CHEMICAL SYNTHESIS AND BIOCHEMICAL STUDIES ON DERIVATIVES OF THE $GABA_A$ RECEPTOR ANTAGONIST, RU5135

by

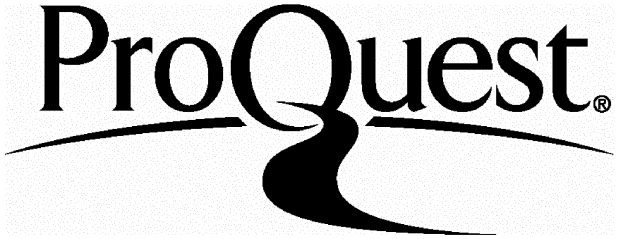
Abigail Rachel Charters

A thesis submitted for the degree of

Doctor of Philosophy of the University of London

Department of Pharmaceutical Chemistry
School of Pharmacy
29/39 Brunswick Square
London
WC1N 1AX

June 1993



ABSTRACT

The $GABA_A$ receptors are fast-acting, ligand-gated chloride ion channels whose function can be allosterically modulated by several classes of therapeutically important drugs such as benzodiazepines, barbiturates and neurosteroids. The $GABA_A$ receptors constitute a large gene family and several complementary DNAs which encode $GABA_A$ receptor subunits have been cloned. Consequently, it has emerged that the mammalian central nervous system may contain several pharmacological subclasses of $GABA_A$ receptors. The characterisation of these subclasses may contribute to the formation of therapeutically selective agents which modulate $GABA_A$ receptor function.

The amidine steroid, RU5135, is a competitive, high affinity antagonist of the $GABA_A$ receptor and the strychnine-sensitive glycine receptor. The compound contains two chemically functional groups which have permitted the synthesis of novel, pharmacologically active derivatives of RU5135. These were isolated by flash chromatography and thin layer chromatography and their structures were analysed by proton nuclear magnetic resonance spectroscopy and electron impact mass spectroscopy.

The *N*- and *O*-derivatives of RU5135 were investigated to determine their respective potencies at membrane-bound $GABA_A$ and glycine receptors and detergent-solubilised $GABA_A$ receptors from bovine brain by the use of radioligand inhibition binding assays. The synthetic derivatives were subsequently immobilised to Sepharose and the resultant RU5135 affinity matrices were characterised with respect to their ability to isolate $GABA_A$ receptors from sodium deoxycholate detergent extracts of bovine cerebral cortex.

For Nan,

with love and happy memories

CONTENTS

Abstract		1
Contents		3
List of Figures		8
List of Tables		10
Abbreviations		12
Acknowledgements		14
Chapter 1	**General Introduction**	16
1.1	Synaptic transmission by amino acids	17
1.2	The GABA receptors	17
1.3	Pharmacological profile of the $GABA_A$ receptor	18
1.3.1	Benzodiazepine modulation of the $GABA_A$ receptor	20
1.3.2	Barbiturate modulation of the $GABA_A$ receptor	22
1.3.3	Neurosteroid modulation of the $GABA_A$ receptor	23
1.3.4	The picrotoxin/TBPS binding site of the $GABA_A$ receptor	24
1.3.5	The avermectin B_{1a} binding site of the $GABA_A$ receptor	25
1.3.6	The Zn^{2+} binding site of the $GABA_A$ receptor	25
1.3.7	Other potential binding sites of the $GABA_A$ receptor	26
1.4	Purification of the $GABA_A$ receptor by benzodiazepine agonist affinity chromatography	27
1.5	Molecular characterisation of the $GABA_A$ receptor	29
1.5.1	The membrane-bound $GABA_A$ receptor	29
1.5.2	The soluble $GABA_A$ receptor	32
1.5.3	The purified $GABA_A$ receptor	32
1.6	Molecular cloning of the $GABA_A$ receptor genes	34

1.7	Heterogeneity of the $GABA_A$ receptor subunits	36
1.7.1	Functional properties of recombinant $GABA_A$ receptors	38
1.7.2	Regional distribution of the $GABA_A$ receptor subunit mRNAs	40
1.7.3	Immunological studies of the $GABA_A$ receptor	42
1.8	Pharmacological properties of the subunits of the $GABA_A$ receptor	44
1.9	Post-translational modification of the $GABA_A$ receptor subunits	46
1.10	The objectives of this study	48
Chapter 2	**Synthesis of the Derivatives of RU5135**	57
2.1	**Introduction**	58
2.1.1	The principles of affinity chromatography	58
2.1.2	Properties of immobilised ligands in affinity chromatography	59
2.1.3	Strategy for synthesis of RU5135 affinity matrices	60
2.1.3.1	Protection of the amidine group of RU5135	61
2.1.3.2	Chemical modification of the C3-hydroxyl group of RU5135	61
2.2	**Materials**	65
2.3	**Methods**	66
2.3.1	Synthesis of RU5135	66
2.3.2	Protection of the amidine group of RU5135	68
2.3.2.1	Reaction of RU5135 with arylsulphonyl chlorides	68
2.3.2.2	Reaction of RU5135 with trityl chloride	69
2.3.2.3	Reaction of RU5135 with benzyloxycarbonyl (cbz) chloride	70
2.3.2.4	Reaction of RU5135 with 9-fluorenylmethoxycarbonyl (fmoc) chloride	71

2.3.3	Reaction of fmoc-RU5135 with 1,4-butanediol diglycidyl ether (BDDE)	72
2.3.4	Deprotection of the amidine group of RU5135 and its derivatives	73
2.3.4.1	Photolytic cleavage of the cbz group	73
2.3.4.2	Base-catalysed cleavage of the fmoc group	73
2.3.5	Derivatisation of RU5135 using diamines	74
2.3.5.1	Reaction of RU5135 with succinyl chloride	74
2.3.5.2	Reaction of RU5135-succinyl chloride with diamines	74
2.3.6	Separation of synthetic compounds by flash chromatography	74
2.4	**Results**	76
2.4.1	Synthesis and chemical characterisation of RU5135	76
2.4.1.1	Synthesis of RU5135	76
2.4.1.2	Chemical characterisation of RU5135	77
2.4.2	Protection of the amidine group of RU5135	78
2.4.2.1	Arginine-type protection groups	78
2.4.2.2	Cbz protection	79
2.4.2.3	Fmoc protection	79
2.4.3	Deprotection of the amidine group of RU5135 and its derivatives	80
2.4.3.1	Photolytic cleavage of the cbz group	80
2.4.3.2	Base-catalysed cleavage of the fmoc group	80
2.4.4	Coupling of fmoc-RU5135 to BDDE	81
2.4.5	Deprotection of fmoc-RU5135-BDDE	81
2.4.6	Derivatisation of RU5135 using diamines	82
2.4.6.1	Reaction of RU5135 with succinyl chloride	82
2.4.6.2	Reaction of RU5135-succinyl chloride with 1,6-diaminohexane	83
2.4.6.3	Reaction of RU5135-succinyl chloride with 1,12-diaminododecane	83

2.5	**Discussion**	107
Chapter 3	**Biochemical Studies on the Derivatives of RU5135**	116
3.1	**Introduction**	117
3.2	**Materials**	119
3.3	**Methods**	120
3.3.1	Preparation of membranes from bovine cerebral cortex and spinal cord	120
3.3.2	Solubilisation of the $GABA_A$ receptor	121
3.3.3	Gel filtration of the $GABA_A$ receptor	118
3.3.4	Determination of protein concentration	121
3.3.5	Radioligand binding assays	123
3.3.5.1	Radioligand binding to membrane-bound receptors	123
3.3.5.2	Radioligand binding to soluble receptors	124
3.3.5.3	Determination of the IC_{50} values for RU5135 and its derivatives on [^{3}H]muscimol specific binding activity to membrane-bound and soluble receptors	124
3.3.5.4	Enhancement of benzodiazepine binding by GABA and neuroactive steroids	125
3.3.6	Preparation of RU5135 affinity chromatography matrices	126
3.3.6.1	Synthesis of the RU5135-epoxy-activated Sepharose 6B affinity matrix	126
3.3.6.2	Synthesis of the RU5135-BDDE Sepharose 6B affinity matrix	127
3.3.6.3	Synthesis of the RU5135-CNBr-activated Sepharose 4B affinity matrix	128
3.3.6.4	Determination of the concentration of immobilised ligand in the affinity matrices by ultra-violet spectrophotometry	129
3.3.7	Affinity chromatography of the $GABA_A$ receptor	130
3.4	**Results**	131

3.4.1	Characterisation of RU5135 binding to $GABA_A$ receptors	131
3.4.1.1	Characterisation of membrane-bound $GABA_A$ receptors	131
3.4.1.2	Characterisation of soluble $GABA_A$ receptors	132
3.4.1.3	Characterisation of membrane-bound, strychnine-sensitive glycine receptors	134
3.4.2	Characterisation of the binding of the synthetic derivatives of RU5135	134
3.4.3	Affinity chromatography of the $GABA_A$ receptor	138
3.4.3.1	Optimisation of experimental conditions	138
3.4.3.2	Determination of immobilised ligand concentration in resins	139
3.4.3.3	Characterisation of the affinities of the derivatised resins for the NaDOC solubilised $GABA_A$ receptor	140
3.4.3.4	Determination of the binding capacity of the derivatised resins in affinity chromatography	141
3.5	**Discussion**	165
Chapter 4	**General Discussion**	178
	Bibliography	186

LIST OF FIGURES

1.1	Chemical structure of selected $GABA_A$ receptors	49
1.2	Chemical structure of modulatory ligands of the $GABA_A$ receptor	50
1.3	Predicted topology of a ligand-gated ion channel subunit	52
2.1.1	The principle of affinity chromatography	63
2.1.2	The chemical structure of RU5135	64
2.4.1	The synthesis of RU5135	84
2.4.2	^1H nmr of RU5135	85
2.4.3	Electron impact mass spectrum of RU5135	86
2.4.4	^1H nmr of 3α-acetoxy-11,17-dioxo-5β-androstane	87
2.4.5	^1H nmr of 3α-acetoxy-11,17-dioxo-16-oximino-5β-androstane	88
2.4.6	^1H nmr of nitrile amide intermediate IV	89
2.4.7	^1H nmr of nitrile carbamic acid intermediate V	90
2.4.8	Protection moieties used for the amidine group of RU5135	91
2.4.9	The synthesis of ligands to be coupled via epoxide groups	92
2.4.10	UV spectrum of photolysed epoxy-activated Sepharose 6B	93
2.4.11	The synthesis of ligands to be coupled via amine groups	94
2.5.1	Base-catalysed elimination of the fmoc group	115
3.4.1	Effect of thiocyanate and chloride ions on the affinity of RU5135 for the membrane-bound $GABA_A$ receptor	144
3.4.2	Effect of RU5135 on the enhancement of [^3H]flunitrazepam specific binding activity by GABA in bovine cerebral cortex membranes	145

3.4.3	Competitive inhibition of [^{3}H]muscimol specific binding activity by RU5135 and fmoc-RU5135 in NaDOC-solubilised $GABA_A$ receptors	146
3.4.4	Competitive inhibition of [^{3}H]muscimol specific binding activity by RU5135, fmoc-RU5135 and RU5135-BDDE in NaDOC-solubilised $GABA_A$ receptors	147
3.4.5	Competitive inhibition of [^{3}H]strychnine specific binding activity by RU5135, fmoc-RU5135-BDDE and RU5135-BDDE in bovine spinal cord membranes	148
3.4.6	Time-dependence of the removal of the fmoc protection moiety from the amidine group of RU5135	149
3.4.7	Competitive inhibition of [^{3}H]muscimol specific binding activity by RU5135, RU5135-succinyl chloride and RU5135-succinyl-6-aminohexane in NaDOC-solubilised $GABA_A$ receptors	150
3.4.8	Competitive inhibition of [^{3}H]muscimol specific binding activity by RU5135, RU5135-succinyl chloride and RU5135-succinyl-12-aminododecane in NaDOC-solubilised $GABA_A$ receptors	151
3.4.9	Competitive inhibition of [^{3}H]strychnine specific binding activity by RU5135, RU5135-succinyl chloride and RU5135-succinyl-12-aminododecane to membrane-bound glycine receptors in bovine spinal cord	152
3.4.10	Competitive inhibition of [^{3}H]muscimol specific binding activity to NaDOC-solubilised $GABA_A$ receptors by RU5135-BDDE-Sepharose 6B	153
3.4.11	Competitive inhibition of [^{3}H]muscimol specific binding activity to NaDOC-solubilised $GABA_A$ receptors by RU5135-succinyl-12-Sepharose 4B	154

LIST OF TABLES

1.1	Summary of the benzodiazepine agonist affinity columns and the experimental conditions used for the purification of $GABA_A$ receptors	53
1.2	Characteristics of the known subunits of vertebrate $GABA_A$ receptors	54
2.4.1	The yields of the intermediate compounds in the synthesis of RU5135	95
2.4.2	Spectral and physical data for RU5135	96
2.4.3	Spectral and physical data for methylbenzene sulphonyl RU5135	98
2.4.4	Spectral and physical data for mesitylene-2-sulphonyl RU5135	99
2.4.5	Spectral and physical data for cbz-RU5135	100
2.4.6	Spectral and physical data for fmoc-RU5135	101
2.4.7	Spectral and physical data for fmoc-RU5135-BDDE	102
2.4.8	Spectral and physical data for RU5135-BDDE	103
2.4.9	Spectral and physical data for RU5135-succinyl chloride	104
2.4.10	Spectral and physical data for RU5135-succinyl-6-aminohexane	105
2.4.11	Spectral and physical data for RU5135-succinyl-12-aminododecane	106
3.4.1	IC_{50} values for RU5135 and its derivatives for the competitive inhibition of [^{3}H]muscimol at the NaDOC-solubilised $GABA_A$ receptor	155
3.4.2	Summary of the time-dependence of removal of the fmoc protection moiety from the amidine group of RU5135	156
3.4.3	Optimisation of the conditions for affinity chromatography using ethanolamine-treated Sepharose 6B	157

3.4.4	Optimisation of the conditions for affinity chromatography using Tris HCl-treated Sepharose 6B	158
3.4.5	Optimisation of the conditions for affinity chromatography using glycine-treated Sepharose 6B	159
3.4.6	UV spectroscopy of immobilised ligands	160
3.4.7	Affinity chromatography of the NaDOC-solubilised $GABA_A$ receptor by RU5135-epoxy-activated Sepharose 6B	161
3.4.8	Affinity chromatography of the NaDOC-solubilised $GABA_A$ receptor by RU5135-BDDE-Sepharose 6B	162
3.4.9	Affinity chromatography of the NaDOC-solubilised $GABA_A$ receptor by RU5135-succinyl-6-Sepharose 4B	163
3.4.10	Affinity chromatography of the NaDOC-solubilised $GABA_A$ receptor by RU5135-succinyl-12-Sepharose 4B	164

ABBREVIATIONS

^{1}H nmr	Proton nuclear magnetic resonance spectroscopy
[^{3}H]PrCC	[^{3}H]Propyl-β-carboline-3-carboxylate
APSA	3-Aminopropanesulphonic acid
AVM	Avermectin B_{1a}
BDDE	1,4-butanediol diglycidyl ether
BSA	Bovine serum albumin
BZ	Benzodiazepine
Cbz	Benzyloxycarbonyl
$CDCl_3$	Deuterated chloroform
cDNA	Complementary DNA
CH_2Cl_2	Dichloromethane
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulphonate
$CHCl_3$	Chloroform
CNBr	Cyanogen bromide
CNS	Central nervous system
d-DMSO	Deuterated dimethylsulphoxide
DAVA	δ-Aminovaleric acid
DBU	Diaza-bicyclo-undecane
DMCM	Methyl-4-ethyl-6,7-dimethoxy-β-carboline-3-carboxylate
DMSO	Dimethylsulphoxide
EDTA	Ethylenediamine tetraacetic acid
EI-MS	Electron impact mass spectroscopy
EtOAc	Ethyl acetate
EtOH	Ethanol
Flu	[^{3}H]Flunitrazepam binding sites
Fmoc	9-Fluorenylmethoxycarbonyl
GABA	γ-Aminobutyric acid

h	Hill coefficient
HEK-293	Human embryonic kidney 293 cells
HEPES	4-(2-Hydroxyethyl)-1-piperazine-ethane sulphonic acid
hplc	High performance liquid chromatography
IC_{50}	Concentration at which 50% of the maximal specific binding is inhibited.
IR	Infra-red
K-phosphate	Potassium dihydrogen phosphate
kDa	Kilodalton
MeOH	Methanol
MHz	Megahertz
Mr	Relative molecular mass
mRNA	Messenger RNA
Musc	[^{3}H]Muscimol binding sites
nAChR	Nicotinic acetylcholine receptor
NaDOC	Sodium deoxycholate
PEI	Polyethylene imine
PKA	Protein kinase A (cyclic AMP-dependent protein kinase)
PKC	Protein kinase C
PMSF	Phenylmethylsulphonylfluoride
ppm	Parts per million
R_f	Ratio of distance moved by compound to distance moved by solvent front.
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBPS	*t*-Butylbicyclophosphorothionate
THIP	4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridin-3-ol
tlc	Thin layer chromatography
TMG	1,1,3,3-Tetramethylguanidine
TMS	Trimethylsilane
UV	Ultra-violet

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. Anne Stephenson at the School of Pharmacy and Dr. Charles Hedgecock at Roussel Laboratories Ltd. for giving me the opportunity to do a Ph.D. and for their patient teaching, advice and encouragement throughout the project. My thanks also go to the members of the Research Department at Roussel Laboratories Ltd., particularly Dr. Stuart Jones for teaching me the rudiments of organic chemistry.

The other people in Lab. 435 gave me motivation, lots of good laughs and, most importantly, hope. They are Chris, Paul, Simon P., Penny, Miroslav and Simon T. (who was in the same boat) and I am grateful to them all for being good friends as well as excellent colleagues.

Life as a Ph.D. student is not effortless and I am indebted to my friends outside Lab. 435, namely Chris, Claire, Sab, Mike, Pete, Steve, Perv, Dude, Adrian and Michelle who filled the social life at SOP with fun and helped to keep my spirits high. Special thanks must also go to Phil, for being there, especially when things looked very brown.

The writing of this thesis could not have been completed without the expertise of Graham Florence of the Computer Unit, to whom I am grateful for for the loan of the computer, nor without the artistic talents of Annie Cavanagh of the Pharmacognosy department who provided superb drawings from my inferior sketches. Technical expertise apart, they are both very much appreciated for their patience and friendship throughout my time at SOP.

I acknowledge the SERC and Roussel Laboratories Ltd. for financial support throughout this project.

Finally, my utmost gratitude must go to my family, especially my parents who have always given me their unconditional love, support (financial or otherwise) and encouragement.

Chapter 1

General Introduction

1.1 Synaptic Transmission by Amino Acids

The understanding of the properties of the central nervous system (CNS) requires the elucidation of the mechanisms of neuronal activity and the molecular basis for the physiological changes which accompany neurotransmission. This characterisation depends upon the identification of the chemical entities which control these mechanisms. By the mid-1960s, the ability of certain amino acids to increase or decrease neuronal firing when applied directly to the exposed cerebral cortex had been reported. γ-Aminobutyric acid (GABA) and glycine inhibited neuronal firing, while L-glutamate and L-aspartate were excitatory. The idea was postulated that these amino acids might play a physiological role as neurotransmitters in the central nervous system.

Neurotransmitters exert their effects by interacting specifically with receptor proteins. The interaction between a fast-acting neurotransmitter and its receptor results in a conformational change in the receptor structure leading to an alteration in ion flux across the cell membrane causing either hyperpolarisation or depolarisation of the cell and, ultimately, a physiological event.

1.2 The GABA Receptors

GABA is now well established as the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) and it is estimated that up to 40% of the synapses may use GABA as their transmitter. There are at least

three different binding sites with which GABA interacts in the mammalian CNS. These are the GABA transport system, the $GABA_A$ and the $GABA_B$ receptors. The binding of GABA to its transport system was found to be dependent on the presence of Na^+ (Enna and Snyder, 1975). The $GABA_B$ receptor is agonised by the GABA analogue, (−)-baclofen. This receptor is a G-protein linked receptor and acts via secondary messenger systems (Bowery, 1989). GABA binding to the $GABA_A$ receptors is competitively blocked by the convulsant alkaloid, (+)-bicuculline. The interaction of GABA with the $GABA_A$ receptors results in the influx of chloride ions, hyperpolarisation of the cell and, thus, inhibition of nerve activity. Additionally, the existence of a third type of GABA receptor, the $GABA_C$ receptor, has been reported which is insensitive to both (+)-bicuculline and (−)-baclofen (Drew *et al.*, 1984).

The $GABA_A$ receptors are fast-acting ligand-gated chloride ion channels and are widely distributed throughout the mammalian CNS (Velasquez *et al.*, 1989). They are also found in a number of peripheral tissues including the intestine and the female reproductive system (reviewed in Ong and Kerr, 1990).

1.3 Pharmacological Profile of the $GABA_A$ Receptor

$GABA_A$ receptor binding *in vitro* in brain using [^{3}H]GABA was first described by Zukin *et al.* (1974) using Na^+-free conditions. This Na^+-independent GABA binding was inhibited by the known $GABA_A$ receptor agonists muscimol and 3-aminopropane sulphonic acid and the antagonist (+)-bicuculline but not by the inhibitors of the GABA transport system, nipecotic

acid and 2,4-diaminobutyric acid. The [^{3}H]GABA receptor binding was comprised of two sites of different affinity (Olsen *et al.*, 1981). The high affinity site had a K_D value in the nanomolar range whereas the low affinity site had a K_D value in the micromolar range. The density of sites, $B_{max.}$, varied with brain region with the low affinity site being the more abundant (Olsen *et al.*, 1981; Van Ness *et al.*, 1982). Electrophysiological studies have shown that channel opening occurs at micromolar GABA concentration (e.g. Simmonds, 1981) and it was suggested that it is the binding to the low affinity sites that is responsible for the physiological events. The high affinity sites may represent a desensitised form of the $GABA_A$ receptor (Sieghart, 1992).

The GABA-gated chloride channel is also activated by the GABA agonists muscimol, isoguvacine, THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) and DAVA (δ-aminovaleric acid). These compounds activate channel openings of similar conductance but with different life times compared to the openings activated by GABA (Barker and Mathers, 1981) (Figure 1.1).

The phthalide isoquinoline alkaloid (+)-bicuculline from plants is the best studied example of a competitive antagonist at the $GABA_A$ receptor (reviewed in Turner and Whittle, 1983). This compound reduces GABA-induced membrane current by eliminating chloride channel activation. Other $GABA_A$ receptor antagonists include (+)-hydrastine, another phthalide isoquinoline alkaloid (Huang and Johnston, 1990), the synthetic pyridazinyl-GABA derivatives, SR 42641 and SR 95531 (Desarmenien *et al.*, 1987, Mienville and Vicini, 1987), and the synthetic amidine steroid RU5135 (Hunt and Clements-

Jewry, 1981) (Figure 1.1).

The $GABA_A$ receptor has a complex pharmacology and several different classes of therapeutically important drugs are known to modulate the current flowing through $GABA_A$ receptors by interacting allosterically with the GABA binding site. These include the anxiolytic benzodiazepines, the anaesthetic, hypnotic and convulsant barbiturates, some neurosteroids, the anthelmintic avermectins, picrotoxin, *t*-butylbicyclophosphorothionate (TBPS) and pentetrazole (Figure 1.2).

1.3.1 Benzodiazepine Modulation of the $GABA_A$ Receptor

Radioligand binding studies have demonstrated the presence of a single, high-affinity benzodiazepine binding site in the brain (Squires and Braestrup, 1977; Mohler and Okada, 1977). The binding of classical benzodiazepine agonists (e.g. diazepam and flunitrazepam) to brain membranes was potentiated by GABA or muscimol and this potentiation was inhibited by (+)-bicuculline. Reciprocally, benzodiazepine agonists were shown to enhance the binding of GABA to the $GABA_A$ receptor (Skeritt *et al.*, 1982) thus demonstrating the close association of the benzodiazepine binding site with the $GABA_A$ receptor. Electrophysiological studies have shown that, in the presence of GABA, benzodiazepine agonists cause an increase in the chloride ion current flowing through the $GABA_A$ receptor channel by increasing the frequency of opening of the channel (Study and Barker, 1981). Inverse agonists of the benzodiazepine receptor (e.g. Ro 15-4513) decrease GABA-induced whole-cell currents and

antagonists (e.g. Ro 15-1788) (Figure 1.2) inhibit the actions of the benzodiazepine agonists. Other non-benzodiazepine ligands act at the benzodiazepine recognition site of the $GABA_A$ receptor, for example, β-carbolines, quinolones, pyrimidines and pyrazoloquinolines. These ligands have similar effects on the GABA-induced chloride current as the benzodiazepines agonists and inverse agonists. Other non-benzodiazepine agonists which act at the benzodiazepine recognition site are Zolpidem and CL 218,872 (Richards *et al.*, 1991).

Receptor ligand autoradiographic studies showed that there were differences between the distribution of high affinity GABA binding sites labelled with [^{3}H]muscimol and high affinity benzodiazepine binding sites labelled with [^{3}H]flunitrazepam (Unnerstall *et al.*, 1981). In particular, in the cerebellum, the high affinity benzodiazepine binding sites were localised in the molecular layer, whereas the high affinity GABA binding sites were found in the granule cell layer. In addition, it was observed that the binding of the non-benzodiazepine ligand, CL 218,872, was biphasic in many brain regions (Squires *et al.*, 1979). The sites with higher affinity for CL 218,872 (type I) predominated overall and particularly in the cerebellum. Type II receptors had lower affinity for CL 218,872 and were enriched in only a few areas including the hippocampus, striatum and spinal cord. A similar distribution of high and low affinity β-carboline binding sites was also found in rat brain. The total number of receptors, $B_{max.}$, labelled by [^{3}H]flunitrazepam and [^{3}H]propyl β-carboline-3-carboxylate ([^{3}H]PrCC) was equal in the cerebellum but in the

hippocampus, only 50% of the [^{3}H]flunitrazepam labelled binding sites were also labelled with [^{3}H]PrCC suggesting the existence of heterogeneous populations of benzodiazepine receptors in the mammalian CNS (Braestrup and Nielsen, 1981).

1.3.2 Barbiturate Modulation of the $GABA_A$ Receptor

It has been shown that barbiturates enhance the binding of GABA and benzodiazepines to their respective recognition sites within the $GABA_A$ receptor complex. Electrophysiological studies have shown that the sedative hypnotic barbiturates enhance the actions of GABA by increasing the mean open time of the chloride channel (Study and Barker, 1981). At higher concentrations, they are able to enhance chloride conductance in the absence of GABA (Bormann, 1988). *In vitro* ligand binding studies have shown that barbiturates enhance benzodiazepine binding to brain synaptic membranes (Leeb-Lundberg *et al.*, 1980).

The convulsant barbiturates inhibit the binding of [^{35}S]TBPS, a chloride channel blocker, to the $GABA_A$ receptor. This inhibition is non-competitive and is reduced allosterically by the GABA antagonists (+)-bicuculline and RU5135 (Squires *et al.*, 1983; Ramanjaneyulu and Ticku, 1984; Trifiletti *et al.*, 1984). Whereas convulsants such as picrotoxinin, TBPS and pentetrazole and some convulsant barbiturates, inhibited [^{35}S]TBPS binding completely, the depressant barbiturates interacted allosterically with the [^{35}S]TBPS binding sites. This indicated that the depressant barbiturates enhance GABA-induced chloride flux

by interacting with a binding site that is distinct from that of GABA, benzodiazepines or picrotoxinin/TBPS (Supavilai and Karobath, 1984).

1.3.3 Neurosteroid Modulation of the $GABA_A$ Receptor

Several steroids, such as the anaesthetic alphaxalone (5α-pregnan-3α-ol-11,20-dione) or the sedative-hypnotic and anxiolytic 3α-hydroxylated, 5α-reduced metabolites of progesterone have been shown to be neuromodulators of the $GABA_A$ receptor complex. Allopregnanolone and allotetrahydro-deoxycorticosterone are among the most potent of the known ligands of the $GABA_A$ receptors in the mammalian CNS, with affinities comparable to those of benzodiazepines and at least a thousand times higher than that of the anaesthetic barbiturate pentobarbital (reviewed in Simmonds, 1991). These findings have prompted speculation that these compounds may act as endogenous ligands for the receptor and produce anxiolytic and sedative effects under physiological conditions such as stress and pregnancy (Paul and Purdy, 1992).

Electrophysiological studies have shown that steroids enhance GABA-activated chloride flux (Harrison and Simmonds, 1984). The mechanism by which they acted was to prolong the mean open time of the chloride channel in a similar way to the barbiturates (Barker *et al.*, 1987). In radioligand binding studies, these steroids enhanced both [^{3}H]muscimol and [^{3}H]flunitrazepam binding to the $GABA_A$ receptor (Harrison and Simmonds, 1984; Harrison *et al.*, 1987; Majewska *et al.*, 1986). However, allopregnanolone enhanced GABA-

activated chloride currents in the presence of maximally effective concentrations of pentobarbital and vice versa (Gee *et al.*, 1987; Morrow *et al.*, 1990). Similar synergistic but non-additive effects of steroids and barbiturates on the inhibition of [^{35}S]TBPS binding have also been reported (Gee *et al.*, 1988; Turner *et al.*, 1989). Furthermore, benzodiazepine antagonists failed to alter the ability of allopregnanolone to potentiate GABA-activated chloride conductance ((Morrow *et al.*, 1990). These results provided evidence for a separate site of action of steroids within the $GABA_A$ receptor complex which is distinct from the binding sites for GABA, benzodiazepines or barbiturates.

1.3.4 The Picrotoxin/TBPS Binding Site of the $GABA_A$ Receptor

Convulsant drugs, such as picrotoxin and TBPS are non-competitive antagonists of the GABA-induced chloride current and are believed to act directly at the chloride channel-gating site by binding to a site located close to it (Van Renterghem *et al.*, 1987). In binding studies, these compounds do not inhibit GABA receptor binding and do not displace benzodiazepines from their high affinity binding sites (Olsen, 1982).

GABA and the compounds which mimic or facilitate the effects of the $GABA_A$ receptor (e.g. benzodiazepine agonists, barbiturates and steroids) allosterically inhibit [^{35}S]TBPS binding by reducing its binding affinity. Compounds reducing the efficacy of GABA at $GABA_A$ receptors, such as some convulsant β-carbolines, enhanced [^{35}S]TBPS binding affinity (Gee ,1988). Thus, it was confirmed the picrotoxin/TBPS binding site was an important modulatory

site of the $GABA_A$ receptor protein.

1.3.5 The Avermectin B_{1a} Binding Site of $GABA_A$ Receptors

Avermectin B_{1a} (AVM) is a macrocyclic lactone from *Streptococcus avermitilis* with potent insecticidal and anthelmintic properties. Studies have shown that there is a high affinity binding site for [^{3}H]AVM on brain membranes and that this binding site exhibits a series of complex allosteric interactions with binding sites for GABA, benzodiazepines, barbiturates and TBPS. AVM-stimulated [^{35}S]TBPS binding and high affinity [^{3}H]AVM binding was modulated by $GABA_A$ receptor agonists and antagonists in a chloride ion-dependent manner (Drexler and Sieghart, 1984a, 1984b). These results indicate a close association of AVM binding sites with the $GABA_A$ receptor complex and suggest that these sites are not identical with the GABA, benzodiazepine, barbiturate or picrotoxinin/TBPS sites.

1.3.6 The Zn^{2+} Binding Site of the $GABA_A$ Receptor

Zn^{2+} inhibition of the $GABA_A$ response of neurons has been observed in a variety of species including lobster muscle (Smart and Constanti, 1982) and frog dorsal root ganglion cells (Akaike *et al.*, 1987). In chick spinal cord neurons, it was found that the GABA-activated current was inhibited by Zn^{2+} and various other divalent cations (excluding Ba^{2+}, Ca^{2+} or Mg^{2+}) when applied extracellularly, but that these cations were ineffective when applied intracellularly. This inhibition by cations was mediated via a common, saturable

recognition site and the dose-response curve for the inhibition of the GABA response by Zn^{2+} was unaffected by benzodiazepines, barbiturates or steroids (Celentano *et al.*, 1991). However, in primary cultures of rat superior cervical ganglion cells, Zn^{2+} antagonised GABA-gated currents in cells from young animals, but not from adults, indicating a developmental switch with respect to Zn^{2+} sensitivity in the functional properties of $GABA_A$ receptors in this preparation (Smart and Constanti, 1990). These diverse effects of Zn^{2+} may be of functional importance, since Zn^{2+} is present in many synaptic vesicles in the vertebrate CNS (Xie and Smart, 1991).

1.3.7 Other Potential Binding Sites of the $GABA_A$ Receptor

There is pharmacological and electrophysiological evidence to suggest that the general anaesthetic propofol (2,6-diisopropylphenol) and the anxiolytic, anti-convulsant and sedative-hypnotic clomethiazole exert some of their actions through the $GABA_A$ receptor complex (Hales and Lambert, 1991; Hales and Lambert, 1992). These compounds dose-dependently potentiated GABA-activated chloride currents and, at higher doses, directly activated the channel by increasing the chloride conductance of the membranes in a (+)-bicuculline-sensitive manner. They were found to allosterically inhibit [^{35}S]TBPS binding and to enhance [^{3}H]muscimol binding but they had no effect on [^{3}H]flunitrazepam binding or on the barbiturate enhancement of [^{3}H]flunitrazepam binding (Moody and Skolnick, 1989). These observations indicate that clomethiazole and propofol interacts with a site distinct from that

of other sedative-hypnotics such as benzodiazepines and barbiturates, but additional studies are necessary in order to clarify the interactions of these drugs with the $GABA_A$ receptor.

1.4 Purification of the $GABA_A$ Receptor by Benzodiazepine Agonist Affinity Chromatography

The $GABA_A$ receptor is an integral membrane protein and a prerequisite for its purification is the solubilisation of the protein with the retention of its radioligand binding activity. The first attempts at solubilisation were performed in the late 1970s and early 1980s by several groups (reviewed in Stephenson, 1988 and Stephenson and Barnard, 1986). It was found that both the GABA and benzodiazepine binding sites were readily extracted by a variety of non-denaturing detergents such as sodium deoxycholate (NaDOC) at a near-physiological salt concentration and Triton X-100 with 1 M KCl. However, in the presence of these detergents, barbiturate regulation of benzodiazepine binding was not detectable (Stephenson *et al.*, 1982). The zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) has been shown to solubilise $GABA_A$ receptors which retain their [^{35}S]TBPS binding activity and the barbiturate potentiation of benzodiazepine binding although the total number of benzodiazepine binding sites solubilised with this detergent was reduced compared to solubilisation with NaDOC (Stephenson and Olsen, 1982, Sigel and Barnard, 1984, King *et al.*, 1987). However, the CHAPS-solubilised receptor was unstable and the allosteric interactions between the barbiturate and

benzodiazepine binding sites were lost upon storage (Stephenson and Olsen, 1982). [^{35}S]TBPS binding activity and barbiturate enhancement of benzodiazepine binding were, however, stable when CHAPS solubilisation was performed in the presence of an extract of natural brain lipid supplemented with cholesterol hemisuccinate (Bristow and Martin, 1987). The availability of these stable, soluble preparations facilitated the isolation of the $GABA_A$ receptor.

The $GABA_A$ receptor has been purified by means of benzodiazepine agonist affinity chromatography where a benzodiazepine agonist, e.g. Ro 7-1986/1 (Sigel *et al.*, 1983, Sigel and Barnard, 1984, Schoch *et al.*, 1984, Kirkness and Turner, 1985, Stauber *et al.*, 1987) was attached via a spacer arm to an insoluble matrix support. The purification of the protein involved the application of detergent-solubilised extracts of brain membranes to the benzodiazepine agonist affinity column. This was washed extensively to remove non-specifically bound proteins. The receptor was bio-specifically eluted with a water-soluble benzodiazepine e.g. clorazepate, (Sigel *et al.*, 1983) which was itself removed by ion-exchange chromatography (Sigel *et al.*, 1983), gel filtration (Olsen *et al.*, 1984) or diafiltration (Tallman, 1984). The purification procedure yielded a highly purified product in essentially one chromatographic step (Sigel *et al.*, 1983; Martini *et al.*, 1982; Olsen *et al.*, 1984; Taniguchi and Kuriyama, 1984). Although the structure of the benzodiazepine columns used for the isolation of the $GABA_A$ receptor by various groups is essentially the same, i.e. a benzodiazepine agonist coupled to an insoluble support by a long, straight chain spacer arm, the specific structure has varied. A number of

different benzodiazepine agonists have been employed and the length and charge of the spacer arm has been varied. The structures of some of these affinity columns are compared in Table 1.1

The purified receptor showed the pharmacological characteristics of the crude soluble extract from which it was isolated. Therefore, where NaDOC was used, the purified receptor contained GABA and benzodiazepine binding sites (Sigel *et al.*, 1983; Stauber *et al.*, 1987). Furthermore, the allosteric interaction between the GABA and benzodiazepine binding sites was found in the purified receptors. When CHAPS was used as the solubilising detergent, a high affinity binding site for [^{35}S]TBPS was co-purified with the high affinity binding sites for GABA and benzodiazepines (Sigel and Barnard, 1984). Barbiturate enhancement of benzodiazepine binding in these receptors was also retained. The stoichiometry of the binding sites for these ligands within the $GABA_A$ receptor complex varied according to the conditions employed for its solubilisation and purification (reviewed in Stephenson, 1988), however, the binding site stoichiometry for the $GABA_A$ receptor *in vivo* is still unknown.

1.5 Molecular Characterisation of the $GABA_A$ Receptor

1.5.1 The Membrane-Bound $GABA_A$ Receptor

The size of the membrane-bound $GABA_A$ receptor was determined by irradiation inactivation studies. The calculated molecular sizes ranged from 50 kDa (Paul *et al.*, 1981), 90-100 kDa (Doble and Iversen, 1982) to 200-220 kDa

(Chang *et al.*, 1981, Chang and Barnard, 1982). The variation in these values probably reflects the differences in experimental procedure and may relate to the determination of the size of a single subunit, multiple subunits or to the whole receptor itself (Stephenson, 1988).

Identification of the constituent proteins of the $GABA_A$/benzodiazepine receptor was initially accomplished by the photoaffinity labelling of the benzodiazepine binding sites with [^{3}H]flunitrazepam. A membrane protein of molecular weight 51 kDa was photolabelled in all brain regions (Mohler *et al.*, 1980). Further investigations revealed that a heterogeneous population of proteins were irreversibly labelled by [^{3}H]flunitrazepam with different brain-regional distributions. A 55 kDa protein was found to be prominent in the hippocampus and striatum (Sieghart and Karobath, 1980) and further bands of 53 and 59 kDa have also been demonstrated (Sieghart *et al.*, 1983). However, in the cerebellum, only the 51 kDa photolabelled protein was detected. The photolabelling of all these proteins by [^{3}H]flunitrazepam could be enhanced by GABA and this enhancement was inhibited by (+)-bicuculline indicating that all these benzodiazepine binding sites were associated with $GABA_A$ receptors (Sieghart and Karobath, 1980).

Photolabelling of the 51 kDa protein was selectively inhibited by the non-benzodiazepine ligand CL 218,872. This observation and the predominance of the 51 kDa protein in the cerebellum, where type I receptors are enriched (see Section 1.3.1), indicated that the 51 kDa protein was associated with type I benzodiazepine receptors. In contrast, photolabelling of the 53, 55 and 59 kDa

proteins was inhibited by CL 218,872 but at concentrations higher than those required to inhibit photolabelling of the 51 kDa protein, indicating that these proteins may be associated with type II benzodiazepine receptors (Sieghart *et al.*, 1983; Eichinger and Sieghart, 1986).

Ro 15-4513 was shown to be an alternative photoaffinity label for benzodiazepine receptors (Mohler *et al.*, 1984). This compound is the azide derivative of Ro 15-1788 (flumazenil), an antagonist, but it, itself, is a negative modulator of the $GABA_A$/benzodiazepine complex and is, therefore, an inverse agonist. It has been shown that [^{3}H]Ro 15-4513 labelled proteins in rat brain sections which were of similar molecular weight to those labelled by [^{3}H]flunitrazepam and with similar brain-regional distributions. However, in the cerebellum, [^{3}H]Ro 15-4513 labelled an additional protein of molecular weight 57 kDa. This photoaffinity labelling was not inhibited by flunitrazepam, CL 218,872 or β-carbolines but was displaced by Ro 15-1788. Autoradiographic evidence pointed to the localisation of this 57 kDa protein in the cerebellar granule cell layer (Sieghart *et al.*, 1987).

The GABA agonist muscimol has also been shown to irreversibly photolabel the membrane-bound $GABA_A$ receptor (Asano *et al.*, 1983; Cavalla and Neff, 1985). The labelling occurred in a time- and concentration-dependent manner and was inhibited by GABA agonists and antagonists. It was found that a protein of molecular weight 52 kDa was labelled in rat cerebellar synaptosomal membranes (Cavalla and Neff, 1985).

1.5.2 The Soluble $GABA_A$ Receptor

Sucrose density gradient centrifugation and gel filtration of the solubilised receptor determined the size of the NaDOC-solubilised bovine and rat receptor as 240 kDa and that of the CHAPS-solubilised receptor as 290 kDa (Mamalaki *et al.*, 1989). These values were found to be not significantly different from each other and it was concluded that the molecular size of the $GABA_A$ receptor from bovine and rat was the same irrespective of the detergent conditions used. Additionally, it was shown that following solubilisation with CHAPS, the GABA, benzodiazepine and TBPS binding sites co-migrated in chromatographic and sedimentation experiments. The addition of Triton X-100 to the soluble receptor to obscure TBPS binding did not alter the hydrodynamic properties of the receptor and it was concluded that, in NaDOC, the TBPS binding site was not dissociated from the receptor complex but that the TBPS binding activity was inhibited by the detergent itself (King *et al.*, 1987).

1.5.3 The Purified $GABA_A$ Receptor

The size of the purified receptor from rat and bovine brain was estimated to be 230 kDa by sucrose density gradient centrifugation and gel filtration which corresponded well to the value found for the soluble receptor (Mamalaki *et al.*, 1989).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified receptor revealed two polypeptides with molecular

weights of 53 kDa and 57 kDa which were named the α and the β subunits, respectively (Sigel *et al.*, 1983). The same subunit composition was isolated under all purification conditions (Sigel *et al.*, 1983; Sigel and Barnard, 1984; Schoch *et al.*, 1984).

Photoaffinity labelling of the purified receptor with [^{3}H]flunitrazepam showed that it was the α subunit which was preferentially photolabelled but that radioactivity could be incorporated into the β subunit if higher concentrations of [^{3}H]flunitrazepam were used (Sigel *et al.*, 1983). The molecular weight of the photoaffinity labelled α subunit corresponded to the protein into which the photoaffinity label was predominantly incorporated in membranes described by the same group. This protein was compared to the 51 kDa protein which was photoaffinity labelled with [^{3}H]flunitrazepam by Mohler *et al.* (1980) and Sieghart *et al.* (1983). The discrepancies among the molecular weights were attributed to the differences in the SDS-PAGE techniques employed by the various workers (Sigel *et al.*, 1983).

Photoaffinity labelling of the purified receptor with [^{3}H]muscimol showed that the incorporation of the radioactivity was predominantly into the β subunit. However, radioactivity was also incorporated into the α subunit albeit at lower efficiency (Casalotti *et al.*, 1986). It was suggested that the photoaffinity labelling of the β subunit by [^{3}H]muscimol may represent the high affinity GABA binding site and the α subunit may correspond to the low affinity GABA binding site (Casalotti, 1988).

1.6 Molecular Cloning of the $GABA_A$ Receptor Genes

The cloning of complementary DNAs (cDNAs) encoding $GABA_A$ receptor subunits was first reported by Schofield *et al.* (1987). The approach that was employed was to obtain several non-contiguous partial amino acid sequences of the purified receptor by enzymatic cleavage and high performance liquid chromatography (h.p.l.c.). From these, oligodeoxyribonucleic acid probes were constructed and used to screen both a bovine and a calf cerebral cortex cDNA library to isolate $GABA_A$ receptor cDNAs which contained the entire protein-coding region of the α and β subunits.

The deduced protein sequences of the α and the β polypeptides showed several common features. They both had 25-30 residues at the N-terminal which was characteristic of the cleavable signal peptide for integral membrane proteins. The hydropathy plot for each of the polypeptides showed the presence of four hydrophobic putative membrane-spanning domains (M1-M4, Figure 1.3). Each subunit contained potential extracellular N-glycosylation sites, two for the α and three for the β subunit. The β subunit also contained a potential intracellular site for phosphorylation by protein kinase A (PKA). The α subunit was found to contain 456 amino acids (Mr 44 000) and the β subunit contained 474 amino acids (Mr 55 000) (Schofield *et al.*, 1987). These values correlated well with those predicted from the protein chemistry studies on the respective deglycosylated subunits (Mamalaki *et al.*, 1987). The two amino acid sequences were homologous and it was suggested that both subunits had evolved from a

common ancestral gene (Schofield *et al.*, 1987).

It is predicted that the trans-membrane topology of the subunits is that the four transmembrane spanning regions are arranged such that the N-terminus and the C-terminus are both extracellular (Schofield *et al.*, 1987). With this arrangement, the most divergent region between the two polypeptides occurs in the large cytoplasmic domain between M3 and M4 (Figure 1.3). It is also predicted that the N-terminal domain contains the ligand binding sites for GABA and the allosteric modulatory drugs (Schofield *et al.*, 1987).

This model for the structure of the individual subunits of the $GABA_A$ receptor was similar to that of the nicotinic acetylcholine receptor (nAChR), another ligand-gated ion channel. Indeed, it was found that there was a degree of amino acid sequence homology between the $GABA_A$ receptor α and β subunits and all the known subunits of the nAChR particularly in the M2 region and in the N-terminal domain. A structural feature in the N-terminal regions of ligand-gated ion channel subunits is the "cys-loop" which is a predicted β-structural loop formed by a disulphide bond between two conserved cysteine residues in the N-terminal region. This structure is present in all the known subunits of the peripheral and neuronal nAChR, the strychnine-sensitive glycine receptor and the recently cloned $5\text{-}HT_3$ receptor (Criado *et al.*, 1986; Grenningloh *et al.*, 1987; Maricq *et al.*, 1991). These structural similarities and the degree of sequence homology between the subunits of the $GABA_A$ receptor, the nAChR and the strychnine-sensitive glycine receptor led to the hypothesis of the existence of a ligand-gated ion channel superfamily (Schofield *et al.*,

1987; Grenningloh *et al.*, 1987).

The muscle nAChR was the first member of the superfamily whose quaternary structure was elucidated (reviewed in Unwin, 1989). The five-subunit stoichiometry was determined by preparative gel electrophoresis (Lindstrom *et al.*, 1979) and by quantitative amino-terminal analysis of the purified receptor (Raftery, *et al.*, 1980). Given the degree of homology among the polypeptides of the nAChR, the $GABA_A$ receptor and the strychnine-sensitive glycine receptor, the latter two receptors would be expected also to have five subunits. Indeed, this has been shown for the strychnine-sensitive glycine receptors by chemical cross-linking of the glycine receptor subunits (Langosch *et al.*, 1988).

1.7 Heterogeneity of the $GABA_A$ Receptor Subunits

Since the initial cloning of the cDNAs encoding α and β subunits by Schofield *et al.* (1987) several groups have reported the cloning of additional cDNAs encoding $GABA_A$ receptor polypeptides. To date, fourteen genes have been identified in mammals including six α subunits, α1-α6, three β subunits, β1-β3, three γ subunits, γ1-γ3, one δ subunit and one ρ subunit. In addition, a fourth β subunit which has an alternative splice variant has been identified in chick brain (β4 and β4') (Bateson *et al.*, 1991). Splice variants of the γ2 subunit have also been found, the short form, γ2S and the long form, γ2L, adding to the heterogeneity of the $GABA_A$ receptor subunits (Whiting *et al.*, 1990; Kofuji *et al.*, 1991). Each subunit shows the same structural characteristics, i.e. they all possess four putative transmembrane regions, a large extracellular N-terminus

containing the characteristic cys loop and a large intracellular domain between M3 and M4 (Figure 1.3). Characteristic features of the subunits are summarised in Table 1.2. Amino acid sequence identity within a subunit family (e.g. α1-α6) is greater than 75% whereas identity among subunit families is 30-40%. $GABA_A$ receptor α subunits are as homologous with the α subunit of the inhibitory glycine receptor (35%) as they are with the $GABA_A$ receptor β subunit but subunits of both the $GABA_A$ and inhibitory glycine receptors show less homology with those of the nAChR α subunits (15-20%) (Schofield *et al.*, 1987; Grenningloh *et al.*, 1987). There is only low if any amino acid sequence homology among the $GABA_A$, strychnine-sensitive glycine receptor and the nAChR and members of the ionotropic glutamate receptor family which are excitatory ligand-gated ion channels that have been recently cloned (e.g. Hollmann *et al.*, 1989; Sommer and Seeburg, 1992).

The current view is that the pharmacological and biophysical diversity of $GABA_A$ receptor subtypes arises from the assembly of differing combinations of the 15 (or more) subunits into pentameric structures (reviewed in Burt and Kamatchi, 1991). Thousands of combinations are, therefore, possible. To date, the exact subunit composition of any native $GABA_A$ receptor is unknown. The best evidence from which the *in vivo* subunit combinations can be elucidated has come from three approaches. The first is a comparative study of the functional properties of recombinant receptors of known combinations of subunits expressed transiently in mammalian cells or in *Xenopus* oocytes. The second approach is to study the distribution of the various subunit mRNAs in

the brain using both *in situ* hybridisation and Northern blot analysis. Thirdly, immunological techniques such as immunocytochemistry and immunoaffinity chromatography have been employed using sequence-specific antibodies in order to detect specific subunit-containing $GABA_A$ receptors in brain and to identify other subunit types with which they might co-exist (Duggan *et al.*, 1991; Thompson *et al.*, 1992; Pollard *et al.*, 1993).

1.7.1 Functional Properties of Recombinant $GABA_A$ Receptors

Investigations into the functional properties of cloned receptor-channel complexes require expression systems on which quantitative electrophysiological measurements of receptor activation can be made (reviewed in Dingledine *et al.*, 1990). Expression of $GABA_A$ receptors was initially achieved following injection of total brain messenger RNA (mRNA) into *Xenopus* oocytes, allowing electrophysiological analysis using the voltage-clamp method (Smart *et al.*, 1983; Houamed *et al.*, 1984). This was later extended to the expression of the mRNAs encoding the individual $GABA_A$ receptor α and β subunits (Schofield *et al.*, 1987). Alternative expression systems, e.g. human embryonic kidney (HEK) 293 cells, have been employed for the transient expression of $GABA_A$ receptor subunits. Here, comparatively large quantities of receptor protein are produced and this system is appropriate for biochemical studies, such as the determination of pharmacological specificity via radioligand binding assays (e.g. Pritchett *et al.*, 1988).

Early expression studies of $GABA_A$ receptor subunits in *Xenopus* oocytes

showed that expression of a single subunit yielded GABA-gated chloride channels although these responses were weak (Blair *et al.*, (1988). Furthermore, it was shown that co-expression of the α1 and β1 subunits gave rise to the expression of functional receptors which had robust GABA-activated chloride channels. This GABA response was inhibited in a reversible manner by (+)-bicuculline. The GABA-activated current was also sensitive to picrotoxin. Benzodiazepine and barbiturate potentiation of the GABA response was evident, however, the potentiation by benzodiazepines (clorazepate) was much reduced compared to the native receptor (Schofield *et al.*, 1987). Later studies showed that no consistent potentiation of the channels by benzodiazepines could be shown and, therefore, it was postulated that other types of $GABA_A$ receptor subunit existed (Levitan *et al.*, 1988a).

The discovery of a cDNA encoding the γ2 subunit provided a solution to this paradox (Pritchett *et al.*, 1989b). Co-expression of the γ2 subunit with α1 and β1 subunits in HEK 293 cells resulted in chloride channels which exhibited flunitrazepam potentiation and the presence of high-affinity benzodiazepine binding sites (Pritchett *et al.*, 1989b). [^{3}H]Flunitrazepam binding to the membranes of the transfected cells was inhibited by diazepam, clonazepam or methyl-4-ethyl-6,7-dimethoxy-β-carboline-3-carboxylate (DMCM) and the rank order of potency of these compounds was similar to that found in native $GABA_A$ receptors (Pritchett *et al.*, 1989b). The γ3 subunit has been reported to have similar properties to the γ2 subunit in expression studies also performed on transfected HEK 293 cells (Knoflach *et al.*, 1991).

GABA-gated chloride channels which are formed by the expression of single subunit cDNAs are weak with the exception of the ρ subunit cloned from the retina (Cutting *et al.*, 1991). This subunit forms robust, homo-oligomeric GABA-gated chloride channels upon expression of the subunit in *Xenopus* oocytes (Cutting *et al.*,1991).The GABA response in these receptors was insensitive to (+)-bicuculline but was inhibited by picrotoxin. In addition, the GABA activation was not modulated by pentobarbital or by benzodiazepines. (Shimada *et al.*, 1992). These studies supported earlier findings that the expression of bovine retinal mRNA in *Xenopus* oocytes resulted in a (+)-bicuculline-insensitive $GABA_A$ receptor response (Polenzani *et al.*, 1991; Woodward *et al.*, 1992). It was postulated that these chloride channels may represent the $GABA_C$ receptors (see Section 1.2; Drew *et al.*, 1984). Recently, the pharmacology of another GABA receptor chloride channel in retina has been reported. The chloride channel in rat retinal bipolar cells had a similar pharmacological profile to that of the recombinant ρ1 subunit except that it had reduced sensitivity to picrotoxin suggesting that this tissue may also contain a heterogeneous population of GABA-activated chloride channels (Fiegenspan *et al.*, 1993).

1.7.2 Regional Distribution of $GABA_A$ Receptor Subunit mRNAs

The distribution of the various $GABA_A$ receptor subunit mRNAs has been investigated by several groups using Northern blot analysis and *in situ* hybridisation techniques. Northern blot analysis gives a crude indication as to

the presence or absence of a particular mRNA in a defined brain region whereas *in situ* hybridisation delineates the cellular localisation of specific mRNAs.

Expression patterns for α1-α6, β1-β3, γ1-γ3 and δ subunit mRNAs in adult rat brain have been determined by *in situ* hybridisation. Each mRNA displayed a unique distribution ranging from ubiquitous for α1 subunit mRNA to discrete localisation for α6 subunit mRNA. Some neuronal populations displayed complex patterns of co-expressed $GABA_A$ receptor subunit-specific mRNAs whereas others only expressed a few subunit mRNAs (Wisden *et al.*, 1992).

The mRNAs for the more abundant subunits, e.g. α1, β2, β3 and γ2, appear to be present in many rat brain regions. There is a correlation between the localisation of the γ2 subunit and high-affinity benzodiazepine agonist binding which is consistent with the apparent role of this subunit in benzodiazepine modulation of the $GABA_A$ receptor as discussed previously (see section 1.7.1). In contrast, the distribution of the δ subunit appears to be associated with high-affinity [^{3}H]muscimol specific binding activity. It is present in low abundance in the olfactory bulb, the thalamus and the hippocampus but it is concentrated in the cerebellar granule cells (Shivers *et al.*, 1989). The expression of the α6 subunit mRNA is confined to the cerebellar granule cells (Luddens *et al.*, 1990).

1.7.3 Immunological Studies of the $GABA_A$ receptor

Immunoaffinity chromatographic isolation of $GABA_A$ receptors has provided important information about the subunit composition of native $GABA_A$ receptors. By employing site-directed, subunit-specific antibodies immobilised to a support matrix, it has been possible to isolate populations of receptor which contain at least one common subunit. Other subunits which may co-exist in the receptor complex have then been investigated by Western blotting analysis using subunit-specific antibodies. To date, successful immunoaffinity purification of the $GABA_A$ receptor has been achieved using polyclonal antibodies which were raised to amino acid sequences of cloned α subunits (Fuchs *et al.*, 1990; Duggan *et al.*, 1991; Zezula and Sieghart, 1991) or with monoclonal antibodies raised to the Ro 7-1986/1 affinity column-purified $GABA_A$/benzodiazepine receptor (Park *et al.*, 1991).

The information obtained from these studies is consistent with predictions of receptor populations from the results of *in situ* hybridisation experiments in that many different combinations of subunits exist in native $GABA_A$ receptors. Using immunoaffinity columns in which anti-α1, anti-α2 or anti-α3 $GABA_A$ receptor subunit polyclonal antibodies were immobilised to Sepharose 4B, it was shown that, although the immunoreactivity for the specific α subunit antibody that was used for purification was enriched in the immunoaffinity-purified receptor from calf cortex, immunoreactivity with the other α subunit-specific antibodies was also present. Furthermore, it was shown that within these

preparations, immunoreactivity with the anti-γ2 1-15 cys subunit antibody was present (Duggan *et al.*, 1992). Thus, it was concluded that the γ2 subunits co-exists with each population of α1, α2 and α3 subunit-containing receptor complex and that there are minor populations of $GABA_A$ receptors that contain α1α2, α1α3 or α2α3 subunit pairs within the same oligomer in the cerebral cortex (Duggan *et al.*, 1991). Similar studies on the cerebellum indicated that most of the $GABA_A$ receptors present in this region contained one α subunit type (either α1 or α6) but a small population do exist which contain both α1 and α6 subunits (Pollard *et al.*, 1993). As predicted from the *in situ* hybridisation studies, immunoreactivity for the α6 subunit could not be detected in the cerebral cortex (Pollard *et al.*, 1993). Immunocytochemical studies supported these findings. Thompson *et al.* (1992) showed that, in sections of rat brain, α6 subunit immunoreactivity was present in the granule cells of the cerebellum only.

Studies investigating the pharmacology of the immunoaffinity purified receptors showed that the receptor purified by anti-α1 subunit antibody affinity chromatography exhibited high affinity for [^{3}H]flunitrazepam and that this benzodiazepine binding was displaced with high affinity by CL 218,872 indicating an enrichment of type I benzodiazepine pharmacology. Conversely, anti-α3 antibody purified receptors had high affinity for flunitrazepam but low affinity for CL 218,872 indicating enrichment for type II benzodiazepine pharmacology (Zezula and Sieghart, 1991). These observations were consistent with the expression studies which investigated the benzodiazepine pharmacology

conferred on recombinant receptors by α subunits (Section 1.8).

Although a wealth of information has been obtained from the immunological techniques, there are still no pentameric subunit combinations of the $GABA_A$ receptor which are known to exist *in vivo*. Until such information becomes available, it is difficult to address the questions of regulation of expression, mode of assembly and the function of the modulatory sites of the $GABA_A$ receptor *in vivo*.

1.8 Pharmacological Properties of the Subunits of $GABA_A$ Receptors

Different α subunit variants create GABA/benzodiazepine binding sites of the previously established type I or type II pharmacology (see section 1.3.1) when combined with β1 and γ2 subunits (Pritchett *et al.*, 1989a). The α1 subunit is associated with type I pharmacology and α2 and α3 subunits confer type II pharmacology. The α5 subunit bestows an unusual type II pharmacology on the receptor in that the recombinant receptor exhibits very low affinity for Zolpidem. The α6 subunit has a unique benzodiazepine pharmacology in that recombinant receptors formed by α6β2γ2 co-expressed in HEK 293 cells have high affinity for benzodiazepine antagonists (Ro 15-1788) and inverse agonists (Ro 15-4513) only (Luddens *et al.*, 1990). Electrophysiological studies comparing α1β2γ2 and α6β2γ2 isoforms substantiated these findings by showing that the GABA-activated chloride current was reduced by Ro 15-4513 in both recombinants but only in the α1 subunit-containing species was the agonist flunitrazepam able to increase the current (Kleingoor *et al.*, 1991). There

is some evidence that the α4 subunit cloned from rat brain exhibits a similar pharmacological profile to the α6 subunit when expressed with β2 and γ2 in HEK 293 cells in that it has high affinity for Ro 15-4513 but fails to bind the benzodiazepine agonists (Wisden *et al.*, 1991).

The γ2 subunit confers high affinity benzodiazepine binding on recombinant receptors (Pritchett *et al.*, 1989; Section 1.7.1). In contrast to the effects of the γ2 subunit on recombinant receptors, the γ1 subunit confers reduced flunitrazepam potentiation of the GABA response on channels formed in HEK 293 cells by co-expression of α1, β1 and γ1 $GABA_A$ receptor subunits compared to similar combinations with the γ2 subunit. The ability of the β-carbolines to reduce GABA responses was decreased or changed to a potentiation in various combinations with the γ1 subunit in HEK 293 cells (Von Blankenfield *et al.*, 1990). There was also unusual potentiation of the GABA response to the benzodiazepine antagonist flumazenil (Ro 15-1788) in α1β1γ1 constructs expressed in oocytes (Ymer *et al.*, 1990). This unusual pharmacology of the γ1 containing receptors studied led to the suggestion that these combinations do not exist in native $GABA_A$ receptors (Ymer *et al.*, 1990). Similar observations have been cited for the δ subunit in that substitution of this subunit in expression studies for the γ2 subunit abolishes benzodiazepine binding (Shivers *et al.*, 1989).

1.9 Post-Translational Modification of the $GABA_A$ Receptor Subunits

Different neurons may perform different patterns of post-translational modification on their $GABA_A$ receptors which have profound effects on the efficacy of transmission at GABAergic synapses. Phosphorylation is the best-studied post-translational modification of the $GABA_A$ receptors.

Cloning of the cDNAs which encode the subunits of the $GABA_A$ receptor revealed that the amino acid sequences contain consensus sequences for phosphorylation by PKA, protein kinase C (PKC) and tyrosine kinases. These consensus sequences are present in the putative cytoplasmic loops between M3 and M4 (Section 1.6, Figure 1.3) but the number and position of phosphorylation sites varies among the different subunits. All β subunit cDNAs isolated to date contain a conserved consensus phosphorylation site for PKA and all γ subunits have a conserved consensus tyrosine kinase phosphorylation site. In addition, the γ2L subunit contains a PKC consensus sequence within the eight amino acid insert (Whiting *et al.*, 1990; Kofuji *et al.*, 1991). This suggests that alternative splicing of the γ2 subunit may provide a novel mechanism for conferring PKC sensitivity on the $GABA_A$ receptor.

Experiments studying the phosphorylation of the $GABA_A$ receptor *in vitro* showed that it is a substrate for a variety of protein kinases. It has been reported that an unidentified protein kinase associated with purified preparations of $GABA_A$ receptor can phosphorylate the α subunit (Sweetman *et al.*, 1988). In addition, two distinct types of β subunit can be differentially phosphorylated by

PKA and PKC. PKA phosphorylates a 58 kDa protein and PKC phosphorylates a 56 kDa subunit (Browning *et al.*, 1990). Studies on phosphorylation of fusion proteins prepared from the cytoplasmic, M3-M4 regions of subunits have shown that this region of the β1 subunit is a substrate for PKC and PKA (Moss *et al.*, 1992a). Similar studies involving fusion proteins of the cytoplasmic loops of γ2S and γ2L subunits have shown that they are substrates for PKC but not for PKA (Moss *et al.*, 1992a). These results suggest that identical receptor subtypes may be differentially regulated by PKC and PKA.

The effects of phosphorylation on the functional modulation of $GABA_A$ receptors have also been investigated. In HEK 293 cells transfected with various combinations of $GABA_A$ receptor subunits and the catalytic subunit of PKA, phosphorylation reduced the amplitude of the GABA response in α1β1γ2 or α1β1 recombinant receptors (Moss *et al.*, 1992b). Similar results were obtained from studies on isolated brain membrane vesicles (Leidenheimer *et al.*, 1991). PKC was also found to down-regulate the GABA-gated chloride current in *Xenopus* oocytes expressing a variety of αβ and αβγ2 recombinant receptors (Sigel *et al.*,1991).

There is limited evidence available for regional heterogeneity in $GABA_A$ receptor glycosylation patterns and its functional significance (Sweetnam and Tallman, 1986). However, there is evidence to suggest that β subunits may be *N*-glycosylated to different degrees in the intact $GABA_A$ receptor (Buchstaller *et al.*, 1991). Recent investigations suggest the existence of differentially *O*-glycosylated α5 subunits in rat brain (Sieghart *et al.*, 1993). However, the

localisation and possible function of the glycosylation variants requires further investigation.

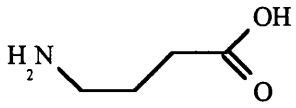
1.10 The Objectives of This Study

When this project was begun in October 1989, several subunits of the $GABA_A$ receptor had been cloned and some had been pharmacologically characterised. However, there was evidence to suggest the presence of benzodiazepine-insensitive $GABA_A$ receptors such as autoradiographic and photoaffinity labelling studies performed using Ro 15-4513 (as discussed in Section 1.5.1) and the apparent mismatch in percentages of [^{3}H]muscimol binding sites compared to [^{3}H]flunitrazepam binding sites purified by benzodiazepine agonist affinity chromatography (Stephenson, 1987). This may now be partially explained by the discovery and pharmacological characterisation of the α6 and α4 subunits in 1990 and 1991 respectively (Section 1.8). However, the discovery of these subunits does not exclude the possibility that other subunits exist which confer benzodiazepine agonist insensitivity on $GABA_A$ receptors. Additionally, $GABA_A$ receptors may exist which have no benzodiazepine-sensitivity at all such as those containing αβδ or αβ alone. The aim of this project was, therefore, to develop a novel method for the isolation of the $GABA_A$ receptor by affinity chromatography using a high affinity ligand which was specific for the GABA recognition site, thereby detecting all $GABA_A$ receptor subtypes irrespective of their benzodiazepine pharmacology.

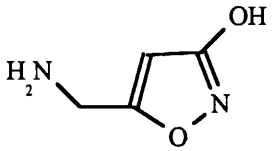
Figure 1.1

Chemical Structure of Selected $GABA_A$ Receptor Ligands

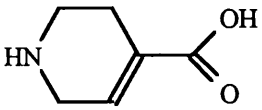
Agonists



GABA

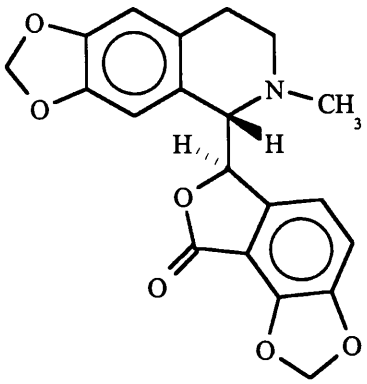


Muscimol

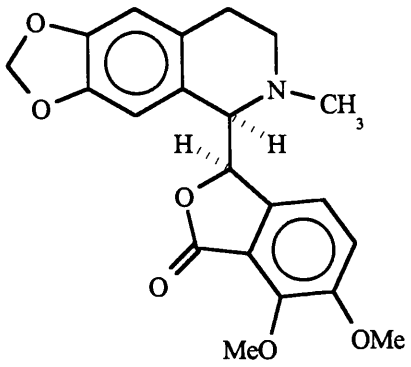


Isoguvacine

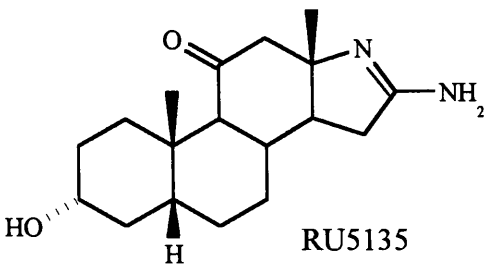
Antagonists



(+)-Bicuculline



(+)-Hydrastine

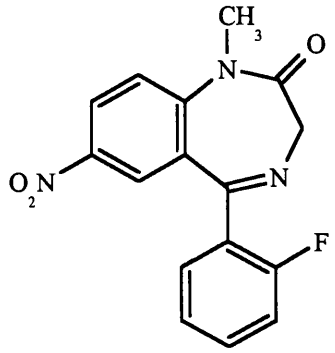


RU5135

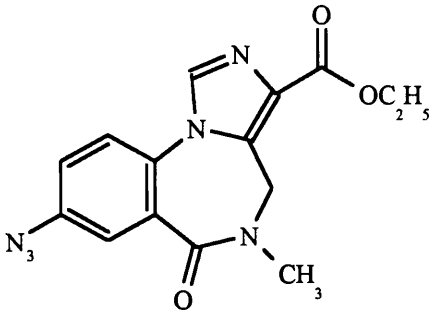
Figure 1.2

Chemical Structure of Modulatory Ligands of the $GABA_A$ Receptor

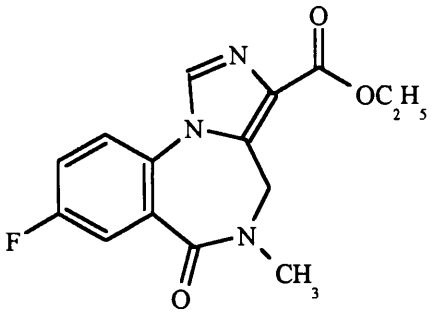
Benzodiazepines



Flunitrazepam
(agonist)

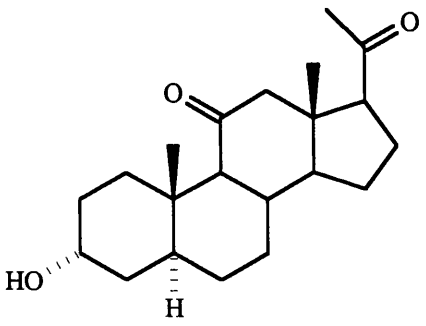


Ro 15-4513
(inverse agonist)

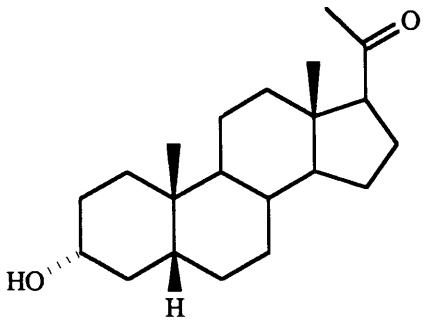


Ro 15-1788
(antagonist)

Neurosteroids

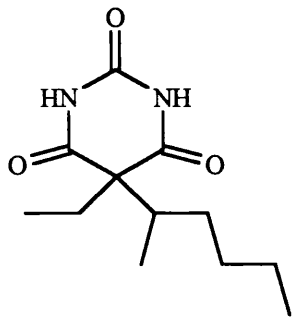


Alphaxalone

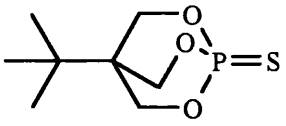


Pregnanolone

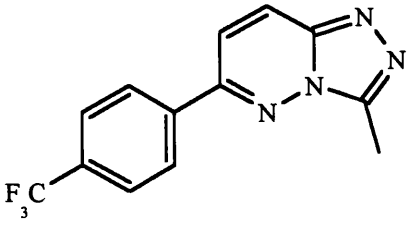
Others



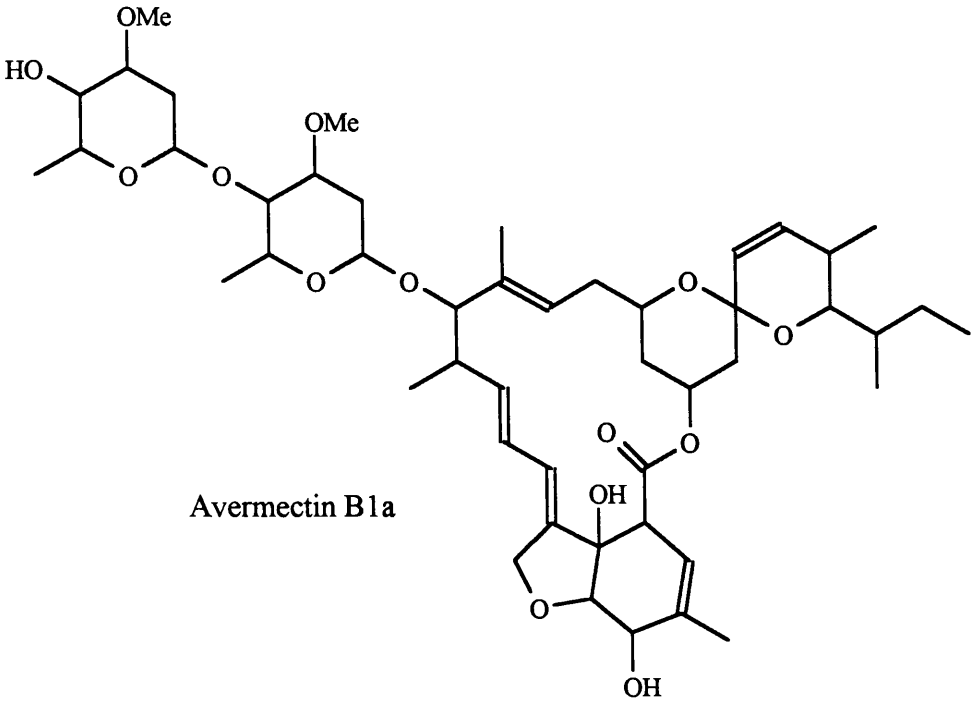
Pentobarbital



TBPS



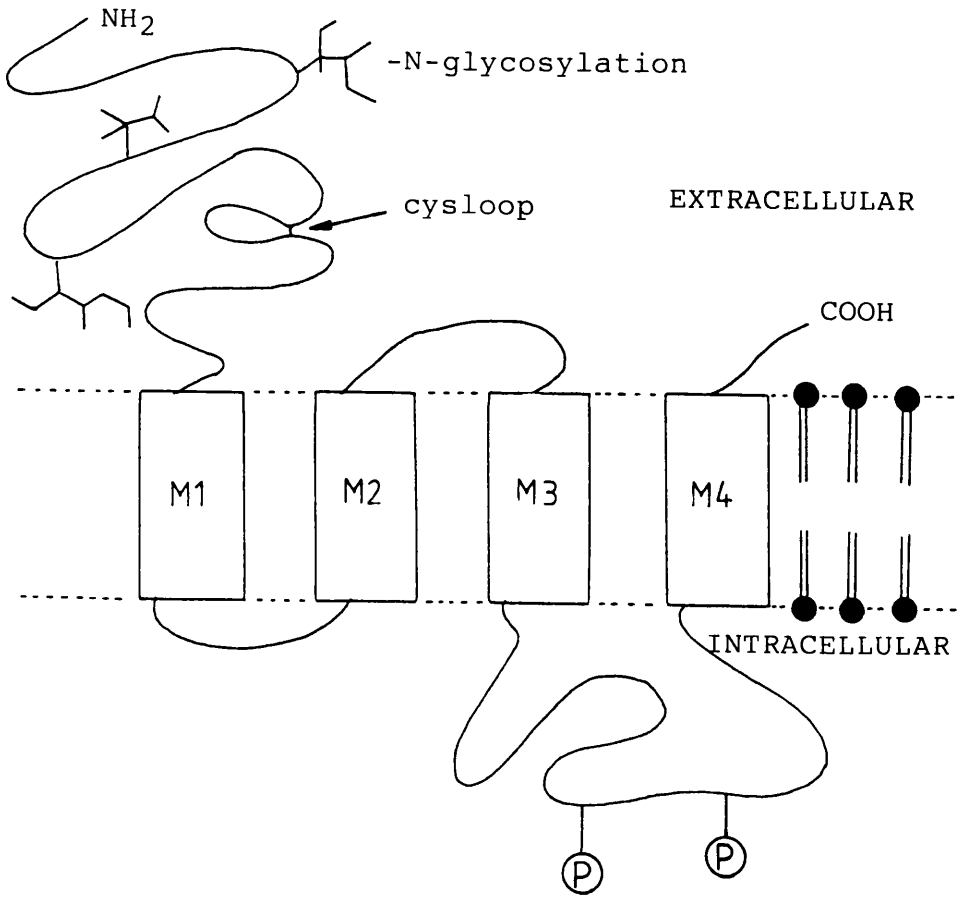
CL 218,872



Avermectin B1a

Figure 1.3

Predicted Topology of a Ligand-Gated Ion Channel Subunit



Pentameric subunit structure representing the arrangement of the transmembrane regions around the central ion channel.

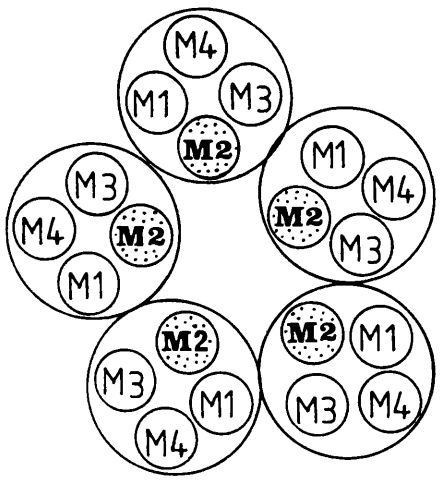


Table 1.1

Summary of the Benzodiazepine Agonist Affinity Columns and the Experimental Conditions used for the Purification of $GABA_A$ Receptors

Ligand	Spacer Arm		Detergent used for solubilisation/purification[a]	References
	Length (atoms)	Net Charge		
Ro 7-1986/1	15	+	NaDOC/Triton X-100	Sigel *et al.*, 1983
Ro 7-1986/1	15	+	CHAPS	Sigel & Barnard, 1984
Ro 7-1986/1	15	+	Triton X-100	Kirkness & Turner, 1985
Ro 7-1986/1	13	0	NaDOC/Triton X-100	Stauber *et al.*, 1987
Ro 7-1986/1	13	0	Triton X-100	Stauber *et al.*, 1987
Ro 7-1986/1	7	0	Triton X-100	Gavish & Snyder, 1981
Delorazepam	12	0	Triton X-100	Martini *et al.*, 1982
1012-S	17	0	Nonidet P-40	Taguchi & Kuriyama, 1984

[a]Where only one detergent is given, the extraction and purification procedures were performed under identical conditions.

Table 1.2

Characteristics of the Known Subunits of Vertebrate $GABA_A$ Receptors

Subunit	Mr[a]	Selected Properties	References
α1	48 800	Confers type I BZ pharmacology when expressed with βγ2 subunits. Overall most abundant mRNA.	Schofield *et al.*, 1987 Sequier *et al.*, 1988 Krestchatisky *et al.*, 1989
α2	48 000	Confers type II BZ pharmacology when expressed with βγ2 subunits. Loci include Bergmann glia of cerebellum.	Krestchatisky *et al.*, 1989
α3	52 000	Confers type II BZ pharmacology when expressed with βγ2 subunits. Least conserved sequence amongst species.	Malherbe *et al.*, 1990
α4	65 000	Expression of this subunit with β2γ2 subunits results in receptors which bind BZ inverse agonists (Ro 15-4513) and antagonists but are insensitive to agonists.	Ymer *et al.*, 1989 Wisden *et al.*, 1991
α5	48 400	Confers unusual type II BZ pharmacology when expressed with βγ2 subunits; the recombinant receptors have an unusually low affinity for Zolpidem. Alternative nomenclature[b].	Krestchatisky *et al.*, 1989 Malherbe *et al.*, 1990 Pritchett & Seeburg, 1990

α6	48 500	Expression of this subunit with β2γ2 subunits results in receptors which bind BZ inverse agonists (Ro 15-4513) and antagonists but are insensitive to agonists. Discrete localisation in cerebellar granule cells.	Luddens *et al.*, 1990 Kato, 1990
β1	51 400	mRNA less abundant overall than β2 and β3.	Schofield *et al.*, 1987 Lolait *et al.*, 1989 Ymer *et al.*, 1989
β2	52 000	mRNA more abundant overall than β3.	Ymer *et al.*, 1989
β3	52 000	Alternative nomenclature[c].	Lolait *et al.*, 1989 Ymer *et al.*, 1989
β4, β4'	53 000 (chick)	Alternative splice variants. To date, found only in chick.	Bateson *et al.*, 1991
γ1	54 000	Contributes atypical BZ response in recombinant receptors composed of αβγ1.	Ymer *et al.*, 1990
γ2S, γ2L	48 000	Essential for typical BZ sensitivity. Contributes Zn^{2+} insensitivity. Alternative splice variants; long form contains new PKC site which affects ethanol interactions.	Pritchett *et al.*, 1989 Shivers *et al.*, 1989
γ3	52 500 (mouse)	Contributes typical BZ sensitivity. mRNA distribution similar to γ2 but less abundant.	Knoflach *et al.*, 1991
δ	48 500	Distribution consistent with BZ agonist-insensitive receptors.	Shivers *et al.*, 1989

ρ1	48 000 (human)	Localised in retina. Forms robust homo-oligomeric Cl^- channels.	Cutting *et al.*, 1991

[a]Relative molecular mass of the non-*N*-glycosylated polypeptide predicted from the amino acid sequence deduced from the cDNA cloned from rat brain (unless otherwise stated).
[b]This subunit has been termed α4 (Krestchatisky *et al.*, 1989).
[c]This subunit has been termed β2 (Lolait *et al.*, 1989).
BZ = benzodiazepine.

Chapter 2

Synthesis of the Derivatives of RU5135

2.1 INTRODUCTION

This chapter will describe the chemical synthesis and analysis of RU5135 and its derivatives. Some of the criteria of affinity chromatography and basic objectives in the synthesis of the derivatives of RU5135 will be discussed in this Introduction.

2.1.1 The Principles of Affinity Chromatography

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance immobilised on an insoluble support matrix (Figure 2.1.1). Thus, proteins are isolated by this technique on the basis of their biological activity and low abundance proteins can be purified by the order of several thousand-fold.

Many ligand-gated ion channel proteins have been isolated by affinity chromatography, for example, the inhibitory glycine receptor using immobilised strychnine (Pfeiffer *et al.*, 1982) and the $GABA_A$ receptor by benzodiazepine agonist affinity chromatography as described in Section 1.4. The nature of the technique allows the isolation of receptors with the retention of the binding site for the immobilised ligand, therefore allowing both biochemical and pharmacological characterisation of the receptor in its isolated state. Although this is a powerful technique in the study of neurotransmitter receptors, it requires the availability of ligands with the appropriate characteristics.

2.1.2 Properties of Immobilised Ligands in Affinity Chromatography

A ligand used for affinity chromatography should satisfy several criteria. These are:

1) The ligand should have high affinity for its specific binding sites.

2) The interaction of the ligand with its specific binding site must be reversible.

3) Chemical modification of the ligand, such that its high affinity for its specific binding site is retained, must be possible.

Although there are many ligands which interact with the GABA recognition site of the $GABA_A$ receptor (Figure 1.1), most of them do not fulfil the above criteria as their affinity for the $GABA_A$ receptor is not sufficiently high. However, RU5135 (3α-hydroxy-11-oxo-16-imino-17-aza-5β-androstane) is an antagonist of the $GABA_A$ receptor and of the inhibitory glycine receptor (Hunt and Clements-Jewry, 1981; Simmonds and Turner, 1985; Curtis and Malik, 1985). It binds reversibly and with high affinity to these receptors (IC_{50} = 11 nM and 6 nM respectively, Olsen, 1984). It is a chemically bifunctional molecule and has the structure shown in Figure 2.1.2.

Although relatively few structure activity studies have been performed involving RU5135, it is thought that the compound interacts with the GABA recognition site of the $GABA_A$ receptor via the region of the molecule between the 11-carbonyl and the amidine group. In support of this, Wermuth *et al.* (1987) reported that *N*-alkylation of GABA analogues caused a reduction in

binding affinity but if the amine was replaced by an amidine function then binding affinity was restored. The bulky steroidal substituent had no apparent effect on the affinity of the molecule for the $GABA_A$ receptor. The C3-hydroxyl group may, therefore, be a potential site of immobilisation such that the affinity of the compound for the $GABA_A$ receptor is not significantly reduced.

2.1.3 Strategy for Synthesis of RU5135 Affinity Chromatography Matrices

RU5135 is a chemically bifunctional compound with a hydroxyl group at the C3 position in addition to the amidine group in ring D (Figure 2.1.2). Various support matrices are commercially available which immobilise ligands via different functional groups, however, those which react with hydroxyl groups may also be capable of forming covalent bonds through amidine functional moieties, for example, epoxy-activated Sepharose 6B. This matrix is synthesised by coupling 1,4-butanediol diglycidyl ether (BDDE) to Sepharose 6B in the presence of sodium hydroxide and sodium borohydride (Sundberg and Porath, 1974). The product is a matrix containing long (12 atoms), hydrophilic spacer arms with a free epoxide group which can react with hydroxyl, thiol or amine groups. The amidine moiety of RU5135 probably exists as a tautomeric mixture, thus, it is possible that this may react with an epoxide group. Therefore, a prerequisite for the synthesis of RU5135 affinity chromatography matrices is either the chemical protection of the amidine group of RU5135 or the functionalisation of the hydroxyl moiety under conditions where the support matrix does not react with the amidine group.

2.1.3.1 Protection of the Amidine Group of RU5135

Protection methods which are used specifically for the amidine group are not well characterised. However, there are many methods available for the protection of amine groups at the α position and within the side chains of amino acids during peptide synthesis. Arginine is a basic amino acid containing a guanidine function in its side chain and for which there are specific protection methods available, for example, arylsulphonamide formation (Paleos *et al.*, 1974; Yajima *et al.*, 1978) or reaction with trityl chloride (Greene, 1981). The amidine group is similar in structure and physical character to the guanidine group and, therefore, these methods are of potential use for the protection of RU5135.

Other protection methods available are those applied to the primary amine groups of α-amino acids. These include the benzyloxycarbonyl (cbz) group and its substituted benzyl derivatives (Hanessian and Masse, 1977; Amit *et al.*, 1974; Barltrop and Schofield, 1965) and the 9-fluorenylmethoxycarbonyl (fmoc) group (Carpino and Han, 1972; Paquet, 1982; Albericio *et al.*, 1990).

2.1.3.2 Chemical Modification of the C3-Hydroxyl Group of RU5135

An alternative approach to the synthesis of RU5135 affinity chromatography matrices is to modify or activate the C3-hydroxyl group of RU5135 so that it can be immobilised to an affinity matrix while not reacting with the amidine group. Acid chlorides will react with hydroxyl groups forming

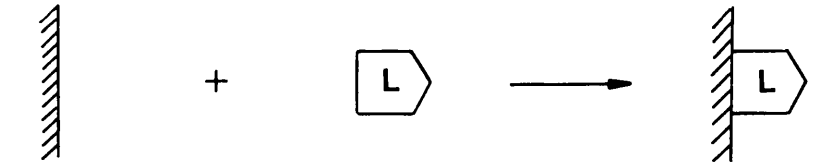
stable ester linkages while giving salts with amidines. Therefore, it may be possible to react a diacyl chloride with the C3-hydroxyl group of RU5135. The free acyl chloride may then be reacted with one of the primary amines of a diaminoalkane which could subsequently be coupled to cyanogen bromide (CNBr) -activated Sepharose 4B.

The chemical syntheses and analyses of derivatives of RU5135 for use in ligand affinity chromatography are reported here.

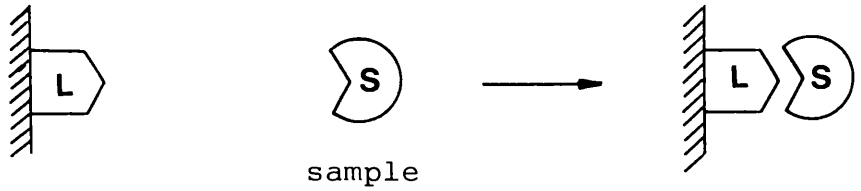
Figure 2.1.1

The Principle of Affinity Chromatography

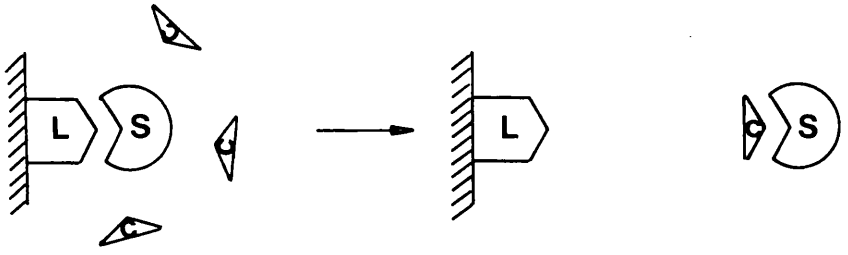
1) Immobilise the ligand



2) Adsorb sample



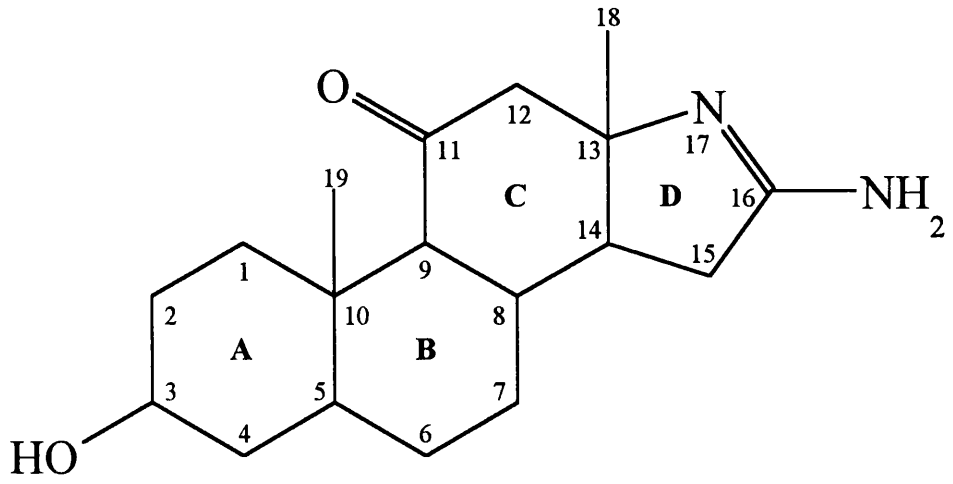
3) Desorb bound sample with competing ligand



4) Remove competing ligand by gel filtration or ion-exchange chromatography

Figure 2.1.2

The Chemical Structure of RU5135



The numbering of the atoms in this molecule complies with the IUPAC conventional rules for the nomenclature of steroids which are described in *Eur. J. Pharmacol.* **186** 429-458.

2.2 MATERIALS

RU5135, 3α-acetoxy-11,17-dioxo-5β-androstane and 3α-acetoxy-11,17-dioxo-16-oximino-5β-androstane were kindly provided by Roussel Laboratories Ltd., Swindon, U.K. Methylbenzenesulphonyl chloride, mesitylene-2-sulphonyl chloride, trityl chloride, benzyloxycarbonyl chloride, 9-fluorenylmethoxycarbonyl chloride, 1,1,3,3-tetramethylguanidine, 1,4-butanediol diglycidyl ether, succinyl chloride, 1,6-diaminohexane, 1,12-diaminododecane, diaza-bicyclo-undecane and ethanolamine were all of analytical grade and were purchased from Aldrich Ltd., Dorset, U.K. Kieselgel and t.l.c. plates were from Koch-Light Ltd. All other chemicals, including solvents, were of analytical grade and were purchased from standard commercial sources.

2.3 METHODS

2.3.1 Synthesis of RU5135

The synthesis of RU5135 was carried out according to the methods described by Jouquey and Hunt (1985). The reaction scheme is shown in Figure 2.4.1.

i) 3α-acetoxy-11,17-dioxo-5β-androstane (I) (5 g) was dissolved in dichloromethane (25 ml). *t*-Butyl nitrite (5 ml) was added and the mixture cooled to 0°C. A solution of potassium *t*-butoxide (2 g) in *t*-butyl alcohol (15 ml) was added dropwise to the reaction over 30 min. A clear red solution was obtained and the mixture was stirred for a further 2 min at 0°C. A solution of 5% (v/v) HCl in methanol (20 ml) was added dropwise over 25 min at 0°C. Immediately after this addition, the mixture was washed with water (2 x 25 ml), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The brown/yellow oil was resuspended in ethyl acetate (25 ml) and heated to reflux. The mixture was cooled and the product crystallised with diethyl ether. The cream solid was filtered, washed with diethyl ether and dried in a vacuum oven to constant weight.

ii) Thionyl chloride (50 ml) was cooled to 0°C and 3α-acetoxy-11,17-dioxo-16-oximino-5β-androstane (II) (5 g) was added gradually over 15 min. The mixture was stirred for 1 h at 0°C. The yellow solution obtained was evaporated to dryness under reduced pressure, taken up into anhydrous benzene (20 ml) and re-evaporated to dryness under reduced pressure. This process was

repeated in order to dispel any residual thionyl chloride. This acid chloride intermediate (III) was used for the next stage of the synthesis without purification.

iii) The acid chloride (III) was dissolved in acetone (100 ml) and cooled to 0°C. Concentrated ammonia (40 ml) was added dropwise to the mixture. The reaction was stirred for 30 min at 0°C then 1 h at room temperature. The mixture was poured into ice/water (250 ml) and extracted with dichloromethane (3 x 70 ml). The extracts were pooled, washed with water (2 x 50 ml), dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The product (IV) was recrystallised from a 3:1 mixture of acetone/diisopropyl ether.

iv) The nitrile amide (IV) (1 g) was stirred in ethanol (25 ml) and water (7.5 ml) and cooled to 0°C for 15 min. Sodium hypochlorite (15% (v/v), 2.5 ml) was added dropwise and the mixture stirred at 0°C for 90 min. The reaction mixture was poured into water (50 ml) to give a white emulsion. This was acidified with a few drops of concentrated hydrochloric acid and extracted with dichloromethane (3 x 15 ml). The extracts were combined, washed with water (1 x 15 ml), dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure. The product (V) was washed with diisopropyl ether, filtered and dried in a vacuum oven for 30 min at 40°C.

v) The nitrile carbamic acid (V) (1 g) was dissolved in methanol (20 ml). Aqueous sodium hydroxide (10 M, 2.5 ml) was added dropwise and the mixture refluxed for 1 h. The mixture was cooled to room temperature and water (15 ml) was added to dissolve the precipitate of Na_2CO_3 which had

formed. The methanol was evaporated under reduced pressure. The product (RU5135) was recrystallised from ice-cold water and dried in a vacuum oven at 40°C to constant weight. The structure was confirmed by standard analytical methods.

2.3.2 Protection of the Amidine Group of RU5135

The products of the protection reactions are shown in Figure 2.4.8. All structures were verified by standard analytical methods.

2.3.2.1 Reaction of RU5135 with Arylsulphonyl Chlorides

Method A

Aryl sulphonamide formation was carried out according to the method of Schnabel and Li (1960). RU5135 (40 mg) was dissolved in a solution of 4 M NaOH (0.22 ml) and acetone (1.78 ml) and cooled to 0°C. Arylsulphonyl chloride (20 mg) was dissolved in acetone (1 ml) and this was added dropwise to the steroid mixture. The solution was then stirred at 0°C for 2 h. The acetone was removed by evaporation under reduced pressure. The residue was washed with diethyl ether to remove traces of arylsulphonyl chloride and the product was extracted into ethyl acetate. This was washed with 10% (v/v) HCl (1 x 3 ml) then water (5 x 3 ml). The organic phase was dried over $MgSO_4$, filtered and evaporated under reduced pressure.

Method B

RU5135 (0.1 mmol) and arylsulphonyl chloride (0.1 mmol) were stirred

in pyridine (1 ml) at 25°C under an atmosphere of nitrogen for 24 h. The pyridine was evaporated under reduced pressure and the residue stirred with diethyl ether to dispel traces of arylsulphonyl chloride. This was decanted and the residue was taken up in dichloromethane. This was washed with 5% (w/v) $NaHCO_3$ (3 x 2 ml). The organic phase was dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure.

2.3.2.2 Reaction of RU5135 with Trityl Chloride

Method A

RU5135 (0.1 mmol) was dissolved in dimethylformamide (2 ml) with sodium hydride (0.1 mmol) and stirred at room temperature. Trityl chloride (0.1 mmol) dissolved in dimethylformamide (1 ml) was added dropwise. The mixture was stirred for 90 min at room temperature. The solution was diluted with ethyl acetate (3 ml) and then washed with 5% (w/v) $NaHCO_3$ (3 x 3 ml). The organic phase was dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure.

Method B

RU5135 (0.1 mmol) and trityl chloride (0.2 mmol) were stirred in pyridine (1 ml) at room temperature under an atmosphere of nitrogen for 18 h. The pyridine was evaporated under reduced pressure. The residue was washed with diethyl ether (2 ml) and taken up into dichloromethane. It was washed with 5% (w/v) $NaHCO_3$ (3 x 3 ml), the organic phase was dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure.

2.3.2.3 Reaction of RU5135 with Benzyloxycarbonyl (Cbz) Chloride

Method A

RU5135 (0.1 mmol) was dissolved in 1 M NaOH, 50% tetrahydrofuran (1 ml). Cbz chloride (0.1 mmol) was added and the mixture stirred at room temperature for 4 h. The mixture was extracted with ethyl acetate, reduced to a minimum volume and subjected to flash chromatography using ethyl acetate as solvent as described in 2.3.5.

Method B

RU5135 (0.1 mmol) was stirred in 0.1 M Na_2CO_3 (1 ml) at 0°C. Cbz chloride (0.1 mmol) dissolved in dimethylformamide (1 ml) was added dropwise to the reaction mixture at 0°C. The reaction was stirred at the same temperature for 1 h, then allowed to warm to room temperature and stirred for a further 4 h. The solution was diluted with distilled water (2 ml) and extracted with ethyl acetate (2 x 2 ml). The organic layer was separated and washed with distilled water (3 x 1 ml), dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure. The residue was dissolved in a minimum volume of ethyl acetate and separated by flash chromatography using ethyl acetate as the solvent as described in 2.3.6.

Method C

RU5135 (0.1 mmol) was dissolved in pyridine (1 ml) and cooled to 0°C. 1,1,3,3-tetramethylguanidine (TMG) (0.1 mmol) was added and the mixture was allowed to stir for 5 min. Cbz chloride (0.1 mmol) was added and the mixture

was stirred at 0°C for 4 h under an atmosphere of nitrogen. The reaction was dissolved in dichloromethane (2 ml) and washed with water (3 x 2 ml). The organic phase was dried over $MgSO_4$, filtered and evaporated under reduced pressure.

Method D

RU5135 (0.1 mmol) was stirred in dichloromethane (1 ml) at room temperature. Diaza-bicyclo-undecane (DBU) (0.1 mmol) was added and the mixture was stirred for 5 min at room temperature. Cbz chloride was added to the solution and the reaction was allowed to continue, with stirring, for a further 2 h. The dichloromethane was evaporated under reduced pressure; the residue was dissolved in a minimum amount of ethyl acetate and subjected immediately to flash chromatography using ethyl acetate as the solvent as described in 2.3.6.

2.3.2.4 Reaction of RU5135 with 9-Fluorenylmethoxycarbonyl (Fmoc) Chloride

Method A

RU5135 (0.1 mmol) was stirred in anhydrous dioxan (1 ml) under an atmosphere of nitrogen at room temperature. Fmoc chloride (0.1 mmol) and Na_2CO_3 or $NaHCO_3$ (0.25 mmol) were added and the reaction was continued for 2, 4, 6 or 24 h at room temperature. The reaction was followed by thin layer chromatography (tlc) using ethyl acetate as the solvent system. The product was isolated by flash chromatography as described in 2.3.6.

Method B

RU5135 (0.1 mmol) was stirred in dichloromethane (1 ml) at room temperature under an atmosphere of nitrogen. TMG (0.11 mmol) was added and the mixture stirred for 10 min. Fmoc chloride (0.11 mmol) was added and the reaction stirred for 30 min. The mixture was washed with water (3 x 2 ml), the organic phase dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure. The residue was dissolved in a minimum volume of ethyl acetate and subjected to flash chromatography using ethyl acetate as the solvent as described in 2.3.6.

2.3.3 Reaction of Fmoc-RU5135 with 1,4-butanedioldiglycidyl ether (BDDE)

This method was adapted from that of Vretblad (1976). Fmoc-RU5135 (0.05 mmol) and 1,4-butanediol diglycidyl ether (0.05 mmol) were stirred in 1 M Na_2CO_3, pH 10, 50% (v/v) dioxan (1 ml) for 16 h. The reaction mixture was extracted into chloroform (2 ml) and the extract washed with water (3 x 2 ml). The organic phase was dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure. The residue was dissolved in a minimum volume of ethyl acetate and subjected to flash chromatography using ethyl acetate as the solvent as described in 2.3.6.

2.3.4 Deprotection of the Amidine groups of RU5135 and its Derivatives

2.3.4.1 Photolytic Cleavage of the Cbz Group

Cbz-RU5135 (0.1 mmol) was dissolved in ethanol/water (2:1 v/v) (5 ml) and irradiated at λ = 254 nm for 4 h using a medium pressure, mercury lamp at 40-45°C. The solution was cooled, acidified and concentrated to approximately 0.5 ml under reduced pressure. A white solid was formed upon the addition of diethyl ether (3 ml) to the solution. This was filtered and dried in a vacuum oven at 40°C to constant weight.

Epoxy-activated Sepharose 6B (1 ml) was subjected to photolysis in an identical fashion. It was then cooled, packed into 1 mm path length quartz cuvettes and UV spectrophotometry was performed as described in 3.3.4.4. The spectrum of this resin was compared to that of the identical resin which had not undergone photolysis.

2.3.4.2 Base-Catalysed Cleavage of the Fmoc Group

This was performed according to the method of Carpino and Han (1972). A solution of fmoc-RU5135 (0.1 mmol) was stirred in ethanolamine (1 ml) at room temperature for 0.5, 1, 3, 6 or 12 h. The mixture was poured into ice-cold, distilled water (10 ml) and the residue filtered. This cream/white residue was washed extensively with diethyl ether (6 x 3 ml) and the white solid obtained was filtered and dried in a vacuum oven at 40°C to constant weight.

2.3.5 Derivatisation of RU5135 using Diamines

2.3.5.1 Reaction of RU5135 with Succinyl Chloride

RU5135 (0.2 mmol) was dissolved in pyridine (1 ml). Succinyl chloride (0.22 mmol) was added and the reaction stirred at 70°C for 6 h. The pyridine was evaporated under reduced pressure, the residue taken up in a minimum volume of ethyl acetate and subjected to flash chromatography as described in 2.3.6.

2.3.5.2 Reaction of RU5135-Succinyl Chloride with Diamines

RU5135-succinyl chloride (0.2 mmol) was dissolved in pyridine (1 ml). Either 1,6-diaminohexane or 1,12-diaminododecane (0.3 mmol) was added and the reaction was stirred at room temperature for 8 h. The pyridine was evaporated under reduced pressure, the residue was taken up into a minimum volume of ethyl acetate and subjected to flash chromatography as described in 2.3.6.

2.3.6 Separation of Synthetic Compounds by Flash Chromatography

Kieselgel (5 g) was suspended in ethyl acetate and packed in a glass column (1.5 cm x 30.0 cm). It was washed with 3 column volumes of ethyl acetate. The sample was loaded onto the column and eluted with ethyl acetate under pressure. Fractions (1 ml) were collected and the elution of the products was monitored by performing analytical tlc on the fractions. The fractions which

contained products were pooled, evaporated to dryness under reduced pressure and subjected to spectral analyses in order to ascertain their structures.

2.4 RESULTS

A small sample of RU5135 was kindly provided by Roussel UCLAF, however, due to the nature of this study, it was necessary to synthesise more RU5135. Although a significant amount of time was invested in the synthesis, for the purposes of the present study, this should be considered routine work.

2.4.1 Synthesis and Chemical Characterisation of RU5135

2.4.1.1 Synthesis of RU5135

The synthesis of RU5135 was performed according to the methods of Jouquey and Hunt (1985) shown in the reaction scheme in Figure 2.4.1. In brief, 3α-acetoxy-11,17-dioxo-5β-androstane (I) was treated with *t*-butyl nitrite in alkaline medium which gave the keto-oxime (II). This was rapidly converted to the acid chloride derivative (III) upon reaction with thionyl chloride at 0°C. This compound was converted to the corresponding amide (IV) with ammonium hydroxide and this intermediate then underwent Hoffmann rearrangement at low temperature in the presence of sodium hypochlorite to give intermediate V. This was cyclised to the amidine (RU5135) using methanolic sodium hydroxide at reflux temperature.

The synthesis was first carried out on a small scale (1 g) and then on a preparative scale (5 g). The yields of the small scale preparation were lower than the values reported by Jouquey and Hunt (1985) but those obtained for the preparative scale synthesis were in good agreement with the reported yields

(Table 2.4.1). A single recrystallisation was necessary to give a product with high purity (> 97%, measured by hplc).

2.4.1.2 Chemical Characterisation of RU5135

The characterisation of RU5135 by proton nuclear magnetic resonance spectroscopy (^{1}H nmr) and electron impact mass spectroscopy (EI-MS) was facilitated by the chemical characterisation of its synthetic intermediates. The spectroscopic data available on the amidine steroid at the beginning of this study was limited. However, alterations in the spectra of the intermediates and the relation of these alterations to the structures could be investigated. These data were used to assign the peaks in the ^{1}H nmr spectrum of RU5135 and this was used as the basic reference spectrum for the assessment of all experiments involving the derivatisation of RU5135 (Figure 2.4.2). Additional information for the assignation of the peaks in the ^{1}H nmr spectrum of RU5135 was provided by 2D-nmr experiments which were used to determine the coupling between signals. The spectral data for RU5135 are given in Table 2.4.7. Trimethylsilane (TMS) was the internal standard in all ^{1}H nmr experiments.

2.4.2 Protection of the Amidine Group of RU5135

The protection of the amidine group of RU5135 was performed according to the various methods described in Section 2.3.2. The chemical structures were verified by standard analytical procedures and are summarised in Figure 2.4.8.

2.4.2.1 Arginine-type Protection Groups

For the arylsulphonamides, the yield of the reaction varied according to the conditions of the reaction. It was found that when the reaction of RU5135 with arylsulphonyl chlorides was performed in aqueous NaOH and acetone, products were detected by t.l.c. using dichloromethane/methanol (9:1 v/v) which were UV sensitive and were easily purified by flash chromatography. The yields of these products were 45% and 55% and spectral analyses verified the structures as those of methylbenzenesulphonyl-RU5135 and mesitylene-2-sulphonyl-RU5135 respectively (Figure 2.4.8). The physical and spectral characteristics of these compounds are given in Tables 2.4.3 and 2.4.4 respectively.

When the identical reactions were performed in the presence of the weak base pyridine, there was no protection reaction but merely the formation of the hydrochloride salt of RU5135. Here, the steroidal structure was found to have separated into the aqueous phase instead of the organic layer. The ^{1}H nmr of this compound had none of the characteristic signals of the protection group. This was also true for the reactions of RU5135 with trityl chloride whether the

reaction was performed in pyridine or in dimethylformamide containing sodium hydride.

2.4.2.2 Cbz Protection

The efficiency of the reaction of RU5135 with cbz chloride varied according to the conditions of the reaction. The maximum yield was achieved when the reaction was performed in an aqueous solution of 1 M NaOH in tetrahydrofuran when the yield of the powdery, white solid was 63-72% (Section 2.3.2.3.A) whose structure was confirmed as being cbz-RU5135 (Figure 2.4.8) by spectral analyses. The use of a strong base (TMG or DBU, Sections 2.3.2.3.C and 2.3.2.3.D) gave a lower yield of 55% for the same product; when a weaker base was used (1 M Na_2CO_3) the yield was lower still at 40% (Section 2.3.2.3.B). The physical and spectral characteristics of cbz-RU5135 are given in Table 2.4.5.

2.4.2.3 Fmoc Protection

The fmoc derivative of RU5135 was synthesised using a weak base (Na_2CO_3 or $NaHCO_3$) in dry 1,4-dioxan (Section 2.3.2.4.A). The yield of this reaction was very low (7-12%) and was not increased by either using a stronger base (TMG, Section 2.3.2.4.B) or by increasing the time of reaction. The yield was slightly higher with Na_2CO_3 than with $NaHCO_3$ (8-12% versus 7-10%) and the cream/white powder was easily purified by flash chromatography. This reaction was continued for a relatively long time (4 days) and it was shown that

the crude mixture contained the starting material (RU5135) and the elimination product of fmoc chloride, dibenzofulvene. The physical and spectral data for fmoc-RU5135 are shown in Table 2.4.6.

2.4.3 Deprotection of the Amidine Group of RU5135 and its Derivatives

2.4.3.1 Photolytic Cleavage of the Cbz Group

The photolytic cleavage of the N-cbz group from the amidine group of RU5135 was performed as described in 2.3.4.1. It was found that the deprotection reaction was very efficient giving a yield of 95% of deprotected RU5135. Since this reaction would ultimately be performed on cbz-RU5135 which was covalently attached to epoxy-activated Sepharose 6B, the photolytic reaction was carried out on a suspension of the resin itself. The effect of photolysis was assessed by UV spectrophotometry. It was found that this had a profound effect on the spectrum of the resin as shown in Figure 2.4.6. Photolysis of the Sepharose caused an increase in absorbance at $\lambda = 230$ nm and a decrease in absorbance at $\lambda = 328$ nm. Before photolysis, the $\lambda_{max.}$ for Sepharose 6B was 270 nm.

2.4.3.2 Base-Catalysed Cleavage of the Fmoc Group

The cleavage of the fmoc moiety from the amidine group of RU5135 was achieved in high yield (94%) using ethanolamine which is a relatively weak

base. Purification of the deprotected RU5135 was relatively simple due to the difference in solubility of fmoc-RU5135 and the deprotected compound. Washing with diethyl ether and filtering removed residual fmoc-RU5135 and dibenzofulvene (the elimination product of the fmoc group) with minimal losses of RU5135. The time course of the cleavage reaction was investigated and the yield of RU5135 was found to reach a maximum after 5 h. There was no further increase in yield after this time even when the reaction was allowed to continue for up to 12 h. The effect of ethanolamine on epoxy-activated Sepharose 6B was not tested because this compound is used to block the excess unreacted epoxide groups in the resin and this reaction is well documented (Sundberg and Porath, 1974).

2.4.4 Coupling of Fmoc-RU5135 to BDDE

This reaction was found to occur in relatively high yields of 55-60% resulting in a cream/white powder. The product was readily soluble in ethyl acetate which facilitated its purification by flash chromatography using this as solvent. The physical and spectral data are given in Table 2.4.7.

2.4.5 Deprotection of Fmoc-RU5135-BDDE

This reaction was performed in ethanolamine as described in 2.3.4.2. The product was a white solid which was readily soluble in ethyl acetate. It was not visible on t.l.c. plates under UV light but was detected as a single spot after spraying with 10% H_2SO_4 and charring. The physical and spectral data are

shown in Table 2.4.8.

2.4.6 Derivatisation of RU5135 using Diamines

The general reaction scheme for this method of synthesis is shown in Figure 2.4.11. The advantage of this method was that there was no need for protection of the amidine group prior to coupling due to the fact that the reaction between tertiary amines and acid chlorides is much slower than that of acid chlorides with alcohols. Therefore, it was possible to use the acid chloride derivative of a dicarboxylic acid as a link between the C3-hydroxyl group of RU5135 and one of the primary amines of a diaminoalkane. This produced a covalently linked spacer arm with a free primary amine which could potentially be coupled to a support matrix and an additional advantage was that the length of this spacer arm can be varied with relative ease.

2.4.6.1 Reaction of RU5135 with Succinyl Chloride

This reaction was achieved in the presence of pyridine at 70°C in good yield (73%). The starting material and product could not be detected under a UV lamp due to the absence of an UV absorbing group. However, the compounds were visualised on tlc plates after spraying with 10% H_2SO_4 and charring. The product was separated from residual starting materials by flash chromatography using ethyl acetate as the solvent. The physical and spectral data for RU5135-succinyl chloride are shown in Table 2.4.9.

2.4.6.2 Reaction of RU5135-Succinyl Chloride with 1,6-Diaminohexane

This reaction was achieved in the presence of pyridine at room temperature in high yield (69%). The progress of the reaction was monitored by t.l.c and the compounds were visualised by spraying with 10% H_2SO_4 and charring. The product was separated by flash chromatography using ethyl acetate as the solvent. The physical and spectral data are shown in Table 2.4.10.

2.4.6.3 Reaction of RU5135-Succinyl Chloride with 1,12-Diaminododecane

This reaction was achieved in the presence of pyridine at room temperature in high yield (75%). The progress of the reaction was monitored by t.l.c and the compounds were visualised by spraying with 10% H_2SO_4 and charring. The product was separated by flash chromatography using ethyl acetate as the solvent. The physical and spectral data are shown in Table 2.4.11.

Figure 2.4.1

The Synthesis of RU5135

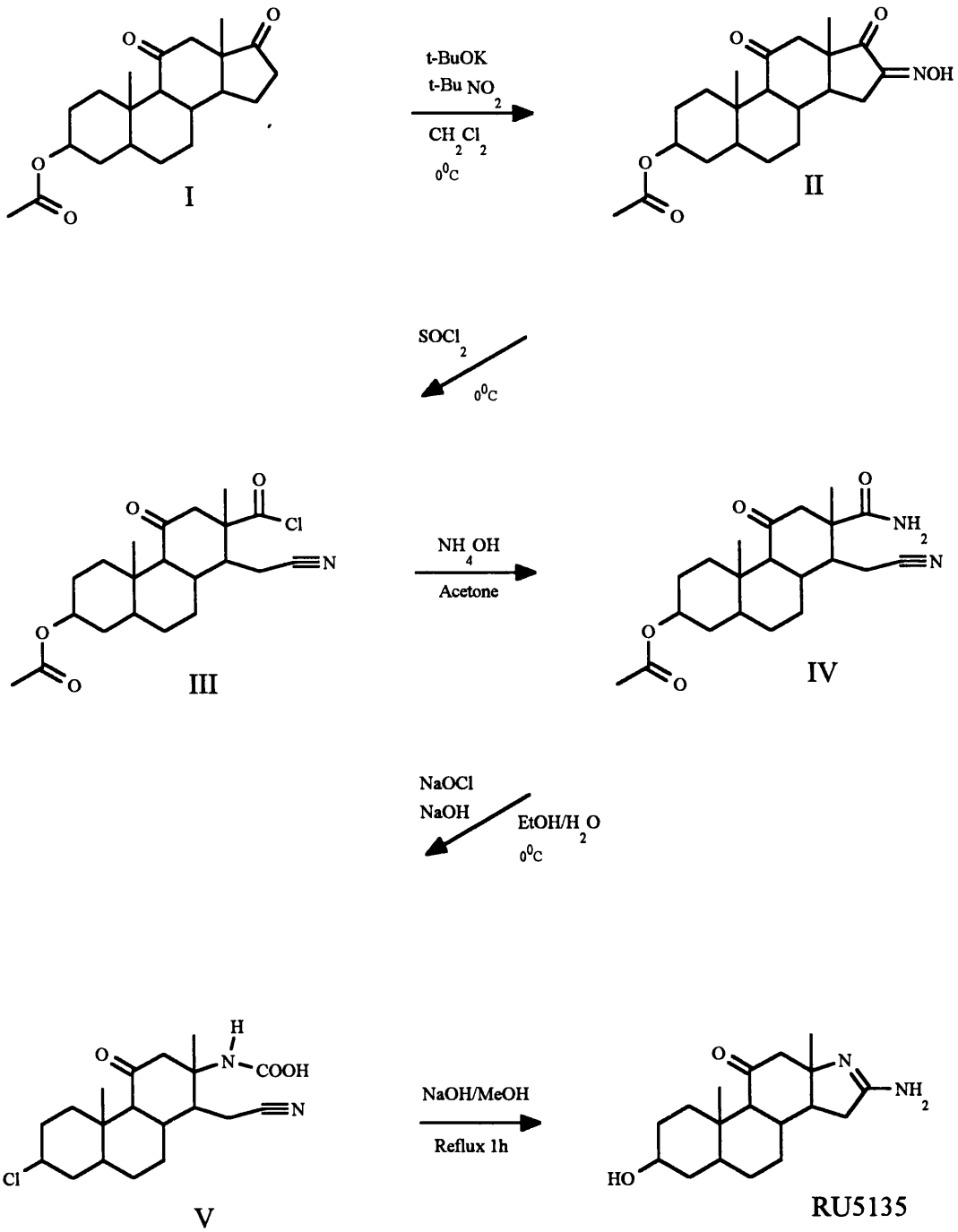


Figure 2.4.2 **^{1}H nmr of RU5135**

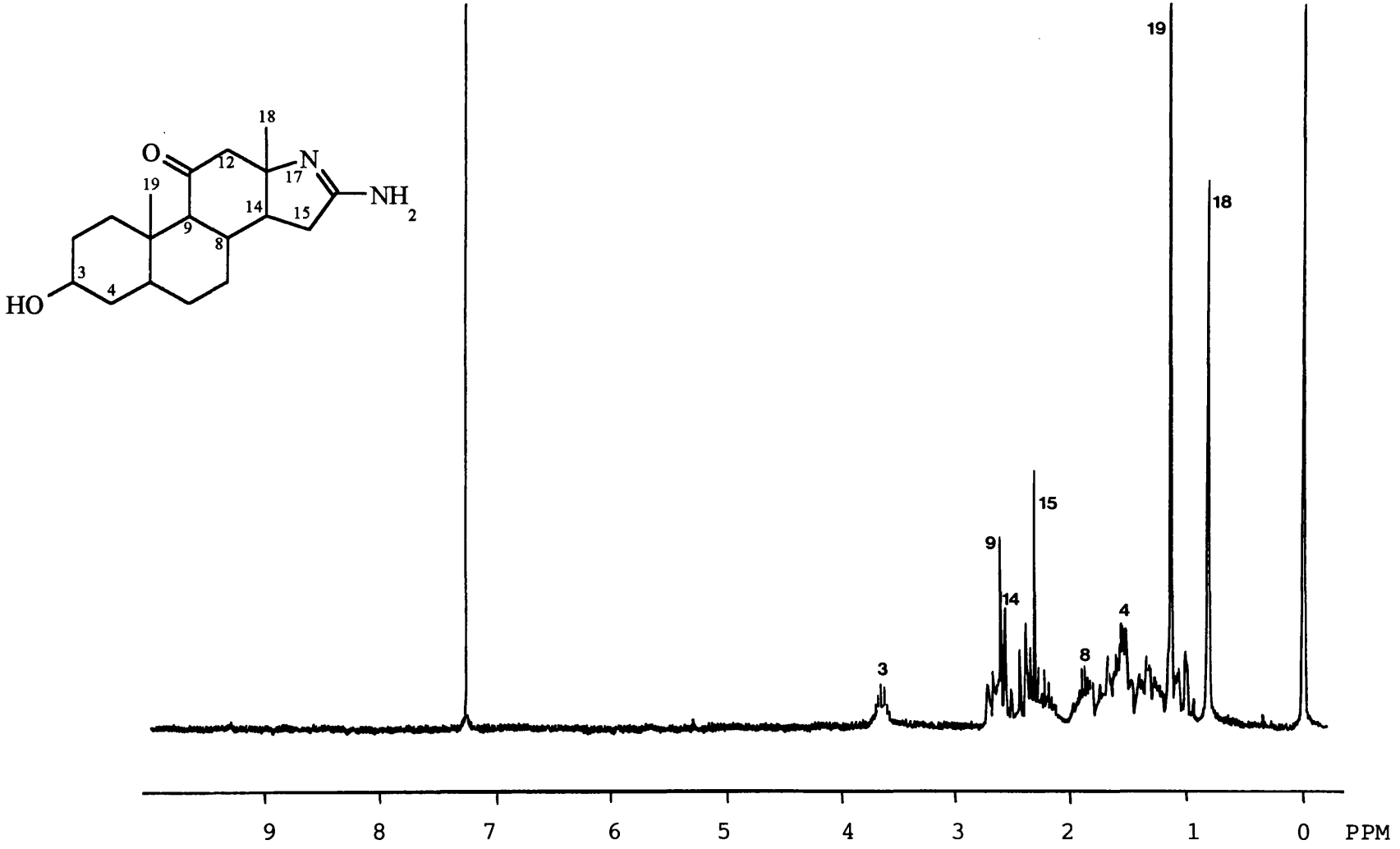


Figure 2.4.3 **Electron Impact Mass Spectrum of RU5135**

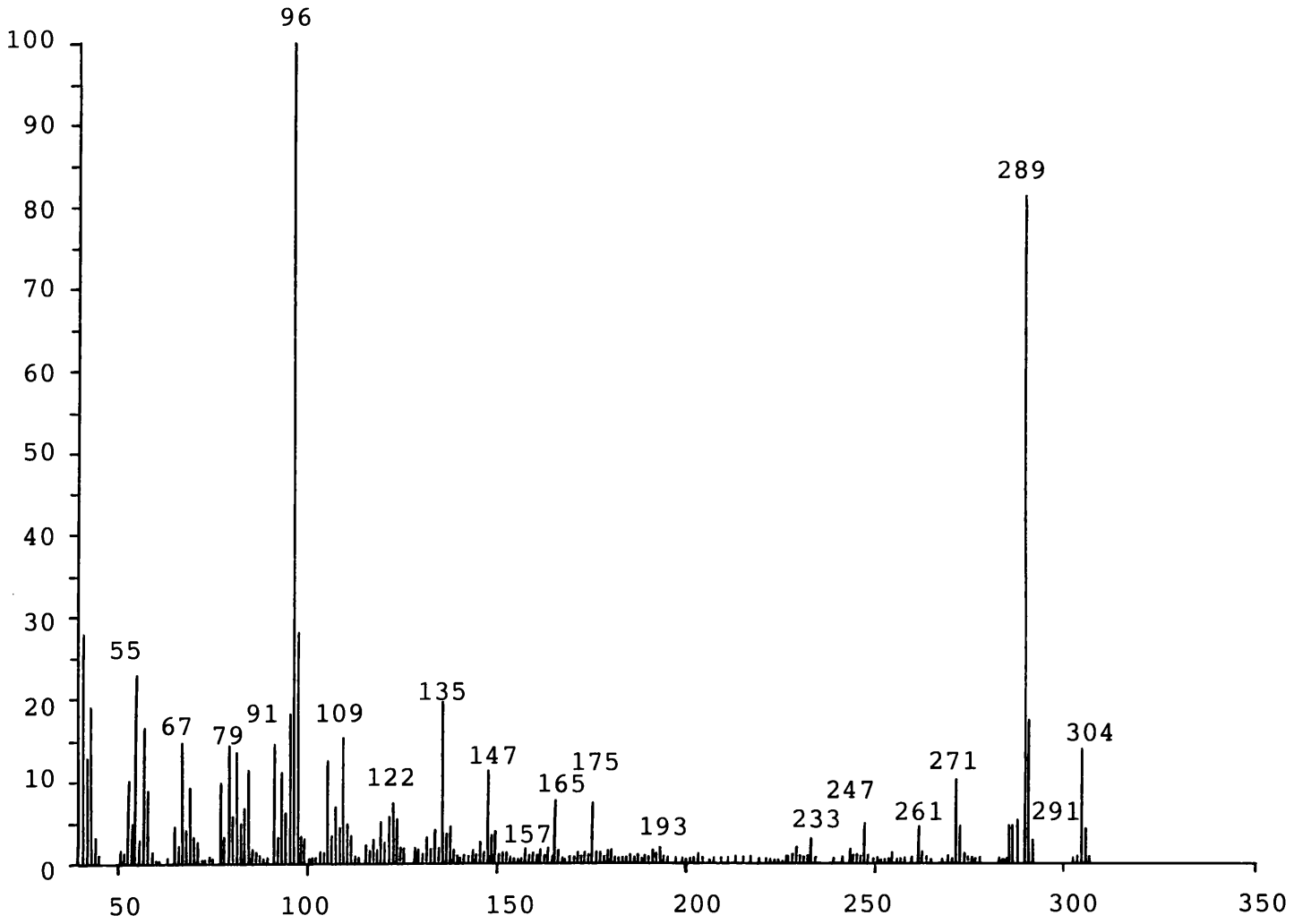


Figure 2.4.4 **^{1}H nmr of 3α-Acetoxy-11,17-dioxo-5β-androstane**

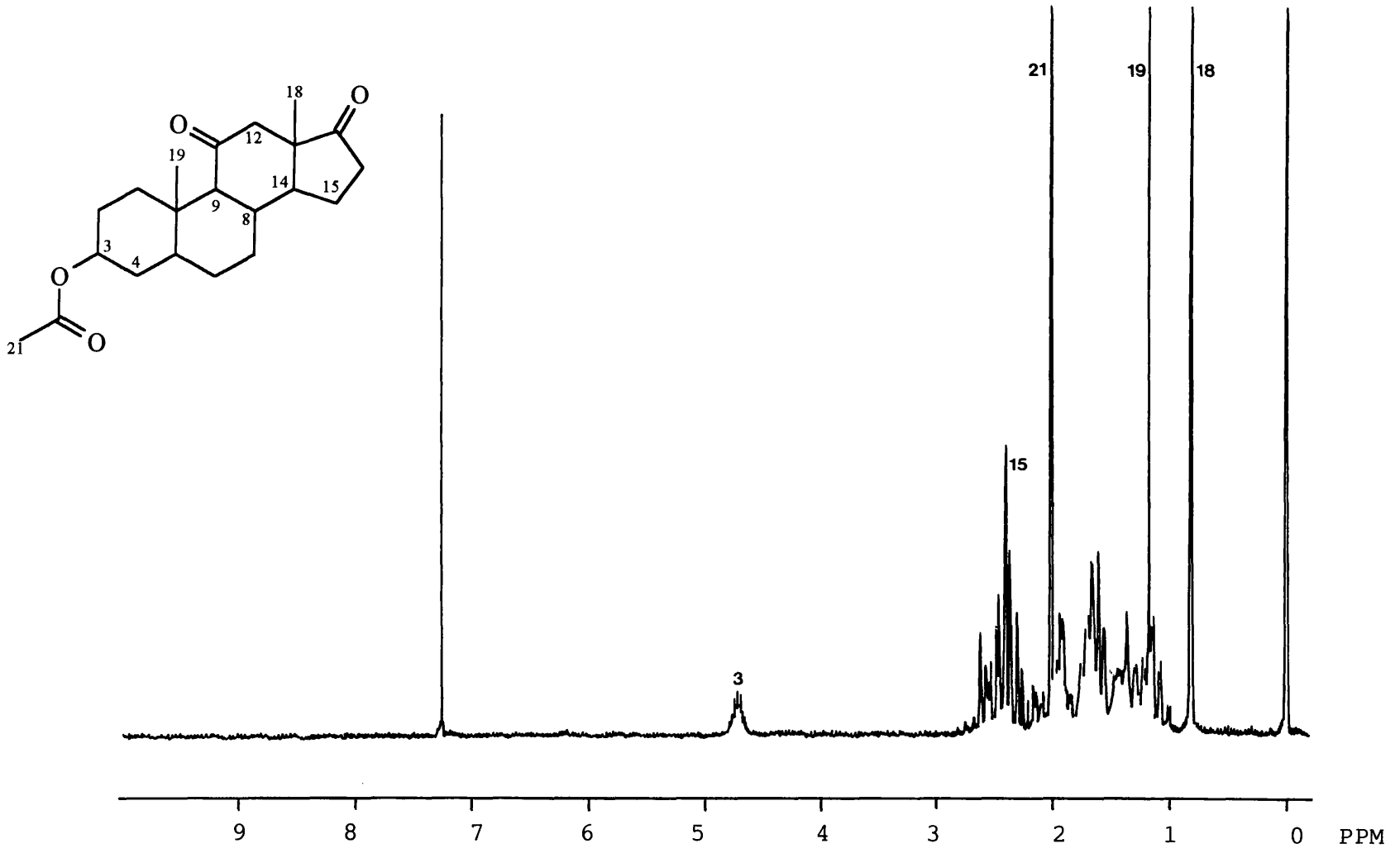


Figure 2.4.5 **^{1}H nmr of 3α-Acetoxy-11,17-dioxo-16-oximino-5β-androstane**

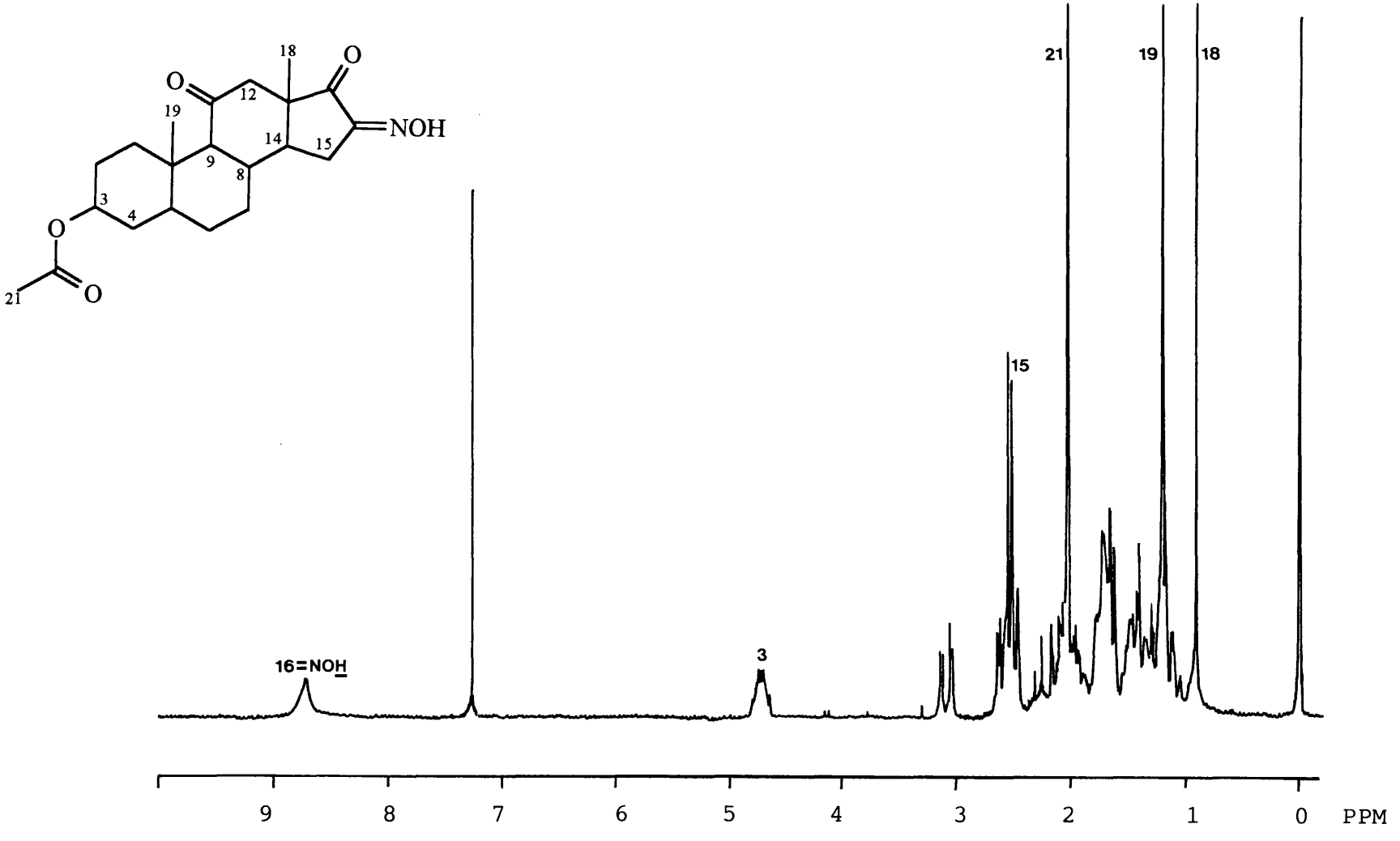


Figure 2.4.6 **^{1}H nmr of Nitrile Amide Intermediate IV**

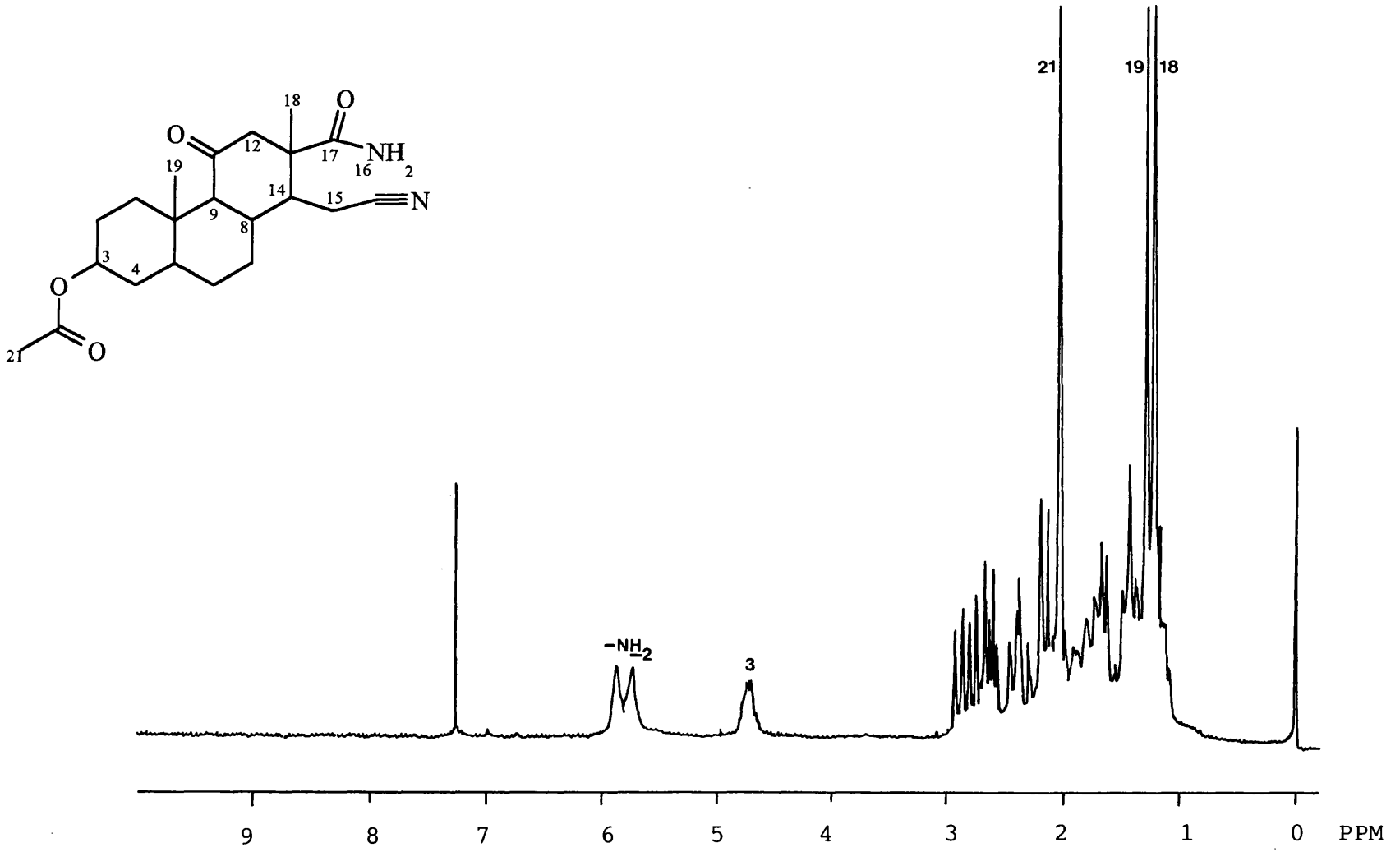


Figure 2.4.7 **^{1}H nmr of Nitrile Carbamic Acid Intermediate V**

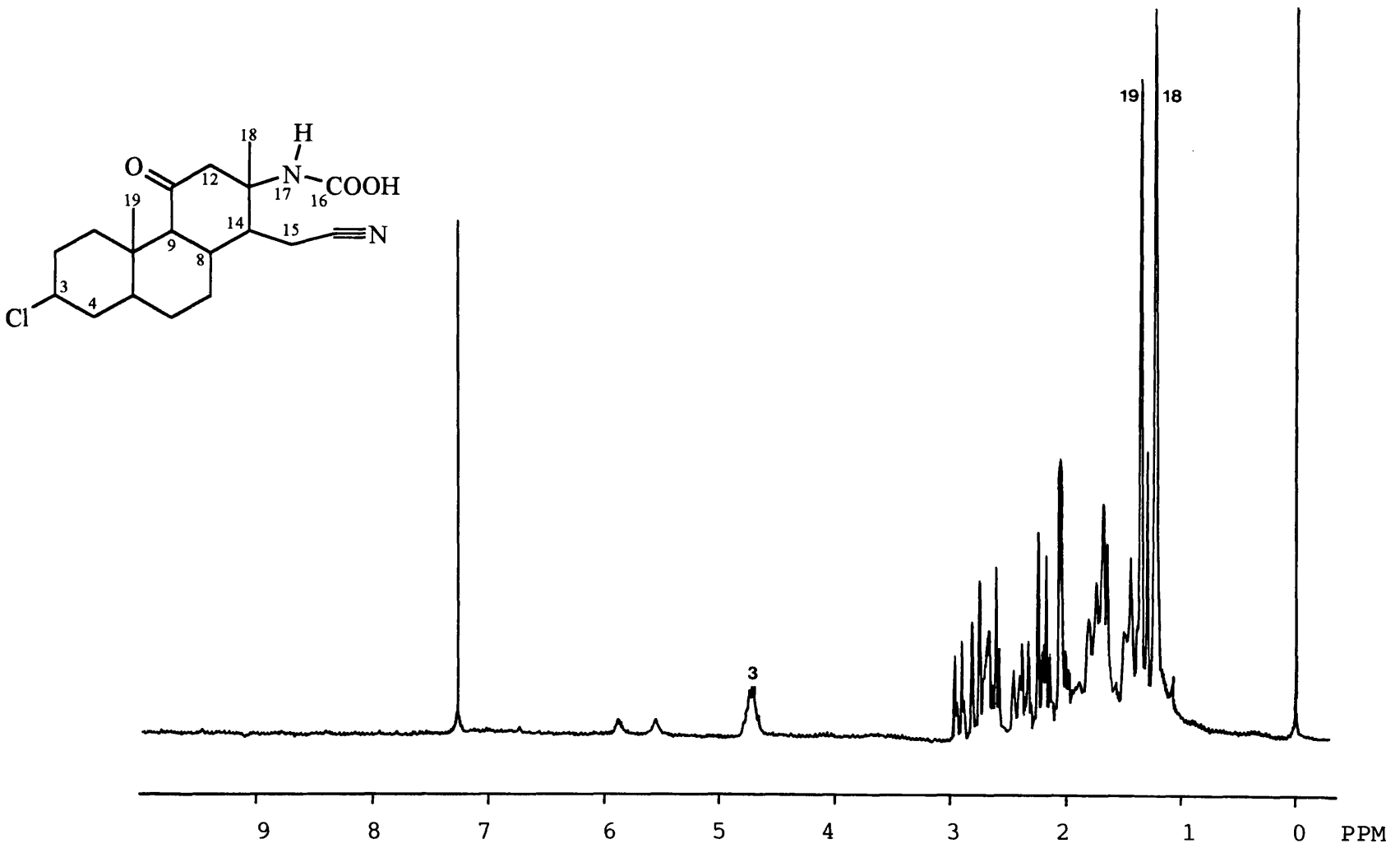
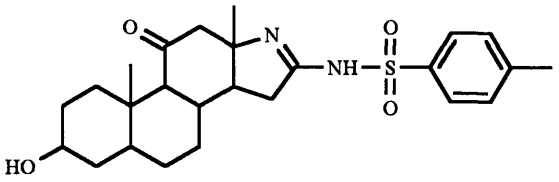
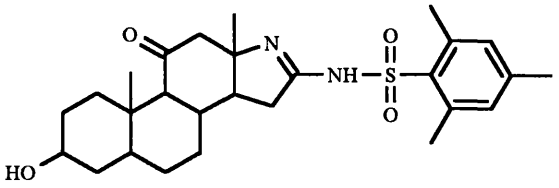


Figure 2.4.8

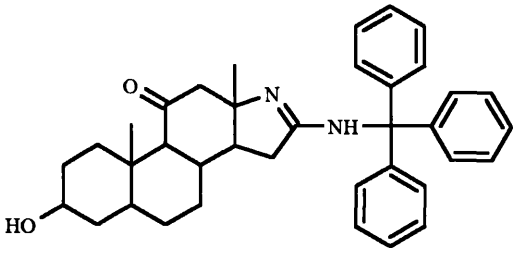
Protection Moieties used for the Amidine Group of RU5135



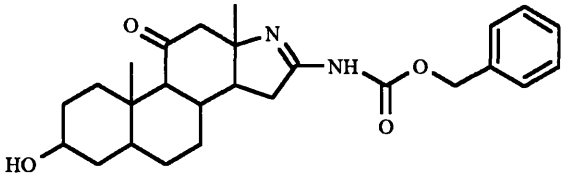
Methylbenzene sulphonyl-RU5135



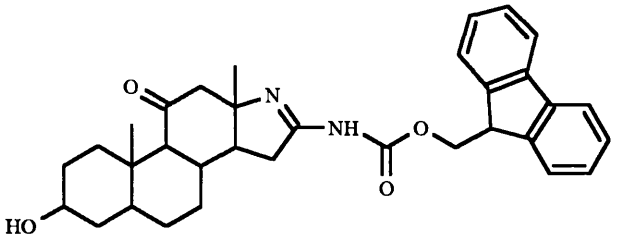
Mesitylene-2-sulphonyl-RU5135



Trityl-RU5135



Cbz-RU5135



Fmoc-RU5135

Figure 2.4.9

The Synthesis of Ligands to be Coupled Via Epoxide Groups

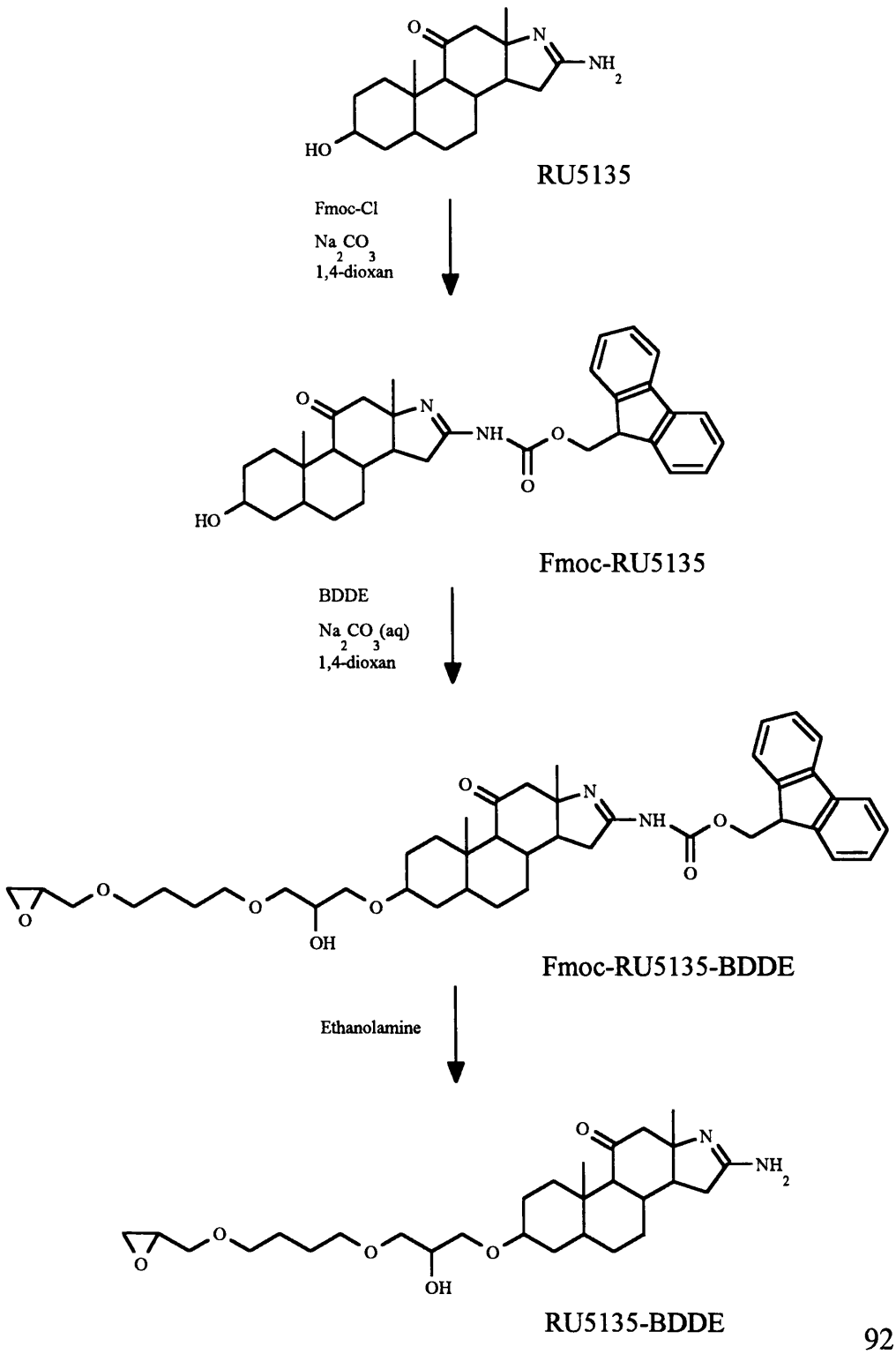


Figure 2.4.10 **UV Spectrum of Photolysed Epoxy-Activated Sepharose 6B**

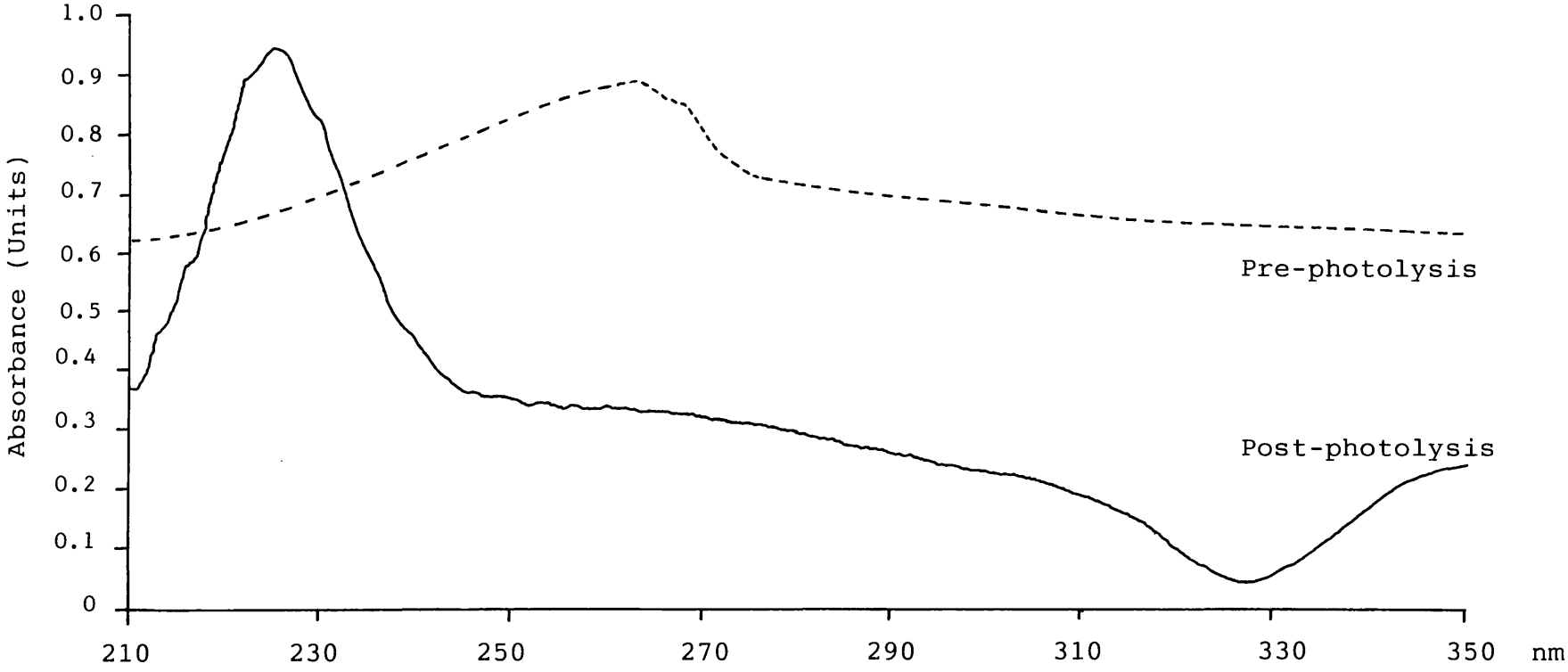


Figure 2.4.11

The Synthesis of Ligands to be Coupled Via Amine Groups

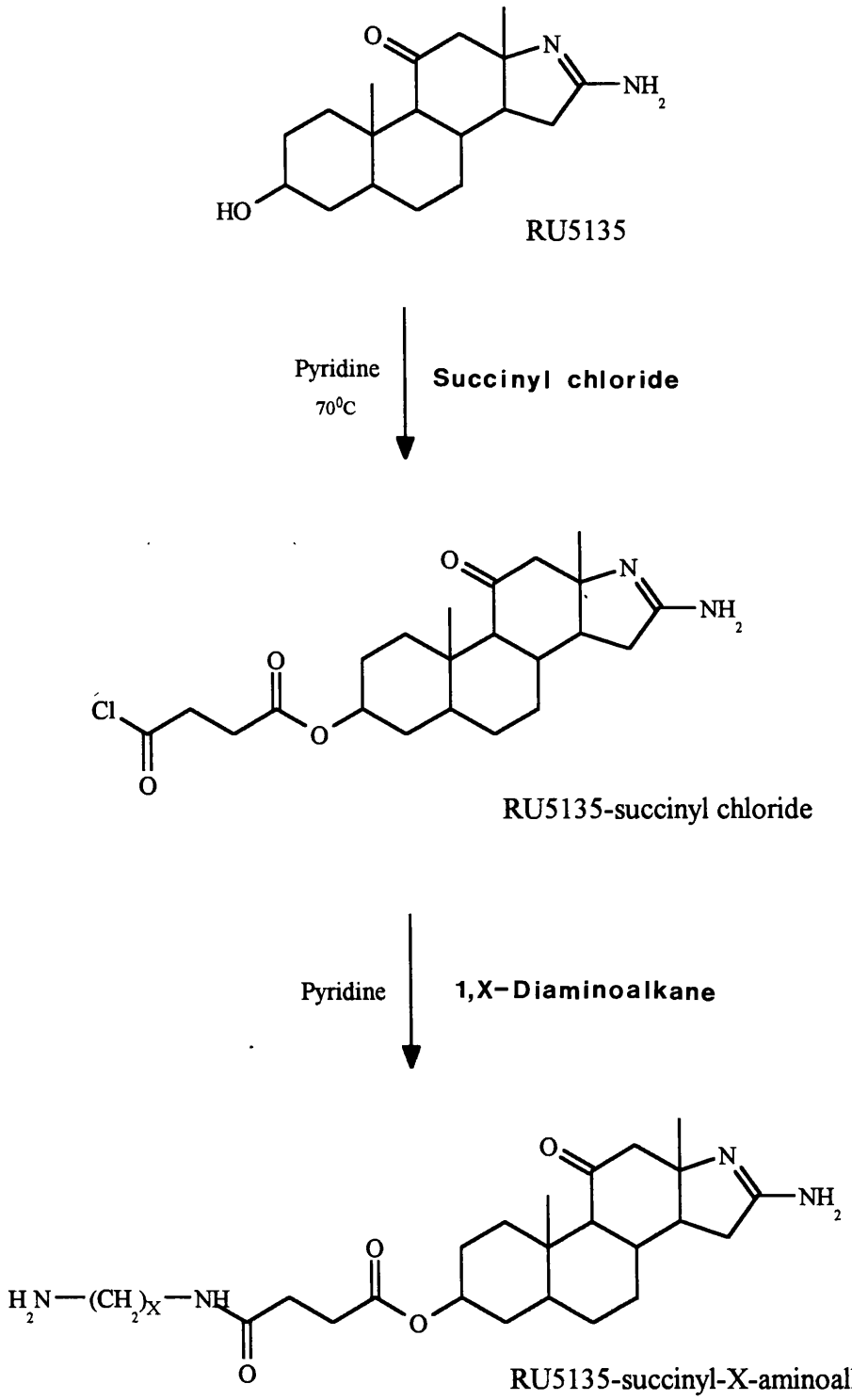


Table 2.4.1

The Yields of the Intermediate Compounds in the Synthesis of RU5135

Compound	Literature Yield[a]	Yield in This Study
II	75%	74%
III	87%	ND
IV	92%	72%[b]
V	81%	90%
RU5135	66%	52%

a These values are quoted from Jouquey and Hunt (1985).
b This value was obtained with respect to the amount of compound II as starting material.
ND = Not Determined.

Table 2.4.2

Spectral and Physical Data for RU5135

R_f (EtOAc) = 0.0

R_f (CH_2Cl_2/MeOH, 9:1 v/v) = 0.0

EI-MS: m/z peaks at

304	(15%) ($M^+ + 1$, $C_{18}H_{27}O_2N_2$)		
289	(85%)	147	(15%)
271	(12%)	135	(20%)
261	(5%)	122	(8%)
247	(5%)	109	(15%)
175	(10%)	96	(100%)
165	(10%)		

See Figure 2.4.3.

Anal. Calcd. for $C_{18}H_{28}N_2O_2$: C 71.1, H 9.2, N 9.2, O 10.5
Found C 72.4, H 9.0, N 8.9, O 9.7

200 MHz ^1H nmr ($CDCl_3$) (See Figure 2.4.2)

δ (ppm)	Integration	Multiplicity	Inference
0.82	3H	s	3H-18
1.15	3H	s	3H-19
2.29	2H	s	2H-15
2.52	1H	m	1H-14
2.58	1H	s	1H-9
3.65	1H	m	1H-3

IR (KBr disc, surface film): $V_{max.}$ at:
3300, 2900, 2850, 1695, 1650, 1590 cm^{-1}

It should be noted that the ^1H nmr analyses of some of the derivatives of RU5135 were performed in d-DMSO due to the increased solubility of the compounds in this solvent compared to $CDCl_3$. As the chemical shifts of the protons vary slightly in different solvents, depending on the relative polarity of the solvents, the ^1H nmr spectral data for RU5135 in d-DMSO is also given here. However, for clarity and since these data are purely for reference purposes, only the signals which may shift significantly upon derivatisation of RU5135 have been quoted.

200 MHz ^1H nmr (d-DMSO)

δ (ppm)	Integration	Multiplicity	Inference
0.61	3H	s	3H-18
1.04	3H	s	3H-19
3.38	1H	m	1H-3

Table 2.4.3

Spectral and Physical Data for Methylbenzenesulphonyl RU5135

R_f (CH_2Cl_2/MeOH, 9:1 v/v) = 0.5

Anal. Calcd. for $C_{25}H_{28}N_2O_4S$: C 65.4, H 6.1, N 6.1, O 13.9, S 7.1
Found C 64.8, H 6.7, N 6.3, O 13.8, S 8.4

200 MHz ^1H nmr (d-DMSO)

δ (ppm)	Integration	Multiplicity	Inference
0.88	3H	s	3H-18
1.07	3H	s	3H-19
2.21	3H	s	$-SO_2-Ar-C\underline{H}_3$
3.54	1H	m	1H-3
7.37-7.50	4H	m	Aromatic H

Table 2.4.4

Spectral and Physical Data for Mesitylene-2-sulphonyl RU5135

R_f (CH_2Cl_2/MeOH, 9:1 v/v) = 0.6

Anal. Calcd. for $C_{27}H_{38}N_2O_4S$: C 66.4, H 7.8, N 5.7, O 13.1, S 6.9
Found C 65.9, H 7.6, N 6.0, O 13.0, S 7.5

200 MHz ^1H nmr (d-DMSO)

δ (ppm)	Integration	Multiplicity	Inference
0.88	3H	s	3H-18
1.06	3H	s	3H-19
2.29	6H	s	mesitylene CH_3
3.65	1H	m	1H-3
7.02	2H	s	Aromatic H

IR (KBr disc, surface film): $V_{max.}$ at
3400, 2920, 2830, 1780, 1600, 1300, 1190 cm^{-1}

Table 2.4.5

Spectral and Physical Data for Cbz-RU5135

R_f (CH_2Cl_2/MeOH, 9:1 v/v) = 0.7

EI-MS: m/z peaks at
438 (15%) ($M^+ + 1$, $C_{26}H_{33}O_4N_2$)
423 (5%)
379 (10%)
304 (45%)
289 (15%)

Anal. Calcd. for $C_{26}H_{34}N_2O_4$: C 71.2, H 7.8, N 6.4, O 14.6
Found C 70.9, H 8.0, N 6.1, O 15.0

200 MHz ^1H nmr ($CDCl_3$)

δ (ppm)	Integration	Multiplicity	Inference
1.00	3H	s	3H-18
1.13	3H	s	3H-19
3.68	1H	m	1H-3
5.13	2H	s	NHCOOC<u>H</u>$_2$Ph
7.31-7.39	5H	m	Aromatic H

IR (KBr disc, surface film): $V_{max.}$ at:
3580, 3120, 3060, 1800, 1480 cm^{-1}

Table 2.4.6

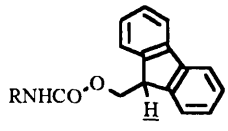
Spectral and Physical Data for Fmoc-RU5135

R_f (EtOAc) = 0.5

EI-MS: m/z peaks at
528 (4%) ($M^+ + 1$, $C_{33}H_{37}O_4N_2$)
303 (40%)
288 (15%)

Anal. Calcd. for $C_{33}H_{38}N_2O_4$: C 75.3, H 7.2, N 5.3, O 12.2
Found C 74.9, H 7.1, N 5.4, O 12.6

200 MHz ^1H nmr ($CDCl_3$)

δ (ppm)	Integration	Multiplicity	Inference
1.00	3H	s	3H-18
1.12	3H	s	3H-19
3.65	1H	m	1H-3
4.38	2H	d	NHCOOC<u>H</u>$_2$Fl
4.54	1H	t	
7.36-7.81	8H	m	Aromatic H

IR (KBr disc, surface film): $V_{max.}$ at:
3400, 2920, 2860, 1700, 1450, 1300 cm^{-1}

Table 2.4.7

Spectral and Physical Data for Fmoc-RU5135-BDDE

R_f (EtOAc) = 0.8

EI-MS: m/z peaks at
726 (5%) ($M^+ + 1$, $C_{43}H_{56}O_8N_2$)
524 (15%)
502 (5%)
303 (35%)

Anal. Calcd. for $C_{43}H_{56}N_2O_8$: C 70.9, H 7.7, N 3.8, O 17.6
Found C 71.1, H 7.6, N 4.0, O 17.3

200 MHz ^1H nmr ($CDCl_3$)

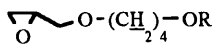
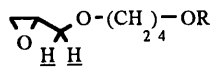
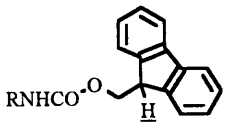
δ (ppm)	Integration	Multiplicity	Inference
1.00	3H	s	3H-18
1.13	3H	s	3H-19
1.57	8H	m	
3.61-3.71	7H	m	
4.38	2H	d	NHCOOCH$_2$Fl
4.54	2H	t	
7.36-7.81	8H	m	Aromatic H

Table 2.4.8

Spectral and Physical Data for RU5135-BDDE

R_F (EtOAc) = 0.3

EI-MS: m/z peaks at
504 (5%) ($M^+ + 1$, $C_{28}H_{45}O_6N_2$)
303 (20%)

Anal. Calcd. for $C_{28}H_{45}N_2O_6$: C 66.4, H 9.1, N 5.5, O 19.0
Found C 68.0, H 8.5, N 6.3, O 17.2

200 MHz ^1H nmr ($CDCl_3$)

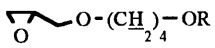
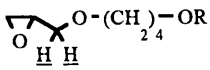
δ (ppm)	Integration	Multiplicity	Inference
0.88	3H	s	3H-18
1.14	3H	s	3H-19
1.57	4H	m	
3.61-3.71	7H	m	

Table 2.4.9

Spectral and Physical Data for RU5135-Succinyl Chloride

R_f (EtOAc) = 0.35

EI-MS: m/z peaks at
422 (5%) ($M^+ + 1$, $C_{22}H_{31}O_4N_2Cl$)
303 (20%)

200 MHz ^1H nmr ($CDCl_3$)

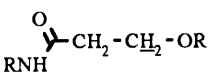
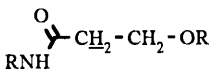
δ (ppm)	Integration	Multiplicity	Inference
0.84	3H	s	3H-18
1.19	2H	s	3H-19
3.45-3.50	2H	q	
4.00-4.15	2H	q	

Table 2.4.10

Spectral and Physical Data for RU5135-Succinyl-6-Aminohexane

R_f (EtOAc) = 0.6

EI-MS: m/z peaks at
501 (5%) ($M^+ + 1$, $C_{28}H_{60}O_4N_4$)
387 (10%)
303 (30%)

Anal. Calcd. for $C_{28}H_{60}N_4O_4$: C 65.1, H 11.6, N 10.9, O 12.4
Found C 64.9, H 10.9, N 10.9, O 13.3

200 MHz ^1H nmr ($CDCl_3$)

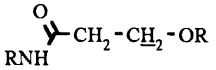
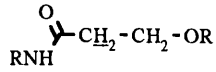
δ (ppm)	Integration	Multiplicity	Inference
0.84	3H	s	3H-18
1.19	2H	s	3H-19
1.25	9H	m	Alkyl H
2.63-2.66	4H	m	-C<u>H</u>$_2$-NH_2
3.45-3.50	2H	q	
3.90-4.05	2H	q	

Table 2.4.11

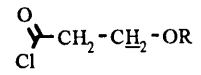
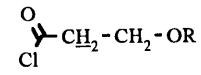
Spectral and Physical Data for RU5135-Succinyl-12-Aminododecane

R_f (EtOAc) = 0.7

EI-MS: m/z peaks at
585 (5%) ($M^+ + 1$, $C_{35}H_{72}O_4N_4$)
387 (10%)
303 (25%)

Anal. Calcd. for $C_{35}H_{72}O_4N_4$: C 68.6, H 11.8, N 9.2, O 10.5
Found C 68.4, H 10.2, N 9.7, O 11.7

200 MHz ^1H nmr ($CDCl_3$)

δ (ppm)	Integration	Multiplicity	Inference
0.86	3H	s	3H-18
1.21	3H	s	3H-19
1.27	15H	m	Alkyl H
2.60-2.65	3H	m	$-C\underline{H}_2-NH_2$
3.48-3.51	2H	q	
3.90-4.05	2H	q	

2.5 DISCUSSION

The synthesis of RU5135 has been achieved and the results found in this study for the large scale synthesis agreed well with the reported findings of Jouquey and Hunt (1985) (Table 2.4.1). For the smaller scale preparation (1 g), the yields were slightly lower than expected and this may be due to losses occurring from the reaction volume which represent a larger proportion of the total in the small scale reaction.

Each of the intermediates of the synthesis of RU5135 (Figure 2.4.1) were subjected to spectral analysis and the resultant spectra (Figures 2.4.4 to 2.4.7) were employed to reconcile the structure of RU5135 with its ^{1}H nmr spectrum as there was an extremely limited amount of physical data available on the structure of RU5135 at the beginning of this study. The major difference among these spectra was the shift of the 3H singlet at δ 0.82 ppm signal. This signal shifted as the structure of ring D of the steroid backbone altered during the synthesis of RU5135 whereas the chemical shift of the 3H singlet at δ 1.15 ppm remained constant. The signal at δ 0.82 ppm was, therefore, attributed to the C18-methyl group and the signal at δ 1.15 ppm was assigned as the C19-methyl group. Other signals in the ^{1}H nmr of RU5135 were assigned by 2D-nmr experiments which determined which signals were coupled.

The initial strategy for the protection of the amidine group of RU5135 involved the use of compounds which are used for the protection of the guanidine function of arginine in peptide synthesis. The methylbenzenesulphonyl

(or toluenesulphonyl) and mesitylene-2-sulphonyl groups have both been used for the protection of arginine (Greene, 1981). The protection of the amidine group of RU5135 was achieved using these compounds but the yields of the reactions were lower than those which have been obtained for the protection of arginine (Schnabel and Li, 1960) (Section 2.4.2.1). This may be due to the reduced solubility of RU5135 compared to arginine. Indeed, RU5135 was found to be insoluble in a number of non-polar organic compounds but soluble in solvents such as dimethylformamide and pyridine. Despite the presence of the steroidal backbone, RU5135 was relatively polar in its behaviour. As can be seen from the ^{1}H nmr data for both of these compounds, there was a shift in the signal of the C18-methyl group but the C19-methyl signal remained constant. This showed that the reaction between RU5135 and the arylsulphonyl chlorides had occurred at the amidine group and not at the C3-hydroxyl group since the shift of the hydrogen at position C3 remained constant (Tables 2.4.3 and 2.4.4; Figure 2.4.2). Protection of the amidine group of RU5135 with trityl chloride was unsuccessful and this may be due to the fact that pyridine or sodium hydride were not strong enough bases to deprotonate the amidine group, hence the result was the formation of the salt of RU5135.

The protection of RU5135 by either cbz or fmoc was achieved by reaction in the presence of a mild base. The yield of the cbz protection reaction was approximately ten times that of the fmoc protection reaction, however, the yields for both reactions were lower than those reported for the protection of the α-amine groups of amino acids (Carpino and Han, 1972; Paquet, 1982). It is

likely that the reason for this difference in yields for the carbamate formation is that the fmoc group is very base-labile compared to the cbz group. Cleavage of the fmoc group is achieved in the presence of relatively weak organic bases such as ethanolamine, morpholine or piperidine (Carpino and Han, 1972). The amidine group of RU5135 is a relatively strong base and, therefore, it was considered possible that this is strong enough to cause the elimination of the fmoc group. This theory was confirmed by the fact that when the reaction between RU5135 and fmoc chloride was performed in the presence of Na_2CO_3 and dry 1,4-dioxan at room temperature for a prolonged period (4 days), spectral analysis of the crude reaction mixture showed the presence of only RU5135 and dibenzofulvene (the elimination product of fmoc chloride). An identical reaction was performed on fmoc-RU5135 purified by flash chromatography (in the absence of RU5135) and, after 4 days, there had been no detectable elimination of the fmoc group. This strongly suggests that the elimination of fmoc is caused by RU5135 itself. It is likely that the mechanism of the elimination reaction is bimolecular rather than intramolecular since fmoc-RU5135 is stable upon isolation and no detectable breakdown of this product was evident after storage at 4°C for 6 weeks. The elimination of the fmoc group occurs via the mechanism shown in Figure 2.5.1 (Carpino and Han, 1972).

The amidine group of RU5135 was successfully derivatised with four different protection groups. The *N*-derivatised compounds were isolated in high purity (as determined by analytical tlc and ^1H nmr) by flash chromatography. This method of isolation was found to be superior to recrystallisation for these

compounds due to the small reaction scales and the amounts of products synthesised. All of these derivatives were potentially of use as ligands for immobilisation to epoxy-activated Sepharose 6B in the synthesis of RU5135 affinity chromatography matrices. However, for these compounds to be used as immobilised ligands it would be necessary to remove the protection moiety after the ligand had been coupled to the affinity matrix. Therefore, it was important to consider the effect of the deprotection reactions and conditions on the affinity matrix backbone (Sepharose). The arylsulphonyl protection groups are removed by the action of strong acids such as hydrofluoric acid and trifluoroacetic acid (Yajima *et al.*, 1978), but the cbz and fmoc groups are removed under much milder conditions (photolysis and treatment with mild base, respectively). For this reason, cbz-RU5135 and fmoc-RU5135 (despite its low synthetic yield) were chosen as ligands to be investigated for their immobilisation to epoxy-activated Sepharose 6B.

Attempts were made to try to increase the yield of the fmoc protection reaction by utilising stronger organic bases such as TMG and DBU. However these were unsuccessful because the bases caused the elimination of fmoc chloride themselves. However, the fmoc-RU5135 that was synthesised could be isolated relatively easily by flash chromatography and was stable upon storage. Additionally, although the yield of the fmoc chloride with RU5135 in the presence of Na_2CO_3 and 1,4-dioxan reaction was low, it was possible to re-isolate the unreacted starting material from the reaction vessel by filtration and, therefore, recycle the RU5135. In this way it was possible to increase the

amount of fmoc-RU5135 produced from a single batch of RU5135.

It has been shown that the cbz group and its derivatives can be cleaved from the amino groups of peptides by irradiation with U.V. light from a medium pressure, mercury lamp (Barltrop and Schofield, 1965). This method has also been used to remove the cbz group from other types of compound such as sugars (Hanessian and Masse, 1977). The removal of the cbz group from RU5135 was achieved by photolysis in an aqueous medium using a similar method. The product of the photolytic reaction (RU5135) was re-isolated in high yield showing that the deprotection was successful.

Since the object of this study was to covalently attach the protected compound to epoxy-activated Sepharose 6B and then perform the cleavage reaction on the immobilised ligand, it was necessary to ascertain the effects of irradiation by U.V. light on the structure of the epoxy-activated Sepharose 6B. Therefore, an identical photolysis reaction was performed on this underivatised resin and the result was assessed by U.V. spectrophotometry. Photolysis of the affinity matrix caused a shift in the $\lambda_{max.}$ value from 270 nm to 230 nm as shown in Figure 2.4.10. The absorbance baseline level of the matrix prior to photolysis was higher than that of the matrix following photolysis, however, this may simply reflect slight differences in concentration of the resins caused by packing into cuvettes. The photolytic degradation of the matrix may be due to the raised temperature which occurs when the reaction vessel is irradiated for prolonged periods of time rather than the direct action of U.V. light. It is also not known whether photolysis causes chemical changes in the spacer arm or in

the Sepharose backbone itself, however, degradation did occur and, therefore, was likely to have a detrimental effect on the potential use of this resin in affinity chromatography. This method, therefore, was not used in subsequent experiments involving the synthesis of the RU5135 affinity chromatography matrices.

The cleavage of the fmoc group from RU5135 was achieved by performing the method of Carpino and Han (1972) which involves the use of ethanolamine. The deprotected RU5135 was isolated in high yield (95%) and the product was isolated on the basis of its decreased solubility with respect to its fmoc derivative. This was a particularly useful method due to the fact that inactivation of the excess reactive groups in epoxy-activated Sepharose 6B and CNBr-activated Sepharose 4B both require treatment with ethanolamine. Therefore, despite the low yield of the reaction of RU5135 with fmoc chloride, the fmoc group was deemed to be the protection method of choice due to the mild reaction conditions involved in its removal from the amidine group of RU5135. Additionally, the presence of the fmoc moiety increased the solubility of RU5135 dramatically. This had the advantage that solvents of low boiling points could be used in subsequent reactions which could be dispelled easily at low temperatures or under vacuum.

BDDE is used to couple to Sepharose 6B to produce epoxy-activated Sepharose 6B by standard methods described by (Sundberg and Porath, 1974). Identical methods were utilised in this study to couple fmoc-RU5135 to the free spacer arm. This reagent has a free epoxide group at each end of its chain

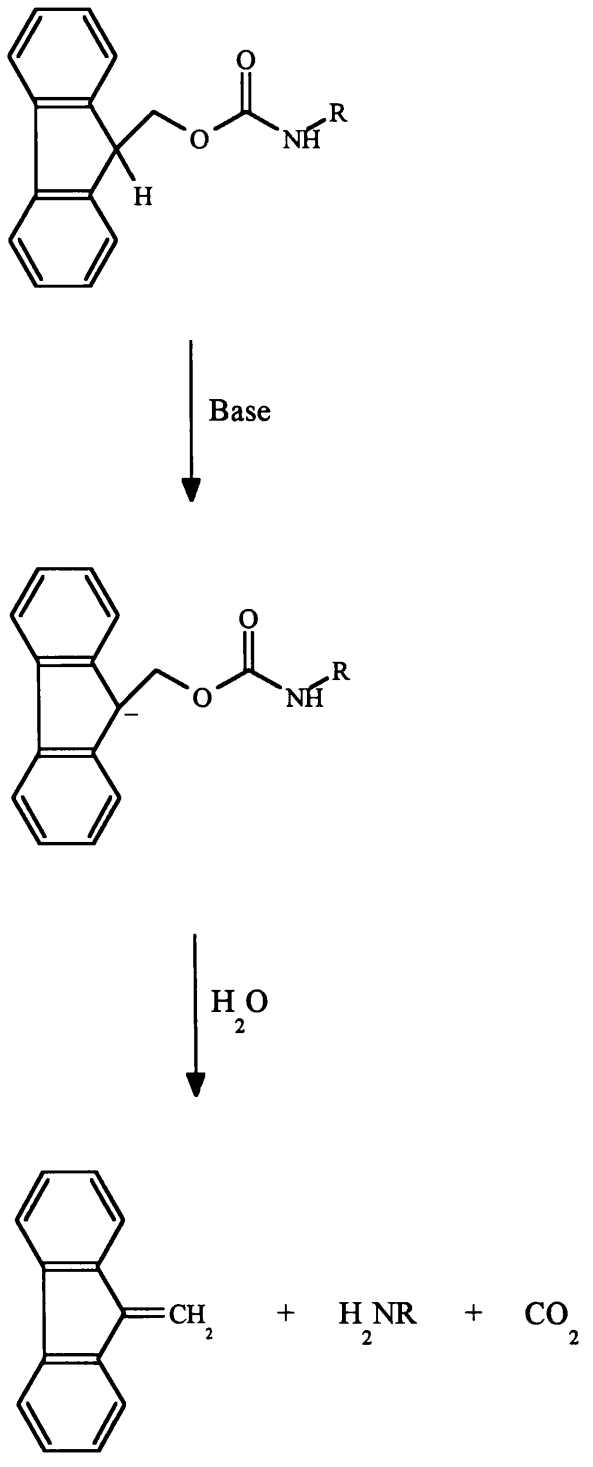
structure which can react with the 3-hydroxyl function of fmoc-RU5135. In order to overcome the possibility that one molecule of BDDE could react with two molecules of RU5135, an excess of the former compound was present in the reaction mixture. In this way, it was ensured that only the desired compound was synthesised.

An alternative method of synthesis of the affinity column was developed in order to assess the effect of the length of the spacer arm on the ability of the resin to function as an affinity column. This method has the advantage that the problematic amidine protection step is avoided. The synthesis was achieved by utilising the acid chloride derivative of succinic acid. Succinyl chloride is a diacyl chloride and was reacted with the C3-hydroxyl group of RU5135 giving rise to the RU5135-succinyl chloride derivative (Figure 2.4.11). The acid chloride was reacted with a primary amine of either 1,6-diaminohexane or 1,12-diaminododecane with the diamine present in an excess thus leaving a free primary amine which could be coupled to CNBr-activated Sepharose 4B (Cuatrecasas, 1970). This reaction was achieved in relatively high yield. However, difficulties arose in following the reaction due to the lack of an UV absorbing group in either the starting material or the product. After investigation, it was found that the reaction could be followed by spraying the tlc plate with 10% H_2SO_4 and charring on a hotplate. The presence of an alkyl moiety on the 3-hydroxyl group of RU5135 improved the solubility in organic solvents and thus, the separation of the product was relatively simple by flash chromatography using ethyl acetate as the solvent.

In summary, several derivatives of RU5135 have been synthesised, purified and analysed. Some of these compounds were potentially useful for employment as immobilised ligands in RU5135 affinity chromatography matrices for the isolation of the $GABA_A$ receptor. Studies on their structure-activity relationships may also provide an insight into the characteristics of the interaction of the antagonist with its specific binding site in the $GABA_A$ receptor complex.

Figure 2.5.1

Base-Catalysed Elimination of the Fmoc Group
Reproduced from Carpino and Han (1972).



Chapter 3

Biochemical Studies on the Derivatives of RU5135

3.1 INTRODUCTION

This chapter will describe the biochemical analyses of the derivatives of RU5135 whose chemical syntheses were reported in Chapter 2. The characteristics of the binding of RU5135 to the membrane-bound $GABA_A$ receptor will be discussed in this Introduction.

The pharmacological actions of RU5135 include sedation and somnolence at low doses with convulsions at sublethal dosage (Hunt and Clements-Jewry, 1981). These observations may be related to the finding that RU5135 is a powerful $GABA_A$ receptor antagonist (Hunt and Clements-Jewry, 1981; Olsen, 1984; Simmonds and Turner, 1985). Radioligand binding studies have shown that RU5135 inhibits [^{3}H]muscimol and [^{3}H]GABA specific binding activity with high affinity (Olsen, 1984; Hunt and Clements-Jewry, 1981). Equilibrium analysis of this inhibition indicated that RU5135 was acting at the same site as [^{3}H]muscimol and [^{3}H]GABA since there was no evidence for cooperativity between the antagonist and agonist binding sites (Hunt and Clements-Jewry, 1981). Further evidence is provided by recent research which was performed to investigate the interactions of RU5135 and the TBPS binding site at the $GABA_A$ receptor. These studies provided further evidence that RU5135 is a competitive antagonist by examining the ability of RU5135 to antagonise GABA modulation of TBPS binding in rat brain (Cadoni and Gee, 1992). In the same studies, RU5135 was found to have no significant action at the putative neurosteroid binding site of the $GABA_A$ receptor.

It has been shown that RU5135 also binds reversibly and with high

affinity to the strychnine-sensitive glycine receptor (Hunt and Clements-Jewry, 1981; Simmonds and Turner, 1985). RU5135, therefore, apparently fails to distinguish between the two inhibitory receptors and this might infer that the antagonist binding sites of the $GABA_A$ and the strychnine-sensitive glycine receptor share some structural homology (Olsen, 1984).

It has been shown that the affinity of antagonists at the $GABA_A$ receptor can be altered by performing the radioligand binding assay in the presence of various anions. Enna and Snyder (1977) found that the potency of (+)-bicuculline was increased in the presence of thiocyanate, iodide and nitrate by approximately 10-fold. These anions, however, did not alter the potencies of agonists. It had been suggested that the receptor may exist in interconvertible conformational states with selective high affinity for agonists and antagonists respectively and that these anions exert their influence on the antagonist state of the receptor. Olsen (1984) has shown that the affinity of RU5135 for membrane-bound $GABA_A$ receptors is enhanced when assayed in the presence of thiocyanate ions. This is potentially a useful phenomenon in affinity chromatography where the affinity of the ligand for the $GABA_A$ receptor may be increased by the composition of the environment.

The chemical structure of RU5135 and its relationship to pharmacological activity was discussed in detail in Section 2.1. In this chapter, the effects of the derivatisation of RU5135 on the affinity of the compound for the $GABA_A$ and strychnine-sensitive glycine receptor and their use as immobilised ligands in the affinity chromatographic isolation of the $GABA_A$ receptor will be discussed.

3.2 MATERIALS

[Methylene-^{3}H]muscimol (7-20 Ci/mmol) was purchased from Amersham International (UK). [Methyl-^{3}H]flunitrazepam (80-85 Ci/mmol) and [Benzene ring-^{3}H]strychnine (23 Ci/mmol) were from New England Nuclear (Dupont). Flunitrazepam was purchased from Sigma (UK). Sodium deoxycholate and Triton X-100 were from Koch-Light Laboratories (UK). GF/B glass fibre filters were from Whatman. Fmoc-chloride, 1,4-butanediol diglycidyl ether, 1,12-diaminododecane, 1,6-diaminohexane, 1,4-dioxan and ethanolamine were purchased from Aldrich (UK). RU5135 was a gift from Roussel Laboratories Ltd. Sephadex G-25, epoxy-activated Sepharose 6B, Sepharose 6B, cyanogen bromide-activated Sepharose 4B and pregnanolone were from Sigma (UK). Optiphase "Hi Safe" scintillation fluid was from LKB. Fmoc-RU5135, fmoc-RU5135-BDDE, RU5135-BDDE, RU5135-succinyl chloride, RU5135-succinyl-6-aminohexane and RU5135-succinyl-12-aminododecane were synthesised as described in Chapter 2 and were purified to analytical grade. All other chemicals were of analytical grade and were from standard commercial sources.

3.3 METHODS

3.3.1 Preparation of Membranes from Bovine Cerebral Cortex and Spinal Cord

Bovine brains and spinal cord were collected from the slaughter house and transported to the laboratory on ice (less than 60 min). Cerebral cortex was dissected out and stripped of the meninges before being frozen rapidly in liquid nitrogen. Both tissues were stored at −80°C until use.

Membranes were prepared by the method of Sigel *et al.* (1983). Cerebral cortex or spinal cord was thawed, chopped and homogenised in a Waring blender at 4°C in 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), pH 7.4, 0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.02% (w/v) NaN_3, 1 mM benzamidine, 10 mg/l soybean trypsin inhibitor, 0.1 mM phenylmethylsulphonylfluoride (PMSF) (Buffer A). The homogenate was centrifuged at 1 000 x g for 15 min at 4°C. The supernatant was collected and centrifuged at 27 000 x g for 45 min at 4°C. The pellet (P1) was resuspended using a glass-Teflon homogeniser in the same buffer as above except that sucrose and PMSF were omitted, (Buffer B). The homogenate was centrifuged at 27 000 x g for 45 min at 4°C and the pellet (P2) was resuspended in buffer B. The suspension was used either directly in membrane radioligand binding assays (Section 3.3.5.1) or for membrane protein solubilisation (Section 3.3.2).

3.3.2 Solubilisation of the $GABA_A$ Receptor

The method used was that of Sigel *et al.* (1983). The P2 membrane pellet from bovine cortex was resuspended in buffer B (Section 3.3.1) at 12-16 mg protein/ml to which 3.5 M KCl and 20% (w/v) NaDOC were added to final concentrations of 150 mM and 0.5% (w/v) respectively. Solubilisation was continued, with stirring, for 10 min at 4°C and the extract was centrifuged for 75 min at 100 000 x g at 4°C. The supernatant was the source of crude soluble protein in all subsequent experiments.

3.3.3 Gel Filtration of the Soluble $GABA_A$ Receptor

Sephadex G-25 (30 g) was swollen in 10 vol. distilled H_2O at 100°C for 45 min. The gel was cooled to 4°C and poured into a perspex column (3.0 cm x 53.0 cm) and equilibrated with either 50 mM Tris/HCl or 10 mM K-phosphate, pH 7.4, containing 1 mM EDTA, 0.02% (w/v) NaN_3, 0.5% (v/v) Triton X-100 and either 150 mM KCl or 500 mM KCl or 150 mM KSCN (Buffer C) which had been degassed.

Calibration of the column was carried out by dissolving Blue Dextran (10 mg) and [^{3}H]glycine (10 µl) in buffer C (10 ml). This volume was equivalent to that volume of crude soluble protein to be desalted. This was applied to the column at a rate of 2 ml/min and fractions of 4 ml were collected. These fractions were assayed by ultra-violet spectrophotometry at λ = 540 nm. Additionally, aliquots (10 µl) of each fraction were counted for radioactivity.

The degassed crude soluble protein (10 ml) was applied to the Sephadex G-25 column under gravity at a flow-rate of approximately 2 ml/min. The column was eluted with buffer C at a rate of 2 ml/min and fractions of 4 ml were collected. Protein-containing fractions were pooled and these were used as the source of soluble protein in all subsequent experiments.

3.3.4 Determination of Protein Concentration

The protein concentration of membrane-bound and soluble samples was performed using the method of Lowry *et al.* (1951). The assay reagent was prepared by mixing 1% (w/v) $CuSO_4$ (1 ml) with 2% (w/v) Na-tartrate (1 ml) and 2% (w/v) Na_2CO_3 in 0.1 M NaOH, 0.5% (w/v) SDS (98 ml). A known volume of protein sample was diluted to a final volume of 200 µl and assay reagent (1 ml) was added. After incubation for 10 min at room temperature, Folin Ciocalteu reagent (100 µl, diluted 1:1 with H_2O) was added and each tube vortexed immediately. The reaction was allowed to continue for 30 min and the absorbance was read at λ = 750 nm. The protein content of each sample was calculated with reference to a standard curve determined with bovine serum albumin (BSA).

3.3.5 Radioligand Binding Assays

3.3.5.1 Radioligand Binding to Membrane-Bound Receptors

Bovine cerebral cortex or spinal cord membranes were prepared as described in Section 3.3.1. Endogenous GABA or glycine was removed by subjecting the membranes to freeze-thaw cycles (Olsen *et al.*, 1981). The membranes were frozen for 12 h at –20°C, thawed, homogenised using a glass-Teflon homogeniser and centrifuged at 27 000 x g for 45 min at 4°C. They were then resuspended in buffer B (Section 3.3.1). The cycle was repeated twice and the membranes were suspended to 1 mg protein/ml in 20 mM K-phosphate, pH 7.4, 1 mM EDTA, 0.02% (w/v) NaN_3 for use in the radioligand binding assays and protein concentration determination. Membranes were incubated (160 µl) in triplicate with radioactive ligand (20 µl) whose final concentrations were 10 nM, 1 nM and 1 nM for [^{3}H]muscimol, [^{3}H]flunitrazepam and [^{3}H]strychnine respectively. To parallel sets of samples, either buffer (20 µl) to determine total binding or the unlabelled ligand (10^{-3}M GABA, 10^{-4}M flunitrazepam or 10^{-4}M strychnine) (20 µl) was added to determine non-specific binding. Incubations were carried out for 30 min for [^{3}H]muscimol or 45 min for [^{3}H]flunitrazepam and [^{3}H]strychnine all at 4°C. Bound and free ligands were separated by filtration under vacuum onto Whatman GF/B filters. Filters were washed with 20 mM K-phosphate, pH 7.4 (3 x 4 ml), dried and placed into scintillation vials. Optiphase "Hi Safe" scintillant (4 ml) was added to each vial and the samples were counted in an LKB liquid scintillation counter.

3.3.5.2 Radioligand Binding to Soluble Receptors

Crude soluble protein was desalted into Buffer C according to the procedure described in Section 3.3.3. The concentration of protein after gel filtration was 2.0-2.5 mg protein/ml. This was diluted to 1 mg protein/ml and incubated (160 µl) with radioactive ligand (20 µl) at final concentrations of 10 nM and 4 nM for [^{3}H]muscimol and [^{3}H]flunitrazepam respectively. To parallel sets of samples, either buffer (20 µl) to determine total binding or unlabelled drug (10^{-3}M GABA or 10^{-4}M flunitrazepam) (20 µl) were added to determine non-specific binding. Incubations were carried out for 45 min at 4°C. Bound and free ligands were separated by filtration under vacuum onto Whatman GF/B filters which had been pre-soaked for at least 1 h in 0.3% (v/v) polyethylene imine (PEI). These filters were washed with 20 mM K-phosphate, pH 7.4, (3 x 4 ml), dried and placed into scintillation vials. Optiphase "Hi Safe" scintillant (4 ml) was added to each vial and counted in an LKB liquid scintillation counter.

3.3.5.3 Determination of the IC_{50} Values for RU5135 and its Derivatives on [^{3}H]Muscimol Specific Binding Activity to Membrane-Bound and Soluble Receptors

Radioligand binding assays were performed according to the methods described above. Membrane-bound or soluble protein was incubated in the presence of [^{3}H]muscimol and the competing ligand in the concentration range

10^{-4} M to 10^{-10} M at 0.5 $\log_{10}$ unit intervals. Non-specific binding was measured in the presence of 10^{-4} M GABA. Bound and free ligand was separated by the method described in Sections 3.3.5.1 or 3.3.5.2.

In some experiments, the aim was to determine the IC_{50} value for the competing ligand while it was covalently attached to the resin. In these cases, the derivatised and control resin were prepared in the immobilised ligand concentration range 0.2 mM to 0.2 μM at 0.5 $\log_{10}$ unit intervals as measured by the method described in Section 3.3.6.4. These were employed, in the radioligand binding assay on soluble protein, in the same way as the competing ligands described above. During the incubation period of 1 h, the samples were shaken constantly until the moment of filtering. Filters were subsequently treated in an identical manner to that which is described in Section 3.3.5.2.

3.3.5.4 Enhancement of Benzodiazepine binding by GABA and Neuroactive Steroids

Radioligand binding assays were performed according to the methods described in 3.3.5.1. Bovine cerebral cortex membranes were incubated with 1 nM [^{3}H]flunitrazepam in the presence of GABA, pregnanolone or RU5135 at concentrations in the range 10^{-4} M to 10^{-10} M at 0.5 $\log_{10}$ unit intervals. Non-specific binding was measured in the presence of 10^{-4} M flunitrazepam. Bound and free ligands were separated by the method described in Section 3.3.5.1.

All IC_{50} and EC_{50} values quoted are expressed as the mean ± S.D. of n determinations.

3.3.6 Preparation of RU5135 Affinity Chromatography Matrices

3.3.6.1 Synthesis of the RU5135-epoxy-activated Sepharose 6B Affinity Matrix

The synthesis of the RU5135 epoxy-activated Sepharose 6B affinity matrix was performed according to the method of Vretblad (1976). Epoxy-activated Sepharose 6B (1 g, 3 ml) was swollen in 50 ml of distilled H_2O at room temperature for 15 min. It was washed on a sintered glass funnel with distilled H_2O (3 x 10ml) and transferred to 0.1 M Na_2CO_3, pH 10, 50% (v/v) dioxan (10 ml) which contained 5.3 mg (6.0 μmol, final concentration 2 μmol/ml resin) fmoc-RU5135 (Section 2.4.2.3). The gel was rotated end-over-end at room temperature for 16-24 h. The resin was washed on a sintered glass funnel with 50% (v/v) dioxan (3 x 10 ml) and distilled H_2O (3 x 10 ml). Excess epoxide groups were inactivated and the fmoc group removed by reaction with 1 M ethanolamine (10 ml) for 4 h at room temperature. The gel was washed on a sintered glass funnel with 1 M ethanolamine (3 x 10 ml) and distilled H_2O (3 x 10 ml). The resin (1 ml) was poured into a Biorad Poly Prep^R column (0.8 cm x 4.0 cm) and equilibrated with buffer C (Section 3.3.3) before use in affinity chromatography (Section 3.3.7). The concentration of ligand in the resin was determined by ultra-violet spectrophotometry as described in Section 3.3.6.4.

A control resin was prepared in an identical manner except that fmoc-RU5135 was omitted from the coupling reaction.

3.3.6.2 Synthesis of the RU5135-BDDE Sepharose 6B Affinity Matrix

The synthesis of the RU5135-BDDE Sepharose 6B affinity matrix was performed according to the method of Sundberg and Porath (1974). Sepharose 6B (1 g, 3 ml) was swollen in distilled H_2O (50 ml) at room temperature for 15 min. It was washed on a sintered glass funnel with distilled water (3 x 10 ml) and transferred to 0.1 M NaOH, pH 11, containing 2 mg/ml $NaBH_4$, 50% (v/v) dioxan (10 ml) and 6.8 mg (6.0 µmol, final concentration 2 µmol/ml resin) fmoc-RU5135-BDDE (Section 2.4.4). The resin was rotated end-over-end at room temperature for 16-24 h. The gel was washed on a sintered glass funnel with 50% (v/v) dioxan (3 x 10 ml) and distilled H_2O (3 x 10 ml) and transferred to 1 M ethanolamine for 4 h at room temperature. It was washed on a sintered glass funnel with 1 M ethanolamine (3 x 10 ml) and distilled H_2O (3 x 10 ml). The affinity resin (1 ml) was poured into a Biorad Poly Prep^R column (0.8 cm x 4.0 cm) and equilibrated with Buffer C (Section 3.3.3) before use in affinity chromatography (Section 3.3.7). The concentration of ligand on the resin was determined by ultra-violet spectrophotometry as described in Section 3.3.6.4.

A control resin was prepared in an identical manner except that fmoc-RU5135-BDDE was omitted from the coupling reaction.

3.3.6.3 Synthesis of the RU5135-CNBr-Activated Sepharose 4B Affinity Matrix

The synthesis of the RU5135-CNBr-activated Sepharose 4B matrix was performed according to the method of Cuatrecasas (1970). CNBr-activated Sepharose 4B (1 g, 3 ml) was swollen in 1 mM HCl (200 ml) for 15 min at room temperature and washed on a sintered glass funnel with 1 mM HCl (5 x 40 ml) and 0.1 M $NaHCO_3$, pH 8.3 (10 ml). The gel was transferred immediately to 0.1 M $NaHCO_3$, pH 8.3 (10 ml) containing either 7.5 mg (6.0 μmol, final concentration 2 μmol/ml resin) RU5135-succinyl-12-aminododecane (Section 2.4.6.3) or 2.5 mg (6.0 μmol, final concentration 2 μmol/ml resin) RU5135-succinyl-6-aminohexane (Section 2.4.6.2). The gels were rotated end-over-end at room temperature for 2 h. The resins were washed on a sintered glass funnel with distilled H_2O (3 x 10 ml) and transferred to 1 M ethanolamine (10 ml) for 2 h at room temperature. They were washed on a sintered glass funnel with distilled H_2O (3 x 10 ml). The resins (1 ml) were poured into Biorad Poly Prep^R columns (0.8 cm x 4.0 cm) and equilibrated with buffer C (Section 3.3.3) before use in affinity chromatography (Section 3.3.7). The concentration of ligand on the resin was determined by ultra-violet spectrophotometry as described in Section 3.3.6.4.

A control resin was prepared in an identical manner except that RU5135-succinyl-12-aminododecane and RU5135-succinyl-6-aminohexane were omitted from the coupling reaction.

3.3.6.4 Determination of the Concentration of Immobilised Ligand in the Affinity Matrices by Ultra-violet Spectrophotometry

In order to determine the extinction coefficient for the ligands to be coupled to the affinity resins, they were each dissolved in absolute ethanol at 10 mM concentrations. These were used as the stock solutions of each ligand. The spectra of these solutions were scanned from λ = 900 nm to λ = 190 nm to determine the $\lambda_{max.}$ of each ligand. A range of concentrations from each of these stock solutions was prepared (10 mM to 0.1 μM at 1 $\log_{10}$ unit intervals) and the absorbance of each of these was measured at the determined $\lambda_{max.}$. The extinction coefficient for each ligand was determined using the Beer-Lambert Law:

$$A = \varepsilon cl$$

A = absorbance,

ε = extinction coefficient for the particular compound,

c = concentration,

l = path length.

The concentration of immobilised ligand was determined as follows. The gels were diluted 1:1 in distilled H_2O and packed into quartz cuvettes (1 mm path length). The absorbance of each (at $\lambda_{max.}$) was measured relative to the respective control resin in a Perkin-Elmer Lambda 15 dual-beam spectrophotometer. The difference in absorbance between the derivatised and control resin was used to calculate the concentration of ligand present on the affinity matrix.

3.3.7 Affinity Chromatography of the $GABA_A$ Receptor

Affinity columns were prepared and equilibrated by the methods described in Section 3.3.6. Soluble, desalted protein (Section 3.3.3) was applied to both the derivatised and the control affinity matrices (Section 3.3.6) at a rate of 3 ml/h and recirculated overnight at 4°C. The flow-through was collected and assayed for [^{3}H]muscimol and [^{3}H]flunitrazepam specific binding activity. The column was washed at a rate of 3 ml/h with a known volume of Buffer C (Section 3.3.3). This was also assayed for [^{3}H]muscimol and [^{3}H]flunitrazepam specific binding activity. Elution of the columns was performed using buffer C (Section 3.3.3) containing 5 mM GABA applied at a rate of 3 ml/h. Fractions (1 ml) were collected and each fraction was assayed for specific [^{3}H]flunitrazepam binding activity (Section 3.3.5.2).

In some experiments, purification was attempted by a batch method. This involved placing derivatised resin (100 µl) in an eppendorf tube and adding soluble, desalted protein (1 ml). The sample was rotated end-over-end at 4°C for 1 h. After the incubation period, the resin was allowed to settle and the supernatant was removed and assayed for the presence of [^{3}H]muscimol and [^{3}H]flunitrazepam specific binding activity as described in Section 3.3.5.2. A control resin was treated in the same way and a third eppendorf was incubated which contained soluble, desalted protein (1 ml) and Buffer C (100 µl) alone.

3.4 RESULTS

3.4.1 Characterisation of RU5135 Binding to $GABA_A$ Receptors

In order to validate the potential use of RU5135 as an immobilised ligand in affinity chromatography, the action of RU5135 on the radioligand binding properties of the $GABA_A$ receptor in the membrane-bound and soluble state was characterised. Thus, the affinity of RU5135 for the $GABA_A$ receptor was defined under the experimental conditions of RU5135 affinity chromatography of the $GABA_A$ receptor. The binding properties of RU5135 at the $GABA_A$ receptor were defined by performing a number of competitive inhibition and enhancement studies on the membrane-bound and the NaDOC-solubilised receptor.

3.4.1.1 Characterisation of Membrane-Bound $GABA_A$ Receptors

Radioligand binding assays were performed according to the method described in Section 3.3.5.1. It was found that RU5135 inhibited specific [^{3}H]muscimol binding to $GABA_A$ receptors in bovine cerebral cortex membranes in a dose-dependent manner with high affinity (IC_{50} = 15 ± 2 nM, Hill coefficient (h) = 1.03, n = 4) in the presence of chloride ions at a concentration of 150 mM. The potency of RU5135 was enhanced when thiocyanate ions at the same concentration were present in the assay system (IC_{50} = 5 ± 3 nM, h = 0.90, n = 4) (Figure 3.4.1). The affinity of RU5135 was higher than that of GABA which displaced [^{3}H]muscimol specific binding activity with IC_{50} = 44

Calculation of K_i of RU5135 inhibition of GABA enhancement of [^{3}H]flunitrazepam binding:

Read directly from the graph and assume that 50% of the maximal stimulation by GABA causes 130% enhancement,

then IC_{50} = antilog −6.58
= 2.6×10^{-7} M
= 260 nM

Therefore, using Cheng-Prusoff equation, K_i = 130 nM

± 7 nM (n = 3). GABA enhanced [^{3}H]flunitrazepam specific binding activity in a dose-dependent manner (EC_{50} = 0.10 ± 0.04 μM, n = 3) with a maximum enhancement of approximately 160% and this enhancement was inhibited by RU5135 (IC_{50} = 5.6 ± 2.3 μM, n = 3)*(Figure 3.4.2). This effect of RU5135 was found to be in direct contrast to that of 5α-pregnane-3α-ol-20-one (pregnanolone, a 3α-hydroxy steroid). This drug has been shown to potentiate the muscimol enhancement of [^{3}H]flunitrazepam binding in a dose-dependent manner (Harrison *et al.*, 1987). In this study, RU5135 alone inhibited benzodiazepine agonist binding at relatively high concentrations (IC_{50} = 80 ± 11 μM, n = 3) whereas pregnanolone was able to potentiate [^{3}H]flunitrazepam specific binding activity (EC_{50} = 0.7 ± 0.1 μM, n = 3) with a maximal stimulation of approximately 150%.

3.4.1.2 Characterisation of Soluble $GABA_A$ Receptors

Radioligand binding assays were performed according to the methods described in Section 3.3.5.2. It was found that RU5135 inhibited specific [^{3}H]muscimol binding to NaDOC-solubilised $GABA_A$ receptors extracted from bovine cerebral cortex membranes in a dose-dependent manner and with high affinity (IC_{50} = 4 ± 2 nM, n = 15) in the presence of 150 mM chloride ions. The Hill coefficient (1.03) was not significantly different from unity ($P > 0.05$, Student's t-test). The affinity of RU5135 in solubilised $GABA_A$ receptors was significantly higher than that determined for the membrane-bound receptors (IC_{50} = 15 ± 2 nM, n = 3). Increasing the concentration of KCl from 150 mM to 500

mM did not have a significant effect on the IC_{50} or *h* of RU5135 for the $GABA_A$ receptor.

Similar experiments were performed to measure the affinity of RU5135 for the $GABA_A$ receptor in the presence of 150 mM KSCN. NaDOC solubilised protein was desalted into buffer C (Section 3.3.3) containing 150 mM KSCN and radioligand binding studies were performed as described in Section 3.3.5.2. Although the results of these studies always showed a shift of the dose-response curve to the left of that obtained for a similar experiment performed in parallel in the presence of 150 mM KCl (IC_{50} = 2 ± 1 nM, n = 5), these shifts in affinity were found to be insignificant when analysed statistically ($P > 0.05$, Student's t-test).

GABA also inhibited specific [^{3}H]muscimol binding in a dose-dependent fashion with high affinity (IC_{50} = 22 ± 13 nM, n = 3) but this value was not significantly different from that observed in membranes (IC_{50} = 44 ± 7 nM, n = 3) ($P > 0.05$, Student's t-test). GABA enhanced specific [^{3}H]flunitrazepam binding in a dose-dependent manner (EC_{50} = 0.50 ± 0.01 μM, n = 3) with a maximum enhancement of approximately 150% and this enhancement was completely inhibited by RU5135 (IC_{50} = 75 ± 12 μM, n = 3). No enhancement of either [^{3}H]muscimol or [^{3}H]flunitrazepam specific binding activities by pregnanolone in the soluble extracts could be detected.

3.4.1.3 Characterisation of Membrane-Bound, Strychnine-Sensitive Glycine Receptors

Radioligand binding assays were performed according to the method described in Section 3.3.5.1. It was found that RU5135 inhibited [^{3}H]strychnine specific binding to bovine spinal cord membranes in a dose-dependent manner with high affinity (IC_{50} = 9 ± 8 nM, h = 0.9, n = 6) in the presence of chloride ions at a concentration of 150 mM. The apparent affinity of RU5135 for the strychnine-sensitive glycine receptor decreased upon storage of the spinal cord membrane preparation on ice over a few hours (2-6 h) and, therefore, in subsequent experiments, following five freeze-thaw cycles, the membrane preparation was resuspended in buffer B (Section 3.3.1) and centrifuged twice and used immediately in radioligand binding assays.

3.4.2 Characterisation of the Binding of the Synthetic Derivatives of RU5135

The chemical structure of RU5135 and its relationship to the pharmacological activity of the compound was discussed in detail in Section 2.1. and it was suggested that the region of the molecule which is responsible for binding to the GABA recognition site of the $GABA_A$ receptor was the C11-C17 region (Figure 2.1.1). However, for the purposes of this study, it was necessary to investigate the effects of the derivatisation of the amidine and C3-hydroxyl groups of RU5135 on the affinity of the compound for the $GABA_A$ receptor and, therefore, validate the potential use of these derivatives as immobilised

ligands in affinity chromatography. The results of these studies are presented here.

It was found that derivatisation of the amidine group of RU5135 with fmoc produced a thousand-fold decrease in its affinity for the NaDOC-solubilised $GABA_A$ receptor (IC_{50} = 3 090 ± 555 nM, h = 1.05, n = 5) (Figure 3.4.3). The coupling of fmoc-RU5135 to BDDE did not have a significant effect on the low affinity of fmoc-RU5135 for the $GABA_A$ receptor (IC_{50} = 7 590 ± 1 200 nM, h = 0.95, n = 3). Following removal of the fmoc group by treatment with ethanolamine, the affinity of the *O*-derivatised RU5135 was increased (IC_{50} = 44 ± 26 nM, h = 1.1, n = 3). However, the affinity of RU5135-BDDE was significantly lower than that of RU5135 (Figure 3.4.4). These results are summarised in Table 3.4.1.

The rate of removal of the fmoc group from fmoc-RU5135 was investigated in order to define the optimum reaction time for the greatest efficiency of deprotection. This was achieved by performing studies on the competitive inhibition of [^{3}H]muscimol binding activity at various stages of the reaction, thereby determining the IC_{50} value of the reaction mixture at various times and, thus, monitoring the time-dependence of the deprotection reaction. The detailed methods are given in the legend to Figure 3.4.6.

The rate of removal of the fmoc protection moiety by ethanolamine from RU5135 was found to decrease with time. It was shown that the ability of the ligand to inhibit specific [^{3}H]muscimol binding activity increased with time reaching a plateau after 5 h. Even when the reaction was allowed to

continue for 24 h the affinity of the ligand did not significantly change from that found after 5 h. Therefore, in subsequent experiments involving the removal of fmoc from the amidine group of RU5135, the standard reaction time was taken as being 5 h. The IC_{50} values for each time point are summarised in Table 3.4.2 and the relationship between the IC_{50} value of the ligand and time is shown in Figure 3.4.6.

Derivatisation of RU5135 with succinyl chloride resulted in a compound with affinity that was lower than that of RU5135 (IC_{50} = 22 ± 6 nM, h = 0.99, n = 3) and further derivatisation with 1,6-diaminohexane and 1,12-diaminododecane resulted in compounds with lower affinities (IC_{50} = 48 ± 14 nM, h = 0.88, and 45 ± 16 nM, h = 1.00, n = 3, respectively) but these were not significantly different from that of RU5135-succinyl chloride or from RU5135-BDDE (Figures 3.4.7 and 3.4.8).

Similar effects were observed for the inhibition of [^{3}H]strychnine binding by the derivatives of RU5135. It was shown that the affinity of RU5135 was reduced by the presence of the fmoc moiety (IC_{50} = 39 ± 15 μM, h = 0.97, n = 4). Following the reaction of fmoc-RU5135 to BDDE and the subsequent removal of the fmoc group with ethanolamine, it was shown that the affinity of the compound was increased (IC_{50} = 55 ± 18 nM, h = 0.9, n = 3). However, the affinity of RU5135-BDDE was significantly lower than RU5135 for the glycine receptor (Figure 3.4.5). *O*-derivatisation of RU5135 with succinyl chloride significantly reduced the affinity of the ligand for the inhibitory glycine receptor (IC_{50} = 38 ± 12 nM, h = 1.09, n = 3) and further derivatisation with 1,12-

diaminododecane reduced it further (IC_{50} = 68 ± 22 nM, h = 0.89, n = 3) (Figure 3.4.9). The affinity of the diamine derivative was significantly lower than that of the succinyl chloride derivative ($P \leq 0.01$, Student's t-test).

Control experiments were carried out using BDDE, diamines, fmoc chloride, succinyl chloride, ethanol and ethanolamine to ensure that the affinities of the RU5135 derivatives observed were not due to the protection moiety, the spacer arm or contaminants from the reactions. In all of these cases, the IC_{50} values were found to be greater than 1 mM.

A summary of the affinities of all the synthetic derivatives of RU5135 for the NaDOC-solubilised $GABA_A$ receptor is shown in Table 3.4.1.

3.4.3 Affinity Chromatography of the $GABA_A$ Receptor

3.4.3.1 Optimisation of Experimental Conditions

Initial investigations into the isolation of the $GABA_A$ receptor by RU5135 affinity chromatography indicated that there was an appreciable amount of non-specific binding to the support matrix. This was shown by running derivatised and control columns in parallel (Section 3.3.7). The large amount of non-specific binding present was obscuring the detection of any specific binding to the derivatised column, therefore, it was essential to overcome the problem.

In order to minimise the amount of non-specific binding to the affinity columns, a number of experiments were performed on control resins. These were resins that had been prepared by the method described in Section 3.3.6, in the absence of ligand, except that the excess epoxide groups were inactivated using either 1 M ethanolamine, 1 M glycine or 1 M Tris HCl. These gels were then used in affinity chromatography according to the methods described in Section 3.3.6. Different buffer systems and KCl concentrations were employed in order to diminish the amount of non-specific binding. The experimental conditions and the results from typical experiments are given in Tables 3.4.3 to 3.4.5.

From the results of the optimisation experiments it can be seen that, in the cases of the ethanolamine and Tris HCl treated resins, K-phosphate buffers had greater effect in reducing the non-specific binding to Sepharose than Tris HCl when affinity chromatography was performed in the presence of 150 mM

KCl. HEPES buffer systems containing 150 mM KCl did not reduce the amount of non-specific binding significantly compared to K-phosphate buffer. When the buffer system was Tris HCl, however, the results were inconsistent. This was also the case when the epoxide groups in the resin were blocked with glycine. Therefore, Tris-HCl buffers and glycine-treatment of the matrix were not used as standard conditions in subsequent experiments. It was found that the non-specific binding could be further reduced by increasing the salt concentration in the buffer to 500 mM (see Table 3.4.3. columns 5 and 6). Thus, in all subsequent experiments, the excess reactive groups in the affinity matrices were blocked by treatment with ethanolamine and the standard buffer employed for affinity chromatography was 10 mM K-phosphate, pH 7.4, containing 500 mM KCl, 1 mM EDTA, 0.02% NaN_3 and 0.5% (v/v) Triton X-100.

3.4.3.2 Determination of Immobilised Ligand Concentration in Resins

The concentration of ligand in the derivatised resins was determined by the method described in Section 3.3.6.4. The $\lambda_{max.}$ values determined for fmoc-RU5135 and fmoc-RU5135-BDDE were 301 nm and 295 nm respectively, and were in good agreement with values published by Milligen^R for peptide synthesis. The $\lambda_{max.}$ for RU5135-succinyl-6-aminohexane and RU5135-succinyl-12-aminododecane were 256nm and 243 nm respectively. These values enabled the calculation of the concentrations of the immobilised ligands by the method given in Section 3.3.6.4 and the results are summarised in Table 3.4.6.

The efficiency of the coupling reaction differed between the two types

of Sepharose used. The epoxy-activated Sepharose 6B and the Sepharose 6B gave an efficiency of coupling of 10% compared to the CNBr-activated Sepharose 4B which reacted with 100% of the ligand present in the coupling reaction. Since the coupling reaction for all resins was performed in the presence of 2 μmol ligand (Section 3.3.6), the concentration of immobilised ligand for the epoxy-activated Sepharose 6B and Sepharose 6B resins was 0.2 μmol/ml resin and 2.0 μmol/ml resin for the CNBr-activated Sepharose 4B. The efficiency of the coupling of fmoc-RU5135-BDDE to Sepharose 6B or fmoc-RU5135 to epoxy-activated Sepharose 6B was not increased by increasing the concentration of the ligand in the coupling solution, increasing the time of reaction or by increasing the temperature at which the reaction was performed. These results are summarised in Table 3.4.6.

3.4.3.3 Characterisation of the Affinities of the Derivatised Resins for the NaDOC-Solubilised $GABA_A$ Receptor

The binding affinities of the derivatised resins were determined by performing competitive inhibition studies using the derivatised resin as the competing ligand. The affinity of the derivatised resin could then be compared with that of the control resin and that of the supernatant from the resin suspension. This would give some indication as to whether the ligand was covalently attached to the matrix or held there by a hydrophobic interaction. It was found that Sepharose 6B derivatised with fmoc-RU5135-BDDE was able to inhibit specific [^{3}H]muscimol binding to the NaDOC-solubilised $GABA_A$

receptor in a dose-dependent manner with an apparent IC_{50} = 4 ± 0.5 μM (n = 4) relative to the underivatised resin (■; Fig. 3.4.10). This was in contrast to the supernatant of the resin suspension which did not inhibit [^{3}H]muscimol specific binding activity (□). Neither the resin nor the supernatant inhibited [^{3}H]flunitrazepam specific binding activity significantly.

The CNBr-activated Sepharose 4B resin, when derivatised to either RU5135-succinyl-6-aminohexane or RU5135-succinyl-12-aminododecane, appeared to inhibit specific [^{3}H]muscimol binding activity in a dose-dependent manner with relatively high affinity (IC_{50} = 300 ± 50 nM, n = 3) (Figure 3.4.11). However, the supernatants of these resins inhibited specific [^{3}H]muscimol binding activity with similar affinity suggesting that the ligands were not covalently bound to the matrix. Indeed, after several washing cycles, it was shown that the resin lost its apparent ability to bind the [^{3}H]muscimol binding site specifically and the amount of receptor bound approached non-specific levels indicating that the ligand which inhibits [^{3}H]muscimol specific binding activity was progressively removed from the matrix (Figure 3.4.11).

3.4.3.4 Determination of the Binding Capacity of the Derivatised Affinity Chromatography Matrices

These experiments were performed as described in section 3.3.7. Desalted soluble protein was loaded onto the columns at a rate of 3 ml/h and recirculated overnight. The affinity resins were washed and the filtrate and the washes were assayed for [^{3}H]muscimol and [^{3}H]flunitrazepam specific binding activity. This

enabled the assessment of the affinity resin to purify the $GABA_A$ receptor. In all experiments the derivatised resins were run in parallel with a control resin which had been prepared in an identical way but in the absence of RU5135 or its derivatives. Optimal conditions as determined in 3.4.3.1 were employed for all experiments which involved the use of the affinity resins.

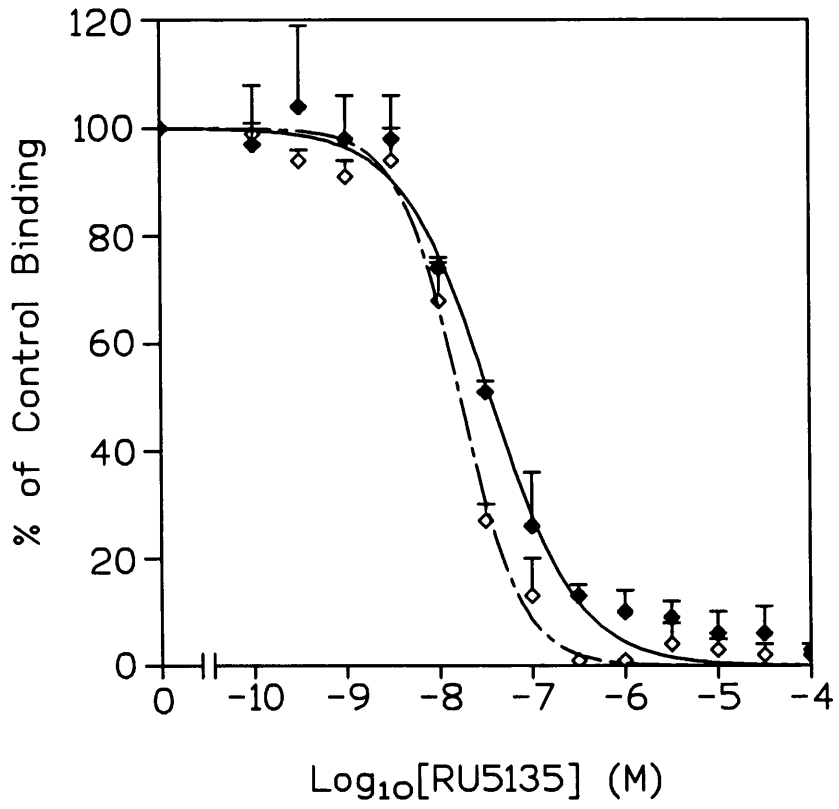
It was found that for the epoxy-activated Sepharose 6B and Sepharose 6B, the amount of receptor specifically bound after recirculation and washing was approximately 20% in both the derivatised and control resins. This value was not significantly altered by preparing the resins and loading the NaDOC extract within the same day. The reduction of the flow rate for the application of the NaDOC solubilised extract to the column also had no effect on the binding capacity of the derivatised resin. No significant difference between the numbers of [^{3}H]muscimol sites and [^{3}H]flunitrazepam binding sites which adhered to either derivatised or control columns could be found (Tables 3.4.7 and 3.4.8). Additionally, there was no significant elution of specific [^{3}H]flunitrazepam binding activity from the matrix.

In the case of the CNBr-activated Sepharose 4B columns, there was an apparent retention of [^{3}H]muscimol binding sites to the derivatised affinity resin compared to that of the control resin. This was in direct contrast to the results found for [^{3}H]flunitrazepam binding sites where only approximately 20% were bound. This was true for both the ligands which were immobilised to CNBr-activated Sepharose 4B (Tables 3.4.9 and 3.4.10). However, it was shown that desalting of the filtrate from these columns immediately after the affinity

chromatography step resulted in the reappearance of the [^{3}H]muscimol binding sites. The [^{3}H]muscimol specific binding activity recovered by desalting was typically 65-70%.

Figure 3.4.1

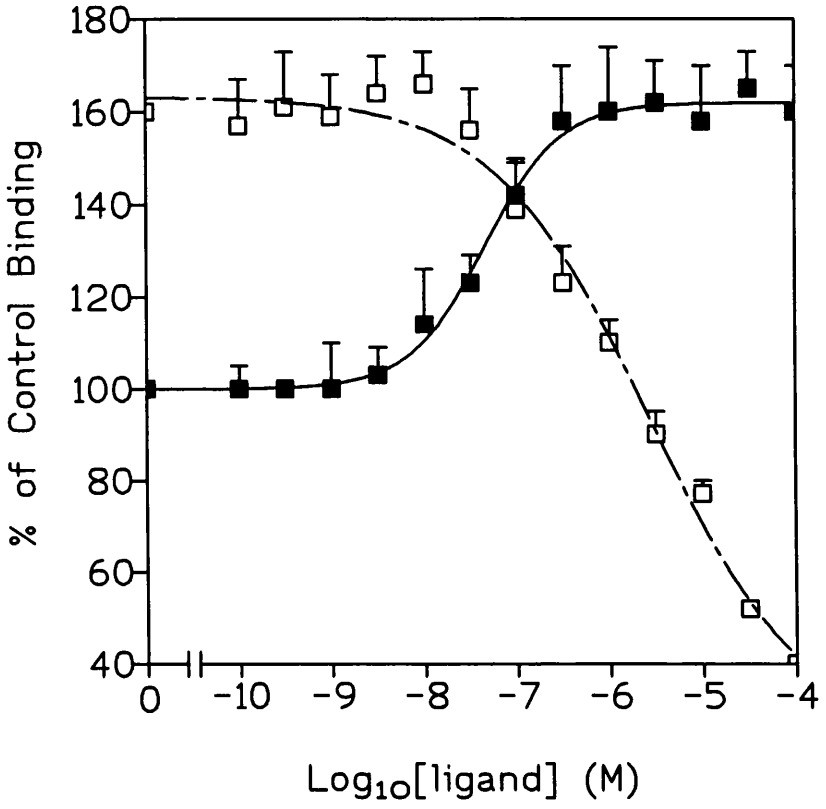
Effect of Thiocyanate and Chloride Ions on the Affinity of RU5135 for the Membrane-Bound $GABA_A$ Receptor



The competitive inhibition of [^{3}H]muscimol binding by RU5135 in membrane-bound $GABA_A$ receptors was measured in the presence of 150 mM KCl (♦) or 150 mM KSCN (◊). Non-specific binding was determined by an identical incubation in the presence of 10^{-4} M GABA. Total binding was defined as the radioactivity bound in the absence of RU5135. Control binding was, therefore, defined to be the difference between total and non-specific binding. All values are expressed as a percentage of control binding. The percentage control binding for each concentration of RU5135 was determined in triplicate and expressed as mean ± SEM. The graphs are representative of 4 independent experiments. The results were analysed statistically by an unpaired student's t-test in the Graphpad Instat® software package. $P \leq 0.05$. All competitive inhibition experiments performed in this study were analysed in a similar manner.

Figure 3.4.2

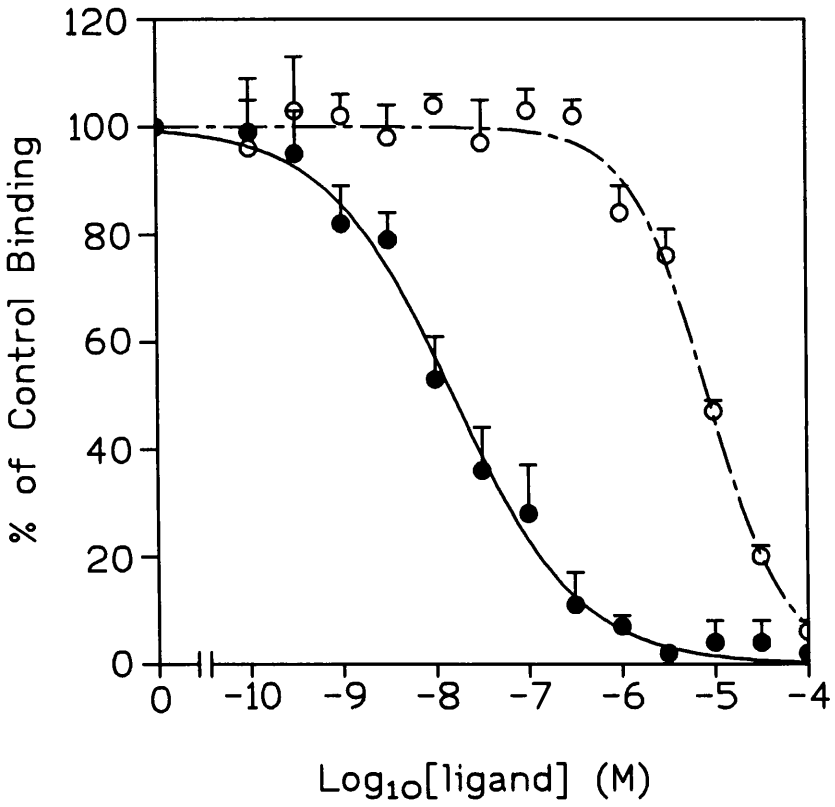
Effect of RU5135 on the Enhancement of [^{3}H]Flunitrazepam Specific Binding Activity by GABA in Bovine Cerebral Cortex Membranes



The enhancement of [^{3}H]flunitrazepam specific binding activity by GABA (■) was performed according to the method described in Section 3.3.5.4. The concentration of GABA at which maximal enhancement occurred was 10^{-6} M and, thus, the effect of RU5135 on the enhancement of [^{3}H]flunitrazepam specific binding activity by 10^{-6} M GABA was measured (□). The percentage control binding for each concentration of ligand was determined in triplicate and the points are expressed as the mean ± SEM as described in the legend to Figure 3.4.1.

Figure 3.4.3

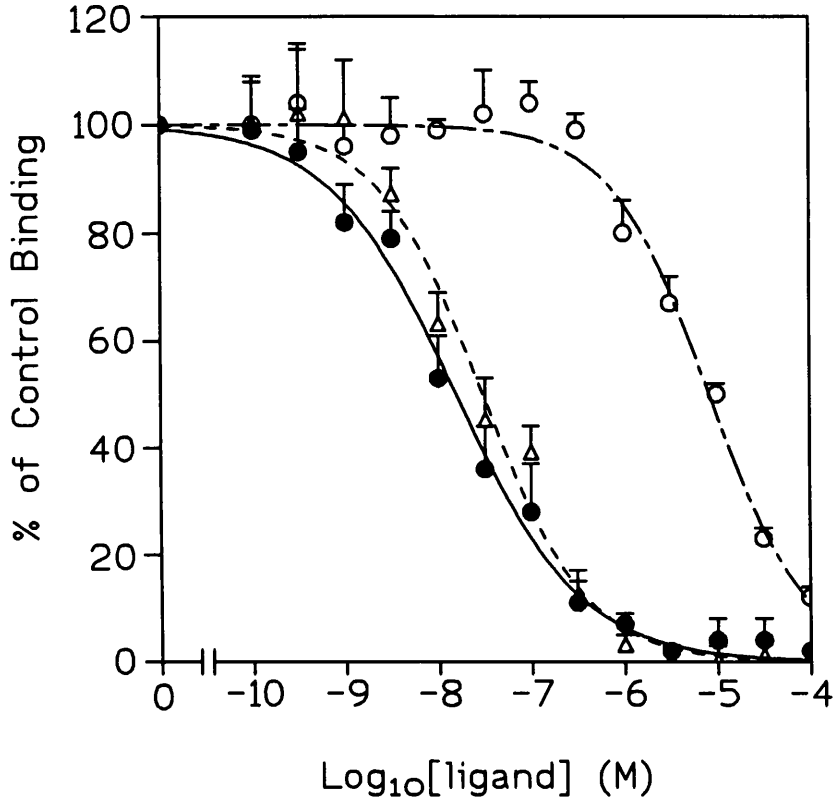
Competitive Inhibition of [^{3}H]Muscimol Specific Binding Activity by RU5135 and Fmoc-RU5135 in NaDOC-Solubilised $GABA_A$ Receptors



The competitive inhibition of [^{3}H]muscimol specific binding activity by RU5135 (●) and fmoc-RU5135 (o) was performed in the presence of 150 mM KCl. The percentage control binding for each concentration of ligand was determined in triplicate and the points are expressed as the mean ± SEM as described in the legend to Figure 3.4.1. The graphs are representative of five independent experiments. $P < 0.001$.

Figure 3.4.4

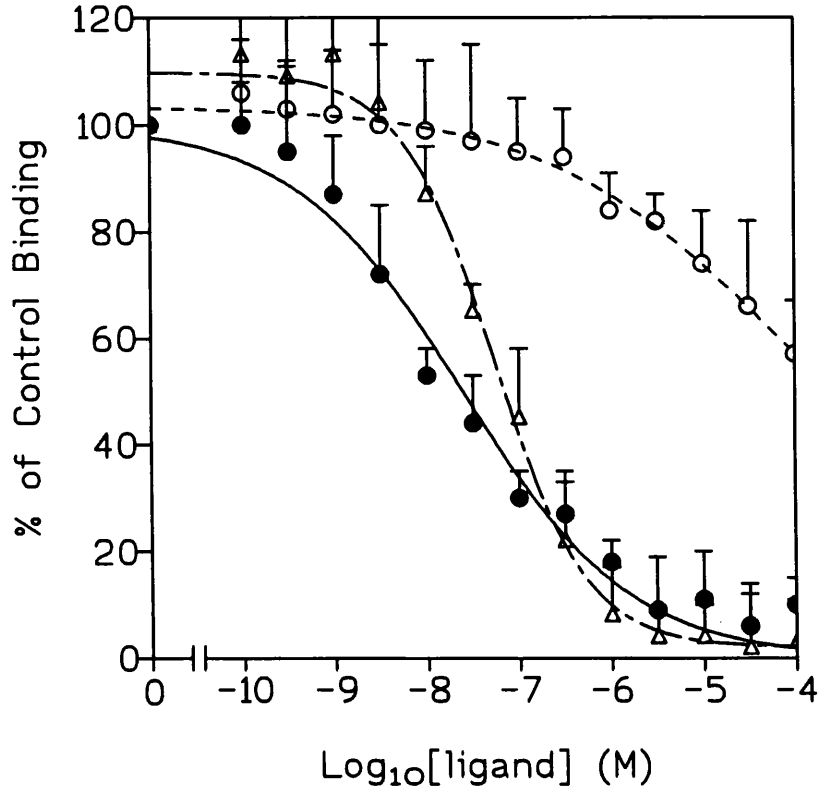
Competitive Inhibition of [^{3}H]Muscimol Specific Binding Activity by RU5135, Fmoc-RU5135-BDDE and RU5135-BDDE in NaDOC-Solubilised $GABA_A$ Receptors



The competitive inhibition of [^{3}H]muscimol by RU5135 (●), fmoc-RU5135-BDDE (○) and RU5135-BDDE (△) was performed in the presence of 150 mM KCl. The percentage specific binding for each concentration of ligand was determined in triplicate and the points are expressed as the mean ± SEM as described in the legend to Figure 3.4.1. The curves are representative of three independent experiments.

Figure 3.4.5

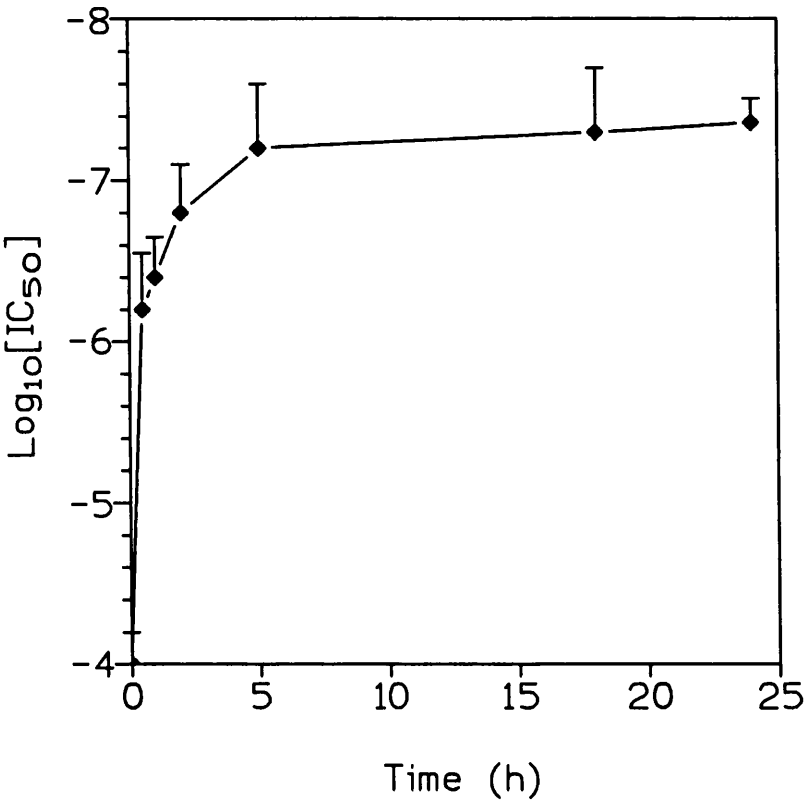
Competitive Inhibition of [^{3}H]Strychnine Specific Binding Activity by RU5135, Fmoc-RU5135 and RU5135-BDDE in Bovine Spinal Cord Membranes



The competitive inhibition of [^{3}H]strychnine specific binding activity by RU5135 (●), fmoc-RU5135 (○) and RU5135-BDDE (△) was performed in the presence of 150 mM KCl. Membranes were subjected to five freeze-thaw cycles before assay to remove the endogenous glycine from the preparation. The percentage control binding for each concentration of ligand was determined in triplicate and the points are expressed as the mean ± SEM as described in the legend to Figure 3.4.1. The graph is representative of six independent experiments.

Figure 3.4.6

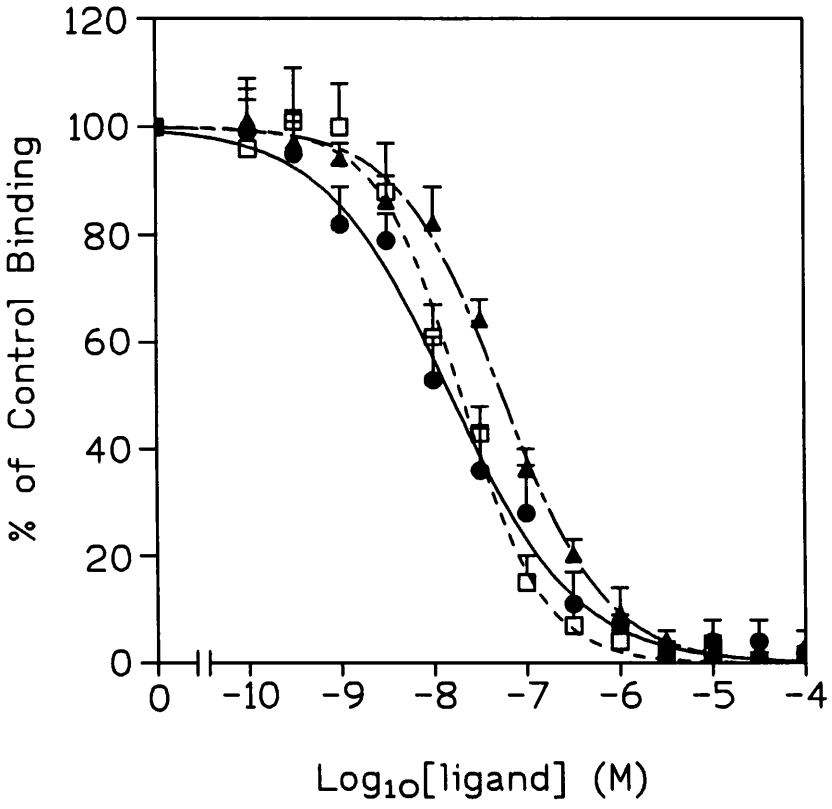
Time-Dependence of the Removal of the Fmoc Protection Moiety from the Amidine Group of RU5135



The time-dependence for the competitive inhibition of [^{3}H]muscimol by the reaction mixture was measured. The reaction was terminated by filtration of the solid Na_2CO_3 and dilution of the mixture to 10^{-4} M, with respect to the concentration of fmoc-RU5135, with ethanol. This was then diluted serially and a radioligand binding assay performed to construct an inhibition curve for each of the time points of the reaction. The relative IC_{50} values for each time point were determined and summarised in Table 3.4.2.

Figure 3.4.7

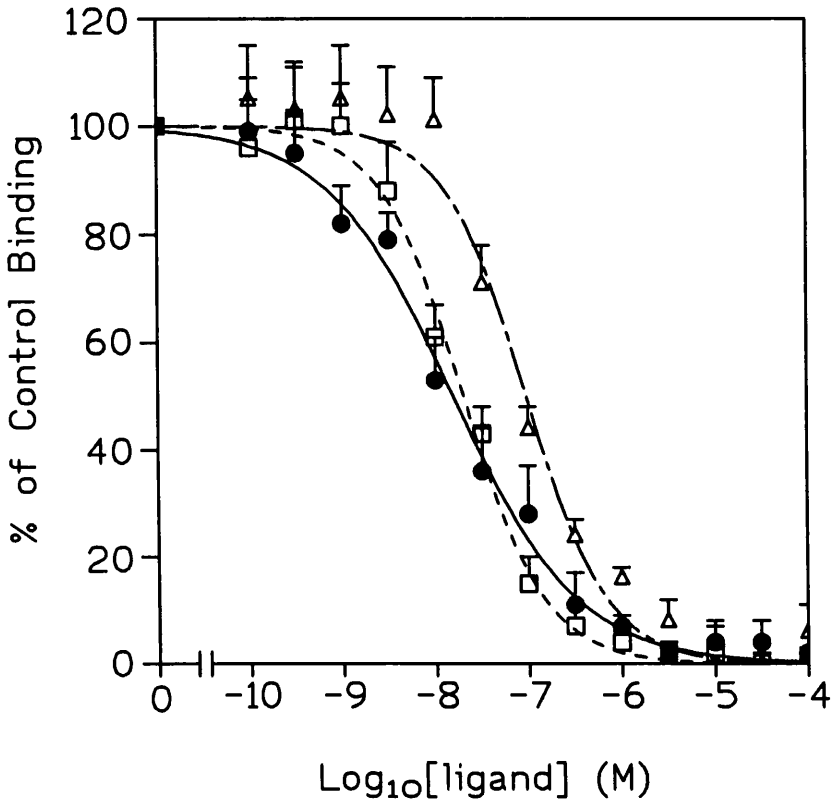
Competitive Inhibition of [^{3}H]Muscimol Specific Binding Activity by RU5135, RU5135-Succinyl Chloride and RU5135-Succinyl-6-Aminohexane in NaDOC-Solubilised $GABA_A$ Receptors



The competitive inhibition of [^{3}H]muscimol by RU5135 (●), RU5135-succinyl chloride (□) and RU5135-succinyl-6-aminohexane (▲) was performed in the presence of 150 mM KCl. The percentage specific binding for each concentration of ligand was determined in triplicate and the points are expressed as the mean ± SEM as described in the legend to Figure 3.4.1. The curves are representative of three independent experiments.

Figure 3.4.8

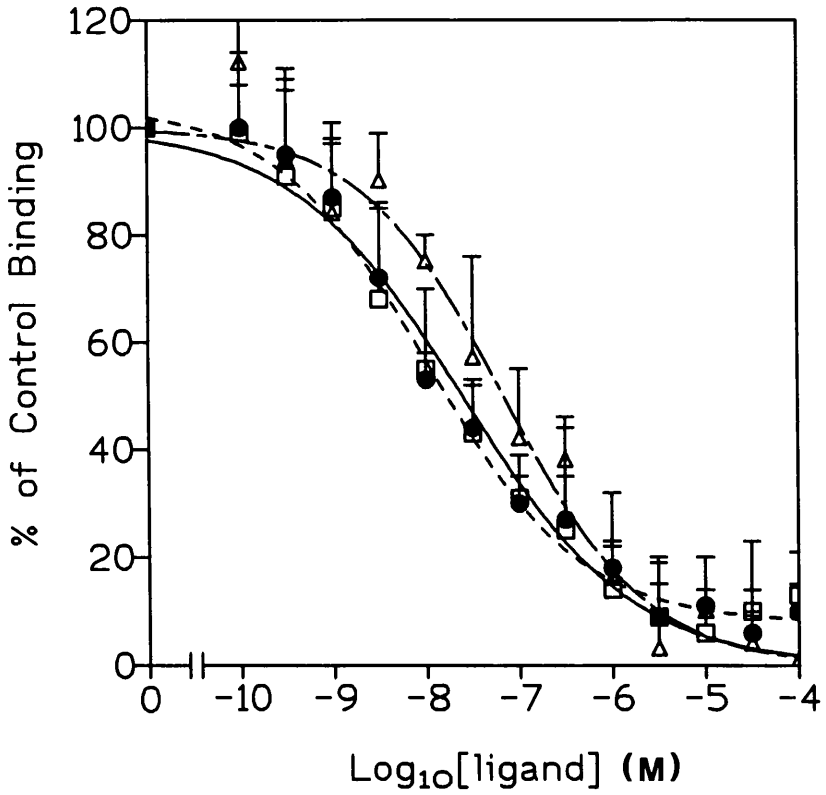
Competitive Inhibition of [^{3}H]Muscimol Specific Binding Activity by RU5135, RU5135-Succinyl Chloride and RU5135-Succinyl-12-Aminododecane in NaDOC-Solubilised $GABA_A$ Receptors



The competitive inhibition of [^{3}H]muscimol by RU5135 (●), RU5135-succinyl chloride (□) and RU5135-succinyl-12-aminododecane (△) was performed in the presence of 150 mM KCl. The percentage specific binding for each concentration of ligand was determined in triplicate and the points are expressed as the mean ± SEM as described in the legend to Figure 3.4.1. The curves are representative of three independent experiments.

Figure 3.4.9

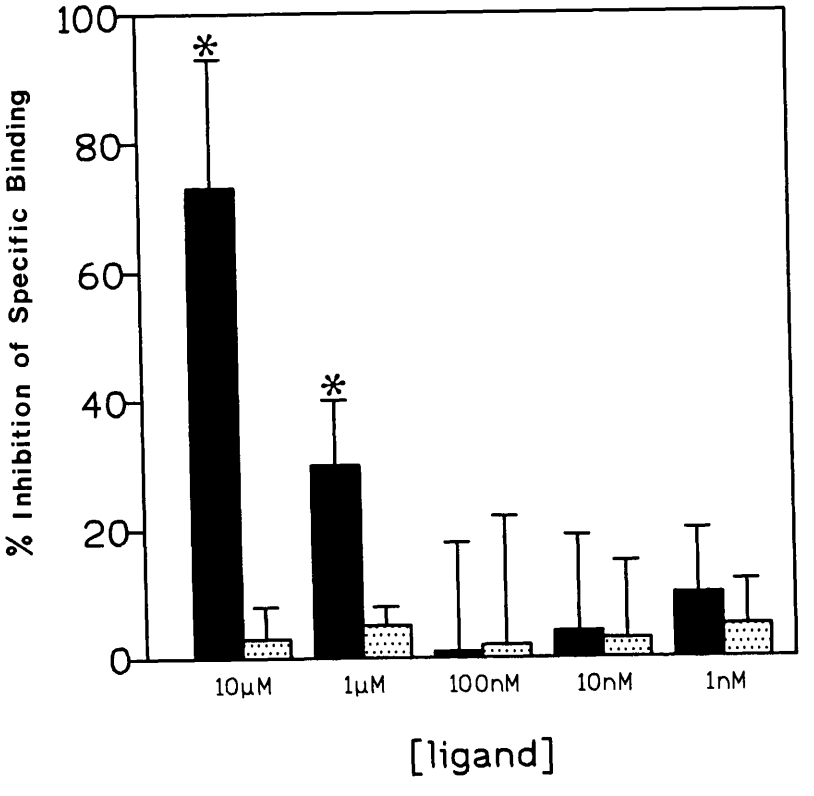
Competitive Inhibition of [^{3}H]Strychnine Specific Binding Activity by RU5135, RU5135-Succinyl Chloride and RU5135-Succinyl-12-Aminododecane to Membrane-Bound Glycine Receptors in Bovine Spinal Cord



The competitive inhibition of [^{3}H]strychnine by RU5135 (●), RU5135-succinyl chloride (□) and RU5135-succinyl-12-aminododecane (△) was performed in the presence of 150 mM KCl. The percentage specific binding for each concentration of ligand was determined in triplicate and the points are expressed as the mean ± SEM as described in the legend to Figure 3.4.1. The curves are representative of three independent experiments.

Figure 3.4.10

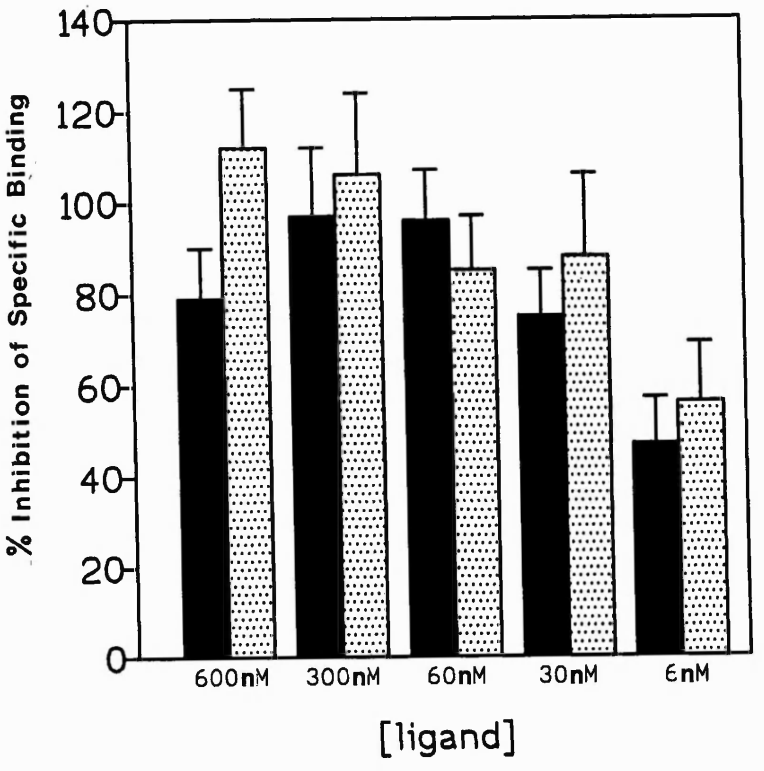
Competitive Inhibition of [^{3}H]Muscimol Specific Binding Activity to NaDOC-Solubilised $GABA_A$ Receptors by RU5135-BDDE-Sepharose 6B



The competitive inhibition of [^{3}H]muscimol binding by RU5135-BDDE-Sepharose 6B (derivatised) was measured in the presence of 500 mM KCl. The competitive inhibition of [^{3}H]muscimol binding by Sepharose 6B (control) was also measured under identical conditions. The percentage of inhibition of specific binding is expressed as the difference between the total binding inhibited by the derivatised matrix and the total binding inhibited by the control matrix (■). Similar experiments were performed under identical conditions to measure the competitive inhibition of [^{3}H]muscimol binding activity by the supernatants of the derivatised and control matrices. The percentage of inhibition of specific binding is expressed as the difference between the total binding inhibited by the supernatant from the derivatised matrix and the total binding inhibited by the supernatant from the control matrix (□). The graph is representative of three independent experiments. (* $P \leq 0.01$).

Figure 3.4.11

Competitive Inhibition of [^{3}H]Muscimol Specific Binding Activity to NaDOC-Solubilised $GABA_A$ Receptors by RU5135-Succinyl-12-Sepharose 4B



The competitive inhibition of [^{3}H]muscimol binding by RU5135-succinyl-12-Sepharose 4B (derivatised) was measured in the presence of 500 mM KCl. The competitive inhibition of [^{3}H]muscimol binding by Sepharose 4B (control) was also measured under identical conditions. The percentage of inhibition of specific binding is expressed as the difference between the total binding inhibited by the derivatised matrix and the total binding inhibited by the control matrix (■). Similar experiments were performed under identical conditions to measure the competitive inhibition of [^{3}H]muscimol binding activity by the supernatants of the derivatised and control matrices. The percentage of inhibition of specific binding is expressed as the difference between the total binding inhibited by the supernatant from the derivatised matrix and the total binding inhibited by the supernatant from the control matrix (□). The graph is representative of three independent experiments.

Table 3.4.1

IC_{50} Values for RU5135 and its Derivatives for the Competitive Inhibition of [^{3}H]Muscimol Specific Binding Activity at the NaDOC-Solubilised $GABA_A$ Receptor

LIGAND	IC_{50} (nM)
RU5135	4.0 ± 2.0
Fmoc-RU5135	4 090 ± 555
Fmoc-RU5135-BDDE	5 580 ± 1 200
RU5135-BDDE	44 ± 26
RU5135-succinyl chloride	22 ± 6
RU5135-succinyl-6-aminohexane	48 ± 14
RU5135-succinyl-12-aminododecane	45 ± 16

Soluble protein was assayed as described in 3.3.6. NaDOC solubilised extract was desalted into buffer C containing 500 mM KCl and diluted in the assay such that the final concentration of KCl was 150 mM. This was incubated in the presence of 10 nM [^{3}H]muscimol with ligands in the concentration range 10^{-4} to 10^{-10} M at 0.5 $\log_{10}$ unit intervals. Non-specific binding was determined in the presence of 10^{-4} M GABA. All values are expressed as the mean ± S.D. of at least three independent determinations.

Table 3.4.2

Summary of Time-Dependence of the Removal of the Fmoc Protection Moiety from the Amidine Group of RU5135

TIME (h)	IC_{50} (nM)
0.5	631 ± 120
1	398 ± 85
2	168 ± 76
5	60 ± 25
18	44 ± 16
24	43 ± 24

These experiments were performed as described in the legend to Figure 3.4.5. Competitive inhibition curves for the inhibition of [^{3}H]muscimol specific binding activity by the reaction mixture at each time-point were constructed and the IC_{50} values were taken directly from these. Non-specific binding was determined in the presence of 10^{-4} M GABA. All values are expressed as the mean ± S.D. of at least three independent determinations.

Table 3.4.3

Optimisation of the Conditions for Affinity Chromatography using Ethanolamine-treated Sepharose 6B.

	10 mM Tris-HCl 150 mM KCl		10 mM KH_2PO_4 150 mM KCl		10 mM KH_2PO_4 500 mM KCl	
Ligand	Musc	Flu	Musc	Flu	Musc	Flu
Applied	100	100	100	100	100	100
Filtrate	30	31	81	68	37	60
Bound	**70 ± 8**	**69 ± 9**	**19 ± 5**	**32 ± 6**	**63 ± 9**	**40 ± 4**
Wash	360	11	>19	2	>63	22
Remainder Bound	**34 ± 6**	**58 ± 3**	**0 ± 8**	**30 ± 5**	**0 ± 5**	**18 ± 5**

Musc = [^{3}H]muscimol binding sites.
Flu = [^{3}H]flunitrazepam binding sites.

The results are representative of at least three independent experiments. NaDOC-solubilised protein was prepared by the method described in 3.3.2. and desalted into the respective buffer consisting of the relevant buffer and salt concentration as described. Affinity chromatography was performed as described in Section 3.3.7. The protein collected from the column is referred to as the filtrate. The columns were then washed with the same desalting buffer with at least 10 column vol. This is referred to as the wash.

All values are expressed as the percentage of the total amount of specific binding activity applied to the columns.

Table 3.4.4

Optimisation of the Conditions for Affinity Chromatography using Tris HCl-treated Sepharose 6B.

	10 mM Tris HCl 150 mM KCl		10 mM KH_2PO_4 150 mM KCl		10 mM KH_2PO_4 500 mM KCl	
Ligand	Musc	Flu	Musc	Flu	Musc	Flu
Applied	100	100	100	100	100	100
Filtrate	18	64	78	65	97	61
Bound	**92 ± 9**	**36 ± 5**	**22 ± 7**	**35 ± 8**	**3 ± 5**	**39 ± 4**
Wash	16	11	0	6	>3	18
Remainder Bound	**76 ± 9**	**25 ± 7**	**22 ± 8**	**29 ± 6**	**0 ± 2**	**21 ± 6**

Musc = [^{3}H]muscimol binding sites.
Flu = [^{3}H]flunitrazepam binding sites.

These results are representative of at least three independent experiments and were performed as described in the legend to Table 3.4.4.

All values are expressed as the percentage of the total amount of specific binding activity applied to the column.

Table 3.4.5

Optimisation of the Conditions for Affinity Chromatography using Glycine-treated Sepharose 6B.

	10 mM Tris HCl 150 mM KCl		10 mM KH_2PO_4 150 mM KCl		10 mM KH_2PO_4 500 mM KCl	
Ligand	Musc	Flu	Musc	Flu	Musc	Flu
Applied	100	100	100	100	ND	ND
Filtrate	83	23	86	43	ND	ND
Bound	**17 ± 7**	**77 ± 5**	**14 ± 9**	**57 ± 6**	ND	ND
Wash	0	6	7	6	ND	ND
Remainder Bound	**17 ± 8**	**71 ± 9**	**7 ± 12**	**23 ± 9**	ND	ND

Musc = [^{3}H]muscimol binding sites.
Flu = [^{3}H]flunitrazepam binding sites.
ND = Not determined.

These results are representative of at least three independent experiments and were performed as described in the legend to Table 3.4.4.

All values are expressed as the percentage of the total amount of specific binding activity applied to the column.

Table 3.4.6

U.V. Spectroscopy of Immobilised Ligands

RESIN	LIGAND	$\lambda_{max.}$ (nm)	IMMOBILISED LIGAND CONC$^{N.}$
Epoxy-activated Sepharose 6B	Fmoc-RU5135	301	0.2 μmol/ml
Sepharose 6B	Fmoc-RU5135-BDDE		10% efficiency
CNBr-activated Sepharose 4B	RU5135-succinyl-6-aminohexane	256	2.0 μmol/ml
	RU5135-succinyl-12-aminododecane		100% efficiency

The concentration of immobilised ligand was measured as described in 3.3.7. and is expressed as the amount of ligand per ml swollen resin. The percentage efficiency of the coupling reaction was determined on the basis of the amount of ligand available for reaction that was present in the coupling solution.

Table 3.4.7

Affinity Chromatography of the NaDOC-Solubilised $GABA_A$ Receptor by RU5135-Epoxy-Activated Sepharose 6B

Ligand	Musc		Flu	
Column	Deriv.	Control	Deriv.	Control
Applied	100	100	100	100
Filtrate	48	62	58	65
Bound	**52 ± 7**	**38 ± 9**	**42 ± 4**	**35 ± 9**
Wash	25	18	26	12
Remainder Bound	**27 ± 8**	**20 ± 12**	**16 ± 10**	**23 ± 8**

Musc = [^{3}H]muscimol binding sites.
Flu = [^{3}H]flunitrazepam binding sites.
Deriv. = Derivatised resin (RU5135 immobilised).
Control = Control resin (no RU5135 present).

NaDOC solubilised protein was prepared by the method described in 3.3.2. and desalted into buffer C (3.3.3) consisting of the relevant buffer and salt concentration as described. The soluble extract (5 ml) was applied to the columns at a flow rate of 3 ml/h and recirculated overnight. The protein collected from the column is referred to as the filtrate. The columns were then washed with the same desalting buffer with at least 10 column vol. This is referred to as the wash.

All values are expressed as the percentage of the total amount of activity applied to the columns.

Table 3.4.8

Affinity Chromatography of the NaDOC-Solubilised $GABA_A$ Receptor by RU5135-BDDE-Sepharose 6B

Ligand	Musc		Flu	
Column	Deriv.	Control	Deriv.	Control
Applied	100	100	100	100
Filtrate	57	78	69	83
Bound	**43 ± 8**	**22 ± 12**	**31 ± 11**	**17 ± 9**
Wash	ND	ND	ND	ND
Remainder Bound	ND	ND	ND	ND

Musc = [^{3}H]muscimol binding sites.
Flu = [^{3}H]flunitrazepam binding sites.
Deriv. = Derivatised resin (RU5135 immobilised).
Control = Control resin (no RU5135 present).
ND = Not determined.

NaDOC solubilised protein was prepared by the method described in 3.3.2. and desalted into buffer C (3.3.3) consisting of the relevant buffer and salt concentration as described. The soluble extract (5 ml) was applied to the columns at a flow rate of 3 ml/h and recirculated overnight. The protein collected from the column is referred to as the filtrate. The columns were then washed with the same desalting buffer with at least 10 column vol. This is referred to as the wash.

All values are expressed as the percentage of the total amount of activity applied to the columns.

Table 3.4.9

Affinity Chromatography of the NaDOC-Solubilised $GABA_A$ Receptor by RU5135-Succinyl-6-Sepharose 4B

Ligand	Musc		Flu	
Column	Deriv.	Control	Deriv.	Control
Applied	100	100	100	100
Filtrate	27	86	86	79
Bound	**73 ± 12**	**14 ± 5**	**14 ± 7**	**21 ± 9**
Wash	1	6	14	10
Remainder Bound	**72 ± 10**	**8 ± 4**	**0 ± 3**	**11 ± 7**

Musc = [^{3}H]muscimol binding sites.
Flu = [^{3}H]flunitrazepam binding sites.
Deriv. = Derivatised resin (RU5135 immobilised).
Control = Control resin (no RU5135 present).

NaDOC solubilised protein was prepared by the method described in 3.3.2. and desalted into buffer C (3.3.3) consisting of the relevant buffer and salt concentration as described. The soluble extract (5 ml) was applied to the columns at a flow rate of 3 ml/h and recirculated overnight. The protein collected from the column is referred to as the filtrate. The columns were then washed with the same desalting buffer with at least 10 column vol. This is referred to as the wash.

All values are expressed as the percentage of the total amount of activity applied to the columns.

Table 3.4.10

Affinity Chromatography of the NaDOC-Solubilised $GABA_A$ Receptor by RU5135-Succinyl-12-Sepharose 4B

Ligand	Musc		Flu	
Column	Deriv.	Control	Deriv.	Control
Applied	100	100	100	100
Filtrate	18	60	69	68
Bound	**82 ± 13**	**40 ± 10**	**31 ± 7**	**32 ± 8**
Wash	5	12	12	7
Remainder Bound	**77 ± 10**	**28 ± 10**	**19 ± 9**	**25 ± 6**

Musc = [^{3}H]muscimol binding sites.
Flu = [^{3}H]flunitrazepam binding sites.
Deriv. = Derivatised resin (RU5135 immobilised).
Control = Control resin (no RU5135 present).

NaDOC solubilised protein was prepared by the method described in 3.3.2. and desalted into buffer C (3.3.3) consisting of the relevant buffer and salt concentration as described. The soluble extract (5 ml) was applied to the columns at a flow rate of 3 ml/h and recirculated overnight. The protein collected from the column is referred to as the filtrate. The columns were then washed with the same desalting buffer with at least 10 column vol. This is referred to as the wash.

All values are expressed as the percentage of the total amount of activity applied to the columns.

3.5 DISCUSSION

Previous studies have shown that RU5135 is a competitive $GABA_A$ receptor antagonist in rat cerebral cortex membranes (Olsen, 1984; Hunt and Clements-Jewry, 1981). Due to the nature of this research, it was essential to characterise the binding of RU5135 to $GABA_A$ receptors both in intact and NaDOC-solubilised bovine cerebral cortex membranes. The results of this study show that RU5135 displaces [^{3}H]muscimol specific binding activity from both membrane-bound and NaDOC-solubilised bovine cerebral cortex $GABA_A$ receptors in a dose-dependent manner and with high affinity (Sections 3.4.1.1 and 3.4.1.2). The high affinity is comparable to data published elsewhere where the IC_{50} value for the competitive inhibition of [^{3}H]muscimol specific binding activity by RU5135 was found to be 11.0 nM in rat brain (Olsen, 1984). However, the affinity of RU5135 for the NaDOC-solubilised bovine cortical $GABA_A$ receptor was significantly different from that of the membrane-bound receptor and this may be due to increased efficiency of removal of GABA from the NaDOC-solubilised $GABA_A$ receptor by gel filtration compared to the subjection of the membrane-bound receptors to repeated freeze-thaw cycles.

It has been shown that RU5135 blocks the GABA inhibition of [^{35}S]TBPS binding to the picrotoxin/TBPS binding site of the $GABA_A$ receptor in rat brain (Squires *et al.*, 1983). These results, together with observations that RU5135 also inhibited the GABA enhancement of benzodiazepine binding in rat brain (Hunt and Clements-Jewry, 1981), suggested that RU5135 is a competitive GABA antagonist at the $GABA_A$ receptor complex. Similar results

are reported in this study. In bovine cerebral cortex membranes, RU5135 completely inhibited the GABA enhancement of [^{3}H]flunitrazepam binding in a dose-dependent manner. The IC_{50} value for this inhibition (5.6 ± 2.3 μM) was consistent with values published previously in studies on rat cerebral cortex (Hunt and Clements-Jewry, 1981).

RU5135 is a 3α-hydroxy steroid and the 3α-hydroxy moiety is an essential structural feature for $GABA_A$ receptor agonist neurosteroids (Harrison *et al.*, 1987; Gee *et al.*, 1988). However, in this study, pregnanolone (a potent neurosteroid) was found to potentiate benzodiazepine binding. This was in agreement with results published by other workers (Gee, 1988). This was in direct contrast to the effects of RU5135 and, therefore, it was concluded that RU5135 was acting as a GABA antagonist and not interacting with the neurosteroid binding site of the $GABA_A$ receptor in the bovine cerebral cortex. This conclusion has recently been substantiated by observations that RU5135 does not competitively antagonise pregnanolone inhibition of [^{35}S]TBPS binding in rat cerebral cortex (Cadoni and Gee, 1992). Further evidence was provided by the fact that inhibition of [^{3}H]muscimol specific binding activity by RU5135 was measured in NaDOC-solubilised $GABA_A$ receptors from bovine cerebral cortex. Enhancement of [^{3}H]muscimol binding activity by pregnanolone was not detected. It is unlikely that this observation is due to the fact that NaDOC (a steroidal detergent) may be interacting with the putative neurosteroid binding site of the $GABA_A$ receptor since the detergent was always exchanged for Triton X-100 during gel filtration of the soluble extract (Section 3.3.3). Triton X-100

is a detergent of the polyoxyethylene type and is not known to interact with the [^{3}h]muscimol or [^{3}H]flunitrazepam binding sites of the $GABA_A$ receptor. Furthermore, it has been shown previously that the barbiturate binding site was not detectable after solubilisation with NaDOC (Section 1.4; Stephenson *et al.*, 1982). Therefore, the lack of detectable neurosteroid binding to the solubilised receptor is likely to be due to an inactivation of the neurosteroid binding site during solubilisation and/or gel filtration of the $GABA_A$ receptor. This provides further evidence that RU5135 acts at the GABA binding site, not the putative neurosteroid binding site, of the $GABA_A$ receptor.

The presence of thiocyanate ions in the assay buffer served to significantly increase the affinity of RU5135 for the membrane-bound $GABA_A$ receptor from bovine cerebral cortex. These findings were consistent with work performed in rat cerebral cortex where the affinity of RU5135 was also found to increase in the presence of thiocyanate ions compared to chloride ions (Olsen, 1984). It has also been demonstrated that the affinity of the antagonist (+)-bicuculline for the $GABA_A$ receptor is increased when the assay is performed in the presence of thiocyanate but that the affinity of agonists (GABA and 3-aminopropanesulphonic acid, APSA) is unchanged (Enna and Snyder, 1977). The explanation for these phenomena is at present unknown. However, recent evidence has shown that different amino acids in the primary structure of the subunits are important in the binding of agonists and antagonists to the $GABA_A$ receptor (Sigel *et al.*, 1992) and the strychnine-sensitive glycine receptor (Vandenberg *et al.*, 1992). Sigel and co-workers have shown that a single amino

acid substitution at position 64 (Phe to Leu) results in a 2000-fold decrease in the affinity for GABA-dependent chloride channel gating. The affinity of antagonists was also decreased but to a lesser extent (6-fold for bicuculline methochloride and 200-fold for SR 95531). In the case of the glycine receptor, it was shown that two residues (Lys-200 and Tyr-202) were important for strychnine binding but that the agonist binding site is located at Thr-204. Similar experiments performed on the benzodiazepine binding site of $GABA_A$ receptor have shown that a single histidine residue at position 100 is essential for agonist binding but not for the binding of inverse agonists (Wieland *et al.*, 1992).

The presence of thiocyanate ions in the assay buffer also appeared to increase the affinity of RU5135 for the $GABA_A$ receptor compared to chloride ions. Although a similar trend was observed in each individual experiment in that the presence of thiocyanate ions caused a shift in the competitive inhibition curve to the left, the difference in affinities of RU5135 was found to be insignificant when analysed statistically. The increase in affinity was always found to be approximately 2-fold, however, the experiment would need to be repeated a substantial number of times in order that the standard deviations become sufficiently small that the changes are statistically significant.

RU5135 inhibited [^{3}H]strychnine specific binding activity to the membrane-bound, strychnine-sensitive glycine receptor from bovine spinal cord in a dose-dependent manner and with high affinity. The affinity of RU5135 for the glycine receptor was higher in this study than for the membrane-bound

$GABA_A$ receptor which was in agreement to previously published data on rat tissue where the IC_{50} values for both receptor types were comparable (Olsen, 1984). However, estimates of the IC_{50} value for the competitive inhibition of [^{3}H]strychnine specific binding activity in bovine tissue were variable. This may be due to difficulties in the removal of endogenous glycine from the synaptosomal membranes. Indeed, even when the membranes were subjected to five freeze-thaw cycles, there was still variability in the affinity of RU5135 for the glycine receptor. The differences between the reported affinities of RU5135 for the membrane-bound glycine receptor and the $GABA_A$ receptor may reflect the differences in the efficiencies of removal of the respective endogenous agonists from the bovine membranes by the freeze-thawing technique.

The effects of the derivatisation of RU5135 on its affinity for the NaDOC-solubilised $GABA_A$ receptor and the membrane-bound glycine receptor were investigated and the results are reported in Section 3.4.2. Protection of the amidine group in ring D resulted in a dramatic decrease in affinity of the compound for both receptor types. Treatment with ethanolamine to remove the fmoc protection moiety returned the affinity of the compound to the nanomolar range (Figure 3.4.6). The conclusion from this observation was that the amidine region of the molecule is essential for the interaction of RU5135 with the $GABA_A$ receptor and the glycine receptor. The rate of removal of the fmoc group was determined by time course studies in order to determine standard conditions for the removal of the fmoc group following the immobilisation of fmoc-RU5135 or fmoc-RU5135-BDDE to the affinity matrices. Due to the

nature of these studies, it was not feasible to purify the product by flash chromatography and conduct spectral analyses to ascertain the purity of the sample before assaying for the inhibition of specific [^{3}H]muscimol binding activity because of the amount of time involved in performing those procedures. Therefore, the experiments were performed such that the concentration of ligand was always 1 mM with respect to fmoc-RU5135. The assumption was made that the deprotection of one molecule of fmoc-RU5135 resulted in one molecule of RU5135, therefore, at the end of the experiment, the concentration of RU5135 in the reaction mixture would be 1 mM. The basic assumption of this experiment is that the reaction is capable of occurring to 100% efficiency and this is supported by reports that the removal of the fmoc group from amino acids during solid-phase peptide synthesis occurs in near quantitative yield (Albericio *et al.*, 1990). The time course experiments showed that the IC_{50} of the ligand for the $GABA_A$ receptor increased with time and the affinity eventually returned to the nanomolar range. Therefore, as the fmoc group was removed from the amidine group of RU5135, its affinity was restored and this provided further evidence that the amidine function is essential for the high affinity interaction of RU5135 with its binding site in the $GABA_A$ receptor.

The coupling of BDDE to fmoc-RU5135 did not alter the low affinity of this compound for the $GABA_A$ receptor significantly. However, the affinity did return to the nanomolar range following treatment with ethanolamine. The affinity of this product (RU5135-BDDE) was significantly lower than that of the original RU5135. Interestingly, the reaction of RU5135 with succinyl chloride

resulted in a compound which had significantly reduced affinity for the NaDOC-solubilised $GABA_A$ receptor compared to RU5135. Reaction of the acid chloride derivative with a diaminoalkane reduced the affinity of the compound still further. The affinities of these compounds were not significantly different from that of RU5135-BDDE. This may be due to impurities in the small ligand sample used for the radioligand binding assays but this is unlikely since these compounds were purified by flash chromatography and the structure and purity were confirmed by tlc and spectral anlyses. The possibility exists that the presence of a spacer arm at the C3 position influences the interaction of RU5135 with its specific binding site. This study has shown that the C11-C17 region of RU5135 is requisite high affinity $GABA_A$ receptor binding but this does not exclude the possibility of additional influence by the C3-hydroxyl group on the high affinity of the compound for its binding site. The length of the spacer arm had no significant effect on the affinity of the compound for the receptor with comparable affinities for RU5135-BDDE, RU5135-succinyl chloride, RU5135-succinyl-6-aminohexane and RU5135-succinyl-12-aminododecane. Although the affinities of these compounds were reduced, they were sufficiently high that they were of potential use as immobilised ligands in RU5135 affinity chromatography by comparison with benzodiazepine ligands which have been used for the affinity chromatographic purification of the $GABA_A$ receptor (Sigel *et al.*, 1983).

In initial studies on the RU5135 affinity chromatographic isolation of the $GABA_A$ receptor, it was found that the receptor bound non-specifically to the

affinity matrix. Therefore, it was important to reduce this to a minimum before the derivatised matrices were employed. In order to reduce the amount of non-specific interactions between the RU5135 affinity matrices and the NaDOC-solubilised $GABA_A$ receptor, several experiments were performed upon control matrices, the results of which are described in Section 3.4.3.1.

These experiments showed that the affinity chromatography of the $GABA_A$ receptor could be performed in the presence of a high salt concentration (500 mM KCl) and high detergent concentration (0.5% (v/v) Triton X-100) without causing significant loss of [^{3}H]muscimol or [^{3}H]flunitrazepam specific binding activities from the extract of bovine cerebral cortex within the time course of the experiment. This is consistent with previous studies on the isolation of the $GABA_A$ receptor from bovine and rat brain by benzodiazepine agonist affinity chromatography where the [^{3}H]muscimol or [^{3}H]flunitrazepam specific binding activities have remained stable throughout the preparation (e.g. Sigel *et al.*, 1983; Sigel and Barnard, 1984; Stauber *et al.*, 1987). The high salt concentration was especially effective in reducing the amount of binding activity that was non-specifically bound to the affinity matrices. The likely explanation for this phenomenon was because it reduced the number of ionic interactions of the receptor protein with the Sepharose. The high detergent content served to reduce the amount of hydrophobic interactions by ensuring that the protein remained in solution.

The choice of buffer employed in these control experiments was also important. While HEPES and K-phosphate buffers (containing 500 mM KCl and

* It is possible for Tris-HCl to react with the excess epoxide groups in epoxy-activated Sepharose 6b. Therefore, the compound can be used to block these excess groups following the synthesis of an affinity column. However, due to the ionic nature of Tris-HCl, there may be additional ionic charges introduced into the matrix. It is possible that these ionic charges interact with the $GABA_A$ receptor protein causing its retention on the affinity column.

0.5% Triton X-100) maintained the amount of non-specific binding at approximately 20%, Tris-HCl buffers raised the level. This may be due to the fact that Tris-HCl introduced additional ionic charges into the Sepharose. It was shown that when Tris-HCl was used to block the excess reactive groups in the support matrix, non-specific binding of the receptor to the matrix was high (50-60%). This was also true for glycine used as a blocking agent. However, in the latter case, it may be possible that glycine can bind to the $GABA_A$ receptor, albeit with low affinity, thereby retaining it within the matrix. Ethanolamine as a blocking agent did not alter the amount of non-specific binding. Therefore, ethanolamine was employed as the blocking agent in all subsequent experiments with K-phosphate buffer containing 500 mM KCl and 0.5% Triton X-100.

The concentration of immobilised ligand in the affinity matrices might best be determined by chemical analyses such as ^1H nmr or mass spectroscopy. However, this was not possible with the available equipment. Therefore, the technique employed was UV spectrophotometry according to the method described by Sigel *et al.* (1982), the results of which are recounted in Section 3.4.3.2 and summarised in Table 3.4.6. This technique is subjective and depends upon the ability of the ligand of interest to absorb UV light in quantities that are easily detectable. Consequently, for the purposes of this study, a dual beam spectrophotometer was used to measure the difference in absorption between the derivatised matrix and the control matrix. The fmoc group is a relatively strong absorber of UV light due to the number of conjugated bonds in the fluorenyl moiety. Hence, the λ_{max} value was relatively high and, therefore, relatively easy

to detect. However, in the case of the amino derivatives, the absorption was low and the $\lambda_{max.}$ value was also low. The consequence of this was that the effect of light scattering by the Sepharose at low wavelengths ($\lambda < 210$ nm) on the measurement of ligand concentration for the immobilised aminoalkanes was large. Possible over- or under-estimations in the determination of immobilised ligand concentration were minimised by comparison of the spectrum of the immobilised ligand with spectra of a range of known concentrations of the free ligand in solution. In this way, calibration curves were constructed and the immobilised ligand concentrations were calculated from them.

The determination of the binding capacity of the RU5135 Sepharose 6B affinity matrices showed that these affinity matrices were inefficient in specifically isolating the $GABA_A$ receptor via the GABA recognition site. The amounts of [^{3}H]muscimol binding activity retained on the matrices were not significantly higher than the amount retained by the control matrices and the reasons for this were unclear. Therefore, the affinities of the derivatised matrices for the $GABA_A$ receptor were determined by radioligand binding assays according to the methods described in Section 3.3.5.3 and the results are described in Section 3.4.3.3. These experiments were performed in order to ascertain the affinity of immobilised RU5135 for the NaDOC-solubilised $GABA_A$ receptor and they also had the advantage that, by assaying the matrix suspension and the supernatant from that suspension, the covalent attachment of RU5135 to the matrix could be verified. The coupling of RU5135-BDDE to Sepharose 6B reduced the apparent affinity of this ligand by a factor of 100-fold

with respect to the competitive inhibition of [^{3}H]muscimol specific binding activity. The supernatant from this matrix did not inhibit [^{3}H]muscimol binding activity significantly. This suggested that RU5135-BDDE was indeed covalently bound to Sepharose 6B and not merely held there by hydrophobic interaction due to the physical nature of the ligand. It is possible that the reduction in the IC_{50} value of the immobilised ligand occurred because of the structure of the spacer arm. BDDE is a straight 12 atom chain which contains no double bonds. Consequently, the bonds are able to rotate freely and the molecule can adopt a variety of conformations. Given the hydrophobic nature of RU5135, it is possible that the spacer arm folds in such a way as to ensure that RU5135 is within a hydrophobic pocket within the Sepharose matrix instead of in the aqueous surroundings. The implication from this would be that less RU5135 is available for binding the $GABA_A$ receptor and the apparent affinity of the immobilised ligand for the receptor is, therefore, reduced.

Similar analysis of the aminoalkane-derivatised matrices showed that although the matrices inhibited [^{3}H]muscimol specific binding activity with relatively high affinity (IC_{50} = 300 nM), the supernatants from these suspensions also inhibited [^{3}H]muscimol specific binding activity to a similar degree. This suggested that the ligand was not covalently attached to the matrix but was held there by hydrophobic interaction. The estimation of the concentration of the immobilised ligand was high and the efficiency of the coupling reaction for the aminoalkane derivatives of RU5135 was estimated to be quantitative. However, the interpretation of this may lie in the fact that RU5135 is a lipophilic molecule

and the presence of a long hydrophobic spacer arm increases the lipophilicity. It might be envisioned that the aminoalkane derivatives reside in the hydrophobic pockets of the matrix and not in aqueous solution as is required for efficient coupling to take place. This is supported by the fact that several washing cycles were necessary for the complete removal of the ligands from the matrix. Similar phenomena have been previously reported. It has been shown that polyclonal antibodies which have been immobilised to CNBr-activated Sepharose 4B for use in the immunoaffinity chromatographic isolation of $GABA_A$ receptors can be progressively removed from the matrix although it is unclear whether this is due to the breakage of the covalent bonds between the antibody and the matrix or to inefficient coupling reactions (Duggan *et al.*, 1991; Pollard *et al.*, 1993).

Further evidence of "ligand-leakage" was provided by performing affinity chromatographic isolation experiments on the $GABA_A$ receptor (Section 3.3.7). Initial observations intimated that [^{3}H]muscimol binding activity was retained by the matrix, however, these observations were not paralleled by [^{3}H]flunitrazepam binding activity. Subsequent gel filtration of the flow through from the matrix caused the [^{3}H]muscimol binding activity to reappear. These observations provided further support for the theory that the immobilised ligand was not bound covalently to the affinity matrix. The likely explanation for the amount of [^{3}H]muscimol binding sites which had apparently bound the affinity matrix was that the ligand had, indeed, leaked from the matrix, bound to the GABA recognition site of the $GABA_A$ receptor and, hence, inhibited

[^{3}H]muscimol specific binding activity in the eluted protein extract.

Although RU5135-BDDE was covalently bound to Sepharose 6B, it failed to bind the $GABA_A$ receptor with sufficiently high affinity for its isolation. It would appear that, whereas the ligand was immobilised to the Sepharose, its affinity (IC_{50} = 4 μM) was not sufficient to retain the $GABA_A$ receptor. Assays for [^{3}H]muscimol and [^{3}H]flunitrazepam specific binding activity before and after the isolation procedure showed that there was no significant inactivation of the $GABA_A$ receptor dispensing with this as a possible explanation for this occurrence and, therefore, the low affinity of the immobilised ligand is the most likely explanation. It is possible that the *O*-derivatisation of RU5135 results in an alteration in the kinetics of the interaction between RU5135 and its binding site. However, accurate and direct measurement of the kinetics of the binding of this ligand to the $GABA_A$ receptor are difficult in the absence of tritiated RU5135.

Although it has been demonstrated that the derivatives synthesised in this study were unsuccessful as immobilised ligands in the affinity chromatographic isolation of the $GABA_A$ receptor via its GABA recognition site, they may prove to be valuable tools in the future in investigations into the antagonist and agonist binding sites of the $GABA_A$ and strychnine-sensitive glycine receptors. The structure-activity relationships of the ligands at these inhibitory members of the ligand-gated ion channel superfamily may provide further insights into the resemblances of the antagonist binding site of the two receptors and aid subsequent elucidation of their structure and function.

Chapter 4

General Discussion

The aim of this project was to develop a novel method for the isolation of the $GABA_A$ receptor from bovine brain. Prior to the commencement of this study, the $GABA_A$ receptor had only been purified by benzodiazepine agonist affinity chromatography which is dependent on the presence of a high affinity benzodiazepine agonist binding site within the receptor complex. This had led to the cloning of several cDNAs which encoded $GABA_A$ receptor subunits and some of these had been pharmacologically characterised (Section 1.8; reviewed in Olsen and Tobin, 1990). At this time, the γ2 subunit had been cloned and it had been shown that the presence of this subunit was required for high affinity benzodiazepine binding in recombinant receptors expressed in HEK 293 cells (Pritchett *et al.*, 1989b). However, it had also been shown that the type of benzodiazepine pharmacology depended upon the α subunit present in the recombinant receptors (Section 1.8; Pritchett *et al.*, 1989a). The α1 subunit was associated with type I benzodiazepine pharmacology whereas the α2 and α3 subunits were associated with type II pharmacology as defined in Section 1.3.1.

In October 1989, there were no published reports of α subunits which displayed a lack of affinity for benzodiazepine agonists when present in recombinant $GABA_A$ receptors. However, there was evidence to suggest that there were native $GABA_A$ receptors in mammalian brain which were insensitive to benzodiazepine agonists. Autoradiographic studies and photoaffinity labelling experiments using [^{3}H]Ro 15-4513 and [^{3}H]flunitrazepam showed that the pattern of labelling by these two benzodiazepines varied among brain regions with the most striking difference being in the cerebellum where [^{3}H]Ro 15-4513

labelled a protein which was not displaced by benzodiazepine agonists, the β-carbolines or by CL 218,872 (Sieghart *et al.*, 1987). Other evidence came from observations that there was an apparent mismatch between the percentages of [^{3}H]muscimol specific binding sites and [^{3}H]flunitrazepam specific binding sites purified by benzodiazepine agonist affinity chromatography (Stephenson, 1987). These observations may partially be explained by the discovery and pharmacological characterisation of the α6 and α4 subunits which confer benzodiazepine agonist insensitivity on recombinant receptors (Luddens *et al.*, 1990; Wisden *et al.*, 1991). The pharmacological profiles of these subunits in recombinant receptors are discussed in Section 1.8.

The discovery of these polypeptides does not preclude the possibility that other subunits exist which confer benzodiazepine insensitivity on $GABA_A$ receptors. Most importantly, it should be recognised that, to date, the subunit composition of any native $GABA_A$ receptor is unknown. To elucidate this structure would provide essential information for studies into the function and mechanisms of operation of the $GABA_A$ receptor at the molecular level which may be extrapolated to other members of the ligand-gated ion channel superfamily. The aim of this project was, therefore, to develop a novel method for the isolation of the $GABA_A$ receptor by affinity chromatography using a high affinity ligand which was specific for the GABA recognition site. This would contribute a technique for the isolation of native $GABA_A$ receptors irrespective of their benzodiazepine pharmacology. The subunit composition and pharmacological properties of these receptors could then be investigated and

these may provide important information about the $GABA_A$ receptor.

RU5135 was the ligand chosen for immobilisation to affinity chromatography matrices as a consequence of its high affinity for the $GABA_A$ receptor and its chemical structure (Hunt and Clements-Jewry, 1981; Olsen, 1984; Section 2.1). The advantage of this compound was that it was chemically bifunctional and the structure of the molecule was such that the hydroxyl group at the C3 position was a relatively long distance from the region of the molecule that was thought to be important in binding to the GABA recognition site of the $GABA_A$ receptor (Section 2.1). The bifunctionality of the molecule meant that either the amidine group must be protected before coupling to an affinity matrix via the C3 hydroxyl group or the C3 hydroxyl group was modified chemically such that an affinity matrix which does not form covalent bonds with amidine groups could be employed. This was achieved, after extensive investigations, by the protection moiety used in peptide synthesis, fmoc. The fmoc derivative of RU5135 was then either coupled directly to epoxy-activated Sepharose 6B or reacted with the free spacer arm (BDDE) and subsequently coupled to Sepharose 6B. An alternative method of synthesis of the RU5135 affinity matrix involved the chemical modification of the C3 hydroxyl group of RU5135 to the hemisuccinyl chloride derivative. This acid chloride was then reacted with diaminoalkanes of various chain lengths the products of which were coupled to CNBr-activated Sepharose 4B.

These synthetic pathways yielded four derivatives of RU5135 that were of potential use for immobilisation to affinity chromatography matrices and

purification of the $GABA_A$ receptor, namely fmoc-RU5135, fmoc-RU5135-BDDE, RU5135-succinyl-6-aminohexane and RU5135-succinyl-12-aminododecane. The structures of these compounds were purified by flash chromatography and preparative tlc and chemically analysed by ^1H nmr and mass spectroscopy. These compounds were then investigated by biochemical methods to assess their effectiveness as immobilised ligands in affinity chromatography and to gain some insight into the characteristics of the binding of RU5135 to the $GABA_A$ and strychnine-sensitive glycine receptors.

The investigations into the affinities of the various derivatives of RU5135 may provide interesting information about the interaction of RU5135 with its binding site in both the $GABA_A$ and strychnine-sensitive glycine receptors. At the beginning of this project, it was thought that the region in the molecule which was responsible for the interaction with the receptor was that area between C11 and C17 (Figure 2.1.2) and that the hydroxyl group at the C3 position was of little importance. This theory was substantiated by structure-activity relationships of similar compounds which showed that the presence of the amidine group increased affinity for the $GABA_A$ receptor compared to the presence of an amine group in the same position (Wermuth *et al.*, 1987). This study has shown that the C11-C17 region of RU5135 is essential for the binding of this ligand to its binding site in both $GABA_A$ and strychnine-sensitive glycine receptors. We have also provided evidence that the C3-hydroxyl position may participate in the interaction of the ligand with its receptor binding site. The observations in this study show that as the size of the *O*-derivatisation moiety

increases, the affinity of the ligand for the $GABA_A$ receptor decreases. It is suggested, therefore, that although the C11-C17 region of RU5135 is essential for binding to the $GABA_A$ and glycine receptors, the locality around the C3-hydroxyl group is necessary for bestowing high affinity on the ligand. This may become very important in studies which attempt to delineate the agonist and antagonist binding sites of the ligand-gated ion channel receptors. There is evidence to suggest that the amino acid residues necessary for agonist binding to the $GABA_A$ receptor are different from those required for antagonist binding as discussed in detail in Section 3.5 (Sigel *et al.*, 1992). Analogous studies have provided similar information about the agonist and antagonist binding sites of the strychnine-sensitive glycine receptor (Vandenburg *et al.*, 1992). Even in the case of benzodiazepine receptors, it has been shown that a single amino acid substitution in the α subunit can significantly modify the pharmacology of the expressed recombinant receptors (Wieland *et al.*, 1992).

Radioligand binding assays showed that the affinity of the aminoalkane derivatives and of the deprotected fmoc derivatives were sufficiently high that they would retain adequate affinity for the $GABA_A$ receptor for its isolation when immobilised to an affinity chromatography matrix. However, it was shown that the immobilisation of the aminoalkane derivatives to CNBr-activated Sepharose 4B was difficult and it has been suggested that the reason for this may be the hydrophobicity of these molecules as discussed in Section 3.5. Immobilisation of fmoc-RU5135-BDDE to Sepharose 6B followed by deprotection of the ligand using ethanolamine resulted in a large decrease in the

affinity of the ligand for the $GABA_A$ receptor. This may have been due to the hydrophobic nature of RU5135 and the long length of the spacer arm which allowed the ligand to reside in a hydrophobic pocket within the Sepharose backbone as discussed in Section 3.5.

Although some valuable information about the binding of antagonists at the $GABA_A$ receptor was provided by this study, the RU5135 affinity chromatography matrices synthesised were unsuccessful in the isolation of the $GABA_A$ receptor. There is, however, still a requirement for the discovery of a homogeneous population of $GABA_A$ receptors in order to investigate its mode of function, regulation, expression mechanisms and influences on other cellular functions. Information such as this may use immunological techniques to isolate specific subunit containing $GABA_A$ receptors. Major progress has already been made in this field with the isolation of various populations of $GABA_A$ receptors which contain specific α subunits (Pollard *et al.*, 1993). However, these studies have proved difficult because of the relatively low abundance of the $GABA_A$ receptors. They are also restricted by the fact that there is only a limited number of antibodies available. There may be other $GABA_A$ receptor subunits, which have yet to be cloned, present in the immunoaffinity preparations that, since there are no antibodies to them, are undetectable.

Alternative approaches may be to investigate immortalised cell lines which may contain homogeneous populations of $GABA_A$ receptors. There is one report of such a cell line, to date, in which the only α subunit found is the $\alpha 3$ subunit (Noble *et al.*, 1993). This is potentially a very useful tool, however,

extensive further investigations are necessary because it is possible that other α subunits, as yet not cloned, may be present.

The strategy for the elucidation of the structure, functions and mechanisms of native $GABA_A$ receptors requires further investment of time and resources. Only when this is accomplished will the development of selective therapeutic agents for the treatment of anxiety and other neurological disorders be viable.

Bibliography

Albericio F., Cordonier-Knieb N., Biancalana S., Gera L., Masada R.I., Hudson D. and Barany G. (1990)

Preparation and application of the 5-(4-(9-fluorenylmethoxy carbonyl)aminomethyl-3,5-dimethoxyphenoxy)-valeric acid (PAL) handle for the solid phase synthesis of C-terminal peptide amides under mild conditions.

J. Org Chem. **55** 3730-3743

Akaike N., Yakushiji T., Tokutomi N. and Carpenter D.O. (1987)

Multiple mechanisms of antagonism of γ-aminobutyric acid (GABA) responses.

Cell Mol. Neurobiol. **7** 97-103

Amit B., Zehavi U. and Patchornik A. (1974)

Photosensitive protecting groups of amino sugars and their use in glycoside synthesis. 2-Nitrobenzyloxycarbonyl-amino and 6-nitroveratryloxycarbonyl-amino derivatives.

J. Org. Chem. **39** 192-196

Asano T., Sakakibara J. and Ogasawara N. (1983)

Molecular sizes of GABA and benzodiazepine receptor proteins are identical.

FEBS Lett. **151** 277-280

Barker J.L. and Mathers D.A. (1981)

GABA analogues activate channels of different duration on cultured mouse spinal neurones.

Science **212** 358-361

Barker J.L., Harrison N.L., Lange G.D. and Owen D.G. (1987)
Potentiation of γ-aminobutyric acid-activated chloride conductance by a steroid anaesthetic in cultured rat spinal neurones.
J. Physiol. **386** 485-501

Barltrop J.A. and Schofield P. (1965)
Organic photochemistry part II. Some photosensitive protecting groups.
J. Chem. Soc. 4758-4765

Bateson A.N., Lasham A. and Darlison M.G. (1991)
γ- Aminobutyric acid A receptor heterogeneity is increased by alternative splicing of a novel β-subunit gene transcript.
J. Neurochem. **56** 1437-1440

Blair L.A.C., Levitan E.D., Marshall J., Dionne V. and Barnard E.A. (1988)
Single subunits of the $GABA_A$ receptor form ion channels with properties of the native receptor.
Science **242** 577-579

Bormann J. (1988)
Electrophysiology of $GABA_A$ and $GABA_B$ receptor subtypes.
Trends Neurosci. **11** 112-116

Bowery N. (1989)
$GABA_B$ receptors and their significance in mammalian pharmacology.
Trends Pharmacol. Sci. **10** 401-407

Braestrup C. and Nielsen M. (1981)

[^{3}H]Propyl β-carboline-3-carboxylate as a selective radioligand for the BZ_1 benzodiazepine receptor subclass.

J. Neurochem. **37** 333-341

Bristow D. and Martin I.L. (1987)

Solubilisation of the γ-aminobutyric acid/benzodiazepine receptor from rat cerebellum: optimal preservation of the modulatory responses by natural brain lipids.

J. Neurochem. **49** 1386-1393

Brown D.A. and Marsh S. (1978)

Axonal GABA-receptors in mammalian peripheral nerve trunks.

Brain Res. **156** 187-191

Browning M.D., Bureau M., Dudek E.M. and Olsen R.W. (1990)

Protein kinase C and cAMP-dependent protein kinase phosphorylate the β subunit of the purified γ-aminobutyric acid A receptor.

Proc. Natl. Acad. Sci. (USA) **87** 1315-1318

Buchstaller A., Adamiker D., Fuchs K. and Sieghart W. (1991)

N-deglycosylation and immunological identification indicates the existence of β subunit isoforms of the rat $GABA_A$ receptor.

FEBS Lett. **287** 27-30

Burt D.R. and Kamatchi G.L. (1991)

$GABA_A$ receptor subtypes: from pharmacology to molecular biology.

FASEB J. **5** 2916-2923

Cadoni C. and Gee K.W. (1992)

Complex interactions between the steroid derivative RU5135 and the $GABA_A$ receptor complex.

Eur. J. Pharmacol. **227** 147-151

Carpino L.A. and Han G.Y. (1972)

The 9-fluorenylmethoxycarbonyl amino protecting group.

J. Org. Chem. **37** 3404-3409

Casalotti S.O. (1988)

Studies on the molecular characterisation of the GABA/benzodiazepine receptor.

Ph.D. Thesis, University of London.

Casalotti S.O., Stephenson F.A. and Barnard E.A. (1986)

Separate subunits for agonist abd benzodiazepine binding in the γ-aminobutyric $acid_A$ receptor oligomer.

J. Biol. Chem. **261** 15013-15016

Cavalla D. and Neff N.H. (1985)

Photoaffinity labelling of the $GABA_A$ receptor with [^{3}H]munscimol.

J. Neurochem. **44** 916-921

Celentano J.J., Gyenes M., Gibbs T.T. and Farb D.H. (1991)

Negative modulation of the γ-aminobutyric acid response by extracellular zinc.

Mol. Pharmacol. **40** 766-773

Chang L-R and Barnard E.A. (1982)

The benzodiazepine/GABA receptor complex: molecular size in brain synaptic membranes and in solution.

J. Neurochem. **39** 1507-1518

Chang L-R, Barnard E.A., Lo M.M.S. and Dolly J.O. (1981)

Molecular sizes of benzodiazepine receptors and the interacting GABA receptors in the membrane are identical.

FEBS Lett. **126** 309-312

Criado M., Sarin V., Fox J.L. and Lindstrom J. (1986)

Evidence that the acetylcholine binding site is not formed by the sequence α127-143 of the acetylcholine receptor.

Biochemistry **25** 2839-2846

Cuatrecasas P. (1970)

Agarose derivatives for purification of protein by affinity chromatography.

Nature (London) **228** 1327-1328

Curtis D.R. and Malik R. (1985)

Glycine antagonism by RU5135

Eur. J. Pharmacol. **110** 383-384

Cutting G.R., Lu L., O'Hara B.F., Kasch L.M., Montrose-Rafizadeh C., Donovan D.M., Shimada S., Antonarakis S.E., Guggino W.B., Uhl G.R. and Kazazian H.H. (1991)

Cloning of the γ-aminobutyric acid (GABA) ρ1 cDNA: a GABA receptor subunit highly expressed in the retina.

Proc. Natl. Acad. Sci. (USA) **88** 2673-2677

Desarmenien M., Desaulles E., Feltz P. and Hamann M. (1987)
Electrophysiological study of SR 42641, a novel aminopyridazine derivative of GABA: antagonist properties and receptor selectivity of $GABA_A$ versus $GABA_B$ responses.
Br. J. Pharmacol. **90** 287-298

Dingledine R., Myers S.J. and Nicholas R.A. (1990)
Molecular biology of mammalian amino acid receptors.
FASEB J. **4** 2636-2645

Doble A. and Iversen L.L. (1982)
Molecular size of benzodiazepine receptors in rat brain *in situ*: evidence for a functional dimer.
Nature (London) **295** 522-523

Drew C.A., Johnston G.A.R. and Weatherby R.P. (1984)
Bicuculline-insensitive GABA receptors: studies on the binding of (−)-baclofen to rat cerebellar membranes.
Neurosci. Lett. **52** 317-321

Drexler G. and Sieghart W. (1984a)
[^{35}S]*t*-Butylbicyclophosphorothionate and avermectin bind to different sites associated with the γ-aminobutyric acid-benzodiazepine receptor complex.
Neurosci. Lett. **50** 273-277

Drexler G. and Sieghart W. (1984b)
Evidence for association of a high affinity avermectin binding site with the benzodiazepine receptor.
Eur. J. Pharmacol. **101** 201-207

Duggan M.J., Pollard S. and Stephenson F.A. (1991)

Immunoaffinity purification of $GABA_A$ receptor α-subunit iso-oligomers: demonstration of receptor populations containing α1α2, α1α3 and α2α3 subunit pairs.

J. Biol. Chem. **266** 24778-24784

Duggan M.J., Pollard S. and Stephenson F.A. (1992)

Quantitative immunoprecipitation studies with anti-γ-aminobutyric $acid_A$ receptor γ2 1-15 cys antibodies.

J. Neurochem. **58** 72-77

Eichinger A. and Sieghart W. (1985)

Differential degradation of different benzodiazepine binding proteins by incubation of membranes from cerebellum or hippocampus with trypsin.

J. Neurochem. **45** 219-226

Enna S.J. and Snyder S.H. (1975)

Properties of γ-aminobutyric acid (GABA) receptor binding in rat brain synaptic membrane fractions.

Brain Res. **100** 81-97

Enna S.J. and Snyder S.H. (1977)

Influences of ions, enzymes and detergents on γaminobutyric acid receptor binding in synaptic membranes of rat brain.

Mol. Pharmacol. **13** 442-453

Fiegenspan A., Wassle H and Bormann J. (1993)

Pharmacology of GABA receptor Cl^- channels in rat retinal polar cells.

Nature (London) **361** 159-164

Fuchs K., Adamiker D. and Sieghart W. (1990)
Identification of α2- and α3-subunits of the $GABA_A$-benzodiazepine receptor complex purified from the brains of young rats.
FEBS Lett. **261** 52-54

Furuyama T., Sato M., Sato K., Araki T., Inagaki H. and Tohyama M. (1992)
Co-expression of glycine receptor β subunit and $GABA_A$ receptor γ subunit mRNA in the rat dorsal root ganglion cells.
Mol. Brain Res. **12** 335-338

Gavish M. and Snyder S.H. (1981)
γ-Aminobutyric acid and benzodiazepine receptors: co-purification and characterisation.
Proc. natl. Acad. Sci. (USA) **78** 1939-1942

Gee K.W. (1988)
Steroid modulation of the GABA/benzodiazepine receptor-linked chloride ionophore.
Mol. Neurobiol. **2** 291-317

Gee K.W., Chang W.C., Brinton R.E., Coirini H. and McEwen B.S. (1987)
GABA-dependent modulation of the Cl^- ionophore by steroids in rat brain.
Eur. J. Pharmacol. **136** 419-423

Gee K.W., Bolger M.B., Brinton R.E., Coirini H. and McEwen B.S. (1988)
Steroid modulation of the chloride ionophore in rat brain: structure-activity requirements, regional dependence and mechanism of action.
J. Pharmacol. Exp. Ther. **246** 803-812

Greene T. (1981)

Protective groups in organic synthesis.

Wiley-Interscience.

Grenningloh G, Rienitz A., Schmitt B., Methfessel C., Zensen M., Beyreuther K., Gundelfinger E.D. and Betz H. (1987)

The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors.

Nature (London) **328** 215-220

Hales T.G. and Lambert J.J. (1991)

The actions of propofol on inhibitory amino acid receptors of bovine adrenomedullary chromaffin cells and rodent central neurones.

Br. J. Pharmacol. **104** 619-628

Hales T.G. and Lambert J.J. (1992)

Modulation of $GABA_A$ and glycine receptors by chlormethiazole.

Eur. J. Pharmacol. **210** 239-246

Hanessian S. and Masse R. (1977)

Photochemical cleavage of the N-(benzyloxycarbonyl) group in amino sugar derivatives.

Carbohydr. Res. **54** 142-144

Harrison N.L. and Simmonds M.A. (1984)

Modulation of the GABA receptor complex by a steroid anaesthetic.

Brain Res. **323** 287-292

Harrison N.L., Majewska M.D., Harrington J.W. and Barker J.L. (1987)
Structure-activity relationships for steroid interaction with the γ-aminobutyric $acid_A$ receptor complex.
J. Pharmacol. Exp. Ther. **241** 346-353

Hollmann M., O'Shea-Greenfield A., Rogers S.W. and Heinemann S. (1989)
Cloning by functional expression of a member of the glutamate receptor family.
Nature (London) **342** 643-648

Houamed K.M., Bilbe G., Smart T.G., Constanti A., Brown D.A., Barnard E.A. and Richards B.M. (1984)
Expression of functional GABA, glycine and glutamate receptors in *Xenopus* oocytes injected with rat brain mRNA.
Nature (London) **310** 318-321

Huang J-H and Johnston G.A.R. (1990)
(+)-Hydrastine, a potent competitive antagonist at mammalian $GABA_A$ receptors.
Br. J. Pharmacol. **99** 727-730

Hunt P. and Clements-Jewry S. (1981)
A steroid derivative, RU5135, antagonises the GABA/benzodiazepine receptor interaction.
Neuropharmacology **20** 357-361

Jouquey A. and Hunt P.F. (1985)
17-Aza-androstane derivatives.
UK Patent No. GB 2141717A

Kato K. (1990)

Novel $GABA_A$ receptor α subunit is expressed only in cerebellar granule cells.

J. Mol. Biol. **214** 619-624

King R.G., Nielsen M., Stauber G.B. and Olsen R.W. (1987)

Convulsant/barbiturate activity on the soluble γ-aminobutyric acid-benzodiazepine receptor.

Eur. J. Biochem. **169** 555-562

Kirkness E.F. and Turner A.J. (1985)

The γ-aminobutyrate/benzodiazepine receptor from pig cerebral cortex: purification and characterisation.

Biochem. Soc. Trans. **13** 1211-1212

Kleingoor C., Ewert M., Von Blankenfeld G., Seeburg P.H. and Kettenmann H. (1991)

Inverse but not full benzodiazepine agonists modulate recombinant α6β2γ2 $GABA_A$ receptors in transfected human embryonic kidney cells.

Neurosci. Lett. **130** 169-172

Knoflach F., Backus K.H., Giller T., Malherbe P. Pfimlin P., Mohler H. and Trube G. (1992)

Pharmacological and electrophysiological properties of recombinant $GABA_A$ receptors comprising the α3, β1 and γ2 subunits.

Eur. J. Neurosci. **4** 1-9

Knoflach F., Rhyner Th., Villa M., Kellenberger S., Drescher U., Malherbe P., Sigel E. and Mohler H. (1991)
The γ3-subunit of the $GABA_A$ receptor confers sensitivity to benzodiazepine receptor ligands.
FEBS Lett. **293** 191-194

Kofuji P., Wang J.B., Moss S.J., Huganir R.L. and Burt D.B. (1991)
Generation of two forms of the γ-aminobutyric acid $_A$ receptor γ2 subunit in mice by alternative splicing.
J. Neurochem. **56** 713-715

Krestchatisky M., MacLennan A.J., Chiang M-Y, Xu W., Jackson M.B., Brecha N., Sternini C., Olsen R.W. and Tobin A.J. (1989)
A novel α subunit in rat brain $GABA_A$ receptors.
Neuron **3** 745-753

Langosch D., Thomas L. and Betz H. (1988)
Conserved quaternary structure of ligand-gated ion channels; the postsynaptic glycine receptor is a pentamer.
Proc. Natl. Acad. Sci. (USA) **85** 7394-7398

Leeb-Lundberg L.M.F. and Olsen R.W. (1983)
Heterogeneity of benzodiazepine receptor interactions with γ-aminobutyric acid and barbiturate receptor sites.
Mol. Pharmacol. **23** 315-325

Leeb-Lundberg F., Snowman A. and Olsen R.W. (1980)
Barbiturate receptor sites are coupled to benzodiazepine receptors.
Proc. Natl. Acad. Sci. (USA) **77** 7468-7472

Leidenheimer N.J., Machu T.K., Endo S., Olsen R.W., Harris R.A. and Browning M.D. (1991)
Cyclic AMP-dependent protein kinase decreases γ-aminobutyric $acid_A$ receptor-mediated $^{36}Cl^-$ uptake by brain microsacs.
J. Neurochem. **57** 722-725

Levitan E.S., Blair L.A.C., Dionne V.E. and Barnard E.A. (1988a)
Biophysical and pharmacological properties of cloned $GABA_A$ receptor subunits expressed in *Xenopus* oocytes.
Neuron **1** 773-781

Levitan E.S., Schofield P.R., Burt D.R., Rhee L.M., Wisden W., Kohler M, Fujita N., Rodriguez H.F., Stephenson F.A., Darlison M.G., Barnard E.A. and Seeburg P.H. (1988b)
Structural and functional basis for $GABA_A$ receptor heterogeneity.
Nature (London) **335** 76-79

Lindstrom J., Merlie J. and Yogeeswaran G. (1979)
Biochemical proerties of acetylcholine receptor subunits from *Torpedo californica.*
Biochemistry **18** 4465-4470

Lolait S.J., O'Carroll A-M, Kusano K., Muller J-M, Brownstein M.J. and Mahan L.C. (1989)
Cloning and expression of a novel rat $GABA_A$ receptor.
FEBS Lett. **246** 145-148

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951)
Protein measurement with the Folin phenol reagent.
J. Biol. Chem. **193** 265-275

Luddens H., Pritchett D.B., Kohler M., Killisch I., Kienanen K., Monyer H., Sprengel R. and Seeburg P.H. (1990)
Cerebellar $GABA_A$ receptor selective for a behavioural antagonist.
Nature (London) **346** 648-651

Majewska M.D., Harrison N.L., Schwartz R.D., Barker J.L. and Paul S.M. (1986)
Steroid hormone metabolites are barbiturate-like modulators of the $GABA_A$ receptor.
Science **232** 1004-1007

Malherbe P. Sigel E. Baur R. Persohn E. Richards J.G. and Mohler H. (1990)
Functional expression and sites of gene transcription of a novel α subunit of the $GABA_A$ receptor in rat brain.
FEBS Lett. **260** 261-265

Mamalaki C., Stephenson F.A. and Barnard E.A. (1987)
The $GABA_A$/benzodiazepine receptor is a heterotetramer of homologous α and β subunits.
EMBO J. **6** 561-565

Mamalaki C., Barnard E.A. and Stephenson F.A. (1989)
Molecular size of the γ-aminobutyric $acid_A$ receptor purified from mammalian cerebral cortex.
J. Neurochem. **52** 124-134

Maricq A.V., Peterson A.S., Brake A.J., Myers R.M. and Julius D. (1991)
Primary structure and functional expression of the 5-HT_3 receptor, a serotonin-gated ion channel.
Science **254** 254-255

Martini C., Lucacchini A. and Ronca G. (1981)

Specific adsorbents for affinity chromatography of benzodiazepine binding proteins.

Prep. Biochem. **11** 487-499

Martini C., Lucacchini A., Ronca G., Herlia S. and Rossi C.A. (1982)

Isolation of putative benzodiazepine receptors from rat brain membranes by affinity chromatography.

J. Neurochem. **38** 15-19

Mienville J-M. and Vicini S. (1987)

A pyridazinyl derivative of gamma-aminobutyric acid (GABA), SR 95531, is a potent antagonist of Cl^- channel opening regulated by $GABA_A$ receptors.

Neuropharmacology **26** 779-783

Mohler H. and Okada T. (1977)

Benzodiazepine receptor: demonstration in the central nervous system.

Science **198** 849-851

Mohler H., Battersby M.K. and Richards J.G. (1980)

Benzodiazepine receptor protein identified and visuailised in brain tissue by a photoaffinity label.

Proc. Natl. Acad. Sci. (USA) **77** 1666-1670

Mohler H., Sieghart W., Richards J.G. and Hunkeler W. (1984)

Photoaffinity labelling of benzodiazepine receptors with a partial inverse agonist.

Eur. J. Pharmacol. **102** 191-192

Moody E.J. and Skolnick P. (1989)

Chlormethiazole: neurochemical actions at the γ-aminobutyric acid receptor complex.

Eur. J. Pharmacol. **164** 153-158

Morrow A.L., Pace J.R., Purdy R.H. and Paul S.M. (1990)

Characterisation of steroid interactions with γ-aminobutyric acid receptor-gated chloride ion channels: evidence for multiple steroid recognition sites.

Mol. Pharmacol. **37** 263-270

Moss S.J., Doherty C.A. and Huganir R.L. (1992a)

Identification of the cAMP-dependent protein kinase and protein kinase C phosphorylation sites within the major intracellular domains of the β1, γ2S and γ2L subunits of the γ-aminobutyric acid type A receptor.

J. Biol. Chem. **267** 14470-14476

Moss S.J., Smart T.G., Blackstone C.D. and Huganir R.L. (1992b)

Functional modulation of $GABA_A$ receptors by cAMP-dependent protein phosphorylation.

Science **257** 661-665

Myslobodsky M.S. and Kofman O. (1983)

Regular and lasting neocortical spiking produced by systemic administration of a steroid derivative in the rat.

Neuropharmacology **22** 157-164

Noble P.J. Anderson S.M.P. Desouza R. Cross A.J. and Stephenson F.A. (1993)
Identification of the $GABA_A$ receptor α3 subunit in the IMR-32 neuroblastoma cell line.
J. Neurochem. In Press.

Olsen R.W. (1982)
Drug interactions at the GABA receptor-ionophore complex.
Ann. Rev. Pharmacol. Toxicol. **22** 247-277

Olsen R.W. (1984)
γ-Aminobutyric acid receptor binding antagonism by the amidine steroid RU5135.
Eur. J. Pharmacol. **103** 333-337

Olsen R.W. and Tobin A.J. (1990)
Molecular biology of $GABA_A$ receptors.
FASEB J. **4** 1469-1480

Olsen R.W., Bergman M., Van Ness P.C., Lummis S.C., Watkins A., Napias C. and Greenlee D.V. (1981)
γ-Aminobutyric acid receptor binding in mammalian brain: heterogeneity of binding sites.
Mol. Pharmacol. **19** 217-227

Olsen R.W., Wong E.H.F., Stauber G.B., King R.G. and Fisher J.B. (1984)
Biochemical properties of the GABA/barbiturate/benzodiazepine receptor chloride ion channel complex.
In *Neurotransmitter receptors: mechanism of action and regulation* (eds S. Kito, T. Segawa, K. Kuriyama and H.I. Yamamura), Plenum Press, New York, pp. 205-219

Ong J. and Kerr D.I.B. (1990)

GABA-receptors in peripheral tissues.

Life Sci. **46** 1489-1501

Paleos C.M., Varveri F.S. and Gregoriou G.A. (1974)

Some new arenesulphonate leaving groups less reactive than the p-toluenesulphonate group.

J. Org. Chem. **39** 3594-3595

Paquet A. (1982)

Introduction of 9-fluorenylmethoxycarbonyl, trichloroethoxycarbonyl and benzyloxycarbonyl amine protecting groups into O-unprotected hydroxyamino acids using succinimidyl carbonates.

Can. J. Chem. **60** 976-980

Park D., Vitorica J., Tous G. and de Blas A. (1991)

Purification of the γ-aminobutyric $acid_A$/benzodiazepine receptor complex by immunoaffinity chromatography.

J. Neurochem. **56** 1662-1971

Paul S.M. and Purdy R.H. (1992)

Neuroactive steroids.

FASEB J. **6** 2311-2322

Paul S.M., Kempner E.S. and Skolnick P. (1981)

In situ molecular weight determination of brain and peripheral benzodiazepine binding sites.

Eur J. Pharmacol. **76** 465-466

Pfeiffer F., Graham D. and Betz H. (1982)

Purification by affinity chromatography of the glycine receptor of rat spinal cord.

J. Biol. Chem. **257** 9389-9393

Polenzani L., Woodward R.M. and Miledi R. (1991)

Expression of mammalian γaminobutyric acid receptors with distinct pharmacology in *Xenopus* oocytes.

Proc. Natl. Acad. Sci. (USA) **88** 4318-4322

Pollard S., Duggan M.J. and Stephenson F.A. (1993)

Further evidence for the existence of α subunit heterogeneity within discrete $GABA_A$ receptor subpopulations.

J. Biol. Chem. **268** 3753-3757

Pritchett D.B. and Seeburg P.H. (1990)

γ-Aminobutyric $acid_A$ receptor α5 subunit creates novel type II benzodiazepine receptor pharmacology.

J. Neurochem. **54** 1802-1804

Pritchett D.B., Sontheimer H., Gormann C.M., Kettenmann H., Seeburg P.H. and Schofield P.R. (1988)

Transient expression shows ligand gating and allosteric potentiation of $GABA_A$ receptor subunits.

Science **242** 1306-1308

Pritchett D.B., Luddens H. and Seeburg P.H. (1989a)

Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells.

Science **245** 1389-1392

Pritchett D.B., Sontheimer H., Shivers B.D., Ymer S., Kettenmann H., Schofield P.R. and Seeburg P.H. (1989b)
Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology.
Nature (London) **338** 582-585

Raftery M.A., Hunkapiller M.W., Strader C.D.and Hood L.E. (1980)
Acetylcholine receptor: complex of homologous subunits.
Science **208** 1454-1457

Ramanjaneyulu R. and Ticku M.K. (1984)
Binding characteristics and interactions of depressant drugs with [^{35}S]*t*-butylbicyclophosphorothionate, a ligand that binds to the picrotoxin site.
J. Neurochem. **42** 221-229

Richards G., Schoch P. and Haefely W. (1991)
Benzodiazepine receptors: new vistas.
Seminars in the Neurosciences **3** 191-203

Schnabel E. and Li C.H. (1960)
The synthesis of L-histidyl-D-phenylalanyl-L-arginyl-L-tryptophylglycine and its melanocyte-stimulating activity.
J. Am. Chem. Soc. **82** 4576-4579

Schoch P., Haring P., Takacs B., Stahli C. and Mohler H. (1984)
A GABA/benzodiazepine receptor complex from bovine brain: purification, reconstitution and immunological characterisation.
J. Recept. Res. **4** 189-200

Schoch P. Richards J.G. Haring P. Takacs B. Stahli C., Staehelin T. Haefely W. and Mohler H. (1985)
Co-localisation of $GABA_A$ receptors and benzodiazepine receptors in the brain shown by monoclonal antibodies.
Nature (London) **314** 168-171

Schofield P., Darlison M.G., Fujita N., Burt D.R., Stephenson F.A., Rodriguez H., Rhee L.M., Ramachandran J., Reale V., Glencorse T.A., Seeburg P.H. and Barnard E.A. (1987)
Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family.
Nature (London) **328** 221-227

Schofield P., Pritchett D., Sontheimer H., Kettenmann H. and Seeburg P. (1989)
Sequence and expression of human $GABA_A$ receptor $\alpha 1$ and $\beta 1$ subunits.
FEBS Lett. **244** 361-364

Sequier J., Richards J., Malherbe P., Price G. Mathews S. and Mohler H. (1988)
Mapping of brain areas containing RNA homologous to cDNAs encoding the α and β subunits of the rat $GABA_A$ γ-aminobutyrate receptor.
Proc. Natl. Acad. Sci. (USA) **85** 7815-7819

Shimada S., Cutting G. and Uhl G.R. (1992)
γ-Aminobutyric acid A or C receptor? γ-aminobutyric acid rho 1 receptor RNA induces bicuculline-, barbiturate- and benzodiazepine-insensitive γ-aminobutyric acid responses in *Xenopus* oocytes.
Mol. Pharmacol. **41** 683-687

Shivers B.D., Killisch I., Sprengel R., Sontheimer H., Kohler M., Schofield P.R. and Seeburg P.H. (1989)

Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations.

Neuron **3** 327-337

Sieghart W. (1992)

$GABA_A$ receptors: ligand-gated Cl^- ion channels modulated by multiple drug-binding sites.

Trends Pharmacol. Sci. **13** 446-450

Sieghart W. and Karobath M. (1980)

Molecular heterogeneity of benzodiazepine receptors.

Nature (London) **286** 285-287

Sieghart W., Mayer A. and Drexler G. (1983)

Properties of [^{3}H]flunitrazepam binding to different benzodiazepine binding proteins.

Eur. J. Pharmacol. **88** 291-299

Sieghart W., Eichinger A., Richards J.G. and Mohler H. (1987)

Photoaffinity labelling of benzodiazepine receptor proteins with a partial inverse agonist [^{3}H]Ro15-4513: a biochemical and autoradiographical study.

J. Neurochem. **48** 46-52

Sieghart W., Item C., Buchstaller A., Fuchs K, Hoger H. and Adamiker D. (1993)

Evidence for the existence of differential *O*-glycosylated α5 subunits of the γ-aminobutyric $acid_A$ receptor in the rat brain.

J. Neurochem. **60** 93-98

Sigel E. and Barnard E.A. (1984)

A γ-aminobutyric acid/benzodiazepine receptor complex from bovine cerebral cortex. Improved purification with preservation of regulatory sites and their interactions.

J. Biol. Chem. **259** 7219-7223

Sigel E., Mamalaki C. and Barnard E.A. (1982)

Isolation of a GABA receptor from bovine brain using a benzodiazepine affinity column.

FEBS Lett. **147** 45-48

Sigel E., Stephenson F.A., Mamalaki C. and Barnard E.A. (1983)

A γ-aminobutyric acid/benzodiazepine receptor complex of bovine cerebral cortex.

J. Biol. Chem. **258** 6965-6971

Sigel E., Baur R., Malherbe P. and Mohler H. (1989)

The rat β1 subunit of the $GABA_A$ receptor forms a picrotoxin-sensitive anion channel open in the absence of GABA.

FEBS Lett. **257** 377-379

Sigel E., Baur R., Trube G., Mohler H. and Malherbe P. (1990)

The effect of subunit composition of rat brain $GABA_A$ receptors on channel function.

Neuron **5** 703-711

Sigel E., Baur R. and Malherbe P. (1991)

Activation of protein kinase C results in down modulation of different recombinant $GABA_A$ channels.

FEBS Lett. **291** 150-152

Sigel E., Baur R., Kellenberger S. and Malherbe P. (1992)

Point mutations affecting antagonist affinity and agonist-dependent gating of $GABA_A$ receptor channels

EMBO J. **11** 2017-2023

Simmonds M.A. (1981)

Distinction between the effects of barbiturates, benzodiazepines and phenytoin on responses to γ-aminobutyric acid receptor activation and antagonism by bicuculline and picrotoxin.

Br. J. Pharmacol. **73** 739-747

Simmonds M.A. (1991)

Modulation of the $GABA_A$ receptor by steroids.

Seminars in the Neurosciences **3** 231-239

Simmonds M.A. and Turner J.P. (1985)

Antagonism of inhibitory amino acids by the steroid derivative RU5135.

Br. J. Pharmacol. **84** 631-635

Skerritt J.H., Willow M. and Johnston G.A.R. (1982)
Diazepam enhancement of low affinity GABA binding to rat brain membranes.
Neurosci. Lett. **29** 63-66

Smart T.G. and Constanti A. (1982)
A novel effect of zinc on the lobster muscle GABA receptor.
Proc. R. Soc. (London) B **215** 327-341

Smart T.G. and Constanti A. (1990)
Differential effect of zinc on the vertebrate $GABA_A$ receptor complex.
Br. J. Pharmacol. **99** 643-654

Smart T.G., Constanti A., Bilbe G., Brown D.A. and Barnard E.A. (1983)
Synthesis of functional chick brain GABA-benzodiazepine-barbiturate/receptor complexes in mRNA injected *Xenopus* oocytes.
Neurosci. Lett. **40** 55-59

Sommer B. and Seeburg P.H. (1992)
Glutamate receptor channels: novel properties and new clones.
Trends Pharmacol. Sci. **13** 291-296

Sommer B., Poustka A., Spurr N.K. and Seeburg P.H. (1990)
The murine $GABA_A$ receptor δ subunit gene: structure and assignment to human chromosome 1.
DNA and Cell Biology **9** 561-568

Squires R.F. and Braestrup C. (1977)
Benzodiazepine receptors in rat brain.
Nature (London) **266** 732-734

Squires R.F., Benson D.I., Braestrup C., Coupet J., Klepner C.A., Myers V. and Beer B. (1979)
Some properties of specific benzodiazepine receptors: New evidence for multiple receptors.
Pharmacol. Biochem. Behav. **10** 825-830

Squires R.F., Casida J.E., Richardson M. and Saederup E. (1983)
[^{35}S]*t*-Butylbicyclophosphorothionate binds with high affinity to brain specific sites coupled to γ-aminobutyric acid A and ion recognition sites.
Mol. Pharmacol. **23** 326-336

Stauber G.B., Ransom R.W., Dilber A.I. and Olsen R.W. (1987)
The γ-aminobutyric-acid/benzodiazepine-receptor protein from rat brain. Large-scale purification and preparation of antibodies.
Eur. J. Biochem. **167** 125-133

Stephenson F.A. (1978)
Progress towards the understanding of the $GABA_A$ receptor structure.
J. Recept. Res. **7** 43-54

Stephenson F.A. (1988)
Understanding the $GABA_A$ receptor: a chemically gated ion channel.
Biochem. J. **249** 21-32

Stephenson F.A. and Barnard E.A. (1986)
Purification and charcterisation of the brain GABA/benzodiazepine receptor. In *Benzodiazepine/GABA receptors and chloride channels: structural and functional properties.* (Eds R.W. Olsen and J.C. Venter) pp 261-271. Alan R. Liss, New York.

Stephenson F.A. and Olsen R.W. (1982)

Solubilisation by CHAPS detergent of barbiturate-enhanced benzodiazepine-GABA receptor complex.

J. Neurochem. **39** 1579-1586

Stephenson F.A. and Olsen R.W. (1983)

Biochemical pharmacology of the GABA receptor protein complex.

In *Advances in Biochemical Psychopharmacology* (eds P. Mandel and F.V. Defeudis) **37** 71-80

Stephenson F.A., Watkins A.E. and Olsen R.W. (1982)

Physicochemical characterisation of detergent solubilised γ-aminobutyric acid and benzodiazepine receptor proteins from bovine brain.

Eur. J. Pharmacol. **123** 291-298

Study R.E. and Barker J.L. (1981)

Diazepam and (−)-pentobarbital: fluctuation analysis reveals different mechanisms for potentiation of γ-aminobutyric acid responses in cultured central neurons.

Proc. Natl. Acad. Sci. (USA) **78** 7180-7184

Sundberg L. and Porath J. (1974)

Preparation of adsorbents for biospecific affinity chromatography. Attachment of group-containing ligands to insoluble polymers by means of bifunctional oxiranes.

J. Chromatogr. **90** 87-98

Supavilai P. and Karobath M. (1984)

[^{35}S]*t*-Butylbicyclophosphorothionate binding sites are constituents of the γ-aminobutyric acid benzodiazepine receptor complex.

J. Neurosci. **4** 1193-1200

Sweetnam P.M. and Tallman J.F. (1986)

Regional differences in brain benzodiazepine receptor carbohydrates.

Mol. Pharmacol. **29** 299-306

Sweetnam P.M., Lloyd J., Gallombardo P., Madison R.T., Gallagher D.W., Tallman J.F. and Nestler E.J. (1988)

Phosphorylation of the $GABA_A$/benzodiazepine receptor α subunit by a receptor-associated protein kinase.

J. Neurochem. **51** 1274-1284

Swope S.L., Moss S.J., Blackstone C.D. and Huganir R.L. (1992)

Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity.

FASEB J. **6** 2514-2523

Taguchi J.F. and Kuriyama K. (1984)

Purification of γ-aminobutyric acid (GABA) receptor from rat brain by affinity column chromatography using a new benzodiazepine, 1012-S, as an immobilised ligand.

Brain Res. **323** 219-226

Tallman J.F. (1984)

Properties of the purified GABA/benzodiazepine complex.

Clin. Neuropharmacol. **7** 558-559

Thompson C.L., Bodewitz G., Stephenson F.A. and Turner J.D. (1992)
Mapping of $GABA_A$ receptor α5 and α6 subunit-like immunoreactivity in rat brain.
Neurosci. Lett. **144** 53-56

Trifiletti R.R., Snowman A.M. and Snyder S.H. (1984)
Solubilisation and anionic regulation of cerebral sedative/convulsant receptors labelled with [^{35}S]*t*-butylbicyclophosphorothionate (TBPS).
Biochem. Biophys. Res. Commun. **120** 692-699

Turner A.J. and Whittle S.R. (1983)
Biochemical dissection of the gamma-aminobutyrate synapse.
Biochem. J. **209** 29-41

Turner D.M., Ransom R.W., Yang J.S-J and Olsen R.W. (1989)
Steroid anaesthetics and naturally occurring analogues modulate the γ-aminobutyric acid receptor complex at a site distinct from barbiturates.
J. Pharmacol. Exp. Ther. **248** 960-966

Unnerstall J.R., Kuhar M.J., Niehoff D.L. and Palacios J.M. (1981)
Benzodiazepine receptors are coupled to a subpopulation of γ-aminobutyric acid (GABA) receptors: evidence from a quantitative autoradiographic study.
J. Pharmacol. Exp. Ther. **218** 797-804

Unwin N. (1989)
The structure of ion channels in membranes of excitable cells.
Neuron **3** 665-676

Van Renterghem C., Bilbe G., Moss S., Smart T.G., Constanti A., Brown D.A. and Barnard E.A. (1987)
GABA receptors induced in *Xenopus* oocytes by chick brain mRNA: evaluation of TBPS as a use-dependent channel blocker.
Mol. Brain Res. **2** 21-31

Van Ness P., Watkins A., Bergman M., Tourtellotte W. and Olsen R.W. (1982)
γ-Aminobutyric acid receptors in normal human brain and Huntington's disease.
Neurology (N.Y.) **32** 63-68

Vandenberg R.J., Handford C.A. and Schofield P.R. (1992)
Distinct agonist and antagonist binding sites on the glycine receptor.
Neuron **9** 491-496

Velazquez J.L., Thompson C.L., Barnes E.M. and Angelides K.J. (1989)
Distribution and lateral mobility of GABA/benzodiazepine receptors on nerve cells.
J. Neurosci. **9** 2163-2169

Von Blankenfield G., Ymer S., Pritchett D.B., Sontheimer H., Ewert M., Seeburg S.P. and Kettenmann H. (1990)
Differential benzodiazepine pharmacology of mammalian recombinant $GABA_A$ receptors.
Neurosci. Lett. **115** 269-273

Vretblad (1976)
Purification of lectins by biospecific affinity chromatography.
Biochim. Biophys. Acta **434** 169-176

Wermuth C-G., Bourguignon J-J., Schlewer G., Giles J-P., Schoenfelder A., Melikian A., Bouchet M-J., Chantreux D., Molimard J-C., Heaulme M., Chambon J-P. and Biziere K. (1987)
Synthesis and structure activity relationships of a series of aminopyridazine derivatives of γ-aminobutyric acid acting as selective $GABA_A$ antagonists.
J. Med. Chem. **30** 239-249

Whiting P., McKernan R.M. and Iversen, L.L. (1990)
Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site.
Proc. Natl. Acad. Sci. (USA) **87** 9966-9970

Wieland H.A., Luddens H. and Seeburg P.H. (1992)
A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding.
J. Biol. Chem. **267** 1426-1429

Wisden W., Morris B.J., Darlison M.G., Hunt S.P. and Barnard E.A. (1988)
Distinct $GABA_A$ receptor α subunit mRNAs show differential patterns of expression in bovine brain.
Neuron **1** 937-947

Wisden W. Herb A. Wieland H. Keinanen K. Luddens H. and Seeburg P.(1991)
Cloning, pharmacological characteristics and expression pattern of the rat $GABA_A$ receptor α4 subunit.
FEBS Lett. **289** 227-230

Wisden W., Laurie D.J., Monyer H. and Seeburg P.H. (1992)
The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, Diencephalon, Mesencephalon.
J. Neurosci. **12** 1040-1062

Woodward R.M., Polenzani L. and Miledi R. (1992)
Characterisation of bicuculline/barbiturate-insensitive γ-aminobutyric acid receptors expressed in *Xenopus* oocytes. I. Effects of Cl^- channel inhibitors.
Mol. Pharmacol. **42** 165-173

Xie X.M. and Smart T.G. (1991)
A physiological role for endogenous zinc in rat hippocampal synaptic neurotransmission.
Nature (London) **349** 521-524

Yajima H., Takeyama M., Kanaki J. and Mitani K. (1978)
The mesitylene-2-sulphonyl group, an acidolytically removable N^G-protecting group for arginine.
J.C.S. Chem. Commun. **1978** 482-483

Ymer S., Draguhn A., Kohler M., Schofield P.R. and Seeburg P.H. (1989)
Sequence and expression of a novel $GABA_A$ receptor α subunit.
FEBS Lett. **258** 119-122

Ymer S., Draguhn A., Wisden W., Werner P., Keinanen K., Schofield P.R., Sprengel R., Pritchett D.B. and Seeburg P.H. (1990)
Structural and functional characterisation of the γ1 subunit of $GABA_A$/benzodiazepine receptors.
EMBO J. **9** 3261-3267

Zezula J. and Sieghart W. (1991)

Isolation of type I and type II $GABA_A$-benzodiazepine receptors by immunoaffinity chromatography.

FEBS Lett. **284** 15-18

Zukin S.R., Young A.B. and Snyder S.H. (1974)

Gamma-aminobutyric acid binding to receptor sites in the rat central nervous system.

Proc. Natl. Acad. Sci. (USA) **71** 4802-4807