γ-GLUTAMYL-N-MUSTARD PRODRUGS

A thesis submitted by

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ABSTRACT

The γ-L-glutamyl adducts of several bis-2-chloro ethyl and bis-2-bromo propyl-4-phenylene diamine mustards were synthesized by modified literature procedures. Improved yields, in particular were obtained from the conversion of the aromatic bis-2-hydroxy alkyl amines to the corresponding mustard by using bis-2-tosyl ester intermediates.

The enzyme kinetics of the γ-L-glutamyl adducts to the parent mustard and L-glutamic acid are reported. Values of the kinetic parameters K_m and V_{max} for hydrolysis are determined and compared with those of glutathione and glutamine, the enzyme's natural substrates.

The toxic effect of the γ-L-glutamyl adducts against two cell lines are reported and compared with the toxic effects of the parent mustard. One cell line (JB1) obtained from an aflatoxin-B_1 induced hepatoma contains elevated levels of γGT, whereas the other (BL8L) obtained from normal liver, contains low levels of γGT. The ability of the γGT inhibitor serine borate to mediate the toxic effects of the γ-L-glutamyl adducts on the two cell lines is also investigated.

The effect of γ-[N,N -bis-(2-chloro ethyl)-4-phenylene diamine]-L-glutamic acid (γGPDM) on transplanted hepatoma in nude mice is reported.

The results are critically discussed in terms of the possible use of γ-L-glutamyl aromatic mustards as anti hepatoma prodrugs.

TO MY PARENTS AND IND KAUR, MY GRANDMOTHER

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AT-125	L-(2α,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid
BSO	D,L-buthionine-S,R-sulphoximine
DMF	Dimethyl Formamide
DMSO	Dimethyl sulphoxide
DON	6-Diazo-5-oxo-L-norleucine
γ-Glutamyl-AMC	γ-L-Glutamyl-7-amino-4-methyl coumarin
γGBrPDM	γ-L-[N,N-bis-(2-bromo ethyl)-4-phenylene diamine]-glutamic acid.
γG-2-BrPDM	γ-L-[N,N-bis-(2-bromo propyl)-4-phenylene diamine]-glutamic acid
γ-GBrPDMF	γ-L-[N,N-bis-(2-bromo ethyl)-2-Fluoro-4-pheylene diamine]-glutamic acid
γGPDM	γ-L-[N,N-bis-(2-chloro ethyl)-4-phenylene diamine]-glutamic acid
γGPDMCl	γ-L-[N,N-bis-(2-chloro ethyl)-2-chloro-4-phenylene diamine]-glutamic acid
γGPDMF	γ-L-[N,N-bis-(2-chloro ethyl)-2-fluoro-4-phenylene diamine]-glutamic acid
γGT	γ-Glutamyl transferase
HBSS	Hanks balanced salt solution
HBV	Hepatitis B virus
HN2	Bis-2-chloro ethyl amine
HN3	Tris-2-chloro ethyl amine

L-azaserine	O-diazo-acetyl-L-serine
L-OC	L-γ-Glutamyl-(o-carboxy) phenyl hydrazide
MOPS	2-(N-Morpholino) propane-Sulphonic acid
OPT	O - Phathaldialdehyde
PPDM	N,N-bis-(2-chloro ethyl)-4-phenylene diamine
THF	Tetrahydrofuran
TMS	Tetramethylsilane
Tris	2-Amino-2-hydroxymethlpropane-1,3-diol
Ts	Toluene-4-sulphonyl group

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CHAPTER 1

INTRODUCTION

1.1 The Cancer Problem

Cancer has long been a major problem for mankind. Insofar as skeletons recovered from mummies in Egypt show deformations similar to those produced by bone tumours. After arterial disease it is the largest cause of death in the developed countries. It also affects people in the underdeveloped countries, but the incidence there is generally lower.

Cancer is a collective term for over a hundred types of malignant tumours. The latter are defined as a group of abnormally proliferating cells that can arise in any part of the body and spread from their sites of origin. If the growths remain strictly local and non invasive, they are called benign tumours.

Cancer cells arise from a single cell that undergo permanent DNA changes causing it to become relatively undifferentiated and to loose its cell-cell contact inhibition. As a result, many of these altered cells undergo repeated mitosis, at rates faster than those of the surrounding normal cells.

There are two main changes in a cancer cell. The first change is of a regulatory nature. The multiplication of the malignant cell does not obey the normal regulatory mechanisms of the organism and is in a continuous multiplication process. The second change of the cancer cell deals with its relations with the neighbouring cells. Thus, normally cells are limited to specific organs whereas cancer cells can invade other tissues. In addition they can migrate through the blood or the lymphatic system to establish new colonies of cells in other distant organs. This process is called metastasis and is responsible for the highly lethal nature of cancers.

Many factors are believed to be involved in the formation of most tumours. A wide variety of agents can act on the normal cells and transform them into malignant cells. The agents which contribute to carcinogenesis include: Chemicals[1], physical agents[2] (eg. ionizing and non-ionizing radiation), and biological agents (viruses[3], hormones[4]).

The fundamental modes of modern cancer therapy are surgical treatment, radiotherapy, chemotherapy and immunotherapy. By using a combination of two or more of these modes, cure rates of certain cancers have been greatly improved. It has to be realised that surgery, radiotherapy or chemotherapy alone rarely achieves the total destruction of malignant cells essential for a cure. It is often necessary to employ surgical treatment followed by radiotherapy to reduce the tumour size to a level that can be effectively coped with by chemotherapy, which, in turn, would reduce the size of the remaining tumour cells to a small enough level to be completely destroyed by the host immunologic defences.

1.2 Hepatocellulor Carcinoma

Estimates for the world and regional frequencies of hepatocellulor carcinoma varies widely, but it is especially common in Africa, (particularly Mozambique and Senegal) and South East Asia[5]. Most tumours arise in a Cirrhotic liver and the vast majority are associated with hepatitus B virus (HBV)[6-8] infections. There are several routes of entry of the virus, but the most common is by infection from the mother, who is a carrier of the HBV surface antigen (HB_sAG), in the immediate postnatal period[6].

It is also thought that dietary aflatoxin B, produced by a fungus Aspergillus flavus may be an additional cause of hepato carcinoma in Africa[5]. Recently, a high concentration of aflatoxin has been reported in millet beer in Nigeria[9]. The diet in endemic regions consists of dried food (eg. peanuts, maize, corn) which has been stored for prolonged periods of time in a hot climate, conditions suitable for the growth of the Aspergillus fungus.

Evidence that moderate consumption of alcohol by HB_sAg carriers is associated with a significantly higher incidence of hepatocellular carcinoma compared with controls was reported in Italy[10].

Early diagnosis of the cancer is important. Serum alpha-foetoprotein concentrations have been measured[11]. In Taiwan, ceoliac arteriography, CT scanning and ultrasonography have also proved of use[12].

Even if the tumour is detected early, no form of treatment has proved completely effective. This is because most hepatocellular carcinomas are multifocal. The therapeutic agents 5-fluoro uracil and dexorubacin (especially when introduced into the hepatic artery) have given some promising results[5]. Also hepatic artery embolisation has reduced tumuour size, and given some symptomatic relief[13].

1.3 Cancer Chemotherapy

Certain drugs inhibit cell reproduction or selectively destroy reproducing cells without permanent injury to non reproducing cells. The treatment of cancers with drugs of this type is a branch of chemotherapy.

Certain differences have been observed between normal and malignant cells. For example, malignant cells often have greater free radical character,[14] lower pH,[15] tumour associated antigens,[16] tumuour-produced hormone peptides,[16] higher biowater content,[17] higher potassium ion and lower calcium ion concentrations,[18] different potassium isotope ratios,[18] and larger amounts of methylated nucleosides.[19] Further, some types of tumour cells (leukemia cells) require an external source of L-asparagine whereas normal cells do not.[20] These differences are more use in detecting rather than curing cancer. Most drugs in current use inhibit cell division by interfering with either the synthesis or the metabolism of nucleic acids, or with cell mitosis.[21] None of the anti-cancer drugs so far discovered is capable of absolute discrimination between malignant cells and healthy cells that are undergoing fast reproduction such as those of the gastro intestinal tract and the bone marrow. A similar problem occurs in the radiation treatment of cancer. These anti tumour agents therefore produce side effects ranging from nausea and hair loss to suppression of the immune system. More selective toxicity for cancer cells can be achieved either by designing drugs with greater selectivity or by using techniques to restrict drug action to specific cells.

In 1952 Danelli[22] pointed out that drugs can be made more selective by increasing the number of variables on which the drug depends for its activity. Many variations can be achieved by exploiting both the differences in chemical reactivity and physical properties (solubility, polarity) of drugs and the differences between normal and malignant cells (pH, enzyme levels). Further biologically significant moieties such as amino acids[23,24] and carbohydrates[25-27] can be used either to convey or to inactivate the toxic constituent. Use of the latter to achieve higher selectivity for anti-cancer drugs is the principle of latent

chemotherapeutic activity. This implies the design of a drug which is itself inactive but can be converted by a process known to occur in vivo (hydrolysis, reduction, oxidation) into an active form at the tumour site.

Another technique known as combination chemotherapy, involves the administration of several drugs, simultaneously or sequentially, with a view partly to preventing the development of resistance and partly to obtain a synergistic effect. This is a particularly successful approach in the treatment of leukemias and lymphomas.

A given dosage of anti cancer drug only destroys the same percentage of tumour cells.[28] Therefore, considerable difficulty has been experienced by treating solid tumours chemotherapeutically,[29] and there is thus a need for additional novel targeted drugs for these malignancies.

Anti tumour agents can be divided into six classes that reflect their varying mechanism of action. These classes are alkylating agents (discussed in the next section), antimetabolites, antibiotics, hormones, mitotic inhibitors, and enzymes.

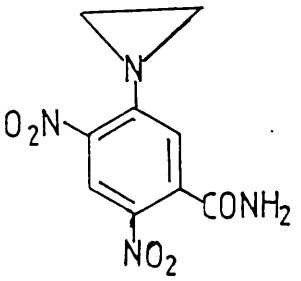
1.4 Alkylating agents

Many anti tumour alkylating agents are known eg. nitrogen mustards, aziridines, methanesulphonates and epoxides and all react with various cellular nucleophiles such as hydroxy-, amino-, mercapto-, or imidazole groups of proteins and nucleic acids. It is the cross linking of nuclear DNA, however, which is the principal biologically significant reaction.[30,31] In general, alkylating agents are not cancer-specific and they also destroy normal cells. This low selectivity together with their high reactivity are factors which have been overcome by chemical modification.

Compounds with at least two alkylating moieties, have shown better anti tumour properties. This led to the hypothesis that cross-linking of the DNA double helix should take place.[32] Evidence in support of this theory has been given,[33] but a few monofunctional compounds such as CB1954[34] [1] also have good anti tumour properties. DNA sites that may be involved in alkylation reactions include the N7-[35] and O6-[36] positions of guanine residues, phosphoryl groups (alkylation of which can be followed by transalkylation of the N-7 position of guanine.[35] Alkylation of nucleophilic centres of other biologically important molecules has never been excluded and methane sulphonates, such as myleran[2], for example react with the thiol group of cysteine in a protein chain.[36]

The alkylating agents have three main biological effects on cells. Firstly a cytostatic effect (low dosage) in which the mitosis of the cell is delayed or entirely prevented. Secondly, a mutagenic effect (medium dosage) in which cell division takes place but the daughter cells are altered with possible development of carcinogenic properties. Thirdly a cytotoxic effect (high dosage) in which cells are irreparably damaged and killed.

Some of the most important anti-tumour alkylating agents are i) Mustine (the hydrochloride of the nitrogen mustard), ii) cyclosphosphamide [3] (the most commonly used nitrogen mustard derivative), iii) Phenylalanine mustard [4] (whose L-isomer melphalan, is at least 5 times more active than the D-isomer, which illustrates the influence of chirality upon biological activity[23,37]), iv) triethylenethiophosphoramide (thio - TE PA), and v) myleran (busulphan).



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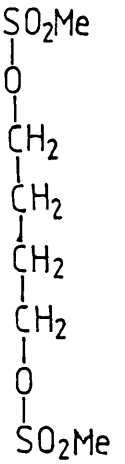

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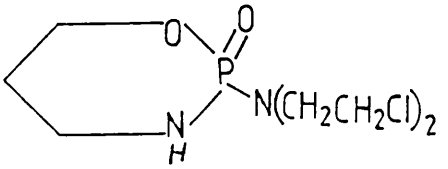

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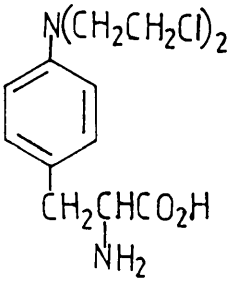


[4]

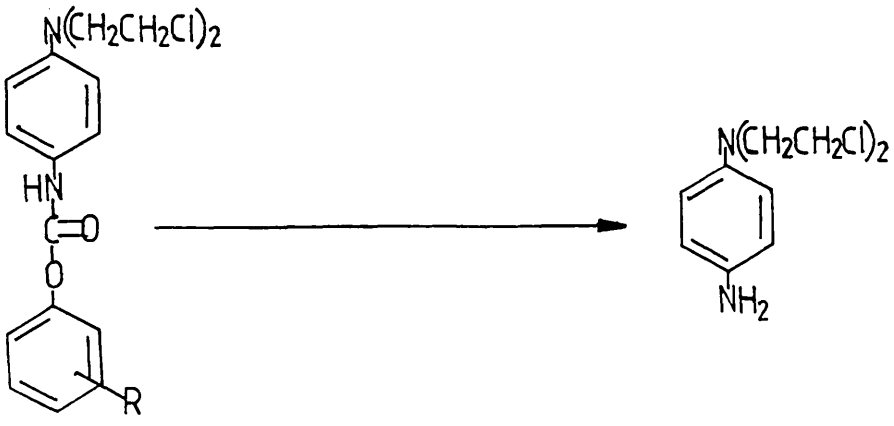
One other important factor in the design of effective drugs is the attachment of the alkylating function to biological carriers such as carbohydrates, amino acids, polypeptides, nucleosides, and steroids. This often facilitates transport of the drug to the tumour site as in the case of Degranol[38] (a D - mannital derivative), melphalan (a glycine derivative), and phenesterin,[39] (a cholesterol ester derivative).

1.5 Nitrogen Mustards

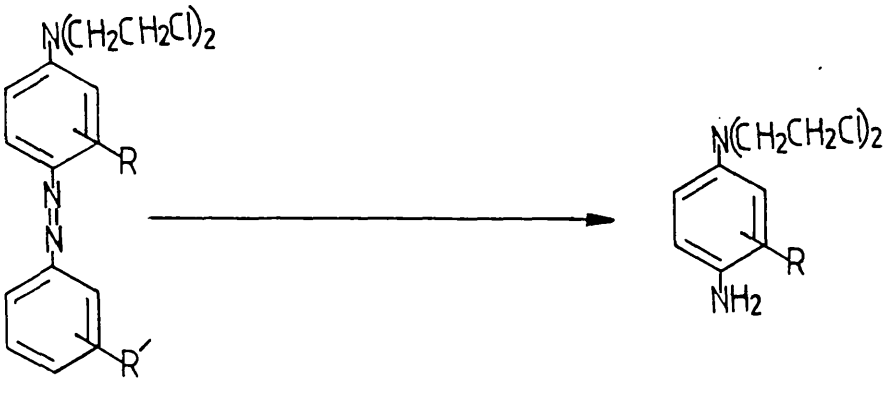
In 1942, Goodman and Gilman[40] studied the pharmacology of nitrogen mustard derivatives and noted their effect on dividing cells. The two compounds tested were tris-2-chloro ethylamine (HN3), which was the first mustard used clinically,[40] and bis-2-chloroethyl amine (HN2) better known as mustine hydrochloride. Subsequently, both compounds were used clinically.[40,41]

The chemical reactivity of the nitrogen mustards depends on cleavage of the halogen atom from the molecule. This reaction is facilitated by the electron releasing capability of the N-atom. Thus, aromatic nitrogen mustards are usually less reactive than aliphatic analogues. To obtain milder-acting agents, Haddow and Ross[42] began the study of the aromatic nitrogen mustards. Subsequently a large number of related compounds have been prepared and some have been successful in clinical use, eg. chlorambucil and melphalan.

To improve the selectivity of aromatic nitrogen mustard drugs, carbamate (urethane) derivatives were synthesized.[43] Incorporation of the electron attracting N-urethane group lowered the reactivity of the mustard moiety. On hydrolysis of the urethane group and loss of CO_2 (scheme 1),



Scheme1



[5]

Scheme 2

the full reactivity of the parent mustard becomes available. High levels of azo reductase in primary liver tumours led to the introduction of azomustards [5]. These compounds are deactivated by the conjugated ring system and therefore show very low toxicity.[44] On reduction by azo reductase, they are converted to the highly toxic parent phenylene diamine mustard (scheme 2). The elaboration of aromatic nitrogen mustards in these ways was extensively studied by Owen[43] and Ross.[30]

1.6 Alkylation reactions of aromatic nitrogen mustards

Alkylation reactions of nitrogen mustards may be defined as the conversion of

$$R - X + Nu^- \longrightarrow R - Nu + X^-$$

Scheme 3

Where R is the alkyl group, X is the leaving group (usually chlorine) Nu^- is the nucleophilic centre of biologically important molecules which then become alkylated.

Scheme 3 can proceed via two mechanisms, SN1 or SN2 mechanisms. The SN1 mechanism involves slow solvent-assisted ionization of the mustard $(R\text{-}X)_{solv}$, followed by rapid reaction of the resulting carbonium ion (R^+solv) with a nucleophilic centre (Nu^-), as shown in scheme 4.

$$(R - X)_{solv} \underset{r.d.s}{\overset{slow}{\rightleftharpoons}} R^{+}_{solv} + X^{-}_{solv}$$

$$Nu^{-} + R^{+}_{solv} \xrightarrow{fast} R - Nu$$

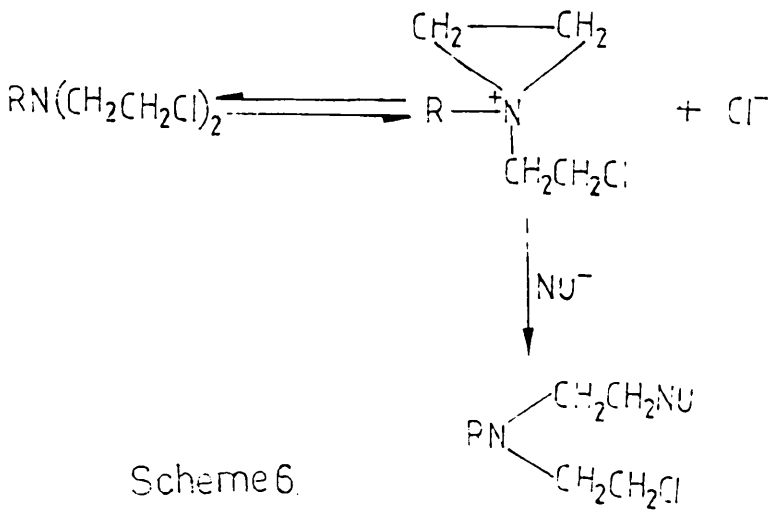
Scheme 4

The SN2 mechanism however involves a concerted alkylation of Nu^{-} by the intact RX as shown in Scheme 5.

$$X - R + Nu^{-} \xrightarrow{r.d.s.} [X \ldots\ldots R \ldots\ldots Nu]^{\ddagger} \longrightarrow R - Nu + X$$

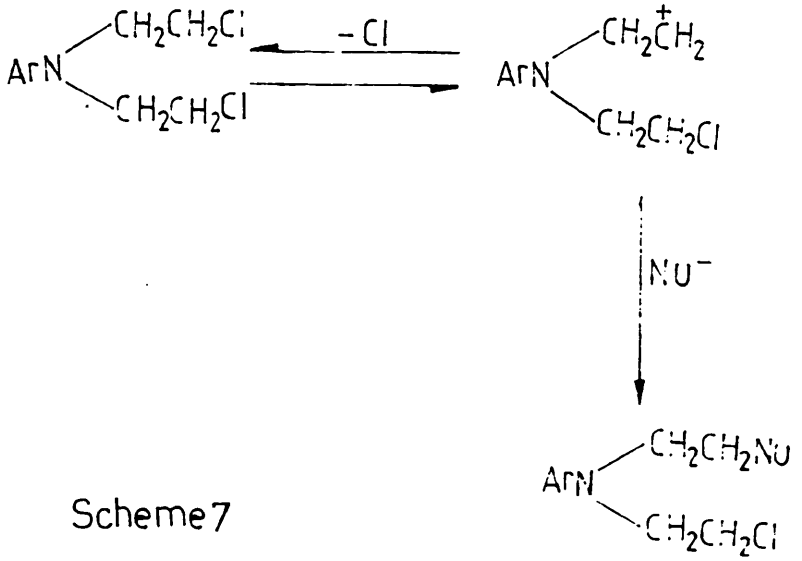
Scheme 5

Aliphatic nitrogen mustards decompose readily in solution to form an aziridinium ion,[45,46] which may then react with the nucleophile (scheme 6).



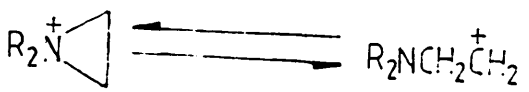
Scheme 6

Initially, aromatic nitrogen mustards were thought to react via a different mechanism. Thus, Ross[47,48] from studies of the hydrolysis of mustards concluded that aziridinium ions were not the reactive intermediates. Instead he concluded these reactions were SN1 with rate limiting formation of carbonium ion intermediates (scheme 7).



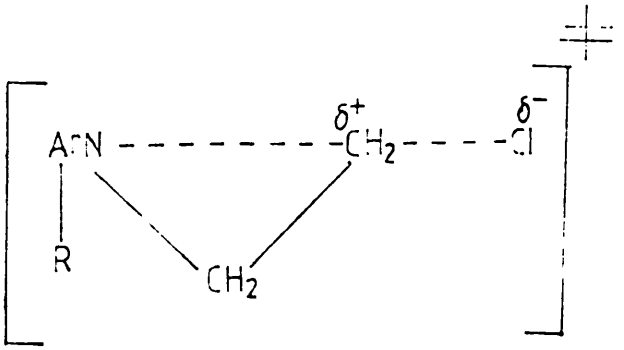
Scheme 7

This mechanism was supported by Bergel,[49] but Leonard[50] believed the carbonium ion was in equilibrium with the aziridinium ion (scheme 8).



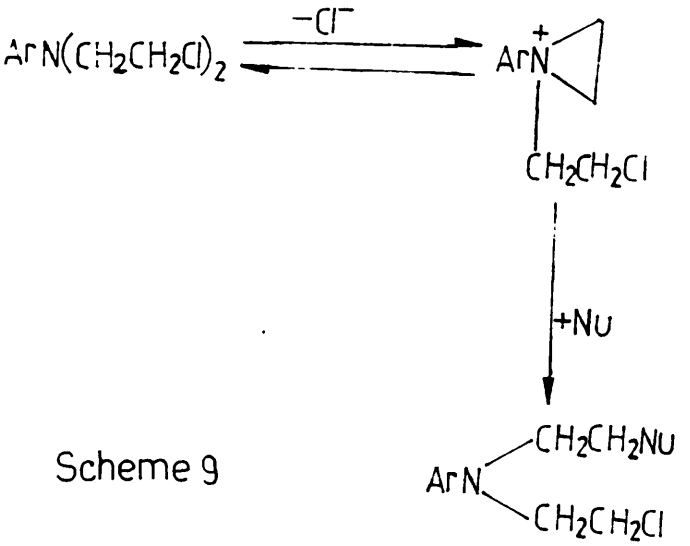
Scheme 8

From the comparative studies of hydrolysis and the acylation of 4 - (4'-nitrophenyl)-pyridine, Bardos[51] et al concluded that both reactions passed through a transition state intermediate [6].



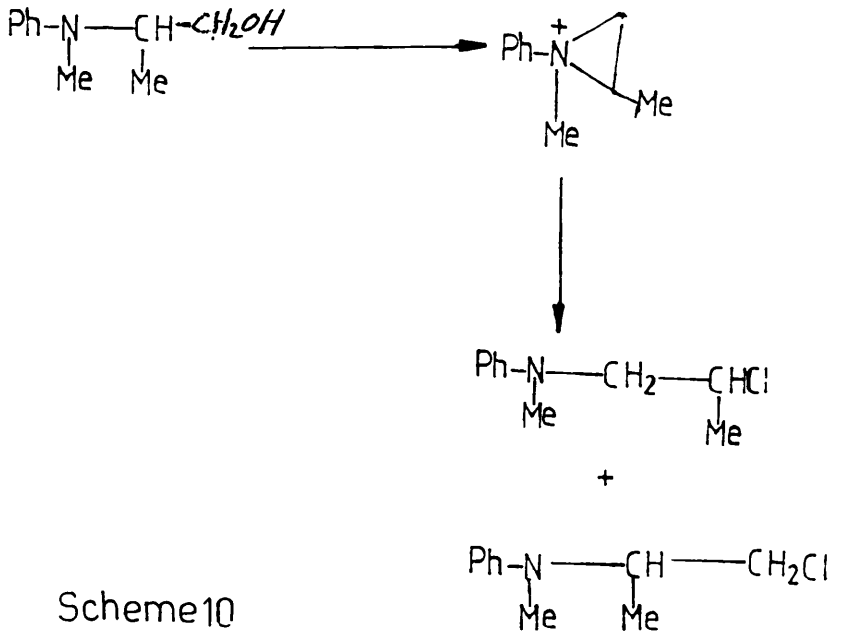
[6]

subsequently all the above mechanisms have been discarded.[35,52,53] The accepted mechanism now is the formation of an aziridinium ion via an intramolecular SN2 reaction similar to that for aliphatic mustards (scheme 9).



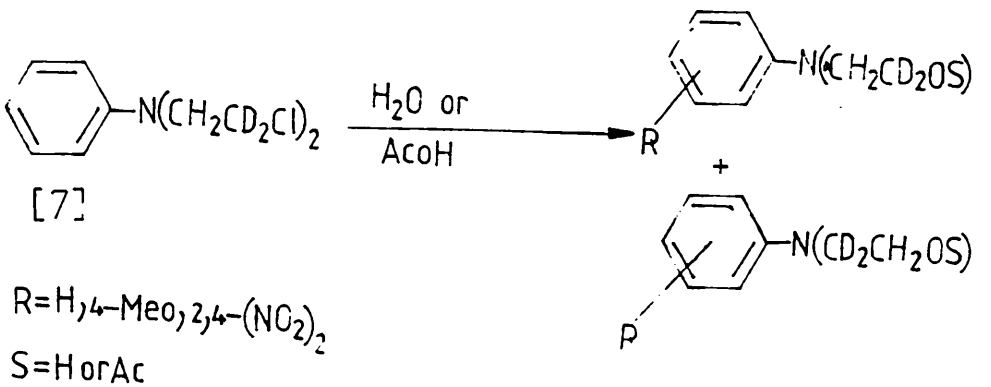
Scheme 9

Owen et al[52] established that [N - methyl - N - (1 - methyl -2-hydroxyethyl] aniline, gave on chlorination predominantly [N-methyl-N-(2-methyl-2-chloro ethyl] aniline plus some unrearranged product (scheme 10), whereas acetolysis gave predominantly [N-methyl-N-(2-methyl-2-acetoxy ethyl] aniline.



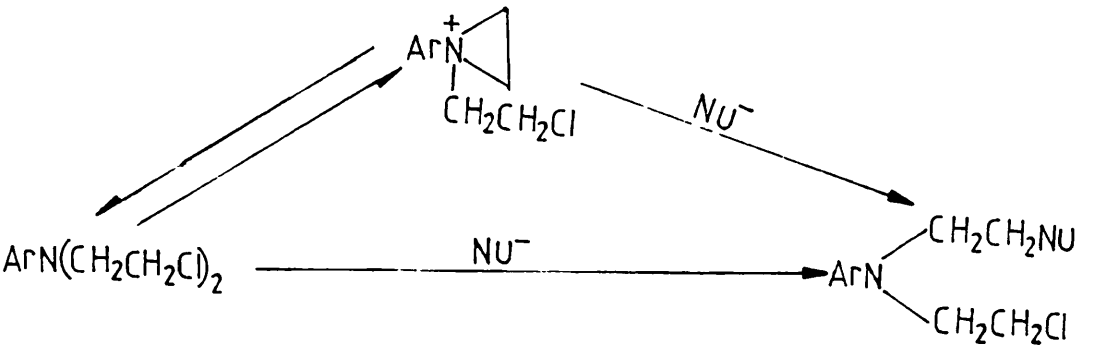
Scheme 10

Scrambling of the deuterium label was observed when the aryl mustards [7] were hydrolysed or acetolysed, proving intermediate formation of an aziridinium ion.[52]



Scheme 11

The treatment with powerful nucleophiles (eg. potassium p-thiocresolate) however, produced no scrambling of the deuterium label which requires direct displacement of Cl^- without intermediacy of the aziridinium ion. Owen and Benn[52] therefore proposed the following mechanism for the reactions of aryl nitrogen mustards (scheme 12).



Scheme 12

The pathway involving the azridinium intermediate is usually preferred, except for reactions with powerful nucleophiles. This dual pathway was confirmed by Sflomas.[54]

1.7 γ-GLUTAMYL TRANSFERASE (γGT)

1.71 Reactions of γGT

γ-Glutamyl transferase catalyses transfer of the γ-glutamyl moiety of glutathione and other γ-glutamyl compounds (the donors) to several acceptors[55,56] normally amino acids and peptides (13). If the γ-glutamyl donor is also the acceptor, auto transpeptidation occurs (14). The enzyme also catalyses the hydrolysis of γglutamyl compounds (15).

γ-glu-x+ acceptor $\rightleftharpoons$ γ-glu - acceptor + H X ..... (13)

γ-glu-x+ γ-glu-X $\rightleftharpoons$ γ-glu - γ-glu-X + Hx ..... . (14)

γ-glu-x+ H_2O ——> glutamate + HX ..... (15)

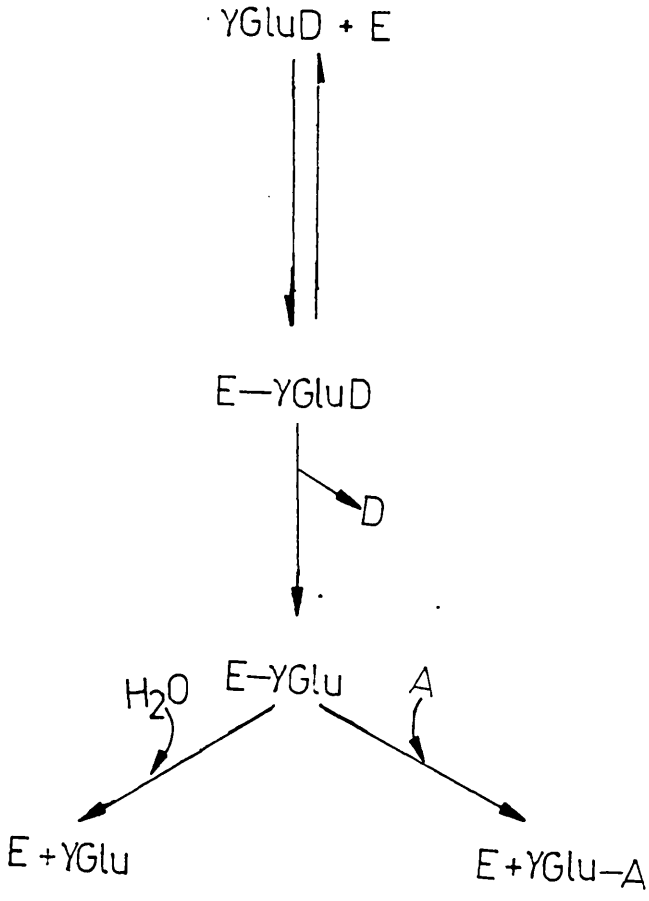
The enzyme transfers γ-glutamyl groups from glutamine, glutathione and other γ-glutamyl derivatives to acceptors. The most active amino acid acceptors include L-cystine[57] and L-glutamine,[58] and the most active di-peptide acceptors are L-methionylglycine, L-glutaminylglycine, L-alanylglycine, L-cystinyl glycine, L-serylglycine and glycylglycine.[59]

Kinetic studies of γGT catalysed transpeptidation and hydrolysis indicate a ping pong mechanism comprised of two half reactions. The first reaction leads to the formation of a covalent γ-glutamyl-enzyme intermediate. This may react with an acceptor to form a γ-glutamyl adduct (γGlu-A) or undergo hydrolysis to glutamic acid[58,59] (scheme 16).

1.72 Occurrence of γGT

γGT was discovered by Hanes et al[60] from sheep kidney. He also demonstrated that it was specific for γ-glutamyl peptides, causing either hydrolysis or transpeptidation reaction of γ-glutamyl peptides to occur.

The enzyme is predominantly membrane bound, the active site being orientated on the outer surface of the cell membrane.[61] The organ with the highest concentration of γGT is the kidney.[62-65] Other sites with substantially lower γGT activity are glandular epithelium of the breast, the primary follicle in the ovary, epididymis and prostate,[66] the bile ducts and the bile canaliculor regions of hepatocytes,[67] jejunal epithelium, choroid plexus of the brain, capillary endothelium[77] and the lower



Scheme 16

epithelium of growing hair follicle.[69] High levels of γGT have been detected in hepatocellulor carcinoma.[70] The levels of γGT in human hepatoma and ascitic fluid has been found to be between 3 to 13 fold as compared to normal liver.[71,72] γGT activity in aflatoxin induced rat hepatoma have been found to be as high as 1000 fold greater than normal rat hepatocytes.[73]

1.73 Physical and chemical properties of γGT

γGT is composed of two sub units 46,000 Daltons and 22,000 Daltons.[74] Results obtained by Tsuji et al[75,76] indicate that the amino terminal portion of the heavy sub unit of γGT contain the portion that anchors the enzyme to the brush border membranes. The hydrolysis reaction catalysed by γGT shows a broad pH optimum rate between 6 and 8 whereas transpeptidation shows an optimum rate between pH 8 and 9.[77,78] The pH optimum for transpeptidation varies for different acceptors.[79]

1.7 4 Possible roles of γGT

Meister suggested in 1973 that γGT plays a key role in the γ-glutamyl cycle, the pathway for the synthesis and degradation of glutathione.[80,81] The combined action of γ-glutamyl cysteine synthetase and then glutathione synthetase[82] produces glutathione from its constituent amino acids. The reactions are considered to facilitate the storage of cellular cysteine as glutathione. γGT catalyses the first step in the decomposition of the glutathione leading to the release of cysteine moiety.

Since intact glutathione is not taken up by cells, it serves as a substrate for membrane bound γGT. The γ-glutamyl residue which is then formed in close association with cell membrane, is then transported into the cell.[83]

McIntyre and Curthays proposed that γGT was also involved in glutathione hydrolysis.[84] Glutathione is transported in blood plasma to the kidney, where it is degraded to its constituent amino acids. Glutathione is resistant to hydrolysis by most proteases and amino peptides. It is thought that the initial step in the degradation of glutathione is catalysed by γGT, which is localised on the external surfaces of the brush border membrane. The γGT catalyzes a hydrolytic rather than transpeptidation reaction. Cook and Peters[85] suggested the γGT is involved in glutamine hydrolysis. They have shown that under physiological conditions and saturating conditions for both glutamine and glutathione, it is glutamine hydrolysis that predominates.

γGT is also involved in the detoxification of foreign compounds. Many strongly electron deficient compounds react with the sulphydryl group of glutathione, to form a glutathione conjugate in a reaction catalysed by glutathione -S- transferase. The γ-glutamyl moeity of the glutathione conjugate is then removed by γGT.[86] Cleavage of the glycine moiety followed by N-acetylation ultimately leads to the formation of mercapturic acid.

1.75 Inhibitors of γGT

The enzyme is inactivated by the irreversible inhibitors O-diazo-acetyl-L- serine (L-azaserine),[86] 6-diazo-5-oxo-L-norleucine (DON),[86] or L-(2β, 5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazole acetic acid (AT-125).[87-89] All of these compounds, are also glutamine antagonists, and they react covalently with γGT at the substrate binding site for the glutamyl portion.

L-serine[90] and D-serine[91] in the presence of borate are both reversible inhibitors of γGT. The serine appears to occupy the active site which is normally involved in binding the α-amine and α-carboxyl groups of glutathione, and the borate anion appears to bridge between an active site hydroxyl group and the hydroxyl group of the serine. In this way the tetrahedral borate complex acts as a transition state analog at the catalytic centre of the enzyme.[91] L-γ-Glutamyl-(o- carboxy) phenyl hydrazide (L-OC) is a tightly bound reversible inhibitor of γGT.[91]

1.76 γGT in Hepatocarcinoma

High serum levels of γGT have been regarded as a sign of hepatobilliary dysfunction.[92,93] In 1972 Fiala et al[94] discovered elevated γGT levels in the liver of rats fed on 3'-methyl-4-dimethylaminobenzene (a liver carcinogen) and subsequently, in transplanted rat hepatomas.[94a] Using histochemical stains Kalengay[95] was able to detect markedly increased γGT levels in Aflatoxin B, induced rat hepatomas. Other measurements have shown up to 1000 fold higher γGT levels in these

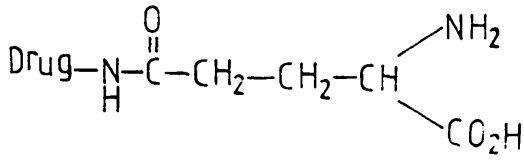
induced hepatomas compared to normal hepatocytes.[73] The findings have been confirmed qualitatively for other experimentally induced rat hepatomas and hepatoma cell cultures.[96,97] Elevated γGT levels have also been reported for human hepatocellular carcinomas.[71,72]

1.8 γGT N-Mustard Prodrugs

As mentioned previously, there is no effective treatment for primary hepatoma. It is particularly difficult to direct chemotherapeutic agents to the hepatoma tissue.

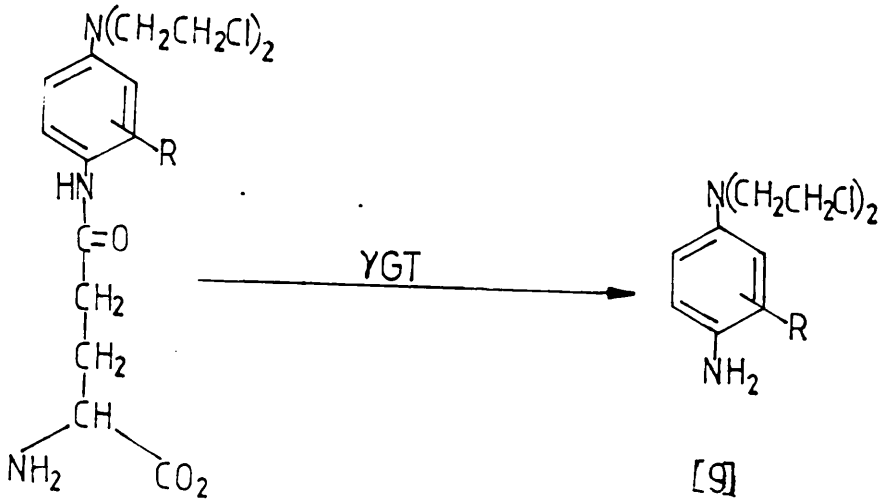
Most anti-cancer drugs eg. N-mustards are relatively reactive and they would decompose before reaching the liver. One difference in hepatoma cells compared to normal hepatocytes is their high γGT activity. In principle, this difference can be utilised to selectively destroy hepatoma cells.

γGT has a relatively broad substrate specificity towards the γ-peptide linkage of glutamic acid [8]. Thus, by linking the cytotoxic agent to the γ-carboxy group of glutamic acid, the agent could be selectively released in the hepatocellulor carcinoma, to localise its pharmacolgical effect. Normal hepatocytes with lower γGT levels would be less likely to release the cytotoxic agent.



[8]

A group of widely used anti-cancer drugs are the aromatic nitrogen mustards, and phenylene diamine mustard [9] is one of the most powerful of these compounds. Its reactivity relates to electron donation by the 4-amino group which increases the basicity of the tertiary nitrogen atom and facilitates the formation of the azridinium ion intermediate. Coupling of this 4-amino group to the γ-carboxyl group of glutamic acid reduces the electron donation. Thus the γ-glutamyl phenylene diamine mustard adduct [10] should be less reactive than the free phenylene diamine mustard. In principle, on reaching the hepatoma tissue [10] would be preferentially converted to the highly toxic parent mustard [9] as outlined in scheme 17.

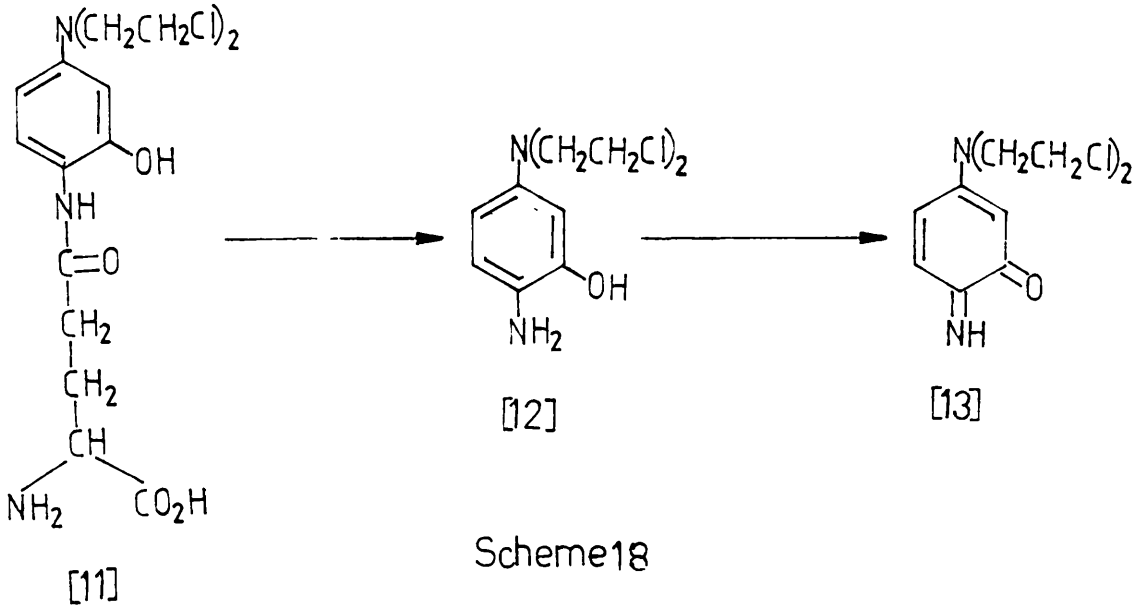


[10] [9]

Scheme17

Further, it is known that many tumours, particularly large ones with ischaemic zones, have an enhanced reductive capability possibly because of the low oxygen tension of the ischaemic zones. This difference from normal cells may also be utilised to enhance the specificity of anti-cancer agents. For example, the specificity of the γ-glutamyl aromatic mustard could be further increased by structural factors leading to activation in a reductive environment,

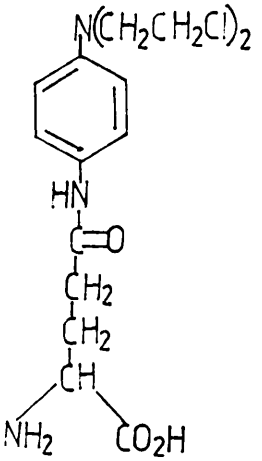
compared to deactivation in an oxidative environment. This goal could be realised by inclusion of the 3-hydroxyl substituent as in [11]. In hepatoma tissue, elevated levels of γGT would preferentially cleave the γ-peptide linkage to release the highly toxic N,N-bis-(2-chloro ethyl)-3-hydroxy-4-phenylene diamine [12]. However, if [12] were to escape from anoxic regions of the hepatoma or the prodrug itself were cleaved at other sites of high γGT, the oxygenated conditions would convert the mustard to the quinone imine [13] (scheme 18). Neither the quinone nor imine group is able to activate the mustard by electron donation, but the quinone-imine moiety, itself may be cytotoxic.



Scheme18

γ-glutamyl prodrugs were first synthesized to show specificity towards the kidney. γ-Glutamyl dopamine was synthesized by With et al[98] as a specific renal vasodilator. This approach enabled lower concentrations of the prodrug to be used, therefore preventing effects such as hypertension caused by large doses of free dopamine.

γ-[N,N-bis-(2-chloro ethyl)-4-phenylene diamine]-glutamic acid [14] was synthesized by Smith et al,[99] as a potential anti-cancer prodrug. They also showed [14] was a substrate for γGT and M.Manson et al[73] showed [14] was more toxic towards γGT positive hepatoma cells than to normal γGT negative hepatocytes.



[14]

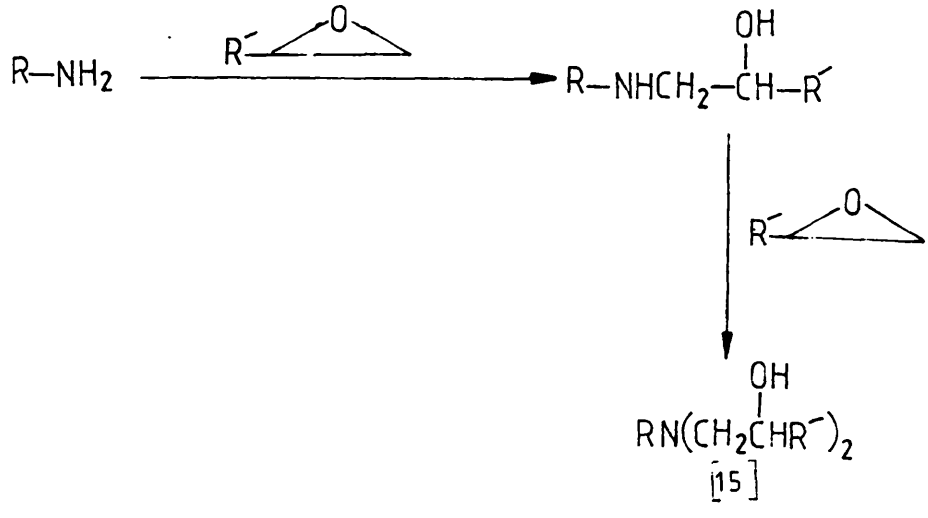
CHAPTER 2

CHEMICAL SYNTHESIS

2.1 Preparation of nitrogen mustards

The preparation of nitrogen mustards usually involves the synthesis of the corresponding bis-(2-hydroxy ethyl) amino derivative [15] followed by halogenation of the diol.

There are three main methods of preparing the bis-hydroxy amines. The most common involves reaction of the amine with an epoxide[100,101] (scheme 19). The epoxide and the amine are heated in a carius tube at a temperature above 150°C. At lower temperatures large amounts of the mono substituted products are produced[47].



Scheme 19

In the case of ethylene oxide it is important to use the theoretical quantities of the epoxide otherwise polymers of type [15a] are obtained.

$$R\text{-}N\text{-}[CH_2(OCH_2CH_2)_nOCH_2CH_2OH]_2$$

[15a]

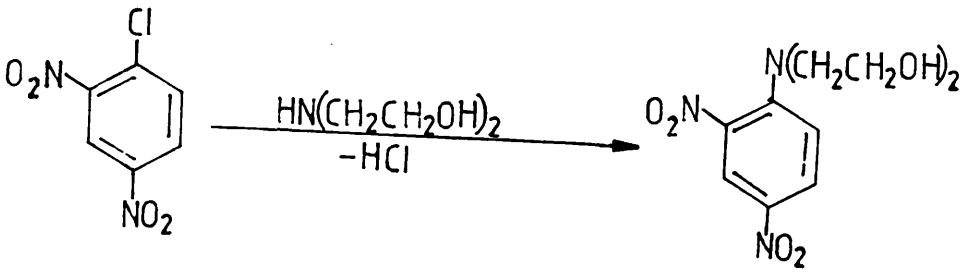
Alternatively the epoxide and amine are stirred at room temperature or below in the presence of 50% aqueous acetic acid. An advantage of this method is that excess ethylene oxide can be used.

Another general method for the synthesis of diols is the reaction of amines with halohydrin in the presence of a base, usually calcium carbonate. The reaction is carried out either by heating under reflux in aqueous media[48] or by heating solvent free at a temperature above 100°C[102]. The base neutralizes the acid co product which would protonate the amine reactant (scheme 20).

$$R\text{-}NH_2 + 2\ X\text{-}CH_2CH_2OH \xrightarrow{-2HX} R\text{-}N\text{-}(CH_2CH_2OH)_2$$

scheme 20

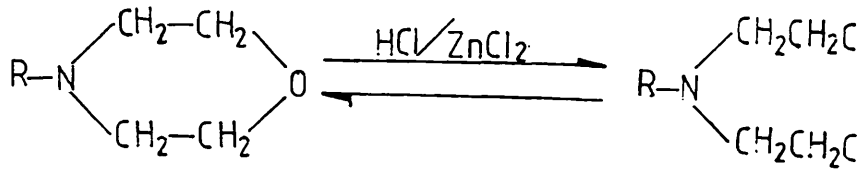
A third method for the formation of bis-hydroxy ethylamines [16] is the reaction of di-ethanolomine with 'active' halogen compounds such as 2,4 - dinitrochlorobenzene. This is usually carried out by heating under reflux in ethanol[103] (scheme 21).



Scheme 21

Replacement of the diol by halogen groups can be achieved in several ways, but certain reagents give cleaner products and in high yields. The most common reagents for chlorination are $POCl_3$, PCl_5, and occasionally $SOCl_2$[48]. Similarly PBr_5 is used for bromination of the diol[48]. 4-Toluenesulphonyl chloride is often used to activate the diol[104-106] followed by displacement of the tosyl esters using either Li Cl or $CaCl_2$.

Nitrogen mustards have also been prepared by directly heating the relevant morpholino derivative [17] with HCl and $ZnCl_2$ at 180°C [107] (scheme 22).



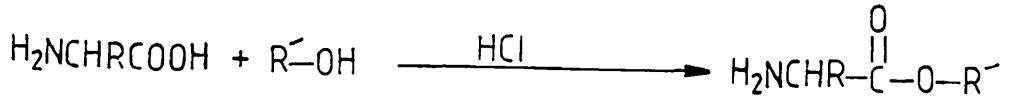
[17]

Scheme 22

Frequently yields from the halogenation step are poor, and the products can only be purified chromatographically.

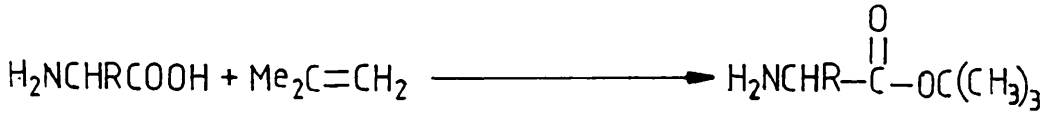
2.2 Protection of glutamic acid

The carboxyl groups of amino acids are usually protected by acid-catalysed esterification with a suitable alcohol[108](scheme 23).



Scheme 23

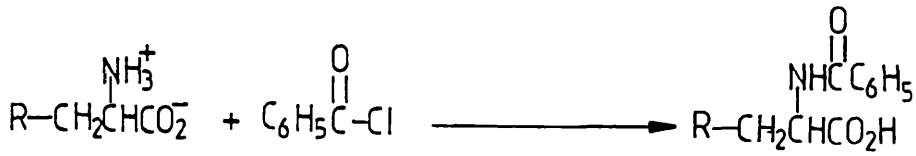
Tertiary butyl esters, however can be obtained by acid (H_2SO_4) catalysed addition of isobutene[109] (scheme 24). One advantage of butyl esters is their relatively easy removal by the action of either HCl in benzene or HBr in acetic acid.



Scheme24

Benzyl esters, first introduced by Bergmann et al[110,111], are also useful protecting group because of their ready removal by low pressure hydrogenolysis over a palladium catalyst[112]. The benzyl ester is normally obtained by esterification in benzene with azeotropic removal of water[113]. Substituted benzyl esters e.g. 4-nitro[114] and 4-methoxy[115] have also been used and can also be cleaved by either catalytic hydrogenation or acid catalysted hydrolysis.

The most common method for the protection of the amino groups of amino acids involves conversion to a urethane derivative by treatment under Schotten-Baumann conditions[116,117], with an appropriate alkyl chloroformate (Scheme 25). Commonly, the N-benzyloxycarbonyl derivative is prepared, which can be removed by hydrogenation[118], anhydrous trifluoroacetic acid[119], sodium in liquid ammonia[120] or HBr in acetic acid[121,122]. Alternatively, the t-n-butyl oxycarbonyl derivative is prepared [123] and this can be removed by HCl in acetic acid.



Scheme 25

The formyl group studied first by Emil Fischer[124], and then by Sheehan et al[125], has also been extensively used for the protection of α-amino-acids. The substrate is usually formylated with formic acid and acetic anhydride and deprotected either with alcohols containing dilute HCl[125] or by oxidation with hydrogen peroxide[126].

2.3 Coupling procedures

The mixed anhydride and the carbodiimide methods are the two most popular procedures for the coupling of amino acids via formation of an amide bond.

2.31 Mixed anhydride method

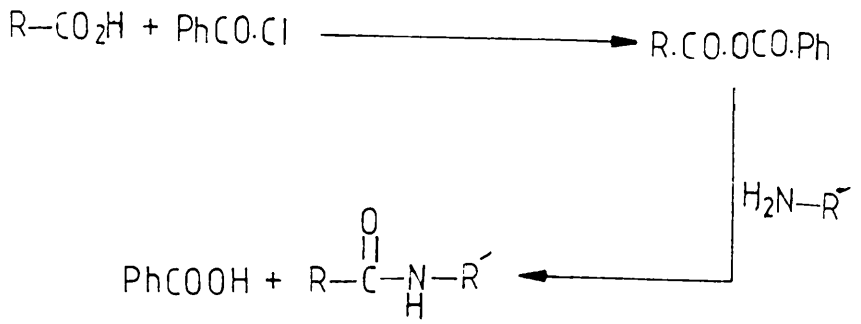
This method was introduced by Wieland[127] using mixed anhydrides of the N-protected amino acid and benzoic acid (Scheme 26), he then reacted the mixed anhydride with an amino acid ester to form the peptide. Nowadays mixed anhydrides derived from ethyl chloroformate (scheme 27) or isobutyl chloroformate are more often used[128].

2.32 Carbodiimide method

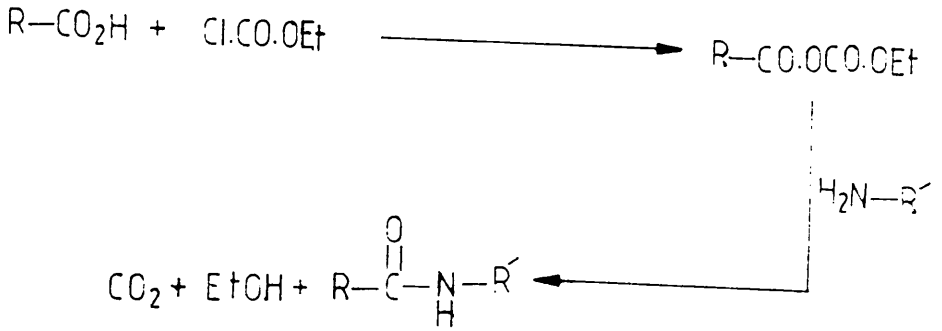
Sheehan and Hess[129] introduced this procedure in 1955 using N,N-dialkylated carbodiimide, or preferably N,N-dicyclohexyl carbodiimide (scheme 28). It quickly became one of the most important coupling methods, because of the simplicity and low tendency to induce racemization.

The reaction is usually carried out in dichloromethane and normally allowed to proceed at room temperature for 4 to 18 hours. The dicyclohexylurea precipitates and can be removed by filtration.

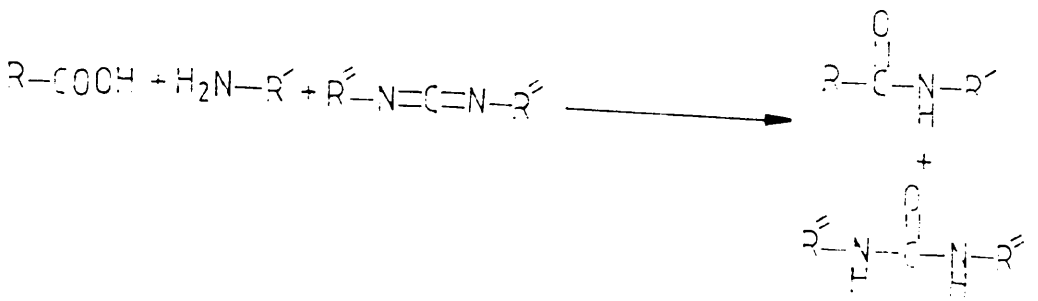
Many other coupling methods for peptides are known. These involve acid chlorides, acid azides, mixed anhydrides derived from inorganic acids, activated esters, isocyanates, acylimadazole and isooxazolium salts. Their details can be found in the text by Schroder and Lubke[130].



Scheme 26



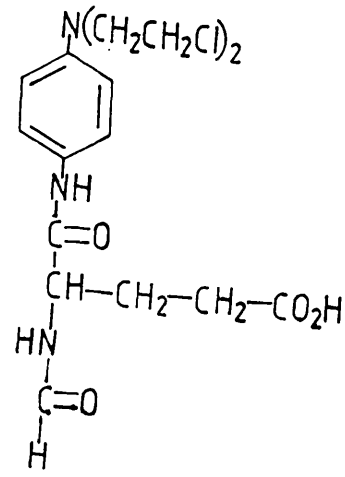
Scheme 27



Scheme 28

2.4 Synthesis of L-glutamyl Mustard Adducts

Johnson[131] attempted the synthesis of ≺-D,L- [N,N-bis-(2-chloro ethyl)-4-phenylene diamine]- ≺-N-formyl glutamic acid [18], using benzyl ester to protect the γ-carboxyl group during coupling. He failed, however, to remove the benzyl ester by hydrogenolysis over Adam's catalyst on 5% palladium/charcoal.



[18]

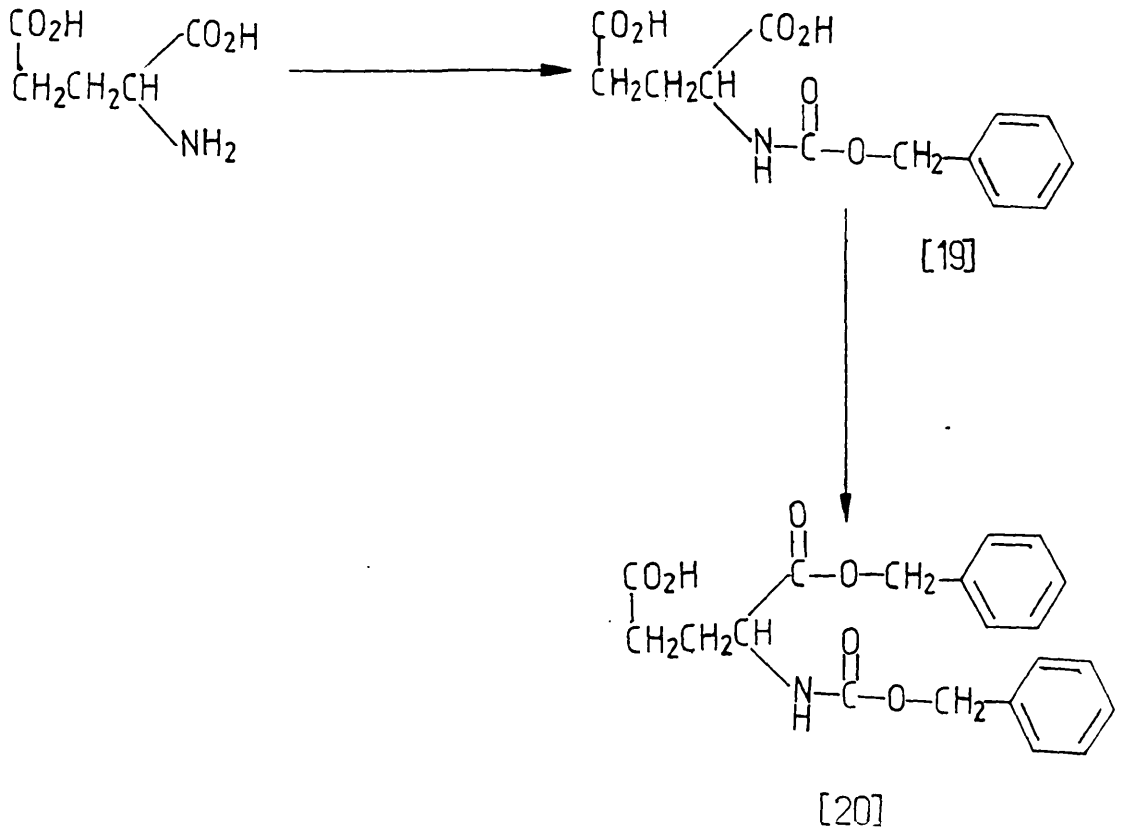
Smith et al[99] synthesized the γ-L- [N,N-bis-(2-chloroethyl)-4-phenylene diamine] - glutamic acid by conventional procedures using ≺-N-t-butyloxycarbonyl and ≺-t-butyl protecting groups which were then removed by acid hydrolysis. The product was isolated as the HCl salt. In our hands, the HCl salt was too hygroscopic to obtain a micro analytically pure sample. The free amine, however, was non-hygroscopic and quite stable at temperatures below -15°C.

The synthesis of γ-L-glutamyl-4-phenylene diamine mustards can be conveniently sub-divided into four steps: 1), synthesis of the glutamic acid fragment; 2), synthesis of the 4-phenylene diamine mustard fragment; 3), coupling of the two fragments; and 4), removal of protecting groups.

2.41 Glutamic Acid Fragment

To ensure coupling proceeds only at the γ-carboxyl function, both the α-carboxyl and α-amino groups of the glutamic acid must be deactivated with suitable protecting groups. As mentioned above, protection can be effected in several ways. Several factors suggested that in the present case protecting groups removable under the mildest conditions would be especially desirable. For example, deprotection under acidic conditions produces amine salts of the γ-L-glutamyl-4-phenylene diamine mustards which are extremely hygroscopic and difficult to purify. Deprotection under basic conditions, however, may result in displacement of the mustard halogens. Accordingly, it was decided at the outset to protect the glutamic acid fragment whith α-N-carbobenzoxy and α-benzyl ester groups because both can be removed by hydrogenolysis under very mild, neutral conditions.

The synthesis of the γ-glutamyl fragment is outlined in scheme 29. It involves two steps, the α-amino group was protected first by formation of the N-carbobenzoxy glutamic acid [19]. This was achieved by reacting L-glutamic acid with a 20% molar excess of benzyl chloroformate in 2 m NaOH at a temperature below 3°C for


CO2H
CO2H
CH2CH2CH
NH2
CO2H
CO2H
CH2CH2CH
O
N
H
C
O
CH2
[19]
O
C
O
CH2
CO2H
CH2CH2CH
O
N
H
C
O
CH2
[20]

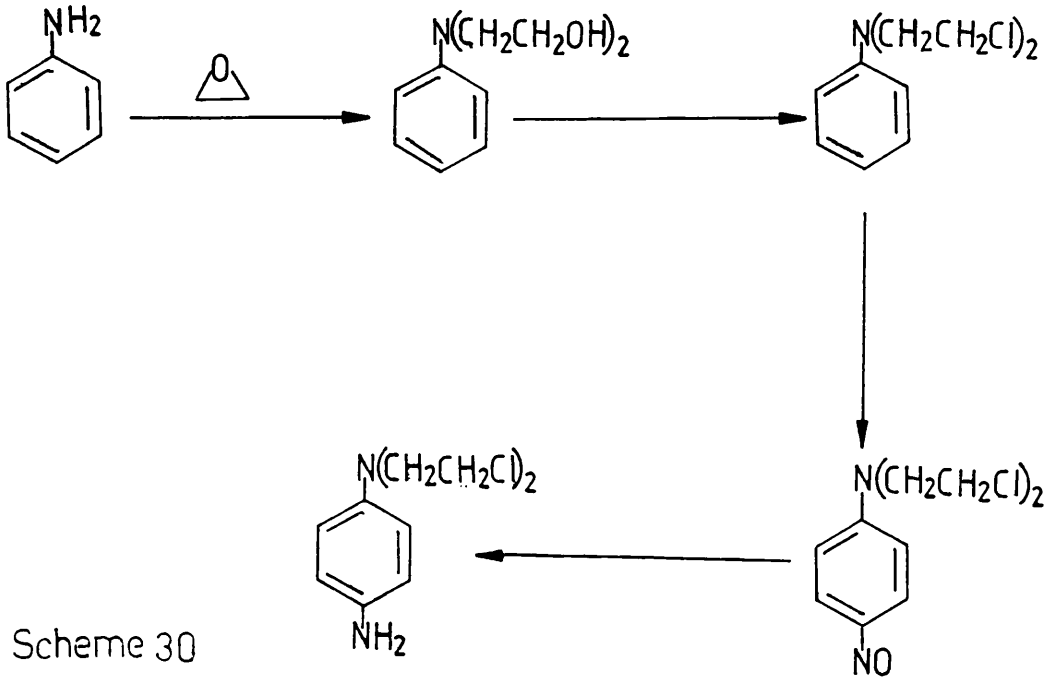
Scheme 29

24h. After washing with ether and acidification to pH 1.5 with conc HCl, the resulting solution was extracted with ethyl acetate which on evaporation gave a white solid of N-carbobenzoxy glutamic acid. This was carried onto the next step without further purification. Yields were usually ca 90%.

The ≺-carboxyl group was then protected by formation of the α-benzyl-N-carbobenzoxy-L-glutamate [20]. This was accomplished by reacting [19] with benzyl bromide. This involved dissolving in DMF and adding an equimolar amount of di-cyclo hexylamine. The mixture was heated to 80°C and an equimolar amount of benzyl bromide added. After removal of the di-cyclohexylamine hydrobromide by filtration, the DMF was removed by washing with water. Removal of the DMF gave a yellow oil of [20] in 62% yield. This compound was used in the coupling step without further purification. The reaction of [19] with benzyl bromide is highly selective, as shown by the 62% yield of [20]. The γ-carboxyl group remains unesterified and therefore available for peptide bond formation. This selectivity arises because the basicity of the α-carboxyl group is lowered by the adjacent α-N-carbobenzoxy substituent to such an extent that only the α-carboxyl group reacts with benzyl bromide. The presence of ten aromatic protons in the ^{1}H-nmr spectrum at a chemical shift of 7.3 relative to TMS shows the presence of two benzene rings in [20].

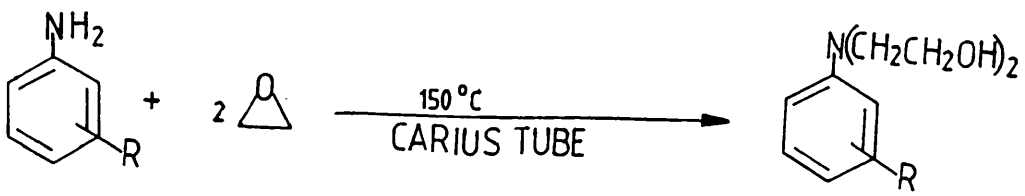
2.42 4-Phenylene Diamine Mustards

These compounds are well known. The usual synthetic route from aniline is outlined in scheme 30. The amino substituent is first converted to the N,N-bis-2-chloroethyl amino substituent. This substituent is ortho/Para directing towards electrophilic reagents, so the 4-amino moiety can be introduced by either nitrosation or nitration followed by reduction.



Scheme 30

The first step in the synthesis involved conversion of the aniline to the corresponding diol by bis hydroxyethylation. This was carried out by reacting the appropriate aniline with a 2-fold molar excess of ethylene oxide in a Carius tube (without solvent) at 150°C for 16-24h. Yields in the region of 90% were obtained.

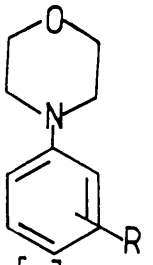


R = H, 3-OMe

Scheme 31

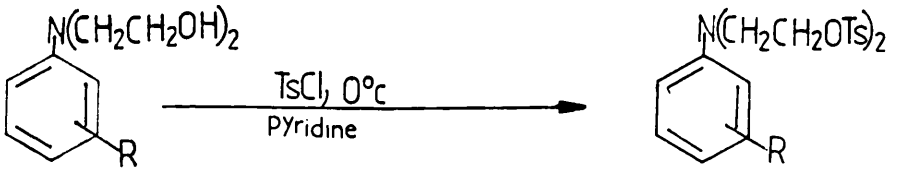
The N,N-bis-(2-hydroxy ethyl) anilines were usually purified by recrystallisation from benzene, but N,N-bis-(2-hydroxy ethyl) -3-methoxy aniline was only obtained as a oil and was carried on to the next step without purification.

The N,N-bis-2-chloro ethyl aniline can be obtained by simple chlorination of the diol. Initially this reaction was carried out by heating under reflux with phosphorus pentachloride in benzene, but yields of the N,N-bis-2-chloroethyl anilines were only 40-45%, and the reaction was not clean. The major by-product appeared to be the N-arylmorpholine [21].



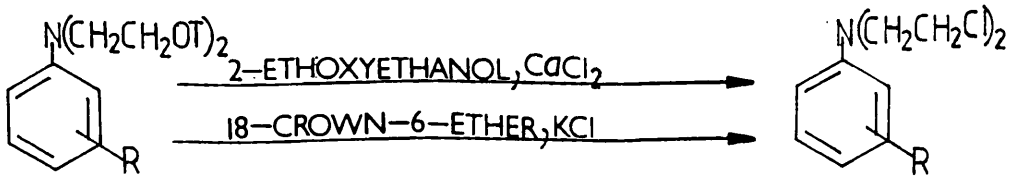
[21]

Subsequently, the diol was first converted to the di-tosyl ester and then to the N,N-bis-2-chloro ethyl aniline. This procedure significantly improved the yield. The tosyl ester formation was carried out by reacting excess tosyl chloride with the relevant diol in pyridine solvent at 0°C (Scheme 32). Yields of the tosyl ester were in the range 85-95%, after purification by recrystallisation from either ethanol or methanol. The ^{1}H-nmr spectrum showed the presence of 13 aromatic protons (8 of which were associated with the two tosyl groups) plus a singlet at δ ca 2.4 corresponding to 6 protons of the two methyl substituents of the tosyl groups.



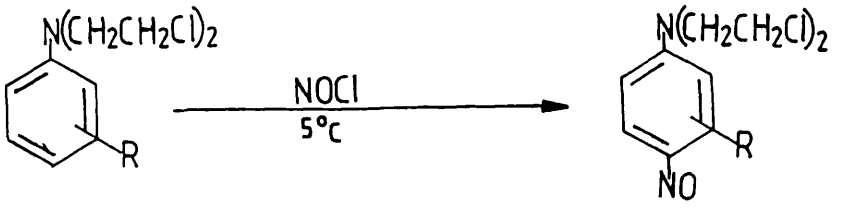
Scheme 32

The tosyl ester was displaced readily by chloride ion either by heating under reflux in toluene in the presence of KCl plus 18-crown-6-ether or by heating with $CaCl_2$ at 120°C for 20 min in 2-ethoxy ethanol (Scheme 33). Both methods gave clean products, but higher yields (ca 85%) were obtained with $CaCl_2$ and this was therefore preferred. The products were normally obtained as solids and purified by crystallisation either from benzene/petroleum ether or ethanol. The ^{1}H-nmr spectra show the absence of either aromatic or methyl protons associated with the tosyl groups.



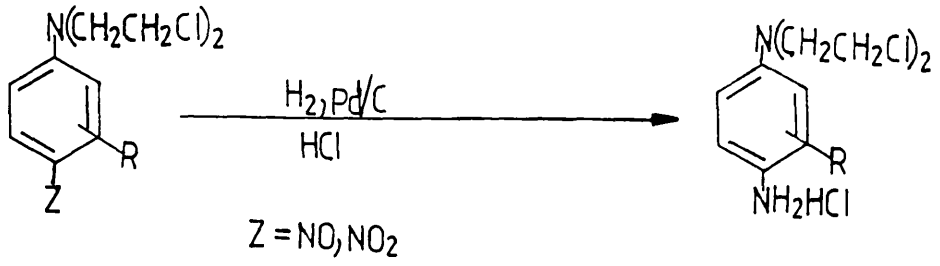
Scheme 33

The N,N-bis-2-chloro ethyl anilines were nitrosated at the 4-position of the aromatic ring using sodium nitrite in concentrated hydrochloric acid at 5°C (Scheme 34). This reaction is virtually 100% regiospecific. The 4-nitroso product was recrystallised from ethanol to give dark green crystals.



Scheme34

Both the 4-nitroso and 4-nitro groups of aromatic nitrogen mustards are reduced to the corresponding amine by hydrogenation at room temperature and pressure over palladium/charcoal catalyst. This method was used because the conditions are mild (Scheme 35). The N,N-bis-2-chloro ethyl-4-phenylene diamine mustard produced, was isolated as the HCl salt after carrying out the hydrogenation in methanol. After the theoretical amount of hydrogen had been taken up, the solution was filtered into a solution of methanolic HCl (made by the addition of acetyl chloride to methanol). The solvent was evaporated to give a yellow solid, which as already mentioned by Owen et al[132], rapidly discoloured on isolation, probably due to polymerisation and/or oxidation. Due to their instability, the phenylene diamine mustards, were rapidly carried on to the next step of the synthesis without further purification. A small amount of N,N-bis-(2-chloro ethyl)-4-phenylene diamine (PPDM) was obtained microanalytically pure by recrystallising several times from methanol/ether. For the HCl salts of the phenylene diamine mustards the ^{1}H-nmr spectra showed a broad band at δ ca 10.0 ppm relative to TMS corresponding to the amine hydrochloride moiety.

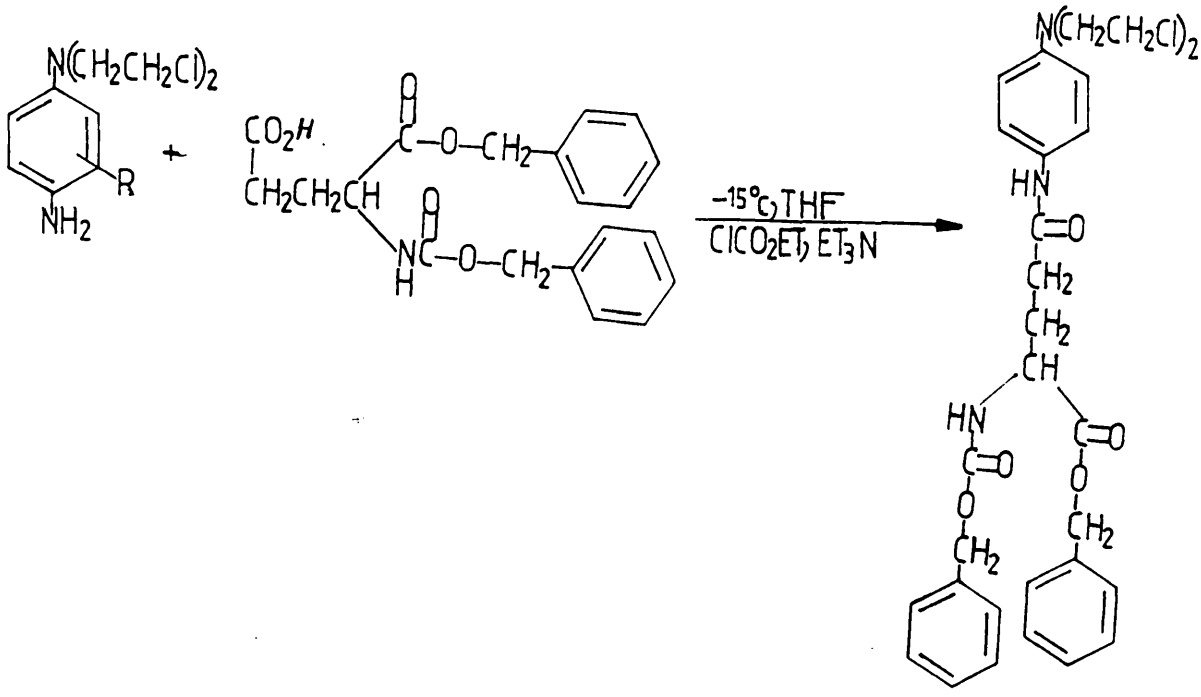


Scheme 35

2.43 Coupling

For all the compounds synthesised, the glutamic acid fragment and the 4-phenylene diamine mustard fragment were coupled using the mixed anhydride procedure.

The 4-phenylene diamine mustard HCl salt was first neutralised with triethylamine in THF and then reacted for 45 min at minus 10-15°C with the mixed anhydride obtained from the protected glutamic acid and ethyl chloroformate in dry THF. It was essential to keep the temperature below -10°C and to exclude moisture to avoid unproductive decomposition or hydrolysis of the mixed anhydride. The pre cooled solution of 4-phenylene diamine mustard in THF or ethyl acetate was added to the mixed anhydride whilst maintaining the temperature below -10°C, and it was found by trial and error that the optimum reaction time for these conditions was 2h. After filtering off the precipitated triethylamine hydrochloride and evaporating the solvents, the resultant brown oil was purified by column chromatagraphy using silica and ethyl acetate/petroleum ether or ether as eluents. This usually gave a solid product which was recrystallised from ethyl acetate/petroleum ether. The yields of purified γ-L-glutamyl mustard adduct were in the region of 45%.

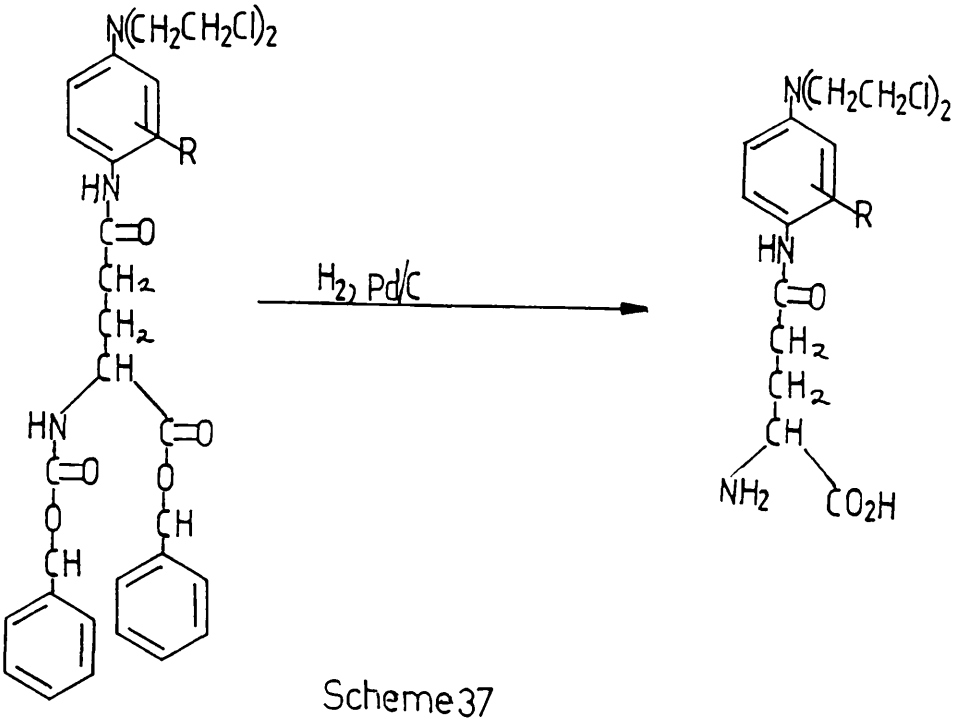


Scheme 36

The ^{1}H-nmr spectra of these adducts showed the presence of 14 aromatic protons, four of which are A'B' coupled and therefore belonging to the phenylene diamine mustard. The remainder formed a poorly resolved multiplet at 7.6 ppm associated with the α-N-carbobenzoxy and α-benzyl ester protecting groups. The IR spectra showed characteristic bonds at 1680 and 1740 cm^{-1}, belonging to the C = 0 of the amide and benzyl ester groups, respectively.

2.44 Removal Of Protecting Groups

The α-benzyl ester and α-N-carbobenzoxy protecting groups were removed by hydrogenolysis at atmospheric pressure and temperature over 5% palladium charcoal catalyst in a mixture of dichloromethane and methanol (1:2.5). The reaction was left stirring overnight, after which the catalyst was filtered off. On removal of the solvents, the γ-L-glutamyl-4-phenylene diamine mustard was obtained as a white or grey solid (Scheme 37). The solids were then recrystallised from a mixture of methanol and ethanol. The ^{1}H-nmr spectra of these solids were devoid of 10 aromatic protons present in the starting material, indicating the loss of the two benzyl protecting groups. Attempts were made to obtain the γ-L-glutamyl mustard adduct as HCl salts by the addition of one equivalent of methanolic HCl following the procedure described previously. On filtration of the recrystallised solid, however, the white solid rapidly transferred into a brown oil. Despite attempts to exclude moisture (e.g. filtration in a dry glove box) a microanalytically pure sample was not obtained. Other salts (e.g. acetate, trifluoroacetate, hydrobromide) of the γ-L-glutamyl mustard adducts were prepared, but all proved extremely difficult to purify. Indeed, the first microanalytically pure sample was obtained by forming the dicyclohexylamine salt of the α-carboxyl group of the γ-L-glutamyl mustard adduct. This was achieved by adding an equimolar amount of dicyclohexylamine to the hydrogenation mixture. On filtration and evaporation of the solvents, a white solid was obtained. After dissolving the solid in a small amount of absolute ethanol and cooling, a white powder dropped out of solution. This proved to be (after spectral analysis) the free γ-L-glutamyl-4-phenylene diamine mustard which was microanalytically pure.



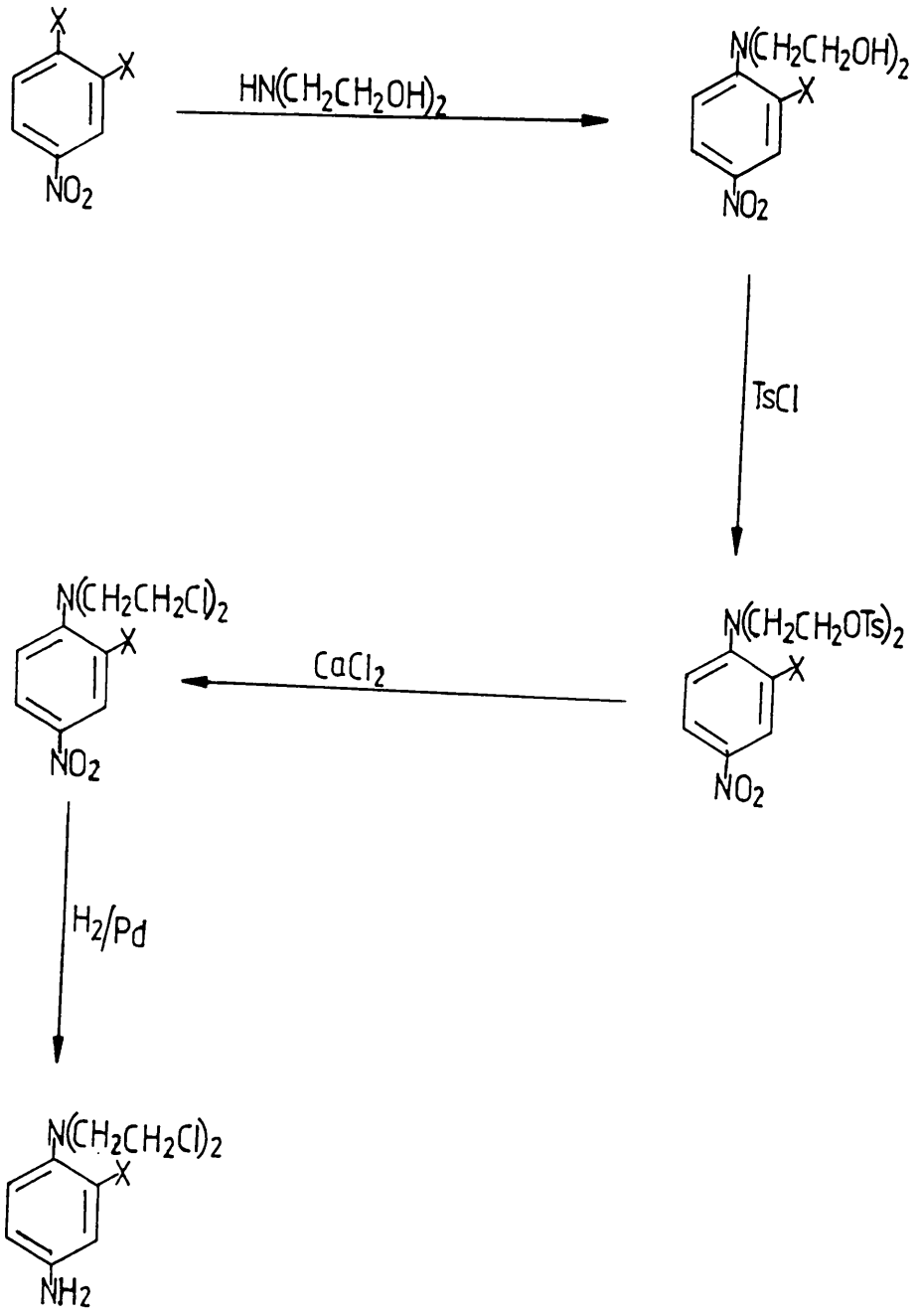
Scheme 37

2.45 Synthesis Of γGPDM Analogues

As reported in Chapter 4, γGPDM itself was cytotoxic to the BL8L (normal) cells. The toxicity of the γ-L-glutamyl-4-phenylene diamine mustards should be reduced by lowering the basicity of the tertiary nitrogen atom of the mustard moiety. This can be achieved by electron withdrawing groups in the benzene ring at the 2- and 6-positions. The nature of these substituents is critical. If too strongly electron withdrawing, the parent 4- phenylene diamine mustard will probably exhibit only feeble cytotoxicity. If bulky, the tertiary nitrogen atom of the mustard moiety may be twisted out of the plane of the benzene ring with a consequential increase in its basicity and therefore cytotoxicity of the γ-L-glutamyl

mustard adduct. Consideration of the factors suggests that the most suitable substituent would be a single fluoro or chloro group. It was therefore decided to synthesise and examine the cytotoxic properties of γ-L- [N,N-bis-(2-chloro ethyl)-2-fluoro-4-phenylene diamine] -glutamic acid and γ-L- [N,N-bis-(2-chloro ethyl)-2-chloro -4-phenylene diamine] -glutamic acid.

The presence of 2-fluoro and 2-chloro substituents lowers the bascicity of aniline, and not surprisingly on reaction with ethylene oxide lower yields (55-65%) of the corresponding diol were obtained. To achieve better yields, an alternative synthesis involving nucleophilic displacement by diethanolamine was employed. This synthesis is outlined in Scheme 38. The 4-nitro and 2-halide substituents activate the benzene nucleus to nucleophilic substitution and the halogen at the 1- position should be amenable to nucleophilic displacement by di-ethanol amine. This procedure was used previously by Medici et al[132] to prepare diols of various chloro substituted 4-nitro anilines. The appropriate 3,4-halogeno nitro benzene was reacted with di-ethanolamine without solvent at 110°C-120°C to give the required diol (Scheme 38). The reaction of diethanolamine with 3,4-dichloro nitro benzene was followed by TLC and required 24h for completion, whereas with 3,4- di fluoro nitro benzene the reaction (again by TLC) was complete in 5h. Further, with 3,4- di chloro-4-nitro benzene, the reaction gave some 3,3'4,4'-tetrachloroazobenzene as a side product. No corresponding tetra fluoro azo side-product formed with 3,4- di fluoro-4-nitrobenzene. Consequently, higher yields of N, N-bis-(2-hydroxy ethyl) amino-2-fluoro-4-nitro benzene (75%) were produced than the corresponding-2-chloro diol (58%).


X
X
NO2
HN(CH2CH2OH)2
N(CH2CH2OH)2
X
NO2
TsCl
N(CH2CH2OTs)2
X
NO2
CaCl2
N(CH2CH2Cl)2
X
NO2
H2/Pd
N(CH2CH2Cl)2
X
NH2

Scheme38

Subsequent steps and yields in the synthesis of γGPDMCl and γGPDMF were similar to those described previously for γGPDM. Thus N,N-bis-(2-hydroxy ethyl) amino-2-fluoro-4-nitro benzene and N,N-bis-(2-hydroxy ethyl) amino-2-chloro-4-nitro benzene were tosylated and the tosyl groups replaced by chloride. The 4-nitro substituent was then reduced by hydrogenation at room temperature and pressure over Pd/C catalyst to give the corresponding 4-phenylene diamine mustard as hydrochloride salt. The mustards, after neutralisation, were then coupled with the protected glutamic acid and finally, the deprotection of the γ-glutamyl substituents was effected by catalytic hydrogenation.

2.5 Synthesis of N,N-(bis-2-bromo ethyl) and N,N-bis-(2-bromo propyl)-4-phenylene diamine mustards

These compounds were synthesised by analogous procedures to those described for the N,N-bis-(2-chloro ethyl)-4-phenylene diamine mustards.

In the synthesis of the mustard fragments, bromination of the N,N-bis-(2-hydroxy ethyl) aniline proved difficult. The PBr_3, PBr_5 and thionyl bromide reagents used previously[47,48] gave low yields (30-55%) and extensive chromatography was required to gain pure products. Reaction of the corresponding N,N-bis-(2-tosyl ethyl) aniline with excess calcium bromide in 2- ethoxyethanol at 120°C for 30 min, followed by evaporation of the solvent, extraction with benzene and then evaporation of benzene gave a white solid of N,N-bis-(2-bromo ethyl) aniline or a yellow solid of

N,N-bis-(2-bromo ethyl)-2-fluoro-4-nitro aniline. These compounds were recrystallised from ethanol or methanol to obtain microanalytically pure samples in yields of ca 80-85%. The ^{1}HNMR spectra indicated the absence of aromatic protons belonging to the tosyl groups and the IR spectra indicated the absence of the S = O stretch.

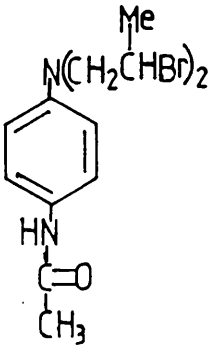
Scheme 39

It was discovered in the synthesis of γ-L- [N,N-bis-(2-bromo ethyl)-4-phenylene diamine] -glutamic acid from N,N-bis-(2-bromo ethyl)-4-nitro aniline, that the best yields were obtained without attempting to isolate the intermediate, HBr salt of the phenylene diamine mustard. This could be due to the increased reactivity of the phenylene diamine mustard associated with the high lability of the bromine atoms. Therefore, the hydrogenation step was carried out by dissolving the N,N-bis-(2-bromo ethyl)-4-nitro or 4-nitroso aniline in the minimal amount of dry ethyl acetate containing the 10% palladium on charcoal. The mixture was stirred whilst hydrogenation occurred, the reaction stopped immediately after the theoretical amount of hydrogen had been taken up, the catalyst filtered off and the filtrate cooled in cardice to -10°C. The filtrate was then added to the already prepared mixed anhydride of protected glutamic acid and ethyl chloroformate in THF and the mixture maintained at a temperature below -10°C. Subsequent steps were the same as those as described previously, for the preparation of γ-L-glutamyl-N,N-bis-(2-chloro ethyl) amino mustards.

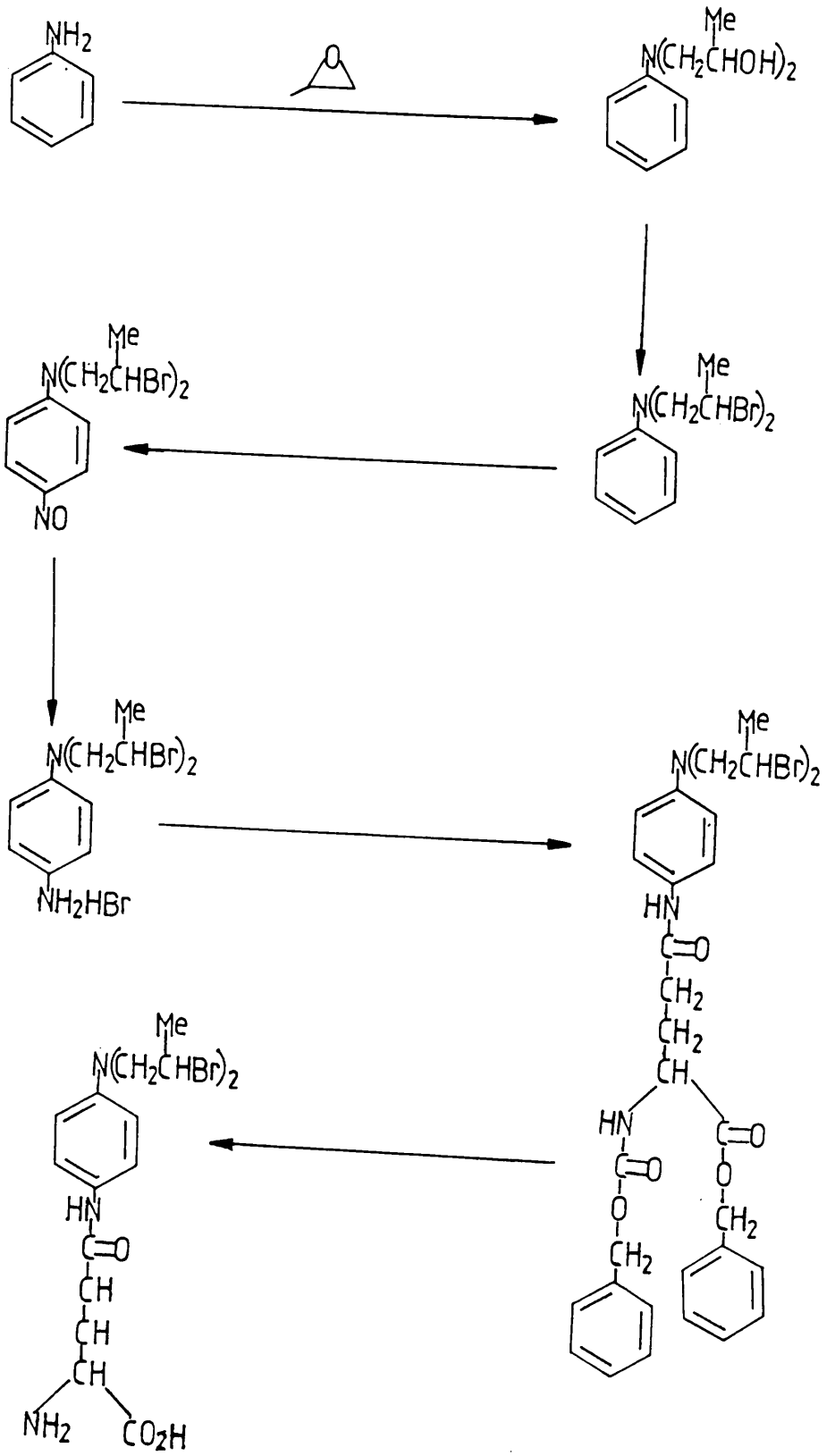
For the synthesis of γ- [N,N-bis-(2-bromo propyl)-4-phenylene diamine] -glutamic acid, the same procedure as for the synthesis of γGPDM was employed at first (Scheme 40). Reduction of the N,N-bis-(2-bromo propyl)-4-nitroso aniline to the corresponding phenylene diamine mustard by catalytic hydrogenation in methanol in the presence of 10% palladium charcoal catalyst gave an impurity however, which proved difficult to remove. The impurity was carried through the remaining steps of the synthesis, giving products which were difficult to purify.

Secondary bromine groups are reknowned for their lability and extra care must be exercised to ensure all solvents are totally dry. The secondary bromine groups are most reactive in the synthesis, after formation of the N,N-bis-(2-bromo propyl)-4-phenylene diamine, due to the elctron donating ability of the 4-amino group. Various polymerised, oxidation and other products could form. Particularly in view of the prolonged reaction time for complete hydrogenolysis.

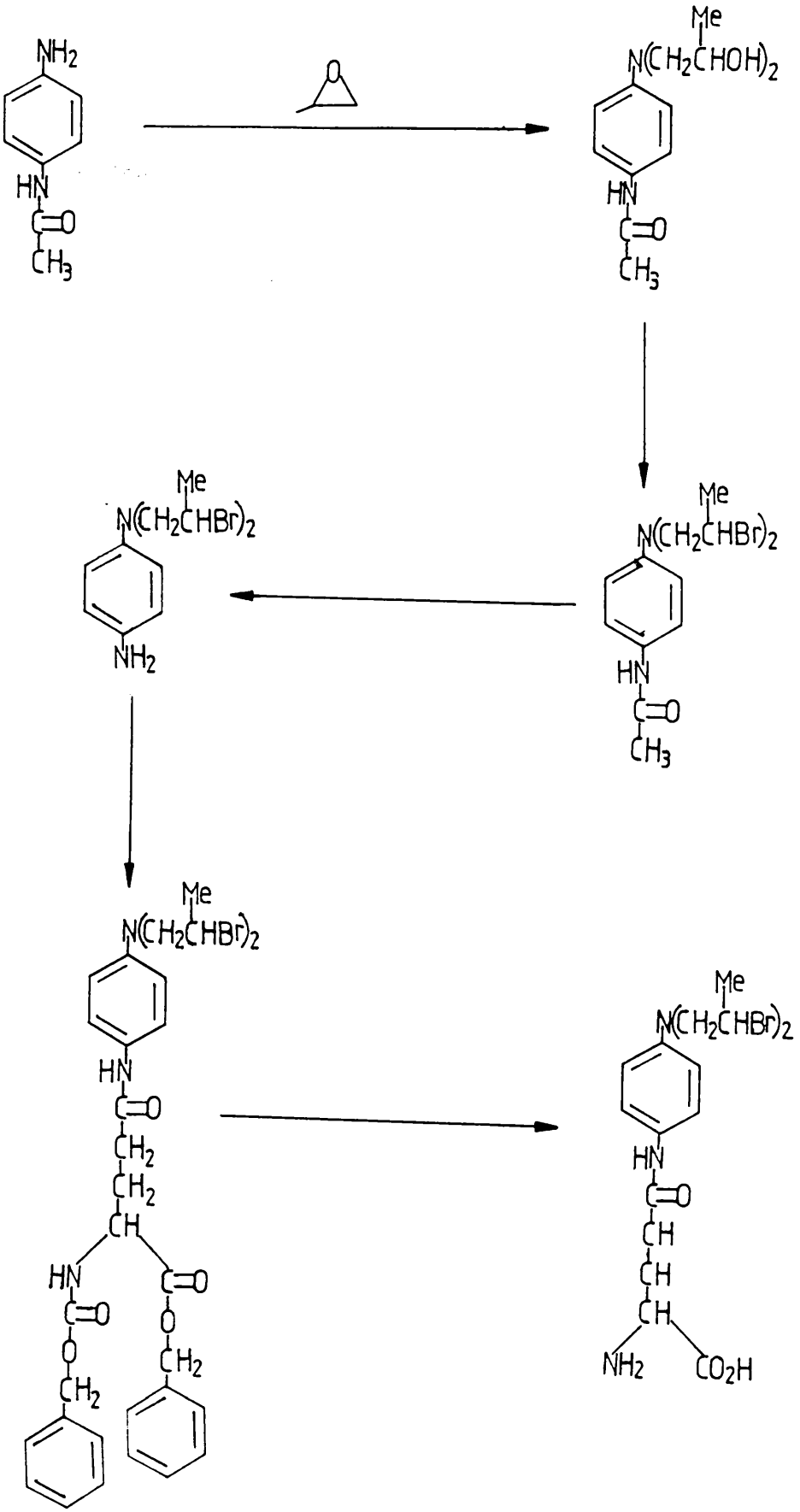
A new synthetic method was required to minimise these problems. They were overcome by forming the N,N-bis-(2-bromo propyl) amino-4-acetanilide [22].



[22]

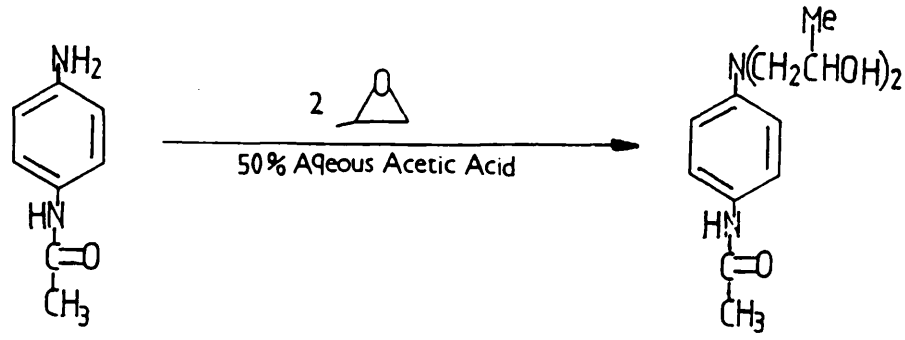


Scheme 40



Scheme 41

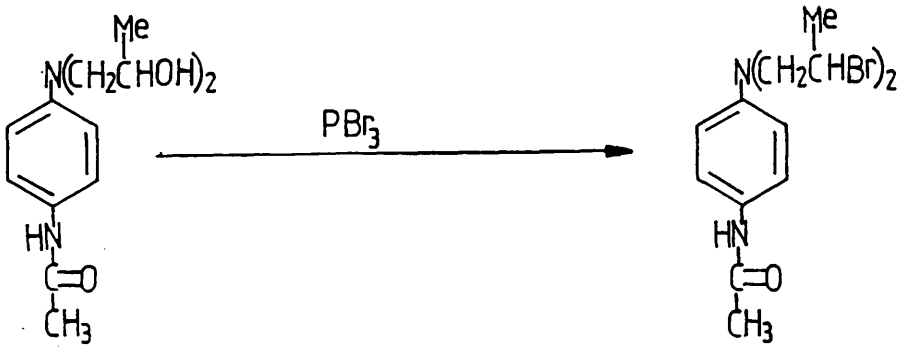
The 4-acetanilide substituent is less electron donating and thus stabilises the mustard moiety. Further, it can be hydrolysed by HBr to give the corresponding N,N-bis-(2-bromo propyl)-4-phenylene diamine as a hydrobromide salt [23]. The revised synthesis of γ-L- [N,N-bis-(2-bromo propyl)-4-phenylene diamine] -glutamic acid is shown in Scheme 41.



Scheme 42

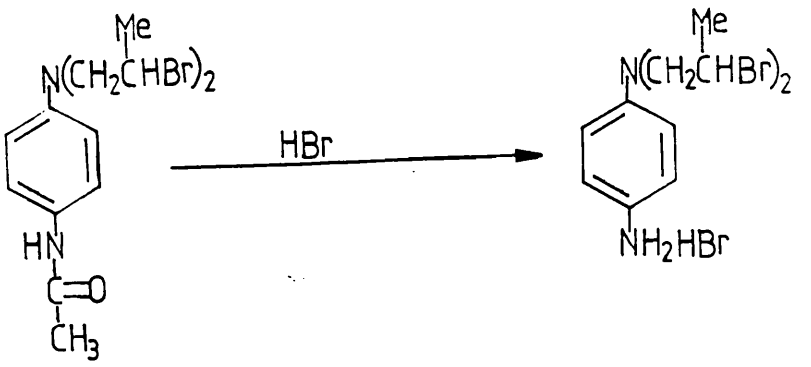
The N,N-bis-(2-hydroxy propyl) amino-4-acetanilide was prepared by reacting 4-amino acetanilide with an 8- fold excess of propylene oxide in 50% aqueous acetic acid at room temperature for 48h. Evaporation of the solvents gave a syrup, which proved resistant to complete purification even by column chromatography on silica. Thus an impure product was obtained as a yellow oil in yields of 63%.

The IR spectrum of this oil gave a broad absorbance at 3400 cm^{-1} indicative of the hydroxyl groups, and the ^{1}H-nmr spectrum showed the presence of 4-aromatic protons with an A'B' splitting pattern at δ = 6.8 ppm and a further 8 protons as a multiplet at δ = 4.2 - 2.8 ppm. Both are consistent with the formation of N,N-bis-(2-hydroxy propyl) amino-4-acetanilide.



Scheme43

The next step in this synthesis would normally be tosylation of the hydroxy functions followed by their displacement by bromides using $CaBr_2$ in 2-ethoxy ethanol. This seemed inadvisable, however, because tosylation of secondary alchols usually gives very poor yields of products. A more traditional method using PBr_3 was therefore employed for the bromination. A solution of the N,N-bis-(2-hydroxy propyl) amino-4-acetanilide in benzene was added to a 3- fold excess of PBr_3. The mixture was heated on a steam bath until the reaction slackened, crushed ice was added to remove any excess PBr_3 and on washing the benzene layer successively with water and aqueous $NaHCO_3$ followed by evaporation of the solvent, a grey solid was obtained. This solid was recrystallised from ethanol to give pure white needles of N,N-bis-(2-bromo propyl) amino-4-acetanilide at a low yield of 32%. The IR spectrum showed the disappearance of the hydroxy absorbance at V = 3400 cm^{-1}.



Scheme43a

The acetyl group was removed by heating the N,N-bis-(2-bromo propyl) amino-4-acetanilide under reflux in 45% HBr for 3h. The solution was neutralised with solid $NaHCO_3$ and then extracted with ether. On addition of 45% HBr in acetic acid dissolved in methanol, N,N-bis-(2-bromo propyl)-4-phenylene diamine hydrobromide precipitated as a white solid. On filtration however, the solid rapidly discoloured. Accordingly, the solid was coupled with the protected glutamic acid immediately and without further purification as described for the γ-L-glutamyl-bis chloro mustards. This gave the γ-benzyl-N-carbobenzoxy-N,N-bis-(2-bromo propyl)-4-phenylene diamine glutamate, as a white solid, but the yield was only 28%.

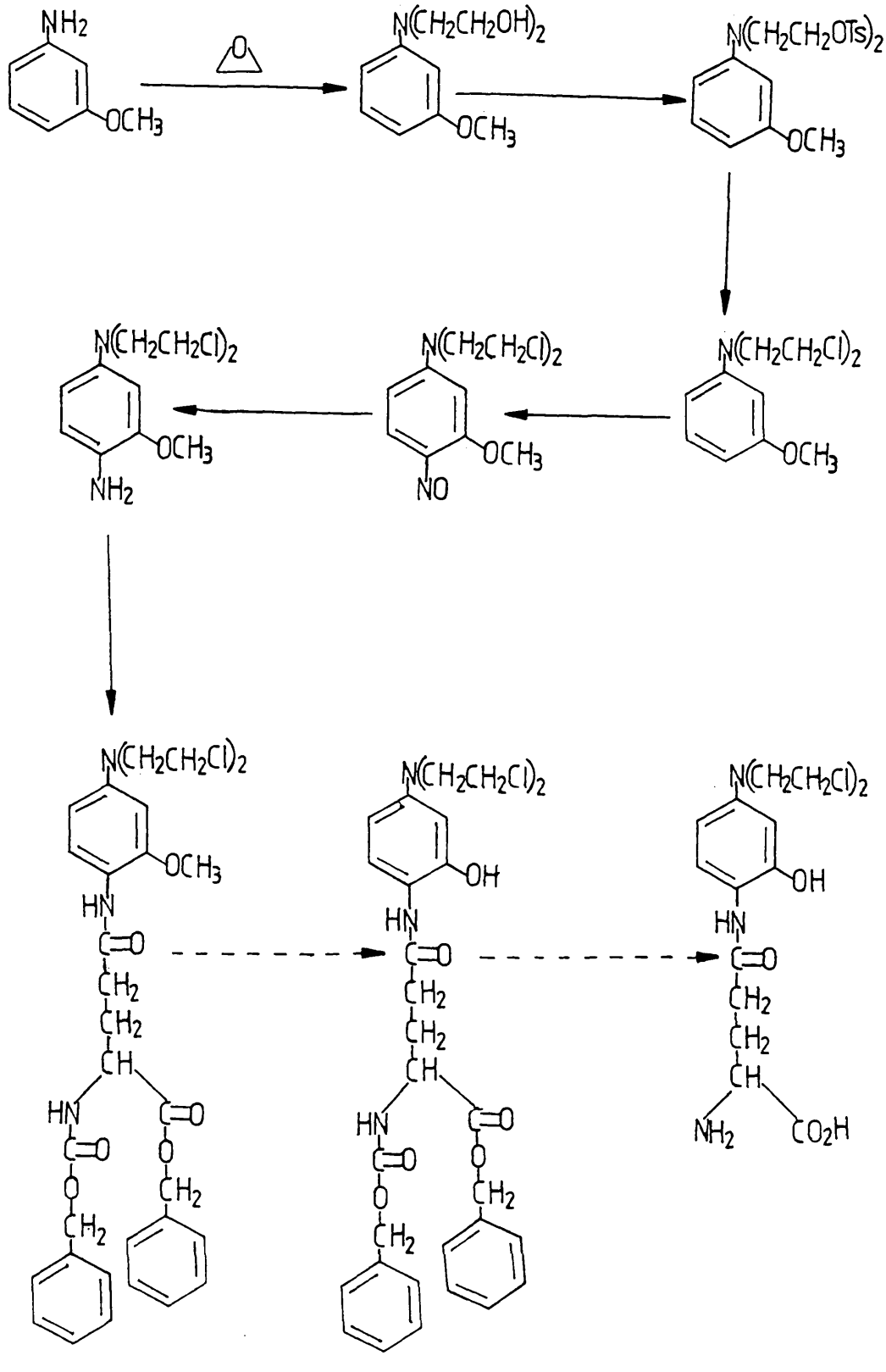
Finally the γ-benzyl protecting groups were removed by catalytic hydrogenolysis in the presence of 10% palladium charcoal in methanol/ dichloromethane as solvent, to give the γ-L- [N,N-bis-(2-bromo propyl)-4-phenylene diamine] -glutamic acid in a yield of 55%.

2.6 Attempted Synthesis of γ-L- [N,N-bis-(2-chloro ethyl) -3-hydroxy-4-phenylene diamine] -glutamic acid [24]

In addition to the usual protection of the α-amino and α-carboxyl groups of glutamic acid, the synthesis of [24] requires protection for the aromatic 3-hydroxy substituent. This protection should not be to bulky otherwise steric interactions may impede coupling of the 4-phenylene diamine mustard with the protected glutamic acid. Further, removal of the protecting group by alkaline hydrolysis is out of question because of the lability of the mustard chlorine groups. Likewise, removal by catalytic hydrogenation is out of question because reduction of the 4-nitroso group to form the 4-phenylene diamine mustard would also remove the 3-hydroxy protecting group.

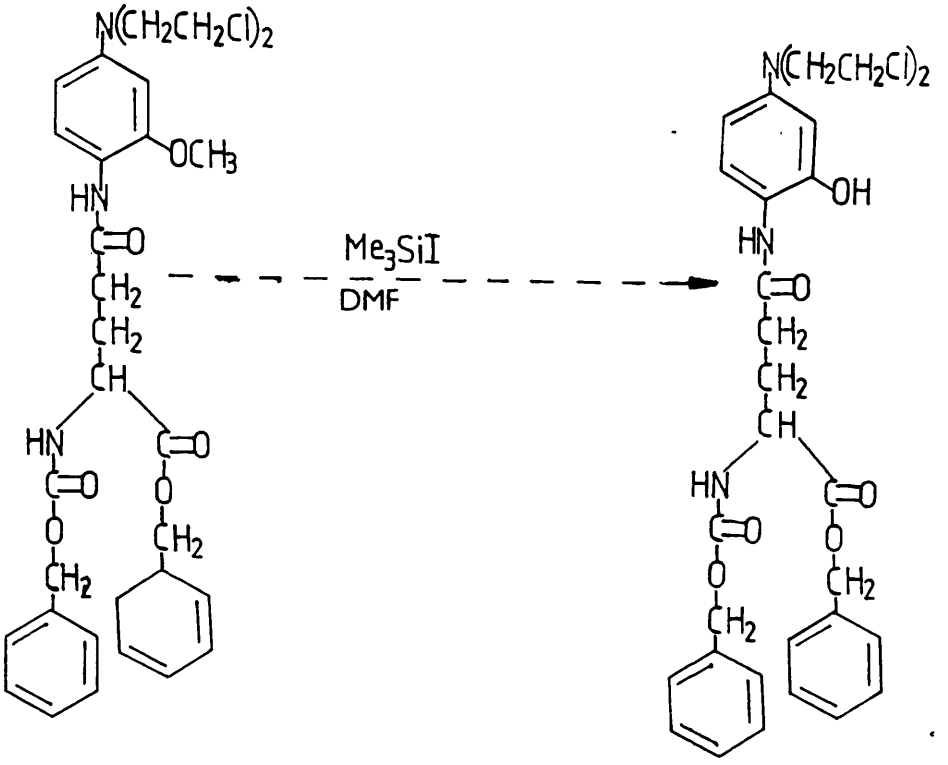
For these reasons, the phenol group was protected by conversion to the methyl ether. Normally methyl ethers are removed by acid hydrolysis, but a mild method of cleavage using Me_3SiI has been reported[133].

The synthesis of the γ-L- [N,N-bis-(2-chloro ethyl) -3-hydroxy-4-phenylene diamine] -glutamic acid followed the same procedure as that for γGPDM, using 3-methoxy aniline as the starting material which was reacted with ethylene oxide (Scheme 44). The 3-methoxy phenylene diamine mustard intermediate was coupled successfully to the protected glutamic acid via the usual mixed anhydride to give γ-L-benzyl-N-carbobenzoxy-N,N-bis-(2-chloro ethyl)-3-



Scheme 44

methoxy-4-phenylene diamine glutamate as a white solid with a yield of 41%. An attempt to remove the methyl protecting group to give the 3-hydroxy analogue (Scheme 45) by dissolving the protected mustard in dry DMF followed by one molar equivalent of Me_3SiI gave a brown oil. On silica TLC using 2-Butanone: Acetic acid: water (8:1:1) as eluent five compounds (one of which was the starting material) were apparent in the reaction mixture. Unfortunately, insufficient time was available to examine these compounds further. A possible reason for several products could be due to concurrent removal of the α-benzyl ester and N-carbobenzoxy groups of the glutamic acid by Me_3SiI.



Scheme45

CHAPTER 3

ENZYMOLOGY

3.10 γ GLUTAMYL TRANSFERASE (γGT) EXTRACTION AND PURIFICATION

The specific activity, purification and yields obtained after each step in the purification are displayed in Table 1. The rat kidneys without ureters and capsules were first homogenised at 4°C in 100 mM mops - HCl buffer containing 1 mM EDTA (pH 7.2) for 2 min in a Waring blender at top speed. The specific activity at this point was 1.02 Umg^{-1} protein. On centrifuging the homogenate at 16,000g for 1h, discarding the supernatant liquid and resuspending the pellets in 100 mM mops-HCl at pH 7.2, the washed membranes were obtained. The specific activity of the membranes was 3.83 U $Mg^{-1}l^{-1}$, but a substantial amount of material had been lost for unknown reasons. As a result, the yield was only 35% compared with a yield of 95% achieved by Cook and Peters[79] using rat kidney, however their specific activity was lower (1.76 $U.Mg^{-1}l^{-1}$). The yield was 23% compared to the literature value for the same step, which was 66.4%. After fractionation with ammonium sulphate, the fraction from 50-100% saturation which was resuspended in a minimal volume of Mops-HCl (pH 7.2) buffer, had a specific activity of 11.8 $u.mg^{-1}l^{-1}$ and a yield of 23%. Both the activity and yield were lower than those achieved by Cook and Peters[79], for the same step.

The above resuspended fraction was loaded on to a phenyl boronate PBA60 column. The boronate ligands bind to the 1,2-Cis diols of carbohydrates. As γGT is a glycoprotein it binds to the column, while all of the non-glycoproteins are eluted. The bound γGT was released from the ligand and eluted by washing the column with 10mM Tris buffer (pH 8.7). All fractions were assayed for γGT activity by using the Smith[140] et al method, and the results are shown in Fig. 1. Fractions No. 210 to 290 were collected and concentrated by ultrafiltration (each fraction consisted of 3. cm^3 of solution).

	VOLUME (cm^3)	TOTAL ACTIVITY (u)	TOTAL PROTEIN (mg)	SPECIFIC ACTIVITY ($umg1^{-1}$)	PURIFICATION (FOLD)	YIELD (%)
HOMOGENATE	160	13,200	12,900	1.02	1.0	100
MEMBRANES	80	4,600	1,200	3.83	3.75	35
EXTRACT	140	3,000	336	9.02	8.85	23
50-100% $(NH_4)_2 SO_4$ + ULTRA-FILTRATION	21	1,190	101	11.80	11.60	9
PBA - 60 + ULTRA-FILTRATION	1.7	928	14	66.30	65.00	7
G200 + ULTRA-FILTRATION	1.4	429	1.8	227.0	223.0	3

TABLE 1

Purification , yield and specific activity of γGT after various stages of the purification procedure

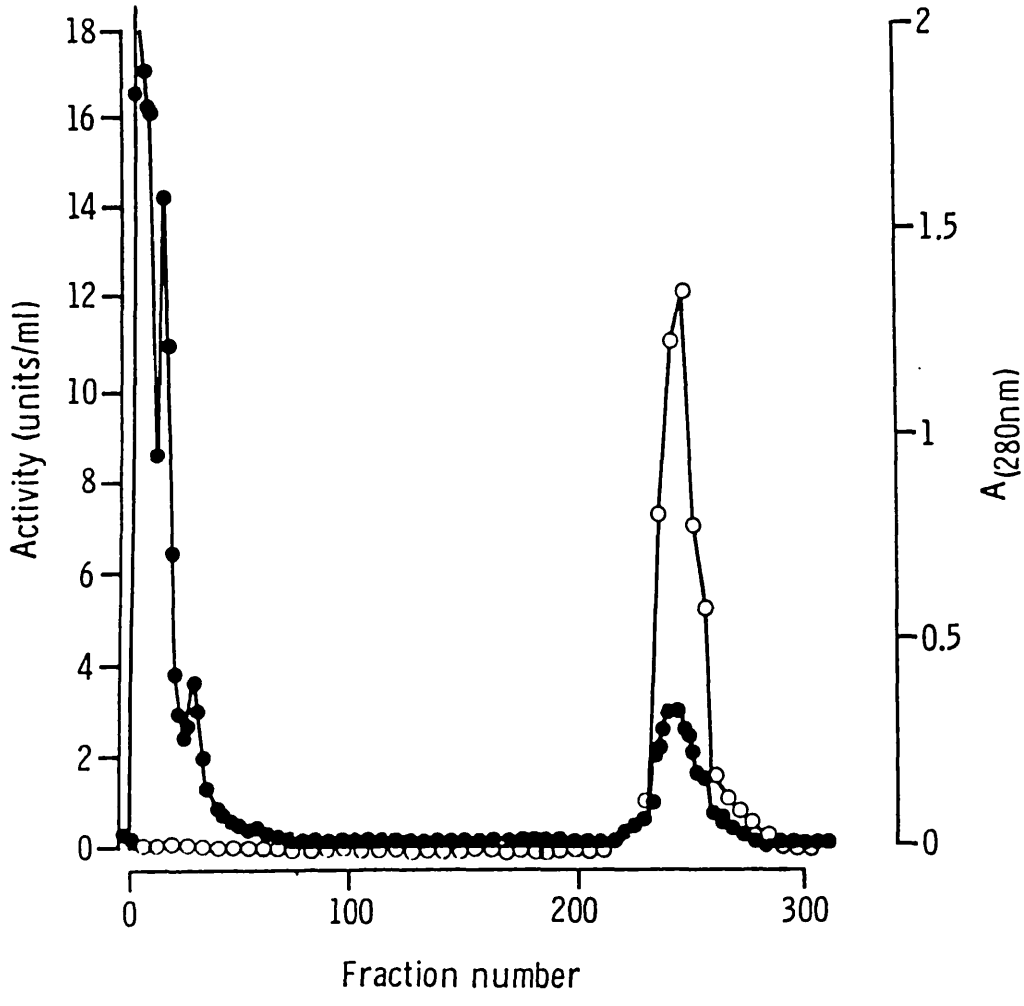


FIG 1

A chromatogram of γGT after passing through a phenyl boronate PBA-60 column. The absorbance at 280 nm (-●-●-) and γGT activity (-o-o-) were measured.

The specific activity of the combined fractions was 66.3 u $mg^{-1}l^{-1}$. This activity is higher than the literature value (43.5 u $\mu g^{-1}l^{-1}$), but the yield (7%) was lower.

Finally, the concentrated γGT was loaded onto a G-200 gel filteration column, which had been pre-equilibrated with 50 mM Tris-HCl (pH 7.5) buffer. Fig. 2 shows the chromatogram of the γGT containing fractions. Fractions No. 45 to 59 were collected and subjected to ultrafilt ration. The specific activity of the purified enzyme was 227 u $mg^{-1},1,^{-1}$ higher than that achieved by Cook and Peters[79] (165 umg^{-1} l^{-1}), although the yield of 3.25% was less than theirs (11.9%). The low yield was attributed to substantial loss of γGT activity when obtaining membranes from the homogenate.

Polyacrylamide gel electrophoresis of the purified γGT is shown in Fig 3. The bands correspond to the following, (A) 30 μg each of molecular mass markers: Phosphorylase b, 94 kdal; bovine serum albumin, 67 kdal; ovalbumin 43 kdal; carbonic anhydrase; 30 kdal; soy bean trypsin inhibitor, 20 kdal and γ-lactoglobulin 14.4 kdal, 5 μg of enzyme samples were pre treated with 0.05M Na_2CO_3, 0.05M dithiothreitol at room temperature for 20 min at 200°C. Electrophoresis was carried out as described[134] with 6% stacking gel and 10% separation gel. The following fractions during the purification were subjected to polyacrylamide gel electrophoresis; (B) Homogenate fraction, (C) membrane fraction, (D) Extract fraction, (E) PBA-60 fraction and (F) G-200 + ultrafilteration fraction.

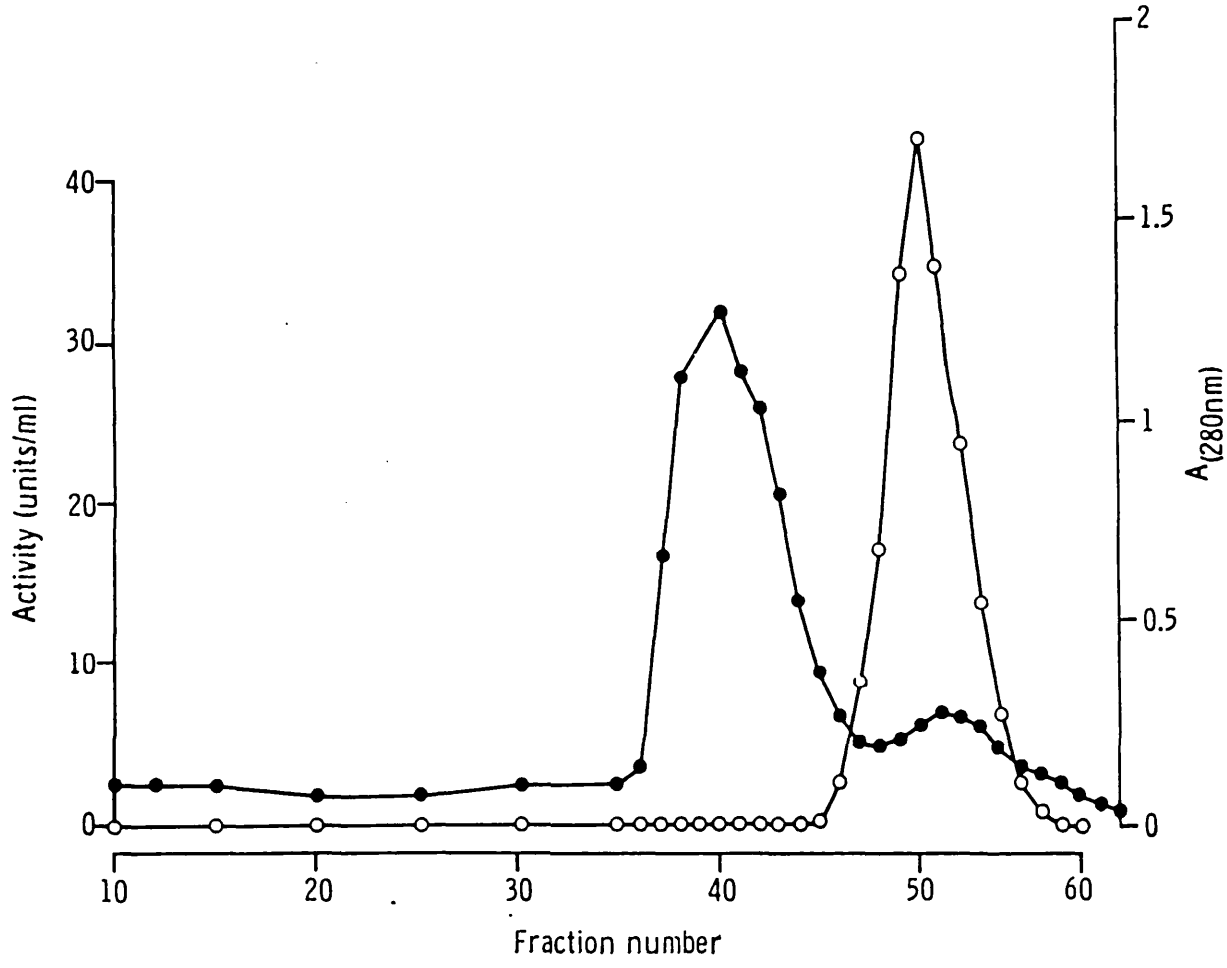


FIG 2

A chromatogram of γGT after passing through a G-200 column. The absorbance at 280 nm (-●-●-) and the γGT activity (-O-O-) were measured.

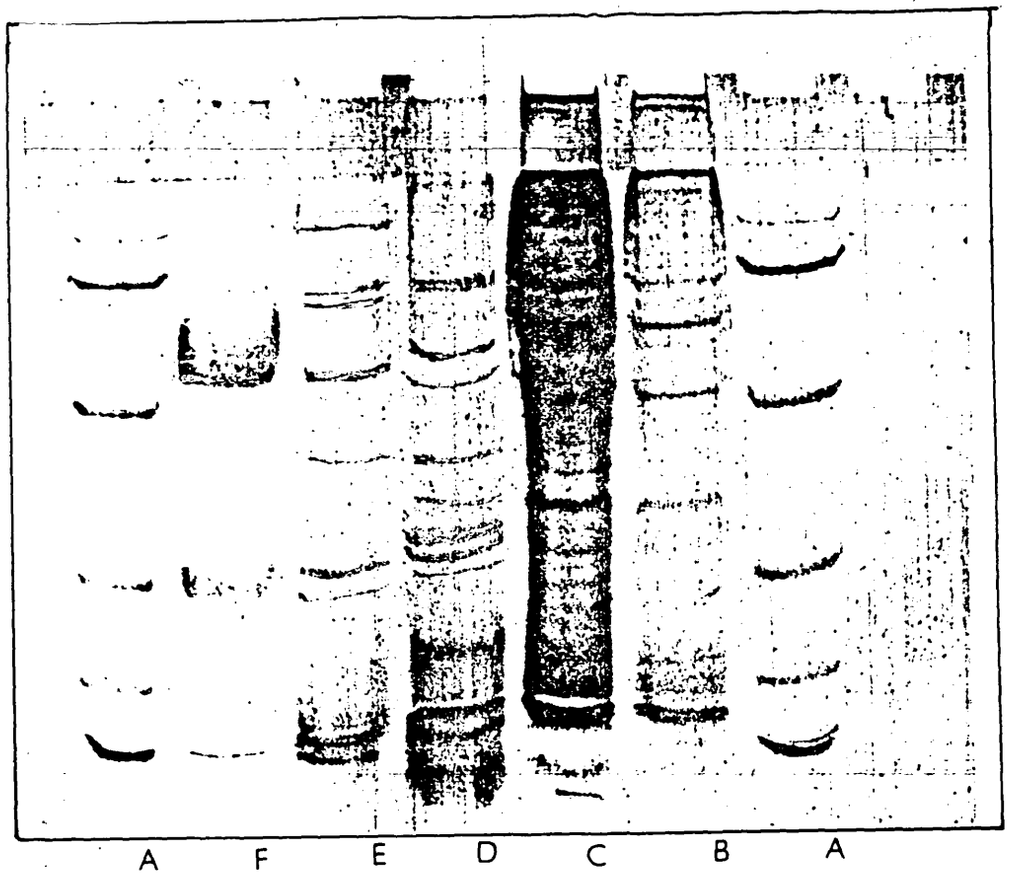


FIG 3

SDS - Polyacrylamide gel electrophoresis of purified YGT.

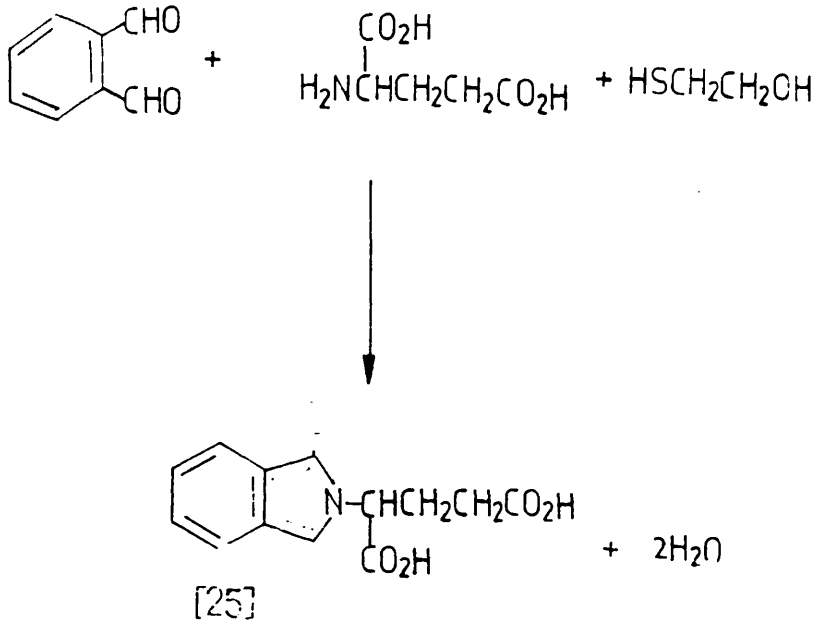
The bands correspond to the following:-

(A) Molecular mass marker.
(B) Homogenate fraction.
(C) Membrane fraction.
(D) Extract.
(E) PBA-60 fraction.
(F) G-200 + ultrafiltration fraction.

The polyacrylamide gel electrophoresis of the purified γGT in the presence of SDS showed two coomassie blue staining bands. These corresponding to the two sub units of γGT with an apparent molecular mass of 26 kdal and 56.2 kdal.

3.11 Enzyme kinetics

The HPLC method of Cook and Peters[135] which is both rapid and sensitive was used to assay γGT activity. It measures the amount of glutamic acid released from a γ-glutamyl substrate by γGT as the OPT-derivative[25]. Thus aliquots (50 µl) of the reaction solutions containing glutamic acid were reacted with O-phthaldialdehyde (50 µl) in alkaline medium and in the presence of 2-mercaptoethanol as reducing agent. This gave the strongly fluorescent OPT-derivative [25] (scheme 46), which was separated and quantified by HPLC. The assay determined the picomoles of glutamic acid present, therefore the assay was calibrated using 0-400 picomoles solutions of authentic glutamic acid.



[25]

Scheme 46

The K_m and V_{max} values for the prodrugs were determined from the experimental data by the Cornish-Bowden linear plot method[136]. This involves plotting $^{[S]}/V\ V_s$ [S]. The point where the graph cuts the x-axis is the $-K_m$ value and the point where the graph cuts the y-axis gives the $^{km}/V_{max}$ values. From these, the k_m and V_{max} values can be calculated.

The comparison of the hydrolysis of γ-L[N,N-bis-(2-chloro ethyl)-4-phenylene diamine]-glutamic acid (γGPDM), γ-L-[N,N-bis-(2-chloro ethyl)-2-fluoro-4-phenylene diamine]-glutamic acid (γGPDMF), γ-L-[N,N-bis-(2-chloro ethyl) -2-chloro-4-phenylene diamine] glutamic acid (γGPDMCl), γ L-[N,N-bis-(2-bromo ethyl)-4-phenylene diamine]-glutamic acid (γGBrPDM), γ-L-[N,N-bis (2-bromo ethyl) 2-fluoro 4 phenylene diamine] glutamic acid (γGBrPDMF), Glutathione and L glutamine by γGT, plotting $\frac{[S]}{V}\ V_s$[S] of the release of glutamic acid are shown in Figs 4-6.

The Cornish-Bowden method was used because it gives more accurate values for the kinetic parameters. The alternative Lineweaver-Burke plot gives undue weight to measurements made at low substrate concentrations, where the results are likely to be most inaccurate.

The k_m and V_{max} values obtained from Figs 4-6 for the five prodrugs (γGPDM, γGPDMF, γGPDMCL, γGBrPDM, γGBrPDMF) together with those for glutathione and glutamine (the enzymes natural substrates), are summarised in Table 2. The values show that all the γ-L-glutamyl aromatic mustards have similar V_{max} values (7.9 to 9.4 μ moles/min/μg protein), which are slightly lower than glutathione (V_{max} = 12.8 μ moles/min/μg protein) but approximately 2.5 fold higher than L-glutamine (V_{max} = 3.5 μ moles/ min/μg protein). The k_m for the γ-glutamyl mustards are of a similar value to that of glutathione but lower than that for L-glutamine. The highest prodrug k_m values applies to γGPDM (k_m = 62 μ m. .) and the lowest to γGPDMF (k_m = 25 μ m. .) which is very close to that of glutathione (K_m = 23 μ m. .). The 2-fluoro-γ-L-glutamyl mustards have the lowest k_m and marginally higher V_{max} values. This suggests that the 2-fluoro compounds are better substrates than either the 2-chloro or unsubstituted compounds. The results in Table 2 also suggest that all of the γ-glutamyl mustard prodrugs are comparable to glutathione as substrates for γGT but are better substrates than L-glutamine.

The rate of spontaneous hydrolysis of the γ-L-glutamyl mustards in Hanks balanced salt solution (HBSS), at 37°C was determined. HBSS is the media in which the prodrugs were exposed to the cell lines.

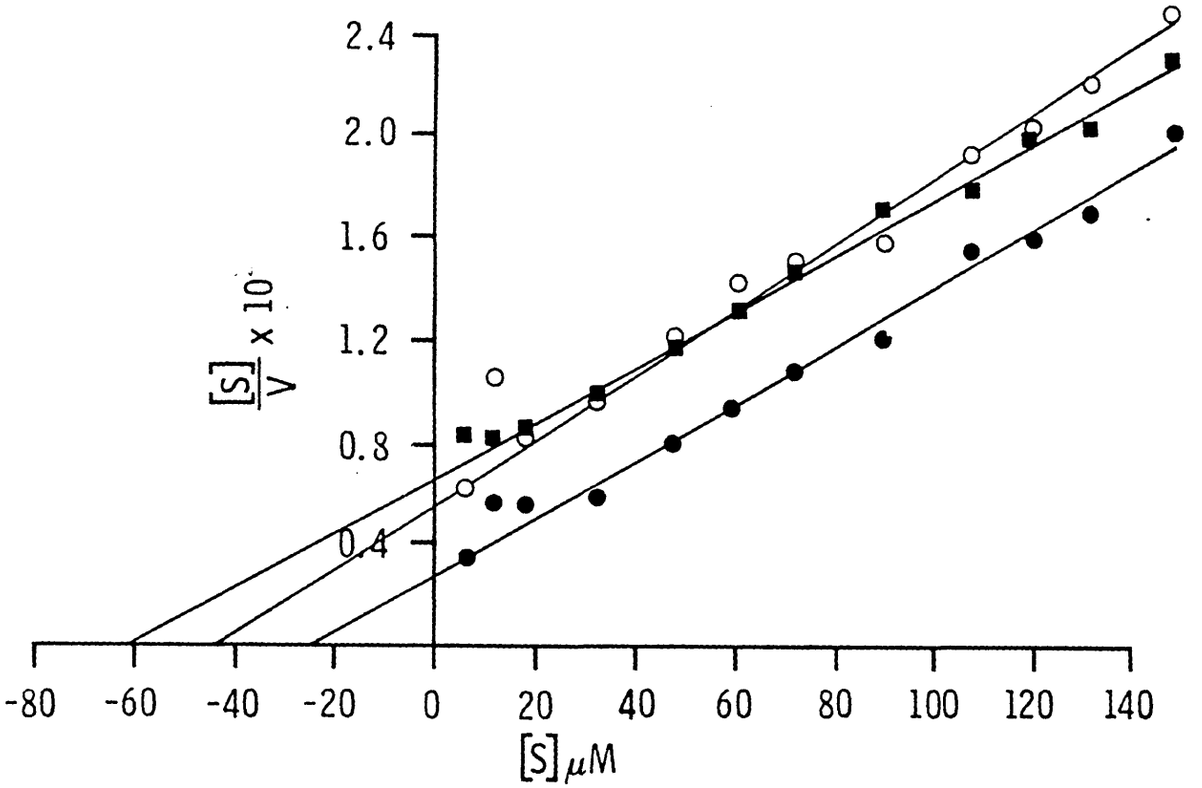


FIG 4

[S]/V versus [S] plots of the release of glutamic acid from YGPDM (-■-■-), YGPDMF (-●-●-) and YGPDMCl (-o-o-) by YGT.

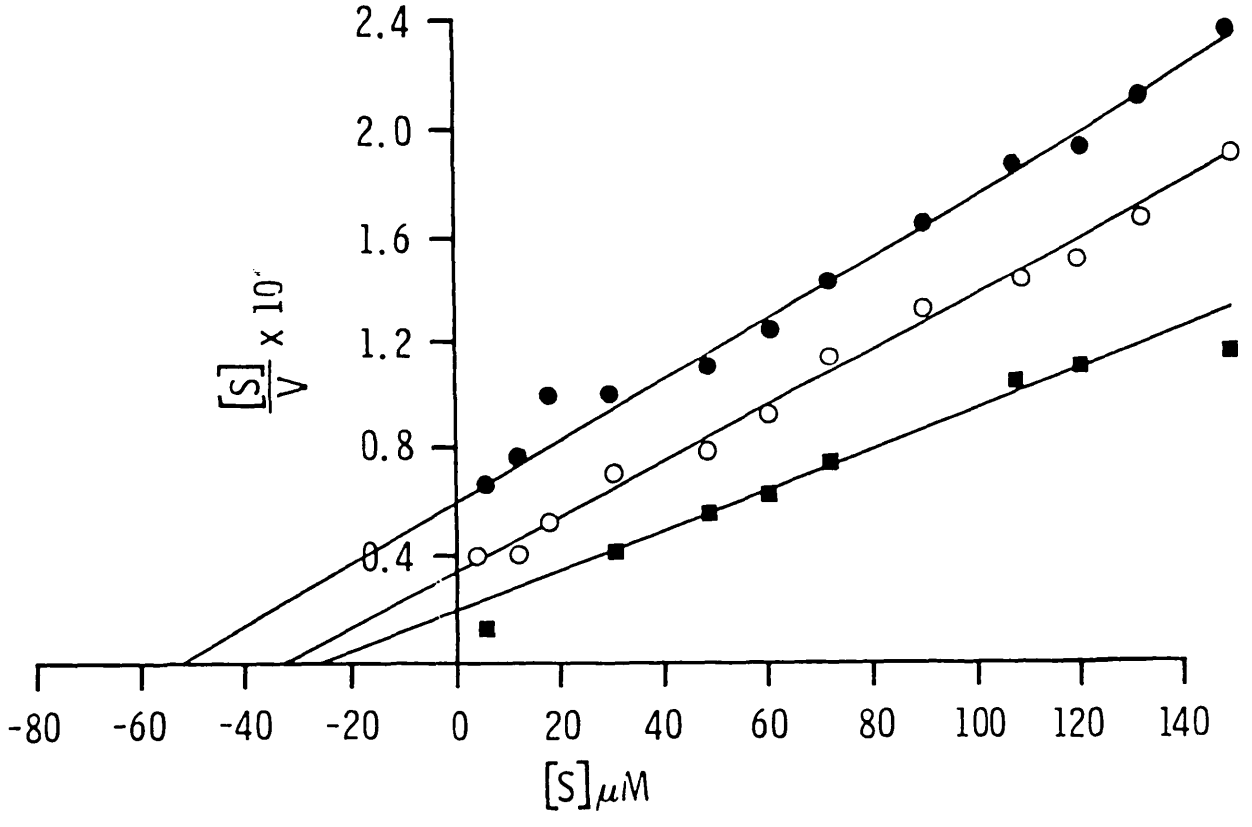


FIG 5

The [S]/V versus [S] plots for YGBrPDM (-●-●-), YGBrPDMF (-o-o-) and glutathione (-■-■-).

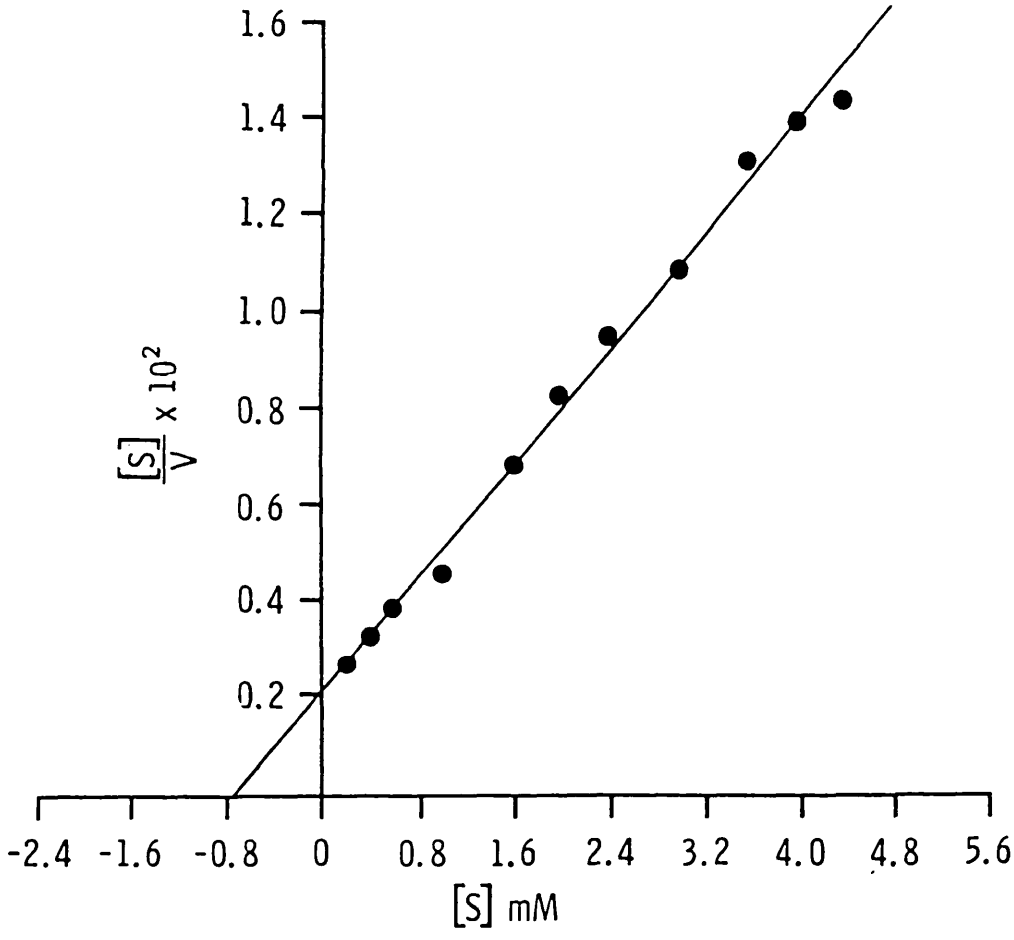


FIG 6

The [S]/V versus [S] plot of the release of glutamic acid from glutamine by γGT.

SUBSTRATE	Km (μM)	Vmax (μMol/min/mg protein)
γGPDM	62	9.4
γGPDMF	25	8.9
γGPDMCl	44	7.9
γGBrPDM	51	8.5
γGBrPDMF	31	9.4
GLUTATHIONE	23	12.3
GLUTAMINE	760	3.5

TABLE 2

Km and Vmax values for five γ-glutamyl mustards, glutathione and glutamine towards γGT

A 150 μ mol solution of the appropriate prodrug was made up in HBSS and left in a water bath at 37°C for 2h. An aliquot (50 μl) was withdrawn and assayed for glutamic acid by the HPLC method described previously and compared with the glutamic acid content of the freshly made solution of the prodrug in HBSS. These experiments showed no formation of glutamic acid and therefore no hydrolysis of the five γ-glutamyl mustards occurred in the absence of γGT. Therefore any toxicity towards the cell lines (see chapter 4) related to hydrolysis of the prodrug by γGT to release the parent mustard or to the γ-L-glutamyl mustard adduct itself.

3.12 Summary

γGT was extracted and purified using the literature procedure[79]. Its specific activity was higher than the published value but the yield was lower. For hydrolysis by γGT, the γ-glutamyl mustards gave slightly lower V_{max} values than glutathione, but larger than L-glutamine. The results suggest that γGT should catalyse the hydrolysis of γ-L-glutamyl mustards at rates comparable with those for the natural substrates of γGT.

CHAPTER 4

CELL CYTOTOXICITY

4.1 Tissue Culture

The fundamental nature of malignancy appears to be a defect within each cell. It follows that the defect can be studied in tissue culture[137-139]. Cells can be removed under suitable conditions from normal or tumour tissues to a dish of growth medium where, under appropriate conditions, they will grow and divide. Since the cells divide mitotically, they produce a clone of genetically identical cells that can be transferred from dish to dish many times. Cultured cells however, change their properties probably by mutation, like transplanted tumours in animals.

Cultured cells are quite fastidious. Many chemical and physical conditions must be correct for growth and division. Those requirements include low molecular weight nutrients and vitamins. Depending on the particular cell type, up to a dozen nutritionally essential amino acids and a variety of vitamins are needed together with inorganic ions such as Na^+, K^+, Ca^{2+}, Mg^{2+}, Fe^{2+}, phosphate and other trace metal elements. The pH of the medium must be carefully controlled close to neutrality and therefore buffers are added. The cells also need oxygen and carbon dioxide.

In addition to low molecular weight nutrients, cultured cells need a variety of proteins called growth factors. It is usual practice to add calf's serum to the media, at about a tenfold dilution. Serum contains many compounds including the necessary growth factors. Sera from different animals promote in vitro cell growth to different extents. Fetal calf serum is particularly good for this purpose.

The two principle physical factors influencing in vitro cell growth are temperature and space. Mammalian cells grow well only between ca 33 and 39°C, and a temperature of 37°C is usually used. Each cell needs a minimum amount of space to grow. When cells are limited in space, for example by a tiny surface or by overcrowding, cells will not grow.

4.2 γGT Activity in BL8L (normal) and JBI (hepatoma) cells

Transpeptidation and hydrolysis activity of γGT in the BL8L and JBI cell lines was measured by the removal of the cells by trypsinisation from culture dishes, the cells were washed and resuspended in PBS. The γGT activity was assayed by the method of Smith et al[140]. The Vmax and Km values were determined, using γ-GAMC as substrate, by the Cornish-Bowden linear plot. This work was carried out in collaboration with Dr F Mansfield. The results are displayed in Table 3.

The results show that the Vmax for transpeptidation by γ-GAMC is 83 times greater for the JBI than the BL8L cell line. For hydrolysis Vmax is 8 times greater for the JBI than the BL8L cell line. Thus, the JBI cells do contain more γGT activity than the BL8L cells. The Vmax values for transpeptidation relative to hydrolysis are 39 and 3.5, respectively for the JBI and BL8 cell. The Km values are lower for the JBI than the BL8L cells in regard to both transpeptidation and hydrolysis.

These results also show that the predominant reaction for both the JBI and BL8L cells is transpeptidation rather than hydrolysis.

	TRANS PEPTIDATION REACTION		HYDROLASE REACTION	
	BL8L	JB1	BL8L	JB1
Vmax (nMol/min/mg protein)	7.4	617	2.1	16.0
Km(μM)	880	360	970	97.0

TABLE 3

The Km and Vmax values for γGT in BL8L and JB1 cells using γ-Glutamyl-AMC as the substrate

4.21 Growth rates of JBI and BL8L Cells

The growth rates of JBI and BL8L cells were measured by the removal of the cells at various time intervals by trypsinisation. The cells were counted by the use of a coulter counter. An average of three plates were used for each reading.

The results from Fig 7 and 8 show that the growth rate of JBI cells is 5.15×10^4 cells/hr whereas for the BL8L cell it is 8.38×10^4 cells/hr. Therefore the average rate of growth of BL8L cells is 1.6 times faster than the JBI cells.

4.22 Cell Cytotoxicities

For the reasons discussed below, the cytotoxicity of the mustards and their 1-glutamyl adducts were examined under two sets of conditions.

Initially pre-confluent cultures of JBI and BL8L cells were treated with several concentrations of the parent mustard, N-N-bis-(2-chloro-ethyl) - 4 -phenylene diamine hydrochloride (PPDM) in Williams media and serum at 37°C for 24 hours, after which the surviving cells were counted using a coulter counter. The experiment was carried out in duplicate and an average was taken. The results, expressed as a percentage of the control to which no PPDM was added are shown in Fig. 9. The plot shows that PPDM is more toxic towards the BL8L than the JBI cells.

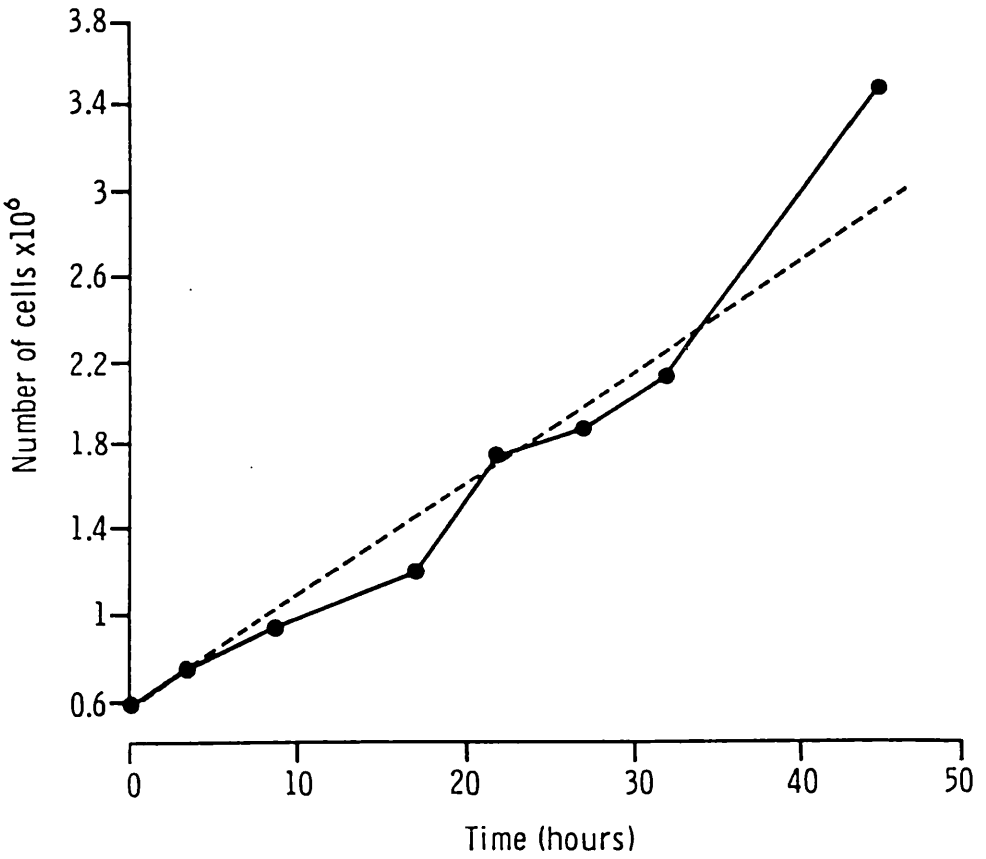


FIG 7

The growth rate of JBI cells.

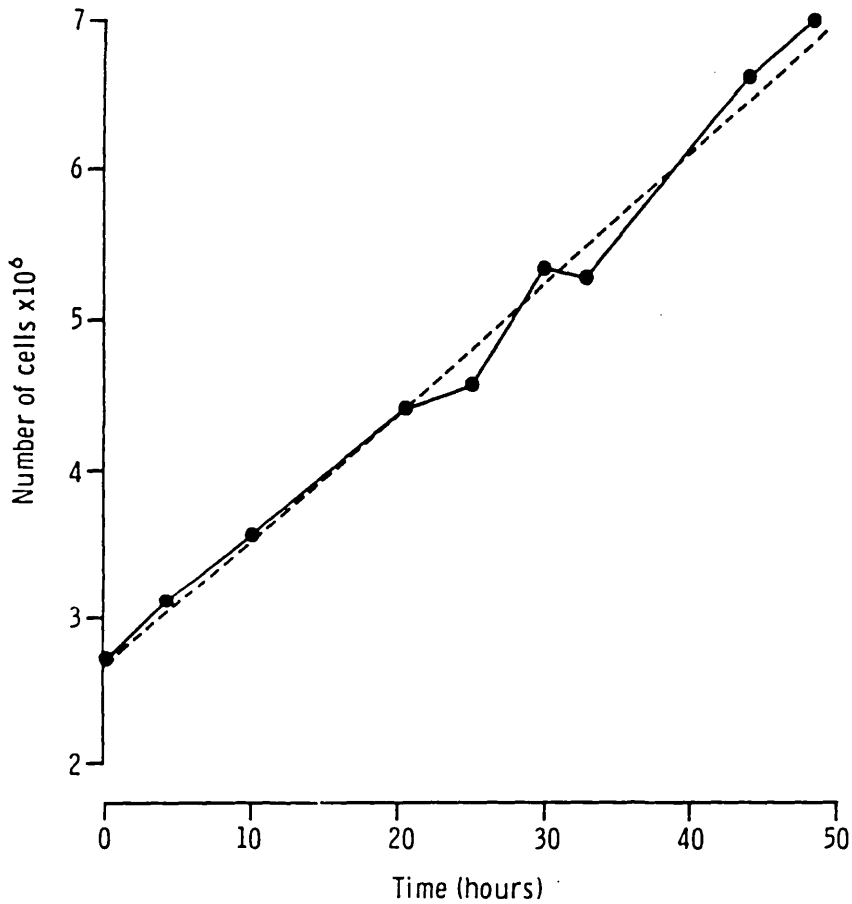


FIG 8

The growth rate of BL8L cells.

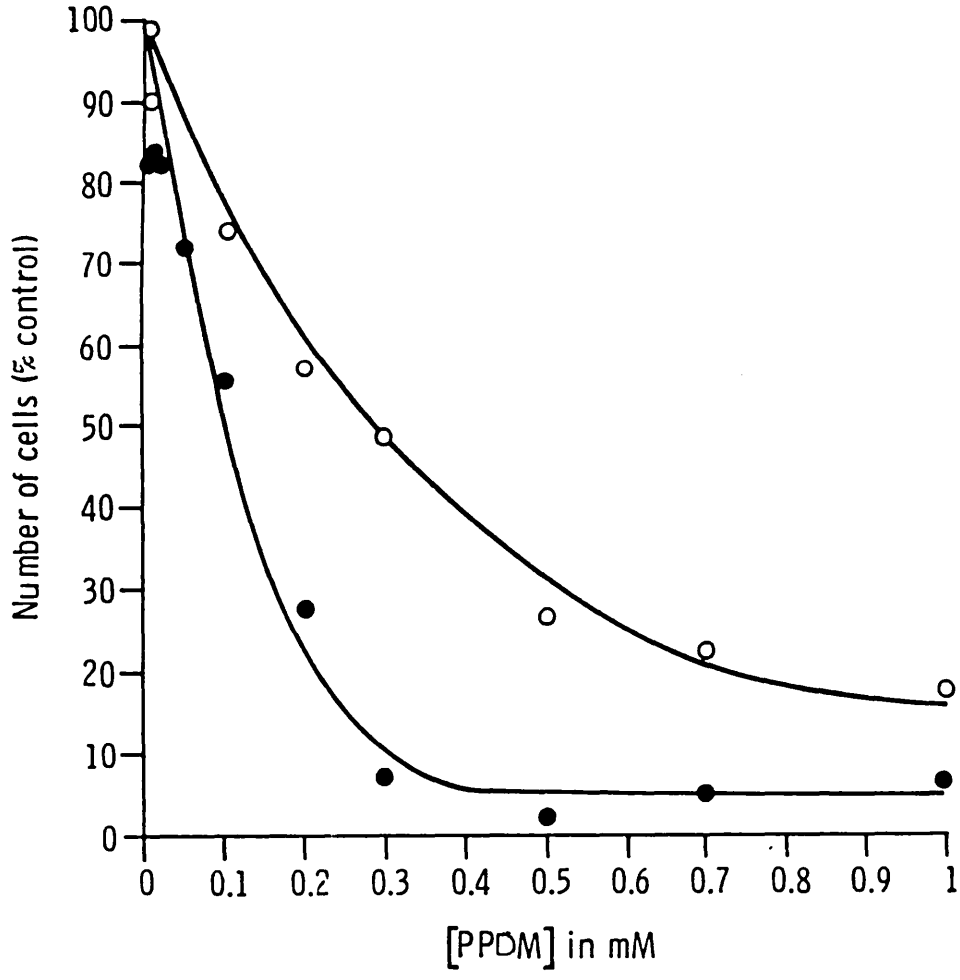


FIG 9

The effect of various concentrations of PPDM (24h exposure) on JBI (–o–o–) and BL8L (–●–●–) cell lines.

As PPDM is a strong alkylating agent, it would also react with the protein, amino acid and other constituents of the media as well as the BL8L and JBI cells. These additional reactions would attenuate the cytotoxic activity of the PPDM at a given concentration. For this reason cytotoxicity experiments were subsequently carried out in Hanks balanced salt solution (HBSS). This saline media containing no serum, maintains tissues and organs in conditions suitable for short experiments in vitro. Apart from various salts to maintain osmotic and ionic equilibrium, HBSS contains glucose as a source of energy. It represents a simple replacement for blood, in which the major ionic components are reproduced.

The pre confluent monolayer cultures of the two cell lines were therefore exposed to various concentrations of the parent mustard (PPDM) for 2h at 37°C in HBSS without serum. After 2h the media was removed, cells washed twice with HBSS (10 cm^3) and supplemented Williams 'E' media added. The cells were allowed to incubate at 37°C for 24h and then counted. The results were expressed as a percentage of the control to which no PPDM was added. The results (Fig. 10) show that the BL8L cells are more susceptible than the JBI cells to the parent mustard. The I_{50} values (drug concentration producing 50% cell death) for PPDM was 20 μm for the BL8L cells compared to 65 μm for the JBI cells. Therefore, PPDM is over three times more toxic towards the BL8L than the JBI cells.

The cells were also treated exactly as described above with varying concentrations of γGPDM for 2h in HBSS, and counted after incubation in supplemented Williams media for 24h. These results (Fig. 11) show that the γGPDM is more toxic towards JBI than BL8L cells.

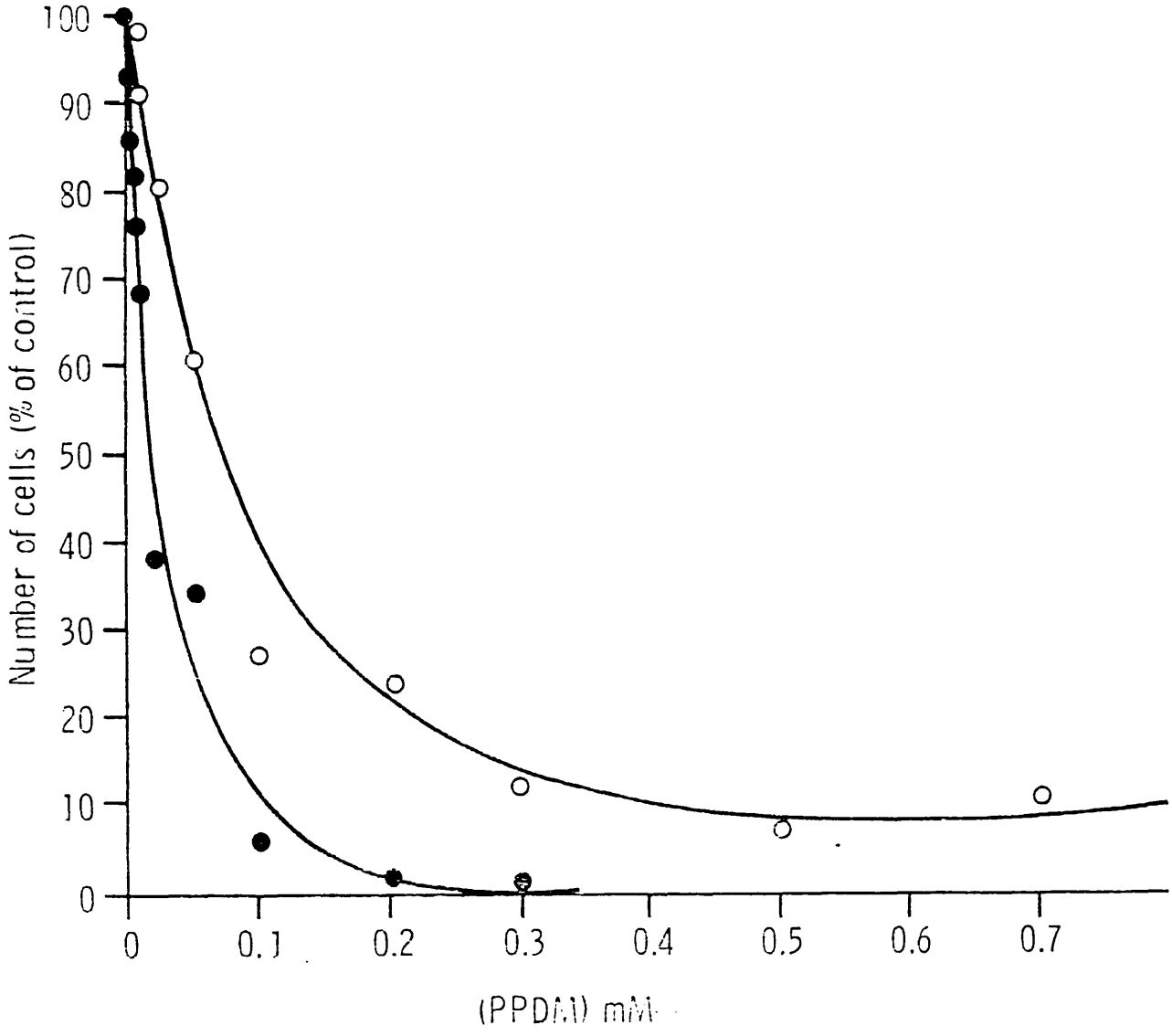


FIG 1Ø

A plot of cell death (as % of control) against various concentrations of PPDM for 2h exposure at 37°C on JBI (-o-o-) and BL8L (-●-●-) cell lines.

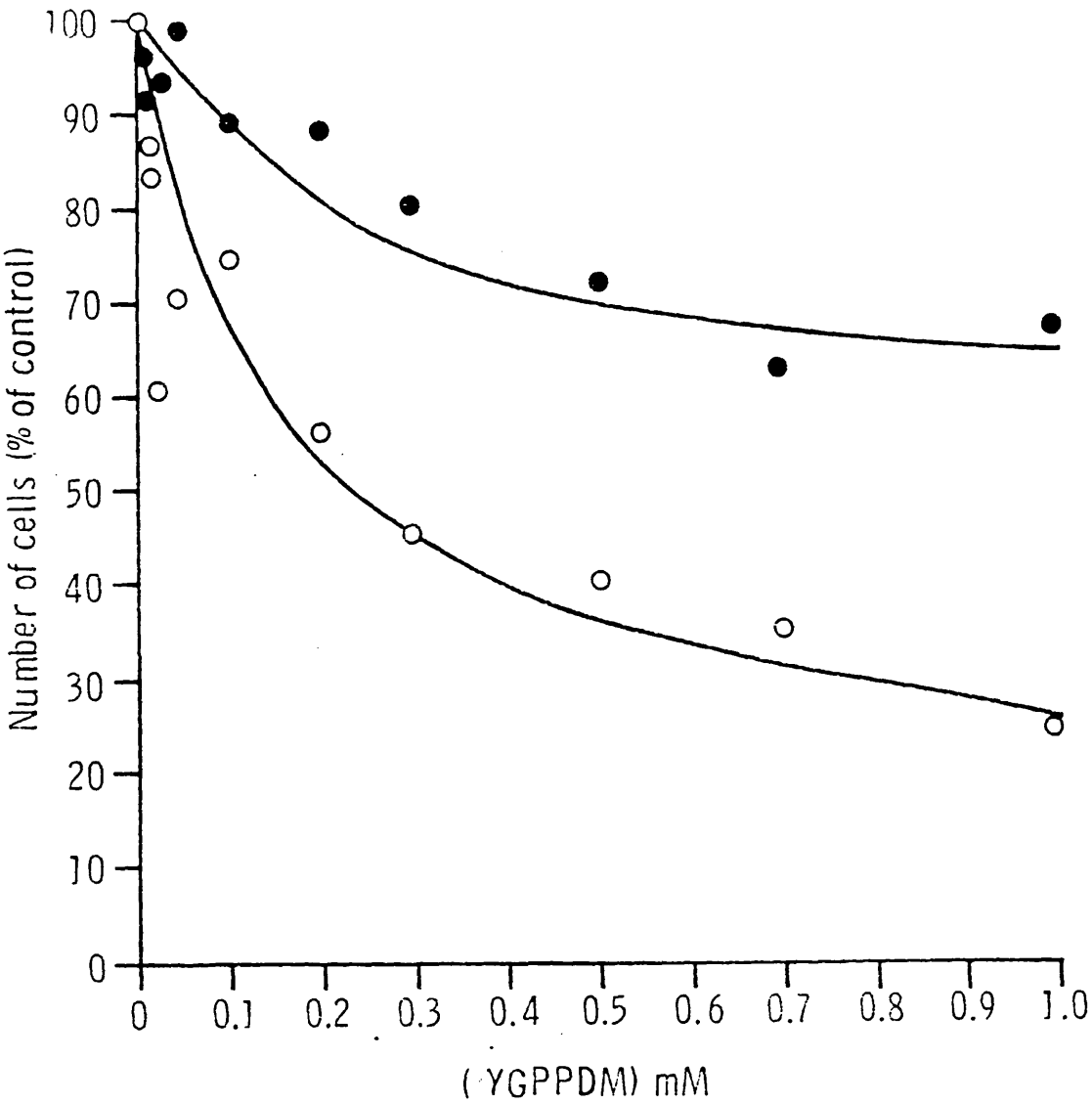


FIG 11

A plot of cell death (as % of control) against varying concentration of YGPDM for 2h exposure at 37°C on JBI (-O-O-) and BL8L (-●-●-) cell lines.

For γGPDM I_{50} value of 220 μm was obtained for the JBI cells compared with an I_{50} of greater than 1.0 mm for the BL8L cells. Because, even high concentrations of γGPDM, failed to destroy 50% of the BL8L cells, I_{20} values (drug concentration killing 20% of cells) are more useful for comparative purposes. The I_{20} = 80 μm for the JBI cells and I_{20} = 250 μm for the BL8L cells.

To determine the optimum time of exposure of the prodrugs to the cell lines, to produce a maximum differential toxicity towards the BL8L and JBI cells, the two cell lines were exposed to 220 μm γGPDM* for various periods of time in HBSS at 37°C. After exposure for the appropriate time, the cells were washed twice with HBSS and left in supplemented Williams media for 24h after which the cells were counted and the results expressed as percentage of the control. These experiments were done in duplicate and the average no of cells was taken. The results are reported in Fig. 12. It is evident that after 1h, 3% of the BL8L cells are destroyed compared to 15% of the JBI cells. After 2h, the observed cell deaths are 20% of BL8L and 50% of JBI cells and after 5h, 45% of BL8L and 70% of JBI cells. Clearly the differential toxicity of the prodrug towards the BL8L and JBI cells appears to decrease with increasing exposure time. An arbitary exposure time of 2h was therefore selected for all further experiments. This time gives good differential toxicity (ie. 2.5 times more JBI than BL8L cells killed), and a sufficiently high percentage of cell deaths to ensure reproducible results. The results in Fig. 12 also show that cell deaths increase with longer exposure to γGPDM up to ca. 5h. Thereafter, the percentage cell deaths are effectively constant. Presumably, the γGPDM prodrug has decomposed in 5h.

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* 220 μm γGPDM corresponds to the I_{50} for JBI cells.

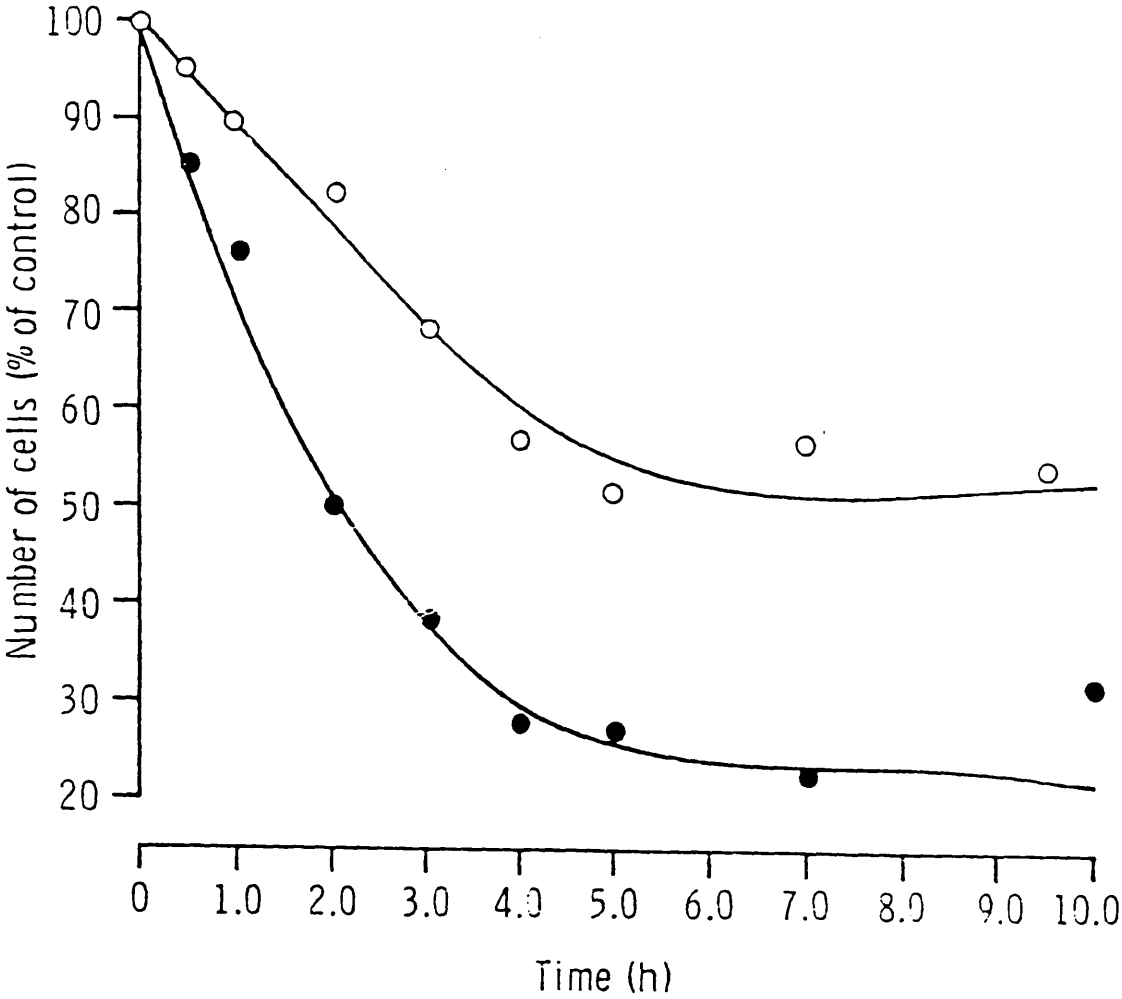


FIG 12

The effect of 220μm YGPDM after various exposure times on JBI (-●-●-) and BL8L (-o-o-) cell lines at 37°C.

Using the standard protocol, the BL8L and JBI cell lines were exposed to all six of the γ-glutamyl prodrugs (γGPDM, γGPDMF, γGPDMCL, γGBrPDM, γGBrPDMF and γG-2-BrPDM) whose synthesis is reported in Chapter 2. Thus, the pre-confluent monolayer of cells were exposed to various concentrations of each prodrug for 2h at 37°C in HBSS media, after which the media was removed and the cells washed twice with HBSS and then incubated in supplemented Williams 'E' media for 24h at 37°C. The remaining cells were counted. The results are shown in Figs. 13-16, and an average of two plates was taken for each concentration. From these graphs I_{20} and I_{50} values for the effect of the prodrugs on the two cell lines were obtained by inspection and these values are reported in Table 4. Both I_{20} and I_{50} values were obtained because in several instances only small effects were evident for the prodrugs on the BL8L cells.

The I_{50} values in Table 4 show that the γ-L-glutamyl dibromo mustards are more toxic to both normal (BL8L) and hepatoma (JBI) cell lines than the corresponding γ-L-glutamyl-dichloro mustards. For the JBI cells, I_{50} values of the γ-L-glutamyl mustards range from 100 to 150 μm, whereas for the dichloro analogues they range from 220 to 270 μm. For the BL8L cells, the I_{50} values vary from 220 to 300 μm, whereas the γ-L-glutamyl dichloro mustards are insufficiently toxic to give I_{50} values for the concentrations studied. From the I_{20} values it is clear that the least toxic γ-L-glutamyl prodrug is γGPDMCl.

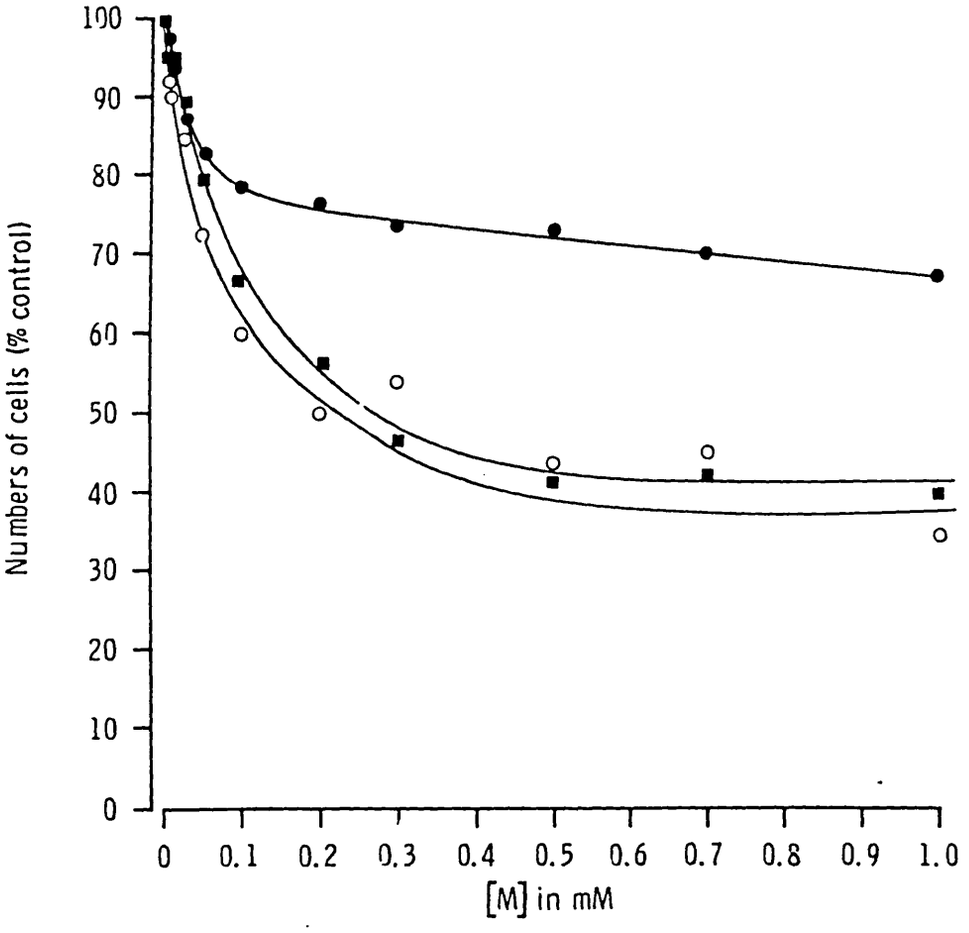


FIG 13

Plot of cell death (as % of control) against prodrug concentration for YGPDM (-●-●-), YGBrPDM (-■-■-) and YGBrPDMF (-O-O-) for the BL8L cell line at 37°C and 2h exposure.

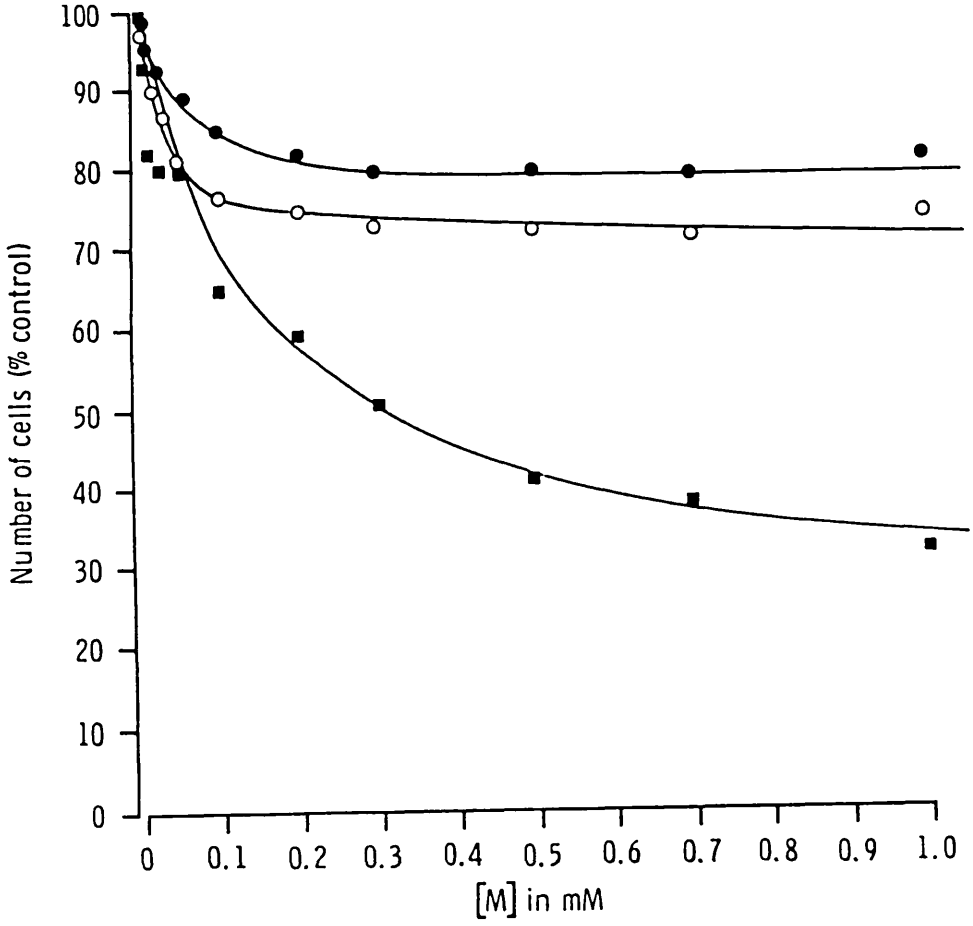


FIG 14

Plot of cell death (as % of control) against prodrug concentration for YGPDMCl (-●-●-), YGPDMF (-○-○-) and YG-2-BrPDM (-■-■-) for the BL8L cell line at 2h exposure and 37°C.

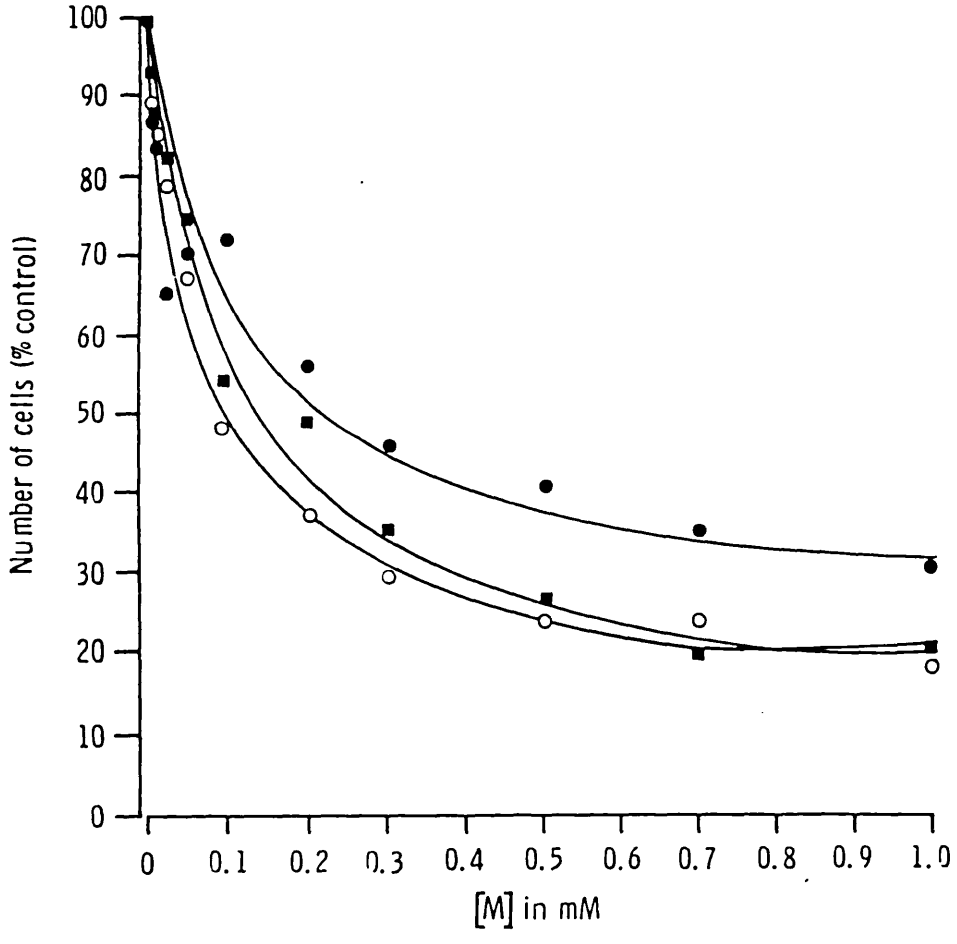


FIG 15

Plot of cell death (as % of control) against prodrug concentration for YGPDM (-●-●-), YGBrPDM (-■-■-) and YGBrPDMF (-o-o-) for the JBI cell line at 2h exposure and 37°C.

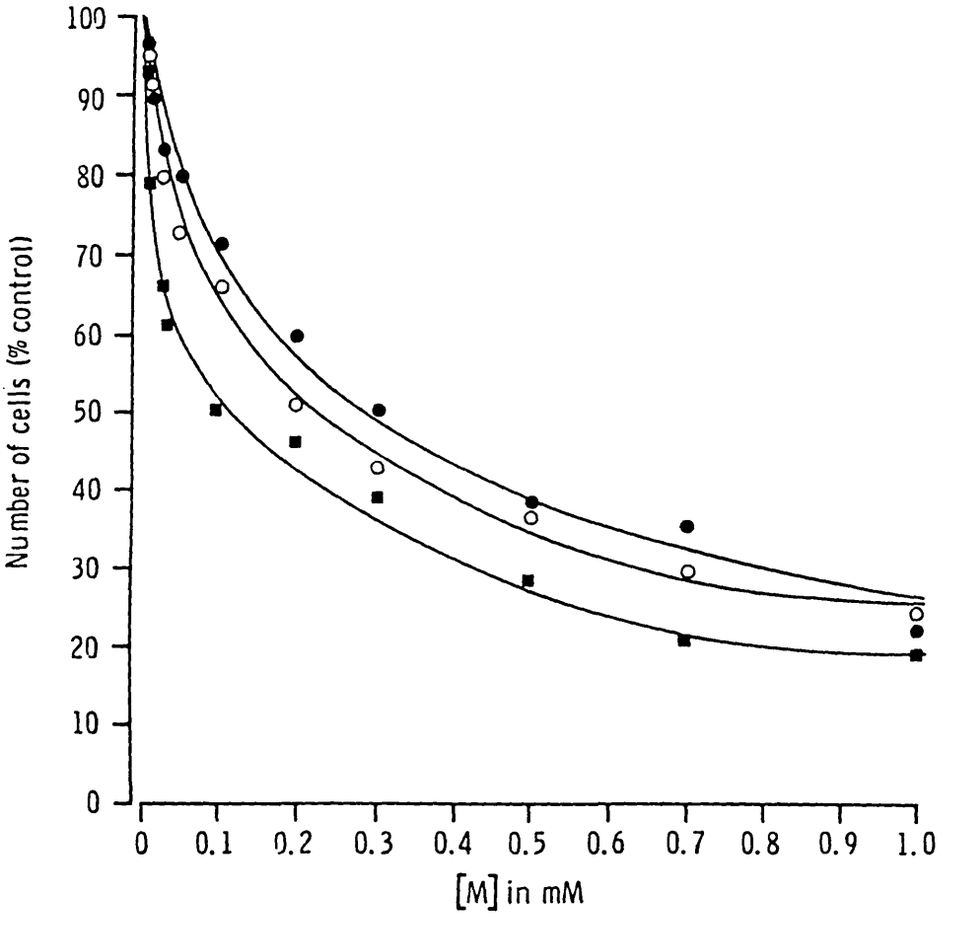


FIG 16

Plot of cell death (as % of control) against prodrug concentration for YGPDMCl (-●-●-), YGPDMF (-o-o-) and YG-2-BrPDM (-■-■-) for the JBI cell line at 37° C and 2h exposure.

	I_{50} (μM)		I_{20} (μM)	
PRODRUG	BL8L	JB1	BL8L	JB1
γGPDM	-	220	75	-
γGPDMF	-	220	50	-
γGPDMCl	-	270	300	-
γGBrPDM	250	150	-	-
γGBrPDMF	220	100	-	-
γG-2-BrPDM	300	110	-	-
PPDM	20	65	-	-

TABLE 4

The I_{50} and I_{20} values for the effect of γ-glutamyl prodrugs on the BL8L and JB1 cell lines .

All of the γ-L-glutamyl prodrugs are more toxic to γGt rich hepatoma JBI cells than normal BL8L cells. However, considerable toxicity towards the BL8L cell line is apparent. This could relate either to the presence of small amounts of γGT in the BL8L cells or to the toxicity of the prodrugs themselves without activation by γGT.

To address these alternative explanations the toxicities of the prodrugs towards the two cell lines was investigated in the presence of 10 mM serine borate, a competitive inhibitor of γGT. These measurements were made by incubating the pre-confluent monolayer of cells for 2h in HBSS at 37°C followed by recovery in supplemented Williams E media for 24h as before. The 2h incubations were carried out in the presence of the serine borate alone and with the addition of the I_{50} concentration of the appropriate γ-L-glutamyl mustard prodrug. The results obtained are summarised in Tables 5-8.
The results show that addition of 10 mm serine borate decreases the toxicities of the γ-L-glutamyl dichloro mustards towards the JBI cells, but has little mediating effect towards the BL8L cells. The effect of 10 mM serine borate on the toxicities of the γ-L-glutamyl dibromo mustards and PPDM is much less noticeable for both, the JBI and BL8L cells. This suggests that all of the γ-L-glutamyl mustards themselves are toxic even without cleavage by γGT. This conclusion applies especially to the more labile dibromo mustard prodrugs.

	CONTROL	SERINE (10mM)	BORIC ACID (10mM)	SERINE BORATE (10mM)	γGDPM (220μM)	γGDPM (220μM) + SERINE BORATE (10mM)
JB1 CELLS (γGT rich) (x 10^4)	204±2.0 (100)	206±6.0 (101)	209±1.0 (99)	195±2.0 (95)	105±3.0 (51)	149±4.0 (73)
BL8L CELLS (γGT poor) (x 10^4)	412±3.0 (100)	423±7.0 (103)	415±1.0 (101)	417±3.0 (101)	318±3.0 (77)	322±7.0 (78)

TABLE 5

The effect of γGPDM (220μM) on BL8L and JB1 cells in the presence and absence of Serine Borate (10mM).

The figures between the parenthesis represent the number of cells as a percentage of the control.

	CONTROL	SERINE (10mM)	BORIC ACID (10mM)	SERINE BORATE (10mM)	γGPDMF (220μM)	γGPDMF (220μM) + SERINE BORATE (10mM)	γGPDMC1 (270μM)	γGPDMC1 (270μM) + SERINE BORATE (10mM)
JB1 CELLS (γGT-rich) ($\times 10^4$)	537±13 (100)	529±6 (99)	532±8 (99)	524±14 (98)	283±9 (53)	422±2 (79)	329±14 (61)	432±5 (80)
BL8L CELLS (γGT-poor) ($\times 10^4$)	464±4 (100)	445±10 (96)	415±11 (89)	428±8 (92)	314±5 (68)	301±4 (65)	338±8 (73)	343±5 (74)

TABLE 6

The effect of γGPDMF(220μM) and γGPDMC1(270μM) on BL8L and JB1 cells in the presence and absence of Serine Borate(10mM). (The figures in parenthesis represent the number of cells as a percentage of control).

	CONTROL	SERINE (10mM)	BORIC ACID (10mM)	SERINE BORATE (10mM)	γGBrPDMF (100μM)	γGBrPDMF (100μM) + SERINE BORATE (10mM)	γGBrPDM (150μM)	γGBrPDM (150μM) + SERINE BORATE (10mM)
JB1 CELLS (γGT-rich) ($\times 10^4$)	470±6 (100)	478±6 (102)	468±4 (99)	464±4 (99)	239±3 (51)	244±3 (52)	231±3 (49)	242±3 (52)
BL8L CELLS (γGT-poor) ($\times 10^4$)	337±8 (100)	354±7 (105)	326±4 (97)	345±13 (102)	136±3 (40)	140±4 (42)	156±5 (46)	160±10 (48)

TABLE 7

The effect of γGBrPDMF(100μM) and γGBrPDM(150μM) on BL8L and JB1 cells in the presence and absence of Serine Borate(10mM). (The figures in parenthesis represent the number of cells as a percentage of control).

	CONTROL	SERINE (10mM)	BORIC ACID (10mM)	SERINE BORATE (10mM)	PPDM (65μM)	PPDM (65μM) + SERINE BORATE (10mM)	γG-2-BrPDM (110μM)	γG-2-BrPDM (110μM) + SERINE BORATE (10mM)
JB1 CELLS (γGT-rich) (× 10^4)	417±14 (100)	428±4 (103)	395±1 (95)	395±2 (95)	200±8 (48)	184±3 (44)	234±8 (56)	244±6 (58)
BL8L CELLS (γGT-poor) (× 10^4)	344±14 (100)	349±7 (101)	331±7 (96)	354±6 (103)	137±6 (40)	136±2 (40)	165±2 (48)	172±4 (50)

TABLE 8

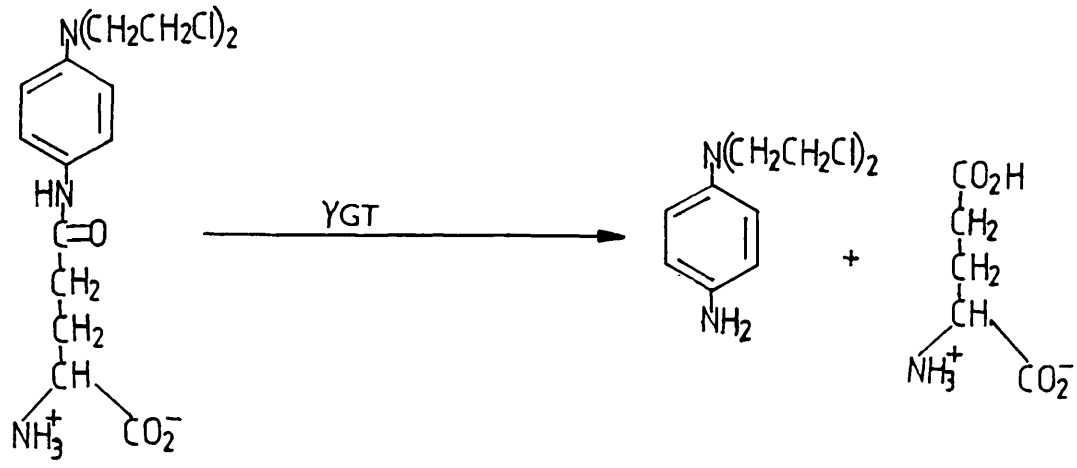
The effect of PPDM(65μM) and γG-2-BrPDM(110μM) on BL8L and JB1 cells in the presence and absence of Serine Borate(10mM). (The figures in parenthesis represent the number of cells as a percentage of control).

4.3 Discussion

4.31 γ-L-Glutamyl-N,N-bis-(2-chloro ethyl)-4-phenylene diamine mustards

The parent mustard (PPDM) was found to be more toxic towards the normal BL8L cells than the hepatoma JBI cells. The chemotherapeutic use of nitrogen mustards depends on their preferential alkylation of the more rapidly proliferating tumour cells. The same factor is responsible for their differential toxicity towards the two cell lines. The regeneration time for the normal rat hepatocyte BL8L cells was almost half that of the rat hepatoma JBI cells. Therefore, PPDM is expected to be more lethal towards the BL8L than the JBI cells, and this is reflected by the I_{50} values which are over three times greater for the JBI than the BL8L cells.

The relative toxicity towards the two cell lines is reversed for the γ-L-glutamyl adduct γGPDM. Thus, γGPDM is more toxic towards the JBI than the BL8L cells, with I_{50} values of 220 μm for the JBI cells and a value greater than 1 mM for the BL8L cells. This reversal of relative toxicity probably reflects the higher γGT activity in the JBI cells compared to the BL8L cells. The γGT enzyme facilities cleavage of the amide bond between the γ-carboxy group of the L-glutamic acid and the 4-phenylene diamine mustard to release the parent mustard PPDM (scheme 47). Since PPDM is expected to be a better alkylating agent than γGPDM, DNA alkylation should be more extensive for the JBI cells with a corresponding greater cytotoxic effect. The differential cytotoxicity of γGPDM on the two cell lines should be greater *in vivo* because normal hepatocytes divide less rapidly than hepatoma cells and are therefore less susceptible to DNA alkylation.



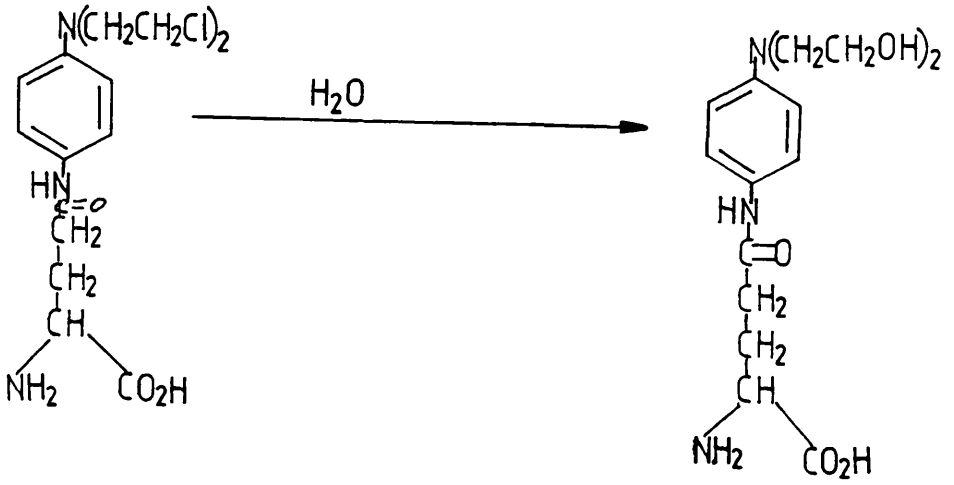
Scheme 47

The BL8L cells contain low levels of γGT and the conversion of γGPDM to the parent PPDM will therefore be lower than in JBI cells. Nevertheless γGPDM is cytotoxic towards the BL8L cells.

In the presence of the γGT inhibitor serine borate, the damaging effect of γGPDM on the JBI cell line is lower. This is possibly due to the lack of γGT activity, thus preventing the formation of the much stronger alkylating parent mustard, resulting in a decrease in damaging effect on the JBI cells. There is little or no difference in the damaging effect of the γGPDM on the BL8L cells with or without the presence of serine borate. It is noticeable that the percentage number of JBI cells destroyed by γGPDM in the

presence of serine borate is similar to the number of BL8L cells destroyed by the prodrug in the absence and presence of 10 mM serine borate. These results suggest that the toxic effects of γGPDM on the BL8L cell line is predominantly due to alkylation by the γ-L-gluatmyl prodrug rather than by small amounts of the parent mustard released by the trace of γGT activity in the BL8L cells. Indeed, data from Ross[30] suggest that even 4-N,N-bis (-2-chloro ethyl) - amino acetanilide (which would be expected to possess similar alkylating capacity as γGPDM) retains some alkylating capacity.

The cytotoxic effects of γGPDM on both the BL8L and JBI cells levels off after 5h. This probably relates to the deactivation of γGPDM by hydrolysis (scheme 48). The optimum exposure time for the cell line experiments was 2h. This seems reasonable bearing in mind the hydrolysis rates of aromatic mustards reported by Ross[30].



Scheme 48

To obtain a prodrug with a better chemotherapeutic ratio (ie. large differential cytotoxicity between BL8L and JBI cells), analogues bearing electron withdrawing aromatic substituents were synthesised and examined. In particular, the effect of electron-withdrawing substituents at the 2-position of γGPDM was investigated. These compounds should be less effective alkylating agents, due to the decreased basicity of the tertiary nitrogen atom of the mustard substituent. The electron withdrawing groups examined were fluorine and chlorine.

The I_{50} of the γGPDMF and γGPDMCl prodrugs towards the JBI cells were 220 and 270 μm, respectively, compared with an I_{50} value of 220 μm for γGPDM, itself. Therefore, there is little difference in their cytotoxicity towards the JBI cells, and all three parent mustards derived from the prodrugs must possess similar alkylating strength. The 4-amino group is a strong electron donator and its activating effect is apparently not strongly mediated by the relatively weak electron withdrawal by the 2-halogeno substituent.

The I_{20} values for γGPDMF and γGPDMCl towards the BL8L cell lines are 50 μm and 300 μm respectively, compared with I_{20} value of 75 μm for γGPDM, itself. Thus, γGPDM and γGPDMF exert similar toxicities towards the BL8L cells whereas γGPDMCl is 4-5 times less toxic.

Experiments in the presence of serine borate inhibitor gave similar results to γGPDM. This suggests that the cytotoxicity of these prodrugs relates to their inherent ability to act as alkylating agents without cleavage to the parent mustard. Thus, the presence of the electron-withdrawing 2-fluoro substituent doesn't appear to significantly reduce this activity.

The results from the enzyme kinetic experiments (Chapter 3) suggest, however, that γGPDMF is a better substrate for the hydrolysis reaction by γGT than either γGPDM or γGPDMCl. It follows that the higher toxicity of γGPDMF towards BL8L cells may relate to more extensive conversion to its active parent mustard by the low levels of γGT in the BL8L cells than for either γGPDM or γGPDMCl. As the BL8L cells are more susceptible to alkylation by the parent mustard, the generation of even marginally higher amounts of parent mustard would have a disproportionate effect on the survival of BL8L cells. It should be pointed out, however, that the results clearly show that γGT in the cell lines possess greater transpeptidation than hydrolytic activity. Thus, the order of substrate suitability of the prodrugs towards the cell lines primarily relates to transpeptidation by the enzyme. It does not follow that γGPDMF is the best prodrug substrate for the transpeptidation reaction of γGT.

4.32 γ-L-Glutamyl-N,N-bis-(2-bromo ethyl and propyl) amino mustards

The ideal prodrug is one which produces a very reactive metabolite with a short half life, so that cytotoxicity is confined to the activating tissue. With this in mind, the γ-L-glutamyl-N,N-bis-(2-bromo ethyl and propyl) amino mustards γGBrPDM, γGBrPDMF and γG-2-BrPDM were also synthesised and their cytotoxicity examined. The 2-bromo substituent is a better nucleofuge than the 2-chloro substituent. It was hoped that the γ-L-glutamyl - N,N-bis-(2-bromo alkyl) amino mustards would themselves be of sufficiently low activity to produce an effective differential effect. As the parent bis-bromo mustard should be highly reactive with hydrolysis proceeding in a matter of seconds hence alkylation might be confined to the liver. This would minimise damage to the bone marrow, which

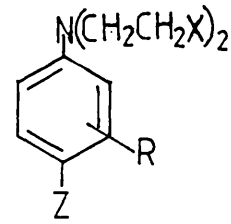
contains very sensitive normal cells. In the liver, dividing malignant cells would be more sensitive to alkylation than non-dividing normal cells.

The I_{50} values for all three γ-L-glutamyl N,N-bis-(2-bromo ethyl) amino and γ-L-glutamyl - N,N-bis-(2-bromo propyl) amino mustards reveal only small differences in their cytotoxicity towards both JBI and BL8L cells. The I_{50} values towards the JBI cells for γGBrPDM, γGBrPDMF and γG-2-BrPDM are 150, 100 and 110 μm, respectively. I_{50} values towards the BL8L cells for γGBrPDm, γGBrPDMF and γG-2-BrPDM are 250, 220 and 300 μm, respectively. Thus, structural differences produced little difference in cytotoxicity towards either cell line, and all of γ-L-glutamyl bis-bromo mustards destroy ca. 2-3 times more JBI cells than BL8L cells. The results imply that these γ-L-glutamyl adducts possess similar alkylating abilities to their parent mustards. This conclusion is supported by the small effect of serine borate inhibitor on the relative cytotoxicities towards the two cell lines. Further, the effects of serine borate inhibition on γ-L-glutamyl - N,N-bis-(2-bromo ethyl and propyl) amino mustards are similar to those for the parent N,N-bis-(2-chloro ethyl) -4-phenylene diamino mustard.

The similar toxicities of γ-L-glutamyl adducts and parent mustards for the N,N-bis-(2-bromo alkyl) compounds could relate to the very good leaving ability of the bromide ion. The mechanism of bromide displacement would be predominantly SNI mechanism compared to SN2 mechanism for displacement of chloride from the N,N-bis-(2-chloro ethyl) amino aromatic mustards. The alkylating capacity of the γ-L-glutamyl bis bromo aromatic mustards may therefore be high.

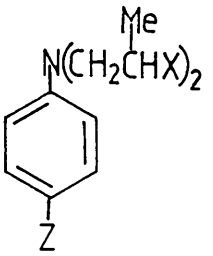
The low differential cytotoxicity of the γ-L-glutamyl -N,N-bis-(2-bromo ethyl and propyl amino mustard prodrugs towards the JBI and BL8L cell lines implies that such compounds will be inefficient anti cancer compounds.

COMPOUND INDEX

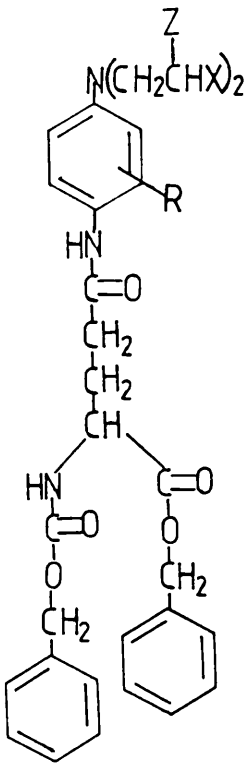


	X	Z	R
[26]	-OH	-H	-H
[27]	-OTs	-H	-H
[28]	-Cl	-H	-H
[29]	-Cl	-NO	-H
[30]	-Cl	$-NH_2$	-H
[32]	OH	NO_2	2-Cl
[33]	-OTs	$-NO_2$	2-Cl
[34]	-Cl	$-NO_2$	2-Cl
[35]	-Cl	$-NH_2$	2-Cl
[38]	-OH	$-NO_2$	2-F
[39]	-OTs	$-NO_2$	2-F

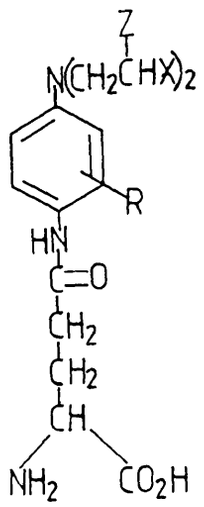
	X	Z	R
[40]	-Cl	$-NO_2$	2-F
[43]	-Br	$-NO_2$	2-F
[46]	-Br	-H	-H
[47]	-Br	-NO	-H
[53]	-OH	-H	$3-OCH_3$
[54]	-OTs	-H	$3-OCH_3$
[55]	-Cl	-H	$3-OCH_3$
[56]	-Cl	-NO	$3-OCH_3$



	X	Z
[50]	OH	-NH-C(=O)-CH3
[22]	Br	-NH-C(=O)-CH3
[23]	Br	-NH2HBr



	X	Z	R
[31]	-Cl	-H	-H
[36]	-Cl	-H	2-Cl
[41]	-Cl	-H	2-F
[44]	-Br	-H	2-F
[48]	-Br	-H	-H
[51]	-Br	-Me	-H
[57]	-Cl	-H	3-OMe



	X	Z	R
[14]	-Cl	-H	-H
[37]	-Cl	-H	2-Cl
[42]	-Cl	-H	2-F
[45]	-Br	-H	2-F
[49]	-Br	-H	-H
[52]	-Br	-Me	-H

CHAPTER 5

EXPERIMENTAL

5.1. GENERAL METHODS

5.11. Instrumentation

Fluorescence measurements were performed on a Perkin Elmer LS-3 fluorescence spectrophotometer. A model 2F coulter counter was used to count cells. Infra-red spectra were recorded on either a Perkin Elmer 88I or a Perkin Elmer 298 grating spectrophotometer. Thin films between sodium chloride plates were used for neat liquids and nujol mulls of solids. Dilute chloroform solutions were also used when required. The spectra were calibrated against polystyrene and ν_{max} is reported in wavenumbers (cm^{-1}). ^1H NMR spectra were recorded on either a Varian EM 360A (60 mHz) or a Jeol (90 mHz) FT spectrometer, as dilute solutions in the appropriate deuterated solvents, with tetramethylsilane as internal standard. Signals are quoted as δ values and described as singlets (s), doublets (d), double doublets (dd), triplets (t), quartets (q), multiplets (m), or broad (br).

Micro analyses were performed by Mr K Jones of Imperial College, London. Mass spectra were recorded by a VG 7070 mass spectrometer, operated by Dr J A Challis or Mr J N Bilton. A Hercule type B5060 EK/O_2 incubator was used for cell culture experiments. HPLC was performed on a Varian model 5000 liquid chromatograph, the OPT-derivatised products being detected with a Pye Unicam PU 4024 fluorescence detector. Melting points of compounds were measured on a Gallenkamp melting point apparatus and are uncorrected.

5.12. Reagents and Materials

Tissue culture material was obtained from Flow Laboratories Ltd, Irivine, Ayrshire. Phenyl boronate matrix PBA 60 and YM-10 ultrafiltration membranes were purchased from Amicon Ltd, Surrey and Sephadex G-200 from Pharmacia (GB) Ltd, Middlesex. The JB1 and BL8L cell lines were a gift from the MRC Toxicology Unit, Carshalton Surrey. Isotone solution was obtained from Coulter Electronics Ltd, Luton. γ-L-glutamyl-7-amino-4-methyl coumarin was purchased from Uniscience, Cambridge Ltd. All other chemicals were purchased from either Sigma Chemicals Co. Ltd, Poole, Dorset, BDH Ltd, Poole or Aldrich Chemicals Co. Ltd, Gillingham, Dorset. Where necessary, they were purified by conventional techniques.

5.2. BIOCHEMICAL METHODS

5.21. Purification of γ-glutamyl transferase (γGT) from rat kidney

This enzyme was purified by the method of Cook and Peters[79]. The ureters and capsules of rat kidneys were removed and the residual tissue was placed in 100 mM mops-HCl buffer containing 1 mM EDTA (pH 7.2). The mixture was then homogenised for 2 minutes in a waring blender at top speed at 4°C. The resulting homogenate was centrifuged at 16000 g for 1 h. The supernatant liquid was discarded and the pellets resuspended in 100 mM Mops-HCl buffer pH (7.2) and pelleted by centrifugation at 16000 g to obtain washed membranes.

These membranes were proteolytically digested by papain which had previously been activated with 10 mM cystine for 30 min at room temperature. The membranes were treated with 1 unit papain per mg membrane protein for 2 h at 37°C and the resulting mixture centrifuged at 16000g for 30 min. The supernatant was fractionated with ammonium sulphate. The fraction from 50-100% saturation was collected by centrifugation at 16000 g and resuspended in a minimal volume of 100 mM Mops-HCl buffer (pH 7.2).

The resuspended fraction was then loaded onto a phenyl boronate PBA-60 column (2.7 cm x 8.6 cm) which had been pre-equilibrated with 100 mM NH_4HCO_3-buffer (pH 8.7). The γGT was eluted with the equilibration buffer + 10 mM Tris (pH 8.7). A typical chromatogram is shown as Fig 1 (Chapter 3). The γGT containing fractions (where each fraction represents 3 cm^3) were combined and concentrated by ultrafiltration via an Amicon YM-10 ultrafiltration membrane. The concentrated γGT was then subjected to gel filtration on a G-200 column (2.2 x 80 cm^3), pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5). A typical chromatogram is shown in Fig 2 (Chapter 3). The γGT containing fractions (3 cm^3) were collected and concentrated by ultrafiltration as before. Table 1 (Chapter 3) summarises the quantities and purification obtained for a typical batch.

5.22 γ-Glutamyl Transferase Assay

The enzyme assay procedure devised by Smith et al[140] was used. A stock substrate solution was prepared by suspending γ-L-glutamyl AMC in 2-methoxyethanol to give a concentration of 10 mM. The suspension was sonicated briefly (20 s) to produce homogeneity.

The stock solution (0.2 cm^3) was then diluted with 9.8 cm^3 of 0.1M ammediol/HCl buffer pH 8.5, containing 20 mM glycyl glycine. The enzyme activity was determined by adding 0.1 cm^3 of enzyme solution to 0.25 cm^3 substrate, and incubating the mixture at 37°C for the appropriate time (5-8 min). The reaction was then stopped by the addition of 2 cm^3 of 50 mM glycine buffer, pH 10.4. The γGT activity was measured fluorimetrically, reading the fluorescence at 440 nM with excitation at 370 nm. A standard fluorescent block was calibrated at these wavelengths against a standard solution of authentic γ-L-glutamyl-AMC.

Assay of γGT hydrolase activity was measured similarly but omitting glycyl glycine from the reaction mixture.

5.23. The HPLC Assay for γ-glutamyl transferase

The HPLC assay of Cook et al[135] was used. Thus to 250 ml of the appropriate dilution of the substrate (γ-glutamyl adducts) in 100 mM mops buffer pH 7.2, 100 µl of the enzyme solution was added and the mixture was left for 6 min at 37°C, after which the reaction was stopped by the addition of 650 µl 0.1M NaOH and cooling the contents over ice. A 50 µl aliquot of terminated reaction mixture of appropriate dilution was mixed with 50 µl of 40 mM OPT/49 mM -mercaptoethanol in 0.1M NaOH. After leaving for 2 min, 50 µl of this solution was injected onto the HPLC column. The sample was chromatographed by reverse-phase chromatography on an ODS-Hypersil column using 300 mM sodium acetate with either 12 or 17% acetonitride (pH 4.85) at a flow rate of 1.5 ml/min. The glutamic

acid eluted at 7 min for 17% acetonitride and 18 min for 12% acetonitrile. The quantity of glutamic acid released was calculated from the peak height by comparison with a calibration curve generated with authentic glutamic acid (p Mol).

The K_M and V_{max} values for the hydrolysis of γ-glutamyl prodrugs were determined by the direct linear pilot method.

5.24. Cell Lines

The two cell lines used, designated BL8L and the JB1 were isolated from male Fischer 344 rats as described by Manson et al[73], and Neale et al[141]. BL8L represents the normal hepatocyte cell line, whereas JB1 represents the hepatoma cell line, containing elevated levels of γGT. Both cell lines were cultured in 90 mm plastic petri dishes containing 10 cm^3 of Williams' Medium E supplemented with 5% calf foetal serum, 2 mm glutamine and 50 μg gentamycin cm^3. They were grown at 37°C in a atmosphere consisting of 95% air 5% carbon dioxide.

The cells were subcultured by trypsinizing for 2 min. The trypsin solution was removed and the plates left in the incubator for 15 min, after which 10 cm^3 of supplemented Williams 'E' media was added and the cells pipetted into the media bottle. The cells were reseeded into plastic petri dishes and left in the incubator at 37°C until the cell lines were confluent or almost confluent.

The cells were subcultured after becoming confluent, and were subsequently passaged several times.

5.25. Cytotoxicity Studies

These experiments were carried out with a pre-confluent monolayer of cells. The media was removed, the monolayer cultures were washed twice with 10 cm^3 of Hanks balanced salt solution (HBSS) and then the appropriate concentration (usually 0-1 mM) of mustard was added in HBSS (10 cm^3). After exposing the cells to the mustard for the desired time at 37°C, the medium was removed, the cells were washed twice in HBSS (10 cm^3), and then allowed to recover in 10 cm^3 of supplemented Williams "E" Medium for 24 h in the incubator at 37°C. Subsequently, the cells were detached from the dishes by trypsinisation, and centrifuged at 2,500 rpm for 5 min. The pellet was recovered and resuspended in 5 cm^3 of phosphate buffered saline and gently homogenised. The cells were then counted on a Coulter Counter. Sterile materials and methods were used throughout these experiments.

5.3. SYNTHETIC METHODS

5.31. N-Carbobenzoxy glutamic acid [19]

Glutamic acid (50g, 0.34 mol) was dissolved in 2M sodium hydroxide (350 cm^3) in a 3 necked round bottomed flask. This solution was cooled to 2-3°C with stirring. Both benzylchloroformate (58 ml, 0.4 mol) and 4M sodium hydroxide (90 cm^3) were simultaneously added dropwise over a period of 30 min. The mixture was left to stir overnight, until the emulsion of the benzylchloroformate had all dissolved. The mixture was washed with ether (3 x 200 cm^3) and

then acidified (at a temperature of 2-4°C) to approximately pH 1.5 with concentrated hydrochloric acid whilst stirring vigorously. The resulting solution was extracted with ethyl acetate (3 x 500 cm^3) and then dried over $MgSO_4$. The solvent was evaporated off to give crude N-carbobenzoxy glutamic acid.

Yield, 84.9g (88.9%); M.P. 118-121°C

ν_{max} (Nujol) 3400, 2700, 1750, 1500 cm^{-1}

δ^1H (Acetone-d_6) 7.25 (5H, s), 6.5 (1H, br, s), 5.0 (2H, s), 4.3 (1H, q), 2.5 (4H, m).

5.32. α-Benzyl-N-carbobenzyoxy-L-glutamate [20]

The method of Nefkens and Nivard[124] was used in the preparation of α-Benzyl-N-carbobenzoxy glutamate.

To N-carbobenzoxy-L-glutamic acid (75g, 0.27 Mol) dissolved in DMF (300 cm^3), was added di-cyclo hexylamine (52.7 cm^3, 0.27 Mol). A white, waxy precipitate was formed, which dissolved on heating the mixture to 80°C. At 80°C benzyl bromide (33.75 cm^3, 0.27 mol) was added to the solution and the mixture stirred for 10 min. A white precipitate was filtered off and the filtrate washed with water (3 x 750 cm^3) to remove DMF. The solution was then dried ($MgSO_4$) and the solvent evaporated under vacuum to give α-benzyl-N-carbobenzoxy L-glutamate as a yellow oil.

Yield 58.7g (62%);

ν_{max} (Neat) 3370, 2900, 1740, 1690, 1520, 750 cm^{-1}

δ^1H ($CDCl_3$) 7.3 (10H, S), 5.7 (1H, br, d), 5.1 (4H, d), 4.1 (1H, q), 2.4 (4H, m).

5.33. N,N-Bis-(2-hydroxy ethyl) aniline [26]

Aniline (14g, 0.15 Mol) and ethylene oxide (15g, 0.3 Mol) were heated at 150°C for 16h in a Carius tube. The contents were extracted with alcohol and crystallised from benzene to give white plates of N,N-bis-(2-hydroxy ethyl) aniline. Yield 25.7g (94.7%); M.P. 56°C, lit 55°C[148].

ν_{max} (Nujol) 3350, 1600, 1500, 1330, 750 cm^{-1};

δH (Acetone-d_6) 7.0 (5H, M), 4.2 (2H, s, br) 3.8 and 3.5 (8H, dt).

5.34. N,N-Bis-(2-toluene-4-sulphonate ethyl) aniline [27]

N,N-Bis-(2-hydroxy ethyl) aniline (2g, 0.011 Mol) and toluene-4-sulphonyl chloride (4.6g, 0.024 Mol) in pyridine (22 cm^3) was stirred ca 0°C for 1h. A further portion (4.6g, 0.024 mol) of the acid chloride was added, and stirring maintained for 1h at 0°C and then a further 1h at ambient temperature. The mixture was cooled, and treated dropwise with sufficient water to decompose the excess sulphonyl chloride. More water (60 cm^3) was added and the resulting suspension was left stirring overnight to

give a pale yellow solid, which was collected, washed with water, and dried to give the crude N,N-bis-(2-toluene-4-sulphonate ethyl) aniline, yield 5g (92.5%). A small amount (0.5g) was recrystallised from ethanol to give white needles of the pure product. M.P. 90-91°C.

ν_{max} ($CHCl_3$) 1600, 1500, 1360, 1100, 800, 750 cm^{-1}

δ'H (Acetone-d_6) 7.5 (8H, m), 6.8 (5H, m), 4.1 and 3.6 (8H, dt), 2.4 (6H, s)

m/z (EI) 489 (M^+, 0.9%), 445 (25.3), 260 (100), 155 (82.3).

Found: C, 58.92; H, 5.51; N, 2.76. $C_{24}\ H_{27}\ N\ S_2\ O_6$ requires C, 58.40; H 5.52; N, 2.86%.

5.35 N,N-Bis-(2-chloro ethyl) aniline [28]

The di tosyl ester (2g, 4.1 m Mol) was dissolved in sodium dried toluene (20 cm^3), containing potassium chloride (0.89g, 0.012 Mol) and 18-crown-6-ether (1.08g, 4.1 μ Mol). The stirred suspension was heated under reflux overnight on an oil bath. The resulting mixture was filtered and the solvent evaporated. The solid obtained was passed through a short column of neutral alumina using benzene as eluent. The eluates were evaporated to yield N,N-bis-(2-chloro ethyl) aniline as a white solid, which was crystallised from benzene-petroleum ether.

Yield 0.59g, (66%) M.P. 45°C (lit 45°C)[48].

ν_{max} (Nujol) 1600, 1500, 770, 650 cm^{-1}

δ'H 7.0 (5H, m), 3.7 (8H, dt);

m/z (EI) 217 (M^+, 10.4%), 170 (31.8), 168 (100).

5.36. N,N-Bis-(2-chloro ethyl)-4-nitrosoaniline [29]

The N,N-Bis-(2-chloroethyl) aniline (2g, 9 m Mol) was dissolved in a warm mixture of conc. HCl (3.9 cm^3) and water (3.7 cm^3). After cooling to 5°C, the mixture was vigorously stirred and a solution of sodium nitrite (0.67g) in water (1.85 cm^3) was added dropwise maintaining the temperature at ca. 5-7°C. After the addition the mixture was stirred for a further 20 min and then ether (40 cm^3 and water (40 cm^3) were added. The aqueous layer was separated and twice extracted with ether (40 cm^3). The ether extracts were dried ($MgSO_4$). Evaporation of the ether gave deep green plates of N,N-bis-(2-chloro ethyl)-4-nitrosoaniline.

Yield 1.77g (78%) M.P. 79-80°C, (lit 79-80°C)[49].

ν_{max} (Nujol) 1600, 1500, 1380, 1100, 820, 710 cm^{-1}

δ'H (Acetone-d_6) 7.4 (4H, m), 4.0 (8H, dt)

m/z EI 247 (m^+).

5.37. N,N-Bis-(2-chloro ethyl)-4-phenylene diamine hydrochloride [30]

A solution of N,N-bis-(2-chloro ethyl)-4-nitrosoaniline (0.8g, 3.2 m Mol) in methanol (180 cm^3) containing 10% palladium-charcoal (0.1g) was stirred under hydrogen until the calculated amount of gas had been absorbed. The solution was filtered into 50% hydrogen chloride in methanol (21 cm^3). On evaporation of the methanol, a yellow solid was obtained which started to go brown. Therefore, it was used immediately in the next stage of the synthesis. The crude yield was 0.7g (81%). A small amount of the brown solid (0.2g) was recrystallised from methanol/ether twice to obtain a pure product as almost colourless plates.

M.P. 245-258 d (lit 250-260 d)[49]

δ'H (DMSO-d_6) 10.1 (3H, s, br), 7.0 (4H, q), 3.7 (8H, s);

m/z (FAB + ve ion) 233 (MH^+-HCl);

Found: C,44.27; H, 5.71, N, 10.23. Calc for $C_{10}H_{15}N_2Cl_3$, C, 44.53; H, 5.57; N, 10.39%.

5.38. γ-Benzyl-N-carbobenzoxy-N-N-bis-(2-chloroethyl)-4-phenylene diamine glutamate [31]

γ-Benzyl-N-carbobenzoxy-L-glutamate (1.0g, 2.74 m Mol) and triethylamine (0.28g, 2.9 m Mol) was dissolved in THF (24 cm^3). The mixture was left stirring and cooled to -15°C with a salt/ice bath. Ethyl chloroformate (0.30g, 2-8 m Mol) in THF (12 cm^3) was added dropwise while stirring, maintaining the temperature below -10°C. This mixture was left stirring for a further 45 min below -10°C, after which a pre-cooled solution of N,N-bis-(2-chloro ethyl)-4-phenylene diamine hydrochloride (0.75g, 2.8 m Mol) and triethylamine (0.28g, 2.8 m Mol) in THF (12 cm^3) was added. The mixture was left stirring for 2 h, after which the solid triethylamine hydrochloride was filtered off and the solvent evaporated from the filtrate. The residual solid was dissolved in dichloromethane (50 cm^3) then washed with water. After drying ($MgSO_4$), the dichloromethane was evaporated off to give a yellow solid, which was recrystallised from ethyl acetate/petroleum ether, to give γ-benzyl-N-carbobenzoxy-N,N-bis-(2-chloroethyl-4-phenylene diamine glutamate as a white solid.

Yield 0.74g (46%); M.P. 121-123°C;

ν_{max} (Nujol) 3370, 1740, 1680, 1520, 1270, 810, 780 cm^{-1};

δ'H (Acetone-d_6) 8.9 (1H, s, br), 7.6 (14H, m), 5.1 (4H, d), 4.4 (2H, m), 3.8 (8H, S), 2.4 (4H, m).

m/z (EI) 585 (M^+);

Found: C, 61.24; H, 5.62; N, 7.13. $C_{30}H_{33}Cl_2N_3O_5$ requires C, 61.43; H, 5.63; N, 7.17%.

5.39. γ-[N,N-bis-(2-chloro ethyl)-4-phenylene diamine]-glutamic acid [14]

γ-Benzyl-N-carbobenzoxy-N-N-bis-(2-chloro ethyl)-4-phenylene diamine glutamate (0.5 g, 0.85 m Mol) was dissolved in a mixture of methanol (25 cm^3) and dichloromethane (10 cm^3). The solution was stirred overnight under hydrogen in the presence of 10% palladium-charcoal catalyst. The catalyst was removed by filtration and the solvents evaporated off. The resulting solid was washed with absolute ethanol to give a white solid of γ-[N,N-bis-(2-chloro ethyl)-4-phenylene diamine]-glutamic acid. For analysis the solid was recrystallised from methanol.

Yield 0.21g (68%), M.P. 159-161°C

ν_{max} (Nujol) 3375, 1680, 1610, 1590, 800 cm^{-1};

δ'H (DMSO-d_6) 7.4 (4H, m), 4.2 (1H, m), 3.65 (8H,t), 2.4 (4H, m);

m/z (FAB + ve ion) 363 (MH^+);

Found: C, 49.45; H, 5.85; N, 11.61. Calc. for $C_{15}H_2Cl_2N_3O_3$ C, 49.72; H, 5.80; N, 11.60%.

5.40. N,N-bis-(2-hydroxy ethyl)-2-chloro-4-nitro aniline [32]

A mixture of 3,4-dichloro nitro benzene (5g, 2.6 m Mol) and di ethanol amine (10 cm^3) was stirred at 118°C for 24 h after which the solution was cooled and partitioned between water (100 cm^3) and dichloromethane (100 cm^3). The organic layer was washed with

water (100 cm^3), and then dried ($MgSO_4$). A orange solid of 3',3', 4,4'-tetrachloro azobenzene was deposited, M.P 154-157°C (lit 158°C). The filtrate was concentrated to a black oil, which was separated by chromatography (silica) using ethyl acetate as eluent, to give an orange oil of N,N-bis-(2-hydroxy ethyl)-2-chloro-4-nitro aniline, which had the same spectral data as that in the literature[132]. Yield 3.96g (58%).

ν_{max} (Neat) 3400, 1600, 1520, 810 cm^{-1}

δ'H (Acetone-d_6) 7.8 (3H, m), 4.0 (2H, s, br), 3.7 (8H, sext).

m/z (EI) 260 (M^+).

5.41. N.N-Bis-(2-toluene-4-sulphonate ethyl)-2-chloro-4-nitro aniline [33]

This compound was synthesised in a similar manner to N,N-Bis-(2-toluene-4-sulphonate ethyl) aniline.

Yield 6.1g (93%); M.P. 110°C;

V_{max} (Nujol) 1590, 1500, 1050, 810, 770 cm^{-1}

δ'H (Acetone-d_6) 7.5 (11H, m), 3.8 (8H, sext), 2.4 (6H, s);

m/z (EI) 568 (M^+, 3.1%), 385 (38.7), 383 (100).

Found: C, 50.57; H, 4.36; N, 4.83. $C_{24}H_{25}ClN_2O_8S_2$ requires C, 50.66; H, 4.40; N, 4.93%.

5.42. N,N,-Bis) (2-chloro ethyl)-2-chloro-4-nitro aniline [34]

A solution of the appropriate di-tosyl ester (4g, 7.0 m Mol), and anhydrous $CaCl_2$ (2g) in 2-ethoxy ethanol (25 cm^3) was stirred at 120°C for 30 min and then allowed to cool. The 2-ethoxy ethanol was removed under vacuum. The aromatic mustard was extracted with benzene (100 cm^3) and the benzene layer was washed with water (100 cm^3) and dried ($MgSO_4$). On evaporation of the solvent, N,N-bis-(2-chloro ethyl)-2-chloro-4-nitro aniline was obtained as an orange oil. It was purified by column chromatography (silica) using benzene eluent. The resulting orange oil had the same spectra as that obtained by Owen et al[132].

ν_{max} (Neat) 1590, 1460, 1380, 1190, 790 cm^{-1}

δ'H (Acetone-d_6) 7.8 (3H, m), 3.8 (8H, m)

m/z (EI) 300 (M^+, 1.80, 298 (5.7), 296 (5.8), 249 (64.3), 247 (100).

5.43. N,N-Bis-(2-chloro ethyl)-2-chloro-4-phenylene diamine hydrochloride [35]

This was synthesised in the same procedure as N,N-Bis-(2-chloro ethyl)-4-phenylene diamine hydrochloride. The yellow solid obtained went rapidly brown, therefore the compound was used in the next step of the synthesis without further purification.

δ'H (DMSO-d_6) 8.3 (3H, s, br), 7.4 (3H, m), 3.5 (8H, m).

5.44 γ-L-[α-Benzyl-N-carbobenzoxy-N-N-bis-(2-chloro ethyl)-2-chloro-4-phenylene diamine]-glutamate [36]

A similar method to that of γ-L-[α-Benzyl-N-carbobenzoxy-N-N-bis-(2-chloro ethyl)-4-phenylene diamine]-glutamate was employed. The final product was obtained as a pale yellow oil which was partially purified by column chromatography (silica) using ether eluent. It was not possible, however, to obtain micro analytically pure samples. Yield 0.78g (39%).

ν_{max} (Neat) 1740, 1690, 1510, 1260, 810, 770 cm^{-1}

δ'H (Acetone-d_6) 9.2 (1H, S, br), 7.4 (13H, m), 5.1 (4H, d), 4.4 (2H, m), 3.5 (8H, S), 2.5 (4H, m);

m/z (EI) 619 (M^+, 0.2%), 513 (0.8), 462 (5.8), 326 (30.6), 282 (43.7), 108 (100).

Accurate m/z (EI) Found: 619.1411000 (M^+), $C_{30}H_{32}Cl_3N_3O_5$ requires 619.1407547.

5.45. γ-L-[N,N-Bis-(2-chloro ethyl)-2-chloro-4-phenylene diamine]-glutamic acid [37]

The same method as that for the synthesis of γ-L-[N,N-Bis-(2-chloro ethyl)-4-phenylene diamine]-glutamic acid was used. Yield 0.2g (62%) M.P. 143-145°C (from ethanol/methanol 7:3).

ν_{max} 3375, 1665, 1600, 800 cm^{-1}

δ'H (DMSO-d_6) 7.0 (3H, m), 4.3 (1H, m), 3.6 (8H, s), 2.4 (4H, m).

m/z (FAB -ve ion) 394 (M^-, 40.6%), 360 (34.0).

Found: C, 45.67, H, 4.96; N, 10.30; $C_{15}H_{20}Cl_3N_3O_3$ requires C, 45.40; H, 5.04; N, 10.59%.

5.46 N,N-Bis-(2-hydroxy ethyl)-2-fluoro-4-nitro aniline [38]

3,4-di fluoro nitro benzene (5g, 0.03 mol) and di ethanol amine (10 cm^3) were stirred together at 110°C for 5 h. The resulting red oil was cooled and dichloromethane (100 cm^3) and water (100 cm^3) added. The water layer was re-extracted twice with dichloromethane (100 cm^3). The combined organic extracts were washed with water (100 cm^3) and dried ($MgSO_4$), on evaporation of the solvent, a yellow solid of N,N-bis-(2-hydroxy ethyl)-2-fluoro-4-nitro aniline was obtained, which was then recrystallised from ethanol to give yellow plates.

Yield 5.8g (75%); M.P. 111-112°C

ν_{max} ($CHCl_3$) 3460, 1610, 1520, 820 cm^{-1}

δ'H (Acetone-d_6) 7.5 (3H, m), 4.2 (2H, S, br), 3.8 (8H, m).

m/z (EI) 244 (M^+, 12.2%), 213 (100), 169 (40.6).

Found: C, 49.31; H, 5.31; N, 11.38. $C_{10}H_{13}FN_2O_4$ requires C, 49.18; H, 5.33; N, 11.48%.

5.47. N,N-Bis-(2-toluene-4-sulphonate ethyl)-2-fluoro-4-nitro aniline [39]

This was synthesised in a similar manner to N,N-Bis-(2-toluene-4-sulphonate ethyl) aniline.

Yield 10.75g (95%); MP 111°C;

ν_{max} (Nujol) 1600, 1510, 1050, 810, 770 cm^{-1}

δ'H (Acetone-d_6) 7.5 (11H, m), 4.2 and 3.8 (8H, dt).

m/z (EI) 551 (M^+, 1.8%), 367 (57), 231 (89), 169 (100).

Found: C, 51.87; H, 4.60; N, 5.02; $C_{24}H_{25}FN_2O_8S_2$ requires C, 52.17; H, 4.53; N, 5.07%.

5.48. N,N-Bis-(2-chloro ethyl)-2-fluoro-4-nitro aniline [40]

The chlorination procedure using calcium chloride and 2-ethoxy ethanol as previously described was used (see section 5.42).

Yield 1.65g (93%; M.P. 67-68°C;

ν_{max} (Nujol) 1600, 1510, 1385, 1195, 780 cm^{-1}

δ'H (Acetone-d_6) 7.6 (3H, m), 3.9 (8H, m).

m/z (EI) 280 (M^+, 10.6%), 233 (34), 201 (35).

Found: C, 42.42; H, 4.00; N, 9.79. $C_{10}H_{11}Cl_2FN_2O_2$ requires C, 42.70; H, 3.91; N, 9.96%.

5.49. γ-L-[α-Benzyl-N-carbobenzoxy-N,N-bis-(2-chloroethyl)-2-fluoro-4-phenylene diamine] glutamate [41]

N,N-Bis-(2-chloro ethyl)-2-fluoro-4-nitro aniline (0.5g. 1.77 m Mol) was dissolved in ethyl acetate (20 cm^3) and hydrogenated in the presence of 10% palladium charcoal catalyst until the theoretical amount of hydrogen had been taken up. The catalyst was removed by filtration and the filtrate cooled to -10°C.

The solution was then added dropwise to a mixed anhydride of the protected glutamic acid, which was prepared as described in section 5.38. The mixed anhydride solution contained α-Benzyl-N-carbobenzoxy glutamic acid (0.66g, 1.76 m Mol), triethylamine (0.19g, 1.9 m Mol) and ethyl chloroformate (0.19g, 1.76 m Mol) all in THF (20 cm^3). The mixture was left to stir for 2 h after which the triethylamine hydrochloride was filtered and the filtrate evaporated. The resulting solid was dissolved in dichloromethane (40 cm^3), and was washed with 1M hydrochloric acid (50 cm^3) and then with dilute sodium bicarbonate solution (50 cm^3), the organic layer was separated, dried ($MgSO_4$) and the solvent evaporated to give a brown oil, which was subjected to chromatographical purification on silica using ether eluent. This gave a white solid of γ-L-[α-benzyl-N-carbobenzoxy-N,N-bis-(2-chloro ethyl)-2-fluoro-4-phenylene diamine]-glutamic acid.

Yield 0.45g (42%); M.P. 104-105°C (from methanol).

ν_{max} (Nujol) 1725, 1680, 1510, 1270, 810 760 cm^{-1}

δ'H (Acetone-d_6) 9.2 (1H, S, br), 7.4 (13H, m), 5.1 (4H, d), 4.3 (2H, m), 3.6 (8H, S), 2.5 (4H, m).

m/z (EI) 603 (M^+, 4.0%), 448 (37), 446 (100), 338 (57)

Found: C, 59.58; H, 5.29; N, 6.92; $C_{30}H_{32}Cl_2FN_3O_5$ requires C, 59.60; H, 5.30; N, 6.95%.

5.50. γ-L-[N,N-Bis-(2-chloro ethyl)-2-fluoro-4-phenylene diamine]-glutamic acid [42]

The deprotection of [41] to give [42] was achieved by hydrogenation in the presence of 10% palladium charcoal catalyst as described in section 5.39. Yield 0.18g (67%). (From ethanol/methanol 7:3) M.P. 147-148°C.

ν_{max} 3380, 1665, 1600, 810 cm^{-1}

δ'H (DMSO-d_6) 7.1 (3H, m), 4.3 (1H, m), 3.7 (8H, S), 2.4 (4H, m).

m/z (FAB + ve ion) 382 (MH^+, 31.1%), 380 (48), 330 (22), 251 (47), 201 (47), 138 (28) 84 (100).

Found: C, 47.42; H, 5.49; N, 10.91. $C_{15}H_{20}Cl_2FN_3O_3$ requires C, 47.37; H, 5.26; N, 11.05%.

5.51. N,N-Bis-(2-bromo ethyl)-2-fluoro-4-nitro aniline [43]

A method similar to that for the synthesis of N,N-bis-(2-chloro ethyl)-2-fluoro-4-nitro aniline was used (see section 5.48). The only difference was the use of calcium bromide in place of calcium chloride.

Yield 1.7g (86%) M.P. 68-69°C from ethanol

ν_{max} (Nujol) 1600, 1520, 1385, 1180, 780 cm^{-1}

δ'H (Acetone-d_6 7.4 (3H, m), 3.8 (8H, m).

m/z (EI) 369 (M^+, 8.3%), 278 (23.6).

Found: C, 32.62; H, 2.94; N, 7.63; $C_{10}H_{11}Br_2FN_2O_2$ requires C, 32.43; H, 2.97; N, 7.57%.

5.52. γ-L-[α-Benzyl-N-carbobenzoxy-N-N-bis (2-bromo ethyl)-2-fluoro-4-phenylene diamine]-glutamate [44]

A procedure similar to that for the corresponding dichloro mustard was used (see section 5.49). This involved reduction of the nitro group by hydrogenation in ethyl acetate, and addition of the phenylene diamine di bromo mustard in situ to the mixed anhydride solution of the protected glutamic acid. Yield 0.37g (48%); M.P. 117-118°C from methanol;

ν_{max} (Nujol) 1730, 1680, 1500, 810, 770 cm^{-1}

δ'H (Acetone-d_6) 8.9 (1H, S, br), 7.4 (13H, m), 5.1 (4H, d), 4.3 (2H, m), 3.6 (8H, m), 2.5 (4H, m).

m/z (EI) 693 (M^+, 0.6%), 585 (1.2), 492 (2.1), 340 (40), 109 (53.0), 91 (100).

Found: C, 51.95; H, 4.62; N, 6.06. $C_{30}H_{32}Br_2FN_3O_5$ requires C, 52.21; H, 4.66; N, 6.10%.

5.53. γ-L-[N,N-(2-bromo ethyl)-2-fluoro-4-phenylene diamine]-glutamic acid [45]

The hydrogenation method for the deprotection of benzyl and carbobenzoxy groups already described previously (section 5.39) was used. Yield 0.08g (61%), M.P. 139-140°C (from ethanol/methanol 7:3).

ν_{max} (Nujol) 3380, 1675, 1600, 800 cm^{-1}

δ'H (DMSO-d_6) 7.2 (3H, m), 4.3 (1H, m), 3.8 (8H, S), 2.4 (4H, m).

m/z (FAB + ve ion) 470 (MH^+, 10.4%), 310 (10.9), 296 (13.6), 245 (11.6), 185 (39.6), 152 (30.2), 93 (82.9), 84 (100).

Found: C, 38.23; H, 4.31; N, 8.89. $C_{15}H_{20}Br_2FN_3O_3$ requires C, 38.38; H, 4.26; N, 8.96%.

5.54. N,N-bis-(2-bromo ethyl) aniline [46]

The bromination procedure using $CaBr_2$ and the appropriate di-tosyl ester in 2 ethoxy ethanol described earlier (section 5.51) was employed.

M.P. 53-55°C (lit 53-55°C)[48]

ν_{max} (Nujol) 1600, 1500, 780 cm^{-1}

δ'H (Acetone-d_6). 7.1 (5H, m), 3.7 (8H, dt)

5.55 N,N-Bis-(2-bromo ethyl)-4-nitroso aniline [47]

The N,N-bis (2-bromo ethyl) aniline (2.9g, 9.3 m Mol) was dissolved in 40% H_2SO_4 (8 cm^3). After cooling to 5°C, the mixture was vigorously stirred and a solution of sodium nitrite (0.67g) in water (2 cm^3) was added dropwise maintaining the temperature below 7°C. After the addition, the mixture was stirred for a further 20 min, water (40 cm^3) was added and the solution was neutralised with sodium carbonate. Ether (40 cm^3) was then added, and the aqueous layer was separated and twice re-extracted with ether (40 cm^3).

The combined ether extracts were dried ($MgSo_4$). Evaporation of the ether gave a deep green oil. This was passed through a short column of silica using ethyl acetate eluent. The resulting solid was recrystallised from methanol to give green plates of N,N-bis-(2-bromo ethyl)-4-nitroso aniline.

Yield 2.1g (68%); M.P. 105°C.

ν_{max} (Nujol) 1600, 1460, 1380, 1110, 810, 710 cm^{-1}

δ'H (Acetone-d_6) 7.8 (2H, d), 7.0 (2H, d), 3.9 (8H, dt).

m/z (EI) 336 (M^+, 47.3%), 334 (25), 241 (100), 213 (76.3).

Found: C, 35.41; H, 3.47, N, 8.14; $C_{10}H_{12}Br_2N_2O$ requires C, 35.71; H, 3.57; N, 8.33%.

5.56. γ-L-[α-Benzyl-N-carbobenzoxy-N-N-bis-(2-bromo ethyl)-4-phenylene diamine]-glutamate [48]

The method used for the preparation of γ-L-[α-Benzyl-N-carbobenzoxy- N,N-bis-(2-chloro ethyl)-2-fluoro-4-phenylene diamine] glutamate was followed (see section 5.52).

Yield 0.16g (42%); M.P. 118-120°C from ethyl acetate/petroleum ether;

ν_{max} (Nujol) 3380, 1735, 1680, 1510, 1280, 810, 770 cm^{-1}

δ'H (Acetone-d_6) 7.5 (2H, d), 7.25 (10H, d), 6.7 (2H, d), 5.1 (4H, d), 4.3 (2H, m), 3.7 (8H, dt), 2.5 (4H, m).

m/z (EI) 675 (M^+, 0.5%), 567 (0.6), 513 (0.4), 107 (18), 91 (100).

Found: C, 53.35; H, 4.87; N, 6.24: $C_{30}H_{33}Br_2N_3O_5$ requires C, 53.33; H, 4.89; N, 6.22%.

5.57. γ-L-[N,N-Bis-(2-bromo ethyl)-4-phenylene diamine]-glutamic acid [49]

The hydrogenation procedure described in section 5.39 was used. The γ-L-[N,N-bis-(2-bromo ethyl)-4-phenylene diamine]-glutamic acid was obtained as a colourless oil which was difficult to purify.

ν_{max} (Nujol) 3370, 1680, 1600, 1590, 800 cm^{-1}

δ'H (DMSO-d_6) 7.4 (2H, d), 7.1 (2H, d), 4.3 (1H, m), 3.7 (8H, m), 2.4 (4H, m).

m/z (FAB +ve) 452 (MH^+ 1.5%), 372 (1.1) 294 (30.7), 18.2 (32.5), 115 (53), 93 (100)

Accurate M/z (FAB + ve) Found 452.00240 (MH^+), $C_{15}H_{21}Br_2N_3O_3$ requires 452.004630.

5.58. 4-N,N-Bis-(2-hydroxy propyl) amino acetanilide [50]

To a cold (0°C) solution of 4-amino acetanilide 3g (0.025 mol) in 50% aqueous acetic acid (25 cm^3) was added propylene oxide 10.9g (0.19 mol) and the solution was allowed to stand for 48 h at room temperature. Evaporation under vacuum gave a purple syrup which was dissolved in ethyl acetate and passed through a short column of silica using ethyl acetate eluent. Evaporation of the eluent gave a pale yellow oil.

Yield 4.1g (63%).

ν_{max} (Neat) 3400, 1660, 1510, 1385, 820 cm^{-1};

δ'H (Acetone-d_6) 6.8 (4H, q), 4.2-2.8 (8H, m), 2.7 (1H, a), 2.1 (3H, a), 1.1 (6H, d).

Accurate m/z (EI) Found: 266.1627000 (M^+), $C_{14}H_{22}N_2O_3$ requires 266.1630428.

5.59. 4-N,N-bis-(2-bromo propyl) amino acetanilide [22]

A solution of N,N-bis-(2-hydroxy propyl amino) acetanilide 3.5g (0.014 mol) in benzene (20 cm^3) was gradually added to phosphorous tribromide 11.4g (0.042 mol) and the mixture then heated on a steam bath until the reaction slackened. Crushed ice (30g) and benzene (100 cm^3) were added, the benzene layer was washed with water (75 cm^3) and 5% sodium bicarbonate solution (75 cm^3). After removal of the benzene under vacuum, the product was crystallised from ethanol to give white needles of 4-N,N-bis-(2-bromo propyl amino) acetanilide.

Yield 1.66g (32%), M.P. 154-155°C

ν_{max} ($CHCl_3$) 1665, 1480, 1390, 800 cm^{-1}

δ'H (Acetone-d_6 7.1 (4H, dd), 4.4 (2H, sext), 3.8 (4H, d), 1.7 (6H, d).

m/z (EI) 391 (M^+, 23.7%), 285 (92.4), 163 (100), 120 (41).

Found: C, 43.18; H, 5.25; N, 7.05; $C_{14}H_{20}Br_2N_2O$ requires C, 42.86, H, 5.10; N, 7.14%.

5.60. N,N-Bis-(2-bromo propyl) phenylene diamine hydrobromide [23]

A solution of 4-N,N-bis-(2-bromo propyl) amino acetonilide (1g, 0.026 Mol) in aqueous 45% hydrobromic acid (20 cm^3) was boiled under reflux for 3 h. After dilution with water (30 cm^3) and neutralisation with solid sodium hydrogen carbonate, the mixture was extracted with ether (4 x 25 cm^3); the combined extracts were dried ($MgSO_4$) and added to a mixture of 45% HBr in acetic acid (5 cm^3) and methanol (10 cm^3). A white precipitate formed, which was filtered off to give an almost white solid of N,N-bis-(2-bromo propyl)-4-phenylene diamine. The solid started to go brown, therefore it was used in the next step of the synthesis without further purification.

Crude yield 0.8g (72%).

ν_{max} (KBr) 3400, 1600, 780 cm^{-1}

δ'H (DMSO-d_6) 10.0 (3H, s, br), 7.0 (4H, dd), 4.5 (2H, sext), 3.8 (4H, d), 1.8 (6H, d).

5.61. γ-L-[α-2-Benzyl-N-carbobenzoxy-N-N-bis-(2-bromo propyl)-4-phenylene diamine] glutamate [51]

The procedure used for the coupling of N,N-bis-(2-chloro ethyl)-4-phenylene diamine with the protected glutamic acid was used (section 5.8).

Yield 0.13g (28%); M.P. 129-130 from methanol.

ν_{max} (Nujol) 3390, 1740, 1690, 1610, 1520, 1270, 820, 780 cm^{-1}

δ'H (Acetone-d_6) 7.3 (14H, m), 5.2 (4H, d), 4.4 (4H, m), 3.8 (4H, d) 2.5 (4H, m), 1.7 (6H, d).

m/z (EI) 702(M^+, 12.1%), 594 (87.3), 475 (94.4).
Found: C, 54.23; H, 5.39; N, 5.69; $C_{32}H_{37}Br_2N_3O_5$ requires C, 54.46; H, 5.26; N, 5.97%.

5.62 γ-L-[N,N-Bis-(2-bromo propyl)-4-phenylene diamine]-glutamic acid [52]

The hydrogenation procedure in the presence of palladium charcoal catalyst, as described in section 5.39 was used for the deprotection of [51] to [52].

Yield 0.11 g (55%)

ν_{max} (Nujor) 3365, 1680, 1600, 1580, 800 cm^{-1}

δ'H (DMSO-d_6) 7.0 (4H,dd), 4.3 (3H,M), 3.7 (4H,M), 2.5 (4H,M), 1.7 (6H,d).

m/z (FAB +ve) 481 (MH^+, 23.2%), 387 (68), 293 (49.3).

5.63 N,N-Bis-(2-hydroxy ethyl)-3-methoxy aniline [53]

The Carius tube method described for the synthesis of N,N-bis-(2-hydroxy ethyl) aniline was used (see Section 5.33). The resulting diol was obtained as a oil and was carried on to the next step without further purification.

Yield 15.6g (91%)

ν_{max} ($CHCl_3$), 3350, 1600, 1340, 750 cm^{-1}

δ'H (Acetone-d_6) 7.1 (1H, m), 6.2 (3H, m) 4.2 (2H, s, br);

m/z (EI) 211 (M^+, 18.2%), 180 (100), 136 (40)

5.64 N,N-Bis-(2-toluene-4-sulphonate ethyl)-3-methoxy aniline [54]

The tosylation procedure described in section 5.34 was used. The resulting di-tosyl product was obtained as a oil, which was purified by column chromatography (silica) using ether eluent. The pure N,N-Bis-(2-toluene-sulphonate ethyl)-3-methoxy aniline was isolated as a colourless oil, after removal of the solvent under vacuum.

Yield 11.8g (83%).

ν_{max} ($CHCl_3$) 1600, 1500 , 1365, 1100, 800 cm^{-1}

δ'H ($CDCl_3$) 7.5 (12H, m), 3.8 (12H, m), 2.4 (6H, s),

m/z (EI) 519 (M^+, 0.6%), 334 (13.9, 193 (40.9), 172 (51.1), 135 (54.1), 91 (100).

Found: C, 57.80; H, 5.59; N, 2.70: $C_{25}H_{29}NS_2O_7$ requires C, 57.90; H, 5.87; N, 2.79%.

5.65. N,N-Bis-(2-chloro ethyl)-3-methoxy aniline [55]

The replacement of the tosyl groups using calcium chloride and 2-ethoxy ethanol was used as described in Section 5.42. The resulting oil was purified by passing through a column of silica using benzene eluent. On evaporation of the solvent N,N-bis-(2-chloro ethyl)-3-methoxy aniline was obtained as a colourless oil.

Yield 1.2g (86%).

ν_{max} (Nujol) 1600, 1500, 770 cm^{-1}

δ'H (Acetone-d_6) 7.2 (1H, t), 6.3 (3H, m), 3.8 (3H, s), 3.6 (8H, m).

m/z (EI) 247 (M^+, 18.8%), 200 (33.2), 198 (100).

Found: C, 5.36; H, 6.30; N, 5.53: $C_{11}H_{15}Cl_2NO$ requires C, 53.23; H, 6.05; N, 5.64%

5.66. N,N-Bis-(2-chloro ethyl)-3-methoxy-4-nitroso aniline [56]

The nitrosation procedure described for the nitrosation of N,N-bis-(2-chloro ethyl) aniline using hydrochloric acid and sodium nitrate was followed (see section 5.36). The resulting green solid was recrystallised from ethanol to give green needles of N,N-bis-(2-chloro ethyl)-3-methoxy-4-nitroso aniline.

Yield 0.8 (65%); M.P. 119-121°C

ν_{max} (Nujol) 1600, 1500, 1340, 1220, 1110, 825 cm^{-1}

m/z (EI) 276 (M^+, 24.3%), 262 (37), 215 (33), 213 (100), 150 (45).

Found: C, 47.78; H, 5.505; N, 10.03; $C_{11}H_{14}Cl_2N_2O_2$ requires C, 47.65; H, 5.05; N, 10.11%.

5.67. γ-L-[α-Benzyl-N-carbobenzoxy-N-N-bis-(2-chloro ethyl) -3-methoxy-4-phenylene diamine] glutamate [57]

This was synthesised in a similar manner to γ-]benzyl-N-carbobenzoxy-N;N-bis-(2-chloro ethyl)-2-fluoro-4-phenylene diamine]-glutamate (see section 5.49).

Yield 0.23 (41%); M.P. (126-128°C)

ν_{max} (Nujol) 3375, 1730, 1690, 1510, 820 cm^{-1}

δ'H (Acetone-d_6) 8.0 (1H, d, br), 7.6 (1H, s), 7.25 (10H, d),
6.2 (3H, m), 5.1 (4.4 d), 3.8 (3H, s), 3.6 (8H, s), 2.4 (4H, m).

m/z (EI) 615 (M^+, 0.9%), 507 (5.3), 458 (9.2), 126 (13.4),
92 (100)

Found: C, 60.12; H, 5.46; N, 6.44; $C_{31}H_{35}Cl_2N_3O_6$ requires
C, 60.39; H, 5.68; N, 6.82%.

CONCLUSION

γ-L-Glutamyl adducts of six aromatic nitrogen mustards were synthesised. These were, three N,N-bis-(2-chloroethyl) -4-phenylene diamine adducts (γGPDM, γGPDMF, γGPDMCl) and three N,N-bis-(2-bromoalkyl) -4-phenylene diamene adducts (γGBrPDM, γGBrPDMF, γG-2-BrPDM) of γ-L-glutomic acid. These compounds were synthesised by modifications and elaborations to published procedures for related compounds. Particular attention was given to obtaining good yields of pure products. The compounds were identified and fully characterised by spectroscopic measurements. In the synthesis, the 2-carboxyl and 2-amino groups of L-glutamic acid were protected by forming a benzyl ester and a N-carbobenyoxy derivative, respectively. Both protecting groups were removed readily by the mild procedure, of hydrogenolysis over palladium charcoal catalyst. The 4-phenylene diamine mustards moieties were synthesised by relatively conventional procedures, these involved either the reaction of the appropriate aniline with an epoxide or the nucleophilic displacement of an activated halogen by diethanolamine. Conventional procedures for halogenating the N,N-bis-(2-hydroxyalkyl) - aniline with PCl_5 or PBr_3 were found to be unsatisfactory. To improve yields and obtain cleaner products, a modified procedure involving tosyl ester formation and replacement of the tosyl group with Cl or Br using $CaCl_2$ or $CaBr_2$ in 2-ethoxy ethanol was developed.

The N,N-bis-(2-halo alkyl) aniline mustards were nitrosated electrophilically and the nitroso or nitro groups reduced by catalytic hydrogenation to give the 4-phenylene diamine mustards.

These compounds were found to be reactive and often unstable, therefore they were immediately coupled to protected glutamic acid residue via the mixed anhydride method using ethyl chloroformate, to give an γ-benzyl-N-carbobenzyoxy glutamate derivative of the 4-phenylene diamine mustard. The parent compounds were obtained by catalytic hydrogenation to remove the γ-benzyl and N-carbobenzyoxy protecting groups.

The enzyme γ-glutamyl transferase was extracted from rat kidney in lower yield but better specific activity than previously reported. By kinetic studies, it was shown that the γ-glutamyl prodrugs are substrates for γGT of comparable activity to glutathione and glutamine the enzyme's natural substrates. The γ-L-N,N-bis-(2-chloro ethyl) -2- fluoro -4- phenylene diamine glutamic acid appeared to be the best prodrug substrate.

The cytotoxic activity of both the γ-L-glutamyl mustard prodrugs and the parent mustard (PPDM) were assessed by their effects on hepatoma (JBI) and normal hepatocyte (BL8L) cell lines. All γ-L-glutamyl mustard prodrugs were found to be more toxic towards the JBI than the BL8L cell lines. This was attributed to the higher levels of γGT in the JBI cells which converted the γ-L-glutamyl mustard prodrugs to their more toxic parent mustards. This was confirmed by independent experiments showing the toxicity of the γ-L-N,N-bis- (2-chloro ethyl) -4-phenylene diamine glutamic acid mustards towards the JBI cells was reduced by the γGT inhibitor serine borate. The γ-L-N,N-bis-(2-bromo alkyl) -4- phenylene diamine glutamic acid mustards were found to be too toxic towards normal BL8L cells to be of any chemotherapeutic use.

The γ-L-N,N-bis -(2-chloro ethyl)-4-phenylene diamine glutamic acid mustards were significantly less toxic towards the BL8L cells than their bromo counterparts yet showed reasonable cytotoxicity towards the JBI cells.

Of all the prodrugs examined γGPDMCl was the least toxic towards the BL8L cells with reasonable toxicity towards the JBI cells. From these experiments, it appears that γGPDMCl probably offers the best chemotherapeutic ratio.

Brief in vivo tests showed that γGPDM had no regressive effect on a transplanted hepatoma in nude mice. Further research is required to establish why the prodrugs are active in vitro but inactive in vivo.

CHAPTER 6

APPENDIX

6.1 In Vivo testing of γGPDM

This work was carried out at the MRC toxicology unit, Carshalton, in collaboration with M Manson, R F Legg and R Verlschoyle.

If immune response is suitably attenuated, a cancer can be induced in vivo by transplanting cells or tumour tissue. Nude (hairless) mice lacking thymus glands and therefore immunologically active T-cells, are normally used for these tests. Many different foreign tumours including those from humans can be grown and tested in this way. Induced hepatomas were obtained by transplanting JB1 cells into the nude mice.

Initially, several concentrations of γGPDM in DMSO were injected intraperitonealy into normal nude mice, to establish the maximum tolerated dose. Single intraperitoneal injections of 0, 5, 10, 25 and 50 mg/kg of γGPDM was used. After ten days all of the mice receiving 50 and 25 mg/kg were dead. The remainder survived and after 2 weeks, they were killed and dissected. The results suggest that γGPDM has a similar toxicity to melphalan, a clinically used aromatic mustard.

Various concentrations of γGPDM were injected intraperitonealy into hepatoma transplanted nude mice. Concentrations of 0 (control), 2.5, 5, 10, 20 and 40 mg/kg of γGPDM in DMSO were used and the experiments were done in triplicates. The animals were weighed prior to injection and following death.

The results summarised in Table9 show that all the 40 mg/kg mice were dead after three days. One 20 mg/kg mouse died after nine days and one 2.5 mg/kg mouse died after nine days due to an excessively large tumour. The remainder were killed after eleven days and dissected for evidence of tumour regresson. There was lack of visual evidence for regression of the hepatoma in any of the nude mice which were dosed with γGPDM as compared with the control.

The results are singularly disappointing. The lack of any apparent chemotheropeutic effect could be due to several reasons. 1) The prodrug may be activated by γGT in either the kidney or another organ more efficiently than the tumour. 2) The chlorine groups of γGPDM may be hydrolysed before reaching the vicinity of the hepatoma. 3) the γGPDM prodrug may be detoxified by, for example, glutathione or glutathione-s-transferase.[143] It has been shown that tumours resistant to alkylating agents also possess higher concentrations of glutathione[144-146], and this may apply to hepatoma cells. L210 leukemia cells resistant to L-phenylalanine mustard (L-PAM) contain two to three fold elevation in both their glutathione and γGT levels compared to L-PAM sensitive leukemia cells[147]. Thus, for all tumours including hepatomas elevated γGT activity may go hand in hand with elevated levels of glutathione.

DOSE	No	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	DAY 10	DAY 11
2mg/kg	1	19g	20g	19g	19g	19g	19g	20g	20g	20g	20g	20g
	2	19	18	18	18	18	18	19	18	18	18	19
	3	18	18	18	18	18	17	17	17	17	killed due to large tumour	
5mg/kg	1	18	18	18	18	18	18	18	18	18	18	17
	2	18	18	17	17	17	17	18	18	18	17	17
	3	19	19	18	18	18	18	18	19	18	18	18
10mg/kg	1	19	21	20	19	19	19	20	20	20	20	20
	2	20	20	20	20	20	20	21	22	22	21	22
	3	18	19	18	18	19	18	18	18	18	18	19
20 mg/kg	1	22	23	20	19	19	19	19	18	17	dead	
	2	19	19	18	18	19	19	19	20	19	19	20
	3	16	17	15	15	15	15	16	16	16	15	16
40mg/kg	1	16	16	15)								
	2	18	17	15)	ALL DEAD AFTER 3 DAYS							
	3	20	19	16)								
0mg/kg CONTROL	1	17	17	17	17	18	17	18	18	18	16	17
	2	21	21	21	21	22	21	22	23	23	21	23
	3	18	18	18	19	20	18	20	20	20	18	19
	4	19	19	19	19	20	19	20	20	17	19	19

TABLE 9

Weight of hepatoma implanted nude mice after a single administration of 0, 2.5, 5, 10, 20 and 40mg/kg of γGPDM.

Some of these difficulties could be overcome by alternative methods of administering the drug or by co-administration of suitable inhibitors, e.g. D,L-buthionine -S,R-sulphoximine (BSO)[146], (to inhibit γ-glutamyl cysteine synthetase) or 2-nitro imidazole. It has been shown that 2-nitro imidazole compounds enhances the cell kill achieved by subsequent exposure to chemotherapeutic agents including melphalan[48-150].

It is clear, however, that pharmocokinetic studies using ^{14}C radio labelled γGPDM are required to explain the inability of γGPDM to regress the transplanted hepatoma.

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