Clarification of nomenclature

Throughout this work compounds are referred to as their γ-L-glutamyl derivatives as opposed to the more correct L-glutamine derivatives,

e.g. γ-N,N-diethyl-L-glutamine = γ-L-glutamyl-diethylamine

and

γ-L-glutaminyl-4-hydroxybenzene = γ-L-glutamyl-4-hydroxyaniline.

This amendment has been made to allow consistency throughout the text for the benefit of the reader.

TO MY PARENTS

γ-GLUTAMYL PRODRUGS

A thesis submitted by

KIM PAULA UPPERTON

in partial fulfilment of the requirements for the degree of Doctor of philosophy of the University of London

February 1989

Department of Chemistry

Imperial College

South Kensington

London SW7

and

Division of Clinical Cell Biology

MRC Clinical Research Centre

Northwick Park Hospital

Harrow

Middlesex HA1 3UJ

ACKNOWLEDGEMENTS

I am indebted to Professors B.C. Challis and T.J. Peters for the enthusiastic encouragement and invaluable guidance throughout the course of this work.

I would like to thank Dr. N.D. Cook, Nirmal and Francis for continued advice, discussions and collaboration.

Thanks are also due to my colleagues at I.C. and in the Division of Clinical Cell Biology, for their help and friendly company.

Finally my sincere appreciation to Audrey Gill for typing this thesis.

ABSTRACT

The possibility of synthesizing γ-L-glutamyl prodrugs as selective cytotoxic agents capable of activation at sites of high GGT content is investigated.

Synthetic strategies have been developed to permit the preparation of a series of γ-L-glutamyl adduct analogues including γ-L-glutamyl amine and γ-L-glutamyl hydroxyaniline derivatives. Attempts to synthesize γ-L-glutamyl adducts of N-(2-chloroethyl)-N-nitrosourea derivatives as putative prodrugs proved unsuccessful and this has been attributed to their instability.

Using model γ-L-glutamyl amine derivatives the kinetics and specificity of the hydrolytic reaction of GGT was examined and the structural requirements of the GGT donor site determined.

The enzyme kinetics of γ-L-glutamyl hydroxyaniline compounds have also been determined and are compared with those of glutamine and glutathione, the enzymes natural donors.

The cytotoxic activity of the γ-L-glutamyl prodrugs as compared with their parent adducts has been assessed by examining their effect on hepatoma cell lines (JB1) and normal hepatocytes (BL8L), rich and deficient in GGT, respectively.

The possibility of redox cycling and free radical formation being involved in the cytotoxic action of 4-hydroxyaniline is also investigated, by observing its effect on oxygen consumption by the cell lines.

CONTENTS

	Page
Title of Thesis	i
Acknowledgements	ii
Abstract	iii
Table of contents	iv
Abbreviations	xi
Compound Index	xii
CHAPTER 1 : INTRODUCTION	1
1.1 Discovery of GGT	2
1.2 Ocurrence of GGT	2
1.3 Physical and chemical properties	3
1.3.1 localization and orientation	3
1.3.2 Purification	4
1.3.3 Structure	4
1.4 Reactions catalysed by GGT	6
1.5 Specificity of donor and acceptor sites	7
1.6 Function of GGT	9
1.6.1 Amino acid transport (γ-Glutamylcycle)	9
1.6.2 Glutathione hydrolysis	11
1.6.3 L-Glutamine hydrolysis	11
1.6.4 The role of GGT in detoxification	12

Page

1.7 GGT in hepatocarcinoma 12

1.8 Incidence of hepatocarcinoma 13

1.9 γ-L-Glutamyl prodrugs 13

1.9.1 N-(2-Chloroethyl)-N-nitrosoureas 15

1.9.1.1 Synthesis of N-(2-chloroethyl)-N-nitrosoureas 17

1.9.2 Hydroxyanilines 21

1.9.3 Synthesis of γ-L-glutamyl prodrugs: an overview 23

1.9.3.1 Protection of glutamic acid 23

1.9.3.2 Coupling procedures 28

i) mixed anhydride 28

ii) carbodi-imide method 30

iii) by phthalylated intermediates 31

CHAPTER 2 : SYNTHESIS 33

2.1 Attempted synthesis of γ-L-glutamyl adducts of N-(2-chloroethyl)-N-nitrosoureas 34

2.1.1 Protection of glutamyl α-carboxyl and α-amino functions 34

2.1.2 Synthesis of α-protected glutamines 37

2.1.3 Synthesis of γ-L-glutamyl ureas 38

2.1.4 Nitrosation of (16) and (17) 39

2.1.5 Deprotection 44

2.1.6 Alternative protecting groups 49

Page

2.2 Synthesis of N-(2-chloroethyl)-N-nitrosourea (30) 52

2.3 Synthesis of model γ-L-glutamyl donor substrates 53

2.4 Synthesis of γ-L-glutamyl hydroxyaniline adducts 57

2.5 Summary 66

CHAPTER 3 : ENZYMOLOGY 68

3.1 Introduction 69

3.2 Purification of GGT 69

3.3 The hydrolysis kinetics and donor specificity of GGT 73

3.3.1 Structural effects of the γ-glutamyl substituent on donor specificity 73

3.3.1.1 Tertiary substrates 78

3.3.1.2 Secondary substrates 80

3.3.2 The effect of the γ-glutamyl structure on donor specificity 89

3.3.3 Donor site of GGT 97

3.4 Hydrolysis of prodrugs by GGT 100

3.4.1 Hydrolysis of α-(4-nitrophenyl)-γ-(N-(2-chloroethyl),N-nitrosocarbamoyl)-L-glutaminate (28) 100

Page

3.4.2 Hydrolysis of the γ-L-glutamyl hydroxyaniline derivatives by GGT 104

3.5 Summary 109

CHAPTER 4 : CYTOTOXICTY TESTS 111

4.1 Introduction 112

4.2 GGT content of the cell lines 112

4.3 Cytotoxity of the parent hydroxyanilines and the γ-L-glutamyl derivatives against BL8L and JB1 cell lines,in vitro 113

4.3.1 Cytotoxicity of the parent hydroxyanilines 113

4.3.1.2 Cytotoxicity of 4-benzoquinone-imine 118

4.3.2 Cytotoxicity of the γ-L-glutamyl hydroxyanilines 120

4.3.2.1 Cytotoxicity of γ-L-glutamyl hydroxyaniline adducts in the presence of serine borate 124

4.3.3 Effect of exposure time on adduct cytotoxicity 126

4.3.3.1 Parent hydroxyanilines 127

4.3.3.2 γ-L-Glutamyl adducts 127

4.4 In vitro cytotoxity of α-(4-nitrophenyl)-γ-(N-(2-chloroethyl),N-nitrosocarbamoyl)-L-glutaminate (28) towards BL8L and JB1 cell lines 131

Page

4.5 Summary 135

CHAPTER 5 : POSSIBLE MODE OF CYTOTOXIC ACTION OF 4-HYDROXYANILINE 139

5.1 Introduction 140

5.2 Effect of 4-hydroxyaniline (68) on oxygen consumption of whole cells 140

5.3 Effect of 4-hydroxyaniline (68) on oxygen consumption of sonicated cells in the presence of NADPH 142

5.4 Conclusions 144

CONCLUSIONS 6 : CONCLUSIONS AND FUTURE WORK 145

CHAPTER 7 : EXPERIMENTAL 153

7.1 General methods 154

7.2 Biochemical methods 155

7.2.1 Purification of GGT 155

7.2.2 Protein determination 156

7.2.3 Assay of GGT activity 156

7.2.4 Assay of γ-glutamyl hydrolase activity: 157

by fluorimetry 157

by UV spectrophotometry 157

by HPLC 157

Page

by ammonia release 158

7.2.5 Tissue culture 159

7.2.6 Assay of GGT activity in cell lines 159

7.2.7 Cytotoxicity testing 160

7.2.7.1 24h exposure to drug 160

7.2.7.2 2h exposure to drug 160

7.2.7.3 Serine borate inhibition 161

7.2.8 Oxygen consumption experiments 161

7.2.8.1 Whole cell measurements 163

7.2.8.2 Sonicated cell measurements 163

7.3 Chemical methods 164

7.3.1 Hydrolysis of α-(4-nitrophenyl)-γ-(N-(2-chloroethyl),N-nitrosocarbamoyl)-L-glutaminate (28) 164

7.3.2 Purification of γ-glutamyl derivatives by ion exchange 164

7.3.3 Synthesis 165

CHAPTER 8 : REFERENCES 189

Page

APPENDIX 199

1) Attempted synthesis of α-N-benzyloxycarbonyl-γ-(N-(2-chloroethyl)-carbamoyl, N-(2-chloroethyl))-L-glutaminate (18) 200

Condensation of α-N-benzyloxycarbonyl-α-benzyl-γ-N-(2-chloroethyl)-L-glutaminate (14) with 2-chloroethyl isocyanate 200

i) In sodium dry toluene 200

ii) In DMSO 200

iii) In a Carius tube 200

iv) using nBuLi 201

2) Attempted synthesis of α-N-benzyloxycarbonyl-γ-(N-(2-chloroethyl)-carbamoyl, N-cyclohexyl)-L-glutaminate (19) 202

Condensation of α-N-benzyloxycarbonyl-α-benzyl-γ-N-cyclohexyl-L-glutaminate (15) with 2-chloroethyl isocyanate 202

i) In dry toluene 202

ii) In DMSO 202

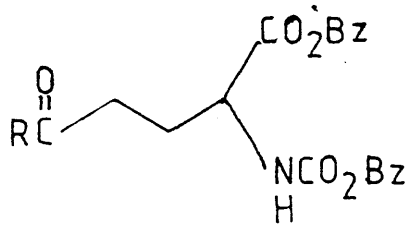
iii) In a Carius tube 202

iv) Using nBuLi 202

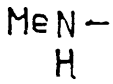
ABBREVIATIONS

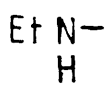
BCNU	1,3-Bis-(2-chloroethyl)-1-nitrosourea.
CCNU	3-Cyclohexyl-1-(2-chloroethyl)-1-nitrosourea.
DMF	Dimethyl formamide.
DMSO	Dimethyl sulphoxide.
DON	6-Diazo-5-oxo-L-norleucine.
EDTA	Ethylenediaminetetra-acetic acid.
γ	Gamma
γ-Glutamyl-AMC	γ-L-Glutamyl-7-amino-4-methyl coumarin.
GGT	γ-Glutamyl transferase.
HBSS	Hanks balanced salt solution.
HBV	Hepatitis B virus.
MeCCNU	3-(Trans-4-methyl cyclohexyl)-1-(2-chloroethyl)-1-nitrosourea.
Mops	2-(N-Morpholino) propane-sulphonic acid.
NADH	Nicotinamide adenine dinucleotide.
NADPH	Nicotinamide adenine dinucleotide phosphate.
OPT	O-Phthaldialdehyde.
PAGE	Polyacrylamide gel electrophoresis.
PBS	Phosphate buffered saline.
THF	Tetrahydrofuran.
Tris	2-Amino-2-hydroxymethylpropane-1, 3-diol.

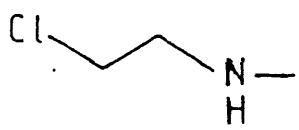
COMPOUND INDEX

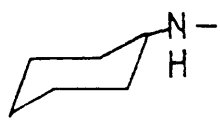


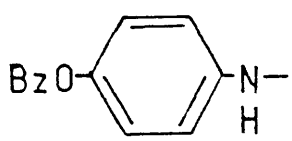
R

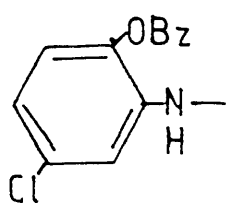
(12) 

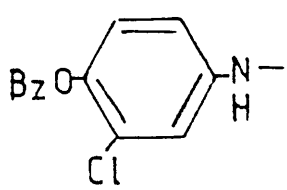
(13) 

(14) 

(15) 

(40) 

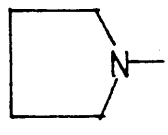
(41) 

(42) 

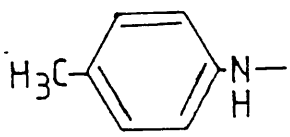
R

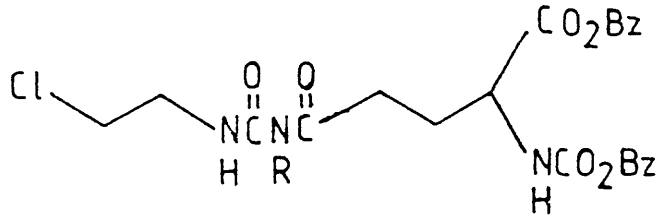
(70) Et_2N—

(71) Me_2N—

(72) 

(73) $tBuN(H)$—

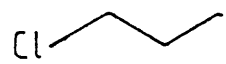
(74) 

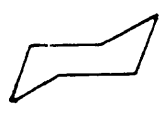


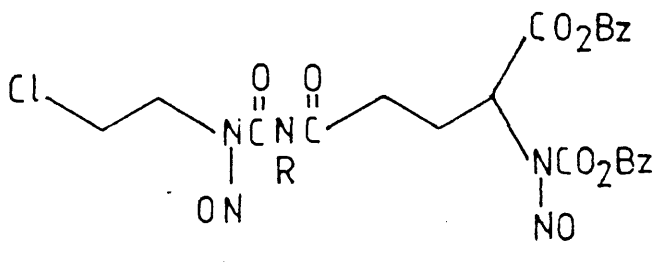
R

(16) Me

(17) Et

(18) 

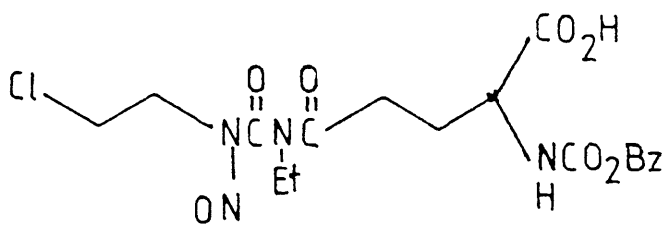
(19) 



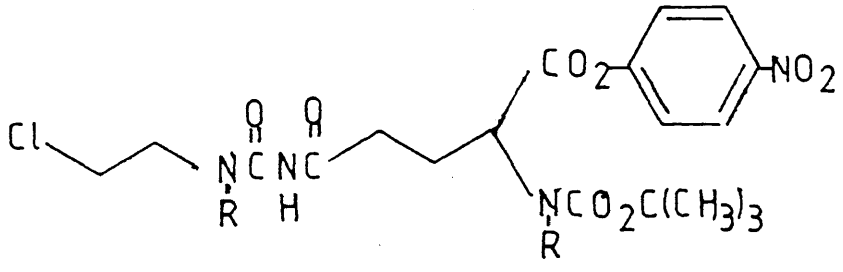
R

(22) Me

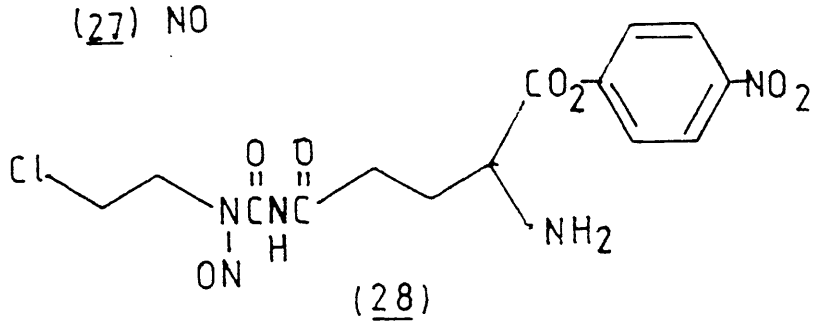
(23) Et



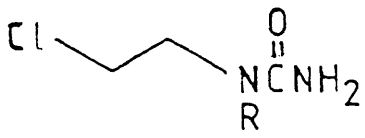
(24)



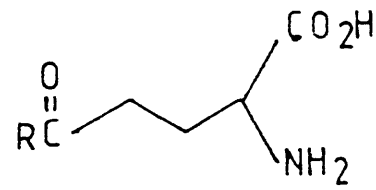
	R
(26)	H
(27)	NO



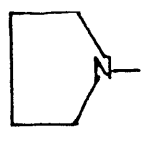
(28)



	R
(29)	H
(30)	NO



R

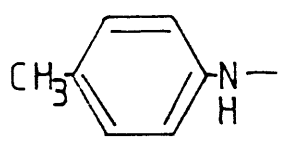
(31) 

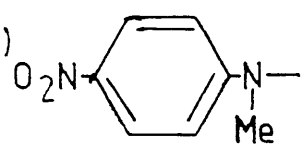
(32) $EtNH-$

(33) Et_2N-

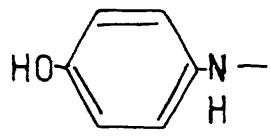
(34) Me_2N-

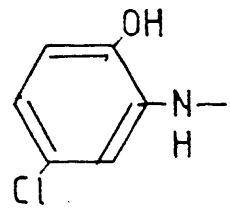
(35) $tBuNH-$

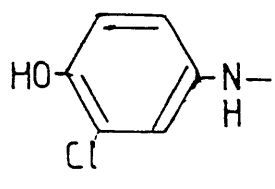
(36) 

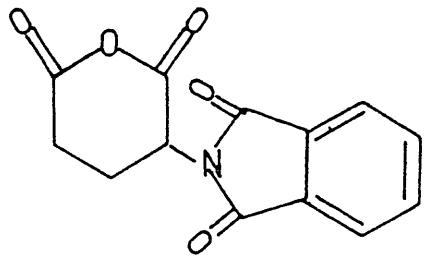
(39) 

R

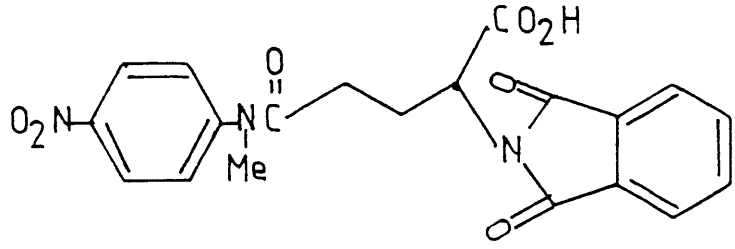
(43) 

(44) 

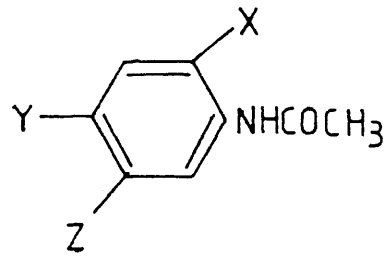
(45) 



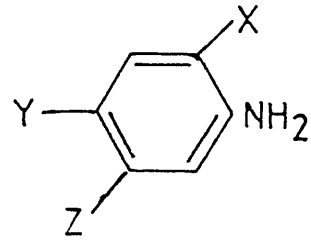
(37)



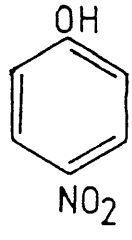
(38)



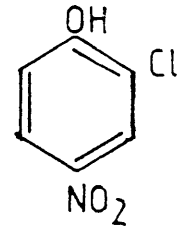
	X	Y	Z
(46)	H	OH	H
(47)	OH	H	Cl
(48)	H	OH	Cl
(49)	H	OBz	H
(50)	OBz	H	Cl
(51)	H	OBz	Cl



	X	Y	Z
(52)	H	OBz	H
(53)	OBz	H	Cl
(54)	H	OBz	Cl
(57)	H	OH	Cl
(68)	H	OH	H
(69)	OH	H	Cl



(55)



(56)

CHAPTER 1

INTRODUCTION

1.1 Discovery of GGT

γ-Glutamyl transferase (GGT) was discovered and named by Hanes et al[1] in 1950, who demonstrated its activity in sheep kidney preparations. They characterized the enzyme and found that it was specific for γ-glutamyl peptides producing either their hydrolysis or transfer of the γ-glutamyl moiety. Subsequently, the enzyme has been isolated and characterized from various mammalian tissues.
The presence of GGT activity in normal human serum was first demonstrated in 1960 by both Szewckzuk and Orlowski[2] and Goldbarg et al[3]. Later Szczeklik et al[4] discovered that GGT activity was increased in the serum of patients with liver diseases including hepatocellular carcinoma.

1.2 Occurrence of GGT

GGT occurs in many anatomical locations of the mammal especially in cells with a secretory or absorptive function. Thus, the kidney has the highest GGT activity followed by the pancreas. In the rat, the pancreas has approximately 20% of the activity found in the kidney, followed by the seminal vesicles, which have approximately 2% of the kidney level[3]. All other tissues have less than 1% the activity found in the kidneys. Nevertheless, histochemical studies of adult human tissues, have localized significant GGT activity within the liver (particularly in the bile ducts and the bile canalicular regions of hepatocytes[5]), within the endothelial or epithelial structures of the spleen, lung, bowel, placenta and thyroid homogenates[3] and in the choroid plexus[6]. Activity has also be demonstrated in the glandular epithelium of the breast, the primary follicle in the ovary, epididymus and prost ate[7] and in jejunal epithelium[8]. Comparative biochemical and histochemical

studies of human foetus, newborn and adult tissues show that foetal brains, lungs and liver (in particular) have much higher GGT activity than similar adult organs, whereas the reverse is true of foetal kidneys[9]. Activity has also been found in the cytosol of certain cells and significant albeit low levels of activity have been found in human blood serum. The origin of serum GGT is thought to be the liver[10] and because of their closely similar biochemical properties[11], it seems probable that soluble GGT in sera is derived from the membrane bound GGT. The increase in serum GGT associated with hepatobiliary but not kidney disease[12] suggests that it originates from the liver. Abnormally high levels of GGT are observed in various tumours, but particularly hepatocellular carcinoma[13,14,15]. The levels of GGT in chemically induced rat hepatoma are often 1000 fold higher than in normal rat hepatocytes[16], and concentrations of GGT in human hepatoma and ascitic fluid have been observed to be 3 to 13 times higher than those of normal adult liver[13,14].

1.3. Physical and chemical properties

1.3.1 Localization and orientation

GGT is a membrane-bound glycoprotein located on the outer surface of the cell membrane. Horiuchi et al[17] deduced the sidedness of the active site and the mode of anchorage of the enzyme in the rat renal brush border membranes on the basis of two observations. Firstly, GGT activity residing in purified brush border vesicles was inhibited by an S-acetyldextran polymer derivative of glutathione which is unable to permeate plasma membranes. Secondly, incubation of the purified vesicles with very low concentrations of papain resulted in limited proteolysis and

release of 80% of the membrane-bound enzyme. Additional information on the molecular organisation of GGT in ascites tumour cells in vitro was obtained by Inoue et al[15] using the impermeable S-acetyldextran glutathione inhibitor the enzyme activity was effectively inhibited.

1.3.2 Purification

Since GGT is bound to plasma membranes, it must be solubilized before purification. Methods used include solubilization with either detergents, organic solvents or proteinases. Usually the solubilized enzyme is then purified by ammonium sulphate precipitation, ion exchange chromatography, lectin affinity, gel filtration chromatography and polyacrylamide gel electrophoresis, although not necessarily in that order. Recently Cook and Peters[19] developed an alternative purification procedure for rat kidney GGT using phenyl-boronate affinity chromatography. This depended on interaction of the boronate ligands with the 1,2 cis-diol groups[20] of GGT, and gave a highly purified homogenous enzyme preparation. Homogenous preparations of GGT have also been obtained from rat hepatoma[21,22], rat pancreas[23], human liver[24] and human primary hepatoma[13].

1.3.3 Structure

The GGT from rat kidney is best characterized. It has two unequal subunits usually referred to as heavy and light. The heavy subunit (Mr 46-60KD) has a 6KD segment at the amino terminus which is rich in hydrophobic amino acids. This is thought to anchor the enzyme in the membrane[25]. The light subunit (Mr 22KD) does not interact with the membrane, and is non-covalently bound to the heavy subunit[26]. Both subunits are necessary for enzymatic activity in

intact membranes[27]. The intact enzyme can be isolated following use of detergents to extract it from the membrane, or the hydrophilic segment of the enzyme can be isolated by treating membrane preparations with papain or bromelain[26]. This releases the hydrophilic segments from the membrane bound hydrophobic tail, it retains full enzymatic activity[26]. More detailed studies of the subunit structures of rat renal GGT reveal that the light subunit from both papain and triton treated GGT have identical molecular weights and amino acid compositions. The heavy subunit obtained with triton, however, is larger than that obtained with papain by 52 amino acid residues located at the amino terminus[28]. These residues are believed to be the membrane-binding segment. The amino terminal residues of the heavy and light subunits of triton solubilized enzyme are methionine and threonine, respectively, whereas those of the papain solubilized enzyme are glycine and threonine, respectively. These differences suggest that the amino terminal region of the heavy subunit is removed by proteolysis[28,29,30].

The γ-glutamyl binding site is thought to reside on the light subunit of GGT[26,29], whereas binding of the acceptor substrate appears to involve both subunits. The isolated heavy subunit is able to bind the inhibitor 6-diazo-5-oxo-L-norleucine (DON), a glutamine analogue which is normally bound to the light subunit on reaction with the intact enzyme[30,31]. Therefore, it appears that both subunits are involved and that the acceptor binding site is located within a cleft between the two.

The structure of the carbohydrate side chains of GGT has been determined for rat kidney GGT[32]. These are both complex and enriched in non-reducing terminal ß-N-acetyl glucosamine residues. Further, GGT purified from different tissues of the same species

are immunologically cross-reactive[33] although several differ in portions of the carbohydrate structure particularly with respect to the sialic acid residues.

1.4 Reactions catalysed by GGT

GGT catalyses the transfer of the γ-glutamyl moiety from glutathione, S-substituted glutamine derivatives and other γ-glutamyl compounds (donors) to several acceptors. When the acceptor is either an amino acid or a dipeptide the process is termed 'transpeptidation' and the product is either a γ-glutamyl amino acid or a γ-glutamyl dipeptide, respectively (Equ 1).

When the acceptor is water, the enzyme catalyses the hydrolysis of the γ-glutamyl donor to form glutamic acid (Equ 2). When the γ-glutamyl donor and acceptor are identical, the process is termed 'autotranspeptidation' (Equ 3).

$$\gamma\text{-Glu} - X + \text{acceptor} \rightleftharpoons \gamma\text{-glu-acceptor} + HX \quad \ldots\ldots(1)$$

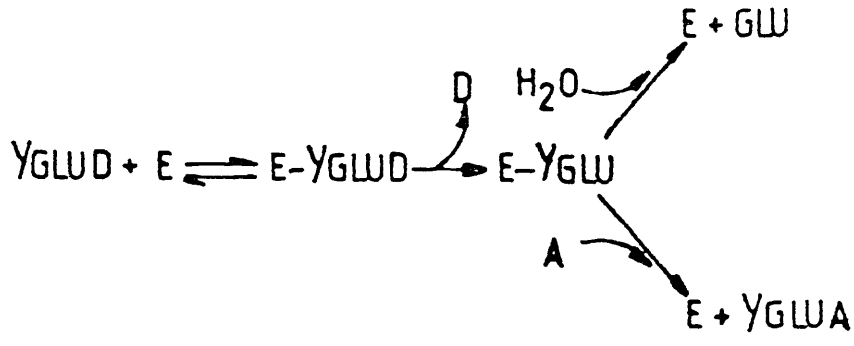
$$\gamma\text{-Glu} - X + H_2O \longrightarrow \text{glutamic acid} + HX \quad \ldots\ldots(2)$$

$$\gamma\text{-Glu} - X + \gamma\text{-Glu} - X \rightleftharpoons \gamma\text{-glu} - \gamma\text{-glu} - X + HX \quad \ldots\ldots(3)$$

All three reactions have been studied. The GGT catalysed hydrolysis exhibits a broad rate maximum between pH 6 and 8[34], whereas optimum transpeptidation occurs between pH 8 and 9[35]. The relative rates of hydrolysis and transpeptidation of a γ-glutamyl substrate therefore depends on the pH as well as the concentration of an acceptor.

Work by Thompson and Meister[36] using L and D γ-glutamyl-4-nitroaniline donors showed the existence of separate donor and acceptor sites. Further, the autotranspeptidation reaction could be avoided either by using the D-donor (because the

acceptor site shows absolute L-specificity), or by using substrate concentrations well below the K_m for the donor activity as an acceptor. When the autotranspeptidation activity is neglible the mechanism for GGT activity can be summarized as shown in Scheme 1.



A= amino acid, peptide, H_2O

D= Donor

Scheme 1

This implies a 'ping-pong' type mechanism with two alternative acceptor substrates i.e., water, producing hydrolysis of the γ-glutamyl donor, or an amino acid or peptide producing transpeptidation. The reactions proceed through a γ-glutamyl enzyme intermediate[37] an example of which has been isolated by Elce[38].

1.5 Specificity of donor and acceptor sites

The specificity of the GGT acceptor site has been studied extensively[36,39,40]. It exhibits absolute L-specificity and all common amino acids and many peptides are acceptable. There is some evidence[19,40] that neutral sulphur containing amino acids (e.g. L-cystine) are the best acceptors. Several dipeptides, however,

are better acceptors than their amino acid constituents. It has also been suggested that the enhanced activity of L-cystine and glycylglycine relates to the low basicity of their α-amino group which provides relatively high concentrations of the neutral acceptor at pH 8.5 where transpeptidation is usually measured[19,40]. The α-carbonyl moiety of the acceptor must also be involved since its absence results in loss of activity.

In contrast the specificity of the donor site has not been well-studied. From kinetic studies McIntyre and Curthoys[34] found that glutathione was a more active natural donor than S-substituted glutathione, and that glutathione disulphide and several γ-L-glutamyl peptides (e.g. γ-glutamyl alanine and γ-glutamyl glutamine) were also donors. Glutamine was found to be ca. 530 fold less active than glutathione[34]. Other suitable model donors included γ-L-glutamyl-4-nitroaniline[41], γ-L-glutamyl -2-naphthylamide[11], and γ-L-glutamyl-7-amino -4-methylcoumarin[42], all of which are commonly used to assay GGT activity. Despite these studies, the influence of donor specificity has not been critically investigated. For example, all of the above donors have either primary or secondary γ-amino substituents and the effect of a tertiary γ-amino substituent is unknown. It is known, however, that both D and L γ-glutamyl donors are active[36,39,43], which has led to the belief that high affinity is more important than stereospecificity.

Several donors have been found to be inactive for reasons which are unknown. These include L-homoglutamine and ß-amino - L- glutaryl - L -α-aminobutyric acid[44-46].

1.6 Function of GGT

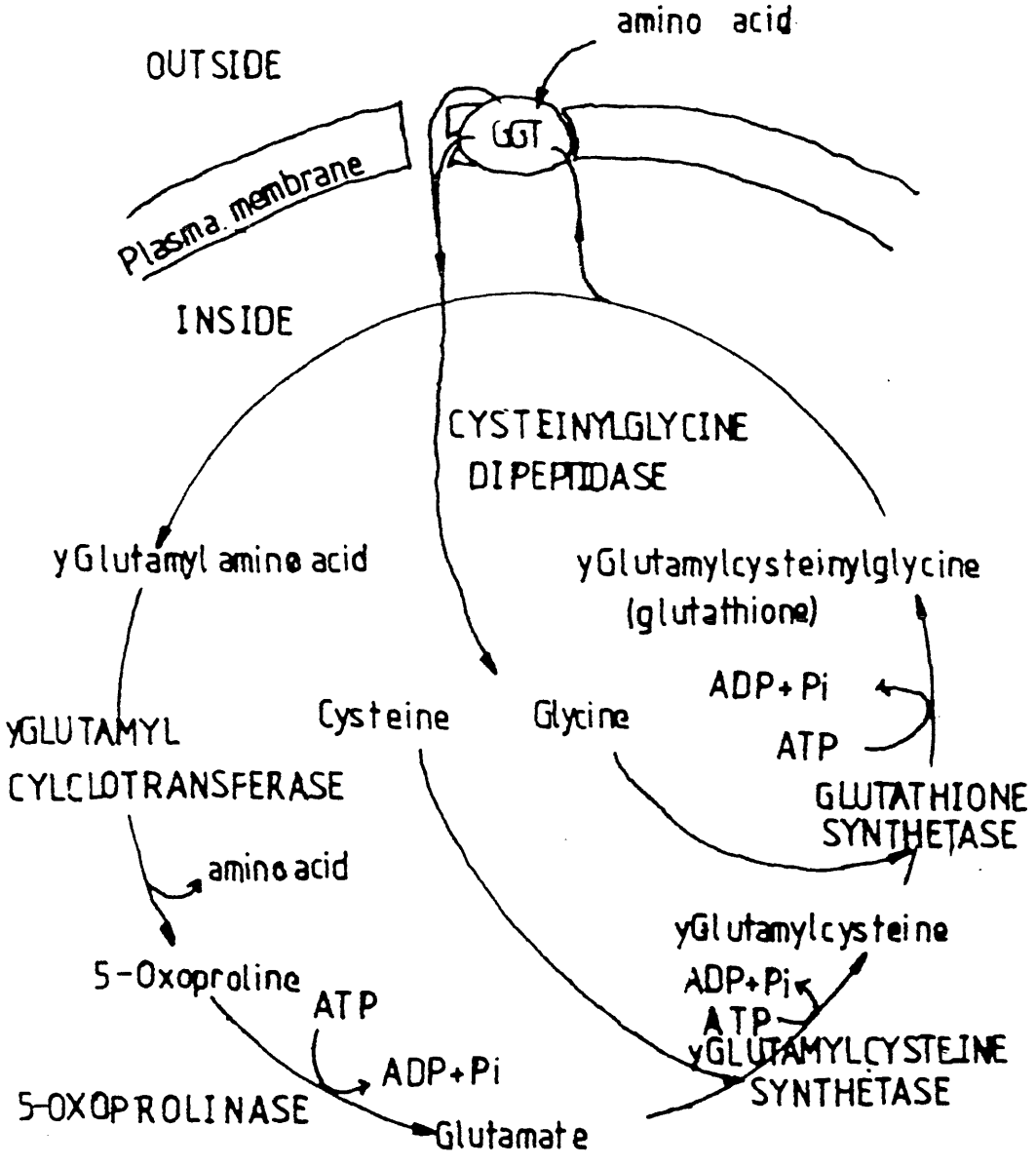
Although GGT was discovered over 30 years ago its physiological role remains uncertain.

1.6.1 Amino acid transport (γ-glutamyl cycle)

In 1973, Meister[47] proposed the existence of a γ-glutamyl cycle responsible for the transport of amino acids across cell membranes in which GGT played a central role (Scheme 2).

He proposed that; (1) GGT transfers the γ-glutamyl moeity of intracellular glutathione to an amino acid acceptor and transports the γ-glutamyl dipeptide across the cell membrane; (2) within the cell, cleavage of the γ-glutamyl dipeptide by γ-glutamyl cyclotransferase yields 5-oxoproline and free amino acids; (3) the cysteinylglycine co-product formed by the initial transpeptidation reaction is subsequently hydrolysed by an intracellular peptidase and; (4) glutathione reforms within the cell from glutamine, L-cysteine and glycine.

Despite widespread acceptance of this cycle as the physiological function of GGT, there is much evidence questioning its validity. Studies by Curthoys and Hughey[48] and McIntyre and Curthoys[34], suggest that hydrolysis rather than transpeptidation is the major reaction catalysed by GGT in the kidney. Further, the localization of GGT activity on the external aspect of the plasma membrane of ascites tumours[18] and on renal brush border membranes[17] is inconsistent with an involvement of GGT in the degradation of intracellular glutathione. Finally recent studies on yeast cells in which GGT was inactivated by 6-diazo-5-oxo-L-norleucine showed no significant difference for the rate of uptake of amino acids and dipeptides compared with unactivated yeast cells[49].


amino acid
OUTSIDE
GGT
Plasma membrane
INSIDE
CYSTEINYLGLYCINE
DIPEPTIDASE
γGlutamyl amino acid
γGlutamylcysteinylglycine
(glutathione)
ADP+Pi
ATP
γGLUTAMYL
CYLCLOTRANSFERASE
Cysteine
Glycine
GLUTATHIONE
SYNTHETASE
amino acid
γGlutamylcysteine
5-Oxoproline
ADP+Pi
ATP
ATP
γGLUTAMYLCYSTEINE
SYNTHETASE
ADP+Pi
5-OXOPROLINASE
Glutamate

Scheme 2

1.6.2 Glutathione hydrolysis

GGT is the only enzyme known to cleave glutathione. This suggests it is involved in the degradation of glutathione and hence, in the regulation of tissue glutathione concentration. The initial steps in this process are the release of glutathione from the liver and its transport via the circulatory system to the kidneys, the major site of its degradation. Since glutathione cannot be taken up intact by cells, McIntyre and Curthoys[50] suggested that it is hydrolysed by GGT into its constituent amino acids which are then absorbed into the kidney cells. Reabsorption of the amino acids and redistribution of L-cysteine for protein synthesis or glutathione resynthesis completes the compartmentalized interorgan metabolism of glutathione. Experimental evidence for such an hypothesis has been provided by Griffith and Meister[44], who found that within 1h urinary glutathione levels in mice increased 3000 fold following injection of the GGT inhibitor γ-L-glutamyl-2-carboxyphenyl hydrazine. Subsequently Anderson et al[51] demonstrated that this effect relates to the lack of glutathione reabsorption by the kidney. McIntyre and Curthoys[50] also showed that patients lacking detectable quantities of kidney GGT had pronounced glutathionurea and glutathionemia. In addition to reclaiming the amino acids from glutathione degradation, GGT can also hydrolyse oxidized glutathione[34] thereby providing a mechanism for the removal of oxidized glutathione from the serum.

1.6.3 L-Glutamine hydrolysis

L-Glutamine is utilized by GGT at a lower rate than glutathione. Nonetheless, recent work by Cook and Peters[52] suggests that L-glutamine is the predominant *in vivo* donor substrate for GGT, because of its higher concentration. Under saturating conditions

for both L-glutamine and glutathione, 65% of the glutamic acid produced by GGT hydrolysis derives from L-glutamine. It is also noteworthy that GGT was first described as a phosphate independent glutaminase[53]. Thus if GGT is primarily hydrolase, it must be an effective glutaminase as well as a glutathionase.

1.6.4 The role of GGT in detoxification

Many foreign compounds, including toxins, are excreted by mammals in the urine or faeces as mercapturic acids. The detoxification of these compounds involves an initial conjugation with glutathione in the liver followed by passage through the kidneys or bile ducts (both of which have high levels of GGT) where they are degraded to mercapturic acids. Removal of the γ-glutamyl group from the glutathione conjugate by GGT, is thought to be the first step in this degradation process[54]. Curthoys and Hughey[48] have summarized evidence for the physiological role of GGT in mercapturic acid formation.

1.7 GGT in hepatocarcinoma

Elevated levels of serum GGT are an indicator of hepatobiliary disfunction[11,55]. The link between GGT and liver carcinogenesis was discovered in 1972 by Fiala et al[15] using rats either fed with the hepatocarcinogen 3-methyl-4-dimethylaminoazobenzene or transplanted with chemically induced hepatomas.

Subsequently Kalengayi et al[56] found an increased GGT activity in focal areas of hepatocytes. A number of other laboratories have detected elevated hepatic GGT levels on dosing rats with various structurally different hepatocarcinogens including aflatoxin. Commonly, GGT activity in rat liver hepatoma is 1000 fold higher than in normal hepatocytes[16].

Human hepatocellular carcinomas also show elevated levels of GGT[13,14,57]. Gerber and Thung[57] reported 80% of human heptocellular carcinomas as GGT positive. Further, GGT released from the cell membrane can be detected in the serum, and the measurement of serum GGT has therefore become a common clinical test for hepatobiliary disfunction. Since elevated serum GGT is generally indicative of hepatobiliary or pancreatic disorders[11], the presence of specific GGT isoenzymes in the serum may detect hepatocellular carcinomas at an early stage.

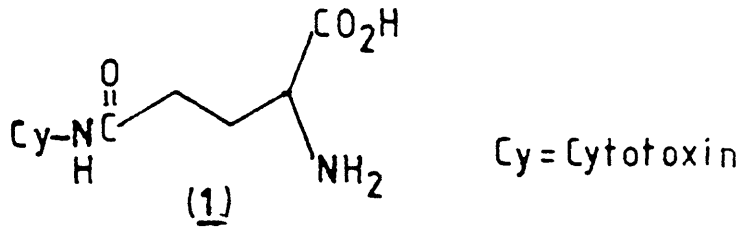
1.8 Incidence of hepatocarcinoma

Hepatocarcinoma is one of the ten most common human cancers with over 250,000 new cases each year[58]. It is one of the most prevalent cancers in Third World countries such as Africa and South East Asia. Usually it is associated with infection by the hepatitis B virus (HBV), but whether HBV is the sole cause of neoplasia is not established. Aflatoxin (a product of the fungus Asperigillus flavus) also induces human hepatocarcinoma and fungal infection by badly stored food such as grain and peanuts is also thought to contribute to primary liver cancers in Third World countries.

1.9 γ-L-Glutamyl prodrugs

As yet no treatment for hepatocarcinoma has proved really effective. Early diagnosis is difficult, so the tumour is often well established before it is detected and corrective resection is usually impossible because the tumours are multifocal. It follows that elevated levels of GGT in hepatocarcinoma are of particular interest as a method of targetting chemotherapeutic agents to the tumors. As discussed in Section 1.5, GGT has a relatively low

specificity towards γ-glutamyl donors. It follows that γ-glutamyl derivatives (1) of pharmacologically active substances may also be substrates and therefore act as organ-specific prodrugs.



(1)

The active cytotoxic agent would be preferentially released from the inactive γ-L-glutamyl derivative (prodrug) at sites of high GGT activity to localize its pharmacological effect.

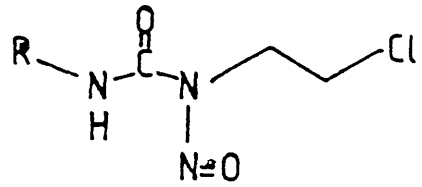
The first γ-L-glutamyl prodrugs were designed to be kidney specific. For example Wilk et al[59] synthesized γ-L-glutamyl dopamine as a specific renal vasodilator. The preferential activation of the γ-L-glutamyl prodrug in the kidney by GGT gave a therapeutic response at lower concentrations than L-dopamine itself, and side effects (e.g. raised blood pressure) were therefore minimized. Manson et al[16] showed that the γ-L-glutamyl derivative of an alkylating mustard analogue of 4-phenylene diamine, γ-[N,N-bis-(2-chloroethyl)-4-phenylene diamine]-L-glutamic acid was activated by GGT positive rat hepatoma cells in vitro. Normal hepatocytes (GGT negative), however were fairly resistant to the prodrug, suggesting that release of the mustard moiety by GGT gave the observed cytotoxic effect in hepatoma cells. Recent work has confirmed that γ-[N,N-bis-(2-chloroethyl)-4-phenylene diamine] -L-glutamic acid is a donor substrate for GGT[60].

These studies imply that γ-glutamyl prodrugs may be selective cytotoxic agents for the treatment of primary hepatoma.

To further investigate their potential, the present study concerned the synthesis and biological properties of some γ-L-glutamyl derivatives of N-(2-chloroethyl)-N-nitrosoureas and hydroxyanilines.

1.9.1 N-(2-Chloroethyl)-N-nitrosoureas

N-(2-Chloroethyl)-N-nitrosoureas are highly active anticancer agents with a broad antitumour activity. Several, such as 1,3-bis-(2-chloroethyl)-1-nitrosourea, BCNU (2); 3-cyclohexyl -1-(2-chloroethyl)-1-nitrosourea, CCNU (3), and 3 - (trans-4-methyl cyclohexyl)-1-(2-chloroethyl)-1-nitrosourea, MeCCNU (4), find application in the treatment of a wide range of neoplasms[61,62].

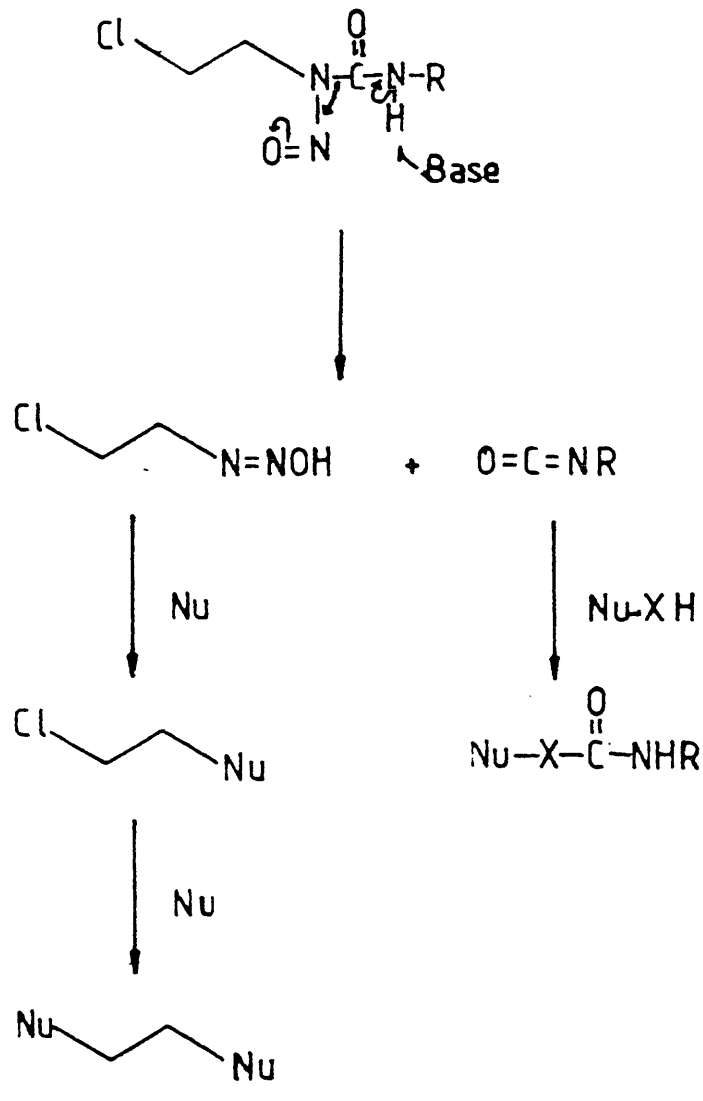


(2) R= $ClCH_2CH_2$ (BCNU)

(3) R= C_6H_{11} (CCNU)

(4) R= trans 4-$CH_3C_6H_{10}$ (MeCCNU)

They decompose spontaneously (Scheme 3) under physiological conditions giving rise to electrophiles derived from 2-chloroethyl diazohydroxide which by alkylation form inter-strand crosslinks in nucleic acids and proteins[63,64]. The other decomposition product



Nu = nucleic acid, proteins etc

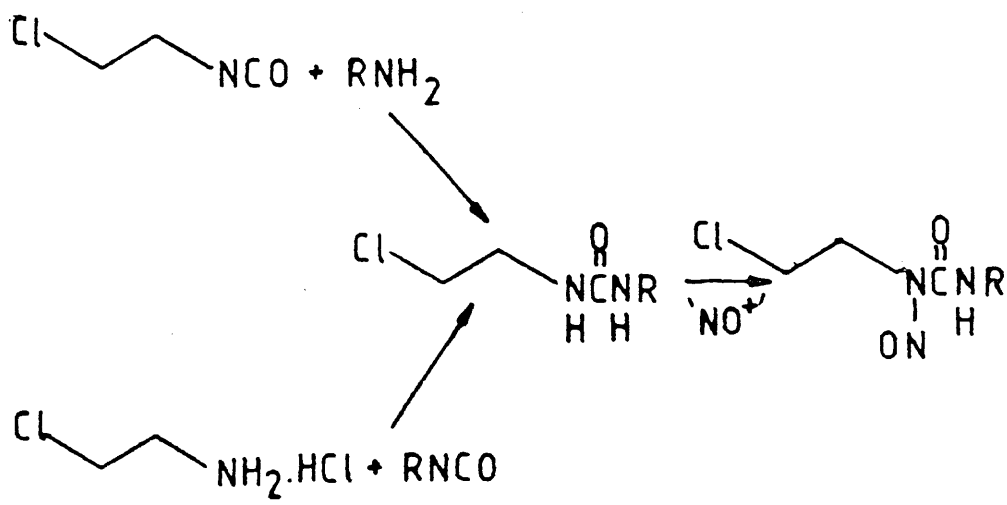
X = S, O, NH etc

Scheme 3

alkyl isocyanate[65] can carbamoylate amino groups in these biological macromolecules.

1.9.1.1 Synthesis of N-(2-chloroethyl)-N-nitrosoureas

Conventional methods for the preparation of N-(2-chloroethyl)-N-nitrosoureas include nitrosation of the corresponding urea with sodium nitrite in aqueous HCl, H_2SO_4 or anhydrous formic acid[61,66-70] or with dinitrogentetroxide[71] (Scheme 4). The ureas in turn are prepared either by the reaction of 2-chloroethyl isocyanate and the appropriate amine or, alkyl isocyanate and 2-chloroethylamine hydrochloride in the presence of non-nucleophlic base in non polar solvents[61,66-70], as shown in Scheme 4.



Scheme 4

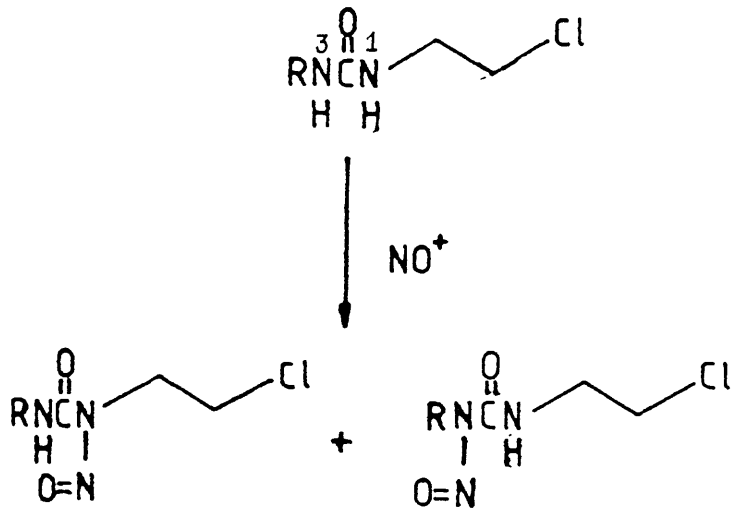
Nitrosation in aqueous systems usually involves the acidification of an aqueous solution of sodium nitrite and the urea at reduced temperatures (0-5°C). Hyde et al[70] were the first to utilize this method for the preparation of N-(2-chloroethyl)-N-nitrosourea, in which 2-chloroethyl urea was nitrosated with sodium nitrite in the

presence of dilute H_2SO_4. Yields from this reaction were found to be low and this was thought to be due to the insolubility of the urea in aqueous systems.

Following the success of N-(2-chloroethyl)-N-nitrosoureas as anticancer agents, more productive methods of synthesis were developed. Johnston et al[66-65] used sodium nitrite in anhydrous formic acid for the nitrosation of many 2-chloroethylurea derivatives.

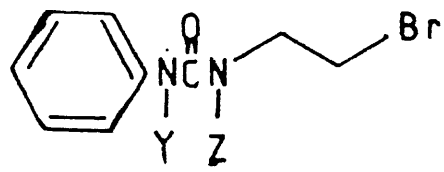
Yields from these reactions were generally higher than those using aqueous systems and formic acid proved to be an excellent solvent for most ureas.

With the preponderance of unsymmetrical 1,3 - disubstituted ureas amongst those used in the synthesis of N-(2-chloroethyl)-N-nitrosoureas, cautious assignment of structure was required - since there are two possible sites of nitrosation; positions 1 and 3 as shown in Scheme 5.



Scheme 5

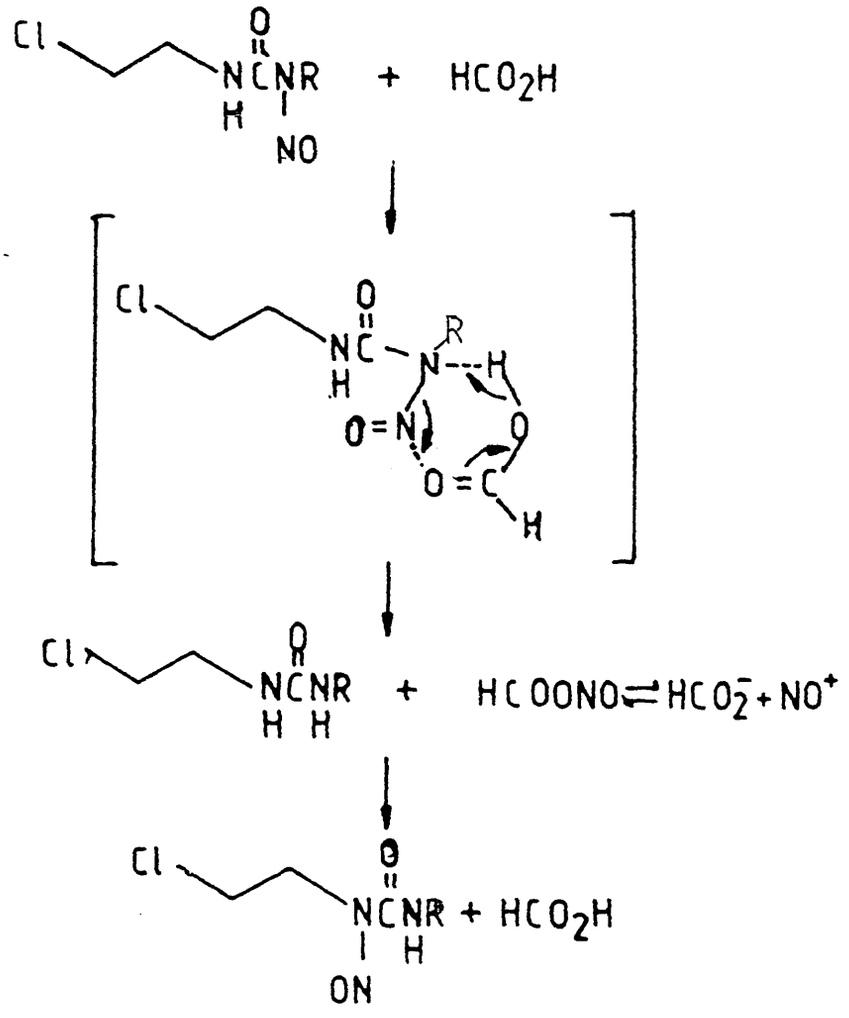
The effect of water on isomer ratio was first observed in the nitrosation of 1-(2-bromoethyl)-3-phenylurea[67], the nitrosation of which with solid sodium nitrite in 85% formic acid gave a 1:1 mixture of isomers (5a) and (b), whereas nitrosation in 98-100% formic acid gave only (5a).



(5a) Z=NO; Y=H

(b) Z=H; Y=NO

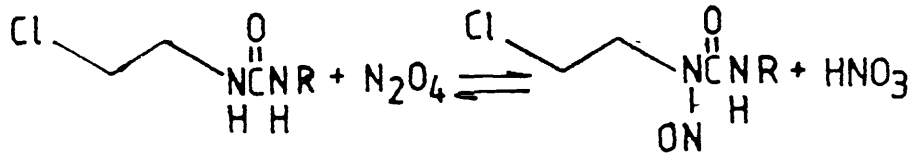
The presence of water had a similar effect on the nitrosation of 1-(2-chloroethyl)-3-phenylurea and 1-(2-chloroethyl)-3-cyclohexylurea[67]. Nitrosation of 1-(2-chloroethyl)-3-cyclohexylurea in undiluted formic acid with an equal volume of aqueous nitrite solution (5-6%) gave a mixture of isomers. However the pure required isomer (1-nitrosourea) could be obtained in high yields by its dissolution in cold, undiluted formic acid, and subsequent reprecipitation by the addition of water. Transfer of the nitroso group in formic acid probably involved the formation of formyl nitrite. Such a mechanism may be depicted as shown in Scheme 6. The isomeric composition may depend on an equilibrium governed by the relative stability of the isomers toward nitroso group abstraction by formic acid. Apparently, as H_2O concentration is increased, there is a point above which abstraction does not occur. In an aqueous system, the formation of isomers may not be reversible, and the isomer ratio may therefore depend primarily on



Scheme 6

their relative rates of formation.

An alternative aprotic nitrosation technique involves the use dinitrogen tetroxide[71] (N_2O_4). The reaction of N_2O_4 with ureas is rapid at 0°C. Generally an inert solvent such as dichloromethane is used. The N_2O_4 is used with an excess of anhydrous sodium acetate, since in the absence of a base the reverse reaction of denitrosation by the nitric acid produced occurs as shown in Scheme 7



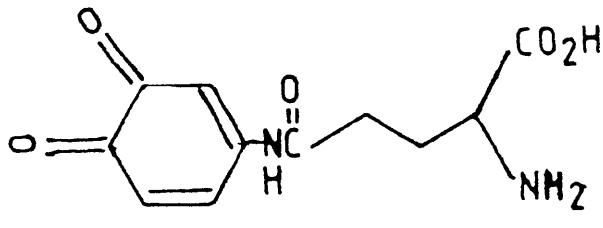


Scheme 7

Although isomer formation may still occur in unsymmetrical 1,3-disubstituted ureas, the very high yields and rapidity of reaction makes it generally the method of choice.

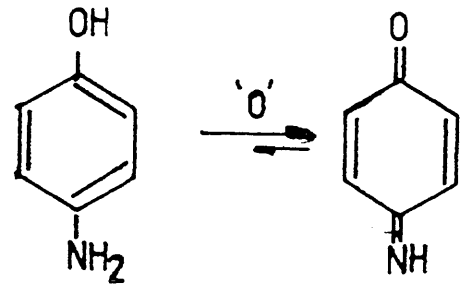
1.9.2 Hydroxyanilines

γ-L-glutamyl-4-hydroxyaniline has been isolated from the gill tissue of a common mushroom, Agaricus bisporsus[72] where it is thought to be the precursor of γ-L-glutaminyl 3,4 benzoquinone (6)



(6)

Compound (6) is known to inhibit various enzymes[73] and to exhibit antitumour activity[74]. γ-L-Glutamyl-4-hydroxyaniline, however, was found to be an inactive cytotoxin[74] in contrast to 4-hydroxyaniline which is both a potent enzyme inhibitor and cytotoxin[75,77]. Calder et al[75] suggested that the cytotoxic activity of 4-hydroxyaniline related to its ready oxidation to benzoquinone-imine (Scheme 8).



Scheme 8

It is possible that the semiquinone radical[76] intermediate is the actual cytotoxin. Conditions which retard the autoxidation of 4-hydroxyaniline (e.g. incubation in acidic media or under nitrogen) decrease the cytotoxicity of the drug towards cell lines[75]

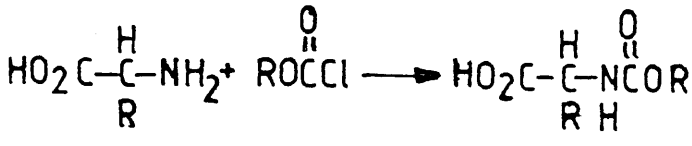
Work by Boekelheide et al[79] has shown that γ-L-glutamyl-4-hydroxyaniline is cleaved by GGT in melanocytes to release 4-hydroxyaniline and effect melanocytotoxicity. This suggests that γ-L-glutamyl-4-hydroxyaniline should be an effective prodrug for GGT positive hepatoma.

1.9.3 Synthesis of γ-L-glutamyl prodrugs - an overview

1.9.3.1 Protection of glutamic acid

Usually, the basic problem in peptide synthesis is one of protecting the amino group. In bringing about the interaction between the carboxyl group of one amino acid and the amino group of different compound, one must prevent interaction between the carboxyl group and the amino group of the same amino acid.

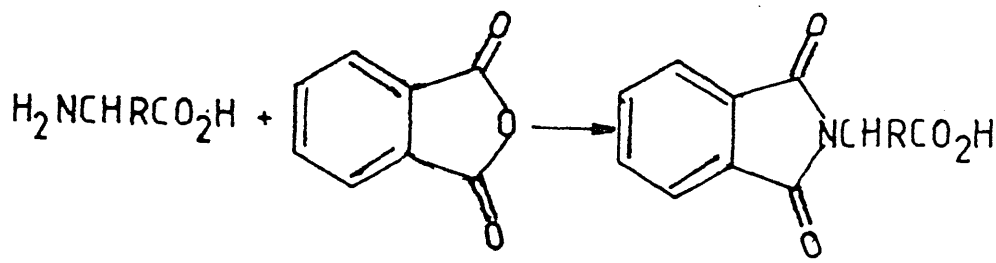
A popular method for protecting the amine moeity of amino acids is by their conversion into a urethane using an appropriate alkyl chloroformate[80] (Scheme 9).



Scheme 9

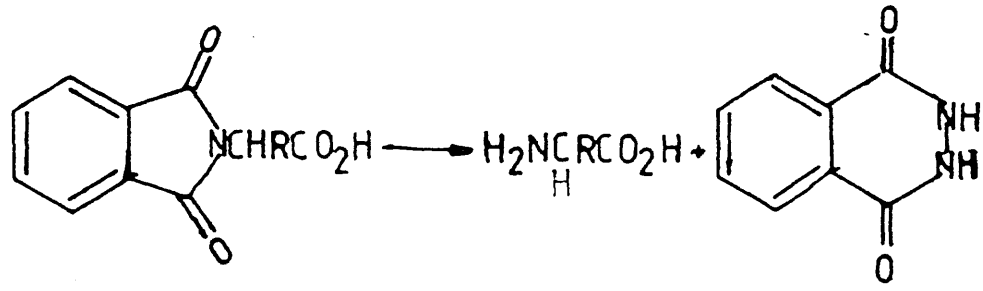
Usually the N-benzyloxycarbonyl derivative is prepared, which can be removed by hydrogenation[80], by abstraction with anhydrous trifluoroacetic acid[81], sodium in liquid ammonia[82] or with HBr in acetic acid[83]. Alternatively, the N-t-butyloxycarbonyl derivative can be synthesized which is removed by HCl in acetic acid[84].

The phthalyl group has also been utilized in a variety of cases as a masking group in the preparation of peptides, particularly γ-glutamyl peptides[85, 86]. Phthalimido-acids are commonly prepared by heating mixtures of the amino acid and phthalic anhydride slightly above the fusion point of the anhydride, or in the presence of solvents such as pyridine or acetic acid (Scheme 10).



Scheme 10

The cleavage of N-alkyl phthalimides is by their treatment with alcoholic hydrazine hydrate followed by HCl, the final products being the phthalhydrazide and the hydrochloride salt of the desired amino acid[85,86] (Scheme 11).

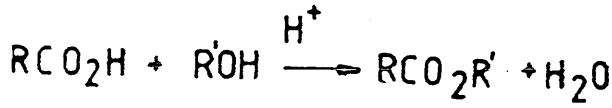


Scheme 11

Other methods used for the protection of amine residues include the use of trityl, tosyl, and trifluoracetyl moeities. Details of their use and removal are given in the text of Schröder and Lubke[87].

Owing to the difficulties arising from the presence of the α-carboxyl group in glutamic acid, protection at this site is also necessary in the synthesis of γ-glutamyl peptides.

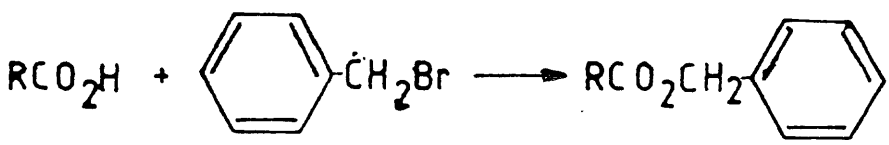
Perhaps the most popular method for protecting carboxylic acids is by their conversion to an ester (Scheme 12).



Scheme 12

Common ester protecting groups employed include ethyl, methyl and tertiary butyl[88], these groups are generally cleaved by alkaline or acidic hydrolysis.

The introduction, in 1932, of the use of the benzyl ester group[89] (removable by low pressure hydrogenolysis) in peptide synthesis made practical the preparation of a wide variety of peptides. The original mode of esterification involved the reaction of the acid with benzyl alcohol with water removal by azeotropic distillation[90]. Later benzyl bromide was introduced as the esterification agent (Scheme 13).

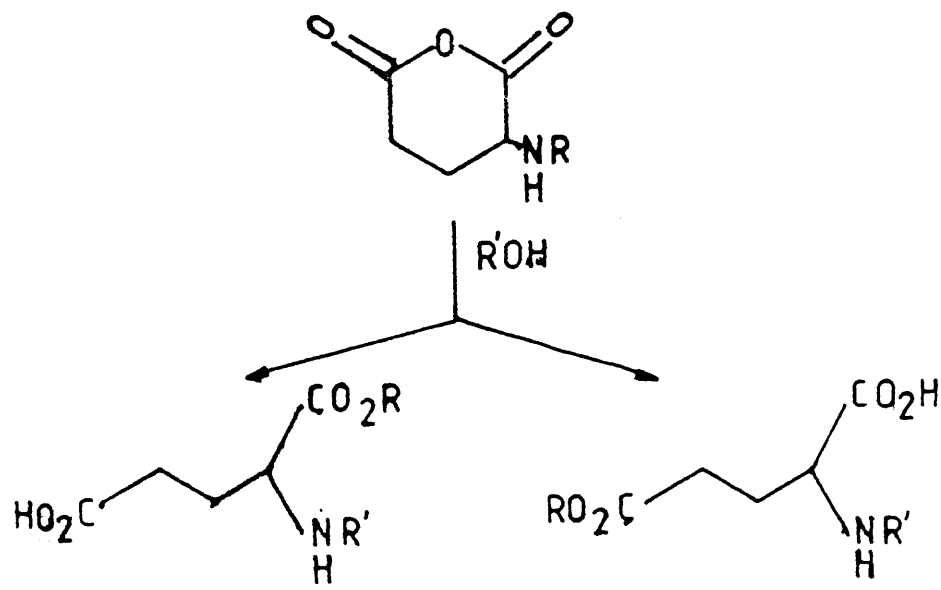


Scheme 13

Substituted benzyl esters such as 4-nitro[91] and 4-methoxy[92] have also been used and these are cleaved by either catalytic hydrogenation or acid catalysed hydrolysis.

Procedures suggested for the preferential masking of the α-carboxyl function of glutamic acid can be ranged under 3 different schemes. The first method is based on the partial hydrolysis of diesters. Sachs and Brand[93] obtained in this way α-benzyl glutamate, by treatment of the corresponding dibenzyl ester with HI. Due to the restricted specificity of the hydrolysis the yield was rather low. Selective splitting of the ester has been achieved in diesters of N-protected glutamic acid, but only if the amino function is blocked by a resistant and bulky trityl residue[92,94].

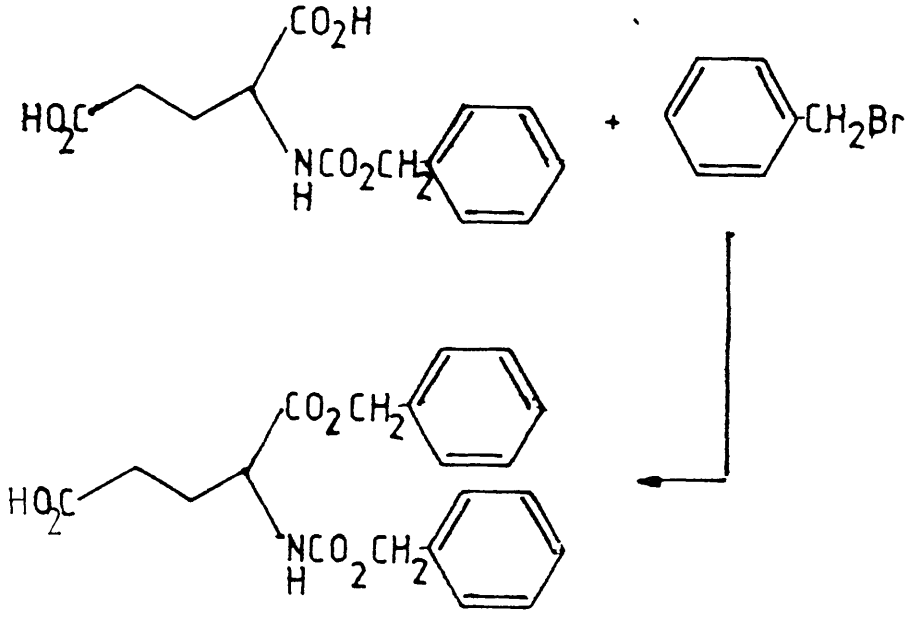
An alternative route arises from the reaction between the N-substituted glutamic anhydride and an appropriate alcohol, facilitated by the addition of a base[95]. Dependent on the nature of the N-substituent, solvent and base, in the main the γ or α-ester will be formed (Scheme 14).



Scheme 14

As the rate of esterification for both carboxyl functions seems to be similar however, this method nearly always leads to the formation of a mixture.

The final method suggested for the synthesis of the α-ester of glutamic acid is that of Nefkins and Nivard[96] involving the reaction of N-benzyloxycarbonyl-L-glutamic acid and benzyl bromide, in the presence of an equimolar amount of a base usually triethylamine or dicyclohexylamine (Scheme 15).



Scheme 15

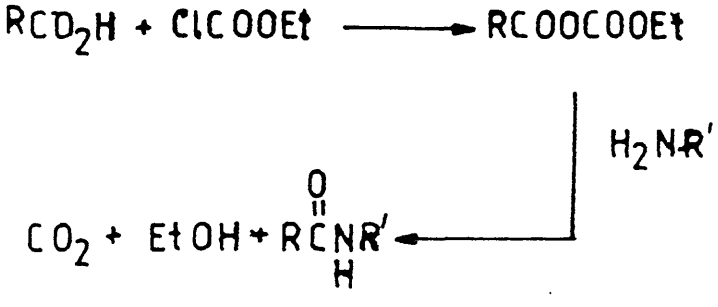
Here the initial protection of the α-amino group with the electron withdrawing benzyloxycarbonyl function, lowers the Pk_a at the α-carboxyl site sufficiently for it to become the most favourable site for esterification. The choice of solvent also influences the shift in the equilibrium between the α and γ-positions, a more polar solvent favouring α-attack. Dicyclohexylamine is often used as the base in this reaction as it causes the precipitation of the α-carboxylate from the α, γ-isomer mixture.

1.9.3.2 Coupling procedures

There are many methods used in the formation of peptide linkages, only three are discussed here.

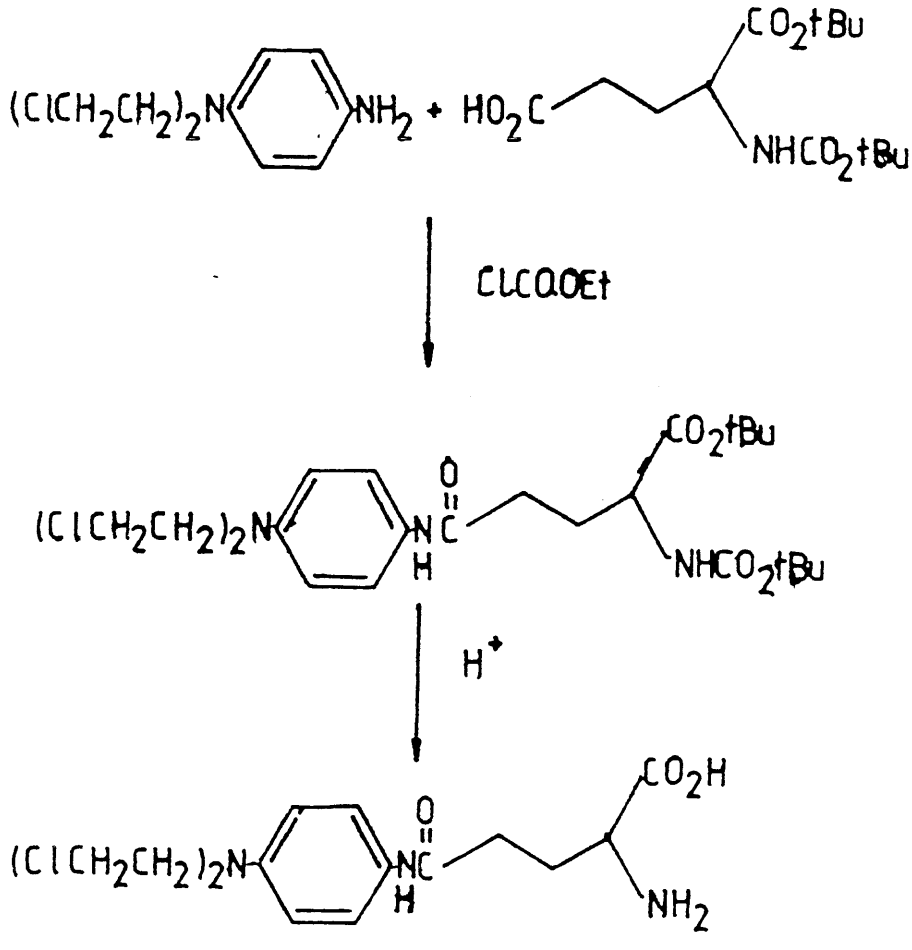
i) Mixed anhydride

This method is perhaps the most widely used coupling procedure in peptide synthesis. It involves the generation of a mixed anhydride at the carboxylic acid site of an amino acid (usually via an alkyl chloroformate such as ethyl[97] or isobutyl chloroformate[98]), which is then condensed with the appropriate amine (or amino acid) (Scheme 16).



Scheme 16

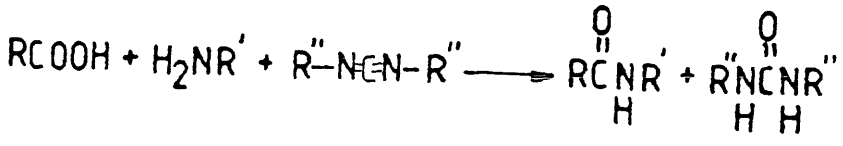
This type of procedure was used to couple α-N-t-butyloxycarbonyl-α-t-butyl-γ-L-glutamic acid with N,N bis-(2-chloroethyl)-4-phenylene diamine in the synthesis of the γ-L-glutamyl prodrug γ-[N,N-bis-(2-chloroethyl)-4-phenylene diamine]-L-glutamic acid. The final deprotected prodrug was then obtained by acidic hydrolysis (Scheme 17).


(ClCH2CH2)2N
NH2
HO2C
CO2tBu
NHCO2tBu
ClCOOEt
(ClCH2CH2)2N
O
NC
H
CO2tBu
NHCO2tBu
H+
(ClCH2CH2)2N
O
NC
H
CO2H
NH2

Scheme 17

ii) Carbodi-imide method

This method was first introduced by Sheehan and Hess in 1955[95]. It involves the use of an N,N dialkylated carbodi-imide, preferably N,N dicyclohexyl carbodi-imide as the coupling reagent (Scheme 18).

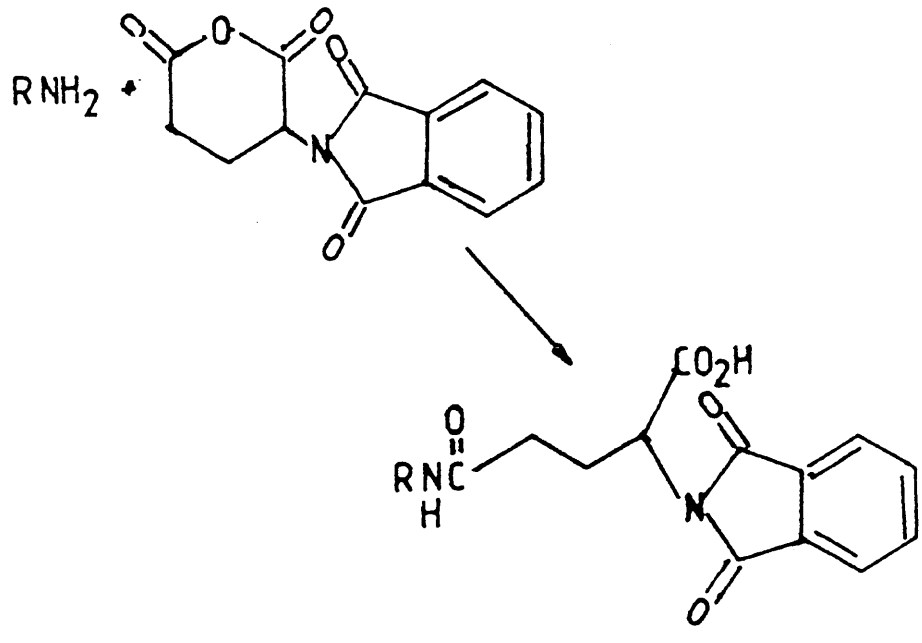


Scheme 18

The reaction is usually carried out in dichloromethane at room temperature over a period of 4-18 hours, after which the precipitate of dicyclohexylurea is removed by filtration. Because of its simplicity and low tendency to induce racemisation it is also a popular coupling procedure.

iii) By phthalylated intermediates

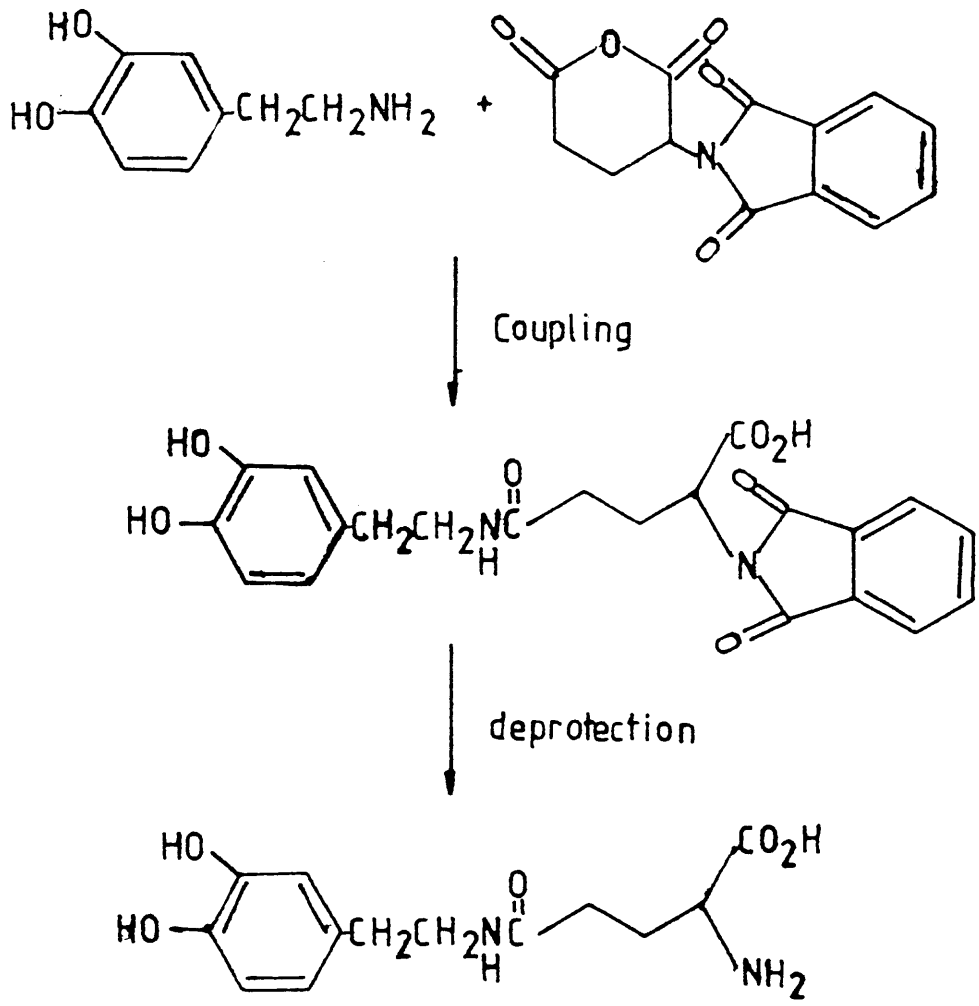
A unique coupling procedure for the synthesis of γ-glutamyl peptides involves the use of N-phthalyl-L-glutamic anhydride as an acylating agent, the peptide link being formed simultaneously with the opening of the ring by an appropriate amine[55]. (Scheme 19).



Scheme 19

The first γ-glutamyl prodrug synthesized, γ-L-glutamyl dopamine[59] was prepared using this coupling technique. In this synthesis unprotected L-dopamine was reacted with N-phthalyl-L-glutamic anhydride to give N-phthalyl-γ-L-glutamyl dopamine. The removal of the phthalyl group using hydrazine hydrate gave the impure

γ-L-glutamyl prodrug, which was subsequently purified by ion-exchange chromatography (Scheme 20).



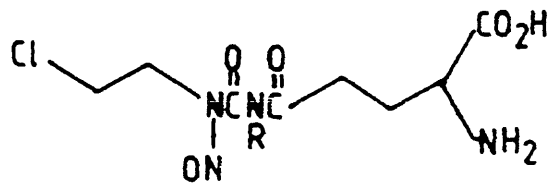
Scheme 20

CHAPTER 2

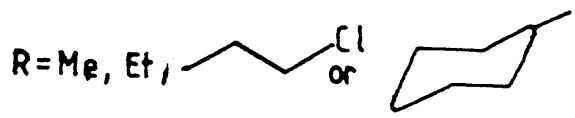
SYNTHESIS

2.1 Attempted synthesis of γ-glutamyl adducts of N-(2-chloroethyl) -N-nitrosoureas

An early goal in the study was the synthesis of the γ-L-glutamyl adducts of N-(2-chloroethyl)-N-nitrosoureas (7). Two of these compounds, of course, are prodrug analogues of the well known anticancer drugs BCNU and CCNU.



(7)



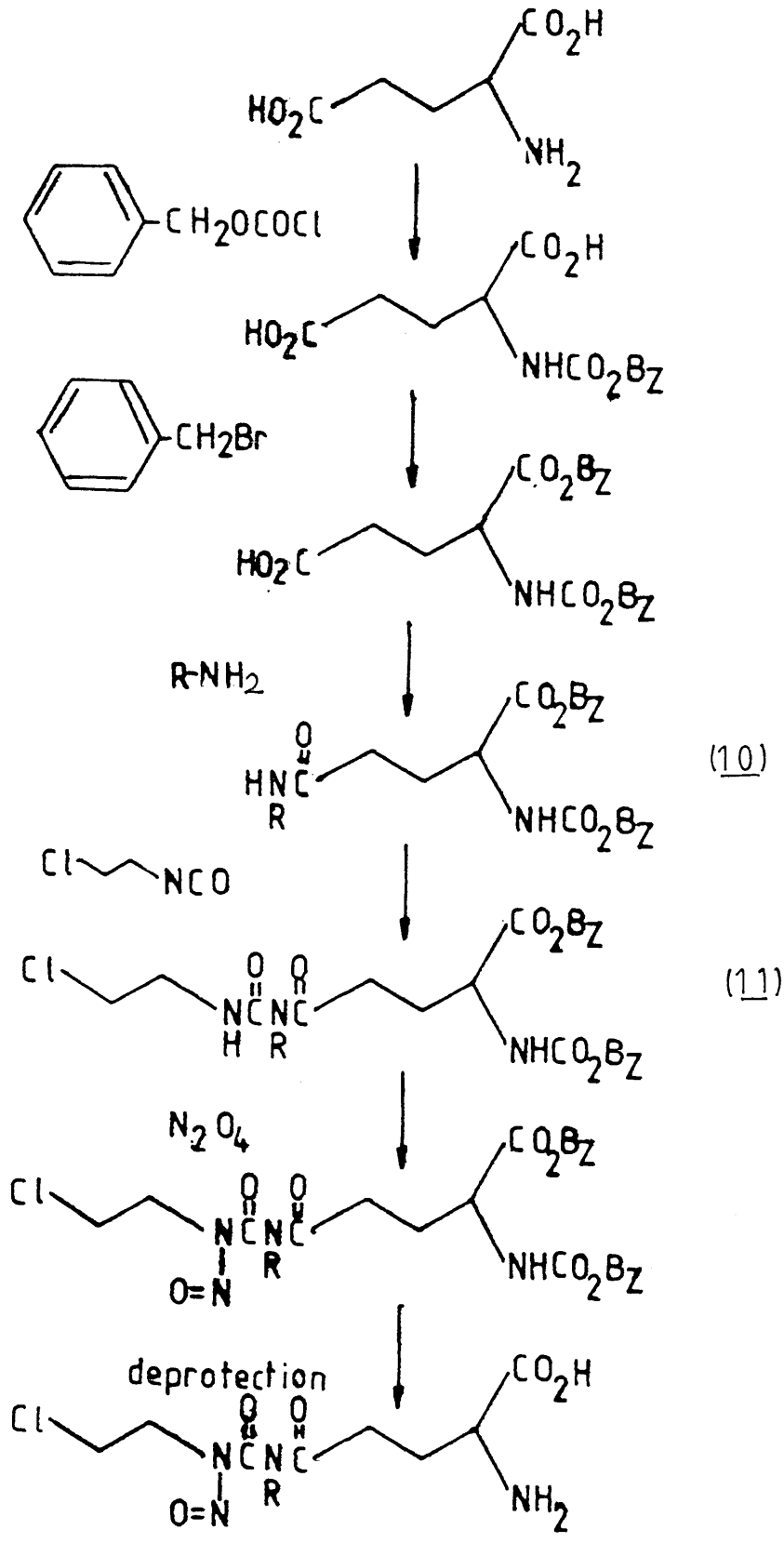
The synthetic strategy selected for (7) is outlined in Scheme 21. Its main features are; (1) α-protection of glutamic acid; (2) preparation of the appropriate glutamine derivative; (3) generation of γ-L-glutamyl urea moieties by condensation of the protected glutamine derivative with 2-chloroethyl isocyanate; (4) aprotic nitrosation of the urea N-atom and; (5) deprotection of the glutamic acid moiety. Because of the lability of N-nitroso group, it was considered essential to introduce this function at a late stage in the synthesis.

2.1.1 Protection of glutamyl α-carboxyl and α-amino functions

Because of the presence of a second carboxyl group in glutamic acid, it was necessary to protect the α-carboxyl function before elaborating glutamic acid at the γ-position. Also the α-amino group of glutamic acid requires protection because it is prone to long chain polymerisations during peptide condensation. Thus

suitable protection of glutamic acid requires selectivity for the α-carboxyl and α-amino groups, stability throughout subsequent reactions yet ability to be removed under very mild conditions to which the final N-nitrosourea is stable (N-nitrosoureas are susceptible to both acid and base catalysed decomposition). Protection of amino and carboxylate groups by trityl moieties would be unsuitable because on nitrosation, the electrophilic nitrosating agent removes the trityl group and the unprotected amine moiety is then deaminated[99]. Similar reservations apply to trialkyl silyl protection, because this group is also cleaved by mild acidic conditions. The advantage of using N-phthalyl-L-glutamic anhydride as a form of internal α-carboxylate protection[85, 86] was considered. This was not pursued experimentally however, because removal of the α-N-phthalyl group after nitrosation without concurrent removal of the N-nitroso group was considered unlikely.

It was therefore decided to protect the α-amino group of glutamic acid with the benzyloxycarbonyl function and the α-carboxyl group by its conversion to the benzyl ester. Both of these protecting groups are stable to nitrosation, and are usually removed by catalytic hydrogenation under mild neutral conditions. Further, protection of the α-amino moiety with the benzyloxycarbonyl function favours subsequent protection at the α-carboxyl group by benzyl bromide, when dicyclohexylamine is used as base. The γ-carboxyl group is then readily available for γ-peptide formation. α-N-Benzyloxycarbonyl-L-glutamic acid (8) was prepared by the reaction between L-glutamic acid and benzyl chloroformate in 2M NaOH at 0°C (see Scheme 21). After washing with ether and acidification to pH 1.5 with concentrated HCl, the resulting solution was extracted with ethyl acetate which on evaporation gave


CO2H
HO2C
NH2
CH2OCOCl
CO2H
HO2C
NHCO2BZ
CH2Br
CO2BZ
HO2C
NHCO2BZ
R-NH2
CO2BZ
HN
C
O
R
NHCO2BZ
(10)
Cl
NCO
CO2BZ
Cl
N
C
O
H
N
C
O
R
NHCO2BZ
(11)
N2O4
CO2BZ
Cl
N
C
O
N
C
O
R
O=N
NHCO2BZ
deprotection
CO2H
Cl
N
C
O
N
C
O
R
O=N
NH2

Scheme 21

a white solid of (8) in yields of up to 77%.

The α-carboxyl group was then protected by formation of α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (9). This was accomplished by reacting (8) with benzyl bromide at 80°C in DMF in the presence of dicyclohexylamine (see Scheme 21). Because of the insolubility of (8) in most solvents it was necessary to use DMF as the solvent, this resulted in subsequent problems in its removal. Very thorough washings with water were required to remove all traces of the DMF. Dicyclohexylamine was used to generate the carboxylate because it precipitates the dicyclohexylammonium salt of the α-ester immediately. Yields of (9) obtained using this procedure were in the order of 60-70%. This compound was used in subsequent steps without further purification.

2.1.2 Synthesis of α-protected glutamines (10)

α-N-Benzyloxycarbonyl-α-benzyl-γ-N-methyl, ethyl, 2-chloroethyl and cyclohexyl L-glutaminate, compounds (12), (13), (14) and (15), respectively, were all prepared using the standard method for condensing the mixed anhydride obtained from reaction between ethylchloroformate and (9) with the appropriate amine (see Scheme 21). The mixed anhydride of ethylchloroformate and (9) was synthesized in situ by the dropwise addition of ethylchloroformate to a solution of (9) in THF at -10°C (Et_3N was added to generate the carboxylate anion). It was essential to keep the reaction temperature below -10°C and to exclude moisture to avoid unproductive decomposition or hydrolysis of the mixed anhydride. Subsequent addition of the appropriate amine at this temperature gave the corresponding α-protected glutamine (10). Because of the instability of the mixed anhydride it was impossible to monitor the reaction by TLC and hence yields from this reaction were found to

vary from 40-60%.

2.1.3 Synthesis of γ-glutamyl ureas (11)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(N'(2-chloroethyl)-carbamoyl, N-methyl)-L-glutaminate (16) and α-N-benzyloxycarbonyl-α-benzyl-γ-(N-(2-chloroethyl)-carbamoyl, N-ethyl)-L-glutaminate (17) were prepared by the reaction of (12) and (13) respectively, with 2-chloroethyl isocyanate. The conditions for urea formation were critical. In particular, it was essential to dry all solvents and reaction vessels thoroughly to prevent polymerisation of 2-chloroethyl isocyanate to its dimers and trimers.

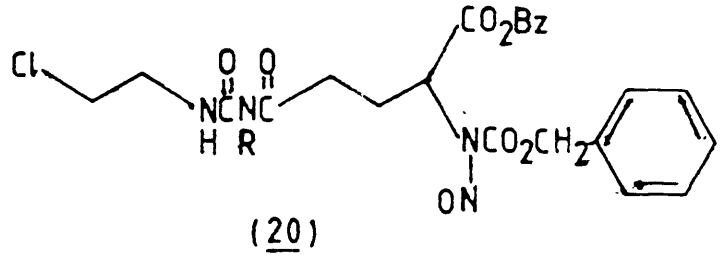
Further, reasonable yields of (16) and (17) (67% and 52%, respectively) could be obtained only by heating (12) and (13), respectively under reflux with a 10 fold excess of 2-chloroethyl isocyanate in sodium dried toluene for 48h.

Attempts to prepare the N-2-chloroethyl and N-cyclohexyl derivatives, (18) and (19), from (14) and (15), respectively by reaction with 2-chloroethyl isocyanate under the same conditions were unsuccessful and starting materials were recovered. A number of different conditions were investigated in an attempt to promote reaction. These included using a larger excess of 2-chloroethyl isocyanate, use of higher boiling point solvents (e.g. xylene) and longer reaction times, all without success. Under even more forcing conditions, such as heating under reflux in DMSO, and under pressure in a Carius tube, the reaction products consisted mainly of degraded starting materials and 2-chloroethyl isocyanate polymers. Similar observations were made by Wiley[100] in an extensive study of the reaction between a number of N-substituted amides and isocyanates. In this work, Wiley found that the use of stringent conditions to promote reaction gave a variety of

indeterminate products and concluded that steric hindrance reduced the reactivity of secondary amides towards isocyanates. Attempted synthesis of (18) and (19) by reaction of (14) and (15), respectively with 2-chloroethyl isocyanate in the presence of n-Buli was also unsuccessful. The reasoning here was that generation of the amide anion of (14) and (15) might overcome the steric hindrance. Similarly, the reaction between the appropriate N-alkyl-N-(2-chloroethyl) urea with the mixed anhydride of (9) and ethylchloroformate, in the presence and absence of n-Buli, were unsuccessful. All reactions conducted in the presence of n-Buli resulted in extensive trimerization of the 2-chloroethyl isocyanate and regeneration of the parent γ-glutamyl amine. This reaction probably proceeds via Scheme 22.

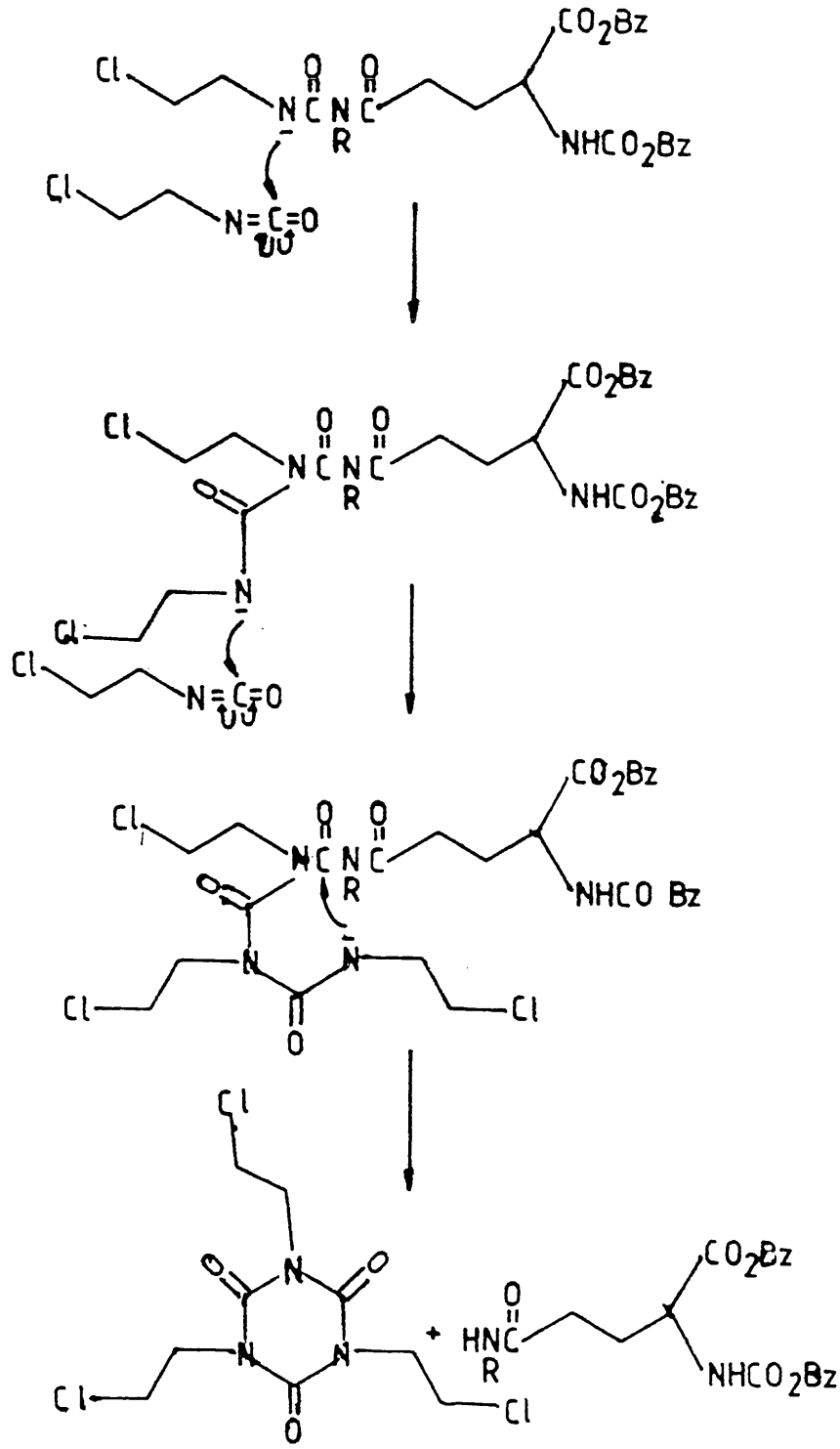
2.1.4 Nitrosation of (16) and (17)

The nitrosation of (16) and (17) was carried out aprotically at 0°C, using N_2O_4 in dry dichloromethane (CH_2Cl_2) containing sodium acetate to keep the solution non-acidic. With equimolar N_2O_4, spectral evidence suggested that nitrosation proceeded initially at the α-amino N-atom to give (20).



(20)

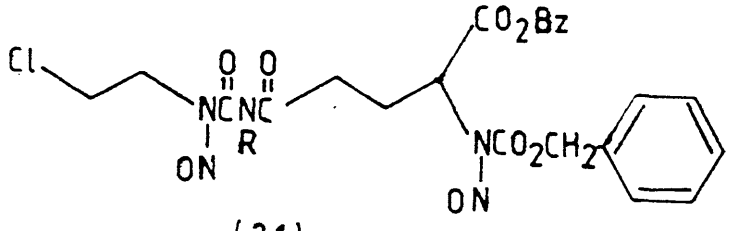
The product (20) gave a similar ^{1}Hnmr spectrum to that obtained from the nitrosation of α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (9). Both spectra showed ca. 0.3 ppm deshielding of the methylene H of the urethane benzyl ester and ca. 0.7 ppm



Scheme 22

deshielding of the methine Hα to the N-nitroso group.

With a 20 fold excess of N_2O_4 and leaving the reaction mixture at 0°C for 5h, the dinitrosated product (21) was obtained.



(21)

(22) R = Me
(23) R = Et

The spectral properties of (22) and (23) are consistent with the structures proposed. Thus their UV/vis spectra in ethanol show 3 bands between 380 and 420 nm, characteristic of N-nitroso compounds and related to n-π* transitions.

The infrared spectra of (22) and (23) in CCl_4 are shown in Figures 1a and 1b respectively. They show the urea C = 0 absorbance at ca. 1720 cm^{-1} compared to 1665 cm^{-1} (for the un-nitrosated precursor) plus N=0 absorbances at ca. 1520 cm^{-1}. The ^1Hnmr spectra of (22) and (23) were recorded in $CDCl_3$. The significant changes observed on nitrosation of (16) and (17) are shown in Table 1.

These results show a 0.3 ppm deshielding of the methylene H of the urethane benzylester and 0.7 ppm deshielding of the methine H α to the N-nitroso group as observed for compound (20). The spectra also show that the methylene protons of the 2-chloroethyl substituent are transformed from a singlet in the un-nitrosated precusor into 2 triplets separated by ca. 0.7 ppm with A_2B_2 symmetry. These observations agree with those for other N-(2-chloroethyl)-N-nitrosoureas as investigated by Johnston et

Figure 1. IR spectra of;

a) α-(N-benzyloxycarbonyl,N-nitroso)-α-benzyl-γ-(N-(2-chloroethyl)-N-nitrosocarbamoyl,N-methyl)-L-glutaminate (22), and b) α-(N-benzyloxycarbonyl,N-nitroso)-α-benzyl-γ-(N-(2-chloroethyl)-N-nitrosocarbamoyl, N-ethyl)-L-glutaminate (23) in CCl_4

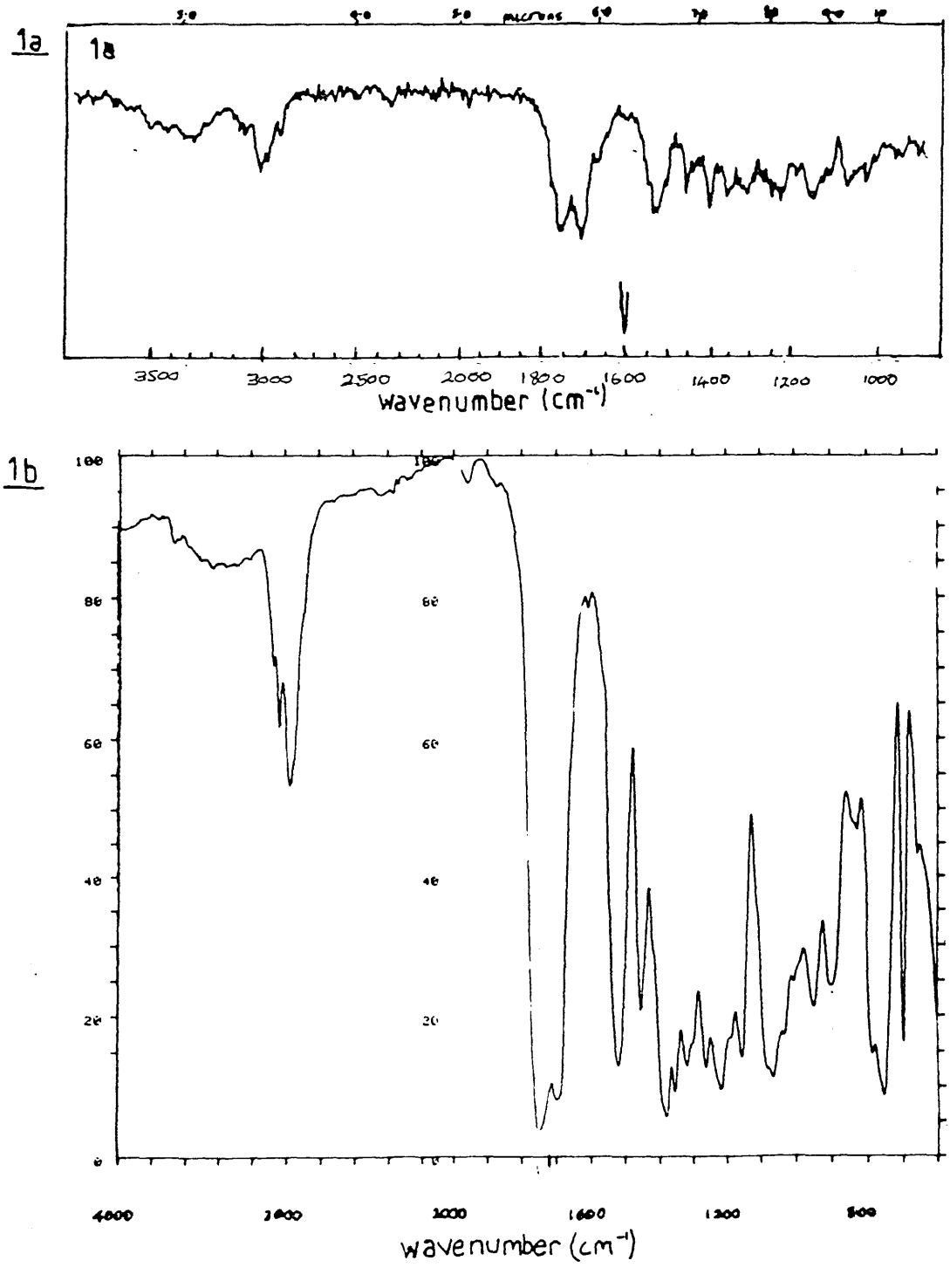
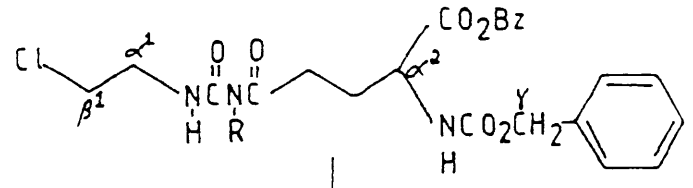
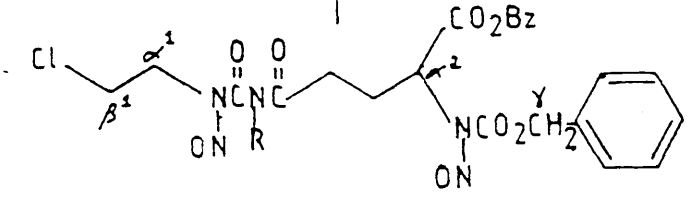


Table 1

Changes in chemical shifts of selected protons on nitrosation of (16) and (17).



R
Me = (16)
Et = (17)



R
Me = (22)
Et = (23)

SUBSTRATE	δHα¹ ppm	δHY ppm	δHα² ppm	δHß ppm
(16)	4.35	5.17	1.9-2.5 (multiplet)	
(22)	5.2	5.4	4.1	3.5
(17)	4.4	5.17	2.0-2.5 (multiplet)	
(23)	5.2	5.4	4.1	3.5

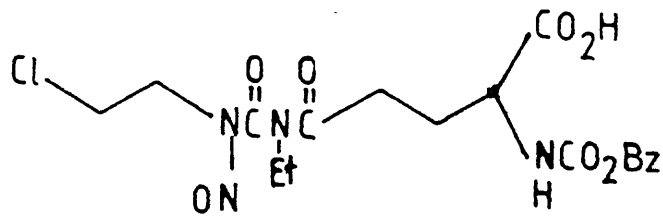
al[67]. The methylene protons α to the N-nitroso group were deshielded by ca. 0.2 ppm. Fast atom bombardment (FAB) mass spectrometry of (22) and (23) gave protonated molecular ions (MH^+) at 548 and 562, respectively.

The most important features of the FAB mass spectra are the presence of the MH^+ and the ions resulting from the loss of 2 N=O. Fragments corresponding to the loss of the benzyloxycarbonyl and benzylic protecting groups were also identifiable as shown for compound (22) in Scheme 23.

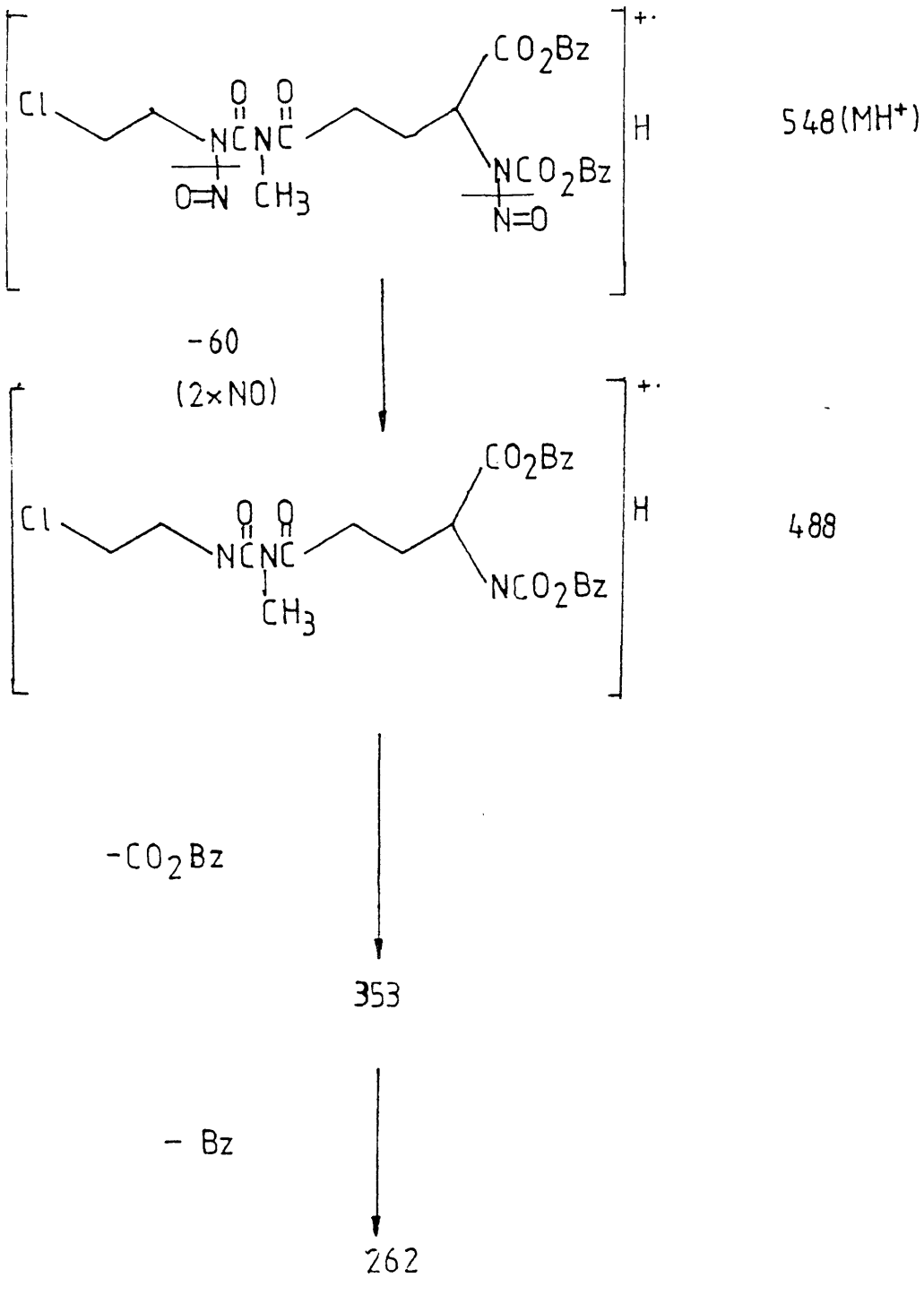
A satisfactory microanalysis could not be obtained for compounds (22) and (23). This reflects the poor stability of the N-nitrosoureas.

2.1.5 Deprotection

The final step of Scheme 21 requires removal of the the α-benzylester and benzyloxycarbonyl groups from (22) and (23). These were to be removed by low pressure hydrogenolysis in an inert solvent using a 10% palladium on charcoal catalyst. The choice of solvent for the hydrogenolysis became critical. In methanol solvent, substantial denitrosation was observed prior to removal of the α-amino and α-carboxylate protection as judged by the loss of the N = O absorbances at λ_{max} ca. 400 and 420 nm, and by ^{1}Hnmr spectroscopy. The hydrogenolysis of (23) in ethanol, however gave a product that was tentatively assigned as (24), if the process was



(24)

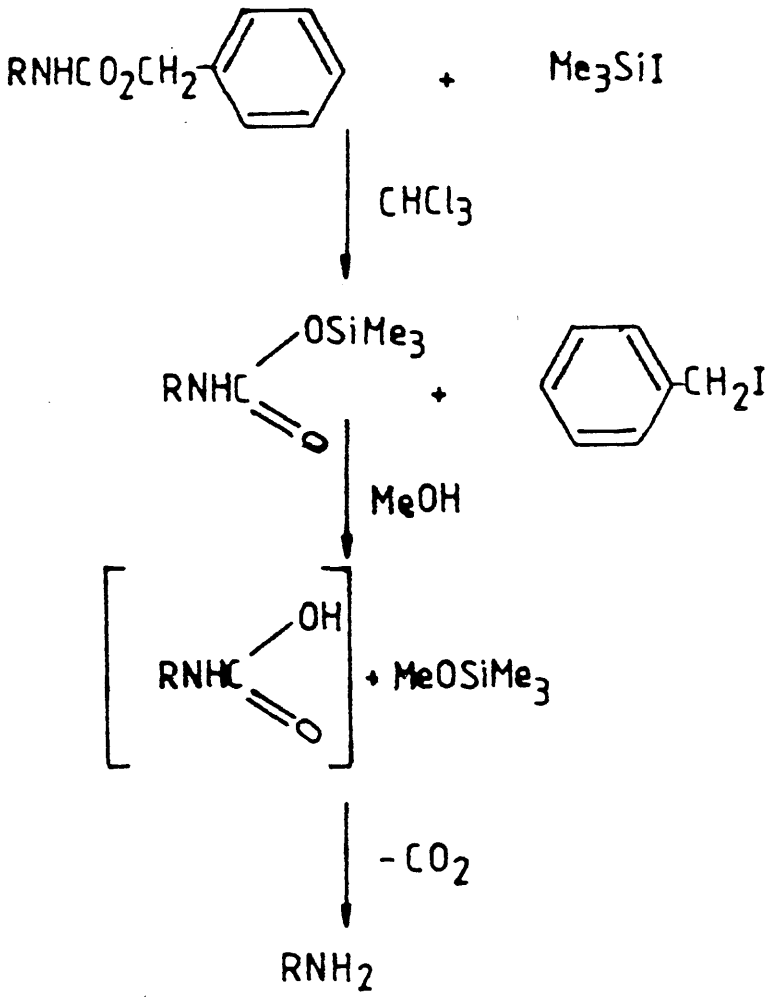


Scheme 23

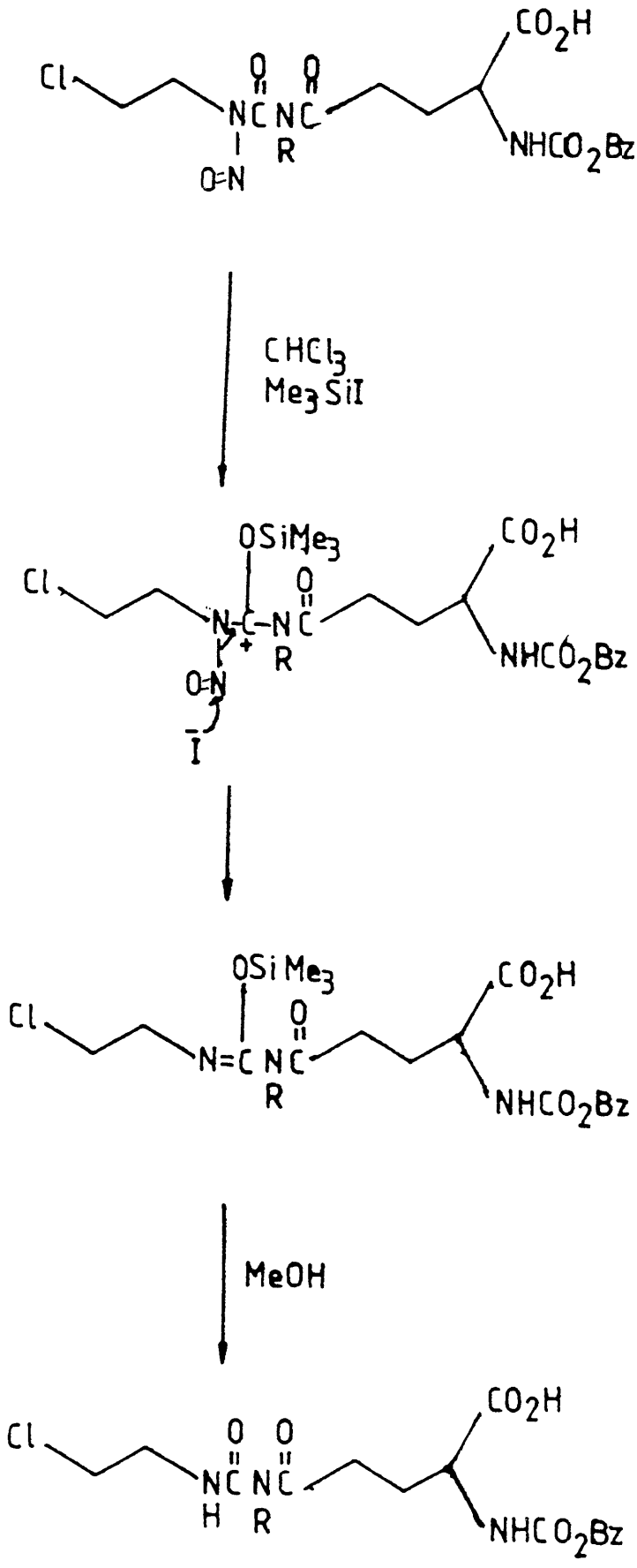
interrupted after the uptake of 0.5 equivalents of hydrogen.

The structure of (24) was determined spectroscopically. Thus the infrared spectra of (24) in CCl_4 showed C=O stretching bands corresponding to a free α-carboxylate group and also a C=O stretch at 1700 cm^{-1} corresponding to the urethane. The C=O stretch corresponding to the N-nitrosourea remained at 1726 cm^{-1} and was also apparent. The ^{1}Hnmr spectra of (24) in $CDCl_3$, showed the loss of a benzylic resonance whilst retaining the triplet A_2B_2 symmetry due to the nitrosation of the N-atom adjacent to the 2-chloroethyl urea moiety. More extensive hydrogenolysis of (24) in ethanol removed all the N-nitroso groups prior to removal of the N-benzyloxycarbonyl group (as judged by UV and ^{1}Hnmr). Using ethyl acetate as solvent, both in the presence and absence of a phase transfer catalyst, resulted in very slow hydrogenolysis and removal of the N-nitroso group preceded removal of the α-N-benzyloxycarbonyl group. Removal of the α-N-benzyloxycarbonyl protection from (24) using other methods (e.g. trimethylsilyliodide, TMSI) were equally unsuccessful, despite suggestions in the literature that cleavage of benzyloxycarbonyl groups from amines by TMSI could be accomplished in 6 min[101]. It has been suggested that treatment of a N-benzyloxycarbonyl compound in chloroform with TMSI gives benzyl iodide and the trimethylsilyl carbonate, the latter can then be converted to the amine by addition of methanol (Scheme 24). Deprotection of (24) using TMSI also gave extensive denitrosation presumably via the pathway of Scheme 25.

Attempts to remove the α-protection from (22) were totally unsuccessful, even removal the α-benzyl group as for (23), resulted in the loss of N-nitroso functions prior to deprotection (as judged by UV and ^{1}Hnmr).


RNHCO2CH2
+
Me3SiI
CHCl3
OSiMe3
RNHC
O
+
CH2I
MeOH
OH
RNHC
O
+ MeOSiMe3
-CO2
RNH2

Scheme 24



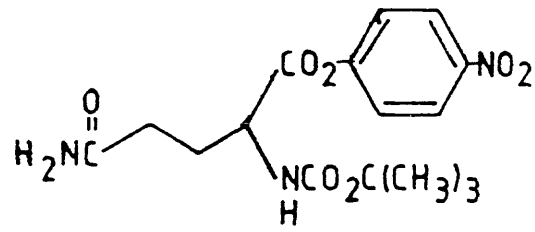
Scheme 25

2.1.6 Alternative protection

Because of the failure to remove both protecting groups from (22) and (23), the use of alternative protection was briefly investigated. Work by Suli et al[102] has shown that the N-t-butyloxycarbonyl group is removed from N-(2-chloroethyl) -N-nitrosocarbamoyl derivatives of polypeptides, by mild acid hydrolysis without substantial loss of the N-nitroso function. α-N-t-butyloxycarbonyl-α-(4-nitrophenyl)-L-glutaminate (25) was obtained commercially and used to investigate the advantages of t-butyloxycarbonyl-N-protection in the synthesis of the N-nitrosourea adducts. The 4-nitrophenyl ester function can also be removed by aqueous hydrolysis. α-(N-t-Butyloxycarbonyl, N-nitroso)-α-(4-nitrophenyl)-γ-(N-(2-chloroethyl),N-nitroso-carbamoyl)-L-glutaminate (27) was synthesized from (25) in a similar way to that described for (22) and (23), as shown in Scheme 26. Thus (25) was heated under reflux in sodium dried toluene with a 10 fold excess of 2-chloroethyl isocyanate. Reasonable yields of (26), (ca. 40%), were obtained over 24h. The relatively shorter reaction time required, probably reflects reaction at the less sterically hindered primary amide. Due to the insolubility of (26) in CH_2Cl_2, aprotic nitrosation by N_2O_4 was carried out in a mixed solvent consisting of THF and CH_2Cl_2 (1:1, v/v). This allowed shorter reaction times than with THF alone. As for compound (20), nitrosation proceeded first at the urethane N-atom. By using 4 molar equivalents of N_2O_4 the di-nitrosated compound (27) was obtained as a yellow oil in yields of 72%. Acidic hydrolysis of (27) with 0.12 M HCl in 95% formic acid at 0°C for 10 min, followed by lyophilisation of the solvent gave (28) as a pale yellow gum in yields of 79%.

The proposed structure for (28) was confirmed spectroscopically.

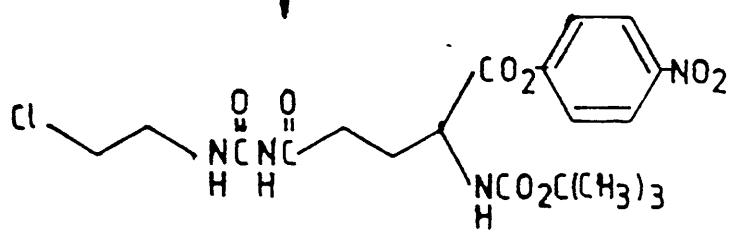
(25)



Cl—N=C=O (×10) /

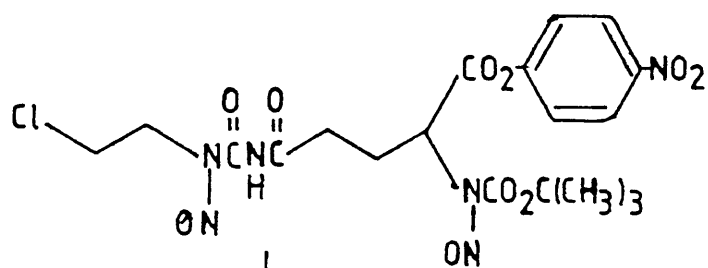
Na dry toluene
reflux 24h

(26)



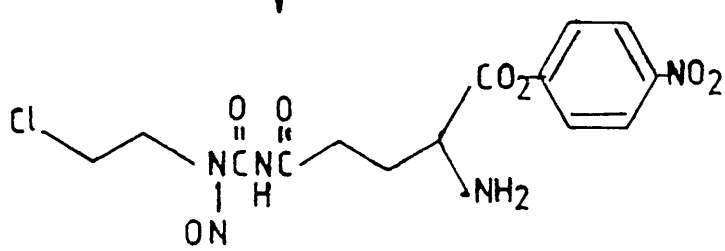
N_2O_4 (4eqs) / NaOAc /
THF: CH_2Cl_2 (1:1)
0°C

(27)



0.12M HCl / 95% HCOOH
10min / 0°C

(28)



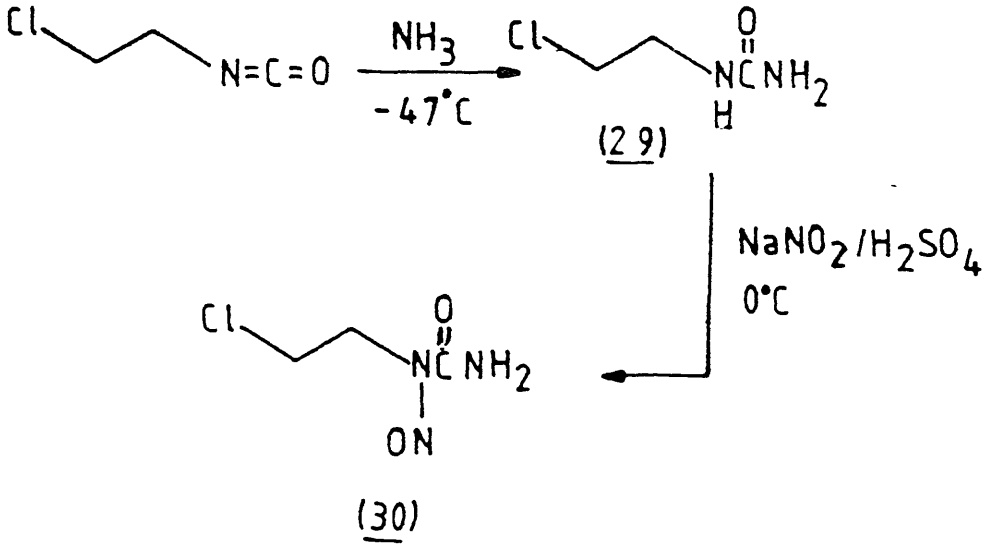
Scheme 26

The UV/vis spectra showed characteristic N-nitroso absorbances between 380 and 420 nm. The infrared spectra of (28) in CCl_4 showed a strong band at 1710 cm^{-1} assigned to the C=O stretch of the N-nitrosourea but unfortunately it was not possible to distinguish between the absorbances at ca. 1488-1520 cm^{-1} assigned to the aromatic C-NO_2 and N-N=O groups. The ^{1}Hnmr spectra of (28) in D_2O clearly showed the absence of the t-butyl resonance at δ1.6 ppm (evident in (27)) whilst retaining the A_2B_2 triplet bands centred at ca. 3.8 ppm and characteristic of the 2-(chloroethyl)-N-nitroso terminus. An accurate mass measurement of compound (28) by FAB (positive ion) spectroscopy gave an MH^+ of 402.0824 amu which compares favourably with the calculated value of 402.0817 amu.

Attempted removal of the α-nitrophenyl ester moiety from (28) by hydrolysis in water over 24h was disappointing. It resulted in extensive removal of the N-nitroso group and concurrent hydrolysis of the peptide bond (see Chapter 3, Section 3.4.1). Conditions effecting removal of the 4-nitrophenyl ester group alone could not be obtained.

2.2 Synthesis of N-(2-chloroethyl)-N-nitrosourea (30)

Compound (30) was synthesized as the parent compound to (28). It was prepared as outlined in Scheme 27.



Scheme 27

The alkylurea (29) was synthesized by bubbling ammonia gas through a solution of 2-chloroethyl isocyanate in ether at ca. -47°C. On allowing the solution to warm to room temperature a white precipitate of (29) was formed which was collected on a filter and recrystallized from ethanol in yields of 80%. Nitrosation of (29) was by $NaNO_2$ in concentrated H_2SO_4 at 0°C, according to the method of Hyde et al[103]. The product (30) was collected on a filter and after washing with water was obtained in 30% yield. The product (30) was characterized by its spectral data and by melting point.

2.3 Synthesis of model γ-L-glutamyl donor substrates

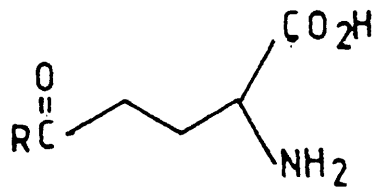
In order to examine the substrate specificity of the GGT donor site a series of γ-L-glutamyl derivatives bearing various substituents were synthesized. The structures of these compounds are summarized in Table 2. With the exception of (39), all were synthesized by reacting the appropriate amine with the mixed anhydride of α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (9) and ethyl chloroformate. The synthesis of this mixed anhydride is described in Section 2.1.2. After isolation and purification, usually by column chromatography, the protection was removed from the α-amino and α-carboxylate groups by low pressure hydrogenolysis in methanol over a 10% palladium on charcoal catalyst (Scheme 28).

The deprotected γ-L-glutamyl donors were invariably recrystallized from aqueous ethanol. Overall yields were in the range of 50-70%. The above method proved unsuccessful for the synthesis of (39), primarily due to the inactivity of N-methyl-4-nitroaniline towards the mixed anhydride intermediate. This inactivity relates to electron withdrawal by the 4-nitro group which reduces the nucleophilic reactivity of the amine moiety. The low temperature required for the mixed anhydride coupling is not conducive to reaction with relatively unreactive amines. The procedure of King and Kidd[85] was therefore adopted for the synthesis of (39). This involved condensation of α-N-phthalyl-L-glutamic anhydride (37) with N-methyl-4-nitroaniline, followed by removal of the α-N-phthalyl protecting group with hydrazine hydrate (Scheme 29). This method allows for the use of elevated temperatures and longer reaction times because the α-N-phthalyl-L-glutamic anhydride is relatively unreactive and resistant to adventitious hydrolysis.

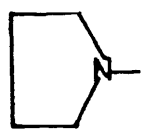
α-N-Phthalyl-L-glutamic anhydride (37) was prepared by a literature procedure[85]. Opening of the anhydride by N-methyl-4-nitroaniline

Table 2

Structures of synthesized model γ-glutamyl donor substrates



R

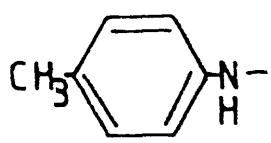
(31) 

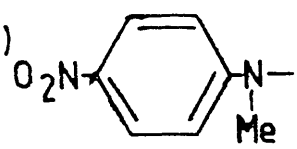
(32) EtN(H)–

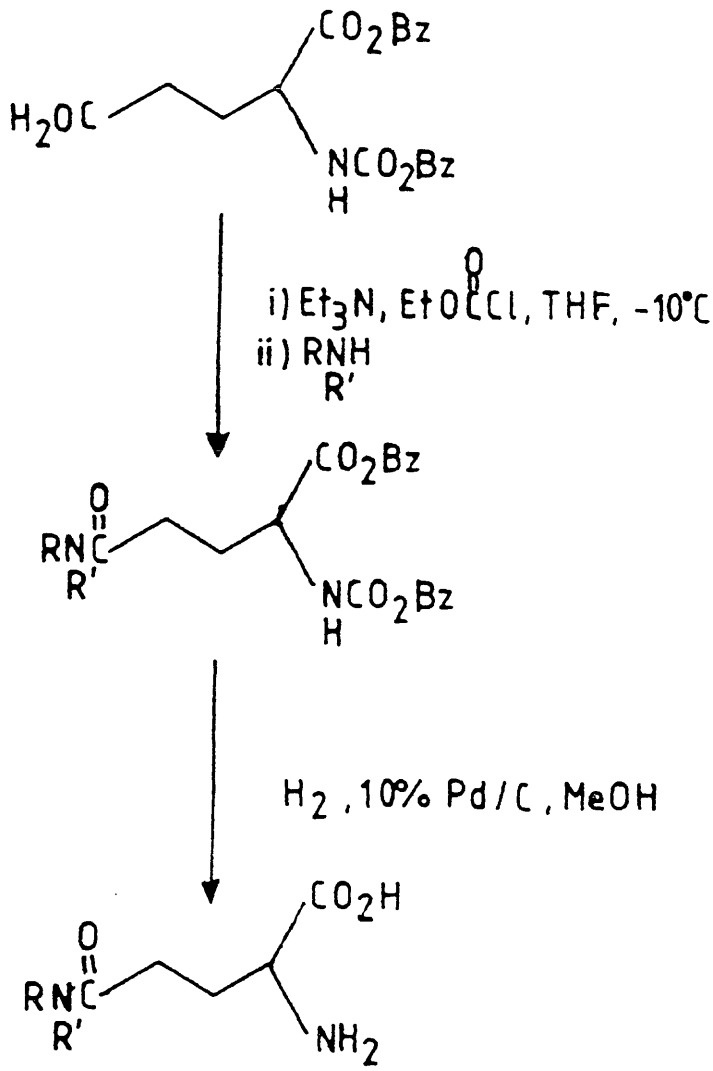
(33) Et_2N–

(34) Me_2N–

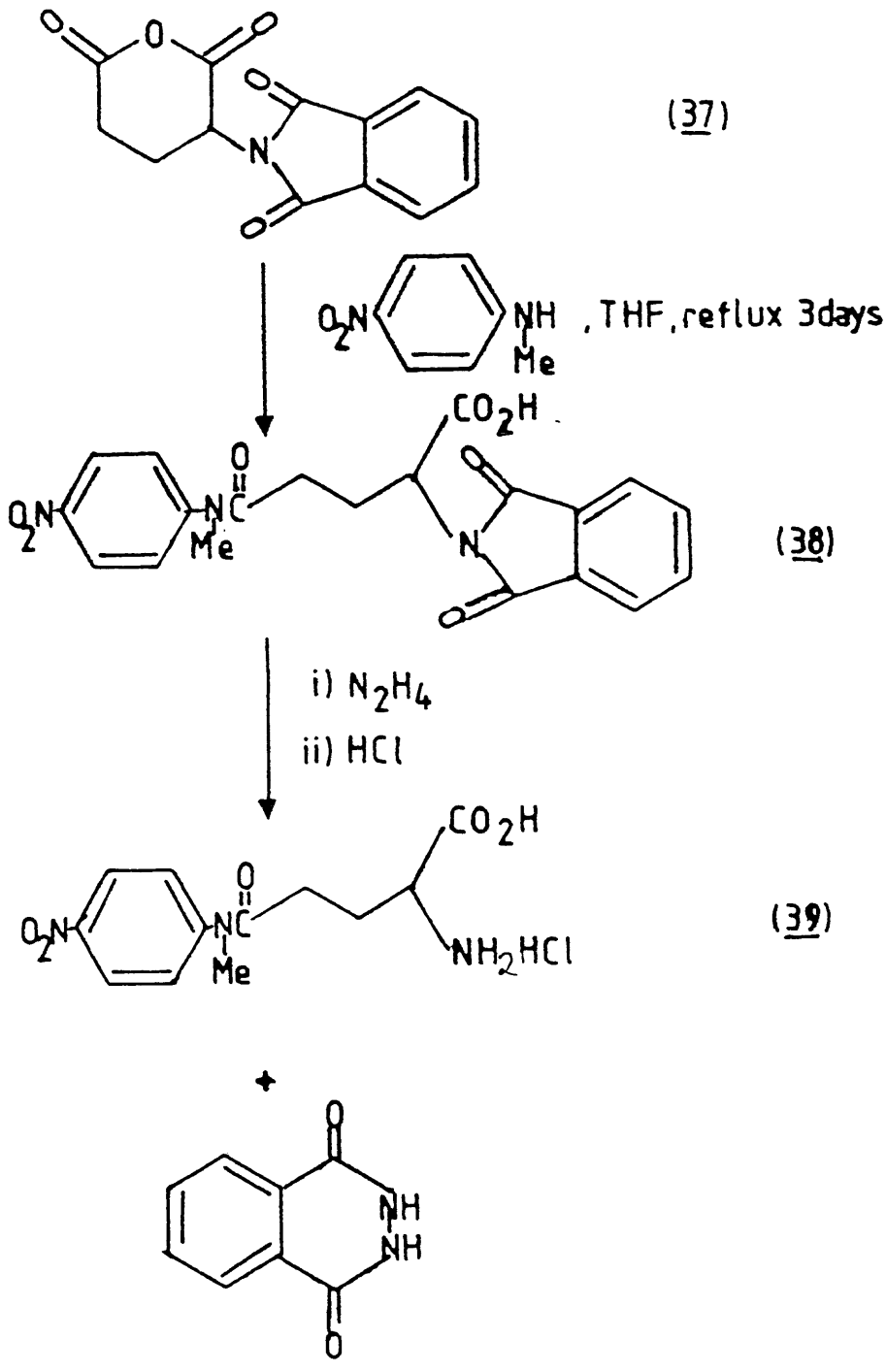
(35) tBuN(H)–

(36) 

(39) 


CO2Bz
H2OC
NCO2Bz
H
i) Et3N, EtOCCl, THF, -10°C
ii) RNH
R'
CO2Bz
RNC
R'
NCO2Bz
H
H2 , 10% Pd/C , MeOH
CO2H
RNC
R'
NH2

Scheme 28



Scheme 29

to give (38), was achieved by heating the two components under reflux in THF. This reaction is unusual, insofar as opening of the α-N-phthalyl-L-glutamic anhydride proceeds regiospecifically at the γ-carbonyl atom. This probably relates to steric hindrance of the α-carbonyl atom by the N-phthalyl substituent. Compound (38) was purified by column chromatography using ethyl acetate to elute less polar co-products followed by methanol to elute (38). Further purification was effected by recrystallization from ethyl acetate and the overall yield was ca. 36%. Removal of the α-N-phthalyl group with hydrazine, following the method of King and Kidd[85] gave very low yields of (39). This was attributed to extensive concurrent cleavage of the γ-peptide bond. A milder procedure was therefore employed using hydrazine hydrate in aqueous methanol in the presence of Et_3N at room temperature for 48 h. Phthalyl hydrazide was removed by filtration after treatment of the reaction solution with HCl. Concentration of the filtrate under reduced pressure followed by the addition of absolute ethanol resulted in the precipitation of (39) .Yields from this milder deprotection step were still low and (39) was obtained in the region of 35% only.

All of the γ-L-glutamyl donor substrates gave spectral properties consistent with the structures proposed. Their purity was confirmed by microanalysis.

2.4 Synthesis of γ-L-glutamyl hydroxyaniline adducts

The strategy adopted for synthesis of these compounds drew heavily on the methods described above. The protected mixed anhydride was readily available and it is clear that it reacts with a variety of amines to give γ-L-glutamyl adducts. The only additional complication anticipated on reaction with hydroxyanilines is

concurrent reaction at both the amino and hydroxy substituents. This can be easily circumvented by appropriate masking of the hydroxy substituent. The γ-L-glutamyl hydroxyaniline adducts synthesized are shown in Table 3, they were prepared as shown in Scheme 30.

These compounds differ only in the position of hydroxy and chloro substituents.

Compounds (43), (44) and (45) were prepared by coupling the appropriate benzyloxyaniline with the mixed anhydride of (9) and ethylchloroformate, followed by the removal of the benzylic protecting groups from the aromatic hydroxy substituent, the α-amino and α-carboxylate group.

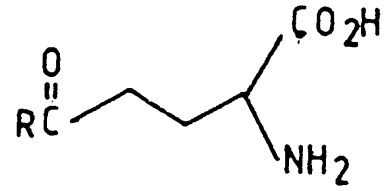
As mentioned above the hydroxy function of the substituted aniline reagent may react concurrently with the mixed anhydride. This function was therefore protected prior to coupling with the mixed anhydride by conversion to the benzyl ether. The benzyl ether protecting group was chosen because it could be cleaved simultaneously with the usual deprotection of the α-glutamyl groups by hydrogenolysis.

The benzyl ethers of the corresponding hydroxyanilines could not be obtained directly by benzylation, because both the hydroxy and amino functions react.

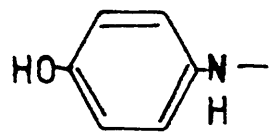
Selective O-benzylation was effected as shown in Scheme 31, by first acylating the amino function to give the corresponding 4-hydroxy, 2-hydroxy-5-chloro and 3-chloro-4-hydroxy acetanilides[104], (46), (47) and (48) respectively, and then by reacting these with benzyl bromide to give, 4-benzyloxy, 2-benzyloxy-5-chloro and 3-chloro-4-benzyloxyacetanilides, (49), (50) and (51), respectively. The N-acyl group was then removed by alkaline hydroylsis to which the benzyl ether is stable to give the

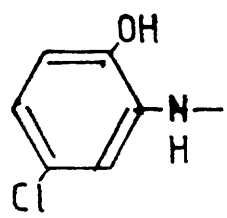
Table 3

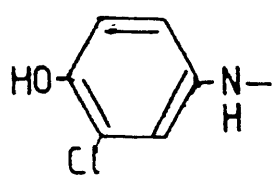
Name and structure of synthesized γ-glutamyl hydroxyaniline derivatives

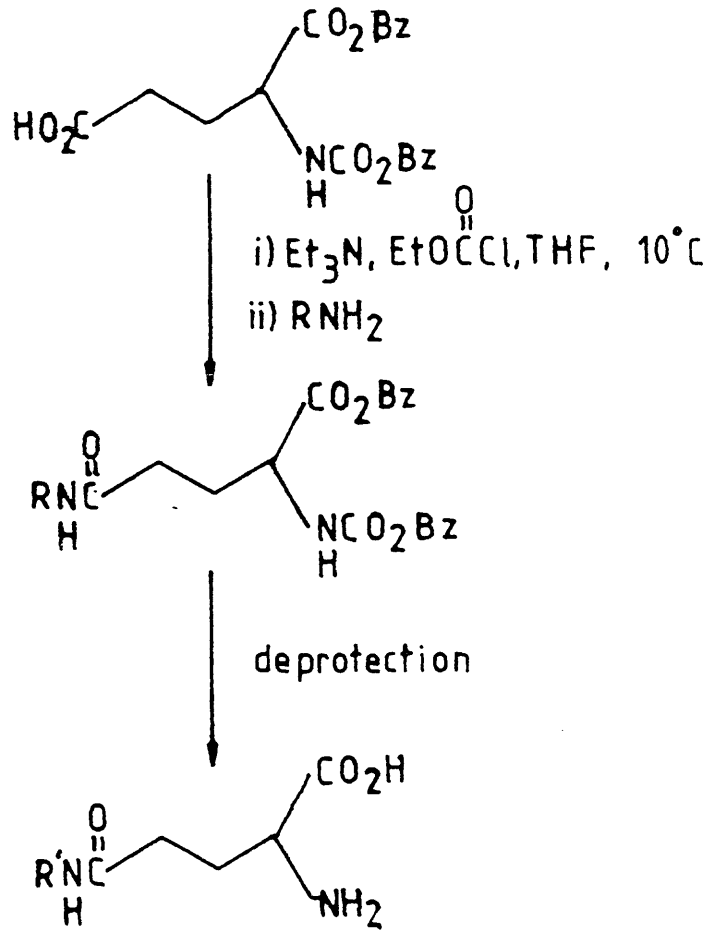


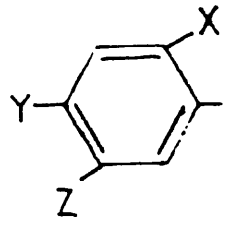
R

(43)  γ-L-glutamyl-4-hydroxyaniline

(44)  γ-L-glutamyl-2-hydroxy-5-chloroaniline

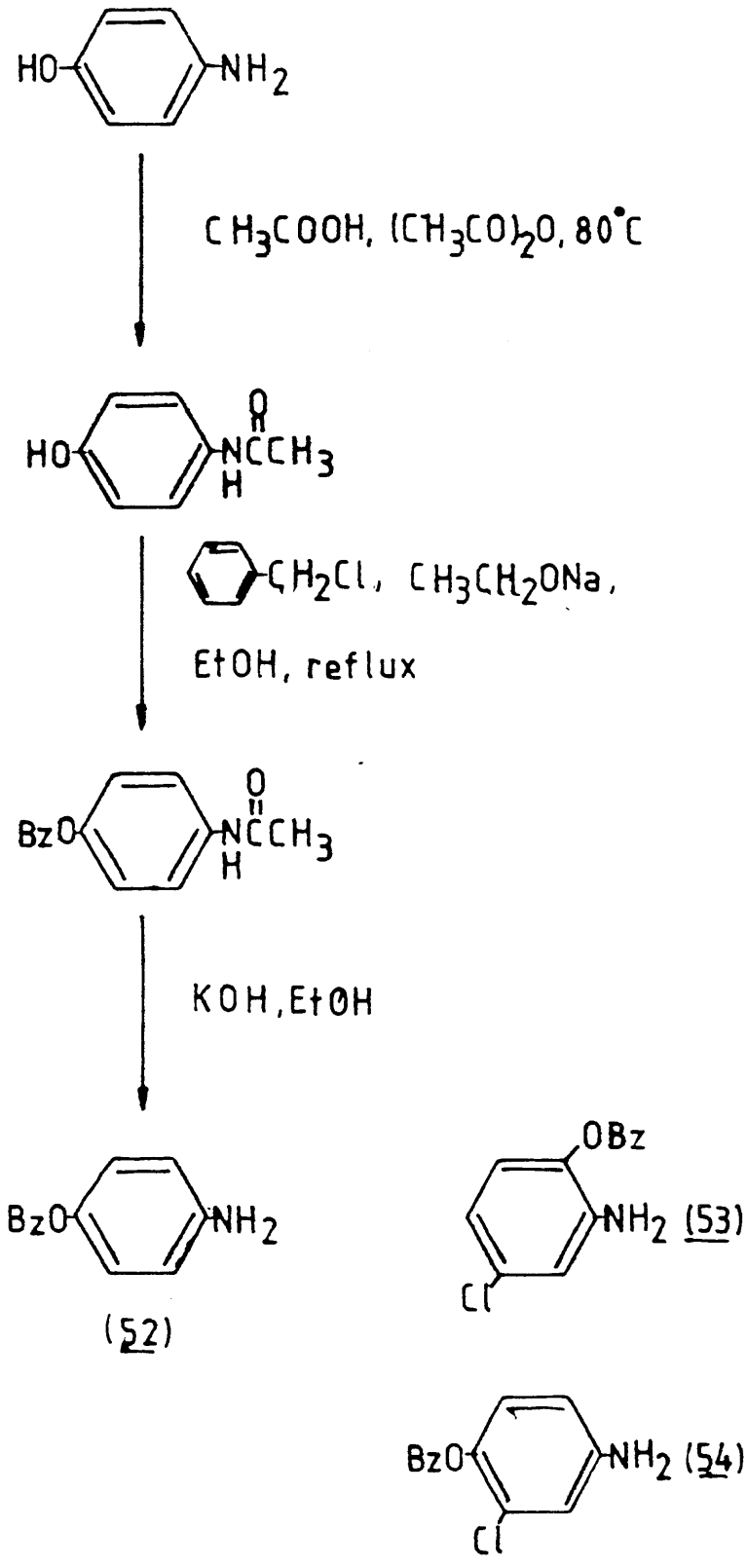
(45)  γ-L-glutamyl-3-chloro-4-hydroxyaniline





	X	Y	Z	
R	H	OBz	H	(40)
	OBz	H	Cl	(41)
	H	OBz	Cl	(42)
R'	H	OH	H	(43)
	OH	H	Cl	(44)
	H	OH	Cl	(45)

Scheme 30


HO
NH2
CH3COOH, (CH3CO)2O, 80°C
HO
O
NCCH3
H
CH2Cl, CH3CH2ONa,
EtOH, reflux
BzO
O
NCCH3
H
KOH, EtOH
BzO
NH2
(52)
OBz
NH2 (53)
Cl
BzO
NH2 (54)
Cl

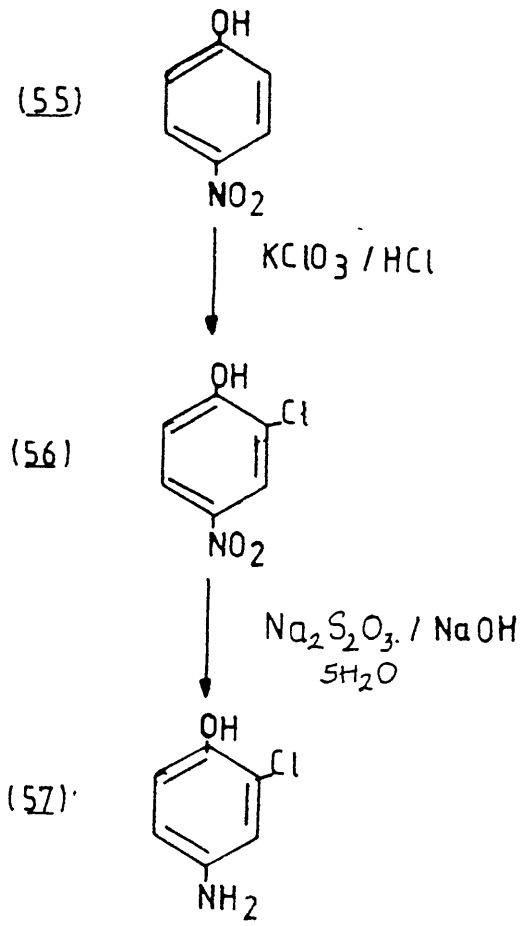
Scheme 31

corresponding benzyloxyanilines (52), (53) and (54) which were recrystallized from hexane in yields of 89, 54 and 60%, respectively. Synthesis of (54), however required additional pretreatment since the parent compound 3-chloro-4-hydroxyaniline (57) was not readily available. Compound (57) was prepared as shown in Scheme 32.

The reaction of (55) with $KClO_3$ in concentrated HCl gave (56) as a yellow paste which was purified by column chromatography with ethyl acetate as eluent in 67% yields. Reduction of (56) by sodium hyposulphite in hot aqueous sodium hydroxide gave (57) which was collected on a filter and recrystallized from water in 74% yield. The parent compound (57) was characterized by its spectral data and also by microanalysis. Synthesis of (54) from (67) was then as outlined in Scheme 31.

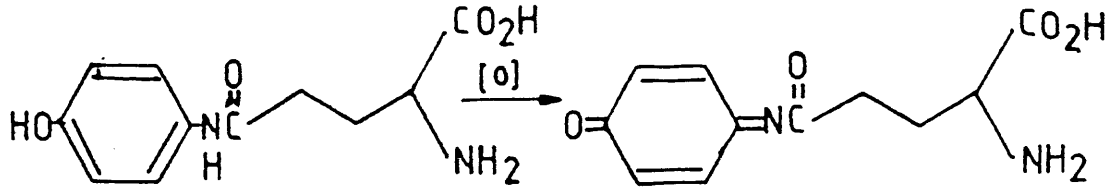
The reaction of the benzyloxyaniline derivatives with the mixed anhydride of (9) and ethylchloroformate (see Scheme 30) was carried out as described previously and gave the corresponding γ-L-glutamyl benzyloxyanilines in yields of 40-60%. Deprotection of (40) and (41) was effected by low pressure hydrogenolysis over a 10% palladium on charcoal catalyst. Because of the poor solubility of the protected compounds in pure alcohol or ethyl acetate, the hydrogenolysis was carried out at 60°C in a mixture of methanol, acetic acid and water (15:5:1 v/v). Both (43) and (44) were obtained as buff coloured powders which darkened on recrystallization from water. This was attributed to adventitious oxidation to their corresponding quinone-imines (Scheme 33).

This problem was overcome by carrying out the recrystallization in deoxygenated water under an argon atmosphere. The yields of (43) and (44) were 42 and 57% respectively. The spectral properties of

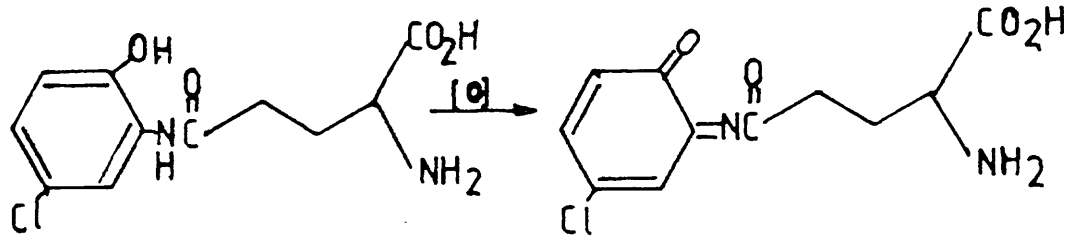


Scheme 32

(43) and (44) were consistent with the proposed structures. Their purity was confirmed by microanalysis.



(43)



(44)

Scheme 33

Hydrogenolysis of (42) under similar conditions in the mixed solvent, ethanol or methanol resulted in the loss of the aromatic chlorine substituent as well as removal of the benzyl protecting groups. This problem was partly overcome by using ethyl acetate as solvent. The hydrogenolysis was slow, however, and after 3 days very small yields of (45) were recovered. This procedure was unpredictable and substantial removal of the benzyl groups was always accompanied by some loss of the aromatic chlorine substituent. Alternative procedures were investigated to remove the benzyl groups by hydrolysis and eventually deprotection was accomplished by reaction with 30% hydrogen bromide (HBr) in acetic acid for 24h at room temperature. The required product (45) was

therefore obtained as the hygroscopic HBr salt. This was purified and neutralised by ion exchange chromatography using Amberlyst 15H resin (in the H^+ form). The HBr was first eluted from the column using water and (45) was eluted with aqueous 0.5 M ammonia solution. Removal of both the water and ammonia by freeze drying gave (45) in 63% yield.

2.5 Summary

Attempts to synthesize γ-L-glutamyl adducts of N^3-sustituted N-(2-chloroethyl)-N-nitrosoureas were wholly unsuccessful.

The protected di-nitrosated precusors, α-(N-benzyloxycarbonyl-N-nitroso)-α-benzyl-γ-(N-(2-chloroethyl)-N-nitrosocarbamoyl), N-methyl and N-ethyl L-glutaminates, (22) and (23), respectively, however were readily prepared. Their synthesis involved the α-protection of L-glutamic acid as α-benzyloxycarbonyl-α-benzyl-L-glutamate (9), condensation of the protected species with the appropriate amine (by a mixed anhydride reaction), reaction of the resulting N-substituted glutamine with 2-chloroethyl isocyanate to give the corresponding urea and finally aprotic nitrosation with N_2O_4.

Attempts to remove both the α-benzyl and α-benzyloxycarbonyl protection from (22) and (23) by hydrogenolysis without simultaneous loss of the N-nitroso group were disappointing.

Interruption of the hydrogenolysis process after a 0.5 equivalent uptake of hydrogen gave a compound that was tentatively assigned as α-N-benzyloxycarbonyl-N-(γ-N-ethyl-L-glutamyl)-N-(2-chloroethyl)-N-nitrosourea (24). Removal of the α-N-benzyloxycarbonyl protection from (24) using TMSI failed.

Preparation of un-nitrosated precusors incorporating slightly larger N^3-substituents such as 2-chloroethyl and cyclohexyl (compounds (18) and (19)) using a similar route to that discussed above proved to be impossible. The reluctance of 2-chloroethyl isocyanate to react with α-N-benzyloxycarbonyl-α-benzyl-γ-N-(2-chloroethyl) and cyclohexyl, L-glutaminates (compounds (14) and (15), respectively) has been attributed to some form of steric hindrance. Other endeavours to condense 2-chloroethyl isocyanate with (14) and (15) including refluxing in high boiling point

solvents , reaction in a Carius tube and the introduction of n-BuLi were equally unrewarding.

The use of alternative glutamic acid protection was briefly investigated using α-N-t-butyloxycarbonyl-α-(4-nitrophenyl)-L-glutaminate as a model. Synthesis of the di-nitrosated compound α-(N-t-butyloxycarbonyl,N-nitroso)-α-(4-nitrophenyl)-γ-(N-(2-chloro ethyl)- N-nitrosocarbamoyl)-L-glutaminate (27), was similar to that of compounds (22) and (23). Deprotection by acidic hydrolysis gave α-(4-nitrophenyl)-γ-(N-(2-chloroethyl)-N-nitrosocarbamoyl)-L-glutaminate (28). The 4-nitrophenyl group could not be removed without substantial loss of the N-nitroso moiety.

Model γ-glutamyl derivatives (except compound (39)) were prepared by reaction of the appropriate amine with the mixed anhydride of (9) and ethyl chloroformate, followed by deprotection by hydrogenolysis. Compound (39) was synthesized by condensing N-phthalyl-L-glutamic anhydride with N-methyl-4-nitroaniline and then deprotecting with hydrazine hydrate.

γ-Glutamyl hydroxyaniline prodrugs (compounds (43), (44) and (45)) were synthesized by reacting the corresponding benzyloxyaniline with the mixed anhydride of (9) followed by deprotection. Deprotection to give (43) and (44) was by hydrogenolysis , and (45) by acidic hydrolysis.

CHAPTER 3

ENZYMOLOGY

3.1 Introduction

The chapter is divided into three sections. The first deals with the purification of γ-glutamyl transferase (GGT), the second reports the specificity and hydrolysis kinetics of model substrates for GGT, and the third reports the kinetics of the hydrolysis of several synthetic γ-glutamyl prodrugs by GGT.

3.2 Purification of GGT

The enzyme GGT was obtained from rat kidneys and purified by the method of Cook and Peters[19]. Rat kidney membranes were treated with papain at 37°C for 2 h. The solubilized GGT was collected as a 50-100% ammonium sulphate fraction and purified by chromatography on PBA-60 phenyl boronate agarose followed by gel filtration on Sephacryl S-200. The enzyme was concentrated by ultrafiltration. Table 4 shows the results of a typical purification.

The use of phenyl boronate affinity chromatography gives highly purified GGT. Phenyl boronate interacts with cis diols of glycoproteins, and facilitates fractionation of the glycoproteins including GGT from the rat kidney extract.The GGT was eluted from the phenyl boronate column with 10 mM Tris/HCl-100 mM NH_4HCO_3 buffer, pH 8.7. The data in Table 4 show that there is substantial loss (ca. 40%) of GGT during the phenyl boronate purification step. This loss of activity may be associated with the long chromatography time. Before elution with 10 mM Tris/HCl-100 mM NH_4HCO_3 buffer the GGT on the column was washed with ca. four column volumes of 100 mM NH_4HCO_3 buffer, pH 8.7 alone, the whole procedure taking about three days. An attempt to shorten the time from binding to elution (by omitting the washing stage and eluting the GGT directly), gave a fairly impure final product as judged by SDS polyacrylamide gel electrophoresis (PAGE).

Plate 1 shows the SDS-PAGE of the purified GGT. Samples were pretreated with 2 cm^3 of mercaptoethanol for 15-30 min and then 25 µl of a solution containing 0.4% Bromophenyl Blue, 2% SDS and 15% glycerol. The mixture was boiled for 2 min at 100°C and loaded on the gel. Electrophoresis was carried out by the method of Neville[105], with a 5% acrylamide stacking gel and an 8% acrylamide separation gel containing 0.1% SDS. Two Coomassie blue-staining bands of Mr = 56 and 28 kD were observed corresponding to the heavy and light subunits of GGT. These Mr values are slightly higher than those cited in the literature[106] where values of Mr = 46 to 50 and 22 to 24 kD, respectively, are usually reported. The difference may relate to the diffuse nature of the glycoprotein bands observed.

Table 4

Purification of γ-Glutamyltransferase from Rat Kidney

Results of typical purification from 94g kidneys

Stage	Volume (cm^3)	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg)	Purification fold	Yield %
Homogenate	210	3630	9345	0.4	1	100
Membranes	160	2920	2640	1.0	3	80
Extract	150	1940	178	11.0	28	53
50 - 100% $(NH_4)_2SO_4$	9	1700	96	18	45	47
PBA-60 + Ultrafiltration	2.5	640	20	33	84	18
G200+ Ultrafiltration	3	3.5	3.0	124	32	10

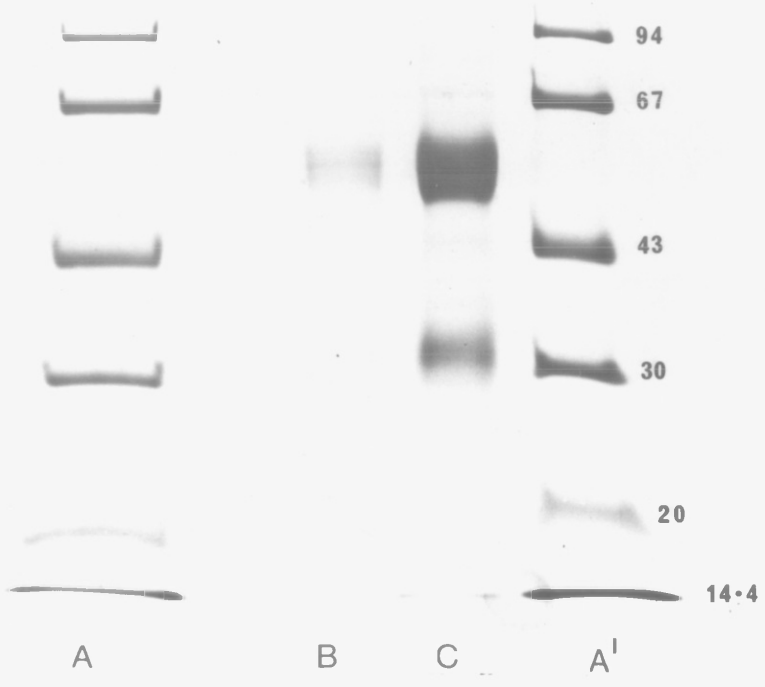


Plate 1. SDS-PAGE of purified GGT.

(A) 3 μg each of molecular mass markers: phosphorylase b, 94kD; bovine serum albumin, 67kD; ovalbumin, 43kD; carbonic anhydrase, 30kD; soybean trypsin inhibitor, 20kD; and γ-lactoglobulin, 14.4kD.

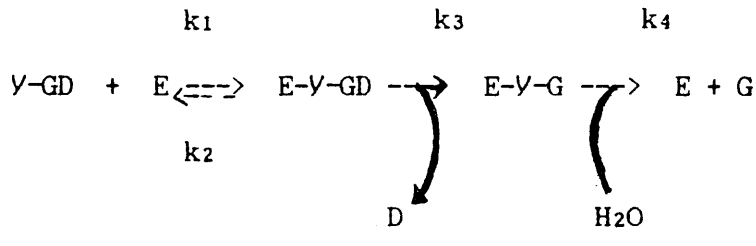
(A') 4 μg each of molecular mass markers (as described above)

(B) 25 μg purified GGT.

(C) 50 μg purified GGT.

3.3 The hydrolysis kinetics and donor specificity of GGT

GGT-catalysed hydrolysis is thought to involve the following steps.Binding of the γ-glutamyl donor (γ-GD) to the enzyme (E), cleavage of the γ-glutamyl donor to form a covalent γ-glutamyl-enzyme intermediate (E-γ-G) followed by breakdown of this intermediate to free enzyme and glutamic acid (G) (Scheme 33). It therefore represents the first half of a ping-pong transpeptidation reaction. The kinetics of the reaction can be measured by the rate of formation of D or the rate of loss of γ-GD.



Scheme 33

In the present study the kinetics of hydrolysis were investigated for several γ-glutamyl analogues and compared with those for the physiological GGT donors, glutathione and glutamine. These data gave information about the donor specificity for GGT.

3.3.1. Structural effects of the γ-glutamyl substituent on donor specificity

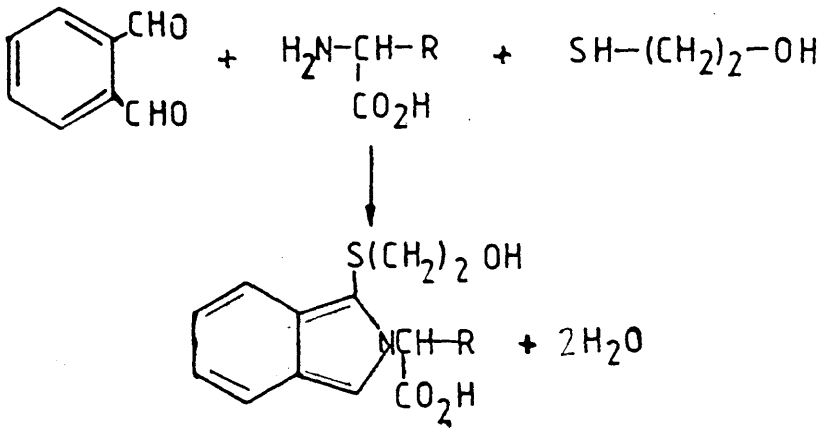
GGT donors previously tested contained either primary or secondary amino groups at the γ-carbonyl bond. Since several of the prodrugs examined in the present study are relatively complex and some contain a tertiary amino group, eight tertiary and secondary

γ-glutamyl derivatives with different steric requirements were synthesized and their donor properties examined and compared with glutathione and glutamine. Several other γ-glutamyl derivatives were purchased and measured similarly.

The structures of the compounds examined are shown in Table 5.

The rates of hydrolysis of all these compounds were determined in 100 mM 3-(N-morpholino)-propane sulphonic acid (Mops) buffer,at pH 7.2 and 37°C. The pH of 7.2 favours the hydrolysis rather than the transpeptidation reaction. It was found that quenching aliquots of the reaction solution in 0.1 M NaOH (as suggested in the literature [107]) produced significant base-catalysed hydrolysis of several of these γ-glutamyl compounds. Accordingly, aliquots were quenched by the addition of 0.65 cm^3 of very hot Mops buffer (pH 7.2) followed by heating at 100°C for 2 min.

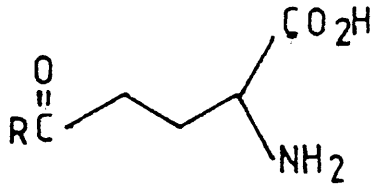
With the exception of (39), (59) and (61), rates of hydrolysis were determined by measurement of the glutamic acid released using the method of Cook and Peters[107]. This involved precolumn derivatization of the L-glutamic acid with o-phthaldialdehyde (OPT) in the presence of 2-mercaptoethanol to produce a fluorescent OPT derivative as shown in Scheme 34.



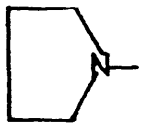
Scheme 34

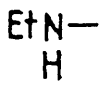
Table 5.

Name and structure of the γ-glutamyl compounds examined for substrate specificity, towards GGT.



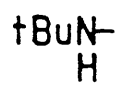
R

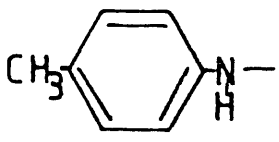
(31)  γ-L-Glutamyl-pyrrolidine

(32)  γ-L-Glutamyl-ethylamine

(33) Et_2N- γ-L-Glutamyl-diethylamine

(34) Me_2N- γ-L-Glutamyl-dimethylamine

(35)  γ-L-Glutamyl-t-butylamine

(36)  γ-L-Glutamyl-4-methylaniline or γ-L-glutaminyl-4-methylbenzene

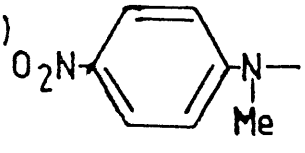
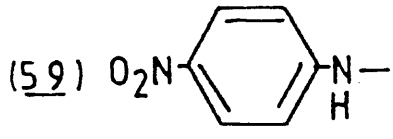
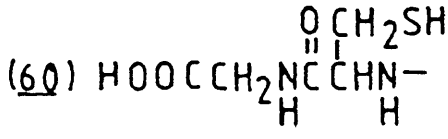
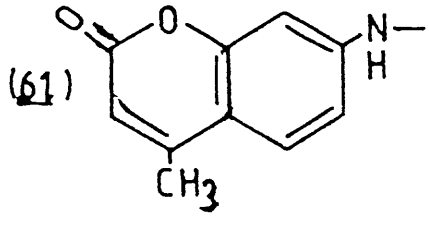
(39)  γ-L-Glutamyl-N-methyl-4-nitroaniline or γ-(N-methyl-L-glutaminyl)-4-nitro benzene

Table 5 cont'd

	R	
(58)	H_2N-	L-Glutamine
(59)		γ-L-Glutamyl-4-nitroaniline or γ-L-Glutaminyl-4-nitrobenzene
(60)		Glutathione
(61)		γ-L-Glutamyl-7-amino-4-methylcoumarin

The OPT derivatized sample was analysed by HPLC using an ODS Hypersil column with 0.3 M aqueous sodium acetate: 17% acetonitrile buffer (pH 4.85) eluent as described in the Experimental chapter. The OPT-derivative of L-glutamic acid elutes at ca. 8 min. For the hydrolysis of (58), this procedure gave an unidentified co-eluting product which interfered with the quantitation, but satisfactory analyses were achieved by eluting with 0.3 M aqueous sodium acetate : 12% acetonitrile buffer (pH 4.85), with which the OPT - derivative of L-glutamic acid eluted at ca. 18 min.

For the hydrolysis of (39) and (59), rates were obtained spectrophotometrically by the increase in a absorbance at λ_{max} = 405 nm[116] corresponding to the formation of N-methyl-4-nitroaniline and 4-nitroaniline,respectively [108]. The rates of hydrolysis of (61) were followed by the fluorimetric method of Smith et al[109].

The compounds were assayed by the addition of 100 µl of enzyme solution to 250 µl of the test substrate dissolved in Mops buffer (pH 7.2), followed by an appropriate incubation period at 37°C and finally quenching the reaction with boiling Mops buffer (pH 7.2). Substrate concentration, enzyme concentration and incubation times in each case were determined by the test substrates activity as a GGT donor. The usual conditions employed were a 1/1000 dilution of purified enzyme and incubation periods at 37°C from 6-8 min. For effective substates such as (60) and (61), maximum substrate concentrations of 0.15 mM and 0.6 mM, respectively allowed V_{max} to be attained with the usual amount of enzyme and incubation period. For compound (36), a maximum concentration of 2 mM was required and for compounds (32) and (58), a maximum substrate concentration of 5 mM. For compound (35) a maximum substrate concentration of 3.0 mM was used but both higher enzyme

concentrations (1/100) and longer incubation times (10 min at 37°C) were required to obtain V_{max}.

The tertiary compounds (31), (33) and (34) were assayed using a 1/100 enzyme dilution, 10 min incubation at 37°C and substrate concentrations up to 100 mM. Compounds (59) and (39) were assayed by the direct addition of 10 µl of a 1/100 enzyme dilution and undiluted enzyme dilution , respectively, to a thermostatted cuvette at 37°C containing 990 µl of substrate solution and monitoring the change in absorbance at λ_{max} 405 nm. For (59) substrate concentrations of up to 0.8 mM were used, and for (39) up to 10 mM.

The results were expressed as Hanes plots, a typical example of which is shown for compound (32) in Figure 2, where K_m is given by the intersept at the x-axis (equal to $-K_m$) and V_{max} is obtained from the intersept at the y-axis (equal to K_m/V_{max}). The intersepts were determined by linear regression of the experimental data using the computerized method of Cornish Bowden[110].

3.3.1.1 Tertiary substrates

The detection limit for glutamic acid by the OPT/HPLC method is 20 pmole. Hydrolysis of the tertiary compounds (31), (33) and (34) monitored by this method gave no detectable glutamic acid release (compared with blanks containing no enzyme). This suggests that even at substrate concentrations of up to 100 mM the rate of hydrolysis of these compounds is less than 0.059 µ moles/min/mg protein. One other tertiary compound (39), was also found to be inactive as a substrate for GGT. No increase absorbance at 405 nm (corresponding to the formation of N-methyl-4-nitroaniline),

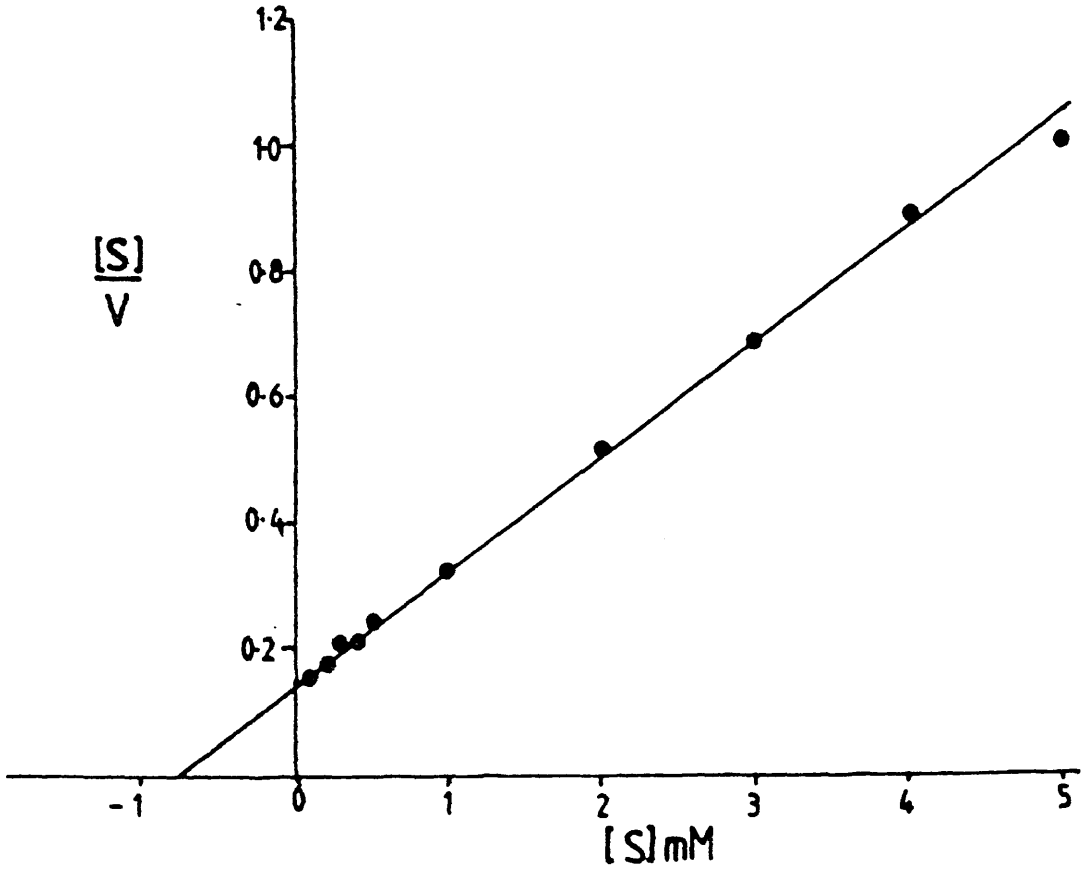


Figure 2. Plot of $\frac{[S]}{V}$ versus [S] for the GGT-catalysed hydrolysis of γ-L-glutamyl-ethylamine (32) at 37°C and pH 7.2

was evident for concentrations of (39) up to 10 mM, against blanks containing no enzyme . The absence of significant GGT-catalysed hydrolysis for (39) is especially interesting. It shows that even tertiary compounds bearing good leaving groups are ineffectual donor substrates for GGT. Thus the nucleofugacity of the expelled amine fragment is unimportant, because N-methyl-4-nitroaniline (pK_a ca. 0.99) is a much better leaving group than dialkylamine (pK_a ca. 10.5). This conclusion is discussed in more detail in the following section.

From these results, it would appear that tertiary compounds are ineffectual substrates for hydrolysis by GGT.

Other experiments summarized in Figure 3 showed that the hydrolysis of the secondary compound (61), when present in 10-fold excess relative to (33) is not inhibited by the addition of (33). Conversely, the results in Figure 3, show that the diethylamine derivative (33) accelerates the hydrolysis of (61). This suggests that whilst inactive as donor substrates, tertiary compounds such as (33) may be active as acceptors for the transpeptidase activity of GGT.

3.3.1.2 Secondary substrates

Hydrolysis of all the γ-L-glutamyl donors bearing secondary N^3-substituents (e.g. (32), (35), (36), (58), (59), (60), and (61)), were catalysed by GGT. The experimental data for the hydrolysis of these compounds are summarized in Table 6. These results reveal the necessity to alter the substrate concentration ranges for the different compounds in order to obtain reliable V_{max} values. Values of K_m and V_{max} obtained from the experimental results in Table 6 are summarized in Table 7. The K_m values show that (32) and (35) are poorer donor substrates (higher K_m) than the

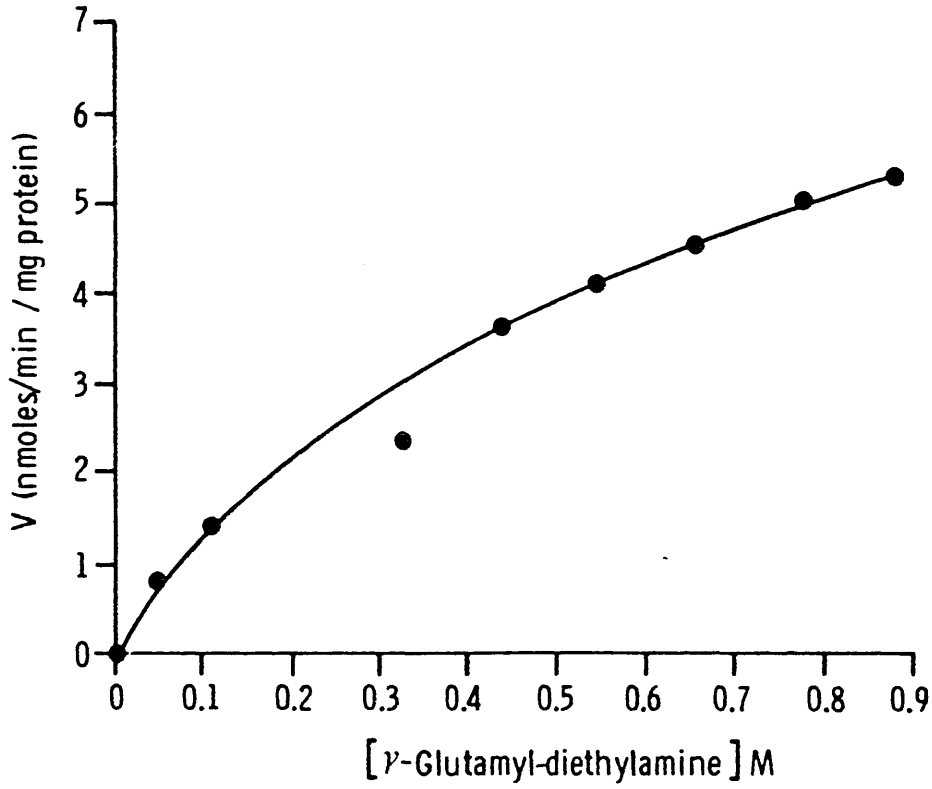


Figure 3. Plot showing the effect of various concentrations of γ-L-glutamyl-diethylamine (33) on the hydrolysis of γ-L-glutamyl-7-amino-4-methylcoumarin (61) by GGT at 37°C and pH 7.2.

Table 6

Experimental data from the GGT catalysed hydrolysis of secondary γ-glutamyl derivatives at pH 7.2 and 37°C

Substrate	Substrate concentration (μM)	V (μmol/min/mg protein)
L-Glutamine (58)	200	0.91
	400	1.14
	600	1.5
	1000	2.12
	1600	2.28
	2000	2.38
	2400	2.5
	3000	2.72
	3600	2.76
	4000	2.85
	5000	2.87
γ-L-Glutamyl 4-nitroaniline (59)	31	5.50
	61	7.80
	82	8.45
	102	9.35
	204	14.2
	304	15.7
	400	20.6
	502	19.25
	790	22.30

Table 6 cont'd

Substrate	Substrate concentration (μM)	V (μmol/min/mg protein)
Glutathione (60)	6	4.73
	30	7.55
	48	8.52
	60	9.30
	72	9.68
	108	10.46
	120	11.0
	132	11.47
	150	12.55
γ-L-Glutamyl-7-amino-4-methylcoumarin (61)	59	3.54
	148	4.85
	208	5.33
	297	5.95
	440	7.08
	504	6.91
	593	7.67
	652	7.87
	741	7.8
γ-L-Glutamyl-ethylamine (32)	100	0.6
	200	1.11
	300	1.39
	400	1.85
	500	2.02
	1000	2.98
	2000	3.89
	3000	4.35
	4000	4.5
	5000	4.67

Table 6 cont'd

Substrate	Substrate concentration (μM)	V (μmol/min/mg protein)
γ-L-Glutamyl-t-butylamine (35)	70	0.41
	140	0.66
	350	0.70
	71	0.11
	1430	0.16
	2140	0.21
	2860	0.22
γ-L-Glutamyl-4-methylaniline (36)	8	2.84
	26	6.70
	43	10.50
	86	11.50
	171	13.90
	214	14.30
	430	14.10
	600	14.80
	1710	15.90
	2140	16.90

Table 7

Values of K_m and V_{max} for various donor substrates of GGT at pH 7.2 and 37°C.

Figures in parentheses indicate 95% confidence limits.

ND. Not detectable <0.059 μmoles/min/mg protein

Substrate	K_m(μM) $\frac{k_4k_3 + k_4k_2}{k_1k_4 + k_1k_3}$	V_{max} (μmol/min/mg protein) $\frac{k_3k_4}{k_3 + k_4}$
L-Glutamine (58)	660 (324-1290)	2.2 (1.6 - 3.1)
γ-L-Glutamyl-4-nitroaniline (59)	116 (99 - 260)	26.4 (21.2 - 31.9)
Glutathione (60)	15 (7.5 - 33.5)	11.9 (10.2 - 15.6)
γ-L-Glutamyl)-7-amino-4-methylcoumarin (61)	135 (81 - 195)	9.18 (8.2 - 10.1)
γ-L-Glutamyl-pyrrolidine (31)	ND	-
γ-L-Glutamyl-ethylamine (32)	804 (756 - 894)	5.4 (5.3 - 5.6)
γ-L-Glutamyl-diethylamine (33)	ND	-
γ-L-Glutamyl-dimethylamine (34)	ND	-
γ-L-Glutamyl-t-butylamine (35)	1251 (514 - 3720)	0.32 (0.25 - 0.57)
γ-L-Glutamyl-4-methylaniline (36)	36.3 (21.4 - 43.5)	5.4 (5.3 - 5.6)
γ-L-Glutamyl-N-methyl-4-nitroaniline (39)	ND	-

native glutamine donor (58), although the difference is not large. Compound (36), however, is a more effective substrate (lower K_m) than (58) . These results suggest that the steric requirement of the donor substrate is not an important factor influencing specificity. It follows that inability of GGT to catalyse the hydrolysis of the tertiary compounds is unlikely to arise from steric hindrances. Work by Cook et al,[111] on the effect of deuterium oxide (D_2O) on the hydrolysis of (61) by GGT showed K_m increased with increasing D_2O concentration.This could be attributed to an isotopic hydrogen exchange by either the donor, the enzyme or both. This finding is consistent with the involvement of hydrogen bonding in the binding of the donor with the enzyme. It is therefore conceivable that the γ-N-H group is necessary for donor activity because of H-bonding with the active site of the enzyme.

The results in Table 7 show that the most rapidly hydrolysed secondary donor substrates contain good nucleofuges with electron withdrawing capability, such as 4-nitroaniline (e.g. compound (59)). Conversely, the slowest hydrolysed donors contain poor nucleofuges with electron donating capability such as t-butylamine (e.g.compound (35)).Further inspection reveals that the ease of hydrolysis of the donor substrate relates to the basicity (pK_a) of the nucleofuge and inter alia to its stability. The linear relationship between the pK_a of the nucleofuge and $V^{app}/_{max}$ is shown in Figure 4, where the $V^{app}/_{max}$ was estimated as the rate of L-glutamic acid formation with 6mM each substate (>9xK_m in each case). Nonetheless $V^{app}/_{max}$ values only change by a factor of ca. 25 for a change in basicity (k_a) of 10^{10}. This may relate to the

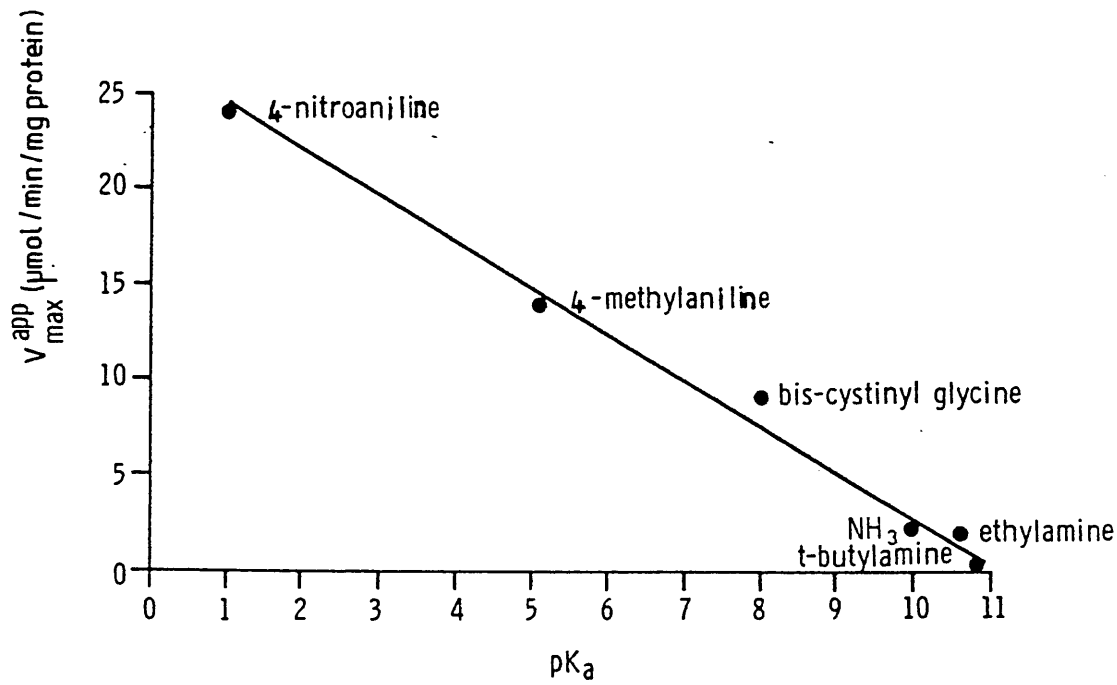


Figure 4. Dependence of V^{app}_{max} on nucleofuge basicity (pK_a) for various γ-glutamyl donors.

composite nature of V_{max} (equ 4). This has 2 limiting

$$V_{max} = \frac{k_3 k_4}{k_3 + k_4} \quad \text{.....equ 4}$$

approximations depending on the relative magnitudes of k_3 and k_4. For $k_3 > k_4$ equ 4 approximates to equ 5. This clearly cannot be

$$V_{max} = \frac{k_3 k_4}{k_3} = k_4 \quad \text{.....equ 5}$$

true because k_4 is the same for all compounds (k_4 is the rate coefficient for the decomposition of E-γ-G to products (Scheme 33) and is therefore independent of the γ-glutamyl substituent),and V_{max} varies. For $k_3 < k_4$, equ 4 approximates to equ 6. It

$$V_{max} = \frac{k_3 k_4}{k_4} = k_3 \quad \text{.....equ 6}$$

follows that the variation of k_3 with pK_a can never exceed that for V_{max} . Thus the nucleofugacity of the γ-glutamyl substituent appears to exert only a small effect on the rate of hydrolysis of the γ-glutamyl donor. It follows that formation of the GGT-donor substrate complex (k_1 in Scheme 33) is the important factor determining the rate of donor substrate hydrolysis.

In terms of binding of the γ-glutamyl compounds to the enzyme there does not appear to be any clear relationship between the structure of the adduct and K_m. K_m is normally assumed in simple one-substrate cases to reflect the dissociation constant (K_d) of the substrate. This does not apply, however, for a ping-pong enzyme such as GGT where the K_m equation (equ 7) is complex. Equ 7

$$K_m = \frac{k_4 k_3 + k_4 k_2}{k_1 k_4 + k_1 k_3} \quad \text{..... equ 7}$$

involves a k_3 term that is substrate dependent and thus K_m values for various substrates cannot be compared and assumed to reflect their reactive affinities for the donor site (except in cases where

V_{max} values are similar and therefore k_3 must also be similar).

3.3.2 The effect of the γ-glutamyl structure on donor specificity

To examine structural effects other than the nucleofugacity of the expelled fragment on donor specificity for the GGT enzyme, the hydrolysis of some other glutamines and amino acids was also studied. The names and structures of the compounds studied are given in Table 8.

Hydrolysis of (63) and (64) was investigated to examine the structural importance of the α-glutamyl substituents. The importance of carbon chain length was investigated via (65) and (66) (i.e. one CH_2 unit more and less, respectively than L-glutamine (58)). The stereospecificity of γ-glutamyl substituents was examined by measuring the hydrolysis of D- glutamine (62).

The hydrolysis of all these compounds was followed by measuring the release of ammonia using a modified procedure of Fawcett and Scott[112] as described below. This used the Berthelot reaction in which ammonia gives a blue colour with phenol and alkaline hypochlorite in the presence of a nitroprusside catalyst. The method is very dependent on pH, reagent concentrations, temperature and the time of colour development. Aliquots of the reaction solutions were added to Mops buffer (pH 7.2), and the reagent concentrations adjusted to give maximum sensitivity (ca. 10 fold higher than reagent concentrations reported in the literature[112]). The absorbance of the assay solution showed λ_{max} at 630 nm, assuming a maximum value after 20 min at 37°C. The calibration curve using NH_4HCO_3 was linear up to 40 nmol NH_3 (Figure 5). Subtraction of the absorbance for blanks*, (obtained by using the reaction solution without enzyme) gave the concentration of ammonia released. The results were reproducible to within 10%.

---

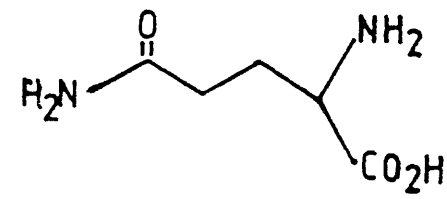
*Since the substrate compounds may generate ammonia and an absorbance at λ_{630}, by non-enzymatic hydrolysis, and since distilled water may also contain ammonia, the absorbance generated by a blank of the reaction solution in which the enzyme was absent was subtracted from the absorbance generated in the presence of enzyme.

In initial experiments, 21.43 mM solutions of each substrate (in Mops buffer, pH 7.2) were treated with a 1/70 enzyme dilution and the whole incubated for 24 min at 37°C. After quenching in the usual way, Berthelot reagent was added and the absorbance at 630nm determined. The results obtained from both the reaction solutions and blanks are shown in Table 9. It is evident that (65) and (66) give no detectable NH_3 release. Since the limit of detection is ca. 4 nmol NH_3, the rates of hydrolysis of (65) and (66) by GGT at 37°C at concentrations up to 21.43 mM are less than 97 nmoles/min/mg protein. Substrates (62)-(64) readily generated NH_3 using a range of concentrations up to and including 21.43 mM as shown in Table 10. The Hanes plots of the data obtained for (62) and (63) are shown in Figure 6 and the kinetic parameters for all substrates are summarized in Table 11.

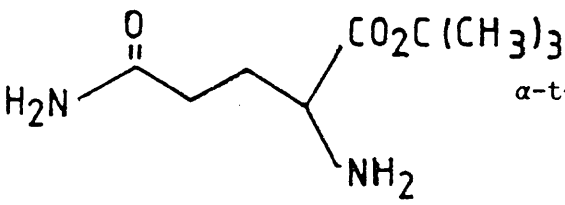
As noted above, neither (65) nor (66) hydrolyse at a significant rate in the presence of GGT. This shows that chain length is an important factor in binding of the donor substrate to the enzyme. It suggests that donor substrates are bound by either the α-amino or α-carboxyl groups in such a way that further interaction by the enzyme occurs at the γ-carbonyl group of the donor. It is also

Table 8.

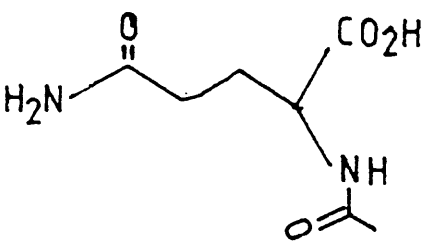
Name and structure of substituted glutamine and other compounds used to investigate the effect of γ-glutamyl structure on donor specificity.

(62) 

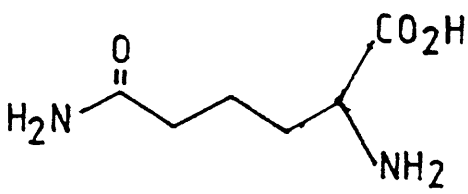
D-Glutamine

(63) 

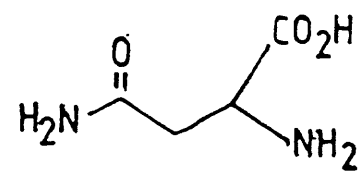
α-t-Butyl-L-glutamine

(64) 

α-N-Acetyl-L-glutamine

(65) 

L-Citrulline

(66) 

L-Asparagine

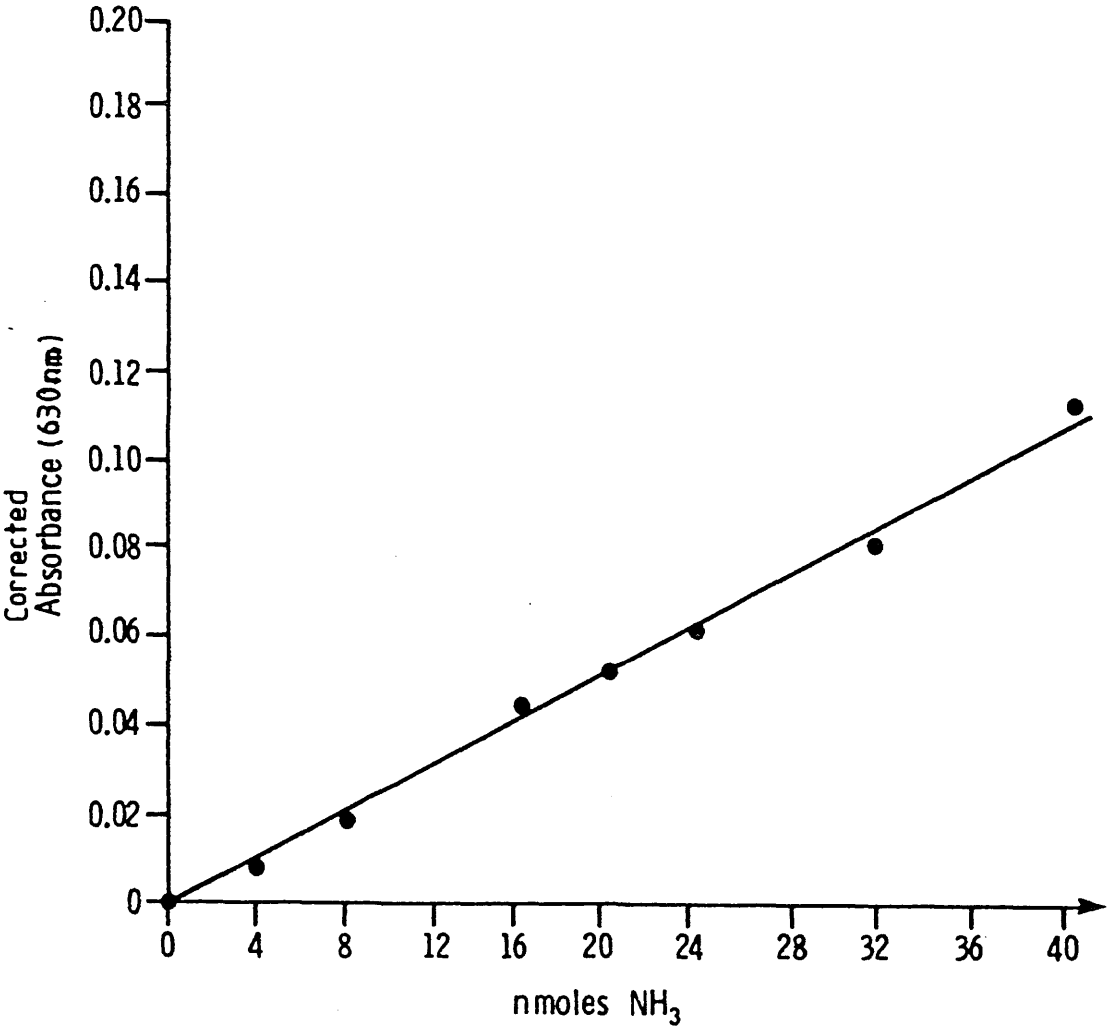


Figure 5. Calibration curve for the colorimetric determination of NH_3 obtained by incubating NH_4HCO_3 with Berthelot reagent for 20 min at 37°C

Table 9

Hydrolysis of glutamine derivatives by GGT at pH 7.2 and 37°C.

Substrate (21.43 mM)	Absorbance (λ630 nm)	Blank Ab (λ630 nm)	Difference (Ab-blank)	V (µmol/min/mg protein)
D-Glutamine (62)	0.91	0.076	0.115	0.935
α-t-Butyl-L-glutamine (63)	0.175	0.092	0.083	0.704
α-N-Acetyl-L-glutamine (64)	0.033	0.026	0.077	0.060
L-Citrulline (65)	0.011	0.011	-	-
L-Asparagine (66)	0.017	0.019	-	-

Table 10

Experimental data for the GGT catalysed hydrolysis of glutamine compounds (62) - (64) at pH 7.2 and 37°C.

Substrate concentration (mM)	V (μmole/min/mg protein)		
	D-glutamine (62)	α-t-Butyl-L-glutamine (63)	α-N-Acetyl-L-glutamine (64)
0.71	0.267	0.093	0
1.43	0.364	0.126	0.017
2.14	0.432	0.200	0.013
2.86	0.573	0.219	0.017
4.28	0.763	0.352	0.030
7.14	0.836	0.439	0.047
10.00	0.996	0.461	0.032
14.30	1.093	0.723	0.043
21.43	1.094	0.737	0.062

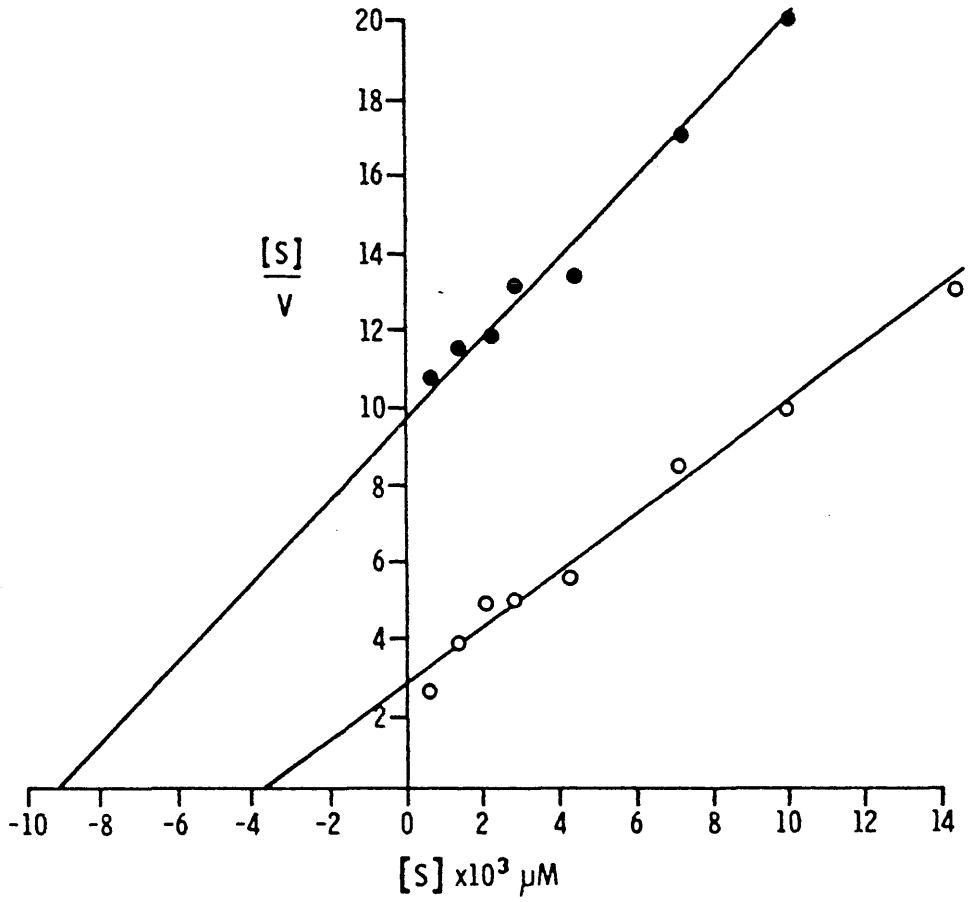


Figure 6. Plots of $\frac{[S]}{V}$ versus [S] for the GGT-catalysed hydrolysis of α-t-butyl-L-glutamine (63),(●) and D-glutamine (62),(○) at 37°C and pH 7.2.

Table 11

Values of K_m and V_{max} for the hydrolysis of glutamine derivatives at pH 7.2 and 37°C.

Figures in parentheses indicate 95% confidence limits.

ND. Not detectable, V_{max} <0.097 μmoles/min/mg protein.

Substrate	K_m (μM)	V_{max} (μmol/min/mg protein)
D-glutamine (62)	3940 (2147-5500)	1.03 (0.72 - 1.44)
α-t-Butyl-L-glutamine (63)	9250 (4330-14480)	1.37 (1.09 - 1.58)
α-N-Acetyl-L-glutamine (64)	ND	-
L-Citrulline (65)	ND	-
L-Asparagine (66)	ND	-

clear that the hydrolysis of (62) is catalysed by GGT with a value of K_m ca. 5 fold larger than that for the L-isomer (58) (see Table 7), but a V_{max} similar for both compounds. Thus, the GGT-catalysed hydrolysis has low (if not negligible) stereospecificity, which suggests that only one of the α-functional groups is involved in binding of the γ-glutamyl donor. Further, the results in Table 10 show that (64) gives a small release of NH_3, but the results were variable and accurate K_m and V_{max} values could not be determined; at the concentrations of (64) examined, its rate of hydrolysis cannot exceed 97 nmoles/min/mg of protein, the limit for the NH_3 assay. It therefore seems reasonable to conclude that ammonia release from (64) is not catalysed by GGT. This suggests that the α-amino group is involved in binding of the donor substate to GGT active site. Conversely modification of the α-carboxyl group to the t-butyl ester (e.g. compound (63)) increases K_m 20 fold without significantly changing V_{max}. This also suggests that binding of the donor substrate involves only the α-amino group and not the α-carboxylate function.

3.3.3. Donor site of GGT

The results of the preceding 2 sections allow firm conclusions about the structure of substrates for GGT to be drawn. Thus, active donor substrates must possess: 1) a primary or secondary γ-substituent (i.e. a free NH proton); 2) a five atom backbone chain; and 3) a free α-amino group. It also appears that the enzyme recognises the γ-glutamyl portion of the donor and that the γ-substituent is not involved in binding. A donor site for GGT consistent with the above results is depicted in Figure 7. The group Z represents the amino acid residue of GGT which forms the covalent γ-glutamyl-enzyme intermediate and X represents the

variable adduct portion of the donor substrate. The main features of Figure 7 are primary sites of interaction with the donor substrate at the α-amino group and the γ-peptide proton. The present results give no information about interaction with the γ-carboxyl group but suggest the α-carboxyl moiety is not involved in binding.

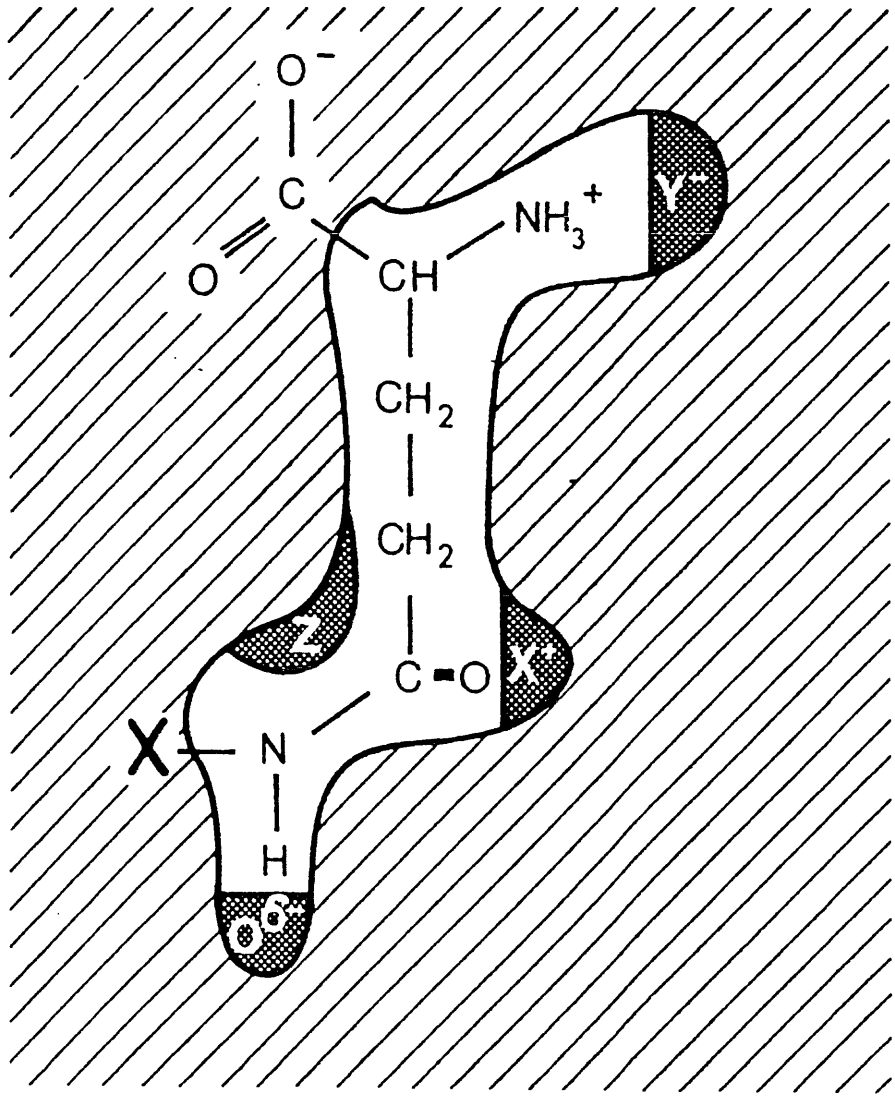


Figure 7. Schematic diagram of the donor site of GGT. X represents the variable adduct portion of the donor substrate. Z represents the group in the active site which forms the covalent γ-glutamyl enzyme intermediate. Y^-,X^+ and $O^{\delta-}$ represent charged species within the donor site that may interact with the donor substrate.

3.4 Hydrolysis of prodrugs by GGT

As reported in Chapter 2, Section 2.1, it proved impossible to synthesize the N-(2-chloroethyl)-N-nitrosourea-γ-L-glutamyl compounds because of their instability. This was an important consideration in the original goal of synthesizing tertiary N-nitroso derivatives as putative prodrugs for GGT. However from the results above it is evident that tertiary N-nitroso compounds are unlikely to be donor substrates for GGT due to the lack of the γ-N-H moiety.

In the course of the synthetic work α-(4-nitrophenyl)-γ-(N-(2-chloroethyl)-N-nitrosocarbamoyl)-L-glutaminate (28), with protection on the α-carboxyl group had been isolated. Because of its availability, it was therefore decided to briefly investigate its hydrolysis in the presence of GGT.

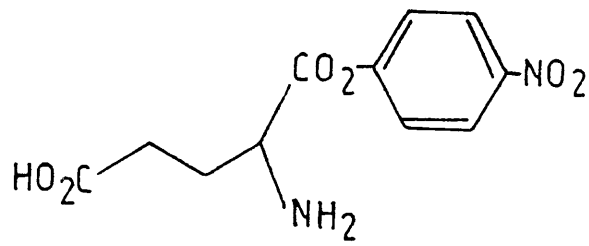
3.4.1 Hydrolysis of α-(4-nitrophenyl)-γ-(N-(2-chloroethyl)-N-nitrosocarbamoyl)-L-glutaminate (28)

Compound (28) has all the requirements for substrate activity as deduced in Sections 3.3.1.1-3.3.2; in particular, it bears both the α-NH_2 and the γ-NH moieties. The hydrolysis of (28) in the presence of GGT was therefore made at 37°C in Mops buffer (pH 7.2). The reactions were followed using HPLC to measure release of N-(2-chloroethyl)-N-nitrosourea (30) at λ_{max} = 245 nm using an ODS Hypersil column and methanol:water (1:1 v/v) mixture as eluent. For a flow rate of 1 ml/min, the N-(2-chloroethyl)-N-nitrosourea gave a retention time of ca. 4.3 min. The reaction could not be quenched by base or the addition of boiling Mops (pH 7.2) because (30) is unstable under these conditions. Aliquots of the reaction solutions were therefore quenched by the addition of ice-cold Mops

buffer (pH 7.2) and keeping these solutions at 0°C prior to analysis. This procedure does not denature the enzyme, but it reduces the rate of hydrolysis and conclusions about the extent of GGT catalysis can be made.

Preliminary experiments showed that extensive base-catalysed hydrolysis of (28) occurred at pH 7.2 and 25°C in the absence of GGT. Thus it was not feasible to obtain satisfactory kinetic data for the hydrolysis of (28) catalysed by GGT.

A typical HPLC assay for the base-catalysed hydrolysis of (28) at pH 7.2 and 25°C is shown in Figure 8. The substrate (28) (3 mM) was dissolved in methanol:water (1:1 v/v) at pH 7.2 and incubated in a water bath at 25°C. At timed intervals, aliquots (50 µl) were withdrawn from the reaction solution and then injected onto the HPLC column. Two major peaks (A and B) are evident on the chromatogram, the first (A) eluting at ca. 4.3 min (30), the second (B) eluting at 8 min and both increasing with incubation time. Although it was not confirmed using authentic materials, peak (B) probably corresponds to the α-4-nitrophenyl-L-glutamate co-product (67) of the hydrolysis reaction.



(67)

The formation of (30) from the hydrolysis of (28) at 25°C and pH 7.2 followed pseudo first order kinetics (equ 8). Thus plots of $\ln(c_\infty - c_t)$, (where c_∞ and c_t refer to concentration at infinite time and concentration at a certain time, respectively) versus time were linear as shown in Figure 9. A value of k_o = 4.2 (± 0.3) x

$$\text{Rate} = k_o\ (28) \quad \ldots\ \text{equ 8}$$

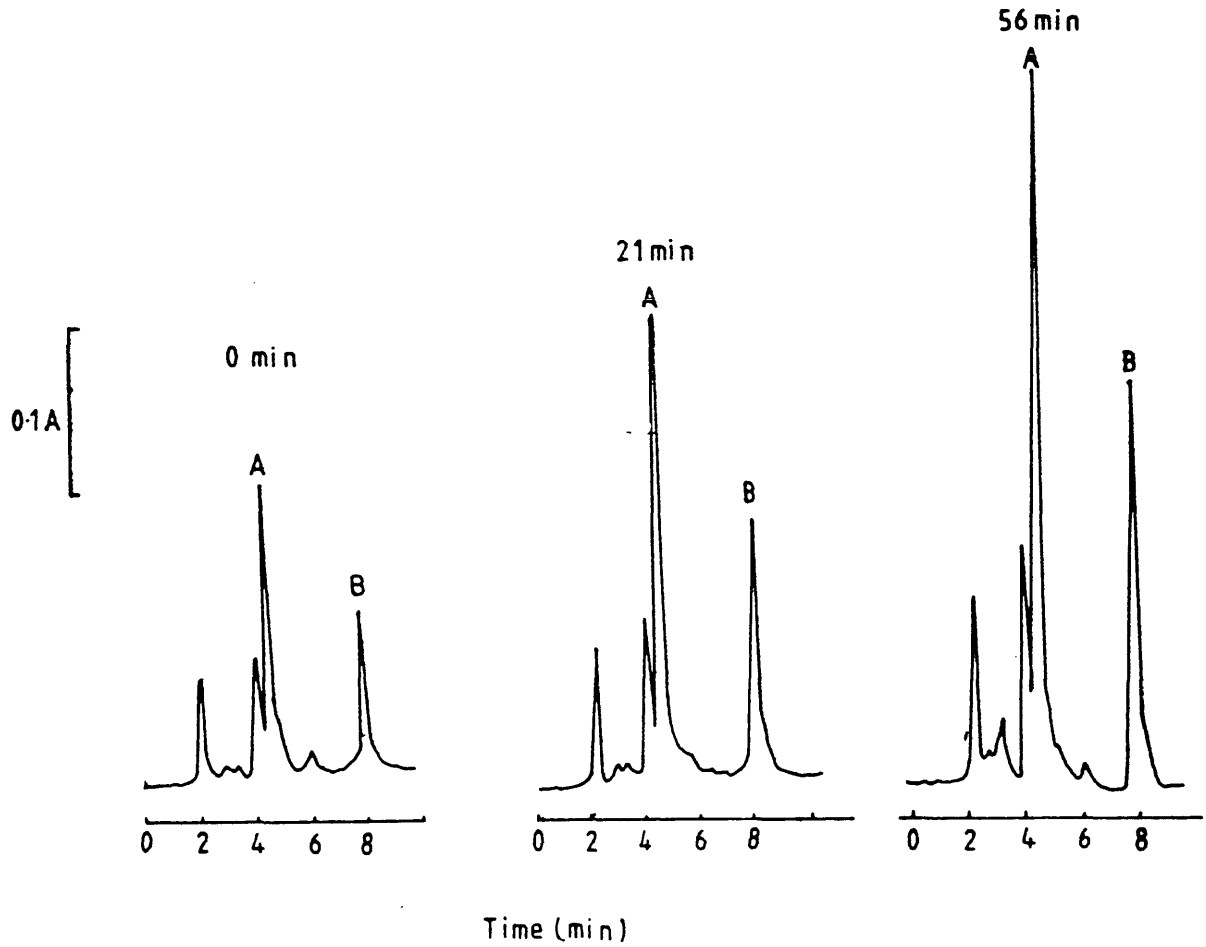


Figure 8. HPLC assay for the base catalysed hydrolysis of (28) over 56 min at 25°C and pH 7.2, using methanol:water (1:1 v/v) eluent.

λ_{max} = 245 nm

A = N-(2-chloroethyl)-N-nitrosourea (retention time = 4.3 min).

B = Possibly α-4-nitrophenyl glutamate (retention time = 8 min).

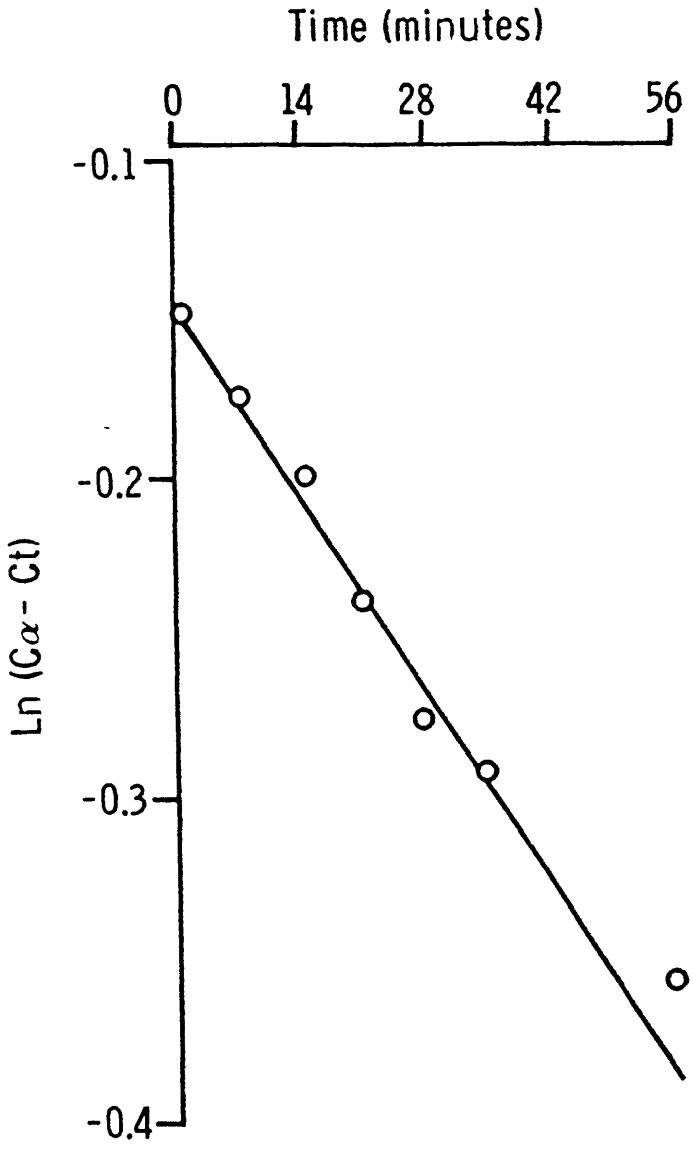


Figure 9. Pseudo-first order plot for the formation of N-(2-chloroethyl)- N-nitrosourea from hydrolysis of (28) at 25°C and pH 7.2.

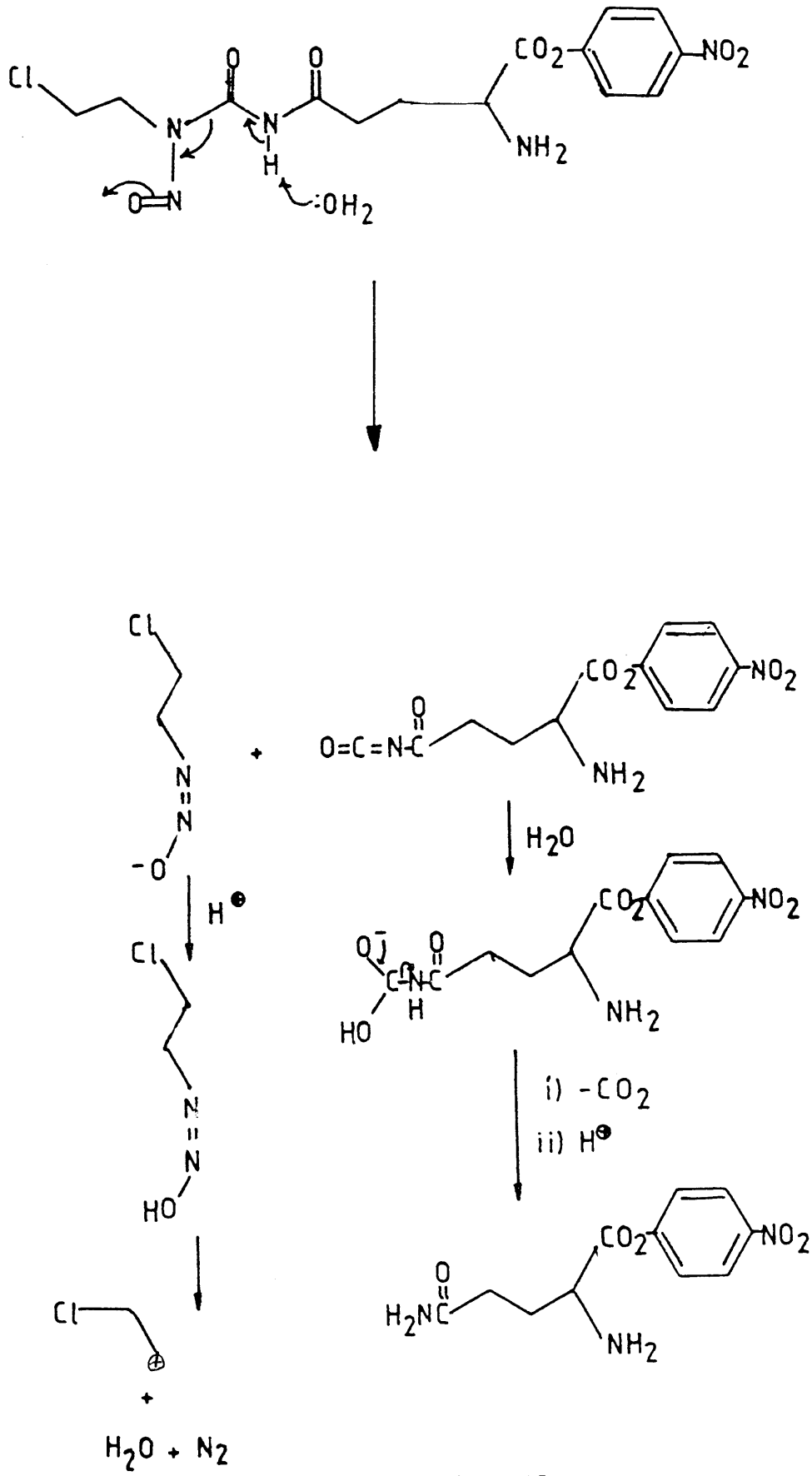
Initial substrate concentration, ca. 3 mM.

10^{-2} min^{-1} (i.e. $t_{1/2}$ ca. 17 min) is obtained from Figure 9. Careful examination of the HPLC chromatograms reveal that the N-(2-chloroethyl)-N-nitrosourea product only accounts for ca. 10% the products from the hydrolysis of (28) after 1h. This suggests that either the product (30) decomposes in the reaction solution or during the HPLC assay, or that substrate (28) decomposes via several pathways. No decomposition of authentic (30) was apparent, however, on standing in aqueous methanol (1:1 v/v,pH 7.2) at 25°C for 60 min. It is therefore concluded that the major decomposition pathway of (28) does not produce (30). As discussed in Chapter 1, N-nitrosoureas are known to decompose under physiological conditions to alkyl diazohydroxides plus isocyanuric acid. It seems very likely that an analogous reaction applies to the decomposition of compound (28) to give the products shown in Scheme 35.

3.4.2. Hydrolysis of the γ-L-glutamyl hydroxyaniline derivatives by GGT

Rates of GGT-catalysed hydrolysis of the γ-L-glutamyl hydroxyaniline derivatives at pH 7.2 and 37°C were measured using the OPT/HPLC assay for L-glutamic acid described previously (Section 3.3.1). The experimental results obtained are summarized in Table 12 and Hanes plots to deduce K_m and V_{max} values from the reported data are shown in Figure 10. The values of K_m and V_{max} obtained by linear regression analysis[110] are tabulated in Table 13.

Compared with the native donor glutathione (60) (see Table 7) both (43) and (44) have larger K_m values than (60) whereas (45) has a lower K_m than (60). The V_{max} values for (44) and (45), are very



Scheme 35

Table 12

Rates of GGT catalysed hydrolysis of γ-L-glutamyl hydroxyaniline derivatives at pH 7.2 and 37°C.

Substrate	Substrate concentration (μM)	V (μmol/min/mg protein)
γ-L-Glutamyl-4-hydroxyaniline (43)	9	0.74
	14	1.03
	29	1.93
	57	2.74
	70	2.93
	140	3.39
	200	3.46
	290	3.95
	430	4.09
	570	4.05
γ-L-Glutamyl-2-hydroxy-5-chloroaniline (44)	43	2.44
	57	3.10
	70	3.50
	140	4.71
	200	5.69
	290	5.82
	430	7.49
	570	7.78
γ-L-Glutamyl-3-chloro-4-hydroxyaniline (45)	3	1.96
	9	5.11
	14	6.46
	29	7.60
	70	10.70
	100	9.61
	120	10.25
	200	10.23

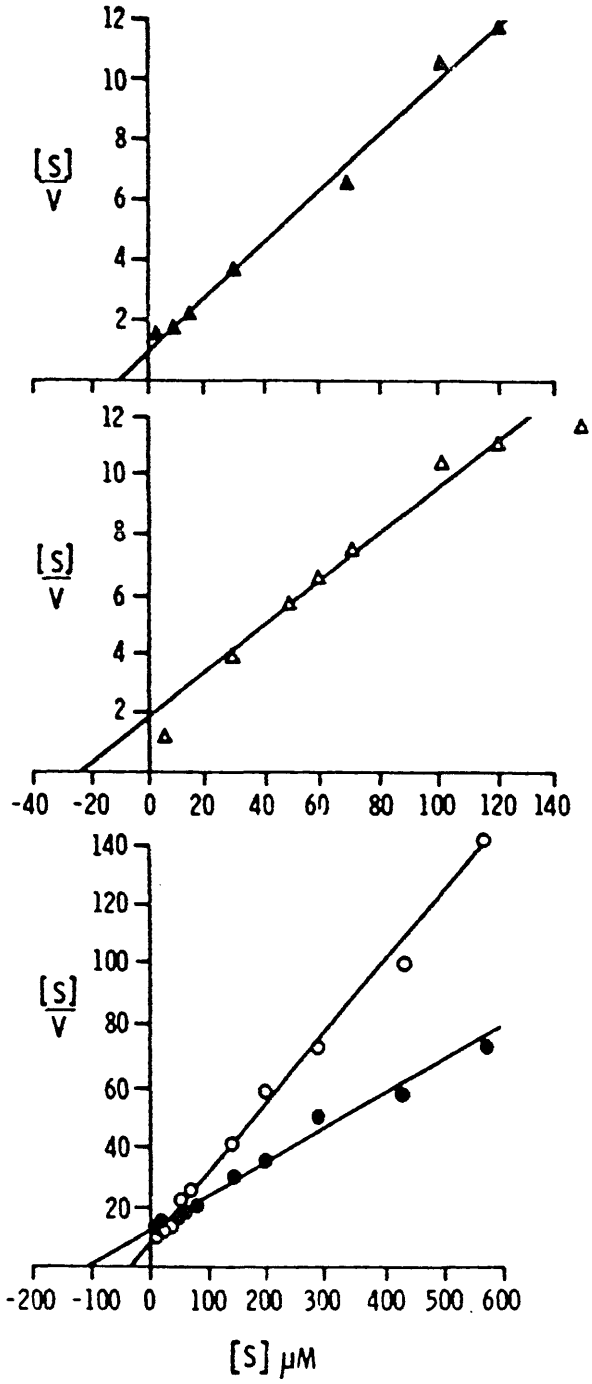


Figure 10. Plots of $\frac{[S]}{V}$ versus [S] for the GGT-catalysed hydrolysis of γ-glutamyl hydroxyaniline prodrugs and glutathione at 37°C and pH 7.2.

(○), γ-L-Glutamyl-4-hydroxyaniline (43).

(●), γ-L-Glutamyl-2-hydroxy-5-chloroaniline (44).

(▲), γ-L-Glutamyl-3-chloro-4-hydroxyaniline (45).

(△), Glutathione (60).

Table 13

Kinetic constants for the GGT catalysed hydrolysis of γ-Glutamyl hydroxyaniline derivatives at pH 7.2 and 37°C.

Kinetic parameters were determined by the direct linear plot method[110].

Figures in parantheses indicate 95% confidence limits.

Substrate	K_m (μM)	V_{max} (μmol/min/mg protein)
γ-L-Glutamyl-4-hydroxyaniline (43)	43 (35 - 53)	4·47(4·3-5)
γ-L-Glutamyl-2-hydroxy-5-chloroaniline (44)	125 (92 - 174)	9.7 (8 - 11)
γ-L-Glutamyl-3-chloro-4-hydroxyaniline (45)	10.6 (5.7 - 16.7)	10·6(10-13)

similar to that for (60). Thus, the k_3 component of K_m must be of similar magnitude for (44),(45) and (60). It follows that the signficantly larger K_m obtained for (44) probably relates to steric hindrance by the 2-hydroxy function being close to the active site of GGT. The V_{max} for (43) is ca. half that of the chlorinated compounds (44) and (45). This may relate to reduced electron withdrawing ability of the unchlorinated 4-hydroxylaniline residue in (43) as reflected by the lower basicity of the corresponding anilines (e.g. pK_a = 5.48 [113], 4.58* and 3.78* for 4-hydroxyaniline, 3-chloro-4-hydroxyaniline and 2-hydroxy-5-chloroaniline, respectively). All three γ-L-glutamyl hydroxyaniline donor substrates are activated by GGT with higher V_{max} values and lower K_m values than the glutamine donor (58) (See Table 7). This implies that GGT catalyses cleavage of these prodrugs at rates comparable with the enzyme's native donors.

*Estimated by comparison of the pK_a for aniline[113] with 3-chloro aniline.

3.5. Summary

GGT was purified to near homogenity. The enzyme was found to activate hydrolysis of a wide variety of substrates including those (e.g. (35) and (61)) with relatively bulky groups attached to the γ-peptide bond. All of the compounds with tertiary γ-N-atoms (e.g. (31), (33), (34) and (39)) were found to be inactive substrates. Their inactivity has been related to the absence of a γ-N-H atom to engage in hydrogen bonding with the active site of the enzyme. Otherwise, GGT seems to be able to activate donor subsrates of diverse structure. Thus cyclic compounds (e.g. (59)),long chain and branched chain adducts (e.g.(60) and (35), respectively) are all

activated by GGT. There appears to be little correlation between the K_m determined for the various substrates and the structure of the γ-glutamyl substituent which also has little influence in binding of the donor substrate to the enzyme. Although the γ-glutamyl substituent is probably not involved in the recognition of the donor substrate by the enzyme, it influences the rate of hydrolysis slightly, thus γ-glutamyl substituents with electron withdrawing properties were more rapidly hydrolysed than those with electron donating properties. Many of the tertiary compounds examined (e.g. (34), (33) and (31)) have very poor leaving groups which may contribute to their inactivity as substrates. This cannot be the critical factor, however, because compound (39) incorporating a good leaving group N-methyl-4-nitroaniline (pK_a = 0.55[113]), was also inactive as a substrate. The leaving group ability of the γ-glutamyl substituent influences the hydrolysis rate slightly, but it is probably the formation of the GGT-donor substrate complex that controls the overall rate of hydrolysis.

The GGT enzyme appears to be selective for the γ-glutamyl portion of the substrate. For substituted glutamine analogues, the α-amino group is important in the binding of the substrate. Thus the enzyme appears to recognise the γ-glutamyl peptide structure by the γ-peptide proton and the α-amino group.

The N-nitrosourea prodrug (28) was found to be too unstable to obtain data on GGT-catalysed hydrolysis. The γ-glutamyl hydroxyaniline derivatives (43)-(45) are all substates for GGT, and they hydrolyse at rates comparable with the enzymes natural donors (58) and (60). Compound (45) was shown to be a particularly effective donor substrate.

CHAPTER 4

CYTOTOXICITY TESTS

4.1 Introduction

The biological activity of the γ-L-glutamyl prodrugs described in Chapter 3 was investigated *in vitro* using cell lines possessing relatively high levels of GGT. The cytotoxicity of the γ-L-glutamyl adduct was compared with that of the parent drug that would be released by enzymatic action of GGT. The measurements were made using a rat hepatoma cell line, high in GGT activity, and comparing the results against a normal rat hepatocyte cell line.

4.2. GGT content of the cell lines

The cell lines used were designated BL8L and JB1. Both were isolated from male Fischer 344 rats as described by Manson et al[16]. BL8L is derived from the normal hepatocyte cells, whereas JB1 is derived from the hepatoma cells. The cell lines were grown in 10 cm^3 of supplemented Williams 'E' medium (as described in the Experimental, Section 7.2.5) at 37°C in a Hareous incubator under an atmosphere of 95% air, 5% CO_2.

Since the GGT levels in both cell types varies with time, the differential GGT activity between the two cell lines was determined. It was necessary to remove the cells from the tissue culture plate by a method other than trypsinization because trypsin (a proteolytic enzyme) may interfere with the membrane bound enzyme. Thus, once the cells had grown to confluency, the Williams 'E' media was removed and replaced with Mops/HCl buffer (100 mM, pH 7.2) and the cells were scraped from the plate using a 'rubber policeman'. After washing by centrifugation (2500 g x 5 min) the cells were finally solubilized in Mops/HCl buffer (100 mM, pH 7.2) containing 1% Triton X100. Their GGT activity was then assayed by the fluorimetric method of Smith et al[109]. These measurements were made on several occasions using cultures at different passage

numbers. The typical results shown in Table 14 suggest that the concentration of GGT in the cell lines does not vary markedly from cell passage to cell passage. Further the BL8L cells clearly contain barely detectable levels of GGT whereas the JB1 cells contain ca. 200 fold higher levels. The results suggest that activation of γ-L-glutamyl prodrugs by GGT should be more extensive in hepatoma cells than normal hepatocytes.

4.3. Cytotoxicity of the parent hydroxyanilines and their γ-L-glutamyl derivatives against BL8L and JB1 cell lines in vitro

These tests were carried out by treating a preconfluent or near confluent monolayer of the two cell lines with various concentrations of the parent hydroxyaniline or the γ-L-glutamyl derivative in supplemented Williams 'E' media (as described in the Experimental, Section 7.2.7.1). Controls were prepared using a similar volume of Williams 'E' media without drug. After incubating the cell samples for 24h at 37°C, the medium was removed, the surviving cell fraction was harvested with trypsin and then counted on a Coulter Counter. The 24h incubation period was arbitrary but all the drugs were found to be active over this time period.

4.3.1. Cytotoxicity of the parent hydroxyanilines

The cytotoxicity of the parent hydroxyanilines against the BL8L and JB1 cell lines are shown in Figures 11 and 12, respectively. From these graphs I_{50} values (drug concentration for 50% cell death) for the effect of the prodrugs on the 2 cell lines were obtained by inspection and these values are reported in Table 15.

From these results it can be seen that the two 4-hydroxyanilines have similar toxicities towards the BL8L cell lines: their I_{50}

Table 14

GGT activities in JB1 and BL8L all lines

The specific activities are for a confluent tissue culture dish of each cell line and are the mean of 3 experiments.

Cell type	Passage No.	Specific Activity μmoles/min/mg protein
BL8L	27	$5.53 \pm 0.3 \times 10^{-4}$
BL8L	41	$5.26 \pm 0.9 \times 10^{-4}$
JB1	17	0.1104 ± 0.01
JB1	29	0.106 ± 0.005

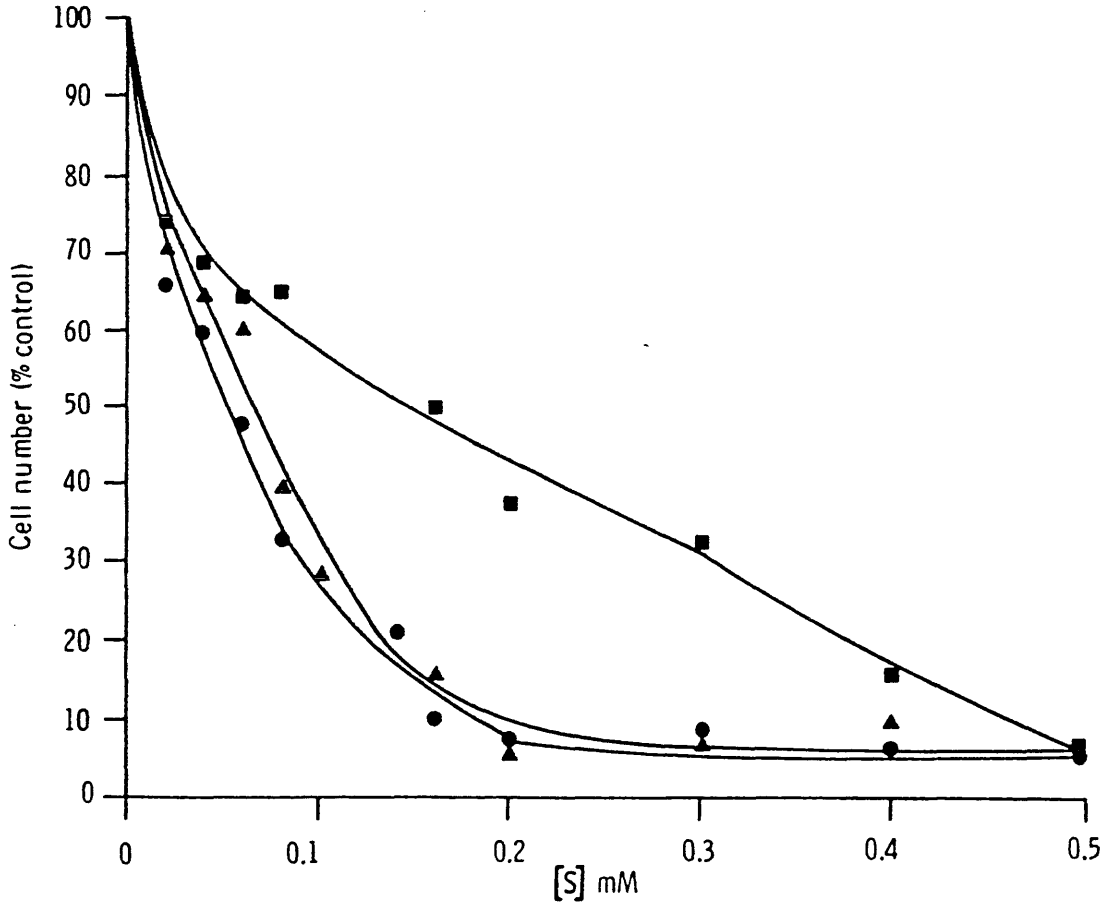


Figure 11. Plot of cell death (as % of control) against concentration of parent hydroxyaniline [S], for BL8L cell lines for 24h exposure at 37°C.

(●), 3-Chloro-4-hydroxyaniline (57).

(▲), 4-Hydroxyaniline (68).

(■), 2-Hydroxy-5-chloroaniline (69).

The results are the mean of 2 experiments.

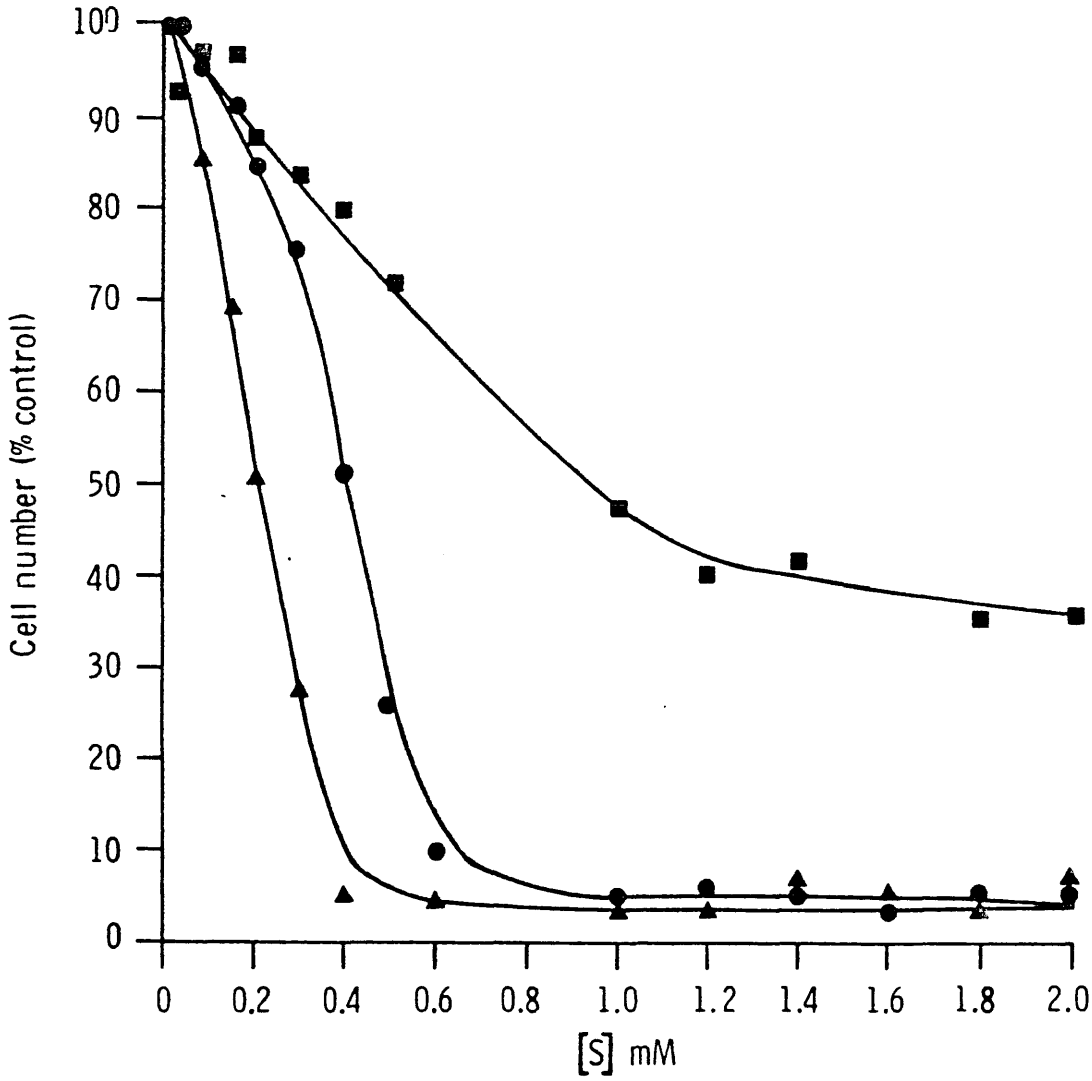


Figure 12. Plot of cell death (as % of control) against concentration of parent hydroxyaniline [S],for JB1 cell lines for a 24h exposure at 37°C.

(●), 3-Chloro-4-hydroxyaniline (57).

(▲), 4-Hydroxyaniline (68).

(■), 2-Hydroxy-5-chloroaniline (69).

The results are the mean of 2 experiments.

Table 15

The I_{50} values for the effect of the parent hydroxyanilines on BL8L and JB1 cell lines.

Preconfluent monolayers were exposed to the drugs for 24h at 37°C.

Drug	I_{50} mM	
	BL8L	JB1
3-Chloro-4-hydroxyaniline (57)	0.05	0.4
4-Hydroxyaniline (68)	0.062	0.21
2-Hydroxyaniline (69)	0.14	0.92

values being 50 μM for 3-chloro-4-hydroxyaniline (57) and 62 μM for 4-hydroxyaniline (68). 2-Hydroxy-5-chloroaniline (69), however has a significantly higher I_{50} of 0.14 mM (i.e., ca. 3 fold higher than the 4-hydroxyanilines).

The cytotoxicity of the parent hydroxyanilines on the JB1 cells is seen to decrease in the order (68) (I_{50} = 0.21 mM) > (57) (I_{50} = 0.4 mM) > (69) (I_{50} = 0.92 mM). The 2-hydroxy-5-chloroaniline is the least toxic as before, and unlike the 4-hydroxyaniline derivatives which produce 100% cell death at the highest concentrations, it only gives a maximum of ca. 70% cell death. It is also apparent from these results that the BL8L cell lines are more susceptible than the JB1 cell lines to the parent hydroxyanilines.

For the above experiments, particularly with the 4-hydroxyanilines (57) and (68), a colour change from pink to brown was observed on adding the substrates to the culture media. This may be indicative of oxidation of the substrates to their benzoquinone-imine analogs. The colour change was less pronounced for (69).

It has been suggested that the benzoquinone-imine form of hydroxyaniline derivatives is responsible for their cytotoxic effects[79]. The above colour changes suggest that the cytotoxicities of the hydroxyanilines examined parallel their ease of oxidation to the quinone-imine . The cytotoxicity of the quinone-imine form was therefore examined directly, using the 4-hydroxyaniline analog (68).

4.3.1.1. The cytotoxicity of 4-benzoquinone-imine

The formation of 4-benzoquinone-imine from (68) was determined by UV spectrophotometry. The UV spectra of (68) in Mops buffer (100 mM, pH 7.2) has an absorbance maximum of 290 nm (Figure 13).

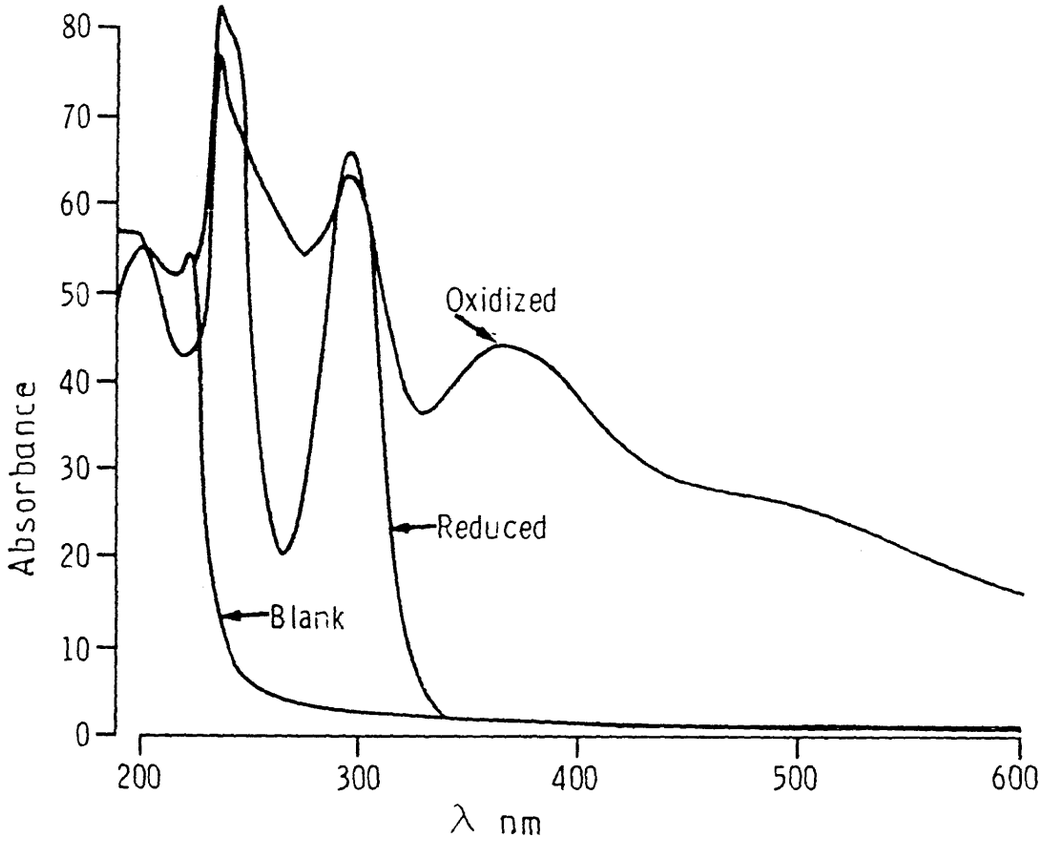
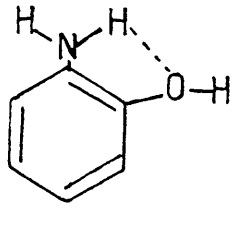


Figure 13. The UV spectra of 4-hydroxyaniline (1 mM), and 4-benzoquinone-imine in Mops/HCl buffer (100 mM, pH 7.2) at 25°C.

On oxidation a new maximum at 370 nm with a shoulder at 480 nm developed (Figure 13). Addition of a few crystals of $NaBH_4$ to the cuvette regenerated the original spectrum. This illustrates the facile redox chemistry of (68).

The cytotoxicity of (68) and 4-benzoquinone-imine was compared by exposing BL8L cells to several concentrations of fresh (68) and to (68) that had been left to oxidize in supplemented Williams 'E' media for 24h. The results (Figure 14) show I_{50} = 78 μM for fresh (68) and 44 μM for 4-benzoquinone-imine. The oxidation potential for (68) is ca. E_o = 0.728V[76]. The oxidation potential for 2-hydroxyanilines is expected to be higher because of stabilization of the reduced form by intramolecular hydrogen bonds, as shown below.



This concurs with the lower cytotoxicity observed for (69) towards both the JB1 and BL8L cell lines.

4.3.2 Cytotoxicity of the γ-L-glutamyl hydroxyanilines

The cytotoxicity of the γ-L-glutamyl hydroxyaniline adducts on both

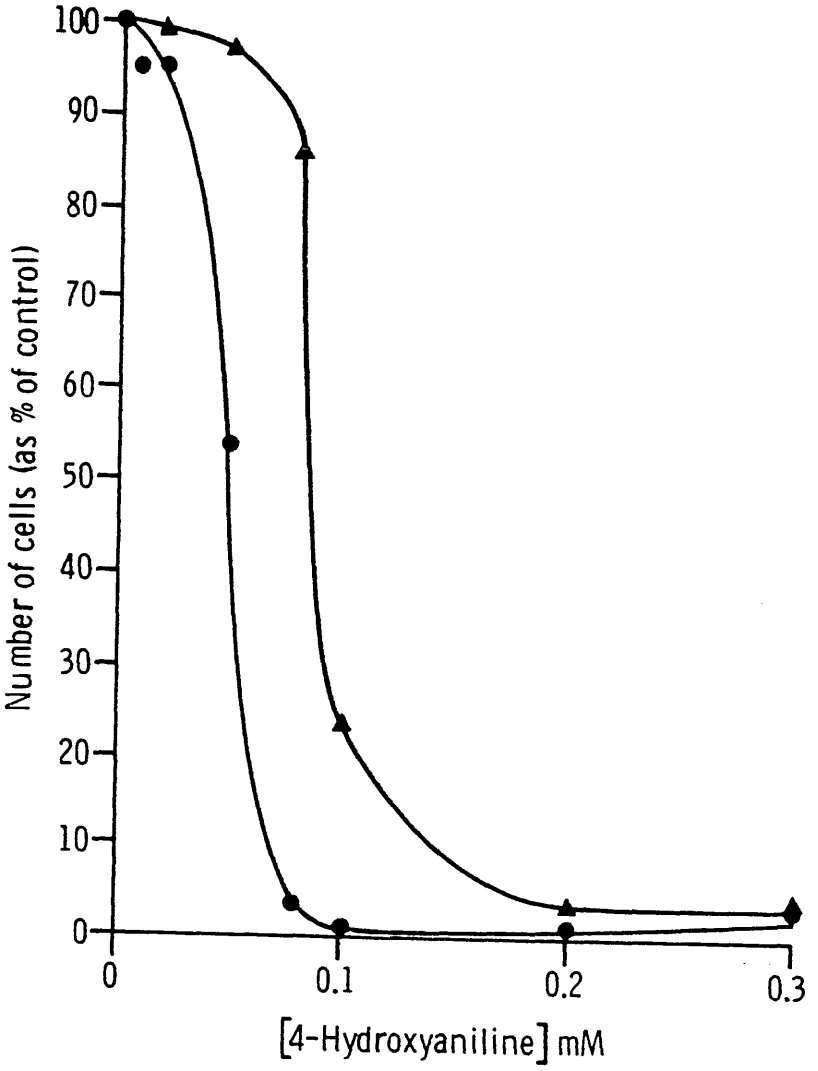


Figure 14. Cytotoxicity of fresh and oxidized 4-hydroxyaniline (68), towards BL8L cell lines for a 24h exposure at 37°C.

(▲), Freshly prepared 4-hydroxyaniline

(●), 24h oxidized 4-hydroxyaniline

The results are the mean of 2 experiments.

the BL8L and JB1 cell lines for 24h exposure, is shown in Figure 15.

An increased susceptibility of the JB1 cell lines to all of the γ-L-glutamyl prodrugs, relative to the BL8L cell lines is apparent. This is a reversal of the cytotoxicity observed for the parent hydroxyanilines on the two cell lines (see Section 4.3.1). It is consistent, however, with the hypothesis that increased levels of GGT in the JB1 cell lines should generate larger amounts of hydroxyanilines from the prodrug adducts.

The I_{50} values obtained for the effect of the prodrugs on the JB1 cell lines are given in Table 16. I_{50} values were not attained for the BL8L cell line. It is clear that γ-L-glutamyl-3-chloro-4-hydroxyaniline (45) is the most potent prodrug towards the JB1 cell lines, with an I_{50} similar to that obtained for 3-chloro-4-hydroxyaniline itself.

The I_{50} values for γ-L-glutamyl-4-hydroxyaniline (43) and γ-L-glutamyl-2-hydroxy-5-chloroaniline (44) are 4 fold and 10 fold higher, respectively, than (45). Thus, the relative cytotoxicities of these γ-L-glutamyl adducts are somewhat different from those of the parent hydroxyanilines. This is not too surprising, however, since the cytotoxic effect of the γ-L-glutamyl adducts will also depend on their rate of cleavage by GGT. In Chapter 3, it was shown that (45) gave a lower K_m value and was hydrolysed by GGT twice as fast as (43). Since the cytotoxicity of their corresponding parent hydroxyanilines are very similar (<2 fold difference), the faster cleavage of (45) plays an important part in determining its potency. Compound (44) although hydrolysed by GGT at a similar rate to (45), has a very large K_m value and also the least toxic parent compound. This appears to be reflected in its lower cytotoxicity (Figure 15).

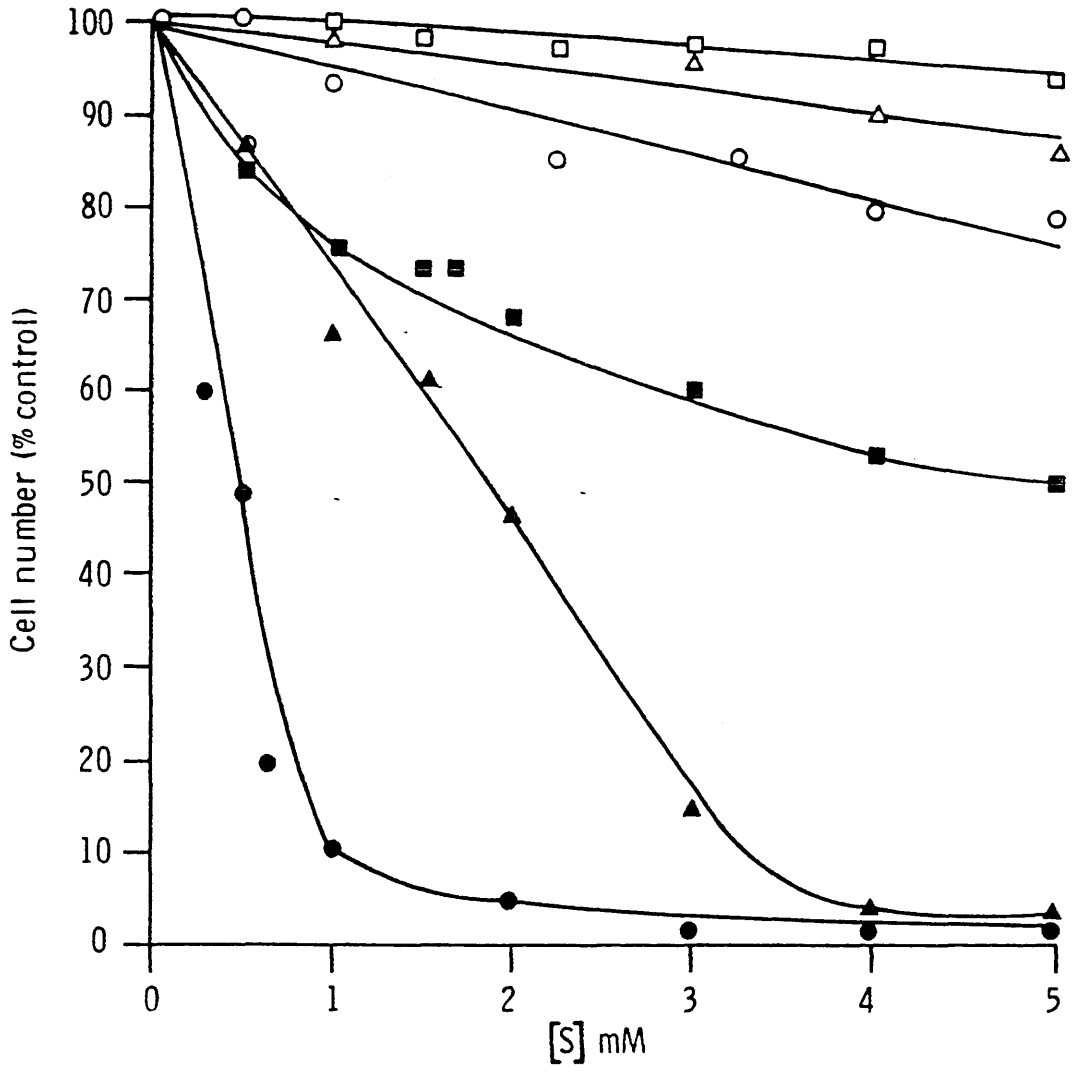


Figure 15. Plot of cell death (as % of control) against concentration of γ-L-glutamyl hydroxyanilines [S],for JB1 (closed symbols) and BL8L cell lines (open symbols) for a 24h exposure at 37°C.

(▲),(△), γ-L-Glutamyl-4-hydroxyaniline (43).

(■),(□), γ-L-Glutamyl-2-hydroxy-5-chloroaniline (44).

(●),(○), γ-L-Glutamyl-3-chloro-4-hydroxyaniline (45).

The results are the mean of 2 experiments.

Table 16

The I_{50} values for the effect of γ-L-glutamyl hydroxyaniline derivatives on JB1 cell lines.

Preconfluent monolayer cultures were exposed to the drugs for 24h at 37°C.

Prodrug	I_{50} (mM)
γ-L-Glutamyl-4-hydroxyaniline (43)	1.9
γ-L-Glutamyl-2-hydroxy-5-chloroaniline (44)	4.75
γ-L-Glutamyl-3-chloro-4-hydroxyaniline (45)	0.47

The effect of the prodrugs on the BL8L cell lines is less pronounced. Although I_{50} values are not attained, there does appear to be a small linear toxic response of the cells to increasing concentrations of prodrug. To test whether the observed toxicity was due to the prodrug itself being toxic or due to low levels of GGT in the BL8L cell line or in the serum used in the incubation media activating the drug, the effect of serine borate inhibition was examined.

4.3.2.1. Cytotoxicity of γ-L-glutamyl hydroxyaniline adducts in the presence of serine borate

Serine borate inhibits GGT by interacting with the γ-glutamyl binding site[114]. Thus the inherent cytotoxicity of γ-L-glutamyl adducts can be determined by carrying out the experiments in the presence of serine borate. The BL8L and JB1 cell lines were exposed for 24h to I_{50} concentrations of each γ-L-glutamyl adduct (in supplemented Williams 'E' media) in the presence and absence of 10 mM serine borate. The cells were then trypsinized and counted in the usual manner.

For comparison, the cell lines were also exposed to the media alone, and to 10 mM boric acid and 10 mM serine both separately and combined.

The results (summarized in Table 17) show that serine borate largely eliminates γ-L-glutamyl adduct toxicity towards both the BL8L and JB1 cell lines. This confirms that the adducts are relatively weak cytotoxins without activation by GGT and that the slight cytotoxic response observed on application of the prodrugs to the BL8L cell line is probably due to the small amounts of GGT present in the cell line or possibly in serum.

The results in Table 17 also show that serine borate has a slight

Table 17

Effect of serine borate on the toxicity of γ-glutamyl hydroxyaniline derivatives on JB1 and BL8L cell lines. Results are the mean of 3 experiments. Figures in parentheses indicate the number of cells surviving as a pecentage of the control.

Prodrug	Cell Type	No of cells (x 10^4)					
		No additions (control)	Prodrug	Prodrug + serine borate (10 mM)	Serine borate (10 mM)	Serine (10 mM)	Boric acid (10 mM)
γ-L-Glutamyl-3-chloro -4-hydroxyaniline (45) (0.5 mM)	JB1	46 ± 6	18 ± 2	37 ± 2	37 ± 4	52 ± 7	42 ± 2
		(100)	(39)	(78)	(79)	(113)	(92)
	BL8L	86 ± 4	87 ± 1	78 ± 3	81 ± 7	84 ± 6	70 ± 0.5
		(100)	(101)	(91)	(94)	(97)	(82)
γ-L-Glutamyl-4-hydroxy aniline (43) (2 mM)	JB1	70 ± 1	24 ± 6	44 ± 4	48 ± 4	66 ± 3	66 ± 4
		(100)	(34)	(63)	(69)	(94)	(94)
	BL8L	32 ± 3	31 ± 2	26 ± 2	28 ± 7	34 ± 2	30 ± 3
		(100)	(97)	(81)	(87)	(106)	(93)
γ-L-Glutamyl-2-hydroxy -5-chloroaniline (44) (5 mM)	JB1	33 ± 2	19 ± 4	30 ± 1	29 ± 1	30 ± 1	32 ± 1
		(100)	(56.3)	(89)	(87)	(90)	(92)
	BL8L	117 ± 9	101 ± 8	97 ± 0.4	92 ± 1	117 ± 8	108 ± 2
		(100)	(86)	(83)	(79)	(100)	(92)

inhibitory effect on cell line growth. The numbers of cells in the presence of serine borate is 10-30% less that of the control. The inhibition is more pronounced for the JB1 than the BL8L cell lines, which suggests that the JB1 cell lines may require high levels of GGT for growth. Neither serine nor boric acid alone have a significant effect on the growth of either cell line.

4.3.3. Effect of exposure time on prodrug cytotoxicity

Because the *in vivo* lifetime of the γ-L-glutamyl adducts is probably less than 24h, the *in vitro* assessment of cytotoxicity against cell lines is best carried out over shorter time periods. Shorter *in vitro* tests were therefore carried out using Hanks Balanced Salt Solution (HBSS) as the incubation media. HBSS contains a combination of salts and glucose to maintain the osmotic and ionic equilibrium of cells and to provide them with a source of energy for briefest survival in the absence of serum. The use of HBSS avoids adventitious cleavage of the γ-L-glutamyl adducts by GGT normally present in serum. Further, Chahal et al[115] have shown that serum in the incubation media may react with and reduce the potency of electrophilic cytotoxic agents.

For the shorter time period cytotoxic tests the confluent cell lines were washed twice with HBSS (to remove all traces of serum) before exposure to the drugs in HBSS at 37°C. After an appropriate time, the drug was removed and the cells again washed twice with HBSS and then incubated for 24h in the usual supplemented Williams 'E' media. Subsequently, the cells were trypsinized and counted as before.

4.3.3.1 Parent hydroxyanilines

The usual incubation time for *in vitro* cytotoxicity tests is between 1 and 2h. Both JB1 and BL8L cell lines were therefore exposed to several concentrations of the parent hydroxyanilines for 2h in HBSS. The 2h cytotoxicity of the parent hydroxyanilines on the BL8L cell lines is reported in Figure 16. Clearly all three of the parent hydroxyanilines examined induce cell death and I_{50} values obtained are summarized in Table 18. Compounds (57) and (68) are the most toxic and (69) less so.

The 2h cytotoxicity of the parent hydroxyanilines on the JB1 cell lines is summarized in Figure 17 and the I_{50} values are listed in Table 18. Compounds (57) and (68) have similar I_{50} values whereas (69) is so much less toxic that the I_{50} value could not be determined.

These results are not strictly comparable with the 24h *in vitro* tests (see Section 4.3.1) because of the different media used. They do however, show similar trends in that the BL8L cells are more susceptible than the JB1 cell lines to the cytotoxic agents, and that (69) is the least cytotoxic.

4.3.3.2 γ-L-Glutamyl adducts

Neither (43) nor (45) showed measurable cytotoxicity to either JB1 or BL8L cell lines over 2h even for adduct concentrations up to 5 mM. Since the parent hydroxyanilines show cytotoxicity over 2h (see Section 4.3.3.1), it seems probable that very little cleavage of the γ-L-glutamyl adduct occurs in 2h. It follows that cleavage by GGT is the rate-limiting process for cytotoxic expression by the γ-L-glutamyl adducts.

Exposure of JB1 cell lines to 1 mM (45) (the concentration at which 90% cell kill was observed over 24h), for various periods of time

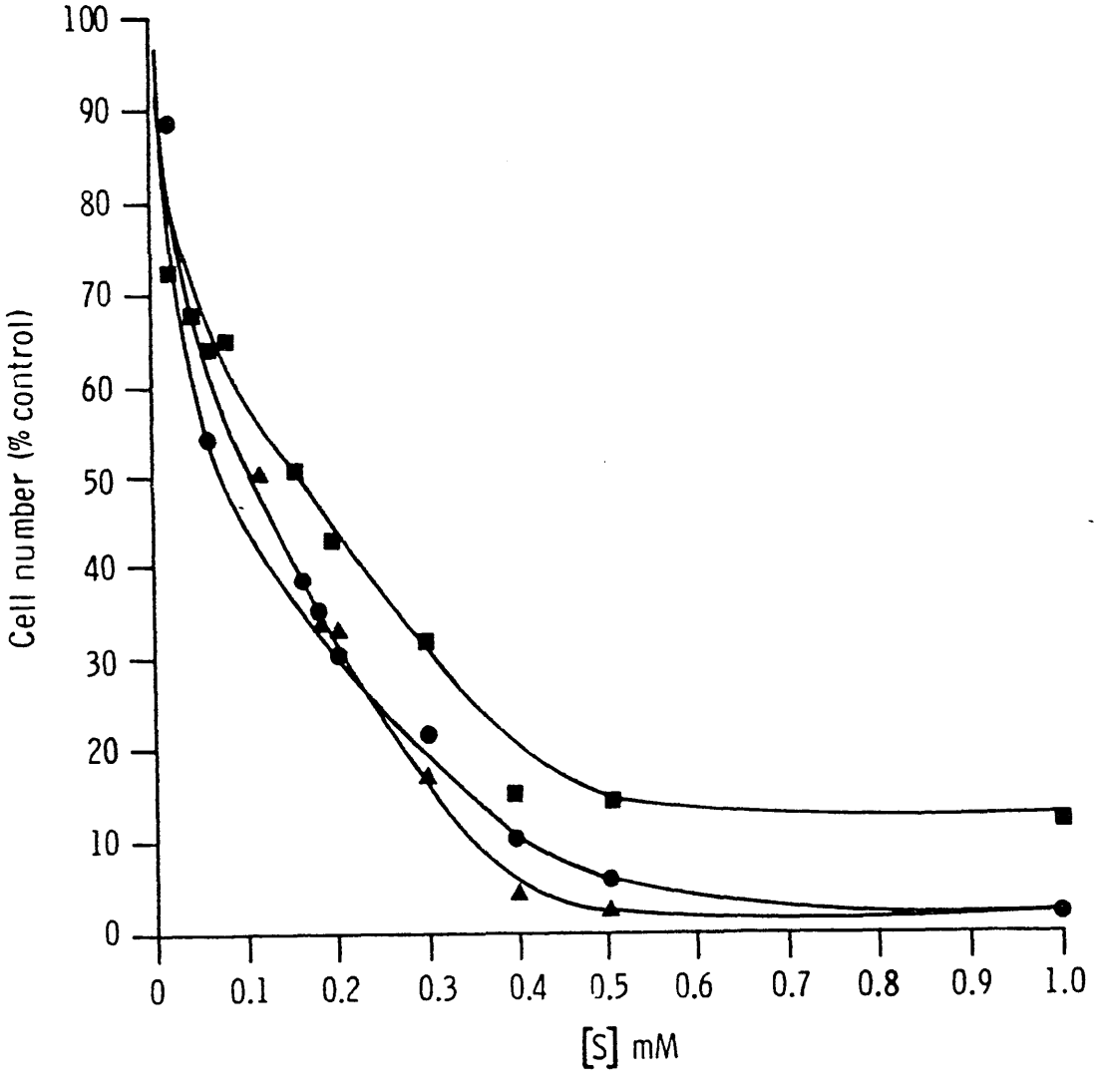


Figure 16. Plot of cell death (as % of control) against concentration of parent hydroxyaniline [S], for BL8L cell lines for a 2 h exposure at 37°C.

(●), 3-Chloro-4-hydroxyaniline (57).

(▲), 4-Hydroxyaniline (68).

(■), 2-Hydroxy-5-chloroaniline (69).

The results are the mean of 2 experiments.

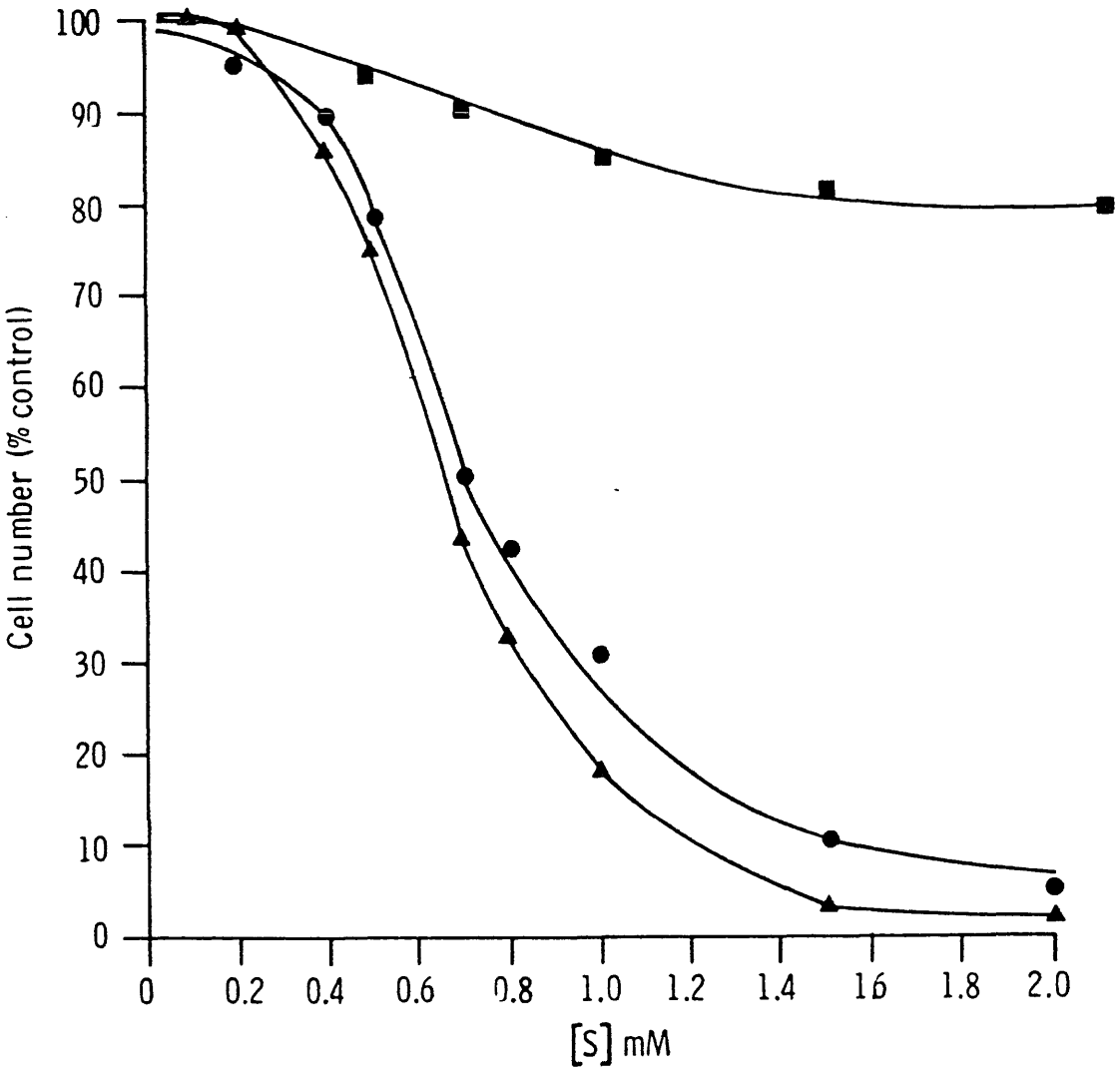


Figure 17. Plot of cell death (as % of control) against concentration of parent hydroxyaniline [S],for JB1 cell lines for a 2h exposure at 37°C.

(●), 3-Chloro-4-hydroxyaniline (57).

(▲), 4-Hydroxyaniline (68).

(■), 2-Hydroxy-5-chloroaniline (69).

The results are the mean of 2 experiments.

Table 18

The I_{50} values for the effect of the parent hydroxyanilines on BL8L and JB1 cell lines.

Preconfluent monolayers were exposed to the drugs for 2h at 37°C in HBSS.

Drug	I_{50} (mM)	
	BL8L	JB1
3-Chloro-4-hydroxyaniline (57)	0.075	0.675
4-Hydroxyaniline (68)	0.10	0.65
2-Hydroxy-5-chloroaniline (69)	0.15	>2

up to 6h in HBSS at 37°C was also observed to have little cytotoxic effect on the cell lines.

4.4. In vitro cytotoxicity of N-(2-chloroethyl)-N-nitrosourea (30) and α-(4-nitrophenyl)-γ-(N-(2-chloroethyl),N-nitrosocarbamoyl)-L-glutaminate (28) towards BL8L and JB1 cell lines

In view of concurrent work by Chahal et al[115] showing that the cytotoxicity of alkylating agents is reduced by their interaction with the serum content of supplemented Williams 'E' media, these experiments were carried out in HBSS at 37°C.

Further, because the stability of N-nitrosoureas in biological systems is low [116], the experiments were conducted over 2h. BL8L and JB1 cell lines were treated with the drugs as described in Section 4.3.3. The results obtained for N-(2-chloroethyl)-N-nitrosourea are shown in Figure 18, and for (28) in Figure 19. The corresponding I_{50} values are summarized in Table 19. It is evident that the BL8L cell line is more susceptible than the JB1 cell line to both N-(2-chloroethyl)-N-nitrosourea and its γ-L-glutaminate adduct. The I_{50} values for the BL8L and JB1 cell lines are 0.2 mM and 0.35 mM respectively, for N-(2-chloroethyl)-N-nitrosourea and 0.3 mM and 0.5 mM, respectively for (28). Thus (28) does not appear to differentiate between the 2 cell lines and its toxicity is very similar to that of the parent N-nitrosourea.

The cytotoxicity of (28) towards both cell lines was also examined in the presence of the serine borate inhibitor of GGT. The cell lines were exposed to 0.5 mM (28), in the presence and absence of 10 mM serine borate in HBSS for 2h, and then left to recover in supplemented Williams 'E' media for 24h. The surviving cell fraction was then trypsinized and counted as before. The results

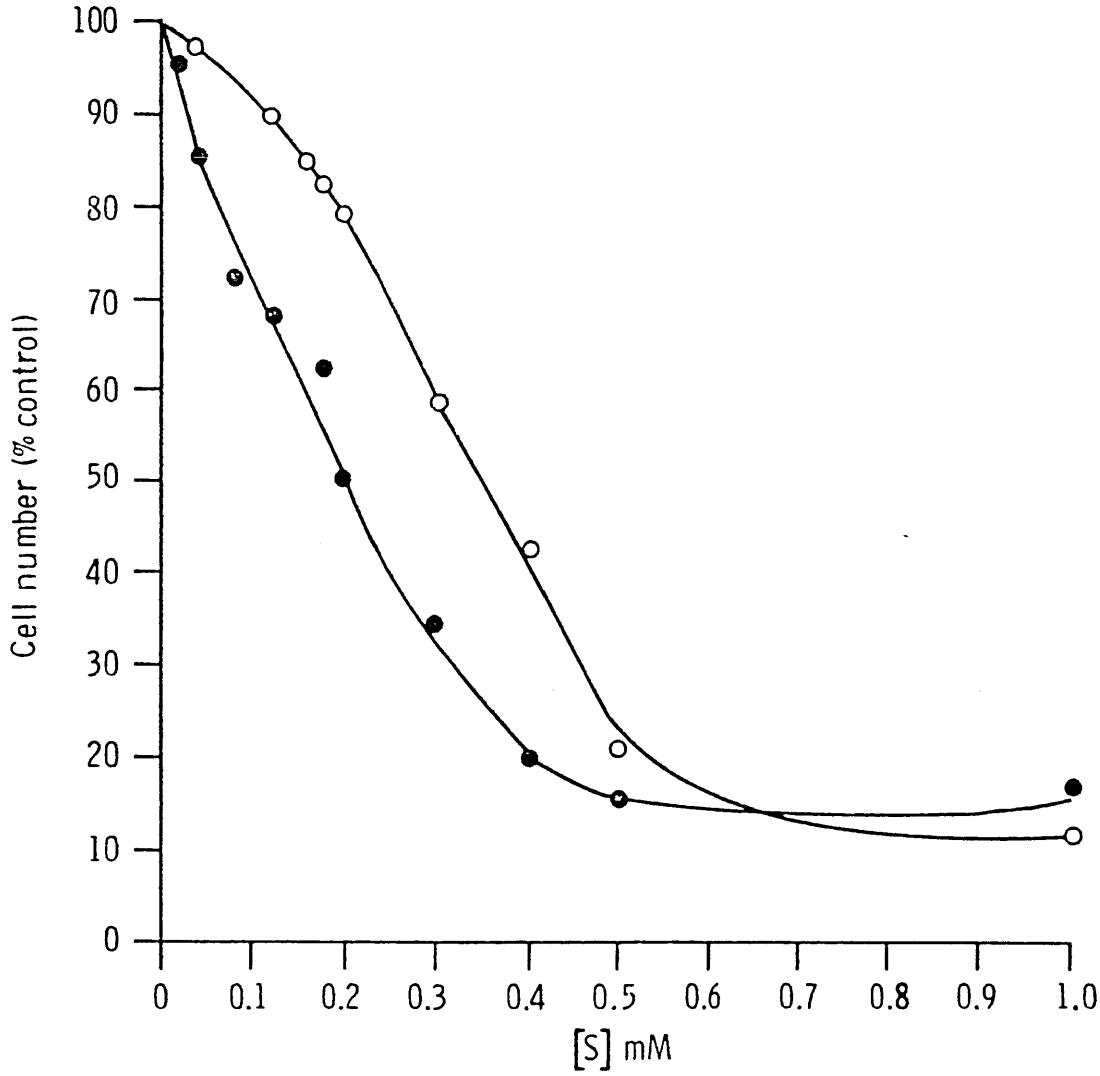


Figure 18. The effect of various concentrations of N-(2-chloroethyl)-N-nitrosourea (30) on JB1 (O) and BL8L (●) cell lines, for a 2h exposure at 37°C.

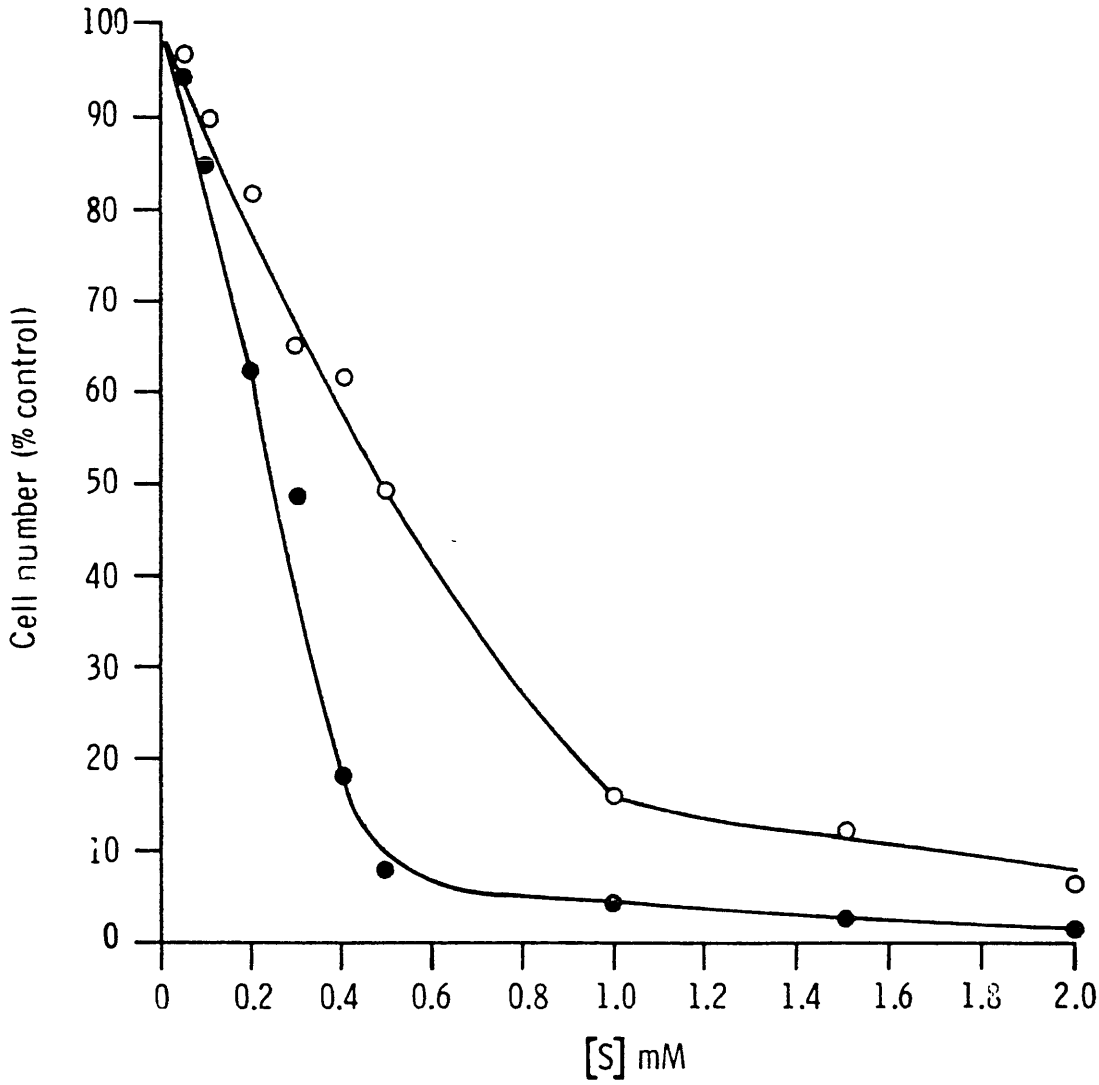


Figure 19. The effect of various concentrations of α-(N-nitrophenyl)-γ-(N-(2-chloroethyl), N-nitrosocarbamoyl)-L-glutaminate (28) on JB1 (○) and BL8L (●) cell lines, for a 2h exposure at 37°C.

Table 19

The I_{50} values for the effect of N-(2-chloroethyl)-N-nitrosourea (30) and α-(nitrophenyl)-γ-(N-(2-chloroethyl), N-nitrosocarbamoyl)-L-glutaminate (28) on BL8L and JB1 cell lines.

Preconfluent monolayer cultures were exposed to the drugs for 2h at 37°C in HBSS.

Drug	I_{50} (mM)	
	BL8L	JB1
N-(2-chloroethyl)-N-nitrosourea (30)	0.2	0.35
α-(4-nitrophenyl)-γ-(N-(2-chloroethyl), N-nitrosocarbamoyl)-L-glutaminate (28)	0.3	0.5

(Table 20) show that serine borate has no effect on the toxicity of (28) towards either the BL8L or the JB1 cell lines. Thus, the observed cytotoxicity of (28) is not related to its activation by GGT. The unselective toxicity of (28) may relate to its instability (see Chapter 3, Section 3.4.1). Even in the absence of GGT, (28) decomposes spontaneously at 37°C to give N-(2-chloroethyl)-N-nitrosourea among other products.

Cell kill by the N-nitrosourea derivatives was observed only on prolonged incubation. This concurs with suggestions that alkylating agents exert their cytotoxic effect by interaction with DNA. Thus, the major toxic effects occur during cell division when the DNA unwinds for replication. It follows that the enhanced cytotoxic susceptibility of the BL8L cell line relative to the JB1 cell line, may reflect their relative rates of proliferation. Chahal et al [115] found that the BL8L cell line proliferates more than twice as rapidly than the JB1 cell line.

4.5. Summary

The JB1 cell lines used in this investigation contained ca. 200 fold more GGT activity than the BL8L cell lines. This difference was sustained on passage from one incubation to another.

The *in vitro* cytotoxicity tests show that the parent hydroxy anilines are toxic to both the BL8L and JB1 cell lines over both 2h and 24h incubation. Over 2h, however, an I_{50} value could not be observed for (69) at the concentrations used. In general the 4-hydroxyanilines (57) and (68) were more cytotoxic and to similar extents than the 2-hydroxyaniline derivative (69).

The active form of the hydroxyanilines is probably the oxidized quinone-imine product. The lower cytotoxicity of (69) compared to (57) and (68), probably reflects it higher oxidation potential.

Table 20

Effect of serine borate on the toxicity of 0.5 mM (28) towards JB1 and BL8L cell lines. Results are the mean of 3 experiments. Figures in parentheses indicate the number of cells surviving as a percentage of the control.

	No additions (control)	(28) (0.5 mM)	(28) + Serine borate (10 mM)	Serine borate (10 mM)
JB1 cells ($\times 10^4$)	49 ± 1	21 ± 2	23 ± 2	43 ± 3
	(100)	(44)	(48)	(89)
BL8L cells ($\times 10^4$)	126 ± 14	8 ± 1	18 ± 0.2	118 ± 7
	(100)	(6)	(12.0)	(94)

All of the parent hydroxyanilines are more toxic towards the BL8L than the JB1 cell lines. The reason for this difference is not fully understood. It may relate to the faster proliferation rate of BL8L cells relative to the JB1 cells and their consequential increased vunerability to attack by electrophilic agents. Another factor may be different extents of glutathione detoxification of the parent hydroxyanilines by the 2 cell lines. The JB1 cell lines probably contain higher glutathione levels than the BL8L cell lines. The sigmoidal shape of the JB1 cytotoxicity curves, particularly for the 2h tests is consistent with this hypothesis.

All of the γ-L-glutamyl hydroxyaniline adducts were more cytotoxic towards the JB1 cell lines than the BL8L over 24h. This is opposite to the effect of the parent hydroxyanilines. Since all of the γ-L-glutamyl hydroxyaniline adducts are substrates for GGT (see Chapter 3, Section 3.4.2), their increased toxicity towards the JB1 cells must reflect the higher levels of GGT activity. Compound (45) was found to be the most potent prodrug tested, and this prodrug was also found to be the best substate for GGT. Compound (44) was found to be the least toxic.

Serine borate largely nullifies the toxicity of the γ-L-glutamyl hydroxyaniline adducts towards the JB1 cells, which supports the conclusion that cleavage of the compounds by GGT is required in order to produce cytotoxicity. Further, over 2h, the γ-L-glutamyl hydroxyaniline adducts showed no cytotoxicity towards the cell lines. This also suggests that the rate of cleavage of the adducts by GGT is an important factor in producing cytotoxicity.

Both N-(2-chloroethyl)-N-nitrosourea and the corresponding γ-L-glutaminate adduct (28) proved more cytotoxic towards BL8L than JB1 cells. Both compounds showed similar cytotoxicities, and the cytotoxicity of (28) was not affected by serine borate. Clearly

(28) does not differentiate between the GGT content of two cell lines and its cytotoxicity does not result from activation by GGT. This is good indication that (28) decomposes spontaneously to generate 2-chloroethyl alkylating moieties. Further, cell kill by N-(2-chloroethyl)-N-nitrosourea and (28) was not apparent until at least one cell division had taken place. This suggests that the enhanced susceptibility of the BL8L cells to the N-(2-chloroethyl)-N-nitrosourea compounds relates to their faster regeneration. The protective effect of higher glutathione levels in JB1 cells may also be a contributing factor.

CHAPTER 5

POSSIBLE MODE OF CYTOTOXIC ACTION OF 4-HYDROXYANILINE

5.1. Introduction

Many quinone anticancer drugs generate free radicals and active oxygen species through redox reactions. Quinone-imine compounds behave similarly. In this chapter the possibility of 4-hydroxyaniline being involved in redox cycling and free radical formation is addressed.

This was done by investigating the effect of 4-hydroxyaniline on oxygen consumption by whole and homogenized cell lines.

5.2 Effect of 4-hydroxyaniline (68) on oxygen consumption of whole cells

For the experiments involving intact cells, the oxygen uptake of trypsinized and washed JB1 hepatoma cells (0.5-1.0 mg/cm^3) in the presence of (68) (0.1 mM) and Williams 'E' media at 20°C, was measured in a Clark type electrode. The total volume of the reaction mixture was 2 cm^3 and details of the contents and the results are reported in Table 21. Whole cells in the absence of added (68) gave an endogenous oxygen consumption rate of 3.4 μmoles,h^{-1}, mg protein^{-1}. The rate was reduced to zero in the combined presence of mitrochondrial inhibitors antimycin A (10μg/cm^3) and Rotenone (22 μM). Addition of(68) (1 mM) to this solution gave an O_2 consumption rate of 0.8 μmoles,h^{-1}, mg protein^{-1}. A control experiment containing Williams 'E'media, the mitrochondrial inhibitors and (68), but where the intact cells were replaced by PBS, showed no O_2 uptake. Since the endogenous oxygen consumption of the JB1 cells was zero in the presence of the two mitrochondrial inhibitors, the stimulation induced by (68) is unlikely to arise from mitrochondrial respiration. This suggests that the site of O_2 uptake is the cytochrome P_{450} enzyme system as reported for other anticancer quinone type drugs[117].

Table 21

Stimulation of O_2 consumption by 4-hydroxyaniline (68) in intact JB1 cells

The control consisted of Williams 'E' buffer (1.5 cm^3), PBS (0.3 cm^3), (68) (1 mM in 0.19 cm^3 PBS), and rotenone (22 μM)/antimycin A (10 $\mu g/cm^3$) mixture (10 μl in DMSO).

Reaction mixture	Oxygen consumption (μmoles/h/mg protein)
Control	0
Williams'E' media (1.5 cm^3)	0
+ 0.3 cm^3 whole cells (0.5-1.0 mg/cm^3)	3.4
+ 10 μl rotenone (22 μM)/Antimycin A (10 $\mu g/cm^3$) mixure	0
+ 0.19 cm^3 4-hydroxyaniline (1 mM)	0.8

5.3. Effect of 4-hydroxyaniline (68) on oxygen consumption of sonicated cells in the presence of NADPH

To test the above conclusion further, O_2 consumption by sonicated cell suspensions in the presence of NADPH (a substrate in the cytochrome P_{450} pathway) was investigated. JB1 cells were trypsinized, washed and then suspended in 1 cm^3 of ammediol buffer (0.1 M, pH 8.2)*. The cells were sonicated for 10s before use and O_2 consumption was monitored in a Clark type electrode at 20°C. The sonicated cell suspension, 1 cm^3 (0.75 mg/cm^3) was added to 0.5 cm^3 of ammediol buffer (pH 8.2) in the electrode chamber and the exogenous O_2 consumption monitored. NADPH (7 mmol) in ammediol buffer (0.1 cm^3) was then added and oxygen uptake again monitored. Finally (68) (1 mM) in PBS (0.2 cm^3) was added and the stimulation in O_2 consumption examined. The results are shown in Figure 20.

The addition of sonicated JB1 cells alone gave an endogenous O_2 consumption rate of 163 nmoles,h^{-1}, mg $protein^{-1}$. This rate was almost doubled (i.e. 310 nmoles,h^{-1}, mg $protein^{-1}$) in the presence of NADPH (7 mM) and increased by ca. 5 fold in the presence of both NADPH (7 mM) and (68) (1 mM). A control experiment in which the sonicated JB1 cells were replaced with 1 cm^3 ammedoil buffer (0.1 M pH 8.2) gave no observable O_2 consumption. Thus, the increased consumption observed in the sonicated cell experiment was not due to the aqueous oxidation of (68) itself. An experiment using NADH in place of NADPH in the presence of sonicated cells in ammediol buffer (0.1 M, pH 8.2) showed no increase in O_2 consumption on addition of (68) (1 mM). This also suggests that increased O_2 consumption observed in the NADPH dependant reaction is not mitrochondrial.

*Since the optimal activity of NADPH dependent O_2 consumption by other quinone type drugs requires a pH between 8.0 and 8.5[118].

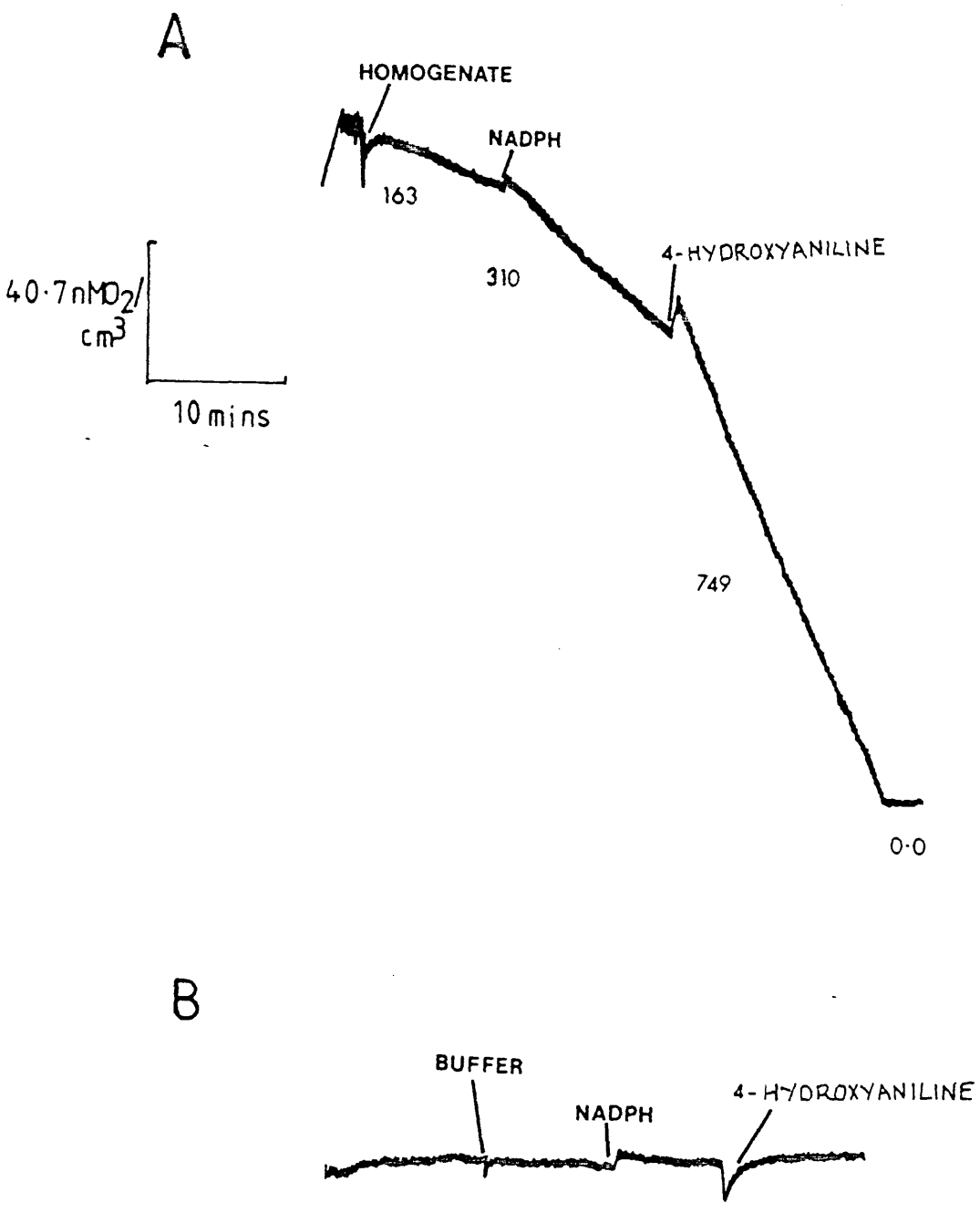


Figure 20. Stimulation of endogenous NADPH O_2 consumption by 4-hydroxyaniline.

(A) Sonicated cells.

(B) Control.

5.4 Conclusions

The above results show that oxygen consumption by JB1 cells is increased in the presence of (68). Further, they also suggest that the increase in O_2 consumption is not due to mitrochondrial respiration.

Many clinically useful antitumour drugs such as adriamycin, daunorubicin and mitomycin[119] contain a quinone or quinone-like moiety which has been implicated in their anticancer activity. The quinone moiety is able to generate free radical semiquinone intermediates and active oxygen species through redox processes[120]. Compound (68) is readily oxidized to a quinone-imine[120] which is expected to behave similarly to a quinone. The present results show that (68) stimulates NADPH - dependent O_2 consumption in sonicated JB1 cells. This may relate to electron transfer from NADPH to molecular O_2. Because of its presence in many cell compartments, NADPH cytochrome P_{450} reductase is regarded as the major enzyme catalysing redox cycling of drugs, although other enzymes may be important[117]. It is not known however, whether the production of oxygen radicals or NADPH depletion within the cell is the critical toxic event of redox cycling. Nonetheless, the bioreduction of the quinone-imine form of (68) by different electron transferring enzymes may be the mechanism by which (68) exerts its cytotoxicity.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions and future work

The possibility of synthesizing γ-L-glutamyl prodrugs for use as selective cytotoxic agents capable of activation at sites of high GGT activity has been investigated. The kinetics and specificity of the hydrolytic reaction of GGT has been examined and the structural requirements of the GGT donor site determined.

An early goal in the study was the synthesis of the γ-L-glutamyl adducts of N^3-substituted N-(2-chloroethyl)-N-nitrosoureas as putative prodrugs for GGT. However due to technical difficulties in their synthesis, particularly in the choice of protecting groups and subsequent deprotection, this proved impossible. In the attempted synthesis of N^3-substituted γ-L-glutamyl-N-(2-chloroethyl)-N-nitrosoureas, L-glutamic acid was first protected at its α-carboxyl and α-amino groups by forming the benzylester and N-benzyloxycarbonyl derivatives, respectively. Subsequent steps in the synthesis involved condensing the α-protected glutamic acid with an appropriate secondary amine (via a mixed anhydride reaction), reaction of the resulting N-substituted glutamine with 2-chloroethyl isocyanate to give the urea, aprotic nitrosation (with N_2O_4), and finally deprotection via catalytic hydrogenolysis over a palladium charcoal catalyst.

Problems were initially encountered when attempting to condense 2-chloroethyl isocyanate and N-substituted glutamines incorporating large N-substituents such as cyclohexyl and 2-chloroethyl moieties. A number of procedures were used in order to promote this reaction including using excess isocyanate, refluxing in high boiling point solvents such as xylene and DMSO, conducting the reaction under pressure in a Carius tube and the use of n-BuLi to generate the anion of the N-substituted glutamine, all of which failed. Similar observations have been shown in work by Wiley[100]. His explanation

for the unreactivity of secondary amides towards isocyanate is that the presence of a secondary group on the N-atom exerts some form of steric hindrance that prevents condensation.

The reaction between isocyanate and N-substituted glutamines bearing smaller N-substituents such as methyl and ethyl, however, could be made to proceed and hence the nitrosated derivatives of α-N-benzyloxycarbonyl-α-benzyl-(N-(2-chloroethyl)-carbamoyl, N-ethyl and N-methyl)-L-glutaminates were prepared. Aprotic nitrosation (with N_2O_4) of the N-methyl and ethyl derivatives was observed to occur at both the N-atom of the 2-chloroethyl terminus and the N-atom of the urethane side chain protection.

Attempts to remove the α-benzyl and α-benzyloxycarbonyl protection by hydrogenolysis over a 10% palladium on charcoal catalyst without simultaneous loss of the required N-nitroso group was unsuccessful, although it was possible to remove the α-benzyl protection from the N-ethyl derivative without substantial loss of the N-nitroso group.

Concurrent work into the structural requirements of the donor site of GGT has since shown that these compounds would probably be inactive as substrates for GGT due to the absence of a γ-peptide proton (as discussed below).

An investigation into the structural requirements of the GGT donor site was made by synthesizing a number of model γ-L-glutamyl adducts, some tertiary in nature and some secondary with various degrees of steric hindrance, and examining their activation by GGT. The model γ-L-glutamyl adducts, with the exception of (39), were prepared by the reaction of the appropriate amine with the mixed anhydride of α-N-benzyloxycarbonyl-α-benzyl-L-glutamate and ethyl chloroformate, followed by deprotection by hydrogenolysis. The compound (39) was synthesized by condensing

N-phthalyl-L-glutamic anhydride with N-methyl-4-nitroaniline, followed by removal of the phthalyl protecting group with hydrazine hydrate.

The enzyme GGT was extracted from rat kidney according to the method of Cook and Peters[19], involving the use of a phenyl boronate column.

Activation of the model γ-L-glutamyl derivatives by GGT was measured as the release of glutamic acid.

By kinetic studies it was found that all secondary compounds were substrates for GGT but that tertiary compounds were not. It has been suggested that this inactivity is due to the absence of a γ-N-H peptide proton.

The adduct structure, does not appear to be important in determining the selectivity of the substrate (providing a γ-peptide proton is present). Thus ring structures, long chain and branched-chain adducts were tolerated by the enzyme. The non-selectivity of the donor site has been reported previously[39]. Meister and co-workers[39] have shown that a number of substituted glutathiones and γ-glutamyl peptides may act as donors for the enzyme.

In this work attempts to analyze the structure required for binding at the enzyme active site have been made, and no correlation between the K_m determined for the various substrates and the adduct structure has been found.

It is apparent from the K_m expression, that in the case of a ping-pong enzyme the K_m does not approximate to the K_d for the substrate and is therefore not a reflection of its affinity for a substrate. However, of the variety of structures tested not one caused total abolition of donor activity except when the donor lacked a γ-peptide proton. This would suggest that the adduct is

not important in the primary binding of the substrate.

Conversely the enzyme does appear to be selective for the γ-glutamyl portion of the substrate. Using substituted glutamine analogues, it has been shown that the α-amino group is important in binding the substrate. The results from this work suggest that the enzyme recognises the γ-glutamyl peptide structure by the peptide bond proton and possibly the γ-carboxyl group and α-amino group. These findings have allowed the tentative "mapping out" of the GGT donor site and provide a useful tool with which to design future prodrugs.

In accordance with these findings an attempt was made to synthesize a secondary γ-L-glutamyl-N-(2-chloroethyl)-N-nitrosourea as a plausible prodrug. The synthetic route to such a compound was very similar to that used in the attempted preparation of the N^3-substituted, γ-L-glutamyl-N-(2-chloroethyl)-N-nitrosoureas, that is, reaction of the appropriate α-protected glutamine with isocyanate to give the corresponding urea, aprotic nitrosation with N_2O_4 and finally deprotection. Because of earlier problems encountered in the synthesis of the γ-L-glutamyl derivatives of the N^3-substituted nitrosoureas - particularly in the deprotection step, different protecting groups were used in its preparation. The α-carboxyl group of the γ-glutamyl moiety was protected with a nitrophenylester and the α-amino with a t-butyloxycarbonyl (BOC), (α-N-t-butyloxycarbonyl-α-(4-nitrophenyl)-L-glutaminate (25) was obtained commercially). Again there were problems in the final deprotection step. Although it was possible to remove the BOC protection at the α-amino group by acidic hydrolysis, subsequent attempts to remove the α-nitrophenylester from the α-carboxyl moiety proved unsuccessful and resulted in the simultaneous loss of the N-nitroso function. Since the partially protected compound

(28), possessed all the pre-requisites for substrate activity, namely a free γ-peptide proton and α-amino group, its activation by GGT was investigated. Unfortunately the instability of (28) in aqueous systems, which resulted in its spontaneous decomposition made it impossible to measure its effectiveness as a substrate for GGT and exposure of the drug to normal hepatocyte (BL8L) and hepatoma (JB1) cell lines resulted in no selective cytotoxic action.

γ-L-glutamyl hydroxyaniline derivatives were synthesized as alternative prodrugs for GGT. Their synthesis involved the protection of the γ-glutamyl moiety using the usual α-benzylic protecting groups, mixed anhydride coupling with the appropriate hydroxyaniline (protected at the hydroxy function), and finally deprotection. Compounds (40) and (41) were deprotected by hydrogenolysis over a palladium charcoal catalyst. Deprotection of (42) by this method resulted in the loss of the chlorine moiety and thus its deprotection was effected by acidic hydrolysis using a HBr/acetic acid mixture (40% HBr).

All three γ-L-glutamyl hydroxyaniline derivatives were found to be effective substrates for GGT, those incorporating chlorine groups in their adducts being the most rapidly hydrolysed.

The cytotoxic activity of the γ-L-glutamyl hydroxyaniline prodrugs and their parent hydroxyanilines was assessed by their effect on hepatoma (JB1) and normal hepatocyte (BL8L) cell lines.

The γ-L-glutamyl hydroxyaniline derivatives showed a selective cytotoxic response towards the JB1 cell line only, whereas the parent adducts were cytotoxic to both cell lines. Since all three prodrugs were shown to be substrates for GGT, their observed selectivity towards JB1 cells may be directly attributable to the

high GGT activity of the JB1 hepatoma cell line. The decrease in toxicity mediated by the GGT inhibitor serine borate supports the hypothesis that the prodrugs are activated in-situ by GGT. Furthermore, the reduced toxicity observed in the presence of the enzyme inhibitor and towards the BL8L cell lines (GGT poor), demonstrates that the prodrugs are of low toxicity compared to the enzyme activated form. Thus, these compounds offer a distinct advantage over conventional prodrugs where in many cases the prodrug itself is found to be slightly toxic.

The main disadvantage of using the γ-glutamyl hydroxyaniline derivatives as prodrugs for GGT is that relatively long exposure times to the cell lines were required for their cytotoxic effect to be observed. This is probably a reflection of their rate of hydrolysis by the enzyme.

It has been observed that the incorporation of electron withdrawing groups into the adduct of γ-L-glutamyl derivatives increases their rate of hydrolysis by GGT, and indeed the γ-glutamyl - chloro - substituted hydroxyaniline derivatives were observed to be hydrolysed more rapidly than their unsubstituted counterpart. Hence, it may be possible to increase the rate of delivery of the cytotoxic adduct to the cell lines by incorporating more electron withdrawing groups into the adduct and this is an avenue for future investigation.

In examining the possible mode of cytotoxic action of 4-hydroxyaniline it was observed that it is the quinone-imine form that is the most cytotoxic and that the drug stimulated a NADPH dependent oxygen utilisation in sonicated cells. This stimulated oxygen consumption may result from augmented transfer of an electron from NADPH to molecular oxygen, which results in the

formation of reactive oxygen species which may exert the cytotoxic effect.

Such a mechanism has been postulated for a number of quinone type antitumor agents[119] and more recently for quinone-imines[122,123]. NADPH cytochrome P-450 reductase localized on the endoplasmic reticulum is generally believed to be the terminal one-electron reductant of quinoid compounds. This type of reduction results in the formation of semi-quinone radicals or, upon redox cycling, in reductive activation of O_2. Although the evidence for redox cycling or processes in the cytotoxic mechanism of 4-hydroxyaniline is incomplete - future work could examine the possibility further. For example, the influence of reactive O_2 species such as H_2O_2 and superoxide on cytotoxicity could be investigated by the addition of enzymes such as catalase (removes H_2O_2) or superoxide dismutase (removes superoxide). Further the reduction of the quinone-imine to semi-quinone radicals could be examined by ESR.

An understanding of the mechanisms by which quinone-imines exert their toxicity could lead to the design of more selective quinoid antitumor agents.

CHAPTER 7

EXPERIMENTAL

7.1 General methods

Tissue culture materials were obtained from Flow Laboratories Ltd., Irvine, Ayrshire, and Isoton II solution from Coulter Electronics Inc. Cell lines used (designated BL8L and JB1) were obtained from the MRC Toxicology unit, Carshalton and were grown in an Haraeus B5060EKO_2 incubator.

All other reagents were obtained from commercial sources unless otherwise stated. All solvents and reagents were purified where necessary, by standard techniques[124]. HPLC analyses were conducted on a Varian Model 5000 instrument coupled to a Pye Unicam PU 4024 fluorescence detector. Fluorimetric measurements were made on a Perkin Elmer 204 fluorescence spectrophotometer. UV-visible measurements and spectra were recorded on either Perkin Elmer 550 or LKB 4050 spectrophotometers calibrated with a Holmium filter. Centrifugations were carried out in an MSC-coolspin centrifuge, model 6L.

Infra-red spectra were measured on either a Perkin Elmer 88I spectrophotometer or a Perkin Elmer 298 grating spectrophotometer both calibrated against polystyrene. ^{1}H-nmr spectra were recorded with a Jeol FX-90Q/90 MHz spectrometer using tetramethylsilane as the internal standard. Mass spectra were provided by the Chemistry Department (IC) service using a VG 7070 instrument, or by the SERC mass spectrometry centre, Swansea.

Melting points were measured on a Gallenkamp hot-stage apparatus and are uncorrected. Elemental microanalyses were provided by Mr. K. Jones of the Chemistry Department, Imperial College.

7.2 Biochemical Methods

7.2.1 Purification of γ- glutamyl transferase (GGT)

GGT was obtained from rat kidney and purified by the method of Cook and Peters[19]. Frozen kidneys were thawed and the ureters and capsules removed. The remaining tissue was then homogenized 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 resulting homogenate was then centrifuged (16000g) for 1 hour. The supernatant liquid was discarded and the pellet resuspended in 100mM Mops/HCl buffer (pH 7.2) and pelleted by centrifugation at 16000g to obtain washed membranes. The membranes were subjected to partial proteolytic digestion by papain which had previously been activated with 10 mM cysteine for 30 min at room temperature. The membranes were treated with 1 unit papain per mg membrane protein for 2h at 37°C and the mixture then centrifuged at 16000g for 30 min. The supernatant was fractionated with ammonium sulphate. The fraction from 50-100% saturation was collected by centrifugation at 16000g and resuspended in a minimal volume of 100 mM Mops/HCl buffer (pH 7.2). The resuspended fraction was then chromatographed on a PBA-60 phenyl boronate column (2.7 x 8.6 cm) pre-equilibrated with 100mM NH_4HCO_3 buffer (pH 8.7). The column was washed with approximately 4 column volumes of the equilibration buffer and the GGT eluted with equilibration buffer containing 10 mM Tris, pH 8.7. The enzyme was concentrated with an Amicon YM-10-ultra filtration membrane and then loaded onto a gel filtration column (G-200, 2.2 x 80 cm) pre-equilibrated with 50 mM Tris/HCl buffer (pH 7.5). Fractions containing the peak activity were concentrated by ultrafiltration as before. The purity of the enzyme was measured by polyacrylamide gel electrophorosis (8% acrylamide) in the

presence of SDS[105]. Two coomassie blue staining bands were observed, corresponding to the subunits of the enzyme.

The protein content of the enzyme was measured as in Section 7.2.2 and its activity assayed as in Section 7.2.3. Table 4 (Chapter 3) summarises quantitative details for a typical purification of GGT by this method.

7.2.2 Protein determination

Protein was measured by the method of Bradford[125], with bovine serum albumin as a standard, on a Perkin Elmer 550 spectrophotometer.

7.2.3 Assay of GGT activity

GGT activity was assayed by the fluorimetric method of Smith et al[109], on a Perkin Elmer 204 fluorescence spectrophotometer. A stock substrate solution of γ-L-glutamyl-7AMC (10 mM) in methoxyethanol was prepared and sonicated for 20s to obtain a homogeneous suspension. The stock solution (0.2 cm^3) was diluted with 9.8 cm^3 of 0.1 M ammediol/HCl buffer, pH 8.5, containing 20 mM glycylglycine and 0.1% (w/v) Triton X-100. Enzyme activity was assayed by the addition of enzyme solution (0.1 cm^3) to the diluted substrate solution (0.25 cm^3) in 6 cm^3 glass tubes and incubating at 37°C for ca. 6-8 min. The reaction was quenched by the addition of 2 cm^3 of ice-cold 0.05 M glycine buffer, pH 10.4 (2cm^3). Fluorescence of the resultant solution was read at 470 nm, with excitation at 340 nm and converted to nanomoles of GGT by reference to a standard fluorescence block, previously calibrated with 7AMC. Suitable enzyme and substrate blanks were run with each batch of assays.

7.2.4 Assay of γ-glutamyl hydrolase activity

By fluorimetry

Hydrolysis of γ-L-glutamyl-7AMC by GGT was assayed as in Section 7.2.3 but at pH 7.5 and omitting glycylglycine from the reaction mixture.

By UV spectrophotometry

Hydrolyis of γ-L-glutamyl-4-nitroanilide (59) and γ-L-glutamyl -N-methyl-4-nitroanilide (39) by GGT was monitored spectrophotometrically[108] on a Perkin Elmer 550 spectrophotometer. Enzyme solution (10 µl) was added directly to a cuvette containing substrate (0.99 cm^3 in Mops buffer, pH 7.2). For (59) concentrations of 0.8 mM were used and for (39),10 mM. The increase in absorbance at 405 nm was then monitored. The cuvette was thermostatted at ± 0.1°C.

By HPLC

Hydrolysis of other γ-glutamyl donors by GGT was measured by the HPLC method of Cook and Peters[107] on a Varian Model 5000 instrument coupled to a Pye Unicam PU 4024 fluorescence detector. Enzyme activity was determined by adding an aliquot of the enzyme solution (0.1 cm^3) to the substrate (0.25 cm^3 in 100 mM Mops buffer, pH 7.2) in a 6 cm^3 glass tube and incubating the mixture at 37°C for ca. 6-8 mins, depending on the particular substrate. The reaction was terminated by the addition of boiling Mops buffer, pH 7.2 (0.65 cm^3), followed by immersion of the tube in boiling water for 2 min. An aliquot (50 µl) of the terminated reaction mixture was mixed with an aqueous solution of 40 mM o-phthaldialdehyde (OPT)/49 mM mercaptoethanol (50 µl), to convert the glutamic acid liberated

to its OPT derivative. After 2 min the resulting OPT derivatized sample was chromatographed by reverse phase liquid chromatography on an ODS Hypersil column (150 nm x 5 mM) with an eluent consisting of 300 mM sodium acetate: 17% acetonitrile (pH 4.85), at a flow rate of 1.5 cm^3/min. The fluorescent OPT-glutamic acid derivative eluted at approximately 8 min. It was detected with a Pye Unicam PU 4024 fluorescence detector. The assay was calibrated against standard solutions of glutamic acid (0-200 pmoles) after their conversion to their OPT derivatives by an analogous procedure, plotting peak height against glutamic acid concentration.

By ammonia release

Substituted glutamine derivatives were assayed by ammonia release using a modified procedure of Fawcett and Scott[112].

Enzyme solution (0.1 cm^3 in Mops buffer, pH 7.2) was added to the substrate solution (0.25 cm^3 in Mops buffer, pH 7.2) in 6 cm^3 glass tubes and the mixture incubated at 37°C for 20 min. The reaction was terminated by immersing the tubes in boiling water for 2 min. After cooling 0.3 cm^3 of nitroprusside solution (62.5g phenol, 0.315g sodium prusside per litre) was added followed immediately by 0.3 cm^3 alkaline hypochlorite (30g NaOH, 12.5 cm^3 sodium hypochlorite, per litre). The final volume was 0.95 cm^3. The reaction solutions were stirred vigorously and then re-incubated at 37°C for 20 min to develop a blue colour, the absorbance of which was measured at 630 nm on a Perkin Elmer 550 spectrophotometer. The yield of ammonia was estimated from a calibration curve generated with standard solutions (0-40 nmol) of ammonium bicarbonate (Figure 5, Chapter 3).

7.2.5 Tissue culture

Two cell lines designated BL8L and JB1 were used. BL8L is a dividing cell line derived from a maintenance hepatocyte culture isolated from a normal adult male Fischer 344 rat. JB1 is a dividing cell line derived from an aflatoxin-induced hepatocellular carcinoma in an adult male Fischer 344 rat.

The cell lines were grown in 90 mm plastic tissue culture dishes in 10 cm^3 of Williams 'E' medium supplemented with 5% foetal calf serum, 2 mM glutamine and 50 µg gentamycin/cm^3. The incubations were carried out at 37°C in a 5% CO_2, 95% air atmosphere in an Heraeus B5060EK/O_2 incubator.

The cells were subcultured in the following way. The media was first removed from the plates by aspiration, and the cells trypsinized with 7 cm^3 of a trypsin:phosphate buffer saline (PBS) mixture (0.025% (w/v) trypsin) for 1 min. The trypsin: PBS mixture was then removed by aspiration and the residue incubated for 5 min. The cells were then resuspended in fresh Williams 'E' media (10 cm^3), diluted 75% (v/v) with supplemented Williams 'E' media and replated (10 cm^3 per plate).

7.2.6 Assay of GGT activity in the cell lines

The media was aspirated from samples of the cell lines cultivated as described in Section 7.2.5, and replaced with 5 cm^3 of 100 mM Mops buffer (pH 7.2). The cells were scraped from the plate and pelleted by centrifugation (2500g, 5 min) at 4°C. The pellet was resuspended in ca. 5 cm^3 of 100 mM Mops/HCl buffer (pH 7.2) containing 1% (w/v) Triton X-100. The GGT activity of this solution was measured as in Section 7.2.3 and its protein content as in Section 7.2.2.

Measurements were made for a preconfluent monolayer of cells.

Experiments were conducted in triplicate and a mean taken. Table 14 (Chapter 4) shows typical results obtained for the assay of GGT activity in both cell lines.

7.2.7 Cytotoxicity testing

All of these experiments were conducted on preconfluent monolayers of cells.

7.2.7.1 24h Exposure to drug

The media was removed by aspiration from plates of cell lines cultured as described in Section 7.2.5. The cells were then exposed to a range of concentrations of the drug (in 10 cm^3 of supplemented Williams 'E' buffer). An equal volume of supplemented Williams 'E' buffer without drug was added to separate plates to act as controls. The plates were then incubated for 24h at 37°C in a 5% CO_2, 95% air atmosphere.

After incubation the medium was removed by aspiration and replaced with 10 cm^3 of trypsin: PBS mixture (as in Section 7.2.5). The cells were then further incubated for 10-15 min, transferred to centrifuge tubes and finally centrifuged (2500g, 5 min) at 4°C to obtain a pellet. The pellet was resuspended in 5 cm^3 of Isoton II solution (Coulter Electronics Inc), homogenized gently, diluted as appropriate (usually 1/100) and counted (Coulter Counter model ZF, aperature set to 32, attentuation to 1.) Each drug was tested in duplicate and each dilution counted in triplicate and the mean taken.

7.2.7.2 2h Exposure to drug

The procedure was similar to that of Section 7.2.7.1 except cells were exposed to the drug for 2h in 10 cm^3 of Hanks Balanced Salt

Solution (HBSS), were washed before and after exposure with HBSS (2 x 10 cm^3) and were allowed to recover for 24h in supplemented Williams 'E' medium prior to counting.

7.2.7.3. Serine borate inhibition

Inhibition of GGT activity by serine borate was determined by the method of Tate and Meister[114]. The media from cell cultures was removed by aspiration and the residual cells exposed to an appropriate concentration of the γ-glutamyl prodrug (usually the I_{50} concentration in Williams'E; medium) in the presence and absence of serine borate (10mM). The final volume was 10 cm^3. For comparative purposes cells were grown as normal in 10 cm^3 Williams 'E' media, and also in the presence of serine (10 mM) and boric acid (10 mM) separately. All plates were then incubated for 24h at 37°C in a 5% CO_2, 95% air atmosphere and subsequently trypsinized and counted as in Section 7.2.7.1.

For 2h drug exposure experiments the cells were washed before and after exposure with HBSS (as in Section 7.2.7.2) and tested for inhibition by 10 mM serine borate in HBSS (10 cm^3) in the presence and absence of the prodrug.

The controls comprised cells grown normally in Williams'E' media, (10mM) and in the presence of serine and boric acid acid (10mM) separately as discussed above.

All experiments were conducted in triplicate and a mean taken. Table 22 shows typical results for the effect of serine borate inhibition on the toxicity of γ-L-glutamyl-4-hydroxyaniline (43) on BL8L and JB1 cell lines.

7.2.8 Oxygen consumption experiments

The oxygen content of reaction mixtures incorporating JB1 cell

Table 22

Effect of serine borate on the toxity of γ-L-glutamyl-4-hydroxyaniline towards BL8L and JB1 cell lines for 24h exposure.

(Results are the mean of 3 experiments, figures between parentheses indicate the number of cells surviving as a percentage of the control).

	No of cells ($x10^5$)			
Cell type	No. Additions (control)	γ-L-glutamyl -4-hydroaniline (2 mM)	γ-L-glutmyl -4-hydroxyaniline (2 mM & serine/ borate (10 mM)	Serine borate (10mM)
JB1	7.01 ± 0.13	2.4 ± 0.27	4.4 ± 0.13	4.82 ± 0.4
	(100)	(34)	(63)	(69)
BL8L	3.2 ± 0.03	3.0 ± 0.16	2.75 ± 0.2	2.8 ± 0.18
	(100)	(93)	(85)	(87)

lines was determined polarographically with a Clark type eletrode (Rank Bros). The oxygen saturation of water at 20°C was taken as 285 nmol/cm^3 [126]. Protein content of the cell samples was determined as in Section 7.2.2.

7.2.8.1 Whole cell measurements

For whole cell measurements, samples of JB1 cells were trypsinized (as in Section 7.2.7.1), centrifuged (2500g, 5 min) at 4°C, washed with PBS (5 cm^3) and finally centrifuged as above. The cells were then resuspended in PBS (1 cm^3) and stored on ice before use.

Whole cells, 0.3 cm^3 (0.5-1.0 mg/cm^3) were added to supplemented Williams 'E' media (1.5 cm^3) in the electrode chamber, and endogenous oxygen consumption at room temperature measured over ca. 5 min. Endogenous oxygen consumption was first reduced to zero by the addition of a 10 µl mixture of rotenone (22 µM) and antimycin A (10 µg/cm^3) in DMSO . 4-Hydroxyaniline (1 mM) in PBS (0.2 cm^3) was then added, and the oxygen consumption at room temperature measured over 10 min. For controls, PBS (0.3 cm^3) was used in place of whole cell suspension.

7.2.8.2 Sonicated cell measurements

For sonicated cell measurements, samples of JB1 cells were trypsinized (as in Section 7.2.7.1), centrifuged (2500g, 5 min) at 4°C, washed with 5 cm^3 of ammediol buffer (0.1 M, pH 8.2), centrifuged again (2500g, 5 min) at 4°C and then resuspended in 1 cm^3 of ammediol buffer (0.1 M, pH 8.2). The cells were sonicated for 10s before use.

Sonicated cell suspension, 1 cm^3 (0.75 mg/cm^3) was added to 0.5 cm^3 of ammediol buffer (0.1 M, pH 8.2) in the electrode chamber and the endogenous oxygen consumption at room temperature measured

over ca. 10 min. A solution of NADPH (7 x 10^{-3} M) in ammediol buffer (0.1 cm^3) was added and the stimulated oxygen uptake monitored at room temperature for ca. 15 min. Finally 4-hydroxyaniline (1.0 mM) in PBS (0.2 cm^3) was added and the increased oxygen consumption again measured at room temperature over 20 min.

7.3 Chemical Methods

7.3.1 Hydrolysis of α-(4-nitrophenyl)-γ-(N-(2-chloroethyl), N-nitrosocarbamoyl)-L-glutaminate (28)

Hydrolysis of (28) at 25°C was examined by the measurement of N-(2-chloroethyl)-N-nitrosourea release, by HPLC on a ODS Hypersil column (5 x 150 mm) using a Varian Model 5000 liquid chromatograph with a Pye Unicam PU 4010 UV detector. No precolumn was used. Authentic N-(2-chloroethyl)-N-nitrosurea was used to calibrate the assay. A solution of (28) (2 mM) in methanol:water (1:1 v/v) was prepared and kept at 25°C in a waterbath. At timed intervals, aliquots (50 µl) of the reaction solution were taken and injected directly onto the HPLC. Elution of N-(2-chloroethyl)-N-nitrosourea. (Retention time = ca. 4.3 min) was by methanol:water (1:1 v/v, pH 7.2) at a flow rate of 1 cm^3/min. The eluent was monitored at λ_{max} 245 nm. Figure 8 (Chapter 3) shows a typical HPLC trace for the hydrolysis of (28) at 25°C.

7.3.2 Purification of γ-glutamyl derivatives by ion exchange

L-Glutamic acid impurity was removed fom all putative γ-glutamyl

donor substrates by ion exchange using Amberlite IRA-45 resin in the acetate form. Amberlite IRA-45 (200 g) was converted to the acetate form by washing the resin successively with water(1000 cm), HCl (5 M, 1000 cm^3), water (until neutral), NaOH (5 M, 1000 cm^3), water (until neutral) and finally sodium acetate (10 M, 1000 cm^3). Before use the resin was washed again with water (4 column volumes). The γ-glutamyl derivatives were loaded and eluted from the column using distilled water, L-glutamic acid is retained. The fractions containing the purified substrates were collected and concentrated by freeze drying.

7.3.3. Synthesis

α-N-Benzyloxycarbonyl-α-benzyl-L-glutamate (9)

α-N-Benzyloxycarbonyl-α-benzyl-L-glutamate was prepared following a literature procedure[96]. This involved the reaction of L-glutamic acid with benzylchloroformate in aqueous alkali to give the α-N-benzyloxycarbonyl-L-glutamic acid derivative, which was subsequently reacted with benzylbromide to give the α-protected ester in yields of 60-70%, $\underline{\upsilon}_{max}$ (Neat) 3370, 2900, 1740 (C=O,ester), 1690 (C=O, urethane), 750 cm^{-1} (C-H, monosubstituted benzene ring); $\underline{\delta}_H$ ($CDCl_3$) 2.4 (4H, m, CH_2CH_2), 4.1 (1H, q, CH), 5.1 (2H, s, $C\underline{H}_2C_6H_5$), 5.7 (1H, d, NH), 7.3 ppm (10H, s, $2C_6H_5$).

α-N-Benzyloxycarbonyl-α-benzyl-γ-N-methyl-L-glutaminate (12).

α-N-Benzyloxycarbonyl-α-benzyl-L-glutamate (3 g, 8.1 mmol) and triethylamine (1.12 cm^3, 8.1 mmol) were dissolved in dry tetrahydrofuran (THF), (100 cm^3) and cooled to -10°C. Ethyl chloroformate (0.7 cm^3, 7.3 mmol) in dry THF (3 cm^3) was added dropwise with stirring and the temperature maintained at -10°C.

Subsequently the solution was stirred for 30 min at -10°C. Methylamine (0.38 cm^3, 8.1 mmol) in dry THF (3 cm^3) was added dropwise with stirring to the mixed anhydride, and the mixture left to warm to room temperature. The THF was removed under vacuum and the residue dissolved in dry dichloromethane (100 cm^3). The solution was then washed with 0.1 HCl (3 x 100 cm^3), 5% sodium carbonate (2 x 100 cm^3) and finally with water (100 cm^3). After drying over anhydrous $MgSO_4$, removal of the solvent under vacuum gave α-N-benzyloxycarbonyl-α-benzyl-γ-N-methyl-L-glutaminate as a white solid which was recrystrallized from toluene. Yield 2.1g (68%), m.p. 98°C, ν_{max} (nujol) 3330 (NH), 1740 (C=O, ester), 1700 (C=O, urethane), 1635 (C=O, amide), 735 and 700 cm^{-1} (C-H monosubstituted benzene ring); δ_H ($CDCl_3$), 1.9-2.4 (4H, m, CH_2CH_2), 2.7 (3H, d, CH_3), 4.4 (1H, m, CH), 5.1 (2H, s, $C\underline{H}_2C_6H_5$), 5.17 (2H, s, $C\underline{H}_2C_6H_5$), 5.625 (1H, d, NH), 7.35 ppm (10H, s, $2C_6H_5$); m/z (EI) 384 (M^+), 276 (M^+-OCH_2Ph).

<u>α-N-Benzyloxycarbonyl-α-benzyl-γ-(N -(2-chloroethyl)-carbamoyl, N-methyl)-L-glutaminate</u> (<u>16</u>)

α-N-Benzyloxycarbonyl-α-benzyl-γ-N-methyl-L-glutaminate (0.96g, 2.49 mmol) was suspended in sodium dried toluene (150 cm^3) and 2-chloroethyl isocyanate (2.13 cm^3, 25 mmol) added. The solution was boiled under reflux for 48h and following removal of the solvent under vacuum, <u>α-N-benzyloxycarbonyl-α-benzyl-γ-(N -(2-chloroethyl)-carbamoyl, N-methyl)-L-glutaminate</u> was obtained as a yellow oil. The oil was purified by column chromatography on silica using ethyl acetate as eluent. Yield 0.82g (67%), (Found: C, 58.86; H,5.80; N,8.50. $C_{24}H_{28}N_3OCl$ requires C,58.84; H,5.72; N,8.58%); ν_{max} ($CHCl_3$) 1740 (C=O, ester), 1700 (C=O, urethane), 1665 cm^{-1}(C=O, urea); δ_H ($CDCl_3$), 1.9-2.5 (4H, m, CH_2CH_2), 3.1

(3H, s, CH_3), 3.6 (4H, s, $ClCH_2CH_2$), 4.4 (1H, m, CH), 5.1 (2H, s, $C\underline{H}_2C_6H_5$), 5.17 (2H, s, $C\underline{H}_2C_6H_5$), 5.6 (1H, d, NH), 7.35 ppm (10H, s, $2C_6H_5$); m/z (FAB +ve ion) 490 (MH^+), 492 (MH^++2).

<u>α-(N-Benzylycarbonyl,N-nitroso)-α-benzyl-γ-(N -(2-choroethyl)-N - nitrosocarbamoyl), N-methyl)-L-glutaminate</u> (<u>22</u>)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(N-(2-chloroethyl)-carbamoyl), N-methyl)-L-glutaminate (2.50g, 5.1 mmol) and sodium acetate (2.1g, 25.5 mmol) in dry dichloromethane (100 cm^3) was cooled to 0°C Liquid N_2O_4 (6.5 cm^3, 0.1 mol) was added and the solution stirred at 0°C for 5 h. The reaction solution was filtered and the filtrate washed successively with water (100 cm^3, 0°C), 5% Na_2CO_3 (100 cm^3, 0°C) and water (100 cm^3, 0°C). The organic fraction was separated, dried over anhydrous $MgSO_4$ and the solvent removed under vacuum at room temperature. The residual yellow oil was purified by flash column chromatography on silica using ethyl acetate: 40-60°C petroleum ether (1:1) as eluent. Yield 1.63g (58%), $\underline{\lambda}_{max}$ (EtOH) 400, 420nm (indicative of N-N=0); $\underline{\nu}_{max}$ (CCl_4) 1750 (C=0, ester and urethane), 1720 (C=0, urea), 1520 cm^{-1} (N=0); $\underline{\delta}_H$ ($CDCl_3$), 2.0-2.7 (4H, m, CH_2CH_2), 3.3 (3H, s, CH_3), 3.5 (2H,t,CH_2Cl), 4.1 (2H,t,CH_2N), 5.0 (2H, s, $C\underline{H}_2C_6H_5$), 5.2 (1H, m, CH), 5.4 (2H, s, $C\underline{H}_2C_6H_5$), 7.3 ppm (10H, m, $2C_6H_5$; m/z (FAB, + ve ion) 548 (MH^+), 550 (MH^++2), 488 (MH^+-2N=0), 353, 262. A satisfactory microanalysis could not be obtained for this compound.

<u>α-N-Benzyloxycarbonyl-α-benzyl-γ-N-ethyl-L-glutaminate</u> (<u>13</u>)

α-N-Benzyloxycarbonyl-α-benzyl-γ-N-ethyl-L-glutaminate (<u>13</u>) was prepared in a similar manner to that described for the synthesis of (<u>12</u>), using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (2g, 5.4 mmol), triethylamine (0.75 cm^3, 4.8 mmol), ethylchloroformate (0.46

cm^3, 4.8 mmol) and ethylamine (0.24 cm^3, 5.4 mmol). The product was recrystallized from ethyl acetate to give a white crystalline solid. Yield 1.29g (60%), m.p. 111°C (lit.,[126] 111-112°C); ν_{max} (nujol) 3300 (NH), 1730 (C=O, ester), 1700 (C=O, urethane), 1640 cm^{-1} (C=O, amide); δ_H ($CDCl_3$) 1.05 (3H,t, $CH_2\underline{CH_3}$), 1.86 (2H, m, CH_2), 2.15 (2H, m, CH_2), 3.20 (2H, q, $\underline{CH_2}CH_3$), 4.35 (1H, m, CH), 5.00 (2H,s,$\underline{CH_2}C_6H_5$), 5.10 (2H,s,$\underline{CH_2}C_6H_5$), 7.35 (10H,s,$2C_5H_6$), 7.6 (1H,d,NH); m/z (EI) 318 (M^+).

<u>α-N-Benzyloxycarbonyl-α-benzyl-γ-(N-(2-chloroethyl)-carbamoyl, N-ethyl)-L-glutaminate</u> (<u>17</u>)

<u>α-N-Benzyloxycarbonyl-α-benzyl-(N-(2-chloroethyl)-carbamoyl, N-ethyl)-L-glutaminate</u> (<u>17</u>) was prepared in a similar manner to compound (<u>16</u>), using α-N-benzyloxycarbonyl-α-benzyl-γ-N-ethyl -L-glutaminate (<u>13</u>) (1g, 2.5 mmol) and 2-chloroethyl isocyanate (2.1 cm^3, 25 mmol). The desired product (<u>17</u>) was obtained as a yellow oil. Yield 0.82g (52%), (Found: C, 59.46; H, 5.80; N, 8.24. $C_{25}H_{30}N_3O_6Cl$ requires C, 59.58; H, 5.95; N, 8.34%); ν_{max} ($CHCl_3$) 1730 (C=O, ester), 1700 (C=O, urethane), 1660 cm^{-1}(C=O, urea); δ_H ($CDCl_3$) 1.1 (3H, t, CH_3), 2.0-2.5 (4H, m, CH_2CH_2), 3.6 (6H, s + q, $ClCH_2CH_2$ and $\underline{CH_2}CH_3$), 4.4 (1H, m, CH), 5.1 (2H, s, $\underline{CH_2}C_6H_5$), 5.17 (2H, s, $\underline{CH_2}C_6H_5$), 5.45 (1H, d, NH), 7.35 ppm (10H, s, $2C_6H_5$); m/z (FAB +ve ion) 504 (MH^+).

<u>α-(N-Benzyloxycarbonyl,N-nitroso)-α-benzyl-γ-(N-(2-chloroethyl) -N-nitrosocarbamoyl, N-ethyl)-L-glutaminate</u> (<u>23</u>).

<u>α-(N-Benzyloxycarbonyl,N-nitroso)-α-benzyl-γ-(N-(2-chloroethyl) -N-nitrosocarbamoyl, N-ethyl)-L-glutaminate</u> (<u>23</u>) was prepared as for compound (<u>22</u>) from α-N-benzyloxycarbonyl-α-benzyl-γ-(N-(2-chloroethyl)-carbamoyl,N-ethyl)-L-glutaminate (<u>17</u>) (2.1g,

4.35 mmol), sodium acetate (1.8g, 20 mmol) and liquid N_2O_4 (5.5 cm^3, 86 mmol). The product was obtained as a yellow oil. Yield 1.78g (78%), λ_{max} (EtOH) 383.9 (sh), 400.5 and 417 nm (indicative of N-N=O), ν_{max} (CCl_4) 1747 (C=O ester and urethane), 1720 (C=O, urea), 1519 cm^{-1} (N=O); δ_H ($CDCl_3$), 1.2 (3H,t,CH_3), 1.9-2.6 (4H, m, CH_2CH_2), 3.5 (2H, t, CH_2Cl), 3.8 (2H, q, CH_2CH_3), 4.1 (2H, t, CH_2N-N=O), 5.0 (2CH, s, $CH_2C_6H_5$), 5.2 (1H, m, CH), 5.4 (2H, s, $CH_2C_6H_5$), 7.3 ppm (10H, m, $2C_6H_5$); m/z (FAB +ve ion), 562 (MH^+), 502 (MH^+-2N=O), 427.

α-N-Benzyloxycarbonyl-N-(γ-N-ethyl-L-glutamyl)-N′-(2-chloroethyl)-N′-nitrosourea (24)

α-(N-Benzyloxycarbonyl,N-nitroso)-α-benzyl-γ-(N-(2-chloroethyl) - N-nitrosocarbamoyl, N-ethyl)-L-glutaminate (23) (0.25g, 0.45 mmol) was dissolved in ethanol (10cm^3) and 50 mg of 10% palladium charcoal catalyst was added. The solution was hydrogenated until 0.5 molar equivalents of hydrogen had been taken up. Hydrogenation was then terminated and the solution filtered. Removal of the solvent at room temperature under vacuum gave a yellow oil which was purified by flash chromatography on silica using dichloromethane as the eluent. Evaporation of the solvent gave α-N-benzyloxycarbonyl-N-(γ-N-ethyl-L-glutamyl), N′-(2-chloroethyl)-N′-nitrosourea as a viscous yellow oil. Yield 60 mg (30%), λ_{max} (EtOH) 403.3, 420 nm (indicative of N-N=O); ν_{max} (CCl_4) 3400 (NH), 1726 (C=O, urea), 1700 (C=O, urethane), δ_H ($CDCl_3$) 1.2 (3H, t, CH_3), 2.0 (2H, m, CH_2), 2.5 (2H, m, CH_2), 3.2 (1H, m, CH), 3.55 (2H, t, CHCl), 3.8 (2H, q, CH_2CH_3), 4.15 (2H, t, CH_2N-N=O), 5.1 (2H, s, $CH_2C_6H_5$), 7.3 ppm (5H, s, C_6H_5). A satisfactory microanalysis could not be obtained for this compound.

α-N-Benzyloxycarbonyl-α-benzyl-γ-N-(2-chloroethyl)-L-glutaminate (14)

α-N-Benzyloxycarbonyl-α-benzyl-γ-N-(2-chloroethyl)-L-glutaminate (14) was prepared in a similar manner to that described for compound (12) using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (1g, 2.7 mmol), triethylamine (0.27 cm^3, 2.7 mmol), ethylchloroformate (0.23 cm^3, 1.67 mmol), and 2-chloroethylamine hydrochloride (0.31g, 2.7 mmol). The desired product was recrystallized from toluene. Yield 0.73g (63%), m.p. 112-114°C; υ_{max} (nujol) 3380 (NH), 1750 (C=O, ester), 1700 (C=O, urethane), 1650 (C=O, amide), 760 cm^{-1} (C-Cl); δ_H ($CDCl_3$) 2.0 - 2.4 (4H, m, CH_2CH_2), 3.6 (4H, m, CH_2CH_2Cl), 4.4. (1H, m, CH), 5.1 (2H, s, $C\underline{H}_2C_6H_5$), 5.17 (2H, s, $C\underline{H}_2C_6H_5$), 7.35 ppm (10H, s, $2C_6H_5$); m/z (EI) 432 (M^+).

α-N-Benzyloxycarbonyl-α-benzyl-γ- N-cyclohexyl-L-glutaminate (15)

α-N-Benzyloxycarbonyl-α-benzyl-γ-N-cyclohexyl-L-glutaminate (15) was prepared as for compound (12), using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (1g, 2.7 mmol), triethylamine (0.27cm^3, 2.7 mmol), ethylchloroformate (0.23 cm^3. 1.67 mmol) and cyclohexylamine (0.31 cm^3, 2.7 mmol). The product was recrystallized from toluene. Yield 0.65g (54%), m.p. 140°C, (Found: C, 69.05; H, 7.23; N, 6.40. $C_{26}H_{32}N_2O_5$ requires: C, 69.02; H, 7.1; N, 6.2%); υ_{max} (nujol) 3330 (NH), 1735 (C=O, ester), 1700 (C=O, urethane), 1635 (C=O, amide); δ_H ($CDCl_3$) 1.0 - 2.2 (14H, m, CH_2CH_2 and CH_2(2), (3), (5) cyclohexyl ring), 3.65 (1H, m, CH(1) cyclohexyl ring), 4.4 (1H, m, CH), 5.1 (2H, s, $C\underline{H}_2C_6H_5$), 5.17 (2H, s, $C\underline{H}_2C_6H_5$), 7.35 ppm (10H, s, $2C_6H_5$); m/z (EI) 452 (M^+).

α-N-t-Butyloxycarbonyl-α-(4-nitrophenyl)-γ-(N-(2-chloroethyl)-carbamoyl)-L-glutaminate (26).

To α-N-t-butyloxycarbonyl-α-(4-nitrophenyl)-L-glutaminate (5g, 14 mmol) suspended in sodium dried toluene (500 cm^3), 2-chloroethyl isocyanate (11.6 cm^3, 140 mmol) was added. The solution was boiled under reflux for 14h. After cooling to room temperature, the solvent was removed on a rotary film evaporator under vacuum, to leave α-N-t-butyloxycarbonyl-α-(4-nitrophenyl)-γ-(N-(2-chloroethyl)-carbamoyl)-L-glutaminate as a white solid, which was recrystallized from ethyl acetate. Yield 3.35g (49%), m.p. 154-155°C, (Found: C, 48.3, H, 5.3; N, 11.78. $C_{19}H_{25}N_4O_8Cl$ requires: C, 48.3, H, 5.3; N, 11.8%); ν_{max} (nujol) 3330, 3310, 3300(NH), 1760 (C=O, ester), 1700 (vb) (C=O, urethane), 1680 (C=O, urea), 1530 and 1350 cm^{-1} (C-NO_2); δ_H (DMSO -d^6) 1.45 (9H, s, $(CH_3)_3$); 2.2 (2H, m, $NCH_2\underline{CH_2}CH$), 2.55 (2H, t, $N\underline{CH_2}CH_2$), 3.6 (4H s, $ClCH_2CH_2N$), 4.4 (1H, m, CH), 6.6 (1H, s (b), NH), 7.3 (2H, d, Ar-H(b)), 8.3 (2H, d, Ar-H (3) and (5)), 8.1 (1H, s (b), NH), 9.7 ppm (1H, s, NH)); m/z (EI) 472 (M^+).

α-(N-t-Butyloxycarbonyl, N-nitroso)-α-(4-nitrophenyl)-γ-(N-(2-chloroethyl-N-nitrosocarbamoyl)-L-glutaminate (27).

α-N-t-Butyloxycarbonyl-α-(4-nitrophenyl)-γ-(N-(2-chloroethyl)-carbamoyl)-L-glutaminate (26) (1.9g, 3.8 mmol) was dissolved in 200 cm^3THF: dichloromethane (1:1 v/v). The solution was cooled to 0°C and sodium acetate (2.63g, 32 mmol) added while stirring vigorously, followed by liquid N_2O_4 (1 cm^3, 15.2 mmol) added slowly over 10 min. The mixture, after stirring for 4h at 0°C, was then filtered and removal of the solvent from the filtrate under vacuum at room temperature gave a yellow oil. The oil was purified by flash chromatography on silica using dichloromethane:ethyl acetate

(9:1, v/v) as eluent to give α-(N-t-butyloxycarbonyl,N-nitroso)-α-(4-nitrophenyl)-γ-(N-(2-chloroethyl)-N-nitrosocarbamoyl)-L-glutaminate (27), still as a yellow oil. Yield 1.54g (72.3%), λ_{max} (EtOH) 400, 420 nm (indicative of N-N=O); υ_{max} (nujol) 3330 (NH), 1770 (C=O, ester), 1750 (C=O,urethane), 1720 (C=O, ester), 1750 (N-N=O/C-NO), 1350 cm^{-1} (C-NO_2); δ_H ($CDCl_3$) 1.6 (9H, s,$(CH_3)_3$), 2.3 (2H, m, $CH_2\underline{CH_2}CH$), 2.9 (2H, t, $\underline{CH_2}CH_2$), 3.5 (2H, t, $ClCH_2$), 4.15 (2H, t, CH_2-N-N=O), 5.6 (1H, q, CH), 7.2 (2H, d, Ar-H (2) and (6)), 8.3 (2H, d, Ar-H (3) and (5)), 9.4 ppm (1H, s, NH); m/z (FAB +ve ion), 531 (MH^+, 0.3%).

α-(4-Nitrophenyl)-γ-(N-(2-chloroethyl) -N-nitrosocarbamoyl) -L-glutaminate (28)

α-(N-t-Butyloxycarbonyl, N-nitroso)-α-(4-nitrophenyl)-γ-(N-(2-chloroethyl) - N-nitrosocarbamoyl)-L-glutaminate (27) (0.1g, 1.88 mmol) was suspended in 0.12 M HCl in 98% HCO_2H (3.2 cm^3) and then stirred vigorously at 0°C for 10 min. The mixture was concentrated by vacuum evapouration at room temperature and the residue, after dilution with water (2 cm^3) was then washed with ethyl acetate (15 cm^3). The aqueous fraction was separated and freeze-dried to give α-(4-nitrophenyl-γ-(N-(2-chloroethyl)-N-nitrosocarbamoyl)-L-glutaminate (28) as a pale yellow sticky solid. Yield 60mg (79%), λ_{max} (EtOH), 400, 420 nm (characteristic of N-N=O); υ_{max} (CCl_4) 1775 (C=O, ester), 1710 (C=O), 1526, 1488 (N-N=O/CNO_2), 1350 cm^{-1} (C-NO_2); δ_H(D_2O) 2.45 (2H, m, $NCH_2\underline{CH_2}CH$), 3.0 (2H, t, $N\underline{CH_2}CH_2$), 3.5 (2H, t, $Cl\underline{CH_2}$), 4.05 (2H, t, CH_2), 4.5 (1H, m, CH), 7.4 (2H, d, Ar-H (2) and (6)), 8.25 (2H, d, Ar-H (3) and (5)), 9.4 ppm (1H, s, NH); FAB accurate mass measurement. (Found, 402.0824, requires; 402.0817).

N-2-chloroethylurea (29).

Ammonia gas was bubbled through a solution of 2-chloroethyl isocyanate (2 cm^3, 24 mmol) in ether (200 cm^3) at ca. -47°C. A white precipitate formed almost immediately and, after allowing the reaction mixture to warm to room temperature, the precipitate was collected by filtration and recrystallized from ethanol to yield N-2-chloroethylurea as white crystals. Yield 2.50g (85%), m.p. 102-104°C (lit.,[103] 99-101°C); ν_{max} (nujol) 3373 (NH), 1652 cm^{-1} (C=O, urea); δ_H (DMSO-d^6) 3.15 (2H, t, $ClCH_2\underline{CH_2}N$), 3.5 (2H, t, $Cl\underline{CH_2}CH_2N$), 5.45 (2H, s(b), NH_2), 6.1 ppm (1H, t (b), $CH_2\underline{NH}$); m/z (EI) 122 (M^+).

N-(2-Chloroethyl)-N-nitrosourea (30)

N-(2-chloroethyl)-N-nitrosourea was prepared by a literature procedure[103]. This involved the nitrosation of N-2-chloroethylurea (29) (15g, 12 mmol) in 96% H_2SO_4 by $NaNO_2$ (0.85g, 12.4 mmol). The product was precipitated and collected on a filter and washed well with water. Yield 0.56g (30%), m.p. 79°C (lit.,[103] 75-78°C); λ_{max} (EtOH) 394.5 and 412 nm (indicative of N-N=O); ν_{max} (nujol) 3396 and 3243 (NH), 1736 cm^{-1} (C=O, urea); δ_H ($CDCl_3$) 3.5 (2H, t, $Cl\underline{CH_2}$), 4.15 ppm (2H, t, CH_2N-N=O); m/z (FAB +ve ion) 152 (MH^+, 7.6%).

γ-L-Glutamyl-ethylamine (32)

α-N-Benzyloxycarbonyl-α-benzyl-γ-N-ethyl-L-glutaminate (13) (5g, 12.5 mmol) was dissolved in methanol (20 cm^3) and 10% palladium on charcoal catalyst (100 mg) added. The mixture was hydrogenated at atmospheric pressure and ambient temperature overnight. The catalyst was removed by filtration and the filtrate vacuum evaporated to dryness to give γ-L-glutamyl-ethylamine as a white

crystalline solid. This was then recrystallized from aqueous alcohol. Yield 1.7g (79%), m.p. 215°C (lit.,[127] 217-219°C), (Found: C, 49.97; H, 8.10; N, 15.96. Calc for $C_7H_{14}N_2O_3$: C, 48.26; H, 8.10; N, 16.08%); ν_{max} (nujol) 3310 (NH), 1640 cm^{-1} (C=O); δ_H (DMSO-d^6) 1.00 (3H, t, $CH_2C\underline{H}_3$), 2.03 (2H, m, CH_2), 2.26 (2H, m, CH_2), 3.08 (2H, q, $C\underline{H}_2CH_2$), 3.63 ppm (1H, t, CH); m/z (FAB +ve ion) 175 (MH^+)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(N, N-diethyl)-L-glutaminate (70)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(N, N-diethyl)-L-glutaminate (70)

was prepared in a similar manner to that described for compound (12) using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (5g, 13.4 mmol), triethylamine (1.88 cm^3, 13.4 mmol), ethylchloroformate (1.2 cm^3, 12.7 mmol) and diethylamine (1.4 cm^3, 13.4 mmol). The product was obtained as a yellow oil. Yield 3.74g (70%), ν_{max} 3330 (NH), 1740 (C=O, ester), 1700 (C=O, urethane), 1640 cm^{-1} (C=O, amide); δ_H ($CDCl_3$) 1.2 (6H, dt, $2CH_2C\underline{H}_3$), 2.0-2.4 (4H, m, $NC\underline{H}_2C\underline{H}_2$), 3.2 (4H, m, $2C\underline{H}_2CH_3$), 4.4 (1H, m, CH), 5.1 (2H, s, $C\underline{H}_2C_6H_5$), 5.17 (2H, s, $C\underline{H}_2C_6H_5$), 7.36 ppm (10H, s, $2C_6H_5$); m/z (EI) 426 (M^+).

γ-L-Glutamyl-diethylamine (33)

α-Benzyloxycarbonyl-α-benzyl-γ-(N,N-diethyl)-L-glutaminate) (70) (31.74g, 8.78 mmol) was hydrogenated over palladium on charcoal catalyst as for compound (32). γ-L-Glutamyl-diethylamine, obtained as a white solid was recrystallized from aqueous alcohol (1:4 v/v). Yield 1.54g (95%), m.p. 117-118°C, (Found: C, 53.48; H 19.26; N, 13.74. $C_9H_{18}N_2O_3$ requires: C, 53.20; H, 9.36: N, 13.8%); ν_{max} (nujol) 1655 cm^{-1} (C=O, amide); δ_H (D_2O) 0.9 (6H, dt, $2C\underline{H}_3CH_2$),+ 2.0 (2H, t, CH_2), 2.4 (2H, m,CH_2), 3.2 (4H, dq, $2C\underline{H}_2CH_3$), 3.6 ppm (1H, t, CH); m/z (FAB +ve ion) 203 (MH^+).

α-N-Benzyloxycarbonyl-α-benzyl-γ-(N,N-dimethyl)-L-glutaminate (71)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(N,N-dimethyl)-L-glutaminate (71) was prepared in a similar manner to that described for compound (12), using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (2.32g, 6.25 mmol), triethylamine (0.87 cm^3, 6.25 mmol), ethylchloroformate (0.54 cm^3, 5.69 mmol), and dimethylamine (0.41 cm^3, 6.25 mmol). Compound (71) was isolated as a clear yellow oil. Yield 1.63g (65.5%), υ_{max} (CCl_4) 3330 (NH), 1740 (C=O, ester), 1700 (C=O, urethane), 1650 cm^{-1} (amide); δ_H ($CDCl_3$) 2.0-2.5 (4H, m, $CH_2\underline{CH_2}$), 2.85 (6H, d, $2CH_3$), 4.36 (1H, m, CH), 5.1 (2H, s, $C\underline{H_2}C_6H_5$), 5.17 (2H, s, $C\underline{H_2}C_6H_5$), 7.36 ppm (10H, s, $2C_6H_5$); m/z (EI) 398 (M^+), 91.

γ-L-Glutamyl-dimethylamine (34)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(N, N-dimethyl)-L-glutaminate (71) (1.63 cm^3, 4 mmol) was hydrogenated over a palladium on charcoal catalyst as for compound (32) to give γ-glutamyl dimethylamine as a white solid. This was then recrystallized from aqueous ethanol (1:4 v/v). Yield 0.4g (56%), m.p. 139°C, (Found: C, 48.21; H, 8.05; N, 16.0. $C_7H_{14}N_2O_3$ requires C, 48.27; H, 8.1; N, 16%); υ_{max} (nujol) 1647 cm^{-1} (C=O, amide); δ_H (D_2O) 2.0 (2H, m, $CH_2\underline{CH_2}CH$), 2.45 (2H, t, CH_2), 2.8 (3H, s, CH_3), 2.9 (3H, s, CH_3), 3.7 ppm (1H, t, CH); m/z (FAB +ve ion) 175 (MH^+, 100%).

α-Benzylester of α-N-benzyloxycarbonyl-γ-L-glutamyl pyrrolidine (72)

The α-benzylester of α-N-benzyloxycarbonyl- γ-L-glutamyl pyrrolidine (72) was prepared in a similar manner to that described for compound (12), using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (5g, 13.4 mmol), triethylamine (1.88 cm^3, 13.4 mmol),

ethylchloroformate (1.2 cm^3, 12.7 mmol) and pyrrolidine (1.1 cm^3, 13.4 mmol). The α-benzylester product of α-N-benzyloxycarbonyl-γ-L-glutamyl pyrrolidine was purified by column chromatography on silica using ethyl acetate and obtained as a clear oil. Yield 3.34g (58.5%), ν_{max} (CCl_4) 3250 (N H), 1730-1710 (C=O, urethane and ester), 1630 cm^{-1} (C=O, amide); δ_H ($CDCl_3$) 1.825 (4H, m, CH_2(3) and (4) pyrrolidine ring), 1.8-2.3 (4H, m, $C\underline{H}_2C\underline{H}_2$), 3.4 (4H, dt, CH_2 (2) and (5) pyrrolidine ring), 5.07 (2H, s, $C\underline{H}_2C_6H_5$), 5.17 (2H, s, $C\underline{H}_2C_6H_5$), 6.0 (1H, d, NH), 7.32 ppm (10H; s, $2C_6H_5$); m/z (EI) 414 (M^+).

γ-L-Glutamyl pyrrolidine (31)

The α-benzyl ester of α-N-benzyloxycarbonyl-γ-L-glutamyl pyrrolidine (72) (3.36g, 7.87 mmol) was hydrogenated over 10% palladium on charcoal catalyst as for compound (32). After filtration of the catalyst the γ-L-glutamyl pyrrolidine was recrystallized from aqueous ethanol to give a white powder. Yield 0.97g (62%), m.p. 163°C, (Found: C, 54,16; H, 8.16, N, 13.85. $C_9H_{16}N_2O_3$ requires C, 54.0; H, 8.0, N, 14.0%); ν_{max} (nujol) 1659 cm^{-1} (C=O, amide), δ_H (D_2O 1.8 (4H, m, CH_2 (3) and (4) pyrrolidine ring), 2.075 (2H, m, $CH_2C\underline{H}_2CH$), 2.45 (2H, t, N $C\underline{H}_2CH_2$), 3.3 (4H, dt, CH_2(2) and (5) pyrrolidine ring), 3.7 ppm (1H, t, CH); m/z (FAB +ve ion) 201 (MH^+).

α-N-Benzyloxycarbonyl-α-benzyl-γ-(t-butyl)-L-glutaminate (73)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(t-butyl)-L-glutaminate (73) was prepared as described for compound (12), using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (3.13g, 8.44 mmol), triethylamine (1.17 cm^3, 8.4 mmol), ethylchloroformate. (0.73 cm^3, 7.68 mmol) and t-butylamine (0.89 cm^3, 8.44 mmol). The product was

purified by column chromatography on silica using an ethyl acetate: (40-60°C) petroleum ether mixture (1:1 v/v) as eluent. The product was obtained as a clear oil. Yield 3.33g (58%), ν_{max} (CCl_4) 3277 (NH), 1730 - 1710 (C=O, ester and urethane), 1630 cm^{-1} (C=O, amide); δ_H ($CDCl_3$) 1.3 (9H, s, $(CH_3)_3$), 1.9-2.5 (4H, m, NCH_2CH_2CH), 4.35 (1H, m, CH), 5.1 (2H, s, $CH_2C_6H_5$), 5.8 (1H, d, NH), 7.32 ppm (10H, s, $2C_6H_5$); m/z (EI) 4.26 (M^+).

γ-L-Glutamyl-t-butylamine (35)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(t-butyl)-L-glutaminate (73) (3.33g, 9.4 mmol) was hydrogenated over a 10% palladium on charcoal catalyst as for compound (32) and the product recrystallized from an acetone:ethanol mixture (1:4 v/v) to give γ-L-glutamyl-t-butylamine as a white crystalline solid. Yield 0.95g (61%), m.p. 190-194°C (d), (Found: C, 53.4; H, 8.76; N, 13.8. $C_{18}H_{30}N_2O_5$ requires C, 53.46; H, 8.9, N, 13.86%); ν_{max} (nujol) 3277 (NH), 1630 cm^{-1} (C=O, amide); δ_H (D_2O) 1.15 (9H, s, $(CH_3)_3$), 2.67 (2H, m, NCH_2CH_2), 2.4 (2H, t, NCH_2CH_2), 3.6 ppm (1H, t, CH); m/z (FAB +ve ion) 203 (MH^+, 100%).

α-N-Benzyloxycarbonyl-α-benzyl-γ-(4-methylphenyl)-L-glutaminate (74)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(4-methylphenyl)-L-glutaminate (74), was prepared in a similar manner to that described for compound (12). Using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (1.89g, 5.1 mmol), triethylamine (0.7cm^3, 5.1 mmol), ethylchloroformate (0.43 cm^3, 4.53 mmol) and 4-methylaniline (0.546g, 8.1 mmol). The product was recrystallized from toluene. Yield 1.2g (51%), m.p. 131-133°C, (Found: C, 70.43; H, 6.1; N, 6.1. $C_{27}H_{28}N_2O_5$ requires C, 70.40; H, 6.1; N, 6.11%); ν_{max} (nujol) 3282

(NH), 1728 (C=O, ester), 1690 (C=O, urethane), 1647 cm^{-1} (C=O, amide); δ_H (DMSO-d^6) 1.7 - 2.5 (7H, m, CH_2CH_2 and CH_3Ar), 4.1 (1H, m, CH), 5.0 (2H, s, $CH_2C_6H_5$), 5.1 (2H, s, $CH_2C_6H_5$) 7.75 ppm (14H, s and q, $2C_6H_5$ and C_6H_4); m/z (EI) 460 (M^+).

γ-L-Glutamyl-4-methylaniline, (γ-L-Glutaminyl-4-methylbenzene) (36)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(4-methylphenyl)-L-glutaminate (74) (1.2g, 2.6 mmol) was hydrogenated as for compound (32) over a 10% palladium on charcoal catalyst and the product γ-L-glutamyl-4-methylaniline recrystallized from aqueous methanol (1:4 v/v). Yield 0.4g (78%), m.p. 185°C, (Found: C 61.22; H, 6.84; N, 11.85. $C_{12}H_{16}N_2O_3$ requires C, 61.0; H, 6.77; N, 11.86%); υ_{max} (nujol) 1632 cm^{-1} (C=O, amide); δ_H (D_2O) 2.0 - 2.15 (5H, m + s, CH_2 and CH_3Ar), 2.4 (2H, t, $OCCH_2$), 3.7 (1H, t, CH), 7.15 ppm (4H, s, C_6H_4); m/z (FAB +ve ion), 237 (MH^+)

α-N-Phthalyl-L-glutamic anhydride (37)

α-N-Phthalyl-L-glutamic anhydride (37) was prepared by a literature procedure[85]. This involved reaction of the diethylester of L-glutamic acid in ether with phthalic anhydride to give diethyl-α-N-(2-carboxybenzoyl)-L-glutamate, followed by boiling under reflux with ethanolic HCl to effect ring closure to diethyl-α-N-phthalyl-L-glutamate. α-N-Phthalyl-L-glutamic acid was then obtained by hydrolysis in boiling acetic acid and HCl. Ring closure to α-N-phthalyl-L-glutamic anhydride was achieved by reaction with acetic acid for 5 min at 100°C. Yields were in the order of 50%, m.p. 196-197°C (lit.,[85] 195-200°C), (Found: C, 60.13; H, 3.47; N, 5.37. Calc for $C_{13}H_9O_5N$: C, 60.63; H, 3.47; N, 5.41%); υ_{max} (nujol) 1829 and 1760 (C=O, anhydride), 1720 cm^{-1} (C=O, lactam); δ_H ($CDCl_3$) 2.1 - 3.4 (4H, m, CH_2CH_2), 5.1 (1H, q, CH),

7.85 ppm (4H, m, C_6H_4); m/z (EI) 215 ($M^+ - CO_2$).

α-N-Phthalyl-γ-(N-methyl-L-glutaminyl)-4-nitrobenzene (38)

α-N-Phthalyl-L-glutamic anhydride (37) 8.72g, 37 mmol) and N-methyl- 4-nitroaniline (8.72g, 57 mmol) were boiled under reflux in dry THF (100 cm) for 3 days. The solvent was removed under vacuum and the resulting pale yellow oil purified by column chromatography on silica , using ethyl acetate first to elute unidentified impurities and methanol to elute α-N-phthalyl-γ-(N-methyl-L-glutaminyl)-4-nitrobenzene (38). Evaporation under vacuum of solvent from the methanol fraction gave (38) as yellow solid, which was recrystallized from ethyl acetate. Yield 4g (36%), m.p. 142°C ; ν_{max} (nujol) 1770 and 1700 (C=O, lactam), 1630 (C=O, amide), 1590 and 1340 (CO_2^-), 870 cm^{-1} (C-H, disubstituted benzene ring); δ_H (DMSO-d^6) 1.8-2.2 (4H, m, CH_2CH_2), 2.75 (3H, s, CH_3), 3.8 (1H, m, CH), 7.0 (2H, d, Ar-H (2) and (6)), 7.3 (4H, s, C_6H_4), 7.6 ppm (2H, d, Ar-H (3) and (5)); m/z (FAB +ve ion) 434 ($MH^+ + Na^+$).

γ-L-Glutamyl-N-methyl-4-nitroaniline, hydrochloride salt; (γ-(N-methyl-L-glutaminyl)-4-nitrobenzene, hydrochloride salt) (39)

α-N-Phthalyl-γ-(N-methyl-L-glutaminyl)-4-nitrobenzene (38) (0.5g, 1.2 mmol) and triethylamine (0.18 cm^3, 1.32 mmol) were dissolved in methanol (3 cm^3) and 90% hydrazine hydrate (0.05 cm^3, 1.32 mmol) in 50% v/v aqueous methanol (2 cm^3) added. The reaction solution was left standing at room temperature for 2 days and then the solvent was removed under vacuum. The residue was suspended in water (10 cm^2) and acidified to pH 4 with 1 M HCl. After stirring for 3h, the phthalylhydrazide precipitate was filtered off and the filtrate concentrated under vacuum to about 2 cm^3. Absolute ethanol (5 cm^3)

was then added and the γ-L-glutamyl-N-methyl-4-nitroaniline ,hydrochloride salt (39) separated out as pale yellow crystals. These were then recrystallized from absolute ethanol. Yield 0.13g (35%), m.p. 135-136°C, (Found: C, 45.37; H, 4.58; N, 13.42. $C_{12}H_{14}N_3O_5HCl$ requires : C, 45.49; H, 4.74 N, 13.3 %); ν_{max} (nujol) 3460 (NH), 1645 (C=O, amide), 1590 (CO_2^-), 1340 (CO_2^- band II), 855 cm^{-1} (C-H, para substituted benzene ring); δ_H (D_2O) 2.1 (2H, t, CH_2), 2.4 (2H, m, CH_2), 3.25 (3H, s, CH_3), 3.62 (1H, t, CH), 7.5 (2H, d, Ar-H (2) and (6)), 8.25 ppm (2H, d, Ar-H (3) and (5)); m/z (FAB +ve ion) 282 (MH^+, 100%).

4-Hydroxyacetanilide (46)

4-Hydroxyacetanilide was prepared by a literature procedure[104]. This involved the reaction of 4-hydroxyaniline (10g, 92 mmol) with acetic acid (6.0 cm^3, 92 mmol) and acetic anhydride (10.5 cm^3, 96 mmol) at 80°C for 40 min. The 4-hydroxyacetanilide was recrystallized from water. Yield 8.1g (58.5%), m.p. 166°C, (lit.,[127] 168°C), (Found: C, 63.52; H, 6.1; N, 9.30. Calc. for $C_8H_9NO_2$: C, 63.58; H, 5.96; N, 9.27%); ν_{max} (nujol); 3300 (NH), 3300-3000 (OH), 1650 (C=O), 870 cm^{-1} (arom C-H); δ_H (DMSO-d^6) 1.1 (3H, s, CH_3), 5.75 (2H, d, Ar-H (3) and (5)), 6.45 (2H, d, Ar-H (2) and (6)), 8.2 (1H, s, NH), 8.7 ppm (1H, s, OH).

4-Benzyloxyacetanilide (49)

4-Benzyloxyacetanilide was prepared by a literature procedure[128]. This involved heating 4-hydroxyacetanilide (46) (8g, 53 mmol) under reflux with benzylchloride (6.1 cm^3, 53 mmol) and sodium ethoxide in ethanol. The product was recrystallized from water. Yield 10.7g (83.8%), m.p. 122-124°C , ν_{max} (nujol) 3300-3200 (NH), 1650 (C=O, amide), 850 cm^{-1} (Ar-H); δ_H ($CDCl_3$) 2.2

(3H, s, $COCH_3$), 5.05 (2H, s, $C\underline{H}_2C_6H_5$), 6.9 (2H, d, Ar-H (3) and (5)), 7.3-7.5 (7H, s + d, C_6H_5 and Ar-H (2) and (6)); m/z 241 (M^+, 23%).

4-Benzyloxyaniline (52)

4-Benzyloxyaniline was also prepared by a literature procedure[128], involving the alkaline hydrolysis of 4-benzyloxyacetanilide (49) (5g, 16.6 mmol) in ethanolic KOH. The product was recrystallized from hexane. Yield 3.8g (89.6%), m.p. 54°C (lit.,[127] 54-55°C); ν_{max} (nujol) 3300 and 3180 (NH_2), 1250 (C-O), 840 cm^{-1} (arom C-H); δ_H ($CDCl_3$) 2.85 (2H, s (b), NH_2), 5.0 (2H, s, $C\underline{H}_2C_6H_5$), 6.6 (2H, d, Ar-H (2) and (6)), 6.8 (2H, d, Ar-H (3) and (5)), 7.36 ppm (5H, m, C_6H_5); m/z (EI) 199 (M^+), 91 ($CH_2C_6H_5$).

α-N-Benzyloxycarbonyl-α-benzyl-γ-(4-benzyloxyphenyl)-L-glutaminate (40)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(4-benzyloxyphenyl)-L-glutaminate (40), was prepared in a similar manner to compound (12) using α-N-benzyloxycarbonyl-α-benzyl-L-glutaminate (7g, 19 mmol), triethylamine (2.6 cm^3, 19 mmol), ethylchloroformate (1.6 cm^3, 17.2 mmol) and 4-benzyloxyaniline (52) (3.8g, 19 mmol). The product was recrystallized from ethyl acetate : 40-60°C petroleum ether (4:1 v/v). Yield 4.10g (40%), m.p. 141-143°C, (Found: C, 71.4; H, 5.82; N, 5.1. $C_{33}H_{32}N_2O_6$ requires C, 71.7; H, 1, 5.8; N, 5.3%); ν_{max} (nujol) 3280 (NH), 1740 (C=O, ester), 1700 (C=O, urethane), 1650 (C=O, amide); δ_H ($CDCl_3$) 1.9-2.4 (4H, m, CH_2CH_2), 4.15 (1H, m, CH), 4.9 (2H, s, $C\underline{H}_2C_6H_5$), 4.95 (2H, s, $C\underline{H}_2C_6H_5$), 5.0 (2H, s, $C\underline{H}_2C_6H_5$), 6.75 (2H, d, Ar-H (3) and (5)), 7.1 - 7.4 ppm (17H, s + d, 3 C_6H_5 and Ar -H (2) and (6)); m/z (FAB +ve ion) 553 (MH^+).

γ-L-Glutamyl-4-hydroxyaniline, (γ-L-glutaminyl-4-hydroxy benzene)(43)

A solution of α-N-benzyloxycarbonyl-α-benzyl-γ-(4-benzyloxyphenyl)-L-glutaminate (40) (4g, 7.25 mmol) in a mixture of methanol:acetic acid:water (15:5:1 v/v) containing 500mg of 10% palladium on charcoal catalyst, was hydrogenated at 60°C for 90 min and then overnight at room temperature. The solution was filtered, the filtrate evaporated to dryness under vacuum and the residue recrystallized from water (under argon) to give γ-L-glutamyl-4-hydroxyaniline as buff crystals. Yield 0.7g (42%), m.p. 207-209°C (d) (lit.,[127] 225°C), (Found: C, 55.22; H, 5.88; N, 11.76. Calc for $C_{11}H_{14}N_2O_4$: C, 55.46; H, 5.88, N, 11.76°C); υ_{max} (nujol) 3270 (OH), 1645 cm^{-1} (C=O, amide); δ_H (NaOD-d^6) 1.8 (2H, m, CH_2); 2.25 (2H, t, $C\underline{H}_2CH_2$), 3.15 (1H, t, CH), 6.45 (2H, d, Ar-H (3) and (5), 6.9 ppm (2H, d, Ar H (2) and (6)); m/z (FAB +ve ion) 239 (MH^+, 74%).

2-Hydroxy-5-chloroacetanilide (47)

2-Hydroxy-5-chloroacetanilide (47) was prepared in a similar manner to that described for compound (46), using 2-hydroxy-5-chloroaniline (10g, 69 mmol), acetic acid (4 cm^3, 6.9 mmol) and acetic anhydride (7.9 cm^3, 83.5 mmol). The 2-hydroxy-5-chloroacetanilide was isolated and then recrystallized from aqueous methanol (4:1 v/v). Yield 9.3g (72%), m.p 175-178°C (lit.,[127] 185°C), (Found : 51.7; H, 4.3; N, 7.53. Calc. for $C_8H_8NO_2Cl$: C, 51.75; H, 4.31; N, 7.55%); υ_{max} (nujol) 3800 (NH), 1664 cm^{-1} (C=O, amide); δ_H (DMSO-d^6) 2.15 (3H, s, CH_3), 6.95 (2H, Ar-H (3) and (4)); 8.0 (1H, d, Ar - H(6)), 9.3 ppm (1H, s, NH); m/z (EI) 185 (M^+), 143 (M^+ - CH_3CO, 100%).

2-Benzyloxy-5-chloroacetanilide (50)

2-Benzyloxy-5-chloroacetanilide was prepared by an adaptation to the literature procedure[128] used for compound (49), by refluxing 2-hydroxy-5-chloroacetanilide (47) (5g, 27 mmol) with benzylchloride (3.11 cm^3, 27 mmol) for 1.5h in a solution of sodium (0.62g, 27 mmol) in ethanol (40 cm^3). Most of the solvent was then removed by distillation (30 cm^3) and the residue poured into 160 cm^3 of cold water from which white crystals of 2-benzyloxy-5-chloroacetanilide (50) separated. After isolation the crystals were recrystallized from methanol:water (4:1 v/v). Yield 7.26g (98%), m.p. 104°C, (Found: C, 65.47; H, 5.06; N, 5.02. $C_{15}H_{14}NO_2Cl$ requires C, 65.33, H, 5.08; N, 5.08%); ν_{max} (nujol) 3300 (NH), 1664 (C=O, amide), 1600 and 1520 cm^{-1} (Ar - H); δ_H ($CDCl_3$) 2.15 (3H, s, CH_3), 5.1 (2H, s, $CH_2C_6H_5$), 6.9 (2H, m, Ar - H (3) and (4)), 7.4 (5H, s, C_6H_5), 8.4 ppm (1H, d, Ar - H (6)); m/z (EI) 275 (M^+), 233 (M^+ - CH_3CO).

2-Benzyloxy-5-chloroaniline (53)

2-Benzyloxy-5-chloroaniline (53) was prepared by adaptation of the literature procedure[128] used for compound (52). Thus 2-benzyl-5-choroacetanilide (50) (5g, 18 mmol) was hydrolysed in 70 cm^3 of 90% ethanolic KOH (10.2g, 18 mmol) for 15h at reflux temperatures. Most of the solvent was then removed by distillation and the residue extracted with ether (3 x 100ml). Removal of the ether by evaporation under vacuum gave 2-benzyloxy-5-chloroaniline as pale yellow crystals. Recrystallization of (53) from cyclohexane gave white crystalline plates. Yield 1.58g (61%), m.p. 70°C, (Found : C, 66.53; H, 5.12; N, 5.89. $C_{13}H_{12}NOCl$ requires C, 66.8; H, 5.13; N, 5.99%); ν_{max} (nujol) 3473 and 3378 (NH), 1615 and 1502 cm^{-1} (Ar - H); δ_H ($CDCl_3$) 4.2 (2H, s (b), NH_2) 5.3 (2H, s,

$C\underline{H}_2C_6H_5$), 7.0 (3H, m, Ar - H (3), (4) and (6)), 7.7 ppm (5H, s, C_6H_5); m/z (EI) 233 (M^+, 31.3%), 235 (M^+, 10.4).

α-N-Benzyloxycarbonyl-α-benzyl-γ-(2-benzyloxy-5-chlorobenzene)-L-glutaminate (41).

α-N-Benzyloxycarbonyl-α-benzyl-γ-(2-benzyloxy-5-chlorobenzene)-L-glutaminate (41) was prepared in a similar manner to that described for compound (12) using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (3.17g, 8.6mmol), triethylamine (1.2 cm^3, 8.6 mmol), ethyl chloroformate (0.77 cm^3, 8.11 mmol) and 2-benzyloxy-5-chloroaniline (53) (2g, 8.6 mmol). The product was purified by column chromatography on silica using ethyl acetate: (40-60°C) petroleum ether (3:2 v/v) as eluent. Yield 2g (40%), m.p. 105°C, (Found: C, 67.2; H, 5.3; N, 4.77. $C_{33}H_{31}N_2O_6$ Cl requires C, 67.5; H, 5.3; N, 4.77%); υ_{max} (nujol) 3290 (NH), 1725 (C=O ester), 1690 (C=O, urethane), 1650 (C=O, amide), 720 and 690 cm^{-1} (Ar - H); δ_H ($CDCl_3$) 2.4-3.0 (4H, m, $NC\underline{H}_2C\underline{H}_2$), 5.4 (1H, m, CH), 5.5 (2H, s, $C\underline{H}_2C_6H_5$), 5.6 (2H, s, $C\underline{H}_2C_6H_5$), 5.65 (2H, s, $C\underline{H}_2C_6H_5$), 6.1 (1H, d, NH), 7.4 (2H, m, Ar - H (3) and (4)), 7.7 (15H, m, $3C_6H_5$), 8.2 ppm (1H, d, Ar - H (6)); m/z (EI) 586 (M^+), 478 (M^+ - $PhCH_2OH$), 91 (100%).

γ-L-Glutamyl-2-hydroxy-5-chloroaniline,(γ-L-Glutaminyl-2-hydroxy-5-chlorobenzene) (44)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(2-benzyloxy-5-chlorophenyl)-L-glutaminate (41) (1.5g, 2.5 mmol) was hydrogenated as described for compound (43) over 10% palladium on charcoal catalyst. After filtration of the catalyst, evaporation of the filtrate under vacuum gave γ-L-glutamyl-2-hydroxy-5-chloroaniline (44) as a buff coloured solid which was recrystallized (under argon) from water.

Yield 0.43g (57%), m.p. 190-194°C (d), (Found: C, 45.84; H, 5.27; N, 9.27. $C_{11}H_{13}N_2O$ Cl H_2O requires C, 45.59; H, 5.16; N, 9.6%); ν_{max} (nujol) 2340 (NH/OH), 1655 cm^{-1} (C=O, amide); δ_H (D_2O) 2.2 (2H, m, CH_2), 2.65 (2H, t, $NC\underline{H}_2CH_2$), 4 (1H, t, CH), 7.1 ppm (3H, m, Ar-H (3), (4) and (6)); m/z (FAB +ve ion) 273 (MH^+, 30.6%), 275 (MH^+, 10.4).

2-Chloro-4-nitrophenol (56)

2-Chloro-4-nitrophenol (56) was prepared by literature procedure[129]. This involved reaction of 4-nitrophenol (5g, 36 mmol) with $KClO_3$ (1.5g, 12.5 mmol) in concentrated hydrochloric acid. The desired product was purified by column chromatography silica using ethyl acetate as eluent. 2-Chloro-4-nitrophenol was obtained as a pale yellow solid. Yield 4.2g (67.5%), m.p. 110°C (lit., [129] 111°C), (Found: C, 41.29; H, 2.22; N, 8.10. Calc. for $C_6H_4NO_3Cl$: C, 41.49, H, 2.31; N, 8.07%); ν_{max} (nujol) 3390 (OH), 1580 (NO_2), 1330 cm^{-1} (NO_2), δ_H ($CDCl_3$) 6.2 (1H, s, (br), OH), 7.12 (1H, d, Ar - H (6)), 8.14 (1H, d, Ar - H (5)), 8.30 ppm (1H, d, Ar - H (3)); m/z (EI) 173 (M^+, 100%), 175 (M^+, 34.1).

3-Chloro-4-hydroxyaniline (57)

3-Chloro-4-hydroxyaniline (57) was prepared by a literature procedure [129]. This involved reduction of 2-chloro-4-nitrophenol (56) (1.2g, 6.9 mmol) in hot, 1.3 M aqueous sodium hydroxide (20 cm^3). The product was recrystallized from water. Yield 0.73g (74%), m.p. 150°C. (lit., [129] 150-151°C), (Found: C, 50.15, H, 4.10, N, 9.71. Calc. for C_6H_6NO Cl: C, 50.17; H, 4.18; N, 9.76%); ν_{max} (nujol) 3170 (OH), 3300, 3260,3160 cm^{-1} (NH_2); δ_H (DMSO-d^6) 4.7 (2H, s(b), NH_2), 6.4 (1H, d, Ar - H (5)), 6.6 (1H, d, Ar - H (6)), 6.7 (1H, d, Ar - H (2)), 8.9 (1H, s(b), OH); m/z (EI) 143

(M^+, 100%), 145 (M^+, 31.8).

3-Chloro-4-hydroxyacetanilide (48)

3-Chloro-4-hydroxyacetanilide (48) was prepared by adaptation of the literature procedure[104] used for compound (46), reacting 3-chloro-4-hydroxyaniline (57) (1.40g, 9.8 mmol) with acetic acid (0.56 cm^3, 9.8 mmol) and acetic anhydride (1.1 cm^3, 11.7 mmol). The 3-chloro-4-hydroxyacetanilide (48) was isolated and recrystallized from water as white crystals. Yield 1.04g (57%), m.p. 140°C. (lit.,[127] 144°C). (Found: C, 51.69; H, 4.29; N, 7.52 $C_8H_8NO_2$ Cl requires, 51.75; H, 4.31; N, 7.55%); ν_{max} (nujol) 3165 (OH), 1627 (C=O, amide), 1420 cm^{-1} (O-H bending); δ_H (DMSO-d^6) 2.0 (3H, s, CH_3), 6.8 (1H, d, Ar -H (6)), 7.15 (1H, d, Ar - H (5)), 7.6 (1H, s, Ar - H (2)), 9.7 ppm (1H, s(b), OH); m/z (EI) 185 (M^+, 34.2%), 187 (M^+, 11.2).

3-Chloro-4-benzyloxyacetanilide (51)

3-Chloro-4-benzyloxyacetanilide (51) was prepared by adaptation of the literature procedure used for compound (49)[128], by refluxing 3-chloro-4-hydroxyacetanilide (48) (1.04g, 5.6 mmol) and benzyl bromide (0.68 cm^{-3}, 5.6 mmol) for 1.5h in a solution of sodium (0.13g, 5.6 mmol) in ethanol (15 ml). Removal of most of the solvent by distillation and pouring the residue into 32 cm^3 of cold water gave 3-chloro-4-benzyloxyacetanilide as white cyrstals that were recrystallized from water. Yield 1.36g (88%), m.p. 140°C, (Found: C, 65.37; H, 5.01; N, 5.05. C_{15} H_{14} NO_2 Cl requires C, 65.33; H, 5.08; N, 5.08%); ν_{max} (nujol), 3279 (NH), 1658 cm^{-1} (C=O, amide); δ_H (DMSO-d^6) 1.95 (3H, s, CH_3), 6.8 ppm (1H, d, Ar - H (2)); m/z (EI) 275 (M^+, 8%), 277 (M^+, 2.8), 91.

3-Chloro-4-benzyloxyaniline (54)

3-Chloro-4-benzyloxyaniline (54) was prepared by adaptation of the literature procedure used for compound (52)[128]. This involved the alkaline hydrolysis of 3-chloro-4-benzyloxyacetanilide (51) (2.63g, 9.5 mmol) in a solution of KOH (5.35g, 9.5 mmol) in 40 cm^3 of 90% ethanol. Most of the solvent was removed by distillation and the residue extracted with ether (3 x 100 cm^3). Removal of the ether by evaporation under vacuum gave 3-chloro-4-benzyloxyaniline which was recrystallized from cyclohexane. Yield 1.2g (54%), m.p. 64°C, (Found: C, 66.55; H, 5.02; N, 5.88. $C_{13}H_{12}NO$ Cl requires. C, 66.8; H, 5.14; N, 5.99%); ν_{max} (nujol) 3400, 2926, 2856 (NH_2),1630,1501 cm^{-1} (Ar-H); δ_H ($CDCl_3$) 3.85 (2H, s(b), NH_2),5.05 (2H, s, $C\underline{H}_2C_6H_5$), 6.7 (3H, m, Ar - H (2), (5), and (6)), 7.4 ppm (5H, s, C_6H_5); m/z (EI) 233 (M^+, 31.3%), 91.

α-N-Benzyloxycarbonyl-α-benzyl-γ-(3-chloro-4-benzyloxyphenyl) -L-glutaminate (42)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(3-chloro-4-benzyloxyphenyl -L-glutaminate (42) was prepared in a manner similar to that for compound (12), using α-N-benzyloxycarbonyl-α-benzyl-L-glutamate (1.91g, 5.14 mmol), triethylamine (0.72 cm^1, 5.14 mmol), ethyl chloroformate (0.4 cm^3, 4.87 mmol) and 3-chloro-4-benzyloxyaniline (54) (1.2g, 5.14 mmol). The product was purified by column chromatography on silica using ethyl acetate: (40-60°C) petroleum ether (3:2 v/v) as eluent. Yield 2.76g (58%), m.p. 134-135°C, (Found: C, 67.31; H, 5.22; N, 4.80. $C_{11}H_{13}N_2O_4$ Cl requires C, 67.5; H, 5.28; N, 4.77%); ν_{max} (nujol) 3290 (NH), 1728 (C=O, ester), 1688 (C=O, urethane), 1658 cm^{-1} (C=O, amide); δ_H ($CDCl_3$) 1.7-2.6 (4H, m, $NC\underline{H}_2C\underline{H}_2$), 4.3 (1H, m, CH), 5.05 (6H, d, $3C\underline{H}_2C_6H_5$), 5.8 (1H, d(b), NH), 6.85 (1H, d, Ar - H (6)), 7.3 (16H, m, $3C_6H_5$

and Ar - H (5)), 7.6 (1H, d, Ar - H (2)), 8.15 ppm (1H, s(b), NH); m/z (EI) 586 (M^+, 1.1%), 478 (M^+- $PhCH_2OH$), 91 (100).

γ-L-Glutamyl-3-chloro-4-hydroxyaniline, (γ-L-glutaminyl-3-chloro-4-hydroxybenzene) (45)

α-N-Benzyloxycarbonyl-α-benzyl-γ-(3-chloro-4-benzyloxyphenyl)-L-glutaminate (42) (0.16g, 0.27 mmol) was dissolved in 30% HBr in glacial acetic acid (4 cm^3) and stirred vigorously for 24h. The solvent was removed by evaporation under vacuum to yield a solid residue, probably the hydrogen bromide salt of (45). The residue was disssolved in H_2O and then purified by ion exchange on an Amberlite resin (protonated from). The HBr was eluted first with distilled H_2O and then (45) with 0.5 M aqueous ammonia. Removal of the solvent by freeze drying gave a white solid which was washed successively with methanol (5 cm^3) to give γ-L-glutamyl-3-chloro-4-hydroxyaniline (45). Yield 47mg (63%), m.p. 197-200°C (d), ν_{max} (nujol) 1658 cm^{-1} (C=O, amide); δ_H (D_2O), 2.15 (2H, m, CH_2), 2.5 (2H, t, NCH_2CH_2), 3.7 (1H, t, CH), 6.9 (1H, d, Ar - H), 7.1 (1H, d, Ar - H), 7.4 ppm (1H, d, Ar -H (2)), m/z (FAB +ve ion) 273 (MH^+, 9.2%), 275 (MH^+, 2.9), 143 (2.2). A satisfactory microanalysis for this compound was not obtained.

CHAPTER 8

REFERENCES

1. C.S. Hanes, F.J.R. Hird, and F.A. Isherwood, Nature., 1950, 166, 288-292.

2. A. Szewczuk, and M. Orlowski, Clin. Chim. Acta., 1960, 5, 680-688.

3. J.A. Goldbarg, O.M. Friedman, E.P. Pineda, E.E. Smith, R. Challerji, E.H. Stein and A.M. Rutenburg, Arch. Biochem. Biophys., 1960 91, 61-70.

4. E. Szczeklik, M. Orlowski, and A. Szewczuk, Gastroenterology., 1961, 41, 353-359.

5. A.Z. Albert, M. Orlowski, and A. Szewczuk, Nature., 1961, 191, 762-768.

6. A.Z. Albert, M. Orlowski, A.Z. Rzucidlo, and J. Orlowska, Acta. Histochem., 1966, 25, 312-320.

7. A.Z. Albert, J. Orlowska, M. Orlowski, and A. Szewczuk, Acta. Histochem., 1964, 18, 78-89.

8. A.M. Rutenburg, H. Kim, J.W. Fischbein, J.S. Hanker, H.L. Wasserkrug, and A.M. Seligman, J. Histochem. Cytochem., 1969, 17, 517-526.

9. A.Z. Albert, Z. Rzucidlo, H. Sterzyk, Acta. Histochem., 1970, 37, 34-39.

10. L. Naftalin, V.J. Child, D.A. Morley, D.A. Smith, Clin. Chim. Acta., 1969, 26, 297-300.

11. S.B. Rosalki, Adv. Clin. Chem., 1975, 17, 53-107.

12. J.B. Whitfield, R.E. Pounder, G. Neale, and D.W. Moss, Gut., 1972, 13, 702-708.

13. P. Selvariay, K.A. Balasubramanian, P.G. Hill, Enzyme., 1981, 57-63.

14. T.J. Peters, C.A. Seymour, G. Wells, F. Fakunle, and G. Neale, Brit. Med. J., 1977, 1576-1577.

15. S. Fiala, A.E. Fiala, and B. Dixon, J. Natn. Cancer. Inst., 1972, 48, 1393 - 1401.

16. M.M. Manson, R.F. Legg, J.V. Watson, J.A. Green, and G.E. Neal, Carcinogenesis, 1981, 2, 661-670.

17. S. Horiuchi, M. Inoue, and Y. Morino, Eur. J. Biochem., 1978, 87, 429 - 437.

18. M. Inoue, S. Horiuchi, and Y. Morino, Eur. J. Biochem., 1977, 78, 609-615.

19. N.D. Cook, and T.J. Peters, Biochem. Biophys. Acta., 1985, 828, 205-212.

20. 'Boronate Ligands in Biochemical Separations' Amicon Corporation, 1981.

21. N. Taniguchi, J. Biochem (Tokyo), 1974, 75, 473.

22. J.L. Ding, G.D. Smith, and T.J. Peters, Biochim. Biophys. Acta., 1981, 661, 191-198.

23. B. Nash, Proc. Fed. Am. Soc. Exp. Biol., 1980, 39, 1866.

24. N.E. Huseby, Biochem. Biophys. Actu., 1977, 483, 46-56.

25. Y. Matsuda, A. Tsuji, and N. Katunuma, J. Biochem., 1983, 93, 1427-1433.

26. R.P. Hughey, and N.P. Curthoys, J. Biol. Chem., 1976, 25, 7863-7870.

27. S.J. Gardell, and S.S. Tate, J. Biol. Chem., 1981, 256, 4799-4804.

28. H. Tsuji, Y. Matzula, and N. Katunuma, J. Biochem (Tokyo)., 1980, 87, 1567.

29. S.S. Tate, and M.E. Ross, J. Biol. Chem., 1977, 256, 4799.

30. S. Horiuchi, I. Masayasu, and Y. Morino, Biochem. Biophys. Res. Commun., 1978, 80, 873-878.

31. S. Horiuchi, M. Inoue, and Y. Morino, Eur. J.Biochem., 1980, 105, 93-102.

32. K. Yamashita, A. Hitoi, Y. Masuda, A. Tsuji, N. Katunuma, and A. Kobata, J. Biol, Chem., 258, 1983, 1098-1107.

33. T. Miura, Y. Matsuda, A.Tsuji, and N. Katunuma, J.Biochem., 1981, 93, 135-147.

34. T.M. McIntyre, and N.P. Curthoys, J. Biol. Chem., 1979, 254, 6499-6504.

35. S.S. Tate, and A. Meister, Proc. Natl. Acad. Sci. U.S.A., 1974, 71, 3329.

36. G.A. Thompson, and A. Meister, Biochem. Biophys. Res. Commun., 1976, 71, 32.

37. A.M. Karkowsky, and M. Orlowski, J. Biol. Chem., 1978, 253, 1574-1581.

38. J.S. Elce, 'Functions of Glutathione in Liver and Kidney', Springer, Heidelberg, 1980.

39. S.S. Tate, and A. Meister, J. Biol. Chem., 1974, 249, 7593-7602.

40. N.D. Cook, and T.J. Peters, Biochim. Biophys. Acta., 1985, 832, 142-147.

41. M. Orlowski, and A. Meister, Biochim. Biophys. Acta., 1963, 73, 679-681.

42. G.D. Smith, J.L. Ding, and T.J. Peters, Anal. Biochem., 1979, 100, 136-139.

43. A. Thompson and A. Meister, J. Biol. Chem., 1979, 254, 2956.

44. O.W. Griffiths, and A. Meister, Proc. Natl. Acad. Sci. USA., 1979, 76, 268-272.

45. R.J. Bridges, O.W. Griffiths, and A. Meister, J. Biol. Chem., 1980, 25, 510.

46. G.G. Glenner, J.E. Folk, and P.J. McMillon, J. Histochem. Cytochem., 1982, 10, 481.

47. A. Meister, Science., 1973, 180, 33-39.

48. N.P. Curthoys, and R.P. Hughey, Enzyme., 1979, 24, 383-403.

49. G.M. Payne, and J.W. Payne, BiochemJ., 1984, 218, 147-155.

50. T.M. McIntyre, and N.P. Curthoys, Int. J. Biochem., 1980, 12, 545-551.

51. M.E. Anderson, R.J. Bridges, A. Meister, Biochem. Biophys. Res. Commun., 1980, 96, 848-853.

52. N.D. Cook, and T.J. Peters, Biochim. Biophys. Acta., 1986, 884, 207-210.

53. S.S. Tate, and A. Meister, J. Biol. Chem., 1975 250, 9530-9533.

54. D.P. Jones, P. Moldeus, A.H. Stead, K. Ormstad, H. Jornwall, and S. Orrenius, J. Biol. Chem., 1979, 254(8, 2787-2792.

55. M.Orlowski, Arch. Immunol. Ther. Exp., 1963, 11, 1-61.

56. M.M.R. Kalengayi, G. Ronchi, V.J. Desmet, J. Natl. Cancer. Inst., 1975 55, 579-588.

57. M.A. Gerber, and S.N. Thung, Am. J. Pathol., 1980, 98, 394-400.

58. Report of a World Health Organisation Meeting, 'World Health Organisation Technical Report Series', No. 694, Geneva, 1983.

59. S. Wilk, H. Mizoguchi, and M. Orlowski, J. Pharmacol. Exp. Ther., 1978, 206 (1), 227.

60. G.D. Smith, P.K. Chakravarty, T.A. Conners, and T.J. Peters, Biochem. Pharmacol., 1984, 33, 527.

61. J.A. Montgomery, J. Med. Chem., 1980, 23, 1063.

62. C. Hansch, A. Leo, C. Schmidt, P.C.C. Jow, and J.A. Montgomery, J. Med. Chem., 1980, 23, 1095.

63. K.W. Kohn, and N.W. Gibson, I.A.R.C. Sci. Publ., 1986, 70, 155-62.

64. J.W. Lown, L.M. McLaughlin, and Y.M. Change, Bioorg. Chem., 1978, 7, 97.

65. A.C. Sartorelli, 'American. Chem. Society', A.C.S. Symp, Series 30, 1976.

66. T.P. Johnston, G.S. McCaleb, J.A. Montgomery, J. Med. Chem., 1963, 6, 669.

67. T.P. Johnston, G.S. McCaleb, and J.A. Montgomery, J. Med. Chem, 1966,9 , 892.

68. T.P. Johnston, G.S. McCaleb, and J.A. Montgomery, J. Med. Chem, 1975, 18, 104.

69. M. Colvin, R.B. Brundrett, W. Cowens, L. Jardine, and D.B. Ludlum, Biochem. Pharm., 1976, 25, 695.

70. K.A. Hyde, E. Acton, W.A. Skinner, L. Goodman, J. Greenburg, and B.R. Baker., J. Medical and Pharmaceutical Chem., 1962. 5, 1-14.

71. E.H. White, J. Am. Chem. Soc., 1955, 77, 6008.

72. R.F. Weaver, K.V. Rajagopalan, and P. Handler, J. Biol. Chem., 1971, 246, 2010-2014.

73. R.F. Weaver, K.V. Rajagopalan, and P. Handler, P. Jeffs, W.L. Byrne, and D. Rosenthal, Proc. Natl. Acad. Sci. USA., 1970, 67, 1050-1056.

74. S.F. Vogel, L.A.K. Kemper, S.J. McGarny, and D.G. Graham, Am. J. Pathol., 1975, 78, 33-48.

75. N.K. Hayworth, and S.M. Lavin, Life. Sci., 1985, 36, 2039-2046.

76. J.L Webb, 'Enzyme and Metabolic inhibitors', Academic Press, New York, 1966, Vol III.

77. Y. Kurata, H. Tsuda, S. Tamano, and N. Ito, Can. Lett., 1985, 28, 19-25.

78. I.C. Calder, C.C. Finder, G.R. Green, K.N. Ham, and J.D. Tange, Brit. Med. J., 1971, 4, 518-521.

79. K. Boekelheide, D.G. Graham, P.D. Mize, and E.H. Koo, J. Invest. Derm., 1980, 75, 322-327.

80. M. Bergmann, and L. Zervas, Ber., 1932, 65, 1192.

81. F. Weygand, and W. Steglich, Z. Naturforsch., 1959, 14B, 472-473

82. R.H. Sifford, and V. Du Vigneand, J. Biol. Chem., 1935, 108, 753.

83. D. Ben and I. Shai, J. Org. Chem., 1954, 19, 62.

84. E. Fischer, and Warburg, Ber., 1905, 38, 3997.

85. F.E. King, and D.A.A. Kidd, J. Chem. Soc., 1949, 4, 3315-3319.

86. J.C. Sheehan, and V.S. Frank, J. Am. Chem. Soc., 1949, 71, 1856.

87. B. Schröder, and K. Lubke, 'Methods of peptide synthesis', Academic Press, 1965, Vol 1.

88. E. Fischer, Ber., 1905, 38, 4173.

89. M. Bergmann, L. Zervas, and W.F. Ross, J. Biol. Chem., 1935, 111, 245.

90. R.B. Merrifield, and D.W. Wooley, J. Am. Chem. Soc., 1958, 80, 6635.

91. R. Schwyzer, and P. Siber, Helv. Chim. Acta., 1959, 42, 972.

92. F. Weygand, and K. Hunger, Z. Naturfo Brsch. 1958, 136, 50

93. H. Sachs, and E. Brand, J. Am. Chem. Soc., 1953, 75, 4610.

94. G. Amiard, R. Heymes, and L. Velluz, Bull. Soc., Chim., 1956, 97.

95. M. Bergmann, L. Zervas, and L. Salzmann, Ber., 1933, 66, 1288.

96. G.H.L. Nefkens, and R.J.F. Nivard, Red. Trav. Chim. Pays-Bas., 1964, 83, 199.

97. J.K. Vaughan, and R.L. Osato, J. Amer. Chem. Soc., 1952, 74, 676.

98 J.C. Sheehan, and G.P. Hess, J. Am. Chem. Soc., 1955, 77, 1067.

99. J.R. Milligan, PhD Thesis, University of London, Imperial College, 1984.

100. P.F. Wiley, J. Am. Chem. Soc., 1949, 71, 3746-3748.

101. R.S. Lott, V.S. Chauhan, and C.H. Stammer, J. Chem. Soc. Chem. commun., 1979, 495.

102. H.Suli-Vargha, K. Medzihradszkey, Int. J. Pept. Protein. Res., 1984, 23, 650-6.

103. K.A. Hyde, E. Adon, W.A. Skinner, L. Goodman, J. Greenberg, and B.R. Baker, J. Med. Chem., 1962, 5, 1-14.

104. T.N. Ghosh, B. Banerjee, and S.L. Lasker, J. Indian. Chem. Soc., 1944, 21 354-356.

105. D.M. Neville Jr, J. Biol. Chem., 1971, 246, 6328-6334.

106. S.S.Tate, "Enzymatic Basis of Detoxication", ed. W.B. Jakeby Academic Press, New York, 1980, vol. 2, p. 95

107. N.D. Cook, and T.J. Peters, Biochem. Soc. Trans., 1985, 13, 1226-1227.

108. M. Orlowski, and A. Meister, Biochem. Biophys. Acta., 1963, 73, 679-681.

109. G.D. Smith, J.L. Ding, and T.J. Peters, Anal. Biochem., 1979, 100, 136-139.

110. A. Cornish-Bowden, W.R. Porter, and W.F. Trager, J. Theor. Biol., 1978, 74, 163-175.

111. N.D. Cook, K.P. Upperton, B.C. Challis, and T.J. Peters, Biochem.Biophys.Acta., 1987, 914, 240-245.

112. J.K. Fawcett, and J.E. Scott, J. Clin. Path., 1960, 13, 156-159.

113. Perrin 'Dissociation constants of organic bases in aqueous solutions', IUPAC supplement 1972.

114. S.S. Tate, and A. Meister, Pro. Natl. Acad. Sci. USA., 1978, 75, 4806 - 4809.

115. N. Chahal, personal communication.

116. M.J. Colvin, and K.A. Brundrett, 'Nitrosoureas, current status, new developments', 1981, Chapt. 5, 51-67.

117. H. Kappus, Biochem. Pharm., 1986, 35, 1-6.

118. N.R. Bacher, S.L. Gordon, and M.V. Gee, Mol. Pharm., 1977, 13, 901-910.

119. N.R. Bacher, S.L. Gordon, and M.V. Gee, Cancer Res., 1978, 38, 1745-1750.

120. A. Begleiter, Biochem. Pharm., 1985, 34, 2629-2636.

121. I.C. Caulder, A.C. Yong, R.A. Woods, and C.A. Crowe, Chem. Biol. Interact., 1979, 27, 246-254.

122. G. Powis, E.M. Hodnett, K.S. Satone, K.L. See, and D.C. Melder, Cancer Res., 1987, 47, 2363-2370.

123. R. Van de Stratt, J. DeVries, and N.P.E. Vermeules, Biochem. Pharm., 1987, 36, 613-619.

124. D.D. Perrin, W.L.F. Armarego, and D.R. Perrin, 'Purification of Laboratory Chemicals', Pergamon, Oxford, 1982, Second Edn.

125. M.M. Bradford, Anal. Biochem., 1976, 72, 248-254.

126. J.B. Chappel, Biochem. J., 1964, 19, 225-237.

127. Chapman and Hall, 'Dictionary of Organic Compounds' J. Buckingham, New York, London, Toronto, 1982, fifth Edn, Vol 3.

128. W.R. Boehme, J. Am. Chem. Soc., 1953, 75, 2502.

129. W.G. Christiansen, J. Am. Chem. Soc., 1923, 45, 2193.

APPENDIX

1)Attempted synthesis of α-N-benzyloxycarbonyl-α-benzyl-γ-(N-(2-chloroethyl)-carbamoyl, N-(2-chloroethyl))-L-glutaminate (18)

Condensation of α-N-benzyloxycarbonyl-α-benzyl-γ-N-(2-chloroethyl)-L-glutaminate (14) with 2-chloroethyl isocyanate

i) In sodium dry toluene

To α-N-benzyloxycarbonyl-α-benzyl-γ-N-(2-chloroethyl)-L-glutaminate (14) (0.15g), 0.35 mmol) in sodium dried toluene (50 ml), a 10 fold excess of 2-chloroethyl isocyanate (0.295 cm^3, 3.5 mmol) was added. The solution was boiled under reflux for 24h and the solvent removed under vacuum. Examination of the residue by TLC (ethyl acetate eluent), ^{1}Hnmr and ir revealed that the product consisted primarily of starting material.

ii) In DMSO

Compound (14) (0.15g, 0.35 mmol) was refluxed with a 10 fold excess of 2-chloroethyl isocyanate (0.295 cm^3, 3.5 mmol) for 24h in DMSO (50 ml). Removal of the solvent under vacuum gave a yellow oil. Examination of the oil by TLC (ethyl acetate eluent) showed the presence of a number of products, these were separated on a silica gel column (ethyl acetate eluent). Examination of the products by ir, ^{1}Hnmr and mass spectrometry showed that none corresponded to the desired compound; α-N-benzyloxycarbonyl-α-benzyl-γ-(N-(2-chloroethyl)-carbamoyl, N-(2-chloroethyl))-L-glutaminate (18).

iii) In a Carius tube

To a Carius tube was added a mixture of (14) (0.5g, 1.2 mmol) and 2-chloroethyl isocyanate (1.023 cm^3, 12 mmol) in sodium dried

toluene (100 ml). The carius tube was sealed and heated at 135°C for 24h. Removal of the solvent under vacuum gave a yellow oil. Examination of the oil by TLC showed that it contained several products, these were separated on a silica gel column (ethyl acetate eluent). Examination of the products by ir, ^{1}Hnmr and mass spectrometry showed that none corresponded to the desired product, compound (18).

iv) Using n-BuLi

Compound (14) (0.5g, 1.2 mmol) was dissolved in THF (100 ml) and kept at a temperature of ca. -78°C (Acetone/CO_2). The system was flushed through with, and kept under an argon atmosphere. n-BuLi (0.75 cm^3, 1.2 mmol) was fed into the solution via a syringe and the whole then left stirring for 30 min at ca. -78^{3}C. 2-Chloroethyl isocyanate (1.023 cm^3, 12 mmol) was then added (via syringe) and the solution allowed to warm to room temperature, with stirring.

The reaction mixture was then quenched with 5M HCl (0.24 cm^3, 1.2 mmol) in H_2O (10 ml) and the THF removed under vacuum. The organic product was then extracted from the aqueous system into CH_2Cl_2 (3 x 50 ml).

Removal of the solvent under vacuum gave a yellow semi-solid which was separated with silica gel (ethyl acetate eluent). Examination of the products by ir, ^{1}Hnmr and mass spectrometry, showed that none corresponded to the desired compound (18).

The experiment was repeated using an equivalent of 2-chloroethyl isocyanate (as opposed to a 10 fold excess) and again a number of indeterminate products were obtained (as judged by ir, ^{1}Hnmr and mass spectrometry).

2) Attempted synthesis of α-N-benzyloxycarbonyl-α-benzyl-γ-(N-(2-chloroethyl)-carbamoyl, N-cyclohexyl)-L-glutaminate (19).

Condensation of α-N-benzyloxycarbonyl-α-benzyl-γ-N-cyclohexyl-L-glutaminate (15) with 2-chloroethyl isocyanate

i) In dry toluene

Method as for compound (14). using α-N-benzyloxycarbonyl-α-benzyl-γ-N-cyclohexyl-L-glutaminate (15), (0.2g, 0.11 mmol) and 2-chloroethyl isocyanate (0.19 cm^3, 6.6 mmol). Again examination of the product by TLC, ir and ^{1}Hnmr showed that it consisted primarily of starting material.

(ii) In DMSO

Method as for compound (14), using (15) (0.2g, 0.66 mmol) and 2-chloroethyl isocyanate (0.19 cm^3, 6.6 mmol). Of the products obtained by separation of the residue on silica gel (ethyl acetate eluent), none corresponded to the required compound (19), (as judged by ir, ^{1}Hnmr and mass spectrometry).

iii) In a Carius tube

Method as for compound (14).

A number of indeterminate products were obtained none of which corresponded to compound (19), (as judged by ir, ^{1}Hnmr and mass spectrometry).

iv) Using *n*BuLi

Method as for compound (14).

Using both a 10 fold excess of 2-chloroethyl isocyanate or an

equivalent, none of the products obtained corresponded to compound (19) (as judged by ir, ^{1}Hnmr, and mass spectrometry).

Biochimica et Biophysica Acta 914 (1987) 240–245
Elsevier

BBA 32910

The donor specificity and kinetics of the hydrolysis reaction of γ-glutamyltransferase

[a] Neil D. Cook, [a] Kim P. Upperton, [b] Brian C. Challis and [a] Timothy J. Peters

[a] *MRC Clinical Research Centre, Division of Clinical Cell Biology, Harrow and* [b] *Department of Chemistry, University of London Imperial College, London (U.K.)*

(Received 13 May 1987)

Key words: γ-Glutamyltransferase; Deuterium oxide; Enzyme kinetics

The donor specificity of the hydrolytic reaction of γ-glutamyltransferase ((5-glutamyl)-peptide : amino-acid 5-glutamyltransferase, EC 2.3.2.2) has been studied by the use of specifically synthesised γ-glutamyl substrates. It was found that a wide variety of γ-glutamylated adducts were hydrolysed by the enzyme. The structure of the adduct was relatively unimportant for donor specificity and the enzyme appears to 'recognise' the γ-glutamyl portion of the donor molecule. In particular the α-amino group and the free proton of the γ-peptide bond appear to be essential for donor activity. The V_{max} of hydrolysis increased proportionally to the electron-withdrawing capacity of the adduct moiety. The rate of formation of γ-glutamyl-enzyme intermediate was therefore dependent upon the structure of the adduct of the γ-glutamyl donor. The results suggest that the enzyme shows little specificity beyond that for γ-glutamyl amides and there is therefore no reason to postulate the presence of a specific glutathione-binding site.

Introduction

γ-Glutamyltransferase ((5-glutamyl)-peptide : amino-acid 5-glutamyltransferase, EC 2.3.2.2) is a plasma-membrane ectoenzyme. It catalyses the transfer of a γ-glutamyl group from donor molecules such as glutathione to a wide variety of acceptor amino acids and dipeptides, forming γ-glutamylated peptides [1–3]. In the absence of a suitable acceptor the enzyme catalyses the hydrolysis of the donor. The enzyme exhibits ping-pong type kinetics [1] and forms a γ-glutamylated-enzyme intermediate [4].

The transpeptidation reaction has been postulated to be involved in amino-acid transport via the γ-glutamyl cycle [5]. γ-Glutamyltransferase is proposed to transfer γ-glutamyl groups from extracellular glutathione to amino acids. The γ-glutamylamino acid is then transported into the cell, where it is cleaved by γ-glutamylcyclotransferase to produce 5-oxo-proline and the free amino acid. However, other workers have forwarded evidence that the predominant action of γ-glutamyltransferase is to catalyse the hydrolysis of glutathione and that the enzyme functions in vivo as a glutathionase [6].

The enzyme also catalyses the hydrolysis of glutamine and was first reported as a phosphate-independent glutaminase [7]. The rate of glutamine hydrolysis is significantly lower than the rate of hydrolysis of glutathione or artificial donors and is therefore usually considered unimportant. However, the results of Dass [8] suggest that

Abbreviation: Mops, 4-morpholinepropanesulphonic acid.

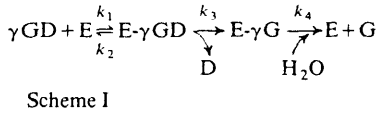
Correspondence: N.D. Cook, MRC Clinical Research Centre, Division of Clinical Cell Biology, Watford Road, Harrow, HA1 3UJ, U.K.

0167-4838/87/$03.50 © 1987 Elsevier Science Publishers B.V. (Biomedical Division)

glutamine utilisation by γ-glutamyltransferase may be significant.

The specificity of the acceptor site has been studied previously, indicating that the best acceptors are likely to be the deprotonated sulphur-containing amino acids [3,9]. However, the specificity of the donor site has not been studied.

The hydrolytic reaction involves the cleavage of the γ-glutamyl donor to form a covalent γ-glutamylated enzyme intermediate followed by the breakdown of the intermediate, giving free enzyme and glutamic acid. It therefore represents the first half of the ping-pong transpeptidation reaction. At initial rates, the reaction can be represented as shown in Scheme I, where G, glutamate; E, enzyme; D, donor ligand, and k_1–k_4 represent the rate constants for each step.



Scheme I

In the present work we report the specificity and kinetics of the above reaction and relate the data obtained to the known physiological γ-glutamyl donors, glutathione and glutamine.

Experimental

Chemicals

γ-Glutamyl 7-amino-4-methylcoumarin and L-glutamic acid were purchased from Cambridge Research Biochemicals (Harston, U.K.). All other chemicals were purchased from either Sigma Chemicals (Poole, U.K.) or BDH (Poole, U.K.).

Preparation of putative γ-glutamyl substrates

γ-Glutamyl substrates were synthesised from a protected glutamic acid derivative [10–12] via a mixed anhydride, the details of the synthesis of these compounds will be published elsewhere. All substrates were determined pure by microanalysis, sharp melting point and fast atom bombardment mass spectrometry.

Measurement of γ-glutamyl hydrolase activity

γ-Glutamyl 7-amino-4-methylcoumarin hydrolysis was measured by the fluorometric method of Smith et al. [13]. γ-Glutamyl-*p*-nitroanilide hydrolysis was measured spectrophotometrically at 405 nm [1]. All other donors were measured by the HPLC method of Cook and Peters [14] or by ammonia release [15].

Purification of γ-glutamyltransferase

γ-Glutamyltransferase was purified from rat kidney by the method of Cook and Peters [3]. Protein was measured by the method of Bradford [16] with bovine serum albumin as a standard.

Results

Effect of deuterium oxide on the hydrolytic reaction of γ-glutamyltransferase

The hydrolytic reaction is slower than the transpeptidation reaction with donors such as γ-glutamyl 7-amino-4-methylcoumarin [3]. As the two reactions probably share the same steps up to the formation of the γ-glutamyl-enzyme intermediate, it is likely that the rate of hydrolytic breakdown of this intermediate (k_4) is the rate-limiting step in the hydrolytic reaction. To test whether the breaking of the bonds of the water molecule is rate-limiting in this step, the reaction was studied in various concentrations of deuterium oxide (^2H_2O). The results are depicted in Fig. 1. There was no apparent change in the V_{max} of hydrolysis due to increasing concentrations of ^2H_2O. The V_{max} is a function of the constants k_3 and k_4, which are the catalytic steps involving the formation and hydrolytic breakdown of the enzyme-γ-glutamyl intermediate. The results suggest that the breaking of the O–H bond on the water molecule is not rate-limiting in the hydrolytic step. There was, however, a significant increase in the K_m, which may be attributable to an isotope-exchange effect with either the donor or the enzyme itself. The results are consistent with some form of hydrogen bonding being involved in the donor substrate-binding site.

Kinetics of hydrolysis of artificial and physiological γ-glutamyl donors

Table IA shows the kinetic parameters determined for the commonly used artificial donors γ-glutamyl-*p*-nitroanilide and γ-glutamyl 7-amino-4-methylcoumarin and the most probable physiological donors, glutathione and glutamine. The

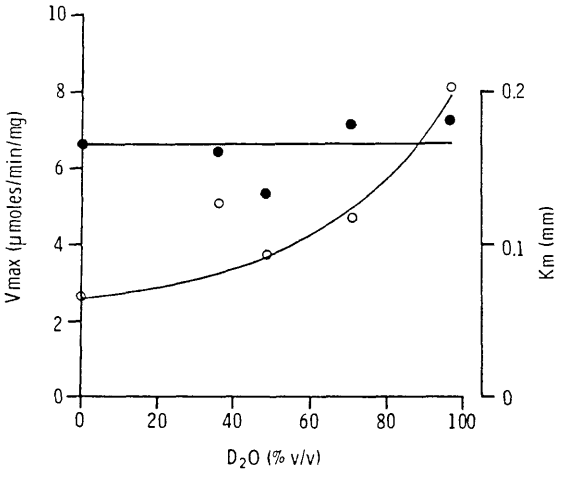


Fig. 1. Effect of ^2H_2O on the V_{max} and K_m of hydrolysis of γ-glutamyl 7-amino-4-methylcoumarin by γ-glutamyltransferase. γ-Glutamyl hydrolase activity was measured at 37 °C in 100 mM Mops buffer (pH* 7.4). K_m and V_{max} were determined with the direct linear plot method [22]. (●, V_{max}; ○, K_m).

V_{max} obtained for both oxidised and reduced glutathione are not significantly different from the artificial donor γ-glutamyl 7-amino-4-methylcoumarin. However, the V_{max} for the artificial donor γ-glutamyl-*p*-nitroanilide is significantly increased, by 2.2-fold, over the former substrates. In contrast, the V_{max} for glutamine hydrolysis is significantly decreased, 5-fold, with respect to glutathione. As the rate constant k_4 is associated with the breakdown of the γ-glutamyl intermediate, we can assume that this rate constant is independent of the donor used. If we make this assumption it would appear that a change in the rate constant k_3 is responsible for the differences observed in the V_{max} with various substrates. The rate of formation of γ-glutamylated enzyme is therefore dependent upon the donor substrate used.

The importance of the adduct to donor specificity

To further probe the importance of the γ-

TABLE 1

KINETIC CONSTANTS DETERMINED FOR ARTIFICIAL AND PHYSIOLOGICAL DONORS OF γ-GLUTAMYLTRANSFERASE

Kinetic parameters were determined by the direct linear plot method [22]. Figures in brackets indicate 95% confidence limits. * Concentration determined as the concentration of γ-glutamyl groups available for reaction. n.d., not detectable.

	K_m (μM) $\frac{(k_4 \cdot k_3)+(k_4 \cdot k_2)}{(k_1 \cdot k_4)+(k_1 \cdot k_3)}$	V_{max} (μmol/min per mg protein) $\frac{k_3 \cdot k_4}{k_3 + k_4}$
(A) Common artificial and physiological donors		
γ-Glutamyl 7-amino-4-methylcoumarin	135 (81–195)	9.18 (8.2–10.1)
γ-Glutamyl-*p*-nitroanilide	166 (99–260)	26.4 (21.2–31.9)
Reduced glutathione	15.0 (7.5–33.5)	11.9 (10.2–15.6)
Oxidised glutathione *	15.2 (11.2–20.9)	8.8 (7.9–10.3)
L-Glutamine	660 (324–1290)	2.2 (1.6–3.1)
(B) Substitution of adduct structure		
γ-Glutamylethylamine	804 (756–894)	5.4 (5.3–5.6)
γ-Glutamyldiethylamine	n.d. (–)	(–)
γ-Glutamyldimethylamine	n.d. (–)	(–)
γ-Glutamyl-*t*-butylamine	1251 (514–3720)	0.32 (0.25–0.57)
γ-Glutamylpyrrolidine	n.d. (–)	(–)
γ-Glutamyl *p*-methylanilide	36.3 (21.4–43.5)	16.1 (14.7–17.2)
γ-Glutamyl *N*-methyl-*p*-nitroanilide	n.d. (–)	(–)
(C) Substitution of glutamyl structure		
D-Glutamine	393 (214–550)	1.03 (0.72–1.44)
L-Asparagine	n.d.	(–)
t-Butyl-γ-carboxyglutamine	925 (430–1440)	1.37 (1.09–1.58)
γ-*N*-Acetylglutamine	n.d.	(–)

glutamyl adduct on the donor specificity a number of putative γ-glutamyl substrates were synthesised as described in Experimental. Seven γ-glutamyl analogues were synthesised to enable us to establish some of the structural requirements of the donor site for the γ-glutamyl adduct. The monoethyl and diethyl derivatives were synthesised to test the effect of steric hindrance around the γ-peptide nitrogen on the hydrolysis reaction. The dimethyl derivative incorporates the lack of a free γ-amide proton with minimal steric hindrance, and the pyrrolidine derivative lacks a γ-amine proton with even less steric hindrance around the peptide bond, as this substituted amine is virtually planar. The *t*-butylamine adduct has a γ-peptide proton and an extremely bulky *t*-butyl group, γ-Glutamyl *p*-methylanilide was synthesised as a substrate similar in structure to *p*-nitroanilide, but with less electron-withdrawing capacity. The donor specificity of these substrates is summarised in Table IB. γ-Glutamylethylamine was found to have a higher V_{max} and K_m than glutamine. As discussed later, the increase in K_m for these substrates is in part due to the change in k_3, as the K_m term for the reaction is complex and does not reflect an approximation to the affinity of the enzyme for the substrate. Most noticeably, substitution of the free hydrogen in the γ-glutamyl peptide bond abolishes donor activity. Hence, dimethyl and diethyl analogues of glutamine were not active as donors. To test whether this inactivity is due to steric hindrance within the active site, γ-glutamylpyrrolidine was prepared. γ-Glutamylpyrrolidine was also inactive as a donor substrate for γ-glutamyltranspeptidase. In order to assess the extent to which steric effects of the adduct may be important to substrate specificity, the extremely bulky *t*-butyl analogue was tested. *t*-Butylglutamine was found to be a substrate with a lowered V_{max} when compared to glutamine and a raised K_m. γ-Glutamyl-*p*-methylanilide was found to be a good donor, although it was hydrolysed less rapidly than the nitroanilide.

It can be seen from Table I that the most rapidly hydrolysed donors contain highly electron-withdrawing groups such as the *p*-nitroanilide adduct, while the poorest donors contain electron-donating groups such as the *t*-butyl adduct. The amount of electron-donating or -withdrawing capacity of the adduct will be reflected in the pK of the amino group of the parent compound. Thus, *p*-nitroanilide has an extremely low pK, while *t*-butylamine has a high pK. The relationship between the pK of the γ-glutamyl adduct and the measured V_{max}^{app} is displayed in Fig. 2. There is a striking correlation between the V_{max}^{app} and the pK_a across the range from 1–12. It is therefore apparent that, while the enzyme will tolerate a diverse range of γ-glutamyl donor compounds, the rate of hydrolysis of the compound is dependent upon the chemical nature of the adduct.

The test the effect of the absence of the γ-proton on the rate of hydrolysis of a γ-glutamyl compound which has a highly electron-withdrawing adduct, γ-glutamyl-*N*-methyl-*p*-nitronilide was synthesised. This compound was found to be inactive as a substrate. In terms of binding of the γ-glutamyl compounds, there does not appear to be any clear relationship between structure and K_m. However, the results of Table I would suggest that the adduct is not important in binding provided a free γ-peptide bond proton is present.

The importance of the γ-glutamyl moiety to donor specificity

To test which portions of the γ-glutamyl structure are important in binding, the hydrolysis of a range of substituted glutamines was studied. The results are summarised in Table IC. Asparagine was not hydrolysed by γ-glutamyltransferase indicating that the side-chain length is important. This

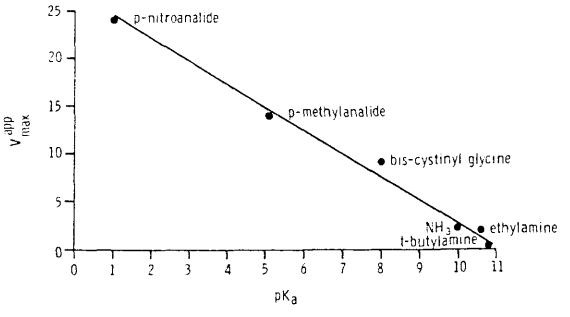


Fig. 2. Relationship between V_{max}^{app} and the pK_a of the leaving group for various γ-glutamyl substrates. V_{max}^{app} was estimated as the rate of glutamic acid produced using 6 mM of each substrate (more than $9 \times K_m$ in each case). Glutamate produced was measured by the HPLC method. pK_a values were obtained from Ref. 23.

would indicate that part of the substrate binding probably occurs at the α-amino or α-carboxy groups. As the enzyme does not display a strict stereospecificity it is likely that only one of these two groups is involved in the primary binding of the γ-glutamyl structure. The results in Table IC suggest that it is the α-amino group which is essential for binding. Substitution of this group completely abolishes donor activity. We were unable to detect ammonia production from *N*-acetylglutamine at concentrations up to 100 mM. Substitution of the α-carboxy group with the bulky *t*-butyl moiety increased the K_m slightly without significantly changing the measured V_{max}. The enzyme therefore appears to bind the α-amino group of the glutamate moiety and presumably another feature involved near the γ-peptide bond. It may be that the free hydrogen atom of the γ-peptide bond is important, as removal of this group results in a loss of activity. Further, diethylglutamine did not inhibit γ-glutamyl 7-amino-4-methylcoumarin hydrolysis when present at concentrations up to 10-fold greater than the substrate concentration. This would suggest that the absence of the hydrogen atom reduces the binding of the compound.

Discussion

In the present work we have studied the donor specificity of γ-glutamyltransferase. In order to probe the donor site, a number of putative γ-glutamyl substrates have been synthesises. The enzyme was found to accept a wide variety of structures, including extremely bulky groups such as γ-glutamyl, *t*-butylglutamine and γ-glutamyl 7-amino-4-methylcoumarin.

The adduct structure appears to be unimportant in determining the selectivity of the substrate. Thus ring structures, long-chained and branched-chain adducts were tolerated by the enzyme. Similarly, neither hydrophobic nor charged groups appeared to be excluded from the hydrolysis reaction. The nonselectivity of the donor site has been reported previously [1,2]. Meister and co-workers have shown that a number of substituted glutathiones and γ-glutamyl peptides may act as donors to the enzyme. In this work we have attempted to analyse the structure required for binding in the enzyme active site. We have found no correlation between the K_m determined for the various substrates and the adduct structure. It is apparent from the K_m expression depicted in Table I that in the case of a ping-pong enzyme the K_m does not approximate to the K_d for the substrate and is therefore not a reflection of the affinity for a substrate. However, of the variety of structures tested, none caused total abolition of donor activity except when the donor lacked a γ-peptide proton. This would suggest that the adduct is not important in the primary binding of the substrate.

The enzyme does, however, appear to be selective for the γ-glutamyl portion of the substrate. Using substituted glutamine analogues we have shown that the α-amino group is important in binding the substrate. The results suggest that the enzyme recognises the γ-glutamyl peptide structure by the peptide bond proton, and possibly the γ-carbonyl group and the α-amino group. The evidence is against a specific donor site specificity other than for γ-glutamyl peptides. There is therefore no reason to suppose that a glutathione-binding site is present, as has been previously postulated [1,2].

We can represent the donor site of γ-glutamyltransferase as depicted in Fig. 3. The enzyme is shown to have primary sites of interaction with the substrate at the α-amino group, the γ-peptide proton and possibly the γ-carbonyl group. The γ-carboxy group and the adduct moiety are portrayed to be essentially outside the active site. The residue Z is meant to represent the amino acid involved in the formation of γ-glutamyl-enzyme intermediate. Although the adduct is not involved in the primary recognition of the donor substrate by the enzyme, it does however determine the rate of formation of γ-glutamyl-enzyme intermediate. The more electron-withdrawing the adduct, the faster the rate of hydrolysis. This is likely to be due to the destabilisation of the γ-peptide bond by the electron-withdrawing groups making the formation of the γ-glutamyl-enzyme intermediate easier. Simulation of the V_{max} equation by substituting various k_3 values at constant k_4 has shown that to produce the range of V_{max} values obtained for the various substrates, k_3 must be varied by at least 100-fold (not shown). There is therefore a significant change in the rate

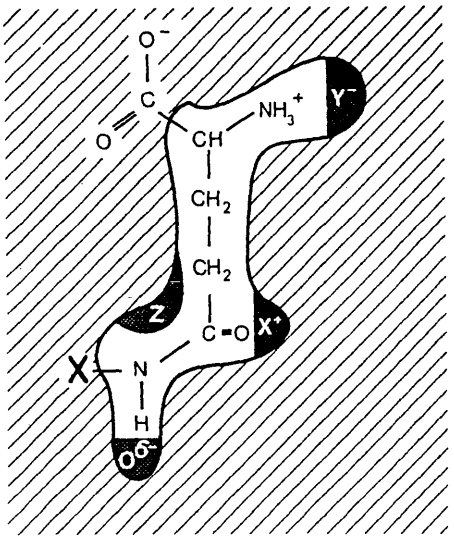


Fig. 3. Proposed map of the donor site of γ-glutamyltransferase. X represents the variable aduct portion of the substrate. Z represents the group in the active site which forms the covalent γ-glutamyl-enzyme intermediate.2e

of formation of γ-glutamyl-enzyme intermediate due to the chemical nature of the adduct used.

The two most likely in vivo donors are glutathione and glutamine. It can be seen from Table I that the V_{max} for glutathione is 4-fold higher than the V_{max} for glutamine. The results of the analogue studies presented here would suggest that there is no reason to suppose that the enzyme is more likely to bind glutathione in preference to glutamine, although the rates of hydrolysis are significantly different. Although glutamine hydrolysis is significantly slower than glutathione hydrolysis, the plasma concentrations of glutamine [17,18] are 100-fold greater than for glutathione [19–21]. As the enzyme appears to be of low adduct specificity, glutamine should compete for binding to the donor site although, once bound, the catalytic hydrolysis is slower than for glutathione. Perhaps of more physiological importance is the fact that the rate of formation of γ-glutamyl-enzyme intermediate with glutamine is 5-fold lower than with glutathione. This may severely affect the steady-state concentration of γ-glutamyl enzyme present. The possible effect of this upon transpeptidation has not been considered, as most workers have not treated glutamine as a significant donor substrate for γ-glutamyltransferase. The simultaneous utilization of glutamine and glutathione by γ-glutamyltransferase is being studied at present to determine the magnitude of competition between these two substrates.

Acknowledgements

We wish to thank Mrs. S. Kingsley for secretarial assistance. N.D.C. is supported by the Cancer Research Campaign. K.P.U. is in receipt of an SERC/MRC CASE award. We also wish to thank Dr. G.D. Smith for his help in deriving the rate equations used in Table I.

References

1 Tate, S.S. and Meister, A. (1974) J. Biol. Chem. 249, 7593–7602
2 Thompson, G.A. and Meister, A. (1971) J. Biol. Chem. 252, 6792–6798
3 Cook, N.D. and Peters, T.J. (1985) Biochim. Biophys. Acta 828, 205–212
4 Elce, J.S. (1980) Biochem. J. 185, 473–481
5 Orlowski, A. and Meister, A. (1970) Proc. Natl. Acad. Sci. USA 67, 1248–1255
6 McIntyre, T.M. and Curthoys, N.P. (1979) J. Biol. Chem. 254, 6499–6504
7 Tate, S.S. and Meister, A. (1975) J. Biol. Chem. 250, 4619–4627
8 Dass, P.D. (1983) Life Sci. 33, 1757–1762
9 Cook, N.D. and Peters, T.J. (1985) Biochim. Biophys. Acta 832, 142–147
10 Bergmann, M. and Zervas, L. (1932) BER. 65, 1194
11 Nefkens, G.H.L. and Nivard, R.J.R. (1984) Recuil. 83, 199–207
13 Smith, G.D., Ding, J.L. and Peters, T.J. (1979) Anal. Biochem. 100, 136–139
14 Cook, N.D. and Peters, T.J. (1985) Biochem. Soc. Trans. 13(6), 1226–1227
15 Fawcett, J.K., Scott, J.E. (1960) J. Clin. Path. 13, 156–159
16 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
17 Jacob, R. and Barrett, E. (1982) J. Chromatogr. 229, 188–192
18 Bos, K.D. and Slump, P. (1985) Clin. Chim. Acta 152, 205–211
19 Teitze, F. (1969) Anal. Biochem. 27, 502–522
20 Hagenfeldt, L., Arvidssan, A. and Larsson, A. (1978) Clin. Chim. Acta 85, 167–173
21 Schulman, J.D., Goodman, S.I., Mace, J.W., Patrick, A.D., Teitze, F. and Butler, E.J. (1975) Biochem. Biophys. Res. Commun. 65, 68–74
22 Cornish-Bowden, A., Porter, W.R. and Trager, W.F. (1978) J. Theor. Biol. 74, 163–175
23 Perrin IUPAC. Dissociation constants of organic bases in aqueous solutions; supplement 1972