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THE DESIGN, SYNTHESIS AND BIOLOGICAL EFFECTS OF NOVEL STEROID PRODRUGS FOR TREATMENT OF GASTROINTESTINAL DISEASES

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B.Sc.

A thesis presented to the University of Dublin

for the degree of Doctor of Philosophy

Based on research carried out under the supervision of

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B.A. (Mod.), Ph.D

at the

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Trinity College Dublin

2008

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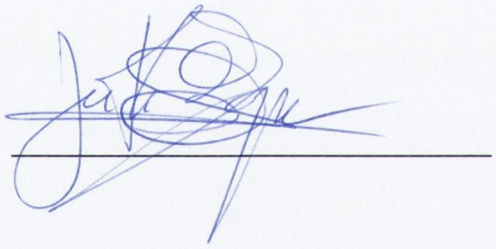

A mis padres

ABSTRACT

The aim of the work described in this thesis was to synthesize and test the biological effects of a novel type of prodrug to deliver steroids to the colon for the treatment of gastrointestinal diseases. Glucocorticoids are used as anti-inflammatory agents in the treatment of inflammatory bowel disease. Unfortunately they undergo absorption from the GIT before reaching the colon and, as a consequence, a high number of systemic side effects are caused in patients. The prodrugs reported here are completely novel and are designed to reach the colon and exploit azoreductase and nitroreductase activity of colonic microflora to release the steroid there.

Prednisolone and hydrocortisone have hydroxyl groups at positions 11, 17 and 21. Due to the different reactivity of these hydroxyl groups position 21 is favoured to undergo an esterification/lactamization. The proposed compounds have an azo group which is designed to undergo reduction in the colon liberating an anilide poised to undergo cyclization at an adjacent ester releasing the steroid. Chapter 2 describes synthetic studies directed towards candidate compounds and models for kinetic studies. Chapter 3 describes *in vitro* kinetic studies on the feasibility of the prodrugs and proof of concept studies in a clinically relevant model of colitis. The kinetic studies revealed that once they have been reduced by the colonic microflora, the cyclization half-life varies between 1.3 and 2.4 hours in aqueous conditions at pH values relevant to the colon. Studies in aqueous solution indicated a moderate stability of prodrugs at pH 7.4. However, an analogous *nitro*prodrug was unexpectedly hydrolyzed at pH 7.4 within two hours. Studies carried out with different strains of *Clostridium perfringes* under anaerobic conditions, revealed that the prodrugs are substrates for human colonic microflora. Colitis mice model studies carried out with the prodrugs demonstrated anti-inflammatory potential for the treatment of IBD and a statistically significant reduction in thymus atrophy compared with prednisolone, probably indicating lower systemic availability. Plasma hydrolysis studies showed that the compounds are good substrates for mouse plasma esterase and would undergo rapid hydrolysis in the mouse if absorbed. A CACO-2 cell model was used to carry out transport studies, which revealed absorption for prednisolone but only secretory transport for the prodrugs tested.

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ABBREVIATIONS

AA	Arachidonic acid
AcOH	Acetic acid
AP	Apical side
5-ASA	5-Aminosalicylic acid or mesalazine
BHI	brain heart infusion
BL	Basolateral side
BOC	*tert*-Butyl carbonyl protecting group
BOC_2O	Di-*tert*-butyl dicarbonate
Bz_2O	Dibenzyl ether
BzBr	Benzyl bromide
CD	Crohn's disease
CFU	Colony forming unit
CMPI	2-Chloro-1-methylpyridium iodide
COSY	Correlation spectroscopy
COX	Cyclooxygenase
DCC	Dicyclohexylcarbodiimide
DBU	Diaza-(1, 3)-bicyclo [5.4.0] undecane
DCM	Dichloromethane
DCU	Dicyclohexyl urea
DIAD	Diisopropyl azodicarboxylate
DMAP	4-Dimethylaminopyridine
DMSO	Dimethyl sulfoxide
DSS	Dextran sodium sulfate
EI	Electron impact
EtOAc	Ethyl acetate

EtOH	Ethanol
GCC	Glucocorticoids
GIT	Gastrointestinal tract
HBSS	Hank's balanced salt solution
HMBC	Heteronuclear multiple bond correlation
HOBt	Hydroxybenzotriazole
HRMS	High resolution mass spectroscopy
IBD	Inflammatory bowel disease
IR	Infrared spectroscopy
K_{obs}	Observed constant of cyclization
M.p.	Melting point
MeOH	Methanol
Mol. wt.	Molecular weight
MPO	myeloperoxydase activity
MS	Mass spectroscopy
MTS	methyl tetrazolium salt
NaOAc	Sodium acetate
NMR	Nuclear magnetic resonance spectroscopy
NSAIDS	Non steroid anti-inflammatory drugs
PABA	*para*-Aminobenzoic acid
PBS	Phosphate buffer solution
PDA UV	Photo diode array ultraviolet
Pd/C	Palladium on activated carbon
PFP	Pentafluorphenol
PG	Protecting group
PGG_2	Protaglandin G_2
PGHS	Prostaglandin H synthase

PLA2	Phospholipase A2
RPM	Revolution per minutes
SA	Salicylic acid
TBAF	Tetrabutyl ammonium fluoride
t-Bu	tert-butyl group
TEER	Trans epithelial electric resistance
TFA	Trifluoro acetic acid
TFAA	Trifluoro acetic acid anhydride
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TxA_2	Thromboxane A_2
UC	Ulcerative colitis
UV	Ultraviolet Spectroscopy

CHAPTER ONE

GENERAL OVERVIEW OF IBD AND PRODRUGS TO COLON DELIVERY

1.1 Introduction

Inflammatory bowel disease (IBD) refers to a variety of inflammatory chronic disorders of unknown cause, which involve a longer or shorter segment of the small intestine and/or colon. Once the symptoms manifest in the patient they can spontaneously appear and disappear at intervals. IBD includes ulcerative colitis (UC) and Crohn's disease (CD) mainly and in 90 % of the cases UC and/or CD diagnosis can be established. In the remaining 10% of the IBD cases diagnosis of UC or CD is not possible; in these situations it is said the cause of the IBD is an indeterminate colitis.

Although IBD pathogenesis is complex and several factors are involved, certain evidence indicates that in its development two phases are involved. The first is an initial strong inflammatory crisis after an aggression or attack over the intestinal mucosa, followed by the second phase of immunological nature which would be responsible for the chronic character of the illness. This second phase seems to be due to an excess immune response, determined genetically, over several luminal antigens, including intestinal microflora (Fiocchi, 1998) (Bouma and Strober, 2003).

An alteration in the epithelial wall function might cause an inappropriate immune response to antigens. A perfect working intestinal mucosa wall is essential to avoid penetration of micro-organisms, bacterial toxins and alimentation antigens through the epithelial walls, that is why alteration in the function could be of vital importance to the chronic development of the process (Panés, 2001).

1.2 Ulcerative colitis

UC was first recognized as a disease entity about 100 years ago, when Wilks and Moxon differentiated it from the infective dysenteries and other diseases which may produce inflammation and ulceration of the colonic mucosa (Wilks and Moxon, 1889).

Although medical treatment has greatly improved during recent decades, it is still far from perfect and in many patients the disease cannot be controlled by medical means. About one in every four or five patients needs to be treated

by radical surgery, usually removal of the entire large bowel (proctocolectomy) with the institution of a permanent ileostomy (Truelove, 1984).

UC is one of the most dangerous diseases of the gastrointestinal tract. The term "ulcerative colitis" is applied to a disease in which a part or the whole of the mucosa of the large bowel becomes diffusely inflamed with a haemorrhagic type of inflammation, which may progress to ulceration. In UC the rectum is in practical terms constantly inflamed and from there the inflammation might spread to the proximal colon and a portion of it or the entire colon might be affected, but always continuously.

The result of this pathological process is that the patient is prone to bleeding per rectum and to diarrhoea, abdominal pain, fever and weight loss. If the inflammation is widespread and severe, the patient suffers from severe bloody diarrhoea and becomes very ill (Rizzello et al., 2002). The disease typically manifests itself in attacks with interposed periods of freedom from symptoms. In addition to the above mentioned symptoms the patients could present with ophthalmologic, skin and joints problems due to the spread of the inflammation to other organs; this process is known as extra-intestinal manifestations.

UC epidemiology occurs all over the world but there are enormous variations in its prevalence. It is most common in the USA, Canada, UK, Scandinavia and other countries of Northern Europe, where the prevalence is about 1 in 1,000-1,500 of the population. The disease is undoubtedly uncommon in Japan although it is currently becoming more prevalent.

In South Africa, the white population suffers from it much the same way as in Europe, but the black population is almost immune. By contrast, UC is far from uncommon in the black population of the USA, which suggests that environmental factors may be more important than genetic. However, in one of the few formal epidemiological studies to be made, the black population of Baltimore was found to have a much lower incidence of the disease than the white population (Truelove, 1984).

1.3 Crohn's disease

Sir Kennedy Dalziel, a Scottish surgeon, was one of the first people to describe what would later come to be known as Crohn's disease. In 1913, he published a paper (Dalziel, 1913) about nine patients with intestinal inflammation with the typical features. However, Crohn's disease is named after the late Dr. Burrill Crohn. He and his colleagues at the Mount Sinai Medical Center in New York City re-emphasized Dalziel's findings in a series of papers in 1932 (Crohn's et al., 1932).

CD is a disorder that can affect any segment of the digestive tube. The gastrointestinal tract becomes thickened, inflamed and swollen. The thickening may lead to narrowing of the hollow digestive tube in that area. Segments of Crohn's inflammation may involve only a few centimetres of the intestine or may be much longer, (Weterman, 1983) over a metre or more. Any part of the tract from the mouth to the back passage can be affected and there may be more than one area of involvement at any one time. The most common site of involvement is the lower ileum, but all or part of the colon may be affected, either alone or with the adjacent ileum. Inflammation in or around the anus is also common. This may take the form of fissures (ulcerated cracks) in the skin of the anal canal, fistulae (small openings discharging pus) around the anus, or tags (swollen but often painless lumps) just outside the anus. Depending on the site of involvement, the intestinal inflammation of CD usually produces abdominal pain and diarrhoea, though sometimes narrowing of the ileum causes some obstruction to onward passage of wastes with episodes of vomiting and constipation. Bleeding may accompany diarrhoea in patients with CD of the colon or rectum. Patients with active CD commonly feel tired and lethargic, and they may run a fever. Thinning of the blood (anaemia) contributes to the tiredness. Sometimes the anaemia, like the fever, just reflects the presence of an inflamed intestine, and will only improve when the CD itself settles spontaneously or with medical or surgical treatment. Disease around the anus is frequently painless unless a local abscess develops (Anagnostides et al., 1991).

Patients with CD can suffer extra intestinal manifestation and ophthalmologic, skin and joints problems will appear. A few other patients

develop inflammation in the liver, but this is usually recognized by blood test rather than symptoms.

Around 1,000,000 people in North America suffer the disease and there are no significant differences between males and females. It can affect people from all ages although it is more common in people under thirty years of age; However a small portion of the affected population suffer the illness between fifty and seventy years of age (Brahme et al., 1975).

1.4 Morphologic characteristic of UC and CD

A few pictures from both diseases describe the nature of UC and CD in different patients at different ages and different parts of the GIT.

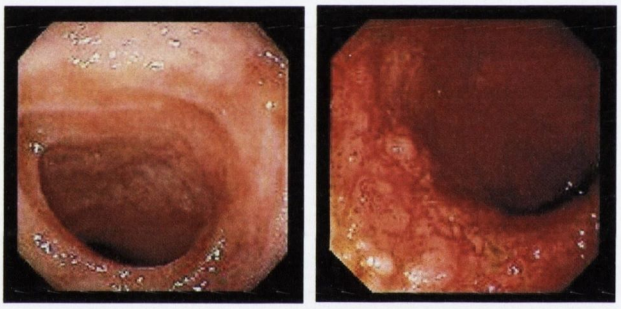


Figure 1.1. CD mucosal features: Left: Normal ileum, right: Mucosal inflammation causes redness, friability (ease of bleeding).

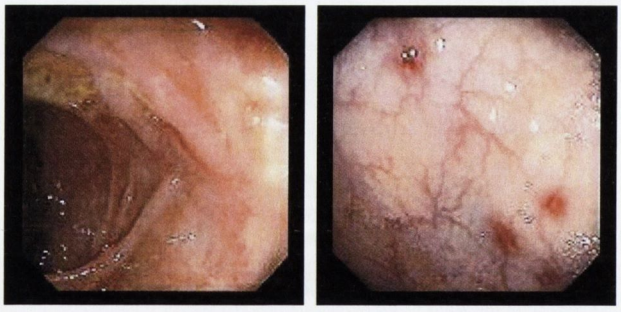


Figure 1.2. Left: The ileum appears normal, right: As commonly seen in CD, there is minimal involvement of the rectum.

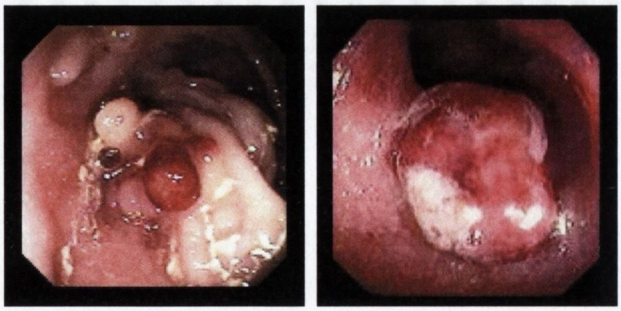


Figure 1.3. Left: Colonic view of the site of a jejunal-colic fistula in a 39 year-old woman with longstanding CD, right: UC the repeated cycle of ulceration, alternating with the deposition of granulation tissue during the healing phase, results in the development of raised areas of inflamed tissue.

To sum up all the differences between the two main diseases of IBD a table with the specific main points on UC and CD is shown next in order to be able to compare their main points:

ULCERATIVE COLITIS	*CROHN'S DISEASE*
Fistulae non frequent	Fistulae frequent
Rectum almost always affected	Rectum generally not affected
Colonic involvement	Involvement from mouth to anus
Continuous	Discontinuous
Diffusion	Segments
Symmetric	Asymmetric
Only mucosa is affected	All wall layers affected

Table 1.1. Ulcerative colitis and Crohn's disease differences

1.5 Treatment of ulcerative colitis and Crohn's disease

As the cause of IBD is unknown (Podolskiy, 2002), (Shanahan, 2002), (Medina et al., 1998), medical treatment has been forced to evolve by a process of trial and error. Fortunately, there have been many controlled

therapeutic trials in this disease and these have defined with considerable precision the uses and limitations of the various agents currently available. There is no medical cure for the disease although most patients can be managed satisfactorily throughout life by medical means; a minority (perhaps about one in every four or five) will require to be treated by radical surgery at some stage in their illness. Only two types of specific treatment have been shown to exert a major influence for induction and maintenance of remission (Cohen et al., 2000) (Rizzello et al., 2003). These are 5-aminosalicylic acid (5-ASA) and glucocorticoids (GCC). Apart from that there are other treatments which are utilized and they are used in the cases where 5-ASA or GCC cannot be used. These drugs are immunosuppressive drugs as such azathioprine, which is used as a maintenance therapy in patients where it is desirable to avoid surgery, 6-mercaptopurin, methotrexate and cyclosporine; biological drugs as infliximab; and finally antibiotics such as metronidazole and ciprofloxacin (O'Morain, 1991).

Combined oral and topical corticosteroid therapy is more effective than either of them alone. Also intravenous administration of water soluble corticosteroids has been helpful in severe attacks. 5-ASA or its prodrug, combined with therapy from corticoids has been proved useful. Mild attacks of colitis are treated by prednisolone in oral dosage of 5 mg four times per day and sulfasalazine (a 5-ASA prodrug) 0.5 g four times per day and topical corticosteroid therapy at night. Moderate attacks are treated by prednisolone orally 10 mg twice daily and topical corticosteroid at night. In patients with severe attacks they can only drink water and prednisolone is administered intravenously in a daily dose of 40 to 60 mg. Antibiotics could be added to the treatment in 1 g intravenous dose and a rectal retention enema of corticoids is used as well. Corticoids are not useful in preventing relapses of the diseases (Schweiz, 1981).

1.5.1 Mesalazine or 5-ASA

Mesalazine or 5-aminosalicylic acid 5-ASA is a salicylic acid derivative (*Figure 1.1*) which has got anti-inflammatory properties and is being used world-wide alone or as a prodrug for the treatment of IBD.

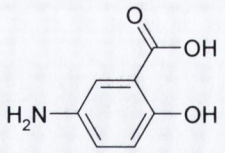


Figure 1.4. 5-Aminosalicylic acid or mesalazine

1.5.2 5-ASA mechanism of action

The mechanism of inflammatory relief of non steroid anti-inflammatory drugs (NSAIDS) was discovered by Dr. John R. Vane in 1971; Vane's theory won him the Nobel Prize for medicine in 1982. He postulated that NSAIDS prevented the enzymatic conversion of arachidonic acid (AA) into prostaglandins (Vane, 1971), molecules that had been found in high concentrations in inflamed tissues of both rats and humans. The enzyme in question is prostaglandin H synthase (PGHS), which has a cyclooxygenase (COX) active site that catalyses the bis-oxygenation of AA into the unstable prostaglandin G_2 (PGG_2) and a peroxidase active site that catalyses the conversion of PGG2 to the more stable prostaglandin H. NSAIDS act on the COX active site of PGHS by competitively or covalently preventing AA from interacting with the necessary residues for catalysis (*Figure 1.5*).

NSAIDS are far more potent inhibitors of PGG_2 production than 5-ASA but they are not effective in the treatment of IBD because they tend to cause intestinal bleeding (Punchard et al., 1992). The increase in the PGG_2 production by the inflamed bowel on removal of 5-ASA treatment suggested that the 5-ASA mechanism of action is also through inhibition of the COX enzyme as suggested in studies carried out by (Rampton et al., 1980). In a model of colitis treated with 5-ASA and inhibitors of thromboxane A_2 (TxA_2) both drugs were equally effective anti-inflammatory agents, suggesting that the anti-inflammatory properties might be through inhibition of TxA_2 production (Vilaseca et al., 1990). There is yet insufficient information on the properties of 5-ASA to suggest what might be a likely mechanism of action. An important factor on the anti-inflammatory action of 5-ASA (or mesalazine) is the concentration of the drug on the local mucosa. Recent studies have shown that all post-operative IBD patients who had 5-ASA levels lower than 20 ng/mg of tissue underwent inflammation again, however

when 5-ASA levels went up to 100 ng/mg of tissue, inflammation was not observed at all (Frieri et al., 1999) so anti-IBD activity is correlated to the mesalazine concentration at the local mucosa.

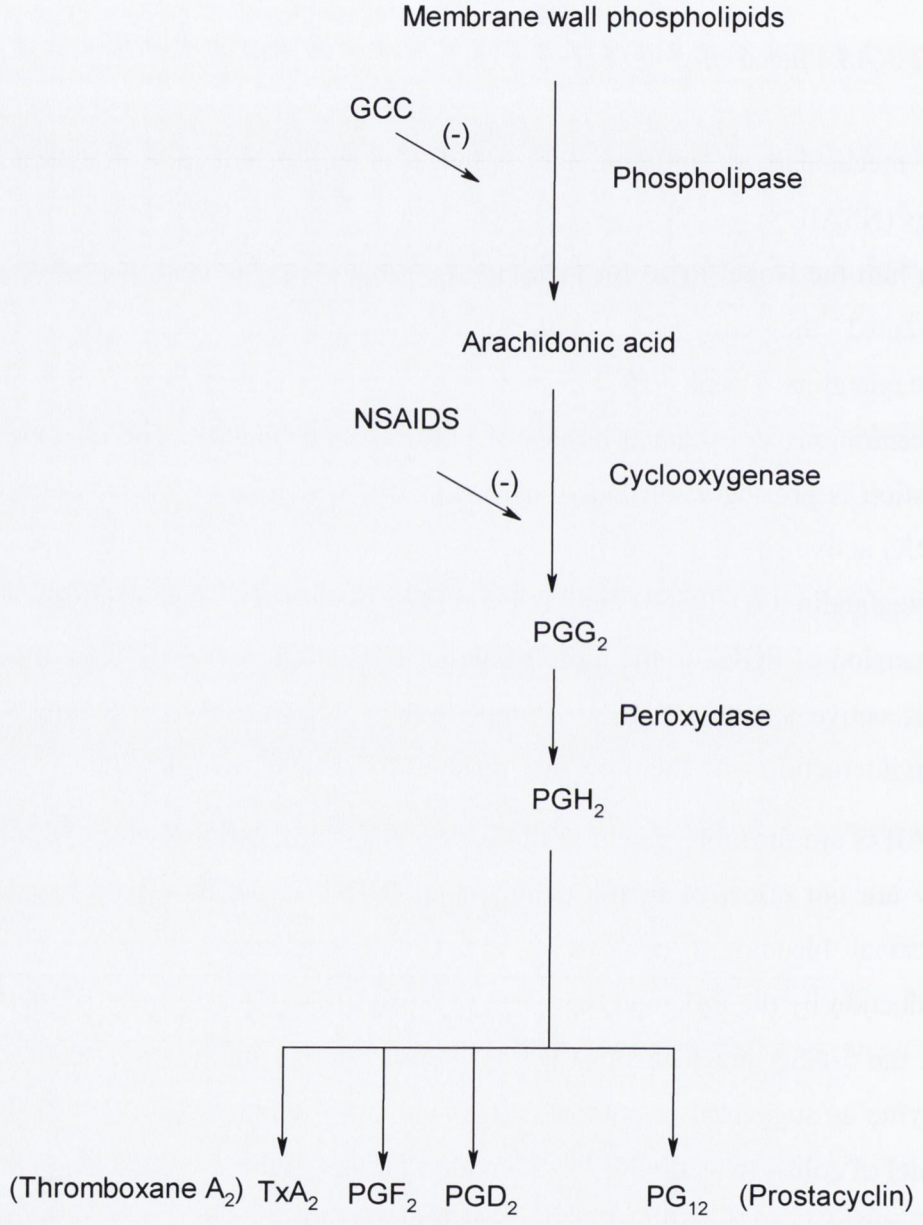


Figure 1.5. Inhibition sites of NSAIDS and GCC in the prostangladin's route

1.5.3 Pharmacokinetics of 5-ASA

In plasma 5-ASA is eliminated by acetylation to N-acetyl-5-ASA and the concentration of administered 5-ASA determines the final time to total plasma clearance which can increase up to 2.4 hours for the highest dosages, (Vree et al., 2000). N-acetyl-5-ASA is eliminated via kidney.

5-ASA in the colon after oral or rectal administration is absorbed by the epithelial cells where some of the 5-ASA undergoes acetylation to N-acetyl-5-ASA and the rest is bound to the cell drug transporter leading to a final drug response after reaching the right concentration (Zhou et al., 1999).

1.5.4 5-ASA side effects

The main adverse effects of 5-ASA are acute pancreatitis (allergies), gastrointestinal symptoms (abdominal pain and diarrhea) (Baker, 2004). Adverse effects of 5-ASA enemas are rare, mild and comprised mainly of anal irritation (Campieri, 2002). However, adverse reaction is found when 5-ASA is administered orally up to 10-20% including headaches and nausea. Kidney damage may be produced as a result of long term use of this drug. Sulphasalazine (a 5-ASA prodrug which will be discussed later) produces frequent and severe adverse effects which are due to the carrier of the 5-ASA, sulphapyridine, which has antibacterial properties.

1.5.5 Glucocorticosteroids

The steroids used in IBD, known as glucocorticoids (GCC), are derivatives of cortisol, a natural occurring steroid produced by the adrenal glands. The first GCC to be given to patients was cortisone, in 1950. The introduction of this potent drug revolutionized the treatment for many chronic diseases, including IBD. Currently the most used GCC are: dexamethasone, prednisone and prednisolone and recently budesonide (F. Richter, 1997), (Mulder and Tytgat, 1993). GCC are steroid hormones with powerful anti-inflammatory effects that have the ability to inhibit all stages of the inflammatory response.

The name glucocorticosteroid derives from early observations that these hormones were involved in glucose metabolism. During times when no food is being taken into the body, glucocorticoids stimulate several processes that serve to increase and maintain normal glucose concentrations in the blood. These processes include:

- Stimulation of glucose production in cells, particularly in the liver.
- Stimulation of fat breakdown in adipose tissues.

- Inhibition of glucose and fat storage in cells.

GCC are only suitable for use as a short term treatment to induce the remission of UC and CD due to the adverse effects during long term treatment (Campieri, 2002), (Schwab and Klotz, 2001).

1.5.6 Glucocorticoids mechanism of action

Studies have shown that GCC desired anti-inflammatory effects are mediated by repression of gene transcription to suppress the production of proteins involved in inflammation process (Barnes, 1998). The mechanism of inflammatory relief of GCC is quite similar to NSAIDS, but they act by interfering with the transcription of enzymes involved in inflammation, activating a group of enzymes known as lipocortins. Lipocortins have been found to inhibit or slow the action of phospholipase A2 (PLA2), a key enzyme involved in the release of arachidonic acid (AA) from the cell membrane (*Figure 1.5*).

AA is type of omega-6 fatty acid. The omega-6 fatty acids in our body often come from vegetable oils and animal meats in our food. Once AA is in our body, it is usually incorporated into our cells membranes. When a cell membrane is damaged or under attack by foreign substances, AA is released from the cell membranes and is converted into substances such as prostaglandins which mediate inflammation. Free AA is converted into inflammatory prostaglandins by COX. Release of AA requires the activation of the enzyme PLA2. As stated previously, lipocortins inhibit PLA2 activity. By activating lipocortins, glucocorticoids cause inhibition of PLA2, thereby inhibiting release of AA and prostaglandin synthesis in the cell. Because lower amounts of inflammatory prostaglandins are synthesized, inflammation is suppressed and damage caused by chronic inflammation is decreased.

1.5.7 Relation between chemical structure and pharmacological properties

Hydrocortisone was the first steroid used to treat IBD but as a consequence of its side effects research to find new synthetic steroids was carried out. Changes in the molecular structure induce changes in the pharmaceutical properties of GCC as a result of changes in pharmacodynamic properties

(affinity, membrane transfer capacity, etc) and pharmacokinetics properties *Figure 1.6* (absorption, protein linkages, excretion, etc). These changes in molecular structure can indeed produce an increase in the anti-inflammatory properties and mineralcorticoid properties ratio (Goodman et al., 1982). That means a good number of actual steroids do not cause serious electrolytic effects to the human body.

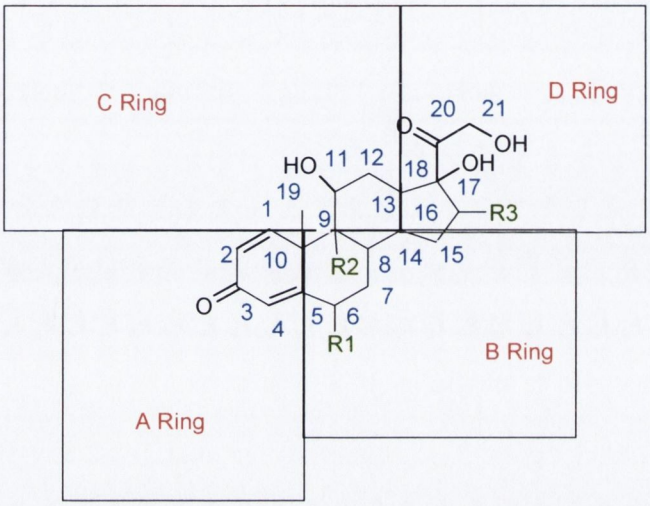


Figure 1.6. GCC chemical structure-activity relation. The thickest lines and green show modification which affect anti-inflammatory properties

Ring A: The presence of the ketone group at C-3 and the alkene group between C-4 and C-5 is essential to anti-inflammatory activity. By introducing an alkene group between C-1 and C-2 an increase in the anti-inflammatory activity is obtained.

Ring B: An increase on the anti-inflammatory properties by methylation on C-6 followed by a decrease in the mineralcorticoid properties. Fluorination on C-6 and/or C-9 also causes an increase in the anti-inflammatory properties.

Ring C: The hydroxyl group at C-11 is essential to the anti-inflammatory properties and irrelevant to the mineralcorticoid activity on steroids.

Ring D: The hydroxyl group in C-17 essential methylation or hydroxylation on C-16 causes an increase in anti-inflammatory effects.

1.5.8 Pharmacokinetics properties of glucocorticosteroids

The pharmacokinetic properties and values of total clearance for each GCC are obviously different. Whereas they all follow a similar pathway of excretion, the GCC are metabolized in the liver where they are hydroxylated. The reduction of the alkene and ketone groups in the A ring leads to the tetrahydroderivatives which are conjugated to glucuronic acid (Kasuya et al., 2000) . These conjugates are excreted via the kidneys. Pharmacokinetic studies carried out by (Bergamaschi et al., 2005) with prednisonone, prednisolone and cortisol in human plasma indicated maximum concentrations start to decrease after three hours.

Budesonide is one of the most recently discovered steroids for the treatment of IBD due to its low adverse effects and its high affinity to the glucocorticoids receptor, the anti-inflammatory response is one of the highest as a consequence (Klotz and Schwab, 2005).

When administered orally 3 to 9 mg/day or rectally 2 mg/100 ml as an enema, the terminal half-life is 2.7 hours and budesonide is metabolized by hydroxylation by an isoenzyme of the cytochrome P450 which is expressed in the epithelial cells and the hepatocytes (Jonsson et al., 1995). Because of the high elimination of budesonide in epithelial cells oral bioavailability is only about 10%. (Spencer and Tavish, 1995), (Mc Keage and Goa, 2002).

1.5.9 Glucocorticosteroids side effects

GCC have got a huge range of adverse effects, when they are used are in long term. Recent studies (Schäcke et al., 2002) suggest that certain side effects are mediated by transactivation (diabetes, glaucoma), transrepression (adrenal insufficiency) and both (osteoporosis). They affect metabolism of proteins, lipids, starch and hydroelectric equilibrium causing the side effects which some of them are mentioned here:

- Softening of the bones, especially hips and spine; mainly with long term therapies but occasionally with short term. Physical inactivity, malnutrition and a low lactose diet increase the risk.
- Reduced immunity.

- Aggravation of diabetes mellitus.
- Cataracts.
- Glaucoma (increase of the eye pressure).
- Reduced potassium levels.
- High blood pressure.
- Ulcers of the stomachs and/or duodenum.
- Rounding of the face as a consequence of lipoid redistribution.
- Adrenal insufficiency.

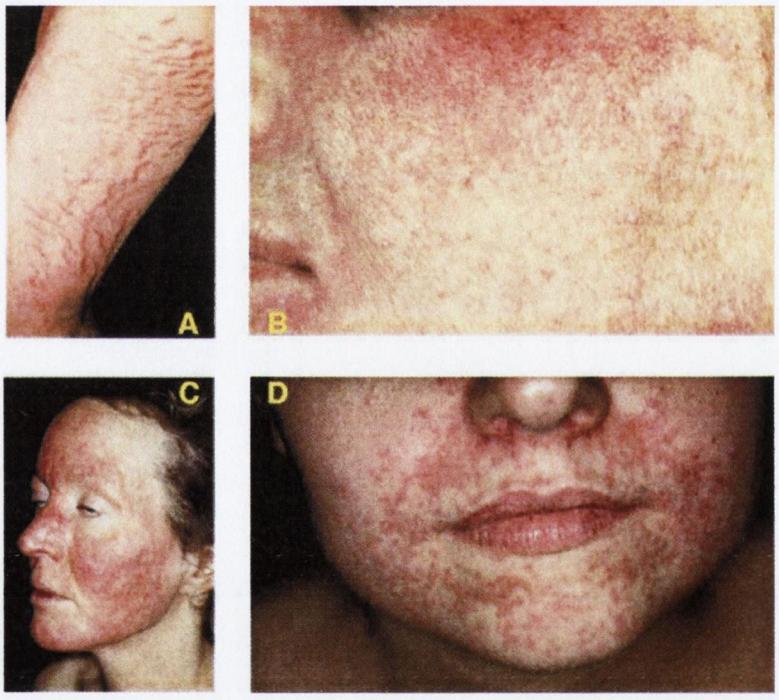


Figure 1.7. Cutaneous side effects of GCC therapy. A: Striae rubrae distensae. B: Skin atrophy. C: Steroid acne. D: Perioral dermatitis.

1.6 Pharmaceutical approaches to colon targeted delivery system

Due to the high absorption rates of the therapeutic drugs for IBD, colon delivery to target these drugs to the affected regions of the bowel seems to be an ideal solution to reduce the potential for the drugs side effects. There are different ways to achieve this delivery such as retarded release formulations and prodrugs.

A prodrug is a non-active derivative of a drug that requires spontaneous or enzymatic transformation *in vivo* to release the active drug. A prodrug usually has better delivery properties than the original drug. Prodrug design must be based on the region to deliver the drug to optimise the prodrug-drug conversion.

Generally, a prodrug is successful for colon drug delivery if is bulky and hydrophilic to minimize absorption from the upper GIT (Sinha and Kumria, 2001). Once reaching the colon it is transformed into a more lipophilic drug molecule and less bulky than before to make the drug available for absorption.

To develop any prodrug delivery system to the colon, intestinal transit time and intraluminal pH profiles must be taken into consideration, especially for retarded release formulations. The orocaecal transit time in patients with CD varies from 75 to 210 minutes (Klotz, 2005) it is delayed compared to healthy subjects (Tursi et al., 2003), which values varies from 75 to 135 minutes.

There are no differences in the pH values of the GIT between IBD patients and healthy subjects. The pH in the small bowel varies between 6.0 to 7.4, in the caecum from 6.3 to 7.4 and finally in the colon from 5.9 to 7.8 (Press et al., 1988). These values are particularly good for the absorption of drugs.

An important tool for developing a prodrug for colon delivery is the enzymatic activity from the colonic microflora enzymes like azoreductase, β-galactosidase, β-xylosidase, nitroreductase, glycosidase deaminase, etc. These enzymes of the colon can be exploited for colon-specific drug delivery.

The bacterial microflora of the stomach and the small intestine is of the order of 10^3–10^4 CFU/ml consisting mainly of gram-positive facultative bacteria (Sinha and Kumria, 2002). These include *Streptococci*, *Staphylococci*, *Lactobacilli* and anaerobic *Veillonella*. The bacterial growth is reduced in this area compared to the colon due to chemical factors (e.g. bile juice, lysozyme) and peristalsis. *Coliforms* and other anaerobic bacteria may be found in low concentration. In the jejunum and the upper ileum very few microorganisms are present. *Lactobacilli* and *Enterococci* can be found there. In the distal ileum, gram-negative bacteria begin to outnumber the gram-positive organisms. The bacterial concentration becomes high. *Coliforms* are

consistently present and anaerobic bacteria such as *Bacteroides, Bifidobacterium*, *Fusobacterium*, and *Clostridium* are found in high concentration (Gorbach et al., 1967). Also, *Sterptococci*, (*Streptococcus faecalis*), *Staphylococci*, *Lactobacilli*, *Clostridium perfringes*, *Veillonella* and at time *Escherichia coli* may be found (Thadepalli et al., 1979). In contrast, flora in the colon are many times greater and is of the order of 10^{13} 10^{14} CFU/ml consisting of mainly anaerobic bacteria (Moore and Holdeman, 1975). The main bacterial population present is that of oxygen-intolerant anaerobic bacteria of various types. The anaerobic bacteria outnumber the aerobes by a factor of 10^2–10^4. As many as 400 different bacterial species are found. The predominant species isolated include *Bacteroides, Bifidobacterium* and *Eubacterium*. Anaerobic gram positive *Cocci, Clostridia, Enterococci* and other species of *Enterobacteriaceae*, *Diphtheroides, Coliforms, Staphylococci, Lactobacillus, Spirochetes,* yeasts, *Proteus, Pseudomonas, Bacillus subtilis, Actinomyces, Borrelia*, *Fusobaterium* and *Clostridium* species are also found. *Bacteroides* species account for nearly 32% of all organisms isolated from the GIT flora. This vast microflora fulfils its energy needs by fermenting various types of substrates that have been left undigested in the small bowel (e.g. di- and polysaccharides, mucopolysaccharides etc).

In conclusion the colon is the perfect site for drug delivery because of its enzymatic activity, the vast majority of the bacterial microflora live there, one third of faecal dry weight consists of bacteria (Simoni et al., 2006) the transit time is long enough to allow the action of the microflora on different prodrugs and the pH conditions are mild.

1.6.1 5-ASA Prodrugs

In the late 1940's Dr. Nana Svartz, a Swedish researcher, proposed linking one of the newly discovered sulpha drugs with 5-ASA to treat rheumatoid arthritis, (Svartz, 1942) at that time thought to be caused by an infection. The patient treated with such a combined drug, named sulphasalazine, happened to have ulcerative colitis as well. The most striking benefit for this patient was control of his colitis. Until the introduction of sulphasalazine in 1942, no medication had been able to control colitis (no medication to cure it has been

found yet). For the next eight years it remained the only drug available for the treatment of colitis, until 1950, when cortisone was introduced. Sulphasalazine continued to be only drug in its class until the early eighties when pure 5-ASA, the active ingredient of sulphasalazine became available.

1.6.1.1 Azo Conjugates

Nowadays aminosalicylates represent drugs of first choice in the treatment of UC and CD. These are only effective in maintaining remission in acute disease and preventing relapse (Klotz, 2005). 5-ASA (also known as mesalazine or mesalamine) has been administrated by enemas or suppositories mainly in patients with UC. Because it was as good as sulphasalazine, 5-ASA proved to be the active moiety of sulphasalazine (Azad Khan et al., 1977). The other moiety of sulphasalazine, sulphapyridine seemed to be the responsible for most of the side effects of sulphasalazine (Chourasia and Jain, 2003). The most frequent side effects of sulphasalazine are gastrointestinal. Nausea and reduced appetite are common but of course these can also be symptoms of IBD. These side effects can sometimes be prevented if you start with a small dose of the drug. It also helps to take the tablets with food.

However, the prodrug allows 5-ASA to reach the affected areas of the colon in a higher concentration than when administrated alone (Klotz, 2005) the need for a carrier for 5-ASA and the need to avoid side effects of sulphapyridine leads to a search for a new type of prodrug for mesalazine. Here there are a few examples of 5-ASA azo conjugates.

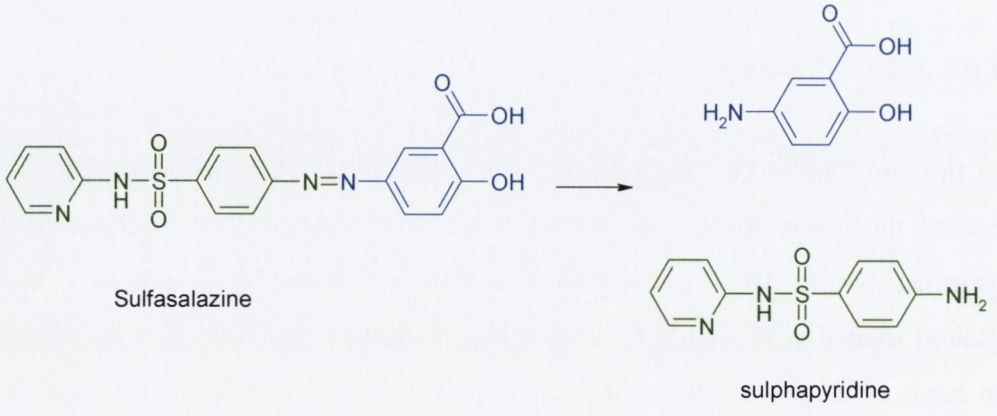


Figure 1.8. Chemical structure of sulphasalazine, showing the cleavage products after bacterial reduction

In *Figure 1.8* we can observe sulphasalazine cleavage to get 5-ASA and sulphapyridine, which have been proved to produce adverse side effects (Liou et al., 2004).

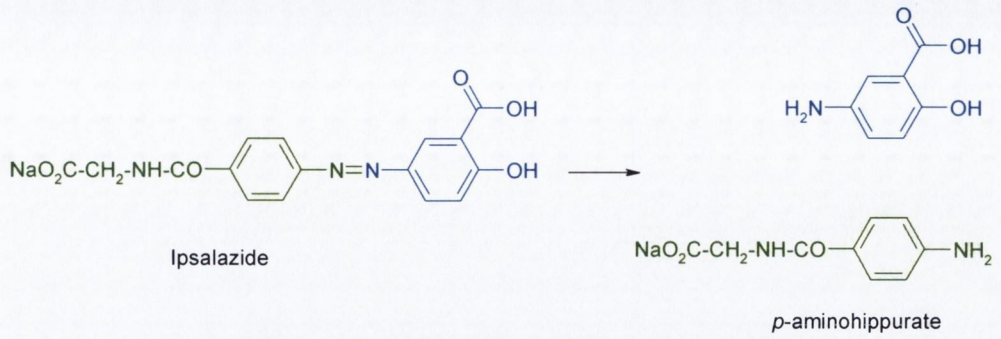


Figure 1.9. Ipsalazide and its components

Ipsalazide has got an inert carrier moiety which only have transport properties as balsalazide shown in *Figure 1.10* below.

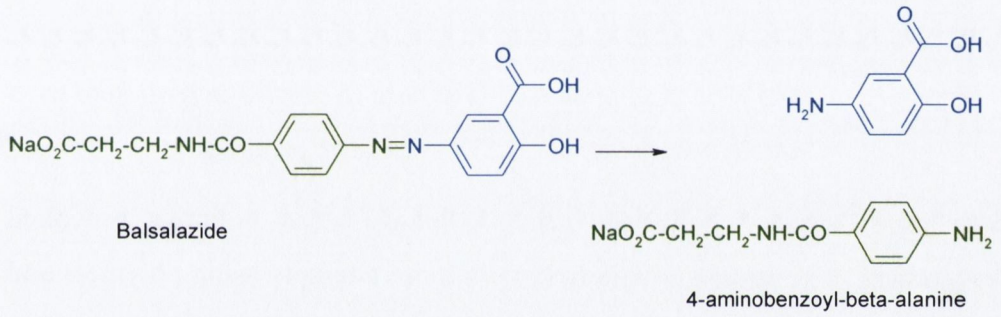


Figure 1.10. Balsalazide and its components

In balsalazide 4-amino-beta-alanine is the inert carrier without therapeutics or adverse properties proved (Travis et al., 1994).

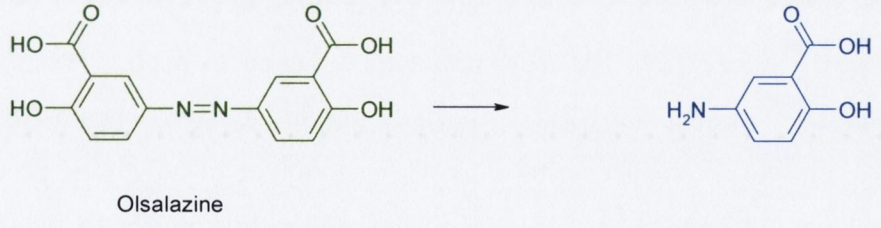


Figure 1.11. One molecule of olsalazine carries two 5-ASA molecules

Olsalazine is another prodrug in which a molecule of 5-ASA acts as a carrier of the other so two molecules of 5-ASA per mol of olsalazine is expected to reach the colon.

1.6.1.2 Polymeric 5-ASA prodrugs

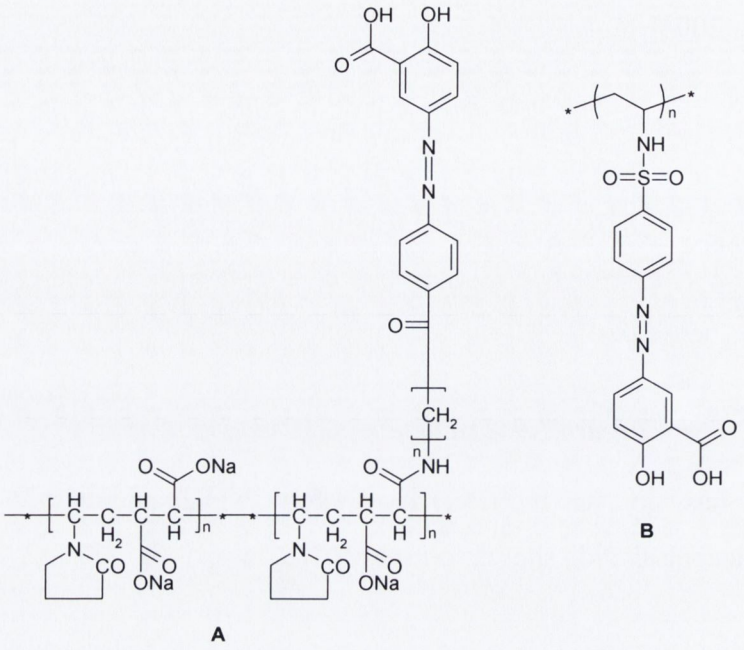


Figure 1.12. A) 5-ASA conjugate linked to poly (1-vinyl-2-pyrrolidone co-maleic anhydride) B) 5-ASA polymeric prodrug

Success in targeted delivery with azo linkage-based prodrugs provoked researchers to go further in this field with more attempts using polymers and the azo bond. (Clader et al., 1996). In *Figure 1.12 [A]* sulphasalazide has been linked to a sulphaniamidoethylene polymer. In spite of maintaining the sulphasalazide's adverse effects this polymeric azo prodrug is more effective against inflammation in patients with mild to moderately severe colitis. A similar polymeric prodrug based on an azo linkage to deliver 5-ASA is shown in *Fig.1.12 [B]*. The azo conjugate is linked to a poly (1-vinyl-2-pyrrolidone co-maleic anhydride). By these polymeric approaches 5-ASA is delivered to the colon successfully. However, this type of polymeric prodrug has got an inconvenience, a huge amount of prodrug needs to be taken because the drug is only 10% of the total prodrug weight and daily requirements of 5-ASA varies from 0.5 g to 3 g.

All these prodrugs rely on the azoreductase activity from the colonic microflora (Nakamura et al., 2002), (Rafii et al., 1990), (Peppercorn and Goldman, 1972). The azo bond is stable in the low pH environment of the stomach and upper GIT. Once in the colon it is reduced by the azoreductases

produced by the colonic microflora releasing 5-ASA as an active moiety along with the carrier molecule.

1.6.1.3 Amino acid conjugates

A different approach to deliver 5-ASA and salicylic acid (SA) to the colon, which is not based on azo linkage, is the formation of peptide bonds between the amino group of the amino acid and the carboxylic acid group of the 5-ASA and SA to form an amino acid conjugate. Because of the polar groups -NH_2- and -COOH- the hydrophilic properties of the prodrug are bigger than the parent drug and the permeability through the GIT membrane is reduced. The amino acids used are mainly non essential such as glycine, tyrosine, methionine, L-alanine, glutamic acid, etc.

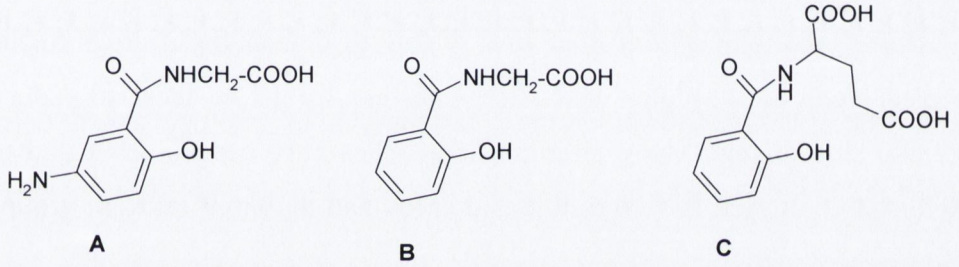


Figure 1.13. A) Glycine conjugate of 5ASA, B) Glycine conjugate of SA, C) Glutamic acid conjugate of SA

The conjugate between 5-ASA and glycine (Jung et al., 1998) showed no release of 5-ASA in the stomach and small intestine. 5-ASA was found in the colonic contents in a 27% and 65% in the caecal contents. When rats were treated with antibiotics the 5-ASA concentration was very low (Leopold, 1995b), (Leopold, 1995a).

When the same approach has been done to SA, the glycine conjugate was absorbed before reaching the colon, so the conjugate with glutamic acid was necessary. In this case the molecular weight was bigger and hydrophilic properties better than in the glycine conjugate. (Nakamura et al., 2002).

1.6.1.4 Latest 5-ASA prodrugs

New conjugates of 5-ASA are being studied in which it is attached to a nitric oxide releasing moiety or ursodeoxycholic acid (Wallace, 2003, Galvez J et al., 2003).

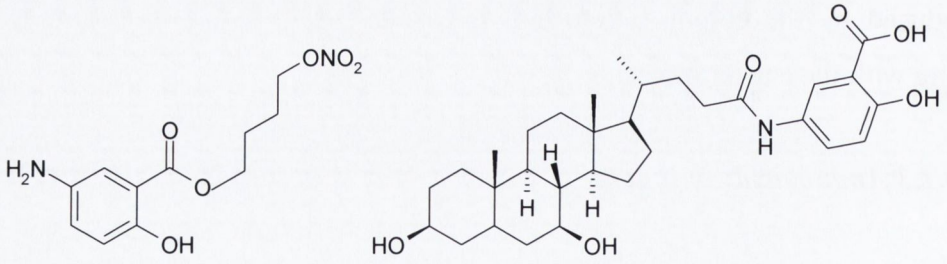


Figure 1.14. Two potential new prodrugs of 5-ASA delivery to the colon

Ursodeoxycholic acid has been found to have protective role in colonic carcinogenesis and causes reduction in the incidence of colonic tumours, but when it is given orally it does not reach the colon (*Figure 1.14*).

1.6.2 Glucocorticosterid prodrugs

As explain above the GCC's used in the treatment for IBD have a huge range of adverse effects that limit their use. A way to deliver these drugs to the affected areas of the colon and large intestine would be ideal to reduce potential side effects. Many different approaches have been investigated to deliver GCC's to the large intestine and colon and the more relevant groups are described next.

1.6.2.1 Metasulphobenzoate salts

Prednisolone metasulphobenzoate has been used topically in patients with distal UC and is more poorly adsorbed than prednisolone 21-phosphate as enema (Mc Intyre et al., 1985). An oral formulation of prednisolone metasulphobenzoate coated in a controlled release matrix was tested (Cameron et al., 2003) showing an improvement in the therapeutic effects over prednisolone although the benefits on the adverse side effects need to be confirmed by formal comparative studies.

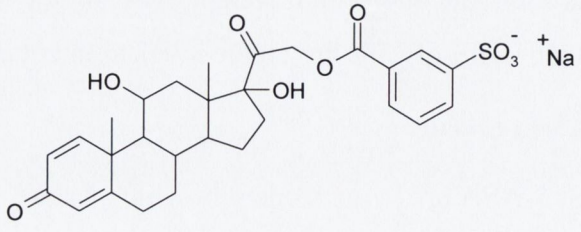


Figure 1.15. Prednisolone metasulphobenzoate salt

1.6.2.2 Phosphate prodrugs

As done with metasulphobenzoate and sulfate salts, phosphate prodrugs are designed to increased the solubility properties of the glucocorticoids.

The phosphate ester was well absorbed in the jejunum in studies (Stuart et al., 1986) carried out using rings prepared from rat jejunum and colon. This study suggested that prednisolone and hydrocortisone 21-phosphates are good substrates for brush border membrane alkaline phosphatase in the jejunum, and the poor uptake in the colon is most likely due to the fact of the lack of this enzyme in the colon.

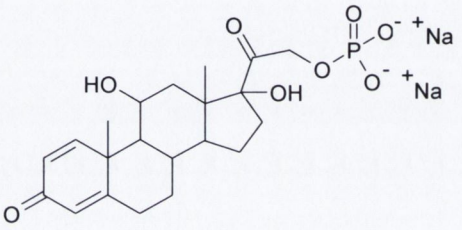


Figure 1.16. Prednisolone 21-phosphate salt

1.6.2.3 Sulfate prodrugs

Prednisolone 21-sulfate sodium salt was synthesized as a colon-specific prodrug of prednisolone with the expectation that it would be stable and non-absorbable in the upper intestine and release prednisolone by the action of sulfatase once it was delivered to the colon. This prodrug was chemically stable at pH 1.2, 4.5, 6.8 and 8. Studies (Jung et al., 2003) suggesting limited absorption and data demonstrate the potential to release the drug in the colon by the action of the microbial sulfatase action.

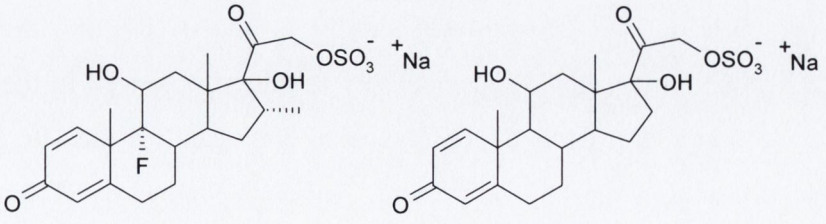
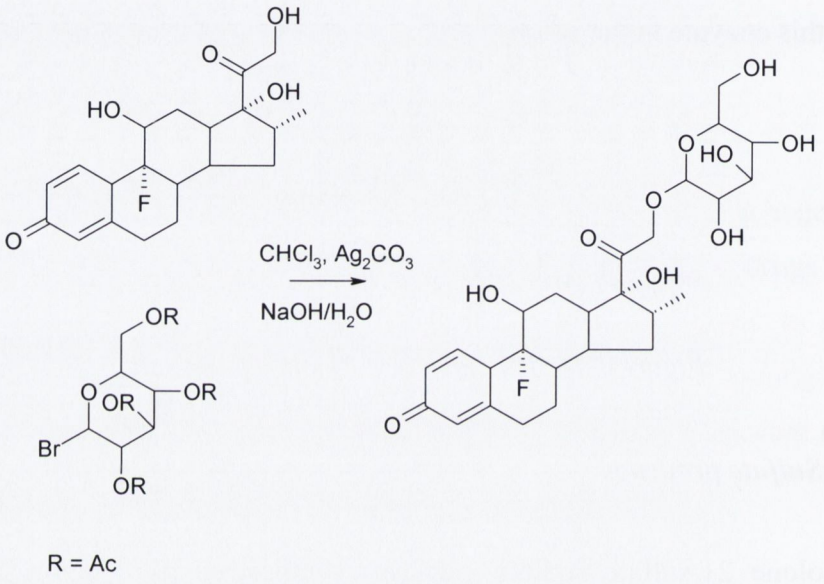


Figure 1.17. Left: Dexamethasone 21-sulfate sodium salt. Right: Prednisolone 21-sulfate sodium salt.

Studies carried out with dexamethasone (Kim et al., 2006) suggested similar properties for the dexamethasone 21-sulfate and even improved the delivery properties.

1.6.2.4 Glycoside conjugates

GCC are attached to sugars by drug glycosides glycosylation reaction from the bromo sugar derivative and the steroid by a modified Koenigs-Knorr reaction (Friend and Chang, 1985).



Scheme 1.1. Koenigs-Knorr reaction

The sugar part of the prodrug is called aglycon and the drug part is called (in this case GCC) aglycon. Glucose, galactose or cellulose are the main sugar moieties used for this purpose. The main enzymes for this prodrug produced by the intestinal microflora are β-D-galactosidase, α-L-arabinofuranosasidase, β-D-xylopyranosidase and β-D-glucosidase (Rubinstein, 1995). The glycosidase activity is shown by the anaerobic colonies, however some glycosidase activity might be observed in the small intestine and some hydrolysis of the glycoside conjugate towards its glycon and aglycon components may be present. Although studies by (Friend and Chang, 1985) with *in vivo* rats model comparing the conjugates to the free steroid when they were administrated orally, the studies demonstrated that the free steroid only reaches the colon in a percentage value of 1% while the glycoside conjugate's percentage value is 60%. The success of this particular

approach must be attributed to the fact of the high hydrophilic properties of the glycosides and the bulky increase of the glycoside conjugate compared to the free steroid, thus the glycoside conjugates are poorly adsorbed by the small intestine walls. Another important factor is the rapid transit time of the prodrug on the small intestine, decreasing the potential of glycosidase activity which may be present on the small intestine.

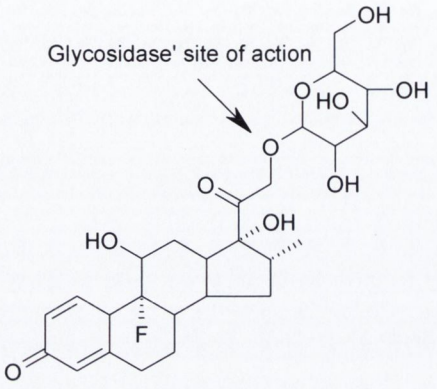


Figure 1.18. Dexamethasone-21-β-glucoside

The next study by (Friend and Chang, 1985) was to compare the kinetics of the hydrolysis depending on the aglycon. They found the prednisolone conjugate of cellulose was hydrolyzed faster than the prednisolone conjugate of glucose which was faster that the prednisolone conjugate of galactose.

1.6.2.5 Glucuronide conjugates

Glucuronide conjugates of steroids show the same similar properties as the previously commentated glycoside conjugates. By attaching glucuronic acid to the steroid the absorption potential of the drug is increased and the prodrug travels through the upper gastrointestinal tract without being absorbed in the intestinal walls.

Once in the large bowel the prodrug relies on the β-D-glucuronidase activity of the intestinal microflora to release the steroid on the right area to be adsorbed. *Figure 1.19* shows the dexamethasone β-D-glucuronide first synthesized by (Haeberling et al., 1993).

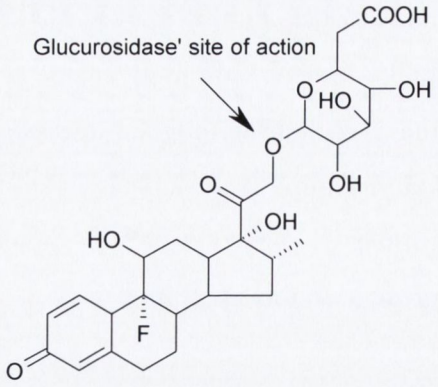


Figure 1.19. Dexamethasone-21-β-glucuronide

The studies carried out by Haeberling showed that glucuronide prodrugs are able to deliver the drugs to the colon and large bowel more specifically and with less risk clearance by the microflora in the stomach and small intestine than the glucoside prodrugs.

Budesonide-β-D-glucuronide conjugate was shown to be more effective than budesonide to treat IBD on rats (N Cui and Fedorak, 1993).

1.6.2.6 Cyclodextrins conjugates

Cyclodextrins are cyclic oligosaccharides consisting of 6-8 glucose units linked by a α-1,4-glucosidic bonds they are stable to gastric acid, salivary and pancreatic enzymes, but the colonic bacteria are able to ferment these oligosaccharides to get their needs of carbon (Antenucci, 1984) and for that reason cyclodextrins are being use to deliver drugs to the colon (Uekama and Irie, 1998).

Steroid cyclodextrin conjugates are prepared by forming the succinate intermediate first and then cyclodextrins are coupled via esterification (Yano et al., 2002).

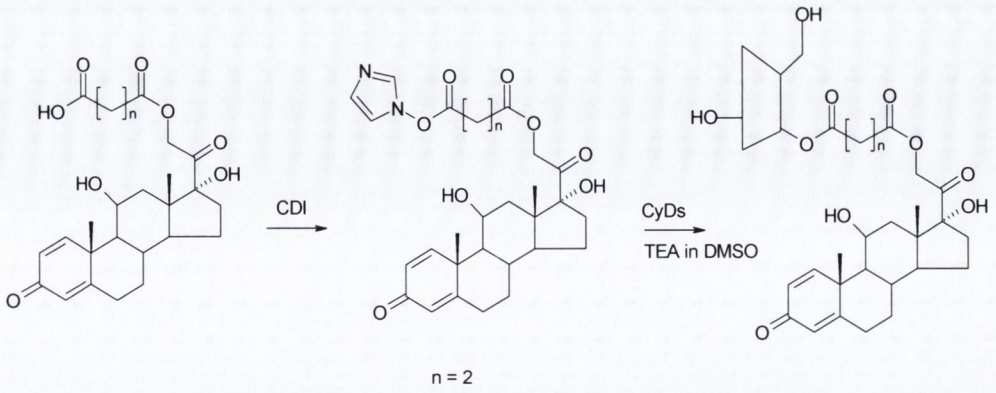


Figure 1.20. Prednisolone cyclodextrin formation

The steroid is protected not only by the covalent linkage but it is protected sterically by being placed in the middle of the cavity formed by the olisaccharides, as shown in *Figure 1.21.*

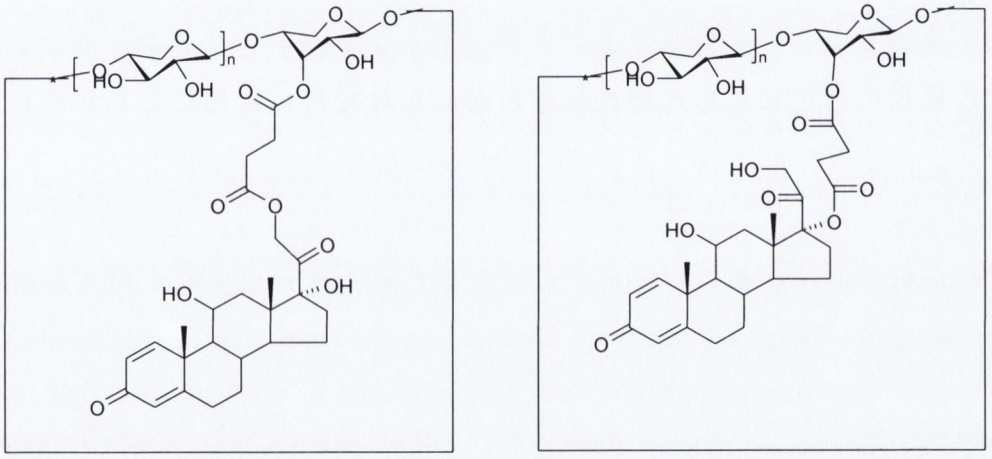


Figure 1.21. Prednisolone cyclodextrin conjugates

The prednisolone percentage release in the colon from these cyclodextrin approaches goes up to 80% indicating the great power of these conjugates as a drug carrier.

1.6.2.7 Dextran conjugates

The difference between dextran conjugates and cyclodextrins conjugates is based on the starch's nature. The steroid is coupled in the same way in both cases, using a succinate spacer. This provides the steroid with a carboxylic acid group to carry out the esterification with the alcohol group from the polysaccharide, and at the same time, allows the esterification by giving space and avoiding steric hindrance.

While cyclodextrins are cyclic oligosccharides, dextrans are linear polysaccharides. A example of steroid-dextran conjugate is shown in *Figure 1.22.*

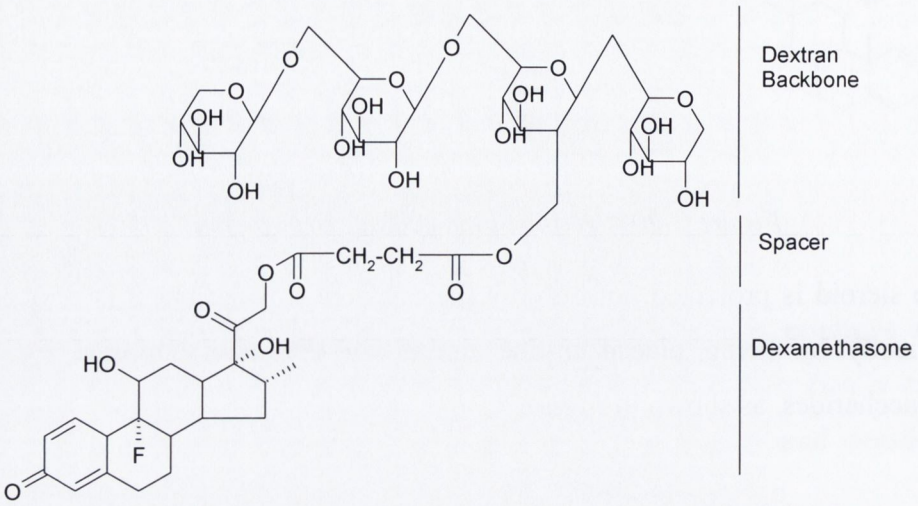


Figure 1.22. Dextran conjugate of dexamethasone

Dextranases are the enzymes responsible for degradation of dextrans. Anaerobic gram-negative bacterias, mainly *Bacteriodes* show a high dextranase activity in the colon, while almost no dextranase activity has been observed on the small intestine (Henhre, 1952). Due to all these reasons, dextrans have drug carrier potential properties and it has been investigated, not only for steroids, but for other drugs such as naproxen and 5-ASA (Sinha and Kumria, 2001).

Dexamethasone has been released in the cecum and the colon in high values from dexamethasone-dextran conjugates (Mc Leod, 1994). One of the problems of the glycosidase approach is that these activities are widespread throughout the GIT resulting in poor targeting.

1.6.2.8 Coated tablets

Attempts to deliver the drug to the colon have been done by coating the drug molecule with polymers that will be degraded in the colon. In these attempts no prodrug systems have been formed because the drug is constantly active. The drug is in tablets, capsules, or pellets and pH sensitive and/or biodegradable polymers are coated, providing a delayed release in the colon.

Another variation is to embed the drug in a polymeric matrix. Once in the colon the polymer is degraded and the drug is released in the affected area.

Masalazine tablets have been coating by amorphous amylase combined with ethylcellulose. Amylose will be degraded by the colonic microflora and ethylcellulose is the structuring agent. Depending on the ratio amylose-ethylcellulose the time of drug release varies. (Wilson and Basit, 2005).

1.7 Design of a new approach to a colon targeted delivery system

At the outset of the project we proposed an innovative strategy for targeting hydroxyl-bearing drugs such as the anti-inflammatory steroids to the colon that overcomes the design flaws in the glycosidase targeting approach. Our design for achieving the site-specific delivery of anti-inflammatory steroids is presented in *Figure 1.23*. The drug is linked via an ester to a carrier group (green), which is connected by an azo-bond to a second carrier group (blue). In the case of nitro conjugate compounds only one carrier (green) is attached to the drug (red). The carrier groups are selected to maximally suppress absorption from the stomach and upper intestine. Our design seeks to exploit the selective reduction of an azo-linker or nitro group in the colon, releasing a chemically unstable latent prodrug that undergoes spontaneous lactamisation liberating the steroid payload. The overall effect is to make the biologically robust ester group, connecting the drug to the carrier group, chemically vulnerable under conditions found only in the colon.

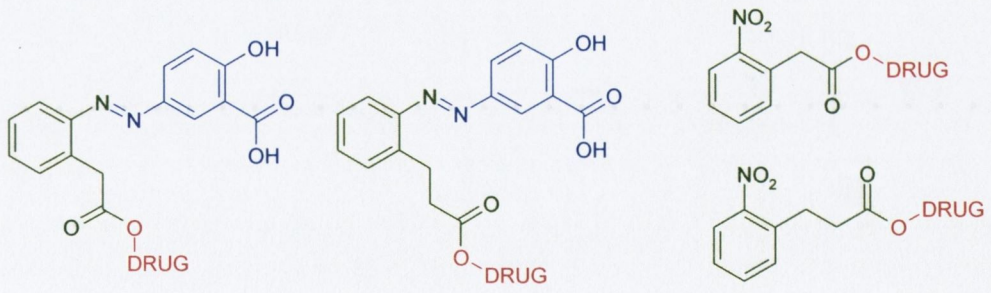
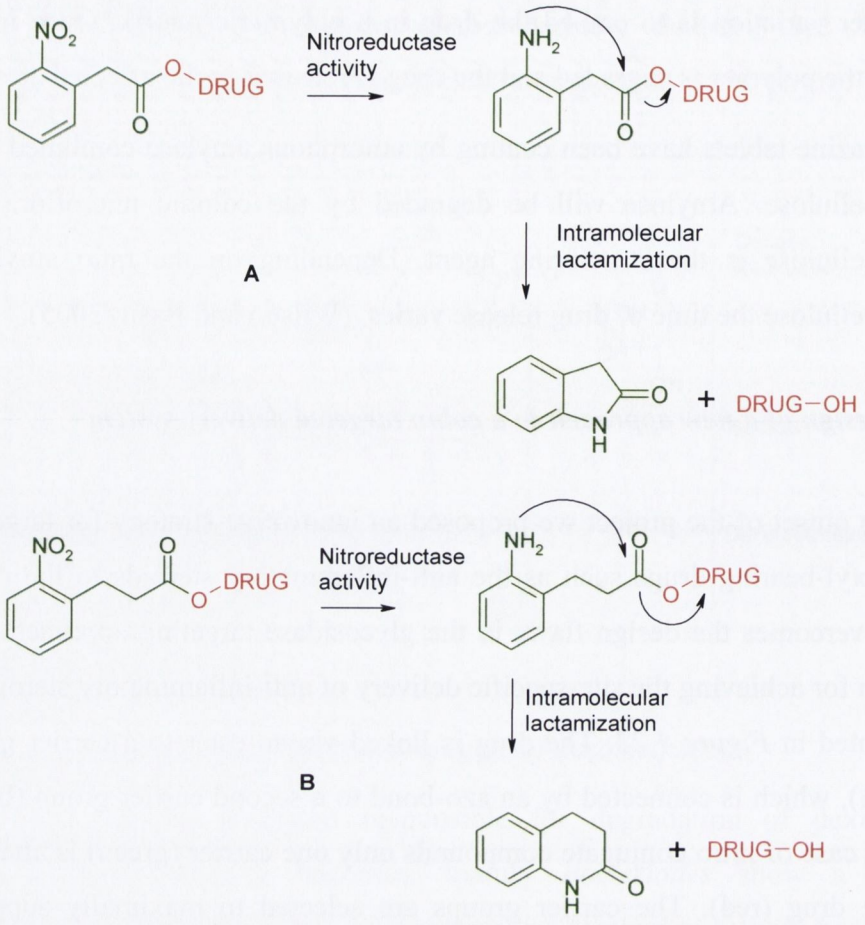
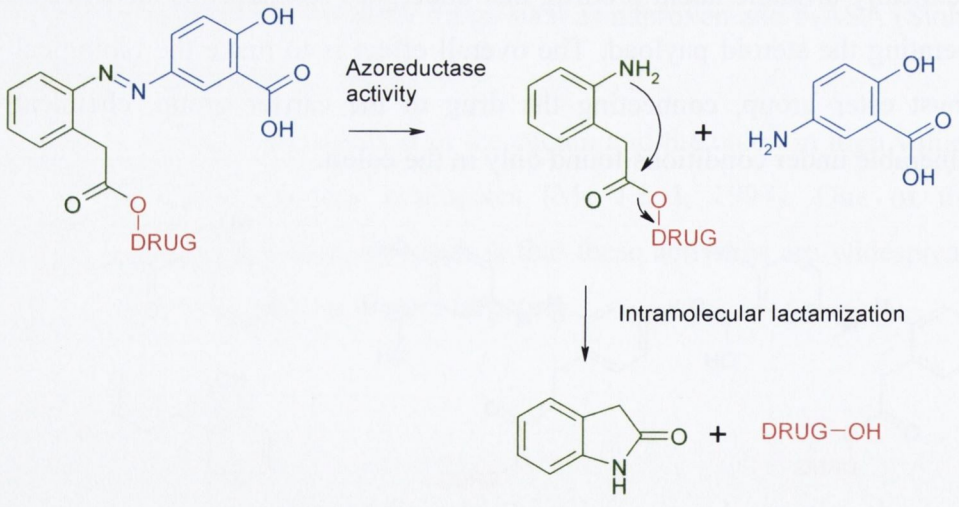


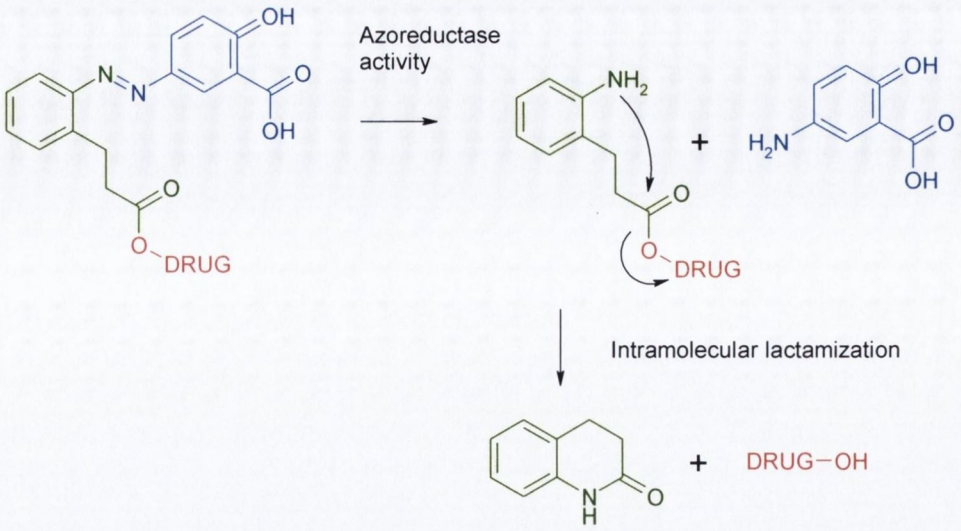
Figure 1.23. Novel colon delivery prodrug systems



Scheme 1.2. Nitroreductase activity and ring closure in phenyl acetic acid (A) and phenylpropionic acid nitroprodrug systems (B)



Scheme 1.3. Azoreductase activity in phenyl acetic acid azoprodrug systems



Scheme 1.4 Azoreductase activity in phenylpropionic acid azoprodrug systems

Among the key strengths of this design are:

- The physicochemical characteristics of the prodrug can be optimised for gastrointestinal penetration to the colon. Gastrointestinal absorption is a function of molecular weight, lipophilicity and polarity; in general, polar, hydrophilic molecules are not well absorbed. Among the possibilities for variation at the 'blue' carrier group are azo linkage to 5-ASA, generating a mutual prodrug of a steroid and 5-ASA which would probably have ideal physicochemical characteristics for passage through the intestine because of its mass, polarity and hydrophilicity.

- Our expectation that azo bond and nitro group reduction would proceed readily was based on the promiscuity of the azo and nitro-reductases present in the colon with respect to the substrate, as evidenced by their ability to efficiently reduce substrates as diverse as nitropyrene (Rafii et al., 1991), ipsalazide, in which the carrier group is p-amino hippurate, balsalzide (p-aminobenzoyl-β-alanine carrier), sulfasalazine (sulfapyridine carrier), (Carceller et al., 2001), 9-aminocamphothecin (Sakuma et al., 2001) and 5-ASA- N-methacrylamide, acryloyloxyethyl and acryloylamido copolymers (Van den Mooter et al., 1994). The presence of the vast microflora in the bowel causes a change in redox potential from – 67±90 mV in the distal small bowel to – 415±72 mV in the right colon (Wilding et al.,

1994). The relative contribution of enzymatic and chemical reduction in the release of azo drugs is not well understood.

- The critical ring closure is dependent on nucleophilic attack by the aniline amino group on the steroidal ester. Although anilines possess low nucleophilicity (compared with aliphatic amines) the high effective molarity of the intramolecular arrangement was expected to promote sufficiently rapid ring closure. This reaction had not been examined under physiological conditions but reports on the intramolecular aminolysis of esters by aniline, at roughly comparable conditions, were highly encouraging with respect to the proposed design. (Kirby et al. 1979) studied the spontaneous cyclization of 2-aminophenyl propionic acid methyl ester, finding that at neutrality (roughly the pH of the colon) and 39°C, the reaction proceeds with an apparent first-order rate constant of $2x10^{-4}$ s^{-1}, corresponding to a first-order half-life of 57 min. Fife and Duddy (1983) reported the ring closure of 2-aminophenyl acetic acid methyl and trifluoro ethyl esters (yielding the indolone) to follow similar kinetics under mildly basic conditions (pH 7–8). This half-life is ideal for drug release in the colon, where residence time tends to be rather extended.

- The proposed ester linkage appeared to be adequately stable under conditions found in the GIT. Esterase activity in the lumen of the GIT is restricted to the pancreatic serine proteases, which exhibit residual esterase activity towards a limited number of substrates, generally esters of aromatic amino acids. Steroidal 21-esters have been shown to be robust in simulated intestinal fluid models. For example (Fleisher et al., 1986) reported a first-order rate constant of 0.003 min^{-1} for the hydrolysis of hydrocortisone-21-succinate in rat intestinal perfusate. This figure corresponds to a half-life of 12 hours which is significantly longer than the expected transit time to the colon. (Jhunjhunwala and Bhalla, 1981) reported the disappearance of less than 5% of hydrocortisone-21-succinate after 24 hours at pH 7 (45°C). The sterically hindered steroidal esters of the proposed design promised to be much less vulnerable towards hydrolysis than hydrocortisone-21-succinate.

- The proposed carrier groups do not possess any known toxicity, although substances in both classes (quinolones and oxindoles) have been intensively studied.

The double prodrug approach outlined here had not previously been investigated for the delivery of any drug to the colon. It seemed to us that the design might find application in the delivery of other drug types to the colon, and as a new concept applicable, in principle, to other drug targeting challenges, though that is not the subject of this thesis.

The overall aim of the project, therefore, was to evaluate the proposed design through the synthesis of glucocorticoidal candidates, the measurement of ring closure kinetics, *in vitro* screening for stability, drug release *in vitro* in the presence of colonic bacteria, and anti-inflammatory efficacy in appropriate animal models.

1.7.1 Technical description

To design and optimize novel systems capable of a two-step release of anti-inflammatory steroids that might be used to target the colon we addressed the following points.

- To synthesise a series of esters of the anti-inflammatory steroids hydrocortisone, and prednisolone at the 21 position for investigation.
- To synthesise azo and nitro-derivates of the amino esters.
- To develop analytical methods for estimation of the rate of ring closure of amino intermediate compounds.
- To estimate the rate of ring closure.
- To measure the stability of the compounds under aqueous conditions (pH 1-8).
- To estimate the rate of reduction of these compounds in the presence of microflora *in vitro*.
- To estimate the *in vivo* efficacy of the compounds using a mouse model of colonic inflammation.

- To measure the permeability of the compounds by absorptive and secretory transport studies across CACO-2 cell lines *in vitro* and its comparison with the free steroid. This will allow assessment of penetration through the GIT. For potential colon-targeting it is evident that compounds of negligible permeability will be sought.

This doctoral thesis has well differentiated strands: Pharmaceutical chemistry and Biology. They include the work packages outlined below.

1.7.2 Pharmaceutical Chemistry

- The synthesis of several new families of novel steroidal esters bearing anilines and azo carriers
- Comprehensive study of the cyclisation kinetics of aminophenyl acetate and propionate esters under simulated physiological conditions.
- Determination of the stability of 21-corticosteroid esters.
- The rate of reduction of steroid azo and nitro conjugates.

1.7.3 Biology

- Evaluation of anti-inflammatory effects of candidate compounds in a mouse model of IBD.
- Insight into the GIT permeability of the azo-compounds and their ability to pass through the GIT.

CHAPTER TWO

DESINGN AND SYNTHESIS OF PRODUGS CONJUGATES

2.1 Introduction

This chapter describes the development of the synthetic routes to the target compounds **1**, **2** and the nitro conjugate, compound **58**. The approach to the synthesis of intermediates generated by microflora reductase activity (compounds **59** and **60**) is also explained.

2.2 Synthetic discussions

Looking at the target azoprodrug structures (**1** and **2**); there are two major synthetic challenges:

1. Formation of the azo linkage between the salicylate and the carrier group.
2. Regioselective attachment of the pro-moiety to the steroid by esterification at the 21-OH group.

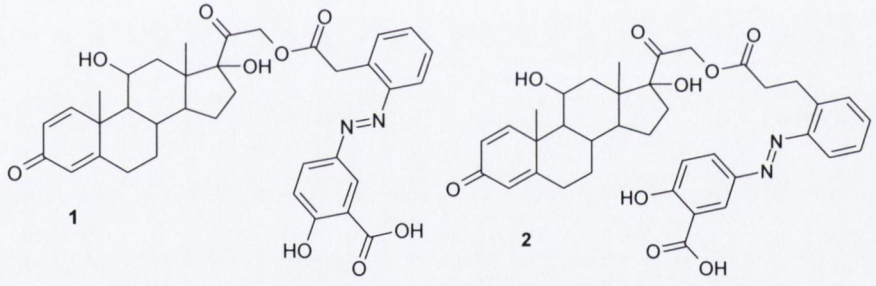


Figure 2.1. Target azo prodrugs

The retrosynthetic analysis of **1** leads us to the two molecules required for its synthesis (*Figure 2.2*) the steroid in this case is prednisolone **3** and the azo carrier **4**. Since prednisolone is commercially available we needed to concentrate firstly on the synthesis of **4** (or its analogue) before examining the esterification reaction at the C-21 alcohol of prednisolone with the correct carboxylic acid group of **4**. This is complicated by the competing steroid hydroxyl groups in positions C-11 and C-17. Fortunately, steroidal esterification generally takes place at the least sterically hindered primary 21-OH-group (Fang et al., 2007). Hydrocortisone was used for development chemistry and to study the lactamization kinetics and it is similar in structure to the more potent steroids of the target prodrugs at the D-ring and 21-OH group.

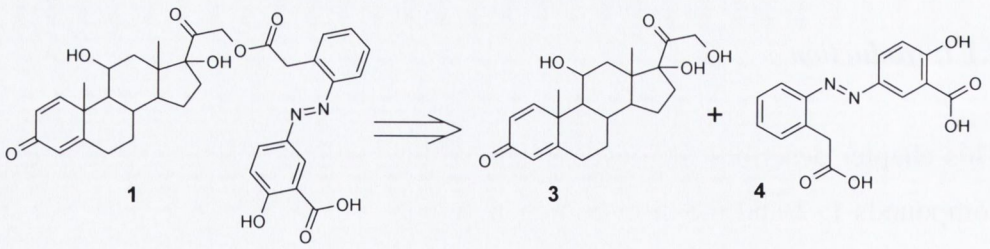


*Figure 2.2. Retrosynthetic analysis of prodrug **1***

Azo carrier **4** has two carboxylic acid groups which are equally exposed to esterification so selective esterification demands an appropriate protecting group. This observation implies an extra disconnection. An azo coupling reaction is required to produce the azo bond as shown in (*Figure 2.3*).

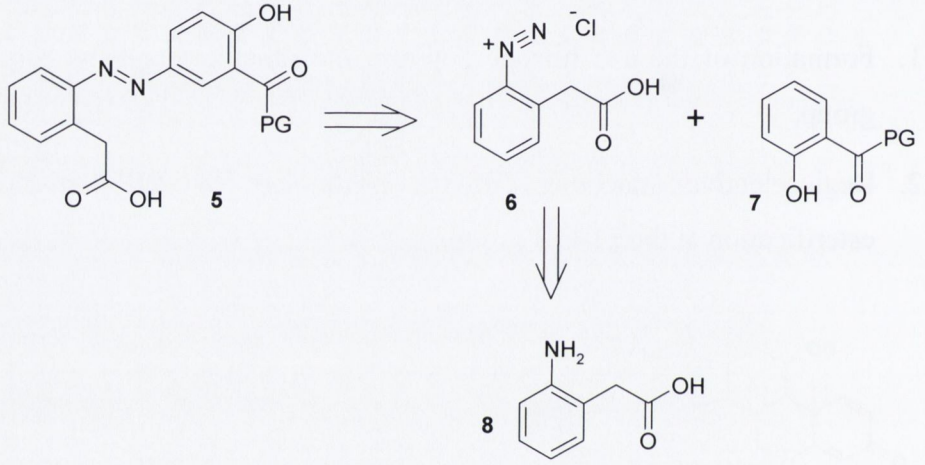


Figure 2.3. Retrosynthetic analysis for prodrug 1 target azo carrier
PG: protecting group

The last consideration was the synthesis of the 2-aminophenylacetic acid **8** because it is not commercially available and the method of first choice was reduction of 2-nitrophenylacetic acid **9** (*Figure 2.4*).

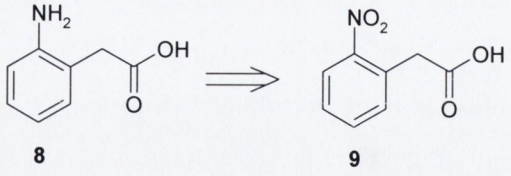


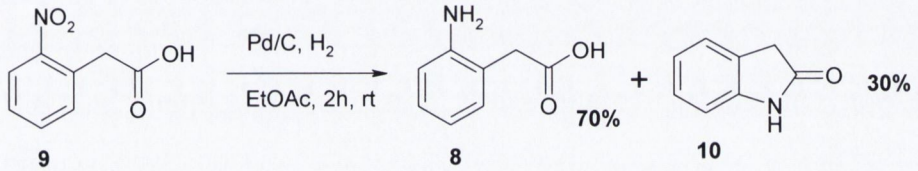
Figure 2.4. Retrosynthetic analysis of 2-aminophenylacetic acid

2.3 Synthesis of prodrug 1

Having introduced the synthetic approach for azo prodrug systems **1** and **2**, the synthesis of compound **1** is detailed in the following sections of this chapter.

2.3.1 Reduction of 2-nitrophenylacetic acid

The reduction of 2-nitrophenylacetic acid **9** was carried out under a hydrogen atmosphere in EtOAc using Pd/C as catalyst at room temperature. Two spots were observed in the TLC plate corresponding to **8** and **10** (after NMR characterization).

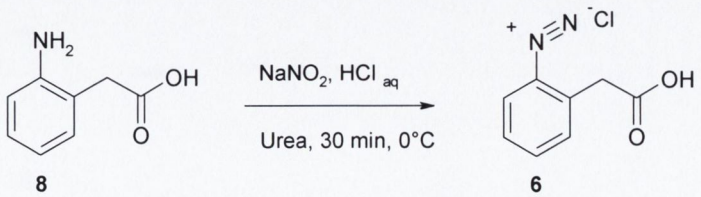


Scheme 2.1. Reduction of 2-nitrophenyl acetic acid

3,4-Dihydro-indol-2-one **10** was produced as an impurity and columned to yield (30%), This indicates that the amino group is prone to undergo cyclization by condensing with the carboxylic acid group, as expected.

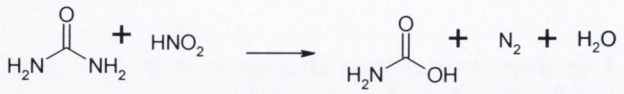
2.3.2 Formation of diazonium salts

Diazonium salts are quite unstable and were stored at 0°C when necessary. Otherwise, to avoid decomposition they were used immediately after formation. An acidic aqueous solution is required and once **8** was dissolved, sodium nitrite was added and the diazotization started to produce the diazonium salt of 2-aminophenyl acetic acid **6**.



Scheme 2.2. Formation of diazonium salts

It is generally found to be important in the synthesis of these salts to add a small amount of urea into the reaction mixture once the reaction is complete to eliminate excess nitrite, otherwise this excess will interfere with the next azocoupling reaction (Leclair and Lizamma, 2003), *Scheme 2.3*.



Scheme 2.3. Elimination of nitrite excess in acidic conditions by urea

2.3.3 Diazocoupling reaction

Azo coupling is the most widely used industrial reaction to produce azo linkages in dyes and pigments (Wang et al., 2003), (Damodaran et al., 2003). The formation of the diazonium salts is carried out in aqueous acidic conditions by adding sodium nitrite. Aromatic diazonium ions act as electrophiles in coupling reactions with activated aromatics such as anilines and phenols under basic conditions. The substitutions normally take place on the *para* position of the activated aromatic ring. When this position is occupied, *ortho* substitution occurs. The pH is important and must be basic in the case of phenols to activate the *para* position and mildly acidic or neutral in the case of anilines. The azo coupling mechanism is shown in *Figure 2.5*.

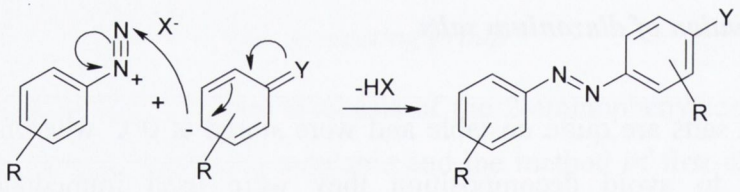
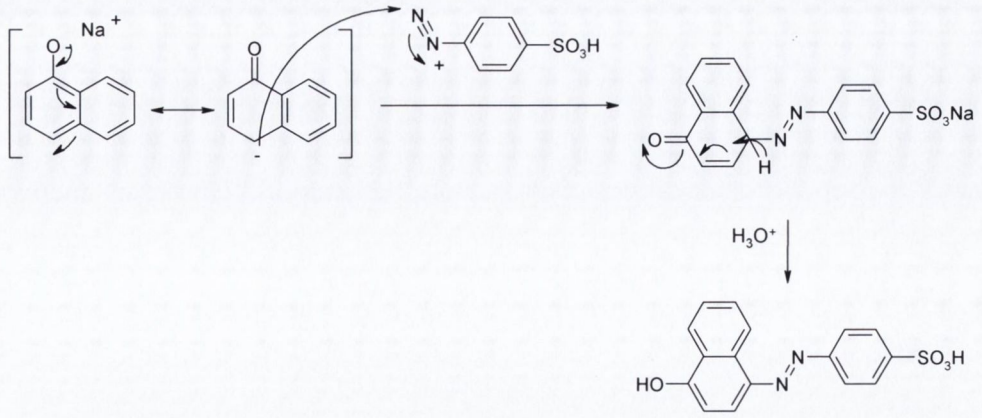


Figure 2.5. Typical azo coupling reaction by diazotazion Y: NH_2 or OH; X: Halogen

Scheme 2.4 shows the mechanism of production of orange 1 dye.



Scheme 2.4. Synthesis of Orange 1 Dye by diazotization.

The diazonium salt of 2-aminophenylacetic acid **6** and the salicylate **7** are in theory the best reagents to produce the azo carrier **5** by azocoupling using diazotization.

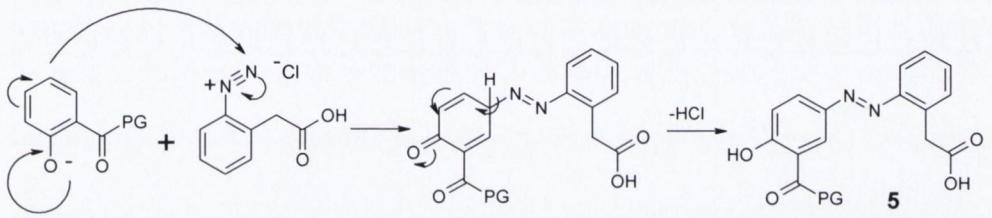
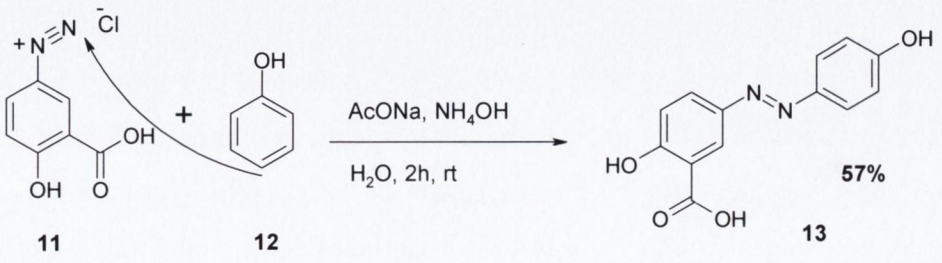


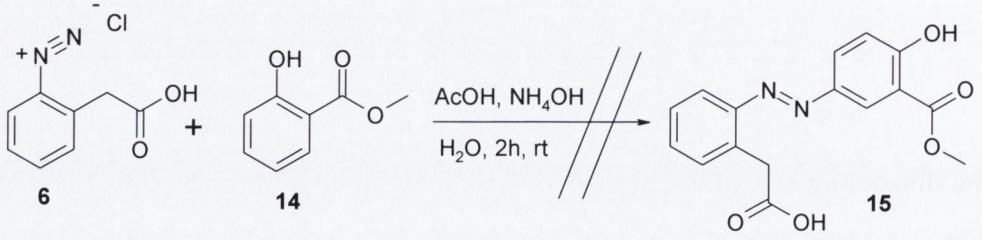
*Figure 2.6. Diazotization azo coupling mechanism to azo carrier **5**.*

In the present case, the demands of the coupling reaction require that the benzene ring of the salicylate must be activated using basic aqueous conditions. Under these conditions, the phenol group will be deprotonated and the *para*-position of the benzene ring will be activated to undergo attack on the diazonium group (*Figure 2.6*). A synthesis of 2-hydroxy-5-(4-hydroxy-phenylazo)-benzoic acid **13** was published by (Leclair and Lizamma, 2003), *Scheme 2.5*. The substituents are located in different positions than in the target product, however similar conditions were used to carry out the synthesis.



*Scheme 2.5. Azo coupling diazotization of 2-hydroxy-5-(4-hydroxy-phenylazo)-benzoic acid **13***

In our case we began using methyl salicylate as a model compound. Evidently, the methyl ester group was not a suitable protecting group because the conditions required for its removal after linking to the steroid would cause hydrolysis between the carrier and the steroid. However, methyl salicylate is commercially available and it gave an idea of how the reaction works for our reagents.

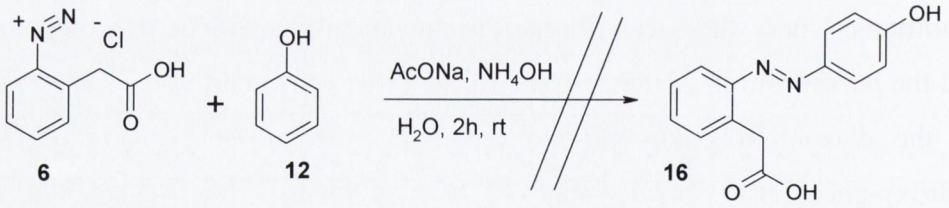


6 14 15

*Scheme 2.6. Failure to synthesize azo carrier **15** by azo coupling*

The diazonium salt of 2-aminophenyl acetic acid **6** was added dropwise into a basic solution of methyl salicylate **14** and the final pH increased to ≥ 10 during the course of reaction, however, we were unable to isolate compound **15** under these conditions.

To understand why the reaction did not work, the diazocoupling reaction was repeated using phenol **12** instead of the methyl salicylate under the same conditions (*Scheme 2.7*).

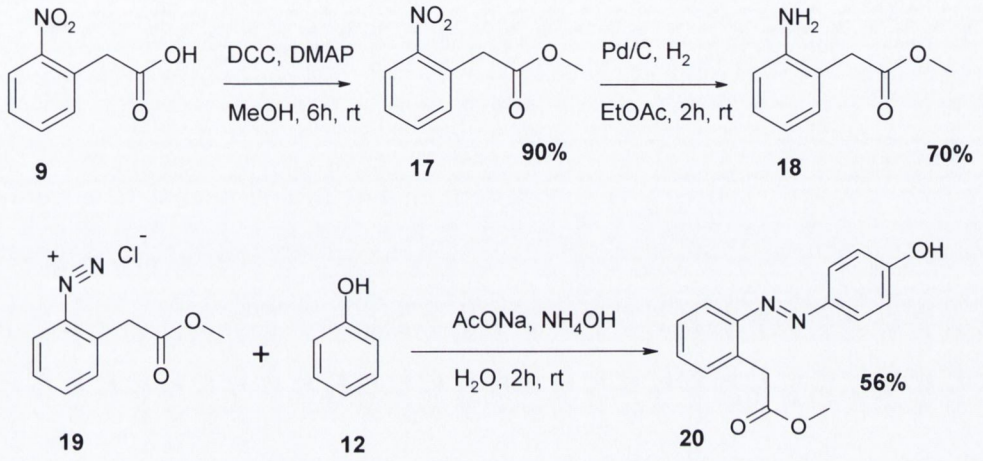


6 12 16

*Scheme 2.7. Failure to synthesize azo carrier **16** by azo coupling*

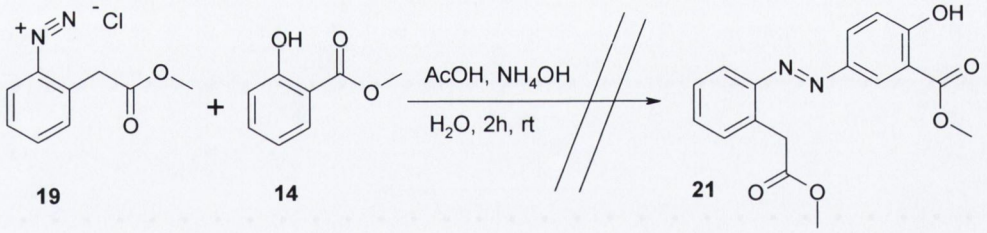
Once again in this case [2-(4-hydroxy-phenylazo)-phenyl] acetic acid **16** was not isolated. This result was surprising because under basic conditions, position 4 of the benzene ring in the phenol is activated, and the potentially deactivating ester group is not present. Therefore the problem appeared to be with the diazonium salt of the 2-aminophenyl acetic acid **6** or **14** or both. To investigate the chemistry further, a series of azocoupling reactions were carried out using alternative substrates to see the effect of substitution on the azocoupling reaction. Esterification was carried out to 2-nitro phenyl acetic acid **9** and the 2-nitrophenylacetic acid methyl ester **17** was then reduced to

form 2-aminophenyl acetic acid methyl ester **18** (*Scheme 2.8*). This was used to form diazonium salt **19**. When diazo coupling was attempted with phenol, an orange spot corresponding to the azo group was observed by TLC analysis. The product was columned yielding [2-(4-hydroxy-phenylazo)-phenyl] acetic acid methyl ester **20** (56%) which was characterized by NMR.



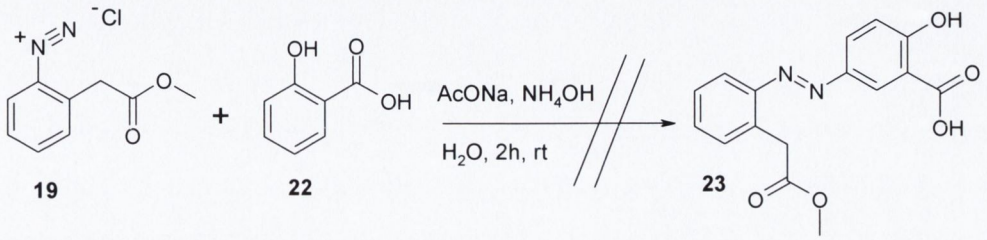
*Scheme 2.8. Synthetic approach for azo carrier **20***

We next attempted to carry out diazocoupling as shown in *Scheme 2.9* using **19** and methyl salicylate **14** to form hydroxy-5-(2-methoxycarbonylmethyl-phenylazo)-benzoic acid methyl ester **21**. However, in this attempt TLC did not show the anticipated azo orange spot.



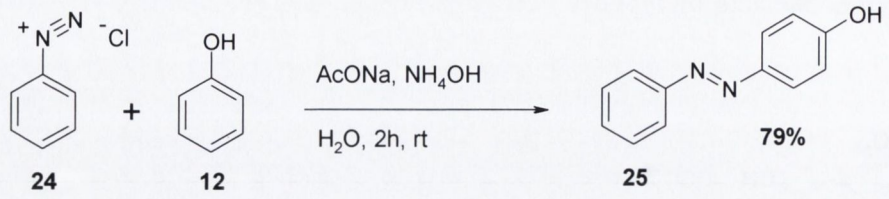
*Scheme 2.9. Failure attempt to synthesize azo carrier **21** by azo coupling*

The reaction was repeated using the unprotected salicylic acid **22** without success (*Scheme 2.10*).



*Scheme 2.10. Failure attempt to synthesize azo carrier **23** by azo coupling*

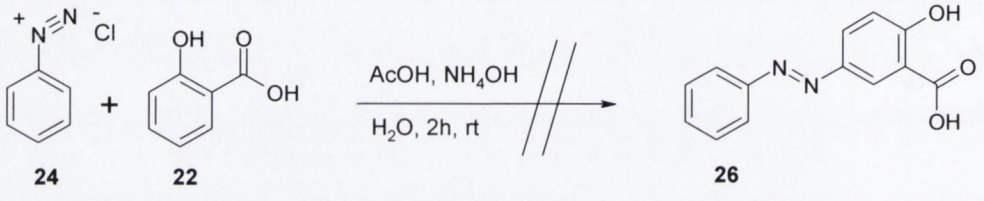
The next attempt was to make the diazonium salt of aniline **24** and use it instead of **19**, to ensure that the conventional reaction worked in our hands, and to examine the influence of the *ortho* ester substituent on the reactivity of the diazonium group.



24 **12** **25**

Scheme 2.11. Diazotization using aniline diazonium salts

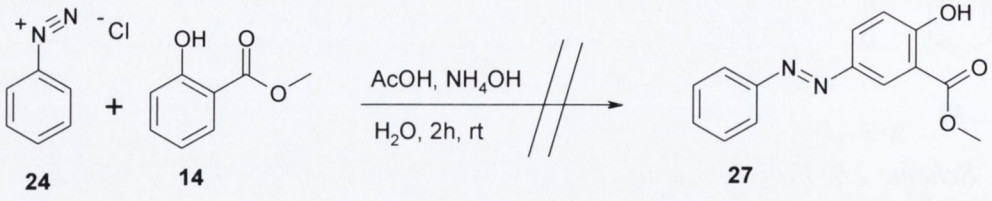
The azo coupling between aniline diazonium salt **24** and phenol **12** gave 4-phenylazo-phenol **25** in excellent yield (79%). We next attempted to repeat the coupling using salicylic acid instead of phenol (*Scheme 2.12*).



24 **22** **26**

Scheme 2.12. Azo coupling attempt of aniline diazonium salt and salicylic acid

We were unable to isolate 2-hydroxy-5-phenylazo benzoic acid **26** using this approach. However, the synthesis of **26** was reported using a very similar method by (Tushar et al., 2001) albeit in poor yield (33%).



24 **14** **27**

Scheme 2.13. Azo coupling attempt of aniline diazonium salt and methyl salicylate

The coupling was also attempted using methyl protected salicylic acid but again with no success. Some conclusions could be made at this point. The diazonium salt of 2-aminophenylacetic acid methyl ester **19** only reacts with phenol **12** (*Scheme 2.8*) to yield **20** (56%), while the diazonium salt of aniline **24** reacts with phenol **12** (*Scheme 2.11*) to yield **25** (79%). Clearly, the *ortho*

ester substituent inhibits azocoupling. Also, having a deactivating group *ortho* to the hydroxyl group in the benzene ring makes the azocoupling more difficult. When reacting the diazonium salt **19** with salicylic acid **22** or methyl salicylate **14** no product is isolated; some small quantities of product can be isolated only when these deactivated systems are coupled with aniline.

In summary the developmental chemistry appeared to indicate that azocoupling by diazotization using diazonium salts is not a useful approach to our target azo carriers because the carboxylic group *ortho* to the phenol-OH group deactivates the benzene ring suppressing the coupling. Furthermore, the *ortho* substituent on the benzene ring probably interferes with coupling too.

A different method of carrying out the azo coupling was required, in which the position of the carboxylic group would not be an impediment to coupling.

2.3.4 Dimerization of benzylamines to form azo conjugates

Searching for different methods to yield the azo carriers lead us to a method based on a radical dimerization mediated by iodine and mercury (II) oxide (Orito et al., 1998).

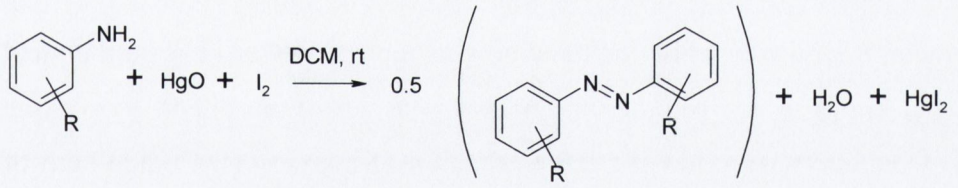
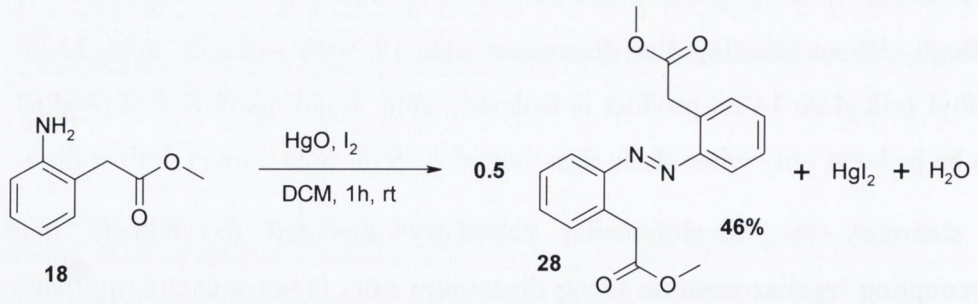


Figure 2.7. Radical dimerization of aniline derivates

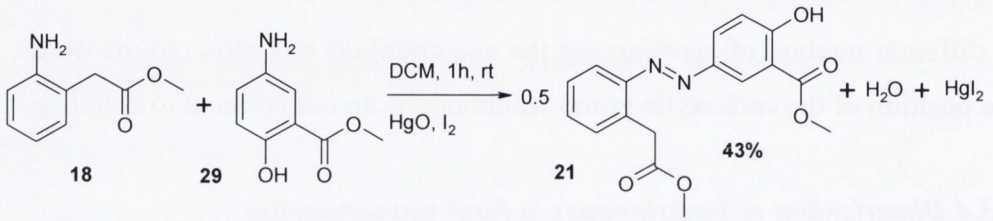
This method was originally used to produce symmetric azo conjugates by dimerization of the amino group. The importance of this procedure lay in the absence of a requirement for benzene ring activation.

Initially we tried the dimerization of **18** using DCM as solvent at room temperature for one hour to check if this method worked with our substrates. When the reaction was carried out on a small scale, the typical orange spot for the azo group was observed, and after flash chromatography [2-(2-methoxycarbonylmethyl-phenylazo)-phenyl] acetic acid methyl ester **28** was isolated (*Scheme 2.14*).



Scheme 2.14. Dimerization of 2-aminophenyl acetic acid methyl ester

After success with the first attempt, the synthesis of **21** was attempted using the methyl ester of 5-ASA **29** (5-ASA itself is insoluble in DCM):



*Scheme 2.15. Formation of asymmetric azo carrier **21** by dimerization*

The reaction was complete in one hour and 2-hydroxy-5-(2-methoxycarbonylmethyl-phenylazo) benzoic acid methyl ester **21** was synthesized (43%) and characterized by NMR. The reaction was complete in a short period of time, using a simple procedure and it yielded the right compound. However when the reaction was repeated at gram scale the yield dropped significantly. Another unsatisfactory aspect of this chemistry was the use of mercury (II) oxide and the production of mercury (II) iodide both of which are toxic. These two points highlighted the need to develop a different method to produce azo carriers.

2.3.5 Azo coupling reaction between substituted anilines and nitrosobenzenes

We next turned our attention to a promising method for azo-synthesis reported by (Schmitt et al., 1989) in which ring activation is not required and the only potential problem with the carboxylic group was a steric one. This coupling approach exploits the dehydration reaction that occurs between primary aromatic amines and nitroso compounds under mildly acidic conditions (*Figure 2.8*). Also the new approach demanded a method for

generating the nitroso reactant. Two different methods were tried for generating the nitroso group:

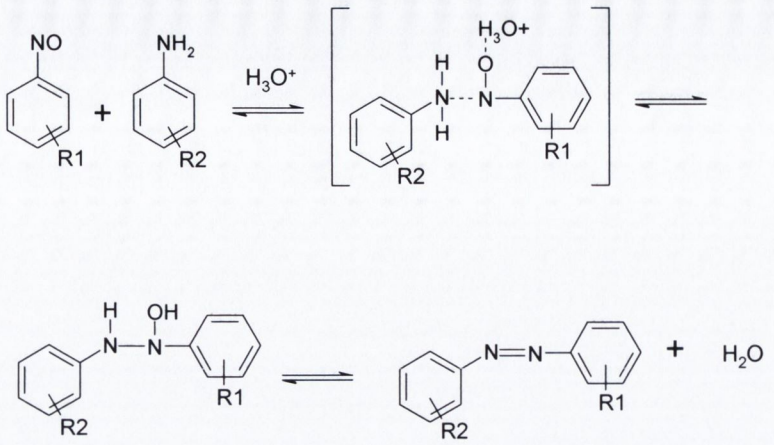
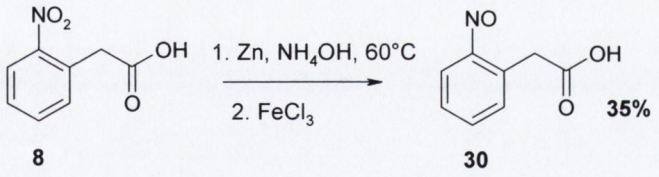


Fig 2.8. Condensation between amino and nitroso groups to form an azo linkage

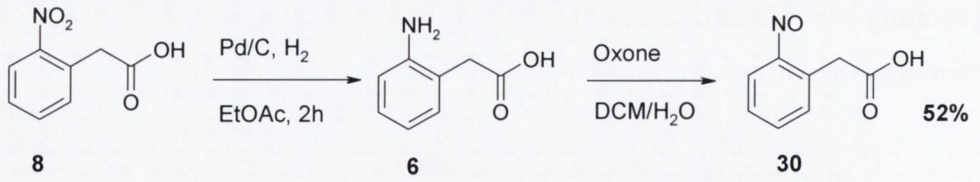
2.3.5.1 Formation of the nitroso group

The first attempt (*Scheme 2.16*) to produce 2-nitrosophenyl acetic acid **30** required zinc dust and ammonium chloride in water at 60°C for six hours (Nutting et al., 1970) followed by addition into an iron (III) chloride solution. The reaction took eight hours to go to completion as indicated by TLC and finally gave **30** in 35% yield after flash chromatography.



Scheme 2.16. 2-Nitrosophenylacetic acid formation by partial reduction

The second procedure investigated (*Scheme 2.17*) employed oxone® as a weak oxidizing agent of the amino group (Crandall and Reix, 1992). The reaction takes place in a biphasic medium: water/DCM. 2-Nitrophenylacetic acid **8** was reduced to **6** and then oxidized to **30**, the reaction monitored by TLC. **30** was characterized by NMR analysis.



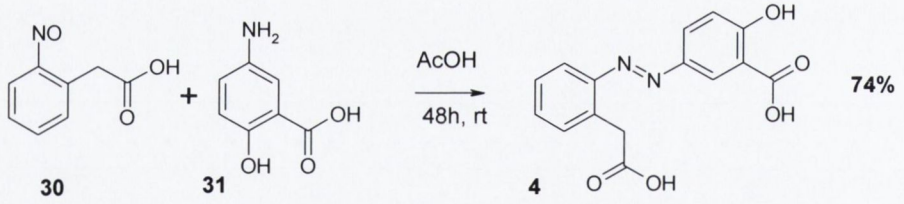
Scheme 2.17. 2-Nitrosophenylacetic acid formation by oxone®

This second way of producing the nitroso group gave **30** in 52% yield from **8**. The reaction took no longer than one hour by TLC, with oxone® as an inexpensive reagent. The advantages of this method were persuasive:

- Better yield
- Shorter reaction times
- Easier work up procedure
- Cheaper reagents

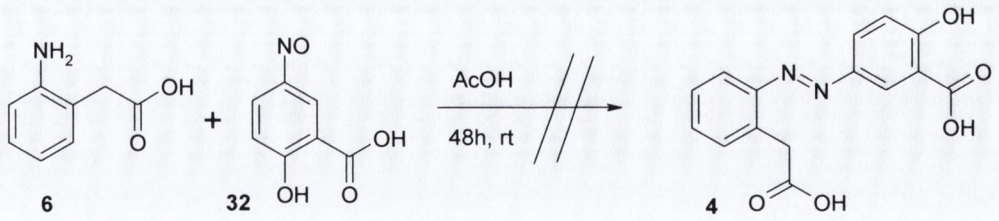
2.3.5.2 Condensation between nitroso and amino groups

Once the synthesis of the nitroso group had been developed, the condensation of the nitroso with the amino group to form the azo carrier was attempted as shown in *Scheme 2.18*. The reaction was performed in glacial acetic acid at room temperature over 48 hours (Priewish and Rück-Braun, 2005).



Scheme 2.18. Condensation between nitroso and amino group to form azo linkage

5-ASA **31** was used to develop the reaction procedure, before the use of an appropriate protective group. Although **31** is quite insoluble in glacial acetic acid, the reaction afforded 5-(2-caboxymethyl-phenylazo)-benzoic acid **4** and the crude mixture was columned to yield **4** (74%) which was characterized by NMR. The condensation between amino and nitroso groups did not show a problem with the substituted benzene ring, and it has generally proven an excellent method for azo carrier synthesis because it can be scaled up to grams in good yield.



Scheme 2.19. 2-Aminophenylacetic acid cyclization inhibits condensation to azo linkage

One important point about the chemical sequence might not be immediately clear, the position of the nitroso and amino groups is very important in this synthesis. In the case of 2-aminophenyl acetic acid under the reaction conditions, condensation competes unsuccessfully with lactamization (*Scheme 2.19*). When arranged as in *Scheme 2.18*, lactamization can't occur because the nitroso donor group is on the phenylacetic acid.

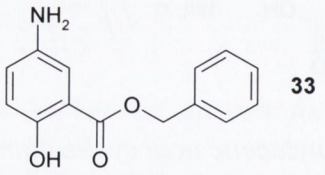
2.3.6 Protecting group selection for the carboxylic acid group of 5-ASA

Since we had now developed a robust approach to azo bond formation, we next needed to think about accommodating a protecting group into the carboxylic acid group of 5-ASA **31** since in the diacid **4**, esterification with the steroid could take place at either acid, and there were no chemical grounds to suspect one would be preferred over the other. The ideal protecting group had to be stable in the acidic conditions in which the azo coupling was carried out and needed to be capable of selective cleavage without affecting the ester link of the azo carrier or the steroid after it has been attached to the steroid. The strengths and weaknesses of some of the protecting approaches considered are described over the next page.

2.3.6.1 Benzyl group

The benzyl ester group shown in *Figure 2.9* would be stable in acidic medium and cleavage could be achieved by reduction to avoid any kind of interaction with the ester bond. The competition between the salicylate acid and phenol groups for esterification would increase the number of steps. However, the real problem seemed the likelihood of reducing the double

bond(s) of the steroid A-ring under the cleavage conditions after esterification of **33** with the steroid.

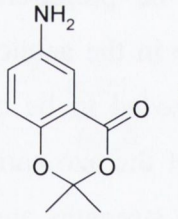


33

Fig 2.9. 5-Aminosalicylic acid protected by benzyl group

2.3.6.2 Dioxin-4-one group

Protecting 5-ASA as dioxin-4-one in **34** seemed ideal *Figure 2.10.* The dioxone (or acetonide) group is stable under acidic conditions and furthermore it masks *both* the acid and phenol group. The acetonide cleavage conditions seemed initially to be incompatible with the ester linkage to the steroid, but we were encouraged by reports that dioxin-4-ones undergo cleavage in the presence of tetrabutyl ammonium fluoride (TBAF) in DCM: the primary 21-ester was expected to be stable under those conditions. Ultimately it would emerge that the selective removal of the acetonide group in the presence of the primary ester is highly problematic (*see later in this chapter*).



34

Fig 2.10. 5-Aminosalicylic acid protected by dioxin-4-one

2.3.6.3 Tert-butyl ester group

The *t*-butyl ester **35** (*Figure 2.11*) is cheap to produce and the cleavage with trifluoro acetic acid (TFA) seemed unlikely to affect the primary ester provided it could be done at low temperature and in a short time. Two disadvantages to this group were: (i) it did not protect the phenol group although it would sterically hinder it from acylation; (ii) migration of the *t*-butyl groups on aromatic rings can occur under acidic conditions.

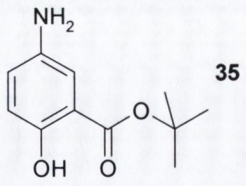
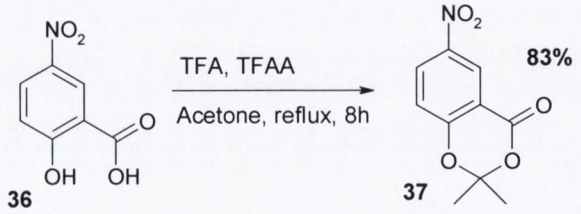


Fig 2.11. 5-Aminosalicylic acid protected by t-*butyl group*

2.3.7 Dioxin-4-one protection/cleavage

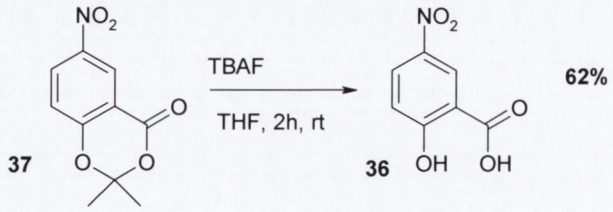
The best of the three options seemed to us, initially at least, to be the dioxin-4-one group, because of its ability to completely mask the salicylate group while prospects for selective removal looked good.



Scheme 2.20. Dioxin-4-one protection conditions

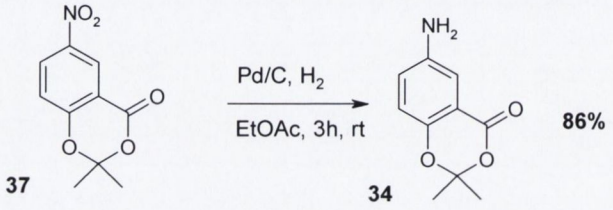
The reaction conditions for acetonide formation *Scheme 2.20* (Swinnen et al., 2005) turned out to be more appropriate to 5-nitro salicylic acid **36** than 5-ASA **31**, because the former is more soluble in acetone. Also, the nitro group is more inert towards side-reaction than the amino group and it is easily converted to amino under reductive conditions.

Compound **37** was obtained by recrystallisation above 20 g scale (70%). Chromatography was used at smaller scale albeit giving higher yields (83%). The time scale for the protection was greatly reduced using a microwave reactor at five grams scale, with the same solvent and reagents over fifteen minutes at 120°C. The reversibility of the protection step which would prove critical in the end was investigated by TLC using a small amount of compound **37**. Deprotection using TBAF in DCM was observed in good yield in a couple of hours (*Scheme 2.21*).



*Scheme 2.21. Dioxin-4-one cleavage by TBAF on the protected nitro compound **37***

TBAF seemed to work well for this system so we were ready to keep going forward in the synthesis and prepare all the starting materials for the azo coupling reaction.

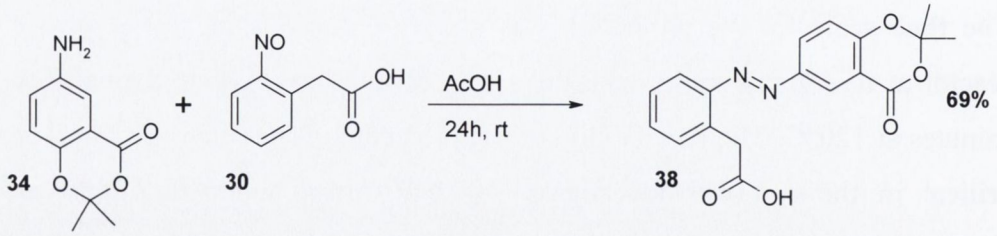


Scheme 2.22. Reduction of 5-nitro dioxin-4-one salicylate

After protection, the nitro compound **37** was reduced to **34** required for the coupling reaction, using the same conditions as before and shown in *Scheme 2.22*.

2.3.8 Azocoupling reaction of dioxin-4-one derivatives

Having prepared compounds **30** and **34** we next investigated whether the dioxinone protection could survive the azocoupling conditions.



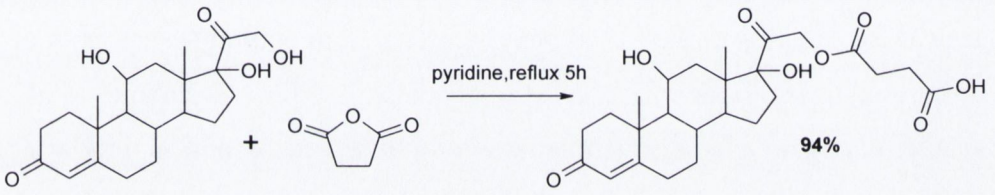
*Scheme 2.23. Condensation between amino and nitroso group to form azo carrier **38***

The reaction was carried out at room temperature and it was complete after twenty four hours to yield the azo carrier **38** (*Scheme 2.23,* 69%). This was characterized by NMR analysis after column chromatography. We were now

ready to explore the coupling of the carrier group to the 21-OH group using cortisone as a model compound.

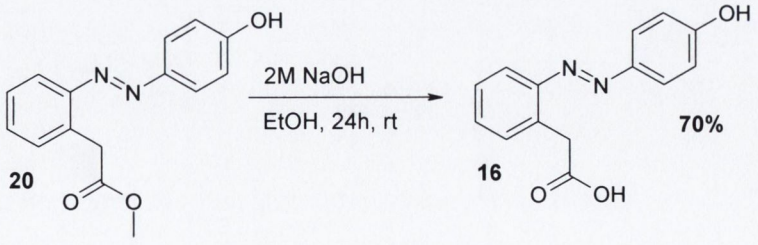
2.3.9 Developing azo carrier and steroid esterification

As already suggested here, the 21-hydroxyl group of the cortico-steroids being, a primary alcohol, is the most amenable to manipulation (including esterification). The 11-hydroxyl group is the most hindered and esterification there is reported to require more forcing conditions with protection of the 21 and 17 groups (Sloan et al., 1978). The 17-hydroxyl group position is more hindered than the 21-position and no competition for esterification was observed between these two hydroxyl groups as shown in *Scheme 2.24* (Fang et al., 2007). Migration of ester groups is known to occur from the 17-position to the 21-position.



Scheme 2.24. Esterification of hydrocortisone at C-21

Previous to developing the azocoupling between anilines and nitrosobenzenes, the esterification step was investigated using the simpler phenol azo product obtained using the diazonium chemistry. In spite of having a phenolic-OH group which could interfere with the esterification the azo carrier, **16** was used because it was the only azo carrier available to us at the time.

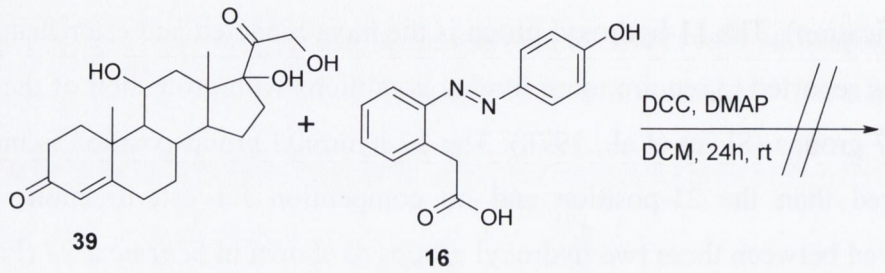


*Scheme 2.25. Azo carrier **20** methyl ester hydrolysis*

We were surprised to find that the azo bond is stable towards basic conditions used for ester hydrolysis. Following treatment of **20** with ethanolic sodium hydroxide (*Scheme 2.25*) the typical carboxylic acid tail on TLC plate was

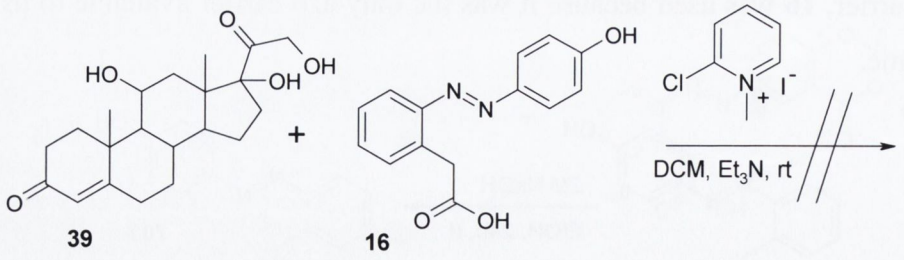
observed at lower Rf value than the ester **20**. The acid **16** was isolated by chromatography and characterized by NMR. Azo carrier **16** was used to investigate coupling to hydrocortisone **39** (*Scheme 2.26*).

Esterification reaction was investigated first using DCC and DMAP in DCM but failed (Forouton and Watson, 1997). The reaction was repeated with HOBt, without success by TLC analysis.



Scheme 2.26. Esterification attempts using DCC and HOBt

2-Chloro-1-methylpyridium iodide salt (CMPI) has been found to be effective for the preparation of carboxylic esters from equimolar amounts of free carboxylic acids and alcohols (Saigo et al., 1977) (Mashraqui et al., 2004). An active acylating intermediate is produced easily and rapidly after initiating the reaction by adding 2.4 equivalents of triethylamine. In the present case, TLC analysis after two hours indicated complete formation of the CMPI-intermediate (*Figure 2.27*). The ester is produced by nucleophilic attack of the alcohol on the carbonyl carbon of the intermediate; however we were unable to isolate the target product using this approach which yielded a complex mixture of products.

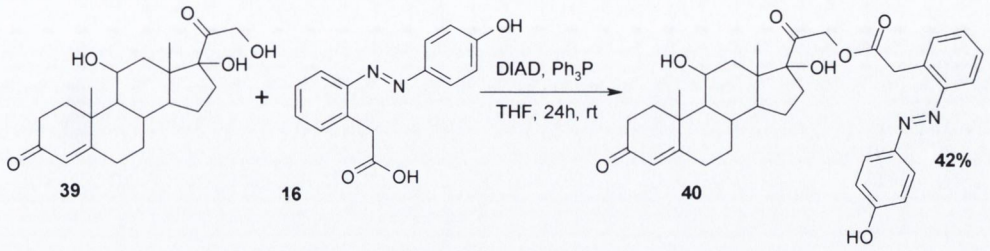


Scheme 2.27. 2-Chloro-1-methylpiridium iodide salt esterification attempt

Pentafluorphenol (PFP) and DCC were used (Babadzhanova et al., 2005), (Ramesh et al., 2006) to form the PFP ester of **16**. However there was no observable reaction with the steroid. Esterification was also attempted using

diaza-(1, 3)-bicyclo [5.4.0] undecane (DBU) and lithium bromide in THF at 80°C. TLC analysis did not reveal any products likely to be the target.

Ultimately the ester **40** could only be isolated using the Mitsunobu approach (Mitsunobu and Yamada, 1967), (Clader et al., 1996) in 42% yield which was characterized by NMR analysis (*Scheme 2.28*).



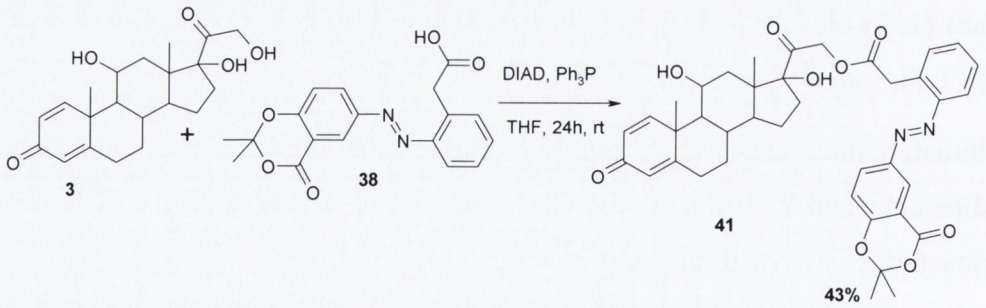
Scheme 2.28. Esterification by Mitsunobu reaction

We were pleased that we had at least produced an ester of the target type, and identified an appropriate coupling methodology. Unfortunately we could not consider investigating **40** as a prodrug because reduction in vivo would liberate 4-hydroxyaniline which is toxic.

2.3.10 Prednisolone esterification to azo carrier 38

At this stage the Mitsunobu reaction had proven to be a reliable esterification method and the nitroso/amino condensation allowed us to synthesize any azo carriers we were likely to need. In this section we describe investigations into the esterification between prednisolone **3** and the dioxin-4-one protected azo carrier, compound **38** *Scheme 2.29*.

The esterification was carried out by Mitsunobu reaction as developed previously, in dry THF as solvent. The reaction was started after adding prednisolone **38**, triphenylphosphine (Ph_3P) and reaching 40°C. At this point diisopropyl azodicarboxylate (DIAD) was added, and the reaction mixture left stirring for one hour before leaving to cool at room temperature with overnight stirring. Purification was the most difficult part of the synthesis, and two flash columns were needed to isolate the dioxin-4-one protected mutual prodrug, compound **41** (40%).



*Scheme 2.29. Dioxin-4-one protected mutual prodrug **41** formation*

Having finally isolated the prodrug **41** and characterized it by NMR (^{1}H-NMR shown in *Figure 2.21*), we began exploring its deprotection to the target compound.

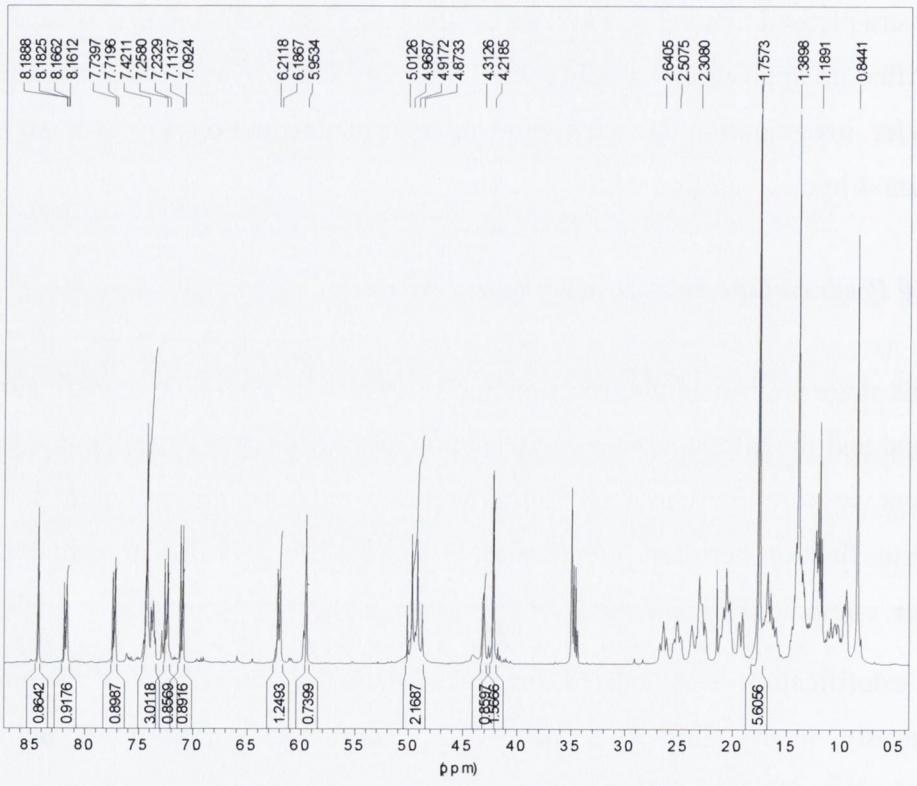
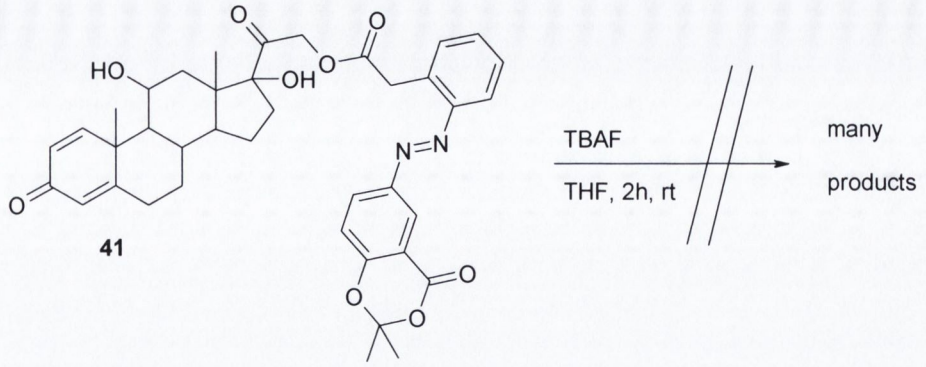


*Figure 2.12.^{1}H-NMR spectrum of dioxone protected mutual prodrug **41***

2.3.11 Dioxin-4-one cleavage experiment

As we described previously, TBAF deprotection of the acetonide of 5-nitrosalicylic acid **37** proceeded smoothly. The same procedure was attempted on the acetonide protected mutual prodrug **41** with high

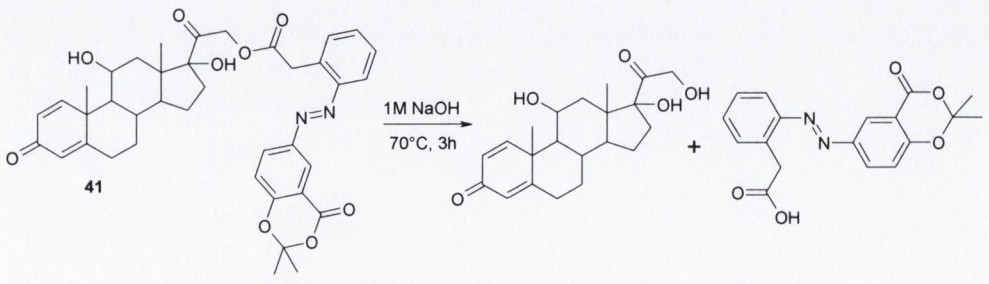
expectations of success although with the possibility of competing de-esterification.



Scheme 2.30. Cleavage failure by TBAF

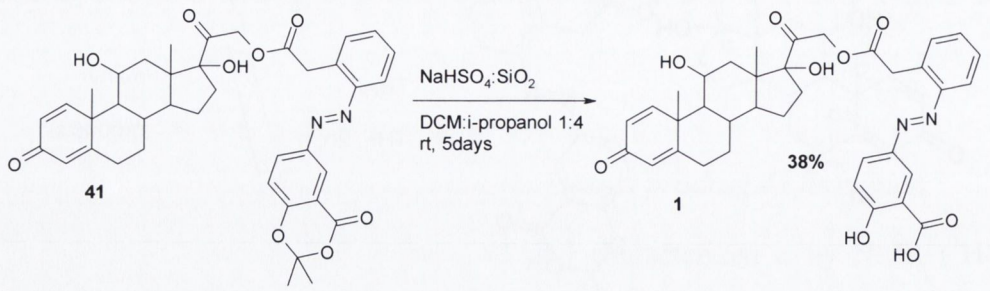
Analysis by TLC following treatment of **41** with TBAF revealed decomposition of the starting material with many spots appearing on the TLC plate. The reaction was repeated at 0°C with a qualitatively similar outcome. Other reagents were investigated including TFA/H_2O (2/1) (Swinnen et al., 2005) but no reaction was observed and starting material remained after eight hours. The reaction was repeated using just TFA but again no deprotection was observed. Deprotection was then attempted using the more aggressive BCl_3 (Fürstner et al., 2001) but a large number of products formed as detected by TLC indicating a lack of selectivity. (Gerrard and Lappert, 1958) point out the high reactivity of boron trichloride and its potential reactivity towards oxygen containing functional groups such as alcohols, ethers, aldehydes, ketones and carboxylic acids. This is probably relevant here.

Treatment with sodium hydroxide 1N solution at 70°C (*Scheme 2.31*) over three hours (Swinnen et al., 2005) caused hydrolysis of the steroid ester linkage rather than the deprotection – a spot corresponding to prednisolone was observed on the TLC plate after analysis of the reaction mixture.



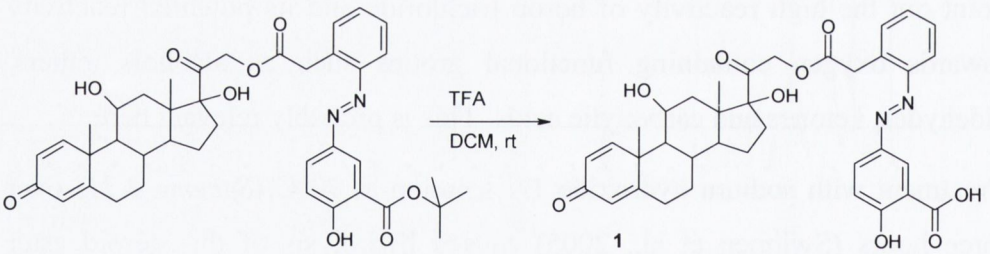
*Scheme 2.31. Hydrolysis of protected prodrug **1***

Ultimately it was found that the deprotection could be accomplished using sodium hydrogen sulfate on silica gel in DCM:isopropanol (Breton, 1997) *Scheme 2.32.*



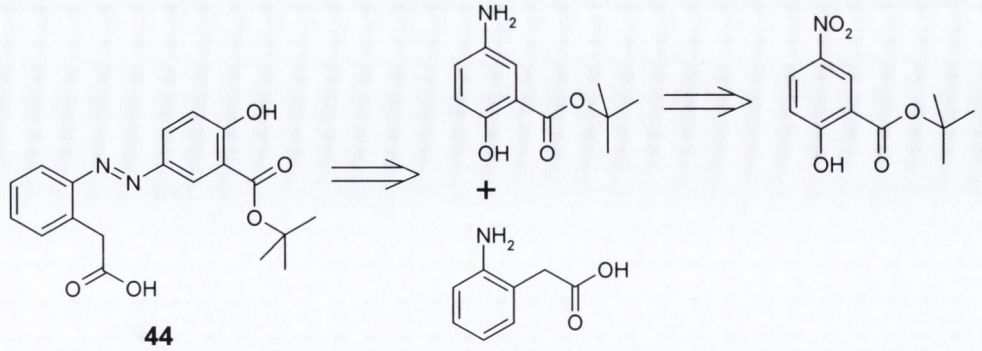
Scheme 2.32. Cleavage of dioxin-4-one group by sodium hydrogen sulfate catalyst

The cleavage took five days and after flash chromatography **1** was obtained in 38% yield. Compound **1** was characterized by NMR. However, while it was satisfying to eventually make the target the acetonide seemed a poor protecting group. The cleavage method took a long time and the yield was very low (*Scheme 2.32*). There didn't seem to be any point in trying to further optimize the deprotection conditions. Instead we decided to go back to the beginning using an alternative *t*-butyl ester protecting group, since this potentially had the virtue of being cleaved under conditions that the primary ester linkage might survive (*Scheme 2.33*).



*Scheme 2.33. Potential tert-butyl ester cleavage to form prodrug **1***

However it was not clear at this point if the *t*-butyl group would survive other chemistries it would encounter along the synthetic route (*Scheme 2.34*).



44

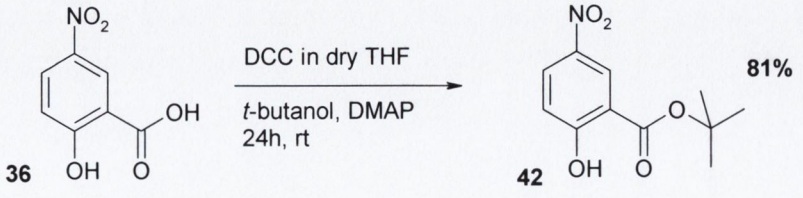
*Scheme 2.34. Retrosynthetic analysis to form azo carrier **44***

2.3.12 Tert-butyl ester protection/cleavage

Having failed to find an efficient deprotection procedure for the dioxinone protected mutual prodrug **41**; we turned our attention to the alternative *t*-butyl protecting group.

Initially protection was attempted on 5-ASA **31** using DCC and DMAP and *t*-butanol as solvent, but no product was formed at all, probably because 5-ASA is highly insoluble under these conditions. 5-Nitrosalicylic acid **36** was used instead because it is soluble in *t*-butanol. However, using the usual coupling conditions the yields were rather poor.

Different attempts with alternative coupling methodologies including PFP, SO_2Cl or CMPI were similarly unsuccessful. A variation on the conventional DCC procedure reported by (Kluger and De Stefano, 2000) was then used successfully. This employed a DCC solution in dry THF *Scheme 2.35* which was added dropwise to a 5-nitrosalicylic acid and DMAP in *t*-butanol solution. This was then left overnight to yield the 5-nitrosalicylic acid *t*-butyl ester. The ester was characterized by ^{1}H and ^{13}C-NMR analysis after purification by chromatography.

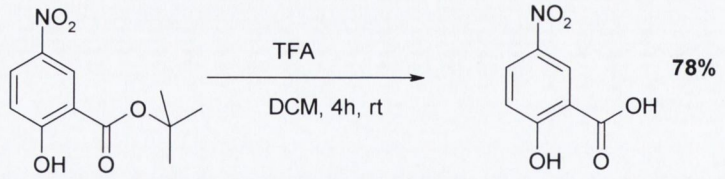


36 **42**

Scheme 2.35. Tert-butyl ester protection conditions

No problems were expected with deprotection but it was checked anyway. Cleavage using TFA in a solution of DCM was carried out in four hours at

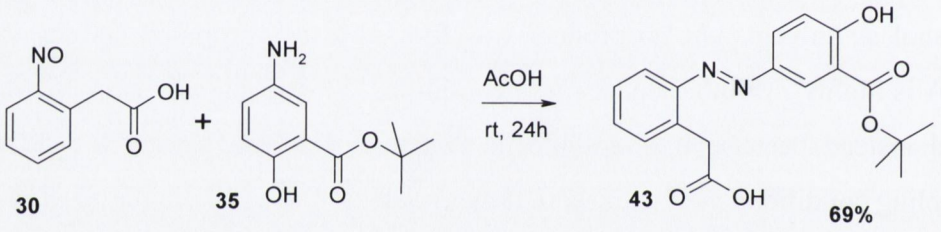
room temperature to yield 5-nitrosalicylic acid **36** as revealed by TLC analysis revealed *Scheme 2.36*.



Scheme 2.36. Cleavage conditions to produce 5-nitrosalicylic acid

2.3.13 Azocoupling of tert-butyl ester protected derivates

Before attempting the synthesis of **43**, 5-nitrosalicylic acid *t*-butyl ester **42** was reduced to 5-amino salicylic acid *t*-butyl ester **35** with Pd/C under hydrogen atmosphere. The condensation between the amino and nitroso groups took place in glacial acetic acid (*Scheme 2.37*).

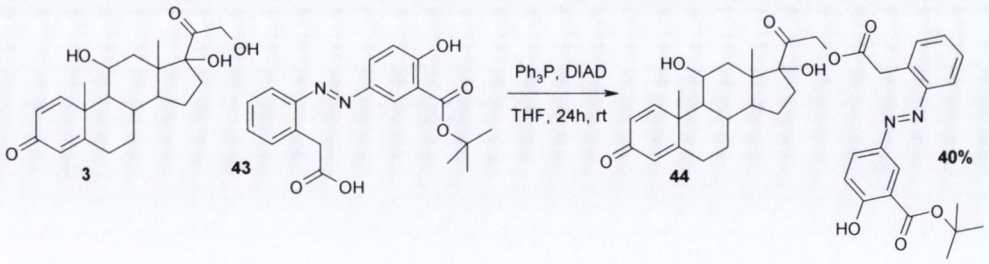


*Scheme 2.37. Condensation conditions to produce azo carrier **43***

We were pleased that the *t*-butyl ester was stable under these acidic conditions; no cleavage was observed after the twenty four hours of reaction time. Azo compound **43** was characterized by ^{1}H and ^{13}C-NMR.

2.3.14 Prednisolone esterification to azo carrier 43

The esterification was performed using the Mitsunobu reaction as before. The protected prodrug was isolated after two columns as orange crystals in around 40% yield. ^{1}H and ^{13}C-NMR analysis was used to characterize **44** (*Scheme 2.38*).

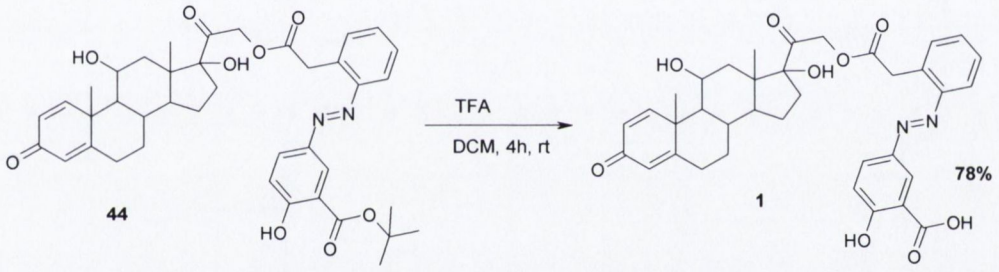


*Scheme 2.38. Mitsunobu reaction between prednisolone **3** and azo carrier **43***

We now turned our attention to the previously problematic deprotection step.

2.3.15 Cleavage of tert-butyl ester group of 45

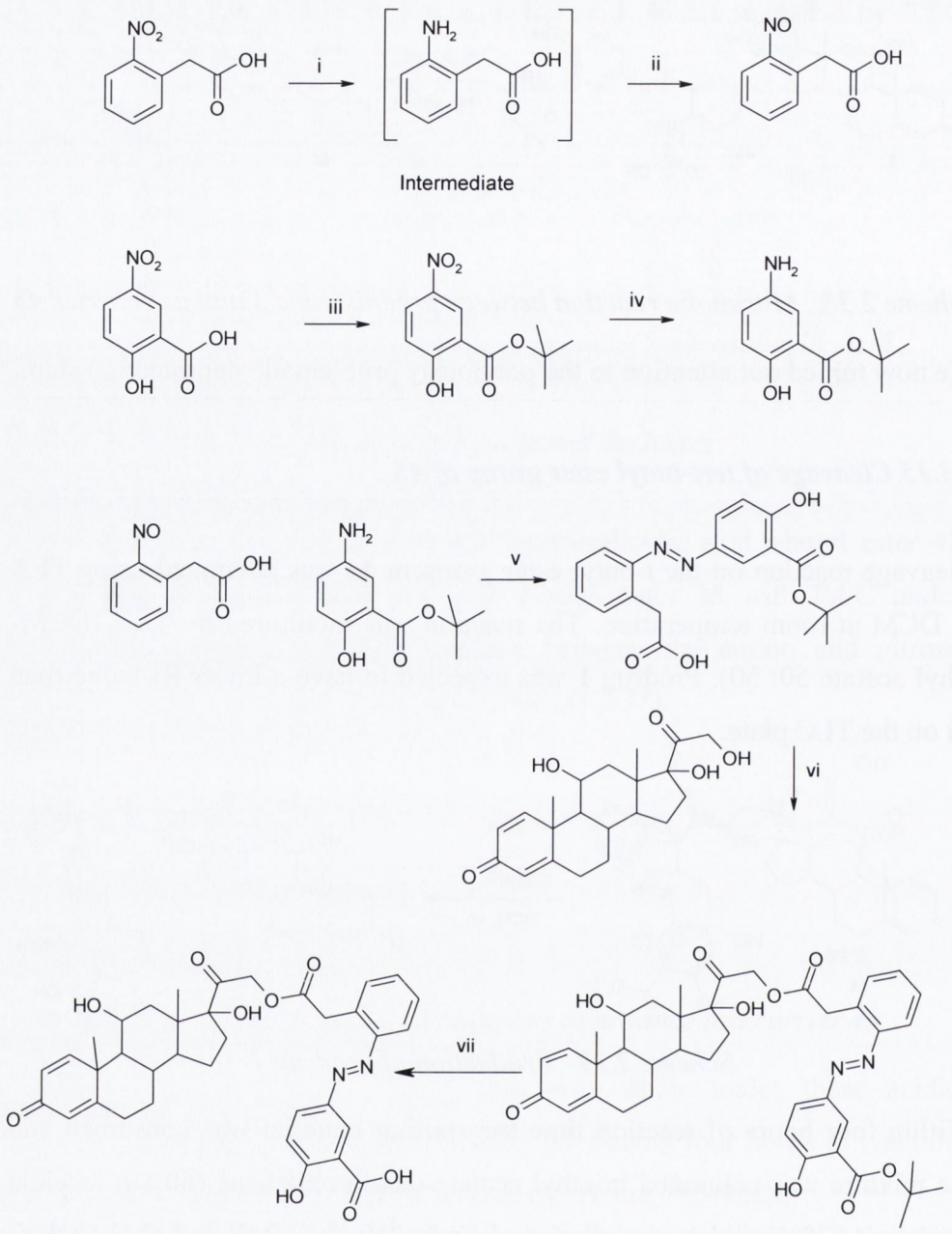
Cleavage reaction on the *t*-butyl ester group in **44** was attempted using TFA in DCM at room temperature. The reaction was monitored by TLC (DCM-ethyl acetate 50: 50). Prodrug **1** was expected to have a lower Rf value than **44** on the TLC plate.



*Scheme 2.39. Production of prodrug **1***

Within four hours of reaction time the starting material was consumed and the mixture was columned in ethyl acetate-dichloromethane (50:50) to yield prodrug **1** (78%) which was characterized by ^{1}H, ^{13}C-NMR and C-H COSY-NMR analysis as shown in *Figure 2.13*.

This excellent yield shows the *t*-butyl ester to be suitable protecting group in the synthesis of the azo prodrugs; the protection reaction is simple and the cleavage is simpler, easier and faster than that of the dioxin-4-one group. The summary of the synthetic route to prodrug **1** is shown next (*Scheme 2.40*).



Scheme 2.40 i: H_2O/ NaOH 2N, Pd/C, H_2; ii: Oxone in DCM/H_2O; iii: DCC in dry THF, DMAP in t-*butanol; iv: Pd/C, H_2; v: AcOH; vi: Ph_3P, DIAD in THF; vii: TFA.*

Characterization of prodrug **1** was done by analyzing the ^{1}H, ^{13}C-NMR spectra shown in *Figure 2.13* and by high resolution mass spectrometry.

The ^{1}H-NMR analysis showed eight protons in the region between 8.50 and 7.04 ppm. Seven are the aromatic protons from the azo carrier group and one is the proton from C-1 of the prednisolone in the multiplet at 7.46 ppm. Prednisolone protons from C-2 and C-4 appear at 6.25 and 6.00 ppm respectively. These assignments were confirmed by the C-H COSY-NMR analysis. Other key protons to be assigned are the two from the CH_2 of the

prednisolone C-22 which appear in the spectrum at 5.01 ppm. Finally the two protons from the CH_2 of the azo carrier group appear as a doublet at 4.31 ppm.

C-H COSY-NMR spectra and comparison between the prodrug **1** and prednisolone ^{1}H-NMR spectra provides information to confirm the peaks at 8.50, 8.05, 7.74, 7.04 and three protons from the multiplet at 7.46 came from the azo carrier. The doublet at 4.31 ppm is shown to come from the azo carrier, in spite of observing the two protons from the CH_2 group as a singlet in the ^{1}H-NMR spectra of the azo carrier **43**.

The ^{13}C-NMR spectrum revealed the right number of CH_3, CH_2, CH and quaternary carbons of the prodrug. Comparison of prednisolone and prodrug **1** (*Figure 2.14*) ^{13}C-NMR spectra revealed a higher chemical shift of the quaternary carbon from C-20 in the prednisolone compared with the prodrug **1**. That can be attributed to the different chemical environment of the C-20 atom in both drugs. Finally the appearance of a new quaternary peak between C-3 and C-5 on the prodrug **1** spectra, corresponding to a carbonyl group of the azo carrier verifies esterification along with the appearance of the new peaks in the right positions.

The high resolution mass spectrum (HRMS), also confirmed esterification with the sodium ion $(M\text{-}Na)^+ = 665.2503$, the expected mass $(M\text{-}Na)^+ = 665.2475$.

Further details of the ^{1}H, ^{13}C-NMR details are presented in the experimental chapter of this thesis.

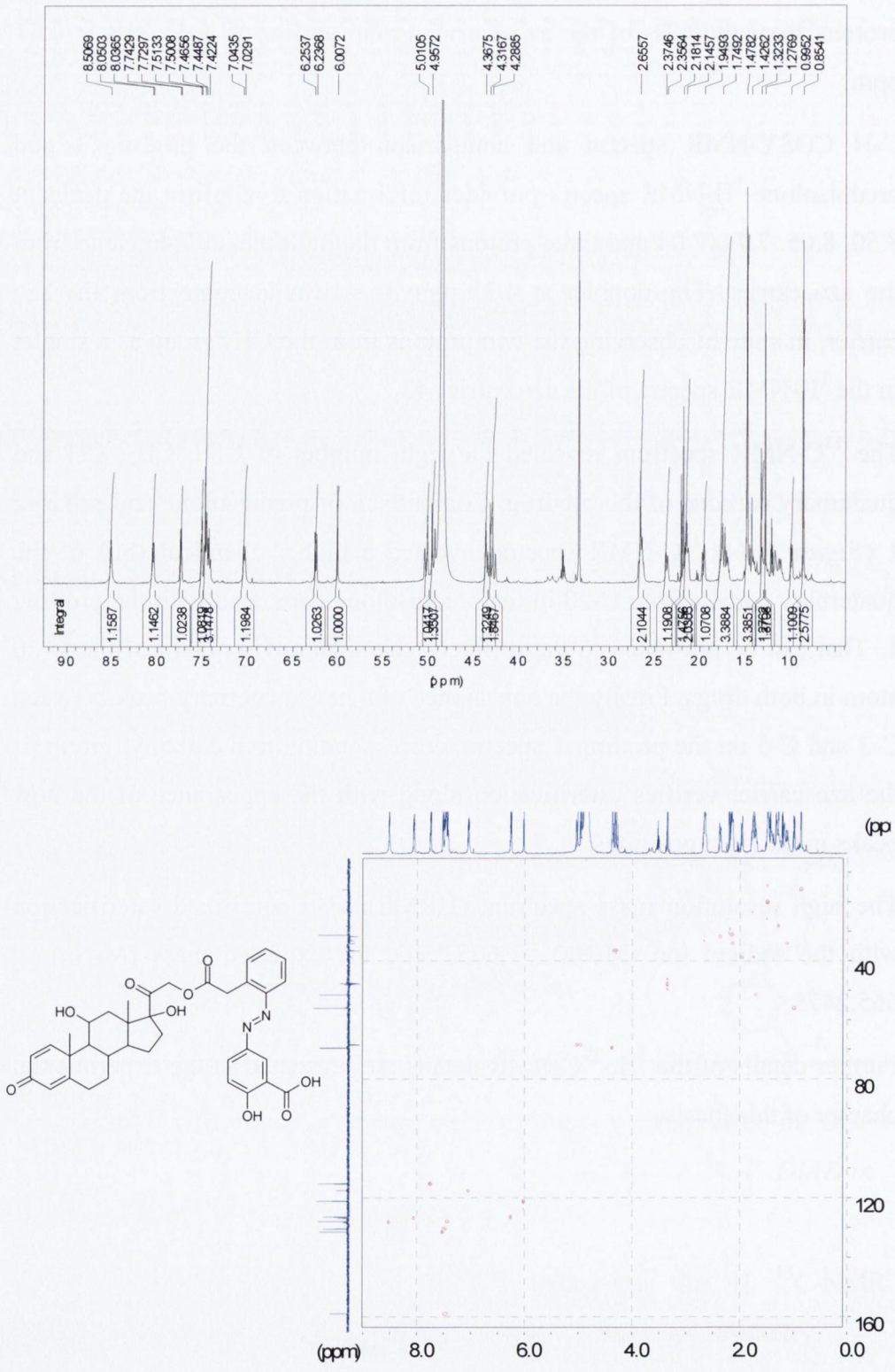


*Figure 2.13. [1]H-NMR and COSY-NMR of prodrug **1***

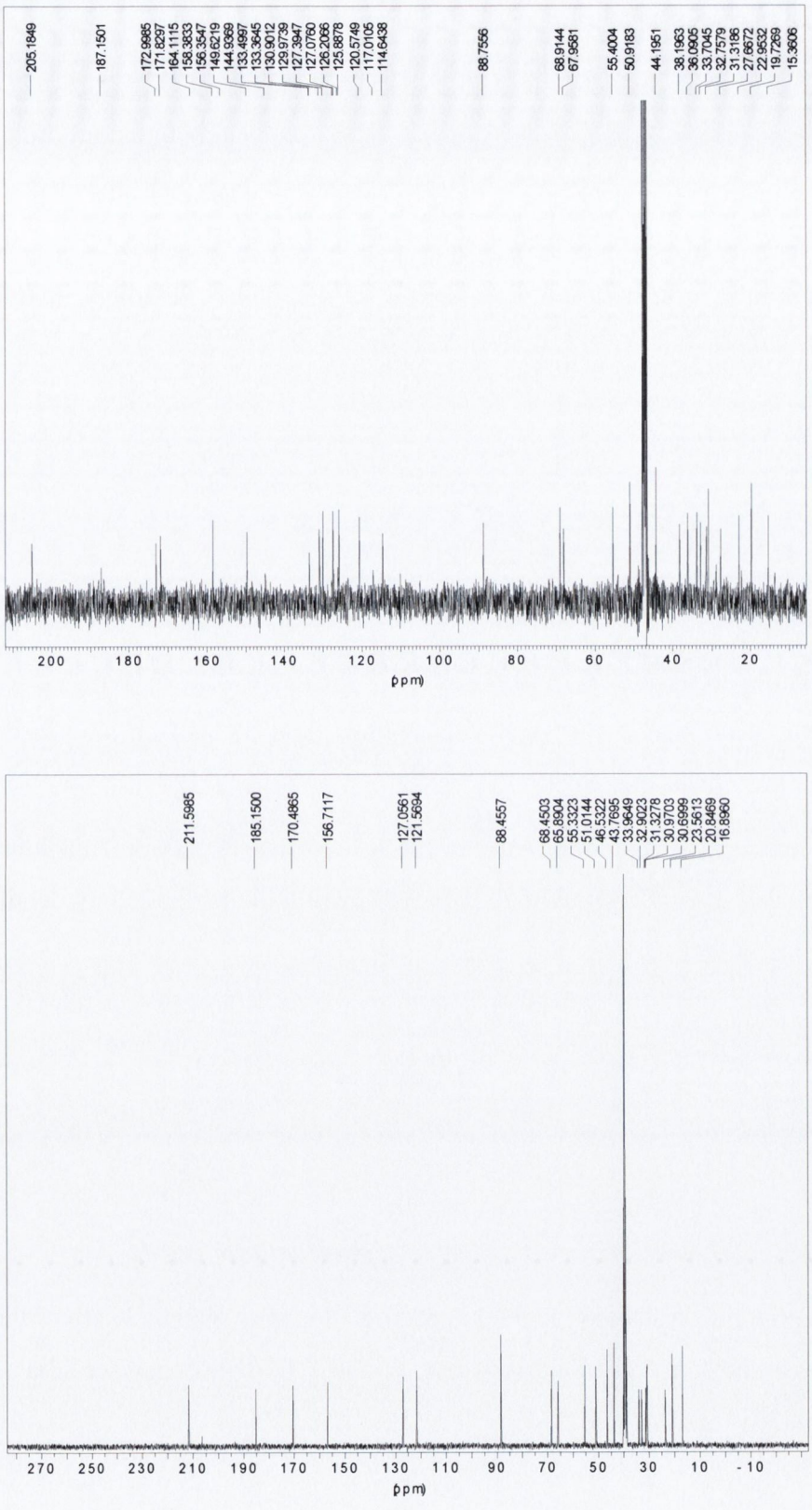
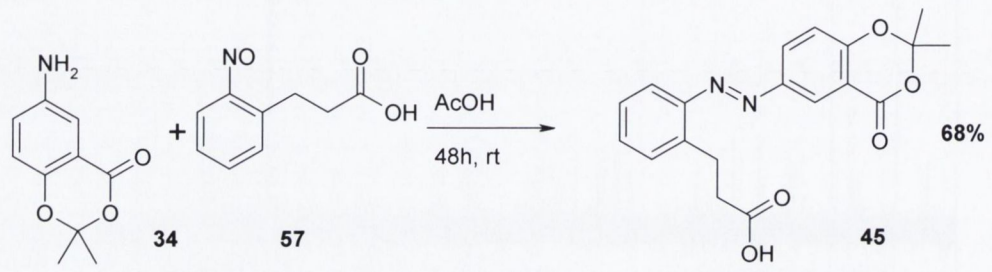


*Figure 2.14. ^{13}C-NMR spectra of prodrug **1** and prednisolone **3***

2.4 Developing prodrug 2 synthesis

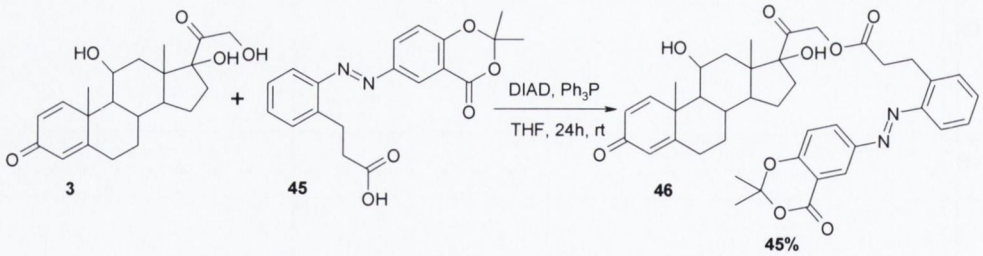
Before starting to describe the proposed synthetic route to prodrug **2** we describe herein the synthesis of a new dioxinone-protected mutual prodrug **46** which unfortunately could not be deprotected satisfactorily (as happened with its analogue **41** and as explained in Section ***2.3.11***). The condensation to form the azo linkage was performed in acetic acid at room temperature (*Scheme 2.41*):



*Scheme 2.41. Condensation to form azo carrier **45***

The synthesis of the 2-nitrosophenylpropionic acid **57** will be described in later sections of this chapter.

Formation of the ester bond between the azo carrier **45** and prednisolone **3** was achieved with a Mitsunobu reaction, following the methodology detailed in Section ***2.3.9*** of the present chapter.



*Scheme 2.42. Dioxone protected mutual prodrug **46***

The dioxinone protected mutual prodrug **46** was isolated after flash chromatography and characterized by ^{1}H and ^{13}C-NMR analyses, the ^{1}H-NMR analysis is shown next (*Figure 2.15*).

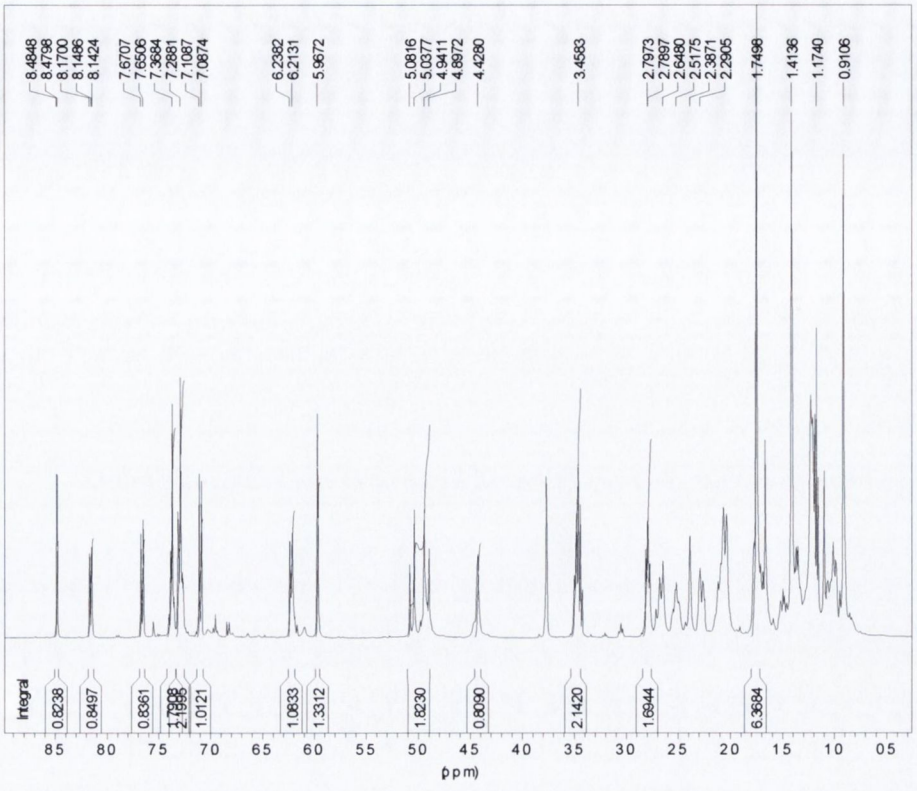
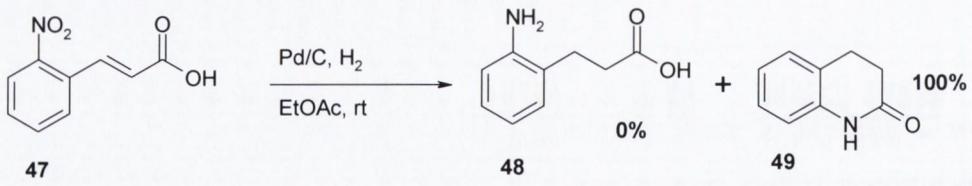


Figure 2.15 Dioxone mutual prodrug ***46*** *^{1}H-NMR spectrum*

2.4.1 Reduction of 2-phenylcinnamic acid

Once a successful approach to prodrug **1** had been developed, it was expected that a similar route could be used to prepare prodrug **2**, since both drugs are similar. Thus it was proposed to use *t*-butyl protection, the key dehydration reaction for azo production and the Mitsunobu reaction for coupling. Because 2-aminophenyl propionic acid **48** is not commercially available it had to be prepared from its commercially available precursor 2-nitrophenyl cinnamic acid **47**. Initially a reduction by Pd/C in ethyl acetate under a hydrogen atmosphere seemed the most logical approach because the nitro and alkene groups are reduced simultaneously. It was planned in the next step to oxidize the amino group to nitroso using oxone®. However as shown in *Scheme 2.43,* when the reduction reaction was attempted we were unable to isolate any of the 2-aminophenyl propionic acid **48** because cyclization to the 3,4-dihydro-1H-quinolin-2-one **49** was very rapid. Generally it was found that 2-aminophenyl propionic acid was more prone to undergo cyclization than 2-aminophenyl acetic acid, at least in organic solvent.



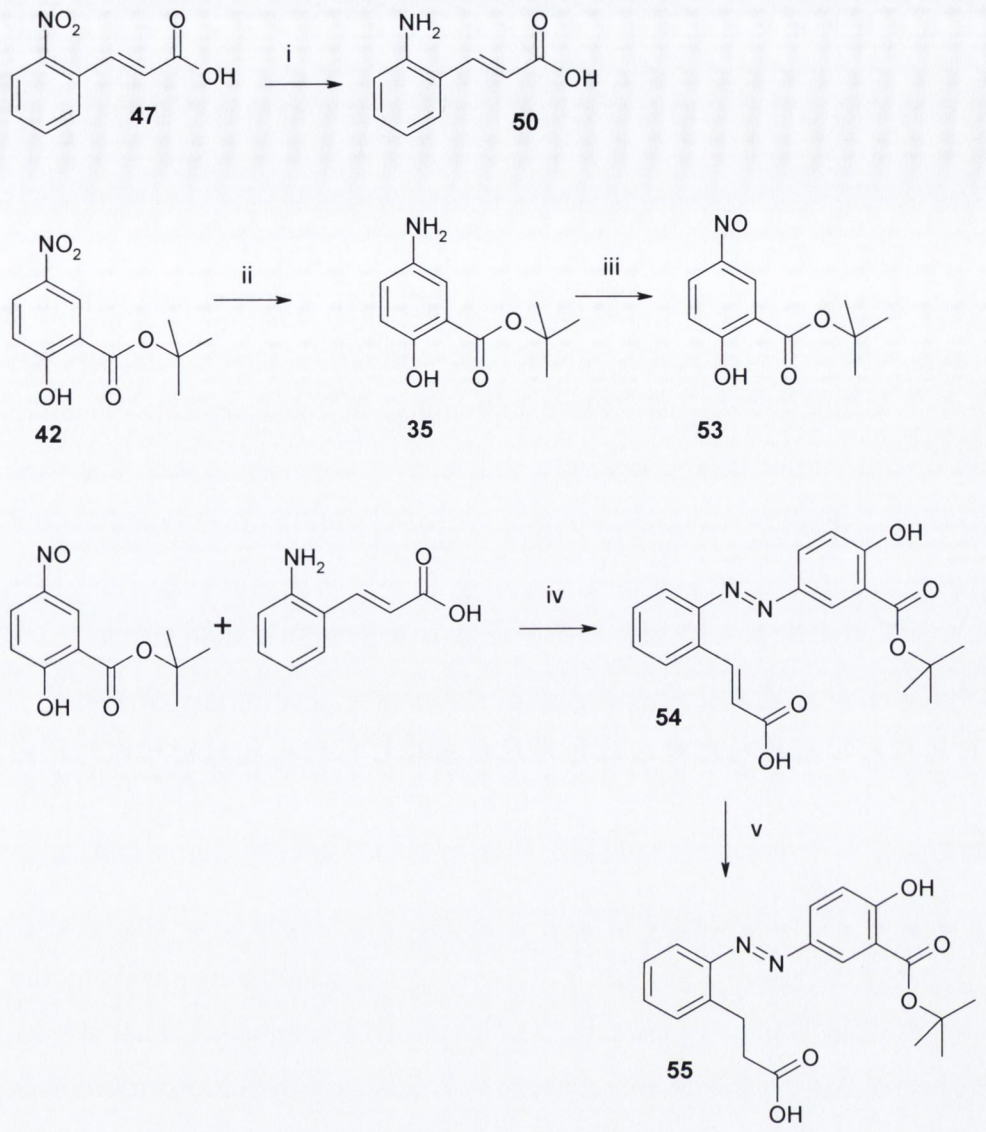
47 48 49

Scheme 2.43. Reduction of 2-nitrocinnamic acid

We needed to find another synthetic approach to prepare the azo carrier for the prodrug **2** and our biggest problem was preventing intramolecular lactamization of 2-aminophenyl propionic acid.

2.4.2 Alternative route to reduction of 2-nitrophenyl cinnamic acid

One strategy for avoiding cyclization would be to take advantage of the *trans* configuration of cinnamic acid. We reasoned that in the *trans* configuration the amino group would be unable to attack the carbonyl group of the acid or the corresponding ester group which could then be left intact until later in the synthesis. For that reason another route to synthesize azo carrier **55** was proposed. It is showed in *Scheme 2.44*.

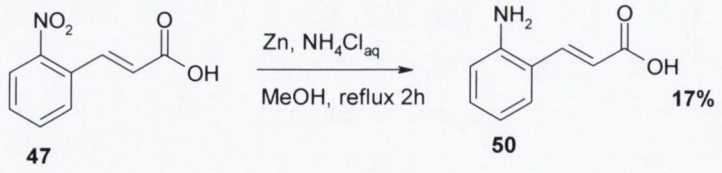


Scheme 2.44. i: Zn, NH_4Cl; ii: Pd/C, H_2; iii: Oxone; iv: AcOH; v: Pd/C, H_2

As shown here the idea was to leave the alkene group until after the azo linkage had been made, precluding the possibility of lactamization. Reduction was possible after introducing the azo group because the azo group cannot cyclize.

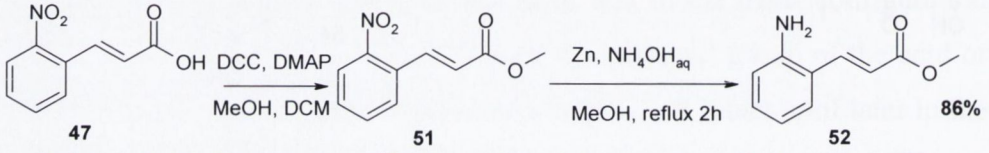
2.4.3 Specific reduction of 2-nitrophenyl cinnamic acid

Selective reduction of the nitro group from 2-nitrophenyl cinnamic acid **47** requires a reducing agent weak enough to leave the alkene intact. The selective reduction method used was previously described by (Heller, 1910).



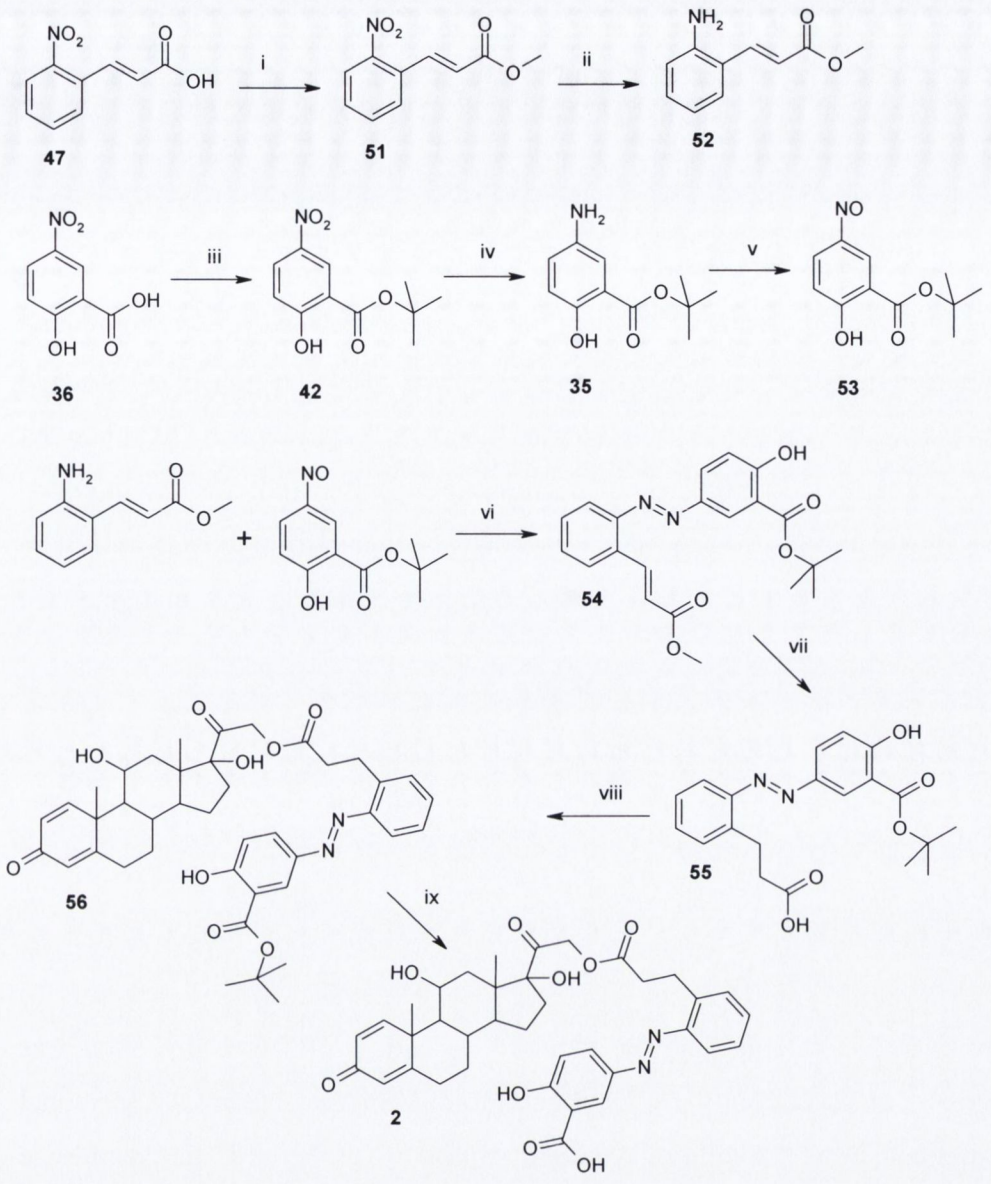
Scheme 2.45. Partial reduction of 2-nitrocinnamic acid

Reduction was carried out with zinc dust and ammonium chloride refluxing for two hours until reaction completion (*Scheme 2.45*). There was, however, a problem with this reaction. The reaction product 2-aminophenyl cinnamic acid **50** was found to be insoluble and it stays with the excess zinc dust making it difficult to work up, resulting in poor yield. In order to address the solubility problem the cinnamic acid was protected as a methyl ester first (*Scheme 2.46*) since this was likely to be more soluble in organic media.



Scheme 2.46. Formation of 2-nitrophenyl cinnamic methyl ester and its selective reduction

In this way the reaction product is easier to isolate than the free acid and the reaction works better. We used the 2-aminophenyl cinnamic acid methyl ester **52** to carry out the azo coupling because in this case the nitroso position is not important since no cyclization is possible with the *trans* alkene group. The synthetic route to the prodrug **2** was then two steps longer than the proposed route in *Scheme 2.47*.

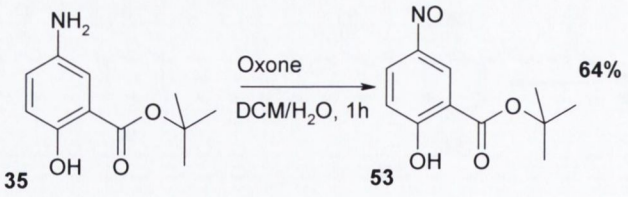


Scheme 2.47. Proposed route towards prodrug **2** *i: DCC, DMAP, MeOH; ii: Zn, NH_4OH; iii: DCC in THF, DMAP in* t*-butanol; iv: Pd/C, H_2; v: oxone®; vi: AcOH; vii: Pd/C, H_2; NaOH 2N; viii: Ph_3P, DIAD in THF; xi: TFA*

2.4.4 First synthetic approach to prodrug 2

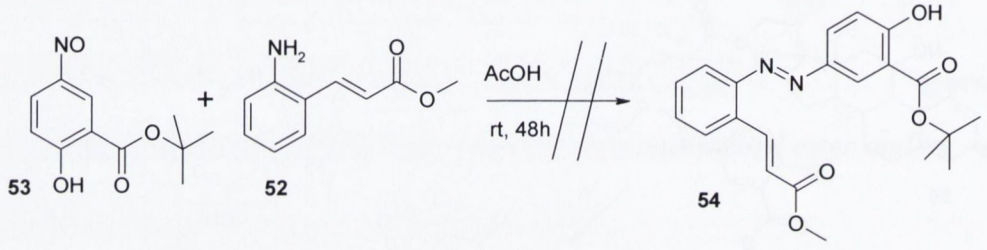
The synthesis of 5-nitroso salicylic acid *t*-butyl ester **53** was required as previously described.

Oxidation of 5-amino salicylic acid *t*-butyl ester **35** with oxone® as in *Scheme 2.48* afforded the corresponding nitroso compound in good yield.



Scheme 2.48. Oxidation of the amino group to nitroso by oxone®

The azo coupling reaction between the appropriate esters, nitroso compound **53** and amino compound **52** did not proceed easily in glacial acetic acid (*Scheme 2.49*) as before. A small orange spot appeared on the TLC plate but the starting materials substantially remained even after reflux for eight hours. The alkene group seems to inhibit the condensation to form the azo linkage in this case compared with the corresponding phenyl acetic acid analogue (*see Scheme 2.37*).



*Scheme 2.49. Condensation to produce azo carrier **54***

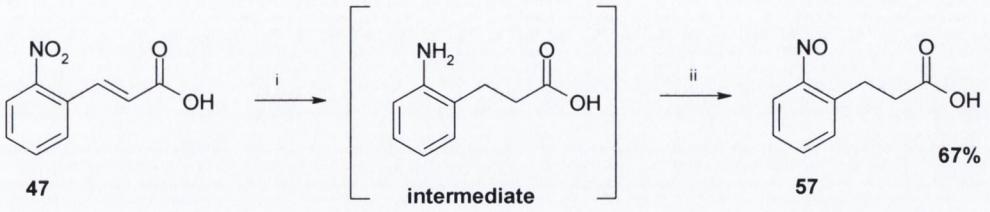
However this reaction and the required hydrolysis of the methyl ester was carried out successfully by Dr Gabor Radics in this laboratory (personal communication). Another synthetic route was devised to synthesize compound **2** due to difficulties in reproducing this chemistry.

2.4.5 Prodrug 2 synthesis

A new synthetic approach to compound **2** was successfully developed with a smaller number of steps.

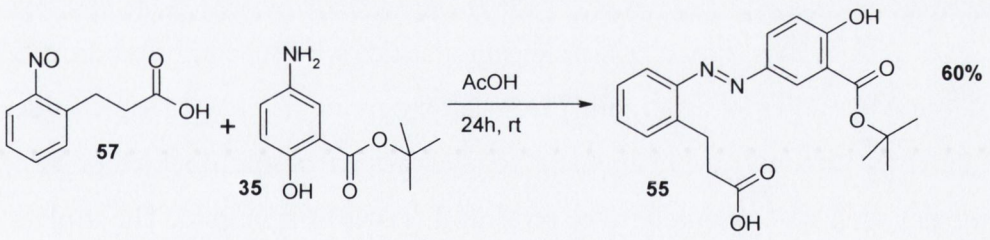
Looking at the synthetic scheme proposed for prodrug **2** (*Scheme 2.47*) it seemed that the main problem was the reduction of the 2-nitrophenyl cinnamic acid **47**. If we could avoid intramolecular cyclization of the 2-aminophenyl propionic acid **48** then we would be able to synthesize 2-nitrosophenyl propionic acid **57** removing several steps in the synthesis, and obtain different starting materials without the alkene group to carry out the nitroso group and amino group condensation to form the azo carrier **55**.

The first suggestion was to carry out the reduction of 2-nitrophenyl cinnamic acid **47** in a different medium, because in organic solvents cyclization is especially rapid. Carrying out the reduction in a basic aqueous solution (Winters and Odasso, 1984) inhibits cyclization because the carboxylate is anionic and suppresses nucleophilic attack *Scheme 2.50.* Pd/C is not as efficient under these conditions and the reaction is slower.



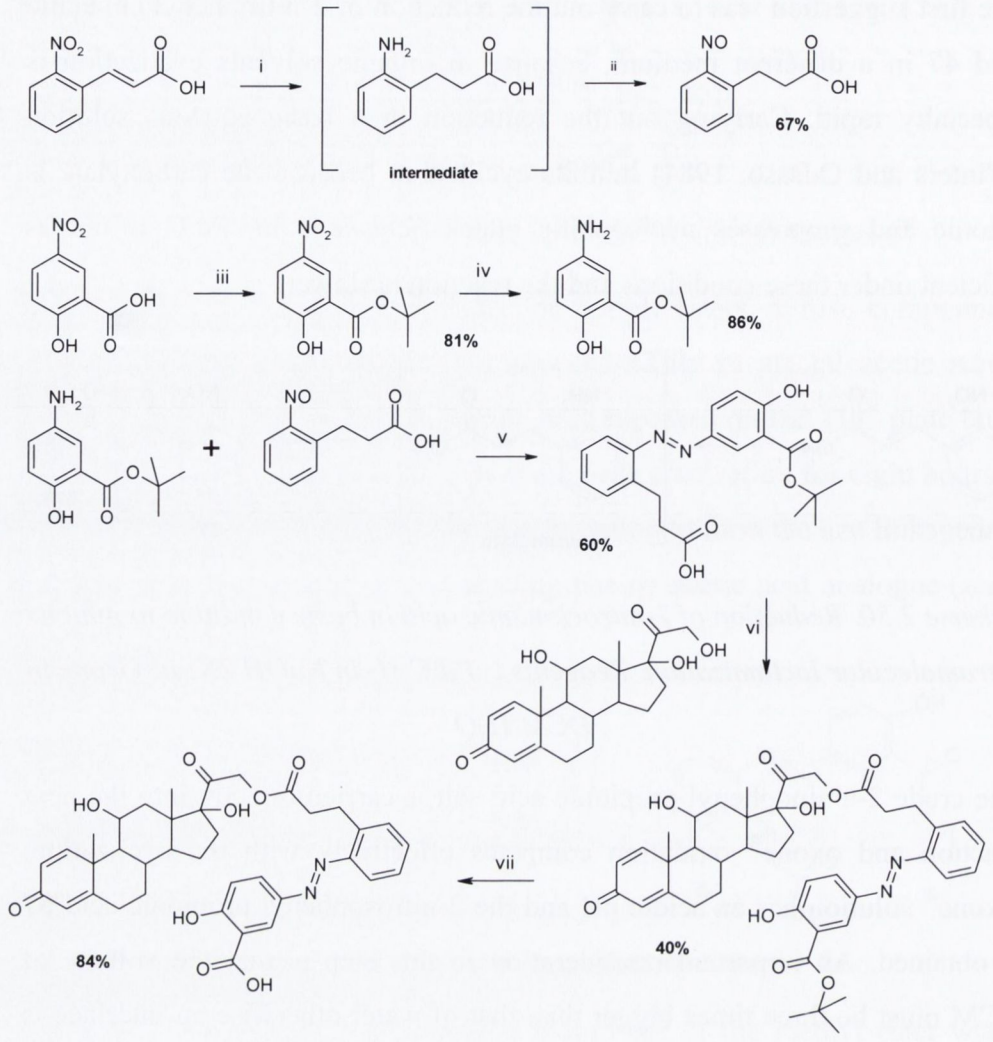
Scheme 2.50. Reduction of 2-nitrocinnamic acid in basic condition to inhibits intramolecular lactamization. Reagents i: Pd/C H_2 in NaOH 2N; ii: Oxone in DCM/H_2O

The crude 2-aminophenyl propionic acid salt is carried directly into the next reaction and oxone® oxidation competes effectively with the cyclization. Oxone® solution has an acidic pH and the 2-nitrosophenyl propionic acid **57** is obtained. An important consideration in this step is that the volume of DCM must be three times bigger than that of water otherwise an interface is formed which appears to trap the product resulting in low yields. Once the 2-nitrosophenylpropionic acid **57** was isolated we were able to effect the condensation to the azo compound to yield the azo carrier **55**. (*Scheme 2.51*)



*Scheme 2.51. Formation of azo carrier **55***

Total synthesis of prodrug **2** as shown in Scheme 2.*52* is four steps shorter than that previously proposed and developed in parallel with Dr Radics.



Scheme 2.52. i: Pd/C, H_2 in NaOH 2N; ii: Oxone®; iii: DCC in THF, DMAP in t*-butanol; iv: Pd/C, H_2; v: AcOH; vi: Ph_3P, DIAD in THF; vii: TFA*

Prodrug **2** was characterized by ^{1}H and ^{13}C-NMR and C-H COSY-NMR (*Figure 2.16)* which show the expected similarities with spectra of prodrug **1**. Prodrug **2** has an extra CH_2 in the carrier group giving rise to two triplets due to the two protons in the aliphatic region instead of the doublet of two protons which was observed in the prodrug **1** ^{1}H-NMR spectrum. The triplets are observed at 3.44 and 2.80 ppm with coupling constants of 7.53 and 8.03 Hz respectively. The two protons from prednisolone C-21 appear at 4.88 ppm, the location of the aromatics protons from the azo carrier and the protons from C-1, C-2, and C-4 of prednisolone in prodrug **2** follow the same order as in the prodrug **1** (*Figure 2.16*).

The ^{13}C-NMR (*Figure 2.17*) in this case shows an extra CH_2 carbon relative to the ^{13}C-NMR of Prodrug **1** and, overall, the correct numbers of CH_3, CH_2,

CH and quaternary carbons were observed. Meanwhile the C-H-COSY (*Figure 2.16*) confirms that one of the protons in the multiplet in the aromatic regions belongs to the C-1 of the prednisolone whereas the two triplets belongs indeed to the two CH_2 groups of the azo carrier linker. Comparison of the ^{13}C-NMR spectrum of prodrug **2** with the prednisolone ^{13}C-NMR spectrum reveals the same changes that were observed in previous comparisons between prodrug **1** and prednisolone. The quaternary carbon at 173.05 ppm in the prodrug **2** ^{13}C-NMR spectrum came from the azo carrier as can be determined from the prednisolone ^{13}C-NMR and prodrug **2** C-H COSY-NMR spectra. HMBC spectrum showed correlation between the quaternary carbon, C-22 (from the carrier) and the protons of C-21 (from prednisolone). Analyzing these spectra it can be assumed that esterification was successful.

Further confirmation was shown from the high resolution mass spectra (HRMS) which, showed the sodium ion $(M\text{-}Na)^+ = 679.2642$, the expected mass $(M\text{-}Na)^+ = 679.2632$.

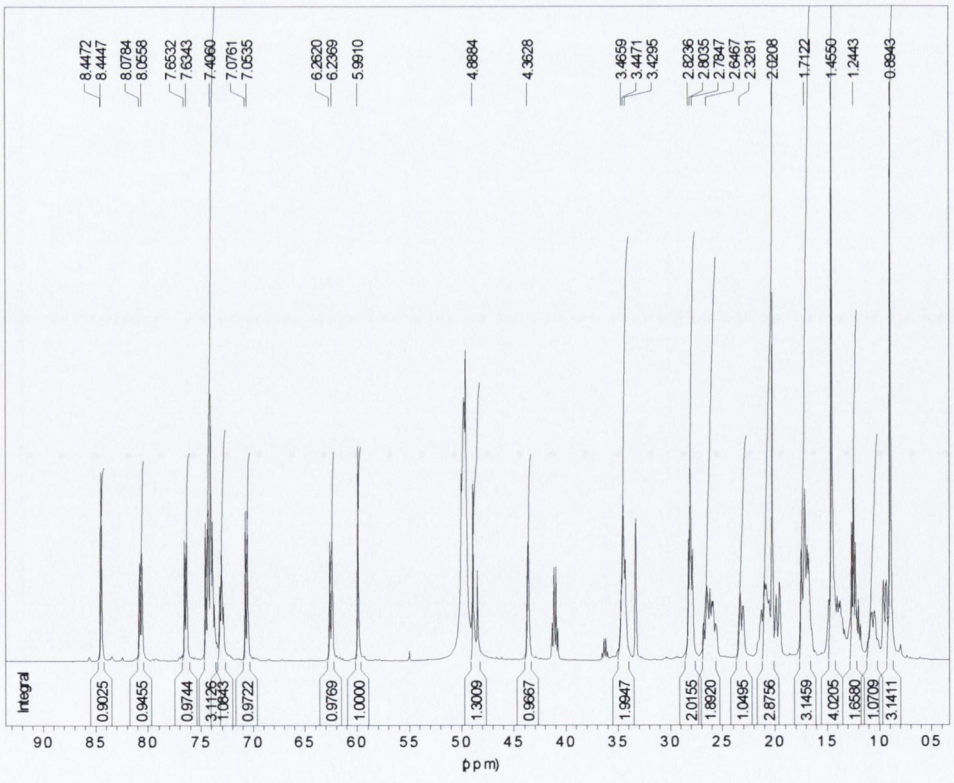


*Figure 2.16. ^{1}H-NMR of prodrug **2***

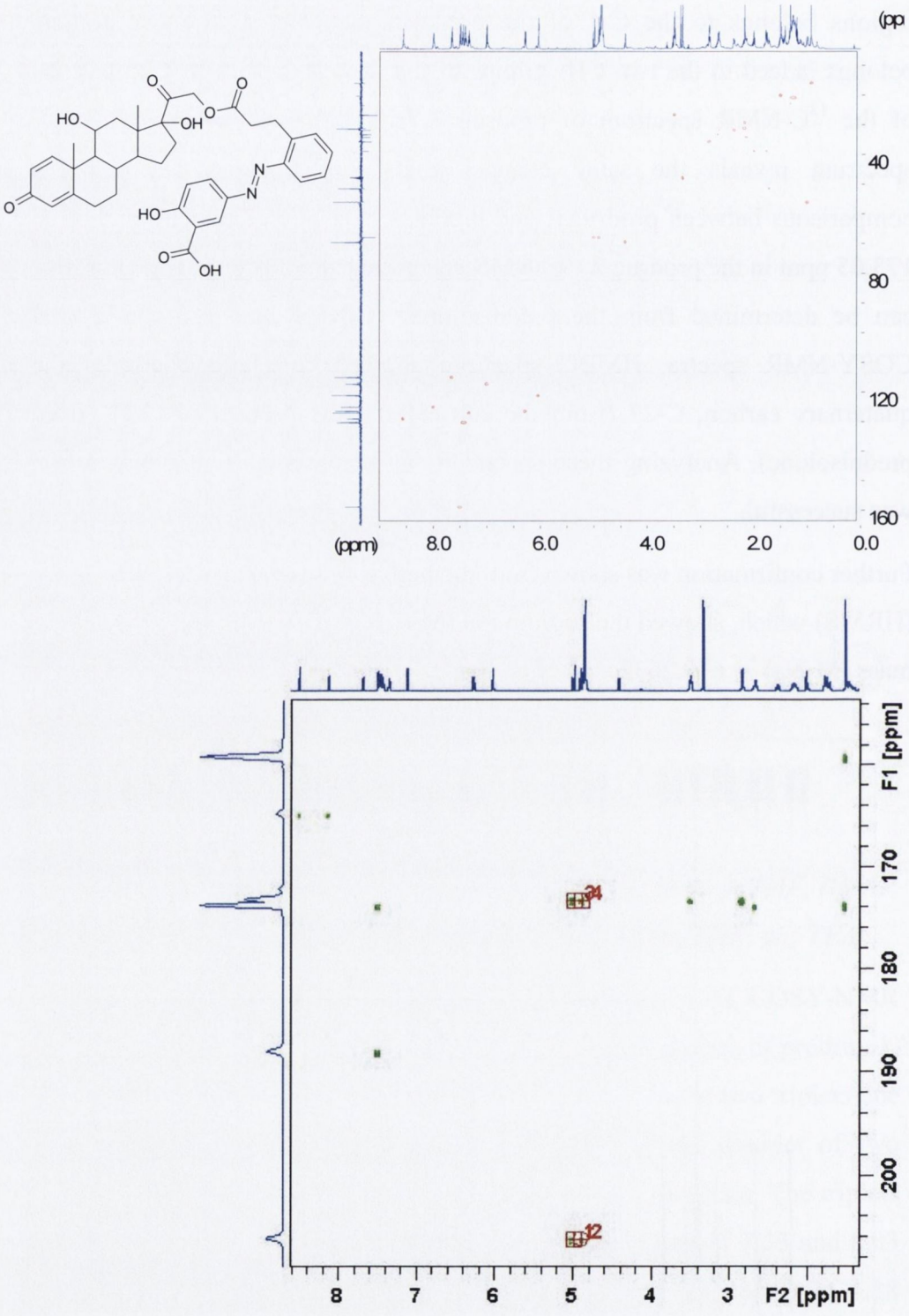


Figure 2.17. CH-COSY and HMBC of prodrug ***2***

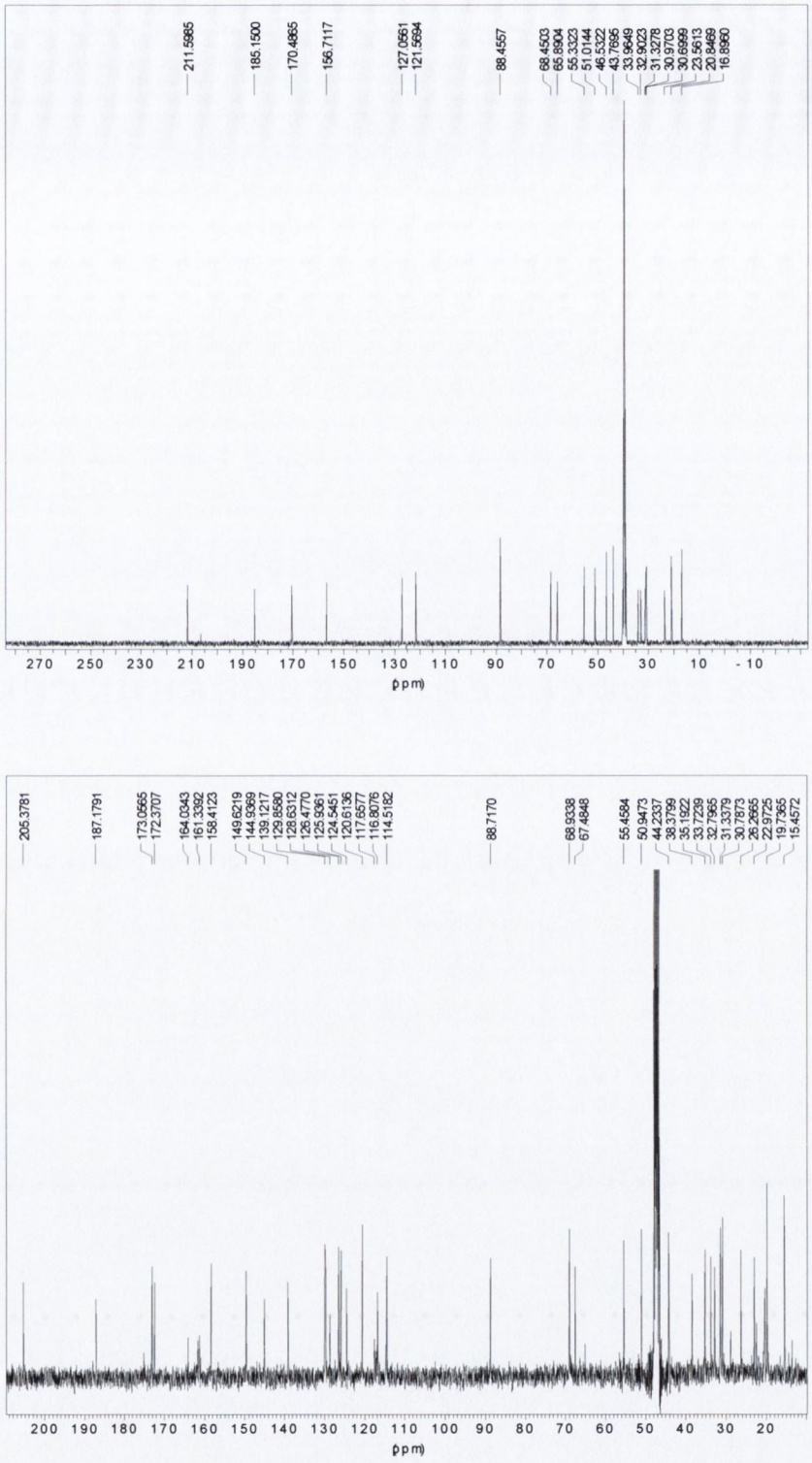
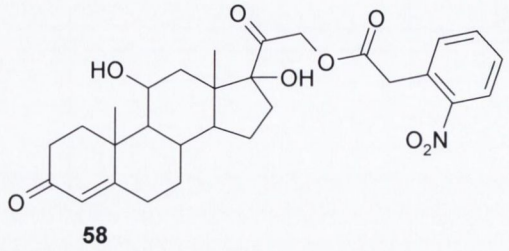


*Figure 2.18. ^{13}C-NMR spectra of prednisolone **3** and prodrug **2***

2.5 Synthesis of nitro conjugate 58

A different type of conjugate to deliver steroids to the colon was developed based on the nitroreductase activity of the colon microflora. In this case the

synthesis was easier than the azo prodrug due to the fact that the carrier attached to the steroid is commercially available.



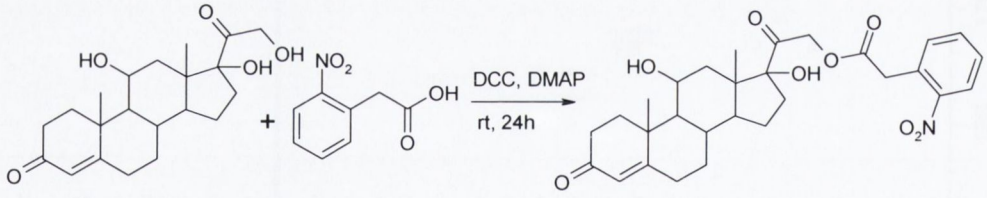
58

*Figure 2.19. Prodrug **58***

Prodrug **58**'s molecular weight is smaller that prodrug **1** and **2** and it has got less polar groups in its structure. However, we are confident it would not be absorbed through the gastrointestinal wall before reaching the colon.

The stability of the ester linkage of compounds **1**, **2** and **58** towards hydrolysis in the gastrointestinal system seems to be a very important factor in the feasibility of the prodrug systems, which was studied in a different chapter of this thesis.

The synthesis of **58** was carried out by esterification between hydrocortisone and 2-nitrophenylacetic acid using DCC, DMAP in DCM at room temperature overnight (*Scheme 2.53*)



39 **9** **58**

Scheme 2.53. Esterification conditions

The esterification was favored in the C-21 hydroxyl group as expected. The reaction was monitored by TLC (DCM: ethyl acetate 60: 40). The final product yield was around 80% after flash chromatography and characterization by ^{1}H and ^{13}C-NMR.

In the ^{1}H-NMR graph below (*Figure 2.19*), we observe the four aromatic protons between 8.15 and 7.46 ppm, and the singlet from the hydrocortisone proton of the C-4 at 5.68 ppm. The two protons of the C-21 give signals between 5.09 and 4.92 ppm. It's important to notice that a singlet is expected here but the distortions in the electromagnetic field caused by the

esterification produce a quadruplet. The same effect is observed in the previous prodrug systems. At 4.44 ppm, a singlet corresponds to the proton of the C-11 and finally, at 4.18 ppm, before the hydrocortisone envelope a singlet corresponding to the CH_2 of the 2-nitrophenylacetic acid. The hydrocortisone envelope is produced between 2.76 and 0.92 ppm.

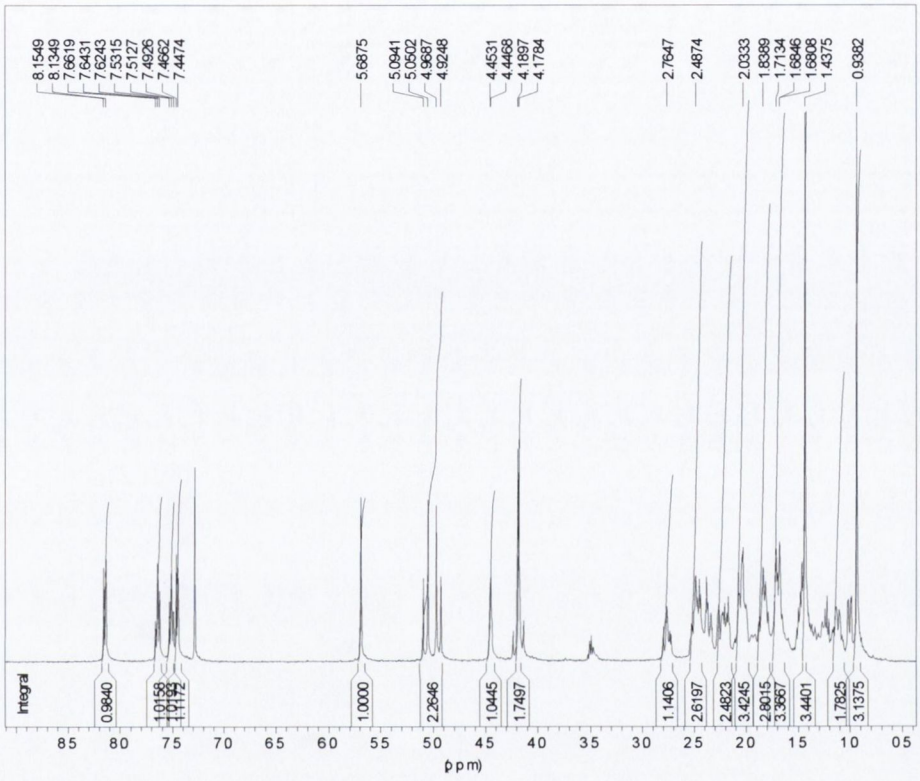


Figure 2.20. Prodrug ***58*** *^{1}H- NMR spectrum*

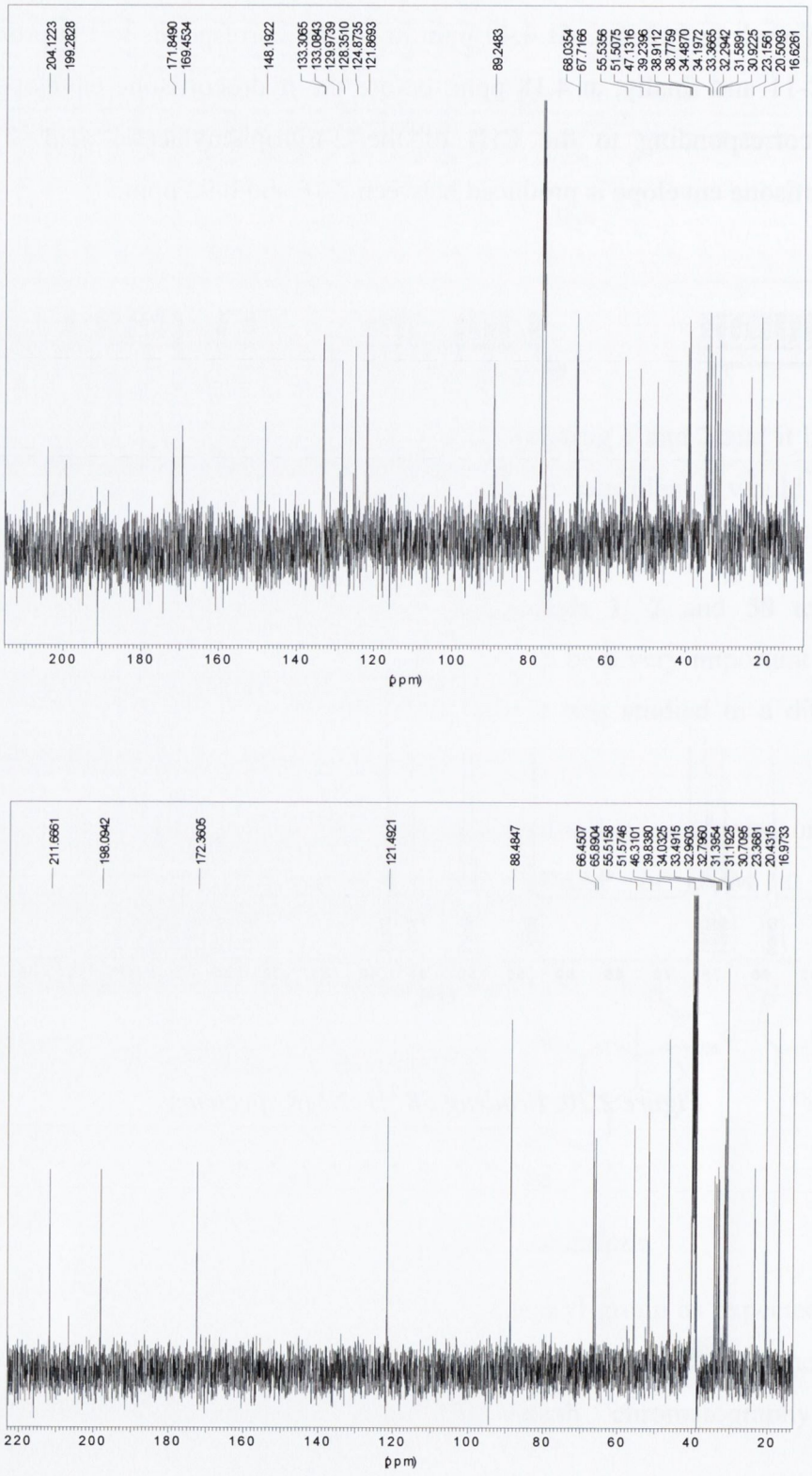


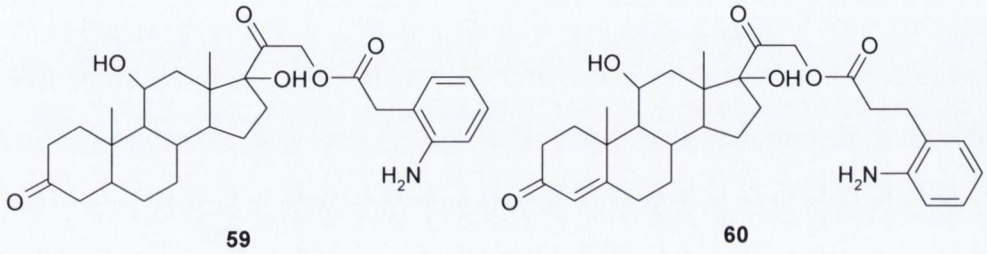
*Figure 2.21. ^{13}C-NMR spectra of prodrug **58** and hydrocortisone **3***

The prodrug **58** ^{13}C-NMR (*Figure 2.20*) shows the right number of CH_3, CH_2, CH and quaternary carbons. Comparison with the hydrocortisone ^{13}C-NMR shows the appearance of the aromatics carbons and the carbonyl carbon

from the 2-nitrophenylacetic acid at 171.84, 148.19, 133.30, 133.08, 129.97, 128.35 and 121.86 ppm. The last difference is the CH_2 carbon extra in the prodrug **58** ^{13}C-NMR spectra compared with the hydrocortisone at 38.77 ppm. Further details are discussed in the experimental chapter.

2.6 Synthesis of reduced intermediates 59 and 60

In order to carry out intramolecular lactamization kinetic studies we needed to synthesize small amounts of the key intermediates expected to be released by colonic bacteria:

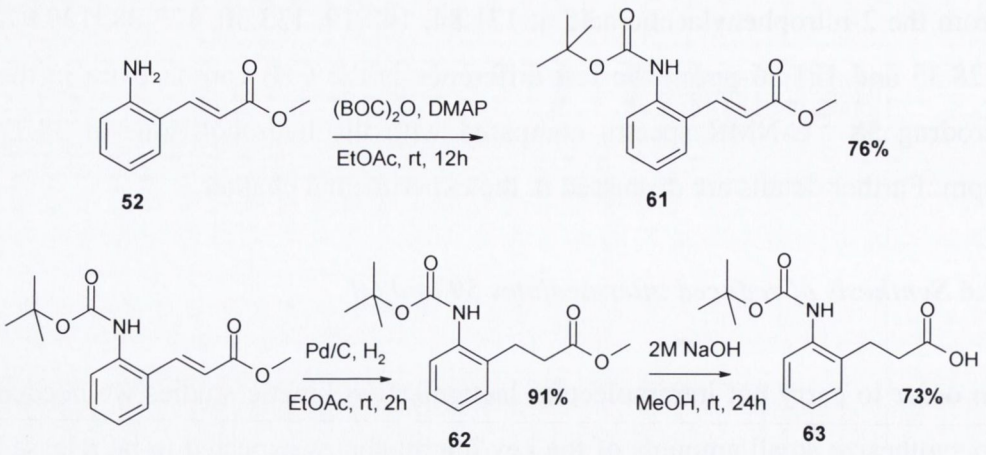


59 **60**

Figure 2.22. Prodrug intermediates after microflora reduction of the azo linkage

Synthesis of **59** could be achieved by reduction of compound **58** with Pd/C (10%) in ethyl acetate the reduction caused the reduction of the double bond in the A ring of the steroid but we did not expect to observe any incidence in the lactamization studies and compound **59** was used as starting material for the cyclization studies. On the other hand, the esterification to form **60** is not practical because of the 2-aminophenyl propionic acid cyclization as well as the likely tendency for competing intermolecular acylation. Protection of the amino group was therefore necessary during synthesis of **60**.

Esterification of 2-nitrophenyl cinnamic acid to its methyl ester **51** was followed by the partial reduction to form **52**, which was isolated and the amino group protected using BOC_2O and DMAP to form **61** after twelve hours at room temperature (*Scheme 2.54*).



*Scheme 2.54. Synthetic route to 2-BOC-aminophenylpropionic acid **63***

Once the amino group was protected, reduction of the alkene group could be performed without cyclization to yield **62**, which was hydrolyzed to the free acid **63** under basic conditions.

After reduction and hydrolysis, the esterification with hydrocortisone was carried out with DCC and DMAP in DCM affording **64** (84%), *Figure 2.22.*

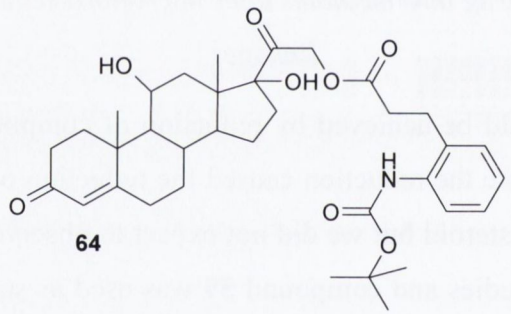


*Figure 2.23. BOC-protected-amino-intermediate of prodrug **2***

BOC group cleavage was carried out at room temperature in DCM/TFA; complete cleavage was observed after two minutes. Cyclization of intermediate **60** was detected by TLC almost immediately after cleavage in organic solvent: the cleavage had to be performed immediately before the kinetic studies so that the real kinetics of the slower aqueous reaction could be monitored in water.

CHAPTER THREE

EVALUATION OF RELEASE KINETICS AND BIOLOGICAL EFFECTS OF THE PRODRUGS

3.1 Introduction

In this chapter, studies were carried out to examine whether the prodrug systems have the potential to reach and release in the colon. The studies carried out in the different prodrug systems are presented in two parts which involve:

1. Kinetic studies: Lactamization kinetic studies of the reduced intermediates of prodrugs **1**, **2** and **58**; studies on the azo and nitro reduction of prodrugs by colonic microflora, and finally, stability studies at different pH values on the prodrugs themselves.
2. Biological and transport studies: The results from a study on a DSS-induced mice colitis model (dextran sulphate sodium DSS) treated with prodrug **1** and **2**, dioxin-4-one protected mutual prodrug **41** and prednisolone as positive control are shown in this part. This study was designed to determine the systemic side effects of the prodrug systems along with their anti-inflammatory properties. The transport of prodrugs **1** and **2** through CACO-2 cells was examined to determine the absorption of **1** and **2** compared with prednisolone **3** with caffeine as control.

3.2 Intramolecular lactamization kinetic studies

It was important to examine the kinetics and catalysis of the cyclization after the nitro or the azo groups (depending on the prodrug system) have been reduced- the faster the intramolecular lactamization, the faster the steroid is expected to be released. Without the intramolecular lactamization reaction the steroid would not be released and the prodrug system would be unsuitable for delivering steroid to the colon.

The systems we tested in this section are shown in *Figure 3.1*. Hydrocortisone was used as a model drug instead of prednisolone. It is important to notice that in compound **59** the double bond on the A ring of the hydrocortisone was reduced in its synthesis as described in chapter 2 section 2.6. However, we suppose the cyclization kinetics would not be affected by

this change because is not close to the functional groups associated with the intramolecular lactamization.

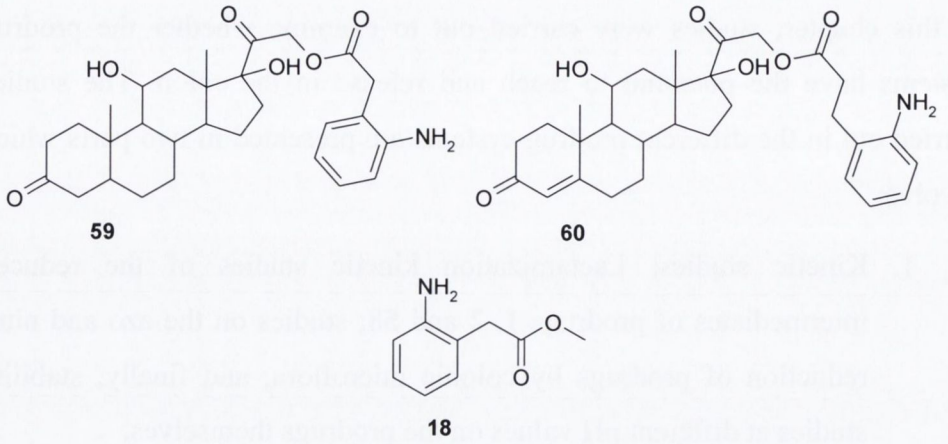


Figure 3.1. Prodrug intermediates after azo linkage reduction

The lactamization studies were carried out in borate buffered solutions at different pH values and the profiles were analyzed as described in the next section. The cyclization reaction of all of the reduced intermediates of the prodrugs tested followed first-order kinetics. For that reason all of the kinetic graphs plot the logarithm of concentration value versus time.

The mechanism of lactam formation of the 2-aminopropionic acid methyl ester **65** to the 3,4-dihydro-1H-quinolin-2-one **49** *Figure 3.2* has been studied and is well known (Kirby and Mujahid, 1979) (Camilleri et al., 1978).

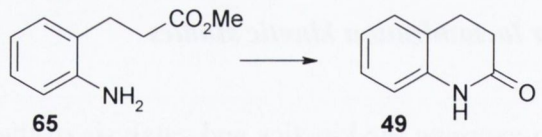


Figure 3.2. Intramolecular lactamization of 2-aminopropionic acid methyl ester

The cyclization of 2-aminophenyl acetic acid methyl ester is susceptible to both general base and acid catalysis but the authors found higher cyclization constants at low pH values and consequently the intramolecular lactamization was faster in acidic conditions. Buffers with pK_a> 6.6 act predominantly as general bases, whereas buffers with pK_a< 6.6 act as general acids. Intramolecular lactamization of compound **65** at low pH could be studied because of the low basicity of the amino group.

The acid catalysis proposed by (Kirby and Mujahid, 1979) assumes that under acidic conditions, compound **65** is present as the conjugate acid in equilibrium with small concentration of the tautomeric form (**66**). In this form the amino group is free and close to the protonated ester, allowing for fast cyclization.

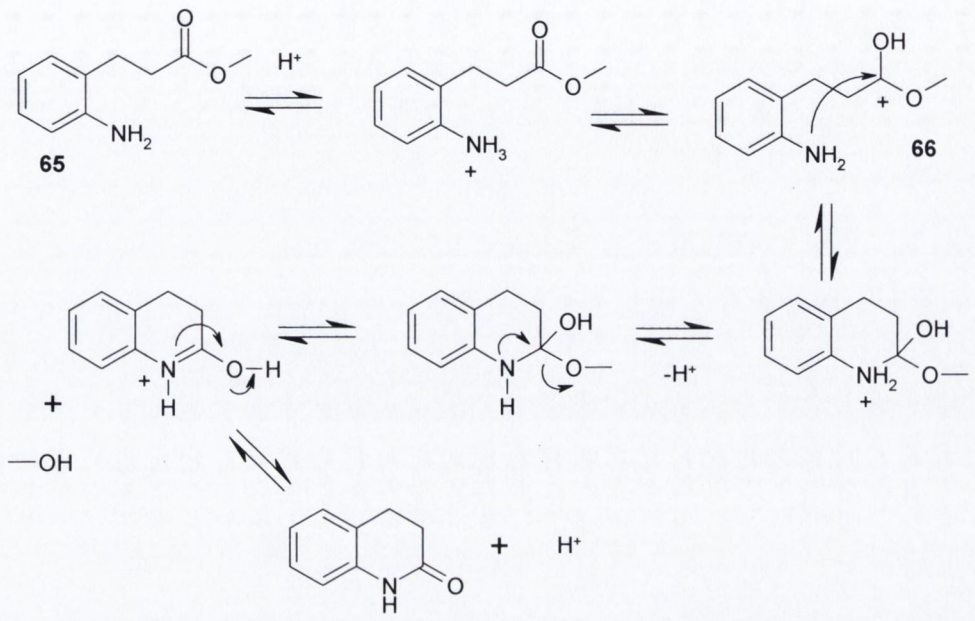


Figure 3.3. Mechanism of intramolecular lactamization under acidic catalysis

The base catalysis of **65** has been explained by the proton removal from tetrahedral intermediate (T^-) which breaks down very rapidly unless it is trapped by the deprotonation of the basic catalysis *Figure 3.4*.

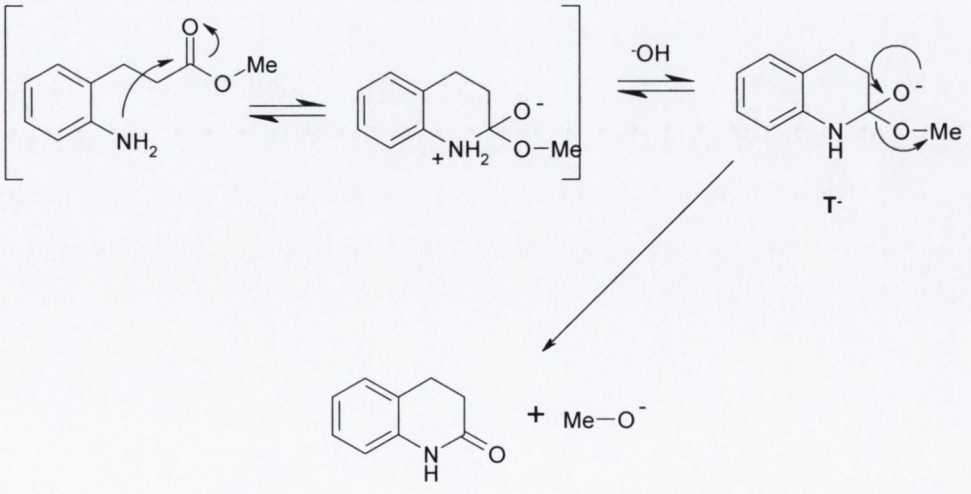


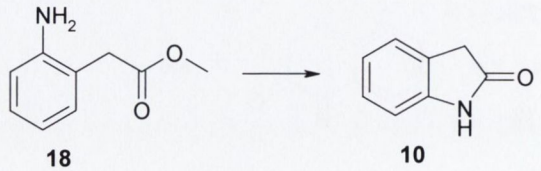
Figure 3.4. Mechanism of intramolecular lactamization under basic catalysis

The mechanisms presented above and proposed by (Kirby and Mujahid, 1979) depend on the low basicity of the amino group from **65**. This means the unprotonated amino group can exist at pH values where significant concentrations of the protonated ester are formed. When the basicity of the amino group increases, the acid catalysis is retarded and general base catalysis *via* intermediate T$^{\pm}$, is thermodynamically favorable. This explains why acid catalysis is not observed in similar systems with more basic amino groups (Fife and DeMark, 1961).

Due to the chemical structure of reduced intermediates **59** and **60** we expected their cyclization to follow the same mechanism as that 2-aminophenylpropionic acid methyl ester **65**. Nevertheless, prior to lactamization studies to reduce intermediates **59** and **60** we carried out a study with 2-aminophenylacetic acid methyl ester **18**.

3.2.1 Intramolecular lactamization of 2-aminophenylacetic acid methyl ester 18

This assay was important because it helped to understand how similar the catalysis of our prodrugs was to that of compound **65**, explained in the previous section. The cyclization reaction of **18** to form 1,3-dihydro-indol-2-one **10** has been studied (Fife and Duddy, 1983) *Figure 3.5*. Following reduction of 2-nitrocinnamic acid methyl ester **51** in ethyl acetate, the only product we obtained was 3,4-dihydro-1H-quinolin-2-one **49.** The slower reaction of the the phenyl acetic-derived compound **18** allowed for its isolation, purification and analysis in aqueous solution. We were not able isolate **65** because the cyclization in organic solvent was completed very quickly. By repeating the lactamization of 2-aminophenyl acetic acid methyl ester **18**, we showed we could do the kinetics and develop methods later on for our prodrug systems.



18 **10**

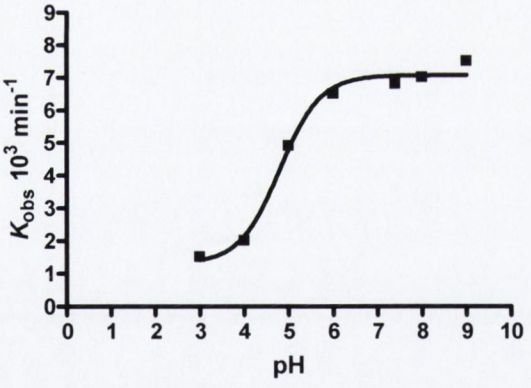
*Figure 3.5. Cyclization of 2-aminophenylacetic acid methyl acid to the 1,3-dihydro-indol-2-one **10***

Different solutions of the anilide **18** at various pH values (borate buffers) and at constant ionic stregth were kept in a water bath at 37°C and aliquots were withdrawn and analyzed by HPLC at discrete time intervals. It was found that the cyclization reactions generally followed pseudo first-order kinetics. The cyclization rate constants were obtained from the slope values of the pseudo first-order kinetic plots [logarithm of the concentration *versus* time (min)]. The results are show in *Table 3.1.*

pH	9	8	7.4	6	5	4	3
$k_{obs}x10^3$ min^{-1}	7.5	7	6.8	6.5	4.9	2	1.5

*Table 3.1. Pseudo-first order rate constants (min^{-1}) for the intramolecular lactamization of **18** at different pH values*

With the data from *Table 3.1* we made a pH-rate profile for the cyclization which is shown below. The curve of the graph was calculated using a sigmoidal extrapolation.



*Graph 3.1. pH-Rate profile for the cyclization of **18** (■) at 37°C. k_{obs} at different pH values for the cyclization of 2-aminophenylacetic acid methyl ester **18***

We observed that the cyclization rate varies with pH. At high pH values the lactamization reaction was faster than at low pH values; at pH 9 lactamization half-life time ($t_{1/2}$) was 1.5 hours while lactamization at pH 3 occurred with a $t_{1/2}$ of 7.7 hours. These results suggest a similar mechanism of cyclization to that of the anilides introduced in the previous section. In the case of 2-aminophenylacetic acid methyl ester **18**, however, the basic catalysis is more pronounced than acidic catalysis and the intramolecular

lactamization was faster at basic pH values. This difference could be attributed to a higher basicity of the amino group than the amino group of **65** or to the fact that a five member ring was formed in this reaction unlike the six-member ring of **65**. Nevertheless it seemed to be clear the cyclization in this case was base catalyzed.

These results looked quite promising for our purposes and the same study was carried out on the reduced intermediate **59**, i.e. the amino ester of hydrocortisone.

3.2.2 Intramolecular lactamization of 59

Using the same experimental conditions as the previous section, the assay was carried out with the reduced intermediate of compounds **1** and **58** (**59**) shown in *Figure 3.6* at 37°C and different pH values using a series of buffers made from borate/citrate/phosphate to give a range of pH from 1.4 to 12, with the same ionic strength value of 0.1. To make these buffers a solution of citric acid monohydrate 0.1 M and boric acid 0.2 M, was mixed with another solution of tri-potassium *ortho*-phosphate 0.1 M at different proportions (see experimental chapter for further details). *Table 3.2* summarises the intramolecular lactamization constants obtained in the assay.

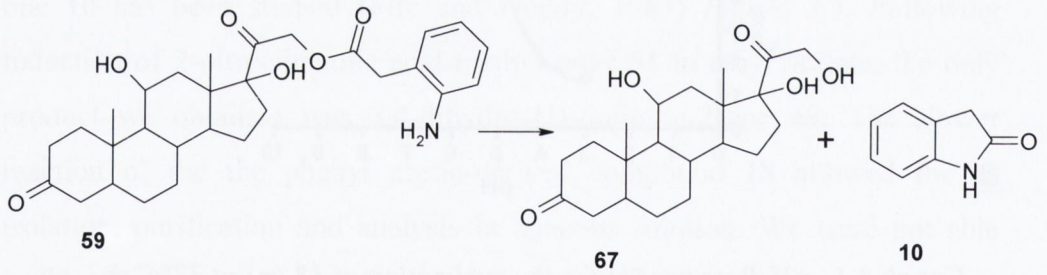
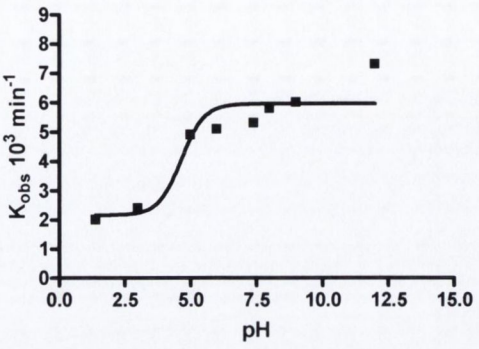


*Figure 3.6. Reduced intermediate **59** intramolecular lactamization reaction*

pH	12	9	8	7.4	6	5	3	1.4
k_{obs} 10^3 min^{-1}	7.3	6	5.8	5.3	5.1	4.8	2.4	2

*Table 3.2. Pseudo first-order rate constants (min^{-1}) for the intramolecular lactamization of **59** at different pH values*

Data from *Table 3.2* was used to make a pH-rate profile for the cyclization of **59** which is shown below. The curve of the graph was calculated using a sigmoidal extrapolation as before. However, clearly this extrapolation does not account for the gradual increase in rate at pH > 7.5.



Graph 3.2. pH-Rate profile for the cyclization of ***59*** *(■)at 37°C.* k_{obs} *at different pH values*

Compound **59**, the expected product of the reduction of prodrug **1** exhibited similar type of behaviour as with the cyclization of **18**. *Graph 3.2* shows the cyclization constants for **59** under basic and acidic conditions. Higher reaction rate constants were obtained at basic pH than in acidic pH values, indicating again that the mechanism of cyclization is similar to that of simple anilide **65** but basic catalysis is favoured (*Figure3.7*). As an example, at pH 9 $t_{1/2}$= 1.9 hours while at pH 3 $t_{1/2}$= 4.8 hours.

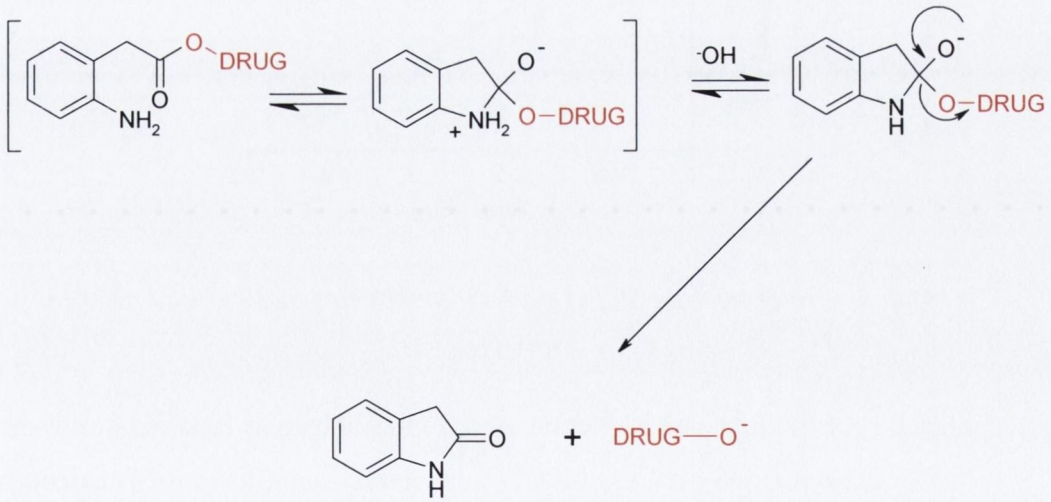
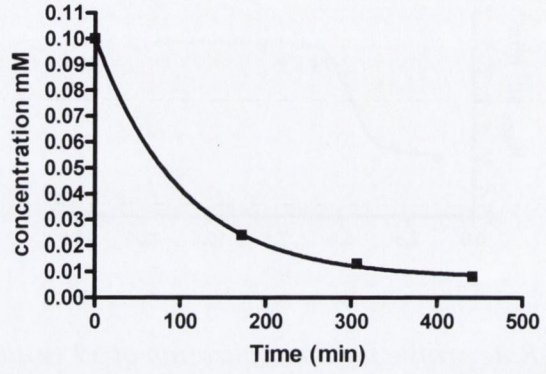


Figure 3.7 Mechanism of intramolecular lactamization of reduced intermediate ***59*** *under basic catalysis*

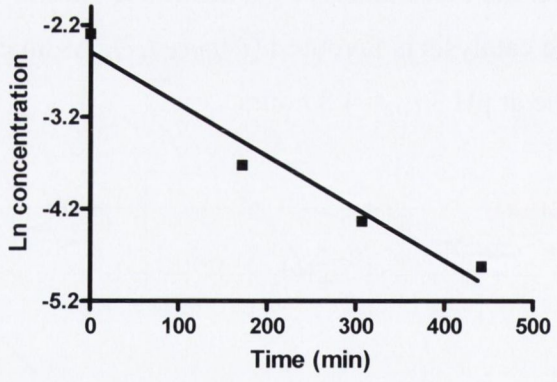
Considering that the colonic pH values of patients with IBD varies between 5.9 to 7.8 (Press et al., 1988) these results suggest that the steroid would be

released in the colon quickly once the reduction of the azo or nitro group had taken place (due to the actions of the intestinal microflora of the colon). We wanted to further examine the intramolecular lactamization of **59** at pH ≥ 6 values. Sample plots obtained in the intramolecular lactamization are shown next:



*Graph 3.3. Intermediate **59** (■) intramolecular lactamization profile at 37°C and pH 12*

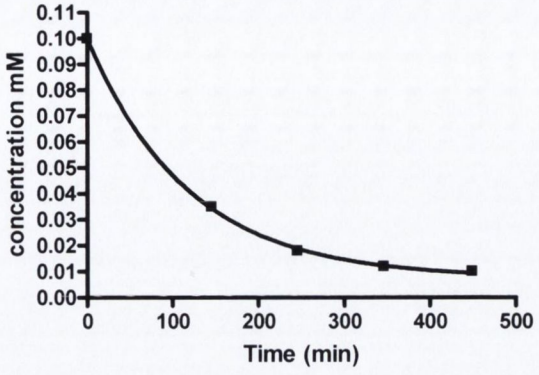
We can see the rate of decay of the reduced derivative from prodrugs **1** and **58** (**59**) by intramolecular lactamization at pH 12 in *Graph 3.3.*



*Graph 3.4. Intermediate **59** (■) cyclization kinetics at 37°C and pH 12 r^2: 0.9596*

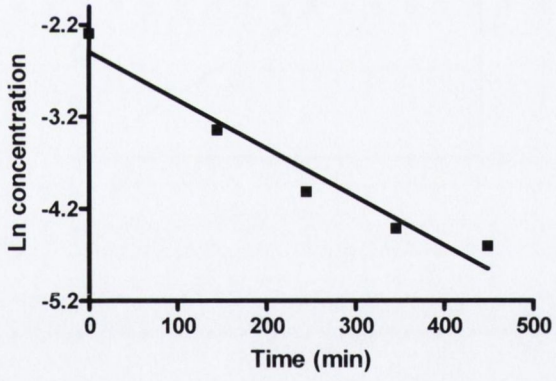
In *Graph 3.4* the first-order kinetics for the intramolecular lactamization were plotted and the slope give us the constant value, that allows us to calculate the half-life time $t_{1/2}$= 1.6 hours. This suggests that the cyclization is fast at pH 12. However, this pH value was not physiologically relevant and further studies were performed in solutions of more appropriate pH.

At pH 8, which is within the normal pH range of the colon, the intramolecular lactamization of **59** is similar to that observed at pH 12, as we can see in the *Graph 3.5*.



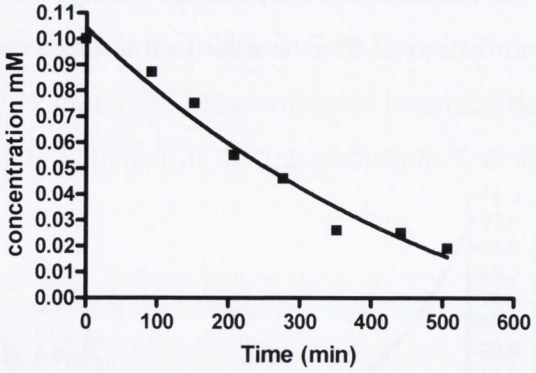
*Graph 3.5. Intermediate **59** (■) intramolecular lactamization profile at 37°C and pH 8*

The reaction again follows pseudo first-order kinetics with a $t_{1/2}$= 2 hours, despite being slower than at pH 12. *Graph 3.6*.



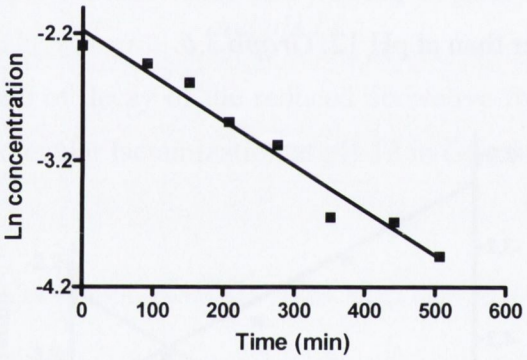
*Graph 3.6. Intermediate **59** (■) cyclization kinetics at 37°C and pH 8 (r^2: 0.9505)*

Intramolecular cyclization at pH 7.4 was considered significant as well and valuable to study and *Graph 3.7* shows the cyclization. The reduced derivative of hydrocortisone was a major product of this process but it was not quantified.



*Graph 3.7. Intermediate **59** (■) intramolecular lactamization profile at 37°C and pH 7.4*

The cyclization profile in *Graph 3.7* seems to be more linear than in the previous cases but the kinetics are still pseudo first-order, as can be observed in *Graph 3.8*.



*Graph 3.8. Plot of Ln concentration of **59** (■) versus time at pH 7.4 (r^2: 0.9657)*

At blood pH $t_{1/2}$= 2.2 hours. We can conclude that intramolecular lactamization seems to be catalyzed in basic conditions with reactions rates increasing as the medium becomes more basic. We also conclude that the half-life ranges from 2-2.4 hours, indicating that the steroid could be released in the colon after azoreductase activity, since the estimated colon transit time in rats is 6-8 hours (David R. Friend, 1984).

3.2.3 Intramolecular lactamization of 60

The expected reduced derivative of compound **2** after azoreductase activity in the colon **60** was synthesized (as described in chapter two section **2.6**), and

its intramolecular lactamization kinetics were studied in PBS at different pH values. In this case we carried out assays in solutions of pH 7.4, 8 and 9.

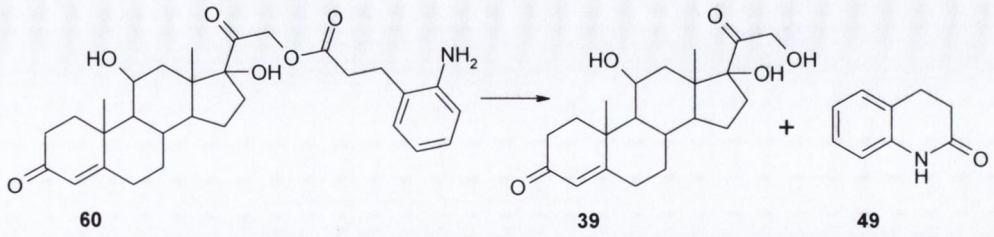


Figure 3.8. Intramolecular lactamization of reduced intermediate ***60***

Different solutions of **60** were kept in a water bath at 37°C and aliquots were withdrawn and analyzed by HPLC at discrete intervals of time. In this prodrug system cyclization is faster in basic media and the suggested mechanism is shown in *Figure 3.9*.

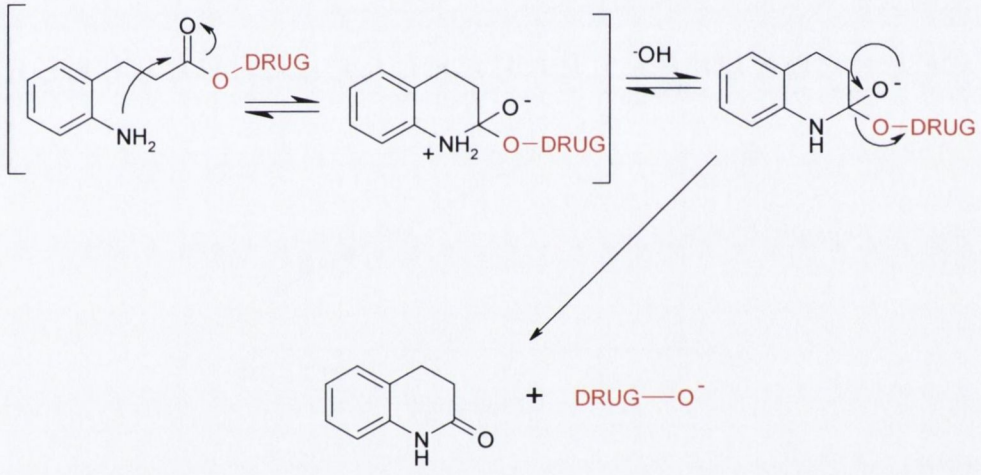
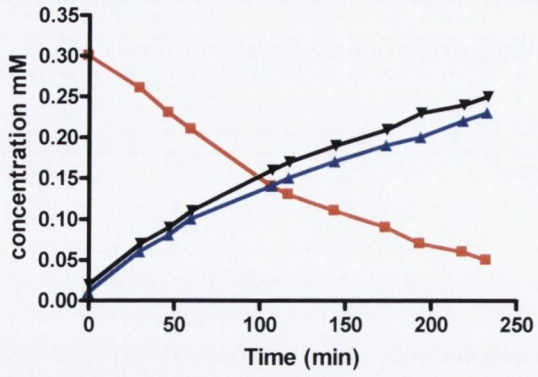


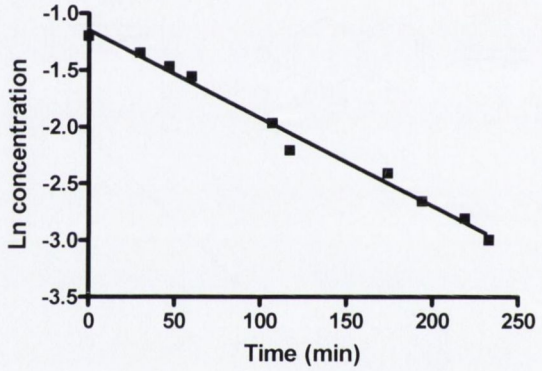
Figure 3.9. Mechanism of the intramolecular lactamization of reduced intermediate ***60*** *under basic catalysis*

The results are discussed below starting with the lactamization at pH 9 as shown in (*Graph 3.9*).



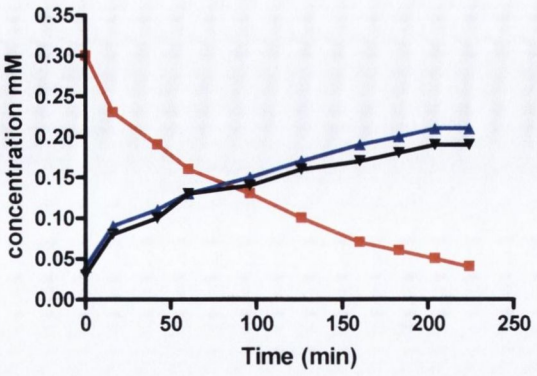
*Graph 3.9. Intermediate **60** intramolecular lactamization profile at 37°C pH 9 (■) reduced intermediate **60** (▲) hydrocortisone (▼) quinolone*

Intramolecular lactamization followed first-order kinetics as we could conclude after analyzing the data and *Graph 3.10* shown below.



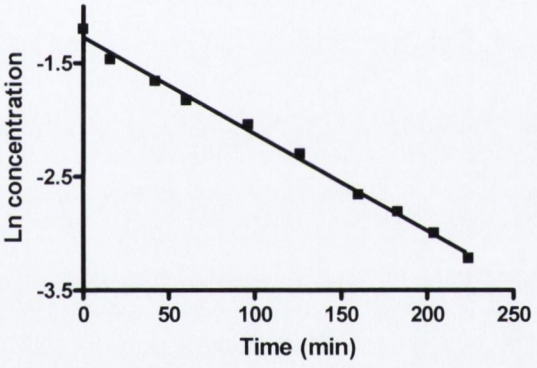
*Graph 3.10. Intermediate **60** (■) cyclization kinetics at 37°C and pH 9 r^2: 0.9886*

The half-life for intramolecular lactamization at pH 9 was 1.4 hours and the intermediate **58** is gone after approximately four hours. Data from the intramolecular lactamization at pH 8 is shown in *Graph 3.11* and a similar behaviour is observed, as expected.



*Graph 3.11. Intermediate **60** intramolecular lactamization profile at 37°C pH 8 (■) reduced intermediate **60** (▲) hydrocortisone (▼) quinolone*

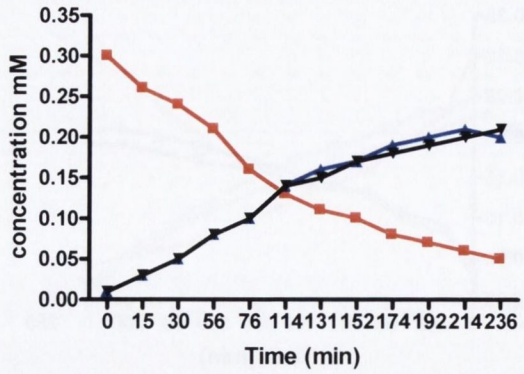
The kinetics were also very similar. However, the half-life time was shorter meaning cyclization was faster than that at pH 9. This result was not expected.



*Graph 3.12. Intermediate **60** (■) cyclization kinetics at pH 8 r^2: 0.9955*

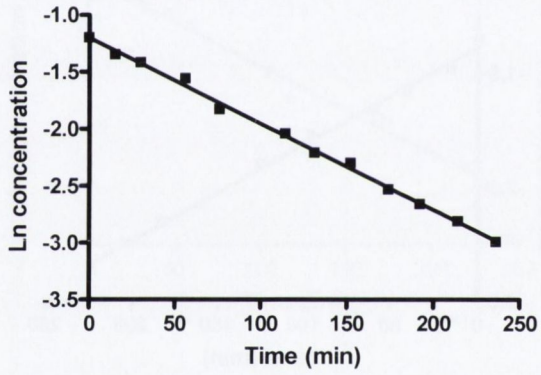
At pH 8 half-life was 1.3 hours, not hugely different to the half-life at pH 9, suggesting similarities in the basic catalysis at these pH values.

Cyclization at physiological conditions (pH 7.4) was examined in *Graph 3.13*, and the plot indicated that the lactamization kinetics were again very similar.



*Graph 3.13. Intermediate **60** intramolecular lactamization profile at 37°C and pH 7.4 (■) reduced intermediate **60** (▲) hydrocortisone (▼) quinolone*

In this profile the intramolecular lactamization was slightly slower than previously as highlighted in *Graph 3.14*. The half-life was 1.5 hours not significantly different to that at pH 8 and 9.



*Graph 3.14. Intermediate **60** (■) cyclization kinetics at 37°C and pH 7.4 r^2: 0.9970*

Intramolecular cyclization of intermediates **59** and **60** was fast and half-lives were no longer than 2.4 hours. However, the intermediate **60** which produced 3,4-dihydro-1H-quinolin-2-one **48** and hydrocortisone **39** after underwent lactamization at a rate that was almost twice as long as that of **59**, which produced 1,3-Dihydro-indol-2-one **10**. When intermediates **60** and **59** were dissolved in organic solvent intramolecular lactamization of **60** was completed almost instantaneously. On the other hand cyclization of intermediate **59** was not.

3.3 Conclusion drawn from the intramolecular lactamization studies

Finally the conclusions we made after these intramolecular lactamization studies were carried out with intermediates **59**, **60** and 2-aminophenylacetic methyl ester **18** were:

1. Compounds **1**, **2** and **58**, once they have been reduced by azoreductase or nitroreductase activity, could undergo intramolecular lactamization in a short period of time (within the orocaecal transit time), making these three systems good potential prodrugs. Although the cyclization of anilides of phenyl acetic and propionic acid esters had been established it was not certain that the same would follow for the steroids.
2. Prodrug **2** is expected to release the steroid faster than prodrugs **58** and **1**, though differences in the rate of steroid release are not hugely significant if we compare the drug half-lives in the cyclization assays.

3.4 Prodrug systems as substrates for colonic bacteria

The next question to be answered was whether the prodrug systems are good substrates for the colonic microflora. In this section nitroreductase and azoreductase activity tests are explained.

3.4.1 Reduction of prodrug 58 by nitroreductase activity

Beginning with the nitroreductase activity, we set up a test using the pure enzyme *E. coli* nitroreductase purchased from Aldrich laboratories. In this test 5-(1-aziridinyl)-2,4-dinitrobenzamide (CB 1954) (*Figure 3.10*) was used as a positive control. The samples were made up in PBS at pH 7.4 and the assay was carried out at 37°C. This pH value was chosen because this is within the pH range in the colon and it is the physiological pH value.

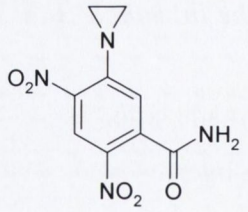
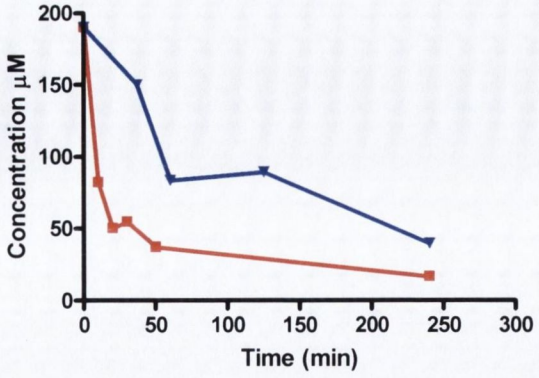


Figure 3.10. 5-(1-aziridinyl)-2,4-dinitrobenzamide (CB 1954)

Time (min)	*Concentration of prodrug* ***58*** *(μM)* *Reduction assay*	*Concentration of prodrug* ***58*** *(μM)* *Negative control*
0	190	190
10	82.3	
20	50.1	
30	54.5	
37		150.1
50	37	
60		83.6
125		89.3
240	16.8	39.9

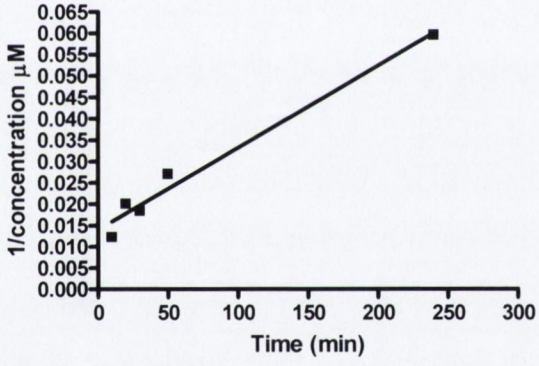
Table 3.3. Prodrug ***58*** *reduction with* E. coli *nitroreductase assay data. The decrease of the concentration in the negative control solution indicated hydrolysis under the assay's conditions.*

The CB 1954 (retention time 5.6 minutes) was reduced completely within 20 minutes indicating the activity of the enzyme in these conditions. Aliquots of the prodrug **58** (retention time 12.1 minutes) were placed on the HPLC system and *Graph 3. 15* illustrate the results.



*Graph 3.15. Prodrug **58** reduction by* E.coli *nitroreductase at 37°C and pH 7.4 (■) prodrug **58** (▼) prodrug **58** hydrolysis in the negative control*

As we can observe, the prodrug **58** was reduced by the *E. coli* nitroreductase and almost all the prodrug had been reduced after four hours. It is important to notice that the kinetics of this reaction are second-order, (the half-life depends on the initial concentration).



*Graph 3.16. Plot of 1/concentration of **58** versus time gives second order rate of **58** at 37°C and pH 7.4 (■) 1/concentration values of **58***

The half-life in this case was 25 minutes indicating that the nitro group of prodrug **58** was potentially prone to be reduced quickly by the intestinal microflora. However, as we can see in *Graph 3.15*, prodrug **58** was not stable under these conditions and the hydrolysis of the ester bond was observed in the negative control test of the assay. Further analyses are shown in the next section of this chapter.

3.4.2 Reduction of prodrug 40 by azoreductase activity

The study was carried out using Direct Blue (an azo dye *Figure 3.11*) as a positive control while the reduction of compound **40** was examined, to estimate the azoreductase activity. Initial assays were carried out with *E. coli* due to its accessibility, but analysis showed no reduction of the azo linkage at all by the bacteria either in aerobic or anaerobic conditions.

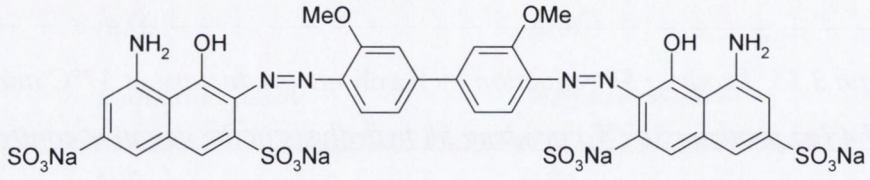


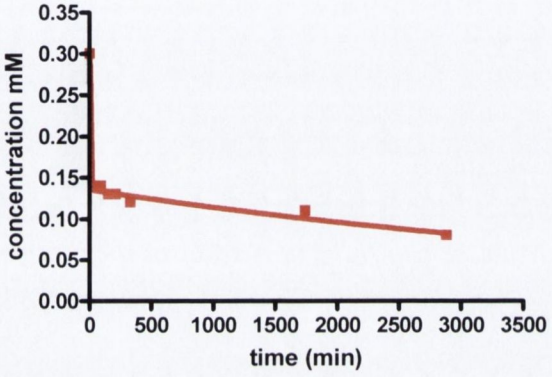
Figure 3.11. Direct Blue an azo dye used as positive control in the azoreductase assays

The assay was repeated using a sonicated solution of *E. coli*. The aim of this assay was to test whether *E. coli* could not reduce the prodrug with its azoreductase enzymes or otherwise if the prodrug could simply not access the enzyme via the membrane wall of the bacteria. The results revealed no azoreductase activity of the *E.coli* contents with the azoprodrug **40** and the positive control, Direct Blue. After these results, it was necessary to search for another type of bacteria with proven azoreductase activity.

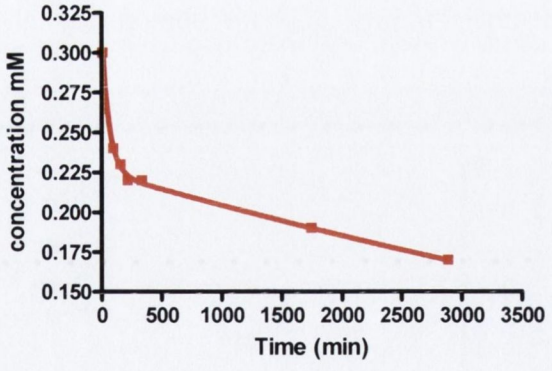
Three strains of *Clostridium perfringes* were used because they were previously shown to contribute to the azoreductase activity of the human colonic microflora (Rafii et al., 1990) and were available at the Trinity Centre for Health Sciences St. James's. The assay was carried out under aerobic and anaerobic conditions, and two different concentrations of brain heart infusion (BHI) were added to the culture with the azoprodrug **40**. The second concentration of BHI was ten times lower than the first BHI solution. This 1/10 dilution was performed to reduced any possible interference in the HPLC analysis due to BHI. The initial observation was the absence of azoreductase activity in aerobic conditions, not only towards the prodrug, but also towards the Direct Blue. On the other hand the *Clostridium perfringes* did reduce the azo linkage under anaerobic conditions confirming the results obtained by (Rafii et al., 1990). Graphs with the reduction profiles are shown below.

Prodrug **40** (mM) in BHI	0.3	0.14	0.13	0.13	0.12	0.11	0.08
Time (min)	0	90	150	210	330	1740	2880
Prodrug **40** (mM) in BHI (1/10)	0.3	0.24	0.23	0.22	0.22	0.19	0.17

*Table 3.4. Data from the azo reduction assay of the prodrug **40** with* Clostridium perfringes *under anaerobic conditions in BHI growth medium (up) and in 1/10 diluted BHI growth medium (down)*



*Graph 3.17. Disappearance of **40** (■) in the presence of* Clostridium perfringes *at 37°C under anaerobic conditions in BHI*



*Graph 3.18. Disappearance of **40** (■) in the presence of* Clostridium perfringes *at 37°C under anaerobic conditions in 1/10 BHI*

The BHI peak did not significantly interfere with the HPLC analysis, but there is a small difference in the graphs. The rate of reduction was lower in the culture with the diluted BHI because the bacteria had fewer nutrients in

the medium. As we can see the slope of the first part of the graphs is smaller in the culture with the diluted BHI than in the one with normal BHI.

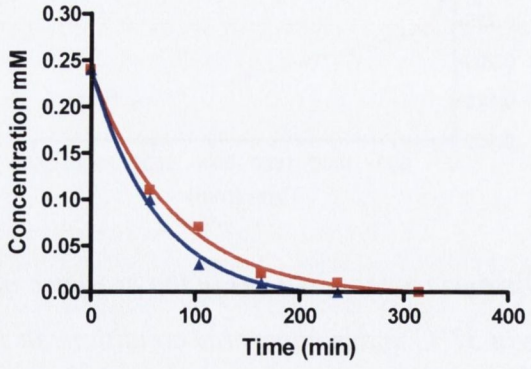
3.4.3 Prodrug 40 as substrate for mouse colonic microflora

We needed to confirm that the mouse microflora had azoreductase activity against azoprodrug **40** prior to examining the prodrug system in live mice. The test was carried out using colon contents of a CD-1 mouse, which were grown on BHI medium, under anaerobic conditions at 37°C.

Prodrug **40** (mM)	0.24	0.11	0.07	0.02	0.01	0
Time (min)	0	57	104	164	237	315
Direct Blue (mM)	0.24	0.1	0.01	0		

*Table 3.5. Data from the azo reduction assay of the prodrug **40** and Direct Blue as positive control under anaerobic conditions with mouse colon contents*

Direct Blue was used as a positive control in the azoreductase assay (Rafii et al., 1990). A negative control containing prodrug **40** and Direct Blue in BHI medium was incubated under the same conditions in the absence of colonic microflora to test the stability of both compounds under experimental conditions.



*Graph 3.19. Disappearance of prodrug **40** (■) in a solution containing colonic contents of CD-1 mice at 37°C under anaerobic conditions in BHI*

Although the reduction products could not be detected by HPLC as is evident in the *Graph 3.19* above the disappearance of prodrug **40** could be attributed

to azoreductase activity because Direct Blue (the positive control) was reduced indicating azoreductase activity (it remains possible that the prodrug disappearance was due to some other event).

3.4.4 Conclusion of the reduction studies

The conclusions after reduction studies in our prodrug system are:

1. In spite of being reduced quickly by the *E. coli* nitroreductase, nitro prodrug **58** was not stable to hydrolysis at 37°C and pH 7.4. Thus, it was not suitable for testing in the DSS-induce colitis mice study, or further development. The reasons for the instability of the nitro compound are obscure.
2. Azo reductase activity took place only under anaerobic conditions and prodrug **40** was a substrate for at least one kind of bacteria known to be present in the human intestine. Although we were not especially interested in compound **40** its reduction did indicate that the analogous compounds **1** and **2** would also be reduced.

3.5 Stability studies

Once the kinetics of the intramolecular lactamization and reduction of the azo linkage and nitro group had been studied, the stability of the prodrug systems was established in order to determine if the prodrug would be hydrolyzed in the pH conditions of the GIT before reaching the colon.

The stability of the different prodrugs shown in *Figure 3.12* was examined. Hydrocortisone was the parent steroid instead of prednisolone in some cases. Compounds were tested under physiological pH conditions (7.4) and in acidic media (pH 2.5) using phosphate salt/buffer solutions with ionic strength (100 mM) at 37°C. Results for the different prodrugs are shown in the following sections of this chapter.

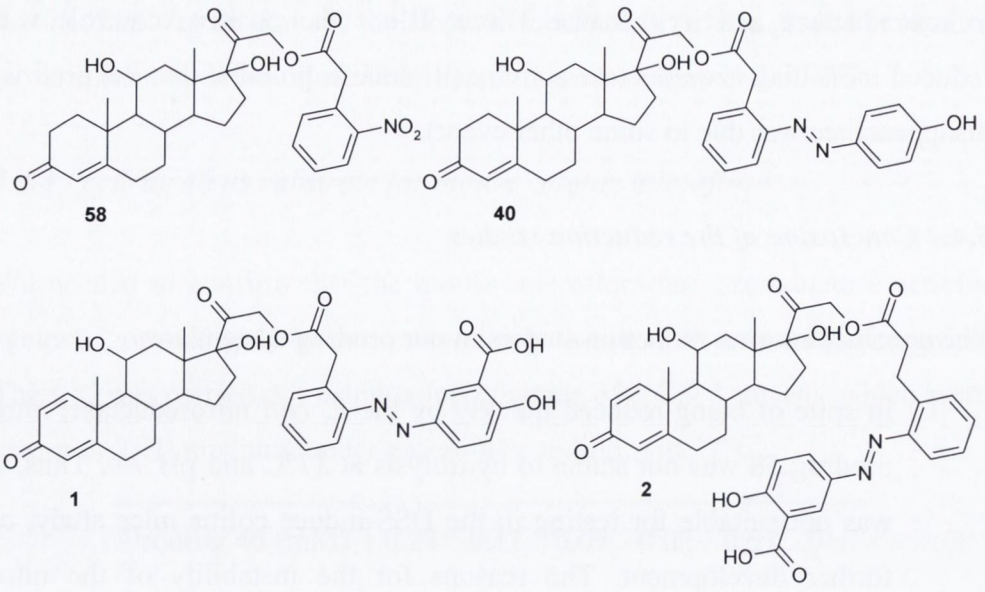
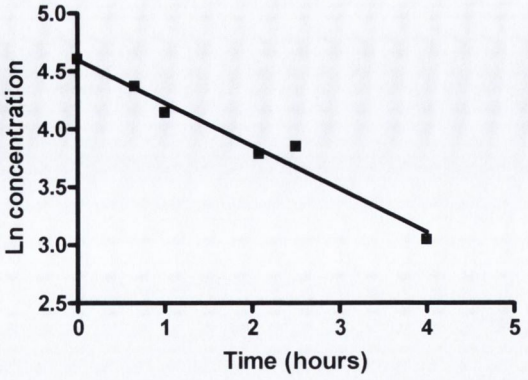


*Figure 3.12. Prodrug systems **1**, **2**, **40** and **58***

Hydrolysis of all prodrugs followed first-order kinetics over several half-lives with exponential decay. Stability data is shown as progress curves and as log concentration versus time.

3.5.1 Compound 58 stability study

Compound **58** was shown to undergo aqueous hydrolysis when the reduction studies were carried out in the previous section. We detected the hydrolysis during analysis of the negative control, which consisted of prodrug **58** in PBS pH 7.4 without *E. coli* nitroreductase, (*Graph 3.15*). In this section, we determined the hydrolysis rate of **58** under aqueous conditions. A stock solution of **58** in ethanol was dissolved in PBS pH 7.4 at 37 °C and aliquots were analyzed by HPLC.

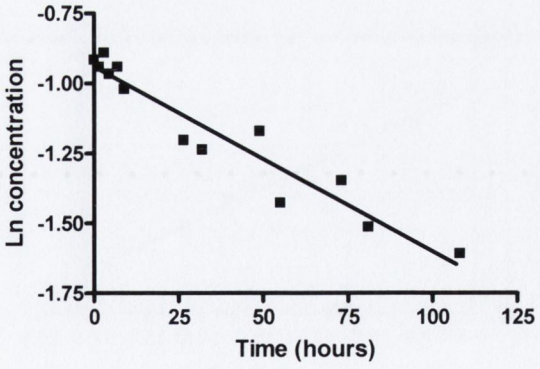


Graph 3.20. Plot of Ln ***58*** *(■) against time (h) at 37°C in PBS (pH 7.4)* r^2*: 0.9691*

The nitroprodrug conjugate **58** underwent hydrolysis rapidly, with a half-life of 1.8 hours. This means that the prodrug system would most likely be hydrolyzed before reaching the colon after oral administration and because of this, it does not seems to be a good prodrug system for delivering steroid to the colon (as we concluded previously).

3.5.2 Prodrug **40** *stability study*

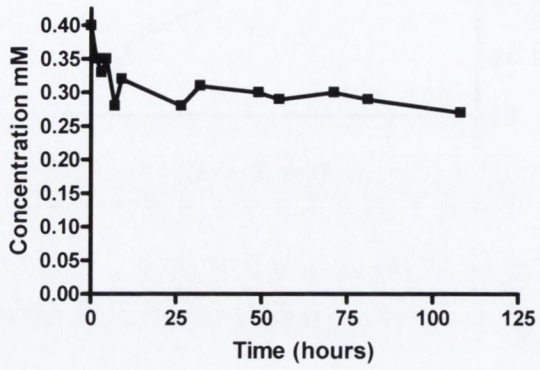
From azo compound **40** stock solution in DMSO different solutions in PBS pH 7.4 and pH 2.5 were prepared and a stability test was carried out at 37°C with remaining compound measured by HPLC.



Graph 3.21. Plot of Ln ***40*** *(■) against time (h) at 37°C in PBS (pH 7.4)* r^2*: 0.9227*

The prodrug **40** was more stable than prodrug **58** giving a $t_{1/2}$ of 104 hours. Therefore we could consider the prodrug **40** stable at pH 7.4 for our purposes.

At pH 2.5 the prodrug **40** was very stable to hydrolysis as *Graph 3.22* indicates. More than half the initial concentration was remaining 108 hours after the studied started.

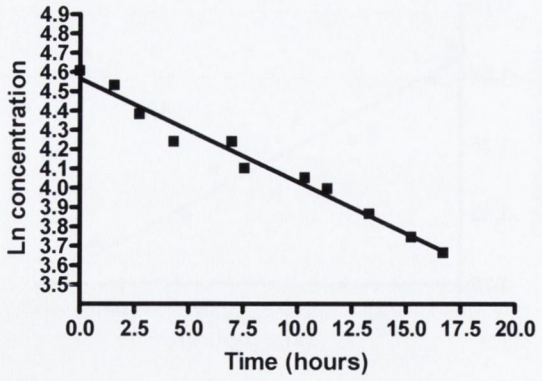


*Graph 3.22. A plot of remaining **40** (■) in PBS at 37°C and pH 2.5*

These results show that the prodrug **40** is quite stable towards aqueous hydrolysis. This would suggest that prodrugs **1** and **2** could be similarly stable. These drugs were analyzed as described below.

3.5.3 Prodrug **1** *stability study*

As before, from a stock solution of compound **1** in DMSO, two sample solutions were prepared in PBS pH 7.4 and 2.5. The stability study was carried out as before.

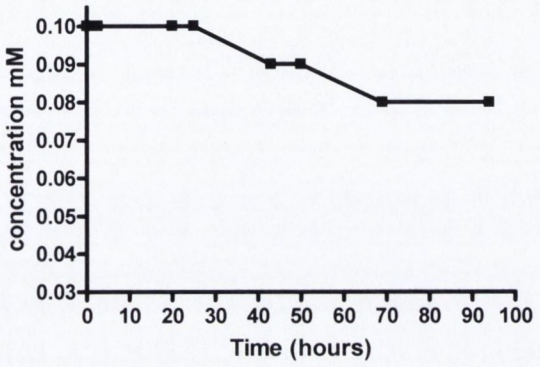


*Graph 3.23. Plot of Ln compound **1** (■) against time (h) at 37°C in PBS (pH 7.4) r^2:0.9748*

Prednisolone was observed confirming hydrolysis and the kinetics are shown in *Graph 3.23* half-life was 12.9 hours at pH 7.4 for prodrug **1**. This value is

quite short compared with the prodrug **40** half-life at the same pH value. However it suggested that the drug might be stable enough to reach the colon before undergoing extensive hydrolysis.

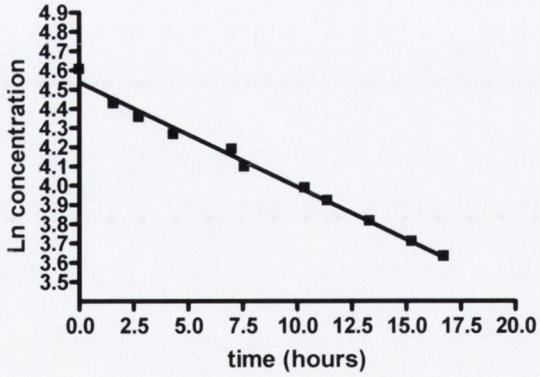
At PBS pH 2.5 no hydrolysis was observed over 25 hours, prodrug **1** concentration changed from 0.1 to 0.08 mM after 94 hours, but prednisolone was not detected by HPLC.



*Graph 3.24. A plot of remaining prodrug **1** (■) in PBS and pH 2.5*

3.5.4 Prodrug 2 stability study

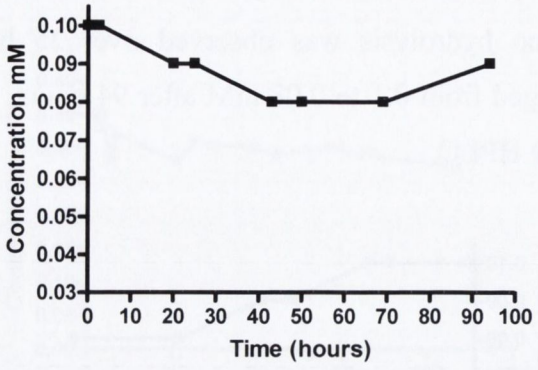
Prodrug **2** stability at pH 7.4 and pH 2.5 was very similar to that of prodrug **1** as we can observe in graphs 3.25 and 3.26.



*Graph 3.25. Plot of Ln **2** (■) against time (h) at 37°C in PBS (pH 7.4)*
r^2:0.9899

In PBS pH 7.4 prednisolone was detected and the prodrug **2** $t_{1/2}$ = 12.7 hours and in PBS pH 2.5 hydrolysis was not observed because no prednisolone was

detected at all. The concentration decreased from 0.1 mM to 0.08 mM over 94 hours and prednisolone was not observed.



*Graph 3.26. A plot of remaining prodrug **2** (■) in PBS at 37°C and pH 2.5*

3.5.5 Conclusions of the stability studies

The conclusions we can take after these analyses are:

1. Unfortunately nitro-substituted steroid ester, compound **58** underwent unexpectedly rapid hydrolysis at pH 7.4 and within two hours half of the initial concentration was already hydrolyzed. Among the possible reasons for this unexpectedly rapid disappearance are: a promotion of aqueous hydrolysis at the ester by the nitro group or, perhaps, by assistance intramolecularly of 21-17 ester migration. Either possibility needs further investigation at a mechanistic level.
2. Azo compound **40** is very stable while prodrugs **1** and **2** are moderately stable at pH 7.4 and stable at pH 2.5. Thus they should undergo little acid-catalysed hydrolysis in the stomach and in the GIT before reaching the colon.

3.6 DSS-induced colitis mice study

In this study the anti-inflammatory effect and systemic side effects of the compounds **1**, **2** and **41** after oral administration were studied using a mouse model of IBD. The prodrugs studied have been designed to avoid absorption in the GIT. The dextran sulfate sodium (DSS) induced colitis model

described here allow us to determine the efficacy of the prodrugs compared with prednisolone and to make some assessment of their absorption.

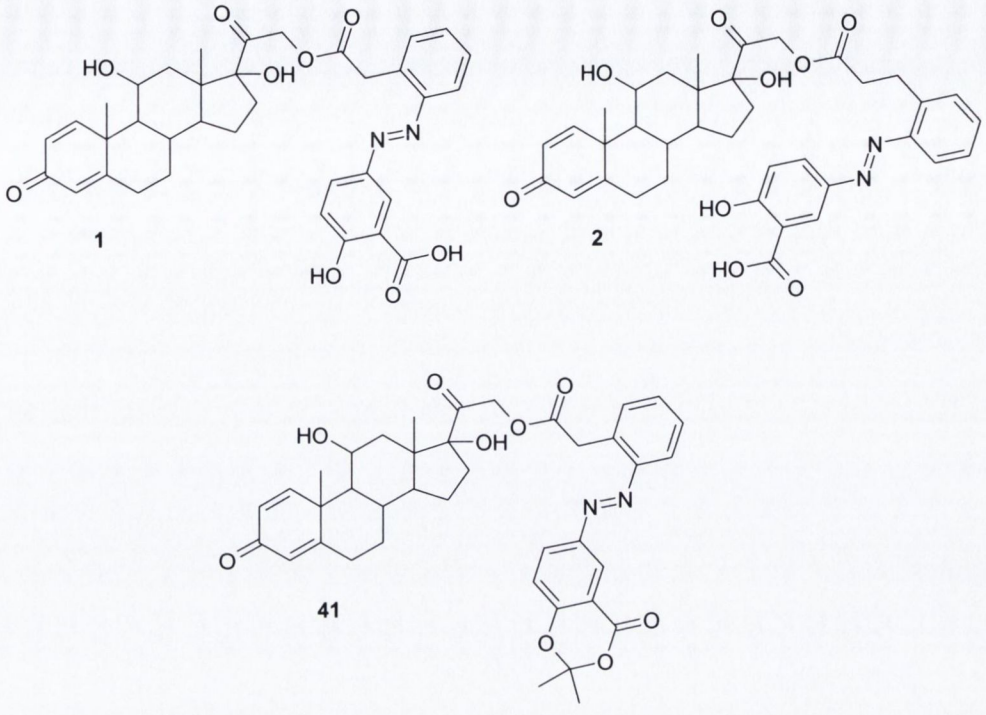


*Figure 3.13. Prodrug **1**, **2** and acetonide protected mutual prodrug **41***

DSS induced colitis is obtained by administration of 5% DSS in drinking water to mice, rats or hamsters over seven days. Colitis is diagnosed by weight loss, shortening of the colon, bloody diarrhoea, neutrophilic infiltration and mucosal ulceration. DSS first induces mucosal injuries and inflammation through a direct toxic effect on epithelial cells, allowing intestinal bacteria to penetrate the injured mucosa and perpetuate mucosal inflammation (Verdu et al., 2000). This is followed by lesioning of the colon and aggregation of lymphoid. The involvement of activated immune cells and inflammatory mediators is demonstrated by an increase in the concentration of mucosal thromboxanes, leukotrienes and interleukins, but the acquired immune system is not involved in the injury since mucosal damage occurs in models using immuno-deficient mice. The role of luminal bacteria in the pathogenesis of this model is still unclear in spite of the increased *bacteriodes* luminal concentration (Okayasu et al., 1990).

DSS induced colitis is a well established model which exhibits many of the symptoms observed in human IBD (Elson et al., 1995) and that makes it a reliable model in the search for new drugs for treating IBD.

The anti-inflammatory and systemic side effects of the different prodrugs were evaluated relative to prednisolone in the present study. Anti-inflammatory effects were studied by comparison of the distal colon length in healthy mice, untreated mice, prednisolone treated mice and prodrug-treated mice. IBD causes shortening of the colon as explain before. Thus, the most effective anti-inflammatory agents associated with reduced distal colon length, indicating less colon tissue damage.

Systemic side effects were studied by comparison of the thymus weight and body weight ratio (Hideki Yano, 2002) in healthy mice, untreated mice, prednisolone-treated mice and prodrug-treated mice, due to the fact that an increase in the concentration of steroid in the bloodstream leads to suppression of the activity of the thymus and the consequent reduction of its weight.

3.6.1 Study design

This study was designed to determine the severity of systemic side effects arising from the different prodrug systems, and its anti inflammatory properties in the treatment of IBD.

Groups of six BALB/c mice per cage were used to study the prodrug systems. The control group, in which colitis had not been introduced, were left untreated. Another group of mice in which colitis was induced by administration of DSS, were left untreated this group acted as a negative control. The positive control group consisted of mice in which colitis was again induced with DSS, but in this group, prednisolone was used as a treatment. The anti-inflammatory effects and systemic side effects of the prodrugs were compared to those of the positive control group.

The study was carried out over six days and the dosage was molar equivalent to five and ten mg of prednisolone per kg of mouse, twice daily. The average weight per group was recorded daily, and at the end of the study, the mice were sacrificed. Blood and faeces samples were taken and kept at – 80°C for future analysis. The parameters measured in this study were:

- Length of the colon

- Thymus body weight ratio T/BW
- Histology of colon damage to determine the disease activity index DAI
- Percentage weight loss

Control, negative control and positive control statistical data were compared with prodrug groups data to determine the anti inflammatory properties of the prodrugs and their systemic side effects.

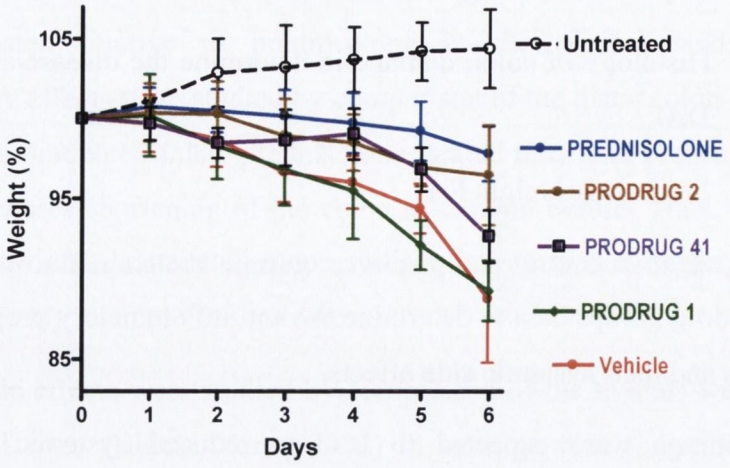
The prodrugs were expected to lead to reduced systemic side effects compared to prednisolone because they are designed to pass through the GIT without being absorbed, (unlike prednisolone which is absorbed).

The anti inflammatory properties of the prodrug systems were expected to be at least as good as prednisolone because, by avoiding absorption, higher concentrations of steroid are expected to reach the damaged areas of the colon.

The study was expected to show whether the design, tested in principle, has any potential in a relevant *in vivo* model. The work was performed at St James's Hospital by Dr Padraic Fallon in consultation with the author.

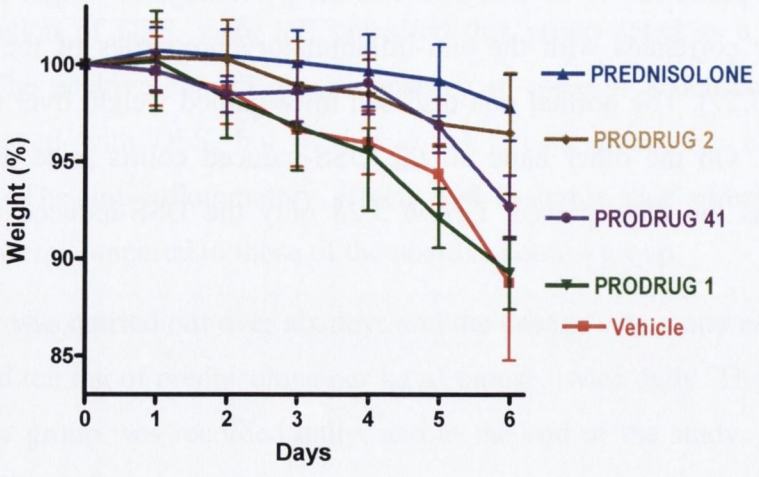
3.6.2 Results

The first parameter to be analyzed was the percentage of weight loss which inversely correlates with the anti-inflammatory properties of the test drug (*Graph 3.27*). The normal non-diseased mice gained weight over the course of study. On the other hand all the DSS-induced colitis mice groups lost weight as the days passed. *Graph 3.28* only the DSS-induced colitis are shown.



Graph 3.27. Body weight loss percentage profile of healthy mice (untreated) and DSS-induced colitis mice treated at 5 mg/kg

Mice which had not been given any drug treatment (i.e. just administered the vehicle) showed a dramatic weight loss of 10%, close to the 15% value at which death occurs. This data provided us with a clear image of the DSS-induced colitis severity after six days. The group of DSS-induced mice treated with prednisolone showed the least weight loss, followed very closely by the group administered prodrug **2**, indicating a strong anti-inflammatory activity for both agents. There was no statistically significant difference between the prednisolone treatment group and the prodrug **2** test group at any time point.

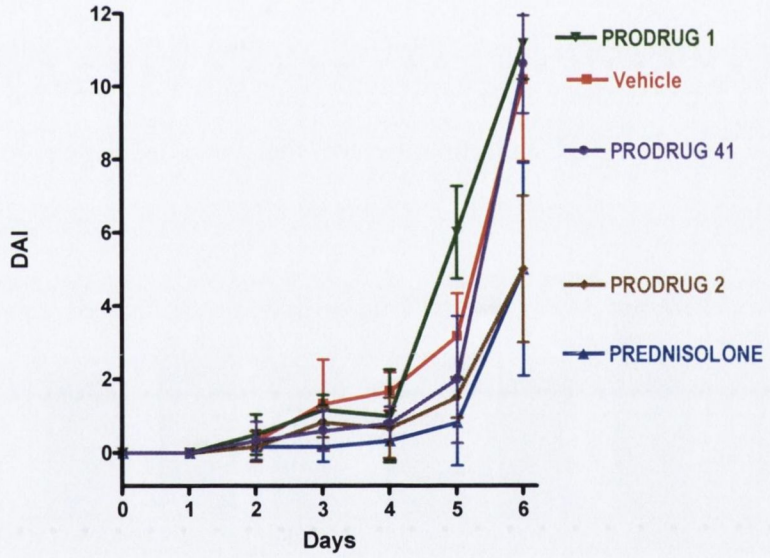


Graph 3.28. Body weight loss percentage profile of DSS-induce colitis mice treated at 5 mg/kg dosage

DSS-induced colitis mice treated with acetonide mutual prodrug **41** lost more than 5% of their original weight, which was intermediate between the weight loss observed in the prednisolone treated and the vehicle administered groups. This indicated possible anti inflammatory effects of compound **41** but the difference with the negative control was not statistically significant.

Finally compound **1** showed no anti inflammatory activity at all because the weight loss observed was practically identical to that of the negative control group (vehicle group).

The next graph (*Graph 3.29*) shows the DAI scores for the treatment groups, higher scores indicates increased damage. It is clear that the anti-inflammatory effects of prednisolone and compound **2** were very similar (i.e. any difference was not statistically significant), and very strong in each case, with relatively little damage to the colon observed in either case.



Graph 3.29. Drug activity index score profile of DSS-induced colitis mice treated at 5 mg/kg

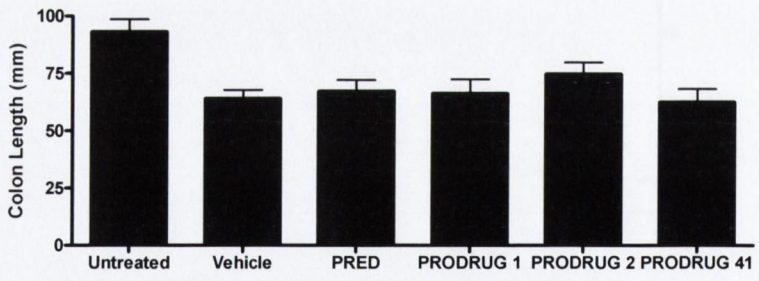
Day 6 DAI- Student's t-test.	
PRED versus Vehicle	$P<0.0061$ reduced DAI
PRO 2 versus Vehicle	$P<0.0018$ reduced DAI
PRO 2 or PRO 41 versus Vehicle	No Significance
PRED versus PRO 1	$P<0.0006$ reduced DAI
PRED versus PRO 2	No Significance
PRED versus PRO 41	$P<0.0054$ reduced DAI

Table 3.6. DAI statistical data

In agreement with graphs 3.27 and 3.28, the group of DSS-induced colitis mice treated with compound **1** scored similarly to the negative control group, indicating significant damage to the colon because of the absence of any anti inflammatory effect. The scores for compound **41** were not statistically different to the negative control group.

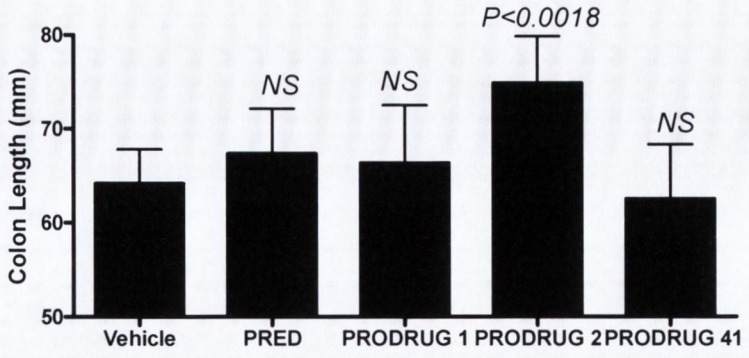
So far, the only system to show anti inflammatory properties was prodrug **2**, which was as effective as prednisolone.

The last parameter by which the anti inflammatory properties of the different prodrug were estimated was the colon length and these measurements are presented in *Graph 3.30*. The colon length of mice in which colitis was not induced by DSS is show as a reference. The colon length of the negative control group was used as reference for the mice in which colitis was induced.



Graph 3.30. Colon length profile of healthy mice (untreated) and DSS-induce colitis mice treated at 5 mg/kg dosage

DSS treatment caused a significant ($P<0.0001$) reduction in colon length in all groups relative to colon lengths of untreated mice. *Graph 3.31* presents the colon length data with the untreated group removed.

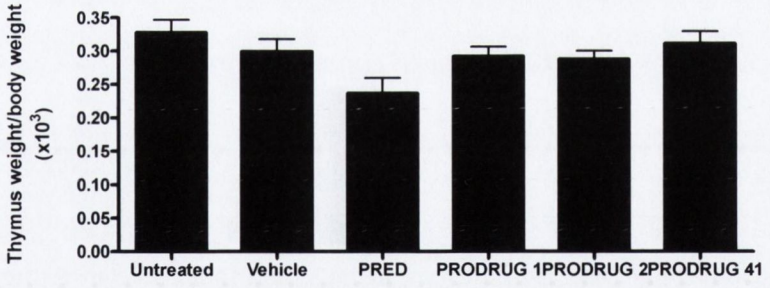


Graph 3.31. Colon length profile DSS-induce colitis mice treated at 5 mg/kg dosage

The *P* values are Student's t-test of difference in colon length versus vehicle group, is important to notice that the compound **2** treatment group had a significant longer colon that in the prednisolone ($P>0.0243$) group

In this analysis prodrug **2** treated DSS-induced colitis mice showed an effect that was better than that of prednisolone with statistical significance. Also it was clear that compounds **1** and **41** were not remedying the reduction in colon length caused by the induced colitis confirming the previous results.

To test the systemic side effects, the thymus weight and body weight ratio is determined as T/BW. The values for this parameter are shown in *Graph 3.32.*



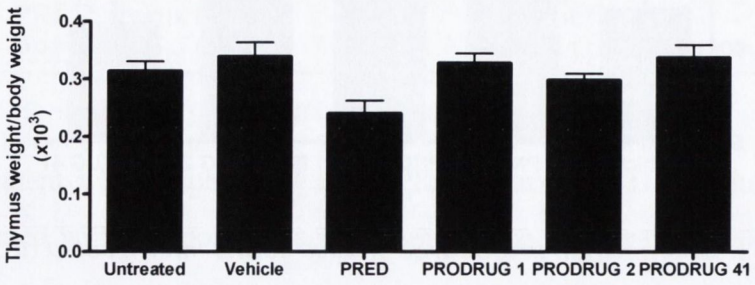
Graph 3.32. Thymus weight body weight ratios (T/BW) profile of healthy mice (untreated) and DSS-induce colitis mice treated at 5 mg/kg dosage

Only prednisolone had significant reduction in T/BW versus untreated mice ($P<0.05$).

The smallest T/BW ratio came from the DSS-induced colitis treated with prednisolone indicating a strong systemic side effect.

Mutual prodrug **41** T/BW was the largest of the DSS-induced colitis mice group, followed by the value from the prodrug **1** treated DSS-induce colitis

mice. Finally the ratio for prodrug **2** was statistically larger than that for prednisolone, indicating reduced systemic side effects. *Graph 3.33* shows the ratios between thymus weight and post treatment body weight.



Graph 3.33. Thymus weight post treatment body weight ratios profile of healthy mice (Untreated) and DSS-induce colitis mice treated at 5 mg/kg dosage

To confirm the good results for prodrug **2** another study was carried out in the same way, with the same parameters collected. But at a higher dose of 10 mg of prednisolone per kg of mouse body weight, only prednisolone and prodrug **2** were studied this time.

The data were qualitatively similar to those obtained at a dosage equivalent of 5 mg per kg of mouse body weight and confirmed the anti inflammatory and weak systemic side effects of prodrug **2**. The data is shown in appendix I.

3.6.3 Conclusions of the DSS-induced colitis mice studies

Prednisolone is widely used in several colitis treatments nowadays because of its anti-inflammatory properties. It was used as our positive control and our studies showed its anti-inflammatory effects as demonstrated in reduced DAI, decreased weight loss and relative maintenance of colon length. On the other hand systemic side effects were apparent, as indicated by reduced thymus weight.

Prodrug **2** was shown to be an anti inflammatory agent that was as effective as prednisolone at a dosage of 5 mg molar equivalent of prednisolone per kg of mouse body weight. However what make the compound **2** a potentially better agent than prednisolone in the treatment of severe colitis is its low levels of systemic side effects as reflected in the T/BW ratio analysis.

Compounds **41** and **1** did not show any anti inflammatory effects after statistical analysis of the parameters studied, and no systemic side effects. These results indicate that neither prodrugs released the prednisolone in the GIT.

After reviewing the results of this study we have to ask ourselves why prodrug **2** releases prednisolone (or at least has similar anti-inflammatory and side-effects) whereas prodrug **1** and **41** do not, when they are similar structurally. Given that neither compound is likely to be absorbed from the GIT (see below) differences in activity seem most likely to arise from differences in their behaviour within the GIT. Either they are reduced at different rates, or their product anilide esters cyclize at different rates. On the face of it the second explanation seems less plausible. Lactamization to give the five member ring lactone 1,3-dihydro-indol-2-one (**10**) proceeds in aqueous solution at the rougly the same rate as its propionate analogue (giving the six member ring lactone 3,4-dihydro-1H-quinolin-2-one **49**). Prednisolone is a product of both processes. Earlier studies on anilide methyl ester cyclizations to 5- or 6-membered lactams were also found to proceed at similar rates. However we saw that in organic solution there is a major difference between the two intermediate anilide esters: cyclizaton to give the six-membered ring was far faster than the five-membered compound. Is the colon more hydrophobic or drier than the buffered aqueous conditions we used as a model? The other possibility is that the propionate acts as a substrate for azo-reductase activity whereas the shorter and perhaps less flexible acetate did not. This explanation seems even less likely given the well established breadth of substrate specificity of azo-reductases, if these are even the causative agents for azo-transformations in the colon.

3.7 Plasma hydrolysis of prodrugs 1 and 2

In the studies carried out using the DSS-induced colitis model the thymus weights of the group of mice treated with azo prodrugs **1** and **41** were statistically similar to those of the untreated group of mice. This indicated that the thymuses of the compounds **1** and **41** treated mice were not affected by systemic prednisolone. One favourable conclusion to this observation was that the prodrugs were not absorbed following absorption; prednisolone

seemed likely to be released in response to metabolic vectors in the liver but especially in blood. It seemed to us that monitoring the stability of the prodrugs in mouse blood plasma would test the validity of this assumption. If, on the other hand, the compounds could be shown to be stable in mouse blood it would be more difficult to support the contention that they had not undergone absorption in the colitis study. Accordingly it was decided to incubate the drugs in mouse plasma and monitor their disappearance kinetics. Blood plasma from BALB/c mice was diluted to 33% with PBS pH 7.4. Stock solutions of prodrugs **1** and **2** were prepared in DMSO. The plasma solutions were kept at 37°C before adding (100 µl) aliquots of the prodrug solutions. Once the prodrugs were added, aliquots were collected every five seconds and mixed with $ZnCl_2$ 2% solution to quench further hydrolysis. The samples were centrifuged and the clear supernatants were analyzed by HPLC. The retention times of prodrugs **1** and **2** were 6.9 and 7.6 minutes respectively, and prednisolone (**3**) retention time was 3.0 minutes. A chromatogram is shown in *Figure 3.14* below illustrates how the hydrolysis was monitored.

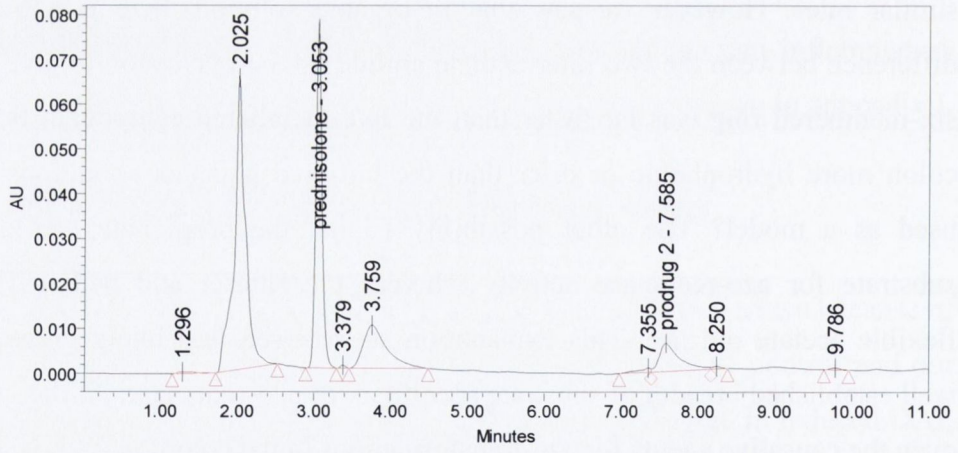
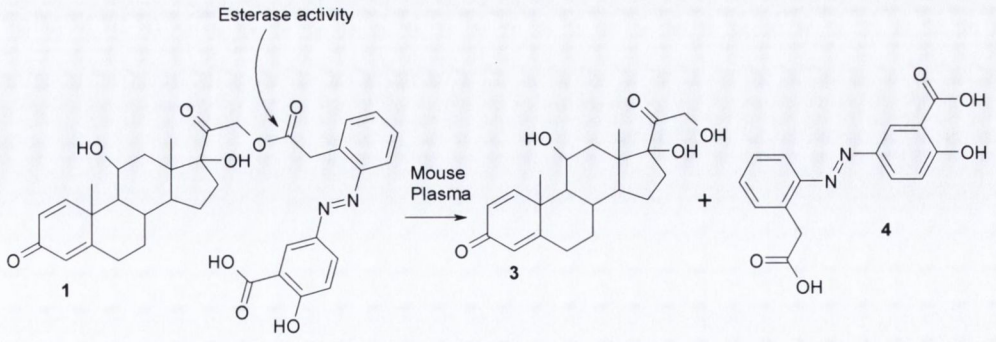


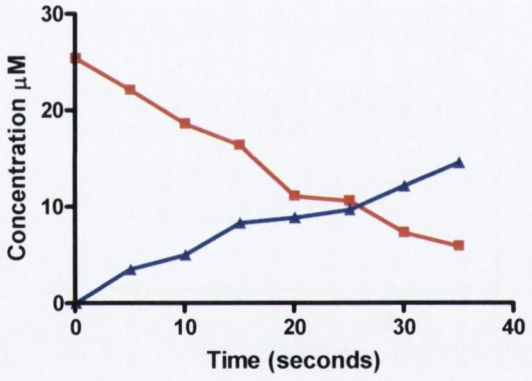
*Figure 3.14. Chromatogram of prodrug **2** plasma hydrolysis after 45 seconds of reaction*

The hydrolysis caused by mouse plasma is illustrated next in the *Scheme 3.1*

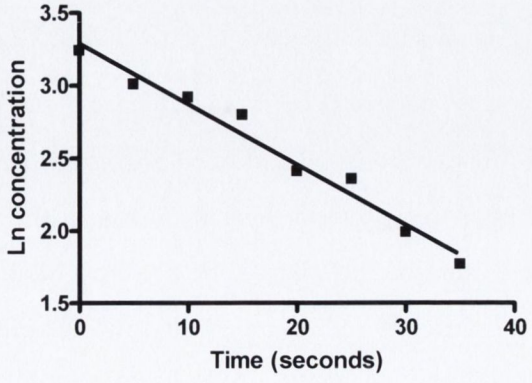


*Scheme 3.1. Ester hydrolysis of the prodrug **1** by mouse plasma*

Both prodrugs were observed to undergo rapid hydrolysis in the mouse plasma solution. Over the same time scale there was a corresponding increase in a peak with the same retention time and PDA UV spectrum as prednisolone. Hydrolysis data for prodrug **2** is presented below first as the straightforward progress curve and then as a first-order Ln plot (Graphs 3.34-3.35):

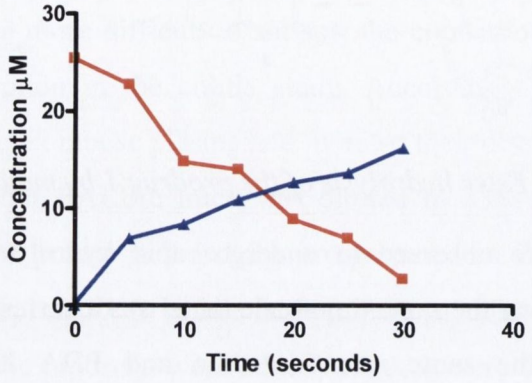


*Graph 3.34. Progress curve for the disappearance of prodrug **2** (■) and the appearance of prednisolone (▲) in 33% mouse plasma (pH 7.4 PBS) at 37°C*

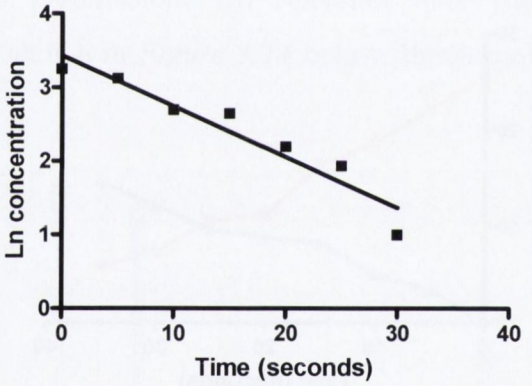


*Graph 3.35. Plot of Ln remaining Prodrug **2**(■) versus time (r^2: 0.9707)*

Prodrug **1** hydrolysis was completed within the same time interval, indicating no significant difference with prodrug **2** in terms of susceptibility to plasma-mediated hydrolysis (Graphs 3.36-3.37).



*Graph 3.36. Progress curve for the disappearance of prodrug **1** (■) and the appearance of prednisolone (▲) in 33% mouse plasma (pH 7.4 PBS) at 37°C*



*Graph 3.37. Plot of Ln remaining prodrug **1**(■) versus time (r^2: 0.9167)*

Both hydrolyses followed pseudo first-order kinetics. Hydrolysis of compounds **1** and **2** under these conditions was complete in one minute. This kind of rapid hydrolysis of a primary ester is expected in highly active mouse plasma solution.

The data indicate that if the prodrugs had been absorbed from the GIT in the colitis model, rapid hydrolysis would have occurred in the blood leading to systemically available steroid. In none of the prodrug treatment groups was there a statistically significant reduction in thymus weight, at least at the lower dose, indicating that none of the prodrugs were available to blood for hydrolysis. In terms of this analysis of course it doesn't matter whether they

were efficacious or not. The data here are consistent with the findings of a CACO-2 cell transport study which will be described next.

3.8 Transport studies

After the results obtained in the DSS-induced colitis mice treated with prodrugs **41**, **1** and **2**, we decided to carry out a study to test the transport of prodrugs **1** and **2** in order to determine if the compounds are likely to resist absorption (as designed) and to explore potential reasons for the differences in activity that emerged in the DSS study.

3.8.1 Introduction

In this study the transport of prednisolone, prodrug **1** and **2** through a CACO-2 cell line was tested and compared. Prior to the transport assay, cytotoxicity of the compounds in CACO-2 cells was estimated using a methyl tetrazolium salt (TMS) proliferative test, to determine the concentrations to use in the transport studies. Two types of transport were examined in this study; absorptive transport from apical to basolateral side (AP>BL) and secretory transport from basolateral to apical side (BL>AP). The work was performed at IBET Labs, Oeiras, Lisbon by the author and Dr Ana Simplicio of IBET under Dr Simplicio's supervision.

3.8.2 Cytotoxicity test

Different concentrations of prodrug **1**, **2** and prednisolone **39** were tested (6.3, 12.5, 50, 100, 200 and 400 μM; four lines each) using the CellTiter 96® Aqueous Assay from Promega (Madison, WI, USA). CACO-2 cells were seeded in a 96 well plate at a concentration of 10^4 cells/cm^2 and incubated until they reached confluence. They were then challenged with the test compounds for two hours. The TMS solution was added and plates were read after one hour at 490 nm for the detection of the formazan resulting from the metabolism of MTS. Readings were also taken at 30, 90 and 120 minutes. Estimates of the rates of cell survival were determined from the differences between the average absorbances in the wells containing the challenged and

the average absorbances in wells containing the unchallenged cells (C=0) (*Table 3.7*).

Lane 1-3 cells+test compound; during the full time of the test; MTS added at time 2h; reading at time 3 h			*Lane 4 cells+test compound replaced by fresh buffer at time 2h. MTS added at the same time; reading at time 3 h*	*Lane 5 test compound; during the full time of the test; MTS added at time 2h; reading at time 3 h*
400	400	400	400	400
200	200	200	200	200
100	100	100	100	100
50	50	50	50	50
25	25	25	25	25
12.5	12.5	12.5	12.5	12.5
6.3	6.3	6.3	6.3	6.3
0	0	0	0	0

Table 3.7. Lane composition for cytotoxicity test

In an additional test, in one lane (4), the challenge solutions were replaced with fresh feeding buffer (for each test compound) before MTS addition.

In lane 5, MTS solution was added to the test compound solutions in the absence of the cells. Results from lane 5 provided evidence of interference by the test compounds since absorbance increased as time passed after MTS addition despite the fact that no cells were present for metabolizing it. The interference was higher with higher concentrations of the test compounds. For this reason tests performed without removal of the test compounds prior to MTS addition were discarded. *Table 3.8* summarizes the survival rate of cells challenged with different concentrations of the test compound for two hours (n=1); based on these results, 50 μM was chosen as the concentration to be used for the donor solution in the transport test.

Concentration μM	*Prednisolone*	*Prodrug 1*	*Prodrug 2*
400	82.0	73.0	68.2
200	84.7	90.8	100.4
100	82.7	85.5	91.9
50	93.5	100.0	97.9
25	96.0	108.5	96.3
12.5	96.5	100.2	91.9
6.3	94.7	93.7	97.8

Table 3.8. Survival rates (%) after challenges by the test compounds

3.8.3 Transport assays results

Compounds were tested in triplicate for transport across a CACO-2 cell monolayer in the AP>BL direction and in the reverse direction. Caffeine was also tested in two wells in the AP>BL direction as a routine control.

Trans-epithelial electric resistance (TEER) was over 450 $\Omega.cm^2$ in all wells used in the experiments prior to the addition of the test compounds.

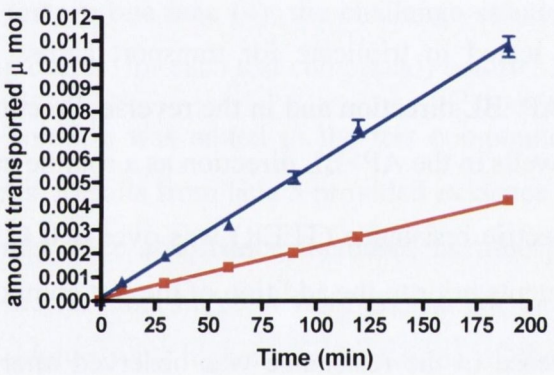
A significant decrease in the resistance was observed after three hours (to about 100 $\Omega.cm^2$) which could indicate damage to the cells; however a recovery to the original values was observed 24 hours after replacing the test solutions with fresh buffer providing evidence that no long term damage was caused to the monolayer the test compounds (raw data in appendix II) by. However one should bear in mind that this reduction in TEER may reflect an absorption enhancing effect due to loosening of the tight junctions as reported in (Mukherjee et al., 2004).

Aliquots were collected after 10, 30, 60, 90, 120 and 180 minutes from the acceptor reservoir and fresh buffer was added to maintain the initial volume. The donor solution was also collected after 180 min.

Time (min)	*Prednisolone concentration mM*		*AP>BL*	*Prednisolone concentration mM*		*BL>AP*
0	0	0	0	0	0	0
10	0.0005	0.0004	0.0005	0.0008	0.0007	0.0009
30	0.0008	0.0007	0.0007	0.0017	0.0019	0.0021
60	0.0016	0.0011	0.0015	0.0029	0.0032	0.0035
90	0.0020	0.0021	0.0019	0.0047	0.0053	0.0056
120	0.0029	0.0027	0.0025	0.0066	0.0077	0.0076
180	0.0044	0.0041	0.0042	0.0099	0.0111	0.0113

Table 3.9. Prednisolone absorptive transport (left) and secretory transport (right) data

The amount transported was determined taking into consideration replenishing volumes.



Graph 3.38. Bidirectional transport of prednisolone. The error bars represent the standard deviation of three determinations(■) prednisolone absorptive transport (AP>BL) (▲) prednisolone secretory transport (BL>AP)

The lines presented in *Graph 3.39* illustrate the bidirectional transport of predinsolone and afforded the apparent permeability coefficient (P_{app}) presented in *Table 3.9*. P_{app} is expressed in cm/s and was determined according to the following equation:

$$P_{app}=J / A_s C_0$$

Where J is the rate of appearance of the compound on the acceptor compartment (μmole/s), C_0 is the initial concentration on the apical side (mM) as determined by the analysis of the donor solution, and A_s is the surface area of the monolayer (cm^2).

The fact that the transport rate is higher in the BL>AP direction suggests a possible efflux mechanism for prednisolone which has previously been observed according to the literature (Dilger et al., 2006). Mass balance suggests an 18% and 6% retention of prednisolone in the cells in the experiments performed in the AP>BL and BL>AP directions respectively. The BL>AP/ AP>BL ratio was 2.7.

		Well	P_{app} *(cm/s)*	*Average*	*SD*	*CI*
AP>BL	***Prednisolone***	**A2**	8.26E-06	7.49E-06	6.62E-07	1.12E-06
		B2	7.09E-06			
		C2	7.13E-06			
BL>AP	***Prednisolone***	**A1**	1.81E-05	2.00E-05	1.9E-06	3.1E-06
		B1	2.00E-05			
		C1	2.18E-05			
	P1	**A2**	1.82E-05	1.56E-05	2.2E-06	3.8E-06
		B2	1.49E-05			
		C2	1.39E-05			
	P2	**A4**	1.47E-05	1.41E-05	2.4E-06	4.1E-06
		B4	1.14E-05			
		C4	1.62E-05			

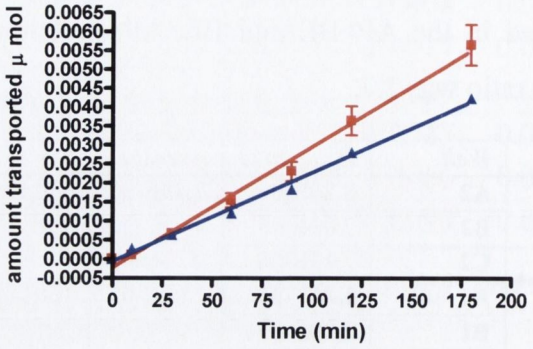
Table 3.10. Apparent permeability coefficient (P_{app}) values

No transport was observed in the AP>BL direction for either prodrug, however some prednisolone was detected in the acceptor compartment but this was attributed to degradation of the test compounds in the HBSS donor solution. Indeed, at the time of the analytical determination, the original donor solutions were found to contain only about 50% of the amount of prodrug added, while several other peaks appeared, including prednisolone.

Time (min)	*Prodrug* ***1*** *concentration mM*		*BL>AP*	*Prodrug* ***2*** *concentration mM*		*BL>AP*
0	0	0	0	0	0	0
10	0.0001	0.0001	0.0001	0.0004	0.0001	0.0003
30	0.0008	0.0006	0.0006	0.0007	0.0005	0.0007
60	0.0014	0.0013	0.0019	0.0013	0.0011	0.0012
90	0.0020	0.0021	0.0028	0.0018	0.0017	0.0020
120	0.0033	0.0032	0.0044	0.0029	0.0028	0.0027
180	0.0050	0.0052	0.0067	0.0043	0.0042	0.0042

*Table 3.11. Data from the secretory transport assay of prodrug **1** and **2***

This observation certainly introduces an error to the determination of the transported amount (*Graph 3.39*) and apparent BL>AP permeability (*Table 3.5*) due to the error in C_0 determination.



*Graph 3.39. Transport of prodrugs **1** and **2**. The error bars represent the standard deviation of three determinations prodrug **1**(■) secretory transport (BL>AP) prodrug **2** (▲) secretory transport (BL>AP)*

3.8.4 Conclusions of the transport through CACO-2 cells studies

It can be concluded that the prodrugs are not likely to be absorbed in the intestinal tract after oral administration as they were not detected in the acceptor compartments when added on the apical side. Moreover there is evidence of efflux since transport was observed when the test compounds were applied to the basolateral side.

Degradation was observed upon solubilisation of the prodrugs in the test buffer Hank's Balanced Salt Solution (HBSS) and therefore no solubility tests were performed. Due to the observed degradation in (HBSS), calibration solutions were prepared in acetonitrile. Nevertheless the tests were still performed using HBSS as transport buffer.

The same solution was completely degraded after two days. Solutions in acetonitrile did not show any sign of degradation after eight days.

CONCLUSIONS AND FUTURE WORK

The synthesis of the azo carriers proposed for conjugation to the steroids proved to be more difficult than expected at the outset. Initial diazotization strategies did not work probably because of interference between with ester side-chain of the carriers. A condensation between nitrosoarenes and anilines was the solution eventually arrived at. Difficulties were also encountered with spontaneous cyclization of some of the materials which limited chemistry approaches especially with the longer cinnamate-derived linkers. The esterification of the carriers to the steroid was not trivial either and a Mitsunobu reaction was the only successful method found, albeit in low yield.

It seems compound **2** is a potential prodrug for the treatment of gastrointestinal diseases. It appears to have similar anti-inflammatory effects to prednisolone in the mouse colitis model but has less impact on thymus weight, indicating that prednisolone delivered this way has lower systemic availability. The data from the mouse study, taken together with its behaviour in mouse blood, indicate that compound **2** was not absorbed from the GIT (as designed). Also, compound **2** is not transported in the CACO-2 model. Puzzlingly, compound **1** is quite similar structurally but it did not show any anti-inflammatory properties suggesting the reduction/cyclization was not completed or it was not rapid enough. There was no thymus atrophy in the compound **1** treatment group either indicating that while it did not have any anti-inflammatory effects, it was not absorbed. The studies by and large validate the original design strategy but further work is clearly called for.

In terms of syntheses, and in order to provide samples for clinical testing, some further work on esterification conditions will be required: the practical potential of the compounds hinges on their synthetic accessibility. There are other interesting targets too from a synthetic and prodrug perspective. One of these would be to use PABA as the terminal acid and amino donor rather that 5ASA. This might address some of the protection and esterification issues in the synthesis and aqueous stability problems. The kinetics studies posed a lot of questions. In particular there is a marked difference between the stability of the phenol-azo compound (**40**) and the salicylate analogues. Compound **40** behaves as expected in aqueous solution- the other compounds exhibited poor

and variable stability. Indeed the stability issue as a whole is important for its relevance to the ability of the compounds to transit the GIT (target the colon) and express their selective activation. We found cyclization in organic solvent at room temperature was completed rapidly for some systems. The relevance to the colon is that conditions there may be drier and more lipophilic than expected (or at least modelled in aqueous solution). Further investigation of the prodrugs would benefit from a realistic model of the colon, including reductase activity. At any rate, the different anti-inflammatory activities of the prodrugs demands attention, given their structural similarity. It is possible (for example) that prodrugs inactive in the mouse model might display activity in a larger animal model where colon residence times are longer. These issues could also be investigated by a predictive colon model. The conclusions on the absorption of the compounds appear sound given collective data, however, analytical studies need to be conducted on the relative distribution of the steroids following oral administration.

In summary we can say that the lead, compound **2**, has promising pharmaceutical and pharmacological characteristics- it is anti-inflammatory and there is significant evidence that it is not absorbed. Future studies need to focus on *how* compound **2** possesses/expresses these characteristics, in other words to determine of it works *in vivo* as it is intended to.

CHAPTER FOUR

EXPERIMENTAL

4.1 Materials

All the chemicals and the enzyme *E.coli* nitroreductase X19284 (recombinant) have been ordered from Sigma Aldrich and Fluka laboratories suppliers.

4.2 General experimental procedures

Melting points:

Uncorrected melting points were obtained using a Stuart® melting point SMP11 melting point apparatus.

Infra red (IR):

Spectra were obtained using a Perking Elmer 205 FT Infrared Paragon 1000 spectrometer. Band positions are given in cm^{-1}. Solid samples were obtained by KBr disk; oils were analyzed as neat films on NaCl plates.

Nuclear Magnetic Resonance (NMR):

^{1}H and ^{13}C spectra were recorded at 27°C on a Bruker Advance II 600 MHz spectrometer (600.13 MHz ^{1}H, 150.91 MHz ^{13}C) and Bruker DPX 400MHz FT NMR spectrometer (400.13 MHz ^{1}H, 100.16 MHz ^{13}C), in either $CDCl_3$ or CD_3OD, (tetramethylsilane as internal standard). For $CDCl_3$, ^{1}H-NMR spectra were assigned relative to the TMS peak at 0.00 δ and ^{13}C-NMR spectra were assigned relative to the middle $CDCl_3$ triplet at 77.00 ppm. For CD_3OD, ^{1}H and ^{13}C-NMR spectra were assigned relative to the centre peaks of the CD_3OD multiplets at 3.30 δ and 49.00 ppm respectively. Coupling constants were reported in hertz (Hz). For ^{1}H-NMR assignments, chemical shifts are reported: shift values (number of protons, description of absorption (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant(s) where applicable, proton assignment).

Mass spectrometry:

High resolution mass spectrometry (HRMS) was performed on a Micromass mass spectrophotometer (EI mode) at the Department of Chemistry, Trinity College.

High performance liquid chromatography (HPLC)

HPLC was performed on a reverse phase 250 mm x 4.6 mm Waters Spherisorb ODS-2, 5 μm column using a Waters Alliance 2695 chromatograph equipped with an autosampler, column oven and dual wavelength detector. The flow rate was 1 ml/min with a mobile phase consisting of 40% phosphate buffer pH 2.5 and 60% acetonitrile at time 0 and grading to 85% acetonitrile at 4 min. Injection volume was 20 μl, and areas determined at 254 nm. The isocratic HPLC method was aqueous phosphate buffer solution pH 2.5 40% and acetonitrile 60%. Flow rate was 1 ml/min.

Chromatographic methods:

Flash chromatography was performed on Merck Kieselgel 60 particle size 0.040-0.063 mm. Thin layer chromatography (TLC), was performed on silica gel Merck F-254 plates. Compounds were visually detected by absorbance at 254 nm and/or vanillin staining.

4.3 General preparation procedures

2-Nitrosophenylpropionic acid ***(57)***

To a solution of 2-nitrophenylcinnamic acid **47** (5 g, 0.0259 mol) in water (100 ml) and two equivalents of 2% NaOH (103 ml), a catalytic amount of Pd/C (10%) was added (5%, 0.25 g). The reaction mixture was stirred under a hydrogen atmosphere at room temperature over 48 hours, after which time TLC (ethyl acetate) analysis showed reaction completion. The reaction mixture was filtered over filter agent to remove the Pd/C (10%) and the solvent was removed under reduced pressure. The crude 2-aminophenylpropionic acid was dissolved in distilled water (50 ml) and DCM (150 ml). Oxone® (31.84 g, 0.0518 mol, 2 equivalents) was dissolved in distilled water (50 ml) and added to the reaction mixture. At this point the reaction pH was 4 so the 2-nitrosophenylpropionic acid was transferred to the organic layer as soon as it was formed. After two hours the reaction mixture was transferred to a separating funnel, the organic layer collected, and the aqueous phase washed with DCM (2 x 25 ml). The organic phase was collected and dried with sodium sulfate. The solvent was removed under reduced pressure to afford 4.12 g of product as dark oil. The crude mixture was flash columned using DCM: ethyl acetate (70: 30) to yield the product as brown crystals (3.48 g, 67%), m.p. 112-114°C.

$IR_{\nu max}$(KBr): 1699.29 cm^{-1}.

^{1}H NMR δ ($CDCl_3$): 7.99 (1H, d, *J* 8 Hz), 7.65 (1H, t, *J* 7.52 Hz), 7.57 (1H, t, *J* 8.04 Hz), 7.43 (1H, m), 3.24 (2H, t, *J* 7.52 Hz), 2.81 (2H, t, *J* 7.52 Hz).

^{13}C NMR δ ($CDCl_3$): 177.96 (C, C-9, C=O), 170.91 (C, C-2), 132.88 (CH, C-5), 131.70 (CH, C-6), 131.63 (C, C-1), 127.30 (CH, C-4), 124.56 (CH, C-3), 34.09 (CH_2, C-8), 27.61 (CH_2, C-7).

5-Nitrosalicylic t-butyl ester ***(42)***

DCC (1.2 equivalents, 6.2 g, 0.030 mol) dissolved in dry tetrahydrofuran (50 ml), was added dropwise over 30 minutes to a stirred solution of 5-

nitrosalicylic acid **36** (5 g, 0.0273 mol) and DMAP (1 equivalent, 3.3 g, 0.0273 mol) in dry *t*-butanol (125 ml). The mixture was stirred overnight and filtered to remove DCU formed and the solvents were removed under reduced pressure to afford the product as a yellow oil. This was flash columned using DCM: ethyl acetate (90: 10) to yield the product as pale yellow crystals (5.29 g, 81%), m.p. 58-60°C (This compound has been made previously (Pandit et al., 2006) but no spectroscopy data was found. The melting point was 81-81.5°C).

IR_{vmax}(KBr): 3417.06, 2118.35, 1715.88 and 1673.77 cm^{-1}.

^1H NMR δ ($CDCl_3$): 11.73 (1H, s), 8.63 (1H, d, *J* 2.52 Hz), 8.26 (1H, dd, *J* 9 and 2.48 Hz), 7.01 (1H, d, *J* 9.04 Hz), 1.61 (9H, s).

^{13}C NMR δ ($CDCl_3$): 168.01 (C, C-2), 166.09 (C, C-7, C=O), 139.24 (C, C-5), 129.54 (CH, C-4), 126.19 (CH, C-6), 117.98 (CH, C-3), 112.95 (C, C-1), 84.45 (C, C-8), 27.57 (3xCH_3, C-9).

*5-Aminosalicylic t-butyl ester **(35)***

To a solution of 5-nitrosalicylic *t*-butyl ester **42** (4.21 g, 0.0176 mol) in ethyl acetate (100 ml) a catalytic amount of Pd/C (10%) was added (0.41 g, 10%). The reaction was stirred under a hydrogen atmosphere for three hours. At that point TLC analysis DCM: ethyl acetate (60: 40) showed reaction completion. The reaction mixture was filtered over celite as a filter agent to remove the Pd/C (10%) and the solvent was removed under reduced pressure, to yield the product as pale green crystals (3.2 g, 86%), m.p. 48-50°C (This compound has been made previously (Pandit et al., 2006) but no spectroscopy data was found. The melting point was 63-64.5°C).

IR_{vmax}(KBr): 3372.92, 1668.16 and 1490.72 cm^{-1}.

^1H NMR δ ($CDCl_3$): 10.49 (1H, s), 7.12 (1H, d, *J* 2.52 Hz), 6.8 (2H, m), 3.5 (2H, NH_2), 1.61 (9H, s).

^{13}C NMR δ ($CDCl_3$): 169.13 (C, C-7), 154.44 (C, C-2), 137.63 (C, C-5), 123.30 (CH, C-4), 117.57 (CH, C-3),

114.71 (CH, C-6), 113.24 (C, C-2), 82.12 (C, C-8), 27.74 (3xCH_3, C-9).

5-[2-(2-Carboxy-ethyl)-phenylazo-]-2-hydroxy-bezoic acid tert-butyl ester (55)

To a solution of 2-nitrosophenylpropionic acid **57** (1.27 g, 6.3 mmol) in glacial acetic acid (20 ml), another solution of 5-aminosalicylic *t*-butyl ester **35** (1 equivalent, 1.32 g, 6.3 mmol) in glacial acetic acid (20 ml) was added dropwise over 15 minutes. The reaction was left stirring for 48 hours until completion by TLC ethyl acetate: DCM (50: 50). The solvent was removed under reduced pressure to afford the product as a black oil. This was flash columned using DCM as mobile phase to yield the product as orange crystals (1.68 g, 72%), m.p. 86-88°C.

IR_{vmax}(KBr): 1700.66 and 1665.87 cm^{-1}.

^{1}H NMR δ ($CDCl_3$): 11.52 (1H, s), 8.46 (1H, s), 8.08 (1H, s), 7.72 (1H, s), 7.42 (3H, m), 7.13 (1H, s), 3.48 (2H, s), 2.82 (2H, s), 1.70 (9H, s).

^{13}C NMR δ ($CDCl_3$): 179.00 (C, C-9, C=O), 169.18 (C, C-16, C=O), 163.79 (C, C-13), 149.57 (C, C-2), 145.09 (C, C-10), 139.21 (C, C-1), 130.54 (CH, C-5), 130.08 (CH, C-6), 127.65 (CH, C-11), 127.08 (CH, C-4), 126.97 (CH, C-15), 118.10 (CH, C-14), 115.07 (CH, C-3), 113.40 (C, C-12), 83.21 (C, C-17), 35.68 (CH_2, C-8), 27.74 (3xCH_3, C-18), 26.54 (CH_2, C-7).

HRMS: Found: $(M\text{-}Na)^+ = 393.1428$

Required: $(M\text{-}Na)^+ = 393.1426$

5-(2-{2-[2-(11,17-Dihydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[α]phenanthren-17yl)-2-oxo-ethoxycarbonyl]-ethyl}-phenylazo)-2-hydroxy-benzoic acid tert-butyl ester ***(56)***

To a solution of azo carrier **55** (1.21 g, 3.3 mmol), prednisolone **3** (1 equivalent, 1.18 g, 3.3 mmol) and PPh_3 (3 equivalents, 2.59 g, 9.9 mmol) in dry tetrahydrofuran (50 ml), DIAD (3 equivalents, 1.9 ml, 9.9 mmol) was added dropwise over 12 minutes after the reaction temperature reached 40°C. Reaction was stirred at 40°C for an hour and then left overnight at room temperature under a nitrogen atmosphere. TLC (DCM) analysis showed completion. The solvent was removed under reduced pressure to afford the product as an orange oil. This was flash columned using DCM: ethyl acetate (50:50). The triphenylphosphine oxide was removed using a second flash column [hexane (200 ml), hexane: ethyl acetate (70: 30)] to yield the product as orange crystals (0.92 g, 40%), m.p. 116-118°C.

IR_{vmax}(KBr): 3435.94, 1724.53, 1658.49 and 1615.49 cm^{-1}.

^1H NMR δ ($CDCl_3$): 8.40 (1H, d, *J* 2 Hz), 8.10 (1H, dd, *J* 8.52 and 2 Hz), 7.68 (1H, d, *J* 8.04 Hz), 7.44 (3H, m), 7.33 (1H, t, *J* 6.52 Hz), 7.09 (1H, d, *J* 8.52 Hz), 6.27 (1H, d, *J* 10.04 Hz), 6.01 (1H, s), 5.00 (1H, d, *J* 17.56 Hz), 4.88 (1H, d, *J* 17.56 Hz), 4.39 (1H, s), 3.49 (2H, t, *J* 9.52), 2.82 (2H, t, *J* 8.04 Hz), 2.66-0.9 (prednisolone envelope, 19H), 1.69 (9H, s).

^{13}C NMR δ ($CDCl_3$): 204.83 (C, C-20, C=O), 186.46 (C, C-3, C=O), 172.51 (C, C-22, C=O), 170.73 (C, C-5), 169.08 (C, C-13', C=O), 163.73 (C, C-10'), 156.77 (CH, C-1), 149.48 (C, C-2'), 145.09 (C, C-7'), 139.31 (C, C-1'), 130.55 (CH, C-5'), 130.00 (CH, C-6'), 129.26 (CH, C-8'), 127.04 (CH, C-2), 126.91 (CH, C-4'), 125.72 (CH, C-12'), 121.65 (CH, C-4), 118.18 (CH, C-11'), 114.99 (CH, C-3'), 113.29 (C, C-9'), 89.20 (C, C-17), 83.27 (C, C-14'), 69.56 (CH, C-11),

67.74 (CH_2, C-21), 54.90 (CH, C-9), 50.87 (CH, C-14), 47.22 (C, C-13), 43.84 (C, C-10), 38.96 (CH_2, C-12), 35.45 (CH_2, C-23), 33.83 (CH_2, C-6), 33.59 (CH_2, C-7), 31.61 (CH_2, C-16), 30.73 (CH, C-8), 27.75 (3xCH_3, C-15'), 26.66 (CH_2, C-24), 23.41 (CH_2, C-15), 20.54 (CH_3, C-19), 16.42 (CH_3, C-18).

HRMS: Found: $(M\text{-}Na)^+ = 735.3283$

Required: $(M\text{-}Na)^+ = 735.3258$

5-(2-{2-[2-(11,17-Dihydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[α]phenanthren-17yl)-2-oxo-ethoxycarbonyl]-ethyl}-phenylazo)-2-hydroxy-benzoic acid ***(2)***

To a solution of *t*-butyl ester protected mutual prodrug **56** (0.9 g, 1.26 mmol) in DCM (3 ml), was added trifluoroacetic acid (3 ml) and the reaction left at room temperature for four hours. TLC analysis using DCM: ethyl acetate (50: 50) showed completion and solvents and trifluoroacetic acid were blown off using nitrogen, to afford the product as orange oil. The crude product was flash columned using DCM: ethyl acetate (50: 50), (100 ml), ethyl acetate (100 ml) and acetone: ethyl acetate (70: 30) to yield the product as an orange crystals (0.7 g, 84%), m.p. 176-178°C.

$IR_{\nu max}$(KBr): 3437.19, 1718.06, 1654.23 and 1587.93 cm^{-1}.

^{1}H NMR δ (MeOD): 8.44 (1H, s), 8.07 (1H, d, *J* 9.03 Hz), 7.65 (1H, d, *J* 7.53 Hz), 7.43 (3H, m), 7.31 (1H, t, *J* 7.53 Hz), 7.07 (1H, d, *J* 9.04 Hz), 6.26 (1H, d, *J* 10.04 Hz), 5.99 (1H, s), 4.88 (2H, d, *J* 17.56 Hz), 4.36 (1H, s), 3.44 (2H, t, *J* 7.53 Hz), 2.80 (2H, t, *J* 8.03 Hz), 2.64-0-89 (prednisolone envelope, 19H).

^{13}C NMR δ (MeOD): 205.37 (C, C-20, C=O), 187.17 (C, C-3, C=O), 173.05 (C, C-22, C=O), 172.05 (C, C-5), 164.03 (C, C-13', C=O), 161.33 (C, C-10'),

158.41 (CH, C-1), 149.62 (C, C-2'), 144.93 (C, C-7'), 139.12 (C, C-1'), 129.85 (CH, C-6'), 128.63 (CH, C-8'), 126.47 (CH, C-2), 125.93 (CH, C-4'), 124.54 (CH, C-12'), 120.61 (CH, C-4), 117.65 (CH, C-11'), 116.80 (CH, C-3'), 114.51 (C, C-9'), 88.71 (C, C-17), 68.93 (CH, C-11), 67.48 (CH_2, C-21), 55.45 (CH, C-9), 50.94 (CH, C-14), MeOD residual peak is masking the peak of C, C-13, 44.23 (C, C-10), 38.37 (CH_2, C-12), 35.19 (CH_2, C-23), 33.72 (CH_2, C-6), 32.79 (CH_2, C-7), 31.33 (CH_2, C-16), 30.78 (CH, C-8), 26.26 (CH_2, C-24), 22.97 (CH_2, C-15), 19.73 (CH_3, C-19), 15.45 (CH_3, C-18).

HRMS: Found: $(M\text{-}Na)^+ = 679.2642$.

Requires: $(M\text{-}Na)^+ = 679.2632$.

*2-Nitrosophenylacetic acid **(30)***

To a solution of 2-nitrophenylacetic acid **9** (5 g, 0.0276 mol) in ethyl acetate (100 ml) a catalytic amount of Pd/C (10%) was added, (5%, 0.25 g). The reaction mixture was stirred under a hydrogen atmosphere at room temperature for two hours, after which time TLC analysis showed reaction to be complete ethyl acetate: DCM (40: 60). The reaction mixture was filtered over celite as a filter agent to remove the Pd/C (10%); the solvent was removed under reduced pressure. The crude 2-aminophenylpropionic was dissolved in DCM (150 ml). Oxone® (2 equivalents, 33.9 g, 0.0552 mol) was dissolved in distilled water (50 ml) and the solution added to the reaction mixture. At this point the reaction pH was 4 so the 2-nitrosophenylpropionic acid was transferred to the organic layer as it was formed. After two hours, the reaction mixture was transferred to a separating funnel, the organic layer collected, and aqueous phase washed (2 x 25 ml) DCM. The organic phase was collected and dried with sodium sulfate. Solvents were removed under reduced pressure to afford the product as dark oil. The crude mixture was

flash columned using DCM: ethyl acetate (70: 30) to yield the product as brown crystals (2.61 g, 52 %), m.p. 116-118°C.

IR_{vmax}(KBr): 1691.86 cm^{-1}.

^1H NMR δ ($CDCl_3$): 9.00 (1H, s, COOH), 7.24 (2H, t, *J* 8.54 and 7.53Hz), 7.04 (1H, t, *J* 7.53 and 7.53Hz), 6.92 (1H, d, *J* 7.53Hz), 3.57 (2H, s).

^{13}C NMR δ ($CDCl_3$): 178.02 (C, C-8, C=O), 141.86 (C, C-1), 127.50 (CH, C-4), 124.77 (C, C-2), 124.15 (CH, C-3), 122.05 (CH, C-5), 109.50 (CH, C-6), 35.84 (CH_2, C-7).

5-(2-Carboxymethyl-phenylazo)-2-hydroxy-benzoic acid tert-butyl ester ***(43)***

To a solution of 2-nitrosophenylacetic acid **30** (1.0 g, 6.1 mmol) in glacial acetic acid (20 ml), a solution of 5-aminosalicylic *t*-butyl ester **35** (1 equivalent, 1.26 g, 6.1 mmol) in glacial acetic acid (20 ml) was added dropwise over 15 minutes. The reaction was left stirring for 48 hours until completion shown by TLC ethyl acetate: DCM (50: 50). The solvent was removed under reduced pressure to afford the product as a black oil. This was flash columned using DCM as mobile phase to yield the product as orange crystals (1.49 g, 69%), m.p.108-110°C.

IR_{vmax}(KBr): 3434.51, 1714.14, 1670.39 and 1587.05 cm^{-1}.

^1H NMR δ ($CDCl_3$): 11.49 (1H, s), 8.37 (1H, d, *J* 2.48 Hz), 7.95 (1H, dd, *J* 9 and 2.48 Hz), 7.76 (1H, d, *J* 8.04 Hz), 7.40 (3H, m), 6.97 (1H, d, *J* 9.04 Hz), 4.10 (2H, s), 1.64 (9H, s).

^{13}C NMR δ ($CDCl_3$): 177.05 (C, C-8, C=O), 169.14 (C, C-15, C=O), 163.83 (C, C-12), 149.40 (C, C-2), 144.84 (C, C-9), 133.26 (CH, C-1), 131.01 (CH, C-5), 130.49 (CH, C-6), 127.82 (CH, C-10), 127.62 (CH, C-4), 127.26 (CH, C-14), 117.99 (CH, C-13), 115.53 (CH, C-3), 113.32 (C, C-11), 83.24 (C, C-16), 37.40 (CH_2, C-7), 27.69 ($3xCH_3$, C-17).

HRMS: Found: $(M\text{-}Na)^+ = 379.1273$

Required: $(M\text{-}Na)^+ = 379.1270$

5-{2-[2-(11,17-Dihydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[α]phenanthren-17yl)-2-oxo-ethoxycarbonylmethyl]-phenylaz}-2-hydroxy-benzoic acid tert-butyl ester ***(44)***

To a solution of azo carrier **43** (0.40 g, 1.1 mmol), prednisolone **3** (1.1 equivalents, 1.18 g, 1.3 mmol) and PPh_3 (3 equivalents, 0.88 g, 3.3 mmol) in dry tetrahydrofuran (50 ml), DIAD (3 equivalents, 0.66 ml, 3.3 mmol) was added dropwise over 12 minutes after the reaction temperature reached 40°C. The reaction was stirring at 40°C for an hour and then left overnight at room temperature under a nitrogen atmosphere. TLC analysis (DCM) showed completion. The solvent was removed under reduced pressure to afford the product as an orange oil. This was flash columned using DCM: ethyl acetate (50:50). After this column the triphenylphosphine oxide was removed and a second flash column was needed hexane (200 ml), hexane: ethyl acetate (70: 30) to yield the product as orange crystals (0.33 g, 43%), m.p. 124-126°C.

$IR_{\nu max}$(KBr): 3436.29, 1726.17, 1659.61 and 1616.09 cm^{-1}.

^1H NMR δ ($CDCl_3$): 11.49 (1H, s), 8.44 (1H, s), 8.05 (1H, d, *J* 9 Hz), 7.75 (1H, d, *J* 7.52 Hz), 7.40 (3H, m), 7.24 (1H, d, *J* 10.04 Hz), 7.06 (1H, d, *J* 9 Hz), 6.25 (1H, s), 5.98 (1H, s), 4.27 (2H, s), 2.68-0.84 (prednisolone envelope, 19H), 1.67 (9H, s).

^{13}C NMR δ ($CDCl_3$): 204.40 (C, C-20, C=O), 186.26 (C, C-3, C=O), 171.36 (C, C-22, C=O), 170.10 (C, C-5), 169.09 (C, C-13, C=O), 163.85 (C, C-10'), 156.25 (CH, C-1), 149.53 (C, C-2'), 145.07 (C, C-7'), 133.25 (C, C-1'), 130.98 (CH, C-5'), 130.53 (CH, C-6'), 129.89 (CH, C-8'), 127.85 (CH, C-2), 127.17 (CH, C-4'), 125.52 (CH, C-12'), 121.79 (CH, C-4), 118.14 (CH, C-11'), 115.14 (CH, C-3'), 113.26 (C, C-9'), 89.17 (C, C-17), 83.30 (C, C-14'), 69.51 (CH, C-11),

67.98 (CH_2, C-21), 54.86 (CH, C-9), 50.86 (CH, C-14), 47.26 (C, C-13), 43.68 (C, C-10), 38.85 (CH_2, C-12), 36.44 (CH_2, C-23), 33.92 (CH_2, C-6), 33.58 (CH_2, C-7), 31.56 (CH_2, C-16), 30.71 (CH, C-8), 27.78 (3xCH_3, C-15'), 23.36 (CH_2, C-15), 20.56 (CH_3, C-19), 16.37 (CH_3, C-18).

5-{2-[2-(11,17-Dihydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[α]phenanthren-17yl)-2-oxo-ethoxycarbonylmethyl]-phenylaz}-2-hydroxy-benzoic acid ***(1)***

To a solution of *t*-butyl ester protected mutual prodrug **44** (0.1 g, 0.15 mmol) in DCM (1 ml), trifluoroacetic acid (1 ml) was added, the reaction was left at room temperature for four hours. TLC analysis ethyl acetate: DCM (50: 50) showed completion and solvents and trifluoroacetic acid were blown off using nitrogen, to afford the product as an orange oil. The crude product was flash columned using DCM: ethyl acetate (50: 50), (100 ml), ethyl acetate (100 ml) and acetone: ethyl acetate (70: 30) to yield of the product as orange crystals (0.078 g, 78%), m.p. 154-156°C.

IR_{vmax} (KBr): 3436.40 and 1654.62 cm^{-1}.

^{1}H NMR δ (MeOD): 8.50 (1H, s), 8.05 (1H, d, *J* 9.04 Hz), 7.74 (1H, d, *J* 7.52 Hz), 7.46 (4H, m), 7.04 (1H, d, *J* 9 Hz), 6.25 (1H, d, *J* 10 Hz), 6.00 (1H, s), 5.01 (2H, d, *J* 17.56 Hz), 4.36 (1H, s), 4.31 (2H, d, *J* 8.52 Hz), 2.68-0.8 (prednisolone envelope, 19H).

^{13}C NMR δ (MeOD): 205.18 (C, C-20, C=O), 187.15 (C, C-3, C=O), 172.99 (C, C-22, C=O), 171.82 (C, C-5), 164.11 (C, C-13', C=O), 158.38 (C, C-10'), 156.35 (CH, C-1), 149.62 (C, C-2'), 144.93 (C, C-7'),133.49 (C, C-1'), 133.36 (CH, C-5'), 130.90 (CH, C-6'), 129.97 (CH, C-8'), 127.39 (CH, C-2), 127.07 (CH, C-4'), 126.20 (CH, C-

12'), 125.88 (CH, C-4), 120.57 (CH, C-11'), 117.01 (CH, C-3'), 114.64 (C, C-9'), 88.75 (C, C-17), 68.91 (CH, C-11), 67.95 (CH_2, C-21), 55.40 (CH, C-9), 50.91 (CH, C-14), MeOD residual peak is masking the peak of C, C-13, 44.19 (C, C-10), 38.19 (CH_2, C-12), 36.09 (CH_2, C-23), 33.70 (CH_2, C-6), 32.75 (CH_2, C-7), 31.31 (CH_2, C-16), 27.66 (CH, C-8), 22.95 (CH_2, C-15), 19.72 (CH_3, C-19), 15.36 (CH_3, C-18).

HRMS: Found: $(M-Na)^+ = 665.2503$.

Requires: $(M-Na)^+ = 665.2475$.

5-Nitrosalicylic acid dioxin-4-one ***(37)***

5-Nitrosalicylic acid **36** (20 g, 1.09 mol) was placed in a 500 ml round bottom flask equipped with a magnetic stirrer and a reflux condenser, trifluoroacetic acid (200 ml) was added followed by trifluoroacetic anhydride (3 equivalents, 45.54 ml, 3.27 mol) and dry acetone (2 equivalents, 16.04 ml, 2.16 mol). The reaction mixture was left at reflux for two hours. Further dry acetone (8 ml) was dropped into the boiling solution every hour (1 equivalent per hour) until reaction was completed within eight hours. The reaction was then concentrated under reduced pressure at about 55°C bath temperature. Toluene was added and removed three times to eliminate any trifuoroacetic acid traces and finally the solid residue was dried under vacuum for one hour at 45°C. The crude brownish solid was recrystallised twice from a mixture of acetone-petroleum ether (1: 4), to yield off-white crystals (20.5 g, 84.1%). (Alternatively flash chromatography can be used to purify the product when smaller scales are used using acetone-petroleum ether (1: 4) or DCM as a mobile phase), m.p. 92-94°C. (This compound has been made previously (Swinnen et al., 2005) but no spectroscopy data was found).

$IR_{\nu max}$(KBr): 1738.92, 1616.23 and 745.94 cm^{-1}

^1H NMR δ ($CDCl_3$): 8.88 (1H, d, *J* 2.51 Hz), 8.46 (1H, dd, *J* 6.52 and 3.01 Hz), 7.16 (1H, d, *J* 9.53 Hz), 1.80 (6H, s, 2xCH_3).

^{13}C NMR δ ($CDCl_3$): 159.82 (C, C-7, C=O), 158.50 (C, C-4), 142.33 (C, C-1), 130.79 (CH, C-6), 125.57 (CH, C-2), 118.09 (CH, C-5), 112.93 (C, C-3), 107.36 (C, C-8), 25.45 (CH_3, C-9 and C-10).

5-Aminosalicylic acid dioxin-4-one ***(34)***

To a solution of 5-nitrosalicylic acid dioxin-4-one **37** (2 g, 8.96 mmol) in ethyl acetate (30 ml) at room temperature, a catalyst amount of Pd/C (10%) (0.5 g) was added. The mixture was stirred under a hydrogen atmosphere until reaction completed by TLC (DCM as mobile phase). The Pd/C (10%) was filtered through celite as a filter agent and the solvent was removed under reduced pressure to yield the product as yellow crystals (1.44 g, 83.2%), m.p. 122-124°C. (This compound has been made previously (Swinnen et al., 2005) but no spectroscopy data was found).

IR_{Vmax}(KBr): 1710.37 (C=O), 3469.02 and 1324.22 (NH_2) cm^{-1}.

^{1}H NMR δ ($CDCl_3$): 7.24 (1H, d, *J* 3.01 Hz), 6.91 (1H, dd, *J* 5.52 and 3.01 Hz), 6.79 (1H, d, *J* 9.04 Hz), 3.61 (2H, s, NH_2), 1.69 (6H, s, 2xCH_3).

^{13}C NMR δ ($CDCl_3$): 161.16 (C, C-7, C=O), 148.15 (C, C-4), 141.36 (C, C-1), 123.38 (CH, C-6), 117.41 (CH, C-2), 113.54 (CH, C-5), 113.51 (C, C-3), 105.75 (C, C-8), 25.20 (CH_3, C-9 and C-10).

HRMS: Found: $(M)^+ = 194.0814$

Requires: $(M)^+ = 194.0814$

[2-(2,2-Dimethyl-4-oxo-4H-benzo[1,3]dioxin-6-ylazo)-phenyl]-aceticacid ***(38)***

To a solution of 2-nitrosophenyl acetic acid **30** (0.5 g, 2.58 mmol) in glacial acetic acid (25 ml) another solution of 5-aminosalicylic acid dioxin-4-one **34** (1 equivalent, 0.42 g, 2.58 mmol) in glacial acetic acid (25 ml) was added. The reaction mixture was stirred vigorously for 48 hours under a nitrogen atmosphere; reaction completion was checked by TLC using ethyl acetate: DCM (50: 50) as mobile phase. The solvent was removed under reduced

pressure and toluene added two times to eliminate any acetic acid traces to afford an orange crude product. This was purified by flash chromatography using DCM to yield product as orange crystals (0.6 g, 89%), m.p. 124-126°C.

IR_{Vmax}(KBr): 1734.84 and 1698.18 cm^{-1}.

^1H NMR δ ($CDCl_3$): 8.52 (1H, d, *J* 2.51 Hz), 8.05 (1H, dd, *J* 8.54 and 2.51 Hz), 7.77 (1H, d, *J* 7.53 Hz), 7.47 (1H, t, *J* 6.57 Hz), 7.43 (2H, m), 7.05 (1H, d, *J* 9.03 Hz), 4.16 (s, 2H), 1.80 (6H, s, 2xCH_3).

^{13}C NMR δ ($CDCl_3$): 177.67 (C, C-8, C=O), 160.42 (C, C-15, C=O), 157.85(C, C-12), 149.66 (C, C-1), 147.86(C, C-9), 133.78 (C, C-2), 131.55 (CH, C-4), 131.45(CH, C-3), 128.93 (CH, C-5), 128.39 (CH, C-14), 126.64 (CH, C-10), 118.14 (CH, C-6), 116.07 (CH, C-13), 113.61 (C, C-11), 106.90 (C, C-16), 37.28 (CH_2, C-7), 25.86 (CH_3, C-17 and C-18).

HRMS: Found: $(M-Na)^+ = 363.0950$.

Requires: $(M-Na)^+ = 363.0957$.

[2-(2,2-Dimethyl-4-oxo-4H-benzo[1,3]dioxin-6-ylazo)-phenyl]-acetic acid 2-(11,17-dihydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[α]phenanthren-17-yl)-2-oxo-ethyl ester ***(41)***

To a solution of azo carrier **38** (1 g, 5.16 mmol) in dry tetrahydrofuran (30 ml) prednisolone **3** (1.2 equivalents, 2.23 g, 6.19 mmol) was added followed by PPh_3 (3 equivalents, 4.11 g, 15.48 mmol) at room temperature. The reaction mixture was stirred under a nitrogen atmosphere and the temperature was raised to 40°C. At this point DIAD (3 equivalents, 3.2 ml, 15.48 mmol) was added dropwise over 12 minutes. The reaction mixture was stirred for two hours. The solvent was removed under reduced pressure to afford the product as crude black oil. Two flash chromatography columns DCM-ethyl acetate (50: 50) and hexane-ethyl acetate (80: 20) were needed to yield the product as orange crystals (1.48 g, 43%), m.p. 136-138°C.

IR_{Vmax}(KBr): 1655.56, 1724.45, 3462.94 cm^{-1}.

^{1}H NMR δ ($CDCl_3$): 8.43 (1H, d, *J* 2.01 Hz), 8.18 (1H, dd, *J* 9.03 and 2.51 Hz), 7.73 (1H, d, *J* 8.03 Hz), 7.43 (2H, m), 7.38 (1H, m), 7.25 (1H, d, *J* 10.04 Hz), 7.11 (1H, d, *J* 8.53 Hz), 6.21 (1H, d, *J* 10.04 Hz), 5.95 (1H, s), 5.01 (1H, d, *J* 17.56 Hz), 4.92 (1H, d, *J* 17.57 Hz), 4.31(1H, s), 4.22 (2H, s), 2.64-0.84 prednisolone envelope, 19H), 1.75 (6H, s, 2xCH_3).

^{13}C NMR δ ($CDCl_3$): 204.67 (C, C-20, C=O), 186.36 (C, C-3, C=O), 171.35 (C, C-22, C=O), 170.53 (C, C-13, C=O), 160.46 (C, C-5), 157.30 (C, C-10'), 156.67 (CH, C-1), 149.24 (C, C-2'), 147.64 (C, C-7'), 134.04 (C, C-1'), 131.66 (CH, C-6'), 131.28 (CH, C-12'), 131.22 (CH, C-4'), 127.88 (CH, C-2), 127.00 (CH, C-5'), 123.35 (CH, C-8'), 121.65(CH, C-4), 117.67 (CH, C-11'), 115.24 (CH, C-3'), 113.17 (C, C-9'), 106.59 (C, C-14'), 89.18 (C, C-17), 69.44 (CH, C-11), 68.19 (CH_2, C-21), 54.88 (CH, C-9), 50.84 (CH, C-14), 47.15 (C, C-13), 43.80 (C, C-10), 38.65 (CH_2, C-12), 37.02 (CH_2, C-23), 33.70 (CH_2, C-6), 33.61 (CH_2, C-7), 31.57 (CH_2, C-16), 30.76 (CH, C-8), 25.41 (CH_3, C-15' and C-16'), 23.39 (CH_2, C-15), 16.24 (CH_3, C-19), 14.81 (CH_3, C-18).

HRMS: Found: $(M-Na)^+ = 705.2784$.

Requires: $(M-Na)^+ = 705.2788$.

*3-[2-(2,2-Dimethyl-4-oxo-4H-benzo[1,3]dioxin-6-ylazo)-phenyl]-propionic acid **(45)***

To a solution of 2-nitrosophenylpropionic acid **57** (1 g, 4.97 mmol) in glacial acetic acid (40 ml) another solution of 5-aminosalicylic acid dioxin-4-one **34** (1 equivalent, 0.9 g, 4.97 mmol) in glacial acetic acid (40 ml) was added. The reaction mixture was vigorously stirred for 48 hours under nitrogen

atmosphere; reaction completion was checked by TLC ethyl acetate: DCM (50: 50) as a mobile phase. The solvent was removed under reduced pressure and toluene (20 ml) added twice to eliminate any traces of acetic acid to afford an orange crude product. This was purified by flash chromatography DCM: ethyl acetate (70: 30) to yield the product as orange crystals (1.17 g, 68%), m.p. 108-110°C.

IR_{Vmax}(KBr): 1703.48 and 1740.04 cm^{-1}.

^1H NMR δ ($CDCl_3$): 8.57 (1H, d, *J* 2 Hz), 8.17 (1H, dd, *J* 6.52 and 2.51 Hz), 7.72 (1H, d, *J* 7.53 Hz), 7.43 (2H, m), 7.15 (1H, d, *J* 9.03 Hz), 3.50 (2H, t, *J* 8.03 and 7.53 Hz), 2.79 (2H, t, *J* 8.03 and 7.53 Hz), 1.81 (6H, s, 2xCH_3).

^{13}C NMR δ ($CDCl_3$): 176.97 (C, C-9,C=O), 160.11 (C, C-16, C=O), 157.37 (C, C-13), 149.39 (C, C-1), 147.64 (C, C-10), 139.80 (C, C-2), 131.16 (CH, C-4), 130.15 (CH, C-3), 129.01 (CH, C-5), 126.99 (CH, C-15), 125.60 (CH, C-11), 117.75 (CH, C-6), 115.20 (CH, C-14), 113.32 (C, C-12), 106.51 (C, C-17), 35.46 (CH_2, C-8), 26.43 (CH_2, C-7), 25.48 (CH_3, C-17 and C-18).

HRMS: Found: $(M-Na)^+$= 377.1102.

Requires: $(M-Na)^+$= 377.1113.

3-[2-(2,2-Dimethyl-4-oxo-4H-benzo[1,3]dioxin-6-ylazo)-phenyl]-propionic acid 2-(11,17-dihydroxy-10,13-dimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3H-cyclopenta[α]phenanthren-17-yl)-2-oxo-ethyl ester ***(46)***

To a solution of azo carrier **45** (0.2 g, 0.56 mmol) in dry tetrahydrofuran (10 ml) prednisolone **3** (0.2 g, 0.85 mmol) was added followed by PPh_3 (0.44 g, 1.68 mmol) at room temperature. The reaction mixture was stirred under a nitrogen atmosphere and the temperature was raised to 40°C. At this point DIAD (0.35ml, 1.68 mmol) was added dropwise over 12 minutes. The reaction mixture was stirred over two hours. The solvent was removed under

reduced pressure to afford the product as crude black oil. Two flash chromatography columns DCM: ethyl acetate (50: 50) and hexane-ethyl acetate (80: 20) were needed to yield the product as orange crystals (0.18 g, 45%), m.p. 130-132°C.

IR_{Vmax}(KBr): 3437.13, 1743.91, 1656.48, 1615.31 cm^{-1}.

^{1}H NMR δ ($CDCl_3$): 8.47 (1H, d, *J* 2.01 Hz), 8.16 (1H, dd, *J* 8.53 and 2.01 Hz), 7.67 (1H, d, *J* 8.03 Hz), 7.38 (2H, m), 7.28 (2H, m), 7.10 (1H, d, *J* 8.54 Hz), 6.23 (1H, d, *J* 10.03 Hz), 5.97 (1H, s), 5.08 (1H, d, *J* 17.57 Hz), 4.94 (1H, d, *J* 17.56 Hz), 4.42(1H, s), 3.45 (2H, t, *J* 6.52), 2.79 (2H, t, *J* 8.03 Hz), 2.68-0.91 prednisolone envelope, 19H), 1.75 (6H, s, 2xCH_3).

^{13}C NMR δ ($CDCl_3$): 204.92 (C, C-20, C=O), 186.44 (C, C-3, C=O), 172.37 (C, C-22, C=O), 170.68 (C, C-13, C=O), 160.45 (C, C-5), 157.25 (C, C-10'), 156.79 (CH, C-1), 149.14 (C, C-2'), 147.61 (C, C-7'), 139.99 (C, C-1'), 131.23 (CH, C-6'), 131.00 (CH, C-12'), 130.05 (CH, C-4'), 127.02 (CH, C-2), 126.93 (CH, C-5'), 123.74 (CH, C-8'), 121.64(CH, C-4), 117.77 (CH, C-11'), 115.16 (CH, C-3'), 113.18 (C, C-9'), 106.57 (C, C-14'), 89.18 (C, C-17), 69.54 (CH, C-11), 67.77 (CH_2, C-21), 54.92 (CH, C-9), 50.88 (CH, C-14), 47.14 (C, C-13), 43.86 (C, C-10), 38.85 (CH_2, C-12), 35.89 (CH_2, C-23), 33.75 (CH_2, C-6), 33.61 (CH_2, C-7), 31.62 (CH_2, C-16), 30.78 (CH, C-8), 26.93 (CH_3, C-15' and C-16'), 25.42 (CH_2, C-24), 23.45 (CH_2, C-15), 16.35 (CH_3, C-19), 14.81 (CH_3, C-18).

HRMS: Found: $(M\text{-}Na)^{+}$= 719.2911.

Requires: $(M\text{-}Na)^{+}$= 719.2945.

*2-Nitrophenyl acetic acid methyl ester **(17)***

To a solution of 2-nitrophenyl acetic acid **8** (5 g, 0.0276 mol) in DCM (50 ml) was added DMAP (1 equivalent, 3.37 g, 0.0276 mol) followed by DCC (1 equivalent, 5.63 g, 0.0276 mol) and methanol (5 equivalents, 5.58 ml, 0.138 mol). The reaction mixture was stirred for three hours until TLC showed reaction was completed. The reaction was filtered to remove the DCU formed and the filtrate was washed with 0.1N HCl (2x 25 ml), saturated aqueous sodium bicarbonate solution (2x 25 ml) and water (2x 25 ml), and dried over sodium sulfate and the solvent removed under reduced pressure. The crude ester was purified by flash chromatography DCM: ethyl acetate (60: 40) to yield the product as a yellow oil (4.8 g, 90%). This compound has been made previously (Fife and Duddy, 1983) but no spectroscopy data was found).

IR_{Vmax}(KBr): 1732.43 cm^{-1}.

^{1}H-NMR δ ($CDCl_3$): 7.94 (1H, d, *J* 8 Hz), 7.4 (1H, t, *J* 7.2 Hz), 7.3 (1H, t, *J* 7.6 Hz), 7.25 (1H, d, *J* 7.6 Hz), 3.89 (2H, s), 3.59 (3H, s).

^{13}C-NMR δ ($CDCl_3$): 170.3 (C=O, C-8), 148.2 (C, C-1), 133.0 (CH, C-4), 132.8 (CH, C-3), 129.2 (C, C-2), 128.0 (CH, C-5), 124.5 (CH, C-6), 51.4 (CH_3, C-9), 38.8 (CH_2, C-7).

*2-Aminophenyl acetic acid methyl ester **(18)***

The 2-nitrophenyl acid methyl ester **17** (1 g, 5.13 mmol) was dissolved in ethyl acetate (20 ml) and a catalyst amount of Pd/C (10%) (0.3 g) was suspended into the solution. The reaction mixture was under a hydrogen atmosphere at room temperature. After two hours TLC ethyl acetate: DCM (60: 40) showed the reaction was complete. Pd/C (10%) was removed by filtration. The solvent was removed under reduced pressure to yield a colorless oil (0.67 g, 67%). (This compound has been made previously (Fife and Duddy, 1983) but no spectroscopy data was found)

IR_{Vmax}(KBr): 1738.10 cm^{-1}

^{1}H-NMR δ ($CDCl_3$): 7.12 (2H, d, *J* 7.53 Hz), 6.77 (1H, td, *J* 7.53 and 1 Hz), 6.72 (1H, d, *J* 8.03 Hz), 3.69 (3H, s), 3.59 (2H, s)

^{13}C-NMR δ ($CDCl_3$): 171.85 (C=O, C-8), 145.19 (C, C-1), 130.67 (CH, C-3), 128.11 (CH, C-5), 118.89 (C, C-2), 118.38 (CH, C-4), 116.06 (CH, C-6), 51.70 (CH_3, C-9), 37.73 (CH_2, C-7).

[2-(4-Hydroxy-phenylazo)-phenyl]-acetic acid methyl ester **(20)**

2-Aminophenyl acid methyl ester **18** (3 g, 0.018 mol) was dissolved in water (40 ml) with concentrated HCl (9 ml) and the solution was cooled in an ice/acetone bath to – 5°C, afterwards sodium nitrite (1 equivalent, 1.32 g, 0.019 mol) in water (1 ml) was added dropwise. At that point the diazonium salt was formed and the solution took a pale yellow color. A spatula of urea was added to neutralize the excess of sodium nitrite (oxidant) and the diazonium salt solution was added dropwise into a solution of phenol (1 equivalent, 1.68 g, 0.018 mol), sodium acetate (2 equivalents, 2.88 g, 0.036 mol) and ammonia solution (5 ml) in water (40 ml), which was at -5°C. After 5 minutes glacial acetic acid (5 ml) was added and the reaction mixture was filtered. The filter cake was dissolved in ethyl acetate and dried over sodium sulfate, the solvent was removed under reduced pressure and the product purified by flash chromatography DCM (100 ml) and DCM: ethyl acetate (80: 20) to yield (2.7 g, 56%), m.p. 168-170°C.

IR_{vmax}(KBr): 3221.84, 1695.48 cm^{-1}.

^{1}H-NMR δ (Acetone): 9.14 (1H, s), 7.86 (2H, d, *J* 8.50 Hz), 7.72 (1H, d, *J* 8 Hz), 7.45 (3H, m, *J* 7.52 Hz), 7.03 (2H, d, *J* 8.50 Hz), 4.16 (2H, s), 3.62 (3H, s).

^{13}C-NMR δ (Acetone): 171.22 (C=O, C-8), 160.29 (C, C-4'), 149.64 (c,C-2), 146.20 (C, C-1'), 134.13 (CH, C-6), 131.13 (CH, C-5), 129.97 (C,C-1), 127.42 (CH, C-4), 124.65 (CH,C-3, C-6', C-2'), 115.38 (CH, C-5'), 114.73 (CH, C-3'), 50.65 (CH3, C-9), 36.41 (CH2, C-7) .

[2-(4-Hydroxy-phenylazo)-phenyl]-acetic acid ***(16)***

The ester **20** (0.266 g, 1 mmol) was dissolved in ethanol (10 ml) under a nitrogen atmosphere and 2N NaOH (2 ml) was added into the mixture and stirred overnight with shielding from light. The reaction mixture was poured into 1N HCl solution and extracted with ethyl acetate. The organic phase was washed with water and brine and dried over magnesium sulfate. The solvent was removed under reduced pressure and the product recrystallised with ethyl acetate: hexane to yield the product as orange crystals (0.2 g, 70%), m.p. 120-122°C (This compound has been made previously (Hutching and Devonald, 1989) but no spectroscopy data was found).

$IR_{\nu max}$(KBr): 3280.04, 1721.07 and 1655.90 cm^{-1}.

^{1}H-NMR δ (Acetone): 10.71 (1H, s), 9.13 (1H, s), 7.88 (2H, d, *J* 8.52 Hz), 7.71 (1H, d, *J* 7.52 Hz), 7.45 (3H, m), 7.01 (2H, d, *J* 8.52 Hz), 4.16 (2H, s).

^{13}C-NMR δ (Acetone): 171.65 (C=O, C-8), 160.20 (C, C-4'), 149.73 (C,C-2), 134.49 (C,C-1'), 131.18 (CH, C-5), 129.91 (CH, C-6, C-1), 127.28 (CH,C-4), 124.69 (CH,C-3, C-6', C-2'), 115.32, (CH, C-5') 114.63 (CH, C-3'), 36.23 (CH2, C-7), 28.51.

HRMS: *Found: (M)* $^{+}$ *= 257.0948*

Required: (M) $^{+}$ *= 257.0926*

[2-(4-Hydroxy-phenylazo)-phenyl]-acetic acid 2-(11,17-dihydroxy-10,13-dimethyl-3-oxo-2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[α]phenanthren-17-yl)-2-oxo-ethyl ester **(40)**

The azo carrier **16** (0.5 g, 1.9 mmol) was added to a solution of hydrocortisone **39** (1 equivalent, 0.68 g, 1.9 mmol) and PPh_3 (3 equivalents, 1.51 g, 5.7 mmol) in dry tetrahydrofuran, under a nitrogen atmosphere and the temperature was raised to 40°C and DIAD (3 equivalents, 1.2 ml, 5.7 mmol) was added dropwise over 12 minutes with vigorous stirring. The mixture was stirred at 40°C for an hour. The reaction was left overnight at room temperature under a nitrogen atmosphere. The volatiles were removed

under reduced pressure to afford the product as an orange oil. This was flash columned DCM: ethyl acetate (50: 50), after that column the triphenylphosphine oxide was removed and a second flash column was needed hexane (200 ml) and hexane: ethyl acetate (70: 30) to yield the product as orange crystals (0.23 g, 42%), m.p. 122-124°C.

$IR_{\nu max}$(KBr): 3437.13, 1721.07, 1655.90 cm^{-1}.

^{1}H-NMR δ (Acetone): 7.76 (2H, d, *J* 8.53 Hz), 7.65 (1H, d, *J* 7.03), 7.33 (3H, m), 6.85 (2H, d, *J* 8.53 Hz), 5.59 (1H, s), 4.90 (1H, d, *J* 17.57 Hz), 4.74 (1H, d, *J* 17.06 Hz), 4.18 (2H, s), 2.60-0.77 (hydrocortisone envelope, 23H).

^{13}C-NMR δ (Acetone): 203.64 (C=O, C-20), 197.00 (C=O, C-3), 171.09 (C=O, C-22), 168.97 (C, C-5), 155.12 (C, C-10'), 149.55 (C, C-2'), 142.60 (C, C-7'), 136.75 (C, C-1'), 133.33 (CH, C-5'), 133.27 (CH, C-6'), 131.45 (C, C-9'), 129.41 (CH, C-8), 128.33 (CH, C-4'), 124.42 (CH, C-12'), 123.74 (CH, C-4), 122.21 (CH, C-11'), 121.26 (CH, C-3'), 88.90 (C, C-17), 67.69 (CH_2, C-21), 66.99 (CH, C-11), 55.69 (CH, C-9), 51.62 (CH, C-14), 46.81 (C, C-13), 38.79 (C, C-10), 38.73 (CH_2, C-1), 38.10 (CH_2, C-12), 34.28 (CH_2, C-23), 33.26 (CH_2, C-6), 33.08 (CH_2, C-2), 32.62 (CH_2, C-7), 31.27 (CH_2, C-16), 31.03 (CH, C-8), 22.94 (CH_2, C-15), 20.04 (CH_3, C-19), 15.97 (CH_3, C-18).

2-Nitrocinnamic acid methyl ester ***(51)***

2-Nitrocinnamic acid **47** (5 g, 0.025 mol) was suspended in DCM (50 ml). DMAP (1 equivalent, 3.5 g, 0.025 mol) was added to the suspension followed by DCC (1 equivalent, 5.9 g, 0.025 mol) and methanol (5 equivalents, 4.12 g, 5.2 ml, 0.129 mol). The reaction was left at room temperature overnight and filtered to remove the DCU formed during esterification. The solvent was removed to afford the product as a yellow oil. This was flash columned

(DCM) to yield the product as a yellow crystals (4.6 g, 88%), m.p. 61-62°C (This compound has been made previously (El-Batta et al., 2007) spectroscopy data was found the ^{1}H and ^{13}C-NMR fit with our spectroscopy data).

IR_{vmax}(KBr): 3422.16 cm^{-1}, 1717.42 cm^{-1}.

^{1}H-NMR δ ($CDCl_3$): 8.13 (1H, d, *J* 16.07 Hz), 8.05 (1H, d, *J* 8.03 Hz), 7.65 (2H, m), 7.55 (1H, t, *J* 8.53 Hz), 6.39 (1H, d, *J* 15.56 Hz), 3.82 (3H, s).

^{13}C-NMR δ ($CDCl_3$): 165.77 (C=O, C-9), 147.82 (C, C-1), 139.71 (CH, C-7), 133.13 (CH, C-4), 130.06 (C, C-2), 129.90 (CH, C-5), 128.67 (CH, C-3), 124.45 (CH, C-6), 122.36 (CH, C-8), 51.56 (CH_3, C-10).

2-Aminocinnamic acid methyl ester **(52)**

A solution of ammonium chloride (2 equivalents, 2.25 g, 0.044 mol) in water (15 ml) was added into a solution of 2-nitrocinnamic acid methyl ester **51** (1 equivalent, 4.6 g, 0.022 mol) in methanol (100 ml). The reaction was stirred and zinc dust (16.5 g, 0.25 mol) was added over 15 minutes and refluxed over two hours. TLC (DCM) showed the reaction was completed and the mixture was hot filtered through celite as a filter agent. The solvent was removed under reduced pressure to afford the product as green oil. This was flash columned DCM: ethyl acetate (60: 40) to yield the product as yellow crystals (3.8 g, 86%), m.p. 55-56°C (This compound has been made previously (El-Batta et al., 2007) spectroscopy data was found the ^{1}H and ^{13}C-NMR fit with our spectroscopy data).

IR_{vmax}(KBr): 1623.13 cm^{-1}

^{1}H-NMR δ ($CDCl_3$): 7.88 (1H, d, *J* 16.07 Hz), 7.40 (1H, d, *J* 7.53 Hz), 7.17 (1H, t, *J* 7.03 Hz), 6.80 (1H, t, *J* 7.53 Hz), 6.73 (1H, d, *J* 8.03 Hz), 6.40 (1H, d, *J* 16.07 Hz), 3.92 (2H, s, NH_2), 3.82 (3H, s).

^{13}C-NMR δ ($CDCl_3$): 167.29 (C=O, C-9), 145.15 (C, C-1), 139.86 (CH, C-7), 130.92 (CH, C-5), 127.62 (CH, C-

3), 119.35 (C, C-2), 118.49 (CH, C-4), 117.17 (CH, C-8), 116.31 (CH, C-6), 51.24 (CH_3, C-10).

5-Nitrososalicylic acid t-butyl ester **(53)**

5-Aminosalicylic acid *t*-butyl ester **35** (0.7 g, 3.3 mmol) was dissolved in and DCM (30 ml). Oxone® (2 equivalents, 2.0 g, 6.6 mol) was dissolved in distilled water (10 ml) and the solution added to the reaction mixture. At this point the reaction pH was 4 so the 2-nitrosophenylpropionic acid was transferred to the organic layer as it was formed. After two hours, the reaction mixture was transferred to a separating funnel, the organic layer collected, and aqueous phase washed (2 x 25 ml) DCM. The organic phase was collected and dried with sodium sulfate. The solvent was removed under reduced pressure to afford of product as dark oil. The crude mixture was flash columned using DCM to yield the product as green crystals (0.43 g, 59 %), m.p. 61-63°C.

$IR_{\nu max}$(KBr): 1673.01 cm^{-1}

^{1}H-NMR δ ($CDCl_3$): 11.98 (1H, s, OH), 8.88 (1H, s), 7.55 (1H, d, *J* 8.03 Hz), 7.02 (1H, d, *J* 9.03 Hz), 1.70 (9H, s).

^{13}C-NMR δ ($CDCl_3$): 168.84 (C, C-2), 167.32 (C=O, C-7), 161.64 (C, C-5), 117.69 (CH, C-4, C-3, C-6), 112.90 (C, C-1), 84.30 (C, C-8), 27.70 (3xCH_3, C-9).

3-(2-t-Butoxycarbonylmethyl-phenyl)-propionic acid methyl ester **(62)**

2-Aminocinnamic acid methyl ester **52** (2.8 g, 0.016 mol) was dissolved in DCM (25 ml) at room temperature. DMAP (1 equivalent, 2 g, 0.016 mol) was added to the reaction mixture followed by BOC-anhydride (1.2 equivalents, 4.2 g, 0.0192 mol). The reaction was left stirring until reaction completion. The solvent was removed under reduced pressure to afford a dark yellow oil. This was dissolved in ethyl acetate (25 ml) and a catalyst amount of Pd/C (10%) (0.6 g) was added. The reaction mixture was left under a hydrogen atmosphere at room temperature. After two hours the reaction was complete by TLC ethyl acetate: DCM (60: 40). The reaction was filtered through celite as a filter agent to remove the Pd/C (10%) and the

solvents removed under reduced pressure to afford the product as a yellow oil. This was columned (DCM) to yield the product as a colorless oil (2.9 g, 67%).

IR_{vmax}(KBr): 1686.84 cm^{-1}

^{1}H-NMR δ ($CDCl_3$): 7.28 (2H, m), 7.07 (1H, t, *J* 7.53 Hz), 6.90 (1H, d, *J* 8.03 Hz), 2.96 (2H, t, *J*, 6.53 Hz), 2.66 (2H, t, *J* 6.53 Hz), 1.51 (9H, s), 1.41 (3H, s).

^{13}C-NMR δ ($CDCl_3$): 168.79 (C=O, C-9), 151.28 (C=O, C-10), 136.56 (C, C-2), 127.53 (CH, C-6), 126.84 (CH, C-5), 125.35 (C, C-1), 123.64 (CH, C-4), 116.41 (CH, C-3), 84.46 (C, C-11), 59.88 (CH_3, C-13), 31.77 (CH_2, C-8), 27.18 (3xCH_3, C-12), 24.95 (CH_2, C-7).

3-(2-t-Butoxycarbonylmethyl-phenyl)-propionic acid **(63)**

To a solution of 3-(2-*t*-butoxycarbonylmethyl-phenyl)-propionic acid methyl ester **62** (0.5 g, 1.7 mmol) in methanol (10 ml), six equivalents of 2N NaOH (5.5 ml, 10.8 mmol) were added to the solution and stirred overnight. The reaction mixture was poured into 1N HCl solution and extracted with ethyl acetate. The organic phase was washed with water and brine and dried over sodium sulfate. The solvent was removed under reduced pressure to yield the product (0.32 g, 73%).

IR_{vmax}(KBr): 1678.31 cm^{-1}

^{1}H-NMR δ ($CDCl_3$): 10.68 (1H, s), 7.61 (1H, s), 7.20 (2H, m), 7.07 (1H, t, *J* 7.52 Hz), 2.90 (2H, t , *J* 7.02 Hz), 2.69 (2H, t, *J* 7.03 Hz), 1.51 (9H, s).

^{13}C-NMR δ ($CDCl_3$): 178.06 (C=O, C-9), 151.28 (C=O, C-10), 135.28 (C, C-2), 128.95 (CH, C-6), 126.61 (CH, C-5, C, C-1), 124.35 (CH, C-4, C-3), 82.50 (C, C-11), 34.09 (CH_2, C-8), 27.87 (3xCH_3, C-12), 27.37 (CH_2, C-7).

3-(2-t-Butoxycarbonylamino-phenyl)-propionic acid 2-(11,17-dihydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxo-ethyl ester **(64)**

To a solution of 3-(2-*t*-butoxycarbonylmethyl-phenyl)-propionic acid **63** (1.4 g, 4.9 mmol) in DCM (20 ml), DMAP (1 equivalent, 0.61 g, 4.9 mmol) and DCC (1 equivalent, 1.0 g, 4.9 mmol) were added to the solution followed by hydrocortisone (1 equivalent, 1.73 g, 4.9 mmol). After three hours the reaction was completed. DCU was removed by filtration. The solvent was removed under reduced pressure to afford a yellowish oil. This was flash columned using DCM: ethyl acetate (60: 40) to yield the product as a off-white crystals (2.8 g, 91%), m.p. 97- 99°C.

IR_{vmax}(KBr): 3449.10, 1724.45 and 1657.08 cm^{-1}.

^{1}H-NMR δ ($CDCl_3$): 7.66 (1H, s, NH_2), 7.33 (1H, s), 7.25 (1H, t, *J* 7.91 Hz), 7.21 (1H, d, *J* 7.53 Hz), 7.12 (1H, t, *J* 7.53 Hz), 5.73 (1H, s), 4.98 (2H, q, *J* 17.69 and 7.91 Hz), 4.5 (1H, s), 2.97 (2H, t, *J* 6.78 Hz), 2.88 (2H, t, *J* 6.58 Hz), 1.57 (9H, s), 2.76-0.99 (hydrocortisone envelope, 23H).

^{13}C-NMR δ ($CDCl_3$): 204.96 (C=O, C-20), 199.83 (C=O, C-3), 172.68 (C=O, C-22), 171.16 (C, C-5), 153.82 (C=O, C-7'), 135.81 (C-2'), 131.56 (C, C-1'), 129.37 (CH, C-6'), 127.06 (CH, C-4'), 124.57 (CH, C-4), 123.66 (CH, C-5'), 122.15 (CH, C-3'), 89.70 (C, C-17), 68.44 (CH_2, C-21), 68.03 (CH, C-11), 60.36 (C, C-8'), 55.99 (CH, C-9), 51.90 (CH, C-14), 47.59 (C, C-13), 39.24 (CH_2, C-12), 34.85 (CH_2, C-1), 34.44 (CH_2, C-2), 33.82 (CH_2, C-23), 32.77 (CH_2, C-7), 32, 06 (CH_2, C-6), 31.42 (CH_2, C-10), 28.30 (3xCH_3, C-9'), 25.53 (CH_2, C-16), 25.39 (CH, C-8), 24. 86 (CH_2, C-24), 23.57 (CH_2, C-15), 20.89 (CH_3, C-19), 16.83 (CH_3, C-18).

HRMS: Found: $(M)^{+}$= 609.3304

Requires: $(M)^+ = 609.3302$

(2-Nitro-phenyl)-acetic acid 2-(11,17-dihydroxy-10,13-dimethyl 3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[α]phenanthren-17-yl)-2-oxo-ethyl ester **(58)**

To a solution of 2-nitrophenylacetic acid **9** (1 g, 5.5 mmol) in DCM (20 ml) DMAP (1 equivalent, 0.7 g, 5.5 mmol) and DCC (1 equivalent, 1.2 g, 5.5 mmol) were added followed by the addition of hydrocortisone **39** (1 equivalent, 2 g, 5.5 mmol) and the reaction mixture was stirred at room temperature for four hours. The reaction mixture was filtrate to remove the DCU formed. The solvent was removed under reduced pressure to afford a yellow oil. This was flash columned using DCM to yield the final product as a white off crystals (2.3 g, 82%), m.p. 96-98°C.

IR_{vmax}(KBr): 3447.46, 1723.57 and 1657.62 cm^{-1}.

^{1}H-NMR δ ($CDCl_3$): 8.15 (1H, d, *J* 8.03 Hz), 7.64 (1H, t, *J* 7.53 Hz), 7.51 (1H, t, *J* 7.53 Hz), 7.46 (1H, d, *J* 7.53 Hz), 5.68 (1H, s), 5.05 (2H, q, *J* 32.62 and 17.57 Hz), 4.46 (1H, s), 4.17 (2H, s), 2.76-0.93 (hydrocortisone envelope 23H).

^{13}C-NMR δ ($CDCl_3$): 204.12 (C=O, C-20), 199.28 (C=O, C-3), 171.84 (C=O, C-22), 169.45 (C, C-5), 148.19 (C, C-2'), 133.30 (CH, C-5'), 133.08 (CH, C-6'), 128.91 (C, C-1'), 128.35 (CH, C-4'), 124.87 (CH, C-4), 121.86 (CH, C-3'), 89.24 (C, C-17), 68.03 (CH_2, C-21), 67.71 (CH, C-11), 55.53 (CH, C-9), 51.50 (CH, C-14), 47.13 (C, C-13), 39.23 (CH_2, C-12), 38.91 (CH_2, C-23), 38.77 (C, C-10), 34.48 (CH_2, C-1), 34.19 (CH_2, C-2), 33.36 (CH_2, C-7), 32.29 (CH_2, C-16), 31.58 (CH, C-6), 30.92(CH, C-8), 23.15 (CH_2, C-15), 20.50 (CH_3, C-19), 16.62 (CH_3, C-18).

HRMS: Found: $(M\text{-}Na)^+ = 548.2247$

Required: $(M\text{-}Na)^+ = 548.2260$

1,3-Dihydro-indol-2-one **(10)**

The 2-nitrophenyl acetic acid methyl ester **17** (0.5 g, 2.6 mmol) was dissolved in ethyl acetate (30 ml) and a catalyst amount of Pd/C (10%) (0.1 g) was suspended into the solution. The reaction mixture was placed under a hydrogen atmosphere at room temperature. After 12 hours, TLC ethyl acetate: DCM (60: 40) showed the reaction was complete and all the 2-aminophenyl acetic acid methyl ester formed underwent cyclization to produce **10**. Pd/C (10%) was removed by filtration. The solvent removed under reduced pressure to afford brownish crystals. This was flash columned (EtOAc: DCM 40: 60) to yield off-white crystals (0.25 g, 73%), m.p. 100-102°C. (This compound has been made previously (Fife and Duddy, 1983) but no spectroscopy data was found the melting point was 105-108°C).

$IR_{\nu max}$(KBr): 3436 and 1618 cm^{-1}.

^{1}H-NMR δ ($CDCl_3$): 9.23 (1H, s), 7.24 (2H, t, *J* 7.53 Hz), 7.05 (1H, t, *J* 7.53 Hz), 6.94 (1H, d, *J* 7.53 Hz), 3.56 (2H, s).

^{13}C-NMR δ ($CDCl_3$): 171.85 (C=O, C-7), 145.19 (C, C-1), 130.67 (CH, C-3), 128.12 (CH, C-5), 118.89 (C, C-2), 116.06 (CH, C-6), 37.73 (CH_2, C-8).

3,4-Dihydro-1H-quinolin-2-one **(49)**

The 2-nitrocinnamic acid **47** (0.5 g, 2.6 mmol) was dissolved in ethyl acetate (30 ml) and a catalyst amount of Pd/C (10%) (0.1 g) was suspended into the solution. The reaction mixture was under a hydrogen atmosphere at room temperature. After one hour, TLC ethyl acetate: DCM (60: 40) showed the reaction was complete and all the 2-aminocinnamic acid formed underwent the cyclization to produce **49**. Pd/C (10%) was removed by filtration. The solvent removed under reduced pressure to yield off-white crystals (0.35 g, 91%), m.p. 118-120°C (This compound has been made previously (Kirby and Mujahid, 1979) but no spectroscopy data was found the melting point was 105-108°C).

$IR_{\nu max}$(KBr): 3449.50 and 1680.03 cm^{-1}.

^{1}H-NMR δ ($CDCl_3$): 8.98 (1H, s), 7.2 (2H, t, *J* 7.53 Hz), 7 (1H, t, *J* 7.53 Hz), 6.85 (1H, d, *J* 8.03 Hz), 2.99 (2H, t, *J* 7.53 Hz), 2.67 (2H, t, *J* 7.03 Hz).

^{13}C-NMR δ ($CDCl_3$): 171.69 (C=O, C-7), 136.81 (C, C-1), 127.48 (CH, C-3), 127.08 (CH, C-5), 123.18 (C, C-2), 122.64 (CH, C-4), 115.05 (CH, C-6), 30.27 (CH_2, C-8), 24.88 (CH_2, C-9).

2-Aminophenyl acetic acid 2-(11,17-dihydroxy-10,13-dimethyl -3-oxo-hexadecahydro-cyclopenta[α]phenanthren-17-yl)-2-oxo-ethyl ester **(59)**

Into a solution of prodrug **58** (1 g, 1.9 mmol) in ethyl acetate (30 ml) a catalytic amount of Pd/C (10%) (0.3 g) was suspended. The reaction mixture was under a hydrogen atmosphere at room temperature. After two hours, TLC ethyl acetate: DCM (60: 40) showed the reaction was complete.The solvent removed under reduced pressure to yield white off crystals (0. 81 g, 86%), m.p. 103-105°C.

IR_{vmax}(KBr): 3436.61 and 1653.59 cm^{-1}.

^{1}H-NMR δ ($CDCl_3$): 7.13 (2H, t, *J* 7.53 Hz), 6.75 (2H, m), 5.1 (1H, d, *J* 17.06 Hz), 4.89 (1H, d, *J* 17-56 Hz), 4.37 (1H, s), 3.72 (2H, s), 2.73-0.92 (reduced hydrocortisone envelope 26H).

^{13}C-NMR δ ($CDCl_3$): 213.04 (C=O, C-20), 211.92.28 (C=O, C-3), 171.04 (C=O, C-22), 144.95 (C, C-2'), 130.76 (CH, C-6'), 128.17 (CH, C-4'), 124.11 (C, C-1'), 118.54 (CH, C-5'), , 116.23 (CH, C-3'), 89.30 (C, C-17), 68.18 (CH_2, C-21), 67.56 (CH, C-11), 59.99 (CH, C-9), 52.05 (CH, C-14), 47.34 (CH, C-4) 47.23 (C, C-5), 46.11 (C, C-13), 37.28 (CH_2, C-12), 34.60 (CH_2, C-23), 33.37 (C, C-10), 31.79 (CH_2, C-2), 30.88 (CH_2, C-1), 26.05 (CH_2, C-7), 25.44 (CH_2, C-16), 25.28 (CH, C-6), 24.46(CH, C-8), 23.21 (CH_2, C-15), 20.59 (CH_3, C-19), 16.60 (CH_3, C-18).

4.4 Lactamization studies

Stock solutions of the reduced derivates **59**, **60** and the lactamization products 1,3-dihydro-indol-2-one **10** and 3,4-dihydro-1H-quinolin-2-one **49** were prepared in concentrations of (10 mM) in EtOH. The lactamization reactions were performed in buffer solutions at different pH values at 37°C and monitored by HPLC analysis.

The buffers were prepared as indicated below:

Solution 1: 0.05 M of citric acid monohydrate (99% ACS, Aldrich) and 0.2 M of boric acid in distilled water.

Solution 2: 0.1 M of tri-potassium *ortho*-phosphate in distilled water. The solutions were mixed in the necessary proportion to achieve the required pH as outlined in *Table 4.1* and made up to (50 ml) with distilled water.

It is possible to make a lineal correlation to make the different solutions at different pH values using the formula below:

Volume of solution 1 (ml) = 6.68 – 0.56 pH

Volume of solution 2 (ml) = –2.00 + 0.8670 pH

Using that linear correlation and making up the solutions until the final volume of (50 ml) with distillated we were able to obtain the same ionic strength value of 0.1 at the different pH values.

pH	1.4	3	4	5	6	7.4	8	9	12
Solution 1 (ml)	5.9	5	4.44	3.9	3.3	2.5	2.2	1.6	0
Solution 2 (ml)	0	0.6	1.5	2.3	3.2	4.4	4.9	5.8	8.4

Table 4.1.preparation of the borate buffers at different pH values

The samples preparations were made up by adding (5 µl) of the particular drug from its stock solutions into (495 µl) of the buffer solutions and the final concentrations were (0.1 mM).

Reduced derivate **59** retention time = 3.8 min.

Reduced derivate **60** retention time = 4.6 min.

1,3-Dihydro-indol-2-one **10** retention time = 1.9 min.

3,4-Dihydro-1H-quinolin-2-one **49** retention time = 2.1 min.

4.5 Nitroreductase studies

A series of stock solutions of *E. coli* nitroreductase 0.73 μg/ml, (0.3 μM) prodrug **58** 5.1 mg/ml (10 mM) NADH 7.1 mg/ml (10 mM) and CB 1954 (10 mM) in PBS pH 7.4 were prepared.

Two samples of 1 ml each to test the nitroreductase activity were prepared:

1. Prodrug **58** from stock solution (15 μl), (200 μl) of NADH from stock solution, (200 μl) of *E. coli* nitroreductase from stock solution and (585 μl) of PBS pH 7.4.
2. CB 1954 from stock solution (15 μl), (200 μl) of NADH from stock solution, (200 μl) of *E. coli* nitroreductase from stock solution and (585 μl) of PBS pH 7.4.

The test was carried out under aerobic conditions at 37°C. Aliquots were analyzed by HPLC using the isocratic method. The reduction of the CB 1954 was completed within 20 minutes, indicating activity of the enzyme under the test conditions.

Prodrug 58 retention time = 12.1 min.

CB 1954 retention time = 5.6 min.

4.6. Azoreductase activity by Clostridium perfringes Experimental

Three different strains of *Clostridium perfringes* were supplied by Dr. Henry Windle from the Institute of Molecular Medicine Trinity Centre for Health Sciences St. James's Hospital were used to test the azoreductase activity towards the prodrug **40**.

The pool of *Clostridium perfringes* was grown on brain heart infusion (BHI) from oxoid. Prior to use the BHI was autoclaved (120°C for 20 minutes) to sterilize. The plates were incubated under anaerobic conditions at 37°C.

Prodrug **40** stock solution was prepared in DMSO 5.6 mg/200 µl (50 mM) then an intermediate solution (6 mM) was made up in DMSO.

Direct Blue stock solution 9.9 mg/200 µl (50 mM).

The dilute BHI was diluted 1/10 using PBS pH 7.4

The different samples tested with the BHI and the *Clostridium perfringes* were prepared as described in the *Table 4.2* below.

Samples	*Dilute 1/10 BHI(ml)*	*BHI (ml)*	*Clostridiums perfringes (ml)*	*Prodrug **40** (µl)*	*Direct Blue (µl)*
1		3.75		250	
2		3.75			10
3		3.75	1		10
4	3.75		1		10
5		3.75	1	250	
6	3.75		1	250	

Table 4.2. Samples preparation for the azoreductase activity test with Clostridium perfringes.

Initial concentration of prodrug **40** on the bacteria culture was 0.3 mM and 5% DMSO (higher concentrations of DMSO are lethal to the bacteria). The aliquots were analyzed by HPLC system after being centrifuged for five minutes at 10,000 rpm, using the isocratic HPLC method described previously at the beginning of this chapter.

Prodrug **40** retention time = 12.4 min.

Direct blue retention time = 4.3 min.

4.6.1 Azoreductase activity by CD-1 colonic microflora

A CD-1 type mouse was sacrificed and the extract from the colon was placed on a plate containing BHI and microbiology grade agar 1.5% (w/v). Prior to use the BHI agar was autoclaved (120°C for 20 minutes) to sterilize.

The plate was incubated under anaerobic conditions (generating using an Anoxomat) at 37°C for 24 hours. After that the bacteria from the colon contents were transferred to a new BHI agar plate to separate them from rest of faeces, the plate was incubated again under anaerobic conditions at 37°C for 24 hours. The bacteria cultures from the clean plate were transferred next to BHI without agar and incubated under anaerobic conditions at 37°C for 24 hours. This was used as bacteria source for the azoreductase assay. Prodrug **40** and Direct Blue stock solutions were made up in DMSO and concentration was 8 mM.

The assay was carried out using Direct Blue as positive control to azoreductase activity and the samples were prepared as described in the table below *Table 4.3*.

Sample	*BHI (µL)*	*prodrug **40*** *(µl)*	*Direct Blue (µl)*	*Bacteria solution (µl)*
Azoprodrug blank	4850	150		
Direct Blue Blank	4850		150	
Azoprodrug Sample	3850	150		1000
Direct blue sample	3850		150	1000

Table 4.3. Contents of the different vessels on the azoreductase with mouse contents assay

The final volume in all the samples was 5 ml and they were 3% in DMSO. Azoprodrug **40** and direct blue final concentration was 0.24 mM.

4.7 DSS-induced colitis mice materials and methods

Prodrug **41**, prodrug **1** and prodrug **2** were synthesized as part of this doctoral thesis project. Prednisolone and DSS were purchased from Sigma-Aldrich laboratories.

BALB/c strain mice were from the Bioresources Unit (Trinity College Dublin). Mice were housed in individually ventilated and filtered cages under positive pressure (Tecniplast, Nothants, UK). Food and water were supplied *ad libitum*. All animal experiments were performed in compliance with Irish Department of Health and Children regulations and approved by the Trinity College Bioresources ethical review board.

4.7.1 Statistical analysis

All *in vivo* experiments were performed with six mice per group. Difference between groups was analyzed by Student's t-test. Colitis scores were analyzed by Mann-Whitney test. P values <0.05 were considered significant. Results on the DIA, colon length and T/BW were expressed as means ± S.E.

4.7.2 Preparation of inflammatory bowel disease model mice

DSS (35-50,000 kDa; MP Biomedicals, OH) was dissolved in the drinking water of the mice. Fresh DSS solution was provided every second day. BALB/c mice were exposed to 5% DSS for six days. The mice were checked every day for morbidity and weight recorded. Induction of colitis was determined by weight loss, faecal blood and, upon autopsy, length of colon. Blood in faeces was detected using a Hemdetect occult blood detection kit (Dipro, Austria).

To quantify induction of colitis a disease activity index (DAI) was determined based on previous studies of DSS-induced colitis (Cooper et al., 1993). DAI was calculated for each mouse daily based on body weight loss, occult blood and stool consistency/diarrhea. A score of 1-4 was given for

each parameter, with a maximum DAI score of 12. Score 0, no weight loss, normal stool, no blood; Score 1, 1-3% weight loss; Score 2, 3-6% weight loss, loose stool, blood visible in stool; Score 3, 6-9% weight loss; Score 4, <9% weight loss, diarrhea, gross breeding. Loose stool was defined as the formation of a stool that readily becomes paste upon handling. Diarrhea was defined as no stool formation. Gross bleeding was defined as fresh blood on fur around the anus with extensive blood in the stool.

4.7.3 Colon histology

At autopsy the length of the colon was measured and a 1 cm section of colon was fixed in 10% formaldehyde-saline. H&E-stained sections were grading based on a scoring system modified from a previous study (Siegmund et al., 2001). Histology scoring was performed in a blinded fashion. A combined score of inflammatory cell infiltration and tissue damage was determined as follows: Cell infiltration: Score 0, occasional inflammatory cells in the LP; 1, increased infiltrate in the LP predominantly at the base of crypts; 2, confluence of inflammatory infiltrate extending into the mucosa; 3, trans-mural extension of infiltrate. Tissue damage: Score 0, no mucosal damage; 1, partial (up to 50%) loss of crypts in large areas; 2, partial to total 50-100% loss of crypts in large areas, epithelium intact; 3, total loss of crypts in large areas and epithelium lost.

4.7.4 Treatment of colonic inflammation

The DSS-induced colitis mice weighting about 18-20 g were allowed 5% DSS water *ad libitum* and (100 µl) of prednisolone and prodrugs **41**, **1** and **2** in 1% solution cremophor and ethanol in water suspension (equivalent to 5 mg of prednisolone per kg or mouse body weight) was orally administrated, twice a day (every twelve hours) to the colitis mice. Six days after the prednisolone and prodrugs treatments, the mice were sacrificed by cervical dislocation. The distal colon segment and the thymus were removed and washed to remove contents of the colon.

The fresh weight of the thymus was measured to obtain the ratios of thymus weight to body weight (T/BW). The damage of the colon specimens were scored as describe in materials and methods.

The distal segments were frozen by liquid nitrogen kept at -80°C for further analysis of myeloperoxidase activity (MPO).

4.8 Plasma hydrolysis studies

Plasma hydrolysis assays were carried out using BALB/c mouse plasma. Three different solutions were prepared:

Solution A: Prodrug **1** and **2** in DMSO 3 mg/ml.

Solution B: PBS pH 7.4 (4 ml) and plasma (2 ml).

Solution C: Zinc sulphate (2%) in distilled water (4 ml) and DMSO (4 ml).

The solution A (100 µl) was poured into solution B at 37°C and that was the hydrolysis sample. Finally aliquots of the hydrolysis sample (250 µl) were withdrawn and added into (500 µl) of solution C to quench the reaction and the final sample was centrifuged at 7000 rpm. The supernatant was injected into the HLPC. The gradient is method described at the beginning of this chapter.

4.9 Transport studies

Compounds were tested in triplicate for transport across a CACO-2 cell monolayer in the AP>BL direction and in the reverse direction.

For the transport studies, cells were grown in Transwell inserts (polycarbonate membrane, 12 mm diameter, 0.4 µm pore size, Corning Costar, NY) at a density of 1.0×10^5 cells/cm^2 and were used at passage 23. The inserts were housed in 12-well plates and the test was performed 22-23 days after seeding.

The test compounds were dissolved in HBSS containing 5% DMSO at (50 µM) and were applied at the donor side. The volumes of the apical and basolateral compartments were respectively (0.5 and 1.5 ml). Caffeine was also tested in two wells in the AP>BL direction as a routine control.

Separations were performed on the gradient HPLC system described previously in this chapter.

The retention times were 3.0 min for prednisolone, 4.8 min for prodrug **1** and 5.9 min for prodrug **2** (the difference in the retention times was due to the fact that the HPLC analysis was carried out in a different system and the column was heated at 30°C).

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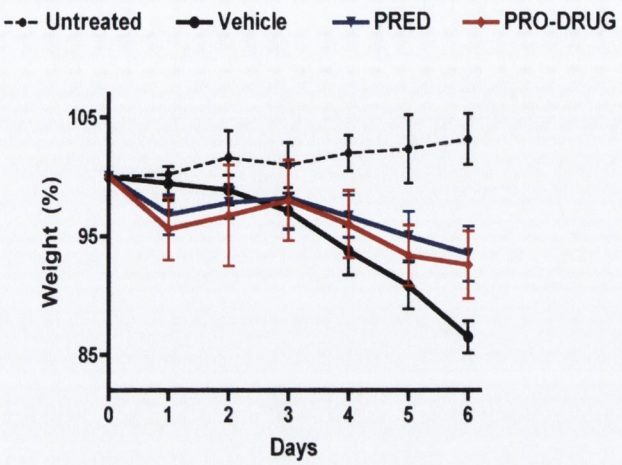
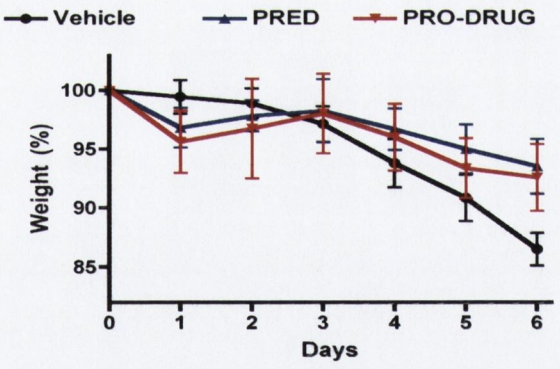
APPENDIX I

STUDY OF COMPOUND 2 IN THE MOUSE DSS-COLITIS MODEL AT 10 mg/kg

DSS-induced colitis mice groups treated with prednisolone and compound 2 at a dosage of 10 mg molar equivalent of prednisolone per kg of mouse body weight



Graph 1. Body weight loss percentage profile of healthy mice (untreated) and DSS-induce colitis mice treated with prodrug 2 and prednisolone at 10 mg/kg dosage



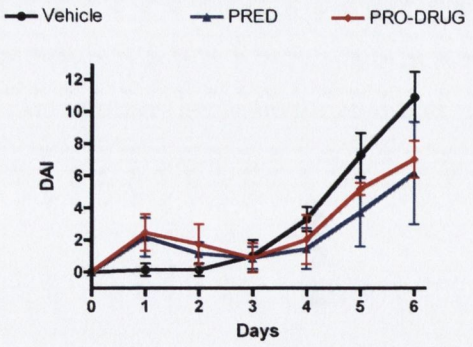
D6: Vehicle versus PRED: P<0.0001
Vehicle versus PRO-DRUG: P<0.001

D5: Vehicle versus PRED: P<0.005
Vehicle versus PRO-DRUG: NS

D4: Vehicle versus PRED: P<0.05
Vehicle versus PRO-DRUG: NS

PRED versus PRO-DRUG. NS difference

Graph 2. Body weight loss percentage profile of DSS-induced colitis mice treated with prodrug 2 and prednisolone at 10 mg/kg dosage



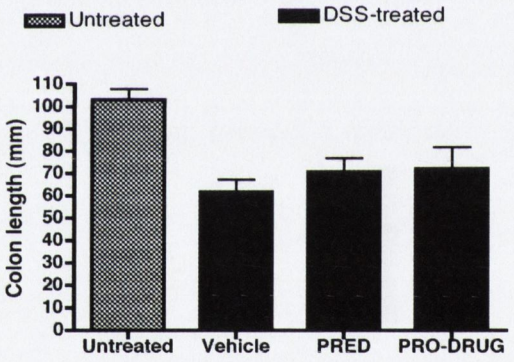
D6: Vehicle versus PRED: P<0.001
Vehicle versus PRO-DRUG: P<0.001

D5: Vehicle versus PRED: P<0.005
Vehicle versus PRO-DRUG: P<0.005

D4: Vehicle versus PRED: P<0.01
Vehicle versus PRO-DRUG: NS

Pred versus PRO-DRUG. NS

*Graph 3. DAI score profile from colon segment of DSS-induced colitis mice treated with prodrug **2** and prednisolone at 10 mg/kg dosage*

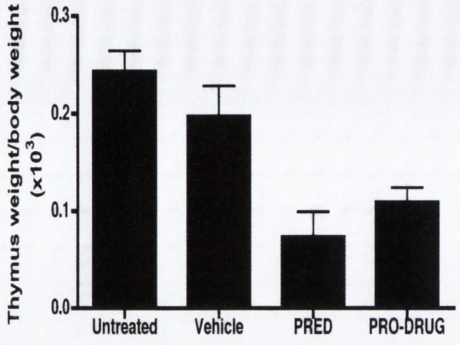


All DSS-treated groups P<0.0001 shorter colons than Untreated group

PRED and PRO-DRUG P<0.05 longer colon than Vehicle-treated.

*Graph 4.. Colon length profile of healthy mice (untreated) and DSS-induced colitis mice treated with prodrug **2** and prednisolone at 10 mg/kg dosage*

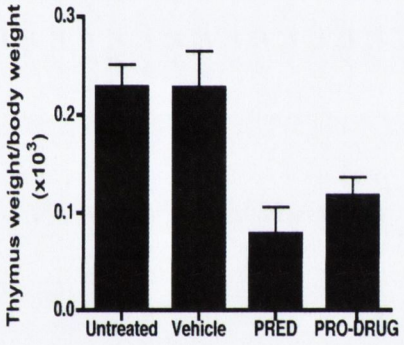
Starting body weight



PRED and PRO-DRUG P<0.0001 smaller thymus than Vehicle.

PRO-DRUG P<0.01 larger thymus than PRED.

Day 6 body weight



PRED and PRO-DRUG P<0.0001 smaller thymus than Vehicle.

PRO-DRUG P<0.01 larger thymus than PRED.

*Graph 5. Thymus weight body weight ratios (T/BW) and thymus weight post treatment body weight ratios profile of healthy mice (untreated) and DSS-induced colitis mice treated with prodrug **2** and prednisolone at 10 mg/kg dosage*

APPENDIX II

TEER MEASUREMENTS

Teer measurements AP>BL transport data

	Well	Time									
		0 min		120 min		180 min		2h after buffer replacement		2h after buffer replacement	
		R (Ω)	TEER (Ω.cm²)	R (Ω)	TEER (Ω.cm²)	R (Ω)	TEER (Ω.cm²)	R (Ω)	TEER (Ω.cm²)	R (Ω)	TEER (Ω.cm²)
Prednisolone	A1	608	512.0	230	137.5	228	135.2	340	247.2	568	476.9
	B1	591	495.3	218	125.7	226	133.3	331	238.3	585	493.7
	C1	623	526.8	228	135.6	223	130.3	328	235.3	590	498.6
P1	A2	615	518.9	228	135.6	242	149.0	335	242.2	535	444.4
	B2	595	499.2	215	122.8	211	118.5	332	239.3	534	443.4
	C2	614	518.0	207	114.9	206	113.6	322	229.4	540	449.4
no cells	A3	84		84		88		91		83	
	B3	89		93		92		88		82	
	C3	91		94		92		88		86	
P2	A4	624	527.8	231	138.5	233	140.2	427	332.8	581	489.7
	B4	619	522.9	221	128.7	230	137.2	346	253.1	556	465.1
	C4	629	532.7	234	141.5	236	143.1	396	302.3	600	508.4

Teer measurements BL>AP transport data

BL>AP	Well	Time									
		0 min		120 min		180 min		2h after buffer replacement		24h after buffer replacement	
		R (Ω)	TEER (Ω.cm²)	R (Ω)	TEER (Ω.cm²)	R (Ω)	TEER (Ω.cm²)	R (Ω)	TEER (Ω.cm²)	R (Ω)	TEER (Ω.cm²)
no cells	A1	82		92		90		83		85	
Caffeine	B1	593	503.2	535	436.2	532	435.2	577	486.4	582	489.4
	C1	599	509.1	554	454.9	547	450.0	582	491.4	544	452.0
Prednisolone	A2	591	501.2	260	165.4	239	146.7	341	254.1	386	296.4
	B2	598	508.1	214	120.1	221	129.0	312	225.5	588	495.3
	C2	612	521.9	218	124.1	205	113.2	327	240.3	697	602.6
P1	A3	590	500.2	201	107.3	201	109.3	309	222.5	446	355.5
	B3	628	537.6	203	109.3	199	107.3	321	234.4	360	270.8
	C3	580	490.4	237	142.8	224	132.0	303	216.6	435	344.6
P2	A4	552	462.8	230	135.9	227	134.9	338	251.1	740	645.0
	B4	567	477.6	274	179.2	269	176.3	347	260.0	666	572.1
	C4	573	483.5	257	162.5	258	165.4	331	244.2	325	236.3

PATENT APPLICATION

GILMER, J.F, MARQUEZ, J, & KELLEHER, D. (2007) Targeting prodrugs for treatment of gastrointestinal diseases. Ireland 2007/0475. 2916/2007 US and Ireland.

Only the first few pages of the patent are presented here for further information the reference is shown above.

Targeting Prodrugs for the Treatment of Gastrointestinal Diseases

FIELD OF THE INVENTION

[0001] The present application relates to compounds and pharmaceutical compositions that may be used for the treatment of gastrointestinal diseases, including, but not limited to inflammatory diseases, such as Inflammatory Bowel Disease (IBD).

BACKGROUND OF THE INVENTION

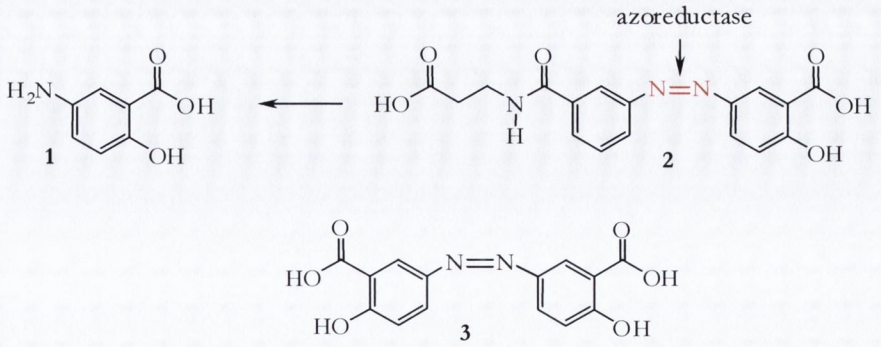
[0002] Drug targeting may be defined as the delivery of a drug to a specific organ, tissue or cell population (Schreier 2001). Still in its infancy, this field offers the prospect of enhancing the efficacy of drug treatment while reducing systemic impact or side effects. Despite the promise of this approach, and ongoing efforts, there have been few successful examples to date due in part to limited understanding of the basic factors underlying drug transport and the expression of potential targeting vectors. Chemical drug targeting involves the deliberate modification of a drug structure (usually bioreversibly) causing it to accumulate in a target tissue; site-specific release from the prodrug is triggered by a chemical or enzymatic condition not present elsewhere in the body.

[0003] The colon is an important challenge to the validity of the drug targeting approach, as conditions in the colon are largely similar to those prevailing elsewhere in the gastrointestinal (GI) system, and the luminal pH gradient through the GI tract is too gradual for effective local drug release on strictly chemical grounds (Bauer, 2001). On the other hand, the colon is an important drug target for the treatment of pathologies of the colon itself, such as inflammatory bowel disease (IBD) and colon cancer,

and for the relief of the chronic constipation that accompanies opioid drug treatment. The colon is also important as a potential portal site for peptide and protein drugs that are not absorbed from other regions of the GI tract or are too unstable in the presence of duodenal proteases to be released there (Saffran, 1986; Bai, 1995).

[0004] One key difference between the colon and small intestine that might be exploited as a vector for site-specific drug release is the luxuriant microflora of the former. The human GI system is home to 400–500 species of bacteria with a total live population of 10^{14} organisms. This is remarkable when compared with the 10^{13} eukaryotic cells that make up the human body. The GI tract has a steadily increasing bacterial concentration gradient on descending from the stomach through the small intestine, followed by an enormous increase at the colon. The bacterial concentration in the small intestine is typically 10^3–10^4 CFU ml^{-1} whereas the concentration in the colon is 10^{11}–10^{12} CFU ml^{-1} and one third of fecal dry weight consists of bacteria (Moore and Holdeman, 1974 and 1975; Simon and Gorbach, 1984). These organisms fulfill their energy needs by fermenting undigested materials entering from the small intestine (particularly polysaccharides) and have for this purpose evolved an elaborate array of enzymes such as azoreductase, glucosidase, β-glucuronidase, β-xylosidase, nitroreductase, galactosidase and deaminase (Scheline, 1973).

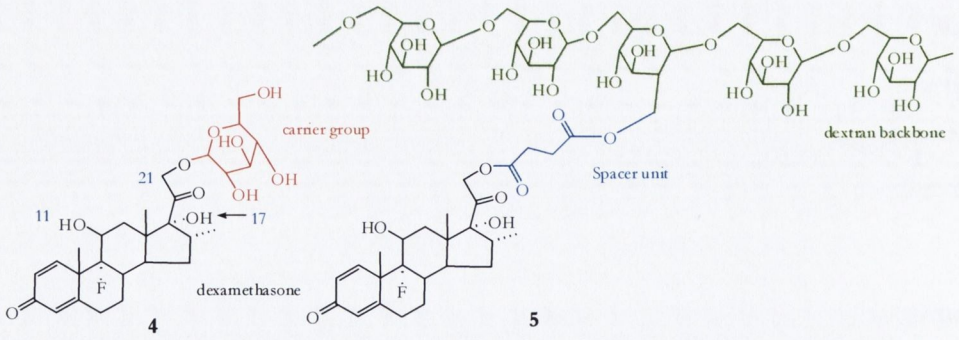
[0005] This abrupt increase in bacterial enzyme expression has been investigated as a means of targeting drugs to the colon, especially those for the treatment of IBD. One successful outcome of these endeavours has been the development of azo-based prodrugs of 5-amino salicylic acid (5-ASA) **1** (*Scheme 1*), which because of their hydrophilicity and polarity pass through the GI system intact before releasing their 5-amino salicylic acid 'payload' upon reduction of the azo linker by azoreductases associated with colonic microflora. Several drugs based on this concept, such as ipsalizide, balsalazide **2** (Chan et al. 1983), sulphasalazine (the prototype) and olsalazide **3** (Willoughby et al., 1982), are in clinical use for the treatment of IBD (Green, 1998).



Scheme 1

[0006] The targeting of other drug types, such as the anti-inflammatory steroids to the colon has been less successful (Sinha & Kumria 2001). Paradoxically, the need for appropriate systems in these cases is more pressing because steroids have multiple systemic side effects when administered orally due to their ready absorption from the stomach and small intestine. Chronic inflammatory bowel disease comprises two major disorders, namely ulcerative colitis and Crohn's disease. Both of these conditions produce significant morbidity in the form of diarrhea, weight loss and potentially serious and life-threatening complications. The incidence in Europe is estimated at up to 80 million/year (Logan, 1998), while the absolute incidence in the United States is believed to be about one million, with 15-30,000 new cases reported annually (DiPirio and Bowden, 1997). It is therefore unsurprising that intense efforts have been made both to unravel the underlying etiology of IBD and develop new therapies, but also to improve existing pharmacotherapy. These efforts have been restricted to 5-ASA derivatives and steroids.

[0007] The efforts to target steroids to the colon have been especially numerous. Technologies that have been investigated include: biodegradable polymers (Basit, 2000), time release systems, coating with pH sensitive materials, gastrointestinal pressure controlled release (Hu et al., 1998) and chemical drug targeting.



Scheme 2

[0008] Approaches belonging to the latter class have revolved around the attachment to steroids of hydrophilic carriers that might act as substrates for bacterial polysaccharide processing enzymes (*Scheme 2*). Examples of carriers investigated include: glycosides (e.g. **4**, References a-g); glucuronides (Haeberlin, 1993; Cui 1994); poly-(L-aspartic acid) derivatives (Leopold, 1995); α, β and γ-cyclodextrin conjugates (Yano, 2002); polymer conjugates (e.g. **5**, McLeod et al., 1994). Although some of these designs are promising, they have thus far met with limited success, due in part to the widespread distribution of glycosidases in the GI tract (resulting in non-site specific release; Sinha & Kumria, 2001), and in some instances, because of slow release characteristics in the colon (up to 12 hours for some polysaccharides; Yang, 2002). The use of the successful azoreductase approach has thus far been restricted to drugs, such as 5-ASA, that bear a primary aromatic amine.

SUMMARY OF THE INVENTION

[0009] Provided herein is a novel strategy for drug-targeting hydroxyl-bearing compounds to the colon. The innovative strategy for targeting hydroxyl-bearing drugs to the colon has been designed to overcome the design flaws in the glycosidase targeting approach. One method for achieving the site-specific delivery of hydroxyl-bearing drugs is schematically presented in *Scheme 3*. In one aspect, the drug is selected from the group that includes an anti-inflammatory drug, an anti-cancer drug, an imaging agent, particularly as used in the imaging of colon diseases; a vaccine, an antigen, an anti-infective drug, a peptide, an antisense molecule and a protein.

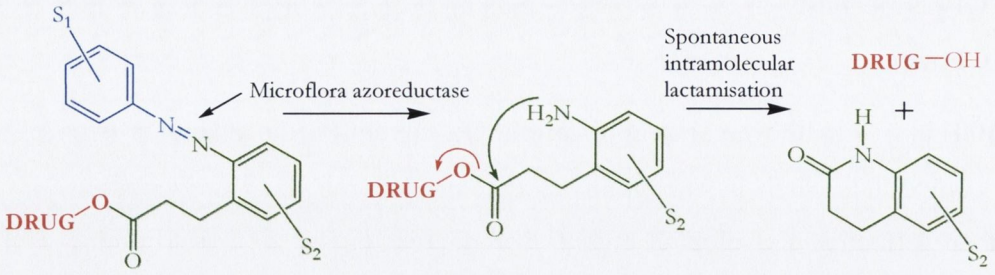
[0010] In one aspect of the present application, the method provides an evaluation of anti-inflammatory effects of candidate compounds in a mouse model of IBD. In another aspect, the method provides insights into the GI permeability of the azo-compounds and their ability to pass through the GI tract.

[0011] In the present application, the inventors discovered a need for compounds, such as the prodrugs disclosed herein, and compositions that are effective as selective agents for decreasing NFκB DNA-binding activity in a patient. In one aspect, there is provided a method comprising administering a therapeutically effective amount of a compound or composition effective to reduce, alleviate or treat various gastrointestinal diseases, including inflammatory bowel disease (IBD).

[0012] In one aspect, the drug is linked via an ester group to a carrier group, which is connected by an azo-bond to a second carrier group. The carrier groups may be directly attached to the azo group or indirectly attached to the azo group. As provided herein, the carrier groups are designed to maximally suppress absorption from stomach and upper intestine. The method exploits the selective reduction of an azo-linker in the colon, releasing a chemically unstable, latent prodrug that subsequently undergoes cyclization, such as lactamization, that liberates the drug payload, such as a steroid. In certain compounds, the cyclization reaction is substantially spontaneous. The overall effect of the design is to make the biologically stable or robust ester group, connecting the drug to the carrier group, chemically vulnerable under conditions found only in the colon.

[0013] Generally the prodrugs of the present application are referred to as "carrier-drug." The "carrier" can comprise compounds such as 5-ASA or para-aminobenzoic acid (PABA). When the carrier has a therapeutic effect, such as 5-ASA, the prodrug is generally referred to as a "mutual prodrug." Such mutual prodrugs can be referred to herein as 5-ASA-drug, wherein the drug can be any appropriate therapeutic agent, including those disclosed herein. Such mutual prodrugs can include, but are not limited to, 5-ASA-ciprofloxacin, 5-ASA-bevacizumab, 5-ASA-prednisolone, 5-ASA-5-ASA, etc. When the carrier does not have a therapeutic effect, such as PABA, the compound can be simply referred to as a "prodrug." Such

prodrugs can be referred to herein as PABA-drug, such as, for example PABA-ciprofloxacin, PABA-bevacizumab, PABA-prednisolone, PABA-5-ASA, etc.



Scheme 3

[0014] It was determined that the physicochemical characteristics of the prodrug can be optimized for gastrointestinal penetration to the colon by varying the nature of the compound, including the substituents S_1 and S_2, wherein one or more of the substituents S_1 and S_2 on the aryl ring may be employed, as disclosed herein.

[0015] Azo bond reduction proceeds readily because it is based on the promiscuity of the azoreductases present in the colon with respect to substrate, as evidenced by their ability to efficiently reduce substrates as diverse as ipsalazide, in which the carrier group is *p*-amino hippurate, balsalzide (*p*-aminobenzoyl-β-alanine carrier), sulfasalazine (sulfapyridine carrier), sterically bulky PAF antagonists (Carceller et al., 2001), 9-aminocamphothecin (Sakuma et al., 2001) and 5-ASA-N-methacrylamide, acryloyloxyethyl and acryloylamido copolymers (e.g. Van den Mooter et al., 1994). The presence of the vast microflora in the bowel causes a change in redox potential from -67±90 in the distal small bowel to –415±72 in the right colon (Wilding et al. 1994). The relative contribution of enzymatic and chemical reduction in the release of azo drugs is not well understood.

[0016] The cyclization or ring closure is dependent on nucleophilic attack by the amine group, such as the aniline amino group on the ester. An example of such ester is a steroidal ester. Although anilines possess low nucleophilicity (compared with aliphatic amines) the high effective molarity of the intramolecular arrangement will promote sufficiently rapid ring closure. Reports on the intramolecular aminolysis of esters by aniline, at roughly comparable conditions, have been described. Kirby et al. (1979)

studied the spontaneous cyclization of methyl 3-(2-aminophenyl)propionate, finding that at neutrality (roughly the pH of the colon) and 39 °C, the reaction proceeds with an apparent first-order rate constant of $2x10^{-4}$ sec^{-1}, corresponding to a first-order half-life of 57 min. Fife and Duddy (1983) reported the ring closure of methyl- and trifluoroethyl (2-aminophenyl)acetate (yielding the indolone) to follow similar kinetics under mildly basic conditions (pH 7–8). These half-lives are ideal for drug release in the colon, where residence time tends be rather extended.

[0017] The ester linkage in the compounds of the present application is highly stable under conditions found in the GI tract. Esterase activity in the lumen of the GI tract is restricted to the pancreatic serine proteases, which exhibit residual esterase activity towards a limited number of substrates, generally esters of aromatic amino acids. Steroidal 21-esters (see *Scheme 2* above for numbering), for example, have been shown to be robust in simulated intestinal fluid models. For example Fleisher et al. (1986) reported a first-order rate constant of 0.003 min^{-1} for the hydrolysis of hydrocortisone-21-succinate in rat intestinal perfusate. This figure corresponds to a half-life of 12 h which is significantly longer than the expected transit time to the colon. Jhunjhunwala (1981) reported the disappearance of less than 5% of hydrocortisone-21-succinate after 24 h at pH 7 (45 °C).

[0018] Gastrointestinal absorption is a function of molecular weight, lipophilicity and polarity; in general, polar, hydrophilic molecules are not well absorbed. Among the possibilities for variation at the carrier group comprising a substituent represented by S_1, for example, are azo linkage linking a carrier, including but not limited to 5-ASA, and a steroid, thereby generating a mutual prodrug of a drug, such as a steroid. Such mutual prodrugs may provide ideal or favourable physicochemical characteristics for passage through the intestine because of mass, polarity and hydrophilicity.

[0019] The sterically hindered steroidal esters disclosed in the present application are much less vulnerable towards hydrolysis than hydrocortisone-21-succinate (see Jhunjhunwala, 1981). Furthermore, the

ester may also be attached on one of several different hydroxyl groups on the steroid nucleus. For example, without being bound by any theory proposed herein, the esters placed at the highly hindered 11β axial hydroxyl group (Figure 2) are more stable than the 21-esters.

[0020] The present application discloses novel systems and compounds that are capable of engaging in a two-step process for releasing drugs, such as anti-inflammatory steroids, that target the colon. The present application also discloses the synthesis of a variety of compounds and their derivatives, including steroidal compounds (e.g. esters of hydrocortisone, dexamethasone or budesonide), nitroimidazoles (e.g. metronidazole), antibiotics, (e.g. quinolines such as nalidixic acid, fluoroquinolones, such as ciprofloxacin or levofloxacin, aminoglycosides, such as amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, tobramycin and apramycin), chemotherapeutics (e.g. leucovorin, topotecan, irinotecan, methotrexate), and antibodies, (e.g. bevacizumab, cetuximab, panitumumab, infliximab). The present application further discloses the measurement of ring closure kinetics, *in vitro* screening for intestinal stability using enzymes and intestinal perfusates, drug release *in vitro* in the presence of colonic bacteria, and therapeutic efficacy, for example, anti-inflammatory efficacy, in appropriate animal models. In one aspect, a series of esters of the anti-inflammatory steroids hydrocortisone, dexamethasone and budesonide at the 21-, 11- and 17 positions are prepared as prodrugs. In another aspect, the application discloses the synthesis of various azo-derivatives of the amino esters.

[0021] In another aspect, there is provided analytical methods for the determination or estimation of the rate of ring closure of the amino intermediate compounds. Also provided herein are methods for estimating the rate of ring closure and determining its dependence on mesomeric effects due to substitution in the carrier group, such as the aniline derivatives. In yet another aspect of the application, there is provided a method for measuring the stability of the compounds under aqueous conditions (at pH 1-8), in the presence of pancreatic serine proteases (*in vitro*) and other enzymes present in the gut and, in the presence of rat intestinal fluid.

In another aspect, there is provided a method for estimating the rate of reduction of these compounds in the presence of microflora *in vitro*. In yet another aspect, there is provided a method for estimating the *in vivo* efficacy of the compounds using a mouse model of colonic inflammation. In another aspect, there is provided a method for measuring the permeability of the compounds as provided herein, using rat perfusion models, which allows the assessment of penetration through the GI tract. For potential colon-targeting, compounds of negligible permeability are employed.