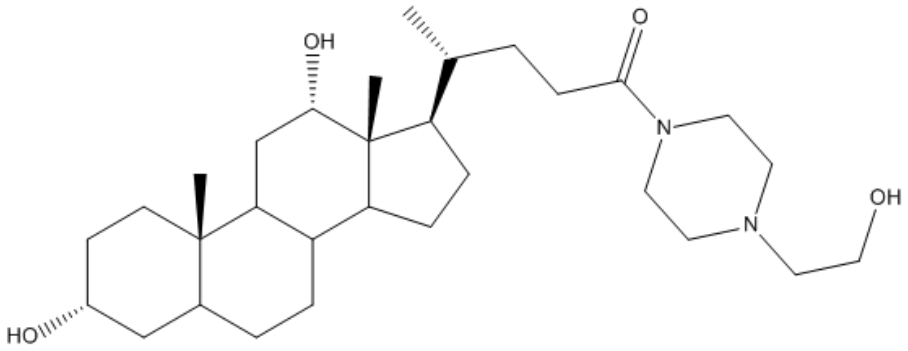
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.65 (s, 3H, 18CH_3), 0.92 (s, 3H, 19-CH_3), 1.00-2.49 (m, 33H, steroidal backbone CH/CH_2), 2.50 (overlapping m, 4H, CH_2), 2.58 (t, 2H, CH_2, J= 5.0), 3.49 (t, 2H, CH_2, J= 2.5), 3.65 (t, 4H, CH_2, J= 5.0) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.5 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 30.2 (CH_2, C2), 30.5 (CH_2, C22), 31.4 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 35.6 (CH_2, C1), 35.8 (CH, C8), 36.4 (CH_2, C4), 40.2 (CH_2, C12), 40.4 (CH, C9), 41.4 (CH, C5), 42.1 (C, C13), 42.7 (CH_2), 45.6 (CH_2), 52.6 (CH_2), 53.1 (CH_2), 56.0 (CH, C17), 56.5 (CH, C14), 57.7 (CH_2), 59.3 (CH_2), 71.8 (CH, C3), 172.1 (CO, C24) ppm.

IR= 3381 (OH), 2916 (alkyl), 2840 (alkyl), 1620 (C=O), 1445, 1258, 1044 (R_2CH-OH) cm^{-1}.

MS (ES) m/z= Found 489.4045 $(M+H)^{+}$; calculated for $C_{30}H_{53}N_2O_3$ 489.4051; 1.2 ppm.

Preparation of (4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-1-[4-(2-hydroxyethyl)piperazin-1-yl]pentan-1-one (compound 95)



Deoxycholic acid (4.0 g 0.01 mol) was dissolved in 1, 4-dioxane (120 mL) with triethylamine (2.70 mL 0.02 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.8 mL 0.005 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 1-(2-hydroxyethylpiperazine) (1.32 mL 0.01 mol) was added and the solution was stirred for 48 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. Solvent was evaporated under reduced pressure and recrystallized from methanol to produce a white powder. TLC EtOAc: MeOH 4:1 R_f 0.2 (single spot).

Yield; 2.41 g, 0.004 mol, 46.8 %.

Melting point: 241.0-243.9 ^{o}C.

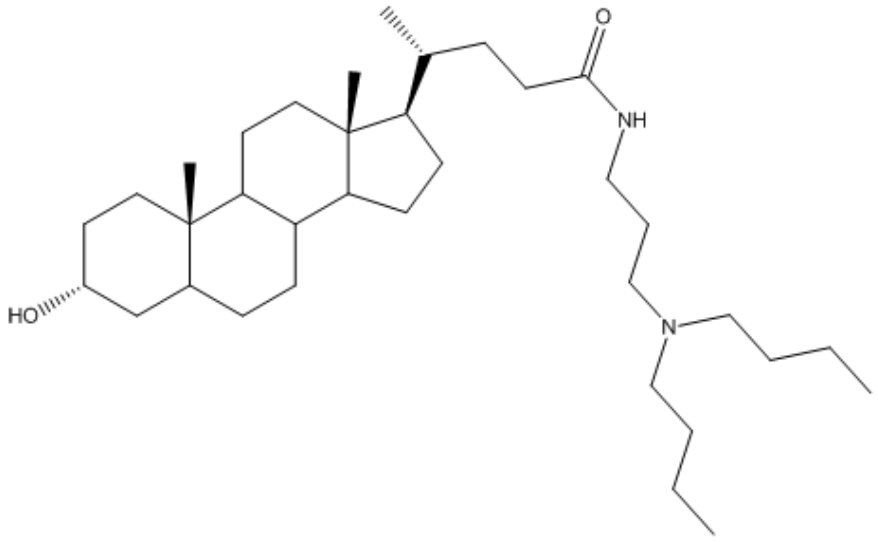
^{1}H NMR ($CDCl_3$/MeOH) (250 MHz) δ= 0.70 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 1.04-2.50 (m, 33H, steroidal backbone CH/CH_2), 2.58 (multiple overlapping multiplets, 6H, CH_2), 3.53 (broad t, 3H, 3-CH/CH_2), 3.65-3.72 (multiple overlapping multiplets, 4 H, CH_2), 3.96 (broad s, 1H, 12-CH) ppm.

^{13}C NMR (d6-DMSO) (250 MHz) δ= 12.4 (CH_3, C18), 17.0 (CH_3, C21), 23.0 (CH_3, C19), 28.5 (CH_2, C16), 29.4 (CH_2, C11), 30.1 (CH_2, C2), 32.8 (CH_2, C23), 33.7 (CH_2, C22), 35.12 (CH, C9), 35.56 (C, C10), 36.22 (CH_2, C1), 45.9 (C, C13), 46.1 (CH, C17), 47.4 (CH, C14), 52.89 (CH_2), 53.43 (CH_2), 58.38 (CH_2), 69.9 (CH, C3), 71.0 (CH_2, C12), 78.33 (CH_2), 78.8 (CH_2), 79.0 (CH_2), 79.3 (CH_2), 171.0 (CO, C24) ppm.

IR= 3407 (OH), 2929 (alkyl), 1620 (C=O), 1454, 1215, 1044 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 505.3994 $(M+H)^+$; calculated fo_r $C_{31}H_{57}N_2O_4$ 505.4000; 1.2 ppm.

Preparation of (4R)-N-[3-(dibutylamino)propyl]-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 96)



Lithocholic acid (0.5 g 0.001 mol) was dissolved in tetrahydrofuran (15 mL) with triethylamine (0.20 mL 0.001 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.2 mL 0.001 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 3-(dibutylamino)-1-propylamine (0.25 mL, 0.001 mol) was added and the solution was stirred for 24 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). This crude material was

further purified using column chromatography (8:2 ethyl acetate/methanol). The compound containing fractions were dried over magnesium sulfate. Solvent was evaporated under reduced pressure to produce a white powder.

Yield; 0.24 g, 0.0004 mol, 33%.

Melting point: 62.9 – 69.3 ^{o}C.

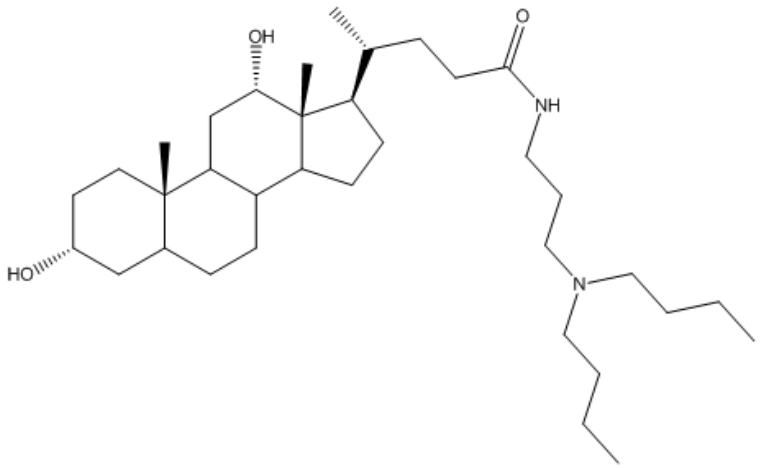
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.64 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 0.94 (m, 6H, CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 1.35 (m, 8H, CH_2), 2.45 (t, 4H, CH_2, J= 5.0), 3.32 (q, 2H, CH_2, J=5.0), 7.51 (broad s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 14.0 (CH_3 CH_2-CH_3), 18.3 (CH_3, C21), 20.7 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 25.1 (CH_2), 26.4 (CH_2, C7), 27.2 (CH_2, C6), 28.2 (CH_2, C16), 28.4 (CH_2), 28.5 (CH_2), 30.4 (CH_2, C2), 30.5 (CH_2, C22), 33.8 (CH_2, C23), 34.5 (C, C10), 35.3 (CH, C20), 36.4 (CH_2, C1), 39.4 (CH_2, C4), 40.1 (CH_2, C12), 40.4 (CH, C9), 42.0 (CH, C5), 42.1 (C, C13), 42.7 (CH_2), 56.1 (CH, C17), 56.4 (CH, C14), 71.6 (CH, C3), 173.6 (CO, C24) ppm.

IR= 3296 (OH), 2916 (alkyl), 2861 (alkyl), 1650 (C=O), 1548, 1441, 1377, 1070, 1066, 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 545.5032 $(M+H)^{+}$; calculated for $C_{35}H_{65}N_2O_2$ 545.5041; 1.6 ppm.

Preparation of (4R)-N-[3-(dibutylamino)propyl]-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide (compound 97)



Deoxycholic acid (0.5 g 0.001 mol) was dissolved in tetrahydrofuran (15 mL) with triethylamine (0.20 mL 0.001 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.2 mL 0.001 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours 3-(dibutylamino)-1-propylamine (0.25 mL 0.001 mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the solution extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). This crude material was further purified using column chromatography (8:2 ethyl acetate/methanol). The compound containing fractions were dried over magnesium sulfate. Solvent was evaporated under reduced pressure to produce viscous oil.

Yield; 0.03g, 0.00005 mol, 4 %.

Melting point: Oil.

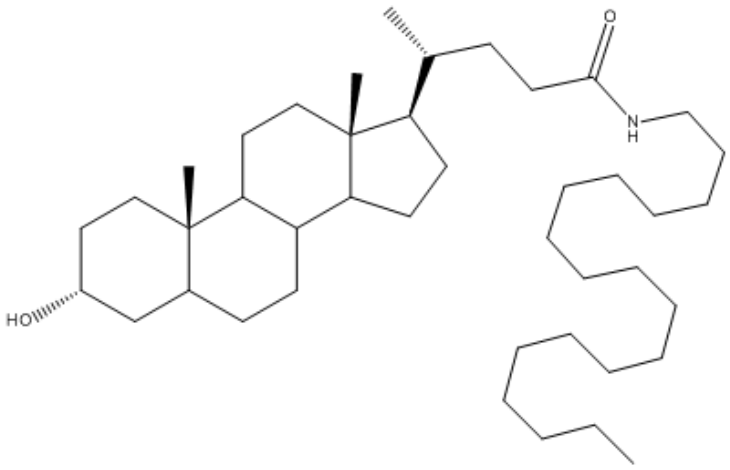
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.67 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 0.91 (m, 6H, CH_3), 1.30-2.22 (m, 33H, steroidal backbone CH/CH_2), 1.48 (m, 8H, CH_2), 2.50 (t, 4H, CH_2, J= 5.0), 3.32 (q, 2H, CH_2, J=5.0), 3.58 (m, 1H, CH), 4.08 (s, 1H, CH), 7.51 (broad s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.7 (CH_3, C18), 14.0 (CH_3), 17.5 (CH_3, C21), 20.6 (CH_2), 23.1 (CH_3, C19), 23.7 (CH_2, C15), 25.0 (CH_2), 26.1 (CH_2), 27.1 (CH_2, C7), 27.5 (CH_2), 28.5 (CH_2, C6), 28.8 (CH_2, C16), 30.4 (CH_2, C11), 31.6 (CH_2, C2), 33.3 (CH_2, C23), 34.1 (CH, C9), 35.3 (C, C10), 36.0 (CH_2, C1), 36.4 (CH, C20), 43.1 (CH, C5), 46.5 (C, C13), 46.9 (CH, C17), 48.2 (CH, C14), 53.2 (CH_2), 53.8 (CH_2), 71.7 (CH, C3), 73.1 (CH_2, C12), 173.7 (CO, C24) ppm.

IR= 3309 (OH), 2925 (alkyl), 2857 (alkyl), 1701 (C=O), 1650, 1445, 1249 (C=O ester stretch), 1044 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 561.4982 $(M+H)^+$; calculated for $C_{35}H_{65}N_2O_3$ 561.4990; 1.4 ppm.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-octadecyl-pentanamide (compound 98)



Lithocholic acid (2.0 g 0.005 mol) was dissolved in tetrahydrofuran (60 mL) with 4-methylmorpholine (2.14 mL, 0.02 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.51 mL, 0.005 mol) was then added dropwise over a ten minute period. Once added, the cold water was

removed and the solution was stirred for 2 hours. After 2 hours octadecylamine (1.43 mL, 0.005 mol) was added and the solution was stirred for a further 24 hours. Water (50 mL) was then added and the solution was extracted with ethyl acetate (3 x 50 mL). The crude product was purified by column chromatography (100 % ethyl acetate). The product containing fractions were dried over magnesium sulfate. The solvent was evaporated under reduced pressure to produce a white powder.

Yield; 0.8 g, 0.001 mol, 23.9 %.

Melting point: 94.8-95.4 ^{o}C.

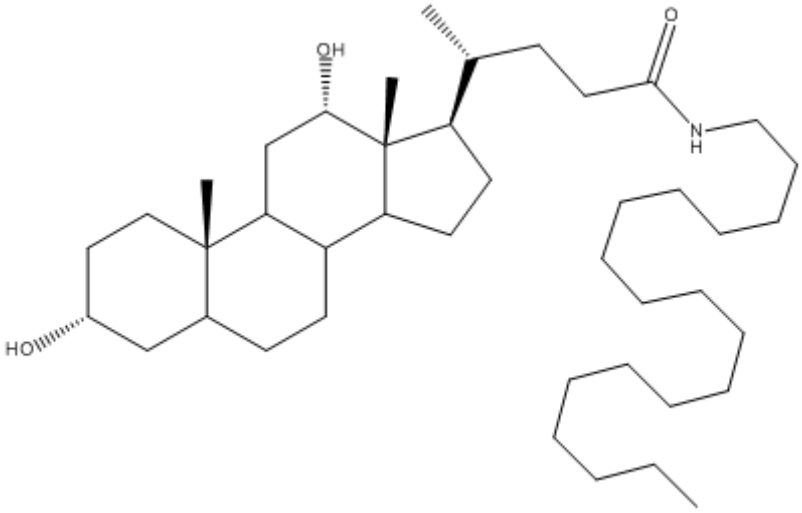
^{1}H NMR (250 MHz) $CDCl_3$ δ= 0.64 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 1.25 (s, 30H, aliphatic CH_2), 3.23 (q, 3H, chain terminal CH_3, J=5.0), 3.64 (m, 1H, 3-CH), 5.36 (broad s, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 20.8 (CH_2, C11), 22.7 (CH_3, C19), 23.3 (CH_2, C15), 24.2 (CH_2), 26.4 (CH_2, C7), 26.9 (CH_2, C6), 27.2 (CH_2, C16), 28.2 (CH_2, aliphatic side chain), 29.3 (CH_2, aliphatic side chain), 29.5 (CH_2, aliphatic side chain), 29.7 (CH_2, aliphatic side chain), 30.5 (CH_2, C2), 31.8 (CH_2, C22), 31.9 (CH_2, C23), 33.7 (C, C10), 35.3 (CH, C20), 35.4 (CH_2, C1), 35.8 (CH, C8), 39.5 (CH_2, C4), 40.2 (CH_2, C12), 40.4 (CH, C9), 42.1 (C, C5), 56.0 (CH, C17), 56.5 (CH, C14), 71.9 (CH, C3), 185.3 (CO, C24) ppm.

IR= 3411 (NH), 3325 (OH), 2912 (alkyl), 2849 (alkyl), 1653 (C=O), 1544, 1464, 1367, 1308, 1040 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 628.6205 $(M+H)^{+}$; calculated for $C_{42}H_{78}NO_2$ 628.6207; 0.6 ppm.

Preparation of (4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-octadecyl-pentanamide (compound 99)



Deoxycholic acid (2.0 g 0.005 mol) was dissolved in 1, 4 dioxane (60 mL) with triethylamine (1.32 mL, 0.01 mol). The solution was cooled for 10 minutes in cold water. Ethyl chloroformate (0.40 mL, 0.003 mol) was then added dropwise over a ten minute period. Once added, the cold water was removed and the solution was stirred for 2 hours. After 2 hours octadecylamine (1.37 g, 0.005 mol) was added and the solution was stirred for a further 48 hours. Water (50 mL) was then added and the solution was extracted with ethyl acetate (3 x 50 mL). The organic layers were combined and washed with saturated sodium hydrogen carbonate solution (3 x 50 mL). The organic layer was dried over magnesium sulfate. The solvent was evaporated under reduced pressure and the product was recrystallized from ethyl acetate to produce a transparent glass like solid.

Yield; 2.32 g, 0.003 mol, 70 %.

Melting point: 54.2 – 57.3 ^{o}C.

^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.68 (s, 3H, 18-CH_3), 0.91 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 1.25 (s, 32H, aliphatic CH_2), 3.22 (q, 2H, CH_2, J= 7.5), 3.61 (m, 1H, 3-CH), 3.98 (1H, 12-CH), 5.53 (broad s, 1H, NH) ppm.

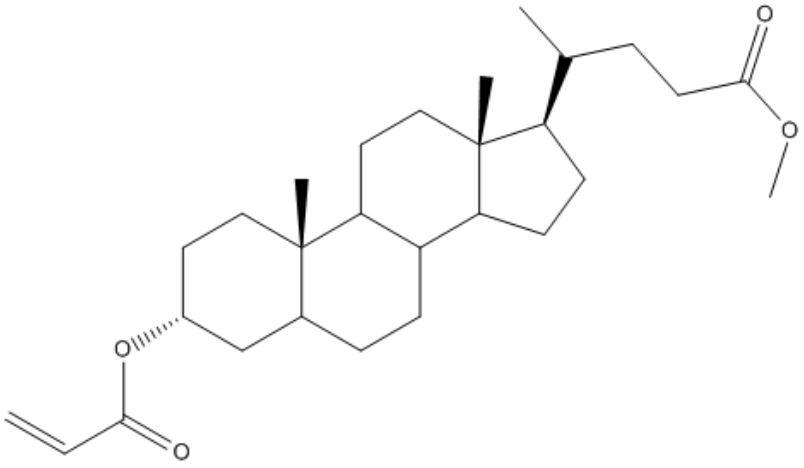
^{13}C NMR (62.9 MHz) δ= 12.7 (CH_3, C18), 14.1 (CH_2), 17.4 (CH_3, C21), 22.6 (CH_2), 23.1 (CH_3, C19), 23.6 (CH_2, C15), 26.1 (CH), 26.9 (CH_2, C7), 28.6 (CH_2, C16), 29.3 (CH_2, C11), 29.5 (CH_2, C2), 29.7 (CH_2), 30.5 (CH), 31.7 (CH_2, C23), 31.9 (CH_2, C22), 33.5 (CH, C9), 33.6 (CH), 34.1 (CH), 35.2 (C, C10), 35.2 (CH), 36.0 (CH_2, C1), 36.4 (CH, C20), 39.5 (CH_2, C4), 42.1 (CH, C5), 46.5 (C, C13), 47.2 (CH, C17), 48.2 (CH, C14), 71.7 (CH, C3), 73.1 (CH_2, C12), 173.4 (CO, C24) ppm.

IR= 3292 (OH), 2912, 2852 (alkyl), 1637 (C=O), 1548, 1189, 1036 (RCHOH) cm^{-1}.

MS (+ESI) m/z= Found 644.5971 $(M+H)^+$; calculated for $C_{42}H_{78}NO_3$ 644.5976; 0.8 ppm.

2.3.2 Manipulations of 3-OH on bile acid derivatives.

Preparation of methyl 4-[(3R,10S,13R,17R)-10,13-dimethyl-3-prop-2-enoyloxy-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoate (compound 100)



Methyl lithocholate (0.25 g 0.0006 mol) was dissolved in chloroform (15 mL) with triethylamine (0.12 mL 0.001 mol). Acryloyl chloride (0.1 mL 0.0009 mol) was added and the solution was stirred for 24

hours. Water (50 mL) was then added and the resultant precipitate was collected by vacuum filtration, washed with water (3 x 20 mL) and dried under vacuum.

Yield; 0.16 g, 0.0003 mol, 57 %.

Melting point: 152.5 – 156.7 ^{o}C.

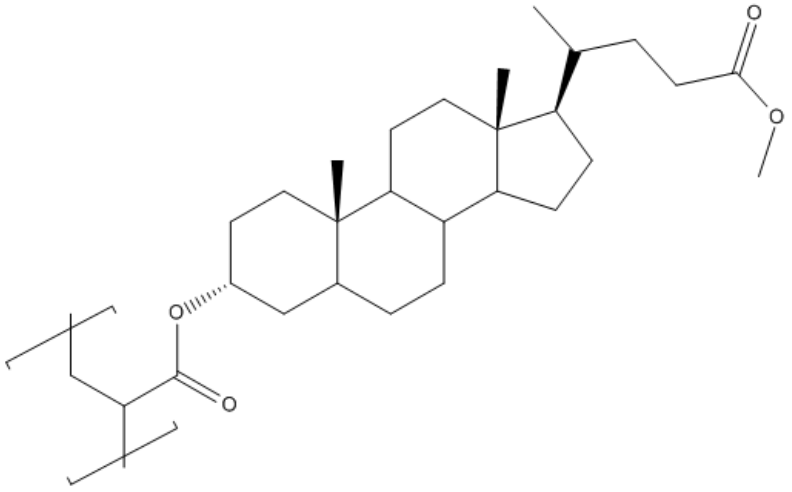
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.58 (s, 3H, 18-CH_3), 0.87 (s, 3H, 19-CH_3), 1.00-2.28 (m, 33H, steroidal backbone CH/CH_2), 3.59 (S, 3H, O-CH_3), 4.73 (m, 1H, 3-CH), 5.74 (dd, 1H, =CH- J = machine not sensitive to distinguish peaks), 6.01 (dd, 1H, =CH J= machine not sensitive enough to distinguish peaks), 6.28 (dd, 1H, =CH, J = 2.5) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 11.0 (CH_3, C18), 17.2 (CH_3, C21), 19.8 (CH_2, C11), 22.3 (CH_3, C19), 23.1 (CH_2, C15), 23.4 (CH_2, C7), 25.3 (CH_2, C6), 26.0 (CH_2, C16), 27.1 (CH_2, C2), 30.0 (CH_2, C22), 32.0 (CH_2, C23), 34.0 (C, C10), 34.3 (CH, C20), 39.1 (CH_2, C12), 40.8 (CH, C9), 42.0 (CH, C5), 54.9 (CH, C17), 55.4 (CH, C14), 73.5 (CH, C3), 128.0 (CH_2 =CH-), 129.1 (=CH_2), 164.7 (CO), 178.1 (CO, C24) ppm.

IR= 2925 (alkyl), 2857 (alkyl), 1735 (C=O), 1714 (C=O), 1428, 1394, 1206, 980, 805 (CH out of plane bending) cm^{-1}.

MS (ES) m/z= Found 462.3574 $(M+NH_4)^+$; calculated for $C_{28}H_{48}NO_4$ 462.3578; 0.8 ppm.

Preparation of poly[(3R,10S,13R,17R)-17-[(1R)-4-(benzylamino)-1-methyl-4-oxo-butyl]-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl] prop-2-enoate (compound 101)



Compound **100** (0.16 g, 0.0003 mol) was dissolved in ethanol (10 mL) with azobisisobutyronitrile (0.005mg). The mixture was heated at reflux for 48 hours. After 48 hours the material was added to water (30 mL) and the resulting precipitate was collected by vacuum filtration, washed with water (3 x 20 mL) and dried under vacuum to produce a white powder.

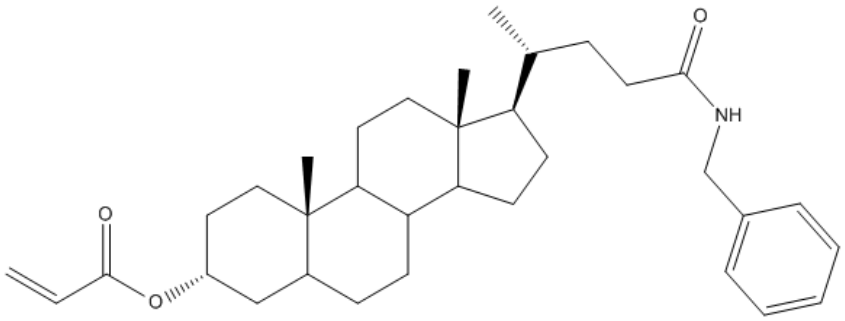
Yield; 0.027 g, 0.0000625 mol, 16.9 %.

^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.64 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 1.10-2.52 (m, 33H, steroidal backbone CH/CH_2), 3.66 (s, 3H, O-CH_3), 4.72 (m, 1H, 3-CH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.2 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.1 (CH_2, C15), 26.3 (CH_2, C7), 27.0 (CH_2, C6), 28.2 (CH_2, C16), 31.0 (CH_2, C22), 31.0 (CH_2, C23), 34.6 (C, C10), 35.3 (CH, C20), 35.8 (CH_2), 40.1 (CH_2, C12), 40.4 (CH, C9), 41.9 (CH, C5), 42.7 (C, C13), 56.0 (CH_2), 56.4 (CH), 56.0 (CH, C17), 56.4 (CH, C14), 174.7 (CO, C24) ppm.

IR= 3423 (NH), 3326 (OH), 2932 (alkyl), 2862 (alkyl), 1724 (C=O), 1448, 1166 (C=O ester stretch), 1020 (R_2CH-OH) cm^{-1}.

Preparation of [(3R,10S,13R,17R)-17-[(1R)-4-(benzylamino)-1-methyl-4-oxo-butyl]-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl] prop-2-enoate (compound 102)



Product from Compound **93** (0.25 g 0.0005 mol) was dissolved in chloroform (15 mL) with triethylamine (0.21 mL 0.002 mol). Acryloyl chloride (0.24 mL 0.002 mol) was added and the solution was stirred for 5 days. Water (50 mL) was then added and the resulting precipitate was collected by vacuum filtration, washed with water (3 x 20 mL) and dried under vacuum.

Yield: 0.023 g, 0.00004 mol, 6.8 %.

Melting point: 123.3 – 127.7 ^{o}C.

^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.64 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.10-2.38 (m, 33H, steroidal backbone CH/CH_2), 4.44 (d, 2H, CH_2 J= 5.0), 4.80 (m, 1H, 3-CH), 5.67 (broad s, 1H, NH), 5.795, (dd, 1H, =CH- J= 10 and 2.5), 6.095 (dd, 1H, =CH J= 10.0 and 17.5), 6.35 (dd, 1H, =CH J= 2.5 and 17.5), 7.29-7.31 (overlapping multiplets, 5H, Aromatics) ppm.

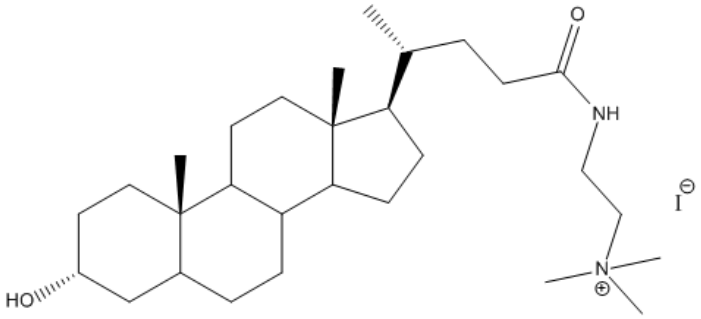
^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 12.0 (CH_3, C18), 18.4 (CH_3, C21), 20.8 (CH_2, C11), 23.3 (CH_3, C19), 24.2 (CH_2, C15), 26.3 (CH_2, C7), 26.6 (CH_2, C6), 27.0 (CH_2, C16), 28.2 (CH_2, C2), 31.8 (CH_2, C22), 32.2 (CH_2, C23), 33.6 (C, C10), 34.6 (CH, C20), 34.6 (CH_2, C1), 35.0 (CH, C8), 35.5 (CH_2, C4), 35.8 (CH_2, C12), 40.1 (CH, C9), 40.4 (CH, C5), 41.9 (C, C13), 42.7 (CH), 43.6 (CH =CH), 56.0 (CH, C17), 56.5 (CH, C14), 74.5 (CH, C3), 127.5 (Ar-CH), 127.8 (Ar-CH), 128.7 (Ar-CH), 129.1 (=CH-), 130.1 (=CH_2), 138.4, (Ar-C), 165.7 (CO), 173.2 (CO, C24) ppm.

IR= 3302 (OH), 2956 (alkyl), 2932 (alkyl), 2862 (alkyl), 1712 (C=O), 1642 (C=O), 1278, 1017 (R_2CH-OH) cm^{-1}.

MS (ES) m/z= Found 520.3782 $(M+NH_4)^+$; calculated for $C_{34}H_{50}NO_3$ 520.3785; 0.6 ppm.

2.3.3 Quaternization of tertiary amines in bile amide derivatives.

Preparation of 2-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]ethyl-trimethyl-ammonium iodide (compound 103)



Compound **62** (0.2 g, 0.0004 mol) was dissolved in chloroform (10 mL) with Iodomethane (0.31 mL 0.002 mol). The solution was left overnight at which point a precipitate had formed which was collected by vacuum filtration, washed with water (3 x 20 mL) and dried under vacuum to produce a white powder. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.212 g, 0.0003 mol, 81.5 %.

Melting point: 212.8 – 216.1 ^oC.

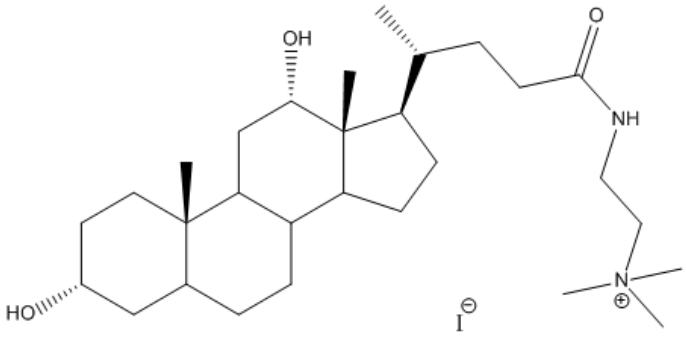
^1H NMR (MeOD) (250 MHz) δ= 0.69, (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.00-2.35 (m, 33H, steroidal backbone CH/CH_2), 3.19, (s, 9H, 3 x CH_3), 3.45 (t, 2H, al-CH_2, J= 7.5), 3.55 (m, 1H, 3-CH), 3.64 (t, 2H, CH_2-NR_3 , J= 7.5) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 11.0 (CH_3, N-CH_3), 12.5 (CH_3, C18), 17.3 (CH_3, C21), 18.8 (CH_2), 21.9 (CH), 23.9 (CH_2), 27.6 (CH), 28.3 (CH), 31.2 (CH), 33.0 (CH), 33.9 (CH), 36.9 (CH_2), 37.2 (CH_2), 41.9 (CH), 43.5 (C, C13), 57.4 (CH, C17), 57.9 (CH, C14), 65.8 (CH_2), 72.4 (CH), 181.2 (CO) ppm.

IR= 3368 (NH), 3245 (OH), 2938 (alkyl), 2852 (alkyl) 1639 (C=O), 1560, 1441, 1258, 1040 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 461.4105 $(M-I)^+$; calculated for $C_{29}H_{53}N_2O_2$ 461.4102; 0.7 ppm.

Preparation of 2-[[(4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]ethyl-trimethyl-ammonium iodide (compound 104)



Compound **63** (0.15 g 0.0003 mol) was dissolved in chloroform (5 mL). Iodomethane (0.22 mL, 0.001 mol) was added and the solution was stirred overnight at ambient temperature. The resultant precipitate was collected *via* vacuum filtration and dried under vacuum overnight. Product was an off-white solid. TLC 100% MeOH, R_f 0.1 (single spot).

Yield 0.09g, 0.0001 mol, 78.9 %.

Melting point: 160.1 – 169.1 ^{o}C.

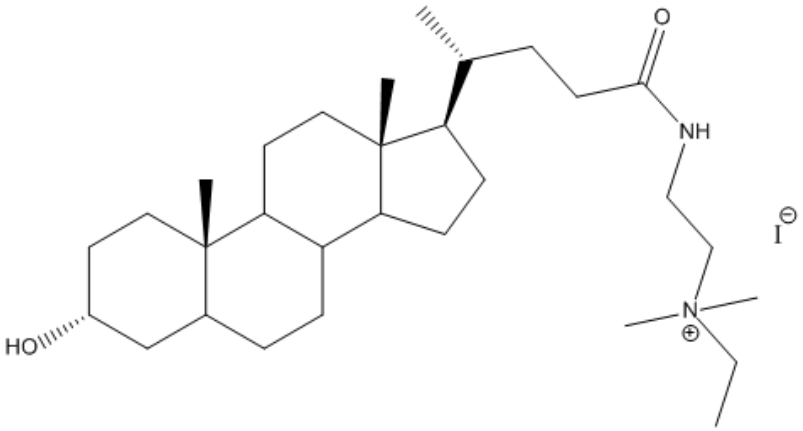
^{1}H NMR (MeOD) (250 MHz) δ= 0.70 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 1.02 (d, 3H, 21-CH_3, J= 5.0), 1.04-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.20 (s, 9H, 3 x CH_3), 3.47 (t, 2H, CH_2, J= 7.5), 3.64 (t, 2H, CH_2, J= 5.0), 3.95 (s, 1H, 12-CH) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 13.2 (CH_3, C18), 17.6 (CH_3, C21), 23.7 (CH_3, C19), 24.8 (CH_2, C15), 27.4 (CH_2, C7), 28.4 (CH_2, C6), 28.7 (CH_2, C16), 29.9 (CH_2, C11), 31.0 (CH_2, C2), 33.0 (CH_2, C23), 33.8 (CH_2), 34.6 (CH_2), 36.4 (CH_2, C1), 36.8 (CH, C20), 37.2 (CH_2, C4), 37.4 (CH, C8), 43.6 (CH, C5), 54.0 (CH), 54.0 (CH), 54.1 (CH), 72.5 (CH, C3), 74.0 (CH_2, C12), 177.3 (CO, C24) ppm.

IR= 3377 (NH), 3249 (OH), 2921 (alkyl), 2861 (alkyl), 1641 (C=O), 1573, 1454, 1373, 1249, 1031 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 477.4060 $(M-I)^{+}$; calculated for $C_{29}H_{53}N_2O_3$ 477.4051; 1.9 ppm.

Preparation of ethyl-[3-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl]-dimethyl-ammonium iodide (compound 105)



Compound **62** (0.2 g 0.0004 mol) was dissolved in chloroform (10 mL). Ethyl Iodide (0.34 mL, 0.001 mol) was added and the solution was stirred overnight at ambient temperature. The resulting precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH, R_f 0.1 (single spot).

Yield; 0.2 g, 0.0003 mol, 76 %.

Melting point: 120.0-123.0 ^{o}C.

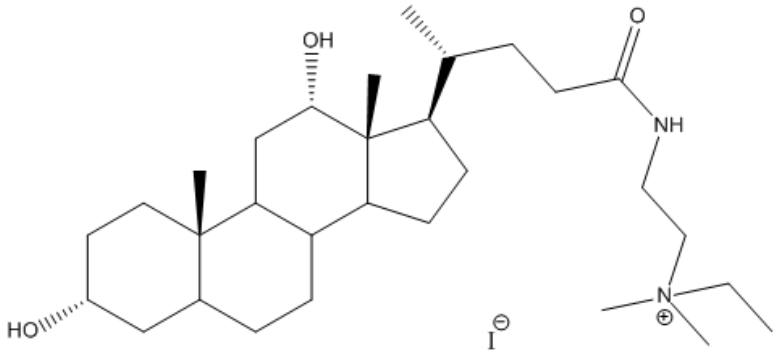
^{1}H NMR (MeOD) (250 MHz) δ= 0.69 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.13 (s, 6H, 2 x CH_3), 3.43 (m, 4H, 3-CH/CH_2), 3.60 (q, 2H, CH_2, J= 7.5) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 8.4 (CH), 12.5 (CH_3, C18), 18.8 (CH_3, C21), 21.4 (CH_2, C11), 21.9 (CH_3, C19), 23.9 (CH_2, C15), 25.2 (CH_2, C7), 28.3 (CH_2, C16), 29.3 (CH_2, C2), 31.2 (CH_2, C23), 33.0 (CH_2), 33.9 (CH_2), 34.2 (C, C10), 35.6 (CH, C20), 36.4 (CH_2, C1), 37.1 (CH, C8), 37.2 (CH_2, C4), 41.5 (CH, C9), 41.9 (CH, C5), 43.5 (C, C13), 43.9 (CH_2), 51.1 (CH_2), 57.3 (CH, C17), 57.9 (CH, C14), 62.4 (CH, C3), 177.3 (CO, C24) ppm.

IR= 3373 (OH), 2942 (alkyl), 2844 (alkyl), 1646 (C=O), 1441, 1270, 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 475.4247 $(M-I)^{+}$; calculated for $C_{30}H_{55}N_2O_2$ 475.4258; 2.3 ppm.

Preparation of 3-[[(4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl-ethyl-dimethyl-ammonium iodide (compound 106)



Compound **63** (0.2 g 0.0004 mol) was dissolved in chloroform (5 mL). Ethyl Iodide (0.62 mL, 0.003 mol) was added and the solution was stirred overnight at ambient temperature. The resulting

precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH, R_f 0.1 (single spot).

Yield; 0.13 g, 0.0002 mol, 52 %.

Melting point: 115.3 – 120.3 ^{o}C.

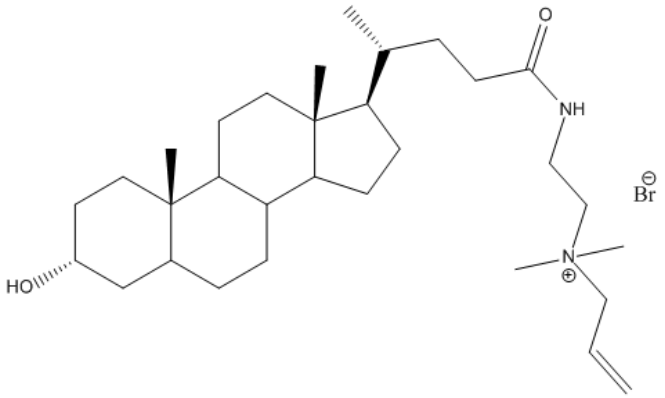
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.70 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 1.02 (d, 2H, CH_2), 1.03-2.38 (m, 33H, steroidal backbone CH/CH_2), 2.21 (m, 6H, 3-CH/CH_2), 3.4 (broad s, 2H, CH_2), 3.45-3.61 (multiple overlapping multiplets, 8H, CH_2) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 8.4 (CH_2), 12.4 (CH_3, C18), 16.9 (CH_3, C21), 21.9 (CH_2), 22.9 (CH_3, C19), 24.0 (CH_2, C15), 26.6 (CH_2, C7), 27.9 (CH_2, C16), 29.1 (CH_2, C11), 30.3 (CH_2, C2), 32.2 (CH_2, C23), 33.1 (CH_2, C22), 34.0 (CH, C9), 34.5 (C, C10), 35.6 (CH_2, C1), 36.0 (CH, C20), 36.4 (CH_2, C4), 36.6 (CH, C8), 42.8 (CH, C5), 55.6 (CH), 57.1 (CH), 63.2 (CH), 71.7 (CH, C3), 73.2 (CH_2, C12), 176.6 (CO, C24) ppm.

IR= 3368 (NH), 3253 (OH), 2921 (alkyl), 2852 (alkyl), 1650 (C=O), 1522, 1445, 1364, 1253, 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 517.4354 $(M-I)^+$; calculated for $C_{32}H_{57}N_2O_3$ 517.4364; 1.9 ppm.

Preparation of allyl-[2-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]ethyl]-dimethyl-ammonium bromide (compound 107)



Compound **62** (0.2 g, 0.0004 mol) was dissolved in a solution of chloroform (10 mL). Allyl bromide (0.27 mL, 0.002 mol) was added and the solution was stirred overnight at which point a precipitate was formed. The precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH, R_f 0.1 (single spot).

Yield; 0.13 g, 0.0002 mol, 54 %.

Melting point: 198.7 – 203.8 ^{o}C.

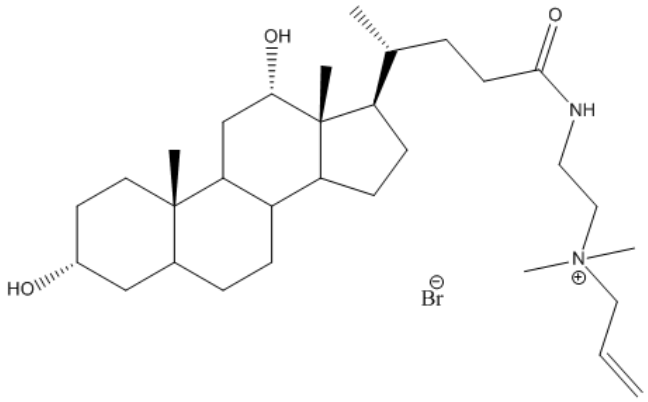
^{1}H NMR (MeOD) (250 MHz) δ= 0.68 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.04-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.12 (s, 6H, 2 x CH_3), 3.39 (t, 2H, CH_2), 3.65 (t, 2H, CH_2), 5.70 (m, 2H, =CH_2), 6.10 (m, 1H, =CH-) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 12.5 (CH_3, C18), 18.8 (CH_3, C21), 21.9 (CH_2, C11), 23.9 (CH_3, C19), 25.2 (CH_2, C15), 27.6 (CH_2, C7), 28.3 (CH_2), 28.0 (CH_2, C16), 33.0 (CH_2), 34.2 (C, C10), 37.1 (CH_2), 41.5 (CH_2), 41.9 (CH_2), 43.8 (CH), 57.3 (CH_2), 57.9 (CH_2), 63.0 (CH_2), 72.4 (CH, C3), 126.0 (=CH_2), 129.8 (=CH-), 177.3 (CO, C24) ppm.

IR= 3266 (OH), 2929 (alkyl), 2848 (alkyl), 1646 (C=O), 1569, 1420, 1066, 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 487.4252 $(M\text{-}Br)^+$; calculated for $C_{31}H_{55}N_2O_2$ 487.4258; 1.2 ppm.

Preparation of allyl-[2-[[(4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]ethyl]-dimethyl-ammonium iodide (compound 108)



Compound **63** (0.15 g, 0.0003 mol) was dissolved in chloroform (5 mL). Allyl bromide (0.20 mL, 0.0016 mol) was added and the solution was stirred overnight at ambient temperature. The resulting precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH, R_f 0.1 (single spot).

Yield; 0.19 g, 0.0001 mol, 53 %.

Melting point: 184.2 – 186.7 ^oC.

^1H NMR (MeOD) (250 MHz) δ= 0.70 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 1.12-2.30 (m, 33H, steroidal backbone CH/CH_2), 3.13 (s, 6H, CH_3), 3.40 (t, 2H, CH_2, J= 7.5), 3.65 (t, 2H, CH_2, J= 5.0), 3.95 (s, 1H, 12-CH), 4.05 (d, 2H, CH_2, J= 5.0), 5.75 (m, 2H, CH_2), 6.10 (m, 1H, =CH-) ppm.

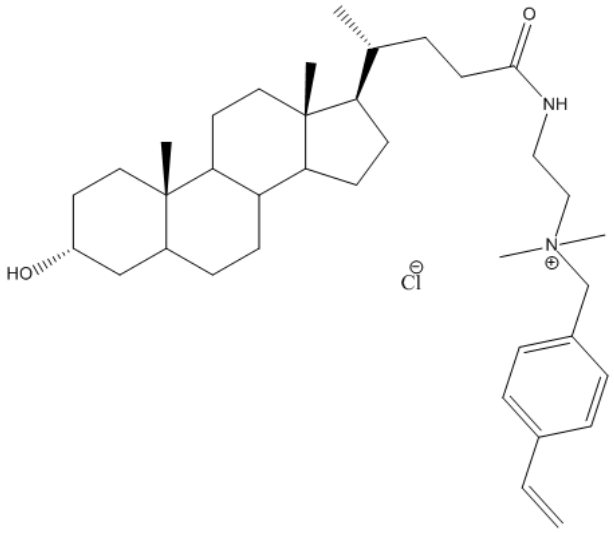
^{13}C NMR (MeOD) (62.9 MHz) δ= 13.2 (CH_3, C18), 17.6 (CH_3, C21), 23.7 (CH_3, C19), 24.8 (CH_2, C15), 27.4 (CH_2, C7), 28.4 (CH_2, C6), 28.7 (CH_2, C16), 29.9 (CH_2, C11), 31.1 (CH_2, C2), 33.0 (CH_2, C23), 33.8 (CH_2, C22), 34.8 (CH, C9), 36.9 (CH), 37.2 (CH), 37.4 (CH), 43.6 (CH, C5), 47.5 (CH,

C17), 48.0 (CH, C14), 63.0 (CH_2), 63.1 (CH_2), 67.9 (CH_2), 68.0 (CH_2) 72.5 (CH, C3), 74.0 (CH_2, C12), 126.1 (=CH_2), 129.8 (=CH-), 177.4 (CO, C24) ppm.

IR= 3415 (NH), 3245 (OH), 2925 (alkyl), 2852 (alkyl), 1646 (C=O), 1441, 1364, 1292 cm^{-1}.

MS (+ESI) m/z= Found 503.4202 $(M-Br)^+$; calculated for $C_{31}H_{55}N_2O_3$ 503.4207; 1.0 ppm.

Preparation of 2-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]ethyl-dimethyl-[(4-vinylphenyl)methyl]ammonium chloride (compound 109)



Compound **62** (0.2 g 0.0004 mol) was dissolved in chloroform (10 mL). Vinyl benzyl chloride (0.64 mL, 0.004 mol) was added and the solution was stirred overnight at ambient temperature. The resulting precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH, R_f 0.1 (single spot).

Yield; 0.07 g, 0.0016 mol, 26.9 %.

Melting point: 143.8 – 148.9 ^oC.

^1H NMR (MeOD) (250 MHz) δ= 0.66 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.10-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.09 (s, 6H, 2x CH_3), 3.41 (t, 2H, CH_2, J= 7.5), 3.73 (t, 2H, CH_2, J=

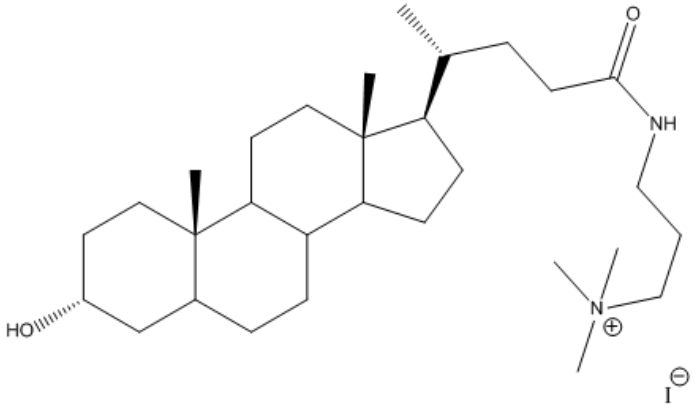
5.0), 4.56 (s, 2H, CH_2), 5.37 (d, 1H, =CH-, J = 12.5), 5.91 (d, 1H, =CH, J= 20.0), 6.80 (dd, 1H, =CH, J= 10.0 and 17.5), 7.57 (dd, 4H, Ar-CH, J= 5.0) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 11.7 (CH_3, C18), 18.0 (CH_3, C21), 21.1 (CH_2, C11), 23.1 (CH_3, C19), 24.4 (CH_2, C15), 26.8 (CH_2, C7), 27.5 (CH_2, C6), 28.4 (CH_2, C16), 30.3 (CH_2, C2), 33.0 (CH_2), 33.9 (CH_2), 35.7 (CH_2), 36.8 (CH), 37.2 (CH), 41.9 (CH), 43.5 (CH), 57.4 (CH, C17), 57.9 (CH, C14), 63.5 (CH_2), 71.6 (CH, C3), 115.8 (=CH_2), 126.9 (Ar-CH), 127.2 (Ar-CH), 133.6 (Ar-CH), 136.2 (Ar-C), 140.8 (=CH-), 176.5 (CO, C24) ppm.

IR= 3339 (NH), 3215 (OH), 2921 (alkyl), 2857 (alkyl), 1667 (C=O), 1646, 1445, 1356, 1070 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 563.4558 $(M-Cl)^+$; calculated for $C_{37}H_{59}N_2O_2$ 563.4571; 2.3 ppm.

Preparation 3-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl-trimethyl-ammonium iodide (compound 110).



Compound **64** (0.5 g 0.002 mol) was dissolved in solution of chloroform (15 mL) and methanol (4 mL). Iodomethane (3.63 mL, 0.02 mol) was added and the solution was stirred for four days at ambient temperature. To induce precipitation the solution was placed in an acetone/dry ice mixture (-78 ^oC). The resulting precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.42 g, 0.06 mol, 64.6 %.

Melting point: 253.8-255.4 ^{o}C.

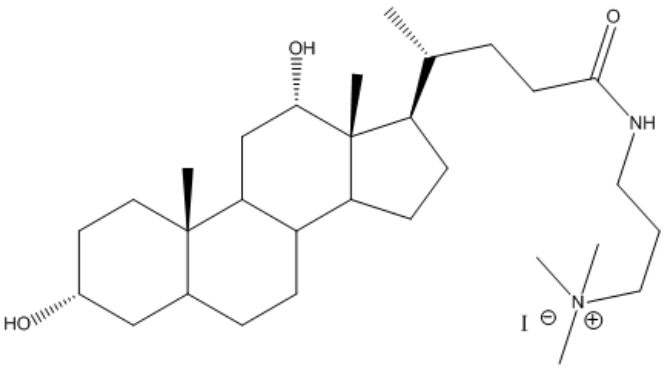
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.69 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.13 (s, 9H, 3 x CH_3), 3.54 (m, 1H, 3-CH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 10.9 (CH_3, C18), 17.3 (CH_3, C21), 20.4 (CH_2, C11), 22.4 (CH_2), 23.0 (CH_3, C19), 23.7 (CH_2, C15), 26.1 (CH_2, C7), 26.8 (CH_2, C6), 28.0 (CH_2, C16), 29.6 (CH_2, C2), 31.6 (CH_2, C22), 32.5 (CH_2, C23), 34.1 (C, C10), 34.9 (CH, C20), 35.4 (CH_2, C1), 35.6 (CH, C8), 35.7 (CH_2, C4), 40.0 (CH_2, C12), 40.4 (CH, C9), 42.0 (CH, C5), 42.4 (C, C13), 52.2 (CH, C17), 55.8 (CH, C14), 56.8 (CH), 64.2 (CH_2), 70.8 (CH, C3), 175.7 (CO, C24) ppm.

IR= 3386 (OH), 2921 (alkyl), 2852 (alkyl), 1641 (C=O), 1552, 1441, 1368, 1253 (C=O ester stretch), 1031 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 475.4256 $(M-I)^{+}$; calculated for $C_{30}H_{55}N_2O_2$ 475.4258; 0.4 ppm.

Preparation of 3-[[(4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl-trimethyl-ammonium iodide (compound 111)



Compound **65** (0.1 g 0.0002 mol) was dissolved in chloroform (5 mL). Iodomethane (0.29 mL, 0.002 mol) was added and the solution was stirred overnight at ambient temperature. The resulting

precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.09 g, 0.0014 mol, 75 %.

Melting point: 150.1 – 153.3 ^{o}C.

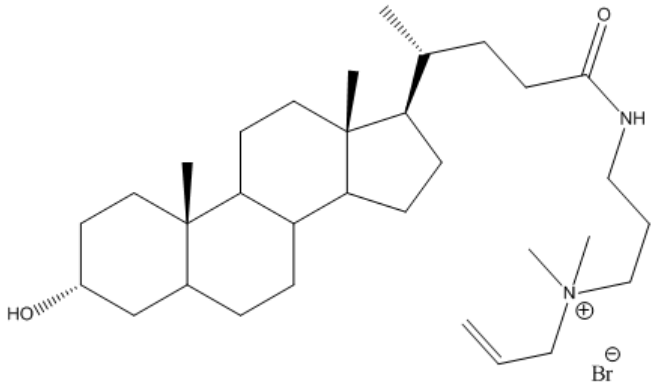
^{1}H NMR (MeOD) (250 MHz) δ= 0.71 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 1.03 (d, 3H, 21- CH_3, J= 7.5), 1.05-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.15 (s, 6H, 2 x CH_3), 3.53 (m, 1H, 3-CH), 3.95 (s, 1H, 12-CH) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 13.2 (CH_3, C18), 17.7 (CH_3, C21), 23.7 (CH_3, C19), 27.4 (CH_2, C7), 28.4 (CH_2, C6), 28.7 (CH_2, C16), 29.9 (CH_2, C11), 31.1 (CH_2, C2), 33.1 (CH_2, C23), 35.3 (C, C10), 37.2 (CH_2, C4), 43.6 (CH, C5), 72.5 (CH, C3), 74.0 (CH_2, C12), 177.3 (CO, C24) ppm.

IR= 3364 (OH), 2933 (alkyl), 2852 (alkyl), 1654 (C=O), 1548, 1437 cm^{-1}.

MS (ES) m/z= Found 491.4195 $(M-I)^{+}$; calculated for $C_{30}H_{55}N_2O_3$ 491.4207; 2.5 ppm.

Preparation of allyl-[3-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl]-dimethyl-ammonium bromide (compound 112)



Compound **64** (0.5 g 0.001 mol) was dissolved in dichloromethane (20 mL). Allyl bromide (1.88 mL, 0.01 mol) was added and the solution was stirred for a further 5 days. No precipitate had been formed so the vial was cooled to -78 ^{o}C and a precipitate formed and collected by vacuum filtration. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.45 g, 0.0007 mol, 71 %.

Melting point: 191.1 – 209.9 ^{o}C.

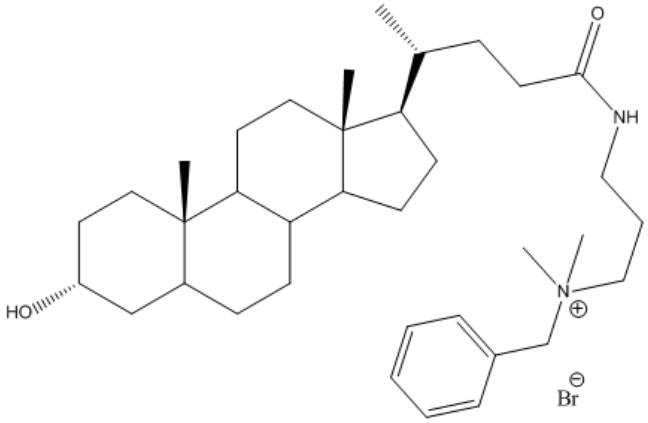
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.68 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.10 (s, 6H, 2 x CH_3), 3.27 (q, 2H, CH_2), 3.54 (m, 1H, 3-CH), 4.01 (d, 2H, CH_2, J= 7.5), 5.74 (t, 2H, CH_2, J= 10.0), 6.08 (m, 1H, =CH-) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 11.1 (CH_3, C18), 17.5 (CH_3, C21), 20.5 (CH_2, C11), 22.5 (CH_3, C19), 23.8 (CH_2, C15), 26.2 (CH_2, C7), 26.9 (CH_2, C6), 27.0 (CH_2, C16), 29.7 (CH_2, C2), 31.7 (CH_2, C22), 32.5 (CH_2, C23), 34.2 (C, C10), 35.0 (CH, C20), 35.4 (CH_2, C1), 35.7 (CH, C8), 35.9 (CH_2, C4), 40.0 (CH_2, C12), 40.4 (CH, C9), 42.0 (CH, C5), 42.4 (C, C13), 55.9 (CH, C17), 56.4 (CH, C14), 61.7 (CH_2), 66.1 (CH_2), 70.9 (CH, C3), 124.8 (=CH_2), 128.0 (=CH-), 175.8 (CO, C24) ppm.

IR= 3411 (NH), 3270 (OH), 2925 (alkyl), 2857 (alkyl), 1646 (C=O), 1543, 1437, 1373, 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 501.4402 $(M-Br)^+$; calculated for $C_{31}H_{55}N_2O_2$ 501.4415: 2.5 ppm.

Preparation of cyclopentylmethyl-[3-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl]-dimethyl-ammonium bromide (compound 113)



Compound **64** (0.5 g 0.001 mol) was dissolved in chloroform (20 mL) with benzyl bromide (1.37 mL, 0.008 mol) and the solution was stirred for 48 hours. The solvent was evaporated under reduced pressure, then re-dissolved in methanol and washed with petroleum ether 60/80 (3 x 20 mL). The solvent was removed under reduced pressure to produce a white powder. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.24 g, 0.0003 mol, 35 %.

Melting point: 145-147.9 ^oC.

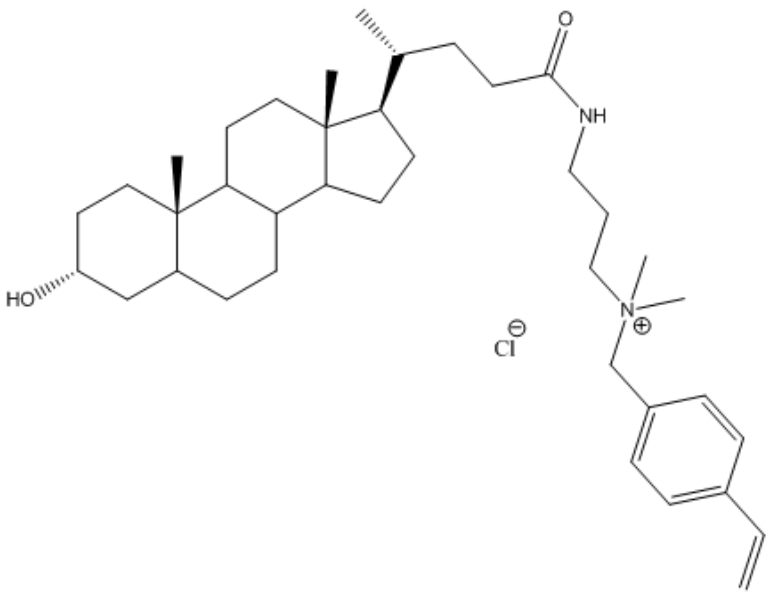
^1H NMR (MeOD) (250 MHz) δ= 0.68 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.05 (s, 6H, 2 x CH_3), 3.54 (m, 1H, 3-CH), 4.56 (s, 2H, CH_2), 7.55 (broad s, 5H, Ar-CH) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 10.9 (CH_3, C18), 17.3 (CH_3, C21), 20.4 (CH_2, C11), 22.4 (CH_3, C19), 22.6 (CH_2, C15), 23.7 (CH), 26.1 (CH_2, C7), 26.8 (CH_2, C6), 27.7 (CH_2, C16), 29.6 (CH_2, C2), 31.6 (CH_2, C22), 32.5 (CH_2, C23), 34.1 (C, C10), 34.9 (CH, C20), 35.3 (CH_2, C1), 35.6 (CH, C8), 35.6 (CH_2, C4), 39.9 (CH_2, C12), 40.3 (CH, C9), 41.9 (CH, C5), 42.3 (C, C13), 55.8 (CH, C17), 56.3 (CH, C14), 61.7 (CH), 67.4 (CH), 70.8 (CH, C3), 127.3 (Ar-CH), 128.8 (Ar-CH), 130.3 (Ar-CH), 132.6 (Ar-C), 175.6 (CO, C24) ppm.

IR= 3253 (OH), 2929 (alkyl), 2865 (alkyl), 1637 (C=O), 1565, 1441 cm^{-1}.

MS (+ESI) m/z= Found 551.4570 $(M-Br)^+$; calculated for $C_{36}H_{59}N_2O_2$ 551.4571; 0.2 ppm.

Preparation of 3-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl-dimethyl-[(4-vinylphenyl)methyl]ammonium chloride (compound 114)



Compound **64** (1.0 g 0.001 mol) was dissolved in dichloromethane (20 mL).Vinyl benzyl chloride (0.89 mL, 0.005) was added and the solution was stirred for 48 hours at ambient temperature. The resulting precipitate was collected by vacuum filtration and was washed with petroleum ether 60/80 (3

x 20 mL). The resultant crude product was dissolved in methanol and again washed with petroleum ether 60/80 (3 x 20 mL). Solvent removed under reduced pressure to produce off-white solid. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 1 g, 0.001 mol, 75 %.

Melting point: > 350 ^{o}C.

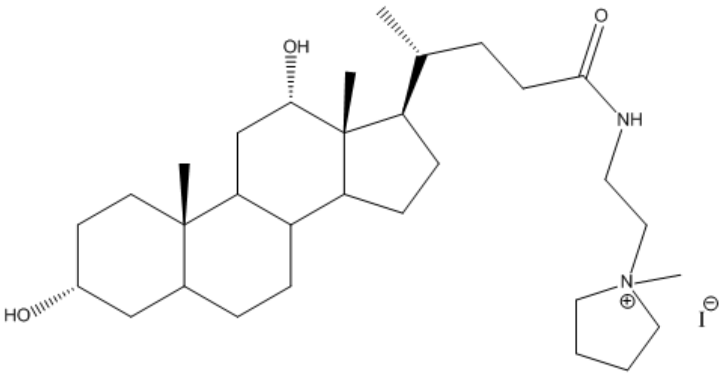
^{1}H NMR (MeOD) (250 MHz) δ= 0.67 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.05 (s, 6H, 2 x CH_3), 4.54 (s, 2H, CH_2), 5.37 (d, 1H, =CH-, J= 12.5), 5.91 (d, 1H, =CH, J=15.0), 6.80 (dd, 1H, =CH, J= 12.5 and 17.5), 7.56 (dd, 5H, Ar-CH, J= 7.5) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 11.9 (CH_3, C18), 18.2 (CH_3, C21), 20.6 (CH_2, C11), 23.3 (CH_3, C19), 24.0 (CH_2, C15), 26.3 (CH_2, C7), 27.1 (CH_2, C6), 28.0 (CH_2, C16), 30.3 (CH_2, C2), 30.9 (CH_2, C22), 30.9 (CH_2, C23), 34.2 (C, C10), 35.1 (CH, C20), 35.3 (CH_2, C1), 35.6 (CH, C8), 36.3 (CH_2, C4), 40.0 (CH_2, C12), 40.2 (CH, C9), 41.9 (CH, C5), 42.4 (C, C13), 55.8 (CH, C17), 56.3 (CH, C14), 70.5 (CH, C3), 178.1 (CO, C24) ppm.

IR= 3356 (OH), 2929 (alkyl), 2852 (alkyl), 1633 (C=O), 1548, 1441, 1031 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 577.4723 $(M-Cl)^{+}$; calculated for $C_{38}H_{61}N_2O_2$ 577.4728; 0.8 ppm.

Preparation of (4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-[2-(1-methylpyrrolidin-1-ium-1-yl)ethyl]pentanamide iodide (compound 115)



Compound **76** (0.1 g 0.0002 mol) was dissolved in chloroform (10 mL). Methyl iodide (0.09 mL 0.0006 mol) was added and the solution was stirred for 1 week. No precipitate formed so solvent was evaporated under reduced pressure. Material was re-dissolved in methanol and washed with petroleum ether 60/80 (3 x 20 mL). Green/brown oil produced. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.08 g, 0.0001mol, 66 %.

Melting point: Oil.

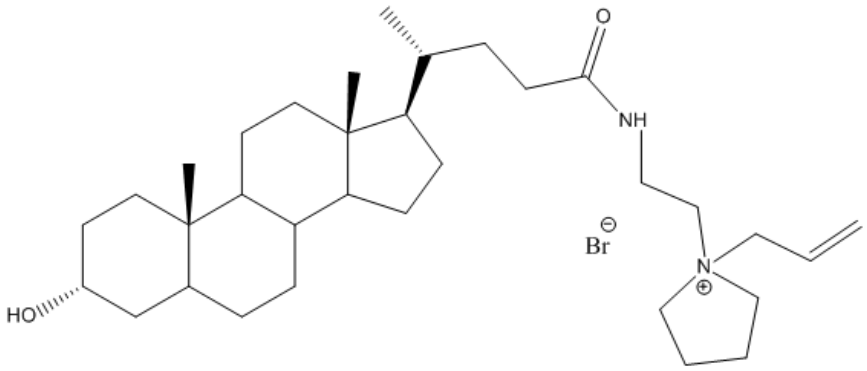
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.70 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 1.00 (d, 3H, 21-CH_3 J= 10.0), 1.20-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.15 (s, 3H, CH_3), 3.50-3.70 (multiple overlapping multiplets, 7H, CH/CH_2), 3.95 (s, 1H, 12-CH) ppm.

^{13}C NMR ($CDCl_3$) (250 MHz) δ= 13.2 (CH_3, C18), 17.7 (CH_3, C21), 22.56 (CH_2), 23.7 (CH_3, C19), 24.9 (CH_2, C15), 27.5 (CH_2, C7), 28.44 (CH_2, C6), 28.7 (CH_2, C16), 29.9 (CH_2, C11), 31.1 (CH_2, C2), 33.0 (CH_2, C23), 33.9 (CH_2, C22), 34.8 (CH, C9), 35.3 (C, C10), 36.4 (CH_2, C1), 36.9 (CH, C20), 37.2 (CH_2, C4), 37.4 (CH, C8), 43.6 (CH, C5), 63.5 (CH_2), 66.1 (CH_2), 72.5 (CH, C3), 74.0 (CH_2, C12), 176.5 (CO, C24) ppm.

IR= 3394 (OH), 2916 (alkyl), 2857 (alkyl), 1646 (C=O), 1530, 1445, 1368, 1253, 1036 (RCH-OH) cm^{-1}.

MS (+ESI) m/z= Found 503.4201 $(M-I)^+$; calculated for $C_{31}H_{55}N_2O_3$ 503.4207; 1.2 ppm.

Preparation of (4R)-N-[2-(1-allylpyrrolidin-1-ium-1-yl)ethyl]-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide bromide (compound 116)



Compound **75** (0.5 g 0.001 mol) was dissolved in dichloromethane (15 mL). Ally bromide (2.79 mL, 0.02) was added and the solution was stirred for 48 hours at 50 ^oC. The resulting precipitate was collected by vacuum filtration and was triturated chloroform (3 x 20 mL). TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.168 g, 0.0002 mol, 27 %.

Melting point: 211.6- 214.8 ^oC.

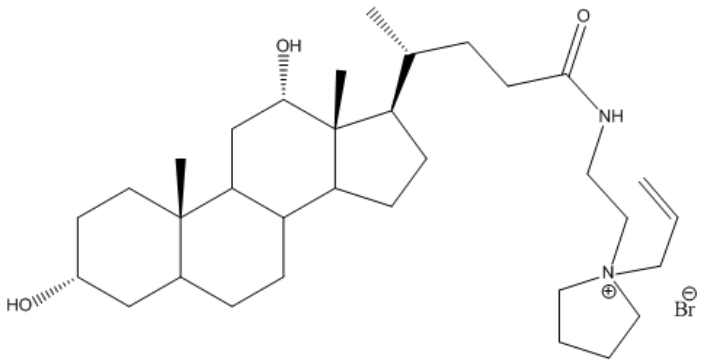
^1H NMR (MeOD) (250 MHz) δ= 0.68 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.62 (overlapping mutliplets, 4H, CH_2), 4.02 (d, 2H, CH_2, J= 7.5), 5.70-5.79 (multiple overlapping multiplets, 2H, =CH_2), 6.11 (m, 1H, =CH-) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 11.0 (CH_3, C18), 17.3 (CH_3, C21), 20.4 (CH_2, C11), 22.3 (CH_3, C19), 22.4 (CH), 23.7 (CH_2, C15), 26.1 (CH_2, C7), 26.8 (CH_2, C6), 27.7 (CH_2, C16), 29.6 (CH_2, C2), 31.4 (CH_2, C22), 32.3 (CH_2, C23), 33.2 (C, C10), 34.1 (CH, C20), 34.9 (CH_2, C1), 35.3 (CH, C8), 35.6 (CH_2, C4), 40.0 (CH_2, C12), 40.3 (CH, C9), 42.0 (CH, C5), 42.4 (C, C13), 55.6 (CH, C17), 56.4 (CH, C14), 57.8 (CH_2), 61.3 (CH_2), 62.1 (CH_2), 70.8 (CH, C3), 125.2 (=CH_2), 127.3 (=CH-), 175.8 (CO, C24) ppm.

IR= 3411 (NH), 3198 (OH), 2925 (alkyl), 2852 (alkyl), 1637 (C=O), 1560, 1441, 1368, 1066 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 513.4405 $(M\text{-}Br)^+$; calculated for $C_{33}H_{57}N_2O_2$ 513.4415; 1.9 ppm.

Preparation of (4R)-N-[2-(1-allylpyrrolidin-1-ium-1-yl)ethyl]-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide bromide (compound 117)



Compound **76** (0.1 g 0.0002 mol) was dissolved in chloroform (10 mL) with allyl bromide (0.08 mL, 0.0006 mol). The solution was stirred for 1 week. The solvent was evaporated under reduced pressure and the product was re-dissolved in methanol and washed with petroleum ether (3 x 10 mL). The solvent was then removed under reduced pressure to produce a white solid. TLC 100% MeOH, R_f 0.1 (single spot).

Yield; 0.1 g, 0.0001 mol, 83 %.

Melting point:176.2 – 179.8 ^{o}C.

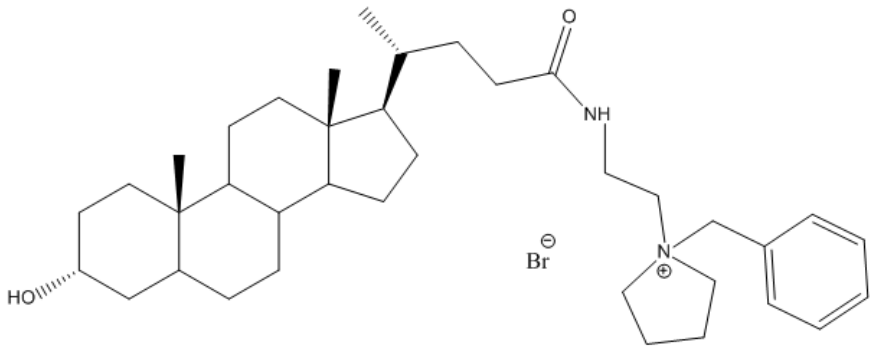
^{1}H NMR (MeOD) (250 MHz) δ= 0.70 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 1.02 (d, 3H, 21-CH_3, J= 5.0), 1.04-2.38 (m, 33H, steroidal backbone CH/CH_2), 2.22 (broad s, 4H, CH_2), 3.515 (q, 2H, CH_2), 3.65 (m, 3H, CH_2/3-CH), 4.01 (broad s, 1H, 12-CH), 4.025 (d, 2H, CH_2 J= 7.5), 5.74 (m, 2H, =CH_2), 6.11(m, 1H, =CH-) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 12.4 (CH_3, C18), 16.8 (CH_3, C21), 21.8 (CH_2), 22.9 (CH_3, C19), 24.0 (CH_2, C15), 26.6 (CH_2), 27.6 (CH_2, C7), 27.9 (CH_2, C6), 28.1 (CH_2, C16), 30.9 (CH_2, C2), 32.2 (CH_2, C23), 33.0 (CH_2, C22), 34.0 (CH, C9), 36.0 (CH_2), 36.6 (CH, C20), 42.7 (CH, C5), 44.8 (C, C13), 47.1 (CH, C17), 58.4 (CH_2), 61.9 (CH_2), 62.8 (CH_2), 71.7 (CH, C3), 73.1 (CH_2, C12), 126.7 (CH_2), 128.9 (CH_2), 177.4 (CO, C24) ppm.

IR= 3305 (OH), 2921 (alkyl), 2857 (alkyl), 1641 (C=O), 1539, 1449, 1040 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 529.4349 $(M-Br)^{+}$; calculated for $C_{33}H_{57}N_2O_3$, 529.4364; 2.8 ppm.

Preparation of (4R)-N-[2-(1-benzylpyrrolidin-1-ium-1-yl)ethyl]-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanamide bromide (compound 118)



Compound **76** (0.5 g 0.001 mol) was dissolved in dichloromethane (20 mL) with benzyl bromide (1.42 mL 0.008 mol) was added and the solution was stirred for 24 hours. The resultant precipitate was collected by vacuum filtration to produce a white powder. TLC 100% MeOH, R_f 0.1 (single spot).

Yield; 0.01 g, 0.0001 mol, 1.5 %.

Melting point: 216.2-220.8 ^{o}C.

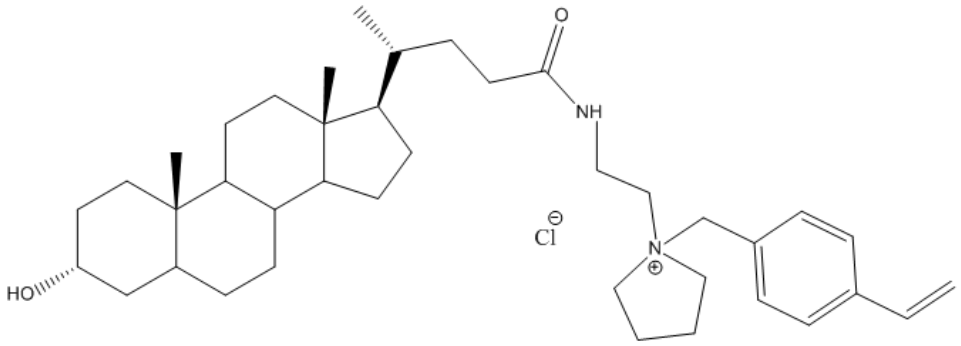
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.66 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.54-3.76 (multiple overlapping multiplets, 7H, 3-CH/CH_2), 4.57 (s, 2H, CH_2), 7.56 (m, 5H, Ar-CH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 10.9 (CH_3, C18), 17.3 (CH_3, C21), 20.4 (CH_2), 20.7 (CH_2, C11), 22.4 (CH_3, C19), 23.7 (CH_2, C15), 27.7 (CH_2, C16), 29.6 (CH_2, C2), 31.5 (CH_2, C22), 32.3 (CH_2, C23), 34.1 (C, C10), 34.9 (CH, C20), 35.3 (CH_2, C1), 35.6 (CH, C8), 35.7 (CH_2, C4), 40.0 (CH_2, C12), 40.3 (CH, C9), 41.9 (CH, C5), 42.4 (C, C13), 55.8 (CH, C17), 56.4 (CH, C14), 57.0 (CH), 61.2 (CH), 70.8 (CH, C3), 127.7 (Ar-CH), 129.0 (Ar-CH), 130.4 (Ar-CH), 132.2 (Ar-C), 175.8 (CO, C24) ppm.

IR= 3343 (NH), 3241 (OH), 2929 (alkyl), 2844 (alkyl), 1667 (C=O), 1535, 1445, 1360, 1070 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 563.4563 $(M-Br)^{+}$; calculated for $C_{37}H_{59}N_2O_2$ 563.4571; 1.4 ppm.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-N-[2-[1-[(4-vinylphenyl)methyl]pyrrolidin-1-ium-1-yl]ethyl]pentanamide chloride (compound 119)



Compound **75** (0.5 g 0.001 mol) was dissolved in dichloromethane (20 mL) with vinyl benzyl chloride (1.74 mL 0.01 mol) was added and the solution was stirred for 48 hours. No precipitate formed so solution was heated at 50 ^{o}C overnight. No precipitate formed so solvent was evaporated under reduced pressure. Material re-dissolved in methanol and washed with petroleum ether 60/80 (3 x 20 mL). White solid produced. TLC 100% MeOH R_f 0.1 (single spot).

Yield: 0.08 g, 0.0001 mol, 12 %.

Melting point: >350 ^{o}C.

^{1}H NMR ($CDCl_3$) (250 MHz) δ= 0.65 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.00-2.24 (m, 33H, steroidal backbone CH/CH_2), 3.56 (multiple overlapping multiplets, 4H, CH_2), 4.57 (s, 2H, CH_2), 5.37 (d, 1H, =CH-, J= 12.5), 5.90 (d, 1H, =CH, J= 17.5), 6.80 (dd, 1H, =CH, J= 12.5 and 17.5), 7.60 (s, 4H, Ar-CH) ppm.

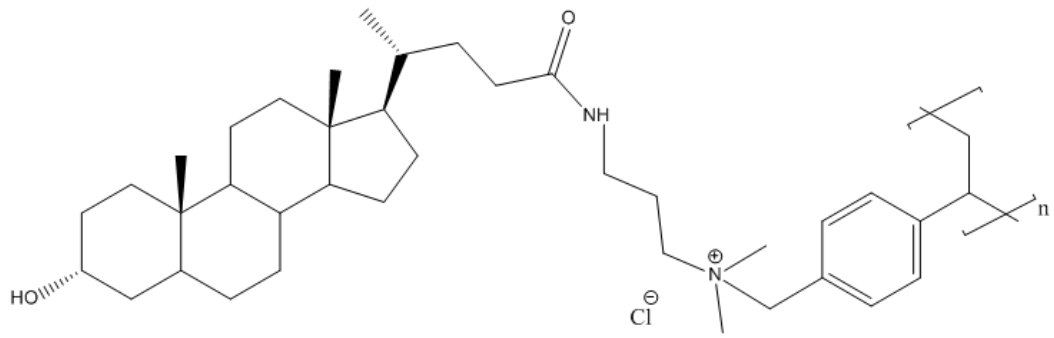
^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 10.9 (CH_3, C18), 17.3 (CH_3, C21), 20.4 (CH_2, C11), 20.7 (CH_2), 22.4 (CH_3, C19), 23.7 (CH_2, C15), 26.1 (CH_2, C7), 27.7 (CH_2, C16), 29.6 (CH_2, C2), 31.5 (CH_2, C22), 32.3 (CH_2, C23), 33.2 (C, C10), 34.1 (CH, C20), 34.9 (CH_2, C1), 35.3 (CH, C8), 35.7 (CH_2, C4), 40.0 (CH_2, C12), 40.3 (CH, C9), 41.9 (CH, C5), 42.4 (C, C13), 55.8 (CH, C17), 56.4 (CH, C14), 57.0

(CH_2), 61.2 (CH), 61.8 (CH_2), 70.8 (CH, C3), 115.0 (Ar-C), 126.6 (=CH), 132.5 (=CH-), 135.4 (Ar-CH), 139.9 (Ar-CH), 175.8 (CO, C24) ppm.

IR= 3360 (OH), 2925 (alkyl), 2852 (alkyl), 1641 (C=O), 1539, 1437, 1368, 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 589.4719 $(M-Cl)^+$; calculated for $C_{39}H_{61}N_2O_2$ 589.4728; 1.5 ppm.

Preparation of poly 3-[[(4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl-dimethyl-[(4-sec-butylphenyl)methyl]ammonium chloride (compound 120)



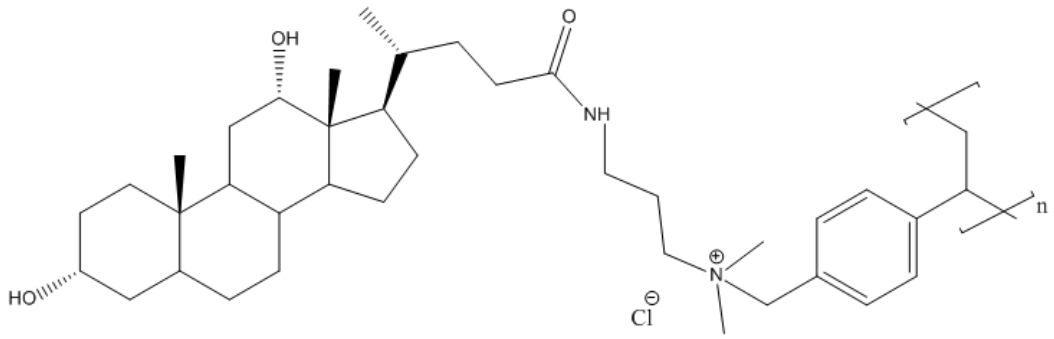
Compound **64** (2.25 g 0.004 mol) was dissolved in chloroform (10 mL). Poly (vinyl benzyl chloride) 60/40 mixture of 3- and 4- isomers (0.25 g, 0.001 mol) was added and the solution was stirred overnight at 50 ^oC. The resulting precipitate was collected by vacuum filtration. The product was a yellow solid which would not dissolve in DMSO, TFA, acetone, 1, 4 dioxane, water or pyridine.

Yield; 0.63 g, 0.001 mol, 20 %.

No NMR achieved due to insolubility of material. Deuterated solvents tried; chloroform, methanol, dimethyl sulfoxide, H_2O, trifluoroacetic acid, acetic acid, acetone, 1, 4-dioxane, pyridine and dimethoxyethane. Presumed crosslinking of material has occurred, resulting in insolubility.

IR= 3270, (OH/NH), 2925 (alkyl), 2865 (alkyl), 1641 (C=O), 1543, 1441 cm^{-1}.

Preparation of poly 3-[[(4R)-4-[(3R,10S,12S,13R,17R)-3,12-dihydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentanoyl]amino]propyl-dimethyl-[(4-sec-butylphenyl)methyl]ammonium chloride (compound 121)



Product from 2/90 (0.75 g 0.001 mol) was dissolved in chloroform (10 mL). Poly (vinyl benzyl chloride) 60/40 mixture of 3- and 4- isomers (0.83 g, 0.005 mol) was added and the solution was stirred overnight at ambient temperature. The resulting precipitate was collected by vacuum filtration and then triturated in hot chloroform (3 x 20 mL). The product was an off-white solid.

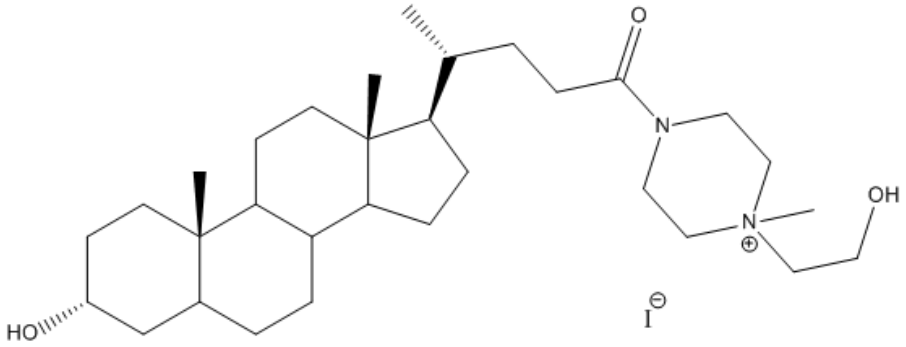
Yield; 0.906 g, 0.001 mol, 81 %.

Melting point: >350 ^{o}C.

^{1}H NMR (MeOD) (250 MHz) δ= 0.71 (broad s, 3H, 18-CH_3), 0.94 (broad s, 3H, 19-CH_3), 3.56 (broad s, 1H, 3-CH), 3.97 (broad s, 1H, 12-CH), 6.55-7.15 (broad s, 4H, Ar-CH) ppm.

IR= 3317 (OH), 2921 (alkyl), 2852 (alkyl), 1641 (C=O), 1445, 1258 (C=O ester stretch), 1040 (R_2CH-OH) cm^{-1}.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-1-[4-(2-hydroxyethyl)-4-methyl-piperazin-4-ium-1-yl]pentan-1-one iodide (compound 122).



Compound **94** (0.2 g 0.0004 mol) was dissolved in chloroform (5 mL). Iodomethane (0.29 mL, 0.002 mol) was added and the solution was stirred overnight at ambient temperature. The resulting precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.01 g, 0.00001 mol, 4 %.

Melting point: 245.3 – 251.3 ^{o}C.

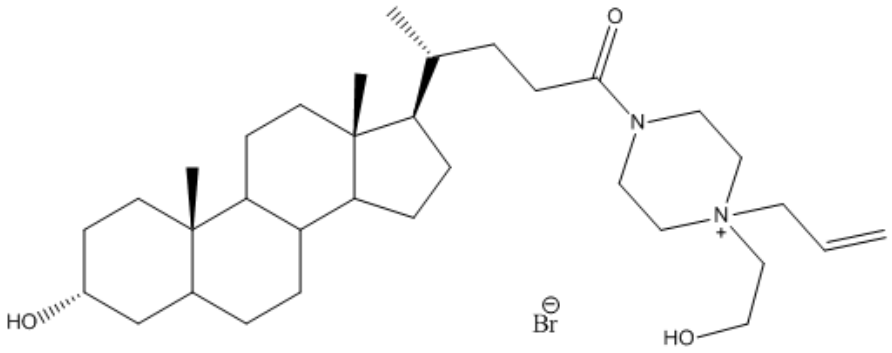
^{1}H NMR (MeOD) (250 MHz) δ= 0.69 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 0.98 (d, 3H, 21-CH_3, J= 5.0), 1.00-2.55 (m, 33H, steroidal backbone CH/CH_2), 3.54-3.65 (multiple overlapping multiplets, 6H, CH_2), 3.97- 4.05 (multiple overlapping multiplets, 6H, CH_2) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 11.5 (CH_3, C18), 16.4 (CH_3, C21), 22.9 (CH_2, C11), 25.9 (CH_2), 27.8 (CH_2), 32.2 (CH), 33.2 (CH), 34.6 (C, C10), 35.1 (CH, C20), 41.1 (CH, C5), 42.4 (C, C13), 44.5 (CH_2), 45.8 (CH), 47.4 (CH), 60.4 (CH_2), 61.3 (CH), 61.8 (CH), 67.1 (CH_2), 70.1 (CH, C3), 71.2 (CH_2), 75.7 (CH_2), 76.3 (CH_2), 80.6 (CH_2), 174.8 (CO, C24) ppm.

IR= 3313 (OH), 2929 (alkyl), 2852 (alkyl), 1607 (C=O), 1466, 1249, 1044 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 503.4195 $(M-I)^+$; calculated for $C_{31}H_{55}N_2O_3$ 503.4207; 2.4 ppm.

Preparation of (4R)-1-[4-allyl-4-(2-hydroxyethyl)piperazin-4-ium-1-yl]-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]pentan-1-one bromide (compound 123)



Compound **94** (0.2 g 0.0004 mol) was dissolved in chloroform (5 mL). Allyl bromide (0.44 mL, 0.003 mol) was added and the solution was stirred overnight at ambient temperature. The resulting precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.02 g, 0.00003 mol, 8 %.

Melting point: 194.9 – 197.8 ^oC.

^1H NMR (MeOD) (250 MHz) δ= 0.70 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 0.98 (d, 3H, 21-CH_3, J= 7.5), 1.00-2.53 (m, 33H, steroidal backbone CH/CH_2), 3.54-3.65 (multiple overlapping multiplets, 6H, CH_2), 4.01 (multiple overlapping multiplets, 6H, CH_2), 4.27 (d, 2H, CH_2, J= 7.5), 5.76 (m, 2H, =CH_2), 6.10 (m, 1H, =CH-) ppm.

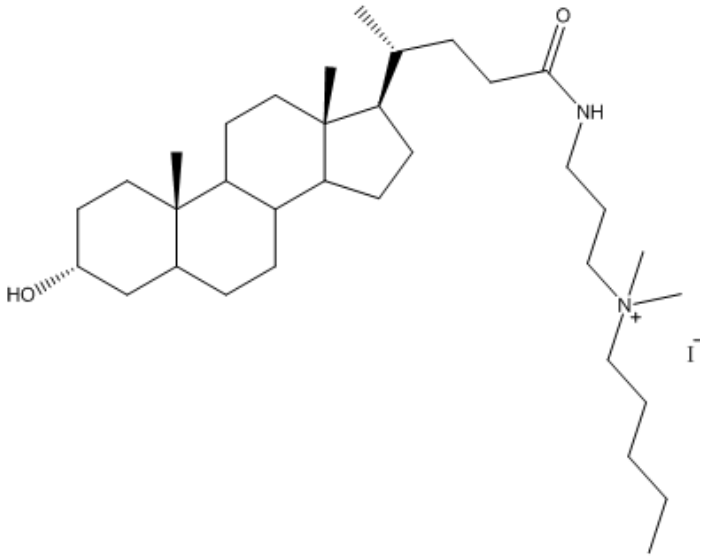
^{13}C NMR (MeOD) (62.9 MHz) δ= 12.5 (CH_3, C18), 18.9 (CH_3, C21), 21.9 (CH_2, C11), 23.9 (CH_3, C19), 25.3 (CH_2, C15), 27.6 (CH_2, C6), 28.3 (CH_2, C16), 29.3 (CH_2, C2), 30.6 (CH_2, C22), 31.1 (CH_2, C23), 32.2 (C, C10), 35.6 (CH, C20), 36.4 (CH_2, C1), 36.9 (CH, C8), 37.2 (CH_2, C4), 41.5 (CH_2,

C12), 41.9 (CH, C9), 56.4 (CH, C17), 57.4 (CH, C14), 57.9 (CH_2), 59.4 (CH_2), 61.6 (CH_2), 63.7 (CH_2), 72.4 (CH, C3), 125.6 (CH), 129.9 (CH), 174.8 (CO, C24) ppm.

IR= 3350 (NH), 3241 (OH), 2938 (alkyl), 2850 (alkyl), 1633 (C=O), 1439, 1244, 1189.78 (C=O ester stretch), 1244 (R_2CH-OH) cm^{-1}.

MS (ES) m/z= Found 529.4349 $(M\text{-}Br)^+$; calculated for $C_{33}H_{57}N_2O_3$ 529.4364; 2.8 ppm.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-1-[4-(2-hydroxyethyl)-4-pentyl-piperazin-4-ium-1-yl]pentan-1-one bromide (compound 124)



Compound **64** (0.5 g 0.001 mol) was dissolved in dichloromethane (20 mL). Iodopentane (1.57 mL, 0.007 mol) was added and the solution was stirred overnight at 40 ^oC. The resulting precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.36 g, 0.0005 mol, 50.7 %.

Melting point: 104.8 – 108.6 ^oC.

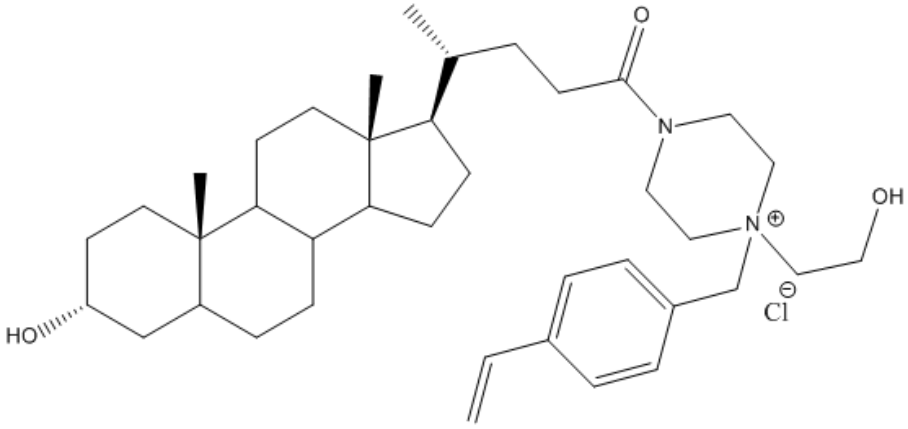
^{1}H NMR (MeOD) (250 MHz) δ= 0.66 (s, 3H, 18-CH_3), 0.92 (s, 3H, 19-CH_3), 0.94 (multiple overlapping multiplets consisting of both CH_3 and CH_2), 1.00-2.38 (m, 33H, steroidal backbone CH/CH_2), 3.08 (s, 5H, CH_3/CH_2). 3.51 (m, 1H, 3-CH) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 11.0 (CH_3, C18), 12.7 (CH_3), 17.3 (CH_3, C21), 20.4 (CH_2, C11), 21.8 (CH_3, C19), 26.1 (CH_2, C7), 29.6 (CH_2, C2), 34.1 (C, C10), 35.4 (CH, C20), 35.7 (CH_2, C1), 40.0 (CH_2, C12), 40.4 (CH, C9), 42.0 (CH, C5),42.4 (C, C13), 49.8 (CH), 55.9 (CH, C17), 56.4 (CH, C14), 70.8 (CH, C3), 175.7 (CO, C24) ppm.

IR= 3449 (OH), 2921 (alkyl), 2857 (alkyl), 1641 (C=O), 1548, 1445, 1364, 1036 (R_2CH-OH) cm^{-1}.

MS (+ESI) m/z= Found 531.4880 M^+; calculated for $C_{34}H_{63}N_2O_2$ 531.4884; 0.8 ppm.

Preparation of (4R)-4-[(3R,10S,13R,17R)-3-hydroxy-10,13-dimethyl-2,3,4,5,6,7,8,9,11,12,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl]-1-[4-(2-hydroxyethyl)-4-[(4-vinylphenyl)methyl]piperazin-4-ium-1-yl]pentan-1-one chloride (compound 125)



Compound **94** (0.2 g 0.0004 mol) was dissolved in chloroform (5 mL). Vinyl benzyl chloride (0.31 mL, 0.002 mol) was added and the solution was stirred overnight at ambient temperature. The resulting precipitate was collected by vacuum filtration and was dried overnight under vacuum. The product was an off-white solid. TLC 100% MeOH R_f 0.1 (single spot).

Yield; 0.1 g, 0.0001 mol, 38 %.

Melting point: 143.6- 146.0 ^{o}C.

^{1}H NMR (MeOD) (250 MHz) δ= 0.680 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.00-2.53 (m, 33H, steroidal backbone CH/CH_2), 3.61 (multiple overlapping multiplets, 6H, CH_2), 5.37 (d, 1H, =CH-, J= 12.5), 5.90 (d, 1H, =CH, J = 17.5), 6.80 (dd, 1H, =CH, J= 12.5 and 17.5), 7.59 (broad s, 4H, Ar-CH) ppm.

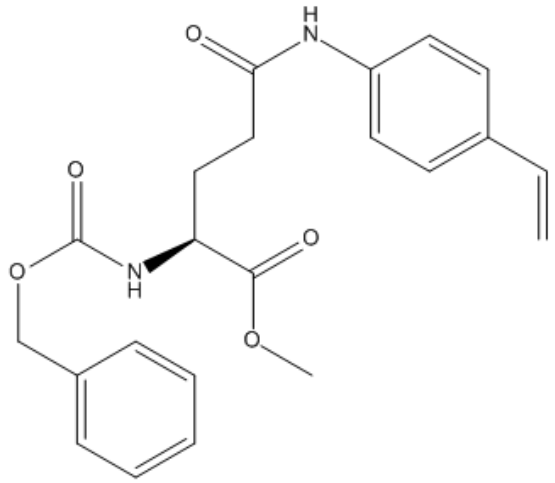
^{13}C NMR (MeOD) (62.9 MHz) δ= 12.5 (CH_3, C18), 18.9 (CH_3, C21), 21.9 (CH_2, C11), 23.9 (CH_3, C19), 25.3 (CH_2, C15), 27.6 (CH_2, C7), 28.3 (CH_2, C6), 29.3 (CH_2, C16), 30.6 (CH_2, C2), 31.2 (CH_2, C22), 32.2 (CH_2, C23), 35.6 (C, C10), 36.4 (CH, C20), 36.8 (CH_2, C1), 37.2 (CH, C8), 40.6 (CH_2, C12), 41.5 (CH, C9), 41.9 (CH, C5), 43.5 (C, C13), 56.5 (CH, C17), 57.4 (CH, C14), 57.9 (CH), 58.1 (CH_2), 58.8 (CH_2), 67.1 (CH), 72.4 (CH, C3), 116.7 (Ar-C), 127.2 (Ar-CH), 128.0 (Ar-CH), 134.9 (Ar-CH), 137.0 (=CH_2), 141.6 (=CH-), 174.8 (CO, C24) ppm.

IR= 3283 (OH), 2925 (alkyl), 2857 (alkyl), 1616 (C=O), 1445, 1044 (R_2CH-OH) cm^{-1}.

MS (ES) m/z= Found 605.4669 $(M\text{-}Cl)^{+}$; calculated for $C_{39}H_{61}N_2O_3$ 605.4677; 1.3 ppm.

2.3.4 Amino acid analogue synthesis

Preparation of methyl 2-(benzyloxycarbonylamino)-5-oxo-5-(4-vinylanilino)pentanoate (compound 126)



From a modified experimental (Carlson et al., 2003) Z-Glutamic acid 1-methyl ester (1.0 g 0.003 mol) was dissolved in dichloromethane (20 mL) with triethylamine (0.40 mL 0.003 mol), N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide hydrochloride (0.64 g, 0.003 mol), vinyl aniline (0.4 mL, 0.003 mol) and DMAP (0.001 g). The solution was left stirring overnight under argon conditions at ambient temperature. The next day dichloromethane (30 mL) was added and the solution was washed with sodium hydrogen carbonate (1 x 30 mL) and monobasic potassium phosphate (1 x 30 mL). Purified by column chromatography (100 % chloroform) for 200 mL then addition of 1 % methanol. Solvent was evaporated under reduced pressure and precipitated by dissolving in methanol and adding dropwise into stirring water. The resultant precipitate was collected by vacuum filtration, washed with water (3 x 20 mL) and dried under vacuum.

Yield; ~20 mg (material dropped; only partially recovered).

Melting point: 145.3 – 147.7 ^{o}C.

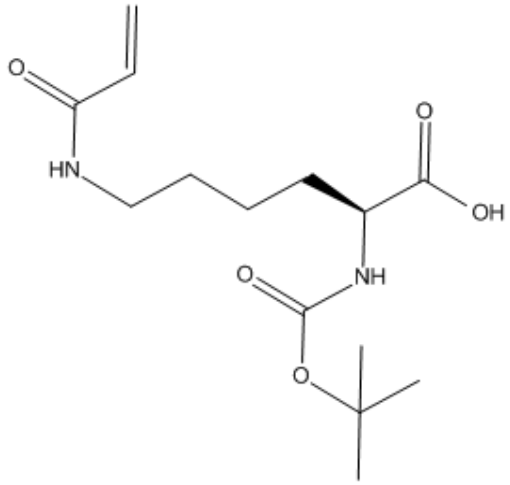
^{1}H NMR ($CDCl_3$) (250 MHz) δ= 2.01-2.41 (overlapping multiplets, 4H, aliphatic CH_2), 3.74 (s, 3H, O-CH_3), 4.44 (m, 1H, CH), 5.11 (s, 2H, CH_2), 5.19 (d, 1H, CH, J= 10.0), 5.675 (d, 1H, CH, J= 17.5), 6.67 (dd, 1H, CH, J=12.5 and 17.5), 7.23-7.55 (overlapping multiplets, 9H, Ar-CH), 8.14 (broad singlet, 1H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 29.6 (CH_3) 33.8 (CH_2), 52.6 (CH_2), 53.3 (CH_2), 67.3 (CH_2), 112.9 (Ar-C), 119.7 (Ar-C), 126.8 (Ar-CH), 128.1 (Ar-CH), 128.3 (Ar-CH), 128.5 (Ar-CH), 133.6 (Ar-CH), 135.9 (Ar-CH), 136.1 (=CH_2), 137.6 (=CH-), 156.7, 170.0, 172.2 (C=O) ppm.

IR= 3293 (NH), 1736 (C=O), 1684 (C=O), 1654 (C=O), 1269, 1241, 1211, 841 (aromatics), 689 (aromatics) cm^{-1}.

MS (ES) m/z= Found 397.1753 $(M+H)^{+}$; calculated for $C_{22}H_{25}N_2O_5$ 397.1758; 1.3 ppm.

Preparation of (2S)-2-(tert-butoxycarbonylamino)-6-(prop-2-enoylamino)hexanoic acid (compound 127)



From literature (Li et al., 2013). Boc-Lys-OH (1 g, 0.004 mol) was dissolved in 10 mL (50/50 mixture acetonitrile/2M NaOH) and was cooled to 10 ^{o}C. Acryloyl chloride (0.5 mL 0.005 mol) dissolved in 5 mL of acetonitrile was then added concurrently with NaOH (15 mL, 0.375 mol) to get the solution to pH 10. Once the correct pH had been achieved, the solution was allowed to warm to ambient temperature and left for a further 2 hours. After 2 hours the solution was acidified to just under pH 7

using 2 M HCl (15 mL, 0.41 mol) and extracted with ethyl acetate (3 x 20 mL). The organic layer was dried over magnesium sulfate. Solvent was evaporated under reduced pressure and triturated with diethyl ether to produce viscous oil.

Yield; 0.16 g, 0.0005 mol, 13 %.

Melting point: Oil.

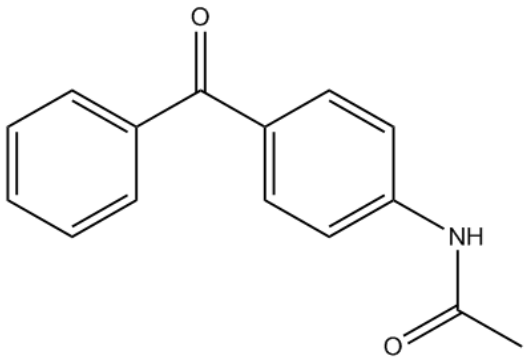
^{1}H NMR (MeOD) (250 MHz) δ= 1.44 (s, 9H, BOC), 3.22 (m, 2H, CH_2), 4.05 (m, 1H, CH), 5.63 (dd, 1H, CH, J= 2.5 and 7.5), 6.22 (dd, 2H, CH_2, J= 2.5 and 7.5) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 28.2 (CH_3 Boc), 32.6, 33.8, 36.4, 44.0 (CH_2 lys-chain), 58.8 (CH), 84.4 (C, Boc), 101.4 (=CH_2), 162.2 (C=O), 172.1 (C=O), 180.3 (C=O) ppm.

MS (ES) m/z= Found 323.1573 $(M+H)^+$; calculated for $C_{14}H_{24}N_2O_5Na$ 323.1577; 1.4 ppm.

2.3.5 Benzophenone derivatives

Preparation of N-(4-benzoylphenyl)acetamide (compound 128)



From a modification of (Bitonti, 1997) 4-aminobenzophenone (1.0 g 0.005 mol) was dissolved in toluene (20 mL) with triethylamine (1.40 mL, 0.01 mol). Acetic anhydride (0.79 mL, 0.007 mol) was then added. Once added, the solution was heated at reflux for 2 hours then added to water (50 mL) wherein a precipitate was formed. The precipitate was collected by vacuum filtration, washed with

water (3 x 20 mL) and dried under vacuum. The crude product was then recrystallized from acetonitrile to produce a white powder.

Yield; 0.41g, 0.0017 mol, 33.8 %.

Melting point: 153.7-155.2 ^{o}C. Lit 151-152 ^{o}C (Peet et al., 1989).

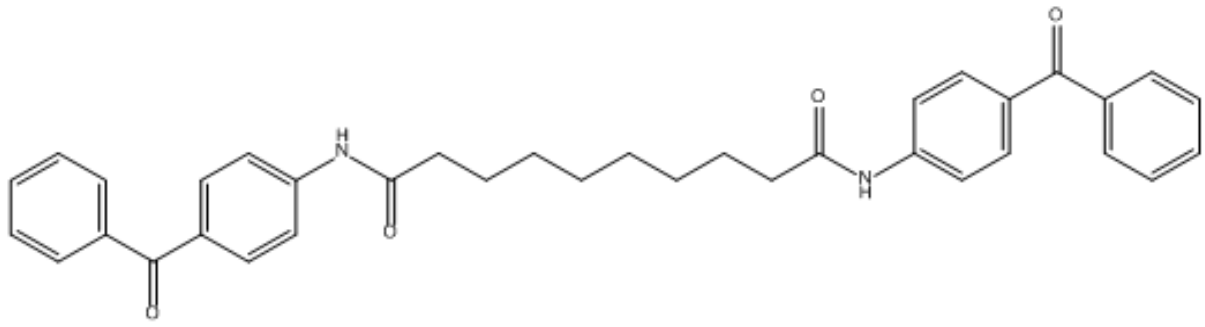
^{1}H NMR (MeOD) (250 MHz) δ= 2.16 (s, 3H, CO-CH_3), 7.52-7.76 (m, 9H, Ar-CH) ppm.

^{13}C NMR (MeOD) (62.9 MHz) δ= 22.4 (CH_3, CO$\underline{C}H_3$), 118.5 (Ar-CH), 127.9 (Ar-CH), 129.2 (Ar-CH), 130.9 (Ar-CH), 131.9 (Ar-CH), 142.9 (Ar-CH), 170.5 (CONH), 196.0 (Ar-CO) ppm.

IR= 3330 (NH), 1701 (C=O), 1513 (aromatic bending), 1407, 1253, 754 (aromatic) cm^{-1}.

MS (+ESI) m/z= Found 240.1022 $(M+H)^{+}$; calculated for $C_{15}H_{14}NO_2$ 240.1024, 0.9 ppm.

Preparation of N, N'-bis(4-benzoylphenyl)decanediamide (compound 129)



Sebacoyl chloride (.34 mL g 0.001 mol) was dissolved in tetrahydrofuran (20 mL) with triethylamine (0.40 mL 0.003 mol). 4-Aminobenzophenone (0.75 g 0.003 mol) and DMAP (~10 mg) was added and the solution was stirred overnight at ambient temperature. Saturated sodium hydrogen carbonate solution (50 mL) was then added and the solution extracted with ethyl acetate (3 x 20 mL). The organic layers were combined and washed with 2 M hydrochloric acid (3 x 20 mL). The organic layer was dried over magnesium sulfate. The solvent was evaporated under reduced pressure and the product was recrystallized from ethanol to produce a white powder.

Yield: Not recorded.

Melting point: 192.8 – 201.6 ^{o}C.

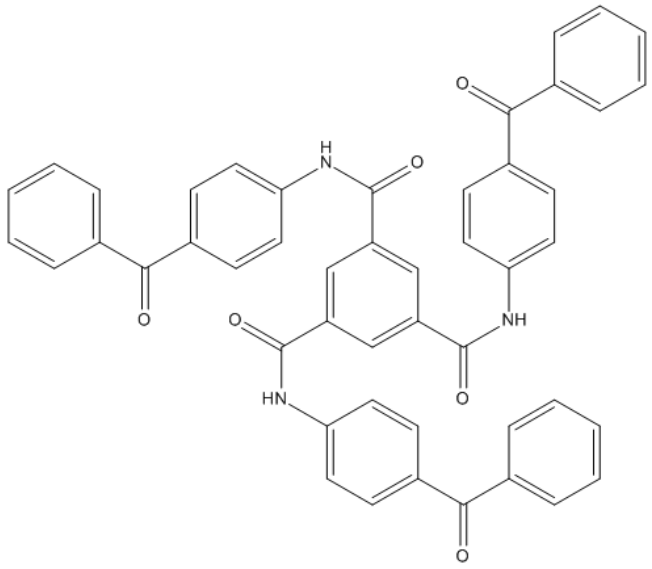
^{1}H NMR (250 MHz) ($CDCl_3$) δ= 1.31 (s, 8H, aliphatic CH_2), 1.61 (broad singlet, 4H, CH_2), 2.35 (t, 4H, CH_2, J= 7.5), 7.55-7.79 (overlapping multiplets, 18H, aromatics), 10.26 (s, 2H, NH) ppm.

^{13}C NMR ($CDCl_3$) (62.9 MHz) δ= 24.8, 28.5, 36.4, 38.4 (CH_2, aliphatic chain), 118.1, 128.4, 129.3, 131.1, 137.5, 143.3 (CH, aromatic), 171.8 (C=O, amide), 194.4 (C=O, ketone) ppm.

IR= 3287 (NH), 2921 (alkyl), 1646 (C=O), 1586 (Aromatic C-H bending), 1398, 1309, 925 (aromatics) cm^{-1}.

MS (+ESI) m/z= Found 561.2740 $(M+H)^{+}$; calculated for $C_{36}H_{37}N_2O_4$ 561.2748; 1.2 ppm.

Preparation of N1, N3, N5-tris(4-benzoylphenyl)benzene-1,3,5-tricarboxamide (compound 130)



From a modification of (Jorgensen and Krebs, 2001) 1, 3, 5-benzenetricarbonyl trichloride (1.0 g 0.005 mol) was dissolved in tetrahydrofuran (20 mL). 4-aminobenzophenone (2.5 g 0.01 mol) was added and the solution was stirred overnight. Solvent was evaporated under reduced pressure and recrystallized from ethanol to produce a white powder.

Yield; 0.65 g, 0.0008 mol, 23 %.

Melting point: 281.4-283.4 ^{o}C.

^{1}H NMR (d6-DMSO) (250 MHz) δ= 7.56-8.08 (overlapping multiplets, 27H, Ar-CH), 8.82 (s, 3H, Ar-CH), 11.00 (s, 3H, NH) ppm.

^{13}C NMR (d6-DMSO) (62.9 MHz) δ= 119.5, 128.5, 129.4, 130.3, 131.0, 132.0, 132.3, 135.1, 137.4, 143.0 (Ar-CH), 164.8, 194.63 (C=O) ppm.

IR= 3228, 3049 (NH), 1586, 1518, 1398, 1313, 848 (aromatic) cm^{-1}.

MS (+ESI) m/z= Found 748.2435 $(M+H)^{+}$; calculated for $C_{48}H_{34}N_3O_6$ 748.2442; 1.0 ppm.

2.4.1 Germination efficacy of sodium taurocholate solution/alternative.

The following is the protocol used by Christian Lowden, Kristian Poole and Amber Lavender for the microbiological testing of the compounds synthesised;

A 2% (w/v) sodium taurocholate comprising double strength thioglycolate germination solution was prepared as Wheeldon *et al* (2008). 200µL was added immediately, in triplicate, to 200µL *C. difficile* NCTC 11204 spores from an original stock suspension of ~10^{6}cfu/mL in microfuge tubes and vortexed. After 1 hour exposure, 600µL sterile distilled water was added to the test samples and vortexed, and then subsequently heated in a water bath to 75°C for 20 minutes. Control samples contained 200µL spore suspensions and 200µL sodium taurocholate germination solution, after 1 hour exposure 600µL sterile distilled water was added to samples, vortexed and were subsequently stored over ice for 20 minutes.

Following heat-shock, serial dilutions were performed and samples were inoculated onto Fastidious Anaerobic Agar comprising 5% (v/v) defibrinated horse blood and 0.1% (w/v) sodium taurocholate. Serial dilutions and inoculations were repeated for control samples.

Method adapted for alternative germination solution consisting 5mL DMSO and 5mL 'Tween 20' in germination solution comprising 2% (w/v) sodium taurocholate and double strength thioglycolate medium.

2.5.1 Unsuccessful Benzophenone reactions

The benzophenone derivatives were measured on a spectrofluorimeter and were found to have an optimum excitation of 310 nm and a fluorescence emission of ~ 360 nm. The benzophenone bile acid analogues (100 mg) were dissolved in chloroform (2 mL). From this solution, 20 μL was removed and put in one well of a 96 well plate, which had previously been filled with a circular disc of PEEK (Polyether ether ketone, fig 25) polymer of a radius to fit neatly into the well plate. The plate was then exposed to 2.02 W/cm^2 for 15 minutes, and then washed by sonication in chloroform 3 times. The fluorescence of the material was then measured to see if any of the material had attached to the polymer surface.

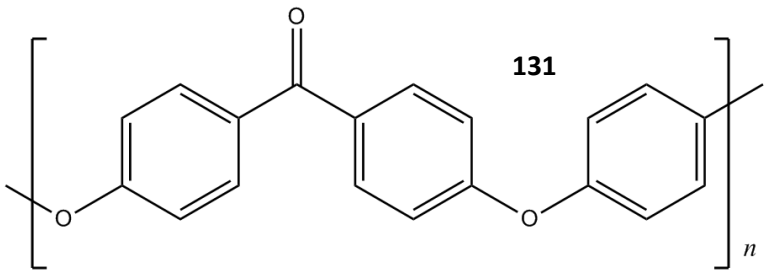


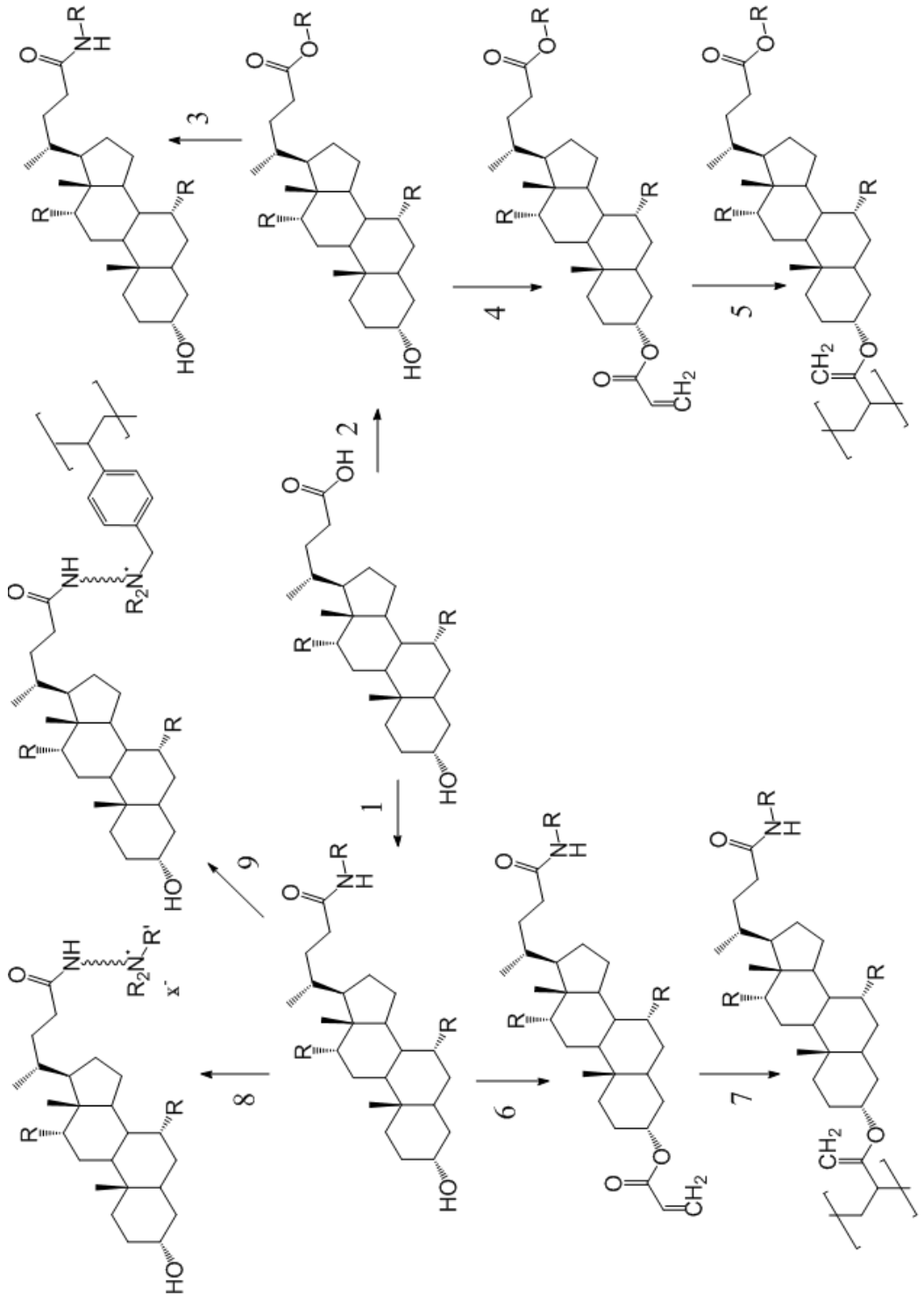
Fig 25: PEEK polymer (compound **131**).

As no fluorescence of an appropriate wavelength was observed after trying to attach the bile amide material to the polymer, this experiment was stopped. A similar set up was tried, with discs, but this time different amounts of material was added (same concentration, 5-40 μL) and the polymer/bile amide solutions were irradiated for differing periods of time, 15, 30 and 45 minutes. To ensure solvents weren't causing an effect, this method was repeated with the following solvents, ethyl acetate, acetonitrile, tetrahydrofuran, ethanol and chloroform. None of these protocols produced any

measurable amount of attachment to the polymeric material, checked by using the spectrofluorimeter to observe any emission.

3.0 Results and discussion:

3.1.1 Overview of project.



Scheme 10: Overall synthesis plan for project

In scheme 10, the overall outline for the project can be seen, broken down into distinct reaction category steps. 1-Mixed anhydride amide bond formation; 2-esterification reaction; 3-aminolysis amide bond formation; 4-Attachment of polymerizable groups to 3-OH position on bile ester; 5-polymerization of attached polymerizable groups; 6-attachment of polymerizable groups to 3-OH on bile amide; 7-polymerisation of attached polymerizable groups; 8-quaternization of tertiary amines; 9-quaternization of tertiary amines using preformed polymeric alkylating materials.

Steroid based materials have been shown to have germinatory ability with regards to the hospital acquired infection *C. difficile.* A set of structural analogues were produced to find an analogue of taurocholate (a known germinant) which was also able to germinate *C. difficile.* That material was then quaternized to produce a material which could possibly germinate the *C. difficile* spore and then destroy the vegetative cell, as the vegetative cell is easier to kill.

As can be seen from scheme 10, there are many different synthetic routes which can be taken to achieve the objective of producing a polymeric, potentially antimicrobial *C. difficile* germinating material.

Some steps limited what could be achieved next, for example, once the material was quaternized, its solubility changed considerably. Attaching an acryloyl group after quaternization would be virtually impossible, due to the ionised material only being soluble in very polar solvents. There was also a problem with the polymerisation of these ionised materials which had a potential polymer group in the alkylating group. The way this was circumnavigated was to use preformed polymeric materials as the alkylating agents.

There didn’t appear to be any difference in reactivity when attaching an acryloyl group to either the bile amide or the methyl ester but it seemed easier to polymerise the ester version of the polymer material than the amide version.

3.2.1Bile amide analysis

The most appropriate way to evaluate whether the synthesis of the steroidal material had been successful was initially through TLC and then through use of proton NMR. The NMR had a tendency to be very complicated due to the overlapping protons in the steroidal rings. However, key functional groups were distinct and thus were able to be used as markers to indicate whether or not the reaction had been successful or not.

It can be seen in fig 26 that there is considerable overlapping of signals in the 1-2 ppm range for bile acids for a proton spectrum acquired at 250 MHz (Tamminen and Kolehmainen, 2001). What can be done at the field strength available in this PhD project is to compare a known NMR spectrum of the starting material (lithocholic and deoxycholic acid) with any potential product which may be formed, or by comparison to known literature data relating to the three bile acids which were manipulated (Waterhous et al., 1985).

There are key peaks in the proton NMR spectrum however, which are readily assignable and make it easier to know whether the reaction has been successfully completed. When using deuterated chloroform there are three methyl peaks in the parent steroid structure, two of which are singlets as they are attached to the steroidal ring at quaternary carbons at positions 18/19, with an integration of 3H each. The final methyl peak is always a doublet as it is coming out from the ring at C21 and is attached to a CH. Other key peaks include the multiplets around 3.6 ppm; this is from the hydrogen that is attached at the 3 position on the steroidal ring, with the 7 position C-H (in deoxycholic) being a much more compact singlet/triplet at 3.95 ppm (Hu et al., 2005). Depending on the type of bile amide prepared, there will usually be a very “rounded” singlet, which is partially split into a poorly resolved triplet at around 5.0-7.0 ppm. This is from the NH in the amide bond. The integration of the amide NH can be compared to the methyl peaks and should be in a ratio of 1:3, suggesting formation of new product. In the ^{13}C NMR spectrum there should only be one peak around the 170 ppm region, as this is from the carbonyl group of the amide. If there is more than one peak present at 170ppm (with there

only being one in the expected structure) then it can be assumed that there is some starting material left and that the material needs to be purified further.

An example of the complexity of the proton NMR is shown in fig 26. The top spectrum shows un-modified lithocholic acid while the bottom spectrum is compound **102**, which has an amide group and an acryloylated 3-OH, thus showing some movements of the key peaks.

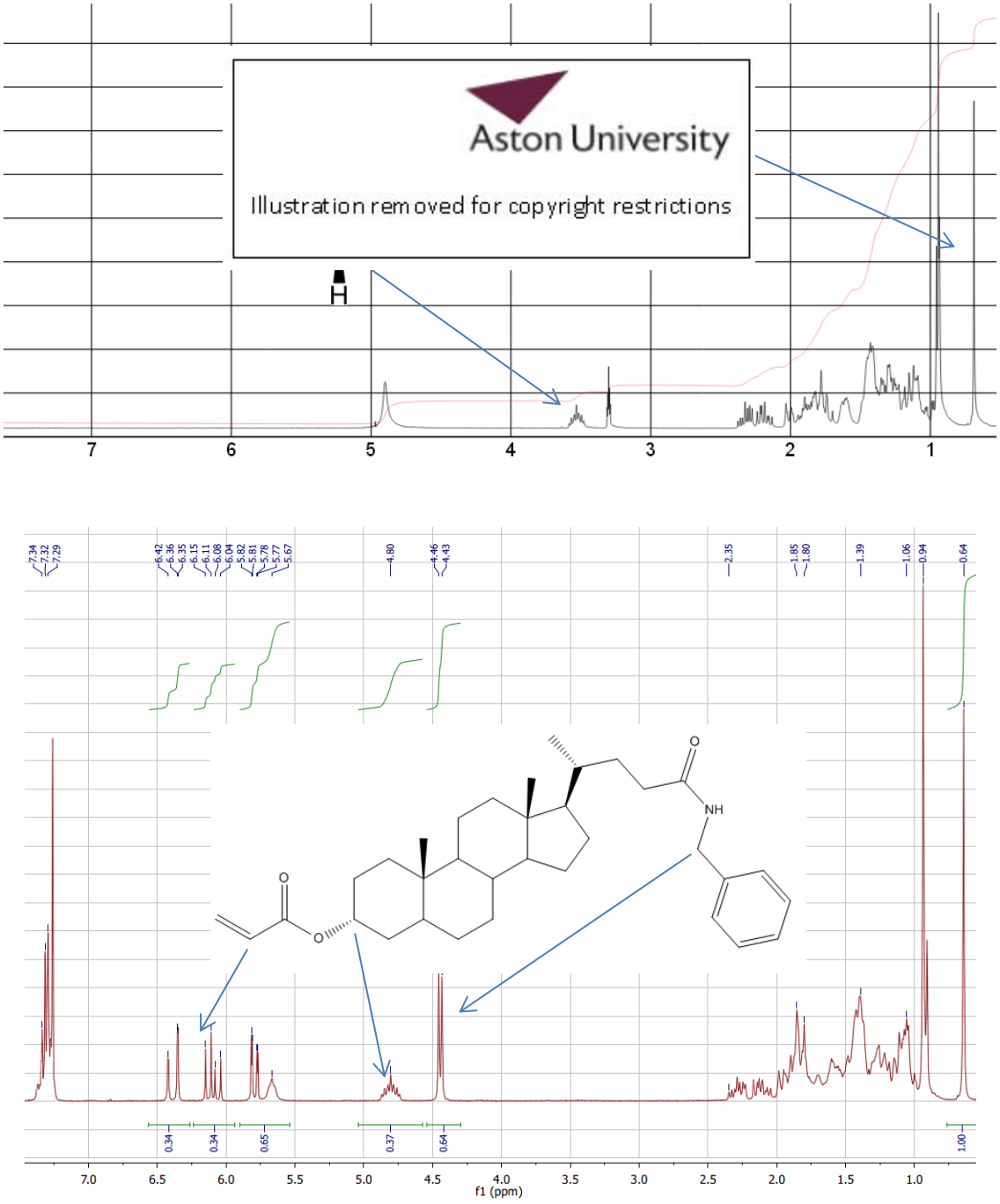
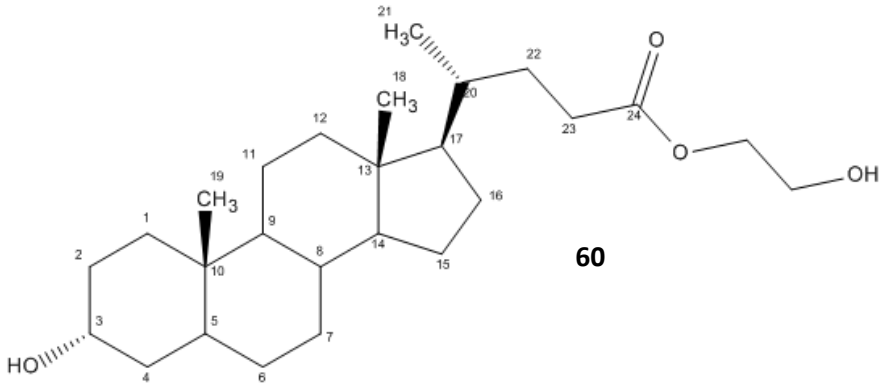


Fig 26: Proton NMR spectrum of lithocholic acid (top) and compound **102** (bottom). The horizontal axis is in ppm.

There is a distinct shift in the 3-CH in the bottom NMR in fig 26. This peak, due to the attachment of the electron-withdrawing acryloyl group has moved a significant distance from 3.5 ppm to 4.8 ppm. Another signal which was an indicator of success was from the amide NH seen at 5.67 ppm on the bottom NMR in fig 26. This peak was usually a broad singlet with shoulders, but wouldn't always

appear in the same place. Both the proton NMR spectra show the complexity of the steroidal hydrogens which is represented by overlapping peaks from ~1.06 to ~2.35. These peaks were consistent and didn't move.



60

Fig 27: Numbered carbons for compound **60**.

Carbon number	^{13}C NMR CPD 62.9 MHz (compound **60**)	^{13}C NMR 75.4 MHz Lithocholic acid (Waterhous et al., 1985)
1	35.8	35.3
2	30.5	30.3
3	71.9	70.5
4	40.1	36.3
5	42.0	41.9
6	27.2	27.1
7	26.4	26.3
8	36.4	35.6
9	41.0	40.2
10	34.5	34.2
11	20.8	20.6
12	40.4	40.0
13	42.7	42.4

14	56.5	56.3
15	24.2	24.0
16	28.2	28.0
17	55.9	55.8
18	12.0	11.9
19	23.3	23.3
20	35.3	35.1
21	18.2	18.2
22	30.9	30.9
23	31.1	30.9
24	174.7	178.1

Table 3: Key peaks of ^{13}C NMR in both lithocholic acid and from compound **60** in $CDCl_3$.

As can be seen from the table above, the interpretation of ^{13}C NMR can be very difficult, due to the numerous peaks, which all have similar ppm values. The reference material found in (Waterhous et al, 1985) is particularly useful for assigning the new peaks on the compounds synthesised. Although many of the peaks here are the same, when other, more complicated side chains are added, the similarity drops, especially if the material becomes quaternized, thus making assignment of quaternized materials very difficult. There can also be a significant variation depending on the deuterated solvent used.

There have been many problems whilst trying to work in this particular area, particularly with initial reactions focussed on the preparation of bile acid amides *via* aminolysis of the corresponding methyl esters. As can be seen from the examples shown in the experimental, the aminolysis yields can be very poor and the reaction times and conditions long and harsh. There does not seem at this point in time, any particular rule as to why certain amides are able to be formed. As long as there is a primary amine, the reaction should take place once a temperature of over 180 ^{o}C has been reached (to overcome ammonium carboxylate salt formation). It was found however, with certain non-polar solvents

(toluene), that aminolysis could be achieved at lower temperature as non-polar solvents have a tendency to disfavour salt formation (Allen et al., 2012). It was also noted, that aminolysis reactions are more likely to work for lithocholic derivatives than for deoxycholic derivatives, and this is presumably because lithocholic is less polar and therefore more soluble in non-polar solvents such as toluene.

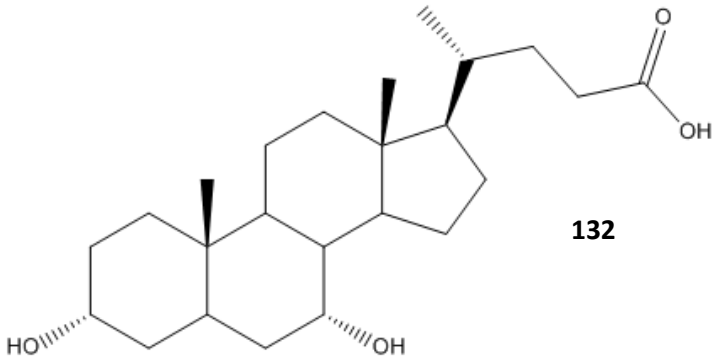
3.3.1 Solubility and reactivity of bile acids.

Due to the amphiphilic nature of the bile acids, solubility was often a key issue encountered when trying to synthesise the bile amides for use in germination of *C. difficile.* The bile acids, particularly deoxycholic acid were only soluble in a range of polar solvents. However, because of the coupling methods used to produce the amides, alcohols were not an appropriate solvent system, so other solvents had to be used, such as THF and 1,4-dioxane.

It had been reported that certain bile acids, under certain conditions could form gels in particular solvents. Literature would suggest that lithocholic acid is more likely to form gels as opposed to deoxycholic and cholic acid because the structure of lithocholic acid is relatively less polar than its counterparts (Pal et al., 2009). Two bile acids can stay "attached" to each other through hydrogen bonding on the carboxylic acid moiety on the bile acid. This was not clearly observed in any of the reactions which were done in this project, but the same literature suggests that it is the ratio of bile acid to amine which is very important.

When working with both lithocholic and deoxycholic derived bile amides, using the ethyl chloroformate method to form the mixed anhydride, it was noted that upon addition of excess water to the tetrahydrofuran or 1, 4-dioxane solution (dependent on bile acid used), that the majority of the amides would precipitate from the aqueous THF/1, 4-dioxane solution and would require minimal purification. It has been noted in the literature (Valkonen et al., 2008) however, that chenodeoxycholic acid will not precipitate out in water. The only difference between this and deoxycholic acid is that the hydroxyl is in the 7 position as opposed to the 12 position. This researcher suggests that it is the

positioning of the hydroxyl group in chenodeoxycholic (fig 28) acid which means it is more able to form stable hydrogen bonds with the solution it is dissolved in than the deoxycholic acid, thus does not precipitate out in polar solvents.



132

Fig 28: Chenodeoxycholic acid (compound **132**).

One unusual observation was the differing solubility of the three bile acids worked on. It was found, for the anhydride method, that lithocholic acid and cholic acid would dissolve readily in tetrahydrofuran. Deoxycholic acid would not dissolve in tetrahydrofuran readily with the yields for the products being significantly lower than if 1, 4-dioxane was used as the solvent. Even when dissolved in the appropriate solvent, the inconsistencies between the yield of bile amide derivatives for both lithocholic acid and deoxycholic acid were large. A specific example would be the formation of the ester of lithocholic and deoxycholic acid with ethylene glycol (compounds 60 and 61). Even though these two reactions were treated in exactly the same way the yields were significantly different. Another situation where this difference is clearly visible is the conversion of the free acid to the methyl ester. In the lithocholic acid reaction, the yield was 96.5 %, an excellent yield, yet, with the deoxycholic version, the yield was 38 %, which is a relatively poor yield.

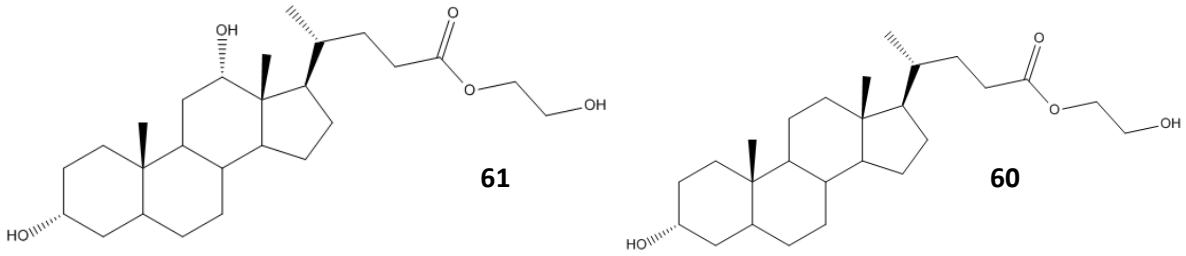
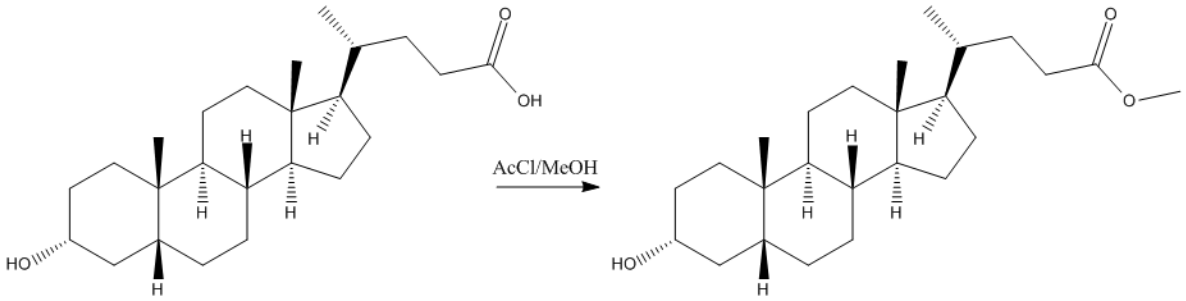


Fig 29: Two similar bile ester products, with very different yields, deoxycholic acid derivative (compound **61**) and lithocholic acid derivative (compound **60**).

3.4.1 Preparation of bile acid esters

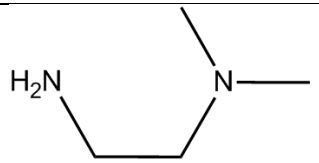
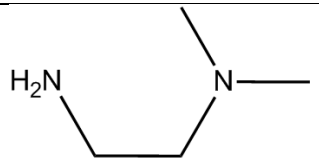
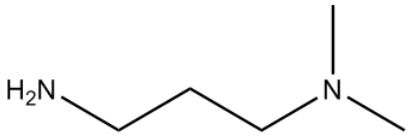
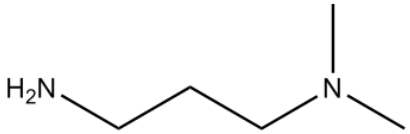


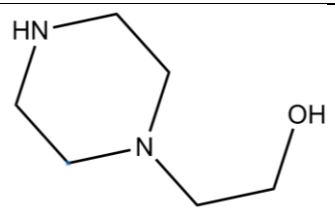
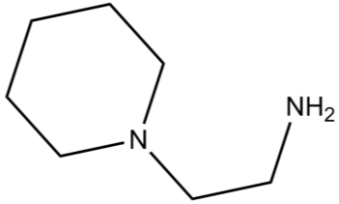
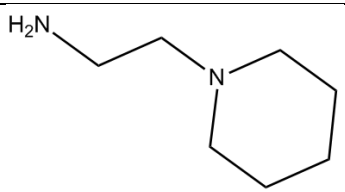
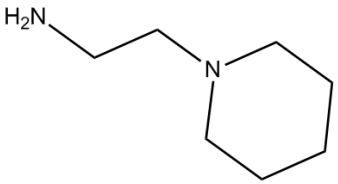
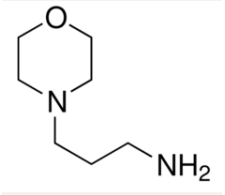
Scheme 11: Esterification of lithocholic acid

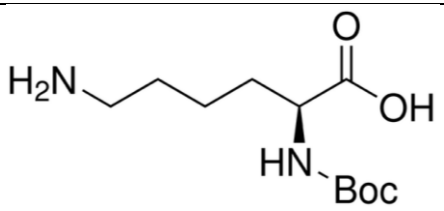
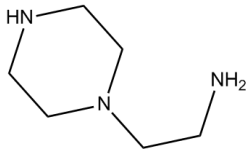
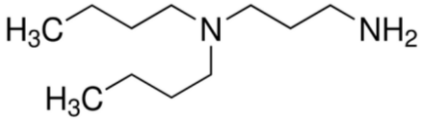
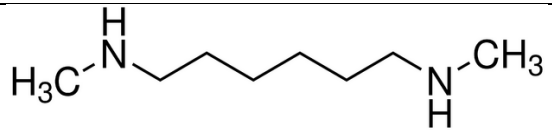
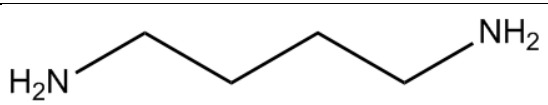
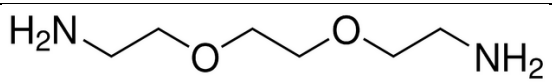
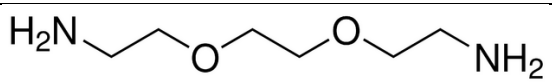
The starting material for the some of the reactions attempted was the preparation of the methyl ester of both deoxycholic and lithocholic acid (scheme 11). This product was then used as a starting material when proceeding with aminolysis reactions and also attachment of polymerizable groups at the 3-OH position. This was achieved in a very straightforward, high yielding reaction (in lithocholic acid), which took less than 1 hour to fully complete.

Attachment of ethylene glycol molecule to the carboxylic acid and therein producing an ester has been successfully synthesised with both lithocholic acid and deoxycholic acid. It is a very easy reaction with high yields and the product precipitates out of solution upon addition of water cleanly. Unfortunately literature suggests that the incorporation of esters as opposed to amides into bile acids nullifies any germinating ability the compounds may have (Howerton et al., 2010).

3.5.1 Aminolysis of bile acid esters, successes and failures.

Compound no.	Parent bile acid	Attachment position/ bond formation	Amine	Temperature ^{o}C	Time	Solvent	Yield
62	Lithocholic acid	Carboxylic acid/amide bond		100	24 hours	toluene	69 %
63	Deoxycholic acid	Carboxylic acid/amide bond		100	24 hours	Toluene	24 %
64	Lithocholic acid	Carboxylic acid/amide bond		140	24 hours	3-dimethylamino-propylamine	69 %
65	Deoxycholic acid	Carboxylic acid/amide bond		100	5 days	3-dimethylamino-propylamine	73 %

	Methyl lithocholate	Methyl ester/amide bond		115	72 hrs.	2-(2-hydroxyethyl) piperazine	N/A
	Methyl lithocholate	Methyl ester/amide bond		150	72 hrs.	(2-aminoethyl) piperidine	N/A
77	Methyl lithocholate	Methyl ester/amide bond		150	48 hours	(2-aminoethyl) piperidine	1.6 %
78	Methyl deoxycholate	Methyl ester/amide bond		70	5 days	Methanol	45 %
	Methyl deoxycholate	Methyl ester/amide bond		150	72 hrs.	Ethylene glycol	N/A

	Methyl Lithocholate	Methyl ester/amide bond		150	72 hours	Ethylene glycol	N/A
	Methyl deoxycholate	Methyl ester/amide bond		150	48 hours	4-(aminoethyl) piperadine	N/A
	Methyl lithocholate	Methyl ester/amide bond		150	24 hours	3-(dibutylamino) propylamine	N/A
	Methyl deoxycholate	Methyl ester/amide bond		70	72 hours	Methanol	N/A
87	Methyl deoxycholate	Methyl ester/amide bond		70	72 hours	Methanol	25 %
88	Lithocholic acid	Carboxylic acid/methyl ester		115	5 days	Toluene	60 %
	Methyl deoxycholate	Methyl ester/amide bond		72	1 week	Methanol	N/A

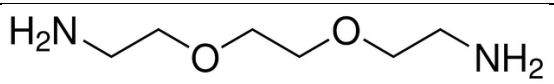
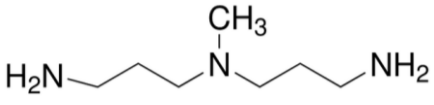
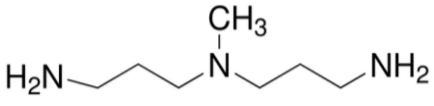
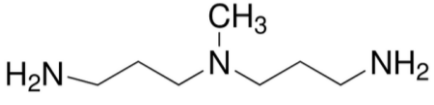
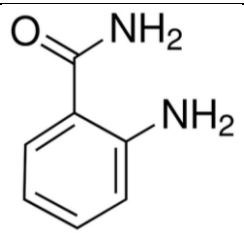
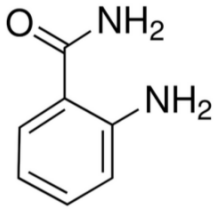
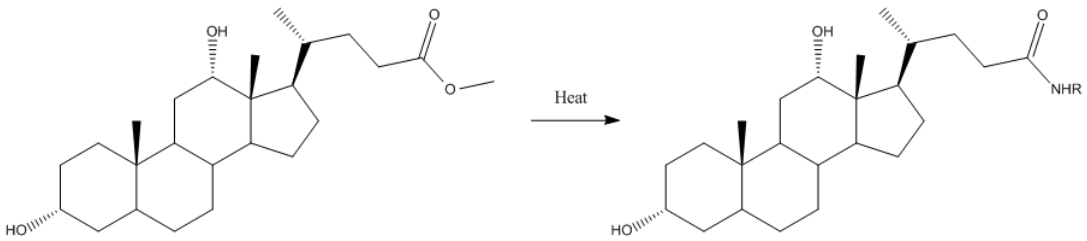
	Cholic acid	Methyl ester/amide bond		115	96 hours	Toluene	N/A
	Lithocholic acid	Methyl ester/amide bond		115	96 hours	Toluene	N/A
	Deoxycholic acid	Methyl ester/amide bond		115	96 hours	Toluene	N/A
	Cholic acid	Methyl ester/amide bond		115	48 hours	Toluene	N/A
	Cholic acid	Methyl ester/amide bond		115	48 hours	Toluene	N/A
	Deoxycholic acid	Methyl ester/amide bond		115	72 hours	Toluene	N/A

Table 4: Showing all experiments tried using aminolysis methodology.

3.6.1 Aminolysis of bile acid esters.



Scheme 12: Aminolysis between methyl lithocholate and an amine.

Various methodologies were used to try and synthesise bile amides which would potentially be used as compounds for the germination of *C. difficile* spores into vegetative cells, which are much easier to kill. The first method of amid bond formation was through the use of aminolysis.

Aminolysis is the reaction of an ester with an amine to produce an amide and an alcohol as a side product. With aminolysis, the prolonged reaction time that it takes to remove all of the starting material usually meant that there were many side reactions going on. This made purification a problem and indicates probable low yields.

With the reaction of methyl lithocholate and 4-(3-aminopropyl) morpholine, TLC indicated that all of the starting material had been consumed. A proton NMR spectrum showed that there was an amide bond present, yet there were so many other unexpected peaks, it was concluded that the reaction had not worked.

When the reaction between methyl deoxycholate/lithocholate and 4-(aminoethyl) piperidine was attempted, it was not successful. There were a large number of spots on the TLC plate after the reaction had been worked up and thus was discontinued. Exactly the same thing happened for the reaction between methyl lithocholate/deoxycholate and 3-(dibutylamino) propylamine. There does not seem to be any clear reason as to why these specific examples did not work, especially as with some of the reactions, the other analogue was successfully synthesised. Apart from the known problems with

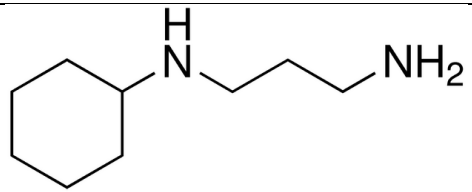
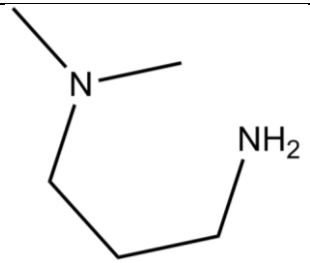
deoxycholic acid, there could be problems with the nucleophilicity of the base or indeed the solubility of the base being very different to that of the parent bile methyl ester.

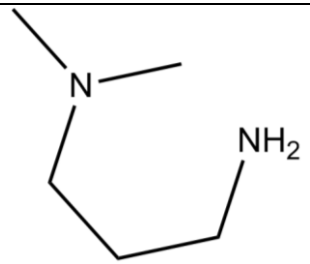
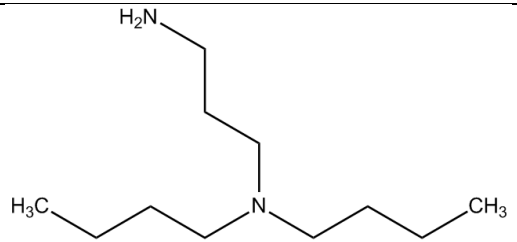
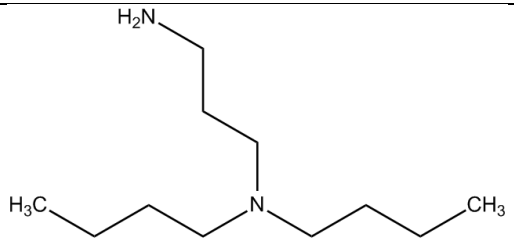
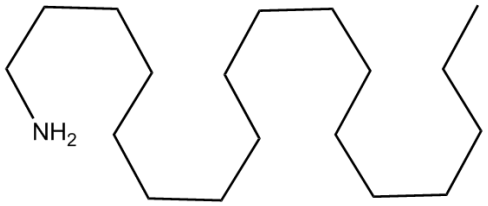
The majority of the aminolysis reactions which used the reacting amine as the solvent did not work (with the exception of compounds **64**, **65** and **77**). Aminolysis reactions were also carried out using toluene as a solvent and there were more successes with this methodology relative to the number of experiments tried using this methodology (compounds **62**, **63**, **88**).

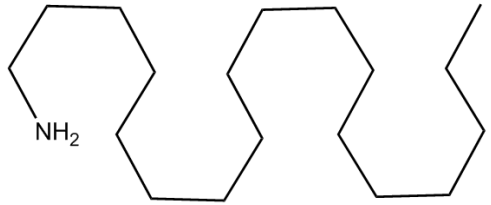
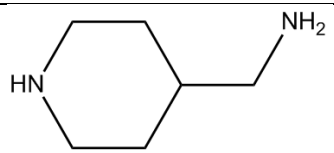
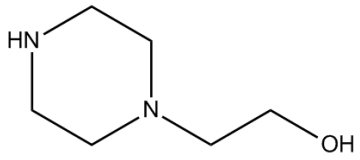
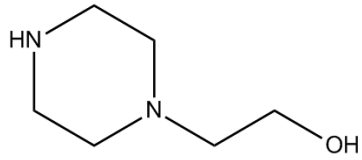
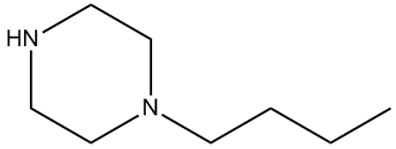
After the failures of these aminolysis reactions had occurred, it was suggested that if a water soluble carbodiimide (N-(3-dimethylaminopropyl)-N'ethylcarbodiimide) was used, the reaction may work. However, like the other carbonyldiimidazole reactions, this was unsuccessful. In this case there simply was no reaction taking place at all, with no consumption of starting materials taking place. The reason for this failure is not immediately clear but as there had been issues with solubility of the bile acids, there may have been some solvent issues which affected the ability of the carbodiimide to form the reactive intermediate.

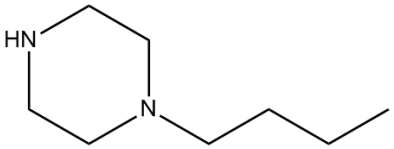
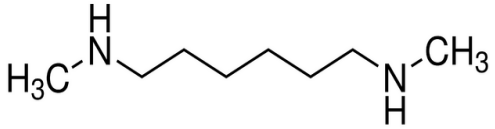
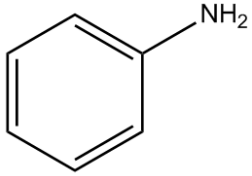
3.7.1 Activation of bile acids and attempted amine coupling.

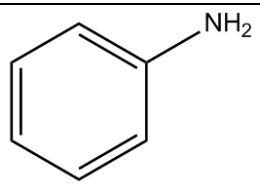
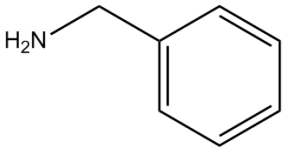
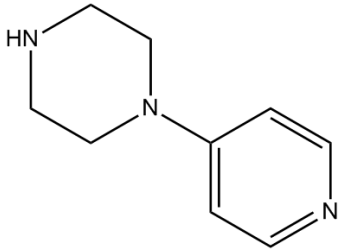
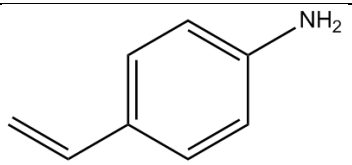
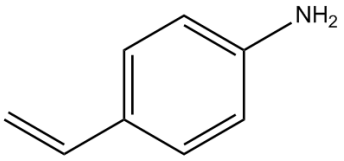
Table 5: Showing all reactions attempted using coupling reagent methodology.

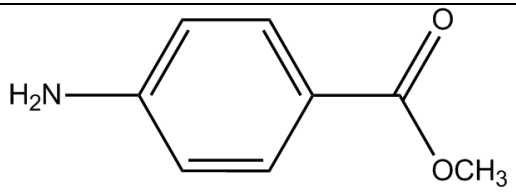
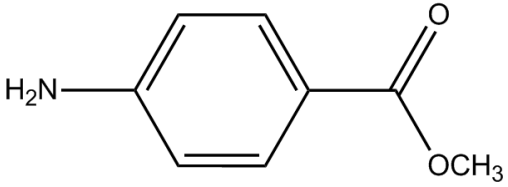
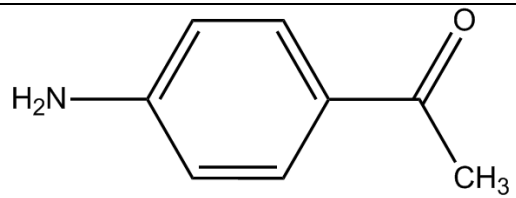
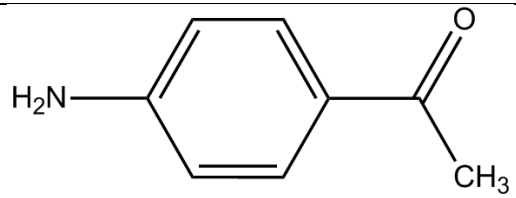
Parent Bile acid	Attachment position/bond formation	Amine	Coupling agent	Time	Solvent	Temperature ^{o}C	Yield	Compound number
Deoxycholic acid	Carboxylic acid/amide bond		N,N' carbonyldiimidazole	4 hours	THF	ambient	N/A	
Deoxycholic acid	Carboxylic acid/amide bond		N,N' carbonyldiimidazole	24 hours		50	N/A	

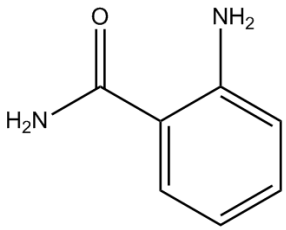
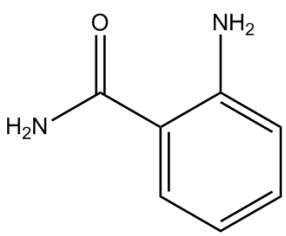
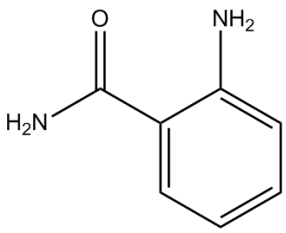
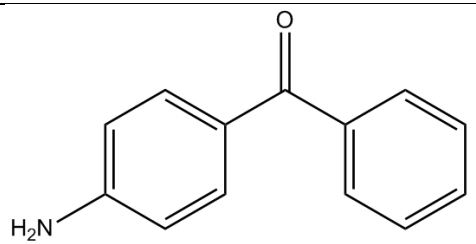
Deoxycholic acid	Carboxylic acid/amide bond		N,N' carbonyldiimidazole	4 hours	DMF	Ambient	N/A	
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	33 %	**96**
Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	48 hours	THF	Ambient	4 %	**97**
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	23.9 %	**98**

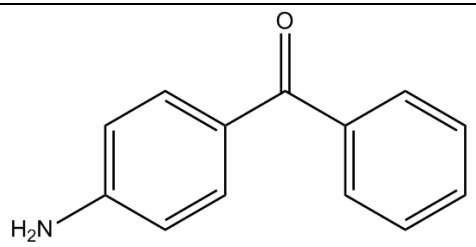
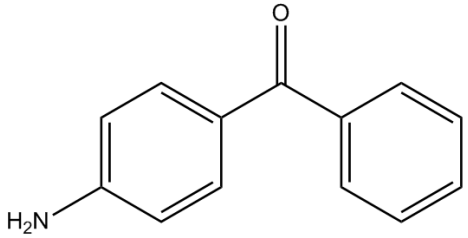
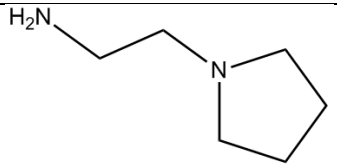
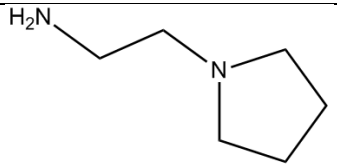
Deoxycholic acid	Carboxylic acid		Ethyl chloroformate	48 hours	1, 4 dioxane	Ambient	70 %	**99**
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	N/A	
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	48 hours	THF	Ambient	43 %	**94**
Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	48 hours	1, 4 dioxane	Ambient	46.8 %	**95**
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	23.5 %	**66**

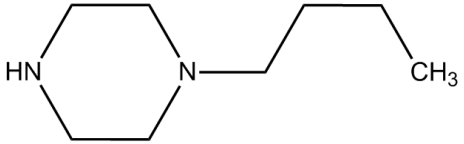
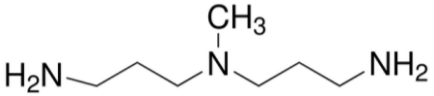
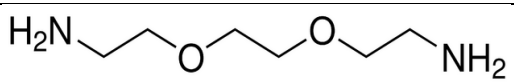
Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	1,4 dioxane	Ambient	6 %	**67**
Lithocholic acid	Carboxylic acid/amide bond	Boc-Lys-OH	Ethyl chloroformate	24 hours	THF	Ambient	24 %	**68**
Deoxycholic acid	Carboxylic acid/amide bond	Boc-Lys-OH	Ethyl chloroformate	24 hours	THF	Ambient	11 %	**69**
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	N/A	
Compound **88**	Amine/amide bond	Methacrylic anhydride		72 hours	Anhydrous DCM	Ambient	N/A	
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	51 %	**70**

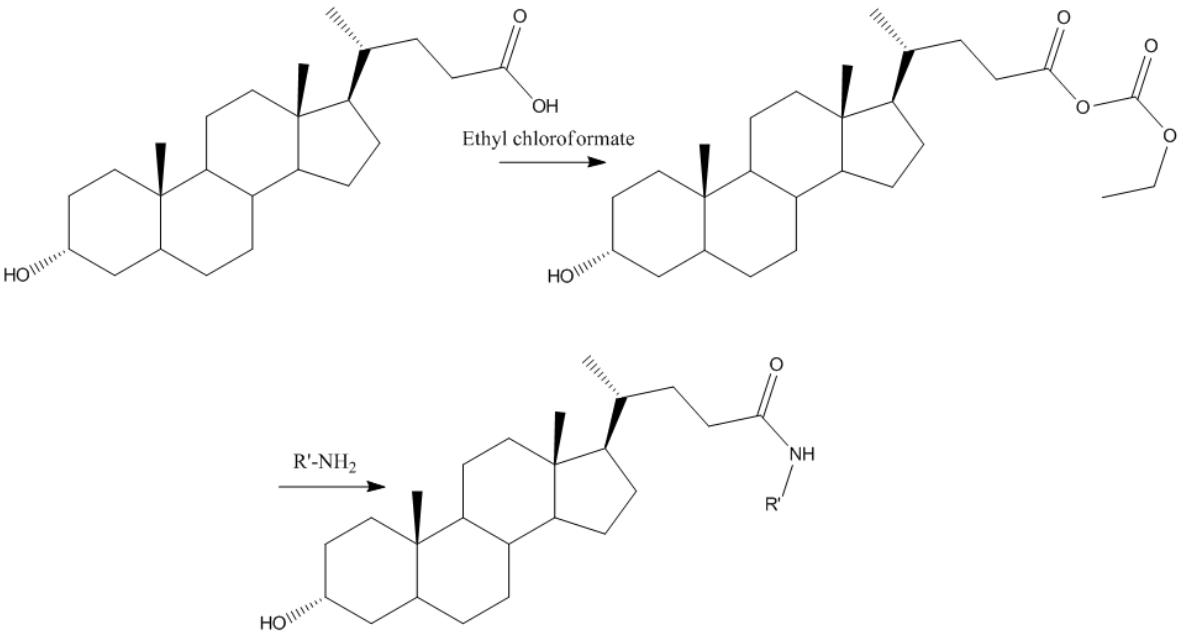
Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	42.8 %	**71**
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	51 %	**93**
Lithocholic acid	Carboxylic acid/amide		Ethyl chloroformate	24 hours	THF	Ambient	4 %	**83**
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	21 %	**72**
Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	1, 4 dioxane	Ambient	Not recorded	**73**

Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	N/A	
Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	1, 4 dioxane	Ambient	21 %	**74**
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	N/A	
Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	48 hours	1, 4 dioxane	Ambient	25 %	**92**

Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	48 hours	THF	Ambient	53 %	**84**
Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	48 hours	1, 4 dioxane	Ambient	68 %	**85**
Cholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	48 hours	1, 4 dioxane	Ambient	26.8 %	**86**
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	19 %	**79**

Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	1, 4 dioxane	Ambient	8 %	**80**
Cholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	1,4 dioxane	Ambient	31 %	**81**
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	Ambient	91.8 %	**75**
Deoxycholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	1,4 dioxane	ambient	49 %	**76**

Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	ambient	N/A	
Lithocholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	ambient	N/A	
Cholic acid	Carboxylic acid/amide bond		Ethyl chloroformate	24 hours	THF	ambient	1.9 %	**89**

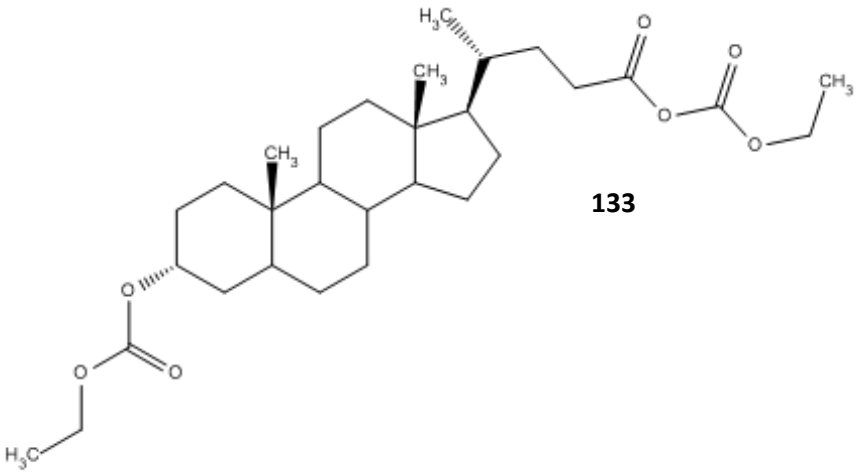


Scheme 13: Activation of lithocholic acid by means of ethyl chloroformate followed by amide formation.

As the aminolysis reactions were not very successful, often with multiple side reactions as well as low yields of desired compound, other coupling methods were investigated, such as DMT-MM and water soluble carbodiimides. The reaction conditions were varied but the reactions were never successful and starting material was always recovered. This was possibly to do with either solubility issues as previously discussed or the possibility that the steroidal moiety was causing some kind of steric hindrance.

Ethyl chloroformate was the only successful coupling reagent which was found for the production of bile amides in this particular project. Ethyl chloroformate can also potentially react with the hydroxyls present on the steroid, producing carbonates. Although it will preferentially react with the carboxylic acid, if the initial mixed anhydride stage is left for too long a period (over the initial 2 hours) or in an excess, the carbonate will form (fig 28). This makes purification from these, already sometimes low yielding reactions even more difficult. The problem was overcome by only adding 0.99 equivalents of ethyl chloroformate. By only adding 0.99 equivalents, it was guaranteed to have some starting material leftover. However, it is easier to remove starting acid by washing with saturated sodium hydrogen

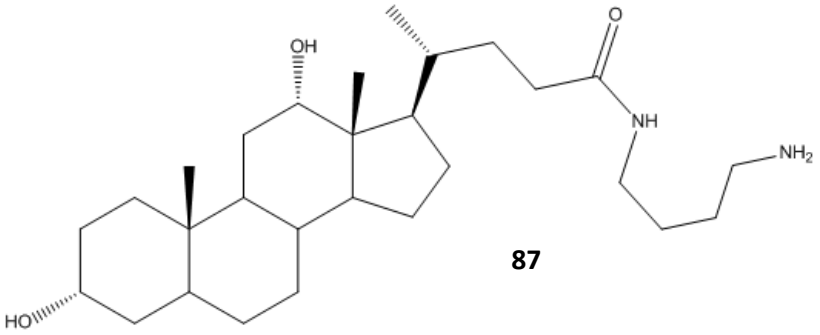
carbonate solution so this was preferable to formation of the carbonate. It has been reported and found that these reaction between acids and ethyl chloroformate type reagents are typically very quick and very exothermic so it was essential to keep the materials cool while the ethyl chloroformate was added dropwise over 10 minutes, otherwise, non-homogeneous reaction products will be formed (Ulmer, 1999). The usual methodology for this was to keep the reaction vessel on ice, apart from when using 1, 4-dioxane, as the solvent would freeze under these conditions, so cold water was used instead.



133

Fig 28: Mixed anhydride of lithocholic acid with an unwanted carbonate formation on the 3-OH position, due to excess ethyl chloroformate (compound **133**).

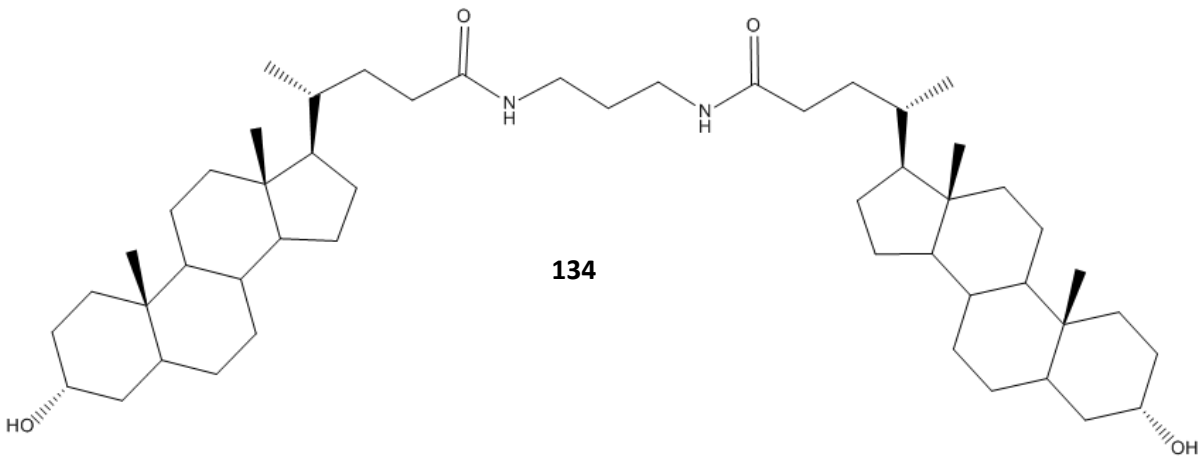
3.8.1 Inverse addition amide formation reactions



87

Fig 29: Compound **87**, an excellent germinator of *C. difficile* spores.

One compound that was tested as a germinant of *C. difficile* spores had a free NH_2 group present (compound **87**). The results received from this were very promising so more compounds with free NH_2 groups were thought to be the way forward. However, this was a difficult synthesis and wasn't successful with either deoxycholic acid or the cholic acid. The problem looks like dimers were formed (fig 30), even though the anhydride was added slowly in inverse conditions, the formation of dimers still occurred or there was such a multitude of compounds from side reactions, that purification was impossible. Although compound **30** was produced by aminolysis with a methyl ester and amine, the same methodology was applied for the other two bile acids, without the same success. This is one of the more unusual situations where the deoxycholic derivative is relatively easily synthesised in comparison to the other two bile acids. Usually, lithocholic and cholic acid derivatives were easier to synthesise/purify than the deoxycholic acid variants.



134

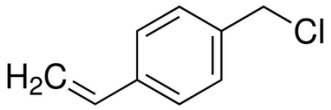
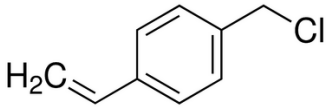
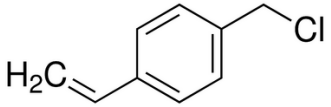
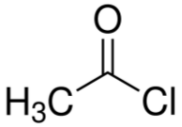
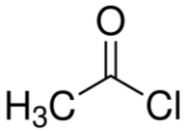
Fig 30: Possible dimerization formation from mixed anhydride induced inverse addition reactions (compound **134**).

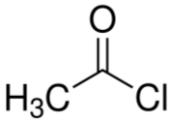
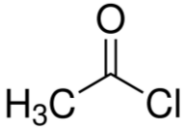
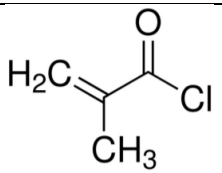
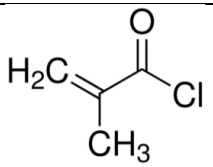
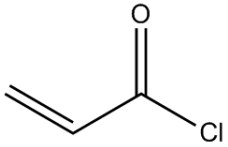
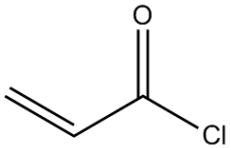
It was found that the ethyl chloroformate induced anhydride method was a reasonable method for the synthesis of bile amides on a 0.5 g scale. To produce enough material for the further quaternization of the material it was decided that a moderate scaling up would be required so as to produce all the required analogues while being as time efficient as possible. It was decided that a 2 g scale would suffice and the reagents were scaled up appropriately. However, it was found that if the reagents were

left in the same molar ratios when scaling up, the reaction would not occur. It was eventually found that for the scaling up of the material, 4 x the equivalent of the base (usually triethylamine) was required for the reaction to occur to a satisfactory yield.

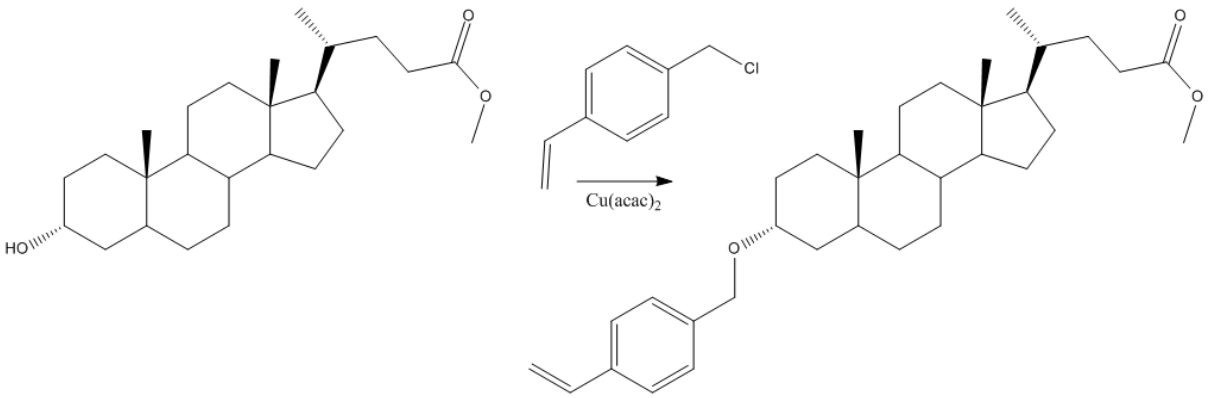
3.9.1Attachment of pendant groups on 3-OH

Table 6: All reactions attempted to manipulate the 3-OH moiety on lithocholic acid.

Parent bile acid	Attachment position/bond formation	Reagent	Base	Catalyst	Temperature ^{o}C	Time	Solvent	Yield
Methyl lithocholate	3-OH, ether			NaI	90	5 hrs.	Acetonitrile	N/A
Methyl lithocholate	3-OH, ether		NaH	NaI	ambient	24 hrs.	Acetonitrile	N/A
Methyl deoxycholate	3-OH, ether			$Cu(acac)_2$	120	24 hours	Vinyl benzyl chloride	N/A
Lithocholic acid	3-OH, ester				Ambient	30 mins	THF	N/A
Lithocholic acid	3-OH, ester				Ambient	30 mins	Ethylene glycol	N/A

Lithocholic acid	3-OH, ester			DMAP	100	24 hours	THF	N/A
Deoxycholic acid	3-OH, ester				100	48 hours	Ethylene glycol	N/A
Compound 61	3-OH, ester				ambient	4 hours	Chloroform	N/A
Lithocholic acid	3-OH, ester			DMAP	ambient	12 hours	Chloroform	N/A
Methyl lithocholate	3-OH, ester				ambient	24 hours	Chloroform	57 % **100**
Compound **93**	3-OH, ester				ambient	5 days	chloroform	6.77 % **102**

3.10.1 Ether linkage



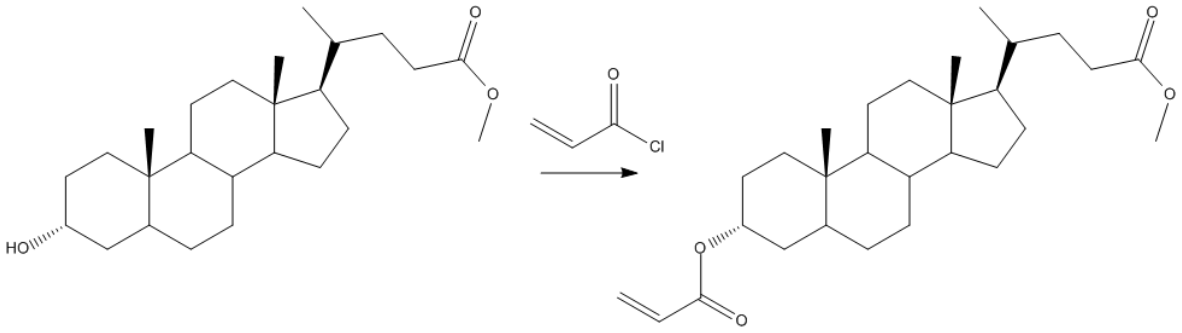
Scheme 14: Attachment of 4-vinyl benzyl chloride to methyl lithocholate.

The ultimate aim of the project was to produce a germinatory, antibacterial polymer bile amide compound which would be able to form a surface. Different methods were investigated to see which methodology would be the most effective at achieving this goal. As literature suggested that 3-OH was less important in the germination of *C. difficile* this was the position which was used as the target for polymer formation.

One failed attempt to attach a group to the 3-OH of lithocholic acid was using $Cu(acac)_2$ as a catalyst to attach an alkyl halide to the hydroxyl group to form an ether bond. Following the literature (Sirkecioglu et al., 2003) this is where heating of the two starting materials with a catalytic quantity of $Cu(acac)_2$ was shown to produce the ether bond in high yields. This was tried with vinyl benzyl chloride and lithocholic acid. In the literature this was successful with benzyl chloride and cholesterol, which are not that different.

Another attempt to form an ether bond on the 3-OH of the lithocholic acid was through the use of sodium hydride (Clayden, 2009). Sodium hydride is a very strong base, which is required to remove the hydrogen from the alcohol group, to produce an alkoxide ion. The vinyl benzyl chloride, which is a suitable electrophile, then reacts with the alkoxide, producing the ether. This reaction was tried several times, with differing concentrations of NaH, none of which was successful.

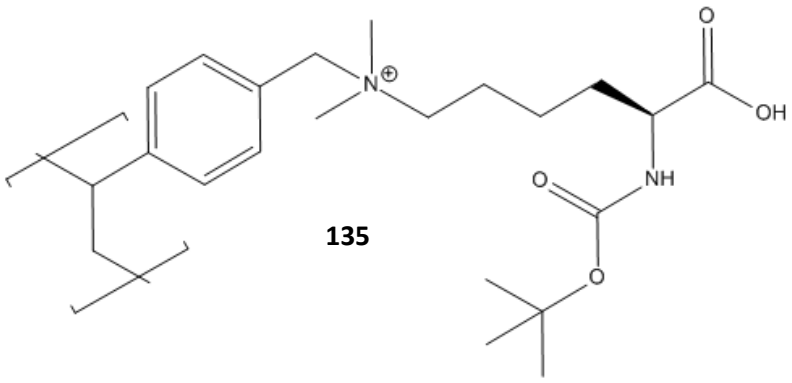
3.11.2 Ester linkage



Scheme 15; Formation of an ester linkage at the 3-OH position on methyl lithocholate, using acryloyl chloride.

To produce a potential polymer by attaching a polymerizable group to the 3-OH several different attempts were made, including the use of both methacryloyl chloride and methacrylic anhydride, which were both unsuccessful in relation to the bile acid structure. This was later found to also be true in the literature (Hu et al., 2005). When acryloylating the 3-OH with acryloyl chloride it was found that the determining factor as to whether the reaction would work or not was the amount of the acryloyl chloride relative to the bile acid/amide. There needed to be at least three times the equivalent amount of the acryloyl chloride for there to be completion of the reaction. Two equivalents was not enough and with this amount there was still a considerable amount of starting material after the same time frame had passed, thus identifying the amount as opposed to time being the critical factor in determining reaction success. No catalyst was used, but some literature suggest this might make a difference (Hu et al., 2005). Although the reaction time was not that long (~3 days), the catalyst may be helpful if the product turned into a commercial venture. The reason that acryloyl chloride was used was because the methacryloyl chloride could not be attached to the 3-OH. This was tried several times with different concentrations, but was not successful, unlike the acryloyl group.

3.12.1 Polymeric compounds.



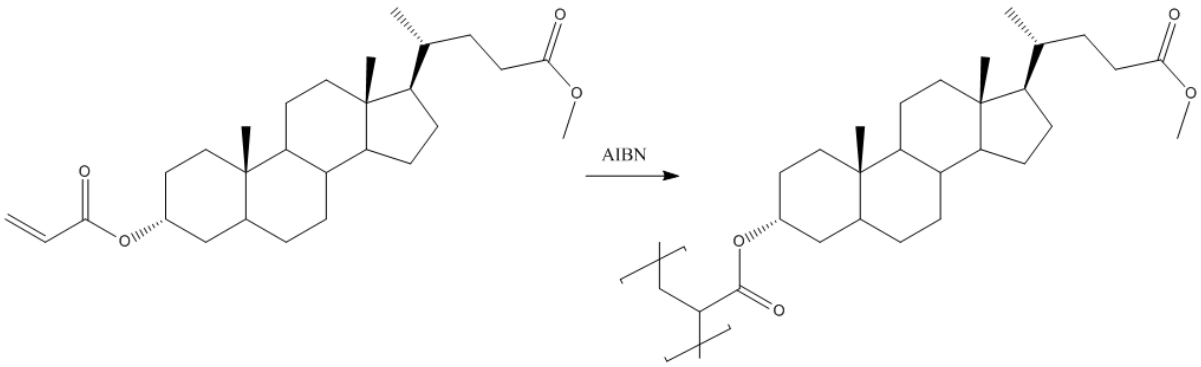
135

Fig 31: A potential polymerised co-germinant, based on L-lysine (compound **135**).

As co-germinants are an important factor in increasing the likelihood of germination, work was done on incorporating a glycine analogue (glycine shown to be the most effective co-germinant) and produce a polymeric version of this material. As it was hoped that introduction of a quaternised ammonium ion would produce an antimicrobial effect, this was initially attempted by using an alkylating preformed polymer to produce the material (**135).**

Boc-N', N'-dimethyl-L-lysine hydrochloride reacted with poly vinyl benzyl chloride for 24 hours. Removal of the solvent produced an insoluble white powder. Thus solution phase analysis was impossible. The fact that the product was completely insoluble yet the starting materials were very soluble in chloroform, would suggest that a reaction had taken place and it could be postulated that the material in fig 31 had been made. Since it was insoluble in most solvents, there is the possibility of crosslinking occurring. One possible type of crosslinking that could have occurred would be *via* polymer bound benzyl ether formation. This type of crosslinking usually requires elevated temperatures and a catalyst however, neither of which were present (Dahan and Portnoy, 2003). More success was found when working on the polymerizable amino acids towards the end of the project, although literature (Howerton et al., 2010) suggests that L-lysine was not a good germinant on its own, by manipulating the side chain something could be produced which might behave like a glycine in terms of co-germinating, due to the fact the stereochemistry was correct and the amino group and the

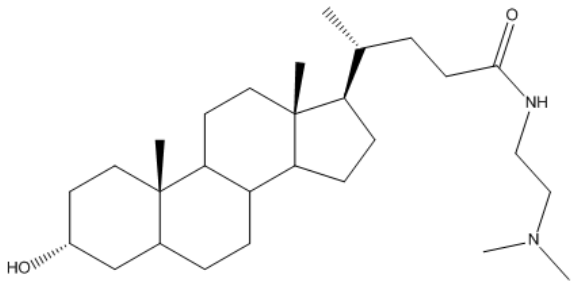
carboxylic acid group were both free, something which again, literature suggests is very important for activity.

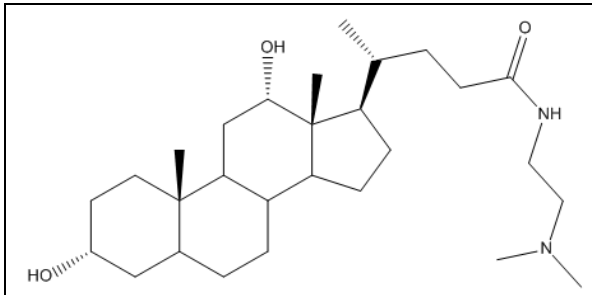
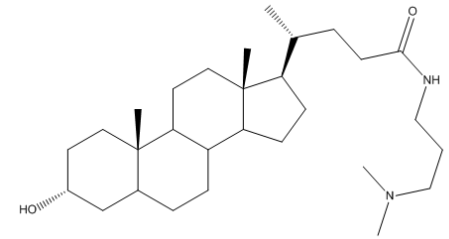


Scheme 16: Compound **101**, a methyl lithocholate which has had an acryloyl group attached at the 3-OH position, which has then been polymerised.

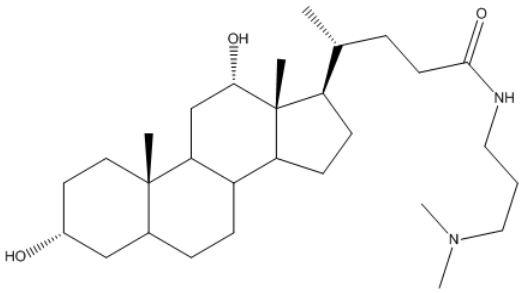
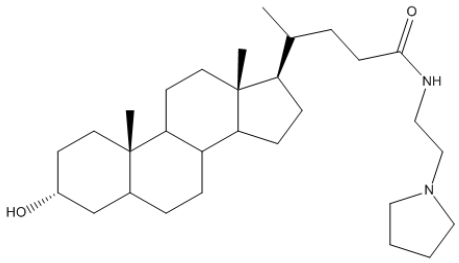
Compound **101** is the polymerised (at 3-OH) methyl ester of lithocholic acid. At room temperature bile acid acrylates are crystalline and are easily polymerizable (Ahlheim et al., 1986). This material was shown in the proton NMR spectrum to have polymerised by the loss of the two doublets from the acryloyl group (at 5.72 and 6.31 ppm respectively) and broadening of all the other steroidal peaks. What was unusual about this compound is the fact that although there was loss of key peaks and broadening of the 3-CH which would be expected, there was distinctly less broadening of the methyl peaks. There are two possible reasons for this, the first being that the polymers were of very short chain lengths, and therefore there was not much broadening of the methyl peaks. The second reason may be because the methyl peaks are so far away from the polymerised end, they still have sufficient mobility to allow relaxation of the ^{1}H nuclei and therefore broadening does not occur.

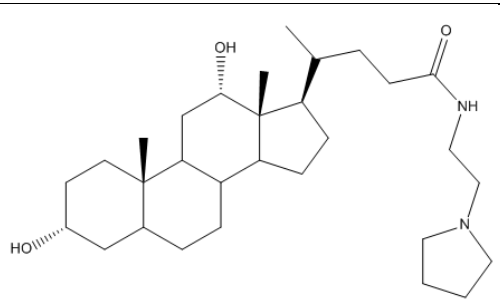
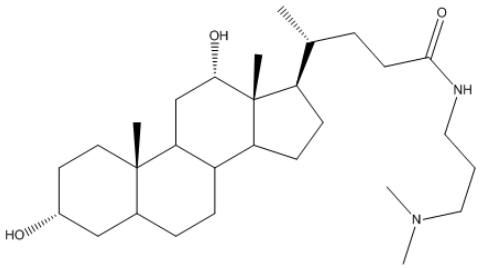
3.13.1 Quaternization of materials to induce antimicrobial activity.

Bile amide	Alkylating agent	solvent	Temperature ^{o}C	time	Yield	Prod compound number
 Compound 62	Iodomethane	Chloroform	ambient	24 hours	81.5 %	**103**
Compound 62	Ethyl iodide	chloroform	Ambient	24 hours	76 %	**105**
Compound 62	Allyl bromide	chloroform	Ambient	24 hours	54 %	**107**
Compound 62	Vinyl benzyl chloride	chloroform	Ambient	24 hours	26.9 %	**109**

 Compound 63	Iodomethane	chloroform	Ambient	24 hours	78.9 %	**104**
Compound 63	Ethyl iodide	chloroform	Ambient	24 hours	52 %	**106**
Compound 63	Allyl bromide	chloroform	Ambient	24 hours	53 %	**108**
 Compound 64	Iodomethane	chloroform	Ambient	24 hours	64.6 %	**110**
Compound 64	Iodopentane	dichloromethane	40	24 hours	50.7 %	**124**
Compound 64	Allyl bromide	dichloromethane	Ambient	24 hours	71 %	**112**
compound 64	Iodopropane	chloroform	ambient	96 hours	N/A	
Compound 64	Iodobutane	DCM	Ambient	48 hours	N/A	

Compound 64	Iodopentane	DCM	Ambient	24 hours	50.7 %	**124**
Compound 64	Iodohexane	DCM	Ambient	24 hours	N/A	
Compound 64	1-bromo-3-phenylpropane	DCM	Ambient	48 hours	N/A	
Compound 64	Cyclopentyl bromide	Chloroform	Ambient	~	N/A	
Compound 64	Benzyl bromide	chloroform	Ambient	48 hours	35 %	**113**
Compound 64	Vinyl benzyl chloride	chloroform	Ambient	48 hours	75 %	**114**
Compound 64	Poly (vinyl benzyl chloride)	chloroform	50	24 hours	20 %	**120**
Compound 64	Chloromethyl pivalate	chloroform	Ambient	96 hours	N/A	

 Compound 65	Iodomethane	chloroform	ambient	24 hours	75 %	**111**
Compound 65	Poly (vinyl benzyl chloride)	chloroform	Ambient	24 hours	81 %	**121**
 Compound 75	Iodobutane	Chloroform	Ambient	48 hours	N/A	
Compound 75	Allyl bromide	chloroform	Ambient	1 week	83 %	**107**
Compound 75	Chloromethyl pivalate	dichloromethane	Ambient	72 hours	N/A	

Compound 75	Vinyl benzyl chloride	dichloromethane	Ambient	48 hours	12 %	**119**
 Compound 76	iodomethane	chloroform	Ambient	1 week	66 %	**115**
Compound 76	Allyl bromide	chloroform	Ambient	24 hours	27 %	**116**
Compound 76	iodobutane	Chloroform	Ambient	48 hours	N/A	
Compound 76	Benzyl bromide	dichloromethane	ambient	24 hours	1.5 %	**118**
	Allyl bromide	dichloromethane	Ambient	96 hours	83 %	**117**

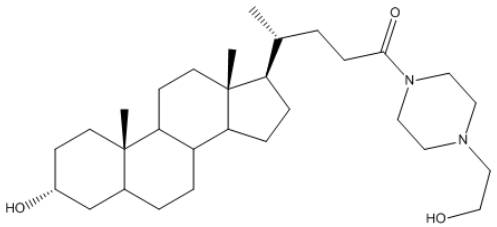
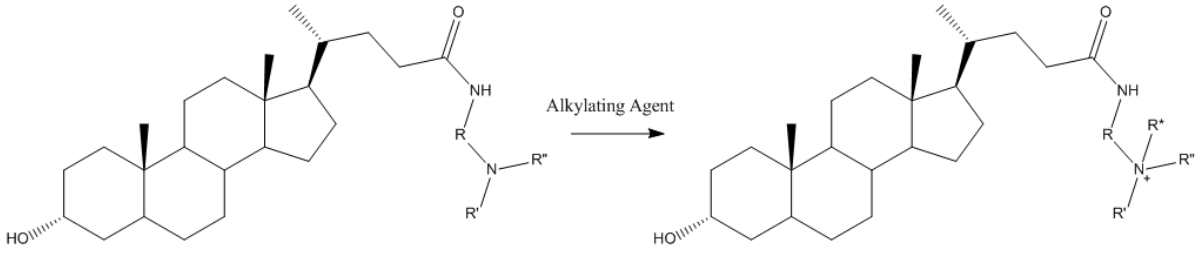
 Compound 94	Iodomethane	chloroform	ambient	24 hours	4 %	**122**
Compound 94	Allyl bromide	chloroform	ambient	24 hours	8 %	**123**
Compound 94	Ethyl iodide	chloroform	Ambient	48 hours	N/A	
Compound 94	Propyl iodide	chloroform	Ambient	2 weeks	N/A	
Compound 94	Butyl iodide	chloroform	Ambient	48 hours	N/A	
Compound 94	Iodohexane	chloroform	Ambient	48 hours	N/A	
Compound 94	Vinyl benzyl chloride	chloroform	Ambient	24 hours	38 %	**125**

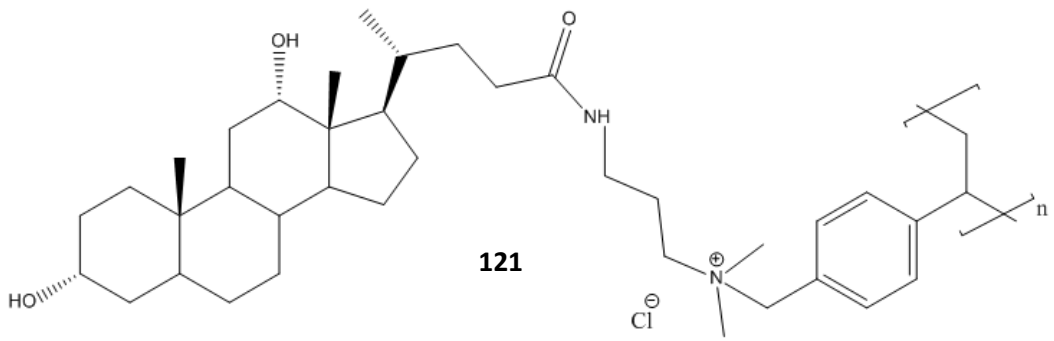
Table 7: All the alkylation reactions with amide analogues of both lithocholic acid and deoxycholic acid.



Scheme 17: Quaternization of a tertiary amine by an alkylating agent.

The purpose of quaternization of the tertiary nitrogen in the compounds was to try and induce some antimicrobial effects. Although the original idea was to produce a material which would initially germinate the *C. difficile* spore then, once it had germinated, the quaternized material would be able to kill it. What has actually happened is that a potential sporicide has been produced. This has the potential added advantage of being non-specific so would kill any bacteria which it came into contact with, which in a hospital setting, is a positive quality.

What was observed, out of a moderate range of differing alkylating agents, that products which had been quaternized with either iodomethane, allyl bromide or vinyl benzyl chloride, had a tendency to precipitate out of the chloroform solution in the purest form, in the quickest time (~1-2 days). It is clearly nothing to do with the counter ion as all three reagents used here precipitated. Iodoethane, iodobutane, iodopentane and iodohexane would always require a work up for them to be a pure product and would generally come out in a much smaller yield.



121

Fig 32: Compound **121**, a quaternized polymeric compound of deoxycholic acid.

Compound **121** (fig 32) is a polymerised, deoxycholic acid quaternized derivative. This compound has not been tested yet as a germinant. When synthesising this compound there was a 10 x equivalent excess of poly(vinyl benzyl chloride) 60/40 mixture of 3- and 4- isomers, therefore, even with 100 % completion, there would have only been 10 % quaternization. Once it had been established that the material was formed it was suggested that a higher loading should be tried. The reaction was completed again with 5 x equivalents of the bile amide. Once again the material precipitated out. To ensure the material was pure, it was washed in hot chloroform (as both starting materials were soluble in chloroform). Once washed the material was dried under vacuum. It was then discovered that this material was insoluble in H_2O, MeOH, DMSO, THF and toluene, so a proton NMR was not achievable. The suggestion for the insolubility would be that crosslinking had occurred. From the inherent structure, the only possible crosslinking that can be suggested is a Williamson ether linkage whereby the free benzoyl chlorides are reacting with the 3-OH to form an ether, thus crosslinking the material, thus making it insoluble (Clayden, 2009). The way to confirm or deny this proposition is through the use of solid state NMR or X-ray diffraction which was not available to the researcher at the time of the project.

Compound **120** was the lithocholic analogue of compound **121**. This was only tried with the higher loading of bile amide to encourage more bile acid pendant groups. However, like compound **64**, there is the possibility that crosslinking has occurred due to insolubility in DMSO, H_2O, TFA, AcOH, acetone, 1, 4-dioxane, pyridine and 1,2-dimethoxyethane. Again, like compound **121**, the only way to confirm the structure is through solid state, but the fact that the material precipitated out after a night in the container would indicate that there has been some quaternization occurring, due to the insolubility of the material in chloroform.

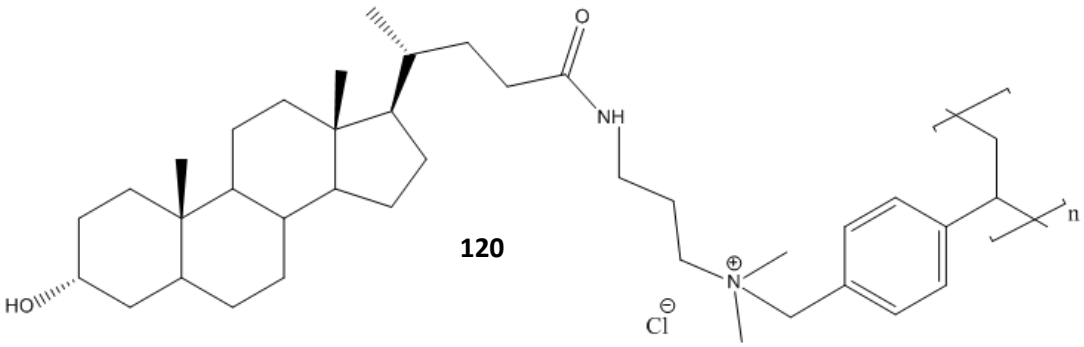


Fig 33; Compound **120,** a potentially antimicrobial steroidal based polymer.

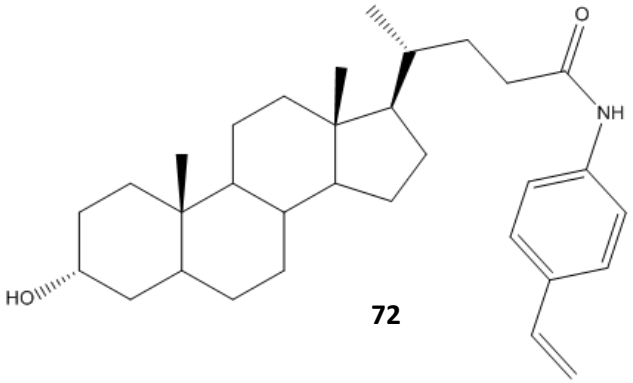
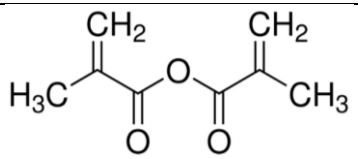
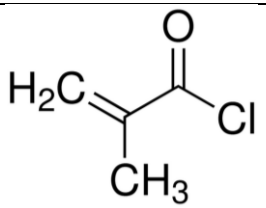
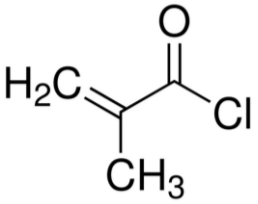
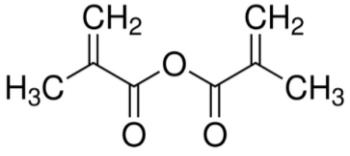


Fig 34: Compound **72**, an aniline derived bile amide.

Compound **72** was synthesised (along with deoxycholic acid analogue) as a potential polymerizable germinant. Although it is yet to be tested, there was some work on trying to polymerise the vinyl group attached to the material using AIBN as a free radical initiator. This was not successful and there was always a significant amount of starting material compared to potential product. Several attempts were made, usually extending the time of exposure to heat and also of adjustment of the amount of AIBN in the reaction vessel, all to no avail. This would seemingly suggest that once the amide functionality is somehow affecting the materials ability to polymerise, or perhaps there are certain steric constraints due to the steroidal material attached to the vinyl aniline.

3.14.1 Synthesis of amino acid analogues

Parent amino acid	Attachment position/bond formation	Reagent	Reagent 2	Catalyst	Temperature ^{o}C	Time	Solvent	Yield	Compound number
Boc-Lys-OH	Amine/amide bond				Ambient		Methanol	N/A	
Boc-Lys-OH	Amine/amide bond			DMAP	Ambient	24 hours	chloroform	N/A	
Boc-Lys-OH	Amine/amide bond		NaOH		Ambient	24 hours	1,4-dioxane	N/A	
Boc-Lys-OH	Amine/amide bond				Ambient	24 hours	1, 4-dioxane	N/A	

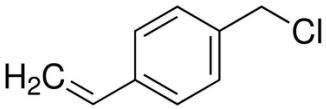
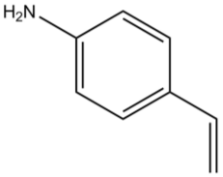
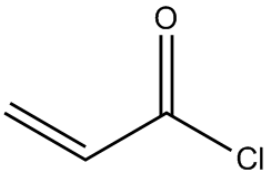
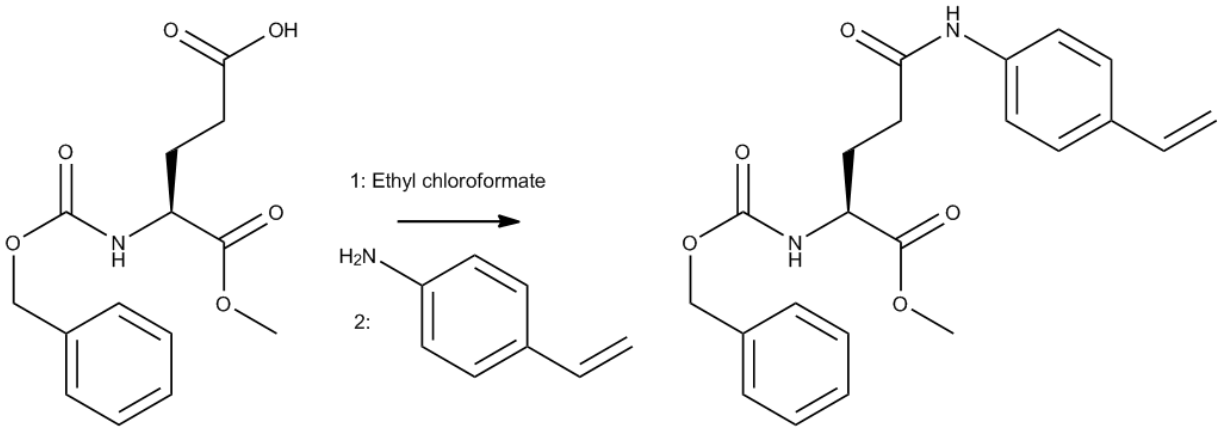
Boc-Ser-OMe	Hydroxyl/ether		Potassium carbonate	NaI	40	24 hours	Acetonitrile	N/A	
Z-Glu-OMe	Carboxylic acid/amide		N-(3-dimethylaminopropyl)-N'-ethyl carbodiimide		Ambient	24 hours	Dichloromethane	>20 mg	**126**
Boc-Lys-OH	Amino/ amide		NaOH		Ambient	2 hours	50/50 acetonitrile/ 2 M NaOH	13 %	**127**

Table 8: All the reactions attempted to make amino acid derivatives.

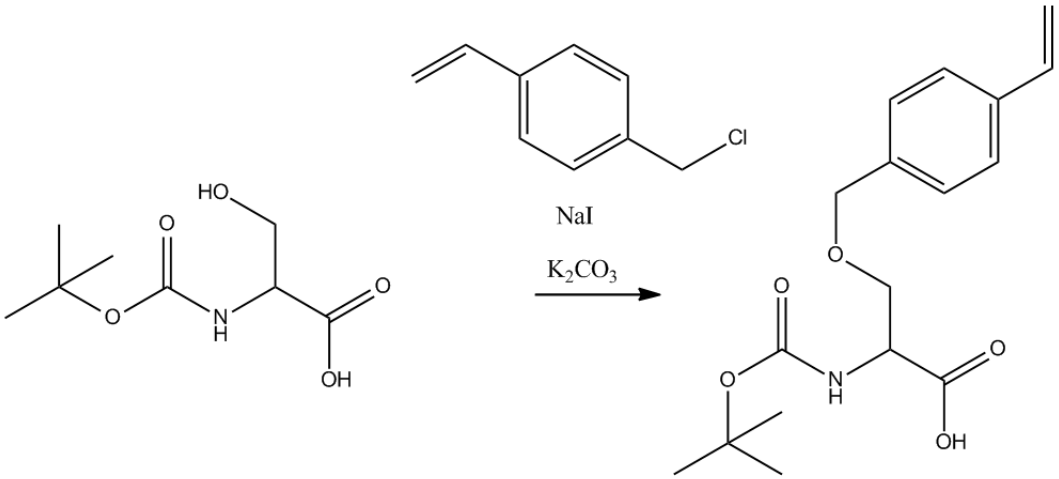
Once a relatively large potential germinatory database had been acquired, towards the end of the project, the focus shifted towards the preparation of polymeric amino acids. Howerton et al, 2010 suggest that glycine is the best co-germinant for germination of *C. difficile* spores so any polymeric analogue of this material would possibly also have some co-germinating ability.

The initial attempt to join a polymerizable moiety to an amino acid was 4-vinyl aniline attaching to Z-Glu-OMe *via* a mixed anhydride. It was observed during the reaction that the material started off colourless but over a period of 24 hours turned darker and darker till the solution was completely brown. A work up was done but no desired product obtained.



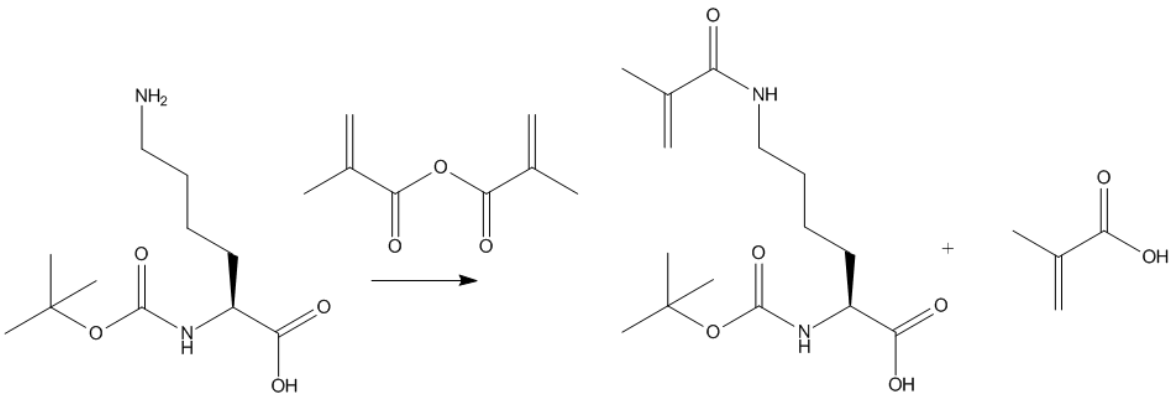
Scheme 18: Synthesis method for a failed coupling between Z-Glu-OMe and 4-vinyl aniline.

The second methodology was also unsuccessful. This involved the coupling of Boc-Ser-OH with vinyl benzyl chloride in a Williamson-ether synthesis with potassium carbonate as the base. After work up, none of the desired product was found. Perhaps the reason for failure was that potassium carbonate was not a strong enough base. If sodium hydride had been used this may have caused the reaction to go to completion.



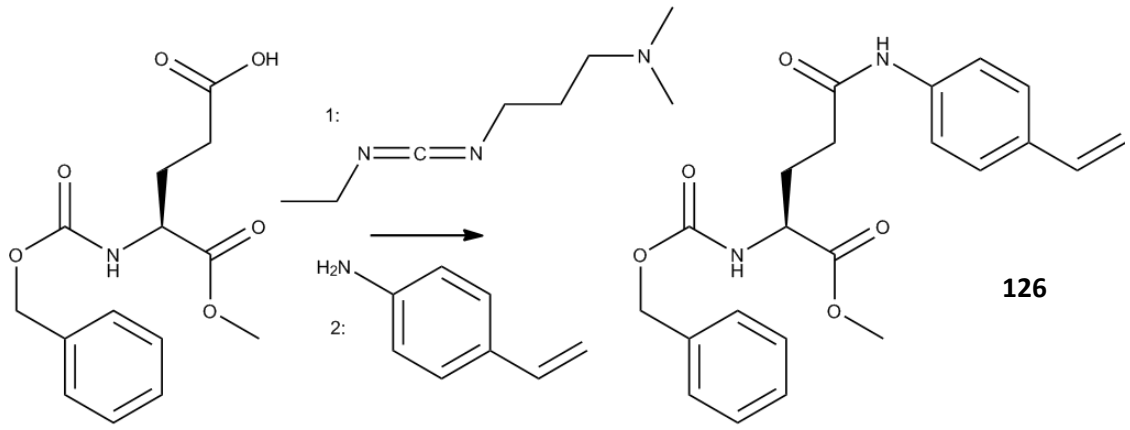
Scheme 19: Attempted coupling of Boc-Ser-OH with vinyl benzyl chloride.

The final attempt to attach an amino acid to a polymerizable group was the reaction of Boc-Lys-OH with methacrylic anhydride. This *in-situ* reaction unfortunately did not work and none of the desired compound was recovered.

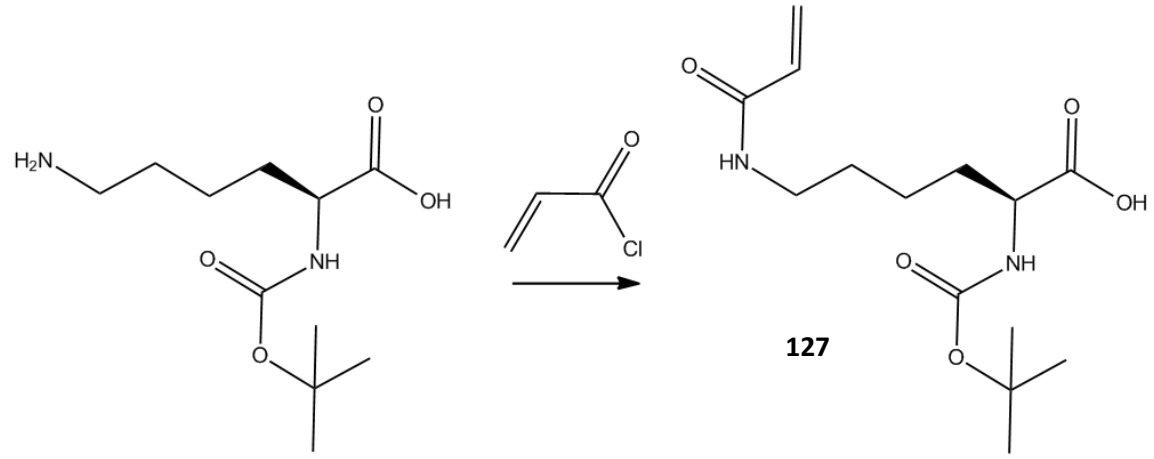


Scheme 20: Attempted coupling of methacrylic anhydride to Boc-Lys-OH.

Compounds **126** and **127** are examples of successful attachment of polymerizable groups to amino acids.



Scheme 21: Synthesis of manipulated amino acid (**126)** using a carbodiimide coupling agent.

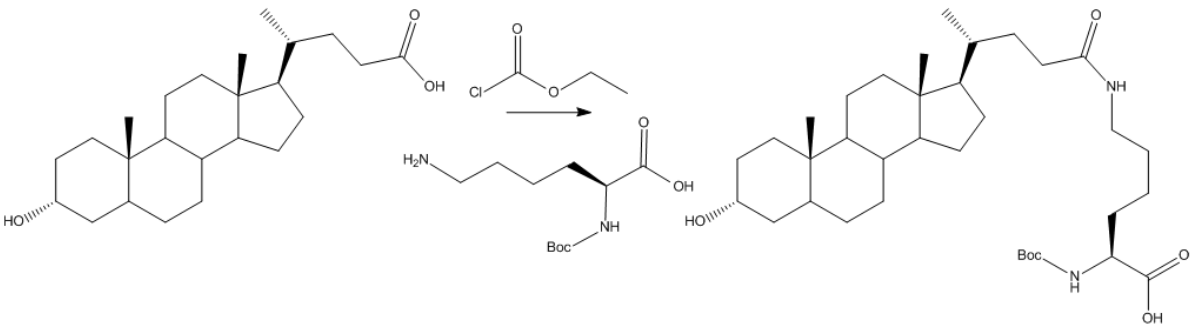


Scheme 22: Compound **127**; a monomeric polymerizable amino acid analogue.

Compound **126** was successfully synthesised from Z-Glu-OMe and 4-vinyl aniline using N-(3-dimethylaminopropyl) N'-ethyl carbodiimide hydrochloride as the coupling agent. This reagent is specifically used for formation of amide bonds, but is more usually used for peptide synthesis. This compound was hard to purify as the proton NMR strongly indicated there was an impurity, which could not be visualised *via* TLC. Eventually it was removed through precipitation in water from methanol. There was an attempt to polymerise the material, but in the short time left, this was not achieved. Given that the bile amide derivative with vinyl aniline attached didn't polymerise, then it may be that vinyl aniline derivatives do not polymerise well.

The synthesis of compound **127** did not require a coupling agent. Acryloyl chloride was used to produce the amide bond. Although it was a low yield, the material was successfully synthesised. There was no time to attempt polymerization of this material. As there has been successful polymerization of acryloyl chlorides with bile acids attached, there may be the possibility of being able to produce a co-polymer containing a co-germinant with a germinant.

As mentioned in the introduction there is a lot of evidence to back up the idea that glycine is a co-germinant of *C. difficile* spores. There was a suggestion of attaching Boc-Lysine-OH to methyl lithocholate through an aminolysis reaction. This produced a TLC with 6 individual spots on the plate, so the reaction was discontinued, as the purification would not be worth the small amount of material recovered. However, this reaction was successful with ethyl chloroformate for both the lithocholic acid and deoxycholic acid, but not with cholic acid.



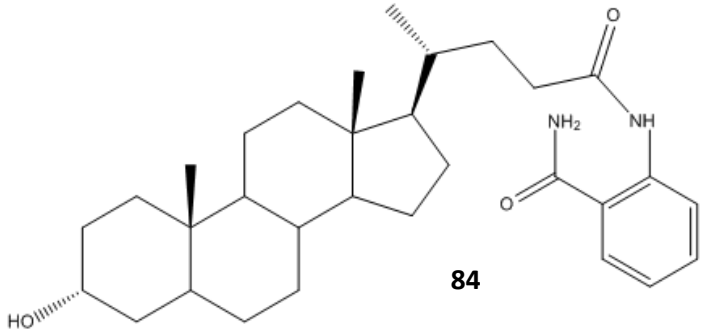
Scheme 23: Mixed anhydride method of attaching an amino acid group to a bile acid.

3.15.1 Bile amides containing fluorescent side chains-anthranilamide

Three bile amide analogues containing 2-aminobenzamide were synthesised to see whether, upon germination testing, staining of the spores or vegetative *C. difficile* cells could be observed under a confocal microscope. This could potentially give more insight into the binding mechanism of the germinants and thus give more data about how germination actually occurs in *C. difficile.*

The three analogues were derivatives of lithocholic acid, deoxycholic acid and cholic acid. Before submission for germinating studies (which was undertaken by microbiologists Kristian Poole and

Amber Lavender), the materials were placed in a 96 well-plate and scanned using a UV spectrometer. From this analysis it was found that the optimum excitation limit was 300 nm and the optimum emission was 464 nm. This result was observed for all three bile amide analogues. If the fluorescent bile acid analogues were to bind to the spores, but not germinate them, this may give some indicator of binding sites on the spore surface. If the bile amide analogue did germinate the spores, it could be a potential germinant. However, under the confocal microscope, after washing, it was found that the material had not bound to the spores or caused germination. This was the same for all three analogues, so research in this particular area was discontinued.

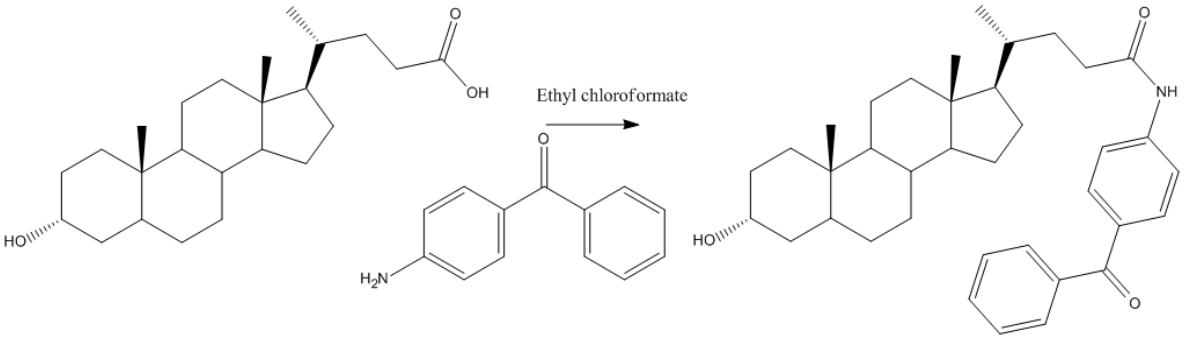


84

Fig 35: A bile amide analogue with 2-aminobenzamide (compound **84**).

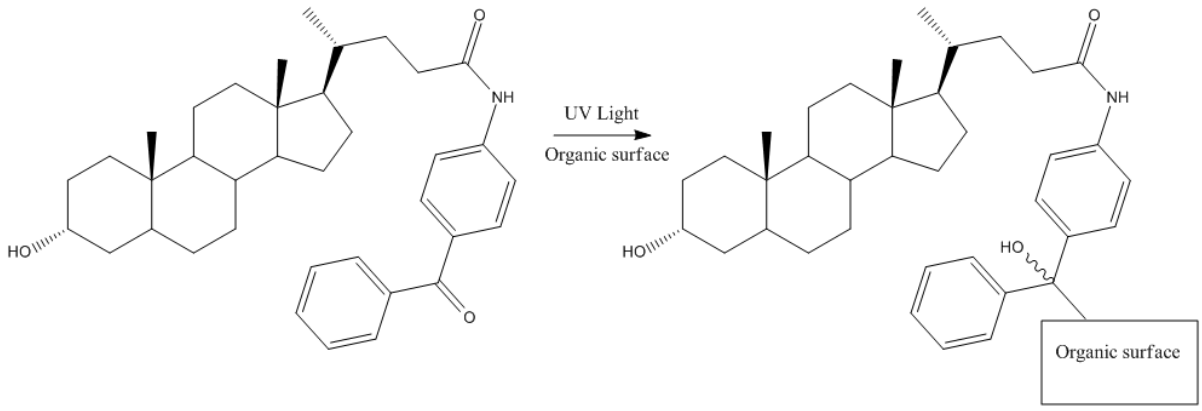
A similar material (compound **91**) also had the presence of the aminobenzamide group and it was hoped the material would be able to be observed interacting with *C. difficile* spores in some way. At the time of writing, no testing on this material had been done.

3.16.1Benzophenone-based materials.



Scheme 24: Synthesis method for a coupling between lithocholic acid and 4-amino-benzophenone.

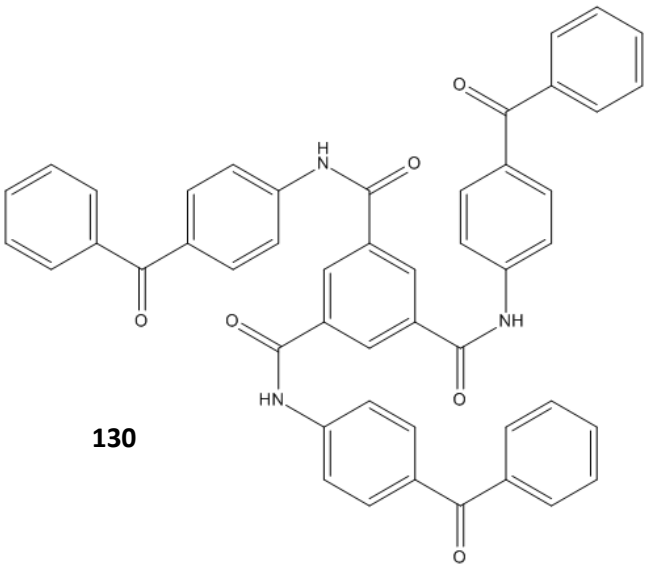
Benzophenones, through a process called hydrogen abstraction, form radicals, which have been shown to chemically attach to any organic material which contains a C-H bond and therefore become incorporated into the material. Thus an entire surface of benzophenone based material could be produced by exposing them to UV radiation and reacting with each other and the surface (Scheme 22). This would be another way of producing a "polymer" like surface and would in theory be a better option, as the material could be reapplied much more easily. The reason for the synthesis of the benzophenone based compounds, including the three bile acid derivatives was based on the literature that exposure of benzophenone moieties to UV radiation initiates hydrogen abstraction. This creates radicals on the benzophenone structure, which can attach to any C-H group, thus providing a surface with potential germinating materials on it.



Scheme 25: Binding of benzophenone based bile amides to organic surface containing C-H bonds.

The fact that no incorporation into the pre-formed polymer structure occurred, suggests several possibilities for not working. The first is that the bile amide with benzophenone and the addition of an amide group next to it, reduced the ability of the benzophenone to undergo hydrogen abstraction, thus no polymer was formed. The second may be the selection of polymer used, which is unlikely as it was a benzophenone based polymer, which would make the most sense to be attached to, as they are inherently attached to each other. Another reason may be length of exposure. The problem with exposure times is that the environment becomes quite warm and therefore there is a substantial loss of solvent, which needs to be replaced.

129



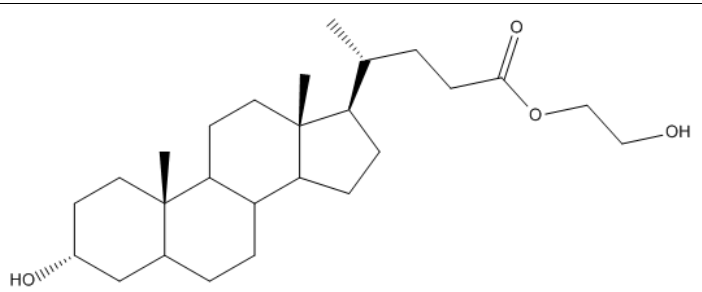
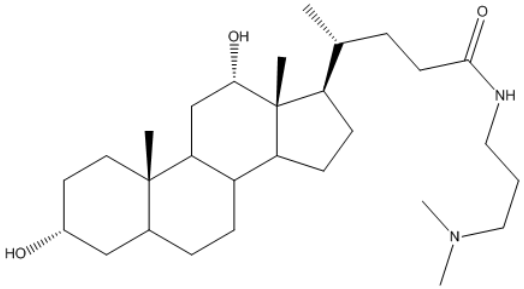
130

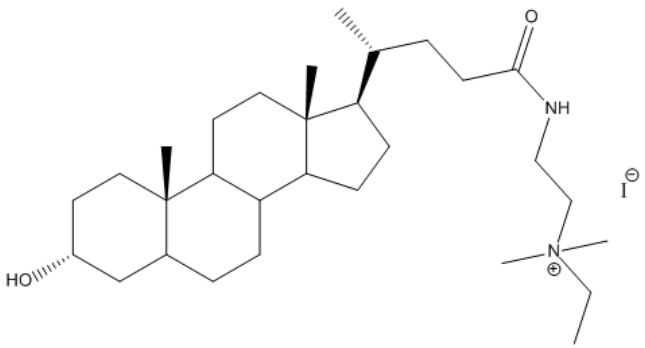
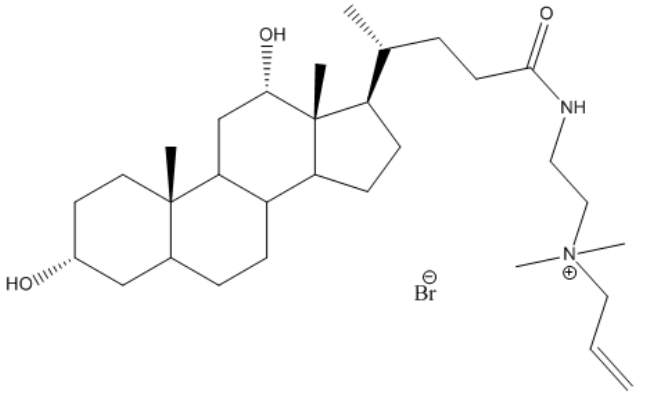
Fig 36; Compounds **129** and **130**, used in the potential production of benzophenone based surfaces.

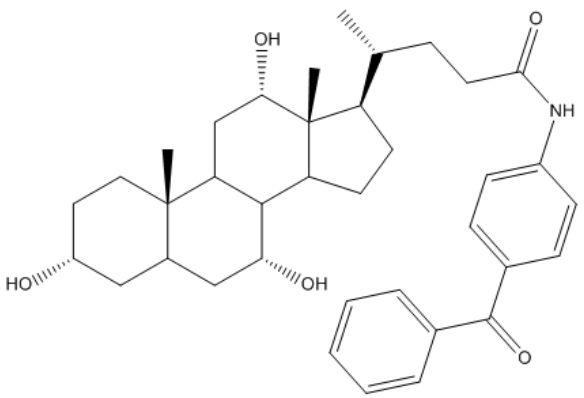
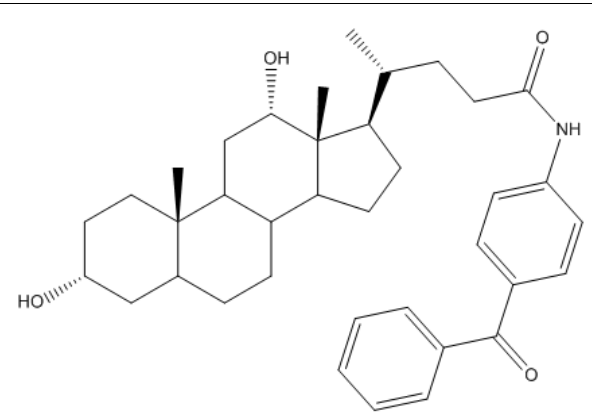
The reason the other two compounds (**129** and **130**) were synthesised was the possibility of attaching these materials to other surfaces, then attaching the bile amide version to it, thus having an *in-situ*

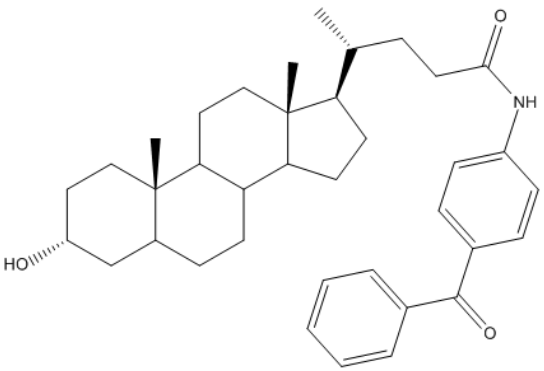
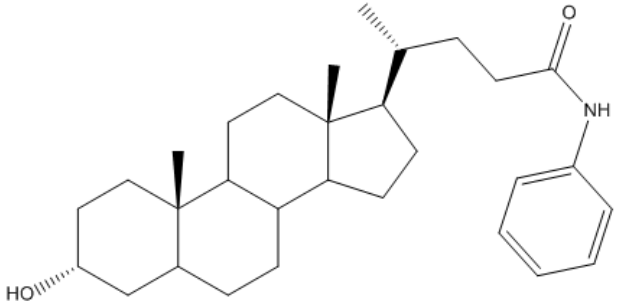
polymer preparation. Due to the lack of hydrogen abstraction on the normal polymer and then the added knowledge of the lack of germinating ability of the material, this idea was discontinued.

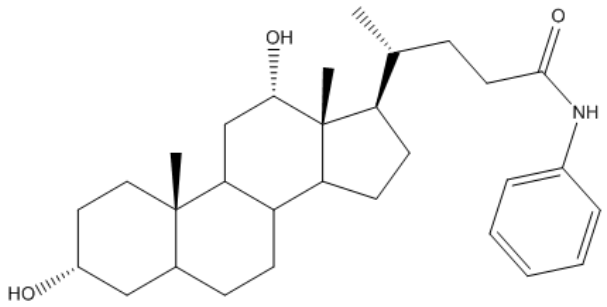
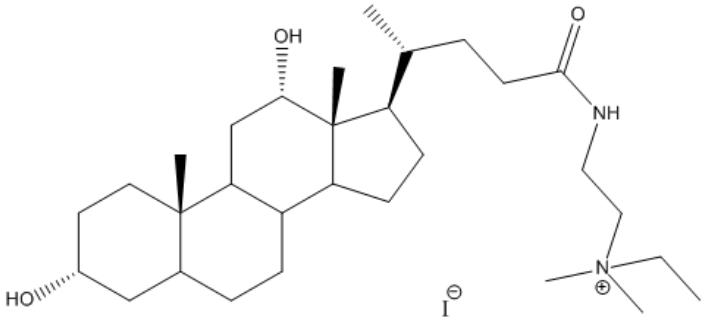
4.1.1 Germination results

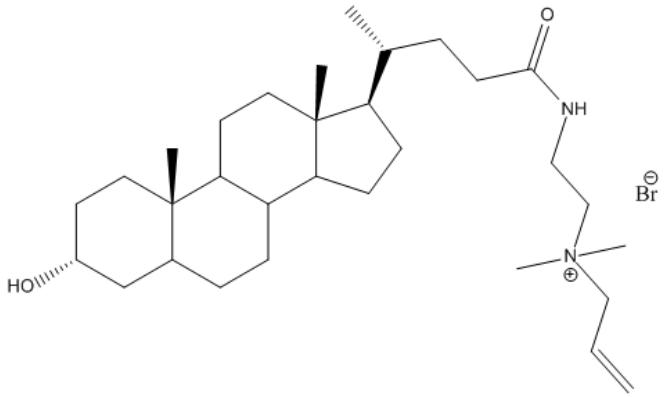
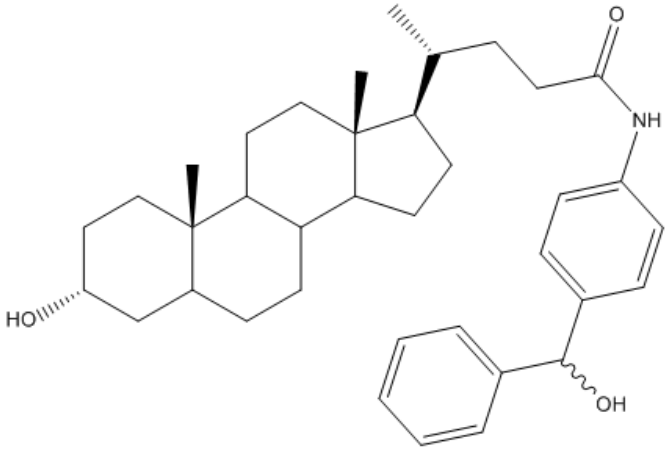
Compound number	Compound structure	Log reduction ice 11204	Log reduction ice 027	Log reduction heat 11204	Log reduction heat 027	Inference
60		0.69	0.92	0.78	0.72	Germinant/potential antimicrobial/sporicide
65		1.22		1.79		Germinant

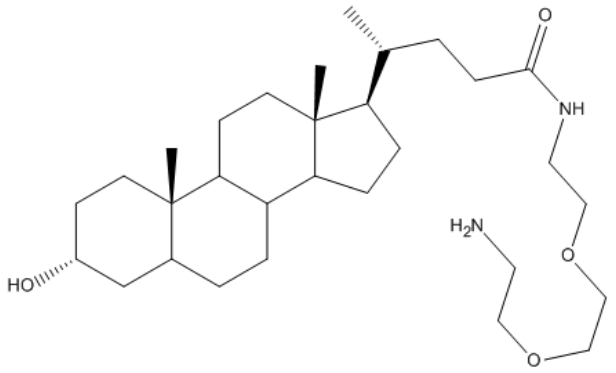
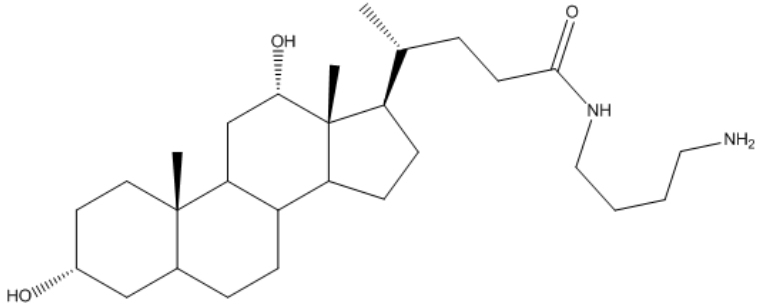
105		0	0	0	0	No activity
108		0	0	0	0	No activity.

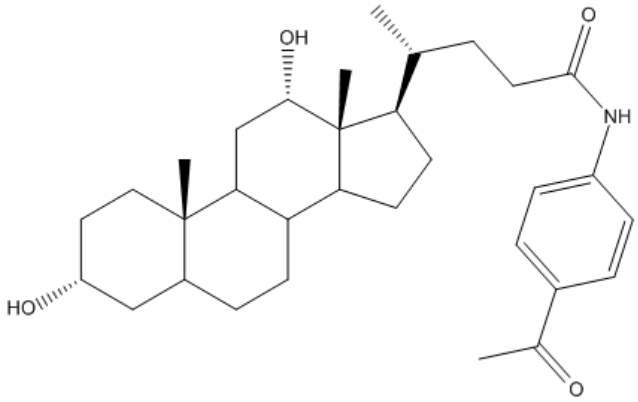
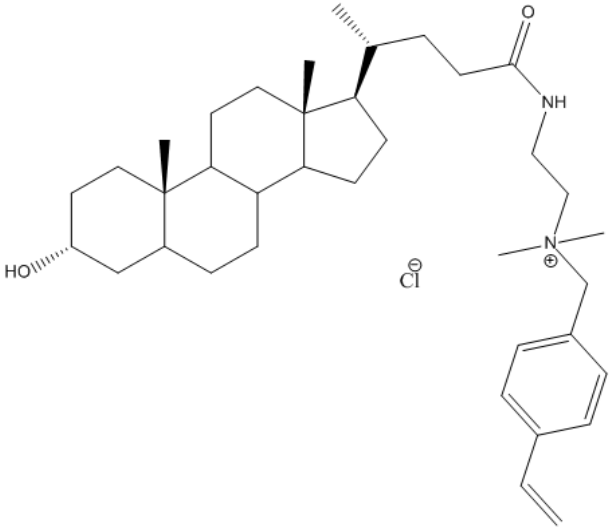
81		0.38	0	Not recorded	Not recorded	Negligible germinatory behaviour.
80		0	0	0	0	No activity

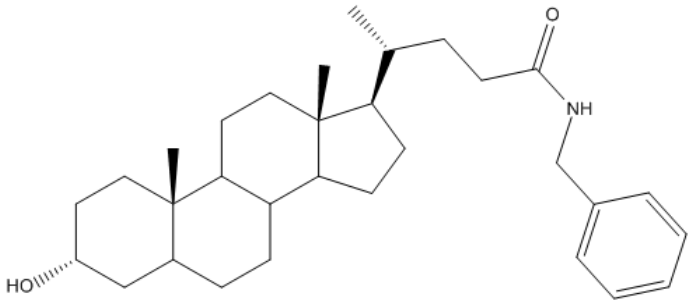
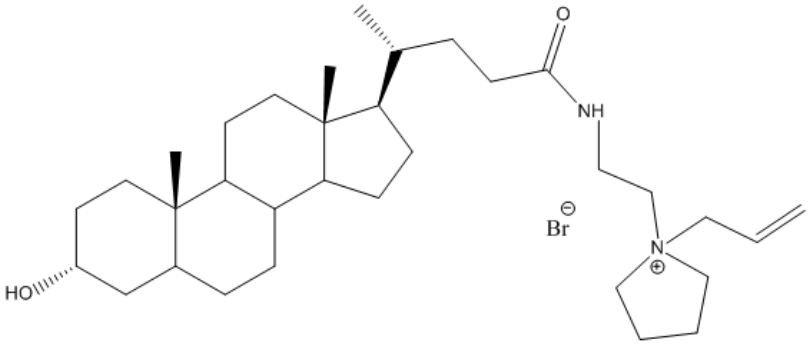
79		0	0	0	0	No activity
70		0	0	0	0	No activity.

71		0	0	0	0	No activity
106		0.75	0	1.38	0	Potential germinant for strain 11204 only

107		0	0	0	0	No activity
82		0.46	N/A	N/A	N/A	Partial germinatory behaviour

88		0	0.45	0	0	Negligible germinating ability.
87		2.62	1.72	3.37	2.91	Good germinant. Probable antimicrobial/sporicide.

92		0.96	0.27	N/A	N/A	Germinant.
109		1.32	1.27	2	1.5	Germinant. Possible antimicrobial/sporicidal activity as well.

93		0	0	0	0	No activity
116		1.05	N/A	0.71	N/A	Germinant of 11204. Also possible antimicrobial/sporicidal activity.

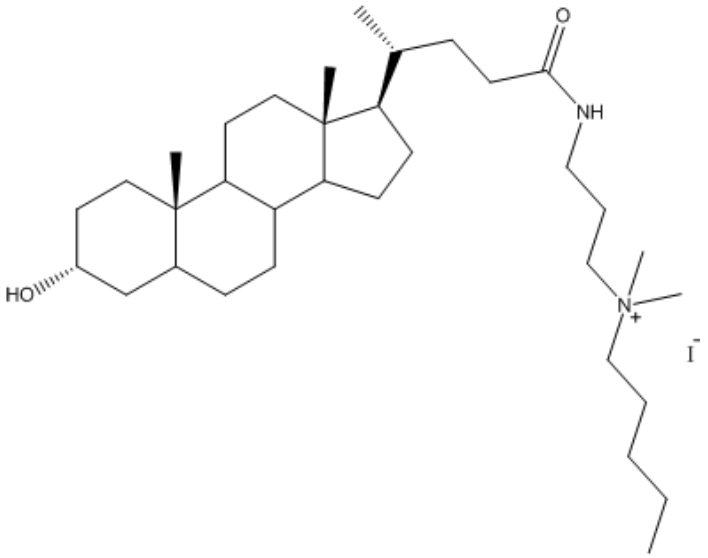
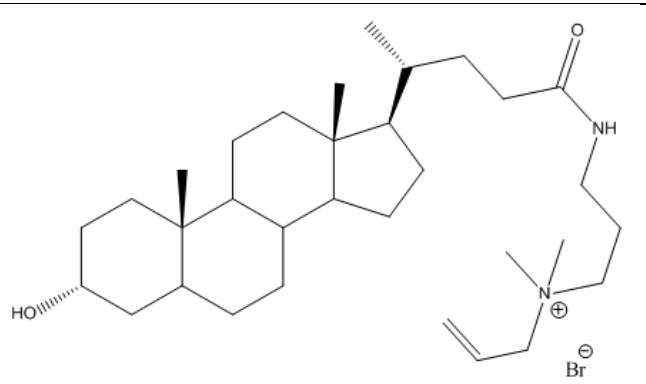
124		N/A	1.58	N/A	2.08	Germinant of 027. Possible antimicrobial/sporicide action.
112		0	0	0	0	No germination activity.

Table 9: Germination results for bile amide derivatives against *C. difficile* spores.

Table 9 gives the log reductions, in both heat and ice of all the compounds which were able to be tested; in due time it is hoped that this database will be increased. The reason for using the ice and heat method was to determine potential antimicrobial ability. After testing, the plates were read to see the number of colonies available. If there was a reduction on the heat, this meant that the compound being tested had germinated the spores and any vegetative cells destroyed by the heat. If there was a reduction in colony numbers on ice this meant that the material was either germinating, then killing the vegetative cells or was simply a sporicide.

Due to the insolubility of certain compounds in certain solvents and the fact that chloroform is not allowed to be used as a solvent for biological testing, the solvents themselves (40% ethanol, 80 % DMSO, 100 % DMSO) were run. It was shown that the use of these solvents did not have an effect on the spores ability to germinate. Before analysis was completed, statistical testing was done on the data, which showed that there was no significant difference between the two different strains tested. Therefore, if there is a difference, this is specifically related to the compound which is being tested.

The method used by the microbiologists is as follows:

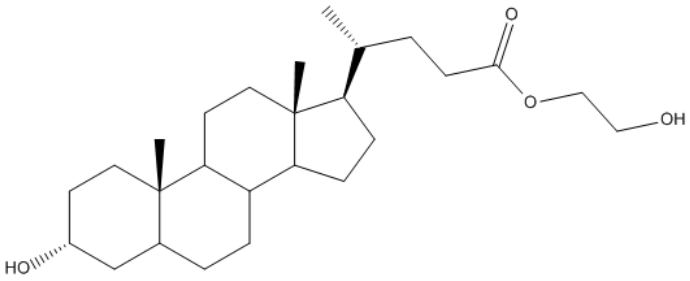
A 2% (w/v) sodium taurocholate comprising double strength thioglycolate germination solution was prepared as Wheeldon *et al* (2008). 200µL was added immediately, in triplicate, to 200µL *C. difficile* NCTC 11204 spores from an original stock suspension of ~10^6cfu/mL in microfuge tubes and vortexed. After 1 hour exposure, 600µL sterile distilled water was added to the test samples and vortexed, and then subsequently heated in a water bath to 75°C for 20 minutes. Control samples contained 200µL spore suspensions and 200µL sodium taurocholate germination solution, after 1 hour exposure 600µL sterile distilled water was added to samples, vortexed and were subsequently stored over ice for 20 minutes.

Following heat-shock, serial dilutions were performed and samples were inoculated onto Fastidious Anaerobic Agar comprising 5% (v/v) defibrinated horse blood and 0.1% (w/v) sodium taurocholate. Serial dilutions and inoculations were repeated for control samples.

Method adapted for alternative germination solution consisting 5mL DMSO and 5mL ‘Tween 20’ in germination solution comprising 2% (w/v) sodium taurocholate and double strength thioglycolate medium.

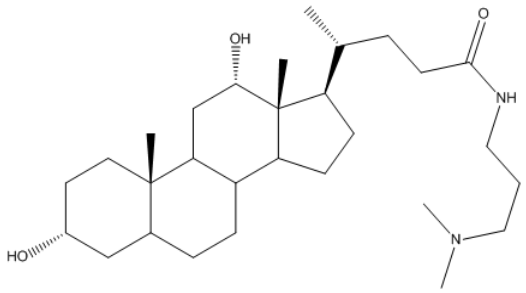
When referring to log reduction of spore counts, 1 log reduction would be equivalent to a 90 % reduction in the spore count, 2 log reduction would be 99 %, 3 log would be 99.9 % and so on.

Compound 60



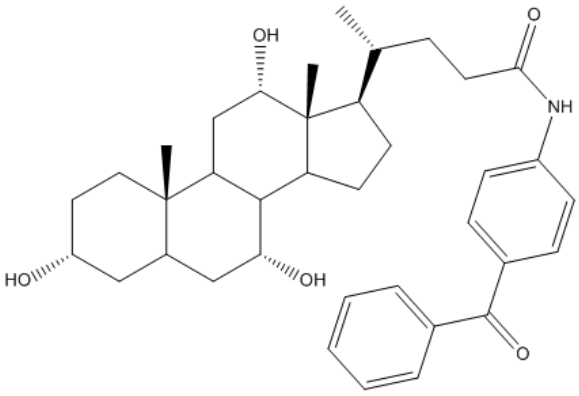
From the literature (Howerton et al., 2010) it would be hypothesised that as this material is both a lithocholic derivative and has a ester bond instead of a amide bond that it would have no *C. difficile* spore germinating ability whatsoever. However, while testing with ice, for strains 11204 and 027 the log reduction in number was 0.69 and 0.92 respectively. This shows that while there is not a huge amount of germination occurring, this particular analogue is a better germinant than lithocholic acid on its own. The effect that the ester bond has on the material’s germinating ability may be somewhat displaced by the presence of the terminal hydroxyl on the end of the ethylene glycol group, which may help to improve germinating ability. With heat there was a larger log reduction in the strain 11204 than in strain 027 (0.78 and 0.72 respectively). The fact that there is similar log reduction (and therefore spore count) in both the heat and the ice would imply there may be some antimicrobial ability, because the ice itself would not be able to kill any vegetative cells. Whether the material is sporicidal or antimicrobial is not clear and could not be proved unless testing on another spore forming bacteria were to be attempted (such as *Bacillus subtilis*).

Compound 65



From the literature (Howerton et al., 2010) it would be hypothesised that as this material is a deoxycholic acid derivative and has an amide bond that it would have some *C. difficile* spore germinating ability. When tested with ice, for strains 11204 (027 not recorded) the log reduction in number was 1.22. With heat 11204 had a log reduction 1.79. The fact that there is a higher reduction with ice would imply there is no antimicrobial ability, and this material is purely a germinant. There is no quaternized nitrogen in this material so there was no expectancy of antimicrobial ability, but as it had an amide bond and two hydroxyls, some germination was expected. As this has moderate germinating ability it should be noted that there are three carbons in the functional chain, as later materials, with differing chain lengths possess different properties.

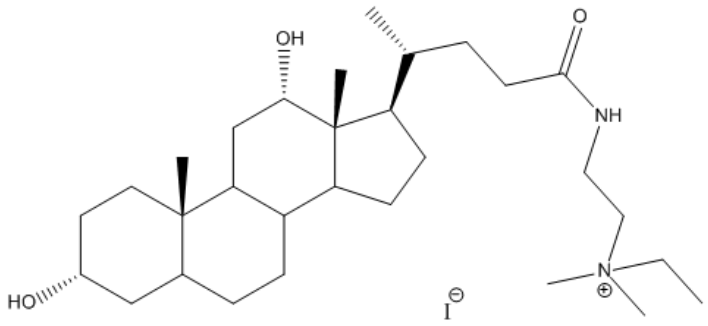
Compound 81



Out of all the three bile acids being worked on in this project, cholic acid has been consistently reported in the literature to be the better germinant, compared with both deoxycholic and lithocholic acid. In the case of strain 11204 this particular cholic acid analogue, the log reduction of the material

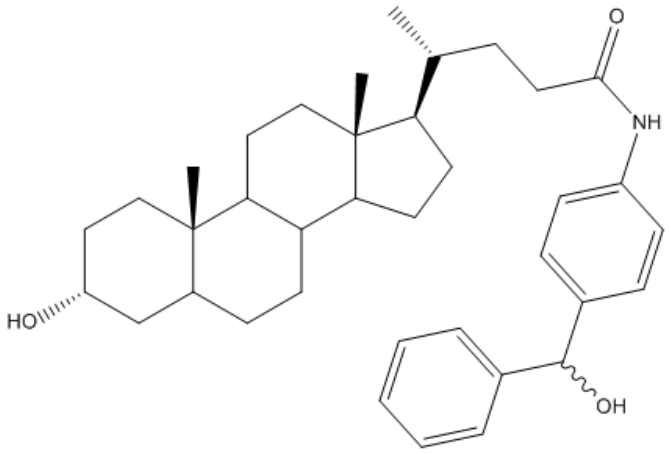
with regard to germination was 0.38, which is insignificant as a germinant. With the 027 strain there was no germinatory ability present whatsoever. This shows that clearly the benzophenone side chain group is a significant inhibitor of the ability of the cholic acid fragment to induce germination.

Compound 106



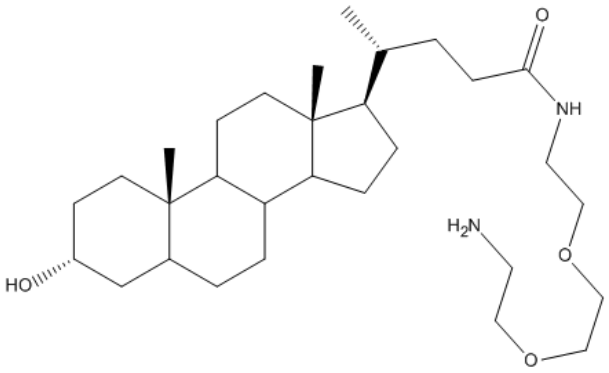
This material is similar to compound **105** but the difference between them is the fact that this is the deoxycholic acid version, as opposed to compound **105** which is the lithocholic acid version. The addition of the extra hydroxyl at position 12 improves the germination properties of this material. There is no improvement with regards to germination of strain 027 but 11204 has a log reduction difference of 0.75 on ice and 1.38 on heat. This seems to go along with the current literature, which specifies that deoxycholic analogues generally, are better than lithocholic acid versions. The fact that there is an improvement, by the addition of an extra hydroxyl, seems to suggest that the counter ion is not playing an important part in the germinatory process, or at least, in this case, iodine is not a problematic counter ion.

Compound 82



This compound is the reduced version of compound **79**, and while that compound had no germinatory effects whatsoever, compound **82** has a log reduction on ice of 0.46 in strain 11204 (027 no significant effect). The only difference between the two compounds is the reduction of the ketone to produce the new hydroxyl group. This extra hydroxyl has clearly had an effect on the germinatory behaviour, even if it is a small effect. It would appear that the introduction of hydroxyls into the side chain can have a positive effect on the promotion of germination. Unfortunately, there was no time to synthesise and test the other analogues of this material to see if this was just an improvement for the lithocholic acid or whether it would be across the entire analogue series.

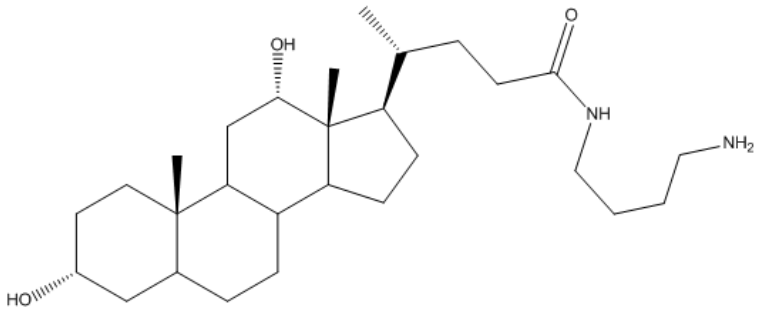
Compound 88



This material only showed log reduction of germination in one of the strains which was tested on. In strain 027 there was a log reduction of 0.45, whereas in 11024 there was no reduction. The fact that

this is the lithocholic version means this would be the least likely to be a germinant, however, it would be a good idea to test the cholic acid version (which has been synthesised), to see if there was any improvement in the germinating ability. It may be that the side chain is too long and flexible so may not be able to interact with the active site, but free NH_2 groups seem to have a positive effect on the germination ability, much like free OH on the side chain do, but the lack of hydroxyls on the steroid structure may be limiting the germinating ability.

Compound 87

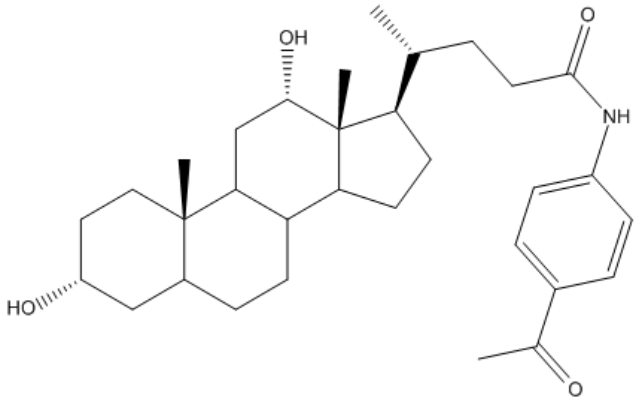


Compound **87** was the most successful germinant produced in this project. On ice for strain 11204 it has a log reduction of 2.62 and for 027 it has a log reduction of 1.72. On heat the log reduction is even higher with strain 11204 having a log reduction of 3.37 and for 027 the log reduction is 2.91. So while this product is clearly an excellent germinant of *C. difficile* spores the large log reduction even on ice, would imply that there is some antimicrobial/sporicidal activity also taking place as there is a large log reduction. The fact that this compound is a deoxycholic analogue ties in with the literature on this compound as a germinant. It is an amide and, similar to earlier compounds with 2 carbons in their side chain (which had no germinatory properties in other compounds). Perhaps the most important factor though is the presence of the free NH_2 terminal group which is similar to compound **31** (which was able to induce some germination). It would appear from this data that the 7 hydroxyl is not essential for germination but that a free amine is. If the analogues were to be synthesised and tested, this may provide more information about the particular importance of hydroxyl positions on this type of compound. The analogues of this compound (lithocholic and cholic) were attempted but proved to be problematic in their syntheses and purification. As the testing wasn't done immediately, the

importance of these molecules was missed and therefore any future work should definitely involve these analogues as a priority.

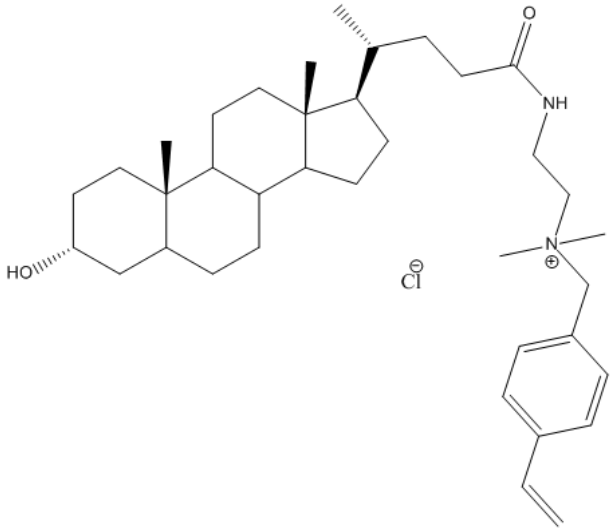
This particular compound is not dissimilar to the compound **57** Lithocholyl-N-2-(2-aminoethyl)amide, which is a proven antimicrobial (Ahonen et al., 2010). The key similarities are the steroid backbone, the amide bond and the free amine group at the end of the side chain. This particular side group may have antimicrobial properties which need to be fully investigated.

Compound 92



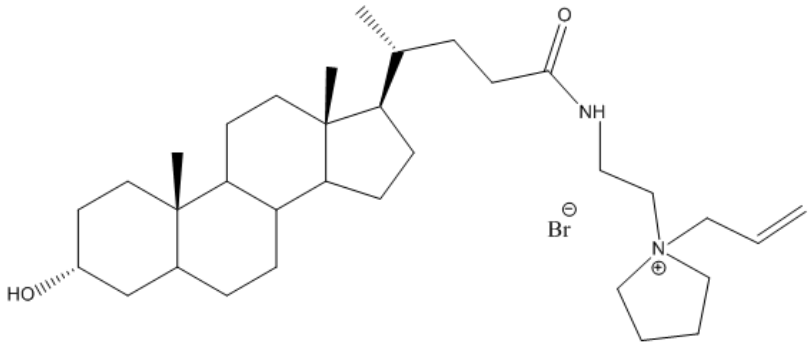
This compound had a log reduction of 0.96 and 0.27 for strains 11204 and 027 respectively. This structure is very similar to compound **71** except for the addition of a methyl ketone, which, evidently, has increased the germinating ability to a small level from no activity. This would imply that the suggestion of the benzene ring being too large to perhaps fit in the binding site was wrong, as there is no other difference between the two compounds. Perhaps the ketone entity, allows for a greater amount of hydrogen bonding, similarly to the free hydroxyl groups, found on other germinating compounds, and it is this hydrogen bonding ability which has increased its germinating ability.

Compound 109



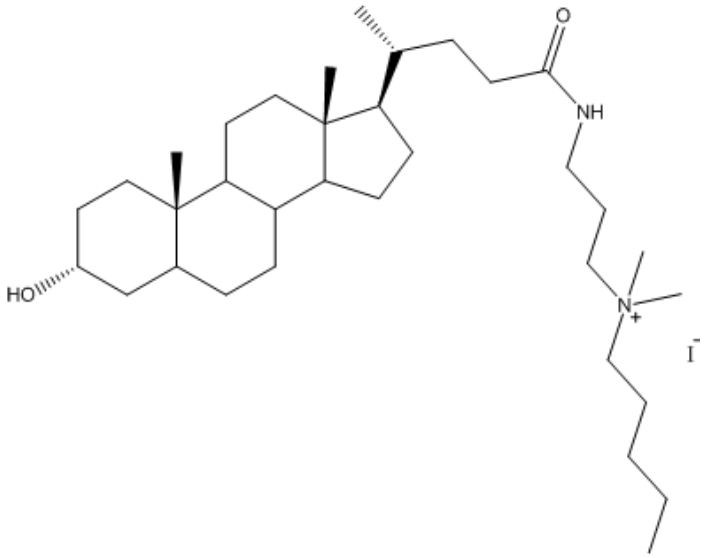
This compound had a log reduction on ice of 1.32 and 1.27 for strains 11204 and 027 respectively. The compound had a log reduction on heat of 2 and 1.5 for strains 11204 and 027 respectively. Due to the fact that there is a large reduction in the ice, coupled with the fact that this material is quaternized, there could be a possibility of this material exhibiting some antimicrobial/sporicidal effects. It cannot be known from the data which is provided whether this material is a germinant-antimicrobial or simply a sporicide. However, testing the material on a bacteria such as bacillus subtilis (a spore forming bacteria), would mean that this ambiguity could be resolved. Due to the fact that this is very similar to compound **107**, with the only difference being the insertion of a benzene ring into the alkylating agent (allyl bromide to 4 vinyl benzyl chloride), and the fact that compound **107** had no germinating ability whatsoever, would imply that this material is a sporicidal agent. Due to the fact that the inherent steroidal structure is clearly not having any antimicrobial effect, and the literature suggesting that *C. difficile* spores are particularly susceptible to chlorine based antimicrobials, it would appear this vinyl benzyl chloride moiety is of particular interest.

Compound 116



This compound displays germinating ability on ice for the 11204 strain, with a log reduction in germination of 1.05 (no germination for 027). It displays a log reduction for the same strain with heat of 0.71. The fact that there is more reduction over ice than heat would suggest, once again, that this compound without the allyl ammonium salt may be a germinant/antimicrobial or a sporicide. The original, un-alkylated version of this compound has not been tested as of yet, but once it has, this will give a clearer understanding as to whether the material is a germinant/antimicrobial or a sporicide. As this material is based on lithocholic acid, the presumption would be at this point that this material is simply a sporicide, due to the lack of lithocholic acid derivatives which have been able to initiate germination in *C. difficile* spores. Presuming it is a sporicide, perhaps it is the fact that the counter ion which is bromine as opposed to chlorine which is limiting its sporicidal activity. If it was possible to alkylate this material with the same alkylating group, but differing counter ion, this would give some insight into the importance of the counter ion. The use of ion exchange resins would be able to do this and thus a fuller investigation of the counter ion could be achieved.

Compound 124



This compound had a log reduction on ice of 1.58 for strain 027 and 2.08 for heat (no data for 11204). The fact that the compound has produced such a relatively large reduction on ice would imply there is some germinant/antimicrobial or sporicidal activity. This activity could only be eluted if the un-alkylated material was tested or the compound was tested on other spore forming bacteria such as *Bacillus subtilis*. The fact that this material is a derivative of the non-germinating bile acid lithocholic acid would suggest some potential sporicidal activity, but more testing would have to be done to be more conclusive.

4.1.2 Overall trends in germination

What the data presented above shows is that the lithocholic amide derivatives are not germinants. It would therefore be better in future to focus on both deoxycholic and cholic acid as potential germinating materials. There appears to be an advantage of having some moiety in the side chain which can become involved in hydrogen bonding (OH and NH). For a more conclusive picture, all of the compounds synthesised should be tested, then more details on specific needs for germination can be explored and refined. To confirm whether the material is only a germinant or whether it has antimicrobial/sporicidal activity then work needs to be done on the compounds using *Bacillus subtilis*.

5.1.1 Future work:

Increasing the number of bile amides for screening would ensure that a more complete structure activity relationship database is produced, as well as continue screening the compounds which have already been synthesised. This increased database should give more information for what features are the most optimal for inducing germination and if quaternized, what alkylating agent produces the best material for antimicrobial activity. Some of the materials already synthesised have been shown to have potential antimicrobial/sporicidal activity, so testing these materials further on other spore forming bacteria such as *Bacillus subtilis,* would enhance the knowledge on whether some sporicidal agents had been produced. From the germination results so far, backed up by literature, it would be suggested to concentrate more on deoxycholic and cholic acid as potential germinants, as there doesn't appear to be any data to back up the use of lithocholic acid as a germinant of *C .difficile*.

Due to the very good germination result of compound **87**, there is a strong argument for more analogues of this type, having a free primary amino group. Although the synthesis of these materials has been difficult, a way around this problem would be to selectively protect one end of the diamine, then use the anhydride method to attach, and then de-protect to produce the free NH_2 group. Using different length chains would perhaps uncover some more interesting information.

Some of the latter work on this project focussed on the production of polymeric bile acid materials. There is a lot more work to be done, but since attachment of a polymerizable group has been relatively easy on the 3-OH (using acryloyl chloride), development of this method should be done. By increasing the number of potential polymerizable functionalities will be able to see if there are any effects on the germinatory ability of the material.

Further work should also involve the testing of the co-germinating materials, in conjunction with known germinatory materials, to see if they can improve germinating ability. Further work should also be done on trying to produce more polymerizable amino acid analogues, to enhance and optimise co-germinating ability using the known literature data about co-germinants.

Unfortunately there were experiments which were unsuccessful, most notably the benzophenone analogue work. All the literature seemed to indicate that this would work, however, after several different attempts, with altering of the conditions, no successful methodology was found. Whether in future there could be a success by manipulating the benzophenone in a different way to induce hydrogen abstraction and thus attachment onto a polymeric surface is yet to be seen. There have been many syntheses of very similar attachments, so there must be something fundamental as to why it won't work when attaching the benzophenone to the bile acid.

6.1.1 Conclusions:

Bile amides have been synthesised in the literature before using several different methodologies. What this project has done, is to optimise the bile amide production using ethyl chloroformate to produce the anhydride, on a 0.5 g scale and then on a 2 g scale.

One of the aims of this project was to produce a structure activity database with regards to germination of *C. difficile* spores. This database has been produced chemically, however, not all of the biological results are available, and therefore a clearer picture of what is important for germination has not been achieved yet.

In terms of polymerizable bile acids and bile amides, this has been achieved after several different methodology attempts were made. There has also potentially been a quaternized, polymeric bile amide, but due to solubility issues, this cannot be confirmed.

The work on production of polymerizable amino acids, for the specific purpose of use as co-germinants has not been described in the literature. This work could have a very important role in the future development of a treatment, should the biological testing come back positively.

While work will continue on a potential polymeric germinating/antibacterial surface there are several precautions that hospitals could make in the meantime. Enhanced infection control methods would

ensure that if there was to be an outbreak, that its chance of spreading to other patients would be severely limited.

As a separate note, it could be suggested that although this work is valid in the laboratory setting, under extremely rigid controls; in the real world there may be problems encountered which the biological experimental protocol cannot account for. One of the limiting factors is that when potential germinants are being tested, this is in an anaerobic environment and therefore, is not a true reflection of what will occur in the real life situation. The only way around this is to potentially develop a new protocol which is more similar to an *in vivo* situation and thus this will give a more accurate reflection of the true germinating nature of the compounds produced.

Due to time limits there are tests which should be done before any commercialisation of the materials occurs. Although a compound may show fantastic properties; great germinant and antimicrobial, this compound could also be extremely toxic. This would need to be investigated as it might have unwanted side effects on people who are continuously exposed to it. Also, the surface would eventually start to break down and would need to be replaced, but it could be potentially quite difficult to monitor if and when that occurs.

An added advantage to this potentially antimicrobial polymer compound is that it would not be specific. The quaternary nitrogen ion is active against a broad range of bacteria and it is unlikely to stay specific to the *C. difficile.* The potential problem is that it may eventually lead to resistance of many different types of bacteria which could pose a real threat to the safety of the human species. The ideal situation would be that there is no antimicrobial activity associated with the compound and that, with a germinatory material and a stringent cleaning regime; the cell life cycle could be broken.

7.1.1 References

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