PREPARATION OF A DUAL-ACTION GERMINANT-BIOCIDE SMART POLYMER AND SOLUBLE AGENT FOR THE ELIMINATION OF *C.DIFFICILE* SPORES

by

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Clostridium difficile is a spore-forming, gram positive bacteria responsible for causing *Clostridium difficile* infection (CDI), the main symptom of which is diarrhoea. It can also cause serious symptoms, for example, pseudomembranous colitis, toxic megacolon and death. CDI spreads through the ingestion of *C. difficile* spores, which are very resistant to cleaning products and can survive on surfaces for many months. Little is known about the germination of *C. difficile* spores; taurocholic acid, a bile acid found naturally in the gut is one of the known germinants, but the exact mechanism of germination is unknown. Other bile acids, such as cholic acid have shown germinating ability, but to a lesser extent.

The aim of this PhD was to create a polymer surface that forced the germination of the *C. difficile* spores upon contact, then to kill the resulting more susceptible vegetative cell. A known germinant of *C. difficile* spores, cholic acid, was derivatised at the carboxyl group in a flexible synthesis to give a range of carboxamide analogues terminating in a quaternised amino function, with or without a polymerisable function. The attachment of polymerisable groups at the hydroxyl functions was also explored.

The cholic acid monomers proved to be resistant to polymerisation. Of the monomeric compounds, however, nine exhibited *C. difficile* spore-germinating activity, two had either sporicidal or germinating and antimicrobial abilities and two other compounds had germinating and sporicidal and/or antimicrobial activity. The results for this series of compounds indicate that germination of *C. difficile* spores is favoured by the presence of a quaternary ammonium function and a two- to four-carbon chain between the cholamide carbonyl and the quaternary centre.

Overall, eleven new compounds exhibited activity against *C. difficile* spores. The incorporation of these compounds into a polymer surface or disinfectant spray has the potential to reduce the risk of infection of CDI by eliminating *C. difficile* from surfaces, in both healthcare and community based settings.

Key Words: Cholic acid, *Clostridium difficile*, antimicrobial, germinants, polymers

To my family

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List of Abbreviations

AIBN	Azobisisobutylonitrile
Ca	Calcium
CDI	Clostridium difficile infection
CDAD	Clostridium difficile associated disease
DCC	N,N'-dicyclohexylcarbodiimide
DCM	Dichloromethne
DIEA	N,N-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
DSC	Disuccinimidyl carbonate
EGDMA	Ethylene glycol dimethylacrylate
EMA	European Medicines Agency
EtOAc	Ethyl acetate
FDA	Food and Drug Adminstrator
GI	Gastro-intestinal tract
HBA	Horse Blood Agar
HCl	Hydrochloric acid
HPA	Health Protection Act
K	Potassium

MeOH Methanol

Na Sodium

NICE National Institute for Health and Care Excellence

NMR Nuclear Magnetic Resonance

RNA Ribonucleic acid

Slp Surface layer protien

TBDMSO *tert*-butyldimethylsilyl oxide

TBDMSi-Cl tert- Butyldimethylsilyl chloride

TBDPSiCl tert-Butylchlorodiphenylsilane

tcdR toxin regulator R

THP Tetrahydropyranyl

TLC Thin layer chromatography

VAT Value added tax

VBC Vinyl benzyl chloride

Zn Zinc

1. INTRODUCTION

1.1 *CLOSTRIDIUM DIFFICILE*, GENERAL BACKGROUND

Clostridium difficile is an anaerobic, Gram positive, spore forming, toxin producing microorganism responsible for causing *C.difficile* infection (CDI), otherwise known as *C.difficile* associated disease (CDAD) (Burns *et al.*, 2010). There are currently in excess of 100 different genetic ribotypes of *C.difficile*. The main symptoms of CDI are diarrhoea and stomach cramps; more serious symptoms are pseudomembranous colitis (inflammation of the colon) (Deneve et al., 2009), which was first reported in 1893 (Rupnik et al., 2009), and toxic mega colon (colonic distension) which can lead to death (Johnson and Gerding, 1998). CDI has been a clinical problem since 1978 (Gerding, 2009), however, the numbers of infections were not considered high until 2005 when the number of cases in the UK reached 46,000 (Duerden, 2011). The number of cases of CDI in Britain has fallen sharply since 2007, with the introduction of the Code of Practice for Infection Prevention and Control during the Health Act 2006 was brought into power after two outbreaks of CDI in which 33 people died ("Health Act 2006," 2006). While this fall in numbers shows how important infection control is, there is growing concern about new strains of *C.difficile* and the lack of treatment offers for CDI.

CDI can only infect a patient when their gut flora is disrupted, usually by broad spectrum antibiotics, such as cephalosporins and other beta-lactam antibiotics and fluoroquinones (Gerding, 2009). Other risk factors for CDI include prolonged hospital stays, immunosuppressants and drugs such as proton pump inhibitors (Deneve et al., 2009). Unusually, treatment for CDI involves further antibiotics, which are vancomycin and metronidazole. Metronidazole is used for mild cases of CDI whereas vancomycin is used for more serious cases, or when metronidazole fails (Rupnik et al., 2009). There have been reports that some *C.difficile* strains, such as ribotype 027, are becoming resistant to vancomycin, causing concern that soon only one treatment will be available for CDI patients (Rupnik *et al*., 2009). Currently, the most common *C.difficile* strain in the UK is 038 (HPA, 2012). A new strain has also recently been reported in Scotland, ribotype 332, which has killed three patients (BBC news, 2013). Whilst there are a lot of alternative treatments advocated by different groups, none have been approved by the National Institute for Health and Care Excellence (NICE) so are unavailable through the NHS.

CDI spreads through contamination of surfaces by *C.difficile* vegetative cells and spores. It can be spread through touch by staff and patients, as well on medical equipment (Vonberg et al., 2008). The spores are highly resistant to a number of cleaning products and hard surface disinfectants. The spores can survive for many months in aerobic conditions (Gerding *et al*., 2008). This allows the infection to spread easily around a hospital setting, causing outbreaks. Vegetative *C.difficile* cells can only survive outside of the body for a few hours and are easily killed by cleaning (Jump et al., 2007). If the vegetative cells are not killed, the stressful conditions experienced by the cell will trigger sporulation.

After a number of outbreaks in the UK, reporting of all CDI cases in patients between the ages of 2 and over became mandatory. Reporting in cases in children under two is not required due to the high prevalence of asymptomatic *C. difficile* in children's gut (Libby *et al*., 1983). The number of cases and deaths related to CDI for every acute trust hospital are available online on the Health Protection Agencies website. The numbers show year on year falls since 2007, however, in 2012, the numbers of cases are starting to level off.

1.2 Prevalence of *C.difficile*

CDI accounts for approximately 25% of all cases of antibiotic associated diarrhoea (Howerton *et al*., 2011). During the months of March 2011 to April 2012 there were 18,005 cases of CDI in acute hospital trusts in England. Of these, 13,836 cases were in patients over the age of 65. The number of cases in hospitals are dropping; between April 2007 to March 2008 there were 45,439 cases reported (Public Health England, 2012). Community based infections are defined as infections acquired within 48 hours after admission to a health care facility, providing the onset of the infection had occurred more than 12 weeks after any release from a hospital or other health care setting. There are, however, more cases of community based infections, (Rodriguez-Palacios et al., 2010) with patients who would be class as low risk (e.g. those who haven't recently been given a course of broad spectrum antibiotics, who are under 65 and have no underlying conditions) contracting CDI (Rupnik et al., 2009). Around 5% of the adult population are asymptomatic carriers of *C.difficile* (Ramirez et al., 2010) and as many as 50% of patients who have been hospitalised for 4 weeks or more (Johnson and Gerding, 1998). The increase in community based CDI is puzzling due to the nature of *C.difficile*. CDI is contracted when there is a disruption to the normal gut flora, which allows *C.difficile* to colonise. Increased numbers in low risk patients could indicate new sources for the infections, such as transmission from domestic animals, or through food, however there have been no such reported cases.

Hospitals have been aware of CDI since 1978, when a voluntary surveillance scheme was set up. In 1990, there were 500 cases of CDI, in 2001 it increased to 20,000 and in 2005 the number of cases reached 46,000 (Duerden, 2011). In June 2003, the first outbreak of CDI involving ribotype 027 occurred in the Stoke Mandeville Hospital, UK. This outbreak had wide reaching consequences, including more priority given to hospital associated infections (HAI). Outbreaks of CDI are further discussed in section 2.1.2.

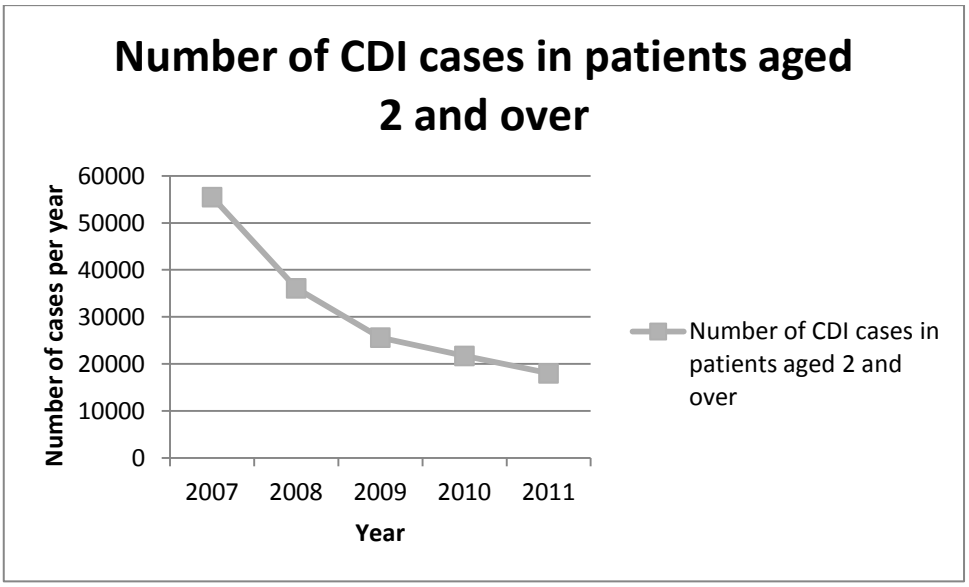


Figure 1 Graph to show the fall in CDI cases in the UK since 2007

1.2.1 TIMELINE OF *C.DIFFICILE*

Pseudomembranous colitis, one of the main complications of CDI was identified in 1893 by John Finney as a "postoperative complication" of a gastroenterostomy performed on a young woman. The cause of this complication was unknown (Rupnik *et al*., 2009). Forty two years later, in 1935, *C.difficile* was described by researchers looking at infant faecal flora. It was thought to be part of a child's normal faecal flora, not a pathogen, due to the prevalence in children up to the age of two. In 1940, Snyder found isolates in children as young as 8 weeks, again, thinking it was part of a child's normal faecal flora (Stark *et al*., 1982). *C.difficile* was not identified as the causative agent for pseudomembranous colitis until 1978. Vancomycin was found to be a very effective antibiotic against *C.difficile* in 1981; however it was also discovered there was a high relapse rate with it too (Rupnik et al., 2009). It wasn't until 1995 that the mechanism of action for *C.difficile* toxins (A and B) was discovered. In 1997, the transcription and toxin production for both toxins were described and in 2001 tcdR, a toxin regulator was discovered (Gerding, 2009). In 2005, a hyper-virulent strain of *C.difficile* was

found, called 027 (Gerding, 2009). Since then, a lot of research has gone into how and why *C.difficile* spores germinate, ways to inhibit germination or cures, such as vaccinations.

1.2.2 Outbreaks of *C.difficile*

There have been many major outbreaks of CDI in British hospitals in the last ten years, two of which occurred in Stoke Mandeville hospital (Healthcare Commission, 2006). The outbreaks led to big changes in infection control protocols in the UK. Other UK outbreaks have occurred in Maidstone and Tunbridge Wells between October 2005 and September 2006. Overall, 500 people were infected with CDI and 60 people died. The contributing factors towards the outbreaks were largely the same as in the Stoke Mandeville outbreak which is discussed further below. There have been outbreaks in the US, Canada and Europe, some of which have been attributed to a certain strain of *C.difficile* (027). The outbreak in Québec was also attributed to the transfer of patients between cities. Outbreaks in Africa and Asia are rare and are usually not caused by stain 027. Due to increased globalisation, the spread of 027 strain is likely to widen, increasing the risks of outbreaks in other parts of the world (Rupnik *et al*., 2009).

1.2.3 Stoke Mandeville

Between 2003 and 2005 there were two outbreaks of CDI in the Stoke Mandeville hospital in Buckinghamshire. During this time, 334 patients were infected with CDI and 33 died. The first hospital wide outbreak occurred between October 2003 and June 2004, where there were 174 new cases and 19 deaths, 16 of which almost certainly contracted CDI whilst at the hospital. The outbreak was so large because patients with CDI were not isolated, leading to contamination of wards. Isolation of patients was not possible due to lack of rooms, other patients being prioritised and lack of focus by senior management to contain the outbreak (Healthcare Commission, 2006).

The second outbreak took place between October 2004 and June 2005. During this outbreak, there were 160 cases and another 19 deaths, of which, 17 of the patients contracted CDI from the hospital. Reasons for the large number of cases lie with the senior management of the hospital, who did not implement advice given by the infection control team or the local health protection unit. This led to the news of the outbreak reaching the Department of Health and the public eye.

The outbreak in Stoke Mandeville is very important in understanding the culture of infection control before 2007. The main reasons for the peak in CDI cases before then were that

healthcare associated infections were not regarded as a priority against other healthcare aspects. Indeed, it was thought that diarrhoea was to be expected alongside antibiotic treatment. Responsibility for the infections were seen to by the infection control staff, not the clinicians, however, as with the Stoke Mandeville outbreaks, advice given by the infection control was not taken seriously by other staff members. This lead to health ministers being made aware of the problems in 2004-5. The introduction of a statutory Code of Practice for Infection Prevention and Control during the Health Act 2006, which was revised in the Health and Social Care Act 2008 to include the independent sector, address many of the issues surrounding infection control. The duties of this act include

" General duty to protect patients, staff and others from HCAI"

"Duty to provide and maintain a clean and appropriate environment for health care"

" Duty to provide adequate isolation facilities"

"Duty to ensure adequate laboratory support".

There was also a national target set by the government of a 30% reduction in CDI cases by 2010-11. The results for this target were achieved and another 30% target reduction was set for 2013. Since the acts, more priority has been given to HAIs, drastically reducing infection rates. Responsibility for infection control has shifted towards clinicians, healthcare board members, chief executives, managers and the government (Duerden, 2011). Infection control still remains a priority, as the risk of antibiotic resistant bacteria causing serious outbreaks in infections is still high.

1.2.4 *C.DIFFICILE* FOUND IN MEAT AND ANIMALS

C.difficile could be considered to be a zoonotic pathogen (Paredes-Sabja and Sarker, 2011). It has been found in farm animals such as cattle, pigs and poultry; domestic animals such as hamsters and guinea pigs, and exotic animals such as elephants and ostriches (Dawson *et al*., 2009; Keessen *et al*., 2011). This suggests that people could be coming into contact with *C.difficile* from places outside of a healthcare setting, something that can be demonstrated by the infection of children with *Escherichia coli* from farm animals (Fairbrother and Nadeau, 2006). However, there have been no reported cases of this kind of transfer of *C.difficile*. Spores have been found many retail meats, with one study in Holland estimating that 20% of ground retail meat and other meat products contained *C.difficile* (Paredes-Sabja and Sarker, 2011). Another study demonstrated that spores could actually survive the recommended

temperatures for cooking meat, suggesting that spores could be ingested through eating a meal (Rodriguez-Palacios *et al*., 2010). Although cases of this kind of transmission have not been reported, there is the possibility of it happening in the future and it does go some way of explaining the increases in the number of community based cases.

1.3 Hypervirulent toxintype III NAP1/027 strain

The strain 027 (or NAP1 as it's known in America) is a particularly virulent strain of *C.difficile*. It has been associated with increased antibiotic resistance, more severe symptoms and longer hospital stays (Rupnik *et al*., 2009). It has an 18bp deletion in the Toxin C gene, a negative regulator, meaning it produces more toxin A and B, the main causes for the symptoms of CDI, than other strains (Deneve et al., 2009). Work is currently ongoing regarding the typing of strains, with reporting of typing results from hospital tests being given to the *C.difficile* Ribotyping Network (CDRN) for England and Northern Ireland. This will give a more accurate idea of the spread of strains throughout England . The number of cases involving the 027 strain is around 20% in Europe, compared to 47% in the USA. The 027 strain was the main strain identified in the Stoke Mandeville outbreaks in 2003 and 2005. Since then, the number of reported cases has dropped dramatically, with the 038 strain being more prevalent in the UK now (HPA, 2012). However, as there is no mandatory reporting of typing of strains, these figures may not be wholly accurate. We can gain evidence from the types of treatments given to patients, as patients with the 027 strain are usually harder to treat (Warren and Guerrant, 2011). If the authors of the above paper are correct in their conclusions that not all isolates of the 027 type are hypervirulent, then we could be exposing patients to unnecessary and expensive treatments if the typing results are taken into consideration when decided treatment for the patient.

1.4 Acquistion of the disease and risk factors

In order to be infected with CDI two main conditions must be met.

1. Exposure and ingestion of *C.difficile* spores must have occurred

2. Normal gut flora has been disrupted, for example, due to the use of antibiotics, immunosupression, old age etc (Gerding, 2009).

If both of these conditions have been met, then the spores can germinate and the infection can establish itself. The infection can have a devastating effect on the body. Vegetative cells multiple and attach themselves to intestinal epithelial cells via surface-layer proteins (S-layer proteins), causing tissue damage. This colonization of the gut initiates an inflammatory

response and causes severe damage to the intestinal epithelium. If left untreated, CDI can lead to bowel perforation, toxic megacolan and even death (Dawson *et al*., 2009).

Risk factors for CDI include the use of broad spectrum antibiotics, such as cephalosporins, clinamycin, fluorquinolones, old age and being on proton pump inhibitors (Deneve et al., 2009). Broad spectrum antibiotics wipe out the natural gut flora allowing *C.difficile* to colonize the gut and the infection to take hold. Old age (over 65 years old) is also a risk factor due to the increased chances of being hospitalized for other, unrelated conditions, increasing the risk of being infected with CDI. Underlying conditions, usually associated with old age, can also cause complications when treating CDI, so increasing the risk of death or serious complications. Old age can also make it harder for the body to fight off infections. Around 70% of all CDI cases are patients aged 65 or over. Note, under twos are not included in CDI statistics due to the prevalence and apparent asymptomatic carriage of *C.difficile*.

1.4.1 Antibiotic use

The sustained over use of antibiotics has led to a massive increase in antibiotic resistant organisms and antibiotic associated diseases. Most antibiotics have now been associated with an increased risk of CDI. Those with the highest risk are clindamycin, cephalosporin and fluoroquinolones (Dawson *et al*., 2009).

Two antibiotics are predominatly used to treat CDI, metronidazole and vancomycin. Due to its cost, metronidazole is used to treat CDI first in cases of mild to moderate infection. Vancomycin is used for more serious cases or when metronidazole is ineffective (Lancaster and Matthews, 2012). As CDI relies on gut flora disruption, restoring normal gut flora as soon as possible is essential. However, this can be difficult to achieve with some patients, with high relapse rates common. One patient in Washington D.C. who had multiple relapses endured a continuous course of oral vancomycin for 31 months, costing $1,030 (£677.85) a month (≈$31,930/£21,013 for the entire course) (Bartlett, 1994).

There is emerging evidence that some 027 strains are becoming resistant to a number of antibiotics, including gatifloxacin and moxifloxacin (Rupnik *et al*., 2009). This presents a massive problem for health care providers. The emergence of a resistant strain could lead to an outbreak of CDI, with increased risk of death due to lack of viable treatments available. The resistance of one strain also means that the resistance of other strains is possible. As only two antibiotics are presently used to treat CDI, any resistant strains could present health care providers with a problematic treatment plan with another serious outbreak probable.

A new antibiotic has recently been approved by the FDA called fidaxomicin which could help in the arsenal against CDI, however the cost of a course of the drug is over 100 times more expensive than a course of metronidazole, and twice the cost of a vancomycin course (Lancaster and Matthews, 2012). If more *C.difficile* strains do become more resistant, then health care providers will be left with little choice but to use fidaxomicin, greatly increasing the cost of treating a CDI patient and putting a big burden on an already stretched NHS budget.

Antibiotic	Induce CDI	Treat CDI	*C.difficile* resistance
Broad spectrum cephalosporin	✓		
2nd and 3rd generation cephalosporin	✓		
Broad spectrum penicillin	✓		N/A
Clindamycin	✓		
Fluoroquinolones	✓		some 027 strains
Ureidopenicillin	low propensity		N/A
Vancomycin		✓	some 027 strains
Metronidazole		✓	

Table 1 Table to show what common antibiotics induce CDI, are used to treat CDI or have shown resistance to *C. difficile*

1.5 GASTROINTESTINAL PHYSIOLOGY

The lower gastrointestinal tract is made up of the small and large intestine. The small intestine is made up of the duodenum, jejunum and ileum, whereas the large intestine is made up of the cecum and colon. Most of the digestion and absorption of food takes place in the small intestine, whereas the large intestines role is to absorb water and carry the food waste form the body. The colon contains a complex microbial community of mainly anaerobic bacteria. This acts as a defence barrier against colonisation of pathogenic bacteria which might enter the body. The gut flora environment is very sensitive and can be effected by changes in diet, age and medication. The changes in the gut due to advancing age are of importance as CDI mainly effects those ages 65 and over. Old age is associated with malnutrition, which will affect the colonic flora environment. This puts the elderly at a higher risk of developing CDI, compared to those aged under 65. *C.difficile* affects the small intestine; however there are different theories for the infections exact mechanism. It is widely accepted that any *C.difficile* cells entering the body will be destroyed by the stomach acid and that the spores will survive.

The spores interact with the bile acids along the GI tract, only germinating when the correct conditions are reached.

1.5.1 Bile acids and Enterohepatic Circulation

Bile acids are found and produced naturally in mammals aiding digestion of food. Primary bile acids, cholate and chenodeoxycholate, are synthesised in the liver whereas secondary bile acids are synthesised though conjugation with either glycine, to produce glycocholic acid, or with taurine, to produce taurocholic acid. They are then secreted into the digestive tract where they facilitate the absorption of fats and cholesterol. The bile acids are also actively absorbed to be reutilised in the liver. Bile enters the cecum at a concentration of approximately 2mM, where it is modified by the normal gut flora. Bile salt hydrolase removes the conjugated amino acid, turning it back into the original bile acid. Here it is further metabolized then actively transported into the cytoplasm and the 7α-hydroxyl is removed, creating lithocholic acid, which solubilises fat for absorption (Sorg and Sonenshein, 2010).

1.5.2 Germination theory

Spore germination is defined as

> "the irreversible loss of spore specific properties ". (Burns *et al*., 2010)

In order for the spores to germinate, they must first come into contact with the correct environment. This is usually occurs in the colon in humans. The spores interact with the germinants around them which triggers germination. The spores then release Zn^{2+}, K^{+}, Na+, dipicolinic acid and Ca^{2+} (Ca-DPA) (Xiao et al., 2011). The role of dipicolinic acid is to provide stability to the spore and also to protect the spore's DNA from any damage that make occur during sporulation (Setlow et al., 2006). The pH of the internal spore rises and there is an uptake of water or rehydration of the core. Potassium ions are reabsorbed, signalling a resumption of metabolic activity. The cortex degrades, there is a loss of spore heat resistance and a transition from phase bright to phase dark occurs. The outgrowth of vegetative bacterium then takes place (Xiao *et al*., 2011).

1.5.3 *C.difficile* Germination

C.difficile spores will only germinate when they encounter very specific conditions inside the body (Xiao *et al*., 2011). The exact mechanism of germination is not known currently as *C. difficile* has a different germination mechanism to other Clostridium species (Sorg and Sonenshein, 2010). *C. difficile* does not have the germination (Ger) receptors which are

common in other Clostridium species and the specific receptors which trigger germination in *C. difficile* are unknown (Lawley et al., 2009).

Germination studies have shown that *C. difficile* germinates in the presence of certain bile acids, namely taurocholic acid, cholic acid and glycocholic acid in vitro (L.J. Wheeldon et al., 2008). The exact mechanism is not known but the theory states that the concentrations of primary and secondary bile acids in the gut play an important role in whether a person is infected with CDI or is a asymptomatic carrier of *C. difficile*.

Bile acids are produced by the body to aid in the absorption of fats and fat soluble vitamins after a meal has been consumed. There are two types of bile acids, primary and secondary. The primary bile acids are cholic acid and chenodeoxycholate which are synthesized from cholesterol. The primary bile acids are conjugated to the amino acids glycine or taurine to make the secondary bile acids taurocholic acid and glycocholic acid. These secondary bile acids are then deconjugated by bile salt hydrolase in the intestinal lumen. The reason for the conjugation then deconjugation is not clear. It is thought that the conjugation of the primary bile acids to the amino acids reduced the toxic effect of the primary bile acids as they travel through the intestines. A reason for the deconjugation in the use of amino acids as a nutrients source. The primary bile acids are then reduced by the enzyme 7-dehydroxylase, a bacterial enzyme, (Hillman, 2004) to deoxycholate and lithocholate which further aid the absorption of fats from a meal (Britton and Young, 2012).

Work carried out by Sorg and Sonenshein has shown that in vitro, *C. difficile* spores germinate in the presence of taurocholic acid and glycine and that they act as co-germinants (Sorg and Sonenshein, 2008). Their work also showed that deoxycholate was also able to trigger germination of the spores but that it was highly toxic to *C. difficile* vegetative cells. This work lead to the theory that if spores are ingested, they will germinate in the presence of the bile acids but the vegetative cells will be killed by deoxycholate. When antibiotics are indroduced to the system, the gut flora is reduced dramatically and deoxycholate is not synthesised, leading to the establishment of *C. difficile* in the gut (Britton and Young, 2012).

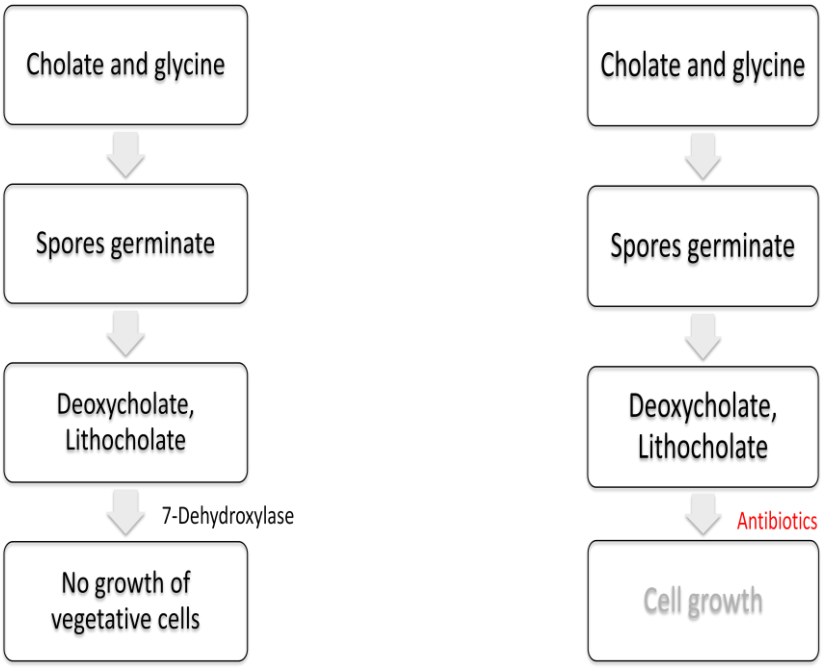


Figure 2 Two flow charts to show one theory of *C. difficile* spore germination. The flow chart on the left shows the germination theory of a patient not on antibiotics whereas the flowchart on the right shows the germination theory of a patient who has been treated with broad spectrum antibiotics.

There are other theories of germination, for example, *C.difficile* spores have four receptor sites, two for taurocholate and two for glycine. The spores bind to taurocholate, which triggers a rapid saturation of the other taurocholate receptor site. The spore then binds to glycine, which again, triggers a saturation of the other glycine site. Once complete, the spore can initiate germination. (Sorg and Sonenshein, 2010).

It has been reported that the optimum concentration of sodium taurocholate to activate spore germination is 0.1-100 $mmol^{-1}$ in vitro which achieved a 2.49-2.62 log reduction of colonies during germination tests. The minimum concentration of taurocholate was found to be 0.1 $mmol^{-1}$. (Wheeldon *et al.*, 2008). There is conflicting evidence surrounding the germinating abilities of all of the cholate derivatives, Wheeldon *et al* state that glycocholate and cholate enhance colony formation but only on agar and also require extensive periods of exposure. A number of amino acids, including glycine are needed for spore germination and a study showed that thioglycollate was more efficient than glycine in germinating spores. Whilst there is some conflict in the theory of spore germination, it is accepted that the *C.difficile* spores use the concentration of bile acids as cues to when to germinate.

1.6 PATHOGENSIS OF *C.DIFFICILE* INFECTION

Due to the difficulty working with *C.difficile*, there are different theories for the mechanism of the infection caused by the spores. It has already been established that *C.difficile* must have a different germination mechanism to other *Clostridium* species due to its lack of Ger receptors.

1.6.1 ENTRY

C.difficile enters the body through the faecal-oral route, meaning spores must be ingested before the infection can take hold. Any vegetative cells will be killed by the stomach acid pH, whereas spores will survive and pass through to the duodenum and onto jejunum, where the concentration of primary bile acids is high.

1.6.2 ADHERENCE

Vegetative *C.difficile* cells binds to intestinal epithelial cells through its surface layer proteins with the aid of its flagella and proteases (Paredes-Sabja *et al*., 2012). The surface layer proteins, especially SlpA, vary between species and are currently not well understood. The flagella aid in the adherence of the cell by penetrating the mucus layer of the epithelial cell surface. The flagella are recognised by the epithelial cells pathogen sensing pathway and triggers an immune response (Solomon, 2013).

1.6.3 EVASION

The production of toxins by *C.difficile* triggers an immune response resulting in a large amount of marcrophages being recruited. A study by Paredes- Sabja *et al*. Lookied at the effect *C. difficile* cells had on Raw 264.7 cells, mouse leukaemic monocyte macrophage cells, in vitro. The have suggested that the spores are recognised by the Raw cells and are indeed phagocytosed by them. The spores, however, appear to be highly resistant to this process, are able to survive and even produce toxins that attach the Raw cells. The spores exosporium contains receptors that are recognised by the Raw 264.7 cells and the spore will be engulfed within 30 minutes. The spore interacts with the phagosomes membrane, eventually disrupting it, killing the cell and allowing the spore to survive. *C.difficile* cells, however, are easily killed by the phagosomes (Paredes-Sabja *et al*., 2012) (Madan and Jr, 2012). If the results of this study are found to be represntative of human cells, this suggests that the spores are able to survive the body's immune response, allowing them to germinate when the conditions are more favourable, which could be 1-4 weeks after CDI treatment.

1.6.4 DAMAGE TO THE HOST

C.difficile cells produce five toxins that damage the host cells, two of which are toxic. Toxins A and B are largely responsible for the symptoms presented with CDI. Both toxins are responsible for the destruction of intestinal epithelial cells (Dawson *et al*., 2009).

Toxin A- Uridine diphosphate glucosylating toxin encoded in the pathogencity locus. Targets intestinal epithelial cells causing neutrophil infiltration, production of signalling proteins, disruption of tight junctions and cell death.

Toxin B- Uridine diphosphate glucosylating toxin encoded in the pathogencity locus. Tcd B causes disruption of tight juctions and cell death. Tcd B can cause infection without Tcd A. (Voth and Ballard, 2005)

Both toxins induce the production of tumour necrosis factor alpha and proinflammatory interleukins. The toxins produced also induce an inflammatory cascade, leading to pseudomembrane formulation in the colon (Madan and Jr, 2012).

Toxin E- An accessory gene in the PaLoc region. Its function is not fully understood but it is though to assist the release of toxins A and B through permeabilization of the cell wall of *C.difficile*.

Toxin R- A regulatory gene, needed for the production of RNA. A sigma factor.

Toxin C- a negative regulator not found in all strains. It controls the amount of toxins A and B released. In strain 027, there is an 18 base pair deletion in toxin C resulting in uncontrolled production in toxins A and B, leading to a more severe form of CDI.

1.6.6 CLINICAL MANIFESTATIONS OF CDI

The main symptom of CDI is diarrhoea, which can range from mild to explosive. This is usually associated with stomach cramps. More serious symptoms are pseudomembranous colitis and toxic mega colon, both of which could lead to death. Pseudomembranous colitis, hardening of the gut wall, is usually diagnosed with colonoscopy whereas toxic mega colon is usually diagnosed with radiographic technique. Complications from CDI can include dehydration, sepsis and intestinal perforation (Deneve et al., 2009; Jump et al., 2007). Symptoms for paediatrics are different and not well understood. There is conflicting evidence about whether babies under two get CDI as 67% of neonates are carriers of *C.difficile*. The symptoms for paediatrics are diarrhoea, colic attacks (crying for an unknown reason), hypermeteorism (excess gas), vomiting and abdominal distension (McFarland et al., 2000).

1.7 Sporulation and Spore Structure

1.7.1 Sporulation

When the cell experiences a highly stressful environment, such as an aerobic environment, it will form a dormant spore to protect itself (Leggett *et al*., 2012). *C.difficile* spores are highly resistant to many environmental attacks and can survive for many months outside of the body. A paper by Leggett *et al*. describes sporulation occuring in seven stages; vegetative cell, asymmetric cell division, engulfment, cortex formation, coat formation and maturation, release.

Asymmetric cell division- the cell divides and forms two compartments, one of which is smaller than the other called the prespore.

Engulfment- a forespore is formed by the mother cell engulfing the prespore. The forespore is now bound in inner and outer membranes.

Cortex formation- a peptidoglycan cortex is formed between the inner and outer forespore membrane.

Coat formation and maturation- the spore coat is formed, as well as dipicolinic acid (DPA), which is responsible for the reduction of water in the forespore. The spore matures.

Release- the spore is released by the lysis of the mother cell. The spores are released into the environment by diarrhoea, which can release in excess of 100 spores and cells per gram of faeces (L.J. Wheeldon et al., 2008). Once released, they can survive on surfaces for many months, until they experience the correct conditions for germination. This can create a vicious cycle of re infection for patients and indeed, relapse rates are high (Leggett et al., 2012).

The spores are able to survive many extreme conditions, including toxic chemicals, pH changes, radiation and desiccation. They can also withstand high temperature; they have been found in meat that has been cooked at recommended temperatures (Rodriguez-Palacios et al., 2010). The spores are also capable of sensing the environment they are in, allowing them to germinant when in favourable conditions (Vonberg et al., 2008). The spores contain a number of features which aid them to survive in stressful conditions, which include having a proteinaceous spore coat with a higher resistance to hydrophillic agents, reduced water content in the spore core and small acid-soluble proteins (SASP) which protects and repairs damaged DNA during germination.

Figure 3 Figure to show the different stages of sporulation of a *C. difficile* bacterial cell

Based on a image from Leggett *et al*. 2012

1.7.2 Spore Structure

The spore structure for *C.difficile* is not fully understood. It is thought to have an exosporium, which, based on studies of other species exosporium, is likely to be made up of mostly protein. Studies have shown that the exosporium of *C.difficile* contains receptors that are recognised by the immune system. It is also thought to be involved in spore attachment to environmental surfaces. The next layer is the spore coat which is made up of a series of thin layers of complex proteins. It is the first barrier to large molecules and chemicals that would kill the spore. The coat must be able to allow the spores germinants through however, not much is known about the spore coat or its mechanisms. Studies have shown that small molecules such as alkylating agents do pass through the coat and ultimately kill the spore. The outer membranes function is unclear as studies have shown it serves no protective function. It is essential for spore formation along with the peptidoglycan cortex and germ cell wall, again, showing no real protective role. The inner membrane has been shown to be highly impermeable, even to very small molecules. It contains the spore's germinant receptors, which are currently unknown in *C.difficile*. The spore's core is dehydrated and contains the spores DNA, RNA, ribosomes, small acid spore proteins and enzymes. The environment of the spore is highly protective, containing a high concentration of DPA (Leggett *et al*., 2012).

Figure 4 Figure to show the structure of a *C. difficile* spore

Based on a image from Leggett *et al.* 2012

1.8 Prevention of CDI

Infection control strategies in most hospitals requires isolation of the patient including use of their own toilet where available. If a room is not available, then it is recommended that a *C.difficile* ward is set up (Vonberg *et al*., 2008). Other infection control measures include hand washing, the use of dedicated cleaning equipment and the use of hard surface disinfectant, for example, 2% w/v hypochlorite bleach. A stool sample is sent off immediately where an assay is carried out to confirm the diagnosis of CDI. *C.difficile* vegetative cells have a very distinctive smell which can be used as a diagnostic test along with the onset of diarrhoea. Treatment of mild to moderate cases usually starts with metronidazole as it is the cheaper option and has a good patient response. If this does not cure the CDI, vancomycin can be used. If the diagnosis is for severe diarrhoea, then vancomycin is used straight away. There is a 20% relapse rate with CDI and the treatment for relapse is to use the same antibiotic that was used previously (Rupnik *et al*., 2009). There are no other recommended treatments for CDI in UK hospitals due to lack of evidence or conflicting data results. One new treatment which has been used with success in the US is a faecal transplant, and which is currently undergoing a randomised trial in the Netherlands. A faecal transplant constists of a stool sample, preferably one from a person who is close to the patient, being put into the bowels of a patient. It is either transplanted via a coloniscope or a nasogastric tube (Mattila et al., 2012). It is seen as a last resort however and is not currently recommended for UK patients.

Probiotics and biotherapies work on the theory of restoring gut flora to normal levels after antibiotic use. The evidence to support this treatment for CDI patients is limited and it is currently not recommended as part of a treatment plan for patients.

The options for treating CDI are very limited. While there are many treatments suggested in the literature, the data sets are very small, conflicting or incomplete. Given the increased and growing antibiotic resistance of *C.difficile*, a new way of tackling this problem is needed.

1.8.1 Contamination and Cleaning

When a patient is diagnosed with CDI, hospital protocol, at the moment, is to isolate patients showing CDI symptoms, if possible (Vonberg *et al*., 2008). Isolation should mean that the patient gets their own en suite room, however this is not always possible. Protocol dictates that it is acceptable to put a number of CDI patients in a room together but this does increase the risk of re-infection. Cleaning should be undertaken using dedicated cleaning equipment which is only used in infected patient's rooms. Cleaning should be undertaken with a 2% v/v hypochlorite solution as it has been shown that spores are killed when bleach is used (Vonberg *et al*., 2008). A number of different agents can be used to kill the spores, for example, a hospital in Nottingham used hydrogen peroxide spray to decontaminate rooms in their high dependency ward (Shapey *et al*., 2008). While this was very effective at killing spores, the room could not be used for a number of hours after the decontamination had taken place, which can be very disruptive on a busy ward. Hydrogen peroxide is also very harmful and an explosion risk. Copper also kills *C.difficile* spores and there was a trial in hospitals using copper equipment. Unfortunately, the hospitals found that people would steal the copper bedpans, equipment etc and with the price of copper increasing, hospitals could no longer afford to restock. Further studies have also shown that copper does not kill the spores, only the vegetative cells (L. J. Wheeldon *et al*., 2008).

Hospital design can play a big role in the spread of spores. A new hospital design in Canada reduced infections considerably by removing corners from rooms. By getting rid of corners and making them curved, cleaners could more effectively clean rooms, corridors and bathrooms. More individual rooms made it easier for isolation to take place and an increased number of sinks reduced CDI rates significantly. Unfortunately, hospital redesign and modification is very expensive, but new hospitals should take these findings into consideration.

1.8.2 New treatments

There are many treatments suggested in the literature of treatment of CDI. One of the newest treatments for CDI which has recently been approved by the FDA and EMA is the use of fidaxomicin, a new class of macrocyclic antibiotics. Trials have shown it to be non-inferior to vancomycin with a slightly lower relapse rate. The HPA has not yet recommended it however, due to the high treatment costs and lack of comparison studies with metronidazole. The NICE summery of fidaxomicin has put the cost of the recommended 10 day treatment cost at £1350 (excluding VAT), whereas the same 10 day treatment course for metronidazole is £2.53 and for vancomycin it is £188.27. Fidaxomicin is the first antibiotic to be recommended for CDI treatment for 30 years, showing the difficulties experienced by researchers investigating new treatments for CDI (Lancaster and Matthews, 2012).

1.9 Bile Acids

Studies have shown that three bile acids germinate *C.difficile* spores. These are cholate, taurocholate and glycocholate (Sorg and Sonenshein, 2010, 2009, 2008; L.J. Wheeldon *et al*., 2008). The structures of these bile salts are shown below along with a structure of cholic acid that has been numbered. It is possible to see that from these structures that all three have a steroid structure with a side chain at position C17 and three hydroxyl groups at positions C3, C7 and C12.

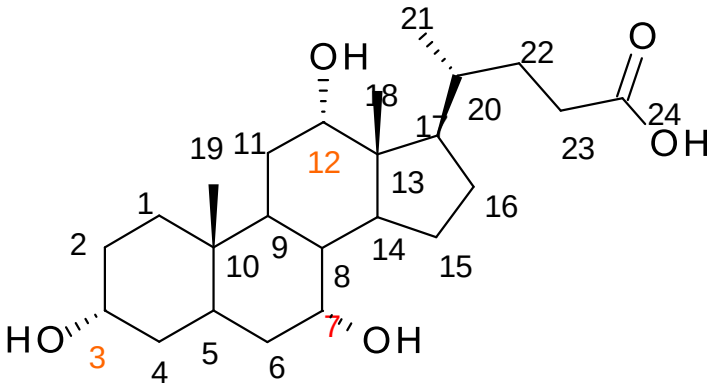


Figure 5 Structure of cholic acid with the carbon atoms numbered

1.9.1 Structure

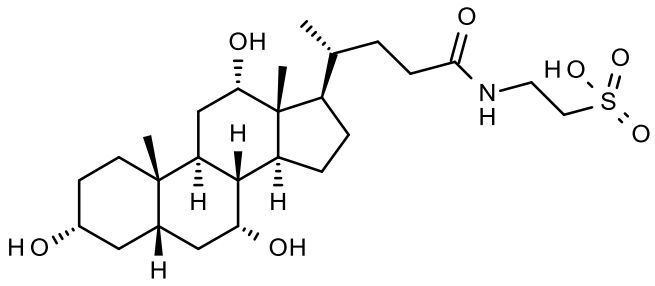


Figure 6 Structure of Taurocholate

Taurocholate is secondary bile acid formed by the conjugation of cholic acid and taurine. It has an amide and a sulfonic acid group for its side chain. It is generally agreed that taurocholate is a "gold standard" germinant for *C.difficile* spores and is routinely used in culture tests (L.J. Wheeldon *et al*., 2008). Recent theories of *C.difficile* germination suggest that taurocholic acid

and glycine act as co-germinants. Spores bind to taurocholic acid first then to glycine (Sorg and Sonenshein, 2010). Ramirez *et al* have recently described that there may be two taurocholate and glycine receptor sites (Ramirez *et al*., 2010). The theory states that as one of the taurocholic aid sites is occupied, a glycine site will also be occupied. This increases the chances of the next taurocholic acid and glycine sites to be occupied, triggering germination. Currently, the receptor sites for *C.difficile* are unknown and there are none of the well known receptor sites which are generally found on other *Clostridium* species (Lawley *et al*., 2009). While taurocholate is the best germinant, manipulation of the structure of taurocholic acid is difficult due to its high solubility in water and low solubility in organic solvents. Work has been carried out on the manipulation of taurocholic acid by a former PhD student without success.

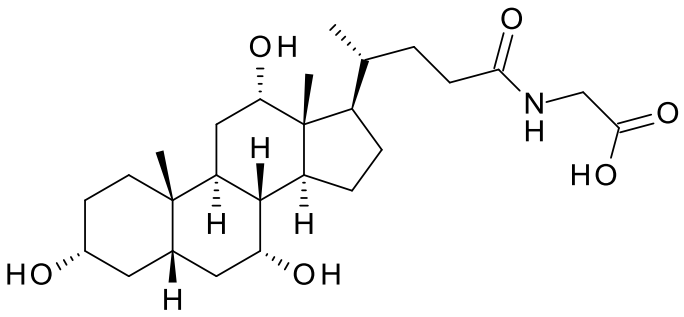


Figure 7 Structure of glycholate

Glycocholate is a secondary bile acid formed by the conjugation of cholic acid and glycine. It has an amide and a carboxylic acid group on its side chain. As with cholic acid, there is conflicting evidence for the germination qualities of this compound. While some have found that glycocholate is a germinant, others state it is a co-germinant (L.J. Wheeldon *et al*., 2008). While there is conflicting evidence concerning spore germination and bile acids, nearly all agree that a rich medium, such as horse blood agar (HBA) agar is needed for germination.

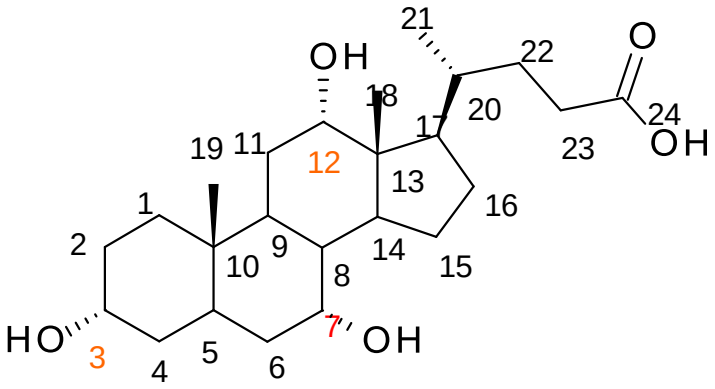


Figure 8 structure of cholic acid

Cholic acid, as shown in figure 8, has a side chain terminating in a carboxylic acid. There is some conflicting evidence concerning cholic acids germinating abilities with some papers saying it is a germinant of spores whereas others say it is a co-germinant. One research group have shown cholic acid to be a germinant but to a lesser extent than taurocholic acid (L.J. Wheeldon *et al*., 2008). It also requires a longer incubation period. There are some disadvantages to cholic acid, for example, it is not soluble in certain solvents, such as chloroform. It is, however, easily converted to the methyl ester form which increases its solubility.

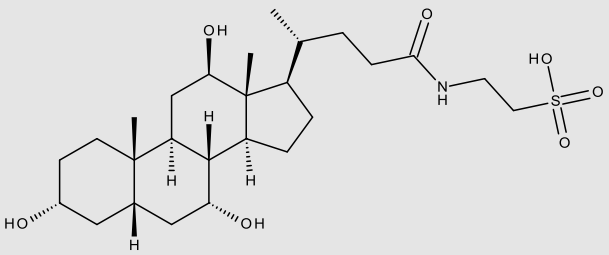
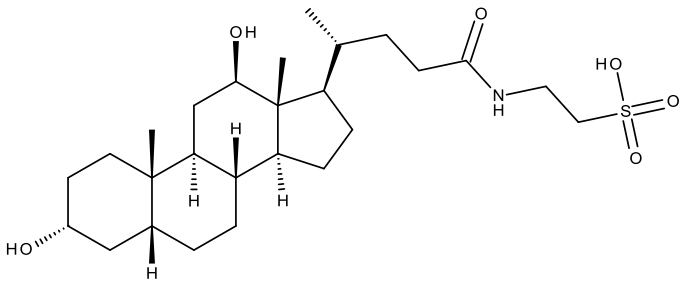
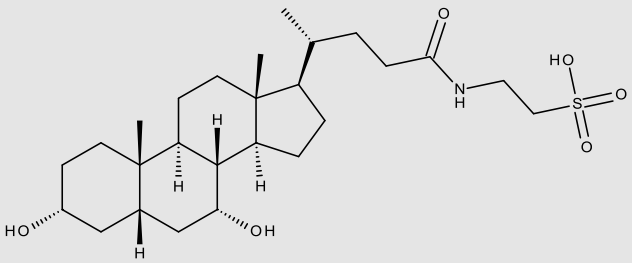
The structure of cholic acid (figure 4) has been labelled in the currently accepted manner. The three hydroxyl groups on the steroid body are on positioned on C3, C7 and C12. These hydroxyl groups are of varying importance for spore germination. Chenodeoxycholate has no C12 hydroxyl present and there is evidence to show that it is an inhibitor of germination (Sorg and Sonenshein, 2009). This suggests that the C12 hydroxyl is essential to spore germination but not to binding, as it is an active competitor to taurocholic acid. Deoxycholate is missing the C7 OH group and there is evidence to show that it still germinates spores, but it then suppresses vegetative cell growth (Britton and Young, 2012). This is interesting as it suggests that the C7-OH is not important for recognition, but is for growth of cells. As there are no natural bile acids with the C3 hydroxyl missing and a synthetic bile acid lacking the C3 hydroxyl has never been reported for *C.difficile* germination, the importance of this hydroxyl is unknown at this point.

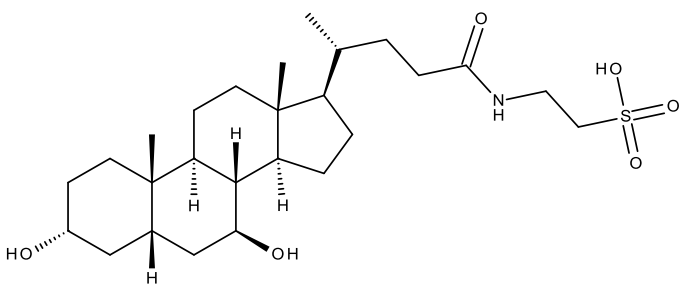
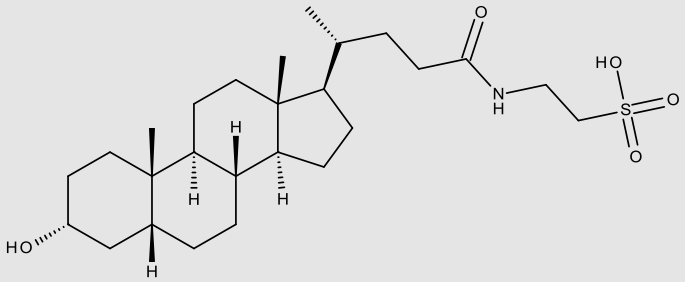
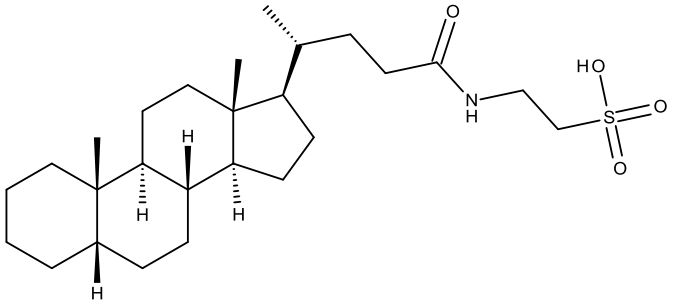
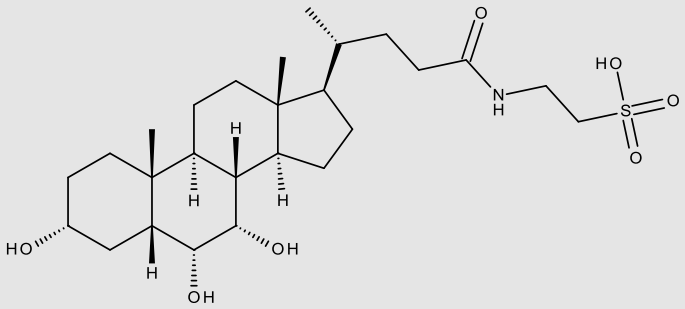
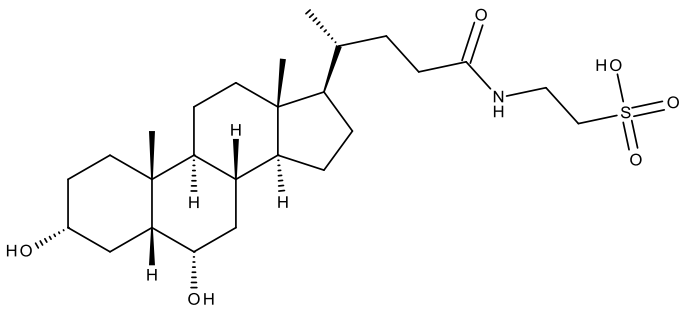
The side chain of the bile salt is also an important feature in germination. The sulfonic acid in taurocholate seems to be of significance as there is agreement that taurocholate germinates spores. It is used in culture tests and was used in diagnostic tests before

immunoassays became widely available. With regards to cholate and glycocholate, there is conflicting evidence to whether they are sole germinants or co-germinants.

1.9.2 Structure Activity

A recent paper has studied the importance of the side chain and hydroxyl groups on taurocholate for the germination of *C.difficile* spores (Howerton *et al*., 2011). Howerton *et al*. made various compounds, each with slight changes in the side chain or a change to the hydroxyl groups. They made 22 compounds altogether, pictured below. They have been arranged into two tables: changes to hydroxyls and changes to side chain.

Manipulation relative to taurocholate	Structure	Activity
Chirality change at C12-OH		No change
Removal of C7-OH		Reduced germination by 70%
Removal of C12-OH		Increased germination by 10%

Removal of C12-OH plus change in chirality at C7-OH		Decreased germination to 3%
Removal of C12-OH and C7-OH		Unable to achieve germination
Removal of all hydroxyls		Unable to achieve germination
Removal of C12-OH and addition of C6-OH		Unable to achieve germination
Removal of C12-OH and C7-OH plus addition of C6-OH		Unable to achieve germination

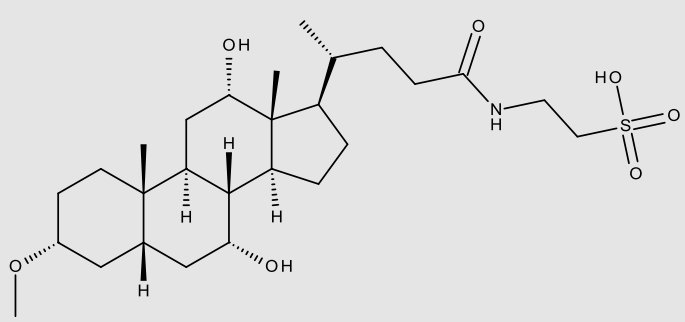
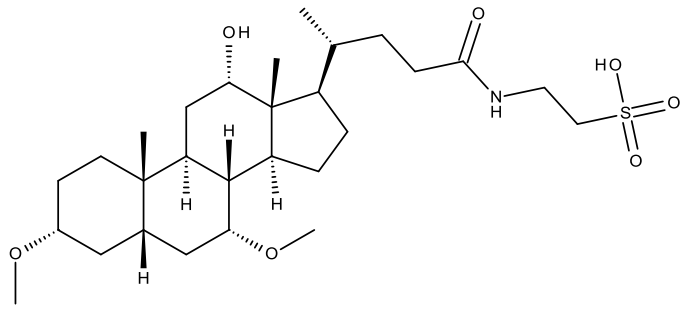
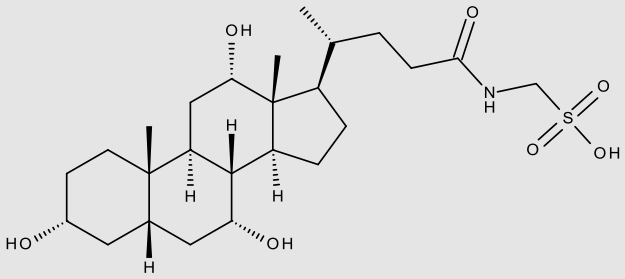
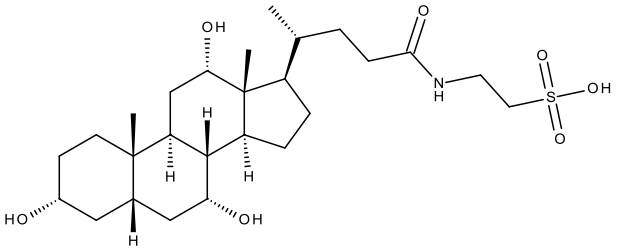
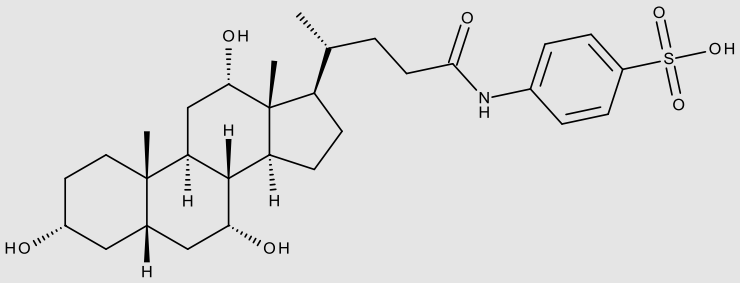
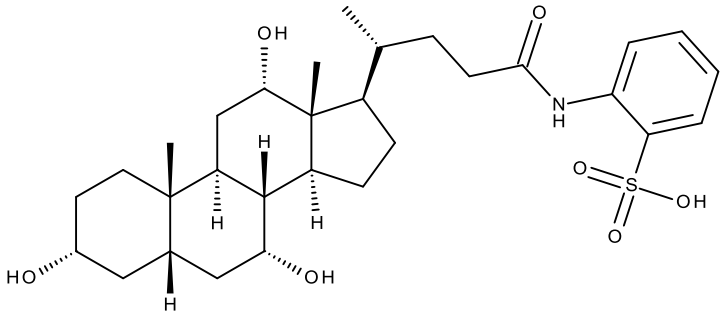
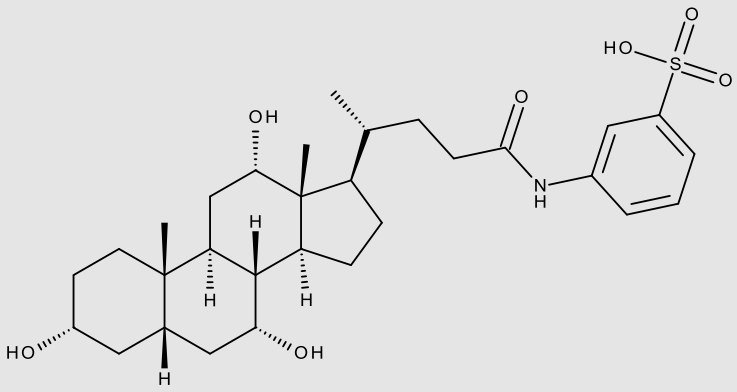
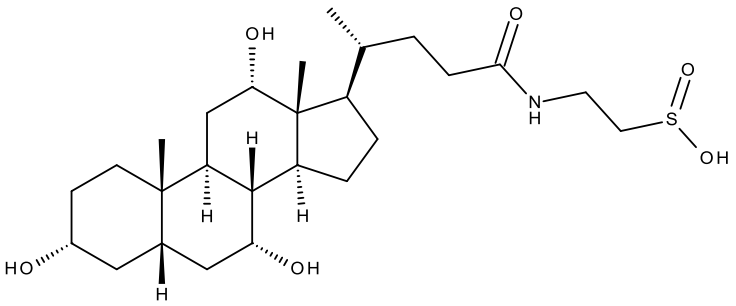
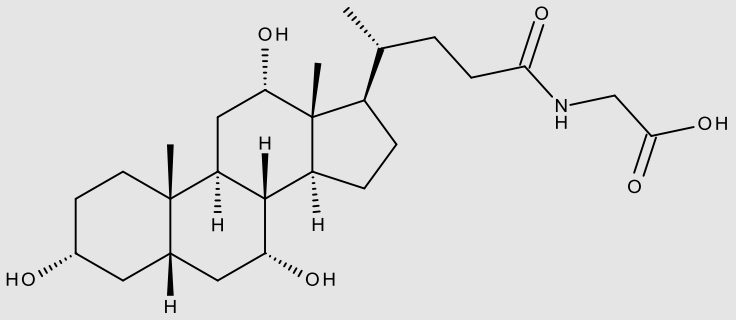
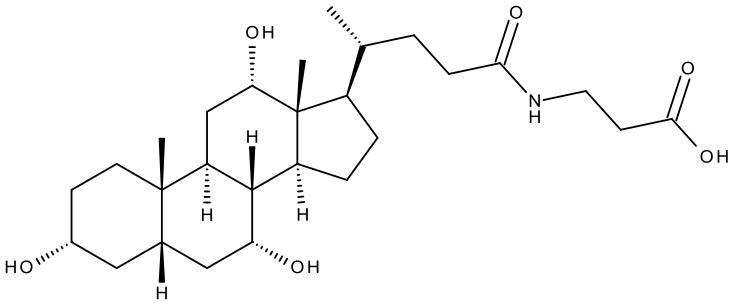
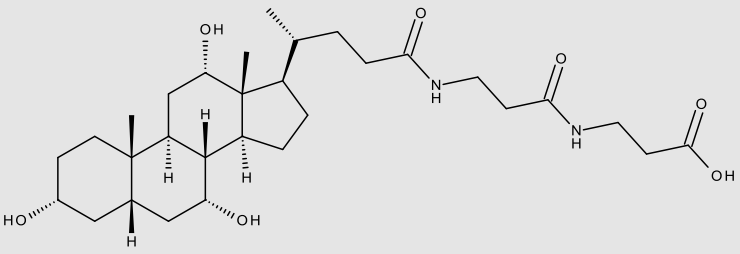
Methylation of the C3 hydroxyl		Unable to achieve or inhibit germination
Methylation of the C3 and C7hydroxyls		Unable to achieve germination

Table 2 Table to show the effect of hydroxyl manipulation and germination of *C. difficile* spores in taurocholate

Manipulation relative to taurocholate	Structure	Activity
Carbon chain shortened by one methylene group		Germination comparable to taurocholate
Carbon chain lengthened by one methylene group		Unable to achieve germination

Replacement of the taurine two carbon unit by a benzene ring		Unable to achieve germination
Replacement of the taurine two carbon unit by a benzene ring and movement of the sulfonic acid group to the ortho position		Unable to achieve germination
Replacement of the taurine two carbon unit by a benzene ring and movement of the sulfonic acid group to the meta position		Unable to achieve germination

Reduction of the sulfonic group to a sulfinic acid		Unable to achieve germination
Shortening the ethyl chain by one carbon and replacement of the sulfonic acid group with a carboxylic acid		Significantly germinate in buffer
Replacement of the sulfonic acid group with a carboxylic acid		Unable to achieve germination
Replacement of the taurine component in the side chain with a dipeptide derived from beta alanine		Unable to achieve germination

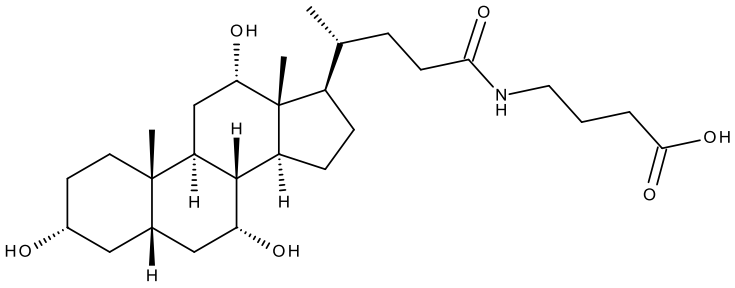
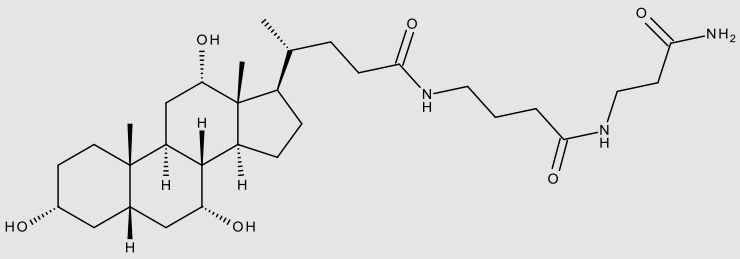
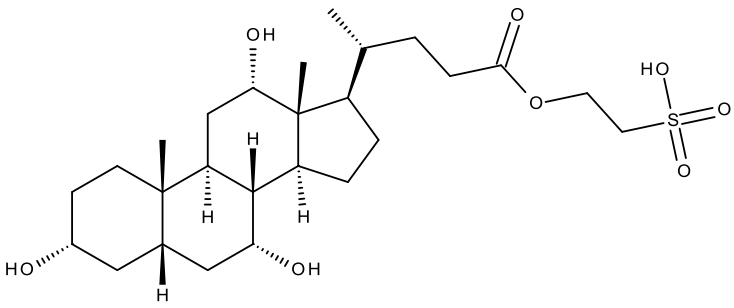
Lengthening the side chain by one carbon and replacement of the sulfonic acid group with carboxylic acid		Unable to achieve germination
Replacement of the taurine component in the side chain with a "dipeptide" derived from gamma-aminobutyric acid terminating in a carboxamide		Unable to achieve germination
Replacement of the amide in the side chain with an ester		Unable to achieve germination

Table 3 Table to show the effecs of side chain manipulation upon the germination of *C. difficile* spores in taurocholate.

It was found that changes in the side chain can make significant differences in the germination activity, with most of them being detrimental. Changes to the hydroxyl groups reduced or abolished the germinating activity. A short summery of the papers findings is given in table 4.

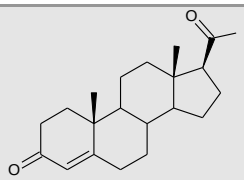
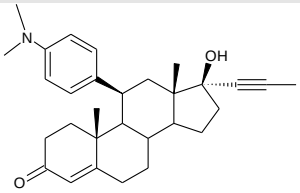
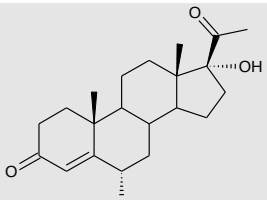
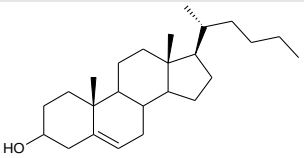
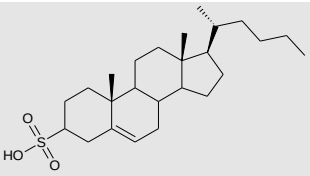
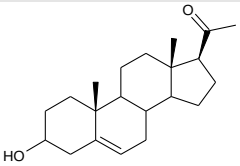
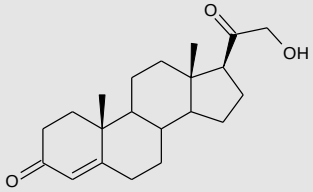
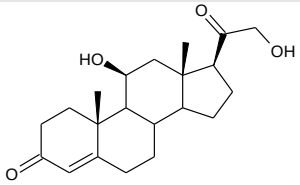
	Modification	Increased germination	No affect on germination	Decreased germination	Role of part
12-OH	Removal			✓	Important for binding and activation of spores
7-OH	Removal			✓	Important for binding and activation of spores
	Change in stereochemistry			✓	
3-OH	Removal			✓	Hydrogen bonding. Essential for the recognition of taurocholate
	Replaced with another hydrogen bond		✓		
C-17 side chain	Shortening alkyl chain		✓		Hydrogen bond ability of amide group is necessary for germination.
	Lengthening chain			✓	
	Bulkier linkages			✓	
	Replacing sulponate group with carboxylate		✓		

Table 4 summary of results from Howerton et al, 2011, that show the detrimental effects of side chain and hydroxyl manipulation of taurocholate and its spore germinating abilities.

Hydrogen bonding seems to be important for both recognition of taurocholate and for the germination of the spores. According to this study, it appears that all three hydroxyls are important, or at least, the hydrogen bonding ability of the hydroxyls. Changing the C3-OH to a methoxy changed the compound's ability to germinate the spores. The authors believe that the C3-OH is essential to the recognition of taurocholate and that its removal decreases germination greatly. One of the shortcomings of this study is that while they suggest the hydrogen bonding ability of the hydroxyl is essential, they do not confirm this with any other type of hydrogen bond donor group, such as an amine. Also, in regards to the other two hydroxyls at C7 and C12, there is no further investigation into the importance of these groups apart from their removal. Questions such as could another group effect the germinating abilities of the compounds remain unanswered. They have also shown that moving the hydroxyls to a different position renders the compound unable to germinate the spores. This is interesting as it suggests there must be a very specific receptor site on the spores for the C7-OH. The authors state that the C7-OH is important for recognition and binding. This is in direct conflict with the theory of deoxycholate, which states that the C7-OH is not important for germination but is important for cell growth. This conflict is indicative of the confusion surrounding the importance of each hydroxyl.

The conclusions reached with the side chain are a bit more conclusive. Side chain length is investigated, showing that a longer chain is detrimental to germination. This seems logical as the compound needs to fit into receptor sites on the *C.difficile* spore, and a longer chain may not fit well. The same goes for a bulkier chain. Again, the important factor for the tail seems to be its hydrogen bond donating ability. Replacing an amide with an ester resulted in no germination, which confirms the importance of the side chains to hydrogen bond. Amides are hydrogen bond donators, whereas esters are not. Replacing the sulfonic group with a carboxylic acid group was also detrimental. This could be due to the hydrogen bonding ability of the sulfonic group.

Liggins at al. Looked at progesterone analogs affects against *C.difficile* germination. They looked at 13 different compounds, the majority of which had no effect on the spores (Liggins *et al*., 2011).

Compound	Structure	Activity
progesterone		Inhibits germination
Mifepristone		Inhibits germination
Medroxyprogesterone		Inhibits germination
Cholesterol		No effect on germination or inhibition compared to progesterone
Cholesterol sulfate		No effect on germination or inhibition compared to progesterone
Pregnenolone		No effect on germination or inhibition compared to progesterone
21-hydroxy pregesterone		Reduces inhibition activity
Corticosterone		No effect on germination or inhibition compared to progesterone

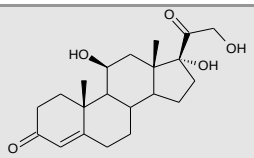
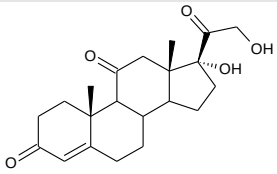
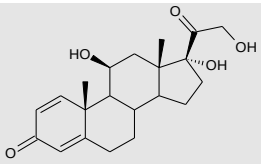
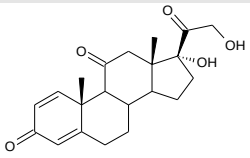
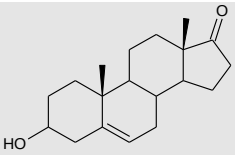
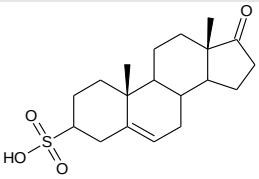
Cortisol		No effect on germination or inhibition compared to progesterone
Cortisone		No effect on germination or inhibition compared to progesterone
Prednisone		Inhibits germination
Prednisolone		Inhibits germination
DHEA		Inhibits germination
DHEA sulfate		Inhibits germination

Table 5 Table to show progesterone analogues effect on *C. difficile* spore germination

The main conclusion of this study was the suggestion that taurocholate, chenodeoxycholate and progesterone bind to the same receptor sites in *C.difficile* spores. Progesterone, however, shows non-cooperative binding. Progesterone was also shown to be 3-fold more active at spore germination inhibition than chenodeoxycholate. The paper also discussed that the spore germination seemed to be inhibited when a ketone group was present instead of a hydroxyl, and that a hydroxyl group at position 17 deactivates any spore germination inhibition. This appears to be true for some of the compounds tested, but does not explain compounds such as DHEA and prednisolone, both of which have ketone and hydroxyl groups present and are both inhibitors. While this paper has made some inroads into the germination of *C. difficle* spores, it does not explain it all.

Copper is known to kill *C.difficile* bacteria and was used in a study by Weaver *et al*. Pure copper is very effective in killing both spores and cells but the disadvantage to this is that the cost of copper can fluctuate so can make it expensive. Copper disrupts the cells membrane by

producing hydroperoxide radicals or it can disrupt the cells electron transport pathway, preventing metabolism from taking place (Weaver *et al*., 2008). Bismuth has also shown antimicrobial properties as shown by Mahony *et al*. various bismuth containing compounds were made and antimicrobial activity was tested for each one. Their activity changed with each compound but they did find some that were very effective at killing *C.difficile* cells. They speculated that sulphur was needed to chelate the bismuth and that the bismuth needed a specific carrier to deliver it to the cells (Mahony *et al*., 1999).

1.10. Hydroxyl Manipulation

There are three hyrdroxyl groups and a carboxylic acid on cholic acid. This means reactions can take place on four sites, making selectivity a problem. As already discussed, the hydroxyl groups on the bile acids have varying degrees of importance regarding germination. A different group on the C12 may render the bile acid incapable of germination and may actually inhibit it. For this reason, in order for test the germinating ability of the new compounds, the hydroxyl groups may have to be protected and then deprotected in order for selectivity to be achieved. Conversely, selectivity may be achieved with careful temperature control and timing for reactions. This approach would be advantageous as it decreases the number of synthetic steps required and limits the possibilities of by-product formation.

1.10.1 Non Protected Hydroxyls

The reactivity series of the hydroxyls to in cholic acid is C3>C7>C12. The C3-OH is a lot less hindered than the C7-OH and the C12-OH due to the presence of other methyl groups on the steroid skeleton. However, there is evidence to show the reactivity changes to C3>C12>C7 when chemistry involving reacting on the C3-OH occurs. If the C3-OH is involved in chemistry to modify the hydroxyl group to a oxo, tosy or silyl group, the C7-OH becomes the most hindered hydroxyl, making the C12OH the more accessible of the two. This change in chemical reactivity could have implications on the germinating ability of any compounds synthesised (Blickenstaff and Orwig, 1969).

1.10.2 Reacting on the C3-OH

Reacting on the C3-OH without protection of the other groups is possible due to the C3-OH being a lot more reactive than the other two hydroxyl groups. There are a few examples of this in the literature. Hirayama *et al* published details of a reaction between methyl cholate and 2-nitro-benzyl-bromide using silver (I)oxide as a catalyst to create an ester on the C3. They achieved good to modest yields, targeting the C3-OH position (Hirayama *et al*., 2003).

Two separate groups have used tert-butyldimethylsilyl chloride to selectively target the C3-OH position (Czajkowska and Morzycki, 2006; Zhang *et al*., 2009) to also create an ester linkage. Both Czajkowska *et al*. and Zhang *et al*. Used TBSMSCl/ imidazole to silyate at the C3 hydroxyl of methyl cholate. The only difference was the time and temperature conditions. Czajkowska *et al*. carried out the experiment at room temperature for 3 hours whilst Zhang *et al*. did it at 10^{o}C and room temperature for 48 hours.

1.10.3 Reacting on the C7 OH

Reacting on the C7-OH was done by Broderick at al. The hydroxyl group at position C7 was oxidised to a carbonyl group (Broderick *et al*., 1998).

1.10.4 Reacting on the C12 OH

Reacting on the C12-OH is difficult due to its reactivity and hindered position. Selective oxidation on the C12 is possible as described by Miljkovic at al. Methyl cholate was oxidised by using anhydrous sodium carbonate and bromine at 0-4^{o}C for 72 hours. Selective oxidation at the C12 position is possible as the reactivity order changes to C12>C7>C3 (MILJKOVIC *et al*., 1996).

1.11 Protected Hydroxyls

Selectively reacting on one hydroxyl group is difficult, especially if you only want to modify the C3-OH. For example, the reaction temperature needs to be controlled in order to slow down the reaction to a suitable pace where it can be monitored easily. Often, the molecular ratios have to be arranged so that there is an excess of starting material, to try and push the equilibrium to favour reacting on one position only. An alternative procedure to the one detailed above is to protect all the hydroxyl groups, then selectively remove the protecting groups and react on the unprotected hydroxyl group. This gives the advantage of using compounds where control of the reaction conditions are more difficult or when the cost of the reactants are high. One disadvantage of using protection groups is that they need to be removed afterwards, without removing anything else.

1.11.1 Protecting all of the hydroxyls

The most common form of hydroxyl protection is acetylation. There are various papers describing this method, all with slight variations. All use acetic anhydride, but there are differences in the rest of the method (A. Brady and K. M. Sanders, 1997; Tochtrop *et al*., 2002; Yoshii *et al*., 2004). Tochtrop *et al* use pyridine and 4-Dimethylaminopyridine (DMAP) with cholic acid to make a triacetylated compound in good yield (94%). Other forms of acetylation include acetylating on the C7 and C12-OH and using tetrahydropyranyl ether (THP) to protect the C3-OH. The tetrahydropyranyl group can then be deprotected in three steps, however, the yield is very low (16%). Fieser used hydrogen chloride in methanol to deprotect the 3OH, using benzene in the recrystallisation method (Fieser and Rajagopalan, 1950). Tochtrop protected the hydroxyl groups in order to work on the side chain so deprotected all of the acetoxy groups using sodium hydroxide.

Starchenkov *et al*. used formic acid to protect hydroxyl groups in cholic acid as the formate esters with the yields for tri-protection at less than 50% and di-protection (C3 and C7) was 77%. Jurcek *et al*. used formic acid as well (Starchenkov *et al*., 2000).

1.11.2 Reacting on the C3-OH

Protecting the C3 hydroxyl can be done in many ways. Hirayama *et al*. used 2-nitrobenzl bromide with methyl cholate to create on ester linkage on the C3, with yields of around 60% but had differences in regioselectivity before chromatographic purification (Hirayama *et al*., 2003). Czajkowska and Morzycki selectivity protected the C3 hydroxyl of methyl cholate using tert-butyldimethylsilyl chloride and imidazole in Dimethylformamide (DMF), which synthesised an ester linkage to the amine. They obtained yields of 92% and removed the *tert*-Butyldimethylsilyl ether (TBDMSO) protecting group using tetrabutylammonium fluoride after manipulating the side chain (Czajkowska and Morzycki, 2006). Yoshii *et al*. protected the C3OH using THP to create a protective ether but recovered yields of only 16%(Yoshii *et al*., 2004). Zhang *et al* also created a ether linkage using tert-butyldimethylsilyl chloride (TBDMSCl) recovering yields of 94% after recrystallization (Zhang *et al*., 2009).

Broderick *et al* selected to work on the C7-OH, creating a ketone (Broderick *et al*., 1998). This was done by first protecting the C3-OH as a hemisuccinate, followed by oxidation of the C7-OH by Jones reagent. Once this was achieved, they acetylated the C3 and C7 hydroxyls in order to manipulate the C12-OH. By first oxidising, then reacting with an amine group, they were able to react $(Boc)_2O$ to the C12-OH and then deprotect the C3 and C7-OH groups.

1.11.3 Semi-protection of the C3-OH and C7-OH

Other authors achieve acetylation on the C3-OH and the C7-OH only and so, have a semi-selective method. Broderick *et al* used pyridine and acetic anhydride with methyl cholate which, left at room temperature, reacted to achieve diacetylation at the C3 and C7 position (Broderick *et al*., 1998). Fieser and Rajagopalan used a mixture of benzene and pyridine with methyl cholate to achieve the same (Fieser and Rajagopalan, 1950). Zhang *et al* used DMAP and triethylamine in dichloromethane to acetylate on the C3- and C7-hydroxyls. Fieser and Rajagopalan also acetylated C3 and C7 but without using DMAP. They used benzene and pyridine, recovering 70% of their product.

1.11.4 Semi-protection ofthe C7 OH and C12 OH

It is also possible to protect two of the hydroxyls while keeping the other one free. For C3 and C7 protection, Cravotto *et al* acetylated the hydroxyls using high intensity ultraviolet light and microwaves stating the concentration of DMAP is essential for selectivity (Cravotto *et al*., 2005). Yoshii *et al* protects C7 and C12 hydroxyls by forming acetates using acetic anhydride but only after the C3 had been protected by THPO (Yoshii *et al*., 2004).

Various papers (Sorg and Sonenshein, 2009; L.J. Wheeldon *et al*., 2008) have shown that the removal of the C12 hydroxyl in a bile acid inhibits germination and Howerton *et al* has shown that there is some importance to the C7 OH in binding and activation of the spores (Howerton *et al*., 2011). Due to the importance of these hydroxyl sites, any chemistry done involving the bile acids will rely heavily on hydroxyl selectivity.

1.12 Manipulation of The Side Chain

For the purposes of this project, manipulation of cholic acids side chain is of equal importance to the work on the hydroxyl groups. In terms of *C.difficile* germination, less is known about the specific groups in the side chain of the bile acids than the importance of the hydroxyl groups, apart for the need for them to be a hydrogen bond donor. While there are very few papers discussing the manipulation of bile acids side chains in regard to *C.difficile*, there are many papers where it is being done for use in building scaffolds. As ultimately the project goals are to synthesis an antimicrobial polymer, it is sensible to add an amine chain to cholic acid, with the aim to later quaternise the end of the chain, creating an antimicrobial warhead. Detailed research in the literature shows there are many possibilities, using a range of different amines.

1.12.1 Cholic acid

Looking firstly at cholic acid, Vallegjo *et al* reacted bases (adenine or adenosine) to the side chain resulting in nitrogenated bile acid derivatives. The yields were low (20-30%) and they had complex mixtures, but if this type of reaction could be modified to create larger yields, it may be possible to react specific amino acids to cholic acid and increase chances of germination (Vallejo *et al*., 2007).

Randazzo *et al* used cholic acid and spermine to create an amine containing chain from the side chain of the bile acid. Tests carried out on the compounds showed that they were antimicrobial against both gram positive and gram negative bacteria. While the yields were low- 30-40%, they have shown that an antimicrobial amine chain can be attached to the side chain which is the main aim of this research (Randazzo *et al*., 2009).

Malik *et al* used TBDPSiCl and TBDMSi-Cl to protect the side chain on cholic acid by creating silyl esters. They found that by using the larger TBDPSi-Cl it was more selective, resulting in a 92% yield (Malik *et al*., 1986). This work could be adapted for the aims of this project and would allow the three hydroxyl groups present on cholic acid to be manipulated without affecting the side chain.

One method used in the literature to form amide bonds is to use ethylchlorocarbonate as a coupling agent. Ethylchloroformated is dripped in to a solution of cholic acid, anhydrous dioxane and tributylamine and left to form a mixed anhydride. This is then followed by the addition of the amine and left to react for three hours to form the amide bond

(Mukhopadhyay *et al*., 2004). This type of reaction is quick and offers good yields with little side reactions.

An amide side chain can also be synthesised when the hydroxyls on cholic acid are protected, as described by a paper in the Journal of the American Chemical Society (Mukhopadhyay *et al*., 2004). Here, the hydroxyls were protected using formic acid to give formate esters, and then the protected cholic acid was put into a solution of dichloromethane, DMAP and dicyclohexylcarbodiimide at 0^{o}C. Dimethylethylenediamine was added after 5 minutes where it was left to react for 18 hours at room temperature to create the amide bond and amine side chain.

1.12.2 Methyl cholate

An amide side chain can also be formed using methyl cholate. Methyl cholate can be preferable due to its increased solubility over cholic acid. An example of a simple manipulation of the side chain of methyl cholated can be seen with Kurosawa *et al* reacting methyl cholate with methylsulfinylcarbanion to create a sulphonyl group. This, however, gave a series of diastereoisomers. They then manipulated it further to create a longer ketone chain on the bile acid (Kurosawa *et al*., 1995). Czajkowska and Morzycki used allyl bromide and sodium hydride to create a six carbon ether chain.

Focussing on amide bond formation, Tamminen and Kolehmainen used ammonia to convert the methyl ester into an amine on methyl cholate as did Miljkovic *et al*.

Pandey *et al* described in a paper from the Journal of the Chemical Society described a method of reacting cholic acid with ethenediamine in the presence of methanol for 48 hours to give an amide side chain (Pandey *et al*., 2002).

1.12.3 Amide bond formation

The amide bond is an essential bond in chemistry and biology, responsible for a lot of biological activity. In biological systems, the amide bond is usually formed by specialist enzymes making the process quick and efficient. Unfortunately, the enzymes are usually much specialised so are unsuitable for some chemical reactions. This presents a problem for chemical reactions, as there are many different chemical ways of synthesising the amide bond and often the most efficient is not always obvious. Synthesis of the amide bond sometimes requires considerable optimisation, making the process time and cost heavy.

Whilst amide bond formation is possible this way in the form of aminolysis and the application of heat, amine degradation can present issues, although this can be prevented with short reaction times and the absence of oxygen (Montalbetti and Falque, 2005).

1.12.4 ACTIVATION

The most common way to form an amide bond is by first activating the carboxylic acid. Activation means that a better leaving group is attached to the compound, allowing for a faster reaction. An example of an activating agent is ethylchloroformate, which is an acyl chloride. The acid chloride of the ethylchoroformate attacks the carbonyl group of cholic acid, creating a mixed anhydride and hydrochloric acid, which can be removed using triethylamine. The mixed anhydride formed is a better leaving group than the carbonyl group so, when attacked by an amine, an amide bond will be formed.

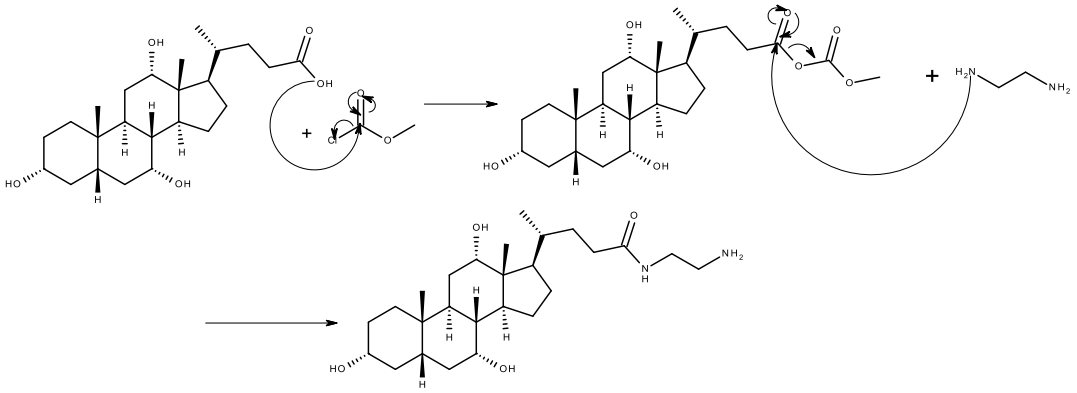


Figure 9 Figure to show the mechanism of action of activation of cholic acid by ethylchloroformate, then a further reaction with diaminoethane to form a cholic acid amide derviative

Other methods of activation include the use of acylimidazoles such as carbonyl diimidazole (CDI). This type of coupling allows a 'one pot' amide bond formation, although the reaction mechanism is more complicated than that of ethylchloroformate.

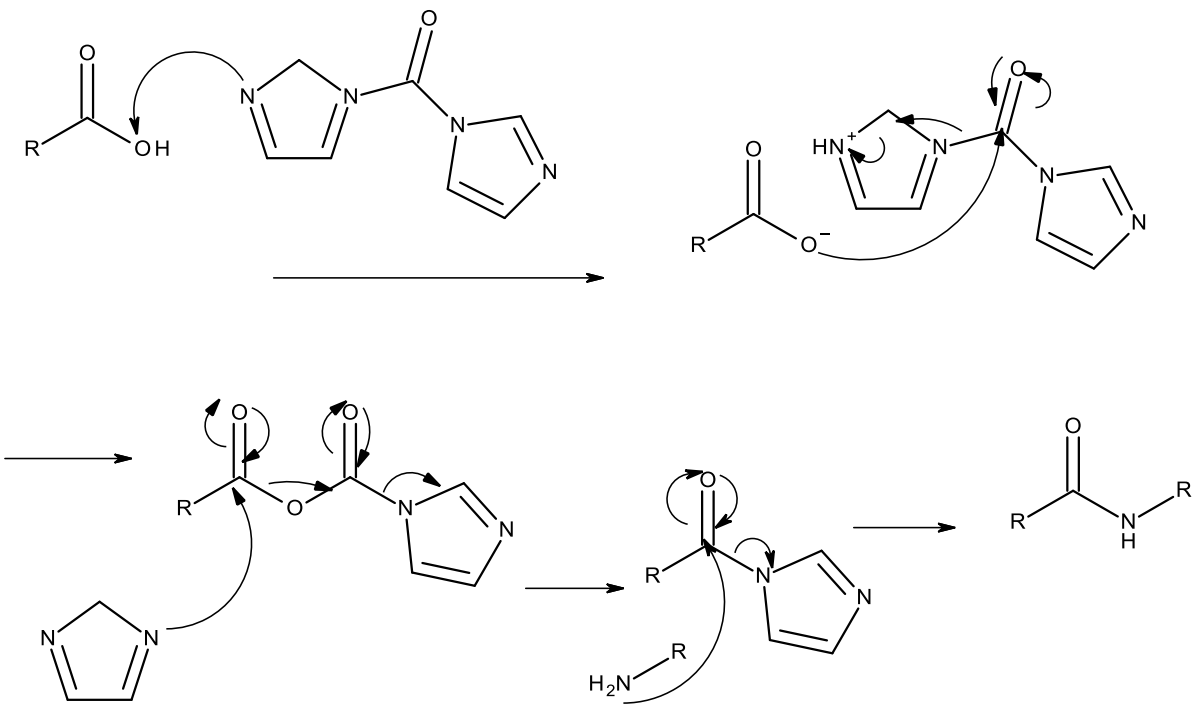


Figure 10 Figure to show the activation of a carboxylic acid by CDI, then aminolysis with an amine to form an amide.

1.12.5 Coupling

Anhydrides can also be used as coupling compounds. Dicyclohexyl carbodiimide (DCC) can be used to form symmetric anhydrides either using heat or by using two equivalents of the acid. Using DCC is a two step reaction, as the anhydride has to be formed first, before the amine can be added. Multiple water washes will remove any urea formed.

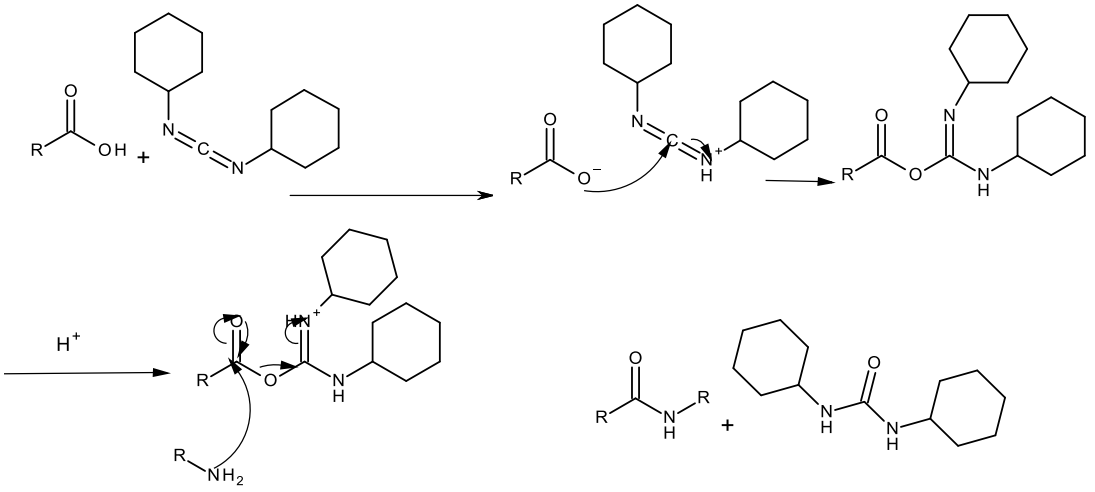


Figure 11 Figure to show the mechanism of action of DCC with a carboxylic acid followed by attack of an amine to form an amide bond.

1.13 Quaternasions

Quaternasions are formed between tertiary amines and alkylating agents. The nitrogen group will have a permanent positive charge whereas the halogen will have a negative charge. Quaternasions require very simple chemistry and a large library of compounds can be created due to the large number of haloalkanes available.

1.13.1 Antimicrobial Activity

Compounds containing a quaternary ammonium have been shown to have antimicrobial qualities to them due to their positive charge (Dhende et al., 2011). Most cell walls are negatively charged; for gram negative bacteria, the lipopolysaccharides in its cell wall give it the negative charge and for gram positive bacteria the negative charge is formed by the peptidoglycan. The positive charge on the quaternised compound disrupts the bacterial cell wall, causing lysis and killing the cell (Charnley et al., 2011).

1.13.2 Quaternary Ammonium Compounds

Quaternary ammonium compounds are synthesised by the alkylation of teriary amines, usually by haloalkanes. Haloalkanes are a group of alkylating agents are compounds containing halogen groups, for example, methyl iodide. They are reactive towards nucleophiles and can be small, for example, ethyl iodide, or large, for example, iodohexane. Quaternary ammonium cations have a permenant positive charge and when coupled with their anion, they are quaternary ammonium compounds or salts (McMurry, 2011).

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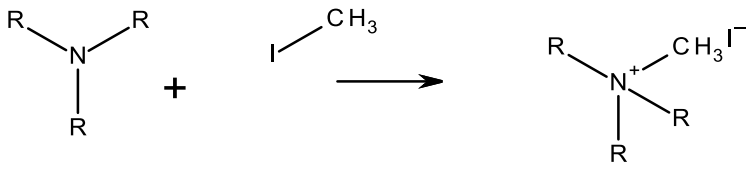


Scheme 1 Scheme to show the mechanism of action of a quaternastion of an teriary amine with a methyl iodide

The compounds do have some unfavourable health effects however. They can cause irritation to both the skin and the respiratory system, and can also cause more extreme symptoms such as skin burns and asthma. Quaternary ammonium compounds are used in many different

applications, for example, some disinfectants. They are very effective compounds but do have disadvantages, especially with prolonged use (Purohit et al., 2000).

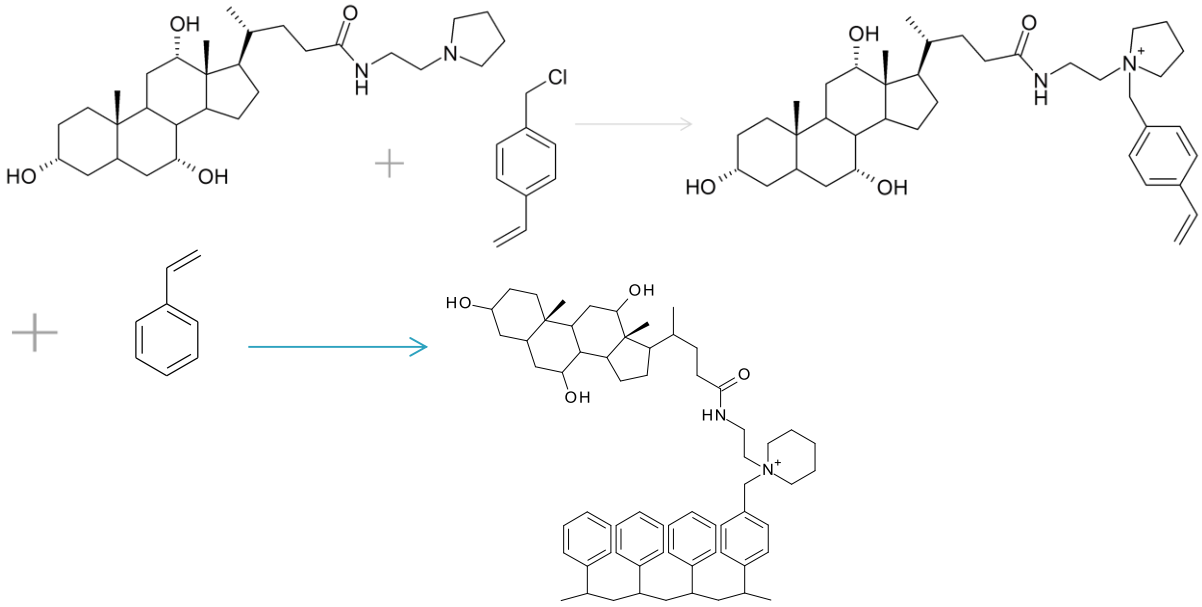
1.14. Polymerisations

Polymers are large molecule made up of smaller molecules (monomers) in repeating units. There are many different polymers beig used today, all with different properties. Copolymers are polymers which two or more repeating units which can be arranged in blocks, random, grafts or alternating units, all of which can drastically alter the properties of the polymer. The sterochemistry of the repeating units can be controlled to give isotactic, where the polymer groups are arranged in the same orientation, syndiotactic, where the groups are arranged in alternate positions, or atactic polymers, where the groups are placed randomly. All of these variants will give the polymers different properties. There is a precedent in literature of using bile acids in polymer synthesis due to their rigid, steroid structure which makes them ideal for liver drug delivery (Zhu and Nichifor, 2002).

1.14.1 Linear Polymers

Linear polymers are single, long, polymer chains, making them flexible and easy to analyse due to their solubility. There is only one carbon-carbon double bond for the initiator to react with. Examples of linear polymers are polystyrene and poly (methyl methacrylate). Linear copolymers are synthesised by reacting two monomers together to create a polymer with different properties.

In the context of this project, the polymers that are most interesting are generally copolymers with vinyl benzyl chloride (VBC) as the point of polymerisation. By attaching VBC to the cholic acid derivative, copolymers or monomers can be synthesised. For example, a derivative such as the one shown in figure 13 can be synthesised and then polymerised with styrene to give a copolymer based on polystyrene.



Scheme 2 the linear polymerisation of a polymerisable cholic acid derivative with polystyrene

By having the cholic acid derivative attached this way, the steroid body of cholic acid is free to move about and all three hydroxyls are free to bind with the *C.difficile* spores. The polymer also contains a quaternised nitrogen giving it an antimicrobial element. As the polymer is linear, it is soluble so can be easily characterised by various analytical techniques, such as NMR spectroscopy.

1.14.2 Cross linked Polymers

Cross linked polymers are more rigid and are harder to characterise once synthesised. Cross linked polymers require at least two polymerisable points on the same compound in order to cross link. Cholic acid has three potential polymerisable points- each of its hydroxyl groups. By attaching polymerisable groups to these, eg, methacrylates, cross linked polymers can be created, either as monomers or copolymers.

An example of a cholic acid monomer is shown in figure 14. All three hydroxyl groups have been reacted on allowing a crosslinked polymer to be synthesised.

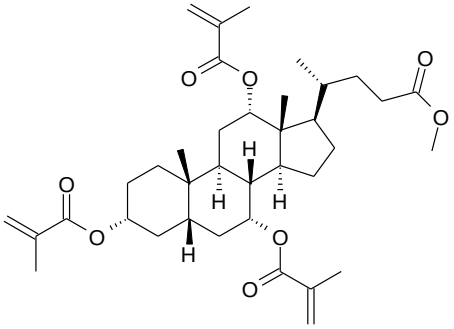
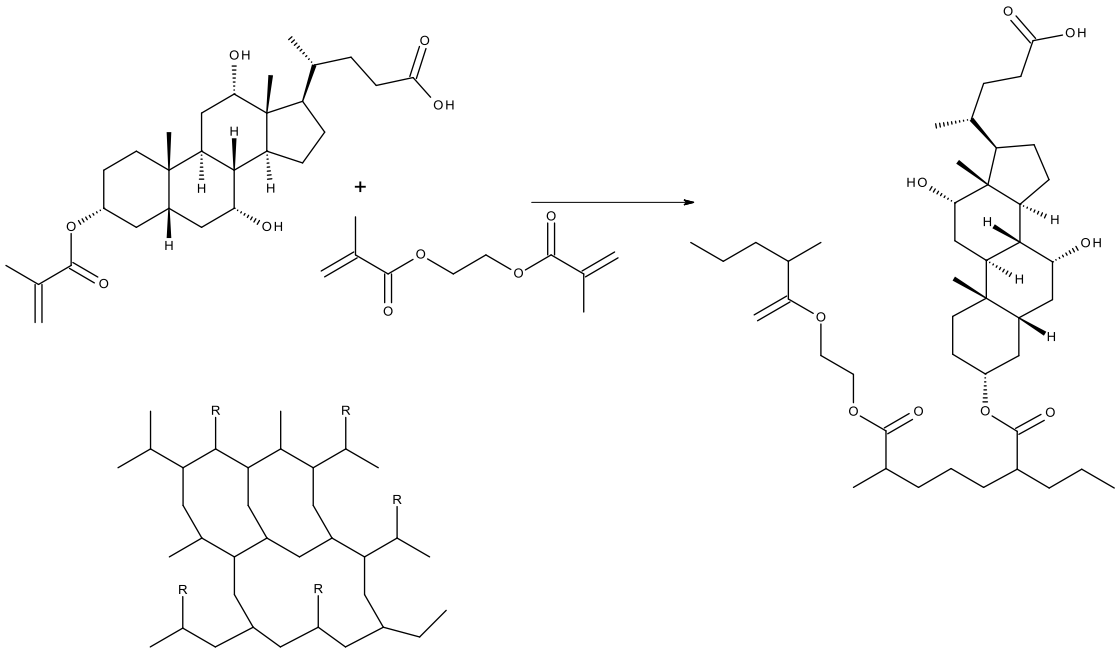


Figure 12 Trimethacrylated cholic acid methyl ester

Another way to create a crosslinked polymer is to use a crosslinker as the copolymer, for example, ethylene glycol dimethacrylate. Here, crosslinking can be achieved and only one hydroxyl group on cholic acid needs to be polymerisable.



Scheme 3 Figure to show an example of the polymerisation of a mono-acryoly cholate with EGDMA to form a cross linked polymer containing a germinating compound.

1.14.3 Antimicrobial Polymers

There are many different types of antimicrobial polymers. Some are specific to a type of bacteria whereas others are not. Polymers containing quaternary ammonium compounds disrupt the negative charge of the bacteria's cell wall and have been proven to kill bacteria upon contact (Charnley *et al*., 2011). The polymers properties are linked with its antimicrobial activity as reviews have found that antimicrobial activity is affected by the length, chemical functionality and alkyl spacer of the polymer. Immobilisation of the cationic group of the polymer increases its antimicrobial activity. Cross linked polymers generally have a reduced antimicrobial activity due to their rigidiness, although it can be increased if the cation chains are long and flexible enough to enter the bacterial cell wall.

The surface properties of the polymer also affect its antimicrobial activity. The hydrophobicity or hydrophillicity of the polymers and its surface charges can affect the way the bacteria adhere to the polymer (Gao *et al*., 2011). As a result of this, a library of novel polymers may have to be synthesized in order to achieve an effective antimicrobial polymer.

Antimicrobial polymers can be made in many different ways; the most common way shown in the literature is the synthesis of a polymer containing a tertiary nitrogen, then quaternisation of the polymer to form a quaternary ammonium compound inside the polymer. This method has the advantage of already having the polymer formed before quaternisation takes place, therefore avoiding any potential polymerisation issues that may arise from having a charged group on a polymerisable compound.

1.14.4 Polymerisable groups on hydroxyls

Attaching polymerisable groups on the three hydroxyl groups of cholic acid is well established in literature. One of the main ways of attaching a polymerisable group on the bile acid is to use methacryloyl chloride. By far the most common procedure for attaching a polymerisable group to the hydroxyls is by attaching a methacrylate or acrylate to the C3-OH. There are a few variations in the method, but the basic method is dissolve methyl cholate in a solvent, e.g. chloroform, add a base (triethylamine) and then to drip methacryloyl chloride in slowly. Depending on the temperture and molar equivalents of the methacryloyl chloride, the reaction will take place on one, two or all three of the hydroxyls. In order to react just on the C3-OH, the most common conditions was putting the solution of methyl cholate and triethylamine on ice at 0^{o}C, dripping in the methyacryloyl chloride, then allowing the temperature to return to room temperature (Benrebouh *et al*., 2001, 2000; Zhang *et al*., 2009, 1998).

Scheme 4 Scheme to show the methacrylation of the C3-H of methyl cholate

Denile and Zhu used the same procedure but converted the 3OH to an oxime first, however converting it to an amine created a mixture of the 3NH_2 stereochemistry. The alpha position had a yield of 56% but the authors found it easier to separate the two stereoisomers after it was reacted with methacryloyl chloride (yield 60%) (Zhu *et al*., 1996). Nichifora *et al* used succinic anhydride to create a polymerizable group on the 3OH position. First, however, the side chain was manipulated with trichloroethylene. After the reaction was complete, (95% yield) the products side chain was converted back into a carboxylic acid with zinc dust and acetic acid (Nichifor *et al*., 2004). Another alternative is using cholic acid, PTSA and diisopropylcarbodiiamide as carried out by Zuluaga *et al* (Zuluaga *et al*., 1999).

1.14.5 Polymerisable groups on the side chain of cholic acid

Polymerisation on the side chain is possible too, but it is not as common. Liu *et al* achieved an 88% yield for their product by first creating a chain on the tail that terminated in an amino group. The amine used was ethylenediamine and a yield of 92% was achieved (Liu *et al*., 2001). The mono-amine derivative was then reacted with acryloyl chloride on ice and left to warm and react at room temperature for 24 hours. By carrying out the reaction on ice, the reaction was slowed enough to allow the acryloyl chloride to react on the amine and not the hydroxyl groups on of cholic acid.Hao *et al* used cholic acid that had a glycoyl side chain attached to it using TEG. They then reacted it with methacryloyl chloride to make a polymerisable tail. Their yields were 65%. Hao *et al*. used the same method but got a yield of 70% (Hao *et al*., 2009). Zhang *et al* also modified the side chain to an amine before attaching a polymerisable group, this time using dimethylamino ethylamine. It was then attached to styrene through a methacryolyl group (Zhang *et al*., 2000).

1.14.6 Polymerisation

Polymerisation of monomers can be carried out in different ways depending on the desired qualities of the polymer. Copolymers (two or more monomers) can allow for the desired polymer properties to be more easily achieved.

1.14.7 Free Radical

During free radical polymerisation, the polymer is built by a radical initiator molecule. The most common way this is achieved is by using azobisisobutyronitrile (AIBN). As it decomposes, it releases nitrogen gas to form two 2-cyanoprop-2-yl radicals. These radicals initiate the polymerisation, creating both linear and cross linked polymers. Free radical polymerisation is usually poorly controlled and are unsuitable if a low molecular weight polymer polymers is desired (Davis *et al*., 1999).

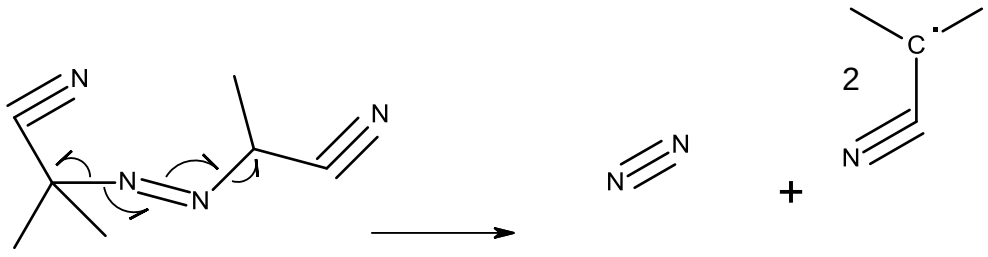


Figure 13 Figure to show the initiation of an AIBN free radical through degradation of AIBN

The general synthesis of a free radical polymerisation is as follows:

The required monomer is dissolved in an appropriate solvent, for example, propanol. A catalytic amount of an initiator, such as AIBN is added. The experimental container is then securely sealed and degassed, before heating to an appropriate temperature. The polymerisation can take between 24- 72 hours to complete, where a precipitate usually forms.

This procedure also works for copolymers.

The first published synthesis of the polymerisation of cholic acid was in 1988. It was carried out in toluene and used p-toluenesulfonic acid as the catalyst (Li et al., 2008).

1.14.8 UV ACTIVATION

UV activation of polymers is again initiated by free radicals, although this time by benzophnone molecules. When exposed to UV light, benzophenone molecules are reduced to hydroxydiphenylmethyl radicals, which can then initiate polymerisation (Scully *et al*., 2008). Benzophenone can inititiate crosslinking in a range of ethylene polymers, including high density polyethylene (Qu, 2002)

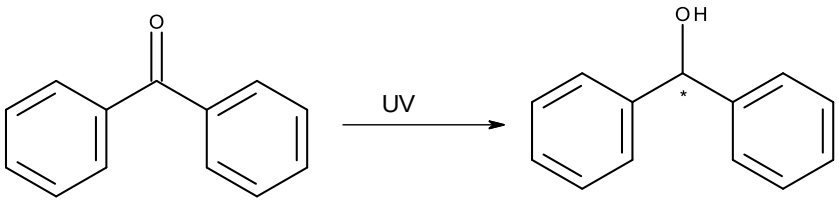


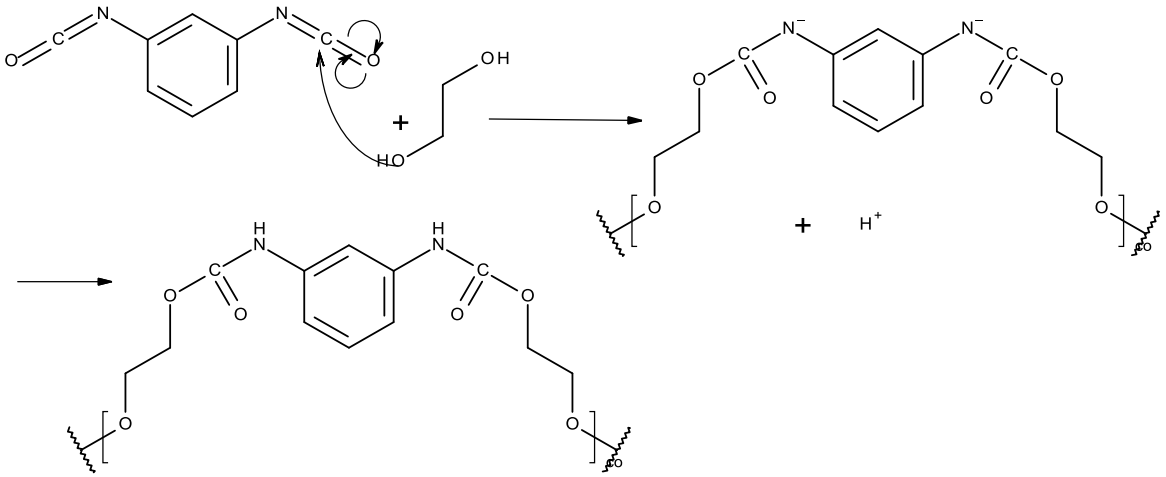
Figure 14 Figure to show the formation of a benzophenone free radical formed in the presence of UV light

In some cases, a solvent is needed to produce the radicals, as the benzophenone captures a hydrogen atom from the solvent to produce the radicals (Adam and Walther, 1996). When using polymers, the benzophenone can extract a hydrogen from the polymer chain to form the ketyl (K*) and polymer alkyl radicals (P*) (Qu, 2002). The amount of UV radiation needed to produce the benzophenone radicals is low; Scully *et al* estimated that the average irradiance of their samples was ~5 W/cm^3.

1.14.9 POLYUREATHANES

Another way to synthese polymers is to use isocyanates to create polyureathanes. Polyureathanes are routinely used to make biomedical equipment, such as catheters, artificial organs and in drug delivery. They can also be used to create coatings and hard plastics. The incorporation of biologically active materials is possible if it has a hydrogen containing group

present (Zdrahala and Zdrahala, 1999). Polyureathanes are prepared by reacting a diisocyanate and a diol. The basic mechanism for the reaction is below (McMurry, 2004).



Scheme 5 Scheme to show the mechanism of action for polyureathane formation between 1,4-cyclohexanediol and methylenebis (phenyl isocyanate)

Cross linked polymers are created when there are more than two hydroxyl groups for the isocyanate to react with.

As polyureathanes are so adaptable, they are idea for the controlled polymerisation of molecules which have specific qualities needed.

1.15 Project Hypothesis

The hypothesis of this project works on the theory that *C. difficile* spores can be forced to germinate in the presence of certain bile acids in unfavourable conditions. The spores of *C. difficile* are extremely difficult to eradicate and are a growing problem in health care settings. Whilst infection control policies have brought down the number of infections since 2007, lack of treatment options and resistance of some strains to one of the antibiotics used to treat the infection mean that the numbers are tailing off and could start to increase again. The vegetative cells of *C. difficile* are much easier to kill than the spores and they also don't survive in an aerobic environment for long. By forcing the germination of the spores in unfavourable conditions, the vegetative cells can be more easily killed and the chances of spreading the infection is greatly reduced.

Quaternary ammonium compounds are known to be antimicrobial against a range of different bacteria. Attaching an diamine to the tail of cholic acid, a known *C. difficile* germinant, and then quaternising the amine, a potential germianting and antimicrobial compound could be synthesised. By incorporating the compound into a polymer, the polymer could be installed in health care settings and help stop the spread of *C.difficile* spores. This in turn could save thousands of lives and decrease the burden of hospital acquired infections on the NHS.

1.15.1 Objectives

The objectives of this project are to synthesise and a polymer containing a manipulated cholic acid derivative against its germinating ability of *C. difficile.* In addition to this, a library of compounds will be synthesised and tested in order to gain further understandings of the germination mechanism of *C. difficile*.

1.15.2 Aim

The aim of this PhD is to synthesis a smart polymer surface which incorporated an antimicrobial warhead in the form of a quaternary nitrogen, and cholic acid, a bile acid that has shown germinating abilities against *Clostridium difficile*. The purpose of this polymer is to stop the spread of *C.difficile* spores throughout health care settings by forcing the spores to germinate in unfavourable conditions, i.e., surface tops, and then the quaternary nitrogen would disrupt the vegetative cells cell wall, causing lysis and death of the cell.

By stopping the spread of the spores, which can live outside the body for months, many thousands of lives coud be saved and the risk of acquiring *Clostridium Difficile* Infection (CDI) would be greatly reduced.

2. EXPERIMENTAL

2.1 INSTRUMENTATION

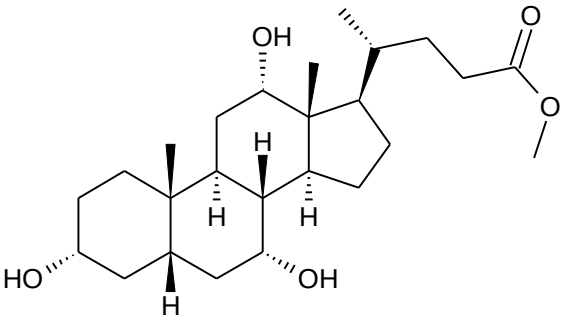
Proton NMR spectra were obtained on a Bruker AC 250 instrument operating at 250 MHz as solutions in $CDCl_3$ and referenced from $\delta CHCl_3$ = 7.26 ppm unless otherwise stated. Carbon NMR spectra were obtained on a Bruker AC 250 instrument operating at 63 MHz as solutions in $CDCl_3$ and referenced from $\delta CDCl_3$ = 77.0 ppm unless otherwise stated. Infrared spectra were recorded as KBr discs on a Mattson 3000 FTIR spectrophotometer or using thermo scientific nicolet 1s5 with ATR attachment as a solid sample. Electrospray mass spectrometry was carried out on a Waters LCT Premier ToF (Time of flight) mass spectrometer. Electrospray mass spectrometry and accurate mass spectrometry was also carried out by the EPSRC National Mass Spectrometry Facility in Swansea with a MAT95 magnetic sector. Melting points were obtained using a Reichert-Jung Thermo Galan hot stage microscope and are corrected.

All chemicals were purchased from Sigma Aldrich.

2.1.1 AMINOLYSIS

Synthesis of methyl cholate (1)

Based on a procedure formed by Fieser *et al*. (Fieser and Rajagopalan, 1950)



Cholic acid (5.0 g, 12.2 mmol) was added to dry methanol (20 mL). Acetylchloride (0.5 mL, 0.04 mmol) was added under argon. The solution was heated at reflux for 45 minutes, then left to cool. Once at room temperature, the solution was cooled further on ice whereupon crystals appeared. The solid material was collected by filtration and washed with small amounts of methanol to give methyl cholate as a white solid. It was dried at room temperature under vacuum to give a white solid.

Yield= 3.548g (68.9%)

TLC: (MeO, R_f=0.86 (single spot)

Melting point= 110.8-111.9 ^{o}C

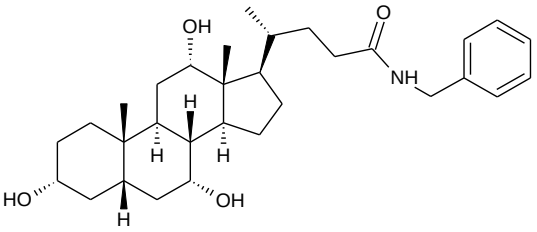
^{1}H NMR ($CDCl_3$) δ ppm: 0.67 (s, 3H, Me-18), 0.88 (s, 3H, Me-19), 0.99 (d, J= 6.0 Hz, 3H, Me-21), 1.0-2.43 (m steroid structure) 2.49 (s, 1H, H-C-C=O), 3.47 (s, 1H, H-C-OH), 3.66 (s, 1H, CH-3), 3.84 (d, 1H, CH-7), 3.95 (s, 1H, CH-12) ppm

^{13}C NMR ($CDCl_3$) δ 174.77(C=O), 73.26 (C12), 68.66 (C7), 52.67 (C3), 51.50 (CH_3), 50.33, (47.05, 46.44, 41.82, 39.47, 39.01, 35.24, 34.70, 31.06, 29.86, 28.17, 27.45, 26.60, 23.17 steroid structure) 22.44 (C19), 17.31 (C21), 12.49 (C18) ppm

MS (+APCI) m/z= 482 (M^+)

IR v = 3399 (OH), 2921, 2869, 1739 (C=O), 1449 cm^{-1}

Synthesis of cholic acid benzyl amide (2)



Method 1. Cholic acid (1 g, 2.44 mmol) was dissolved in DMF (10 ml) under argon. Once the cholic acid had dissolved, diisopropylethylamine (1.7 ml, 9.6 mmol) and disuccinimidyl carbonate (0.7 ml, 2.7 mmol) was added and the reaction was left to stir at room temperature for 19 hours. Benzyl amine (0.5 ml, 4.88 mmol) was added to the solution. After a hour, a white solid had precipitated. The solid product was collected by vacuum filtration and washed with DMF. The product was dried at room temperature under vacuum to give a yellow solid.

Yield= 0.142 g (14.2 %)

Method 2. Methyl cholate (1 g, 2.3 mmol) was placed into a stainless steel pressure vessel along with benzyl amine (1.3 mL, 12.2 mmol) and toluene (5 mL). The pressure vessel was placed into an oil bath and heated to 150^{o}C for 48 hours. The pressure vessel was left to cool before dismantling it. The solution was poured into a round bottomed flask and placed on ice. The solid product was then collected by filtration and dried at room temperature under vacuum. The material was recystallised from ethanol / water to give a white, powdery solid which was dried at room temperature under vacuum under vacuum.

Yield= 0.341g (34.1%)

TLC: (MeOH/DCM 5/1), R_f=0.53 (single spot)

Melting point= 114.1-114.7 ^{o}C

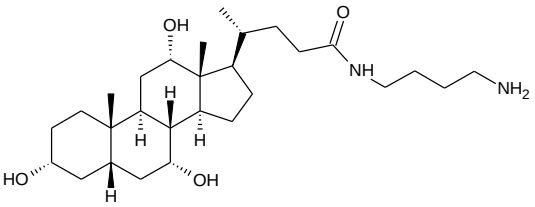
^{1}H NMR (Methanol -d_4) δ ppm: 0.69 (s, 3H, Me-18), 0.91 (s, 3H, Me-19), 1.02 (d, J= 6.5 Hz, 3H, Me-21), 1.0-2.439 (m, steroid structure) 3.36 (m, J=3.4 Hz, 1H, CH-3), 3.78 (s, 1H, CH-7), 3.94 (s, 1H, CH-12), 4.35 (d, 2H, J= 1.9 Hz PH-CH_2), 7.29 (m, J= 6.2Hz, 6H, aromatic ring) ppm

^{13}C NMR ($CDCl_3$) δ 219.45, 218.76, 173.58 (C=O), 154.31 (aromatic ring), 128.69 (aromatic ring), 127.85 (aromatic ring), 127.48 (aromatic ring), 125.07 (aromatic ring), 76.51 (C12), (43.61, 41.92, 39.51, 35.26, 34.69 steroid structure), 26.58 (C19), 17.44 (C21), 12.54 (C18) ppm

MS (+APCI) m/z=Found 497.3578; calculated for $C_{31}H_{48}N_1O_4$ 497.71; -1.4 ppm

IR ν = 3411, 2917, 1644 (C=O), 1540, 1457 cm^{-1}

Synthesis of N-(4-aminobutyl) cholanamide (3)



Methyl cholate (1 g, 2.3 mmol) along with 1,4-diaminobutane (1,4-DAB) (10 mL) was put into a stainless steel pressure vessel and tightly secured. It was placed into an oil bath and heated to 150^{o}C for 48 hours. The pressure vessel was left to cool to room temperature before dismantling it. 30mL chloroform was added to dissolve the product and excess 1,4-DAB which was then poured into a round bottomed flask. The chloroform was taken off using a rotary evaporator and the 1,4-DAB was taken off under reduced pressure rotary evaporation. Dichloromethane (90 mL) was added to precipitate the product. The yellow solid was collected by filtration and dried at room temperature under vacuum.

Yield: 0.3 g, (30.14%)

TLC: (MeOH/DCM 1/5, R_f=0.14 (single spot)

Melting point: 116.4-124.5 ^{o}C

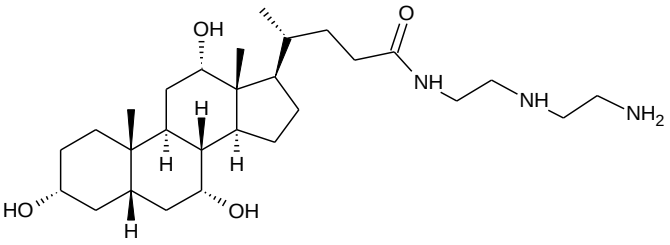
^{1}H NMR ($CDCl_3$) δ ppm: 0.68 (s, 3H, Me-18) 0.89 (s, 3H, Me-19) 1.00 (d, J= 6.3Hz, 3H, Me-21) 1.0-2.43 (m ,steroid structure) 1.22 (dd, J-14.1, 8.5Hz, CH) 2.71 (s, CH_2) 3.28 (m, 1H, CH-3) 3.86 (s, 1H, CH-7) 3.98 (s, 1H, CH-12) ppm

^{13}C NMR (DMSO) δ 172.43 (C=O), 107.85, 70.97 (C12), 66.20 (C7), (46.08, 45.70, 41.35, 40.17, 39.84, 37.97, 35.65, 35.13, 34.87, 34.36, 28.55, 27.51, 27.25, 26.42, 26.20, 22.81, 22.60 steroid ring), 41.71 (CH_2), 40.51(CH_2), 30.38(CH_2), 20.75 (C19), 19.52 (C21), 17.10, 12.31 (C18). ppm

MS (+APCI) m/z= Found 478.3849; calculated for $C_{28}H_{50}N_2O_4$ 478.3771; -1.0 ppm

IR (KBr) v = 3274, 2935, 2898, 2865, 2831, 1736, 1669 (C=O), 1548 cm^{-1}

Synthesis of N-[2-(2-aminomethylamino)ethyl] cholanamide (4)



Methyl cholate (0.5 g, 1.15 mmol) along with diethylenetriamine (2 mL) was put into a round bottomed flask under argon. It was placed into an oil bath and heated to 95 ^{o}C for 48 hours. The flask was left to cool to room temperature. Aceonitrile (15 mL) was added to precipitate the product which was then collected by filtration. The product was purified by solvent extraction between water and chloroform. The chloroform was evaporated under reduced pressure. The solid was dried at room temperature under vacuum.

Yield=0.09 g (18%)

TLC: (MeOH) R_f=0.1 (dominant spot), 0.35 (two spots)

Melting point: 205.6-206.8 ^{o}C

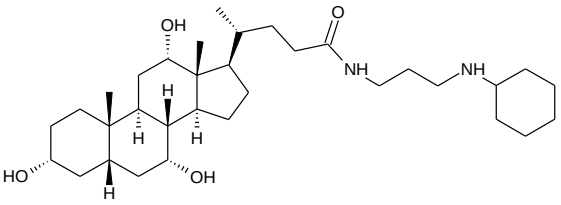
^{1}H NMR ($CDCl_3$) δ ppm: 0.66 (s, 3H, Me-18) 0.87 (s, 3H, Me-19) 0.9 (d, J=6.0Hz, 3H, Me-21) 1.0-2.44 (m, steroid structure) 2.69 (d, J=5.9Hz, CH_2) 2.75-2.88 (m, NH) 3.35 (s, MeOH) 3.47 (m, 1H, CH-3) 3.69 (m, impurity) 3.83 (s, 1H, CH-7) 3.94 (s, 1H, CH-12) 7.01 (s, 1H, NH_2) ppm

^{13}C NMR (DMSO) δ ppm: 172.59 (C=O), 70.98 (C12), 70.41 (C3), 66.22 (C7),(45.70, 41.50, 41.32, 40.49, 40.16, 35.28, 34.35, 32.44, 31.57, 30.38, 28.53, 27.28, 26.18 steroid ring), 22.58 (C19), 17.08 (C21), 12.32 (C18) ppm

MS (+APCI) m/z= Found 493.3958; calculated for $C_{29}H_{53}N_3O_4$ 493.3880; 0.6 ppm

IR (KBr) ν = 3253, 2929, 2865, 1751, 1648 (C=O), 1557 cm^{-1}

Synthesis of N-[3-(cyclohexylamino)propyl] cholanamide(5)



Methyl cholate (2 g, 4.6 mmol) along with cyclohexyl-1,3-propanediamine (4.08 mL) was added to a round bottomed flask under nitrogen. The flask placed in a oil bath at 95 ^{o}C for 48 hours before raising the temperature to 120 ^{o}C for 12 hours. The flask was allowed to cool before chloroform (30 mL) was added to dissolve the product. Solvent extraction between chloroform (30 mL) and water (30 mL) removed any excess amine. This was repeated three times. The chloroform layer was dried over magnesium sulphate and evaporated under reduced pressure to give a solid that was recrystallized from chloroform-petrol 60-80.

Yield = 1.2 g (60%)

Melting point: 104.5-105.8 ^{o}C

^{1}H NMR ($CDCl_3$) *δ* ppm: 0.67 (s, 3H, Me-18) 0.89 (s, 3H, Me-19) 0.98 (d, J= 7.5 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.61- 2.79 (m, ring) 3.26 (m, 1H, C<u>H</u>-3) 3.43 (m, 1H, N<u>H</u>) 3.84 (s, 1H, C<u>H</u>-7) 3.97 (s, 1H, C<u>H</u>-12) ppm

^{13}C NMR ($CDCl_3$) δ ppm: 173.94 (C=O), 71.85 (C12), 68.27 (C7), 56.87 (CH),(46.51, 41.49, 40.08, 39.58, 38.83, 35.38, 31.74, 31.62, 30.41, 29.08, 28.17, 27.61, 26.43 (CH), 26.10 steroid ring), 45.30 (CH_2), 40.68 (CH_2), 25.11 (CH), 34.80 (CH), 33.12 (CH), 23.31, 22.69, 22.51 (C19), 17.57 (C21), 14.17, 12.50 (C18), 11.48 ppm

MS (+APCI) m/z= Found 546.4475; calculated for $C_{33}H_{58}N_2O_4$ 546.4397; 0.4 ppm

IR (KBr) ν = 3265, 3068, 2919, 2853, 1648 (C=0), 1554 cm^{-1}

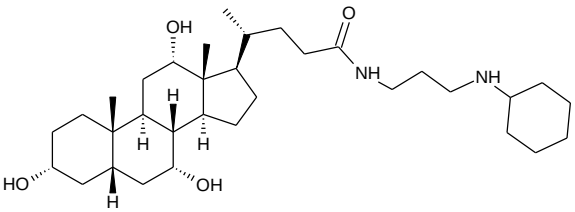
2.1.2 COUPLING CHOLIC ACID

Coupling of cholic acid with ethylchloroformate and various amines

General procedure for the coupling of cholic acid to primary and secondary amines

Cholic acid (0.5 g, 1.2mmol) was dissolved in THF (30 mL) along with triethylamine (2.9 mL, 0.3 mmol). The solution was put on ice for 10 minutes before ethylchloroformate (0.13 mL, 0.013 mmol) was dripped in over 10 minutes. The solution was allowed to react for two hours at room temperature. The required amine (1.2 mmol) was added and left to react for 3 hours. The reaction was quenched with water (30 mL). The mixture was washed with water (3 x 30 mL). The organic layer was dried over magnesium sulphate and the solvent was evaporated under reduced pressure. Solvent extraction between water and ethyl acetate was preformed 3 times before the organic layer was removed on the rotary evaporator. The product was dried at room temperature under vacuum.

Synthesis of N-[3-(cyclohexylamino)propyl] cholanamide (6)



The procedure was followed as the above with the exception of the removal of the two hour wait before adding the amine. Cyclohexylpropanediamine (0.24 mL, 1.56 mmol) added in one portion. The crude material was purified by flash chromatography eluting with methanol.

Yield = 0.36 g (72%)

TLC: (MeOH/EtOAc 1/3, R_f=0.23 (dominant spot), 0.89 (two spots)

Melting point: 104.5-105.9 ^{o}C

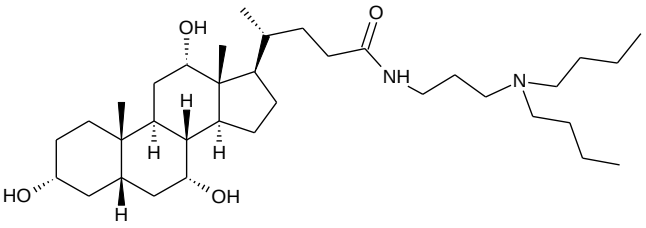
^{1}H NMR ($CDCl_3$) δ ppm: 0.67 (s, 3H, Me-18) 0.89 (s, 3H, Me-19) 0.98 (m, 3H, Me-21) 1.0-2.439 (m, steroid structure) 2.61- 2.79 (m, ring) 3.17 (d, J= 5.0 Hz, 6H, 3C$\underline{H}_2$) 3.43 (m, 1H, C$\underline{H}$-3) 3.64 (s ,1H, C$\underline{H}$-7) 3.77 (s, 1H, C$\underline{H}$-12) 3.87 (s, 2H, C$\underline{H}_2$-ring) 3.97 (s, 2H, C$\underline{H}_2$-ring) 4.01 (s, 2H, CH_2-CH) 4.10 (d, J= 2.5 Hz, 2H, C$\underline{H}_2$-CH) 4.33 (d, J=2.5 Hz, 1H, C$\underline{H}$-NH) 7.07 (m, 1H, N$\underline{H}$-C=O) 7.77 (m, 1H, N$\underline{H}$-ring) ppm

^{13}C NMR ($CDCl_3$) δ ppm: 173.94 (C=O), 73.08 (12), 71.85 (C3), 68.27 (C7), 56.87 (CH), (46.51, 41.49, 40.08, 39.58, 38.83, 35.38, 31.74, 31.62, 30.41, 29.08, 28.17, 27.61, 26.10 steroid ring), 45.30 (CH_2), 40.68 (CH_2), 34.80 (CH_2), 33.12 (CH_2), 26.43 (CH), 25.11 (CH), 23.31, 22.69, 22.51 (C19), 17.57 (C21), 14.17, 12.50 (C18), 11.48 ppm

MS (+APCI) m/z= Found 547.4461; calculated for $C_{33}H_{59}N_2O_4$ 547.4469; -1.5 ppm

IR (KBr) v = 3265, 3068, 2919, 2853, 1648 (C=O), 1554 cm^{-1}

Synthesis of N-[3-(dibutylamino)propyl] cholanamide (7)



The procedure was followed as above with 3-dibutylaminopropylamine (0.22 mL, 1.18mmol) . Purification of the crude product was done dissolving the product in ethyl acetate (10 mL), removing solid impurities by filtration followed by removing the organic solvent. The product was dried at room temperature under vacuum.

Yield= 0.82 g

TLC: (MeOH/EtOAc 1/4, R_f=0.25 (single spot)

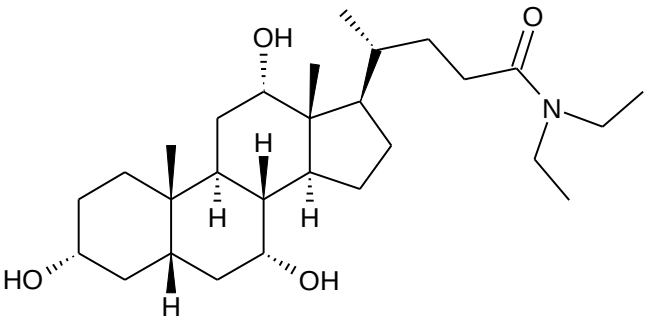
Melting point: 145.1-145.9 ^{o}C

^{1}H NMR ($CDCl_3$) *δ* ppm: 0.65 (s, 3H, Me-18) 0.88 (s, 3H, Me-19) 0.89- 0.97 (m, Me-21) 1.0-2.439 (m, steroid structure) 2.44 (m,butyl chain) 2.55 (m, butyl chain) 3.33 (m, propyl chain) 3.44 (m, 1H, C<u>H</u>-3) 3.84 (s, 1H, C<u>H</u>-7) 3.97 (s, 1H, C<u>H</u>-12) 7.43 (m, 1H, N<u>H</u>) ppm

MS (+APCI) m/z= 577.4931

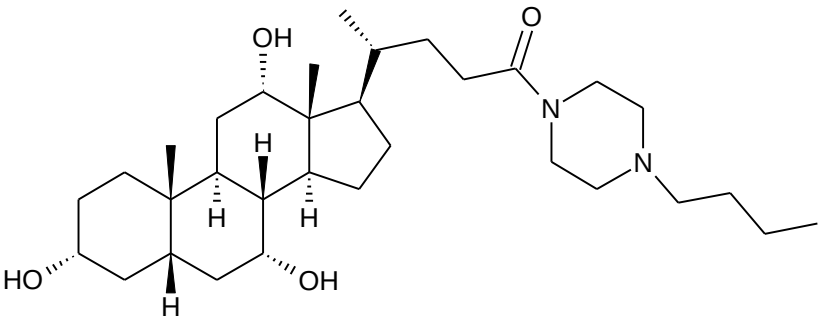
IR (KBr) v = 3299, 3089, 2919, 2853, 2358, 1727, 1642 (C=O), 1545, 1463 cm^{-1}

Attempted Synthesis ofN-[3-(dimethylamino)propyl] cholanamide (8)



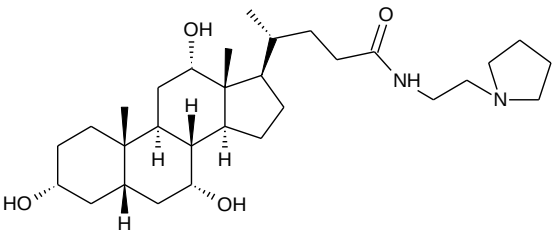
The procedure was followed as above with diethylamine (0.12 mL, 1.6 mmol). The product was found to contain at least four components as judged by TLC.

Attempted synthesis of 1-(4-butylpiperazine-1-yl) cholanone (9)



The procedure was followed as above with butyl piperazine (0.3 mL, 2.1 mmol). The product was found to contain at least three components as judged by TLC.

Synthesis of N-(2-pyrrolidin-1-ylethyl) cholanamide (10)



The procedure was followed as above with 1-(2-aminoethyl) pyrrolidine (0.21 mL, 1.8 mmol).

Yield= 0.74 g (74%)

TLC: (MeOH/EtOAc 1/1, R_f=0.18 (single spot)

Melting point: 96-97.1 ^{o}C

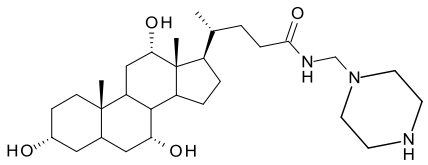
^{1}H NMR ($CDCl_3$) δ ppm: 0.68 (s, 3H, Me-18) 0.89 (s, 3H, Me-19) 1.00 (d, J= 6.2Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.23 (d, J=12.1 Hz, C<u>H</u>$_2$-ring) 2.64 (s 2H, C<u>H</u>$_2$-NH) 3.44 (m 1H, C<u>H</u>-3) 3.84 (s, 1H, C<u>H</u>-7) 3.96 (s, 1H, C<u>H</u>-12) 6.40 (s, 1H, N<u>H</u>) ppm

^{13}C NMR (DMSO) δ ppm: 172.45 (C=O), 79.14, 70.98 (C12), 66.21 (C7), 55.02 (CH_2), 53.58 (CH_2 ring), (46.12, 45.70, 41.49, 37.81, 35.13, 34.35, 32.51, 31.70, 26.18 steroid ring), 41.34 (CH_2) 23.08 (CH_2 ring), 22.59 (C19), 17.07 (C21), 12.30 (C18) ppm

MS (+APCI) m/z= Found 505.3993; calculated for $C_{30}H_{53}N_2O_4$ 505.4000; -1.4 ppm

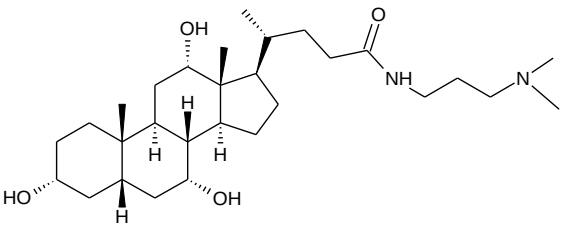
IR (KBr) v = 3287, 2929, 2862, 2155, 1642 (C=O) cm^{-1}

Attempted synthesis of 1-(4-phenylpiperazin-1-yl) cholanone (11)



The procedure was followed as above with 4-aminomethylpiperidine (0.23 mL, 2.0 mmol). The product was found to contain at least four components as judged by TLC.

Synthesis of N-[3-(dimethylamino)propyl] cholanamide(12)



The procedure was followed as above with 3-(dimethylamino)-1-propylamine (0.18 mL, 1.76 mmol).

Yield= 0.62 g (62%)

TLC: (MeOH/EtOAc 1/1), R_f=0.1 (single spot)

Melting point: 163.2-164.0 ^{o}C

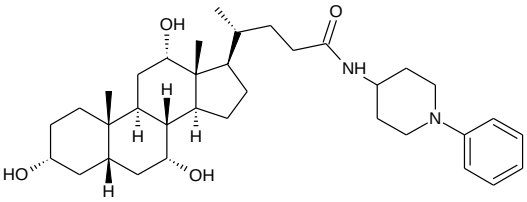
^{1}H NMR ($CDCl_3$) δ ppm: 0.67 (s, 3H, Me-18) 0.87 (s, 3H, Me-19) 0.97 (d, J= 6.2 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.21 (m, C$\underline{H}_2$-NH) 2.33 (s, 6H, 2C$\underline{H}_3$) 3.30 (m, J=5.7Hz, 1H) 3.82 (s, 1H, C$\underline{H}$-7) 3.96 (s, 1H, C$\underline{H}$-12) 6.97 (s, 1H, N$\underline{H}$) ppm

^{13}C NMR (DMSO) δ ppm: 176.07 (C=O), 70.99 (C12), 70.41 (C3), 66.23 (C7), 56.42 (CH_3), (46.25, 44.96, 41.50, 41.31, , 40.14, 35.25, 34.84, 34.36, 32.61, 30.38, 28.51, 27.30, 26.77, 26.17 steroid ring), 45.72 (CH_3), 40.47 (CH_2), 31.52 (CH_2), 22.80, 22.58 (C19), 17.04 (C21), 12.34 (C18) ppm

MS (+APCI) m/z= Found 493.3994; calculated for $C_{29}H_{53}N_2O_4$ 493.4000; -1.2 ppm

IR (KBr) v = 3387, 2932, 2862, 2209, 1991 cm^{-1}

Synthesis of N-(1-phenyl-4-piperidyl) cholanamide (13)



The procedure was followed as above with 4-amino-1-benzylpiperidine (0.34 mL, 1.79 mmol).

Yield= 0.88 g

TLC: (MeOH/EtOAc 1/1), R_f=0.68 (single spot)

Melting point: 79.9-86.5 ^{o}C

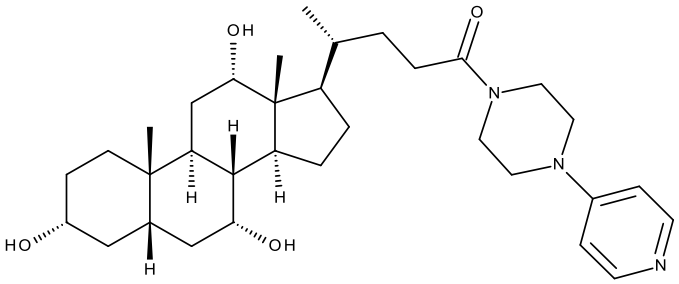
^{1}H NMR ($CDCl_3$) δ ppm: 0.67 (s, 3H, Me-18) 0.89 (s 3H, Me-19) 0.99 (d, J= 6.1 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.83 (d, J=11.9Hz, C$\underline{H}$-NH) 3.43(m, 1H, C$\underline{H}$-3) 3.48 (d, J=3.2Hz, C$\underline{H}$-NH) 3.83 (s, 1H, C$\underline{H}$-7) 3.96 (s, 1H, C$\underline{H}$-12) 5.72 (d, J=8.0Hz, 1H) 7.317 (s, 1H, N$\underline{H}$) ppm

^{13}C NMR (DMSO) δ ppm: 171.78 (C=O), 138.72, 138.63, (128.65, 128.09, 126.77 aromatic ring), 70.98 (C12), 70.41 (C3), 66.21 (C7), 62.15, 51.97, 51.87, (46.12, 45.71, 41.50, 41.33, 40.51, 40.32, 40.17, 35.29, 35.11, 34.85, 34.36, 32.59, 31.73, 31.62, 30.39, 28.53, , 22.77 steroid ring), 27.27 (CH_2 ring), 26.19 (CH2 ring) 22.59 (C19), 17.12 (C21), 14.63, 12.31 (C18) ppm

MS (+APCI) m/z= Found 519.4138; calculated for $C_{36}H_{57}N_2O_4$ 581.4313. -61= benzene ring

IR (KBr) ν = 3279, 3006, 2925, 2810, 2358, 2162, 1782, 1646 (C=O), 1513 cm^{-1}

Synthesis of 1-(3-phenylimidazolidin-1-yl) cholanone (14)



The procedure was followed as above with 1-(4-pyridyl)piperazine (0.24 g, 1.5 mmol).

Yield= 0.523 g

TLC: (MeOH/EtOAc 1/1,) R_f=0.32 (dominant spot), 0.85 (two spots)

Melting point: 139.6-141.2 ^{o}C

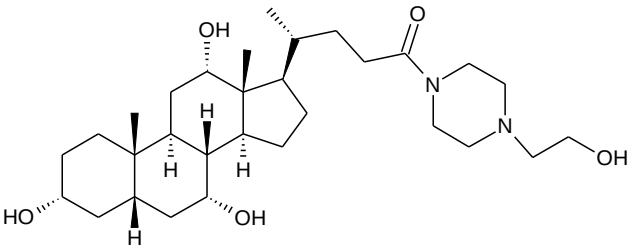
^{1}H NMR ($CDCl_3$) *δ* ppm: 0.68 (s, 3H, Me-18) 0.89 (s, 3H, Me-19) 0.98 (d, 3H, Me-21) 1.0-2.43 (m steroid structure) 2.26 (m, 1H, C<u>H</u>) 3.63 (m, 1H, C<u>H</u>-3) 3.85 (m, 1H, C<u>H</u>-7) 3.98 (s, 1H, C<u>H</u>-12) 4.12 (d, J=6.95 Hz, 2H, C<u>H_2</u>-N) 6.55 - 6.81 (m, 2H, C<u>H_2</u>-N) 8.12 - 8.45 (m, 2H, aromatic ring) ppm

^{13}C NMR (DMSO) δ ppm: 173.30 (C=O), 171.33, 154.17, 149.80, 108.29, 99.48, 71.02 (C12), 70.41 (C3), 66.21 (C7), 59.58, (45.74, 45.74, 45.12, 40.47, 40.14, 35.27, 34.96, 34.35, 30.70, 30.37, 28.49, 26.17 steroid rings), 27.27 (CH_2 ring) 22.77, 22.59 (C19), 17.17, 16.87 (C21), 14.11, 12.28 (C18) ppm

MS (+APCI) m/z= Found 454.3528; calculated for $C_{33}H_{51}N_3O_4$ 553.387. MI-N(Ph)-NCH_2CH_2

IR (KBr) ν = 3396, 3250, 2929, 2859, 2158, 1724, 1593, 1515 cm^{-1}

Synthesis of 1-[4-(2-hydroxyethyl)piperazin-1-yl] cholanone (15)



The procedure was followed as above with 1-(2-hydroxyethyl)piperazine (0.2 mL, 1.5 mmol). Proton NMR analysis showed excess of amine present.

Yield= 0.4 g (80%)

Melting point: 167.8-168.1 ^{o}C

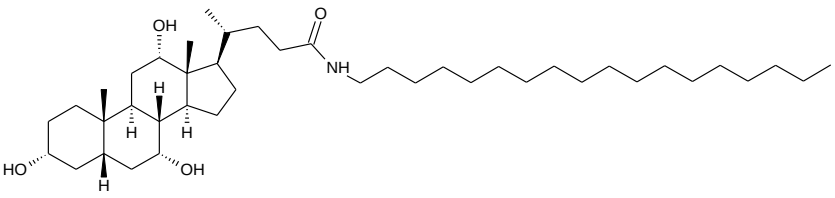
^{1}H NMR ($CDCl_3$) δ ppm: 0.70 (s, 2H, Me-18) 0.90 (s, 2H, Me-19) 1.10 (d, J= 5.3Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.50-2.63 (m, CH-N) 3.48 (m, J=4.9 1Hz, CH-3 and CH) 3.64 (d, J= 5.3Hz, CH-N) 3.86 (s, 1H, CH-7) 3.98 (s, 1H, CH-12) ppm

^{13}C NMR (DMSO) δ ppm: 170.97 (C=O), 154.56, 71.00 (C12), 70.40 (C3), 66.22 (C7), 60.61, 60.12 (CH_2), 58.43 (CH_2), 52.93 (CH_2 ring), 52.80, (46.08, 43.29, 41.48, 35.26, 35.21, 34.83, 34.35, 31.19, 30.39, 29.48, 28.47, 27.29, 26.17 steroid ring), 45.74 (CH_2 ring), 22.81, 22.59 (C19), 17.14 (C21), 14.54, 12.32 (C18) ppm

MS (+APCI) m/z= 521.3943

IR (KBr) v = 3617, 3414, 2916, 2853, 2810, 1687 (C=O), 1624 cm^{-1}

Synthesis of N-octadecylcholanamide(16)



The procedure was followed as above with octadecylamine (0.8 g, 2.9 mmol). Further purification carried out using solvent extraction (x 3)between THF/water (30 mL) and chloroform (30 mL). The organic layer was dried over magnesium sulphate and the solvent was

evaporated under reduced pressure. The product was dried at room temperature under vacuum.

Yield= 0.922 g (90%)

Melting point: 83.6-84.4 ^{o}C

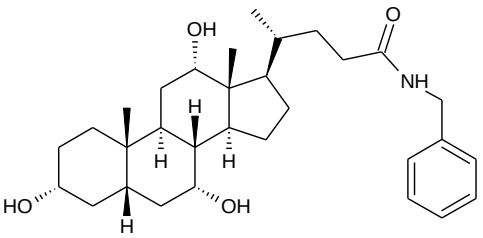
^{1}H NMR ($CDCl_3$) δ ppm: 0.66 (s, 3H, Me-18) 0.87 (d, J= 3.4Hz, 3H, Me-19) 0.97 (d, J= 5.9Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 1.23 (s, 37H, C$\underline{H}_2$-chain) 3.2 (q, J= 7.3, 6.8Hz, C$\underline{H}_2$-N) 3.43 (m, 1H, C$\underline{H}$-3) 3.83 (s, 1H, C$\underline{H}$-7) 3.96 (s, 1H, C$\underline{H}$-12) 5.56 (s, 1H, $\underline{N}$H) ppm

^{13}C NMR (DMSO) δ ppm: 172.90 (C=O), 172.43, 70.98 (C12), 70.40 (C3), 66.21 (C7), 56.68, (46.15, 45.68, 45.08, 41.48, 36.68, 35.12, 34.35, 31.75, 31.26, 30.34, 29.02, 28.67, 27.13, 26.18 steroid ring), 22.79 (CH_2), 22.59 (CH_2), 22.57 (C19), 22.06, 17.08 (C21), 13.92 (CH_2), 12.31 (C18) ppm

MS (+APCI) m/z= Found 660.5923; calculated for $C_{42}H_{77}N_1O_4$ 660.5925; -0.4ppm

IR (KBr) ν = 3299, 2916, 2847, 1648 (C=O), 1642 cm^{-1}

Synthesis of N-benzylcholanamide (17)



The procedure was followed as above with benzylamine (0.12 mL, 1.1 mmol). The product was recrystallised in dichoromethane.

Yield= 0.378 g (74.6%)

Melting point= 114.1-114.7 ^{o}C

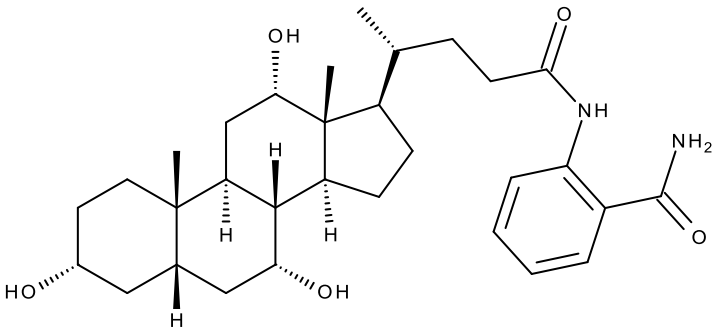
^{1}H NMR (Methanol -d_4) δ ppm: 0.69 (s, 3H, Me-18), 0.91 (s, 3H, Me-19), 1.02 (d, J= 6.3Hz, 3H, Me-21), 1.0-2.43 (m, steroid structure) 3.36 (m, 1H, C$\underline{H}$-3), 3.78 (s, 1H, C$\underline{H}$-7), 3.94 (s, 1H, C$\underline{H}$-12), 4.35 (d, J= 1.9 Hz, 2H, C$\underline{H}_2$-NH), 7.29 (m, J= 6.2Hz, 6H, aromatic ring) ppm

^{13}C NMR (DMSO) δ ppm: 172.55 (C=O),(139.77, 128.17, 127.06, 126.60 aromatic ring), 70.97 (C12), 70.41 (C3), 66.20 (C7), (46.15, 45.71, 41.89, 40.49, 40.16, 35.28, 35.09, 34.86, 34.36, 34.36, 32.49, 31.77, 30.38, 28.53, 27.30, 26.18 steroid ring), 22.61 (C19), 17.06 (C21), 12.31 (C18) ppm

MS (+APCI) m/z= Found 498.3569; calculated for $C_{31}H_{47}N_1O_4$ 498.3578; -1.8 ppm

IR ν = 3411, 2917, 1644, 1540, 1457 cm^{-1}

Synthesis of 2-cholanamidobenzamide (18)



The procedure was followed as above with 2-aminobenzomide (0.5 g, 3.7 mmol). Further seperation of the crude product to purify it was carried out with sodium hydrogen carbonate (30 mL) and 2M hydrochloric acid (30 mL). The organic layer was dried over magnesium sulphate and the solvent was evaporated under reduced pressure. The product was dried at room temperature under vacuum.

Melting point: 103-104 ^{o}C

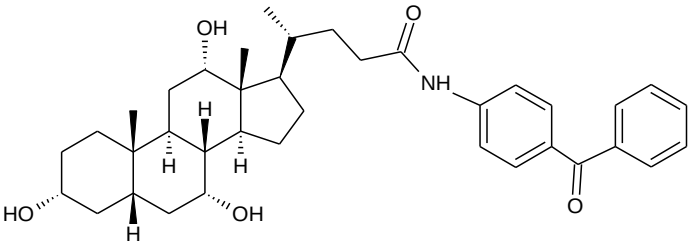
^{1}H NMR ($CDCl_3$) *δ* ppm: 0.69 (s, 3H, Me-18) 0.89 (s, 3H, Me-19) 1.03 (d, J= 6.0Hz, 3H, Me-21) 1.0-2.43 (m steroid structure) 2.10 (s, impurity, acetic acid) 3.47 (m, J=7.9Hz, 1H, C<u>H</u>-3) 3.84 (s, 1H, C<u>H</u>-7) 3.98 (s, 1H, C<u>H</u>-12) 6.25 (s, 4H, N<u>H</u>$_2$) 7.07 (m, ring) 7.52 (d, J= 8.3Hz, ring) 8.62 (d, J= 8.4Hz, 1H, N<u>H</u>) 11.10 (s) ppm

^{13}C NMR ($CDCl_3$) δ ppm: 176.74, 176.03 (C=O), (145.05, 137.37, 133.74, 124.55 aromatic ring), 71.47 (C12), 51.28, 51.00, (46.75, 46.58, 45.75, 45.42, 45.09, 44.75, 44.42, 44.09, 43.75, 40.29, 39.61, 31.43, 27.84 steroid ring), 22.27 (C19), 17.56 (C21) ppm

MS (+APCI) m/z= Found 527.3473; calculated for $C_{31}H_{46}N_5O_5$ 527.3479; -1.2 ppm

IR (KBr) v = 3350, 3220, 2932, 2865, 1721, 1660 (c=C=O), 1612, 1581 cm^{-1}

Synthesis of N-(4-benzoylphenyl)cholanamide (19)



The procedure was followed as above with 4-aminobenzophenone (0.23 g, 1.17 mmol).

Yield= 0.1270 g (25.4%)

Melting point: 125.8-127.6 ^{o}C

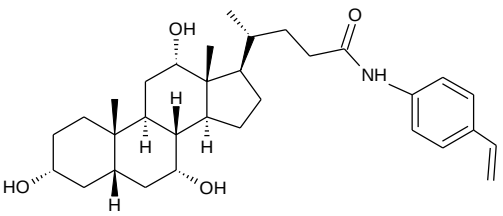
^{1}H NMR ($CDCl_3$) δ ppm: 0.70 (s, 3H, Me-18) 0.90 (s, 2H, Me-19) 1.02 (d, J= 6.3Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 3.48 (m, 1H, CH-3) 3.86 (s, 1H, CH-7) 3.99 (s, 1H, CH-12) 7.37-7.65 (m, 6H, ring) 7.65-7.91 (m, 6H, ring) 8.25 (s, 1N, NH) ppm

^{13}C NMR (DMSO) δ ppm: 194.48 (C=O), 172.42 (C=O), (143.55, 137.59, 132.14, 131.11, 130.99, 129.30, 128.43, 118.16 aromatic rings), 66.21 (C7), (45.72, 40.50, 40.17, 39.84, 39.50, 39.17, 38.84, 38.50, 35.27, 35.12, 34.36, 34.36, 33.56, 31.29, 30.39, 28.54, 27.31, 26.20 steroid ring), 22.60 (C19), 17.13 (C21), 12.34 (C18) ppm

MS (+APCI) m/z= Found 587.3684; calculated for $C_{37}H_{50}N_1O_5$ 587.36; -0.6ppm

IR (KBr) v = 3317, 3098, 2932, 2865, 1967, 1645, 1584, 1521 cm^{-1}

Synthesis of N-(4-vinylphenyl)acetamide (20)



The procedure was followed as above with 4-vinyl aniline (0.42 mL, 3.5 mmol). Further purification with washing product dissolved in ethyl acetate (30 mL) with 2M HCl (30 mL) four times. The organic layer was dried over magnesium sulphate and the solvent was evaporated under reduced pressure. The product was dried at room temperature under vacuum. Proton NMR anaylsis showed the product contained excess 4- vinyl aniline.

TLC: (MeOH/EtOAc 1/1), R_f=0.95 (single spot)

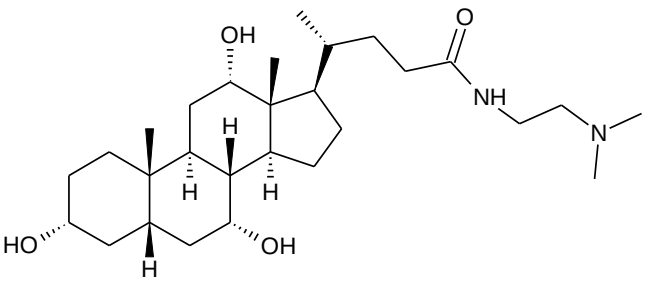
^{1}H NMR ($CDCl_3$) δ ppm: 0.69 (s, 3H, Me-18) 0.90 (s, 3H, Me-19) 1.02 (d, J= 6.0Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.36 (s) 2.62 (s) 3.47 (m, 1H, CH-3) 3.87 (s, 1H, CH-7) 3.99 (s, 1H, CH-12) 5.18 (d, J= 11.5Hz, 1H, CH) 5.67 (d, J=18.0Hz, 1H, CH) 6.61 (dd, 1H, NH) 7.36 (d, J=5.4Hz,aromatic) 7.52 (d, J=8.5Hz, aromatic) ppm

^{13}C NMR (DMSO) δ 171.74 (C=O), (153.40, 139.14, 138.93, 131.73, 131.22, 128.96, 128.86, 128.17, 126.58, 126.47, 125.28, 118.85, 117.95, 112.28 aromatic ring, with excess 4-vinyl aniline) ,(137.30, 136.16, 112.46 C=C), 70.95 (C12), 70.40 (C3), 66.19 (C7), 60.14, (46.06, 45.69, 41.46, 41.34, 40.42, 40.09, 35.27, 35.16, 34.85, 34.35, 33.43, 31.43, 30.35, 28.53, 27.28, 26.16 steroid ring), 22.78, 22.59 (C19), 21.02, 17.11 (C21), 14.48, 12.33 (C18), 10.94 ppm

MS (+APCI) m/z= Found 510.3570; calculated for $C_{32}H_{48}N_1O_4$ 510.3578; -1.5 ppm

IR (KBr) ν = 3429, 3296, 3101, 3044, 2929, 2868, 2364, 1672 (C=O), 1587, 1521, 1508, 1460 cm^{-1}

Synthesis of N-(2-dimethylaminoethyl)cholamide (21)



The procedure was followed as above with cholic acid (5 g, 12.2 mmol), triethylamine (2.9 mL, 28.7 mmol), ethylchloroformate (1.3 mL, 12 mmol) and dimethylethylenediamine (1.3 mL, 14.7 mmol).

Yield=3.2185 g (64 %)

Melting point: 181-182.2 ^{o}C

^{1}H NMR ($CDCl_3$) δ ppm: 0.66 (s, 3H, Me-18) 0.87 (s, 3H, Me-19) 0.98 (d, J=6.0Hz, 3H, Me-21) 1.0-2.43 (m steroid structure) 2.37 (s, 6H, 2C<u>H</u>$_3$) 2.62 (t, 2H, NH-C<u>H</u>$_2$) 3.39 (d, J=5.7Hz, N-C<u>H</u>$_2$ plus C<u>H</u>-3) 3.81 (s, 1H, C<u>H</u>-7) 3.94 (s, 1H, C<u>H</u>-12) 7.17 (s, 1H, N<u>H</u>) ppm

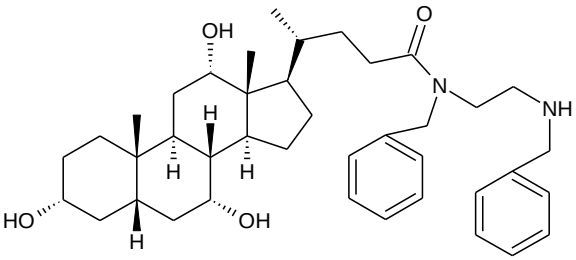
^{13}C NMR ($CDCl_3$) δ ppm:218.02, 178.14, 174.59 (C=O), 73.01, 71.80 (C12), 68.37 (C7), 57.82 (CH_2), (46.54, 46.49, 44.98, 44.63, 41.69, 39.73, 39.59, , 35.62, 35.49, 34.87, 33.15, 31.75, 30.62, 28.30, 27.70, 26.42 steroid ring), 36.26 (CH_3), 23.39, 23.32, 22.58 (C19), 17.55 (C21), 14.73, 12.53 (C18) ppm

MS (+APCI) m/z= Found 479.3835; calculated for $C_{28}H_{50}N_2O_4$ 479.3843 -1.7ppm

IR (KBr) ν = 3484, 3250, 3074, 2925, 2865, 1715, 1633, 1551 cm^{-1}

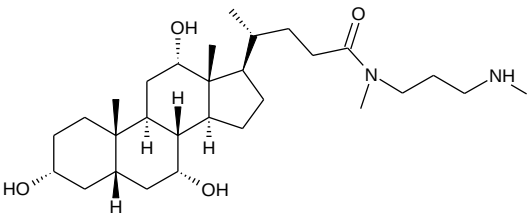
7.1.3 Inverse additions

Attempted synthesis of N-(2-anilinoethyl)-N-phenyl-cholamide (22)



Cholic acid (0.5 g, 1.2 mmol) was dissolved in dry THF (20 mL) along with 4-methylmorpholine (0.28 mL, 3 mmol). The mixture was put in ice for 10 minutes before ethylchloroformate (0.13 mL, 1.0 mmol) was dripped in over 10 minutes. The mixture was allowed to react for 20 minutes at room temperature. In a separate flask, dibenzylethylenediamine (1.2 mL, 4.9 mmol) was dissolved in dry THF (5 mL). The mixed anhydride solution was placed into a 20mL syringe and dripped into the amine solution over 20 minutes. The reaction was left at room temperature for 48 hours. Water (25 mL) was added to precipitate the product. The solid was collected by filtration and dissolved in water before solvent separation (x 3) with ethyl acetate (30 mL). The organic solvent was dried with magnesium sulphate and removed under reduced pressure. Re-crystallisation was attempted in water (10 mL) but TLC analysis in 80/20 chloroform/water showed 4 spots.

Attempted synthesis of N-methyl-N-[2-(methylamino)ethyl] cholamide (23)

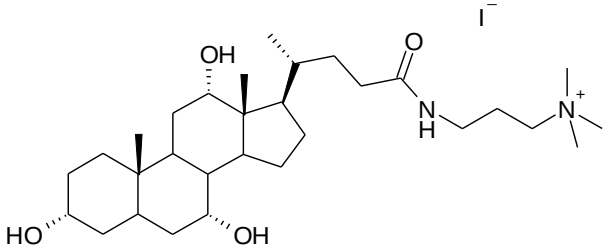


Cholic acid (0.5 g, 1.2 mmol) was dissolved in dry THF (20mL) along with 4-methylmorpholine (0.28 mL, 3.0 mmol). The mixture was put in ice for 10 minutes before ethylchloroformate (0.13 mL, 1.0 mmol) was dripped in over 10 minutes. The mixture was allowed to react for 20 minutes at room temperature. In a separate flask, dimethyl-1,3-propanediamine (0.6 mL, 7.1 mmol) was dissolved in dry THF (5 mL). The mixed anhydride solution was placed into a 20mL syringe and dripped into the amine solution over 20 minutes. The reaction was left at room temperature for 48 hours. Water (25 mL) was added to quench the reaction before solvent separation (x 3) with ethyl acetate (30 mL). The organic solvent was dried with magnesium sulphate and removed under reduced pressure. TLC analysis in 80/20 chloroform/water showed 3 spots.

2.2 QUATERNISATIONS

Each cholic acid derivative was dissolved in either chloroform or dichloromethane along with a tenfold excess of the alkylating agent. The mixtures were left at room temperature for 4-48 hours. Upon the precipitation of the product, the solid was collected by filtration and washed with solvent, dried and purified if necessary.

Synthesis of 3-cholanamidopropyl(trimethyl)ammonium; iodide (24)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1.0 mmol) was dissolved in DCM (25 mL) along with methyl iodide (1.4 mL, 4.3 mmol). The reaction was left for 48 hours where upon a yellow solid formed. The solid was collected by filtration and washed with chloroform (10 mL) before drying at room temperature under vacuum.

Yield= 0.18 g (36%)

TLC: (MeOH), R_f=0.05 (single spot)

Melting point: 134.6-135.4 ^oC

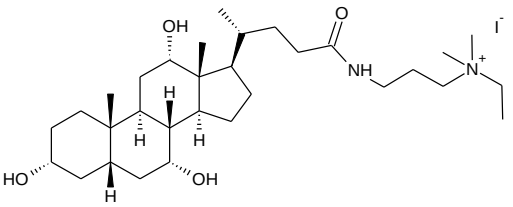
^1H NMR (D_2O) δ ppm: 0.70 (s, 3H, Me-18) 0.90 (s, 3H, Me-19) 0.96 (d, 3H, Me-21) 1.0-2.43 (m, steroid structure) 3.11 (s, 9H, 3C$\underline{H}_3$) 3.30 (ddt, J=17.8Hz, 2H, C$\underline{H}_2$-N) 3.48 (dd, J=10.4, 5.3Hz, 1H, C$\underline{H}$-3) 3.89 (s, 1H, C$\underline{H}$-7) 4.05 (s, 1H, C$\underline{H}$-12) 7.65 (s, 1H, N$\underline{H}$) ppm

^{13}C NMR (DMSO) δ ppm: 212.03, 172.84 (C=O), 79.97, 70.95 (C12), 70.37 (C3), 66.20 (C7), 52.27 (CH_3), 52.18, 45.68 (CH_2), 40.52 (CH_2),(40.43, 40.18, 40.10, 35.27, 34.35, 32.33, 29.95 steroid ring), 22.97, 22.60 (19), 17.11 (C21), 12.31 (C18), 6.83 ppm

MS (+APCI) m/z= Found 507.4150; calculated for $C_{30}H_{55}N_2O_4$ 507.4156; -1.3 ppm

IR (KBr) ν = 3387, 2929, 2862, 1700, 1642 cm^{-1}

Synthesis of 3-cholanamidopropyl-ethyl-dimethyl-ammonium iodide (25)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1.0 mmol) was dissolved in DCM (20 mL) along with ethyl iodide (0.4 mL, 1.3 mmol) and methanol (1 mL). The reaction was left for 1 week before the solvent was removed under reduced pressure. The solid was washed with ether (10 mL) and chloroform (10 mL) before drying. The product was a yellow solid.

Yield =0.267 g (53%)

Melting point: 122.5-123.1 ^oC

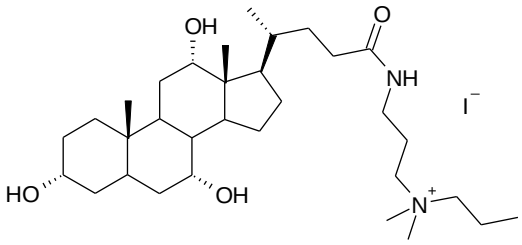
^{1}H NMR (D_2O) δ ppm: 0.72 (s, 3H, Me-18) 0.92 (s, 3H, Me-19) 0.97 (m, 3H, Me-21) 1.0-2.43 (m, steroid structure) 3.04 (s, 6H, C<u>H</u>$_2$) 3.21-3.34 (m, 4H, 2C<u>H</u>$_2$) 3.38 (q, J= 7.3Hz, 2H, C<u>H</u>$_2$-N) 3.44-3.62 (m, 1H, C<u>H</u>-3) 3.89 (s, 1H, C<u>H</u>-7) 4.06 (s, 1H, C<u>H</u>-12) 7.67 (s, 1H, N<u>H</u>) ppm

^{13}C NMR (DMSO) δ ppm: 197.83, 172.88 (C=O), 70.96 (C12), 70.37 (C3), 66.20 (C7), 60.46, 58.59 (CH_3), 49.59, 49.52, (45.96, 41.46, 41.38, 40.51, 40.17, 39.84, 39.51, 39.42, 39.17, 39.01, 38.84, 38.51, 35.47, 35.25, 35.15, 34.87, 34.34, 32.35, 30.35, 28.55, 27.26, 26.20 steroid ring), 45.68 (CH_2), 31.52 (CH_2), 22.77, 22.59 (C19), 22.52 (CH_2), 17.10 (C21), 12.31 (C18), 7.76 ppm

MS (+APCI) m/z= Found 521.4305; calculated for $C_{31}H_{57}N_2O_4$ 521.4313; -1.5 ppm

IR (KBr) v = 3420, 3256, 2913, 2856, 2243, 1639 (C=O) cm^{-1}

Synthesis of 3-cholanamidopropyl-propyl-dimethyl-ammonium iodide (26)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1.0 mmol) was dissolved in DCM (20 mL) along with 1-iodopropane (1.5 mL, 5.0 mmol) and methanol (1 mL). The reaction was left for 1 week before solvent extraction (x 3) between dichloromethane (20 mL) and water (20 mL). The organic layer was dried with magnesium sulphate before being removed under reduced pressure. The product was a white solid which was dried at room temperature under vacuum.

Yield =0.43 g (86%)

TLC: (MeOH), R_f=0.17 (single spot)

Melting point:108.1-108.9 ^{o}C

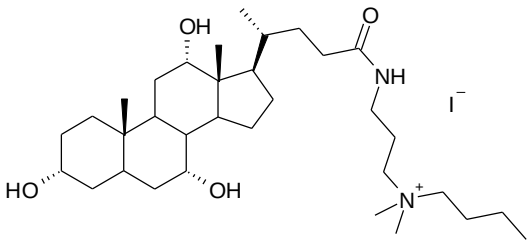
^{1}H NMR (DMSO) δ ppm: 0.57 (s, 3H, Me-18) 0.79 (s, 3H, Me-19) 0.86-0.94 (m, 37H, Me-21 plus 2CH_2) 1.0-2.43 (m, steroid structure) 3.07 (m, 1H, C<u>H</u>-3) 3.15-3.28 (m, 6H, 3CH_2) 3.61 (s, 1H, C<u>H</u>-7) 3.77 (s, 1H, C<u>H</u>-12) 3.99 (d, J=3.5, 1H 3O<u>H</u>) 4.10 (d, 1H, 7O<u>H</u>) 4.32 (d, *J* = 4.34 Hz, 1H, 12O<u>H</u>) 7.88 (t, J=5.7Hz, N<u>H</u>) ppm

^{13}C NMR (DMSO) δ ppm: 172.92 (C=O), 70.97 (C12), 70.37 (C3), 66.20 (C7), 64.37, 61.10, 50.20, 50.16, (45.97, 35.45, 35.15, 34.86, 34.34, 32.37, 30.34, 28.53, 26.20 steroid ring), 45.68 (CH_3), 31.54 (CH_2), 27.26 (CH_2), 22.58 (C19), 17.10 (C21), 15.32 (CH_3), 12.31 (C18), 10.45 (CH_2) ppm

MS (+APCI) m/z= Found 535.4461; calculated for $C_{32}H_{59}N_2O_4$ 535.4469; -1.6 ppm

IR (KBr) ν = 3396, 2935, 2865, 2246, 2124, 1706, 1645 (C=O), 1533 cm^{-1}

Synthesis of 3-cholanamidopropyl-butyl-dimethyl-ammonium iodide (27)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1.0 mmol) was dissolved in DCM (20 mL) along with 1-iodobutane (1.2 mL, 4 mmol) and methanol (1 mL). The reaction was left for 1 week before solvent extraction (x 3) between dichloromethane (20 mL) and water (20 mL). The organic layer was dried with magnesium sulphate before being removed under reduced pressure. The product was a white solid which was dried at room temperature under vacuum.

Yield =0.347 g (69%)

TLC: (MeOH/EtOAc 1/5), R_f=0.23 (dominant spot), 0.9 (two spots)

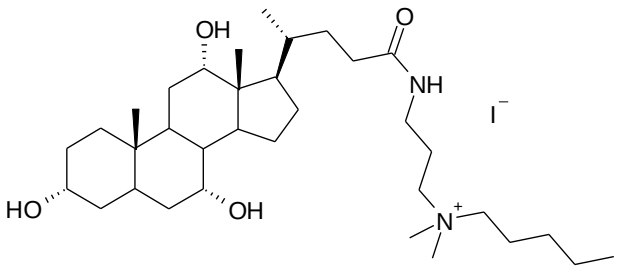
Melting point: 125.8-126.4 ^{o}C

^{1}H NMR (D_2O) δ ppm: 0.68 (s, 3H, Me-18) 0.88 (s, 3H, Me-19) 0.84-1.00 (m, 5H, Me-21 plus CH_2) 1.0-2.43 (m, steroid structure) 3.02 (s, 6H, 3C$\underline{H}_2$) 3.14-3.32 (m, 6H, C$\underline{H}_2$ butane chain) 3.47 (m, 1H, C$\underline{H}$-3) 3.86 (s, 1H, C$\underline{H}$-7) 4.02 (s, 1H, C$\underline{H}$-12) ppm

MS (+APCI) m/z= Found 549.4619; calculated for $C_{33}H_{61}N_2O_4$ 549.4626; -1.2 ppm

IR (KBr) ν = 3378, 2932, 2865, 2358, 2337, 2155, 2009, 1976, 1651, 1633 (C=O), 1539 cm^{-1}

Synthesis of 3-cholanamidopropyl-pentyl-dimethyl-ammonium iodide (28)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1 mmol) was dissolved in chloroform (75 mL) along with 1-iodopentane (1.5 mL) and methanol (1 mL). The reaction was left for 1 week before solvent extraction (x 3) between chloroform (75 mL) and water (70 mL). The organic layer was dried with magnesium sulphate before being removed under reduced pressure. The product was a white solid which was dried at room temperature under vacuum.

Yield =0.077g (77 %)

TLC: (MeOH), R_f=0.21 (single spot)

Melting point: 107.9-108.3^{o}C

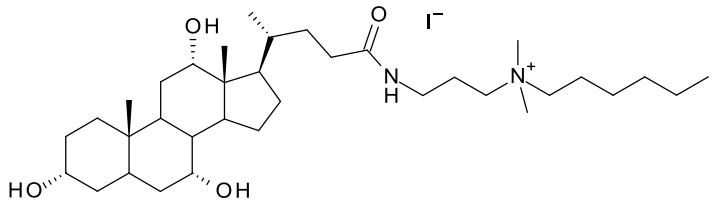
^{1}H NMR (Methanol-d_4) δ ppm: 0.67 (s, 3H, Me-18) 0.83-1.05 (m, 10H, Me-21 plus CH_2) 1.0-2.439 (m, steroid structure) 3.04 (s, 6H, 3C$\underline{H}_2$) 3.47 (m, 1H, C$\underline{H}$-3) 3.76 (s, 1H, C$\underline{H}$-7) 3.92 (d, J=3.3Hz, 1H, C$\underline{H}$-12) ppm

^{13}C NMR (DMSO) δ ppm: 170.89 (C=O), 154.50, 71.01 (C12), 70.42 (C3), 66.22 (C7), 60.58, 57.47, 57.41 (CH_3), 53.14, 52.55, 52.46, 48.55, 46.07, 45.02, 43.30, 41.52, 41.33, 40.93, 35.30, 35.22, 34.85, 29.49, 28.47, 28.35, 27.30, 26.17 steroid ring), 45.74 (CH_3), 40.50 (CH_2), 30.37 (CH_2), 34.34 (CH_2), 31.17 (CH_2), 22.79 (CH_2), 22.57 (C19), 20.03, 17.12 (C21), 14.51, 13.84 (CH_2), 12.29 (C18) ppm

MS (+APCI) m/z= 563.4767

IR (KBr) v = 3381 2929 2871 1645 (C=O) 1533 cm^{-1}

Synthesis of 3-cholanamidopropyl-hexyl-dimethyl-ammonium iodide (29)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1 mmol) was dissolved in chloroform (15 mL) along with 1-iodohexane (2.1 mL, 6.8 mmol) and methanol (1 mL). The reaction was left for 5 days before solvent was removed under reduced vacuum. The product was purified by washing with diethyl ether was collected by filtration and dried at room temperature under vacuum. The product was a orange solid.

Yield =1.74 g

TLC: (MeOH), R_f=0.41 (single spot)

Melting point: 101.7-102.3 ^{o}C

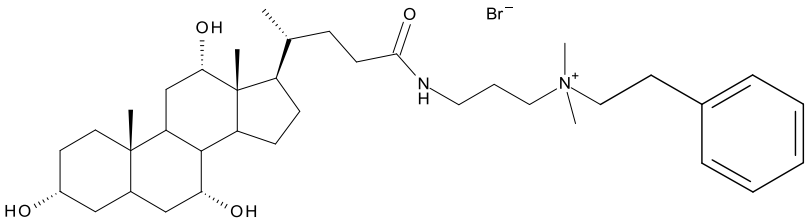
^{1}H NMR (Methanol-d_4) δ ppm: 0.68 (s, 3H, Me-18) 0.90 (d, J= 5.6Hz, 6H) 1.01 (d, J=5.6Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 1.15 (td, J= 7.0, 0.8Hz) 3.04 (s, 6H,3C$\underline{H}_2$) 3.47 (m, 1H, C$\underline{H}$-3) 3.77 (s, 1H, C$\underline{H}$-7) 3.92 (s, 1H, C$\underline{H}$-12) ppm

^{13}C NMR (DMSO) δ ppm: 172.86 (C=O), 70.96 (C12), 70.37 (C3), 66.20 (C7), 62.99, 60.98, 50.12, (45.98, 35.46, 35.13, 30.05, 29.48, 28.54, 27.26, 26.20 steroid ring), 45.68 (CH_3), 34.34 (CH_2), 30.65 (CH_2), 31.55 (CH_2), 25.40, 22.59 (C19), 21.87 (CH_2), 21.59, 17.10 (C21), 13.81 (CH_3), 12.32 (C18), 9.10 (CH_2) ppm

MS (+APCI) m/z= Found 577.4949; calculated for $C_{35}H_{65}N_2O_4$ 577.4939; 1.8 ppm

IR (KBr) v = 3390, 2925, 2859, 1654 (C=O) cm^{-1}

Synthesis of 3-cholanamidopropyl-benzyl-dimethyl-ammonium iodide (30)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1 mmol) was dissolved in chloroform (20 mL) along with 2-phenylethyl bromide (1.36 mL, 5.4 mmol) and methanol (1 mL). The reaction was left for 24 hours before solvent was removed using a rotary evaporator. The resulting solid was washed with diethyl ether (10 mL) and dried under vacuum at room temperature. The product was a white solid.

Melting point: 103.8-105 ^{o}C

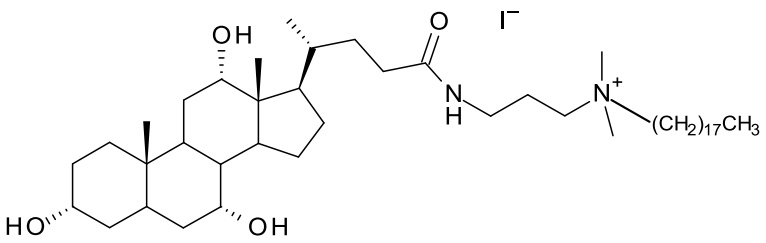
^{1}H NMR (DMSO) δ ppm: 0.58 (s, 3H, Me-18) 0.81 (s, 3H, Me-19) 0.92 (d, J= 5.7 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.49 (p, J = 1.9 Hz, 14H, 2C$\underline{H}_3$) 2.55 – 2.77 (m, 2H, C$\underline{H}_2$-N), 2.99 (s, 8H, 4C$\underline{H}_2$) 3.15 – 3.27 (m, 1H) 3.49 (t, J = 6.6 Hz, 1H, C$\underline{H}$-3) 3.60 (s, 1H, C$\underline{H}$-7) 3.77 (s, 1H, C$\underline{H}$-12) 4.05 (dd, J = 22.2, 3.3 Hz, 1H, 3O$\underline{H}$) 4.10 (m, 1H, 7O$\underline{H}$) 4.32 (d, J = 4.0 Hz, 1H, 12O$\underline{H}$) ppm

^{13}C NMR (DMSO) δ ppm: 172.89 (C=O), (140.23, 128.25, 126.21, 125.98 aromatic ring), 70.96 (C12), 70.38 (C3), 50.13, (45.68, 35.47, 34.85, 34.83, 34.35, 32.36, 31.65, 26.20 steroid ring), 23.54 (CH_2), 22.54 (C19), 17.10 (C21), 12.32 (C18) ppm

MS (+APCI) m/z= 583.4473

IR (KBr) v = 3362, 3059, 3023, 2925, 2859, 1645 (C=O) cm^{-1}

Synthesis of 3-cholanamidopropyl-dimethyl-octadecyl-ammonium iodide (31)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1 mmol) was dissolved in chloroform (15 mL) along with 1-iodo-octadecane (2.4 g, 5 mmol) and methanol (1 mL). The reaction was left for 72 hours before solvent was removed using a rotary evaporator. The resulting solid was washed with diethyl ether (10 mL)before being collected by filtration and dried under vacuum at room temperature. The product was a yellow solid.

Yield =0.30 g (60 %)

Melting point: 115.3-116.4 ^{o}C

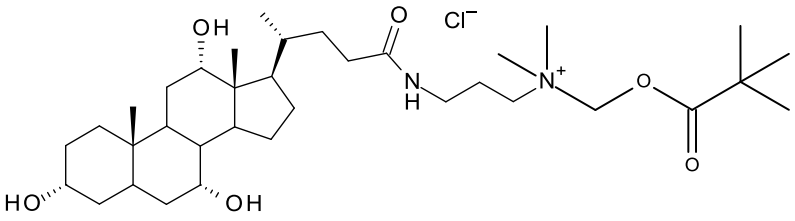
^{1}H NMR (Methanol-d_4) δ ppm: 0.68 (s, 3H, Me-18) 0.84 (s, 3H, Me-19) 1.01 (d, J= 6.0 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 1.26 (s, 9H, 2C$\underline{H}_3$) 2.23 (s, 7H, C$\underline{H}_2$) 3.05 (s) 3.15 (t) 3.27 (m) 3.44 (m, 1H, C$\underline{H}$-3) 3.76 (s, 1H, C$\underline{H}$-7) 3.92 (s, 1H, C$\underline{H}$-12) ppm

^{13}C NMR (DMSO) δ ppm: 172.30 (C=O), 70.98 (C12), 66.21 (C7), (46.21, 45.70, 34.85, 34.35, 31.80, 31.27, 30.34, 29.43, 28.77, 28.51, 27.26, 26.34, 26.17 steroid ring), 22.56 (C19), 22.06, 17.04 (C21), 13.91 (CH_2), 12.29 (C18) ppm

MS (+APCI) m/z= 754.6801

IR (KBr) v = 3362, 2922, 2850, 1639 (C=O) cm^{-1}

Synthesis of 3-cholanamidopropyl-(2,2-dimethylpropanoyloxymethyl)-dimethyl-ammonium chloride (32)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1 mmol) was dissolved in chloroform (20 mL) along with chloromethyl pivalate (1.44 g, 5 mmol) and methanol (1 mL). The reaction was left for 12 hours before solvent was removed using a rotary evaporator. The product was purified by washing with diethyl ether before being collected by filtraction and dried at room temperature under vacuum. The product was a white solid.

Melting point: 127.9-128.3 ^{o}C

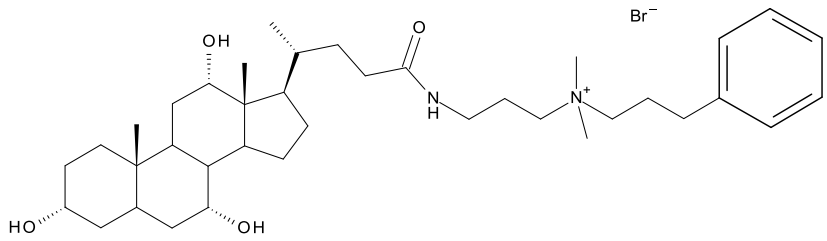
^{1}H NMR (DMSO) δ ppm: 0.57 (s, 3H, Me-18) 0.80 (s, 3H, Me-19) 1.08 (d, J=5.7 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.70 (s, C$\underline{H}_3$-N) 3.05 (s, C$\underline{H}_3$-N) 3.36 (dd, J= 15.7, 8.7Hz, C$\underline{H}$-3) 3.60 (s, 1H, C$\underline{H}$-7) 4.01 (m, 1H, 3O$\underline{H}$) 4.10 (m, 1H, 7O$\underline{H}$) 4.34 (m, 1H, 12O$\underline{H}$) 5.25 (s, 2H) 5.85 (s, 1H) 7.93 (t, J=5.7Hz, 1H, N$\underline{H}$) ppm

^{13}C NMR (DMSO) δ 179.30 (C=O), 172.96 (C=O), 70.96 (C12), 66.20 (C7), 54.42 (CH_3), 47.85, (46.00, 45.70, 41.92, 34.86, 34.35, 27.24, 26.98, 26.43 steroid ring), 24.19, 22.59 (C19), 17.09 (C21), 12.31 (C18) ppm

MS (+APCI) m/z= 607.4669

IR (KBr) v = 3402, 3362, 2932, 2871, 2470, 1760, 1706, 1624 (C=O) cm^{-1}

Synthesis of 3-cholanamidopropyl-dimethyl-(2-phenylethyl)ammonium bromide (33)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1 mmol) was dissolved in chloroform (20 mL) along with 1-bromo-3-phenylpropane (1.52 mL, 5 mmol) and methanol (1 mL). The reaction was left for 12 hours before solvent was removed using a rotary evaporator. The product was purified by washing with diethyl ether (10 mL) was collected by filtration and dried at room temperature under vacuum. The product was a white solid.

Yield =0.83 g

TLC: (MeOH), R_f=0.16 (dominant spot), 0.89 (two spots)

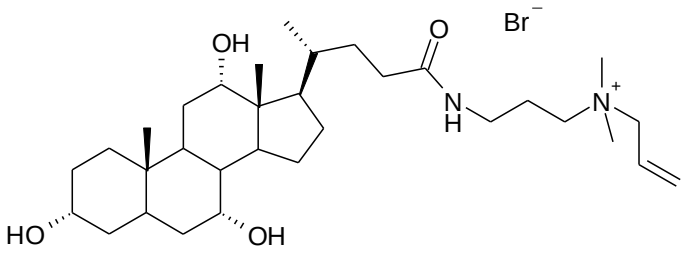
Melting point: 176.8-177.4 ^{o}C

^{1}H NMR (DMSO) δ ppm: 0.58 (s, 3H, Me-18) 0.82 (s, 3H, Me-19) 0.95 (d, J=6.0Hz, 3H, Me-21) 1.10 (t, J=7.0Hz, impurity, diethyl ether) 3.12 (d, J=14.5Hz, 8H) 3.61 (m, 1H, C$\underline{H}$-7) 3.78 (s, 1H, C$\underline{H}$-12) 4.00 (d, J= 2.8 Hz, 1H, 3O$\underline{H}$) 4.10 (d, J= 3.2 Hz, 1H, 7O$\underline{H}$) 4.34 (d, J= 4.1 Hz, 1H, 12O$\underline{H}$) 7.28 (m, 8H, aromatic) 7.93 (m, 1H, N$\underline{H}$) ppm

MS (+APCI) m/z= 611.4411

IR (KBr) v = 3373, 3026, 2929, 2859, 1691 (C=O), 1645 cm^{-1}

Synthesis of 3-cholanamidopropyl-allyl-dimethyl-ammonium;bromide (34)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1 mmol) was dissolved in chloroform (20 mL) along with allyl bromide (1.52 mL, 5 mmol) and methanol (1 mL). The reaction was left for 12 hours before solvent was removed using a rotary evaporator. The product was purified by washing with diethyl ether was collected by filtration and dried at room temperature under vacuum. The product was a white solid.

Yield =0.15 g (30 %)

TLC: (MeOH), R_f=0.32 (one spot)

Melting point: 121.3-121.9 ^{o}C

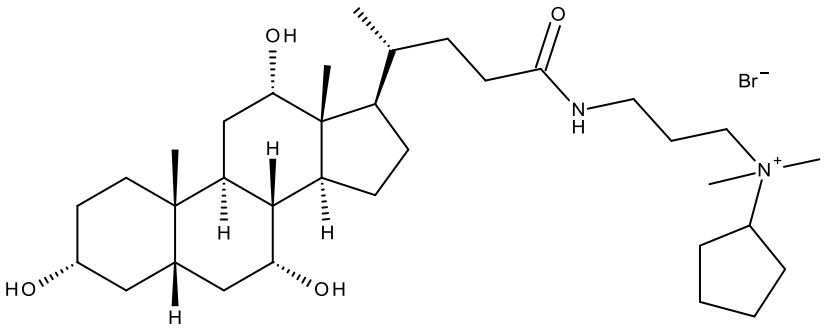
^{1}H NMR (MeOH) δ ppm: 0.68 (s, 3H, Me-18) 0.89 (s, 3H, Me-19) 1.00 (d, J= 6.1 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 3.04 (s, 6H, 2C$\underline{H}_3$) 3.76 (s, 1H, C$\underline{H}$-12) 3.94 (d, J= 7.5 Hz, 3H, C$\underline{H}_2$) 5.68 (d, 1H, C$\underline{H}$=CH) 5.93 – 6.17 (m, 1H, CH=C$\underline{H}$), 7.88 (s, 1H, N$\underline{H}$) ppm

^{13}C NMR (DMSO) δppm: 172.88 (C=O), 127.60 (C=C), 125.76 (C=C), 70.96 (C12), 70.37 (C3), 66.20 (C7), 65.11, 61.12, 49.73, (45.97, 45.68, 35.26, 34.35, 28.53, 27.29, 26.20 steroid ring), 31.55 (CH_2-Br), 25.04 (CH_2), 22.56 (C19), 17.11 (C21), 12.31 (C18) ppm

MS (+APCI) m/z= Found 533.4307; calculated for $C_{32}H_{57}N_2O_4$ 533.4313; -1.1 ppm

IR (KBr) v = 3368, 3077, 2925, 2862, 2710, 1733, 1651 (C=O), 1633, 1466 cm^{-1}

Synthesis of 3-cholanamidopropyl-cyclopentyl-dimethyl-ammonium bromide (35)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1 mmol) was dissolved in DCM (10 mL) along with) of cyclopentyl bromide (0.9 mL, 10 mmol). The reaction was left for 72 hours where the product preciptated out. It was collected by filtration and dried at room temperature under vacuum. The product was a white solid.

Melting point: 102.6-103 ^{o}C

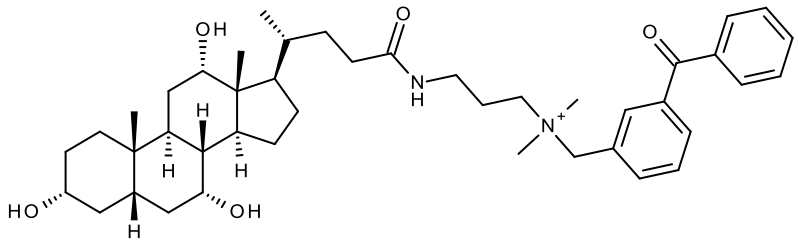
^{1}H NMR (DMSO) δ ppm: 0.57 (d, J=1.4 Hz, 3H Me-18) 0.76-0.97 (m, 6H, Me-19 and Me-21) 1.0-2.43 (m, steroid structure) 2.13 (s, dimethyl) 3.14 (s, 3H) 3.4-3.55 (m, 1H, C<u>H</u>-3) 3.60 (s 1H C<u>H</u>-7) 3.77 (s, 1H, C<u>H</u>-12) 3.94-4.18 (m, 4H, C<u>H$_2$</u>- ring) 4.32 (t , J=3.9Hz, 1H, C<u>H</u>-N) 5.35 (s, 1H, N<u>H</u>) ppm

^{13}C NMR (DMSO) δ ppm: 216.00, 138.38 (C=O), 70.92 (C19), 70.38 (C21), 66.17 (C18), 60.48 (CH-Br), (45.71, 43.19, 41.39, 40.51, 40.17, 36.53, 35.28, 34.89, 34.35, 30.64, 30.37, 30.35, 30.18, 28.51, 26.19 steroid ring), 25.66, 24.51 (CH_2 ring), 22.59 (C19), 21.64, 16.91 (C21), 12.29 (C18) ppm

MS (+APCI) m/z= 562.4448

IR (KBr) v = 3368, 2932, 2865, 2355, 1715 (C=O), 1578, 1460 cm^{-1}

Synthesis of 3-cholanamidopropyl-cyclopentyl-dimethyl-ammonium bromide (36)



N-[3-(dimethylamino)propyl] cholanamide (12) (0.5 g, 1 mmol) was dissolved in DCM (10 mL) along with 4-(Bromomethyl)benzophenone (1 mL, 2.7 mmol). The reaction was left for 72 hours where the product preciptated out. It was collected by filtration and dried at room temperature under vacuum. The product was a white solid.

Yield =0.49 (97 %)

Melting point: 158.8-159.5 ^{o}C

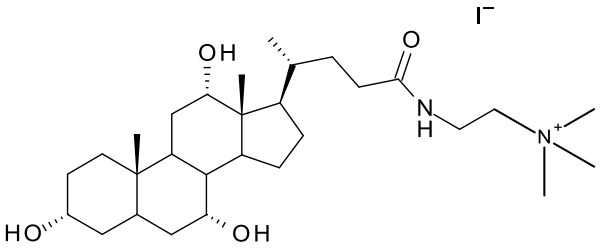
^{1}H NMR (DMSO) δ ppm: 0.58 (d, J=1.4 Hz, 3H Me-18) 0.82 (s, 3H, Me-19) 0.93 (m, 3H, Me-21) 1.0-2.43 (m, steroid structure) 3.03 (s, 3H, C$\underline{H}_3$) 3.61 (m, 1H, C$\underline{H}$-3) 3.78 (s, 1H, C$\underline{H}$-7) 4.02 (s, 1H, C$\underline{H}$-12) 4.12 (t, J = 5.3 Hz, 8H, 2C$\underline{H}_2$) 4.33 (s, 1H, 3O$\underline{H}$), 4.66 (s, 4H, 7O$\underline{H}$), 4.81 (s, 7H), 7.98 – 7.42 (m, 20H, aromatic + N$\underline{H}$) ppm

^{13}C NMR (DMSO) δ ppm: 206.45, 195.31, 173.27, 138.46 (C=O), 136.47, 133.15, 133.10, 132.05, 129.72, 128.66, 111.39, 70.93 (C19), 70.37 (C21), 66.19 (C18), (45.87, 45.69, 41.46, 41.37, 40.49, 40.16, 39.83, 39.49, 39.34, 39.16, 38.99, 38.82, 38.49, 35.25, 34.94, 34.85, 34.34, 30.65, 30.38, 28.51, 27.24, 26.19 steroid ring), 22.75 (CH_2 ring), 22.58 (C19), 21.87, 16.91 (C21), 12.26 (C18) ppm

MS (+APCI) m/z= Found 688.4567; calculated for $C_{43}H_{62}N_1O_6$ 688.4572; -0.7 ppm

IR (KBr) v = 3408, 2935, 2868, 1724 (C=O), 1654, 1596, 1572 cm^{-1}

Synthesis of 3-cholanamidoethylyl(trimethyl)ammonium;iodide (37)



N-[3-(dimethylamino)ethyl] cholanamide (21) (0.5 g, 1.0 mmol) was dissolved in DCM (20 mL) along with methyl iodide (0.3 mL, 2.1 mmol). The reaction was left for 12 hours where upon a solid formed and the solvent had evaporated. Diethyl ether (10 mL) was added to the flask and the solid was collected by filtration before being washed with chloroform (10 mL). The product was dried at room temperature under vacuum.

Yield= 0.314 g (63%)

Melting point: 215.3- 216.1 ^{o}C

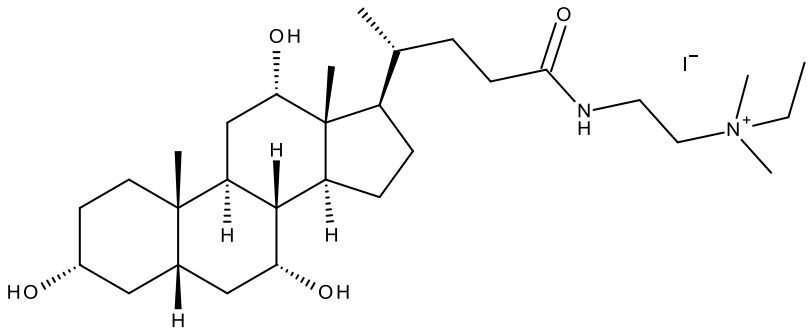
^{1}H NMR (D_2O) δ ppm: 0.58 (s, 3H, Me-18) 0.81 (s, 3H, Me-19) 0.9 (d, 3H, Me-21) 1.0-2.43 (m, steroid structure) 3.08 (s, 9H, 3C$\underline{H}_3$) 3.45 (m, 1H C$\underline{H}$-3) 3.61 (s, 1H, C$\underline{H}$-7) 3.77 (s, 1H, C$\underline{H}$-12) 4.04 (m, 1H C3-O$\underline{H}$) 4.13 (m, 1H, C7-O$\underline{H}$) 4.36 (m, 1H, C12-O$\underline{H}$) 8.33 (s, 1H, N$\underline{H}$) ppm

^{13}C NMR (DMSO) δ ppm: 173.25 (C=O), 70.96 (C12), 70.37 (C3), 66.19 (C7), 52.47 (CH_3), 45.99 (CH_2), (35.28, 35.09, 34.35, 32.92, 32.26, 31.44, 30.25 steroid ring), 26.20, 22.25, 22.59 (19), 17.05(C21), 12.31 (C18) ppm

MS (+APCI) m/z= Found 493.3991; calculated for $C_{29}H_{53}N_2O_4$ 493.4000; -1.8 ppm

IR (KBr) ν = 3423, 3241, 2935, 2862, 2252, 2118, 1663, 1618, 1539 cm^{-1}

Synthesis of 3-cholanamidoethylyl-ethyl-dimethyl-ammonium iodide (38)



N-[3-(dimethylamino)ethyl] cholanamide (21) (0.5 g, 1.0 mmol) was dissolved in DCM (20 mL) along with ethyl iodide (0.4 mL, 1.3 mmol) and methanol (1 mL). The reaction was left for 12 hours before the solvent was removed under reduced pressure. The solid was washed with ether (10 mL) and chloroform (10 mL) before drying. The product was a yellow solid.

Yield =0.280 g (56%)

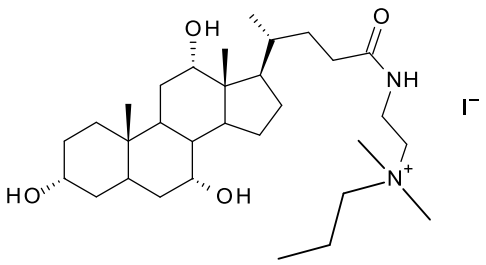
Melting point: 142.3-142.7 ^{o}C

^{1}H NMR (D_2O) δ ppm: 0.86 (s, 3H, Me-18) 0.90 (s, 3H, Me-19) 0.93 (m, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.87 (s, 2H, C<u>H</u>$_2$) 3.04 (s, 4H, 2C<u>H</u>$_2$) 3.24 (q, J= 5.8 Hz, 1H, C<u>H</u>-3) 3.33-3.57 (m, C<u>H</u>2) 3.84 (s, 1H, C<u>H</u>-7) 4.0 (s, 1H, C<u>H</u>-12) 7.61 (s, 1H, N<u>H</u>) ppm

MS (+APCI) m/z= Found 507.4147; calculated for $C_{30}H_{55}N_2O_4$ 507.4156; -1.8 ppm

IR (KBr) v = 3387, 2932, 2862, 2689, 1706, 1648 (C=O), 1533 cm^{-1}

Synthesis of 3-cholanamidopropyl-propyl-dimethyl-ammonium iodide (39)



N-[3-(dimethylamino)ethyl] cholanamide (21) (0.5 g, 1.0 mmol) was dissolved in DCM (20 mL) along with 1-iodopropane (0.5 mL, 1.7 mmol) and methanol (1 mL). The reaction was left for 6 days before the solvent was removed under reduced pressure. The solid was washed with diethyl ether (10 mL) and collected by filtration. Further purification was carried out by washing the crude product with diethyl ether (2 x 10 mL) and chloroform (2 x 10 mL). The product was a white solid which was dried at room temperature under vacuum.

Yield =0.283 g (56%)

Melting point: 108.1-108.9 ^{o}C

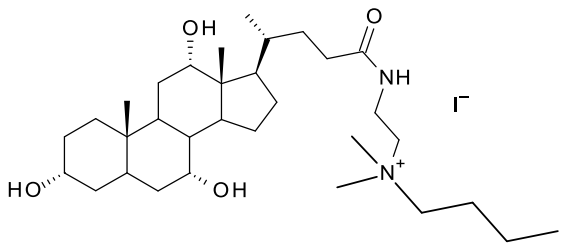
^{1}H NMR (D_2O) δ ppm: 0.64 (s, 3H, Me-18) 0.87 (s, 3H, Me-19) 0.93 (m, 5H, Me-21 + C<u>H</u>$_2$) 1.0-2.43 (m, steroid structure) 2.85 (s, 2H, C<u>H</u>$_2$) 3.06 (s, 3H, C<u>H</u>$_3$) 3.24 (q, J= 5.6, 4.5 Hz, 2H, C<u>H</u>-3 + C<u>H</u>) 3.28-3.60 (m, C<u>H</u>$_2$) 3.83 (s, 1H, C<u>H</u>-7) 3.98 (s, 1H, C<u>H</u>-12) 7.60 (s, 1H, N<u>H</u>) ppm

^{13}C NMR (DMSO) δ ppm: 171.96 (C=O), 148.27, 138.66, 86.11, 79.15, 70.96 (C12), 70.38 (C3), 66.20 (C7), 64.50, (44.22, 41.46, 41.36, 35.11, 34.85, 26.20, 25.09 steroid ring), 45.69 (CH_3), 28.56 (CH_2), 22.77 (C19), 17.06 (C21), 15.34 (CH_3), 12.31 (C18) ppm

MS (+APCI) m/z= Found 521.4303; calculated for $C_{31}H_{57}N_2O_4$ 521.4313; -1.9 ppm

IR (KBr) ν = 3390, 2935, 2862, 2243, 2121, 1651 (C=O), 1536 cm^{-1}

Synthesis of 3-cholanamidoethyl-butyl-dimethyl-ammonium iodide (40)



N-[3-(dimethylamino)propyl] cholanamide (21) (0.5 g, 1.0 mmol) was dissolved in DCM (20 mL) along with 1-iodobutane (0.6 mL, 2 mmol) and methanol (1 mL). The reaction was left for 3 days where a precipitate formed. The crude product was collected by filtration and washed with chloroform (15 mL). The product was a white solid which was dried at room temperature under vacuum.

Yield =0.338 g (67%)

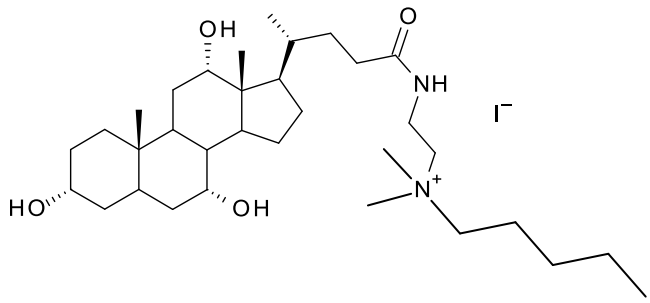
Melting point: 186.4-187.4^{o}C

^{1}H NMR (D_2O) δ ppm: 0.64 (s, 3H, Me-18) 0.84 (s, 3H, Me-19) 0.94 (m, 4H, Me-21+ C<u>H</u>) 1.0-2.43 (m, steroid structure) 2.26 (s, 9H, 3C<u>H</u>$_3$) 2.54 (m, 2H, N-C<u>H</u>$_2$) 3.26-3.31 (m, 2H, C<u>H</u>$_2$) 3.42 (m, 1H, C<u>H</u>-3) 3.82 (s, 1H, C<u>H</u>-7) 3.98 (s, 1H, C<u>H</u>-12)

MS (+APCI) m/z= Found 535.4458; calculated for $C_{32}H_{59}N_2O_4$ 535.4469; -2.1 ppm

IR (KBr) v = 3484, 3432, 3250, 3071, 2922, 2865, 1633 (C=O), 1551 cm^{-1}

Synthesis of 3-cholanamidoethyl-pentyl-dimethyl-ammonium iodide (41)



N-[3-(dimethylamino)ethyl] cholanamide (21) (0.5 g, 1 mmol) was dissolved in DCM (20 mL) along with 1-iodopentane (0.6 mL, 1.9 mmol) and methanol (1 mL). The reaction was left for 3 days where a precipitate formed. The product was collected by filtration, washed with chloroform (20 mL) and dried at room temperature under vacuum. The product was a white solid.

Yield =0.396 g (79 %)

Melting point: 139.6- 141.2 ^{o}C

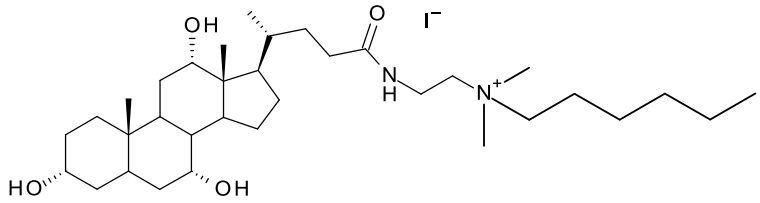
^{1}H NMR (D_2O) δ ppm: 0.64 (s, 3H, Me-18) 0.76 (s, 3H, Me-19) 0.84 (s, 3H, Me-21) 1.0-2.439 (m steroid structure) 2.28 (s, 6H, 2C<u>H</u>$_3$) 2.55 (t, 2H, N-C<u>H</u>$_2$) 3.24 (m, 1H, C<u>H</u>-3) 3.29 (t, 2H, C<u>H</u>$_2$) 3.39 (m, 1H, C<u>H</u>-3) 3.82 (s, 1H, C<u>H</u>-7) 3.98 (s, 1H, C<u>H</u>-12) ppm

^{13}C NMR (D_2O) δ ppm: 172.46 (C=O), 70.99 (C12), 70.40 (C3), 66.21 (C7), 58.32 (CH_3), 48.55, (46.13, 45.18, 36.62, 35.12, 34.85, 34.85, 32.51, 28.52, 28.35, 27.26, 26.18 steroid ring), 45.71 (CH_3), 34.36 (CH_2) 31.71 (CH_2), 30.38 (CH_2), 22.60 (C19), 17.08 (C21), 13.84 12.31 (C18) ppm

MS (+APCI) m/z= Found 549.4618; calculated for $C_{33}H_{61}N_2O_4$ 549.4626; -1.4 ppm

IR (KBr) v = 3484, 3250, 3071, 2925, 2868, 1627 (C=O), 1560 cm^{-1}

Synthesis of 3-cholanamidoethyl-hexyl-dimethyl-ammonium iodide (42)



N-[3-(dimethylamino)ethyl] cholanamide (21) (0.5 g, 1 mmol) was dissolved in DCM (10 mL) along with 1-iodohexane (0.7 mL, 2 mmol) and methanol (1 mL). The reaction was left for 3 days where a precipitate formed. The product was collected by filtration, washed with chloroform (20 mL) and dried at room temperature under vacuum. The product was a orange solid.

Yield =0.82 g

Melting point: 104.2-104.5 ^{o}C

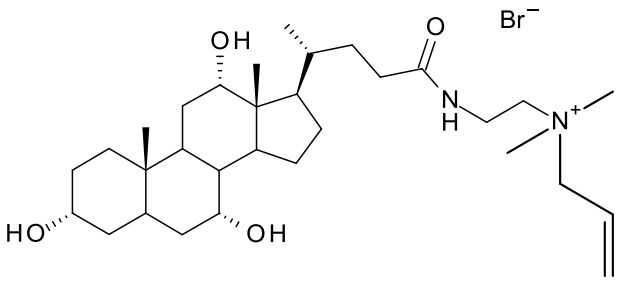
^{1}H NMR (D_2O) *δ* ppm: 0.58 (s, 3H, Me-18) 0.80 (s, 3H, Me-19) 0.87 (m, 10H, Me-21+ 3C$\underline{H}_2$-N) 1.0-2.43 (m, steroid structure) 1.26 (m, 15H) 2.49 (s, 3H, C$\underline{H}_3$-CH_2) 3.04 (s, 5H, 2C$\underline{H}_2$-NH) 3.15 (d) 3.29 (t, J=6.7 Hz, 1H) 3.24 (m, 1H, C$\underline{H}$-3) 3.82(s, 1H, C$\underline{H}$-7) 3.97 (s, 1H, C$\underline{H}$-12) 4.00 (3O$\underline{H}$) 4.08 (7O$\underline{H}$) 4.31 (12O$\underline{H}$) 8.09 (t, 1H, N$\underline{H}$) ppm

^{13}C NMR (D_2O) δ ppm: 172.45 (C=O), 70.98 (C12), 70.40 (C3), 66.21 (C7), 58.32 (CH_3), (46.13, 45.18, 36.62, 35.12, 34.85, 34.85, 32.51, 28.52, 28.35, 27.26, 26.18 steroid ring), 45.71 (CH_3), 34.36 (CH_2) 31.71 (CH_2), 30.38 (CH_2), 22.60 (C19), 17.08 (C21), 12.31 (C18) ppm

MS (+APCI) m/z= Found 563.4771; calculated for $C_{34}H_{63}N_2O_4$ 563.4782; -2.0 ppm

IR (KBr) v = 3393, 3238, 3056, 2925, 2853, 1703, 1651 (C=O), 1606 cm^{-1}

Synthesis of 3-cholanamidoethyl-allyl-dimethyl-ammonium bromide (43)



N-[3-(dimethylamino)ethyl] cholanamide (21) (0.5 g, 1 mmol) was dissolved in DCM (10 mL) along with allyl bromide (0.4 mL, 3.3 mmol) and methanol (1 mL). The reaction was left for 12 hours before solvent was removed using a rotary evaporator. The product was purified by washing with diethyl ether was collected by filtration and dried at room temperature under vacuum. The product was a white solid.

Yield =0.129 g (26 %)

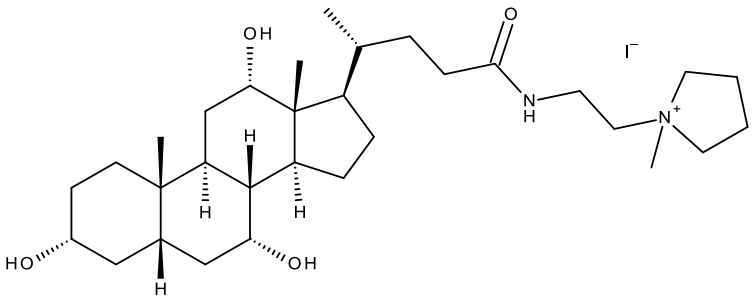
Melting point: 119.1-120.3 ^{o}C

^{1}H NMR ($CDCl_3$) δ ppm: 0.58 (s, 3H, Me-18) 0.81 (s, 3H, Me-19) 0.93 (d, J= 6.0 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 3.03 (s, 6H, 2C$\underline{H}_2$) 3.47 (s, 1H, C$\underline{H}$-3) 3.62 (d, J= 9.2 Hz, 1H, C$\underline{H}$-12) 3.78 (s, 1H, C12-O$\underline{H}$) 4.01 (d, J=2.7 Hz, 3H, C$\underline{H}_2$+ C$\underline{H}_2$-CH=CH) 4.10 (s, 1H, C12-O$\underline{H}$) 5.63 (d, J= 5Hz, 1H, CH=C$\underline{H}_2$) 5.67 (s, 1H) 5.76 (s, 1H, CH= C$\underline{H}_2$) 6.03 (m, 1H, C$\underline{H}$=CH_2) 8.03 (t, 1H, N$\underline{H}$) ppm

MS (+APCI) m/z= Found 519.4146; calculated for $C_{31}H_5N_2O_4$ 519.4156; -2.0 ppm

IR (KBr) v = 3362, 2929, 2862, 1700, 1645 (C=O), 1536 cm^{-1}

Synthesis of N-[2-(1-methylpyrrolidin-1-ium-1-yl)ethyl]cholanamide iodide (44)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in DCM (10 mL) along with of methyl iodide (1.4 mL, 10 mmol). The reaction was left for 60 hours where the product precipitated out. It was then collected by filtration, washed with chloroform (20 mL) and dried at room temperature under vacuum. The product was a white solid.

Melting point: 140.7-141-4 ^{o}C

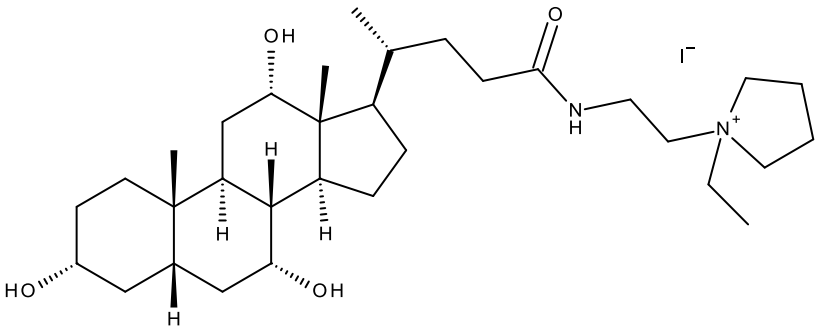
^{1}H NMR (DMSO) δ ppm: 0.58 (s, 3H, Me-18) 0.81 (s, 3H, Me-19) 0.93 (d, J= 5.8Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 3.02 (s, 3H, C<u>H</u>$_3$) 3.20 (m, 1H, C<u>H</u>-3) 3.50 (m) 3.61 (s, 1H, C<u>H</u>-7) 3.77 (s, 1H, C<u>H</u>-12) 4.01 (m, 1H, 3O<u>H</u>) 4.10 (m, 1H, 7O<u>H</u>) 4.33 (m, 1H, 12O<u>H</u>) 8.11 (t, 1H, N<u>H</u>) ppm

^{13}C NMR (DMSO) δ: 173.30 (C=O), 70.96 (C12), 70.37 (C3), 66.19 (C7), 63.86 (CH_2), 61.67 (CH_2 ring), 47.44, (45.98, 45.68, 41.46, 35.09, 34.34, 33.58, 32.25, 31.40, 28.54, 27.26, 26.20 steroid ring), 41.39 (CH_2), 22.76 (CH_2 ring), 22.59 (C19), 20.91, 17.04 (C21), 12.31 (C18) ppm

MS (+APCI) m/z= Found 519.4152; calculated for $C_{31}H_{55}N_2O_4$ 519.4156; -0.8 ppm

IR (KBr) v = 3390, 3274, 2922, 2862, 1651 (C=O), 1533, 1460 cm^{-1}

Synthesis of N-[2-(1-ethylpyrrolidin-1-ium-1-yl)ethyl]cholanamide iodide (45)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in DCM (10 mL) along with of ethyl iodide (0.4 mL, 10 mmol). The reaction was left for 60 hours where the product precipitated out. It was collected by filtration, washed with chloroform and dried at room temperature under vacuum. The product was a yellow solid.

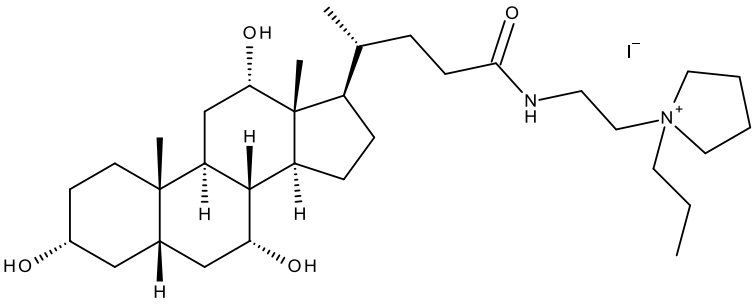
Melting point: 120.7-121.7 ^{o}C

^{1}H NMR (DMSO) δ ppm: 0.57 (s, 3H, Me-18) 0.80 (s, 3H, Me-19) 0.92 (m, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.23 (d, J=12.1 Hz, C$\underline{H}_3$) 2.64 (C$\underline{H}_2$) 3.49 (m, 4H, C$\underline{H}_3$) 3.60 (s, 1H, C$\underline{H}$-7) 3.77 (s, 1H, C$\underline{H}$-12) 4.01 (m, 1H, 3OH) 4.10 (m, 1H, 7OH) 4.34 (m, 1H, 12OH) 5.75 (s, 1H, C$\underline{H}$) 8.08 (s, 1H, N$\underline{H}$) ppm

MS (+APCI) m/z= Found 533.4302; calculated for $C_{32}H_{57}N_2O_4$ 533.4313; -2.0 ppm

IR (KBr) v = 3378, 2929, 2862, 2361, 2158, 2018, 1645 (C=O), 1527 cm^{-1}

Synthesis of N-[2-(1-propylpyrrolidin-1-ium-1-yl)ethyl]cholanamide;iodide (46)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with of 1-iodopropane (3 mL, 10 mmol). The reaction was left for 12 hours where the product

preciptated out. It was collected by filtration, washed with chloroform and dried. The product was a yellow solid.

Yield= 0.947 g

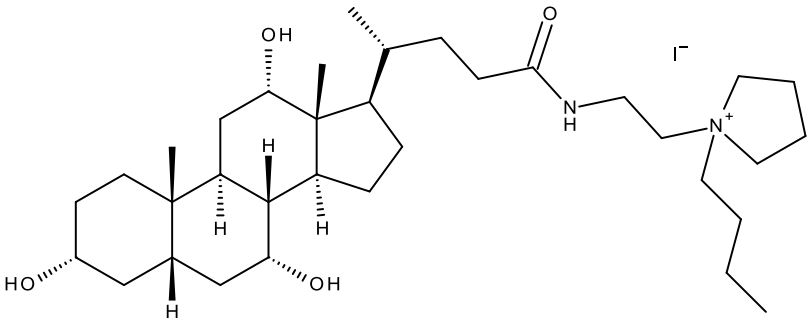
Melting point: 203.8-204.1 ^{o}C

^{1}H NMR (DMSO) δ ppm: 0.58 (s, 3H, Me-18) 0.80 (s, 3H, Me-19) 0.91 (m, 6H, Me-21 + C<u>H</u>$_2$) 1.0-2.43 (m, steroid structure) 3.18 (m) 3.5 (ddt, J=40.0, 13.4, 6.6 Hz, 3H, C<u>H</u>$_3$) 3.61 (s, 1H, C<u>H</u>-7) 3.77 (s, 1H, C<u>H</u>-12) 4.01 (d, 1H, 3O<u>H</u>) 4.10 (d, J= 4.12 Hz, 1H, 7O<u>H</u>) 4.32 (d, J= 3.16 Hz, 1H, 12O<u>H</u>) 8.09 (s, 1H, N<u>H</u>) ppm

MS (+APCI) m/z= Found 547.4463; calculated for $C_{33}H_{59}N_2O_4$ 547.4469; -1.2 ppm

IR (KBr) ν = 3566, 3353, 3217, 2913, 2856, 2246, 2121, 1642, 1527 cm^{-1}

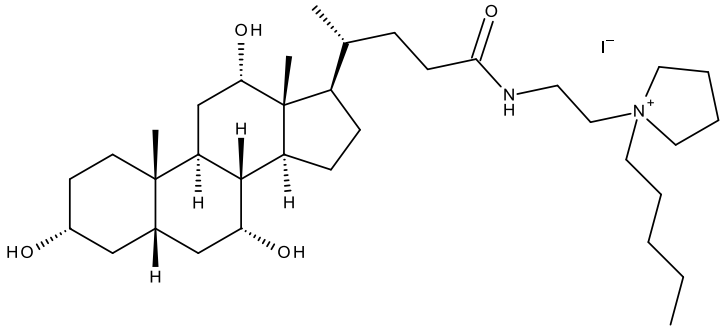
Attempted synthesis of N-[2-(1-butylpyrrolidin-1-ium-1-yl)ethyl]cholanamide iodide (47)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with of 1-iodobutane (1.14 mL, 10 mmol). The reaction was left for 48 hours where the product precipitated out. It was collected by filtration, washed with chloroform and dried at room temperature under vacuum. The product was a white solid.

Proton NMR analysis showed that the product was not the intended compound.

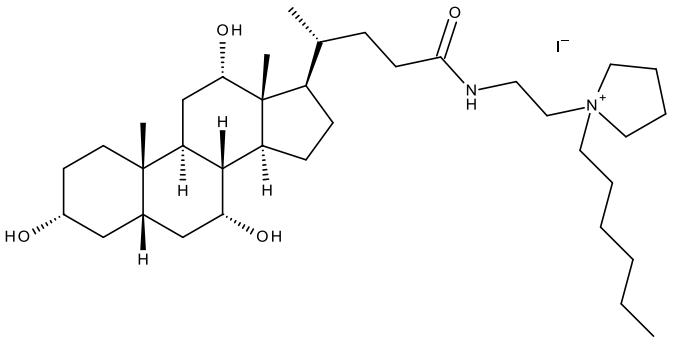
Attempted synthesis of N-[2-(1-pentylpyrrolidin-1-ium-1-yl)ethyl]cholanamide iodide (48)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with of 1-iodopentane (2 mL, 10 mmol). The reaction was left for 96 hours where the product preciptated out. It was collected by filtration, washed with chloroform (20 mL) and dried at room temperature under vacuum. The product was a white solid.

Proton NMR analysis showed it was not the desired compound.

Synthesis of N-[2-(1-hexylylpyrrolidin-1-ium-1-yl)ethyl]cholanamide iodide (49)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with of 1-iodopentane (2 mL, 10 mmol). The reaction was left for 96 hours where the product precipitated out. It was collected by filtration, washed with chloroform (15 mL) and dried at room temperature under vacuum. The product was a yellow solid.

Yield=0.527 g

^{1}H NMR (DMSO) δ ppm: 0.58 (s, 3H, Me-18) 0.81 (s, 3H, Me-19) 0.91 (m, Me-21 + C$\underline{H}_2$) 1.0-2.43 (m, steroid structure) 2.44 (p, J=1.8HZ, 4H, C$\underline{H}_3$) 3.33 (m, C$\underline{H}_2$) 3.49 (m, 8H, C$\underline{H}$-3, C$\underline{H}_2$) 3.61 (m,

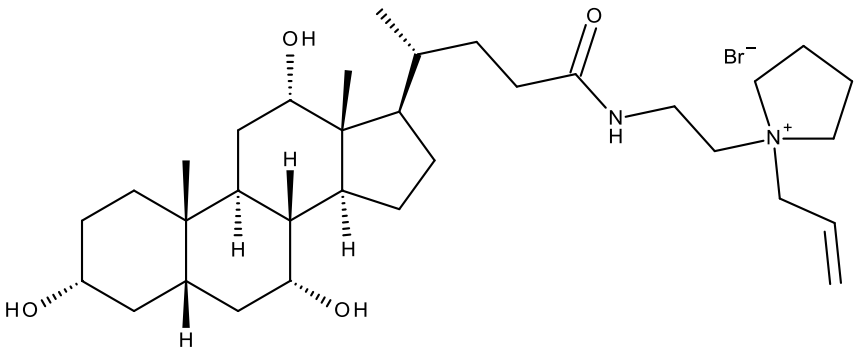
1H, CH-7) 3.77 (s, 1H, CH-12) 4.00 (d, 1H, 3OH) 4.10 (t, 1H, 7OH) 4.30 (s, 1H, 12OH) 7.72 (s, 1H, NH) ppm

^{13}C NMR (DMSO) δ 173.38 (C=O), 70.96 (C12), 70.37 (C3), 70.30, 66.19 (C7), 62.48, 58.76, 56.74 (CH_2 ring), (45.95, 45.69, 40.48, 40.15, 39.81, 39.48, 39.15, 38.81, 38.48, 35.09, 34.34, 32.22, 31.36, 30.71, 28.54, 27.25, 26.21 steroid ring), 25.43 (CH_2 ring), 22.59 (C19), 21.91 (CH_2), 21.15, 17.04 (C21), 13.82 (CH_3), 12.29 (C18) ppm

MS (+APCI) m/z= 589.4926

IR (KBr) v = 3466, 3362, 3186, 3044, 2953, 2922, 1706, 1660, 1624 (C=O), 1530 cm^{-1}

Synthesis of N-[2-(1-allylpyrrolidin-1-ium-1-yl)ethyl]cholanamide bromide (50)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in chloroform (20 mL) along with allylbromide (1.2 mL, 10 mmol). The reaction was left for 96 hours where the product precipitated out. It was collected by filtration, washed with chloroform (20 mL) and dried at room temperature under vacuum. The product was a white solid.

Yield= 0.59 g (60%)

TLC: (MeOH/EtOAc, 1/1, R_f=0.08 (one spot)

Melting point: 186.5-186.9 ^{o}C

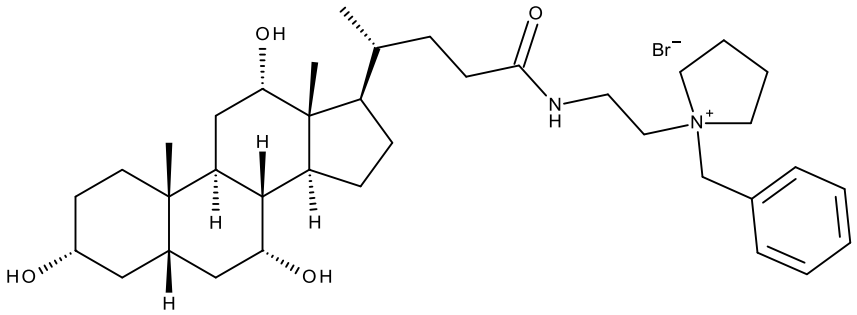
^{1}H NMR (DMSO) *δ* ppm: 0.59 (s, 3H, Me-18) 0.81 (s, 3H, Me-19) 0.95 (d, J= 5.8 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.07 (m) 2.23 (d, J=12.1 Hz, CH_2) 3.27 (m, 3H, CH-3 + CH_2) 3.5 (m, 5H, 2CH_2) 3.61 (s, 1H, CH-7) 3.79 (s, 1H, CH-12) 3.95 (d, J= 7.5 Hz, 1H, CH- allyl bromide) 4.03 (d, 2H, J= 7.1 Hz, 3OH) 4.11 (d, 1H, 7OH) 4.33 (d, 1H, 12OH) 5.66 (t, 2H, CH=CH_2) 6.08 (m, 1H, CH=CH_2) 8.13 (t, 1H, NH) 8.32 (s, 1H) ppm

^{13}C NMR (DMSO) δ ppm: 173.37 (C=O), 127.23 (C=C), 126.28 (C=C), 79.17, 70.94 (C12), 70.36 (C3), 66.18 (C3), 61.74, 60.54, 57.71 (CH_2), (45.94, 45.68, 40.48, 40.15, 40.07, 39.82, 39.73, 39.48, 39.15, 38.81, 38.48, 34.34, 33.11, , 31.39, 30.35, 28.54, 28.53, 27.29, 26.20 steroid ring), 32.18 (CH_2), 22.60 (C19), 21.16, 17.03 (C21), 12.30 (C18) ppm

MS (+APCI) m/z= Found 545.4306; calculated for $C_{33}H_{57}N_2O_4$ 545.4313; -1.3 ppm

IR (KBr) ν = 3472, 3365, 3211, 3050, 2922, 2859, 2458, 2042, 1630 (C=O), 1542 cm^{-1}

Synthesis of N-[2-(1-benzylpyrrolidin-1-ium-1-yl)ethyl]cholanamide bromide (51)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in DCM (15 mL0 along with benzyl bromide (1.2 mL, 10 mmol). The reaction was left for 48 hours where the product precipitated out. It was collected by filtration, washed with chloroform (15 mL) and dried at room temperature under vacuum. The product was a yellow solid.

Yield= 0.59 g

TLC: (MeOH/EtOAc, 1/1), R_f=0.08 (one spot)

Melting point: 165.7-165.9 ^{o}C

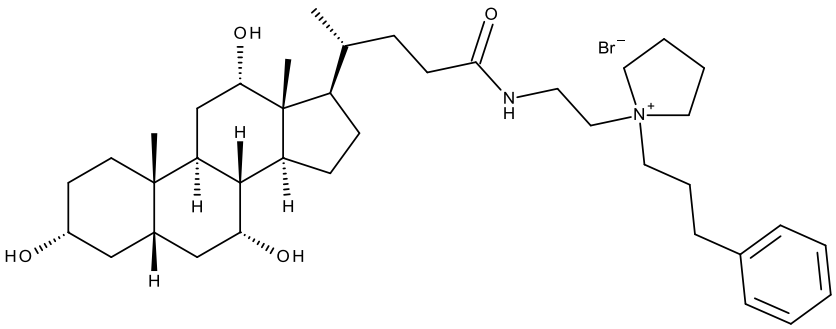
^{1}H NMR (D_2O) *δ* ppm: 0.56 (s, 3H, Me-18) 0.87 (s, 3H, Me-19) 0.92 (d, J= 5.5 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.19 (s) 2.23 (d, J=12.1 Hz, C$\underline{H}_2$) 3.37-3.68 (m, 6H, C$\underline{H}$-3 + C$\underline{H}_2$) 3.75 (s, 1H, C$\underline{H}$-7) 3.98 (s, 1H, C$\underline{H}$-12) 7.38 (s, 1H, N$\underline{H}$) 7.54 (m, aromatic) ppm

^{13}C NMR ($D_{MS}O$) δppm: 173.43 (C=O), (132.61, 129.04, 128.41 aromatic ring), 60.89 (CH_2), 60.86 (CH_2), (45.69, 35.25, 35.10, 34.94, 34.67, 34.34 steroid ring), 22.59 (C19), 20.86, 17.03 (C21), 12.28 (C18) ppm.

MS (+APCI) m/z= 595.4461

IR (KBr) ν = 3368, 3053, 2922, 2862, 2361, 1788, 1706, 1648 (C=O), 1536 cm^{-1}

Synthesis of N-[2-[1-(3-phenylpropyl)pyrrolidin-1-ium-1-yl]ethyl]cholanamide bromide (52)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with 1-bromo-3-phenylpropane (2 mL, 10 mmol). The reaction was left for 12 hours where the product precipitated out. It was collected by filtration, washed with chloroform (10 mL) and dried at room temperature under vacuum. The product was a yellow solid.

Yield=0.986 g (98 %)

Melting point: 140.8-141.3 ^{o}C

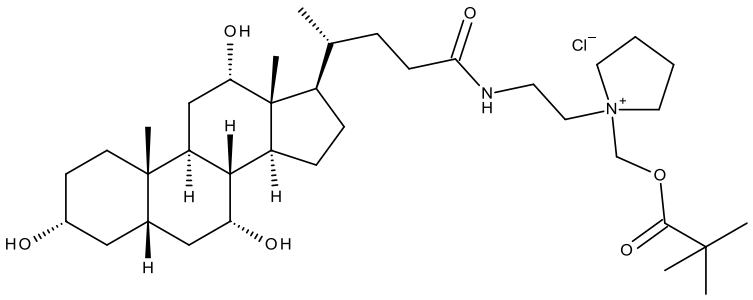
^{1}H NMR (DMSO) δ ppm: 0.58 (s, 3H, Me-18) 0.81 (s, 3H, Me-19) 0.92 (m, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.68, (dt, J=10.9, 7.4Hz, 2H, C$\underline{H}_2$) 3.16-3.58 (m, 9H, C$\underline{H}$-3, C$\underline{H}_2$) 3.60 (s, 1H, C$\underline{H}$-7) 3.77 (s, 1H, C$\underline{H}$-12) 4.02 (m, 1H, 3O$\underline{H}$) 4.10 (m, 1H, 7O$\underline{H}$) 4.33 (m, 1H, 12O$\underline{H}$) 7.12-7.36 (m, 4H, aromatic) 8.11 (t, J=5.5Hz, 1H, N$\underline{H}$) ppm

^{13}C NMR (DMSO) δ ppm: 173.32 (C=O), (140.52, 128.37, 128.37, 128.33, 125.98 aromatic ring), 79.15, 70.97 (C12), 70.38 (C3), 66.20 (C7), 63.84 (CH_2), 61.68 (CH_2), 47.42, (45.98, 45.69, 45.68, 40.48, 40.14, 39.81, 39.48, 39.14, 38.81, 38.47, 35.10, 34.86, 34.35, 33.85, 33.58, 33.36, 32.25, 31.39, 30.34, 28.53, 27.27, 26.20 steroid ring), 34.34 (CH_2), 22.59 (C19), 20.90, 17.04 (C21), 12.30 (C18) ppm.

MS (+APCI) m/z= Found 519.4150; expected 623.478 (MI- $CH_2CH_2C_6H_6$)

IR (KBr) ν = 3387, 3256, 3068, 3026, 2929, 2868, 1651, 1624 (C=O), 1533 cm^{-1}

Synthesis of [1-(2-cholanamidoethyl) pyrrolidin-1-ium-1-yl] methyl 2,2-dimethylpropanoate chloride (53)



N-(2-pyrrolidin-1-ylethyl)cholanamide (10) (0.5 g, 1 mmol) was dissolved in DCM (10 mL) along with chloromethyl pivalate (1.4 mL, 10 mmol). The reaction was left for 72 hours where the product precipitated out. It was collected by filtration, washed with chloroform and (15 mL) dried at room temperature under vacuum. The product was a yellow solid.

Yield=0.335 g (34 %)

Melting point: 121.2-121.8 ^{o}C

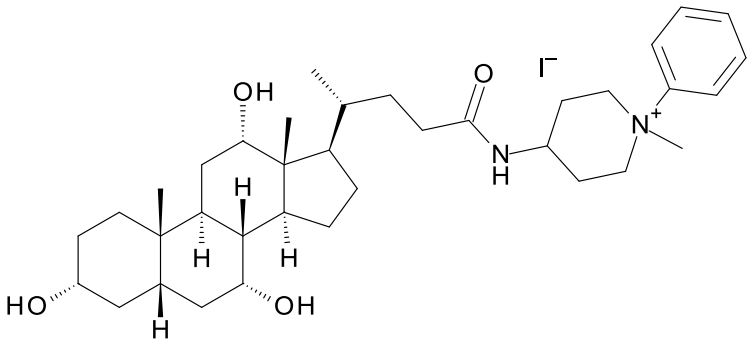
^{1}H NMR (DMSO) δ ppm: 0.58 (s, 3H, Me-18) 0.81 (s 3H Me-19) 0.95 (d, J= 5.7 Hz, 3H, Me-21) 1.0-2.439 (m, steroid structure) 1.11 (s, 3H, CH3) 1.17 (s, 1H, CH) 1.24 (m, 5H) 3.17- 3.47 (m, 4H, 2C$\underline{H}_2$) 3.38 (t, J=9.1 Hz, 1H, C$\underline{H}$-3) 3.87 (s, 1H, C$\underline{H}$-7) 4.03 (s, 1H, C$\underline{H}$-12) 8.66 (s, 1H, N$\underline{H}$) 11.39 (s, 1H) ppm

^{13}C NMR (DMSO) δ 206.43, 179.27, 175.20 (C=O), 173.36, 173.14, 77.70, 70.93 (C12), 70.37 (C3), 69.58, 66.19 (C7), 60.88, 57.80, 53.07 (CH_2), 52.88 (CH_2 ring), (45.71, 37.66, 35.16, 34.85, 34.34, 33.32, 33.29, 32.16, 31.29, 30.65, 30.65, 28.50, 26.97, 26.97, 26.88, 26.42, 26.31, 26.20 steroid ring), 22.57 (C19), 21.81 (CH_2 ring), 17.07 (C21), 12.28 (C18) ppm.

MS (+APCI) m/z= 619.4647

IR (KBr) ν = 3368, 2929, 2874, 2607, 2476, 2249, 2118, 1754, 1709, 1651 (C=O), 1539 cm^{-1}

Synthesis of N-(1-methyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide iodide (54)



N-(1-phenyl-4-piperidyl) cholanamide (14) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with methyl iodide (1.4 mL, 10 mmol). The reaction was left for 72 hours where the product precipitated out. It was collected by filtration, washed with DCM (20 mL) and dried at room temperature under vacuum. The product was a yellow solid.

Yield=0.7 g

TLC: (MeOH), R_f=0.13 (dominant spot), 0.71 (two spots)

Melting point: 165.8-166.7 ^{o}C

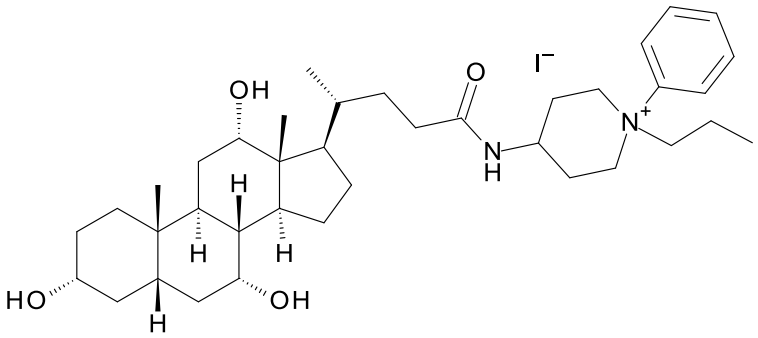
^{1}H NMR (DMSO) *δ* ppm: 0.57 (s, 3H, Me-18) 0.80 (s, 3H, Me-19) 0.92 (d, 3H, Me-21) 1.0-2.43 (m, steroid structure)2.92 (s, 4H, 2C$\underline{H}_2$) 3.03 (s, 1H, C$\underline{H}$) 3.06 (s, 1H, C$\underline{H}$) 3.16 (s, 3H, C$\underline{H}_3$) 3.60 (s, 1H, CH-7) 3.78 (s, 1H, C$\underline{H}$-12) 4.00 (m, 1H, 3OH) 4.10 (S, 1H, 7OH) 4.28 (m, 1H, 12OH) 4.57 (S, 2H, C$\underline{H}_2$) 4.60 (m, 2H, C$\underline{H}_2$) 7.53 (m, 8H, aromatic) 7.79-7.90 (m, 1H, N$\underline{H}$) ppm

^{13}C NMR (DMSO) δ ppm: 172.24 (C=O),(133.08, 128.86, 127.54 aromatic ring), 35.10, 34.35, 22.60 (C19), 17.12 (C21), 12.31 (C18) ppm.

MS (+APCI) m/z= Found 595.4464; calculated for $C_{36}H_{57}N_2O_4$ 595.4469; 0.84 ppm

IR (KBr) v = 3396, 2925, 2862, 2355, 1648 (C=O), 1533 cm^{-1}

Synthesis of N-(1-propyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide iodide (55)



N-(1-phenyl-4-piperidyl) cholanamide (14) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with 1-iodopropane (1.5 mL, 10 mmol). The reaction was left for 72 hours where the product precipitated out. It was collected by filtration, washed with DCM (15 mL) and dried at room temperature under vacuum. The product was a yellow solid.

Yield=0.7 g

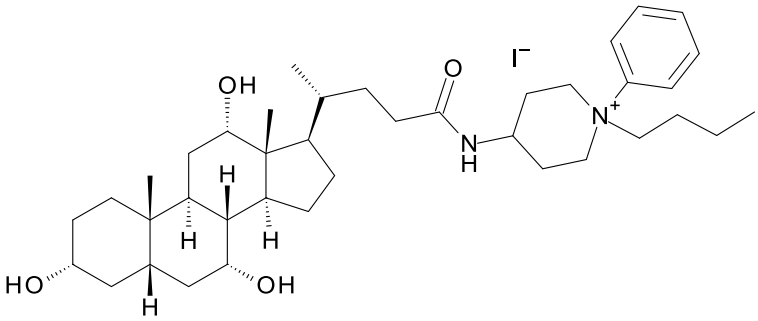
Melting point: 113-113.8 ^{o}C

^{1}H NMR (DMSO) δ ppm: 0.58 (d, J=4.1 Hz, 3H, Me-18) 0.81 (s 3H Me-19) 0.87 (m, 9H, Me-21 + 2CH_3) 1.0-2.439 (m, steroid structure) 2.08 (s, CH_3) 3.01-3.33 (m, 9H, CH-3, CH_2) 3.61 (s, 1H, CH-7) 3.78 (s, 1H, CH-12) 3.92-4.11 (m, 2H, CH_2) 4.60 (d, J=10.3 Hz, 1H) 7.42-7.62 (m, 6H, aromatic ring) ppm

MS (+APCI) m/z=Found 651.5086; expected 609.4625 (MI+ 52)

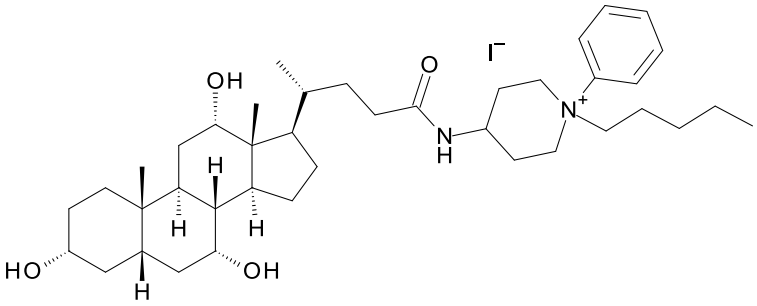
IR (KBr) v = 3393, 2932, 2862, 2361, 2155, 2018, 1642 (C=O), 1533 cm^{-1}

Attempted synthesis of N-(1-butyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide iodide (56)



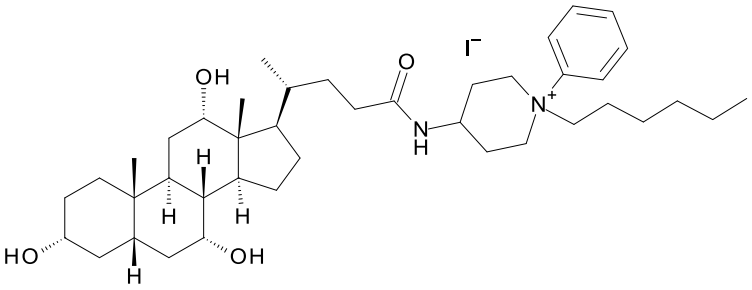
N-(1-phenyl-4-piperidyl) cholanamide (14) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with 1-iodobutane (1.2 mL, 10 mmol). The reaction was left for 72 hours where the reaction did not progress. The reaction was heated to 35 ^{o}C for 48 hours, and then the temperature was raised to 50 ^{o}C for 128 hours. TLC showed the reaction did not progress.

Attempted synthesis of N-(1-pentyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide iodide (57)



N-(1-phenyl-4-piperidyl) cholanamide (14) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with 1-iodopentane (1.5 mL, 10 mmol). The reaction was left for 72 hours where the reaction did not progress. The reaction was heated to 35 ^{o}C for 48 hours, and then the temperature was raised to 50 ^{o}C for 128 hours. TLC showed the reaction did not progress.

Synthesis of N-(1-hexyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide iodide (58)



N-(1-phenyl-4-piperidyl) cholanamide (14)(0.7 g, 1.2 mmol) was dissolved in DCM (15 mL) along with 1-iodohexane (1.5 mL, 10 mmol). The reaction was left for 120 hours where the product did not precipitate. The solvent was removed under vacuum and the product was sonicated then washed with diethyl ether (10 mL). The product was dried at room temperature under vacuum and it a yellow solid.

Yield= 0.55 g (78 %)

TLC: (MeOH), R_f=0.12 (dominant spot), 0.9 (two spots)

Melting point: 126.4-127.8 ^{o}C

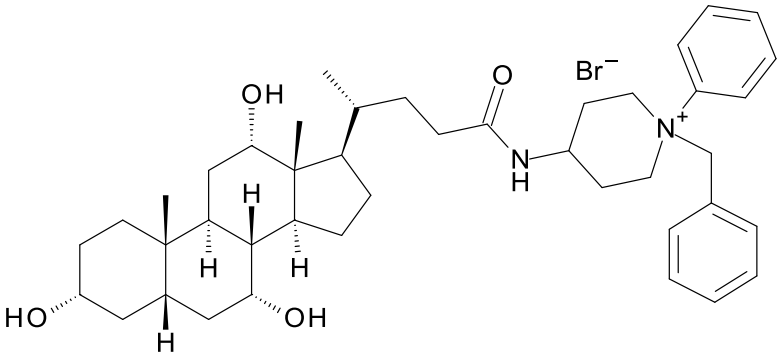
^{1}H NMR ($CDCl_3$) δ ppm: 0.64 (s, 3H, Me-18) 0.86 (s, 3H, Me-19) 0.97 (d, Me-21) 1.0-2.43 (m, steroid structure) 2.04 (s, C$\underline{H}_3$) 2.83 (m,C$\underline{H}_2$) 3.2 (t, 1H, C$\underline{H}_2$-N)3.47(m, 1H, C$\underline{H}$-3) 3.56 (m, 2H, C$\underline{H}_2$) 3.81 (s, 1H, C$\underline{H}$-7) 3.94 (s, 1H, C$\underline{H}$-12) 6.06 (d, J=7.8Hz, 1H, N$\underline{H}$) 7.32 (q, J=4.3, 3.7Hz, 6H, aromatic) ppm

^{13}C NMR (DMSO) δ ppm: 206.44, 171.82 (C=O), (128.73, 128.16, 126.95 aromatic ring), 70.97 (C12), 70.46 (C3), 66.20 (C7), 51.90 (CH_2), (46.11, 45.69, 40.49, 40.16, 35.30, 34.86, 34.35, 34.35, 32.56, 31.72, 30.66, 29.68, 28.53, 27.30, 26.18 steroid ring), 22.59 (C19), 17.11 (C21), 12.31 (C18) ppm.

MS (+APCI) m/z= Found 665.5257; calculated for $C_{41}H_{67}N_2O_4$ 665.5252; 0.77 ppm

IR (KBr) v = 3372, 3296, 3056, 3032, 2932, 2862, 2364, 1642 (C=O), 1530 cm^{-1}

Synthesis of N-(1-benzyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide (59)



N-(1-phenyl-4-piperidyl) cholanamide (14) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with benzyl bromide (1.5 mL, 10 mmol). The reaction was left for 12 days where the product precipitated. The product washed with diethyl ether (10 mL). The product was dried at room temperature under vacuum and it a peach solid. Product is not pure.

Yield= 0.1839 g (18 %)

Melting point: 150.3-150.9 ^{o}C

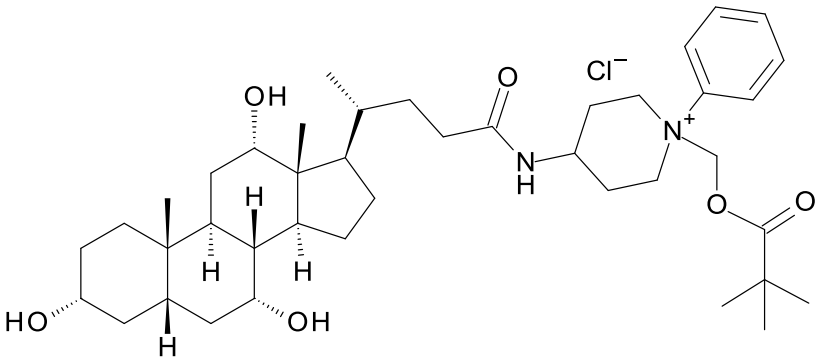
^{1}H NMR (Methanol-d_4) δ ppm: 0.68 (s, 3H, Me-18) 0.90 (s, 3H, Me-19) 0.98 (d, J=6.2 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 3.68 (m, 1H, C<u>H</u>-3) 3.78 (s, 1H, C<u>H</u>-7) 3.92 (s, 1H, C<u>H</u>-12) 4.49 (s, 2H, C<u>H</u>$_2$) 4.54 (s, 4H, 2C<u>H</u>$_2$) 7.19-7.37 (m, 10H, aromatics) 7.45-7.60 (m, 10H, aromatics) 7.89 (s, 10H) ppm

^{13}C NMR (DMSO) δppm: (133.56, 133.08, 130.33, 129.23, 127.46, 127.12, 126.37 aromatic ring), 70.96 (C12), 45.69, 34.35 (CH_2), 27.31, 24.50, 22.59 (C19), 17.04 (C21), 12.30 (C18), 5.85, 1.58 ppm.

MS (+APCI) m/z= Found 671.4770; expected 657.4625 (MI+CH_3)

IR (KBr) ν = 3411, 3329, 3062, 2929, 2859, 2671, 2355, 1654 (C=O), 1536, 1493 cm^{-1}

Synthesis of (4-cholanamido-1-phenyl-piperidin-1-ium-1-yl)methyl 2,2-dimethylpropanoate chloride (60)



N-(1-phenyl-4-piperidyl) cholanamide (14) (0.5 g, 1 mmol) was dissolved in chloroform (15 mL) along with of chloromethyl pivalate (1.4 mL, 10 mmol). The reaction was left for 12 days where the product did not precipitate. The solvent was taken off under vacuum and the product sonicated and washed with diethyl ether (10 mL). The product was dried at room temperature under vacuum and it a yellow solid.

Yield= 0.5215 g (52 %)

Melting point: 135.9-137 ^{o}C

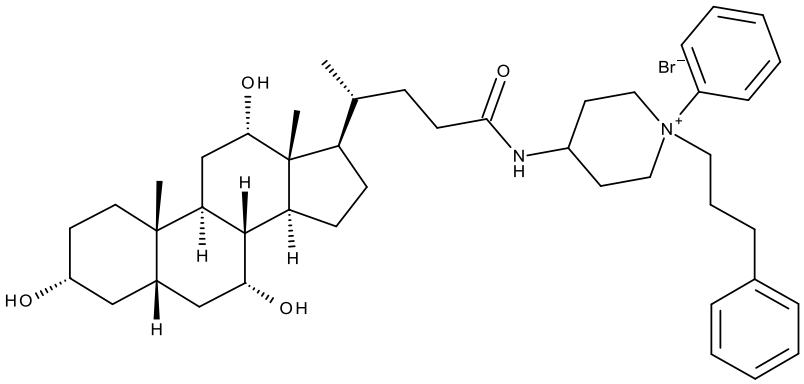
^{1}H NMR (Methanol-d_4) δ ppm: 0.68 (s, 3H, Me-18) 0.92 (s, 3H, Me-19) 1.02 (d, J= 6.1 Hz, 3H, Me-21) 1.0-2.439 (m, steroid structure) 2.99 (d, J=11.4Hz, 1H, C$\underline{H}_3$) 3.78 (s, 1H, C$\underline{H}$-7) 3.97 (s, 1H, C$\underline{H}$-12) 5.75 (s, 2H, O-C$\underline{H}_2$-Cl) 7.24-7.45 (m, 2H, aromatic) 7.55 (m, 1H, N$\underline{H}$) ppm

^{13}C NMR (DMSO) δ ppm: 206.44 (C=O), 175.60 (C=O), 128.69, 69.59 (CH_2),) 45.69, 35.12, 34.35, 30.66, 26.97, 26.32 steroid ring), 22.59 (C19), 17.11 (C21), 12.30 (C18) ppm.

MS (+APCI) m/z= Found 695.4994; calculated for $C_{41}H_{65}N_2O_6$ 695.4993; 0.143 ppm

IR (KBr) v = 3365, 3065, 2935, 2865, 2631, 2525, 2361, 1751, 1642 (C=O), 1542 cm^{-1}

Synthesis of N-[1-phenyl-1-(3-phenylpropyl)piperidin-1-ium-4-yl]cholanamide bromide (61)



N-(1-phenyl-4-piperidyl) cholanamide (14) (0.5 g, 1 mmol) was dissolved in chloroform (15 mL) along with 1-bromo-3-phenylpropane (1.3 mL, 10 mmol). The reaction was left for 12 days where the product did not precipitate. The solvent was taken off under vacuum and the product sonicated and washed with diethyl ether (10 mL). The product was dried at room temperature under vacuum and it a yellow solid.

Yield= 0.79 g

Melting point: 126.1-126.7 ^{o}C

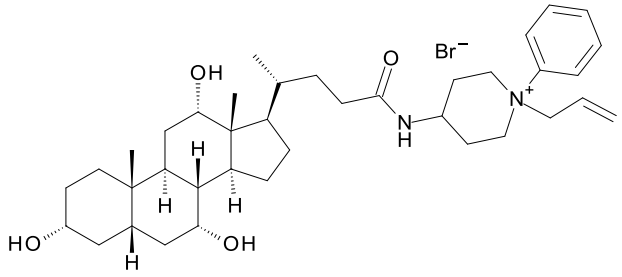
^{1}H NMR (Methanol-d_4) δ ppm: 0.69 (s, 3H, Me-18) 0.91 (s, 3H, Me-19) 0.98 (d, J= 6.2 Hz, 3H, Me-21) 1.0-2.43 (m, steroid structure) 2.98 (d, 2H, C$\underline{H}_2$) 3.43 (m) 3.66 (m, 1H, C$\underline{H}$-3) 3.79 (s, 1H, C$\underline{H}$-7) 3.94 (s, 1H, C$\underline{H}$-12) 4.50 (s, 1H, C$\underline{H}$-N) 7.09-7.41 (m, 7H, aromatic) ppm

^{13}C NMR (DMSO) δ ppm: (140.52, 128.85, 128.43, 128.37, 128.33, 125.98 aromatic ring), 70.99 C12), 70.38 (C3), 66.20 (C7), (45.95, 45.69, 45.69, 40.49, 40.15, 35.27, 34.89, 33.36, 31.62, 30.38, 28.52, 27.31, 26.19 steroid ring), 34.61 (CH_2), 34.36 (CH_2), 33.86 (CH_2), 22.59 (C19), 17.12 (C21), 12.31 (C18) ppm.

MS (+APCI) m/z= Found 699.5087; calculated for $C_{44}H_{65}N_2O_4$ 699.5095; 1.143 ppm

IR (KBr) ν = 3375, 3023, 2932, 2859, 1642 (C=O), 1536 cm^{-1}

Synthesis of N-(1-allyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide bromide (62)



N-(1-phenyl-4-piperidyl) cholanamide (14) (0.5 g, 1 mmol) was dissolved in DCM (15 mL) along with allyl bromide (1.2 mL, 10 mmol). The reaction was left for 4 days where the product precipitated then washed with DCM (25 mL) . The product was dried at room temperature under vacuum and to give an orange solid.

Yield= 0.242 g (24 %)

TLC: (MeOH), R_f=0.24 (one spot)

Melting point: 161.4-161.7 ^{o}C

^{1}H NMR ($CDCl_3$) δ ppm: 0.58 (d, J= 3.6 Hz, 3H, Me-18) 0.81 (s, 3H, Me-19) 0.93 (m, 3H, Me-21) 1.0-2.43 (m steroid structure) 3.18, 3.61 (s, 1H, C<u>H</u>-3), 3.78 (s 1H C<u>H</u>-7), 3.95 (dd, J=16.7, 9.8 Hz 1H C<u>H</u>-12) 4.08 (s) 4.31 (s, 2H, C<u>H$_2$</u>) 4.55-4.68 (m CH=C<u>H$_2$</u>) 5.61-5.83 (m CH=C<u>H$_2$</u>) 7.42-7.62 (m, 6H, aromatic ring) ppm

^{13}C NMR (DMSO) δ ppm: 187.15 (C=O), 133.09 (C=C), (131.49, 130.30, 128.97, 127.16 aromatic ring), 92.56 (C=C), 70.96 (C12), 70.37 (C3), 66.19 (C7), 55.64 (CH_2), (46.01, 41.48, 40.50, 40.16, 39.83, 39.49, 39.15, 38.82, 38.74, 38.50, 37.34, 35.27, 30.66, 28.56 steroid ring), 22.59 (C19), 17.13 (C21), 12.30 (C18) ppm.

MS (+APCI) m/z= Found 621.4620; calculated for $C_{38}H_{59}N_2O_4$ 621.4626; 0.96 ppm

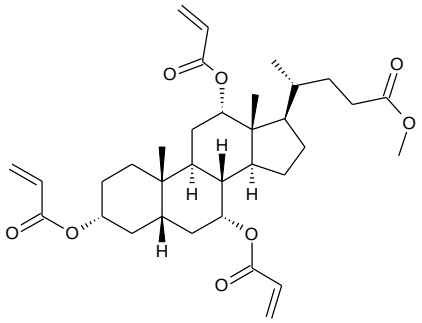
IR (KBr) v = 3378, 3056, 2935, 2856, 2552, 2358, 1639 (C=O), 1533 cm^{-1}

2.3 POLYMERISABLE GROUPS ON THE C3-OH

This work was based on a procedure by Zhang *et al.* (Zhang et al., 1998)

2.3.1 ACRYLOYLATES

3α,7α,12α-triacrylate cholic acid methyl ester (63)



Methyl cholate (4.22 g, 10 mmol) was dissolved in dry chloroform (30 mL). Triethylamine (2.23 mL, 16 mmol) was added and the flask was cooled to ice temperature. Acryloyl chloride (1.3 mL, 15 mmol) in chloroform (10 mL) was dripped in over 30 minutes. The mixture was protected from the light and left at room temperature overnight. More acryloyl chloride (1.3 mL, 15 mmol) in dry chloroform (10 mL) was dripped in over 30 minutes and the reaction was left for 6 hours at room temperature. The flask was maintained at -20 ^{o}C for 5 days. More acryoyl chloride (1.5 mL) was added. The reaction was left for 24 hours at room temperature. The solvent was removed using a rotary evaporator (the temperature was set to 30 ^{o}C) to produce a white solid. Ethyl acetate (50 mL) was added and the solid (salt) was collected by filtration. The ethyl acetate was removed on the rotary evaporator to leave a yellow oil. Flask column chromatography using 95% DCM/ 5% ethyl acetate was used to purify the product, increasing to 9/1 ratio then 3/1 ratio. The solvents were removed by rotary evaporation before the product was dried at room temperature under vacuum.

Yield= 0.2176 g (5 %)

TLC: (DCM/EtOAc 5/1), R_f=0.5 (one spot)

^{1}H NMR ($CDCl_3$) δ ppm: 0.75 (s, 3H, Me-18) 0.83 (s, 3H, Me-19) 0.94 (d, J= 6.0 Hz, 3H, Me-21) 1-2.43 (steroid structure) 2.67 - 2.91 (m, 2H) 3.60- 3.86 (m, 1H) 4.63 (m, 1H, C<u>H</u>-3) 5.03 (s, 1H, C<u>H</u>-7) 5.19 (s, 1H, C<u>H</u>-12) 5.72-5.97 (m, 2H, CH=C<u>H</u>$_2$) 5.99 – 6.52 (m, 3H, CH=C<u>H</u>$_2$) ppm

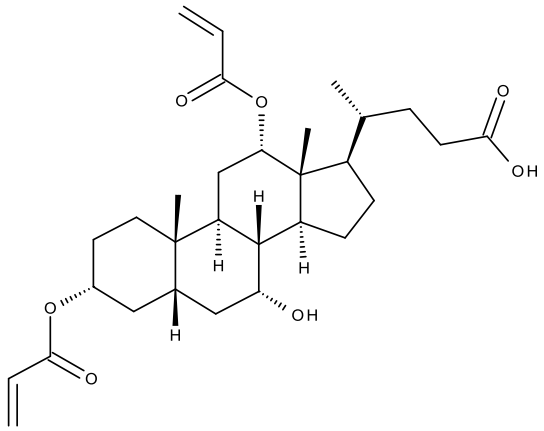
^{13}C NMR ($CDCl_3$) δ ppm: 174.48 (C=O), 169.70(C=O), 169.38(C=O), 169.20(C=O), 165.67(C=O excess), 165.46(C=O excess), 165.38(C=O excess), 165.29, 165.23, 136.42 (C=C), 130.55 (C=C),

130.42, 130.26, 130.18, 129.12, 129.00, 75.43, 74.62, 74.08, 73.92, 70.94 (C12), 70.85 (C3), 51.48, (47.44, 45.23, 45.12, 43.40, 43.32, 43.22, 40.84, 40.74, 39.32, 39.20, 39.14, 38.22, 38.00, 37.87, 34.75, 34.62, 34.55, 34.43, 34.35, 31.29, 30.91, 30.85, 30.74, 28.82, 28.60, 27.17, 26.74, 26.65 steroid ring), 25.46, 25.16, 22.84, 22.5 (C19), 22.34, 17.53 (C21), 17.45, 12.18 (C18), 12.08 ppm.

MS (+APCI) m/z= Found 602.1935; expected 584.3349 (MI+CH_3 +3H)

IR (KBr) ν = 2947, 271, 1715, 1633 (C=O), 1612, 1469 cm^{-1}

Synthesis of 3α,12α diacroylate-7αhydroxycholic acid (64)



This product was collected by column chromatography of the previous product (3α,7α,12α-triacrylate cholic acid methyl ester).

Yield= 0.7744 g (18 %)

TLC: (DCM/EtOAc 5/1), R_f=0.41 (one spot)

^{1}H NMR ($CDCl_3$) *δ* ppm: 0.68 (s, 3H, Me-18) 0.84 (s, 3H, Me-19) 0.95 (d, J= 6.3 Hz, 3H, Me-21) 1-2.43 (Steroid structure) 2.66-2.93 (m, 1H) 3.61-3.93 (s, 3H, C<u>H</u>$_3$) 4.12 (q, J=7.1 Hz, 1H ethyl acetate), 4.64 (tt, J= 10.7, 6.5 Hz, 1H C<u>H</u>-3) 4.99 (s, 1H, C<u>H</u>-7) 5.18 (s, 1H, C<u>H</u>-12) 5.34 (m, 1H) 5.70-5.93 (m, 1H, C<u>H</u>=CH) 5.98-6.52 (m, 2H, CH=C<u>H</u>) ppm

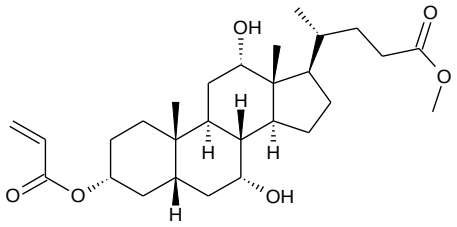
^{13}C NMR ($CDCl_3$) δ ppm: 174.55 (C=O), (169.68, 169.57, 165.78, 165.71, 165.57, 163.18 C=O, including excess acyloyl chloride), (130.66, 130.54, 130.30, 130.19, 130.08, 129.12, 129.00, 128.87 C=C, including excess acyloyl chloride), 75.63, 74.40, 74.28, 74.17, 72.57, 71.79, 71.02 (C12), 67.93 (C7), 60.36, 51.46, 47.46, 47.11, (46.56, 45.20, 45.06, 43.55, 43.40, 42.08, 42.02, 41.18, 41.12, 40.90, 40.84, 39.23, 38.23, 38.10, 35.23, 35.16, 34.96, 34.73, 34.61, 34.52, 34.34,

31.31, 30.98, 30.81, 28.60, 28.17, 28.10, 27.69, 27.51, 27.24, 26.64 steroid ring), 25.51, 25.36, 22.90, 22.53 (C19), 17.41 (C21), 17.32, 12.50, 12.26 (C18), 12.19 ppm.

MS (+APCI) m/z= Found 548.1638; expected 531.3322 (MI+CH_3+ 2H)

IR (KBr) v = 35 26 2944, 2871, 1715, 1639 (C=O), 1612, 1469 cm-1

Synthesis of 3α acetate-7α,12α dihydroxy cholic acid (65)



This product was collected by column chromatography of the product 3α,7α,12α-triacrylate cholic acid methyl ester.

Yield= 0.0232 g

TLC: (DCM/EtOAc 5/1), R_f=0.38 (one spot)

^{1}H NMR ($CDCl_3$) *δ* ppm: 0.70 (s, 3H, Me-18) 0.82 (s, 3H, Me-19) 0.94 (d, J= 6.5 Hz, 3H, Me-21) 1-2.43 (steroid structure) 3.67 (s, 3H, C$\underline{H}_3$) 3.87 (s, 1H, C$\underline{H}$-7) 4.11 (s, 1H, C$\underline{H}$-12) 5.78 (dd, J=10.3, 1.7 Hz, 1H, C$\underline{H}$=CH)6.07 (dd J= 17.3, 10.3 Hz, 1H, CH=C$\underline{H}$) 6.37 (dd, J=17.3, 1.7 Hz, 1H) ppm

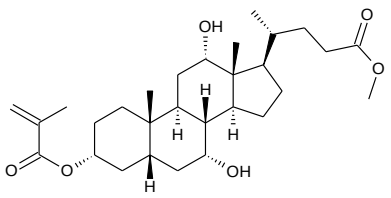
^{13}C NMR ($CDCl_3$) δ ppm: 217.85, 216.22, 215.83, 215.45, 214.86, 214.47, 173.94, 73.08, 71.85 (C12), 68.27 (C7), 56.87, (46.52, 45.30, 41.74, 41.49, 40.08, 39.58, 38.83, 35.37, 34.80, 33.50, 33.13, 31.74, 30.41, 29.10, 28.16, 27.62, 26.43, 26.10 steroid ring), 25.01, 23.32, 22.69, 22.51 (C19), 17.57 (C21), 14.17, 12.50 (C18), 11.48 ppm.

MS (+APCI) m/z= Found 494.16; expected 476.3138 (MI+CH_3 +3H)

IR (KBr) v = 3375, 2959, 2932, 2865, 1712, 1630 (C=O), 1618, 1533 cm^{-1}

2.3.2 METHACRYLATES

Synthesis of 3α methacrylate 7α,12α dihydroxyl cholic acid methyl ester (66)



Methyl cholate (4.22 g, 10 mmol) was dissolved in dry chloroform (30 mL). Triethylamine (2.23 mL, 16 mmol) was added and the flask was put on ice. Methacryloyl chloride (1.45 mL, 15 mmol) in chloroform (10 mL) was dripped in over 30 minutes. The mixture was protected from the light and left at room temperature overnight. The solvent was removed using a rotary evaporator (the temperature was set to 30 ^{o}C) to produce a white solid. Ethyl acetate (50 mL) was added and the solid (salt) was collected by filtration. The ethyl acetate was removed on the rotary evaporator to leave a white solid. Flask column chromatography using 75% DCM/ 25% ethyl acetate was used to purify the product. The solvents were removed by rotary evaporation before the product was dried at room temperature under vacuum.

^{1}H NMR ($CDCl_3$) *δ* ppm: 0.70 (s, 3H, Me-18) 0.83 (s, 3H, Me-19) 0.98 (d, J= 6.1 Hz, 3H, Me-21) 1-2.43 (steroid structure) 3.77 (s, 3H, C$\underline{H}_3$) 3.82 (d, J= 26.9 Hz, 1H, C$\underline{H}$-7) 4.10 (s, 1H, C$\underline{H}$-12) 4.63 (tt, J= 11.2, 4.4 Hz, 1H C$\underline{H}$-3) 5.5 (m, 1H, CH=C$\underline{H}_2$) 5.83 (m, 1H, CH=C$\underline{H}_2$) 6.07 (s, 1H) 6.25 (s, 1H) ppm

^{13}C NMR ($CDCl_3$) δppm: 218.61, 174.68 (C=O), 167.10 (C=O), 136.92 (C), 124.92 (C=C), 74.54, 72.92 (C12), 68.26 (C7), 51.52, 47.26, (46.57, 42.15, 41.23, 39.57, 35.22, 35.13, 34.90, 34.71, 34.42, 31.05, 30.89, 28.44, 27.42, 26.83, 26.69 steroid ring), 23.14, 22.57 (C19), 18.35 (CH_3), 17.37 (C21), 12.57 (C18) ppm.

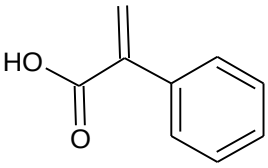
MS (+APCI) m/z= Found 535.3181; expected 476 (MI+CH_3CN+H_2O)

IR (KBr) v = 3599, 3544, 2971, 2935, 2865, 1733, 1703, 1639 (C=O), 1469 cm^{-1}

2.3.3 PHENYL METHACRYLATES

Synthesis of Atropic acid (67)

The method is based on work by Chang *et al.*(Chang et al., 2006)



Potassium hydroxide (19.0 g, 338 mmol) was dissolved in deionised water (40 mL). Tropic acid (15.2 g, 91.6 mmol) was added and the reaction was heated under reflux for 1 hour. The reaction was left to cool to room temperature, then cooled further on ice. Conc. HCl (60 mL) was added to give a white solid. The solid was collected by filtration and washed with water before drying at room temperature under vacuum.

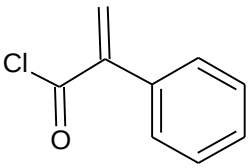
Yield= 7.01 g (36.9 %)

TLC: (MeOH) R_f=0.82 (one spot)

^{1}H NMR ($CDCl_3$) δ ppm: 6.03 (d, J= 1.2 Hz, 1H C<u>H</u>=C) 6.54 (d, J= 1.2 Hz, 1H C=O) 7.26-7.52 (m, 6H aromatic ring) ppm

MS (+APCI) m/z= Found 147.0482; calculated for $C9H_8O_2$ 148.0524; 10.9 ppm

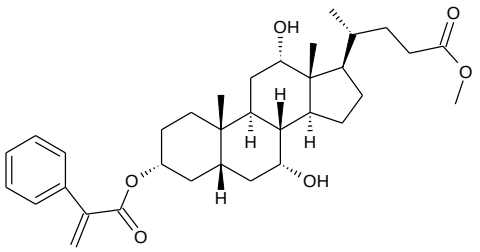
Synthesis of Phenylmethacryloyl chloride (68)



Atropic acid (6) (0.5 g, 3 mmol) was dissolved in toluene (5 mL). Thionyl chloride (10 mL, 6.2 mmol) was added. The reaction was heated to 60 ^{o}C for 4 hours. The reaction was left to cool to room temperature before the solvent was removed to leave a yellow gum.

Yield = 0.6 g

Attempted synthesis of 3α phenylmethacrylate 7α,12α dihydroxy cholic acid methyl ester (69)



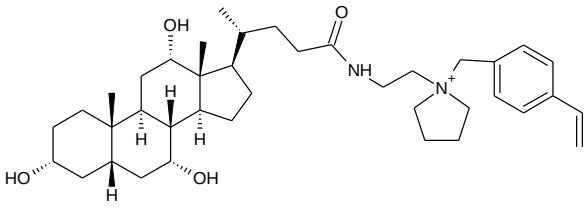
Methyl cholate (0.5 g, 1.15 mmol) was dissolved in dry chloroform (5 mL) along with triethylamine (0.2 mL, 1.72 mmol). The mixture was put on ice. Phenylmethacryloyl chloride (0.6 g, 3.6 mmol) in dry chloroform (5 mL) was dripped in over 7 minutes. The reaction was kept at 4 ^{o}C under argon for 48 hours before the temperature was raised to room temperature for 2 weeks. The reaction was stopped by removing the solvent on the rotary evaporator. Ethyl acetate (50 mL) was added to remove the salt and the solvent was removed to leave a yellow gum. Column chromatography with DCM, then 80/20 methanol/ethyl acetate attempted but good separation was not possible. The solvents were removed by rotary evaporation before the product was dried at room temperature under vacuum.

Proton NMR analysis showed a mixture of starting material and product.

2.3.4 Polymerisable groups on amine chain

2.3.4.1 Preparation of quaternary ammonium cholic acid derivatives containing a vinyl benzyl group

Synthesis of N-[2-[1-[(4-vinylphenyl)methyl]pyrrolidin-1-ium-1-yl]ethyl]cholanamide chloride (70)



N-(2-pyrrolidin-1-ylethyl) cholanamide (10) (0.35 g, 0.7 mmol) was dissolved in chloroform (10 mL). Vinyl benzyl (0.5 mL, 3.5 mmol) chloride was added. The reaction was protected from the light and left to stir overnight. The reaction was heated under reflux for 24 hours. The solvent

was removed under reduced pressure before the product was dried at room temperature under vacuum.

TLC: (MeOH), R_f=0.17, 0.87 (dominant spot) (two spots)

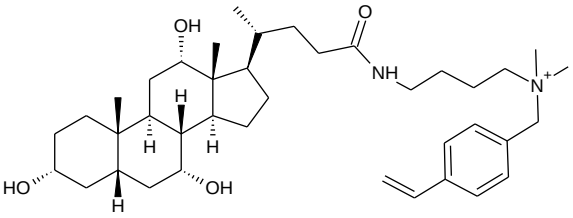
^{1}H NMR ($CDCl_3$) δ ppm: 0.62 (s, 3H, Me-18) 0.85 (s, 3H, Me-19) 0.93 (d, J= 5.9 Hz, 3H, Me-21) 1-2.43 (steroid ring) 3.11 – 3.24 (m, 8H, 2C$\underline{H}_2$), 3.60 (m, 1H, C$\underline{H}$-3), 3.77 (s, 1H, C$\underline{H}$-7), 3.95 (m, 1H, C$\underline{H}$-12), 4.35 (d, J = 4.0 Hz, 2H, C$\underline{H}_2$), 4.54 (s, 2H, C$\underline{H}_2$), 5.38 (d, J = 10.9 Hz, 1H CH=C$\underline{H}_2$), 5.96 (d, J = 17.6 Hz, 1H, CH=C$\underline{H}_2$), 6.80 (dd, J = 17.7, 11.0 Hz, 1H), 8.23 (d, J = 5.2 Hz, 1H, N$\underline{H}$) ppm.

^{13}C NMR (DMSO) δ ppm: 173.40 (C=O), (145.12, 138.80, 132.94, 127.83, 126.57, 104.54 aromatic ring), 135.74 (C=C), 116.15 (C=C), 79.52, 78.99, 78.46, 70.94 (C12), 70.39 (C3), 67.05, 66.21 (C7), 61.14, 60.78, 57.11 (CH_2), 48.55, (45.95, 45.72, 41.48, 41.38, 40.50, 40.42, 40.17, 35.28, 35.13, 34.86, 34.34, 33.10, 32.23, 31.42, 28.51, 27.30, 26.19 steroid ring), 22.58 (C19), 20.86, 17.05 (C21), 14.56, 12.27 (C18) ppm.

MS (+APCI) m/z= 621.4614

IR (KBr) ν = 3350, 3059, 2932, 2862, 1703, 1651 (C=O), 1533 cm^{-1}

Synthesis of 3-acetamidopropyl-dimethyl-[(4-vinylphenyl)methyl]ammonium chloride (71)



N-[3-dimethylamino) propyl] cholanamide (12) (0.35 g, 0.7 mmol) was dissolved in chloroform (10 mL). Vinyl benzyl chloride (0.5 mL, 3.5 mmol) was added. The reaction was protected from the light and left to stir overnight. The reaction was heated under reflux for 24 hours. The product precipitated out, collected by filtration and washed with petrol 40-60 (30 mL). Product was found to be not pure by TLC.

TLC: (MeOH) R_f=0.0.06, 0.84 (dominant spot) (two spots)

^{1}H NMR ($CDCl_3$) δ ppm: 0.65 (s, 3H, Me-18) 0.87 (s, 3H, Me-19) 0.95 (d, J= 6.0 Hz, 3H, Me-21) 1-2.43 (steroid structure) 3.14 (d, 3H, C$\underline{H}_3$) 3.21 (s, 2H, C$\underline{H}_2$) 3.49 (s, 1H, C$\underline{H}$-12), 3.69 (d, J = 19.8 Hz, 1H, C$\underline{H}$-7), 3.90 (s, 1H, C$\underline{H}$-3), 5.38 (dd, J = 11.0, 5.9 Hz, 1H, CH=C$\underline{H}_2$), 5.83 (dd, J = 17.7, 4.8

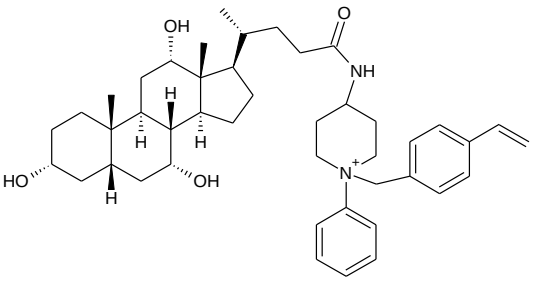
Hz, 1H, CH=C<u>H</u>$_2$), 6.71 (dd, J = 17.6, 10.9 Hz, 1H), 7.30 –7.63 (m, 4H aromatic ring), 8.49 (s, 1H, N<u>H</u>) ppm

^{13}C NMR (DMSO) δ ppm: 172.97 (C=O), (138.80, 135.76, 133.21, 127.38, 126.40 aromatic ring), 116.09 (C=C), 79.52, 78.99, 78.46, 70.97 (C12), 70.39 (C3), 66.20 (C7), 65.85, 61.35, 59.67 (CH_3), 49.22, 48.55, (45.92, 41.48, 41.36, 40.51, 40.18, 35.51, 35.27, 35.18, 34.87, 34.35, 32.31, 28.51, 27.28, 26.20 steroid ring), 45.70 (CH_3), 31.50 (CH_2), 22.79, 22.68, 22.58 (C19), 17.11 (C21), 14.61, 12.29 (C18) ppm.

MS (+APCI) m/z= 609.4619

IR (KBr) v = 3347, 2929, 2862, 1775, 1694, 1627 (C=O), 1551 cm^{-1}

Synthesis of N-[1-phenyl-1-[(4-vinylphenyl)methyl]piperidin-1-ium-4-yl]cholanamide chloride (72)



N-(1-phenyl-4-piperidyl) cholanamide (13) (1.5 g, 3.5 mmol) was dissolved in DCM (20 mL). Vinyl benzyl chloride (1.5 mL, 10.5 mmol) was added. The reaction was protected from the light and left to stir overnight. The reaction was heated under reflux for 24 hours. The product precipitated out, which was then collected by filtration and washed with petrol 40-60 (30 mL). Further purification by solvent extraction (x 3) between petrol 40-60 (10 mL) and methanol (10 mL) left a white solid.

TLC: (MeOH), R_f=0.16 (one spot)

^{1}H NMR ($CDCl_3$) *δ* ppm: 0.57 (s, 32H, Me-18) 0.88 (s, 3H, Me-19) 0.98 (d, J= 6.1 Hz, 3H, Me-21) 1-2.43 (steroid structutre) 3.18 (tt, J = 14.7, 7.3 Hz, 7H, C<u>H</u>$_2$ ring), 3.61 (d, J = 3.7 Hz, 1H, C<u>H</u>-3), 3.77 (d, J = 3.6 Hz, 1H, C<u>H</u>-7), 4.04 (d, J = 3.2 Hz, 1H, C<u>H</u>-12), 4.14 (d, J = 3.3 Hz, 1H, C3-O<u>H</u>), 4.36 (d, J = 4.0 Hz, 1H, C7-O<u>H</u>), 4.53 (s, 1H, C12-O<u>H</u>), 5.38 (d, J = 10.9 Hz, 1H, C<u>H</u>=CH), 5.95 (d, J = 17.7 Hz, 1H, CH=C<u>H</u>), 6.79 (dd, J = 17.6, 11.0 Hz, 1H), 7.73 – 7.43 (m, 4H aromatic ring), 8.07 (t, J = 5.7 Hz, 1H, N<u>H</u>) ppm

^{13}C NMR (DMSO) δ ppm: 172.94 (C=O), (138.80, 135.78, 133.22, 127.43, 126.42, aromatic ring), 116.15 (C=C), 70.95 (C12), 70.37 (C3), 66.19 (C7), 65.83, 61.33, 49.24, (45.94, 45.70, 41.48, 41.36, 40.51, 40.17, 35.50, 35.28, 35.19, 34.87, 34.35, 32.32, 31.51, 30.35, 28.54, 27.29, 26.20 steroid ring), 22.79, 22.67, 22.60 (C19), 17.12 (C21), 12.31 (C18) ppm

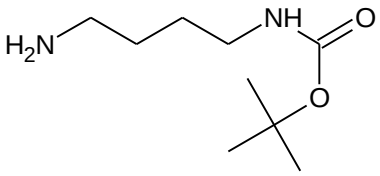
MS (+APCI) m/z= Found 609.4612; expected 683.4782 (MI-73) MI – (vbc + propane)

IR (KBr) v = 3356, 2925, 2862, 2164, 1648 (C=O), 1548 cm^{-1}

7.3.4.3 Boc protection 1,4-diaminobutane

Due to the difficulties of selectively attaching a polymerisable group onto the amine side chain of a modified cholic acid compound, an alternative route was taken. By synthesising a amime chain which had a polymerisable group on one end, the free amine could be coupled to the cholic acid, creating the desired compound. The first step of this synthesis would be to protect one end the diamine, in order to attach a polymerisable group onto the other end. Removal of the protecting group would then allow the free amine to be coupled to cholic acid. The end compound would be cholic acid with an amide side chain terminating in an methacrylate group. The methods are based on the work carried out by Chudzit *et al.* (Chudzik, n.d.)

Synthesis of N-Boc-1,4-butanediamine (73)



1,4-diaminobutane (1 mL, 12.94 mmol) was dissolved in DCM (20 mL) and put on ice. Di-tert-butyl dicarbonate (0.3 g, 1.29 mmol) in DCM (5 mL) was dripped in over 50minutes. The reaction was left at ice temperature for 12 hours. The reaction was washed with water (100 mL) followed by brine (100 mL), before being dried with magnesium sulphate. The solvent was removed under vacuum to leave a yellow solid.

Yield= 0.087g (8.7%)

TLC: (MeOH) R_f=0.07, 0.9 (dominant spot) (two spots)

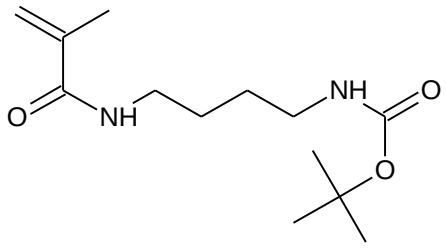
^{1}H NMR ($CDCl_3$) δ ppm: 1.40-1.61 (m, 12H, 3C$\underline{H}_3$) 3.13 (q, J=6.1 H, 2H, C$\underline{H}_2$) 4.55 (s, 1H) ppm

^{13}C NMR (DMSO) δ ppm: 155.57 (C=O), 77.26 (C), 28.36 (CH_3), 28.21, 27.29, 26.93, 26.86 ppm.

MS (+APCI) m/z= Found 289.2126; expected 188. Diamer produced.

IR (KBr) v = 3372 2983 2941 2847 1681 (C=O) 1521 cm^{-1}

Synthesis of tert-butyl N-[4-(2-methylprop-2-enoylamino)butyl]carbamate (74)



Methacrylic anhydride (0.44 mL, 3 mmol) was dissolved in chloroform (8 mL) and put on ice. N-Boc-1,4-butanediamine (78) (0.5 g) in chloroform (1.5 mL) was dripped in over 5 minutes. The reaction was left at ice temperature for 12 hours. Water (20 mL) was added to quench the reaction and the layers were separated. More water (20 mL) was added, the layers separated and this was repeated twice more with sodium hydroxide (20 mL, 1M). The chloroform layer was dried with magnesium sulphate and the majority of it was removed under vacuum. A small amount of petrol 60-80 was used to precipitate the product, which was then collected by filtration and dried to give a white solid.

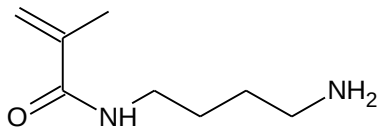
Yield= 0.27g (61 %)

^{1}H NMR ($CDCl_3$) δ ppm: 1.44 (s, 10H, 3C$\underline{H}_3$) 1.48-1.71 (m, 6H) 1.96 (dd, J=1.6, 0.9 Hz, 3H, CH_3) 3.14 (q, J=6.4 Hz) 3.34 (tdd, J=6.6, 5.3, 2.4 Hz, 2H, C$\underline{H}_2$) 4.62 (s, 1H, C$\underline{H}_2$) 5.32 (q, J=1.5Hz, 1H, C$\underline{H}_2$) 5.68 (t, J=1.0 Hz, 1H, C$\underline{H}$=CH) 6.00 (s 1H) ppm

^{13}C NMR (DMSO) δ ppm: 167.30 (C=O), 155.53 (C=O), 140.10 (C=C), 118.58 (C=C), 77.29 (C), 40.51 (CH_2), 40.17(CH_2), 39.84(CH_2), 39.51, 39.17, 39.03, 38.84, 38.51, 28.24(CH_2), 27.00(CH_2), 26.48, 18.65 ppm.

MS (+APCI) m/z= Found 257.1863; calculated for $C_{13}H_{24}N_2O_3$ 256.17; 1.3 ppm

Synthesis of N-(4-aminobutyl)-2-methyl-prop-2-enamide (75)



Tert-butyl N-[4-(2-methylprop-2-enoylamino)butyl]carbamate (79) (0.2 g) was dissolved in DCM (3 mL) and TFA (3 mL) for 5 hours. The solvent was removed under vacuum to leave a clear oil. The product was stored at -20°C.

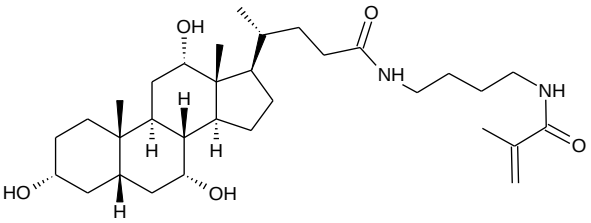
^{1}H NMR ($CDCl_3$) *δ* ppm: 1.96 (s, 3H, C<u>H</u>$_3$) 3.23 (q, J = 6.8 Hz, 2H C<u>H</u>$_2$), 3.86 (s, 2H, C<u>H</u>$_2$), 5.55 (s 1H C<u>H</u>=CH) 5.82 (s, 1H, CH=C<u>H</u>) 7.32 (s, 1H, NH) 7.55 (s, 2H, N<u>H</u>) 13.88 (s, 1H, TFA) ppm

^{13}C NMR ($CDCl_3$) δ ppm: 172.71 (C=O), 164.56, 163.96, 163.36, 162.76, 145.24, 127.37, 123.94, 122.77, 118.17, 113.57, 45.57, 45.24, 44.90, 44.57, 44.24, 43.90, 43.57, 43.27, 32.15, 31.29, 29.72 (CH_2), 23.77 ppm

MS (+APCI) m/z= Found 157.1332; calculated for $C_8H_{17}N_2O_1$ 157.1335; 1.5 ppm

IR (KBr) v = 3409 2930 2863 2111 1641(C=O) cm^{-1}

Synthesis of N-(4-cholanamidobutyl)-2-methyl-prop-2-enamide (76)



Cholic acid (1 g, 2.4 mmol) was dissolved in THF (30 mL) along with triethylamine (0.75 mL). It was put on ice for 10 minutes. Ethylchloroformate (0.13 mL) was dripped in over 10 minutes. It was taken off the ice and left at room temperature for two hours. N-(4-aminobutyl)-2-methyl-prop-2-enamide (80) (0.4 g) was added and left to react for 12 hours. The reaction was quenched with water (30 mL) and separated 3 times. The organic layer was dried over magnesium sulphate, collected by filtration and dried at room temperature under vacuum. NMR showed the reaction had not gone to completion. Further purification attempted but was difficult.

^{1}H NMR ($CDCl_3$) *δ* ppm: 0.68 (d 3H J= 3.1 HZ, Me-18) 0.89 (d 3H J= 2.0 Hz Me-19) 0.982 (d 3H J= 4.3 Hz Me-21) 1-2.43 (steroid structutre) 4.12 (q, 10H, J = 7.1 Hz, CH-3, CH-7), 4.61 (s, 1H, CH-12) 7.27 (s, 1H, NH) ppm

^{13}C NMR (DMSO) δ ppm: 198.77 (C=O), 172.37 (C=O), 116.7 (C=C), 70.96 (C12), 70.40 (C3), 66.21 (C7), 59.71, (46.06, 45.72, 41.49, 41.33, 40.51, 40.17, 39.84, 39.50, 39.17, 39.01, 38.84, 38.50, 35.29, 35.02, 34.85, 34.36, 30.38, 26.18 steroid ring), 22.77, 22.59 (C19), 17.09, 16.91 (C21), 14.06, 12.30 (18) ppm

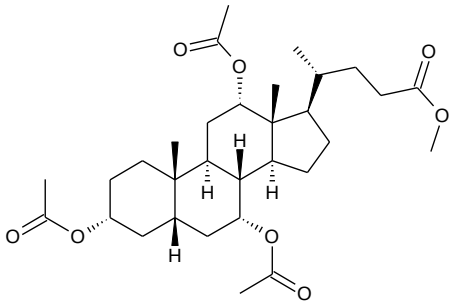
MS (+APCI) m/z= Found 547.4101; calculated for $C_{32}H_{54}N_2O_5$ 546.402; -0.8 ppm

IR (KBr) ν = N/A

2.5 PROTECTING HYDROXYL GROUPS

This work was based on the procedures carried out by Kuhajda *et al.*(Kuhajda et al., 1996)

3α,7α,12α triaetate methyl cholate (77)



Methyl cholate (1 g, 2.4 mmol) was dissolved in dry chloroform (15 mL) along with DMAP (8.6 mg, 0.708 mmol). Triethylamine (1.6 mL, 11 mmol) was added followed by acetic anhydride (1.1 mL, 11.6 mmol). The solution was heated under argon at 80^{o}C for 48 hours. Reaction was left to cool before solvent extractions with sodium bicarbonate (x2) (20 mL), 0.5M HCl (x2) (20 mL) and water (x2) (20 mL). The organic layer was dried using magnesium sulphate removed under reduced pressure and dried to leave a white solid.

Yield= 0.22g (22 %)

TLC: (MeOH) R_f=0.84 (one spot)

^{1}H NMR ($CDCl_3$) *δ* ppm: 0.69 (s, 3H, Me-18) 0.89 (s, 3H, Me-19) 0.97 (d, J= 6.1 Hz, 3H, Me-21) 1-2.43 (steroid ring), 3.66 (d, J = 0.9 Hz, 3H, O-C$\underline{H}_3$), 4.44 (m, 1H, C$\underline{H}$-3), 4.78 (s, 1H, C$\underline{H}$-7), 4.96 (s, 1H, C$\underline{H}$-12) ppm

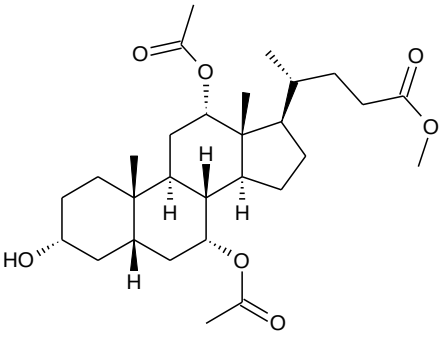
^{13}C NMR ($CDCl_3$) δ ppm: 216.00, 215.29, 214.91, 214.56, 214.33, 214.11, 213.84, 213.50, 213.03, 174.58, 170.58, 170.43, 75.41, 74.11, 70.71, 51.58, 47.36, (45.06, 43.42, 40.93, 37.73, 34.69, 34.35, 31.26, 30.90, 30.77, 28.91, 27.21, 26.90, 25.60 steroid ring), 22.82 (CH_3), 22.59 (C19), 21.68, 21.53, 17.51 (C21), 12.24 (C18) ppm

MS (+APCI) m/z= found 571.3230; expected 548.334 (MI+23)

IR (KBr) ν = 3514 2947 2871 1721 (C=O) cm^{-1}

2.5.1 REMOVAL OF PROTECTING GROUPS

3α hydroxyl 7α,12α diacetate methyl cholate (78)



Triprotected methyl cholate (3.3 g, 5.4 mmol) was dissolved in methanol (30 mL) along with potassium carbonate (1.1 g, 8.21 mmol) was added. The reaction mixture was stirred at room temperature for 3.5 hours. The reaction was stopped by adding acetic acid until the solution turned acidic. Water (30 mL) was added and the flask was put on ice for 1.5 hours to precipitate the product. The product was collected by filtration and dried at room temperature under vacuum.

Yield= 2.13 g (64 %)

TLC: (MeOH), R_f=0.38 (one spot)

^{1}H NMR ($CDCl_3$) δ ppm: 0.72 (s, 3H, Me-18) 0.81 (s, 3H, Me-19) 0.98 (d, J= 6.0 Hz, 3H, Me-21) 1-2.43 (steroid structure) 3.50 (tt, J = 10.6, 4.3 Hz, 1H C<u>H</u>-3), 3.65 (s, 3H, O=C<u>H</u>$_3$), 4.89 (q, J = 3.1 Hz, 1H, C<u>H</u>-7), 5.12 – 5.02 (m, 1H, C<u>H</u>-12) ppm

^{13}C NMR ($CDCl_3$) δ ppm: 214.85, 174.55, 170.64, 119.59, 79.09, 75.42, 71.64, 70.85 (C12), 51.49, 47.32, (45.04, 43.38, 41.02, 38.61, 37.75, 34.84, 34.57, 34.27, 31.33, 30.86, 30.75, 30.42, 28.91, 27.15 steroid ring), 25.53, 25.03, 22.78, 22.55 (C19), 21.62, 21.43, 17.47 (C21), 12.19 (C18) ppm

MS (+APCI) m/z= Found 509.3223; expected 506.3244 (MI+2)

IR (KBr) v = 3447 2925 2868 1724 (C=O) 1430 cm^{-1}

2.6 POLYMERISATION OF COMPOUNDS

79. Polymerisation of 3α,12α diacroylate-7αhydroxycholic acid

3α,12α diacroylate-7αhydroxycholic acid (63) (0.175 g) was dissolved in toluene (2.5 mL). AIBN (0.1 g) was added, along with EGDMA (0.82 mL). 100µl of the solution was pipetted out into a 96 well plate and a PTFE-lined cover was clamped across the top of the plate to seal the wells. It was place in at oven set at 60^{o}C for 12 hours. The products were white discs.

80. Polymerisation of methacrylate 7α,12α dihydroxyl cholic acid

Methacrylate 7α,12α dihydroxyl cholic acid (65) (0.2 g) was dissolved in toluene (3.5 mL). AIBN (0.1 g) was added, along with EGDMA (0.8 mL). 100µl of the solution was pipetted out into a 96 well plate and clamped. It was place in at oven set at 60^{o}C for 12 hours. The products were white discs.

81. Polymerisation of 3α hydroxyl 7α,12α diacetate methyl cholate

3α hydroxyl 7α,12α diacetate methyl cholate (77) (0.5 g) was added to a round bottomed flask along with bis[acetylacetonato]copper (0.02 g) and vinyl benzyl chloride (2 mL). The reaction was heated to 120^{o}C for 4.5 hours. The crude product was purified using column chromatography with petrol 60-80, slowly increasing the amount of ethyl acetate (0-100%). The solvents were removed under reduced pressure and the polymer was dried at room temperature under vacuum.

82. Polymerisation of 3-acetamidopropyl-dimethyl-[(4-vinylphenyl)methyl]ammonium chloride (70)

3-acetamidopropyl-dimethyl-[(4-vinylphenyl)methyl]ammonium chloride (70) (0.5 g) was dissolved in ethanol (4 mL). Styrene (3.9 mL) was added along with AIBN (100 mg). The reaction was degassed and heated to 65^{o}C for 12 hours where a white solid precipitated out. It was purified by dissolving in chloroform (20 mL) and dripping into stirring methanol (100 mL). The product was collected by filtration and dried at room temperature under vacuum to give a white solid.

Proton NMR analysis shows very little incorporation of N-[3- (dimethylamino)propyl] cholanamide (12) into the polymer.

83. 3-acetamidopropyl-dimethyl-[(4-vinylphenyl)methyl]ammonium chloride (70)(0.25 g) was dissolved in ethanol (4 mL) along with AIBN (100 mg). Tert-butyl methacrylate (0.23 mL) was added. The mixture was degassed which argon and heated to 60^{o}C for 4 days. The solution was purified by dripping onto ethyl acetate (75 mL) to give a white powdery solid which was dried at room temperature under vacuum.

Proton NMR analysis showed that no polymerisation had taken place.

84. 3-acetamidopropyl-dimethyl-[(4-vinylphenyl)methyl]ammonium chloride (70) (0.25 g) was suspended in propan-2-ol (6 mL) along with AIBN (100 mg). The mixture was degassed and heated to 70^{o}C for 5 days. The white, cloudy solution was collected by filtration to give a off white solid which was dried at room temperature under vacuum.

No NMR analysis could be undertaken of this polymer due to its insolubility.

85. Polymerisation of N-[2-[1-[(4-vinylphenyl)methyl]pyrrolidin-1-ium-1-yl]ethyl]cholanamide chloride (69)

N-[2-[1-[(4-vinylphenyl)methyl]pyrrolidin-1-ium-1-yl]ethyl]cholanamide chloride (69) (0.5 g) was dissolved in ethanol (4 mL) along with AIBN (100 mg). Tert-butyl methacrylate (0.46 mL) was added. The mixture was degassed with argon and heated to 60^{o}C for 4 days. The solution was purified by dripping onto ethyl acetate(75 mL) to give an off white solid which was dried at room temperature under vacuum.

Proton NMR analysis showed that no polymerisation had taken place.

86. N-[2-[1-[(4-vinylphenyl)methyl]pyrrolidin-1-ium-1-yl]ethyl]cholanamide chloride (69) (0.15 g) was dissolved in ethanol (5 mL) along with AIBN (100 mg). Methacrylate was added (0.8 mL). The mixture was degassed with argon and heated to 60^{o}C for 4 days. An orange solid precipitated out, which was dissolved in chloroform (10 mL) and the solvent was removed under reduced pressure. The polymer was dried at room temperature under vacuum.

Proton NMR analysis shows the product is poly(methyl acrylate). The was no incorporation of N-(2-pyrrolidin-1-ylethyl)cholanamide.

87. N-[2-[1-[(4-vinylphenyl)methyl]pyrrolidin-1-ium-1-yl]ethyl]cholanamide chloride (69)(0.1 g) was dissolved in ethanol (2 mL) along with AIBN (100 mg). The mixture was degassed with argon and heated to 60^{o}C for 2 days. A brown solid precipitated out, which was dissolved in

DCM (10 mL) and the solvent was removed under reduced pressure. The productr was dried at room temperature under vacuum.

Proton NMR analysis showed that no polymerisation had taken place.

88. N-[2-[1-[(4-vinylphenyl)methyl]pyrrolidin-1-ium-1-yl]ethyl]cholanamide chloride (69) was dissolved in 7mL ethanol along with AIBN (100 mg). 2.4mL (23.04mmol) styrene was added. The mixture was degassed and heated to 60^{o}C for 3 days. A white solid precipitated out, which was collected by filtration and purified by dissolving in chloroform and dripping into methanol.

NMR shows no incorporation of product.

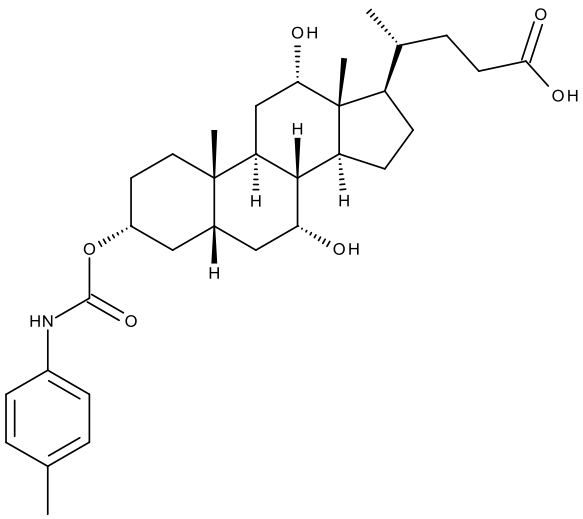
89. Polymerisation of N-[1-phenyl-1-[(4-vinylphenyl)methyl]piperidin-1-ium-4-yl]cholanamide chloride (71)

Synthesis of N-[1-phenyl-1-[(4-vinylphenyl)methyl]piperidin-1-ium-4-yl]cholanamide chloride (70) (0.3 g) was dissolved in proan-2-ol (8 mL) along with AIBN (100 mg). The mixture was degassed with argon and heated to 70^{o}C for 6 days. A white solid precipitated out, which was collected by filtration and dried at room temperature under vacuum.

Proton NMR analysis shows a very short chained polymer was synthesised.

2.7 UREATHANES AND POLYUREATHANES

3α methyl N-(p-tolyl)carbamate 7α 12α hydroxyl cholic acid (90)



Method 1. Cholic acid (1.55 g, 3.8 mmol) was dissolved in THF (10 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with p-tolyl isocyanate (0.53 mL, 4.2 mmol). The reaction was left for 2 days where the product had precipitated out. The solid was collected by filtration and dried at room temperature under vacuum.

^{1}H NMR ($CDCl_3$) δ ppm: 0.70 (s, 3H, Me-18) 0.89 (s, 3H, Me-19) 1.00 (d, J= 6.3 Hz, 3H Me-21) 1.00-2.5 (m, steriod structure) 2.30 (d, 8H, CH_3) 3.86 (s, 1H, C<u>H</u>-7) 4.00 (d, J=3.2Hz, 1H C<u>H</u>-12) 4.56 (t, J=10.7Hz, 1H, C<u>H</u>-3) 6.62 (d, J=7.0Hz, 2H, N<u>H</u>$_2$) 7.03-7.27 (m, 7H, aromatic +C<u>H</u>Cl_3) ppm

MS (+APCI) m/z= 559.3735 (+18)

TLC: (EtOAc, R_f=0.08 (one spot)

Method 2. Cholic acid (1.55 g, 3.8 mmol) was dissolved in THF (10 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with p-tolyl isocyanate (2.6 mL, 21 mmol). The reaction was left for 2 days where the product had precipitated out. The solid was collected by filtration, washed with methanol (10 mL) and dried at room temperature under vacuum. Product was not pure as judged by proton NMR anaylsis.

TLC: (EtOAc) R_f=0.51 (one spot)

^{1}H NMR (DMSO) δ ppm: 0.60 (s, 3H, Me-18) 0.67-0.98 (m, 7H, Me-19 +Me-21 +C<u>H</u>) 1.00-2.5 (m, steriod structure)2.23 (s, 9H, C<u>H</u>$_3$) 3.64 (s) 3.81 (s 1H C<u>H</u>-7OH) 4.13 (dd J=11.4, 3.1Hz, 1H

CH-12) 4.42 (dt J=10.9Hz 1H CH-3) 6.96-7.13 (m, 3H, aromatic) 7.23-7.49 (m, 3H, aromatic) 9.40 (s, 1H) 9.51 (s, 3H) 11.93 (s, 1H) ppm

IR = 3293, 3174, 3029, 2910, 2853, 2722, 1891, 1703, 1636 (C=O), 1587, 1560, 1511 cm^{-1}

Method 3. Cholic acid (1.55 g, 3.8 mmol) was dissolved in THF (10 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with p-tolyl isocyanate (2.6 mL, 19.4 mmol) (reaction carried put twice). One flask was put on ice for 12 hours where the mixture had turned brown and a preciptate had formed. The reaction was quenched with methanol (10 mL), where it turned yellow. The product was collected by filtration and dried. The other flask was heated to 40^{o}C for 12 hours, where it turned purple. The reaction was quenched with methanol (10 mL), where it turned yellow. The product was collected by filtration and dried at room temperature under vacuum.

Ice

TLC: (MeOH), R_f=0.7 (one spot)

^{1}H NMR (DMSO) δ ppm: 0.61 (s 3H Me-18) 0.85 (s 3H Me-19) 0.93 (d, J=6.3 Hz, 3H Me-21) 1.00-2.5 (m steriod structure) 2.24 (s, 20H) 3.18 (s, 2H) 3.65 (, 13H) 3.82 (s, 1H, CH-7) 4.13 (d J=11.6Hz, 1H, CH-12) 4.40 (m, 1H, CH-3) 6.98-7.15 (m, 11H, aromatic) 7.24-7.43 (m, 11,H aromatic) 9.39 (s, 1H) 9.50 (s, 4H) 11.93 (s, 1H) ppm

IR = 3293, 3029, 2983, 2907, 2856, 2725, 1894, 1788, 1700, 1633, 1590, 1508 cm^{-1}

Heat

TLC: (MeOH), R_f=0.7 (one spot)

^{1}H NMR (DMSO) δ ppm: 0.70 (s, 3H, Me-18) 0.77 (d, J= 6 Hz, 3H Me-21) 0.88 (s, 3H, Me-19) 1.00-2.5 (m steriod structure) 2.23 (d, J=5.9Hz, 21H) 3.65 (s, 10H) 4.29 (d, J= 7.9 Hz, 1H, CH-12) 4.39 (m, 1H, CH-3) 4.89 (s, 1H, CH-12) 6.69-7.15 (m, 11H, aromatic) 7.22-7.50 (m, 11H, aromatic) 9.29 (d, J=11.1Hz, 1H) 9.49 (d, J-9.2Hz, 4H) ppm

IR =3705, 3299, 3032, 2910, 2856, 2722, 1894, 1715, 1587, 1508, 1542 cm^{-1}

Method 4. Cholic acid (0.75 g, 1.8 mmol) was dissolved in THF (10 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with p-tolyl isocyanate (1.3 mL, 9.7 mmol) (reaction carried put twice). One flask was put on ice for 12 hours where the mixture had turned brown and a preciptate had formed. The reaction was quenched with methanol, where

it turned yellow. The product was collected by filtration and dried at room temperature under vacuum. The other flask was heated to 40 ^{o}C for 12 hours, where it turned purple. The reaction was quenched with methanol, where it turned yellow. The product was collected by filtration and dried at room temperature under vacuum.

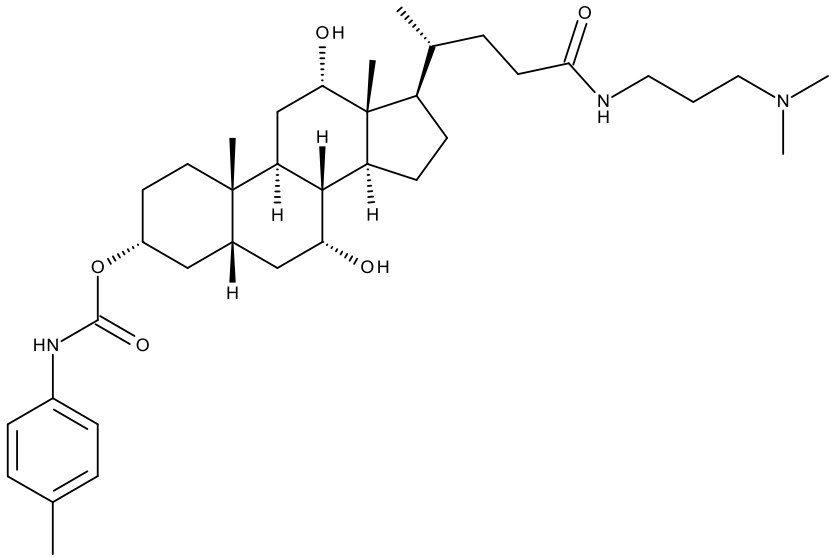
Ice

^{1}H NMR ($CDCl_3$) δ ppm: 0.64 (s, 3H, Me-18) 0.84 (s, 3H, Me-19) 0.96 (d, J= 6.0 Hz, 3H, Me-21) 1.00-2.5 (m steriod structure) 2.26 (d, J=2.3Hz, 10H) 3.72 (s, 6H) 3.80 (s, 1H, CH-7) 3.95 (s, 1H, CH-12) 4.52 (m, 1H, CH-3) 7.04 (dd, J=8.8, 3.0Hz, 8H, aromatic +$CDCl_3$) 7.14-7.42 (m, 5H, aromatic) ppm

Heat

^{1}H NMR ($CDCl_3$) δ ppm: 0.70 (s, 3H, Me-18) 0.91 (d, J= 6.1 Hz, 3H, Me-19) 1.0 (s, 3H, Me-21) 1.00-2.5 (m, steriod structure) 2.22 (s, 17H) 3.51 (s, 24H) 3.87 (s, 1H, CH-7) 4.00 (s, 1H, CH-12) 4.57 (m, 1H, CH-3) 6.96-7.14 (m, 11H, aromatic) 7.28-7.43 (m, 11H, aromatic) 9.49 (s, 4H) ppm

Attempted synthesis of 3α-methyl,N-(p-tolyl)carbomate-7α,12α-hydroxyl,N-[3-(dimethylamino)propyl] cholamide (91)



N-[3-(dimethylamino)propyl] cholamide **(**1.8 g, 3.8 mmol) was dissolved in DCM (10 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with p-tolyl isocyanate (0.53 mL, 4.2 mmol). The reaction was left for 2 days before the solvent was removed under reduced pressure. The solid dried at room temperature under vacuum.

Proton NMR analysis showed no reaction had taken place

Attempted synthesis of 3α-methyl,N-(p-tolyl)carbomate-7α,12α-hydroxyl,N-[3-(dimethylamino)propyl] cholamide (92)

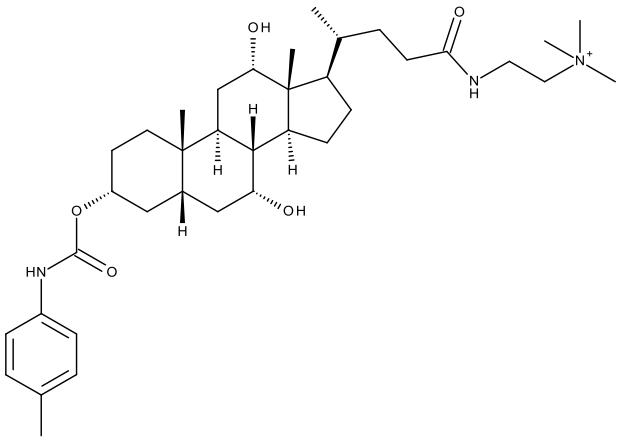
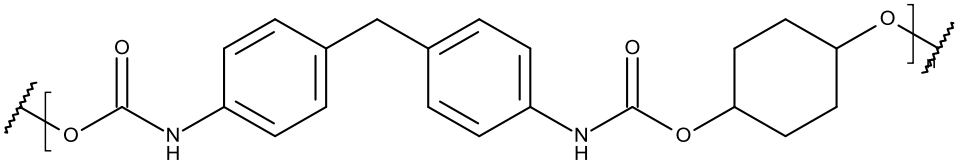


Figure 15

N-[3-(dimethylamino)propyl] cholamide **(**0.25 g) was dissolved in THF (5 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with p-tolyl isocyanate (0.09 mL). The reaction was left for 2 days where a precipitate formed. The solid was collected by filtration and dried at room temperature under vacuum.

Proton NMR analysis showed no reaction had taken place

Poly (N-(4-methoxycyclohexoxy)-4-[[4-[(4-methoxycyclohexoxy)carbamoyl]phenyl]methyl]benzamide) (93)



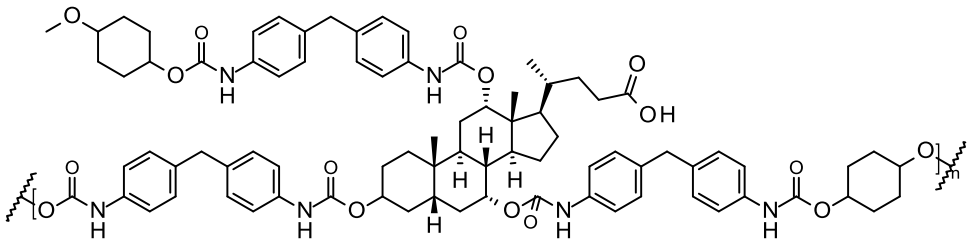
1,4-cyclohexanediol **(**0.9 g, 8 mmol) was dissolved in dry THF (10 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with 4,4-methylenebis (phenyl isocyanate) (1 g, 4 mmol). The reaction was left for 12 hours before being quenched with water (25 mL). A white precipitate formed which was collected by filtration and dried at room temperature under vacuum.

^{1}H NMR (DMSO) δ ppm: 1.17-1.49 (m, 1H, CH) 1.42, 2.09 (m, 7H) 3.37-3.68 (m, 2H, CH_2) 3.78 (s, 1H, C<u>H</u>) 4.45-4.76 (m, 2H, C<u>H</u>$_2$) 7.02-7.14 (m, 2H, aromatic) 7.28-7.41 (m, 2H, aromatic) 9.38-9.54 (m, 1H) ppm

^{13}C NMR (DMSO) δ *ppm*: 70.96, 70.38, 66.18 (CH_2 ring), 63.29, 61.13, 50.64, 50.46, 50.42, 46.02, 45.69, 41.63, 41.48, 32.81, 30.65(CH_2 ring), 25.41, 22.60, 21.85, 21.62, 17.05, 13.91, 12.33 ppm

IR =3317 3120, 3038, 2944, 2859, 1697 (C=O), 1593, 1411 cm^{-1}

Preparation of a polyurethane copolymer of cholic acid and 4,4-methylenebis(phenyl isocyanate (94)



Method 1. Cholic acid (1 g, 2.4 mmol) was dissolved in THF (15 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with 1,4-cyclohexanediol (0.28 g, 2.5 mmol). 4,4-methylenebis (phenyl isocyanate) (2.1 g, 12.25 mmol) was added (reaction carried put twice). One flask was put on ice for 4 hours where the mixture had turned yellow. The reaction was quenched with methanol (15 mL) to form a precipitate. The product was collected by filtration and dried at room temperature under vacuum. The other flask was heated to 45^{o}C for 12 hours, then the reaction was quenched with methanol (15 mL), to form a pink precipitate. The product was collected by filtration and dried at room temperature under vacuum before purification by dissolving in chloroform (10 mL) and dripping in methanol (100 mL) to give a white, powdery solid.

^{1}H NMR ($CDCl_3$) δ ppm: 0.73 (d, J=15.3Hz, 1H) 0.92 (s, 1H) 1.79-1.92 (m, 2H) 3.49 (s, 6H) 3.60-3.81 (m, 4H) 3.88 (s, 2H) 6.51 (s, 1H) 7.10 (d, J=8.3Hz, 1H) ppm

IR = 3305, 2947, 1712, 1636, 1590, 1511 cm^{-1}

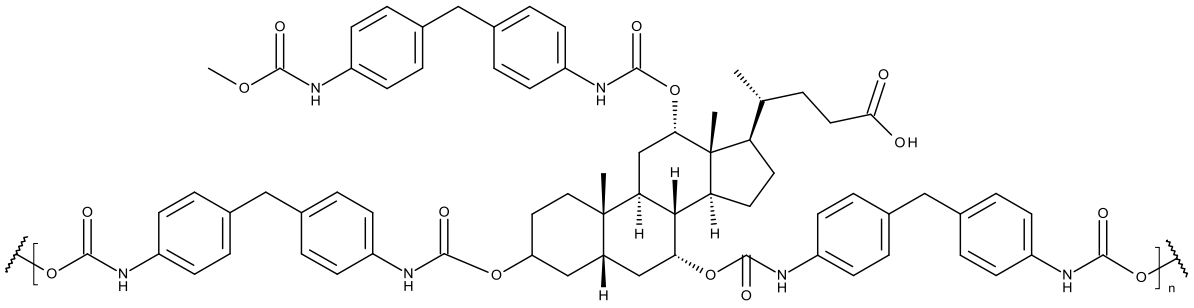
Method 2. Cholic acid (0.5 g, 1.2 mmol) was dissolved in THF (15 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with 1,4-cyclohexanediol (0.28 g, 2.5 mmol). 4,4-methylenebis (phenyl isocyanate) was added (2.1 g, 12.25 mmol). The flask was heated to 40^{o}C for 12 hours, to give a black solution, before more 1,4-cyclohexanediol (0.28 g, 2.5 mmol) was added. After 4 hours the reaction was quenched with methanol (15 mL), to form a light brown precipitate. The product was collected by filtration and dried at room temperature under vacuum before purification by dissolving in THF (10 mL) and dripping in methanol (100 mL) to give a light brown, sticky solid.

^{1}H NMR ($CDCl_3$) δ ppm: 0.59 (s, 3H, Me-18) 0.70 (s, 3H, Me-19) 0.85-0.93 (m, 9H, Me-21 + 2CH_3) 1.00-2.5 (m, steroid structure) 3.17 (d, J=1.9Hz, 2H) 3.77 (d, J=7.0 Hz, 6H) 4.11 (d, J=11.0Hz, 1H) 4.40 (m, 1H, C<u>H</u>-3) 4.72 (s, 3H, C<u>H</u>-7 + CH_2) 7.08 (d, J=8.3Hz, 12H, aromatic) 7.24-7.47 (m, 12H, aromatic +$CDCl_3$) 9.47 (d, J=14.2Hz, 4H) ppm

IR =3302, 2941, 2865, 1697, 1590, 1511 cm^{-1}

Method 3. Cholic acid (5 g, 12.25 mmol) was dissolved in THF (30 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along with 1,4-cyclohexanediol (0.28 g, 2.5 mmol). 4,4-methylenebis (phenyl isocyanate) (2.6 g, 12.25 mmol) was added. The flask was heated to 40^{o}C for 12 hours, before being quenched with methanol (15 mL), to form a yellow solution. The solvents were removed under reduced pressure to give a white solid which was dried at room temperature under vacuum.

Preparation of a polyurethane copolymer of cholic acid and 4,4-methylenebis(phenyl isocyanate) (95)



Method 1. Cholic acid (0.5 g, 1.2 mmol) was dissolved in THF (8 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along 4,4-methylenebis (phenyl isocyanate) (1 mL, 2.48 mmol) was added. The flask was left at room temperature for 4 hours, where it turned black.

The reaction was quenched with methanol (10 mL), to form a light purple solution. The precipitate was collected by filtration and dried at room temperature under vacuum to give a white solid.

^{1}H NMR ($CDCl_3$) δ ppm: 0.70 (s, 3H, Me-18) 0.91 (s, 3H, Me-19) 0.99 (m, 3H, Me-21) 1.00-2.5 (m, steriod structure) 1.65 (s) 348 (s, 1H) 3.88 (s, 2H, CH-7+CH) 3.99 (s, CH-12) 4.57 (m, 1H, CH-3) 6.60 (d, J=11.1Hz, 2H) 7.02-7.16 (m, 4H, aromatic) ppm

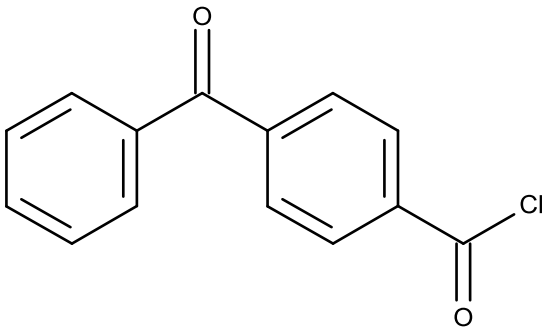
IR = 3308, 3186, 3126, 304,1 2941, 2862, 1700, 1651, 1603, 1521 cm^{-1}

Method 2. Cholic acid (1 g, 2.4 mmol) was dissolved in THF (15 mL). Molybdenum (VI) dichloride dioxide (1.3 mg) was added along 4,4-methylenebis (phenyl isocyanate) (0.3 g, 1.2 mmol) was added. The flask was left at room temperature for 4 hours. The reaction was quenched with methanol (15 mL) and the solvents were removed under reduced pressure to give a white soild which was dried at room temperature under vacuum.

^{1}H NMR ($CDCl_3$) δ ppm: 0.58 (s, 3H, Me-18) 0.84 (s, 3H, Me-19) 0.91 (m, 3H, Me-21) 1.00-2.5 (m, steriod structure) 3.79 (d, J=8.2Hz, 2H) 4.11 (m, 2H, CH-12) 4.00 (tq, J=13.0, 8.0, 6.2 Hz 1H, CH-3) 7.09 (dd, J=8.5,2.7 Hz, 2H, aromatic) 7.33 (d, J=8.2 Hz, 2H) 9.36-9.56 (m, 1H) ppm

2.8 PREPARATION OF THE COMPOUNDS USED IN THE PHOTOCHEMISTRY EXPERIMENTS

Synthesis of 4-benzylbenzoyl chloride (96)



4-benzyl benzoic acid (1 g, 4.42 mmol) was added to a dry flask along with thionyl chloride (3 mL), DMF (0.5 mL) and toluene (13 mL). The flask was heated under reflux for 5 days. The solvent was removed under reduced pressure and the product was re-dissolved in toluene (5 mL) twice and the solvent removed. The product was dried under vacuum at room temperature under vacuum to give a white solid.

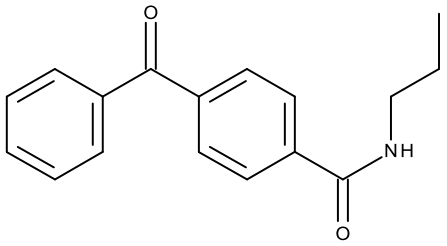
Yield = 1.03 g

TLC: (MeOH) R_f=0.11 (one spot)

^{1}H NMR ($CDCl_3$) δ ppm: 7.50 (tt, *J* = 6.6, 1.5 Hz, 5H, aromatic ring) 7.69 – 7.56 (m, 6H, aromatic ring)

MS (+APCI) m/z= Found 245.0366; calculated for $C_{14}H_{10}Cl_1O_2$ 245.0364; 0.9 ppm

Synthesis of 4-benzoyl-N-propyl-benzamide (97)



4-benzylbenzoyl chloride (0.5 g, 2 mmol) was dissolved in toluene (8 mL). Triethylamine (0.3 mL, 3 mmol) was added along with propyl amine (0.33 mL, 2 mmol) at room temperature and left to react for 2.5 hours. Water (12 mL) was added to preciptate the product, which was

collected by filtration and dried at room temperature under vacuum. The product was recrystallised in acetonitrile (3 mL) to give a white solid.

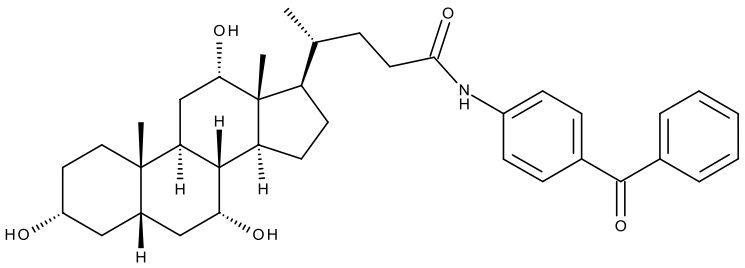
TLC: (MeOH) R_f=0.1 (one spot)

^{1}H NMR ($CDCl_3$) δ ppm: 1.01 (t, J= 7.4 Hz, 3H, C<u>H</u>$_3$) 1.59 – 1.79 (m, 2H, C<u>H</u>$_2$), 3.38 – 3.54 (m, 2H, C<u>H</u>$_2$), 7.42 – 7.70 (m, 3H, aromatic), 7.74 – 7.94 (m, 6H, aromatic)

MS (+APCI) m/z= Found 268.1332; calculated for $C_{17}H_{18}N_1O_2$ 268.1336; 1.5 ppm

IR= 3320, 3032, 2962, 2892, 2874, 2692, 2540, 2510, 2379, 1909, 1860, 1654, 1630, 1557, 1505, 1472 cm^{-1}

Synthesis of N-(4-benzoylphenyl) formamide cholate (98)



Cholic acid (0.5 g, 1.2mmol) was dissolved in THF (30 mL) along with triethylamine (2.9 mL, 0.3 mmol). The solution was put on ice for 10 minutes before ethylchloroformate (0.13 mL, 0.013 mmol) was dripped in over 10 minutes. The solution was allowed to react for two hours at room temperature. 4-aminobenzophenone (0.23 g, 1.2 mmol) was added and left to react for 3 hours. The reaction was quenched with water (30 mL). The mixture was washed with water (3 x 30 mL). The organic layer was dried over magnesium sulphate and the solvent was evaporated under reduced pressure. Solvent extraction between water and ethyl acetate was preformed 3 times before the organic layer was removed under reduced pressure. The product was dried at room temperature under vacuum.

MS (+APCI) m/z= Found 588.3680; calculated for $C_{37}H_{50}N_1O_5$ 588.3684; -0.6 ppm

2.8.1 PHOTOCHEMISTRY EXPERIMENTAL

Reactant 1 and reactant 2 were weighed into a small vial with 1mL solvent. The vial was placed under the UV lamp for a set amount of time. The solution was then subject to TLC, and in some cases, purification and NMR analysis.

Reactant 1	weight	Reactant 2	weight	Solvent	time under UV
16	0.01g	98	0.004g	chloroform	1.2 hours
16	0.01g	98	0.004g	chloroform	1.2 hours
Cholic Acid	0.006g	98	0.004g	DMSO	1 hour
Taurocholic Acid	0.008g	98	0.004g	DMSO	1.2 hours
Cholic Acid	0.006g	98	0.004g	DCM	45 minutes
Taurocholic Acid	0.0118g	98	0.004g	DCM	45 minutes
17	0.0082g	98	0.004g	DCM	45 minutes
Cholic Acid	0.006g	98	0.004g	-	15 minutes
Cholic Acid	0.006g	98	0.004g	Petrol 60-80	15 minutes
Cholic Acid	0.006g	98	0.004g	Acetonitrile	15 minutes
Taurocholic Acid	0.008g	98	0.004g	-	15 minutes
Taurocholic Acid	0.008g	98	0.004g	Petrol 60-80	15 minutes
Taurocholic Acid	0.008g	98	0.004g	Acetonitrile	15 minutes
16	0.01g	98	0.004g	-	15 minutes
16	0.01g	98	0.004g	Petrol 60-80	15 minutes
16	0.01g	98	0.004g	Acetonitrile	15 minutes

Table 6 Table to show amounts of reactants 1 and 2 used, the solvent and reaction times for the UV experiments

Reactant 1	weight	Reactant 2	weight	Solvent	Time under UV
98	0.03g	polystyrene	0.25g	chloroform	1 hour
98	0.0016g	polystyrene	0.25g	toluene	1.5 hours
98	0.004g	polystyrene	0.25g	toluene	2.5hours
98	0.005g	polystyrene	0.25g	Methanol	2 hours
98	0.005g	polystyrene	0.25g	Ethanol	2 hours
98	0.005g	polystyrene	0.25g	Ethyl	2 hours

				acetate	
98	0.005g	polystyrene	0.25g	DCM	2 hours
98	0.005g	polystyrene	0.25g	Acetonitrile	2 hours
98	0.005g	polystyrene	0.25g	Petrol 60-80	2 hours
98	0.005g	polystyrene	0.25g	Petrol 40-60	2 hours
98	0.005g	polystyrene	0.25g	THF	2 hours
98	0.005g	polystyrene	0.25g	DMF	2 hours
98	0.005g	polystyrene	0.25g	Acetone	2 hours
98	0.1g	polystyrene	0.25g	DMF	2.5 hours
98	0.1g	polystyrene	0.25g	Chloroform	2 hours
98	0.1g	polystyrene	0.25g	Acetone	2 hours
98	0.1g	polystyrene	0.25g	Ethyl acetate	2 hours
98	0.1g	polystyrene	0.25g	Methanol	2 hours
98	0.1g	polystyrene	0.25g	Ethanol	2 hours
98	0.1g	polystyrene	0.25g	toluene	2 hours
98	0.1g	polystyrene	0.25g	DCM	2 hours
98	0.1g	polystyrene	0.25g	Acetonitrile	2 hours
98	0.1g	polystyrene	0.25g	Petrol 60-80	2 hours
98	0.1g	polystyrene	0.25g	Petrol 40-60	2 hours

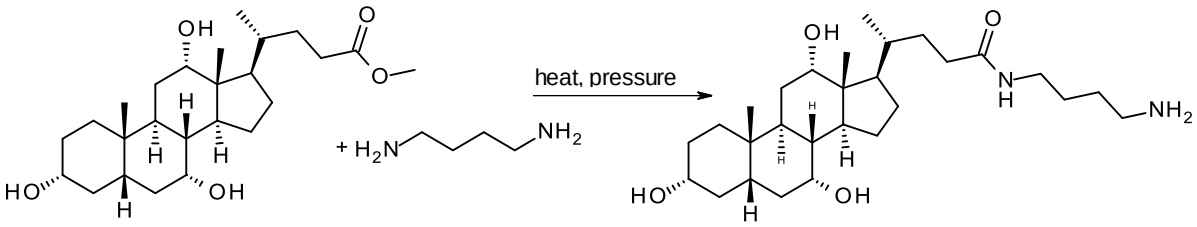
Table 7 Table to show the weight of reactant 1 used, along with the weight of polystryene used, the solvent and reaction times for the UV experiments

3. Results

3.1 Synthesis of Cholic acid amide derivatives

3.1.1 Aminolysis of methyl cholate: Diamines

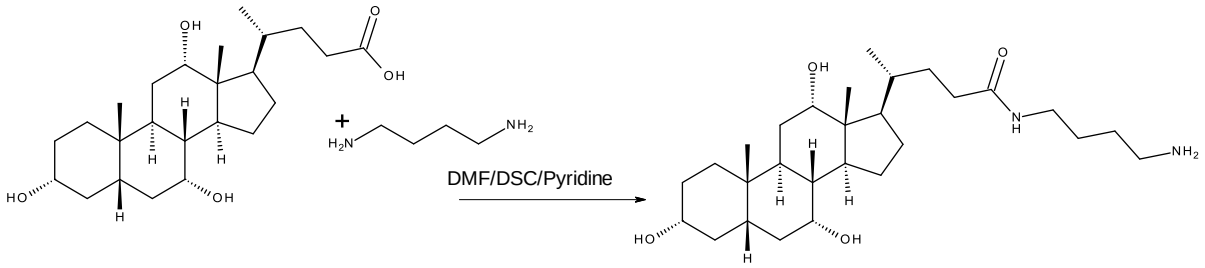
In order to synthesis an antimicrobial cholic acid derivative, an amide tail had to be created. This was achieved with aminolysis of methyl cholate using various amines under a variety of conditions. The general procedure was to add methyl cholate and a large excess of the amine to a pressure vessel and heat.



Scheme 6 Amidation of methyl cholate acid by 1,4-diaminobutane

The results for this procedure were varied. Whilst aminolysis did work for some diamines, the long reaction times and high pressures needed made the reaction difficult to control. Separation of the product from the excess diamine also proved very difficult, reducing the overall yield. Since a pressure vessel was being used, the progress of the reaction could not easily be assessed, meaning reaction times were probably longer than necessary and temperatures higher than needed. Thermal degradation of the diamines could have also occurred although this is arguable due to the relatively short reaction time and limited amount of oxygen present. Overall, this method, whilst successful in low yields was found to be inefficient for the projects needs. The reaction yields are given in table 6.

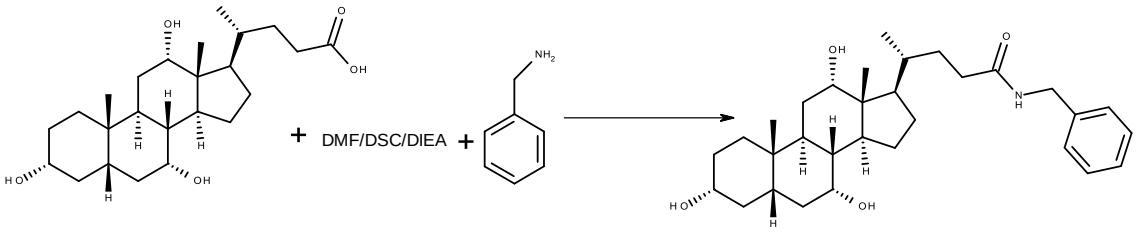
3.1.2 Activation of Cholic acid with disuccinimidyl carbonate



Scheme 7 Amidation of cholic acid via activation with DSC and subsequent reaction with 1,4-diaminobutane

Another method tried was the conversion of cholic acid to the N-hydroxysuccinate ester by means of disuccinimidyl carbonate in the presence of pyridine and then aminolysis with 1,4-diaminobutane. This resulted in low yields and complex mixtures as judged by TLC and NMR spectroscopy. TLC anaylsis showed that the cholic acid had not all been consumed in the reaction, indicating that no, or little of the N-hydroxysuccinate ester had been made so the reaction could not progress.

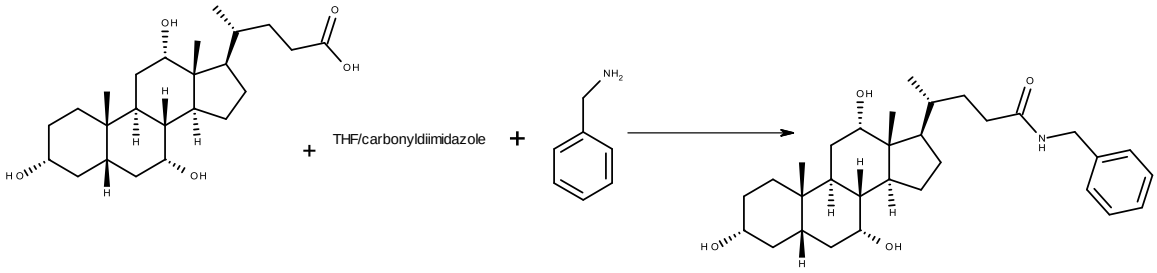
The reaction was repeated with a simpler amine (benzyl amine) as a model and disoproylethylamine instead of pyridine in DMF.



Scheme 8 Synthesis of compound 17 by activation of cholic acid by DSC

This time, the activation step of cholic acid was left for longer than the original experiment (overnight instead of 3 hours) and the aminolysis stage was left for a shorter time (1 hour instead of 48 hours). TLC analysis showed no cholic acid was present and only one component for the product, however, the yield obtained for this reaction was very low, 14% or 0.14 g of the product. Due to the low yields obtained, the use of disuccinimidyl carbonate was also found to be inefficient to the projects needs.

3.1.3 CARBONYL DIIMIDAZOLE ACTIVATION OF CHOLIC ACID



Scheme 9 Synthesis of compound 17 by activation of cholic acid by CDI

Due to the ineffeciencies of the disuccinimidyl carbonate activations, alternative activation methods were tried. Reactions with carbonyldiimidazole, cholic acid and benzyl amine resulted in a number of by products which, even after solvent extraction and acid washes, were still present in the mixture. TLC analysis of the reaction showed that 5 compounds were present in the reaction mixture, including cholic acid. Other issues arose when using carbonyldiimidazole, such as, the imidazoles hydrolytic lability.

Another approach was then taken using N-(3-dimethylaminoprpyl)-N'-ethylcarbodiimide with cholic acid and 3-(dibutylamino) proylamine. The reaction did not go to completion even after 114 hours at room temperature. TLC analysis of the end solution showed a high number of side products so the decision was taken not to procedure any further again, due to ineffieciencies.

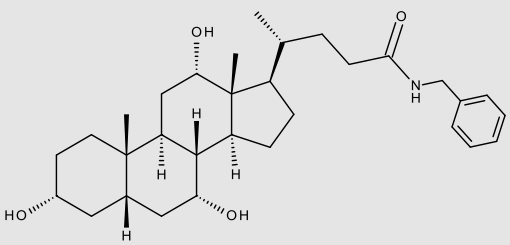
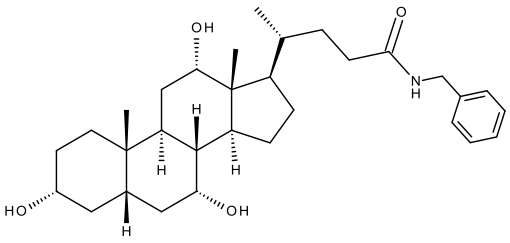
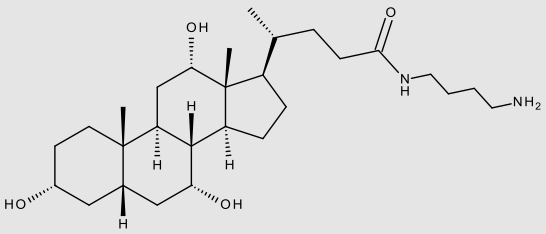
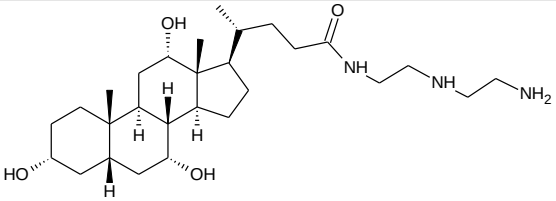
3.1.4 ETHYLCHLOROFORMATE ACTIVATION OF CHOLIC ACID

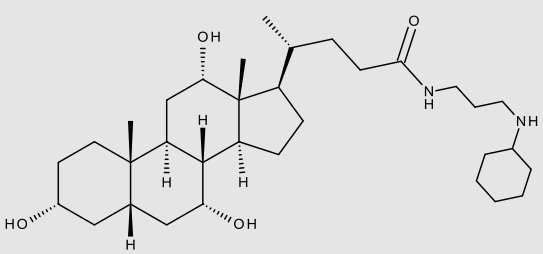
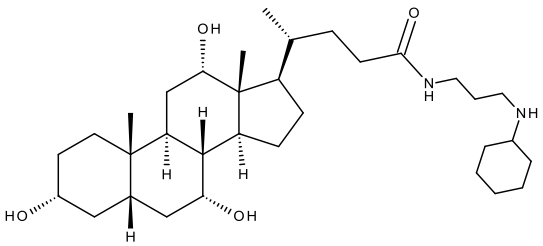
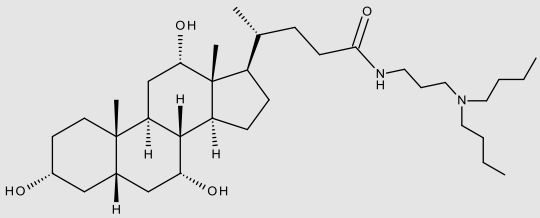
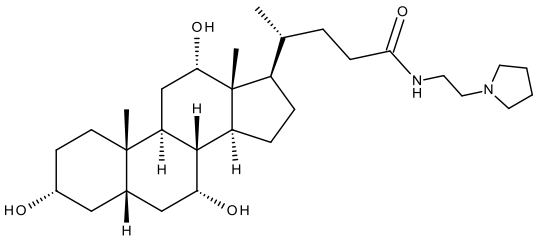
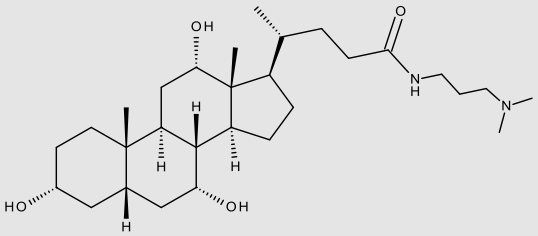
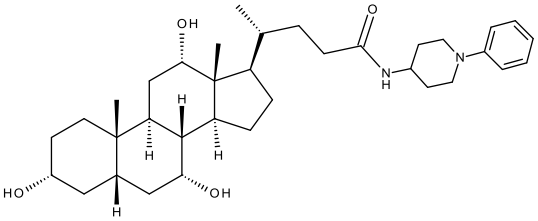
Activating cholic acid with ethylchloroformate was found o be the most successful way for attaching an amine to cholic acid.

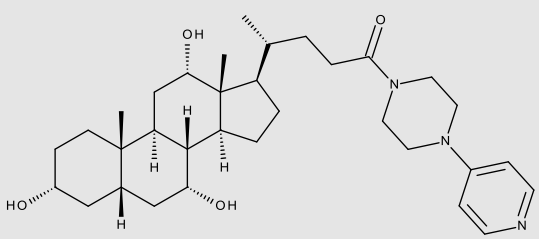
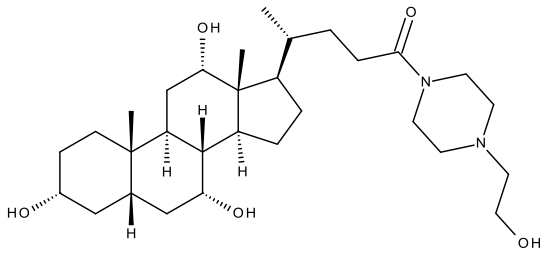
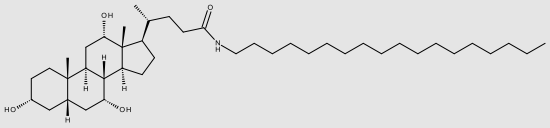
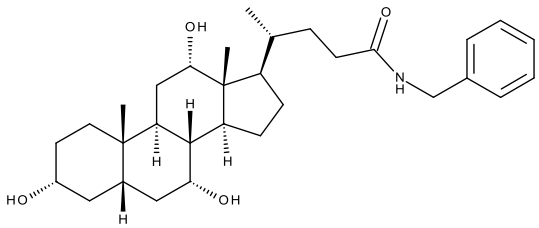
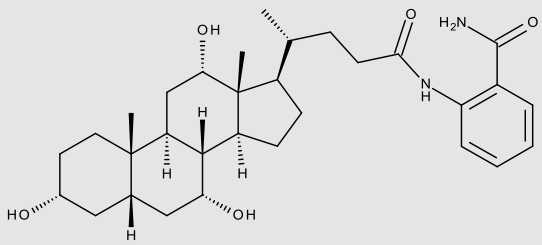
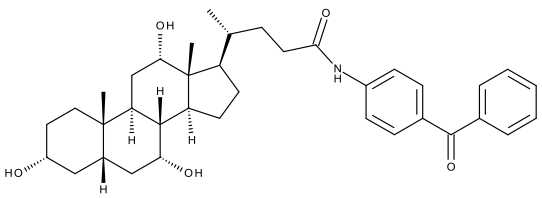
Activation of the acid could be achieved in two hours at room temperature and overall reaction times totalled six hours. There were no adverse reactions in leaving the solution overnight if using cholic acid. The concentration of the sacrificial tertiary amine, however, did seem to have an effect on the reaction. Whilst there was no observed difference in using triethylamine or 4-methylmorpholine, if the concentration of either of them was too low, the reaction would not go to completion. This may be due to the triethylamines role to neutralise any hydrochloric acid produced which allows the reaction to progress. One issue with using ethylchloroformate is the problem with using diamines. It was discovered during the

experimental optimization that both primary and secondary amines would react with the activated cholic acid. This meant that diamines containing two primary amino groups or a primary and a secondary amino group would react at both sites, giving a mixture of amide products. A example of this can be found with N-cyclohexyl-1,3-propanediamine. The activated cholic acid can react in two places to create a dimer. Tertiary amines, however, do not react with the activated site. This allows the use of the diamines with primary amines one end and tertiary amines the other end. The use of these diamines resulted in little purification being needed, good yields and short reaction times.

The yields and methods for all of the cholic acid-amide derivatives synthesised are shown in table 6.

Compound number	Structure	Method	Yield
2		Activation of the carbonyl by disuccinimidyl carbonate	14%
2		Aminolysis	34%
3		Aminolysis	30%
4		Aminolysis	18%

5		Aminolysis	60%
6		Coupling with ethylchloro-formate	72%
7		Coupling with ethylchloro-formate	
10		Coupling with ethylchloro-formate	74%
12		Coupling with ethylchloro-formate	
13		Coupling with ethylchloro-formate	

14		Coupling with ethylchloro-formate	
15		Coupling with ethylchloro-formate	80%
16		Coupling with ethylchloro-formate	90%
17		Coupling with ethylchloro-formate	74%
18		Coupling with ethylchloro-formate	
19		Coupling with ethylchloro-formate	25%

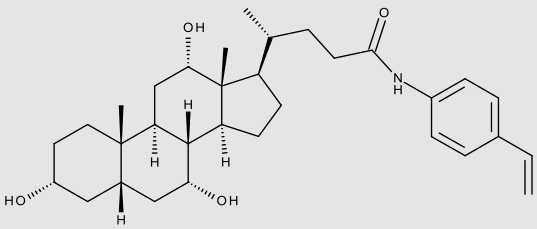
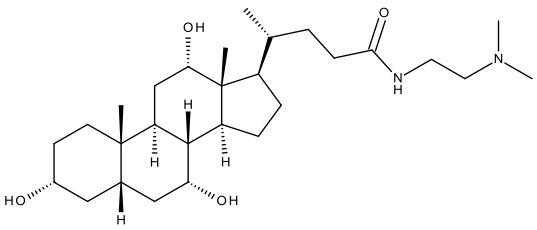
20		Coupling with ethylchloro-formate
21		Coupling with 64% ethylchloro-formate

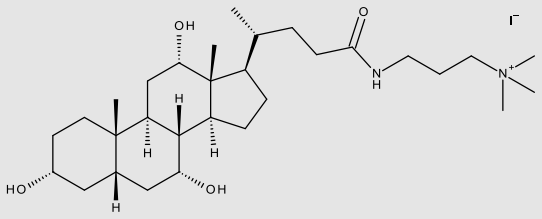
Table 8 Table to show the structures, yields and method used to form amide bonds on cholic acid

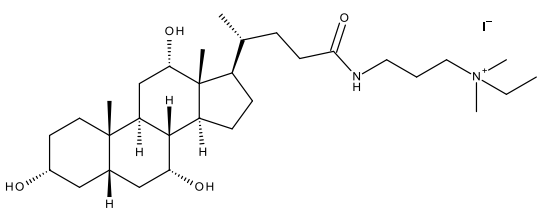
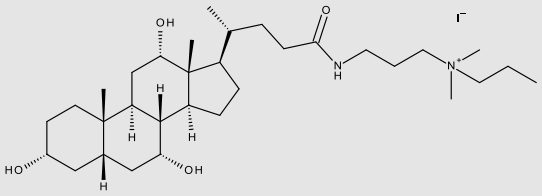
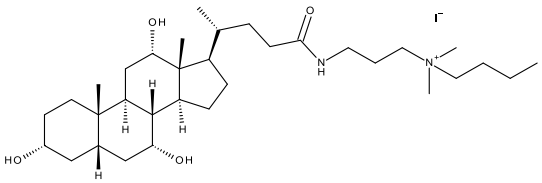
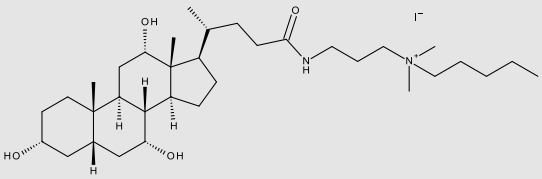
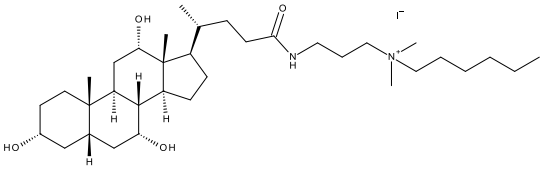
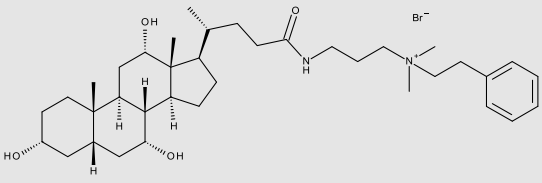
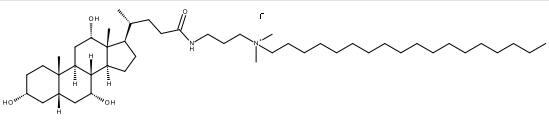
3.2 Quaternisation of cholic acid derivatives bearing a tertiary amine

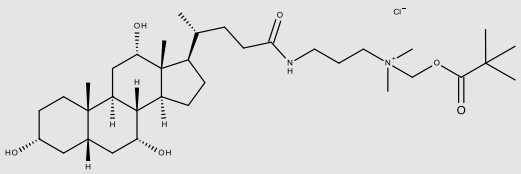
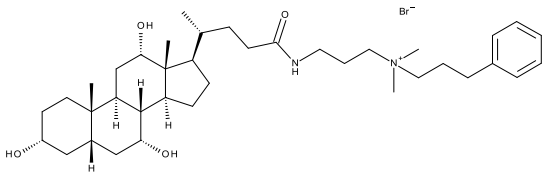
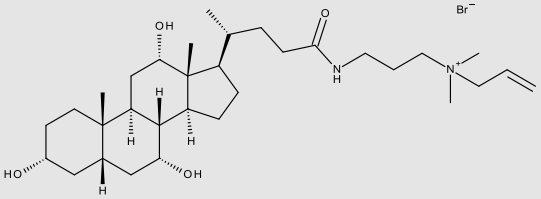
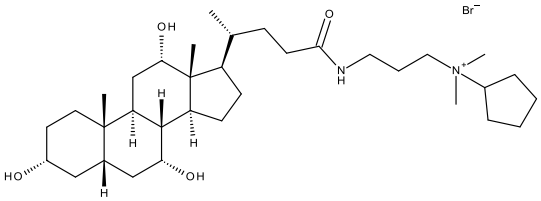
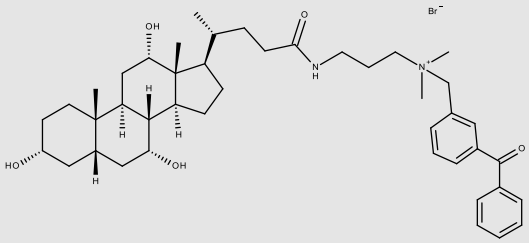
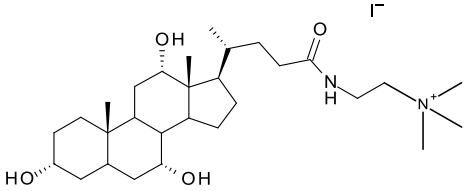
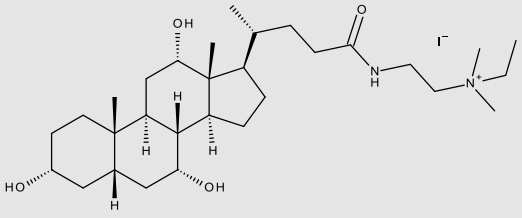
Quaternary amides are well known for their antimicrobial properties. They are straight forward to synthesise and very effective at disrupting cell walls of both gram positive and gram negatve bacteria. The tertiary amine-containing compounds produced in section 2.2 were quaternised with a range of alkyl halides. Generally, the products precipitated from the reaction mixture and were obtained in a pure state by filtration and washing with clean solvent. A small amount of methanol was added to the reaction mixture for those starting materials which did not dissolve in either dichloromethane or chloroform. The products from these reactions did not precipitate out and required the removal of the solvents by rotary evaporation.

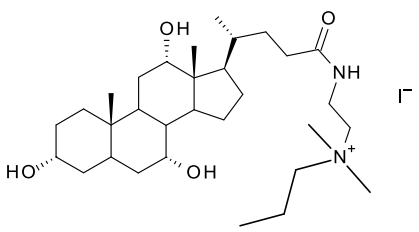
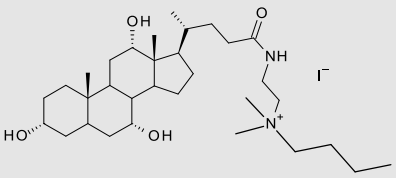
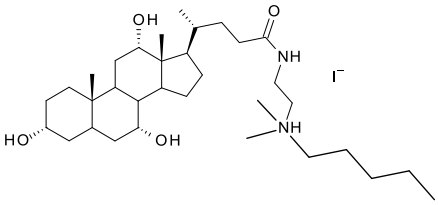
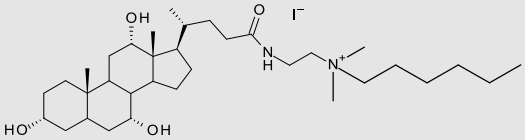
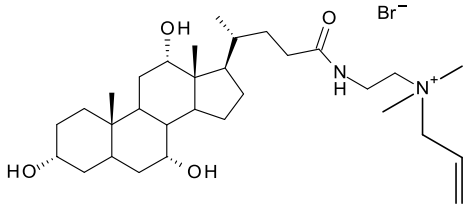
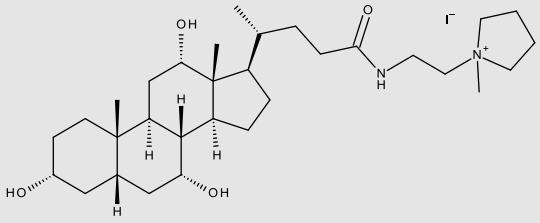
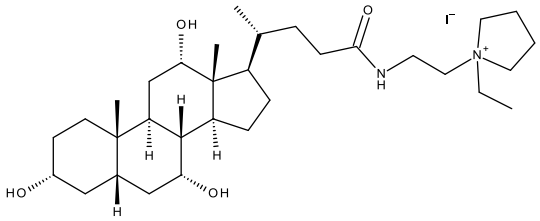
Quaternations were easy to achieve and were relatively straight forward. Problems arose with the longer chained and bulkier alkylating agents. The most problematic were iodobutane, iodopentane and 1-bromo-3-phenylpropane which, when used in a reaction, did not go to completetion. NMR analysis of these alkyl halides was carried out and interpretation of the NMR confirmed their integrity. One reason for the problems with these three alkylating agents might be the length of the chain, however this doesn't explain the successful quaternisations achieved with iodohexane. Steric hinderance, especially with 1-bromo-3-phenyl propane may be an explanation; however, again, there should be no reason why iodobutane and iodopentane were problematic. Unfortunalty, time constrants meant that this problem could not be solved. The use of alternative reaction conditions could determine why under the standard condtions, iodobutane, iodopentane and 1-bromo-3-phenylpropane would not react.

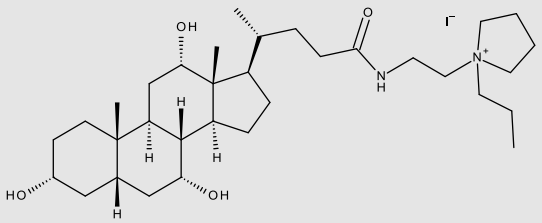
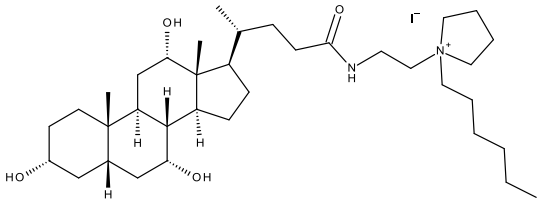
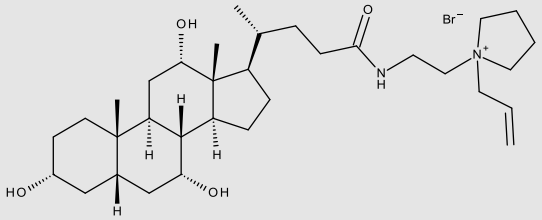
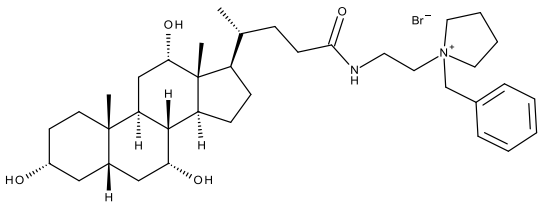
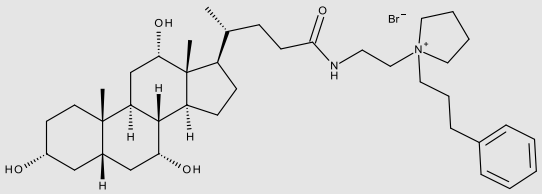
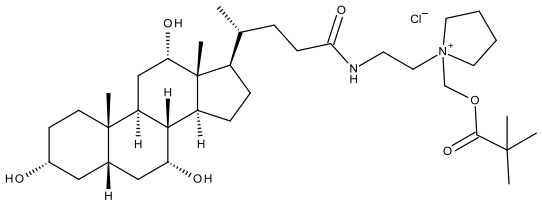
The results and yields for the quaternasations are shown in table 7.

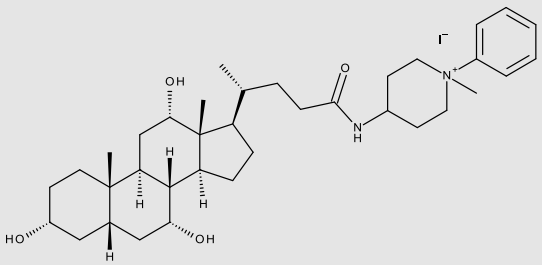
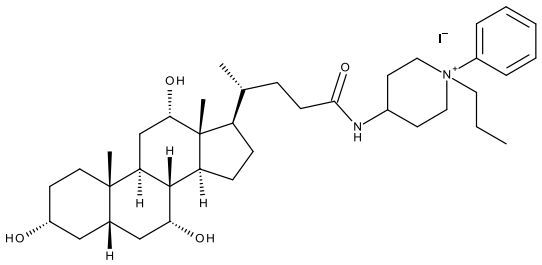
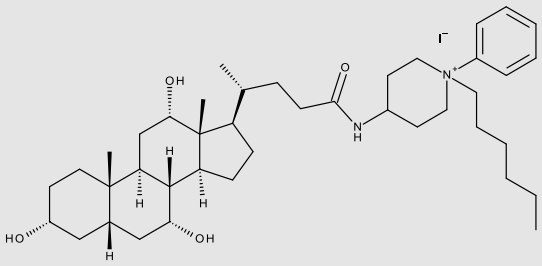
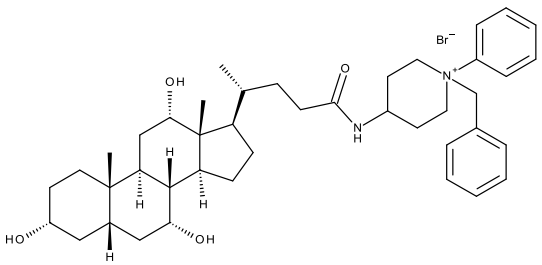
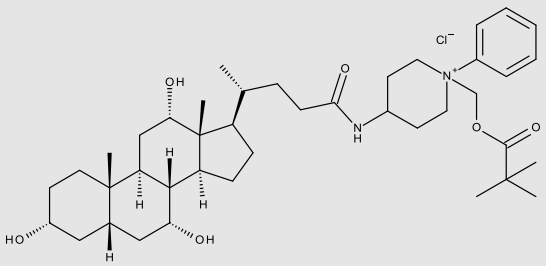
Compound number	Structure	Yield
24		36%

25		58%
26		86%
27		69%
28		14%
29		
30		
31		60%

32		
33		
34		30%
35		
36		96%
37		63 %
38		56 %

39		56 %
40		67 %
41		79 %
42		
43		26 %
44		
45		90%

46		95%
49		
50		
51		
52		
53		34%

54		
55		70%
58		78%
59		18%
60		

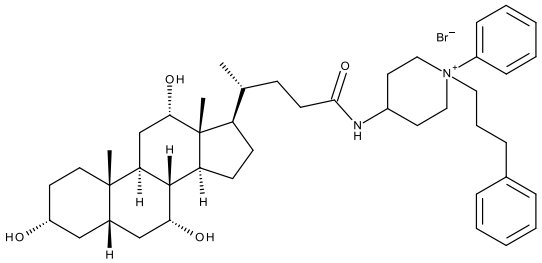
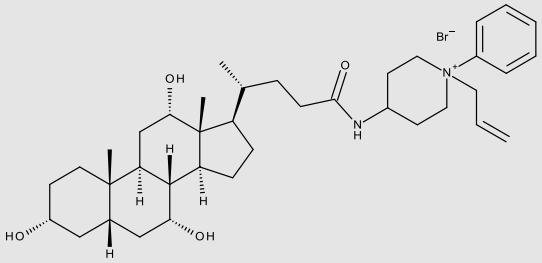
61		
62		24%

Table 9 Table to show the yields of quaterinastions of cholic acid derivatives bearing a tertiary amine

3.3 Interpreting proton NMRs of cholic acid

NMR anaylsis of cholic acid is complicated and sometimes difficult. There are forty hydrogens present, all within close proximity to each other. The peaks for cholic acids steroid body are found between 1ppm and 2.4 ppm. All of the peaks found in this region have been assigned using 2D NMR by Hu *et al*. They are generally not assigned when reporting the NMR analysis in the literature. The three methyl groups present on the steroid body are clearly seen at 0.7 ppm, 0.8 ppm and 0.9 ppm. Generally, these groups do not move when the hydroxyls or side chain have been reacted on. The three hydrogens bonded to the hydroxylated carbon, so H-C3-OH can be seen at 3.48 ppm, the H-C7-OH at 3.9 ppm and the H-C12-OH at 4ppm. These three proton peaks shift when the hydroxyl group is replaced with another functional group. The three hydrogens present on the hydroxyls are present in the steroid body peaks. The reactive series for the three hydroxyls is C3>C7>C12, however this changes once the C3-OH has been modified with to C3>C12>C7. This can be seen in the NMR spectrum and in schemes 5, 6 and 7. When interpreting the NMR of a modified cholic acid compound, it is important to note that the three H-C-OH peaks shift especially when looking for any impurities that may be present.

The solvent used for the NMR spectra can also make analysis difficult. If the NMR is carried out with methanol-d_4, it can be difficult to fully distinguish the H-C3 multiplet due to the water peak from the methanol. This in turn, makes it impossible to carry out the integration for that peak. Some experience is needed to confidently interpret complicated cholic acid derivatives.

Ethyl carbon chains can also be difficult to interpret due to their position in the NMR. The intergration can be sometimes difficult to measure as interference from the steroid body peaks cannot be isolated from the carbon chain peak. In this situation, sensible interpretation of the spectra is needed to ensure it is correct.

3.3 Attaching polymerisable groups to methyl cholate

Attaching methacrylate groups is quite straightforward, however, controlling where the methacryloyl chloride attached onto the methyl cholate's hydroxyls required optimisation. Temperature control played heavily in selectivity of the hydroxyls. At -20^{o}C, the reaction slowed dramatically, virtually stopping. At 4^{o}C, a monomethacrylate was synthesised and at room temperature mono, di and trimethacrylates were synthesised. The positions of the methacrylates can clearly be seen on the proton NMR spectra for the products. The spectrum shown in scheme 5 forms part of a NMR spectrum from methyl cholate in chloroform. The

three hydrogen groups attached to carbons three, seven and twelve can be seen as well as the methyl group. The CH3-OH is at 3.66 ppm, CH7-OH is at 3.84 ppm and the CH12-OH is at 3.95 ppm.

When the C3- hydroxyl is esterified, the reactivity of the hydroxyls towards methacryloyl chloride changes from C3>C7>C12 to C3>C12>C7. This can be seen in the proton NMR spectra. On a mono methacrylolated compound the chemical shift for CH3 is moved left to 4.67 ppm, whereas the other two stay just below 4 ppm.

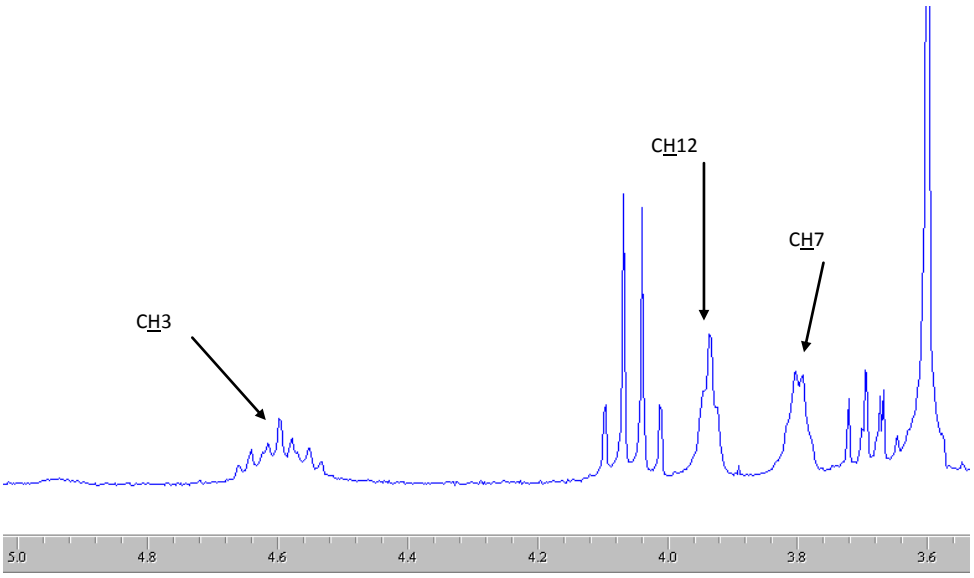


Figure 16 Partial HNMR spectrum of 3α acetate-7α,12α dihydroxy cholic acid (59) showing the positions of the hydrogens on the C3, C7 and C12 carbons

On diacryloylated compounds, the CH12 also shifts to the left at around 5.15 ppm and the CH7 stays at 3.8 ppm.

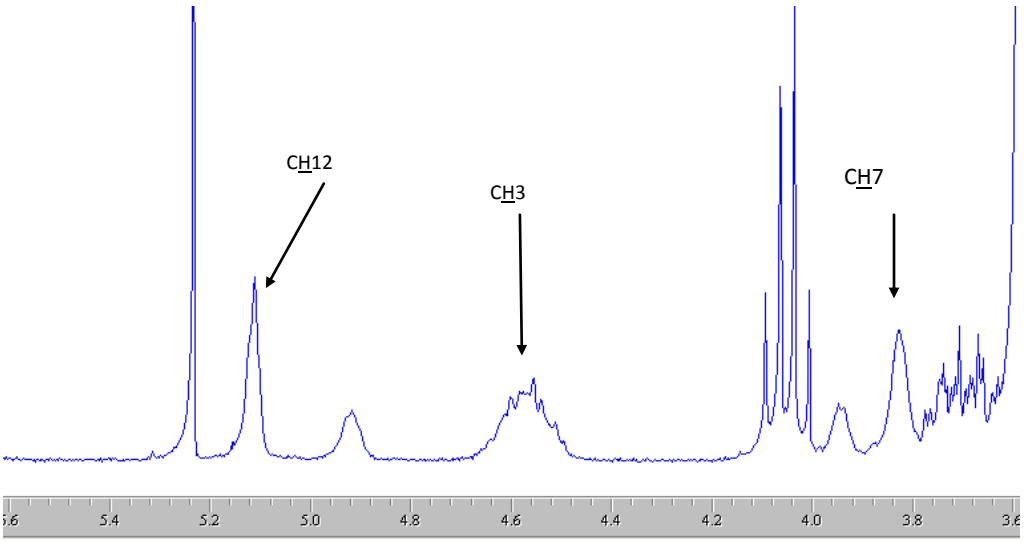


Figure 17 Partial HNMR spectrum of 3α, 12α ,diacetate-7α hydroxy cholic acid to show the movement of the C12 hydrogen once it has been acetaled

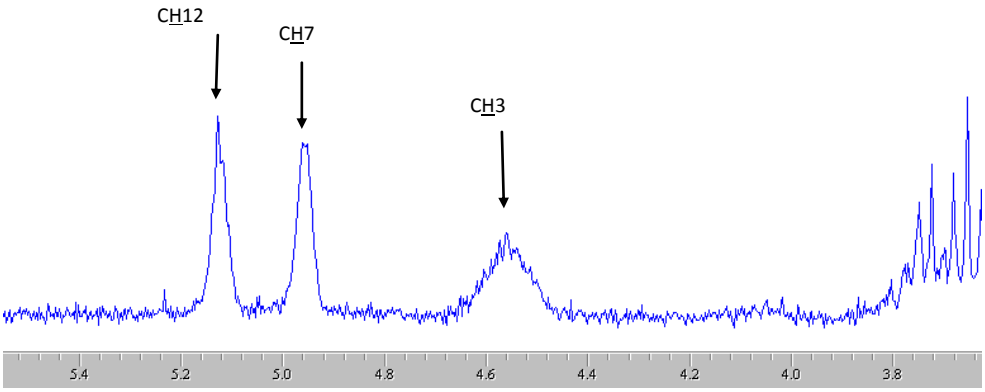


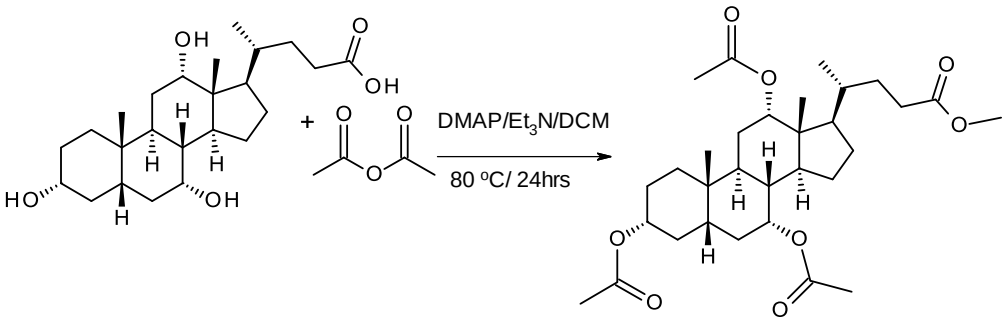
Figure 18 Partial HNMR spectrum of 3α, 12α, 7α triacetate cholic acid showing the new positions of the three hydrogens

Triacryloylation sees all three peaks shift to higher ppm values, with the CH7 being at 4.97 ppm. The changes of reactivity were suggested by Hu *et al* in 2005. They looked at 2D NMR data from previous studies to reach this conclusion, which is consistent with the NMR spectra presented here.

3.4 Acetylation of methyl cholate

Due to the difficulty of selectively reacting on the C3 hydroxyl group on cholic acid or methyl cholate, an alternative approach was taken involving the protection and deprotectio of the hydroxyl groups by acetate groups. This then allowed the C3 protecting group to be removed selectively. The protection and selective deprotection of the hydroxyl groups meant the reaction could be carried out on just the C3 position without the need for low temperatures.

The first step of protecting the hydroxyl groups using acetates were carried out successfully a number of times with good yields. During the experimental optimization process, it was found that the temperature needed totake the reaction to completetion should be 80 ^{o}C and the reaction time is 24 hours. The DMAP concentration was also found to be important; a ration of 1:3 mmol DMAP and cholic acid was needed to take the reaction to completion.



Scheme 10 Scheme to show the synthesis of tri-acetylated methyl cholate

3.4.1 Removal of acetates

Removal of the acetates was achieved using potassium carbonate or trifluoroacetic acid. The reaction was carried out in DCM and monitored by TLC. Potassium carbonate in large quantities, removed more than the C3-acetate group even at low temperatures. By reducing the molar concentration to 1.5 equivalents of the starting material, the selectively to the C3-acetate group decreased. TFA removed the C-acetate group successfully, however, neutralisation and removal of the by-product salt proved difficult. Potassium carbonate was the preferred method of removal of the acetate groups.

3.5 Polymerisations

Polymerisation of the different compounds synthesised in this project sometimes proved difficult. Synthesis of the crosslinked copolymers with EGDMA was carried out in a 96 well plate, creating small polymer discs. Unfortunately, due to the insolubilty of the crosslinked polymers, NMR analysis of the polymer could not be carried out, and the amount of cholic acid incorporation could not be quantified. It could be assumed that due to the method used that all of the cholic acid derivative would be incorporated but due to difficulties with other polymerisations carried out subsequently, it would be very presumptuous to say with any certainty. More difficulties with polymerisations occurred when polymerisation of the quaternised compounds were attempted. When cholic acid derivatives containing vinyl benzyl quaternary ammonium moieties were co-polymerised with styrene, only a small amount of the cholic acid derivative was incorporated into the polymer. One theory behind this could be due to the rates of reaction of the two monomers. It was assumed, as both styrene and the cholic acid derivative have vinylbenzene groups that the rates of reaction would be similar, however, this proved not to be the case, with styrene polymerising a lot faster than the cholic acid monomer. Therefore, in an attempt to improve the chance of the cholic acid derivative incorporation into the polymer, different co-monomers were used, such as tert-butyl methacrylate and methyl acrylate, which are smaller, less bulky monomers. Unfortunately the same problem arose, with very little of the cholic acid derivative being incorporated. Even polymerising the cholic acid based polymerisable derivatives on their own was difficult. The reactions required very long reaction times and often the polymers were insoluble and therefore impossible to analyse by NMR spectroscopy. The fact the starting materials produced insoluble material suggests that a change has occurred but the exact change that happened is difficult to know due to lack of analysis. It would seem that the positive charge on the compounds have interfered in the polymerisation, creating insoluble polymers and slowing the rate of reaction greatly. Changing solvent and increasing the temperature did not seem to make any difference; neither did trying to polymerise it under vacuum. One variation which was not tried was increasing the amount of AIBN to the reaction. The rate of a polymerisation reaction does depend on the amount of initiator present, so the problems found with the polymerisation reactions could simply be down to there not being enough AIBN to create the radicals needed.

Overall, polymerising monomers and copolymers with the cholic acid derivatives was possible but only wih low incorporation and long reaction times. More work needs carrying out on this process to discover exactly what is slowing down the polymerisations to such an extent.

Other analytical techniques, such as solid state NMR could aid in the analysis of the compounds synthesised, giving more confidence in the results.

3.5.1 POLYUREATHANES

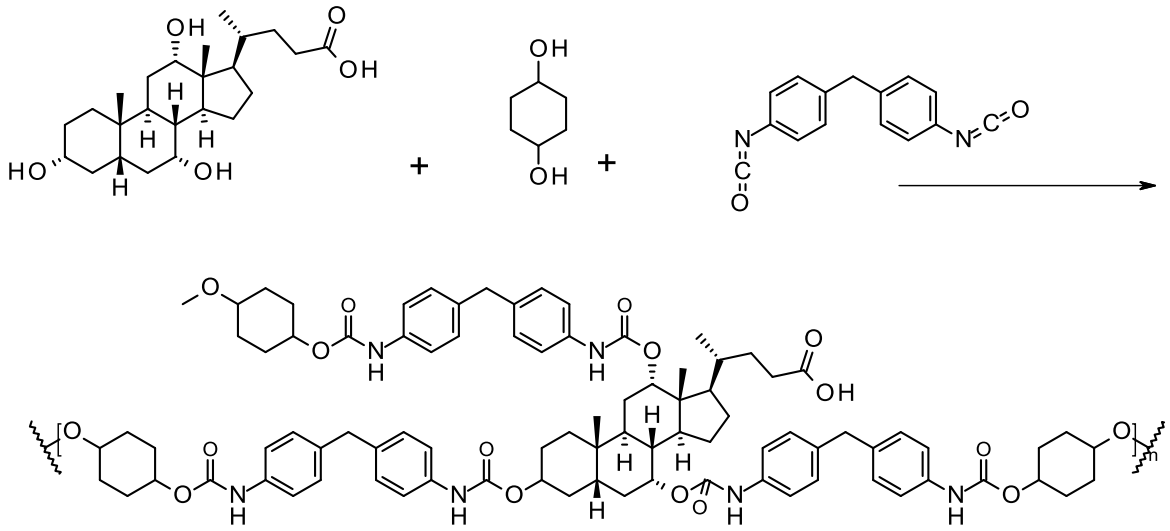


Figure 19 Preparation of a polyurethane copolymer of cholic acid and 4,4-methylenebis(phenyl isocyanate)

Incorporating cholic acid into a polyureathane was successful; however, incorporating cholic acid derivatives was not. This, along with the issues with free radical polymerisation of quaternised cholic acid derivatives suggest that the positive charge on nitrogen is effecting the polymerisation of the compound. Indeed, polymerising quaternary ammonium compounds is not common in the literature. It is more common to quaternise nitrogen that is already incorporated into the polymer. The problems with polymerising monomers with quaternary nitrogens may be due to the positive charge on the compound slowing the reaction rate of polymerisation meaning it is not incorporated into the polymer.

The conditions of the polyureathane experiments were charged several times. Table 8 shows the ratios of cholic acid, cyclohexanediol and 4,4-methylenebis (phenyl isocyanate) used in each experiment.

Polymer number	Cholic acid	Cyclohexanediol	4,4-methylenebis(phenyl isocyanate)
94	1	1	5
94	1	2	5
94	1	0	2
94	5	1	5
94	2	0	1

Table 10 Ratio of cholic acid, 1,4-cyclohexanediol and 4,4-methylenebis(phenyl isocyanate) in THF

Due to the complexities of the NMR spectra for these polymers, it was difficult to assign all of the peaks. By synthesising polymers with simpler isocyanates (p-toyl isocyanate) and by varying the molar equivalents of the cyclohexanediol present, a more accurate prediction could be made as to the composition of the polymers.

Synthesis of compound 93 using method 1

Assignment	1H NMR chemical shif (ppm)
C7-OH unreacted	4.09
C3-OH unreacted	4.13

Table 11 Selected NMR assignments for compound 93

Synthesis of compund 93 using method 1 (repeat)

Assignment	1H NMR chemical shif (ppm)
C7H-OH unreacted	4.09
C3H-OH unreacted	4.28
C3-H	4.40
C7-H	4.71
C12-H	4.89

Table 12 Selected NMR assignments for compound 93

Synthesis of compound 95=4 using method 1

Assignment	1H NMR chemical shif (ppm)
C3-H	4.57
C7-H unreacted	3.88
C12-H unreacted	3.99

Table 13 Selected NMR assignments for compound 94

Synthesis of compound 93 using method 3

Assignment	1H NMR chemical shif (ppm)
C7H-OH unreacted	4.11
C3H-OH unreacted	4.16
C3-H	4.41
C7-H	4.68
C12-H	4.87

Table 14 Selected NMR assignments for compound 94

Synthesis of compound 94 using method 2

Assignment	1H NMR chemical shif (ppm)
C12H-OH unreacted	3.78
C7-H	4.06
C3-H	4.39

Table 15 Selected NMR assignments for compound 94

The concentration of cyclohexanediol seemed to make a difference in the polymer composition. By having a one or no molar equivalent to cholic acid, not all of the hydroxyl groups on cholic acid have been reacted on. By increasingthe ratio to a two molar equivalent, some hydroxyl groups remained un-reacted but some cross linking occurred. It is interesting to note that none of the reactions went to completion. This could be due to steric hindrance as the reaction went on.

Cholic acid has three sites of reaction for this reaction, the three hydroxyl groups. Cyclohexanediol has two reaction sites and 4,4-methylenebis(phenyl isocyanate also has two reaction sites. Table 14 below shows the relative reaction sites for each experiment.

Polymer number	Cholic acid reaction sites	Cyclohexanediol reaction sites	4,4-methylenebis(phenyl isocyanate) reaction sites
94	3	2	10
94	3	4	10
94	3	0	4
94	15	2	10
94	6	0	3

Table 16 Table to show the number of reactive sites for each of the reactants in the polyurethane experiments.

From table 14, it is possible to see that not all of the reactions could have gone to completion as there were not enough reactive sites available. However, even where there are enough reactive sites, the reactions do not go to completion, suggesting that there may be some steric hindrance stopping the reaction from completing.

3.5.2 PHOTOCHEMISTRY

Another potential way to incorporate cholic acid into a polymer was to use photochemistry. A benzophenone group was attached onto the cholic acid on the side chain. The synthesis of the benzophenone chloride starting material was simple offering good yields. Attaching the amine was quick, with the product precipitating with the addition of water and recrystallised in acetonitrile. It was hoped that under UV stimulation that the activated benzophenone moiety would attach to other molecules present in the reaction mixture, in this case, cholic acid or its derivatives.

Cholic acid and a cholic acid derivative were used under the UV lamps along with the benzophenone amine. The starting materials were put into small vials and placed under two different UV lamps. The larger of the two lamps had an intensity of 4.4W/cm^2, whereas the smaller lamp had an intensity reading of 2.02W/cm^2.

Overall, the reactions taken place under these conditions were not successful. This could be because of the "bulkiness" of the cholic acid benzophenone derivative. In order for the radical to be produced, hydrogen extraction must take place. The hydrogen is usually extracted from the solvent, although it can be taken from the polymer. Once the radical has been created, it must be able to move and react with the other compounds present. Due to the shape of the cholic acid benzophenone derivative, movement could be restricted and as

the radical formation is reversible, the radical species may not be able to react with the polystyrene present.

There is some evidence that the presence of oxygen can impede the reaction too, although not to a great extent.

4. Germination Tests

Many of the synthesised compounds were tested for their germinating and antimicrobial abilities against *C.difficile*. Due to the insolubility of some of the compounds in water, DMSO, ethanol and methanol were used to dissolve the compounds. The germination studies were carried out by two project students working in the microbiology lab, Amber Lavender and Kristian Poole. The methods titled the preparation of spore suspensions and heat shock method were written by Amber Lavender.

4.1 Methods

The *C.difficile* reference strain, NCTC 11204 and *C.difficile* ribotype 027 (R20291) (Anaerobic Reference Laboratory, Cardiff, UK) were used during testing.

4.1.2 Preparation of spore suspensions

Spore suspensions of *C.difficile* were prepared following the method proposed by Shetty *et al.* (Shetty et al., 1999). Briefly, Columbia base agar plates were inoculated with the relevant strain of *C.difficile* and incubated for 72 hours anaerobically at 37 ^{o}C (MiniMACS anaerobic cabinet, Don Whitley Scientific, Shipley, UK). Then, the plates were removed and left or 24 hours in aerobic conditions at room temperature. Colonies were then harvested into 20 mL of 50% (w/v) ethanol and 50% saline, and vortex thoroughly. These were stored at 4 ^{o}C until needed.

All experiments were performed in triplicate using spore suspensions containing 1 x10^{7} CFU mL^{-1} spores of *C. diffcile* NCTC 11204 and ribotype 027.

Before use, 1 mL of spores were centrifuged at 13000 rpm for 10 minutes (Spectrafuge 24D; Labnet, Woodbridge, USA). The supernatant was discarded, and the pellet resuspended in 1 mL sterile distilled water and vortex mixed thoroughly.

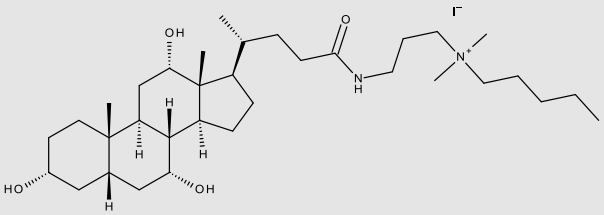
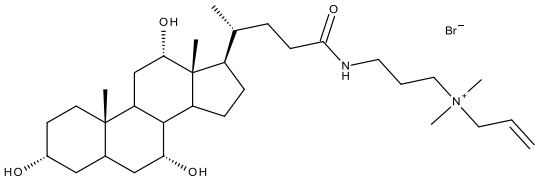
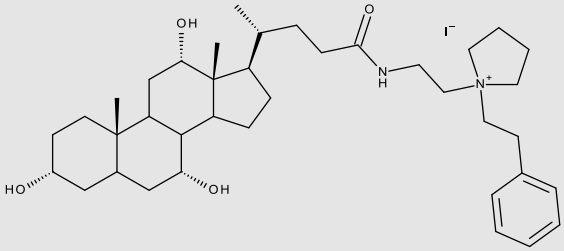
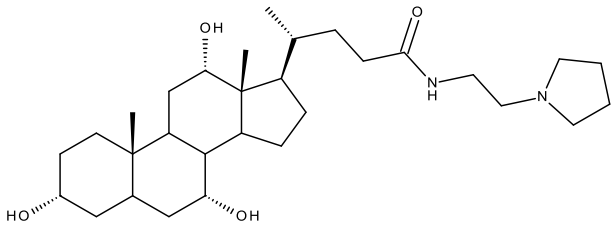
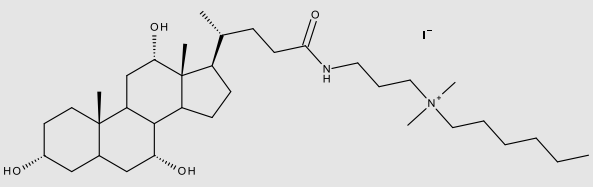
Germination solutions were prepared using 2% (w/v) of the compound in diluent (DMSO, ethanol, methanol, water) plus double strength thioglycollate medium (Oxoid, UK).

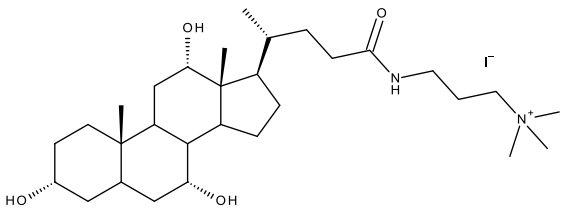
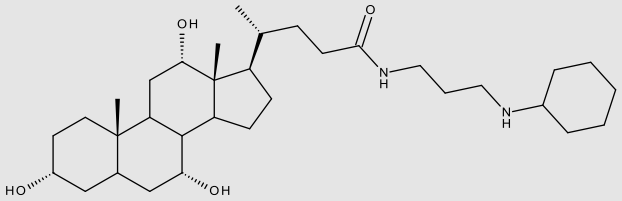
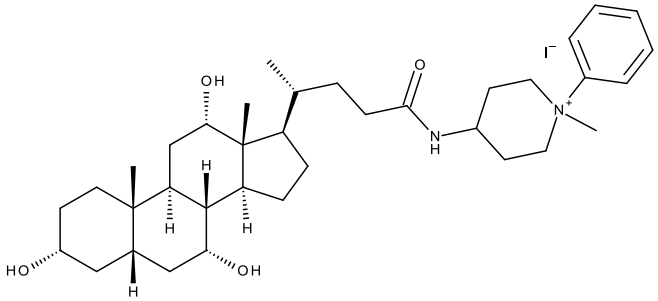
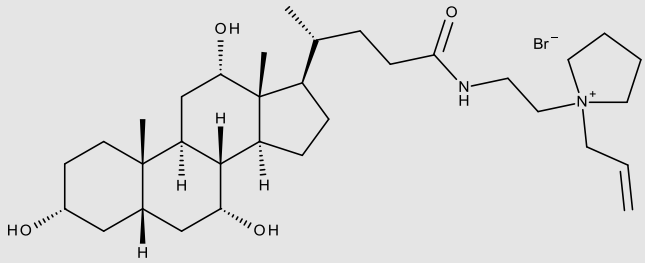
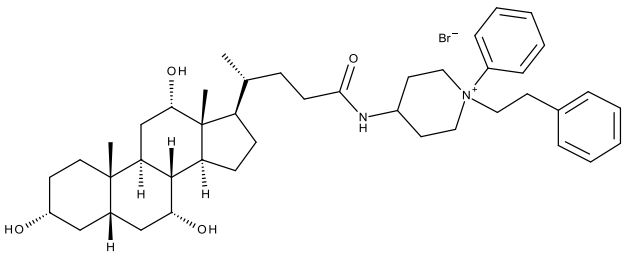
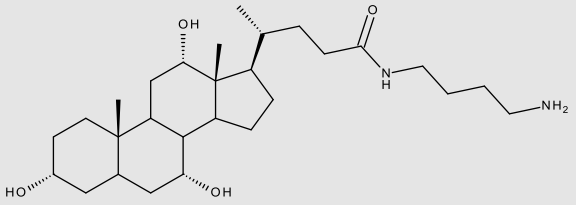
4.1.3Heat shock method

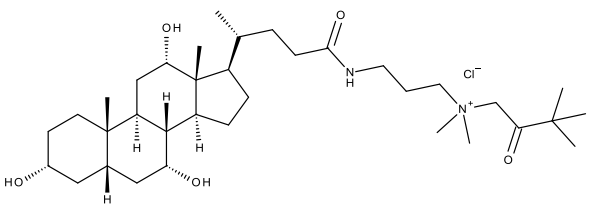
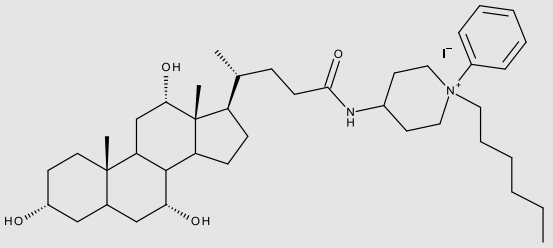
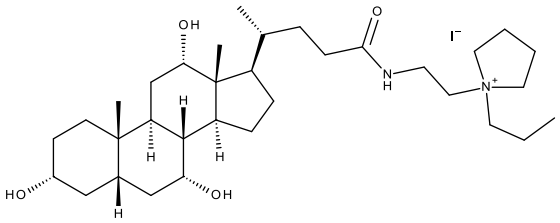
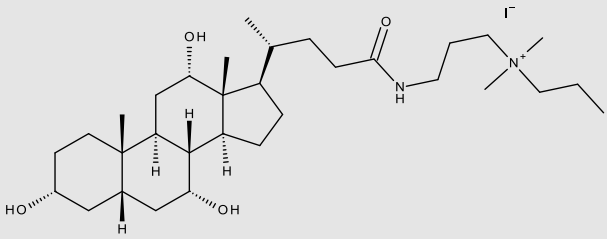
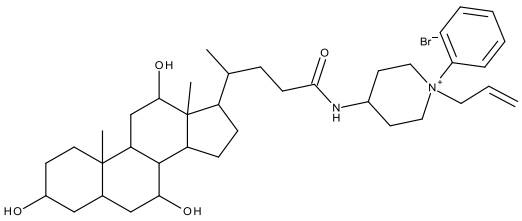
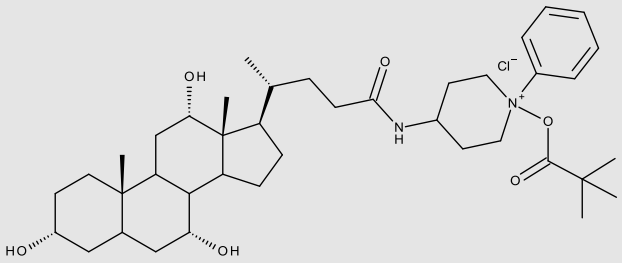
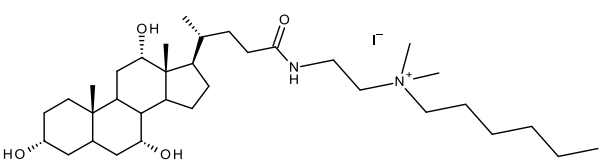
For the heat shock method, 100 µl spores were exposed to 100 µl of the germination solution and incubated at room temperature in air for 1 hour. The entire 200 µl sample was then added to 800 µl sterile distilled water to dilute out the germinant to ineffective concentrations. Samples were placed on heat at 70 ^{o}C for 20 minutes to eliminate any germinated,

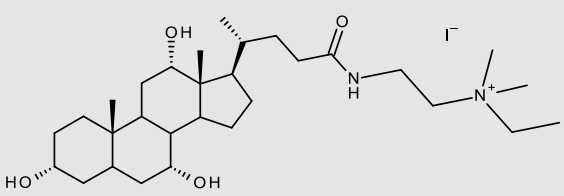
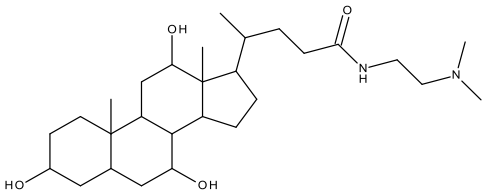
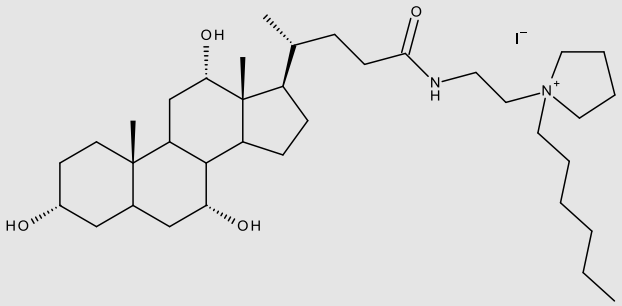
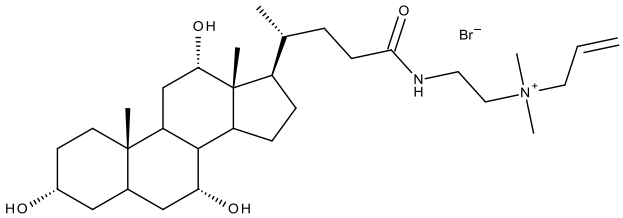
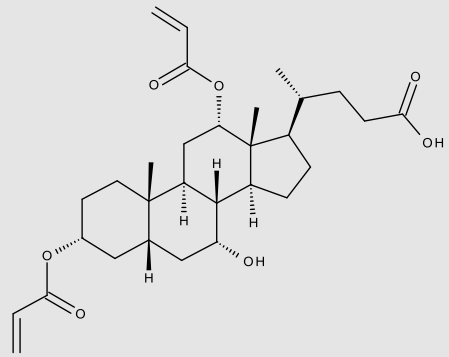
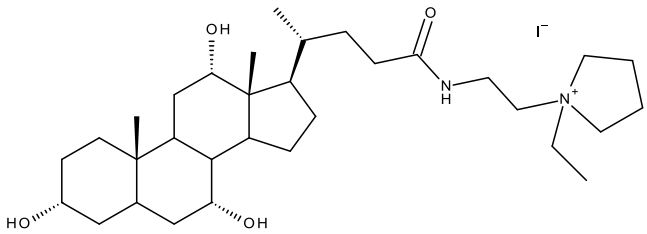
metabolically active spores. Control samples were kept on ice. Solutions were then diluted accordingly using sterile distilled water, and cultured onto fastidious anaerobic agar (Lab M, Bury, UK), supplemented with 0.1% (w/v) sodium taurocholate (ST) and 5% (w/v) defibrinated horse blood using the Miles and Misra method (Miles *et al*., 1938). These were then incubated anaerobically for 48 hours at 37 ^{o}C (MiniMACS anaerobic cabinet, Don Whitley Scientific, Shipley, UK) and the CFU mL^{-1} counted.

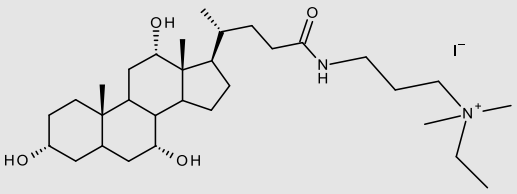
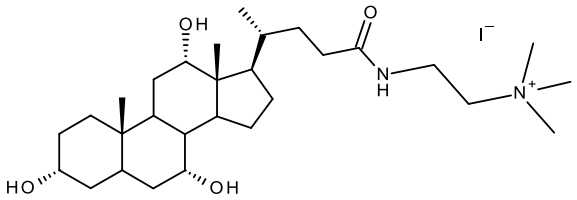
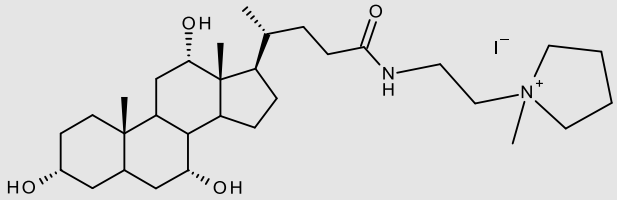
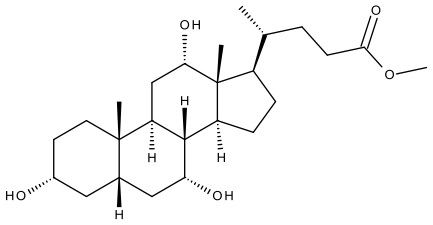
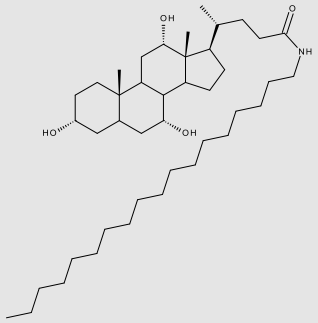
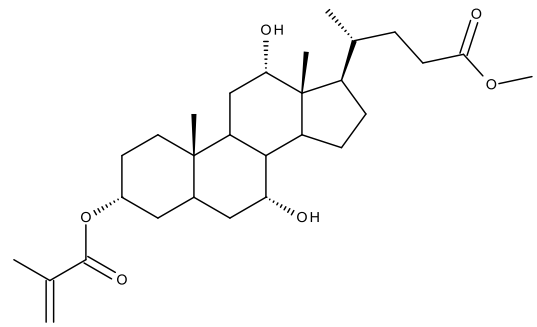
4.2 COMPOUNDS TESTED

Compound number	Compound name	Structure
28	3-cholanamidopropyl-pentyl-dimethyl-ammonium iodide	
34	3-cholanamidopropyl-allyl-dimethyl-ammonium bromide	
52	N-[2-[1-(3-phenylpropyl)pyrrolidin-1-ium-1-yl]ethyl]cholanamide bromide	
10	N-(2-pyrrolidin-1-ylethyl) cholanamide	
29	3-cholanamidopropyl-hexyl-dimethyl-ammonium iodide	

24	3-cholanamidopropyl (trimethyl)ammonium iodide	
6	N-[3-(cyclohexylamino)propyl] cholanamide	
54	N-(1-methyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide iodide	
50	N-[2-(1-allylpyrrolidin-1-ium-1-yl)ethyl] cholanamide bromide	
61	N-[1-phenyl-1-(3-phenylpropyl)piperidin-1-ium-4-yl]cholanamide bromide	
3	N-(4-aminobutyl) cholanamide	

32	3-cholanamidopropyl-(2,2-dimethylpropanoyloxymethyl)-dimethyl-ammonium chloride	
58	N-(1-hexyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide iodide	
46	N-[2-(1-propylpyrrolidin-1-ium-1-yl)ethyl]cholanamide iodide	
26	3-cholanamidopropyl-propyl-dimethyl-ammonium iodide	
61	N-(1-allyl-1-phenyl-piperidin-1-ium-4-yl)cholanamide bromide	
60	(4-cholanamido-1-phenyl-piperidin-1-ium-1-yl)methyl 2,2-dimethylpropanoate chloride	
42	3-cholanamidoethyl-hexyl-dimethyl-ammonium iodide	

45	3-cholanamidoethylyl-ethyl-dimethyl-ammonium iodide	
21	N-(2-dimethylaminoethyl) cholanamide	
49	N-[2-(1-allylpyrrolidin-1-ium-1-yl)ethyl]cholanamide bromide	
43	3-cholanamidoethyl-allyl-dimethyl-ammonium bromide	
59	3α,12α diacroylate-7αhydroxycholic acid	
45	N-[2-(1-ethylpyrrolidin-1-ium-1-yl)ethyl]cholanamide iodide	

25	3-cholanamidopropyl-ethyl-dimethyl-ammonium iodide	
37	3-cholanamidoethylyl (trimethyl)ammonium iodide	
44	N-[2-(1-methylpyrrolidin-1-ium-1-yl)ethyl]cholanamide iodide	
1	Methyl cholate	
16	N-octadecylcholanamide	
69	3α methacrylate 7α,12α dihydroxyl cholic acid methyl ester	

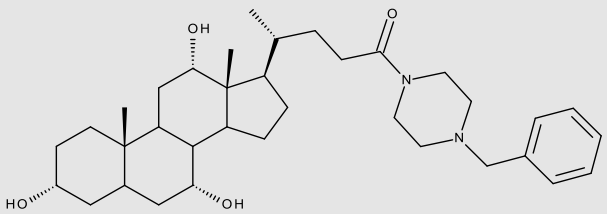
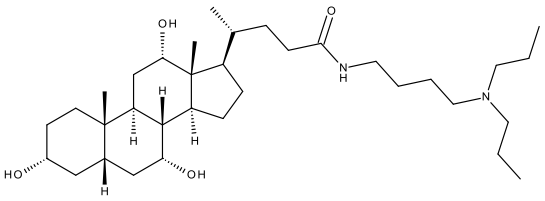
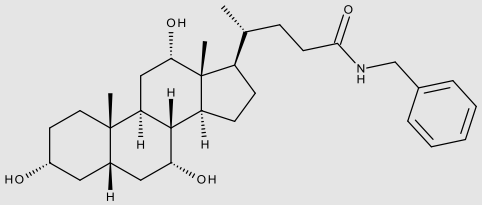
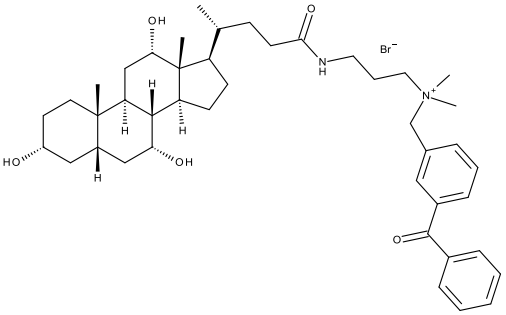
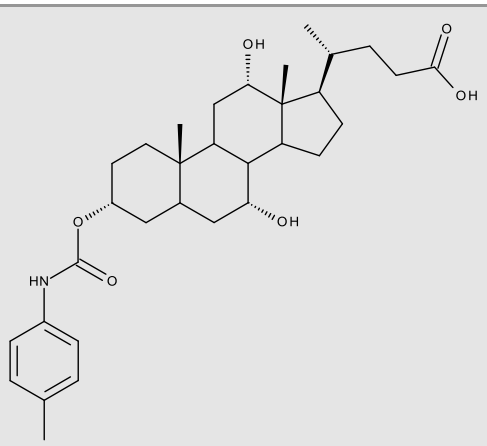
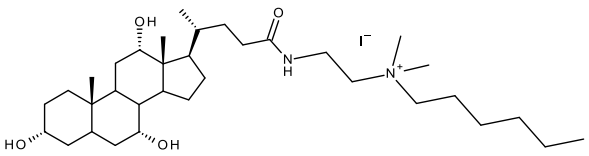
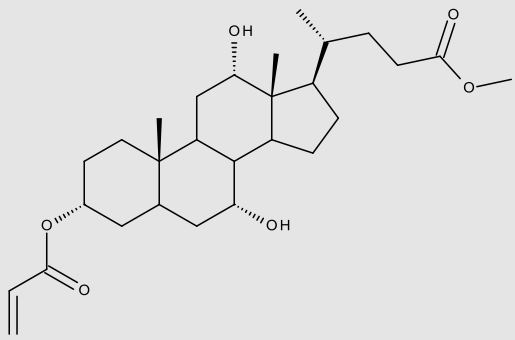
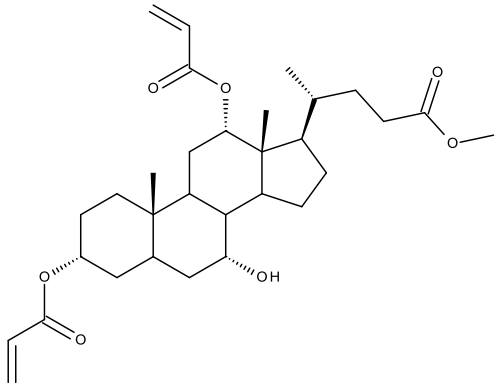
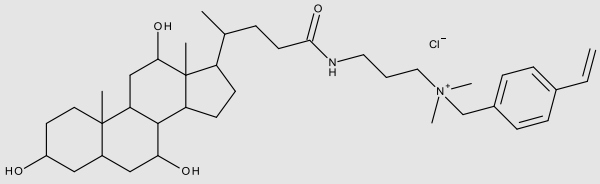
13	N-(1-phenyl-4-piperidyl) cholanamide	
7	N-[3-(dibutylamino)propyl] cholanamide	
2	cholic acid benzyl amide	
36		

Table 17 Table to show the name and structure of each cholic acid dervative tested for the germination of *C. difficile* spores

Some compounds made were not tested due to solubility issues or the yields were too low. These are shown in table 18.

Compound number	Compound name	Structure
93	3α methyl N-(p-tolyl)carbamate 7α 12α hydroxyl cholic acid	
29	3-cholanamidopropyl-hexyl-dimethyl-ammonium;iodide	
60	3α acryloyl-7α,12α dihydroxy cholic acid	
59	3α,12α diacryloyl-7αhydroxycholic acid	
66	3-acetamidopropyl-dimethyl-[(4-vinylphenyl)methyl] ammonium chloride	

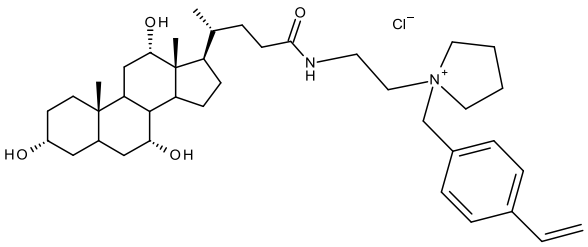
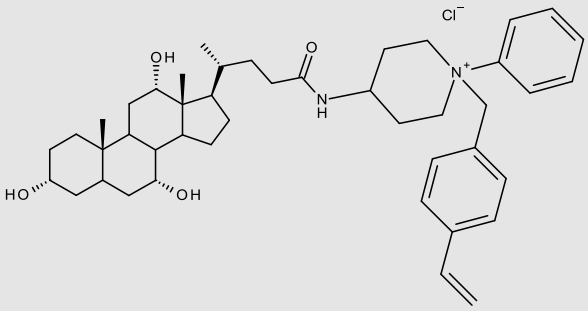
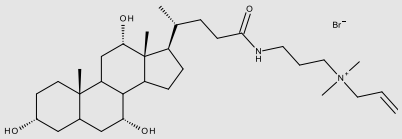
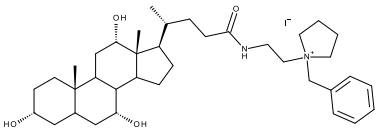
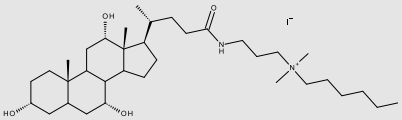
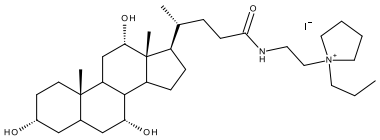
65	N-[2-[1-[(4-vinylphenyl) methyl] pyrrolidin-1-ium-1-yl]ethyl] cholanamide chloride	
67	N-[1-phenyl-1-[(4-vinylphenyl)methyl]piperidin-1-ium-4-yl]cholanamide chloride	

Table 18 Table to show the compounds not tested for germination activity due to low yields

5. GERMINATION RESULTS FOR STRAINS 11204 AND 027

Table 19 shows the results of the germination tests in terms of log reduction on the number of *C. difficile* vegetative cells. A log reduction on heat suggests that the compound is a germinant, whereas a log reduction on ice suggests a sporicide/ germinant-antimicrobial compound. A one log reduction is comparable to cholic acid.

Compound number	Compound name	Structure	> 1 log reduction on heat	> 1 log reduction on ice
34	3-cholan amidopropyl-allyl-dimethyl-ammonium bromide		yes	X
51	N-[2-[1-(3-phenylpropyl) pyrrolidin-1-ium-1-yl]ethyl] cholanamide bromide		yes	yes
29	3-cholan amidopropyl-hexyl-dimethyl-ammonium iodide		yes	x
46	N-[2-(1-propylpyrrolidin-1-ium-1-yl)ethyl] Cholanamide iodide		yes	yes

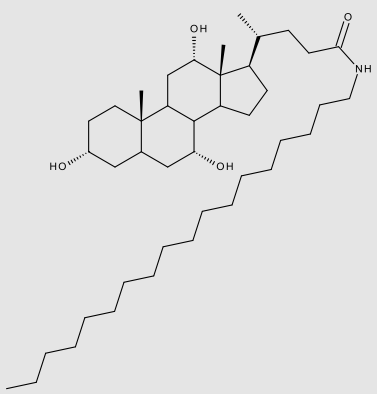
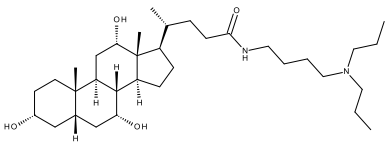
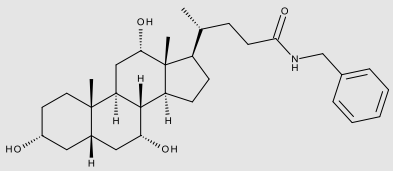
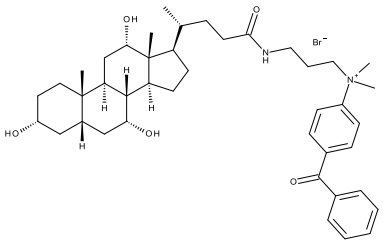
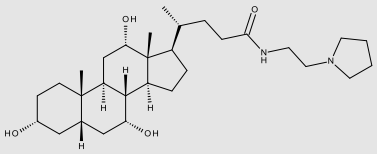
16	N-octadecyl cholanamide		Yes (11204 and 027)	x
7	N-[3-(dibutylamino)propyl] cholanamide		Yes (11204)	Yes (11204)
17	cholic acid benzyl amide		x	Yes (11204)
36			Yes (027)	x
10	N-(2-pyrrolidin-1-ylethyl) cholanamide		Yes (11204)	x

Table 19 Table to show the compounds which showed activity against *C.difficile* spores

5.1 Results of Germination tests

The results from table 19 show the compounds that gave a 1 log reduction or more between the initial spore count and the spore count after the heat and ice treatment. A reduction in the heat count suggests the compound is a germinant, whereas a reduction in the ice suggests a sporicidal or a germinant/ antimicrobial compound. Due to the nature of this test, it is difficult to distinguish between the two.

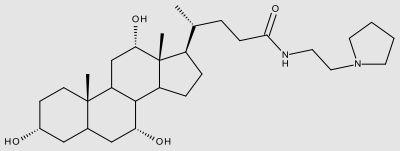
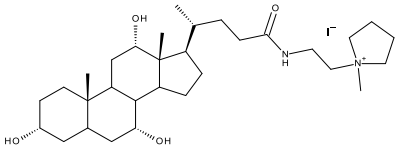
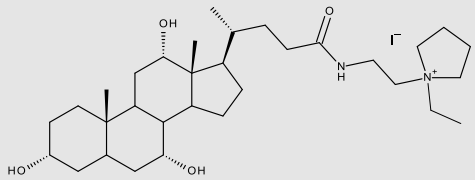
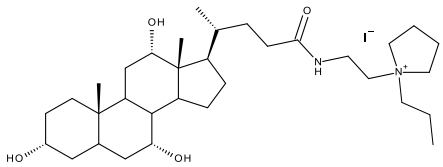
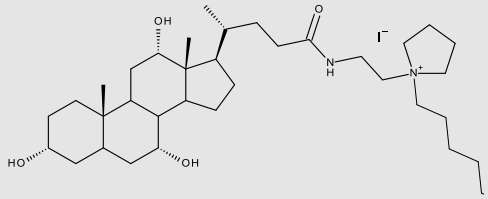
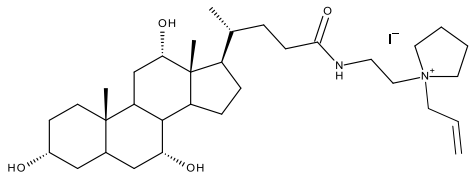
All ten of the compounds synthesised are novel, with three having terminating basic group. Previous research carried out with novel compounds and *C. difficile* spore germination is limited. Howerton et al. synthesised a range of compounds to test against taurocholate, however, these all terminated in an acidic group (Howerton et al., 2011). Other literature shows the importance of hydroxyl groups by either removing or adding more. Currently, there is no published research on compounds terminating in a basic group and their activity against *C. difficile* germination.

Nine of the compounds show germinating abilities on either the 11204 strain or the 027 strain. Looking at the structures, it is difficult to see a pattern between all of them. All of the side chains contain two or more carbons, with a maximum of four. The compounds can be grouped into two groups, however, compounds that contain rings and compounds that contain a 3-4 carbon side chain with two methyl groups.

Six of the germinating compounds feature a quaternary nitrogen in the side chain. This has similarities with the known good germinant, taurocholate, in that this compound has a sulphonate group in its side chain. A sulphonate and a quaternary ammonium group, although of opposite charge, are both polar and strongly electron-withdrawing.

By looking at the families of the compounds, we can also see some patterns emerging. Table 20 shows the germination activiety results for the N-(2-pyrrolidin-1-ylethyl) cholamide (10) compound family. The parent compound showed activity and two of the quaternised compounds, whereas the other four compounds didn't show any activity. The quaternised compounds that showed activity were the N-[2-(1-propyl[yrrolidin-1-ium-1-yl)ethyl]cholananamide iodide (46) and N-[2-[1-(3-phenylmethyl)pyrrolidin-1-ium-1-yl]ethyl]cholanamide bromide (51). Both of these compounds show activity during the heat and ice treatments, whereas the parent compound only shows activity during the heat treatment. The two quaternised compounds have a three carbon chain attached to the quaternary nitrogen. This indicates that this is the ideal lengh of chain for the compound to go

from being a germinant to being a germinant and antimicrobial/sporidical. What is unusal is that the compound N-[2-(1-allylpyrrolidine-1-ium-1yl)ethyl] cholanamide bromide also has a three carbon chain off the quaternary nitrogen but shows no activity against the spores. This suggests that the double bond present is having a negative affect on its germinating ability.

Compound number	**Compound**	**Inactive**	**Active**
10			✓
44		✓	
45		✓	
46			✓
49		✓	
50		✓	

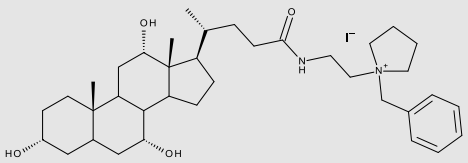
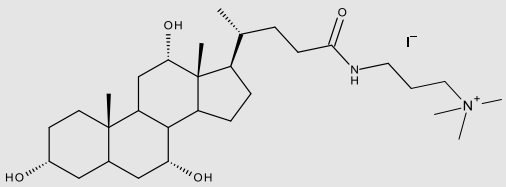
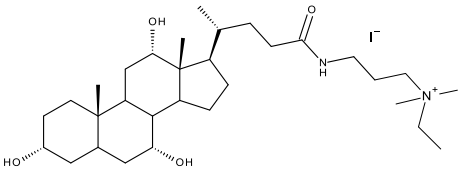
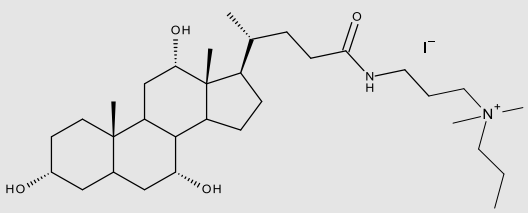
51		✓

Table 20 Table to show the activity of the N-(2-pyrrolidin-1-ylethyl) cholamide family of compounds

Table 21 shows the activity for the N-[3-(dimethylamino)propyl] cholamide (12) family of compounds. With this family it is harder to see a pattern. 3-Cholanamidopropyl-hexyl-dimethyl-ammonium iodide (28), 3-cholanamidopropyl-allyl-dimethyl-ammonium bromide (34) and 3-cholamidopropyl-cyclopentyl-dimethyl-ammonium bromide (36) all showed activity during heat treatment. The shapes and properties of these compounds are all very different. One has a five carbon chain attached to the quaternary nitrogen, one has a three carbon chain and the other has a benzophenone group. Taken in isolation as a family of compounds, there is slight pattern to why these three compounds should all germinate *C.difficile*. The terminal dimethyl ammonium group has to be longer than the propyl chain but smaller than the benzophenone group in order to be active towards the cells.

Compound number	**Compound**	**Inactive**	**Active**
24			✓
25			✓
27			✓

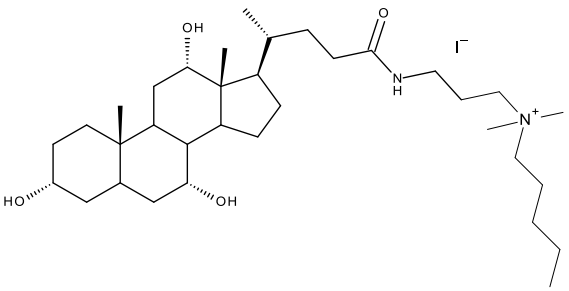
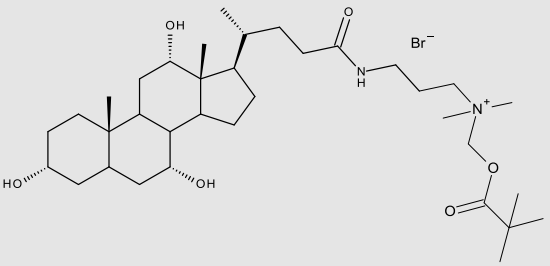
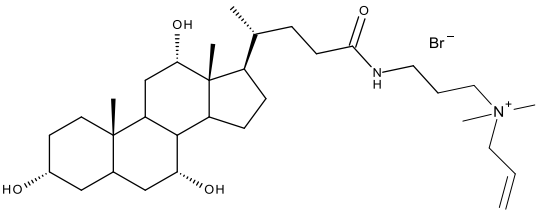
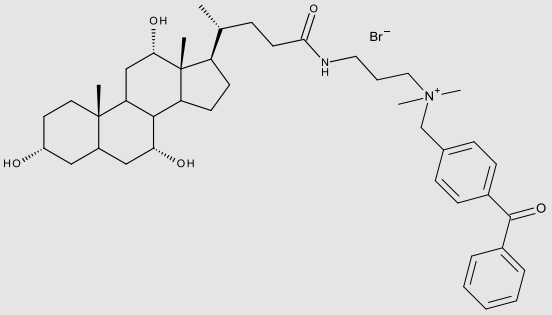
28		✓
32		✓
34		✓
36		✓

Table 21 A table to show the activity for the N-[3-(dimethylamino) propyl] cholamide family of compounds

Table 22 shows the N-(2-dimethylaminoethyl) cholamide (21) compound family that showed no activity in either the heat or the ice treatments. This family of compounds is interesting as it is very similar to the N-[3-(dimethylamino)propyl] cholamide (12) family of compounds. The difference between the two families is the carbon chain length of the amide. The N-[3-(dimethylamino)propyl] cholamide (12) has a three carbon chain length whereas the N-(2-dimethylaminoethyl) cholamide (21) has a two carbon chain length. By shortening the amide chain length by one carbon, all germination activity has been lost. This suggests that the optimum amide chain length for *C.difficile* germination is three carbons.

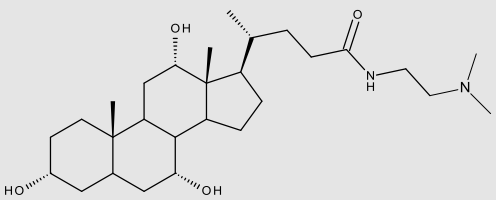
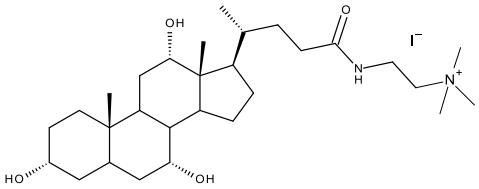
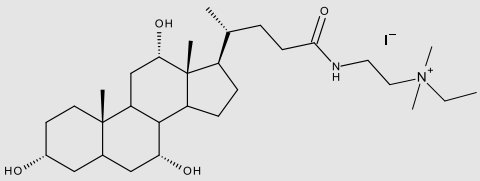
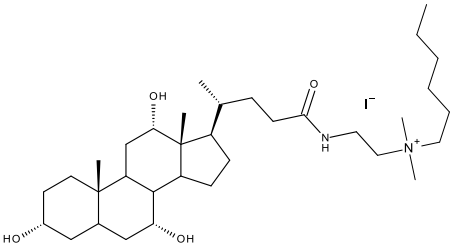
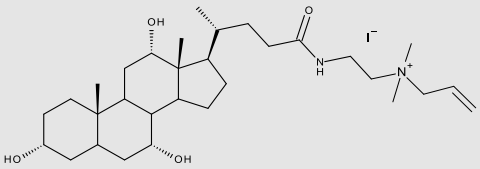
Compound number	Compound	Inactive	Active
21			✓
37			✓
38			✓
42			✓
43			✓

Table 22 A table to show the activity for the N-(2-dimethylaminoethyl) cholamide family of compounds

Table 23 also shows a family of compounds that do not show any germinating abilities. The family of 1-(3-phenylimidazolilidin-1-yl) cholanone (14) compounds is very different to the other compound families. They are a lot bulkier with much more steric hinderence around the quaternary nitrogen center than the other compound families. By looking at the families of compounds and comparing their germinating abilities, it is possible to conclude that the amide chain is very important to the germinating ablity of the compounds, as well as the length of the quaternised chain.

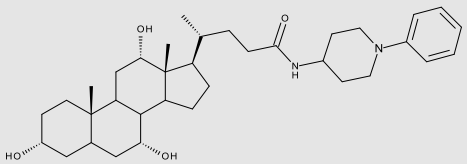
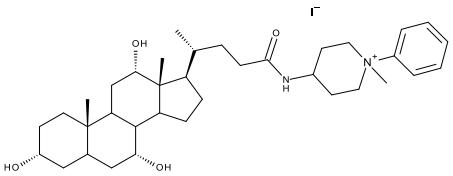
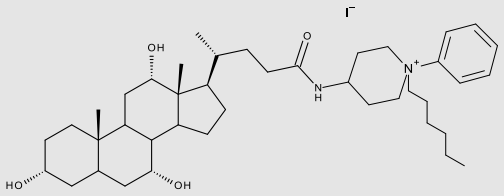
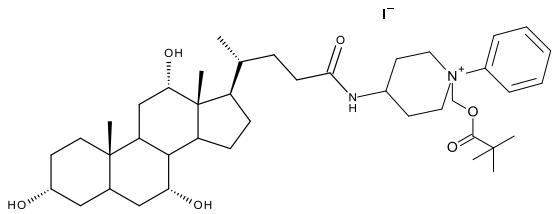
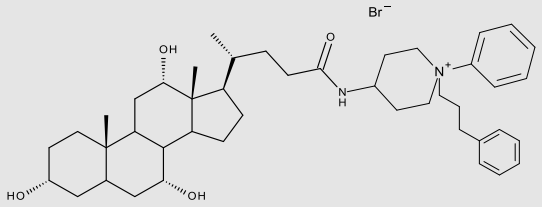
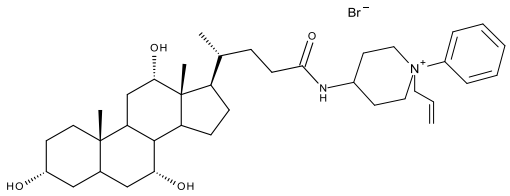
Compound number	Compound	Inactive	Active
14			✓
54			✓
58			✓
60			✓
61			✓
62			✓

Table 23 A table to show the activity for the 1-(3-phenylimidazolilidin-1-yl) cholanone family of compounds

By grouping the compounds into families, small patterns start to emerge. By taking the compounds individually and comparing them to work carried out on other spore germinating compounds, more patterns can be seen.

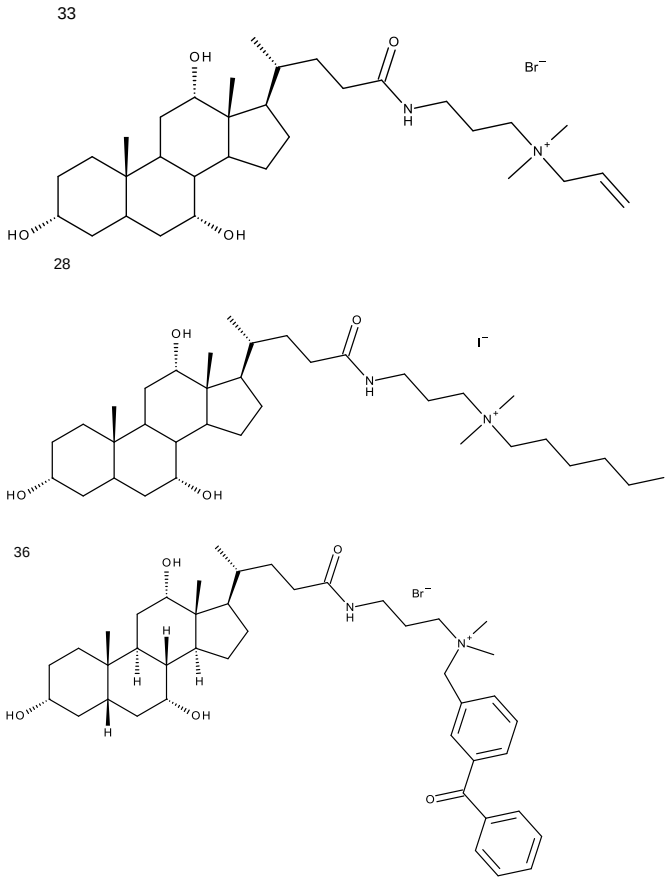


Figure 20 Compounds 33 (top), 28 (middle) and 36 (bottom) from the N-[3-(dimethylamino)propyl] cholamide (12) family

The diaminopropane cholate derivatives are all quaternised and all show reductions on heat treatment, suggesting they are all germinants. Even though the three compounds are quaternised, the postive charge on compounds 33, 28 and 26 do not have any antimicrobial/ sporicidal activity. The size of the compound 36 is large, which makes it unusual as compounds generally have to be small in order to penetrate the spore (Leggett *et al*., 2012). The length of the side chain in these three compounds and their germinating abilities disagrees with the conclusions drawn by a paper by Howerton *et al*. They state that lengthening the side chain is detrimental to the compound's germinating abilities. The compounds synthesised by that group, however, all terminated in either a carboxylic acid group or a sulphonic acid group. These groups could be responsible for the decrease in germinating abilities of their compounds (Howerton *et al*., 2011).

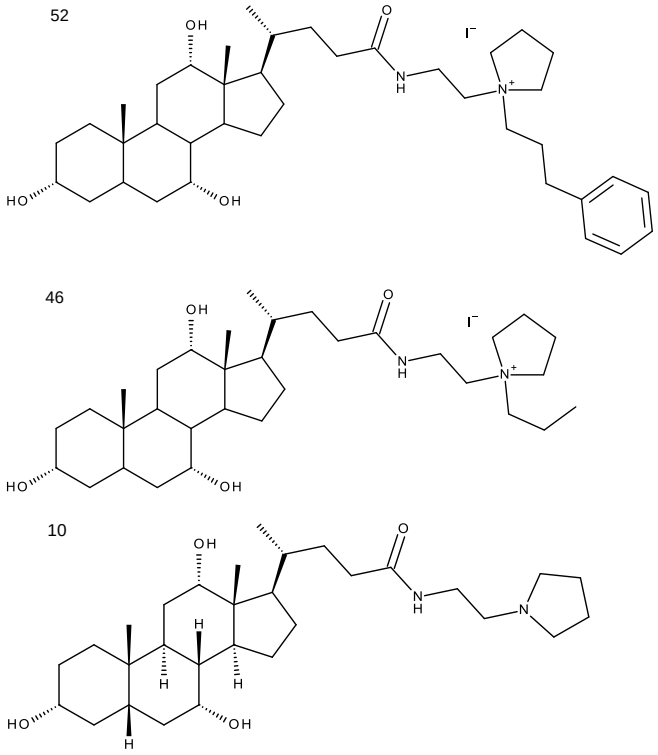


Figure 21 compounds 52 (top), 46 (middle) and 10 (bottom) from the N-(2-pyrrolidin-1-ylethyl) cholamide compound family

The 1-(2-aminoethyl) pyrrolidine cholate derivatives are interesting because the two quaternised compounds show antimicrobial/ sporicidal activity whereas the parent compound only shows germinating abilities. If the parent compound is able to germinate the spore, it is likely that the quaternised versions are acting as antimicrobials, rather than sporicides, but more analysis of these compounds will need to be done to confirm this. The compounds also have a two- carbon chain in the side chain, which is the same as taurocholate. The significance of the length of the chain is interesting as it correlates with the conclusions drawn by Howerton *et al*, where they investigated the effects of modifying taurocholate. One of their conclusions was that altering the length of the ethyl side chain was detrimental to the compound's germinating abilities (Howerton *et al*., 2011). Whilst this set of compounds agrees with Howerton et al, compounds 28, 33 and 36 have a longer side chain, suggesting that the length of the side chain is not as important as first thought.

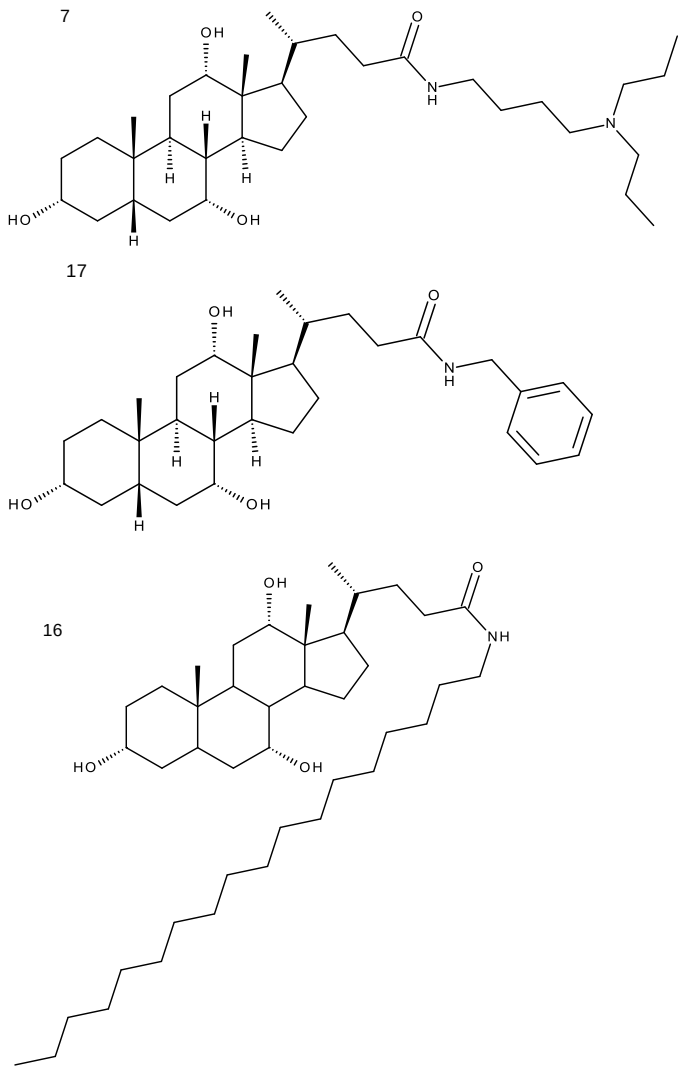


Figure 22 Compound 7 (top) and 17 (middle) and 16 (bottom) which are all unquaternised but show activity against C.difficle

Compound 7 (figure 23) has the longest carbon side chain and has an electron withdrawing group. As there is no positive charge on this compound, therefore, no antimicrobial element, it is easy to jump to the conclusion that this compound is a sporicide. However, as Howerton's works suggests, a positive ammonium is not needed to make the compound antimicrobial. This is also suggestive of compound 17 Figure 23), which, again, has no quaternary ammonium. More tests will need to be carried out in order to see whether it is a sporicide or a germinating and antimicrobial compound. The size of these two compounds and their activity disagrees with the conclusions reached by Howerton *et al*. They found bulkier linkages decreased the germinating abilities of their compounds, possibly because of the size of the spore's active site. So far, all of the work carried out on *C.difficile* germination has stated that the germinant's hydrogen bond donating ability is essential for spore germination. In this

case, there may be some unknown interactions taking place between the spore and germinant, which triggers germination.

Compound 16 is the largest compound and also shows the largest log reductions of all of the compounds. The size of this compound makes it unusual as, compared to the other compounds in this group, it seems too big to fit into an active site to trigger germination. The octadecyl chain in this compound makes this compound very lipophilic and it may be that hydrophobic interactions are important for its interactions with the spore coat and the putative germination site. Another example of hydrophobic interactions and antimicrobial activity can be seen in the work carried out by Kikuchi *et al* with squalamine mimics. Here, they found that the hydrophobicity of the steroid backbone and the basicity of the side chain were the determining factors in its antimicrobial activity. Whilst they were not looking at spore forming bacteria, they did find that the squalamine mimics had antimicrobial activity against gram- positive and gram negative bacteria (Kikuchi *et al*., 1997). This finding supports the finding of this research as some of the non-quaternised compounds have shown antimicrobial activity.

The shape of the side chains of these compounds are very different. Without knowing the active sites of *C.difficile* germination, it is difficult to see a pattern within them. Once the germination active sites have been identified, it will be possible to examine these compounds using molecular modelling software to see how they fit and activate the site.

By comparing these results to the limited work concerning *C.difficile* spore germination, differences in conclusions can be seen. The optimal length of the side chain during this research is between two and four carbons, whereas previous research has shown it to be two carbons long. The hydrogen bond donating ability of the compounds is also important, however, this research has shown compounds with germinating ability and hydrogen bond acceptor groups. All of the research so far agrees that the three hydroxyl groups are essential for germination. Changes to these groups are detrimental to the compounds' germinating abilities, even when the hydroxyl group is kept as a hydrogen bond donator. Overall, without knowing the exact mechanism for *C.difficile* spore germination, it is difficult to know what interactions are occurring between the spores and the germinants. Whilst this research shows ten novel spore germinants, more research is needed in order to understand the essential elements of the germinants.

6. CONCLUSIONS

Once optimised, amidation of the carboxyl of cholic acid using ethylchloroformate was easily achievable. A range of novel amide derivatives have been prepared in good yields. Quaternisation of cholic acid derivatives containing tertiary amino groups was also straightforward. The use of alkyl halides for the quaternisation of cholic acid derivatives containing tertiary amino groups proceeded straightforwardly for the most part. It remains a mystery, however, that 1-iodobutane and 1-iodopentane were unreactive under the standard conditions.

Polymerisation of the quaternary cholic acid derivatives was problematic. The synthesis of copolymers using free radical polymerisations of the quanternised vinyl benzyl compounds and another monomer were unsuccessful. By examining other free radical polymerisations, e.g. the polyureathane synthesis, it is possible to reach the conclusion that the positive charge on the cholic acid derivative monomer slowed the reaction time to such an extent that the derivatives were not incorporated into the polymer. This could be seen with proton NMR analysis of the soluble polymers. Free radical polymerisation of the cholic acid derivatives without another monomer synthesised very low yields of an insoluble polymer. Longer reaction times and varying the temperature of the reaction appeared to make little difference. As the polymers were insoluble, they were hard to analyse by NMR. The insolubility of the product suggests that a polymer did form; however, due to the difficulties experienced in analysing it, it is difficult to say with any certainty what the product is. While the literature shows that VBC based quaternary ammonium compounds can be polymerised, they are generally smaller compounds with little steric hindrance.

Selective reaction on the hydroxyl groups of cholic acid was achievable through temperature control. A low temperature slows down the reaction so that monitoring the reactions progress by TLC is achievable. The reactivity of the hydroxyl groups towards methyacryloyl chloride is C3-OH>C12-OH>C7-OH. This is confirmed by proton NMR analysis and the shifts of the C3, C7 and C12 hydrogens.

Protection and selective removal of acetate groups on the hydroxyl groups of cholic acid and methyl cholate was achievable but required monitoring of the reactions progress.

Synthesis of cholic acid polyureathanes was successful, however, it was not successful with quaternised cholic acid derivatives. The unsuccessful attempts of polymerising the quaternised compounds with both free radical polymerisation and isocyanate polymerisation gives weight

to the theory that the positive charge is affecting the reaction rate of the compound. An advantage of synthesising polyurethanes is that they give a degree of flexibility over the desired properties of the polymer, such as solubility and stiffness.

The UV free radical activation method for the polymerisation of cholic acid derivatives was unsuccessful. UV irradiation did not seem to initiate the radical in order for the reaction to start. This could be due to the strength of the UV bulb or due to the steric hindrance of the compounds.

A number of compounds have shown germinating ability against two different strains of *C.difficile*. The ability of these compounds to reduce the number of viable *C.difficile* spores by 99% (2 log reductions) is a step forward in understanding why *C.difficile* germinates. Given the limited data available on *C.difficile* germination, which mainly concerns taurocholic acid, new compounds that show germinating activity increases the knowledge of germination greatly. As the number of CDI cases plateau, the importance of how *C.difficile* germinates becomes more important.

Due to the properties of the compounds made, the standard test of germination using water was not possible. A novel method of using DMSO in water allowed the compounds to be tested without affecting the result.

These results do show that modifying the hydroxyl groups on cholic acid has a negative effect on germination, which is in line with the literature.

7. FUTURE WORK

One of the main aims in this research was to synthesis a polymer surface in order to force the germination of *C. difficile* spores upon contact. This proved to be very difficult. Furhter optimisation of polymerising quaternary compounds is needed for the research to progress. The quaternary ammonium element of the compound slowed down the reaction rate of the free radical polymerisation to a point where very little of the cholic acid derivative was incorporated. More success has been found in the literature where the polymers have been synthesised first, before then the nitrogen present was quaternised. This could be a new avenue to explore as the rate of reactions with a non quaternised cholic acid derivative should be similar to the reaction rate of a monomer. The polyureathane method of synthesising polymers showed promise and will need developing. The synthesis of the polyureathanes allow for greater flexibility of the desired properties, meaning more polymers could be made. However, the same issues arose when it came to trying to polymerise quaternised derivatives of cholic acid. By optimising the polymerisation synthesis, a wider range of polymers can be made, although this may require the expertise of a polymer engineer.

Another one of the aims was to increase the knowledge of *C. difficile* spore germination by creating a large library of compounds and testing their germinating abilities, in order to gain more information about the germination of the *C.difficile* spores. The germination tests carried out in this study begin to show patterns emerging in some of the compound “families”. By creating a larger library of compounds relating to those already tested, and with different bile acids, a more complete picture of the germination of *C.difficile* spores can be made.

Due to the nature of the testing carried out on the compounds, it is difficult to establish whether a reduction in the ice numbers is due to a germinating and antimicrobial effect or a sporicidal effect. In order to establish this, the compounds will need to be tested against another spore forming bacteria. It if shows activity under ice conditions, then the activity of the compound can be said to be sporicidal. It the compounds show no or little activity against the cells, and then we can assume the compounds show specific effects on *C.difficile*.

In order for the compounds of interest to be used in a health care setting, they need to be tested for their toxicity against human cells. The types of cells used depend on whether the compounds are loose of incorporated into a polymer surface. The toxicity testing becomes more important if the compounds are going to be used as a disinfectant spray or solution. In

this situation, the compounds will have to be tested against a variety of cell lines as the risk of ingestion is higher.

The toxicity testing of a polymer surface will be different as the risk of ingestion of the compounds is low. In this case, toxicity against human epithelial cells may be sufficient. In order for the compounds to be tested, it is likely that more of the compounds will have to be made.

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